

**Clinical pharmacology of netazepide,
a gastrin/CCK₂ receptor antagonist**

**A dissertation submitted to the Institute of Translational Medicine,
Department of Cellular and Molecular Physiology, University of Liverpool,
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by

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Confidential

Dedication

I dedicate this thesis to the late Sir James Black, inventor of β -adrenoceptor and histamine H₂-receptor antagonists, and a good friend and inspirational colleague. We worked together to obtain a licence for YF476 (netazepide), a compound that he referred to as the ‘gold standard’ among gastrin/CCK₂ receptor antagonists. Sadly, he died in March 2010. He would have been excited by the subsequent results of netazepide studies in patients, and even more excited about the recent discovery that TR2-A, the acetyl derivative and prodrug of the main metabolite of netazepide in humans, is a novel gastrin/CCK₂ receptor antagonist and a worthy successor to netazepide.

Jim Black identifies the structure of netazepide in the floor of HMR in October 2009



Foreword

Drug development is rarely straightforward and netazepide (YF476) is no exception. To guide readers through its long chequered history, and to understand better my role in its development, I summarise below the key milestones.

- **1993:** Ferring Pharmaceuticals, Chilworth, England, synthesises YF476 and shows it to be a potent, highly-selective, competitive, orally-active antagonist of gastrin/CCK₂ receptors. Yamanouchi, Japan, joins Ferring to co-develop YF476 (hence the code name YF). The target is gastro-oesophageal reflux disease (GORD).
- **1994:** Huntingdon Life Sciences (HLS), England, starts toxicology studies to support the clinical development of YF476.
- **1996-1997:** Ferring sponsors Hammersmith Medicines Research (HMR) to carry out the first studies of YF476 in healthy subjects. Single doses cause dose-dependent, long-lasting increases in 24-h gastric pH, consistent with antagonism of gastrin/CCK₂ receptors. But, after repeated doses, healthy subjects develop tolerance to the effect of YF476 on gastric pH. Gastroenterologists advise Ferring against developing YF476 because it is widely believed that raising gastric pH ≥ 4 is essential to treat GORD.
- **1998:** Yamanouchi shows that in rats, repeated doses of YF476 cause dose-dependent inhibition of basal and pentagastrin-stimulated gastric acid production and increases in serum gastrin. There is no evidence of tolerance. However, Ferring and Yamanouchi both decide to stop development of YF476 because of tolerance to its effect on gastric pH in healthy subjects.
- **1999:** I start to present the results of YF476 studies in healthy subjects at British Pharmacological Society meetings. Sir James Black contacts me afterwards, because of his long-standing interest in developing a gastrin/CCK₂ receptor antagonist.
- **2000-2003:** James Black Foundation (JBF; funded by Johnson and Johnson, J&J) licenses YF476 from Ferring, and describes it as the 'gold standard' of gastrin/CCK₂ antagonists. Like Yamanouchi, JBF shows that repeated doses of YF476 cause persistent, dose-dependent inhibition of pentagastrin-stimulated gastric acid production in rats. JBF sponsors HMR to do two studies in healthy subjects, to assess the effect of single- and repeated-doses of YF476 on the response to intravenous pentagastrin. These show that YF476 causes dose-dependent and persistent antagonism of the response to pentagastrin, which is consistent with blockade of gastrin/CCK₂ receptors. Despite the favourable pharmacodynamic results, the bioavailability of the oral formulation of YF476 subsequently proves to have been poor.

- **2004:** J&J decides not to fund further development of YF476, and returns it to Ferring.
- **2004:** A Yamanouchi spin-off company licenses YF476 from Ferring, to develop it for treatment of pancreatic cancer, but subsequently returns it because they cannot obtain funding.
- **2006:** HMR founds a subsidiary company called Trio Medicines Ltd (Trio) to license YF476 and continue its development. J&J closes JBF, so Jim Black joins Trio. JBF had tried unsuccessfully for 20 years to invent a gastrin/CCK₂ receptor antagonist worthy of full clinical development. Although results from early trials of JB95008 ('gastrozole') in patients with pancreatic cancer were promising, its development was stopped because it had little or no oral bioavailability, was short acting, and had to be given by continuous intravenous infusion.
- **2006:** The MHRA requests further non-clinical studies of YF476 in order to restart clinical studies: to confirm that the rat and dog were appropriate species for the toxicology studies; and to assess protein binding and metabolism of YF476, and its effect on the hERG channel, ECG intervals and cytochrome p450 enzymes.
- **2007:** HMR manufactures capsules of YF476 for Trio, following the recipe used for the studies sponsored by JFB in 2000–2002, and restarts studies in healthy subjects. Despite favourable pharmacodynamics results showing that YF476 not only suppresses gastric acid secretion as effectively as a proton pump inhibitor (PPI) but also inhibits the trophic effect of the PPI on ECL cells caused by secondary hypergastrinaemia, the formulation proves much less bioavailable than the one used in the first studies in healthy subjects.
- **2007:** The European Medicines Agency designates YF476 an orphan medicinal product (OMP) for treatment of patients with gastric carcinoids in Europe.
- **2009:** The Food and Drug Administration designates YF476 an OMP for treatment of gastric carcinoids in the USA, but only after Trio provides evidence that YF476 not only prevents gastric carcinoids in an animal model but also causes regression of such tumours.
- **2009:** Trio finds out from Yamanouchi that YF476 used in the early clinical studies was spray dried. R5 Research Ltd manufactures a new spray-dried formulation of YF476, which proves to have bioavailability in healthy subjects similar to that of the early formulation.
- **2010:** Start of studies of YF476 for three months in patients with type 1 gastric carcinoids in Liverpool, England, and Trondheim, Norway, and type 2 gastric carcinoids in the USA. WHO reclassifies gastric carcinoids as gastric neuroendocrine tumours (NETs).
- **2011:** WHO accepts netazepide as the generic name for YF476.

- **2013:** I register ‘The Clinical Pharmacology of Netazepide’ for an MD thesis with the University of Liverpool.
- **2012–2015:** I publish in peer-reviewed journals four papers on the clinical pharmacology of netazepide in healthy subjects and a paper on the prevalence of gastric NETs in Europe, USA and Japan, and the rationale for treating them with a gastrin/CCK₂ receptor antagonist.
- **2012–2013:** Netazepide reduces the number and size of type 1 gastric NETs, and normalises tumour biomarkers. Results, which are similar for the two centres, are published in peer-reviewed journals. I am a co-author of both papers.
- **2013:** The MHRA suggests and approves a protocol amendment for dosing patients with type 1 tumours in the Liverpool study with netazepide for a further 12 months, without demanding longer toxicology studies. Norway agrees to compassionate use of netazepide for continued dosing of Trondheim patients. The MHRA grants netazepide a ‘specials licence’ for compassionate treatment of patients with hypergastrinaemia.
- **2013–2015:** Metabolites of netazepide are isolated, synthesised and characterised. Non-clinical studies show that the main metabolite, called TR2, is not quite as potent as netazepide but is more selective and soluble. Also, the acetyl derivative of TR2, called TR2-A, is a prodrug of TR2 and is more soluble than netazepide and TR2. Trio funds non-clinical studies of TR2 and TR2-A.
- **2014–2015:** The MHRA allows pilot studies of TR2 and TR2-A in healthy subjects without first doing specific toxicology studies, because animals, healthy subjects and patients had been exposed to TR2 during studies of netazepide, and because of its good safety profile. In healthy subjects, TR2 is as potent as netazepide, and TR2-A is a prodrug of TR2 and more bioavailable than similar formulations of TR2 and netazepide.
- **2014:** Trio submits an application for a patent for TR2, TR2-A and related compounds.
- **2015:** Trio applies to WHO for eclazepide as the INN for TR2-A.
- **Sep 2015:** Start of toxicology and safety pharmacology studies required to support fast-track development of TR2-A for treatment of patients with hypergastrinaemia and acid-related conditions. The target start-date for studies in healthy subjects is 2016. The target start date for studies in patients is mid 2017.
- **Nov 2015:** The European Patent Office deems Trio’s claim for TR2, TR2-A and related compounds to be novel, inventive and industrially applicable.
- **Nov 2015:** European Medicines Agency designates TR2-A an orphan medicinal product.

Acknowledgements

I have been the principal investigator for all of the studies of netazepide and its metabolites in healthy subjects since 1996, and I have managed the overall development programme since Trio licensed netazepide from Ferring Pharmaceuticals in 2006. I wrote or supervised the writing of the protocols, investigator's brochures, and applications to the research ethics committees and MHRA. More importantly, I interpreted the results of the studies, wrote the manuscripts for publication in peer-reviewed journals, and wrote this thesis. Also, through Trio I have sponsored several non-clinical studies of netazepide and its metabolites, and several studies in patients with gastric NETs or Barrett's oesophagus. I wrote or contributed to the writing of the protocols for the patient studies, and wrote or supervised the preparation of study reports for completed studies.

However, drug development is always a huge team effort by people with various different skills, so I readily acknowledge that many other people have contributed to the development of netazepide and its main metabolite TR2 during the long gestation of netazepide. First and foremost, I thank the healthy subjects and patients who volunteered for studies of netazepide, and my many HMR colleagues – physicians, nurses, pharmacists, data managers, statisticians, project managers, and support staff – who over the years have helped me to carry out the studies or have helped analyse the data that those studies generated. I could not have done the work without them. Second, I thank Ferring for agreeing to license netazepide to Trio, donating their remaining supply of netazepide, and providing company reports of non-clinical studies. Third, I am grateful to the subcontractors and academic collaborators whose services for non-clinical studies – such as pharmacology, toxicology, formulation, and assays – have played an essential role in the development of netazepide. In particular, I thank: Analytical Services International for assaying netazepide and its metabolites in samples from all the clinical pharmacology studies done since 1996; ProSynth for their enthusiasm and commitment towards synthesising and manufacturing TR2 and TR2-A for clinical studies; and Liverpool University for their studies of the effect of netazepide and TR2 and TR2-A on the trophic effects of gastrin. Fourth, I thank my Trio colleagues who have worked with various subcontractors and me to isolate, identify, synthesise, characterise and patent the metabolites of netazepide. Fifth, I thank my supervisors, Professors Mark Pritchard and Andrea Varro, for their support and for keeping me on schedule. Finally, I acknowledge and miss the unwavering passion and commitment of the late Jim Black towards the development of netazepide. I name some of the key individuals in the relevant chapters of this thesis. The names of others are just too many to mention.

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The work in chapters 3–12 is included as in-thesis *Supplementary Material* to provide contextual information only; those chapters are not part of the submission for the degree MD.

Abbreviations

µg	microgram
µmol	micromole
λz	apparent terminal phase rate-constant
°C	degrees Celsius
ALP	alkaline phosphatase
ALT	alanine transferase
AMS	accelerator mass spectrometry
API	active pharmaceutical ingredient
ATP4A	gene that control acids secretion by the parietal cell
AUC	area under the concentration-time curve
AUC _{0-∞}	AUC from zero time extrapolated to infinite time
AUC _{0-t}	AUC from zero time to the time of last quantifiable concentration
bd	twice daily
BLQ	below the limit of quantification
C _τ	trough plasma concentration
CAG	autoimmune chronic atrophic gastritis
CCK	cholecystokinin
CCK ₁	CCK ₁ receptor
CCK ₂	gastrin/CCK ₂ receptor
CCNa	croscarmellose sodium
CgA	chromogranin A
CI	confidence interval
CL/f	apparent total body clearance from plasma
CLC	eosinophil lysophospholipase (Charcot-Leyden crystal protein)
CLDN10	claudin 10
cm	centimetre
C _{max}	maximum observed concentration
CYP	cytochrome P450
ECG	electrocardiogram
ECL cells	enterochromaffin-like cells
ED ₅₀	median effective dose which produces 50% maximum effect
EGF	endothelial growth factor
EMA	European Medicines Agency
ENETS	European Neuroendocrine Tumour Society
ERP27	endoplasmic reticulum protein
F	female
FDA	USA Food and Drug Administration
g	gram
GEP-NENs	gastroentero-pancreatic neuroendocrine neoplasms
GIST	gastrointestinal stromal tumour
GORD	gastro-oesophageal reflux disease
GRA	gastrin receptor antagonist
h	hour
<i>H. pylori</i>	<i>Helicobacter pylori</i>

H ⁺	hydrogen ion
H ⁺ /K ⁺ ATPase	proton pump
H ₂ RA	histamine H ₂ -receptor antagonist
HDC	histidine decarboxylase
hERG	human ether-à-go-go related gene
HMR	Hammersmith Medicines Research
HPLC	high-performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
HR	heart rate
IC ₅₀	concentration of drug causing 50% inhibition of a response
IMPD	investigational medicinal product dossier
INN	international non-proprietary name
ip	intra-peritoneal
iv	intravenous
JBF	James Black Foundation
JLN	Jervell and Lange-Nielsen syndrome
KCNQ1	potassium channel gene that controls acid secretion by the parietal cell
K _d	dissociation constant
kg	kilogram
K _i	inhibition constant
Ki67	marker of cell proliferation
L	litre
LC-PDA-MS	liquid chromatography, photodiode array and mass spectrometry
LLQ	lower limit of quantification
m	metre or month, according to context
<i>m/z</i>	ratio of molecular or atomic mass number and charge number of the ion
MAOB	monoamine oxidase B
MEN-1	multiple endocrine neoplasia type 1
mg	milligram
MHRA	Medicines and Healthcare products Regulatory Agency
min	minute
miR-222	microRNA-222 – involved in initiation and progression of tumours
miRNA	microRNA – regulate expression of several hundred genes
mL	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger RNA – carries DNA code to other parts of the cell for synthesis of proteins from amino acids
MRT	mean residence time
MS	mass spectroscopy
NANETS	North American Neuroendocrine Tumour Society
NETs	neuroendocrine tumours
ng	nanogram
nM	nanomolar
NOAEL	no-observable-adverse-effect level

NOEL	no-observable-effect-level
NoMA	Norwegian Regulatory Agency
NSAID	non-steroidal anti-inflammatory drug
od	once daily
OMP	orphan medicinal product
p27	tumour suppressor and oncogene with potential to cause cancer
PAM	peptidyl-glycine alpha-amidating monooxygenase
PAPPA2	pappalysin 2
pH	the negative logarithm to base 10 of the hydrogen ion concentration
po	by mouth
PPI	proton pump inhibitor
PST	pancreastatin
QTc	QT interval of the ECG corrected for heart rate
REC	Research Ethics Committee
sc	subcutaneous
SCG2	secretogranin II
sd	standard deviation
sem	standard error of the mean
SST	somatostatin
t _{1/2}	half-life
T _{max}	first time of occurrence of C _{max} .
TR2	main metabolite of netazepide in humans
TR2-A	acetyl derivative and prodrug of TR2
UKINETS	United Kingdom and Ireland Neuroendocrine Tumour Society
USA	United States of America
VMAT2	vesicular monoamine transporter 2
V _{z/f}	apparent volume of distribution during the terminal phase
YF476	netazepide
ZES	Zollinger-Ellison syndrome

Abstract

In **non-clinical** studies, netazepide (YF476) is a potent, highly-selective and competitive gastrin/CCK₂ receptor antagonist (GRA), with good oral bioavailability.

In studies involving over **220 healthy subjects**, netazepide:

- at single doses, caused dose-dependent prolonged increases in gastric pH, but repeated doses led to tolerance;
- at single doses, caused dose-dependent inhibition of pentagastrin-induced increases in gastric aspirate volume and acid content, which persisted after repeated doses;
- at single doses, was as effective as the PPI rabeprazole at suppressing pentagastrin-stimulated gastric acid secretion and increasing serum gastrin; the combination was more effective than either treatment alone in suppressing gastric acid secretion;
- reduced plasma CgA – a sign of ECL-cell hypoactivity – whereas rabeprazole increased plasma CgA – a sign of ECL-cell hyperactivity; netazepide also prevented the increase in CgA resulting from rabeprazole-induced hypergastrinaemia;
- at low doses, prevented the increase in plasma CgA resulting from esomeprazole-induced hypergastrinaemia;
- had dose-proportional and linear pharmacokinetics;
- was metabolised mainly to its hydroxy metabolite, TR2, exposure to which was substantial and similar to that of netazepide; and
- was safe and well tolerated after single doses up to 400 mg and repeated doses up to 100 mg twice daily for up to 6 weeks.

In two studies in a total of **16 patients** with autoimmune chronic atrophic gastritis (CAG), achlorhydria, hypergastrinaemia, multiple gastric NETs, and raised circulating CgA, netazepide 50 mg once daily by mouth for 12 weeks:

- reduced the number of tumours and size of the largest one;
- normalised serum CgA, which returned to pre-treatment levels after stopping netazepide, but did not increase serum gastrin further, confirming that all patients had achlorhydria;
- normalised raised mRNA abundancies of gastrin-dependent biomarkers in tumour biopsies, which returned to pre-treatment levels after stopping netazepide; and
- suppressed miR-222 overexpression in biopsies of gastric NETs from CAG patients.

In an extension of the two studies, after an interval off treatment for a mean 14 (range 8–19) months in which tumours regrew and CgA increased significantly, 13 of the 16 patients took netazepide 25 or 50 mg once daily for another 52 weeks. Netazepide eradicated the tumours of 5 patients. One patient was left with one tumour. Netazepide reduced the number and size of the largest tumour in the other patients. The effect on increased CgA and gastrin was the same as in the 12-week studies. Netazepide was well tolerated.

In non-clinical studies, TR2, the main metabolite of netazepide in humans, was not quite as potent as netazepide as a GRA, but was more selective. In healthy subjects, however, TR2 was as potent as netazepide in suppressing pentagastrin-stimulated acid secretion.

Furthermore, in healthy subjects, TR2-A, the acetyl derivative of TR2, proved to be a prodrug of TR2 and more bioavailable than either TR2 or netazepide.

Chapter 1

**Physiology and pathology of gastrin,
gastrin/CCK₂ receptor antagonists, and animal models
of hypergastrinaemia**

Brief history of gastrin and its effect on drug development

Edkins, Head of Physiology, St Bartholomew's Hospital, London, first suggested the existence of 'gastrin' in 1905 when he showed that intravenous injection of pyloric mucosal extracts of stomach stimulated gastric secretion in cats (Edkins 2006); but it wasn't until many years later that the gastrins were isolated and their structure identified (Gregory *et al* 1964; Gregory and Tracy 1964). Meanwhile, Popielski (1920) had shown that histamine stimulates gastric acid secretion in dogs. The discovery by Black *et al* (1972) that the effect of histamine on acid secretion is mediated by histamine H₂ receptors led to the invention of selective histamine H₂-receptor antagonists (H₂RA) and subsequent launch of the first H₂RA, cimetidine, in 1976. Cimetidine and other H₂RAs, such as ranitidine, that followed provided for the first time an effective medical treatment for acid-related diseases. However, patients began to relapse despite continuing H₂RA treatment. Eventually it was realised that H₂RA-induced acid suppression results in an increase in serum gastrin and tolerance to H₂RA treatment (Wilder-Smith *et al* 1990). Pharmaceutical companies, such as Merck, Lilly, and Wyeth, became interested in researching gastrin/CCK₂ receptor antagonists (GRAs). Many were synthesised, but problems with potency, selectivity for the CCK₂ receptor, agonist activity, and oral bioavailability hampered their development. Some GRAs were tested in healthy subjects and patients, but none proved worthy of further development (McDonald 2001; Black and Kalindjian 2002; Herranz 2003; Black 2009).

Interest in GRAs waned when omeprazole and other inhibitors of H⁺, K⁺/ATPase, the proton pump on gastric parietal cells, were launched from 1988 onwards (Olbe *et al* 2003). Proton pump inhibitors (PPIs) proved superior to H₂RAs in healing acid-related conditions and, although PPIs also increase serum gastrin, tolerance does not develop because the proton pump is the final step in gastric acid production. The finding that *H. pylori* causes most cases of peptic ulcer disease (Marshall and Warren 1984), and can be eradicated by a short course of a PPI and a cocktail of antibiotics, was probably another reason for disinterest in researching and developing a GRA for clinical use. After *H. pylori* eradication had been introduced, the need for maintenance therapy for peptic ulcer disease was largely eliminated and gastro-oesophageal reflux disease (GORD) became the main indication for prolonged inhibition of gastric acid. Many millions of patients have taken a PPI since the first one, omeprazole, was marketed in the 1980s. Although in recent years there has been a resurgence of interest in the physiology and pathology of gastrin, especially its trophic effects on the stomach, pancreas and colon, there is still no GRA available for routine clinical use (Malfertheiner *et al* 2006), over a century since Edkin's discovery in 1905 (Modlin *et al* 1997).

Physiology of gastrin

Gastrin is a peptide hormone produced by a single gene and synthesised from a precursor peptide, progastrin, which is processed into progastrin and gastrin peptide fragments of various sizes by sequential enzymatic cleavage. All gastrins have a C-terminal amidated tetrapeptide (Trp-Met-Asp-Phe-NH₂), which acts at a specific G protein-coupled gastrin receptor (also called CCK₂ receptor; formerly known as CCK_B receptor) in the stomach and in the central and peripheral nervous systems. Cloning has shown that CCK₂ receptors in the brain and periphery are identical. The two main circulating forms of gastrin comprise 34 and 17 amino acids (G-34 and G-17, respectively) (Dockray *et al* 2001).

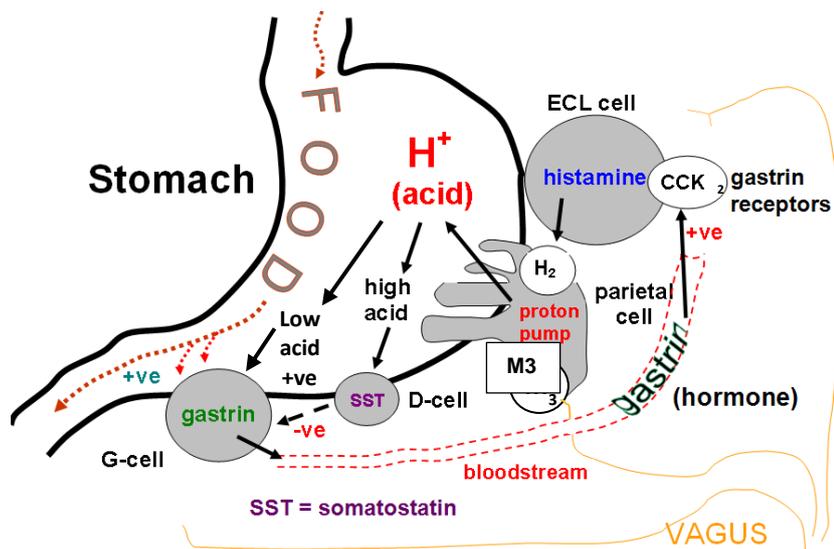
Gastrin is structurally and functionally related to another peptide hormone, cholecystokinin (CCK), which is also produced by a single gene and processed into several molecular forms by sequential enzymatic cleavage (Herranz 2003). The main forms in blood and tissue are CCK-58, CCK-33 and CCK-8. All fragments have at their carboxyl terminus the octapeptide Asp-Tyr(SO₃H)-Met-Gly-Trp-Met-Asp-PheNH₂, which stimulates specific G protein-coupled CCK₁ receptors on pancreatic acinar cells, gall bladder smooth muscle, vagal afferent neurons in the small intestine, and cells in the central nervous system. Thus, the C-terminal tetrapeptide (Trp-Met-Asp-Phe-NH₂) is identical to that of gastrin. The common C-terminal tetrapeptide has hindered research into antagonists selective for the CCK₁ or CCK₂ receptor, and has confounded assays for CCK, because antibodies to CCK may cross react with gastrin.

CCK is produced by I cells in the mucosa of the small intestine. I cells release CCK in response to dietary lipid and protein. CCK is also a neurotransmitter in the central nervous system and in the peripheral nerves innervating the small intestine. CCK stimulates gallbladder contraction and pancreatic enzyme secretion, delays gastric emptying, potentiates insulin secretion, and inhibits food intake (Dockray 2012).

Gastrin is produced by G cells in the gastric antrum. G cells release gastrin in response to food or the thought of food. Gastrin activates gastrin (CCK₂) receptors on enterochromaffin-like (ECL) cells in the gastric oxyntic mucosa to secrete histamine, which in turn stimulates adjacent parietal cells to secrete acid. Parietal cells also express gastrin receptors (Schmitz *et al* 2001), but their role in acid secretion is unclear. Acid secretion is mediated by the proton pump via histamine H₂ and muscarinic M₃ receptors on parietal cells (Aihara *et al* 2003). Somatostatin, a hormone produced by D cells in the stomach, switches off gastrin when intragastric pH falls (Schubert and Peura 2007). Gastric acid secretion is regulated by endocrine, paracrine, and neurocrine mechanisms via at least three signalling pathways: gastrin-histamine (stimulation), CCK₁/somatostatin (inhibition) and neural networks (both

stimulation and inhibition). Different pathways are suppressed or dominate, depending on the circumstances (Chen and Zhao 2010).

Figure 1: Control of gastric acid secretion



Gastrin also causes: proliferation, migration and differentiation of gastric epithelial cells; up-regulates various genes (Table 1) (Watson *et al* 2006; Dimaline and Varro 2007); and stimulates paracrine cascades (Almeida-Vega *et al* 2009), including cytokines, growth factors such as trefoil factor (Kahn *et al* 2003; Tu *et al* 2007), and prostanoids. Muscarinic M₃-receptor knock-out mice fail to develop the trophic response to hypergastrinaemia (Aihara *et al* 2003).

Table 1. Just some of the genes upregulated by gastrin (Watson *et al* 2006)

Gene	Function
Histidine decarboxylase	Acid production
Vesicular monoamine transporter 2	Acid production
Chromogranin A	Acid production
Trefoil family factor 1	Mucosal defence
Regenerating protein 1	Mucosal proliferation
Heparin-binding EGF	Mucosal proliferation
Matrix metalloproteinase family	Proteolysis
Plaminogen activator inhibitor 1 & 2	Proteolysis
Fibroblast growth factor	Cell migration
Ezrin	Parietal cell maturation

Causes and clinical effects of hypergastrinaemia

Circulating gastrin is increased by: hypoacidity due to autoimmune chronic atrophic gastritis (CAG) (Burkitt and Pritchard 2006) or *H. pylori*-induced gastritis (El-Omar *et al* 1997); a gastrinoma in patients with Zollinger-Ellison syndrome (ZES) (Ellison *et al* 2006); acid suppression by H₂RAs, PPIs (Lundell *et al* 2015), potassium-competitive acid inhibitors (Hori *et al* 2011) or vagotomy (Korman *et al* 1972); and mutations of the *KCNQ1* or *KCNE1* potassium channel gene (Rice *et al* 2011; Winbo *et al* 2012, 2013 and 2014) and the *ATP4A* gene (Calvete *et al* 2015), which control acid secretion by the parietal cell, and cysteamine treatment of cystinosis (Dohil *et al* 2010).

CAG hypergastrinaemia leads to ECL-cell hyperplasia and, in some patients, development of gastric neuroendocrine tumours (NETs; formerly known as gastric carcinoids), which are mostly benign but can metastasise. Patients with pernicious anaemia, which is one of the possible clinical presentations of atrophic gastritis, have a nearly seven-fold increased risk of gastric cancer (Vannella *et al* 2013). Patients with hypergastrinaemia caused by genetic mutations of *KCNQ1*, *KCNE1* or *ATP4A* not only develop gastric NETs, but also gastric adenocarcinoma (Rice *et al* 2011; Winbo *et al* 2012, 2013 and 2014; Calvete *et al* 2015).

ZES hypergastrinaemia causes hyperacidity, peptic ulceration and gastric NETs, which have greater potential for malignancy, especially in patients with multiple endocrine neoplasia type 1 (Jensen and Fraker 1994).

H. pylori infection is a major risk factor for peptic ulcer disease (Atherton 2006) and gastric cancer (Helicobacter and Cancer Collaborative Group 2001).

PPI-induced hypergastrinaemia is associated with ECL-cell (Rindi *et al* 2005) and parietal-cell (Stolte *et al* 2000) hyperplasia, fundic gland polyps (Jalving *et al* 2006), increased risk of bone fractures (Yang *et al* 2006), and possibly malignant ECL-cell tumours in rare patients (Jianu *et al* 2012). PPI withdrawal may lead to rebound hyperacidity (Hunfield *et al* 2007) and dyspepsia (Reimer *et al* 2009; Niklasson *et al* 2010).

Gastrin/CCK₂ receptors are also expressed on cells of some patients with pancreatic (Black 2009), colonic (Watson *et al* 2002) and gastric cancer (Goetze *et al* 2013), and Barrett's oesophagus (Haigh *et al* 2003). PPI-induced hypergastrinaemia is associated with advanced neoplasia in Barrett's oesophagus (Wang *et al* 2010).

Thus, there are several potential clinical indications for a gastrin/CCK₂ receptor antagonist.

Classification of gastric neuroendocrine tumours

Gastric neuroendocrine tumours (gastric NETs) are a distinct subtype of the gastroentero-pancreatic neuroendocrine neoplasms (GEP-NENs). Most gastric NETs are gastrin-dependent. GEP-NENs are composed of cells with a neuroendocrine phenotype but because of their rarity their classification has always proved difficult to standardise. However, WHO classified GEP-NENs in 2010 (Rindi *et al* 2010; Klöppel 2011) as follows. First, ‘neuroendocrine’ is now an official label for neoplastic cells expressing neural markers, such as CgA. Second, the term ‘NEN’ encompasses all neuroendocrine tumours, whether they are well or poorly differentiated. Third, all well-differentiated NENs, regardless of whether they behave benignly or develop metastases, are called neuroendocrine tumours (NETs), and graded G1 (Ki67 <2%, equivalent to carcinoids) or G2 (Ki67 2–20%). Ki67 is a marker of cell proliferation. All poorly differentiated neoplasms are termed neuroendocrine carcinomas (NECs) and graded G3 (Ki67 >20%). Subsequently, in an attempt to standardise the diagnosis and treatment of GEP-NENs within Europe, the European Neuroendocrine Tumour Society (ENETS) developed guidelines (Delle Fave *et al* 2012; Salazar *et al* 2012) based on the TNM system (T = size of the primary tumour and whether it has invaded nearby tissue, N = regional lymph node involvement, and M = distant metastases). However, there wasn’t always consensus among the many contributors to the guidelines. The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) have also proposed a TNM classification system for GEP-NENs (Klöppel *et al* 2010).

The stomach gives origin to three distinct types (Table 2) of well-differentiated NET (Rindi *et al* 1993; Scherübl *et al* 2010) and also, but only rarely, to poorly differentiated NECs (Klöppel and Clemens 1996). Gastric NETs were formerly known as gastric carcinoids. In this thesis, the two terms are synonymous.

Table 2. Main characteristics of gastric NETs (Delle Fave *et al* 2012)

Features	Type 1	Type 2	Type 3
Proportion, %	70–80	5–6	14–25
Characteristics	Small (<1–2 cm); 65% multiple; 78% polypoid	Small (<1–2 cm); multiple; polypoid	Unique; large (<2 cm); polypoid and ulcerated
Associated conditions	Chronic atrophic gastritis Pernicious anaemia	Gastrinoma/MEN-1	None
Pathology	Often G1	G1–G2	G3
Serum gastrin	↑	↑	Normal
Gastric pH	↑↑	↓↓	Normal
Metastases, %	2–5	10–30	50–100

Type 1 gastric NETs

About 5% of patients with autoimmune chronic atrophic gastritis (CAG) develop type 1 gastric NETs. They are more common in women. CAG is characterised by chronic inflammation of the gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium, pyloric-type glands and fibrous tissue. That causes oxyntic mucosal atrophy, loss of parietal and chief cells, and achlorhydria. Achlorhydria stimulates G-cell hyperplasia, which results in hypergastrinaemia. Hypergastrinaemia causes growth of enterochromaffin-like (ECL) cells in the gastric corpus that possess gastrin/CCK₂ receptors (Dakin *et al* 2006). Diffuse to micronodular ECL-cell hyperplasia develops and is followed by multiple ECL neoplasms after a latent period of many years (Bordi *et al* 1997). CAG is associated with serum antibodies to parietal cells and intrinsic factor, and is regarded as an autoimmune disease. Intrinsic factor deficiency causes vitamin B-12 malabsorption. Eventually, some patients develop pernicious anaemia (PA) with haematological, gastrointestinal and neurological complications. Patients with PA also have up to a seven-fold increase in gastric adenocarcinoma (Borch 1989; Vannella *et al* 2013), for which hypergastrinaemia might be at least a contributing factor.

The prognosis of type 1 gastric NETs in CAG patients is good, because they are usually G1 grade NETs. However, all type 1 tumours have the potential for malignancy, especially ones that are >2 cm in size, infiltrate the muscularis propria, are angioinvasive and/or are G2 grade (Rappel *et al* 1995).

There have been recent reports of several gene mutations giving rise to gastric NETs. First, Winbo *et al* (2013) reported that patients with the rare Jervell and Lange-Nielsen (JLN) syndrome (congenital hearing loss, long QT interval and high risk of ventricular tachyarrhythmias) also have iron deficiency anaemia, gastric hyperplasia and hypergastrinaemia secondary to hypochlorhydria caused by a mutation of the *KCNQ1* or *KCNE1* potassium channel gene, which is essential for gastric acid secretion. Some patients had gastric carcinoma. Rice *et al* (2011) reported a patient with JLN syndrome who had achlorhydria, hypergastrinaemia and multiple ECL tumours, so the gastric hyperplasia reported by Winbo *et al* (2013) probably represents type 1 NETs too. Furthermore, hypergastrinaemia in such patients appears to be a risk factor for subsequent gastric carcinoma. Winbo *et al* (2014) estimated the mutation-carrier prevalence in Sweden to be about 1:2000-4000. Second, exome sequencing of a family with consanguineous parents and ten children, five of whom had gastric NETs, identified a mutation of the *ATP4A* gene (Calvete *et al* 2015), which encodes the proton pump responsible for acid secretion by gastric

parietal cells. All five children had hypergastrinaemia and three had nodal infiltration, one of whom had gastric adenocarcinoma. All five also had iron deficiency anaemia. These genetic mutations provide strong evidence that hypergastrinaemia has malignant potential.

Type 2 gastric NETs

Type 2 gastric NETs occur in patients with Zollinger-Ellison syndrome (ZES), a condition in which one or more gastrinoma – a gastrin-secreting tumour, usually in the duodenum or pancreas – results in hypergastrinaemia (Ellison *et al* 2006). That causes hyperplasia and hypersecretion of the acid-secreting cells of the stomach, severe peptic ulcer disease, and ECL-cell hyperplasia, which can lead to type 2 gastric NETs (Pellicano *et al* 2006).

Of 106 patients with a gastrinoma (Ellison *et al* 2006), 80 had a sporadic tumour and 26 had a mutation in the multiple endocrine neoplasia gene (MEN-1). Patients with ZES and MEN-1 have a 20–30 fold higher chance of developing a gastric NET than patients with sporadic ZES (Jensen and Fraker 1994). Up to 20% of patients with ZES and MEN-1 develop type 2 gastric NETs. MEN-1 is an autosomal dominant mutation in the gene that codes for menin, a protein that acts as a tumour suppressor. Although hypergastrinaemia is the primary initiator for type 2 NETs, cofactors such as MEN-1 are also required for their development (Richards *et al* 2004). Loss of heterozygosity for MEN-1 is also found in many type 1 and type 2 gastric NETs (D’Adda *et al* 1999). Patients with ZES suffer mainly from the effects of severe ulcer disease – dyspepsia, perforation, bleeding, diarrhoea and weight loss (Roy *et al* 2000).

Gastrinomas and type 2 gastric NETs both have the potential to metastasise.

Type 3 gastric NETs

Type 3 gastric NETs are solitary tumours that develop unrelated to CAG or MEN-1. They are not associated with hypergastrinaemia and occur mainly in men, at a mean age of 55 years (Scherübl *et al* 2010). In most cases type 3 NETs are composed of ECL cells, while EC (serotonin) cell or gastrin-cell tumours are extremely rare (Klöppel and Clemens 1996). Histologically, they are well differentiated, show a trabecular to solid pattern, and in at least one-third of the patients the tumour is already larger than 2 cm at the time of diagnosis, has invaded the muscular layer, shows angioinvasion, and/or has a proliferation rate exceeding 2–5%. In those type 3 NETs, metastases are very likely to be present (Rappel *et al* 1995). In rare cases, type 3 tumours may be associated with a so-called atypical carcinoid syndrome, characterised by cutaneous flushing in the absence of diarrhoea, usually coupled with liver metastases and production of histamine and 5-hydroxytryptophan (Scherübl *et al* 2010).

Poorly differentiated NECs of the stomach (type 4 gastric NENs) are more common in men than in women, aged between 60 and 70 years (Scherübl *et al* 2010). They present as a large

ulcerated lump with symptoms similar to those of adenocarcinomas. Occasionally they harbour an adenocarcinoma component. Hormones cannot be demonstrated and there is no relationship to chronic atrophic gastritis, but in exceptional cases they are associated with MEN-1 (Bordi *et al* 1997). At the time of diagnosis, most of the tumours are already in an advanced stage (tumour diameter >4 cm) and show extensive metastases (Bordi *et al* 1997). Ooi *et al* (1995) and Abraham *et al* (2005) reported multiple large (up to 1.3 cm) ECL-cell tumours in a background of ECL-cell and parietal-cell hyperplasia in patients with hypergastrinaemia, but without ZES. These NETs were thought to result from an abnormality of acid secretion by the parietal cells.

Gastrin/CCK₂ receptor antagonists

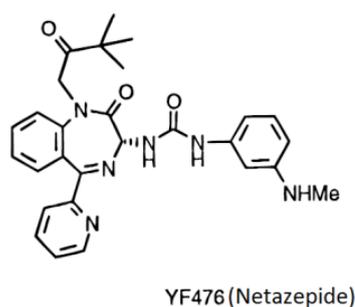
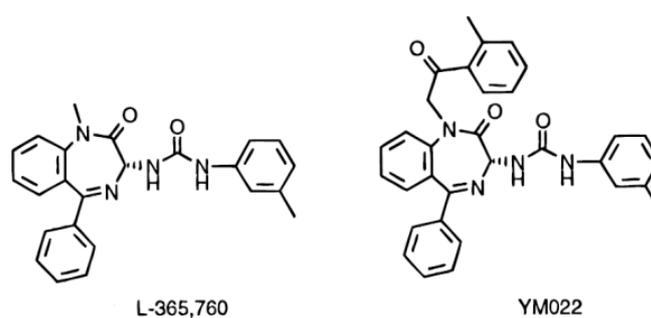
After the discovery in the 1980s that the benzodiazepine-related natural product asperlicin has weak affinity for the CCK₁ receptor, pharmaceutical companies started to search for potent, selective and orally-active benzodiazepine-derived CCK₁ receptor antagonists and CCK₂ receptor antagonists (McDonald 2001; Black and Kalindjian 2002; Herranz 2003).

Combining the elements of diazepam and D-tryptophan produced L-364,718 (devazepide), which was orally-active, had subnanomolar affinity at the CCK₁ receptor and high selectivity compared with the CCK₂ receptor, and lacked agonist activity. In healthy humans, devazepide inhibited CCK-induced gallbladder contraction, and stimulated gastric motility and gastric emptying after food. However, development of devazepide was stopped because it caused gallstones (Iversen *et al* 1991).

Replacing the indol-2-yl-amide of devazepide with an aryl urea moiety at C3 of the 1,4-benzodiazepine, with (*R*) stereochemistry at that position, yielded L-365,260, the first 1,4-benzodiazepine-derived CCK₂ antagonist to possess nanomolar affinity at CCK₂ receptors and reasonable selectivity (140-fold) versus the CCK₁ receptor (Lotti and Chang 1989). Oral L-365,260 was a potent inhibitor of gastrin-stimulated acid secretion in several animal species, with good duration of action. But, it produced only modest and short lasting inhibition of gastrin-stimulated acid secretion in healthy men (Murphy *et al* 1993), and was ineffective in limiting panic attacks in patients (Kramer *et al* 1995). Those results were attributed to its low aqueous solubility and poor oral bioavailability. L-365,260, and related 1,4-benzodiazepine-derived CCK₂ antagonists with better solubility and oral bioavailability, were developed but had the potential to provoke arrhythmias, and were discontinued (Selnick *et al* 1997).

YM022 was the result of research into introducing bulky substituents at N-1 of 1,4-benzodiazepine-based CCK₂ receptor antagonists, to increase receptor affinity (Nishida *et al* 1995). YM022 showed subnanomolar affinity at rat brain CCK₂ receptors, which was more than two orders of magnitude higher than that for rat pancreatic CCK₁ receptors. In rats, intravenous YM022 was a potent inhibitor of gastrin-stimulated gastric acid secretion, and oral YM022 prevented the rebound hyperacidity that occurs after stopping a proton pump inhibitor. However, YM022, like other 1,4-benzodiazepines with bulky substituents at N-1, had low aqueous solubility and had to be formulated as a solid dispersion to achieve adequate oral bioavailability (Yano *et al* 1996). There are no reports of studies of YM022 in humans.

Attempts were made to increase aqueous solubility and oral bioavailability of 1,4-benzodiazepine-derived CCK₂ antagonists by introducing basic groups and either replacing the 5-phenyl group with a 2-pyridyl substituent or replacing the 3-methyl group of the aryl urea moiety with a methylamino group. That resulted in YF476 (netazepide), which had binding affinity at CCK₂ receptors similar to YM022, but 5-fold higher selectivity at CCK₁ receptors (Semple *et al* 1997). Netazepide caused potent inhibition of gastrin-stimulated acid secretion in rats and dogs (Takinami *et al* 1997), and prevented gastric mucosal cell proliferation in rats caused by hypergastrinaemia induced by a proton pump inhibitor (Takemoto *et al* 1998). Duration of antagonism was long after intravenous (Kitano *et al* 2000) or oral (Semple *et al* 1997) administration. Comparison of the results of antagonism after oral and intravenous dosing indicated good oral bioavailability, despite netazepide having poor aqueous solubility. The structure of netazepide and two earlier gastrin/CCK₂ receptor antagonists is shown below.



Animal models of hypergastrinaemia

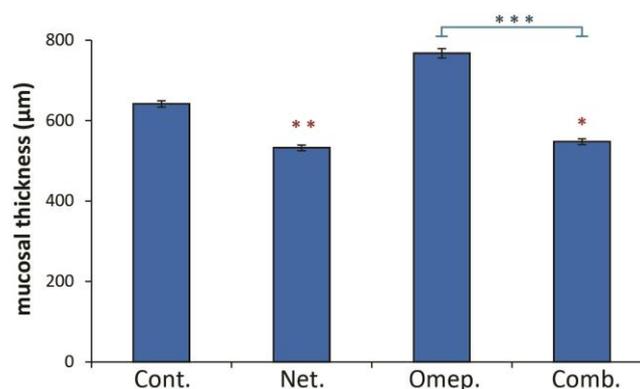
Many animal models have been developed to assess the effect of netazepide and other gastrin/CCK₂ receptor antagonists on the responses to hypergastrinaemia.

PPI-induced hypergastrinaemia in rats

Ding *et al* (1997) assessed the effects in rats of short intravenous infusions of three GRAs on ECL cells stimulated by intravenous gastrin or PPI-induced hypergastrinaemia. Netazepide, which was the most potent compound, caused dose-dependent antagonism of gastrin-evoked HDC activation and blocked the PPI-induced HDC activation and the gastrin- and PPI-induced rise in serum PST, the derivative of chromogranin A (CgA).

Chen *et al* (2000) gave rats netazepide for eight weeks to assess its effect on normal ECL cells and on ECL cells exposed to omeprazole-induced hypergastrinaemia. The outcome measures were ECL-cell morphology, which was assessed by immuno-cytochemistry and electron microscopy, and measurements of serum PST and oxyntic mucosal PST and HDC activity. Netazepide had little if any effect on the density of normal ECL cells but transformed them from slender, elongated cells with prominent projections to small, spherical cells without projections. The Golgi complex, rough endoplasmic reticulum and secretory granules were reduced in size. Serum PST and oxyntic mucosal HDC activity were lowered within hours. Netazepide prevented the effect of omeprazole-induced hypergastrinaemia on ECL-cell activity and density. Omeprazole increased the thickness of the oxyntic mucosa whereas netazepide reduced it, and prevented the increase induced by omeprazole (Figure 3).

Figure 3. Oxyntic mucosal thickness in rats (n = 8) treated for 8 weeks with netazepide and omeprazole, alone and in combination (redrawn after Chen *et al* 2000)

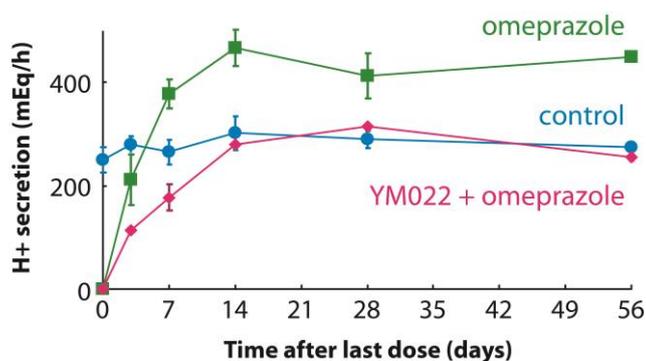


Rebound hyperacidity in rats after stopping a PPI, and its prevention by a GRA

Carlsson *et al* (1986) first showed that omeprazole-induced hypergastrinaemia in rats was followed by increased acid-producing capacity of the gastric mucosa one week after stopping omeprazole. The effect lasted for at least 70 days (Larsson *et al* 1988).

Nishida *et al* (1995) dosed rats for 13 weeks with the gastrin/CCK₂ receptor antagonist, YM022, and the PPI, omeprazole, alone and in combination, and then stopped treatment and assessed the effects for up to 56 days afterwards. 2 hours after the last dose, YM022 and omeprazole each inhibited basal and pentagastrin-induced H⁺ secretion, and increased plasma gastrin. 14 days after stopping omeprazole, the H⁺ responsiveness to pentagastrin was increased compared with control. It lasted at least 56 days and was accompanied by an increase in mucosal cell mass. In contrast, YM022 not only caused long-lasting inhibition of pentagastrin-induced H⁺ secretion but also prevented the hyper-responsiveness to pentagastrin after stopping omeprazole (Figure 4).

Figure 4. Pentagastrin-induced H⁺ secretion in fistula rats after 13-weeks of omeprazole, with and without YM022 (redrawn after Nishida *et al* 1995)



***H. pylori* infection in gerbils**

Netazepide prevented the inflammatory response of the gastric mucosa to *H. pylori* infection in gerbils (Sørdal *et al* 2013).

Barrett's oesophagus in mice

Netazepide prevented hypergastrinaemia-induced lesions in mice similar to those of patients with Barrett's oesophagus (Quante *et al* 2012).

H⁺/K⁺ATPase knock-out mice

Netazepide partly prevented bone loss and deterioration of bone quality in H⁺/K⁺ATPase deficient mice with hypergastrinaemia (Fossmark *et al* 2012; Aasarød *et al* 2015)

Female cotton rats with ECL-cell carcinomas

Netazepide reduced substantially the incidence of ECL-cell carcinomas in female cotton rats that develop such tumours as a result of hypergastrinaemia secondary to gastric hypoacidity (Martinsen *et al* 2003).

Mastomys rodents

Netazepide not only prevented formation of gastric carcinoids accelerated by hypergastrinaemia induced by loxitidine, an insurmountable H₂RA, in Mastomys rodents,

which have a genetic predisposition to such tumours, but also caused regression of formed lesions (Kidd *et al* 2010).

Rats with gastric cancer

Netazepide inhibited gastrin-induced apoptosis in rats with gastric cancer (Cui *et al* 2006). A combination of netazepide and loxidine had synergistic inhibitory effects on development of gastric atrophy and cancer in *H. felis* infected INS-GAS mice, while a proton pump inhibitor had no effects (Takaishi *et al* 2005).

Transgenic mice infected with *H. felis*

miR-222 was overexpressed in the gastric mucosa of transgenic mice with hypergastrinaemia and infected with *H. felis* (Lloyd *et al* 2015). miR-222 targets the tumour suppressor and oncogene p27, which increases cell proliferation, migration and angiogenesis.

NSAID-induced gastric ulcers in rats

Although NSAID-induced gastric ulcers are not associated with hypergastrinaemia, netazepide prevented them as effectively as omeprazole (Webb *et al* 2012).

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Chapter 2

Netazepide: review of non-clinical studies

1. Introduction

ICH Guideline M3 sets out the non-clinical studies of an investigational medicinal product (IMP) that must be done before clinical studies can start. In 1996, when we did the first studies of netazepide in healthy subjects for Ferring (Chapter 3), the following non-clinical studies had been completed: pharmacology; pharmacokinetics; safety pharmacology; toxicity in rats and dogs; embryo-foetal toxicity in rabbits; and genotoxicity. When Trio licensed netazepide from Ferring in 2006, the MHRA asked for additional studies to be done before we could restart clinical studies, because the requirements had changed (ICH M3. R2). The additional studies were: comparative metabolism in rat, dog and human hepatocytes *in vitro*, to confirm that rat and dog are appropriate species for toxicity studies of netazepide; protein binding; hERG channel; ECG intervals; and CYP450 enzymes. Most of those studies were done by Huntington Life Sciences (HLS), Cambridgeshire, England.

This chapter reviews the non-clinical studies published by Ferring and Yamanouchi (Semple *et al* 1997; Takinami *et al* 1997; Takemoto *et al* 1998) and described in unpublished HLS reports and the Investigator's Brochure (2015).

2. Pharmacology

(a) Binding to brain gastrin/CCK₂ and pancreas CCK₁ receptors in rats

Netazepide caused stereoselective and concentration-dependent inhibition of [¹²⁵I]CCK-8 binding to CCK₂ receptors in rat brain, with a K_i of 0.068 nM. When compared with other CCK₂ antagonists, the affinity of netazepide was similar to that of YM022, and 264- and 70-fold higher than that of L-365,260 and CI-988, respectively. K_i of netazepide for the rat pancreatic CCK₁ receptor was 280 nM, giving 4,100-fold greater selectivity for the CCK₂ receptor than the CCK₁ receptor (Semple *et al* 1997).

(b) Binding to cloned canine and human gastrin/CCK₂ receptors

Netazepide caused concentration-dependent inhibition of [¹²⁵I]CCK-8 binding to cloned dog and human gastrin/CCK₂ receptors, with K_i values of 0.62 and 0.19 nM, respectively. The affinity of netazepide for dog gastrin/CCK₂ receptors was 97-fold higher than that of L-365,260 and 14-fold lower than that of YM022. The affinity of netazepide for human gastrin/CCK₂ receptors was 45-fold higher than that of L-365,260, and 4-fold lower than that of YM022 (Semple *et al* 1997).

(c) Pentagastrin-stimulated gastric acid secretion in rats

In anaesthetised rats, intravenous netazepide (0.003–0.1 µmol/kg) inhibited pentagastrin-induced gastric acid secretion, with an ED₅₀ of 0.0086 µmol/kg. Pentagastrin-induced acid

secretion was also suppressed by an intravenous H₂RA (famotidine 0.03–0.3 μmol/kg), with an ED₅₀ of 0.13 μmol/kg. Thus, netazepide was 15 times more potent than famotidine.

(d) Pentagastrin-stimulated gastric acid secretion in Heidenhain pouch dogs

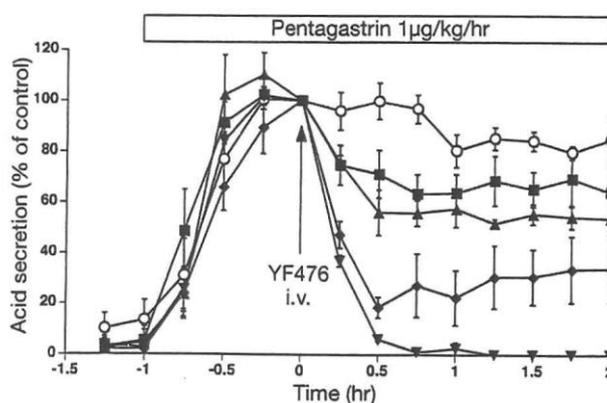
Intravenous netazepide (0.01–0.03 μmol/kg) and famotidine (0.03–0.3 μmol/kg) dose-dependently inhibited pentagastrin-induced gastric acid secretion in Heidenhain pouch dogs, with an ED₅₀ of 0.018 μmol/kg and 0.078 μmol/kg, respectively. Thus, netazepide was four times more potent than famotidine. Oral netazepide (0.01–0.1 μmol/kg) and famotidine (0.1–1 μmol/kg) also dose-dependently inhibited pentagastrin-induced gastric acid secretion in Heidenhain pouch dogs, with an ED₅₀ of 0.020 μmol/kg and 0.092 μmol/kg, respectively. Thus, netazepide was 5 times more potent than famotidine.

(e) Pentagastrin-induced gastric acid secretion in dogs with a gastric fistula

Intravenous netazepide (0.001–0.03 μmol/kg) dose-dependently inhibited pentagastrin-induced gastric acid secretion in beagle dogs with a gastric fistula (Figure 1). ED₅₀ was 0.0023 μmol/kg. The highest dose almost completely inhibited gastric acid secretion.

Figure 1. Effect of intravenous netazepide on pentagastrin-induced gastric acid secretion in dogs with a gastric fistula (Takemoto *et al* 1998)

Netazepide (■ 0.001, ▲ 0.003, ● 0.01, ▼ 0.03 μmol/kg) and vehicle (○) were given intravenously 1 h after the start of pentagastrin infusion. Means ± SEM, n = 4 or 5.

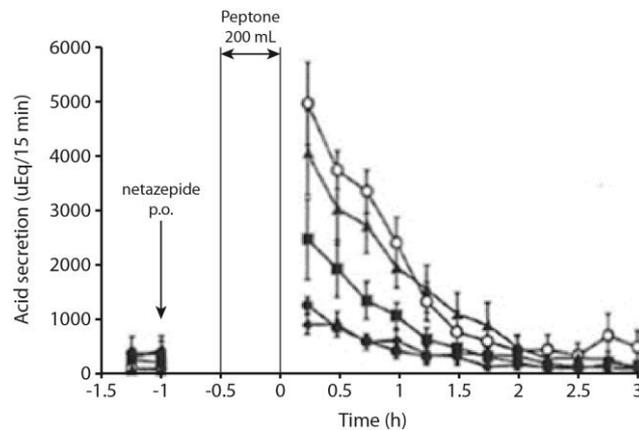


(f) Peptone-induced gastric acid secretion in dogs with a gastric fistula

Oral netazepide (0.03–1 μmol/kg), famotidine (0.3–10 μmol/kg) and omeprazole (3–30 μmol/kg) dose-dependently inhibited peptone-induced gastric acid secretion with an ED₅₀ of 0.11, 0.76 and 4.28 μmol/kg, respectively (Figure 2). The maximum inhibition by netazepide was 74.8% for the 0.3 μmol/kg. In contrast, famotidine and omeprazole almost completely inhibited acid secretion at the highest doses used.

Figure 2. Effect of oral netazepide on peptone (200 ml of 8 %)-induced gastric acid secretion in dogs with a gastric fistula (Takemoto *et al* 1998)

Netazepide (\blacktriangle 0.03, \blacksquare 0.1, \bullet 0.3, \blacktriangledown 1 $\mu\text{mol/kg}$) and placebo (\circ) were given 30 min before peptone introduction. Means \pm sem of 15 min epochs. n = 5 animals



(g) Histamine- and bethanecol-induced acid secretion in anaesthetised rats

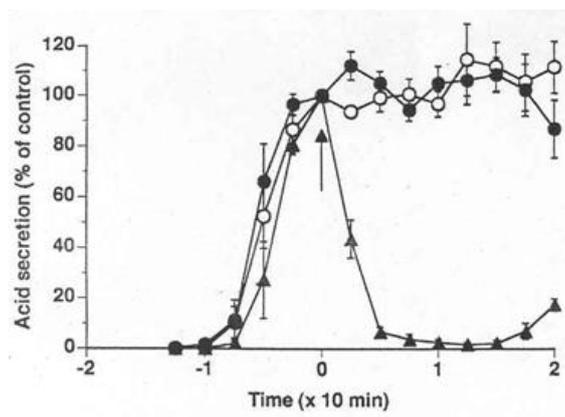
Intravenous netazepide (10 $\mu\text{mol/kg}$) affected neither histamine- nor bethanecol-induced gastric acid secretion in anaesthetised rats.

(h) Histamine-induced gastric acid secretion in Heidenhain pouch dogs

Intravenous netazepide (0.3 $\mu\text{mol/kg}$) did not affect histamine-induced acid secretion in Heidenhain pouch dogs, whereas famotidine (0.3 $\mu\text{mol/kg}$) completely inhibited it (Fig 3).

Figure 3. Effect of netazepide and famotidine on histamine (40 $\mu\text{g/h/kg}$) induced gastric acid secretion in Heidenhain pouch dogs (Takinami *et al* 1997)

Netazepide (\bullet 0.3 $\mu\text{mol/kg}$), famotidine (\blacktriangle 0.3 $\mu\text{mol/kg}$) and vehicle (\circ) were injected intravenously 1 h after histamine infusion. Mean \pm sem. n = 4 animals.



(i) Histamine-induced gastric acid secretion in dogs with a gastric fistula

Intravenous netazepide (0.3 $\mu\text{mol/kg}$) – a dose that completely inhibited pentagastrin-induced gastric acid secretion – had no effect on histamine-induced gastric acid secretion.

(j) Effect of netazepide on HDC activity and gastrin-stimulated acid in rats

A single, large subcutaneous injection of netazepide (300 $\mu\text{mol/kg}$) inhibited histidine decarboxylase (HDC) activity in ECL cells of rats for 8 weeks, and inhibited gastrin-stimulated gastric acid secretion in fistula rats for at least 4 weeks (Kitano *et al* 2000).

(k) Effect of repeated doses of netazepide on gastric acid secretion in rats

Hitherto, only single doses of netazepide had been studied in animals. When our studies in healthy subjects showed that repeated doses (Studies 3 and 4) resulted in partial tolerance to the increase in ambulatory 24-h gastric pH seen after single doses (Study 2), Yamanouchi (unpublished) did the following repeated-dose studies in rats. Netazepide caused persistent inhibition of basal and pentagastrin-stimulated gastric acid secretion and increased serum gastrin. Serum netazepide was constant throughout dosing. Thus, antagonism of gastrin/CCK₂ receptors persists after repeated doses. The increase in serum gastrin is secondary to hypoacidity and does not lead to tolerance.

(a) Effect of 14 days' netazepide on basal gastric acid production and serum gastrin

Netazepide (0.1 or 1 mg/kg) or saline was given to rats subcutaneously (sc) twice daily for 14 days to assess the effect on basal acid secretion and serum gastrin concentrations at 0, 2 and 24 h after the 1 mg/kg dose on Days 1, 7 and 14. On Day 1, netazepide caused dose-dependent inhibition of basal gastric acid secretion (Figure 4) and an increase in serum gastrin, which persisted for 14 days (Figure 5).

Figure 4. Effect of netazepide (YF476 0.1 & 1.0 mg/kg sc) and saline for 14 days on basal acid secretion in anaesthetised rats. Means \pm sem. n = 7 or 8.

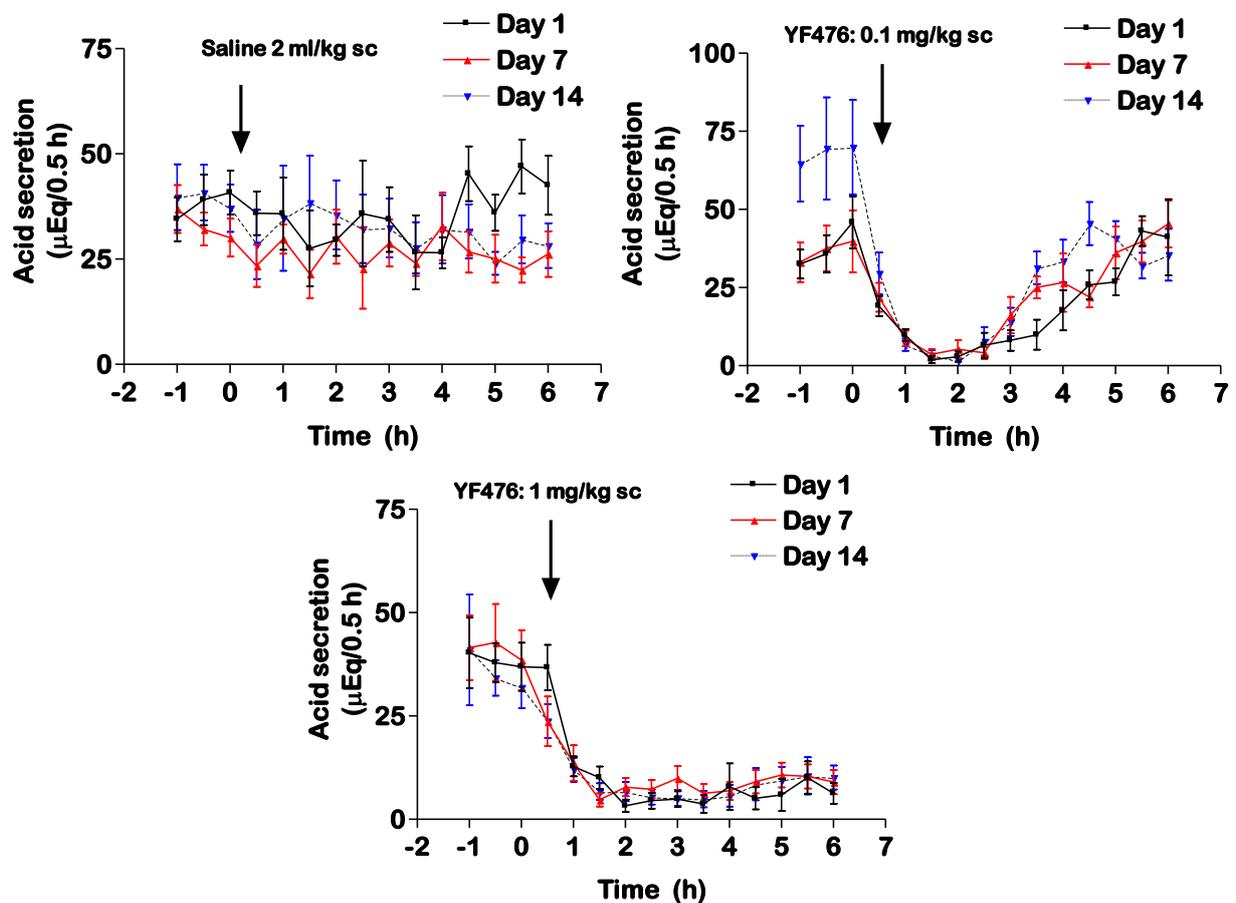


Figure 5. Effect of netazepide (YF476, 1 mg/kg sc) for 14 days on serum gastrin
 Mean \pm sem. n = 5 or 6 rats. *p<0.05 and **p<0.01 compared with saline

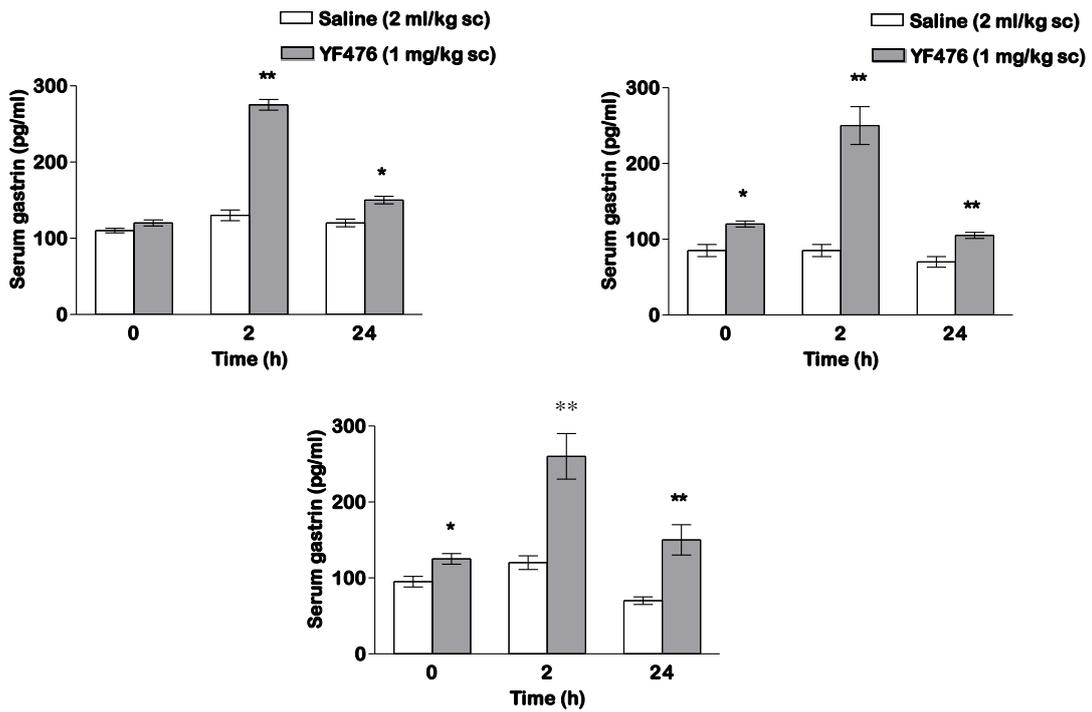
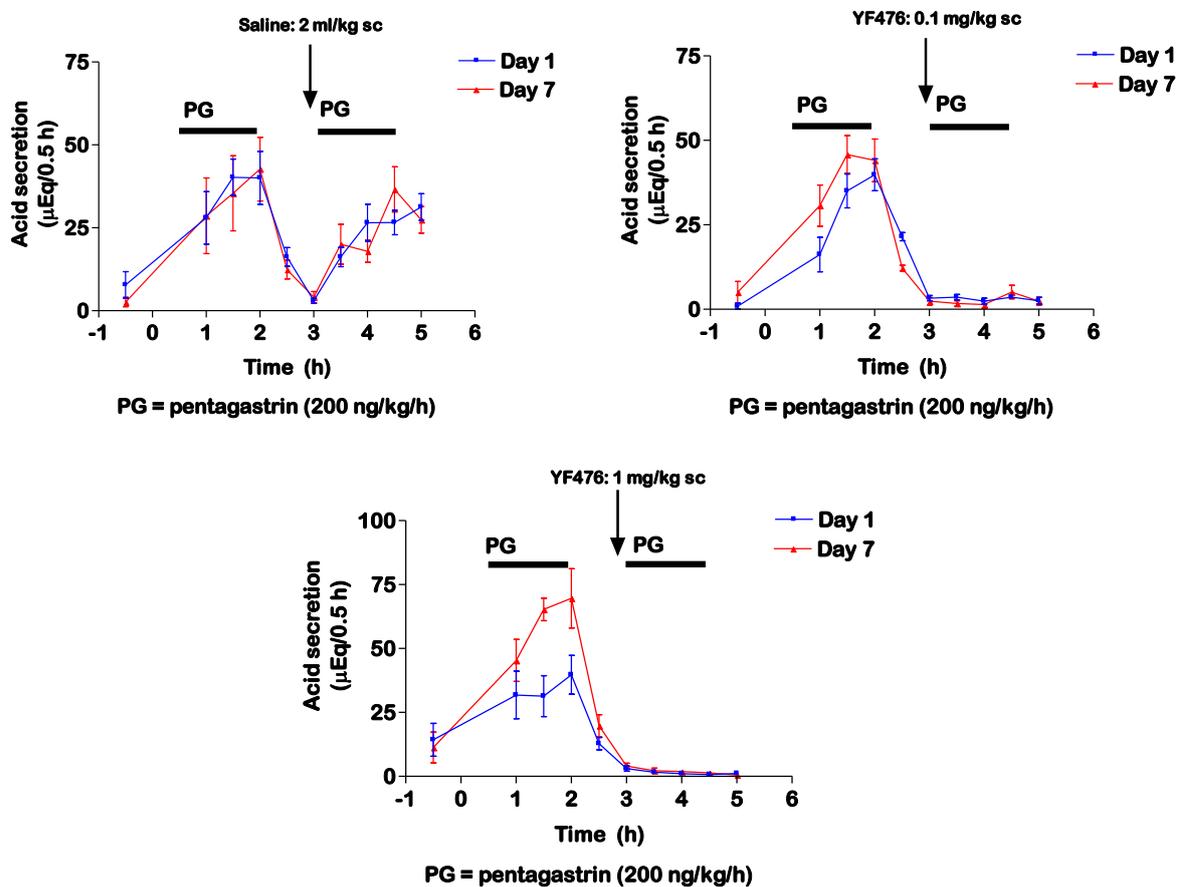


Figure 6. Effect of netazepide (YF476, 0.1 & 1.0 mg/kg sc) and saline for 7 days on pentagastrin (200 ng/kg/h) induced gastric acid secretion in rats
 Means \pm sem. n = 6 or 7



(b) Effect of 7 days' netazepide on pentagastrin-stimulated gastric acid secretion

Compared with saline, netazepide 0.1 and 1 mg/kg s.c. twice daily for 7 days abolished the response to pentagastrin (PG, 200 ng/kg/h) on Days 1 and 7 (Figure 6).

3. Safety pharmacology

(a) General behaviour in mice

The behaviour of mice (3 per dose level) was assessed according to the checklist of Irwin for 6 h and at 24 h after oral netazepide 0.1, 1 and 10 mg/kg. Items included: awareness, mood, motor activity, central nervous system excitation, posture, motor co-ordination, muscle tone, reflexes and autonomic profile. Netazepide did not affect general behaviour.

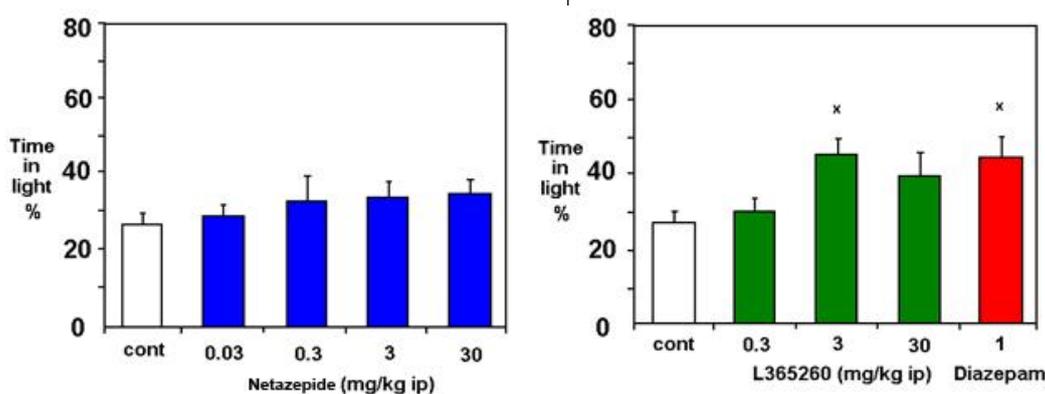
(b) Respiratory and cardiovascular system in dogs

Netazepide 0.1, 0.3, 1.0 and 3.0 mg/kg or saline was given into a femoral vein of pentobarbital-anaesthetised dogs at 10 min intervals. Netazepide affected neither the respiratory rate, blood pressure, heart rate, left ventricular pressure, maximum dp/dt, carotid arterial blood flow, femoral arterial blood flow nor ECG.

(c) Anxiolytic activity in mice

Netazepide (0.03–30 mg/kg), L-365,260 (3 mg/kg), diazepam (1 mg/kg) and vehicle were given intraperitoneally to groups of 10 mice. 30 min later, they were put in a box with light and dark compartments. Exploratory behaviour was observed for 5 min and the percentage time in the light compartment was calculated. Netazepide did not affect the time that animals stayed in the light compartment, whereas L-365,260 (a gastrin/CCK₂ receptor antagonist) and diazepam (a benzodiazepine anxiolytic) both increased it (Figure 7). Thus, netazepide showed no anxiolytic activity, which suggests it does not cross the blood-brain barrier. Indeed, exceedingly low levels of radioactivity were found in all brain regions of the rat after intravenous ¹¹C-netazepide (Wilson *et al* 2001), which confirms that netazepide does not readily cross the blood-brain barrier.

Figure 7. Effect of netazepide, L-365,260 and diazepam on the time that mice stayed in the light compartment. *p<0.05 vs control



(d) Protein binding in human serum

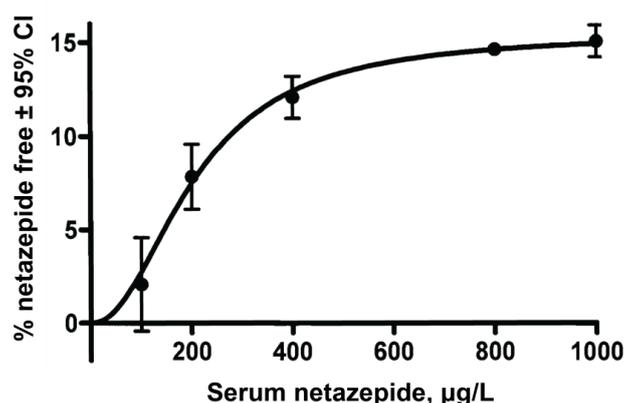
Protein binding by netazepide was assessed in normal human serum *in vitro*. Solutions of 0.1–1 mg/L netazepide were prepared, and protein-bound netazepide was separated from free netazepide in a centri-free micropartition device by centrifugation. Netazepide in the filtrate was assayed. Protein binding decreased with concentration. At the highest concentration tested (1 mg/L), protein binding was about 84% (Table 1; Figure 8).

Table 1. % netazepide unbound to protein in human serum.

Netazepide concentration	0.1 mg/L (n =4)	0.2 mg/L (n =10)	0.4 mg/L (n =10)	0.8 mg/L (n =10)	1 mg/L (n =10)
Mean (SD)	2.07 (1.57)	7.83 (2.43)	12.08 (1.55)	14.61 (0.46)	15.04 (1.18)
Range	1.23–4.43	5.21–13.94	10.25–15.79	14.07–15.35	13.41–17.44

Figure 8. % free netazepide versus total netazepide in serum.

Means \pm 95% confidence interval



(e) hERG ion channel

The effect of netazepide on hERG tail current was assessed in human embryonic kidney cells. Netazepide 1 and 10 μ M caused no detectable channel blockade, whereas 100 μ M caused ~ 31% blockade. Higher concentrations could not be studied because it was not possible to dissolve the netazepide to that extent. Terfenadine 50 nM, a known inhibitor, caused 60% blockade. Thus, the ‘no-observed-adverse-effect level’ (NOAEL) of netazepide can be taken as 10 μ M. IC_{50} could not be determined because of the solubility problem.

In non-clinical pharmacology studies, the inhibitory potency of netazepide at gastrin/CCK₂ receptors was $K_i=0.068$ nM. That concentration is several orders of magnitude lower than the NOAEL in the hERG study of netazepide. In subsequent studies in healthy subjects (Studies 1 and 2), the highest mean C_{max} of netazepide was 704.3 μ g/L, and the highest C_{max} in any subject was 1.209 mg/L. Given that the molecular weight of netazepide is 498.58, 1.209 mg/L corresponds to 2.4 μ M netazepide. Thus, there is about a 4-fold difference in

concentration between the NOAEL of 10 μM in the hERG study and the highest concentration seen in humans at the time. However, at 2.4 μM netazepide, about 84% (2.0 μM) is bound to protein. So, there is a 25-fold difference between the NOAEL (10 μM) and the concentration of free netazepide at the highest recorded C_{max} (0.4 μM).

A study by Redfern *et al* (2003) concluded that a 30-fold margin between C_{max} and hERG IC_{50} may be sufficient. As stated above, the hERG IC_{50} for netazepide could not be determined, because of the solubility problem. However, netazepide 100 μM blocked the hERG channel by 31%, so the IC_{50} is $>100 \mu\text{M}$. There is about a 250-fold difference between 100 μM (the IC_{31}) and the highest recorded C_{max} of free netazepide (0.4 μM).

Given the NOAEL in the hERG study, and the highest C_{max} of free netazepide in subsequent studies in healthy subjects, netazepide appears to have low potential to prolong QTc interval.

(f) CYP450 enzymes

HLS showed that, compared with known inhibitors, netazepide 2.5 $\mu\text{g/mL}$ ($\sim 5 \mu\text{M}$) inhibited CYP3A4, and to a lesser extent CYP2C8 and CYP2C9 in pooled human liver microsomes *in vitro*. Cyprotex, Macclesfield, England, did a more detailed study of pooled human liver microsomes *in vitro* to obtain information about netazepide and CYP3A4, CYP2C8 and CYP2C9 with respect to: IC_{50} ; reversible and irreversible inhibition; and potential to cause drug-drug interactions in patients *in vivo*. The results are shown in Tables 6 and 11. The IC_{50} of netazepide for reversible inhibition was 3.52, 8.37 and 12.1 μM for CYP2C8, CYP3A4 (midazolam) and CYP3A4 (testosterone), respectively (Table 2). Concentrations of netazepide up to 25 μM did not inhibit CYP2C9.

Table 2. Effect of netazepide (5 μM) and selective inhibitors on CYP450 activity in pooled human liver microsomes.

Isoform	Compound	IC_{50} (μM)	SEM
CYP2C8	netazepide	3.52	0.56
	montelukast	0.323	0.05
CYP2C9	netazepide	>25	–
	sulfaphenazole	0.481	0.07
CYP3A4	netazepide	8.37	0.57
	ketoconazole	0.0283	0.001

To assess the likelihood of CYP2C8 and CYP3A4 inhibition in patients *in vivo*, we calculated $[\text{I}]/\text{K}_i$ ratios using IC_{50} values for reversible inhibition by netazepide and C_{max} for total (bound and unbound) plasma concentrations of netazepide from Study 9 of single doses of netazepide 50, 100, 200 and 400 mg in healthy subjects (Table 3). FDA guidelines (2006) state that an

interaction is *likely* if the $[I]/K_i$ ratio is greater than 1, *possible* if between 1 and 0.1, and *remote* if less than 0.1.

Table 3. $[I]/K_i$ ratios calculated with total (bound plus unbound) plasma concentrations of netazepide.

Isoform	IC ₅₀ (μM)	K _i	[I ₁]/K _i	[I ₂]/K _i	[I ₃]/K _i	[I ₄]/K _i
CYP2C8	3.52	1.76	0.18	0.30	0.58	0.80
CYP3A4 (testosterone)	12.10	6.05	0.054	0.09	0.17	0.23
CYP3A4 (midazolam)	8.37	4.19	0.08	0.13	0.25	0.34

$K_i = IC_{50}/2$, and I_1, I_2, I_3 and $I_4 = C_{max}$ for 50, 100, 200 & 400 mg netazepide, respectively, in fasting subjects.

Therefore, the likelihood of a drug-drug interaction based on $[I]/K_i$ ratios for total netazepide is: *possible* for CYP2C8 after netazepide 50–400 mg; *remote* for CYP3A4 (testosterone) after netazepide 50 and 100 mg, and *possible* after 200–400 mg; and *remote* for CYP3A4 (midazolam) after netazepide 50 mg, and *possible* after 100–400 mg

Netazepide 0.5 μM did not cause significant irreversible inhibition of CYP3A4 (midazolam). However, netazepide 5 μM caused 30.2% irreversible inhibition of CYP3A4 (midazolam). Neither CYP3A4 (testosterone) nor CYP2C9 was inhibited by netazepide 5 μM.

The likelihood of an interaction between netazepide and a drug metabolised via CYP2C9 in patients is clearly remote, because netazepide up to 25 μM did not inhibit CYP2C9 *in vitro*. The FDA considers an $[I]/K_i$ ratio >0.1 for the highest proposed clinical dose to be positive, and recommends a study in healthy subjects *in vivo*. At the moment, the highest proposed clinical dose of netazepide is 50–100 mg once daily. On that basis, our results were positive for CYP2C8 at ≥ 50 mg netazepide. They were also positive for CYP3A4 at >50 mg and perhaps >100 mg. Few drugs are metabolised via CYP2C8, and they are unlikely to be co-administered with netazepide, so it may be better to avoid them during early studies of netazepide in patients rather than carrying out a study of CYP2C8 *in vivo*. However, CYP3A4 is more important because about 60% of drugs are metabolised via that enzyme. The method favoured by the FDA to assess the likelihood of interactions requires $[I]/K_i$ ratios to be calculated with steady-state values for C_{max} . The C_{max} values that we used to calculate $[I]/K_i$ ratios were for single doses of spray-dried of netazepide, which we plan to use for studies in patients. We do not have steady-state C_{max} values for that formulation. However, we do have C_{max} values for single and repeated doses of the original spray-dried formulation of netazepide (Studies 1–4). C_{max} values for single doses of the two formulations were similar. Steady-state for the original formulation was reached by Day 3, and there was little increase in exposure by the last day of 7 days of 100 mg twice daily dosing. Therefore, if

steady-state C_{\max} values were used to calculate $[I]/K_i$ ratios, the above conclusions would be much the same.

Mechanism-based or irreversible inhibition is potentially more harmful than reversible inhibition. Netazepide caused some irreversible inhibition of CYP3A4, but the degree of inhibition makes a drug-drug interaction in patients *in vivo* unlikely.

Food increased C_{\max} of 100 mg netazepide by 1.6 fold in healthy subjects (Study 9).

Therefore, netazepide taken with food would reduce the 'safety margin' for interactions.

4. Pharmacokinetics

(a) Rats

Male rats were given single oral doses of netazepide 0.1, 0.3 and 1.0 mg/kg, and an intravenous bolus of netazepide 0.1 mg/kg. Bioavailability was 28, 26 and 26% after 0.1, 0.3 and 1.0 mg/kg, respectively. Thus, bioavailability was independent of dose.

(b) Dogs

Fasted male dogs were given single oral doses of netazepide 0.1, 0.3 and 1.0 mg/kg, and an intravenous bolus of netazepide 0.1 mg/kg. Netazepide concentrations peaked within 2 h after oral doses. Comparison of the terminal half-lives of the oral and intravenous doses suggests that elimination after oral dosing was limited by absorption rate. C_{\max} and AUC increased disproportionately with oral dose. Bioavailability was 27 to 50%.

In a study of oral netazepide 0.1 mg/kg/day for 7 days in fasted male dogs, the rate but not the extent of exposure increased during repeated dosing. Steady state was reached by day 4, and there was little accumulation. Food did not affect bioavailability.

5. Absorption, distribution and excretion

A single oral dose of ^{14}C -netazepide 1 mg/kg was rapidly absorbed by male rats: whole blood and plasma concentrations of parent compound and radioactivity peaked 15 min after dosing. Over half of the radioactivity in blood was in plasma, so there was limited association with red blood cells. Parent compound accounted for over half of total plasma radioactivity. Apparent terminal half-life of unchanged compound and radioactivity was 2.5 and 4.7 h, respectively. Excretion was predominantly faecal (>90%); most of that was biliary.

Tissue concentrations of radioactivity generally peaked 15 min after dosing, and were highest in the gastrointestinal tract and liver. Concentrations in the brain were very low, again confirming that netazepide and any metabolites do not readily cross the blood-brain barrier.

6. Comparative studies of the metabolism of netazepide

(a) Metabolism of ¹⁴C-netazepide by rat, dog and human hepatocytes

In a study to compare the metabolism of ¹⁴C-netazepide by human, rat and dog liver hepatocytes *in vitro*, metabolism of parent netazepide was most efficiently catalysed by human > dog > rat. Phase I and II metabolites were formed. There was no evidence for formation of unique major human metabolites, although the main metabolite was different from that formed by rat and dog. Thus, the rat and dog are appropriate species to assess the potential toxicity of netazepide in humans.

(b) Metabolites in blood and urine after oral administration

Incubation of netazepide with human hepatocytes *in vitro* identified four main metabolites, called A–D. Metabolite A was the main one. The metabolite profiles of netazepide in blood and urine from human, rat and dog were compared to find out if rats and dogs, dosed with netazepide, are exposed to sufficiently high levels of metabolite A to warrant separate investigation and characterisation. The ICH guidance on non-clinical safety studies (ICH M3) states that metabolites with exposures greater than 10% of total drug-related exposure warrant separate investigation and characterisation. In healthy subjects (Study 13), metabolite A was 41% of total drug-related exposure after a single dose of netazepide 500 mg, and 29% after repeated doses of netazepide 50 mg. Metabolite A was almost undetectable in dogs. Rats do produce it, but not to the same extent as humans (Table 9). So, metabolite A does warrant separate investigation and characterisation.

Table 4. Proportions of metabolite A, netazepide, metabolite A /netazepide ratio, and metabolite A mg/kg in human, dog and rat plasma after single or repeated oral doses of netazepide

Dose	500 mg	50 mg/day for 3 days				100 mg/kg/day for 7 days				
Species	Subject 1	Subject 2				Dog			Rat	
Time	7h	Day 1 2h	Day 1 7h	Day 3 2h	Day 3 7h	0.25h	2h	24h	0.25h	2h
Metabolite A (%)	40.9	10.1	27.9	15.2	29.4	0.6	<0.1	nd	6.9	10.2
Netazepide (%)	48.5	81.2	62.1	62.9	51.5	97.2	97.2	84.0	86.1	81.0
Metabolite A /netazepide	0.84	0.12	0.45	0.24	0.57	0.006	0.001	-	0.08	0.12
Metabolite A mg/kg	2.87	0.07	0.20	0.11	0.20	nc	nc	nc	nc	10.2

For humans, a nominal bodyweight of 75 kg was used; nc = not calculable; nd = not detected

(b) Metabolism by hepatocytes from non-rodent species

The previous study showed that the dog is not a suitable species in which to investigate metabolite A. So, another study was done to compare the metabolism of netazepide in hepatocytes derived from four other non-rodent species. In monkey, human, minipig and rabbit hepatocyte incubations, 28 metabolites were found. There was one unique human

metabolite, which was <1% compared to netazepide. The main metabolite in human, monkey and rabbit hepatocytes was metabolite A (Table 5). It was also present in minipig hepatocytes, but it wasn't the main metabolite. So, the minipig, rabbit or monkey would be suitable species in which to study metabolite A.

Table 5. *m/z* values for possible metabolites of netazepide in different species

<i>m/z</i> of [M+H] ⁺	Presence in incubation (% relative to parent compound in T=120 min incubation)			
	rabbit	minipig	monkey	human
517.219	nd	0.05	0.76	0.01
531.235	nd	nd	2.49	0.25
501.225	0.42	8.61	69.1	1.17
529.219	0.03	nd	2.09	0.45
485.230*	0.04	nd	2.26	0.45
515.240 (Metabolite A)	4.12	1.66	242	57.1
517.219	nd	nd	0.49	0.07
531.235	0.12	nd	6.45	1.77
501.222	0.08	2.82	1.38	0.03
515.240	0.02	0.17	0.26	0.13
529.219	0.14	0.02	15.5	2.16
485.230	0.03	nd	1.25	1.24
501.225	nd	nd	nd	0.05
499.245 (Netazepide)	100	100	100	100
531.235	0.01	nd	nd	0.10
517.219	nd	nd	0.15	0.01
531.235	nd	nd	0.67	0.07
501.225	0.04	1.15	0.12	0.02
515.240	nd	1.69	nd	0.12

nd: not detected

* Observed as [M+2H]²⁺ ion with *m/z* 243.118 in the MS spectrum

7. Repeated-dose toxicity studies

(a) Rats

A 13-week toxicity study of netazepide 100, 300, and 1000 mg/kg/day by oral gavage in rats, with 5 or 10 weeks' recovery, showed:

- no effect on body weight, consumption of food or water, clinical signs, haematology or ophthalmoscopy;
- a slight increase in heart weight in some male animals after 1000 mg/kg/day, which was reversible and without changes in histology and routine plasma biochemistry;
- discoloured urine after 300 and 1000 mg/kg/day, and pale faeces after 1000 mg/kg/day;
- minor reversible increases in alkaline phosphatase in some animals (females) after 300 and 1000 mg/kg/day, and some minor reversible increases in alanine transferase in some male animals after 1000 mg/kg/day;

- minor histopathological heart and pancreas lesions in some animals after 1000 mg/kg/day, which are common in laboratory rodents; and
- reversible pharmacological effects after all doses: high serum gastrin; reduction in ECL cells; and increase in G-cells.

Plasma concentrations were higher in females than males. The NOAEL was deemed 100 mg/kg/day. Pharmacokinetic parameters derived from plasma netazepide concentrations in male and female rats are in Table 6.

Table 6. Pharmacokinetic parameters of netazepide in rats

Dose (mg/kg/day)	C _{max} (ng/ml)				AUC ₀₋₂₄ (ng.h/ml)			
	Day 1		Week 13		Day 1		Week 13	
	male	female	male	female	male	female	male	female
100	1187	1750	706	893	5468	7122	4629	5910
300	3463	1976	1162	2398	28683	9645	8150	17809
1000	3905	3261	1410	4410	14894	10678	18955	37225

(b) Dogs

In a preliminary study of netazepide 100 and 500 mg/kg by oral gavage in dogs, plasma concentrations of netazepide after 100 mg/kg were generally higher than those after 500 mg/kg. So, dogs were given netazepide 10, 30 & 100 mg/kg/day by oral gavage for 13 weeks. The findings were:

- no treatment-related changes in veterinary, auditory, ophthalmoscopy and electrocardiography findings;
- no treatment-related changes in safety tests of blood and urine;
- no treatment-related changes in organ weights;
- pancreatic acinar atrophy, with associated ductal-endocrine proliferation and focal degranulation, in 2 of 3 male dogs in each of the low and intermediate dose groups, and in 1 of 3 females in the high dose group; and
- very high serum gastrin concentrations in all treated animals, a pharmacological effect of netazepide. However, the increase in G-cells and reduction in ECL cells seen in rats were not seen in dogs.

The histological changes in pancreas of dogs are consistent with netazepide toxicity, in which case the study did not clearly establish a NOAEL. However, a more likely explanation is that the changes represent nesidioblastosis, a well-recognised finding among laboratory beagle dogs (Katsuta *et al* 1992; Son *et al* 2010), in which case the NOAEL is 100mg/kg/day.

Pharmacokinetic parameters are shown in Table 7.

Table 7. Pharmacokinetic parameters of netazepide in dogs

Dose (mg/kg/day)	C _{max} (ng/ml)				AUC ₀₋₂₄ (ng.h/ml)			
	Day 1		Week 13		Day 1		Week 13	
	male	female	male	female	male	female	male	female
10	86	277	54	494	467	1558	540	3236
30	63	109	211	591	671	909	1482	2186
100	79	145	291	1498	783	1029	3779	10424

8. Embryo-foetal studies

(a) Rats

Netazepide 100, 300 and 1,000 mg/kg/day did not cause maternal toxicity. After 1000 mg/kg/day, there was a higher incidence of incomplete ossification of the supra-occipital, interparietal, parietal, squamous and frontal bones of foetal skulls compared with controls. So, the changes were deemed related to netazepide. Maternal NOAEL was 1,000 mg/kg/day, and foetal NOEL was 300 mg/kg/day.

(b) Rabbits

In a study of netazepide 100, 300, and 1,000 mg/kg/day, there was a reduction in maternal weight gain or weight loss and reduced food consumption after 300 and 1,000 mg/kg/day. Also, there were two deaths (one rabbit died and one was killed) after 1000 mg/kg/day; both deaths were associated with stomach lesions, the cause of which was uncertain. Netazepide had no effect on embryo-foetal survival, growth or development. Maternal NOAEL was 100 mg/kg/day, and foetal NOEL was 1,000 mg/kg/day.

9. Genotoxicity studies

In the Ames test, netazepide showed neither cytotoxic nor mutagenic activity at five concentrations up to 5 mg/plate. The results of a mouse lymphoma L5178y study were equivocal. The assay was done twice in the absence of S9-mix, and three times in the presence of S9-mix. Mutant frequencies in negative control cultures were within normal ranges, and a clear increase in mutant frequency was induced by the positive control chemicals. In the absence of S9-mix, there were no statistically significant increases in mutant frequency at all doses of netazepide tested. However, in 1 of 3 experiments done in the presence of S9-mix, there was a small, but statistically significant increase in mutant frequency after treatment with netazepide 15.6, 125 and 250 µg/mL. The increase was not considered biologically significant.

Netazepide was positive in a chromosome aberration test of cultured human lymphocytes *in vitro*. Positive responses were seen at 45 h after doses as low as 25 mg/mL in the absence of S9-mix, and 500 mg/mL in the presence of S9-mix. But, the results were not reproducible

and were confounded by high toxicity and by statistically significant values that fell within the historical control range.

Therefore, four tests were done *in vivo*: 3 mouse bone marrow micronucleus tests, and an unscheduled DNA synthesis (UDS) assay using rat liver. In the first micronucleus test, netazepide was given as a suspension in 0.5% methylcellulose, orally by gavage, up to 2 g/kg. Although the result was negative, it was compromised because there was no evidence of exposure of the bone marrow to netazepide. In other words, plasma netazepide was not measured, and there was no evidence of bone marrow toxicity. So, the test was repeated with netazepide suspended in 20% hydroxypropyl- β -cyclodextrin at pH 2 to optimise absorption. Doses of 37.5, 150 and 500 mg/kg were given to groups of male and female mice, and bone marrow was analysed 24 and 48 h after dosing. Toxicokinetic analysis showed linear kinetics. There was no effect of any of the doses in male mice. In females, 37.5 and 150 mg/kg also had no effect. However, some female mice had an increase in micronuclei at 48 h after 600 mg/kg. Those micronuclei were large and atypical and were deemed artefact. The results of a third mouse micronucleus test, using the same vehicle, doses and exposure times, with 10 males and 10 females per group, were negative, supporting the view that the micronuclei seen in the second test were, indeed, artefacts. Finally, an assay of unscheduled DNA synthesis in rat liver *in vivo* was also negative.

10. Summary of non-clinical studies, and implications for clinical trials

In non-clinical studies, netazepide proved to be a selective, competitive and potent antagonist of gastrin/CCK₂ receptors *in vitro* and *in vivo*. Single doses inhibited gastric acid secretion in a dose-dependent manner; inhibition persisted after repeated doses. Oral bioavailability of active pharmaceutical ingredient, derived from results of intravenous and oral doses, was 26% and 27–50%, respectively, in rats and dogs.

13-week toxicology studies in rats and dogs did not identify a target organ. Studies to assess genotoxicity and mutagenicity were negative. Netazepide affected neither heart rate nor ECG intervals in conscious dogs. Also, it did not affect hERG channels *in vitro* at concentrations higher than the highest blood concentration seen in healthy subjects. If protein binding is taken into consideration, there was a 25-fold difference between NOAEL (10 μ M) and concentration of free netazepide at the highest recorded C_{max}. Netazepide caused some reversible inhibition of CYP3A4 and CYP2C8 *in vitro*. The likelihood of a drug-drug interaction via those enzymes in trials in patients is remote or possible at the proposed clinical doses of netazepide.

Thus, overall the non-clinical studies support the administration of netazepide to humans for up to 13 weeks. But, until more information is available, in clinical trials, netazepide:

- must not be given to women of child-bearing potential who are at risk of pregnancy; and
- should be given with certain medicines metabolised via CYP3A4 or CYP2C8 only if they cannot be avoided.

Netazepide has low potential to prolong QTc interval in humans. So, a decision about a thorough QTc study can wait until later in development. However, in clinical trials, subjects with prolonged QTc interval should be excluded, and QTc interval should still be monitored.

C_{max} and AUC_{0-24h} at the NOAEL in the species with the lowest exposure (male dogs) were 291 ng/mL and 3,779 ng.h/mL, respectively (Table 6). Exposure to netazepide in humans should not exceed those limits. However, those values may be an underestimate because there were no adverse effects at any dose tested. C_{max} and AUC_{0-24h} at the NOAEL in male rats, which were 706 ng/mL and 4,629 ng.h/mL, respectively, (Table 7) may be better estimates because there were adverse effects at higher doses.

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The work in chapters 3–12 was done before registration for the degree MD. These chapters are included as in-thesis *Supplementary Material* in order to provide contextual information in the development of netazepide. They are not part of the submission for the award of the degree MD.

Chapter 3

Studies 1 and 2. Safety, tolerability, pharmacokinetics, and effect on 24-h gastric acidity of single doses of netazepide, and comparison with placebo and ranitidine, a histamine H₂-receptor antagonist, in healthy subjects

Boyce M, David O, Darwin K, Mitchell T, Johnston A, Warrington S.

Single oral doses of netazepide (YF476), a gastrin receptor antagonist, cause dose-dependent, sustained increases in 24-h gastric pH compared with placebo and ranitidine in healthy subjects. *Aliment Pharmacol Ther* 2012; 36: 181–189.

ABSTRACT

Background

Non-clinical studies have shown netazepide (YF476) to be a potent, selective, competitive and orally active gastrin receptor antagonist.

Objectives

To administer to man for the first time single oral doses of netazepide, to assess their tolerability, safety, pharmacokinetics and effect on 24-h gastric pH.

Design

We did two randomised double-blind single-dose studies in healthy subjects. The first (n=12) was a 6-way incomplete crossover pilot study of rising doses of netazepide (range 0.5–100 mg) and placebo. The second (n=20) was a 5-way complete crossover study of netazepide (5, 25 and 100 mg), ranitidine (150 mg) and placebo. In both trials we collected frequent blood samples, measured plasma netazepide, and calculated pharmacokinetic parameters. In the comparative trial we measured gastric pH continuously for 24 h and compared treatments by percentage time gastric pH ≥ 4 .

Results

Netazepide was well tolerated. Median t_{\max} and $t_{1/2}$ for the 100 mg dose were about 1 and 7 h, respectively, and the pharmacokinetics were dose-proportional. Netazepide and ranitidine each increased gastric pH. Onset of activity was similarly rapid for both. Activity of ranitidine lasted about 12 h, whereas that of netazepide exceeded 24 h. Compared with ranitidine 150 mg, netazepide 5 mg was as effective, and netazepide 25 and 100 mg were much more effective, over the 24 h after dosing.

Conclusions

In man: netazepide is an orally active gastrin receptor antagonist, and endogenous gastrin has a major role in controlling gastric acidity. Studies of repeated doses of netazepide are justified.

Significance of this study

What is already known about this subject?

- Gastrin stimulates gastric acid secretion and growth of ECL cells in the gastric mucosa, which possess gastrin receptors.
- Netazepide (YF476), a gastrin receptor antagonist, has been widely used to assess the physiology and pathology of gastrin in animals, but had not been administered to humans before this study.
- No gastrin receptor antagonist is licensed for routine clinical use.

What are the new findings?

- Single oral doses of netazepide were well tolerated and caused dose-dependent increases in 24-h gastric pH in healthy subjects, consistent with antagonism of gastrin receptors.
- Compared with ranitidine, a histamine H₂-receptor antagonist, activity of netazepide was similarly rapid in onset but was much longer lasting.

How might it impact on clinical practice in the foreseeable future?

- Netazepide is a potential new treatment for acid-related conditions and for the effects of hypergastrinaemia caused by chronic atrophic gastritis or acid suppressants such as proton pump inhibitors.

Introduction

Cholecystokinin (CCK) and gastrin are polypeptide hormones found in the brain and other tissues.¹ CCK stimulates CCK₁ (also called CCK-A) receptors in the gall bladder and pancreas causing contraction and exocrine secretion, respectively. The C-terminal octapeptide is necessary to elicit the full response.² CCK also acts on receptors distinct from CCK₁ receptors, designated CCK₂ (CCK-B) or gastrin receptors, which require only the C-terminal tetrapeptide to elicit a full response.³ The C-terminal tetrapeptide is common to both CCK and gastrin.

Cloning has shown that CCK₂ receptors in the brain and other tissues are identical.⁴ CCK stimulates CCK₂ receptors in the brain, whereas gastrin, which is secreted by G cells in the gastric antrum,⁵ stimulates gastric acid secretion and gastrointestinal cell growth, especially growth of the enterochromaffin-like (ECL) cells in the gastric mucosa.⁶

Food,⁷ gastric distension⁸ and an increase in gastric pH⁹ all cause secretion of gastrin into the circulation. Circulating gastrin stimulates gastrin receptors on ECL cells in the gastric fundus to secrete histamine,¹⁰ which in turn stimulates adjacent gastric oxyntic cells to secrete acid into the stomach lumen. Gastric acid secretion is controlled by (H⁺, K⁺)-ATPase (the proton pump) in response to stimulation of histamine H₂-receptors¹¹ or muscarinic M₃-receptors.¹²

Initially, acid is neutralised by the buffering capacity of food. But as acid secretion continues and digestion proceeds and the gastric contents move into the duodenum, the buffering capacity of the food diminishes and intragastric pH falls. Falling pH stimulates D cells in the gastric antrum to secrete somatostatin, a hormone that switches off gastrin secretion.

Acid secretion is also under nervous control. Food causes central stimulation of the vagus, which leads to gastrin release. The vagus also controls acid secretion via somatostatin.

Many gastrin antagonists have been described.^{13 14} Some have been studied in healthy subjects and patients, but none has progressed beyond early clinical development.¹⁵

Netazepide (YF476) [(3R)-N-(1-(tert-butylcarbonylmethyl)-2,3-dihydro-2-oxo-5-(2-pyridyl)-1H-1,4-benzodiazepin-3-yl)-N'-(3-(methylamino)phenyl)urea] is a benzodiazepine derivative and a novel gastrin receptor antagonist.¹⁶ The pharmacology of netazepide has been assessed in non-clinical studies,^{17 18} as follows. It caused concentration-dependent inhibition of specific binding of the ligand [¹²⁵I]CCK-8 to rat and cloned human¹⁹ gastrin receptors *in vitro*. The affinity of netazepide for rat gastrin receptors was 264 and 70 times higher than that of two other gastrin antagonists, L-365,260²⁰ and CI-988,²¹ respectively, and 4100 times its affinity for rat pancreatic CCK₁ receptors. Netazepide had very little affinity for various other

receptors, such as histamine, muscarinic and benzodiazepine receptors.¹⁷ In anaesthetised rats, intravenous netazepide caused dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion, with an ED₅₀ of 0.0086 µmol/kg, compared with an ED₅₀ of 0.013 µmol/kg for intravenous famotidine. Intravenous netazepide at a high dose of 10 µmol/kg did not affect histamine- or bethanechol-induced acid secretion. In Heidenhain pouch dogs, intravenous and oral netazepide each caused dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion, with ED₅₀ values of 0.018 and 0.020 µmol/kg, respectively.¹⁷ ED₅₀ for intravenous and oral famotidine, a histamine H₂-receptor antagonist (H₂RA), was 0.078 and 0.092 µmol/kg, respectively. In dogs with a gastric fistula, intravenous netazepide dose-dependently inhibited pentagastrin-induced gastric acid secretion, with ED₅₀ 0.0023 µmol/kg. Also in gastric fistula dogs, oral netazepide, famotidine, and the proton pump inhibitor (PPI), omeprazole, each dose-dependently inhibited peptone-induced gastric acid secretion, with ED₅₀ 0.11, 0.76 and 4.28 µmol/kg, respectively.¹⁸ On the basis of those results, netazepide was about 7 and 40 times more potent than famotidine and omeprazole, respectively. Comparison of ED₅₀ for intravenous and oral netazepide indicates that the oral bioavailability was 26–28% in rats and 27–50% dogs. Thus, overall these non-clinical studies show netazepide to be a potent, highly selective, competitive and orally-active antagonist of gastrin receptors. Indeed, netazepide has been described as the ‘gold standard’ for gastrin antagonists,¹⁴ and has been used in many other non-clinical studies, to assess the physiology and pathology of gastrin. A PubMed search currently yields 27 references.

Netazepide was well tolerated in animal toxicology studies (Ferring, Investigator’s Brochure 1997). In 13-week studies, the no-observable-adverse-effect level was 100 mg/kg/day in rats and in dogs. There were increases in gastric G cells and circulating gastrin, and a reduction in gastric ECL cells, consistent with antagonism of gastrin receptors by netazepide. Tests of teratogenicity and mutagenicity were negative.

The favourable pharmacological and toxicological profiles of netazepide and its good oral bioavailability in animals justified studies of netazepide in man. Here we report the first administration of netazepide to man. We did two studies in healthy subjects. The first was a pilot study of single rising oral doses. The second was a placebo-controlled comparative study of single fixed oral doses of netazepide and ranitidine.²² The overall aim was to assess the safety, tolerability and pharmacokinetics of netazepide, and to compare the effect of netazepide on 24-h gastric pH with that of ranitidine. Also, we aimed to assess the relationship between the pharmacokinetics and pharmacodynamics of netazepide.

The non-clinical studies suggested that oral netazepide 10 mg would be as effective as oral ranitidine 150 mg in suppressing gastric acidity.¹⁸ Therefore, for our two clinical studies we chose a range of single doses encompassing 10 mg.

Materials and methods

We did the studies during May–August 1996 at Central Middlesex Hospital, in accordance with the ICH Guideline for Good Clinical Practice and Declaration of Helsinki. Brent Ethics Committee approved the studies. Subjects gave written informed consent.

Treatments

Yamanouchi Pharmaceutical Co, Japan, supplied netazepide (0.5, 5 and 25 mg) capsules and matching placebo capsules. The hospital pharmacy: supplied encapsulated ranitidine 150 mg tablets (Zantac; GlaxoSmithKline) and matching placebo capsules; packed and labelled treatments; and randomised subjects to treatments using sequentially numbered containers.

Study design

Pilot study

Before embarking on a controlled, randomised study of fixed doses, we did a single-dose rising study of netazepide 0.5, 5, 25, 50 and 100 mg by mouth. The design was double-blind, placebo-controlled and incomplete crossover in 2 groups of 6 young healthy men. One group received 0.5, 25 and 100 mg, and the other 5, 50 and 100 mg. The ratio of netazepide:placebo was 4:2 at each dose level. Each subject was resident for 2 nights on 3 occasions and followed up 5–10 days after dosing. We assessed safety and tolerability by vital signs, ECG, safety tests of blood and urine, and adverse events. We took blood at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h after dosing, for assay of plasma netazepide.

Comparative study

The second study was single-dose, double-blind, double-dummy, randomised and 5-way complete crossover in design in 20 healthy young men or women using a reliable method of contraception. Each subject received 5 treatments by mouth: netazepide 5, 25 or 100 mg; ranitidine 150 mg; and placebo. Each subject was resident for 2 nights on 5 occasions and was followed up 5–10 days after the last dose. There were 7 days between doses. We assessed safety and tolerability as in the pilot study, took blood samples at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h after dosing, and collected all urine from 0–24 h after dosing, for netazepide assay. We measured gastric pH continuously for 0.5 h before and 24 h after dosing. Subjects fasted overnight before each dose, which they took with 100 mL water at the same time (0800–0900 h) on each occasion. They rested on their beds for 6 h after dosing and were ambulant thereafter. They ate standard meals at 4, 9, 13 and 22 h after dosing, and drank 150

mL water at 2, 6, 8 and 11 h after dosing. We prohibited alcoholic and caffeinated drinks and smoking while subjects were resident.

Plasma concentrations of netazepide (YF476)

We collected blood in lithium-heparin tubes, and separated plasma by centrifugation (4°C; 800 G for 10 min) and plastic pipette within 15 min of sampling. We stored plasma and urine samples at -20 °C until assay. Huntingdon Life Sciences, England, measured plasma netazepide by a validated liquid chromatographic-tandem mass spectrometric method.²³ The calibration line was linear over the range 0.1 ng/mL (limit of quantification) to 25.0 ng/mL. Intra- and inter-batch precision was <14%, and intra- and inter-batch accuracy was <11% over the entire range.

Measurement of ambulatory gastric pH

We inserted a disposable single-channel antimony internal reference pH electrode, with surface markings of 1 cm (Zinetics Medical, Utah, USA), for a distance of 45–50 cm through the subject's nostril into the stomach. We monitored pH as the sensor passed down the oesophagus, through the gastro-oesophageal sphincter, and into the stomach. Entry of the electrode into the stomach was confirmed by a sharp fall in pH. Next, we withdrew the electrode through the sphincter into the oesophagus, and then repositioned it in the stomach; a sharp rise or fall in pH identified the point at which the sensor crossed the sphincter. We advanced the electrode to a final position 10 cm beyond that point. We recorded intragastric pH every 6 seconds via a menu-driven compact solid-state recorder (Flexilog 2020 24 h recorder; Oakfield Instruments, Oxon, UK); we calibrated the recorder before starting the recording, using pH 4 and 7 buffers. We activated an event marker on the recorder when the electrode was in position, and immediately after dosing. We uploaded recorded data to the Flexisoft II analysis software (Oakfield Instruments) operating within the Microsoft Windows environment, and exported it to other software packages as required.

Statistics

Sample size

For the comparative study, based on our previous experience, 20 subjects were enough to detect 15% increase in pH with 80% power, assuming within-subject coefficient of variation of 0.15 and 5% significance.

Pharmacokinetics

We used WinNonLin to derive pharmacokinetic parameters by standard non-compartmental analysis, and SAS for Windows for statistical analysis. We took maximum plasma

concentrations of netazepide (C_{\max}), and the times at which they occurred (t_{\max}), directly from the experimental data. We calculated area under the plasma concentration-time curve over 0–24 h ($AUC_{0-24\text{ h}}$) using the linear trapezoidal rule, and area under the plasma concentration-time curves to infinite time ($AUC_{0-\infty}$) using the equation: $AUC_{0-\infty} = AUC_{0-24\text{ h}} + \hat{C}_{\text{last}}/\lambda_z$, where \hat{C}_{last} is the predicted plasma concentration of the last measurable sample, and λ_z is the terminal rate constant, determined by log-linear regression analysis of those points that constitute the final, linear phase of the plasma concentration-time curve. We calculated terminal half-life ($t_{1/2}$) as $\ln 2/\lambda_z$. For the comparative study, we used analysis of variance (ANOVA) to assess dose-proportionality of C_{\max} and $AUC_{0-\infty}$. Before doing so, we normalised both to a 100 mg dose and log-transformed them. We included subject and dose as effects in the ANOVA model.

Pharmacodynamics

For the comparative study, we analysed pH data in four intervals: 0–4, 4–9, 9–13 and 13–24 h after dosing. We calculated for each interval: AUC; time $\text{pH} \geq 4$; and percentage time $\text{pH} \geq 4$. We calculated AUC using the trapezoidal rule. The primary outcome measure was percentage time gastric $\text{pH} \geq 4$. We regarded all other pH parameters as secondary. To test for differences among treatments, we subjected each pharmacodynamic parameter to ANOVA using a model that included effects for subject, period, treatment, first-order carryover and treatment-period interaction. To stabilise variance and make data conform to a normal distribution, we log-transformed AUC and arcsin-transformed percentages.²⁴ If treatment was significant in the overall model, we did pairwise comparisons between each active treatment and placebo. In addition, we compared with ranitidine any netazepide doses that were significantly different from placebo. All tests were two-sided, and significance level was $\alpha = 0.05$; we made no adjustments for multiple comparisons.

For graphical presentation, we calculated median pH for successive 2-min periods for individual subjects, to produce smoothed pH-time data. We calculated medians because the data were not normally distributed.

Pharmacokinetic/pharmacodynamic relationship

For the comparative study, we used two methods to assess the relationship between pharmacokinetics (PK) and pharmacodynamics (PD) of netazepide. First, we investigated the dose-response relationship by calculating Pearson correlation coefficients between plasma C_{\max} or $AUC_{0-\infty}$ (with and without logarithmic transformation) and time $\text{pH} \geq 4$ in 24 h. Second, as a measure of the PD effect of netazepide, we subtracted each subject's pH after placebo at each time point ('baseline') from the corresponding pH after each active treatment.

Then we modelled the median of those differences by fitting a fourth-order polynomial curve: we used that equation to simulate a pH profile for each dose. We used a two-compartmental oral model to simulate a profile of netazepide plasma concentrations, which we used with the modelled pH profile to find the parameter that best described the PK/PD relationship.

Results

For the pilot study, we enrolled 15 men, of whom 3 dropped out for social reasons and 12 (mean age 27 y; mean weight 77 kg) completed it. For the comparative study, we enrolled 21 subjects, of whom 1 dropped out for social reasons and 20 (11 men; 9 women; mean age 25 y; mean weight 75 kg) completed it. There were full datasets for statistical and pharmacokinetic analyses.

Tolerability and safety

All treatments were well tolerated. Any adverse events were minor, transient and occurred across treatments. There were no clinically relevant changes in any of the safety assessments.

Pharmacokinetics

Pilot study: Table 1 and Figure 1

Netazepide was rapidly absorbed; median t_{\max} for the 5 dose levels ranged from 0.5 to 0.88 h. The plasma concentration-time curves suggest that netazepide has at least 2 elimination phases: the terminal elimination phase seems to begin about 4–12 h after dosing. Only after the 100 mg dose was the concentration of netazepide above the limit of reliable quantification at 24 h after dosing in every subject. It is likely, therefore, that $t_{1/2}$ values for lower doses do not reflect the terminal elimination phase. The short mean half-lives of about 2 h for the lower doses may represent a more rapid earlier phase of elimination, whereas the higher value of 6.6 h for the 100 mg dose is probably a more accurate estimate of the true terminal elimination half-life of netazepide. That conclusion is supported by the strongly bimodal distribution of the individual half-lives: although the range of values was 1.5–6.9 h, no subject had a value in the range 2.8–4.8 h. Netazepide had dose-proportional pharmacokinetics in the dose range studied: statistical analysis showed no significant evidence against dose-proportionality for either C_{\max} ($p = 0.91$) or $AUC_{0-\infty}$ ($p = 0.97$).

Although there was a greater proportionate increase in C_{\max} and $AUC_{0-\infty}$ from 50 to 100 mg than from 25 to 50 mg, comparisons among treatments included both within- and between-subject comparisons, as the design was incomplete crossover. Netazepide had consistent bioavailability among subjects, as judged by AUC: the coefficient of variation for $AUC_{0-\infty}$ was below 30% at all doses except the 0.5 mg dose. Variation was inevitably higher for that dose because many of the plasma concentrations were close to or below the limit of reliable

quantification. C_{\max} was more variable than AUC_{0-24h} , but the coefficient of variation of C_{\max} was still <60% for all doses.

Comparative study: Tables 1 and 2, and Figure 2

Netazepide was rapidly absorbed: median t_{\max} after the 5, 25 and 100 mg doses ranged from 0.5 to 1 h. Elimination appeared to be biphasic; the terminal elimination phase began ~4–12 h after dosing.

Since only a few blood samples were taken during the terminal phase of elimination, and netazepide concentrations were below the limit of reliable quantification in some of them, the estimate of elimination half-life is unreliable after the lower doses. After the 100 mg dose, plasma concentrations were well above the limit of reliable quantification at all times up to 24 h, so the $t_{1/2}$ of 7.6 h is a more representative value. The values of $t_{1/2}$ for the 5 and 25 mg doses probably represent a faster initial phase of distribution and elimination, rather than the true terminal elimination half-life.

Netazepide had consistent bioavailability among subjects, as judged by the AUC: the coefficient of variation for AUC_{0-24h} was <40% for all doses. Variability in C_{\max} was higher, with a coefficient of variation ranging from 41–87%.

The dose increased in the ratio 1:5:20, while $AUC_{0-\infty}$ and C_{\max} increased in the ratio 1.0:4.7:15.9 and 1.0:4.6:13.0, respectively. Those increases did not deviate significantly from dose-proportionality ($p=0.073$ and $p=0.158$, respectively).

Only 1–2% of netazepide was excreted unchanged in the urine over 24 h.

Pharmacodynamics

Time course of gastric pH: Figure 3

There were characteristic and predictable increases in gastric pH after placebo, corresponding to times at which subjects ate and drank.

Median gastric pH increased quickly after netazepide. After all meals, median gastric pH fell more slowly in subjects on netazepide than those on placebo. Even after breakfast, 24 h after dosing, it remained higher in subjects on netazepide than those on placebo. The trends were similar for all netazepide doses, although the effects on pH were most marked after 100 mg. Although median pH after ranitidine was high in the first 4 h after dosing, it fell more quickly than after netazepide, and, from about 12 h after dosing, it was almost indistinguishable from that after placebo.

Primary response variable: Table 2, Figures 4–5

Netazepide had a clear effect on the primary response variable: it increased percentage time gastric pH ≥ 4 . In all time intervals, mean percentage time pH ≥ 4 for netazepide was significantly higher than for placebo ($p \leq 0.023$). In the interval 0–4 h, mean percentage time pH ≥ 4 for netazepide 5 mg was significantly lower than for ranitidine 150 mg ($p = 0.043$); however, neither netazepide 25 mg nor 100 mg was significantly different from ranitidine. Furthermore, with one exception, mean percentage time pH ≥ 4 was significantly higher for all netazepide doses than for ranitidine during all other time intervals ($p \leq 0.010$). The exception was 13–24 h, during which mean percentage time pH ≥ 4 for netazepide 5 mg did not differ significantly from ranitidine. Netazepide 5 mg was about as effective as ranitidine 150 mg over 0–24 h.

Secondary response variables: Table 3

Netazepide affected mean AUC of gastric pH similarly to percentage time pH ≥ 4 . In all time intervals, AUC was significantly higher than for placebo ($p \leq 0.010$). In the first 4 h after dosing, mean AUC of pH for netazepide 5 mg and 100 mg was significantly lower than that for ranitidine ($p \leq 0.032$): however, it did not differ significantly between netazepide 25 mg and ranitidine. During all other time intervals, mean AUC of pH was significantly higher for all doses of netazepide than for ranitidine ($p \leq 0.032$). There was no carryover effect for time pH ≥ 4 , or for AUC of pH.

Pharmacokinetic/pharmacodynamic relationship: Figures 6–8

Pearson correlation coefficients among PK and PD parameters were weak; the best was between $\log AUC_{0-\infty}$ of netazepide and time pH ≥ 4 , for which r^2 was only 0.16.

The fourth order polynomial curve fitted the placebo-adjusted intragastric pH data well; the trendline for netazepide 100 mg was $r^2 = 0.91$. Comparison of the modelled plasma concentrations of netazepide with the modelled pH difference between netazepide and placebo showed a delay between maximum plasma concentrations and maximum pH. However, when we calculated netazepide concentrations in the peripheral compartment, they fitted the modelled pH profile more closely, although there was still some delay.

Discussion

Single doses of netazepide were well tolerated and safe, and markedly increased gastric pH. Netazepide was rapidly absorbed, with $t_{\max} \sim 1.0$ h. Elimination appeared biphasic, with the terminal phase beginning 4–12 h after dosing. Plasma concentrations after netazepide 100 mg were well above the limit of reliable quantification at all times up to 24 h, so $t_{1/2}$ after that dose (~ 7 h) is probably a true reflection of the behaviour of netazepide. The lower $t_{1/2}$ after smaller

doses probably reflects a faster initial phase of distribution and elimination rather than the true terminal elimination half-life. Netazepide had dose-proportional kinetics at the doses studied, and consistent bioavailability among subjects, as judged by AUC. Only 1–2% of the dose was excreted unchanged in urine, suggesting netazepide is cleared from plasma mainly by hepatic metabolism.

Gastric pH showed only the expected postprandial fluctuations after placebo, whereas all netazepide doses raised pH at some time after dosing. Mean percentage time $\text{pH} \geq 4$ for all netazepide doses was significantly higher than that for placebo or ranitidine. The effect of ranitidine was mainly in the first 4 h after dosing, whereas the effect of netazepide was more sustained and lasted at least 24 h, particularly after the higher doses. Overall, netazepide 5 mg was as effective as ranitidine 150 mg over the 24 h after dosing; netazepide 25 and 100 mg were much more effective.

There was only a weak correlation between plasma AUC and time $\text{pH} \geq 4$ ($r^2 = 0.16$). It appears, therefore, that the effect of netazepide is dependent on other factors in addition to plasma concentrations. The modelled effect trendlines for netazepide 5, 25 and 100 mg showed little difference between the 25 and 100 mg doses, with 25 mg having a near-maximum effect. That suggests 25 mg is at or close to the top of the dose-response curve for suppression of basal and food-stimulated gastric acid secretion.

The effect on pH showed only a loose temporal relationship with plasma netazepide concentrations. Since netazepide does not act in plasma, but via gastrin receptors on ECL cells in the stomach,⁶ that is not surprising. Modelling of netazepide concentration in the second compartment led to a much better, albeit still not perfect, relationship with effect. The model we used was probably too simple, because it assumes that only netazepide binds to gastrin receptors. In practice, gastrin also binds to the receptors, and it is possible that metabolites of netazepide may do so as well. Also, inhibition of gastric acid secretion, for example by H₂RA or PPI, results in an increase in circulating gastrin. Although we did not measure circulating gastrin in our subjects, netazepide does increase it in rats.^{25 26} An increase in circulating gastrin in our subjects may have confounded the modelling results.

Although netazepide is a benzodiazepine derivative, and there are gastrin receptors in the brain, we did not formally test its effect on the central nervous system of our subjects because netazepide showed no activity in an animal model of anxiety, whereas L-365,260 did (Ferring, Investigator's Brochure 1997). However, no subject reported relevant symptoms after netazepide. Also, in a study using positron emission tomography, ¹¹C-netazepide penetrated the brain poorly in rats.²⁷

Most of the gastrin receptor antagonists described have had problems with selectivity, potency or bioavailability.^{13 14 28} Several have been tested in man, but none has been marketed. L-365,260 and CR-2194 were given to healthy subjects to assess their effect on pentagastrin-stimulated gastric acid secretion, but the results were disappointing.^{29 30} L-365,260 and CI-988 have been assessed for anxiolytic activity in healthy subjects and patients.³¹⁻³⁶ Again, results were disappointing. More recently, JB95008, a gastrin antagonist with little oral bioavailability, was given by continuous intravenous infusion to patients with pancreatic cancer in two randomised controlled trials.^{37 38} JB95008 prolonged life compared with placebo, and was as effective as fluorouracil. Gastrin receptors are expressed on human pancreatic cancer cells, which grow when stimulated by gastrin.³⁹

Experiments in healthy subjects, in which food or intravenous gastrin 17 was used to stimulate gastric acid secretion, indicate that gastrin accounts for ~90% of acid secreted after a meal, although the contribution varied among individuals.⁴⁰ Our results support those findings.

Conclusions

Single oral doses of netazepide were well tolerated and safe, and produced a sustained increase in gastric pH, thus highlighting the major role of gastrin in controlling gastric acidity in man. Studies of repeated doses are justified.

Contributors

MB and SW did the studies in healthy subjects, and interpreted the results. AJ, TM and KD analysed the pH data. TM, OD and AJ analysed the PK data. OD and AJ did the PK/PD analyses. MB wrote the draft manuscript, and the others contributed to the final version.

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Table 1. Summary of pharmacokinetic parameters of plasma netazepide

Parameter	Dose of netazepide							
	Pilot study					Comparative study		
	0.5 mg	5 mg	25 mg	50 mg	100 mg	5 mg	25 mg	100 mg
C _{max} (ng/mL) mean (SD)	2.9 (1.7)	19.7 (10.3)	93.6 (42.3)	153.2 (43.1)	397.1 (160.6)	19.3 (9.9)	89.5 (78.0)	251.0 (102.9)
t _{max} (h) median (range)	0.5 (0.5–4.0)	0.75 (0.5–2.0)	0.88 (0.5–4.0)	0.5 (0.5–0.5)	0.8 (0.5–1.5)	1.0 (0.5–2.0)	0.5 (0.5–6.0)	1.0 (0.5–2.0)
AUC _{0–24 h} (ng.h/mL) mean (SD)	3.8 (2.1)	32.0 (7.7)	178.1 (48.1)	283.4 (59.6)	658.2 (182.4)	41.3 (14.7)	193.2 (68.7)	611.7 (144.1)
k _{el} (h ⁻¹) median (range)	0.45 (0.43–0.47) ^a	0.35 (0.26–0.40)	0.38 ^b (0.11–0.46)	0.40 ^b (0.37–0.44)	0.11 ^c (0.1–0.14)	0.33 (0.10–0.52)	0.13 (0.09–0.40)	0.09 (0.09–0.12)
t _{1/2} (h) median (range)	1.55 (1.5–1.6) ^a	2.05 (1.7–2.7)	1.8 (1.5–6.1) ^b	1.7 (1.6–1.9) ^b	6.6 (4.9–6.9) ^c	2.1 (1.3–7.1)	5.5 (1.7–7.3)	7.6 (5.6–7.9)
N	4	4	4	4	8	20	20	20

a, b & c: n = 2, 3 & 6, respectively

Medians were calculated if data were not normally distributed.

Table 2. Back-transformed means (n=20; 95% confidence interval) of percentage time pH \geq 4

Interval after dose	Netazepide 5 mg	Netazepide 25 mg	Netazepide 100 mg	Ranitidine 150 mg	Placebo
0–4 h	44.0*+ (28.2–60.5)	47.5* (27.4–68.1)	48.4* (28.3–68.8)	63.7* (55.5–71.4)	8.4 (1.3–20.8)
4–9 h	55.6*+ (42.0–68.8)	76.1*+ (62.3–87.6)	80.0*+ (67.2–90.3)	32.4* (22.2–43.6)	6.7 (2.8–12.0)
9–13 h	28.1*+ (19.9–37.1)	49.8*+ (36.8–62.9)	51.8*+ (38.7–64.8)	15.9 (9.1–24.2)	9.3 (4.7–15.2)
13–24 h	13.2* (8.0–19.3)	22.8*+ (15.2–31.5)	34.3*+ (24.9–44.4)	9.1 (5.6–13.4)	7.3 (3.7–12.1)

* significant difference from placebo (p<0.05)

+ significant difference from ranitidine (p<0.05)

Table 3. Geometric means (n=20; 95% confidence interval) of AUC for gastric pH

Interval after dose	Netazepide 5 mg	Netazepide 25 mg	Netazepide 100 mg	Ranitidine 150 mg	Placebo
0–4 h	15.5*+ (13.1–18.3)	16.1* (13.3–19.6)	16.4*+ (13.5–20.1)	19.9* (18.3–21.6)	9.8 (8.1–11.9)
4–9 h	19.5*+ (17.4–21.8)	22.7*+ (20.3–25.4)	23.5*+ (19.8–27.2)	16.5* (14.9–18.3)	11.2 (10.2–12.3)
9–13 h	12.6*+ (11.3–14.1)	15.4*+ (13.8–17.1)	15.8*+ (14.0–17.7)	10.7 (9.7–11.8)	9.6 (8.7–10.5)
13–24 h	27.1*+ (24.3–30.2)	31.9*+ (28.4–35.8)	36.2*+ (31.8–41.2)	24.5 (22.1–27.1)	23.7 (21.3–26.3)

* significant difference from placebo (p<0.05)

+ significant difference from ranitidine (p<0.05)

Figure 1. Pilot study: mean plasma concentrations of netazepide after single oral doses of 0.5 mg [•], 5 mg [•], 25 mg [•], 50 mg [•] and 100 mg [•]

See Table 1 for numbers of subjects per dose.

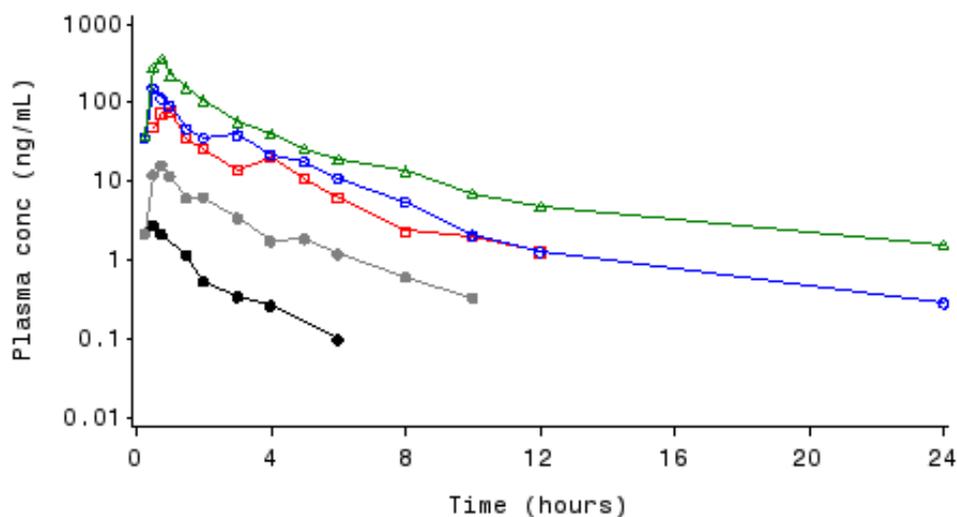


Figure 2. Comparative study: mean (\pm sd; n=20) plasma concentrations of netazepide after single oral doses of 5 mg [•], 25 mg [•] and 100 mg [•].

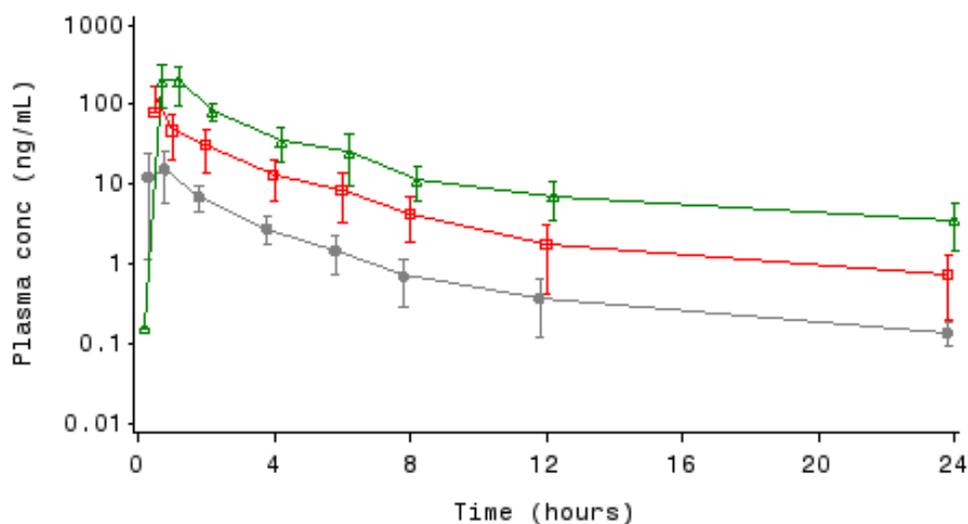


Figure 3. 24-h gastric pH (median; n=20) after single oral doses of (a) netazepide 5 mg , (b) netazepide 25 mg, (c) netazepide 100 mg, and (d) ranitidine 150 mg

For clarity, the profile of each active treatment is compared with that of placebo. Subjects ate standard meals at 4, 9, 13 & 22 h after dosing, and drank water (150 mL) at 2, 6, 8 & 11 h after dosing.

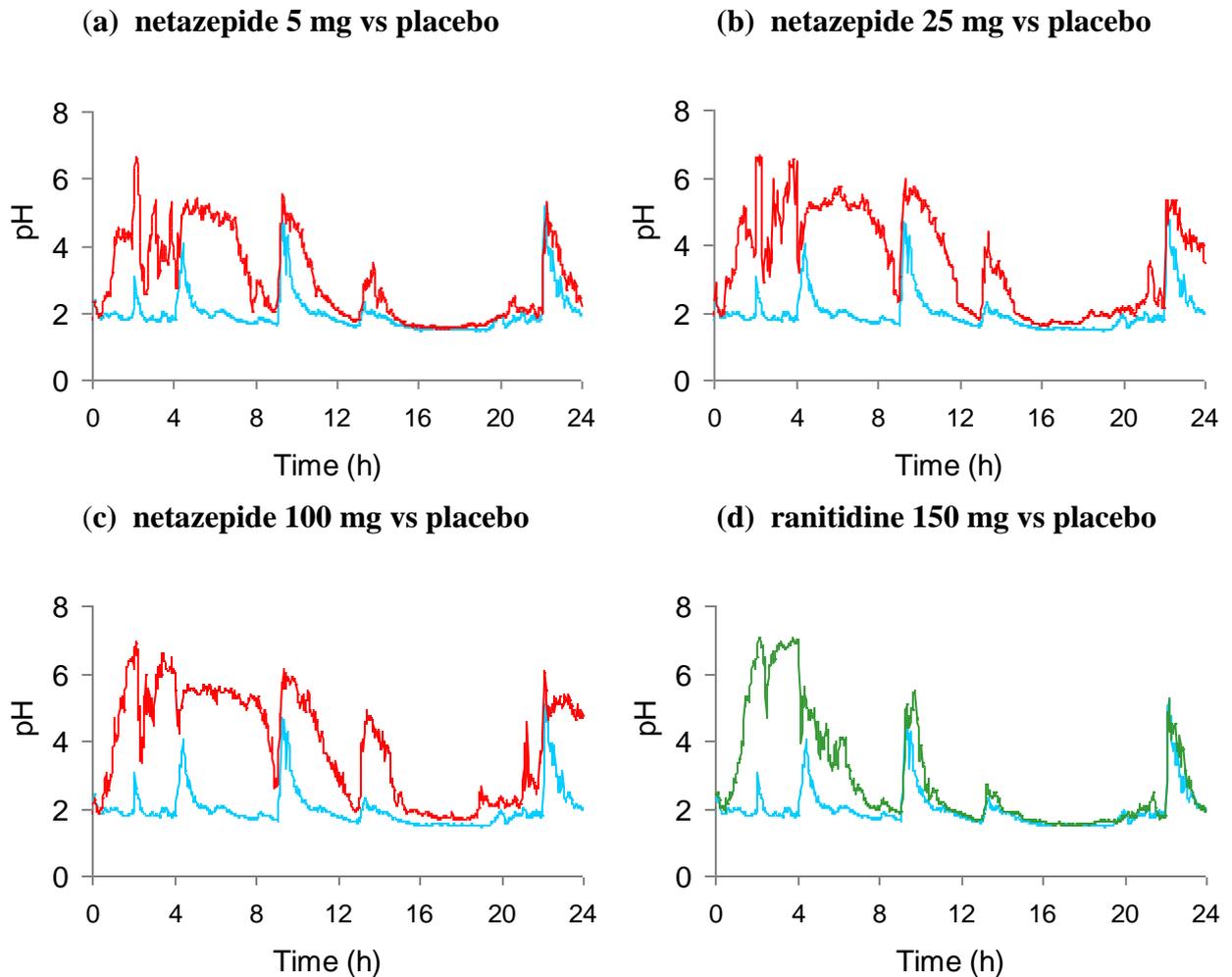


Figure 4. Median (n=20) % time gastric pH ≥ 4 during intervals 0–4, 4–9, 9–13, 13–24 & 0–24 h after single oral doses of netazepide (5, 25 & 100 mg), ranitidine (150 mg) and placebo.

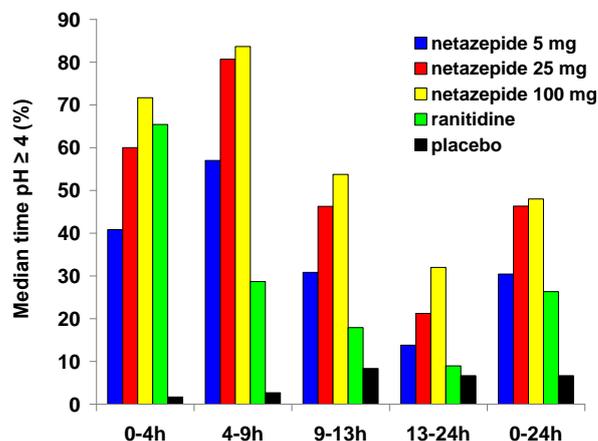


Figure 5. Box-whisker plots (n=20) of % time gastric pH ≥ 4 during (a) 0–4 h, (b) 4–9 h, (c) 9–13 h, (d) 13–24 h and (e) 0–24 h after single oral doses of netazepide (5, 25 & 100 mg), ranitidine (150 mg) and placebo. Mean (+), median, inter-quartile range, and range.

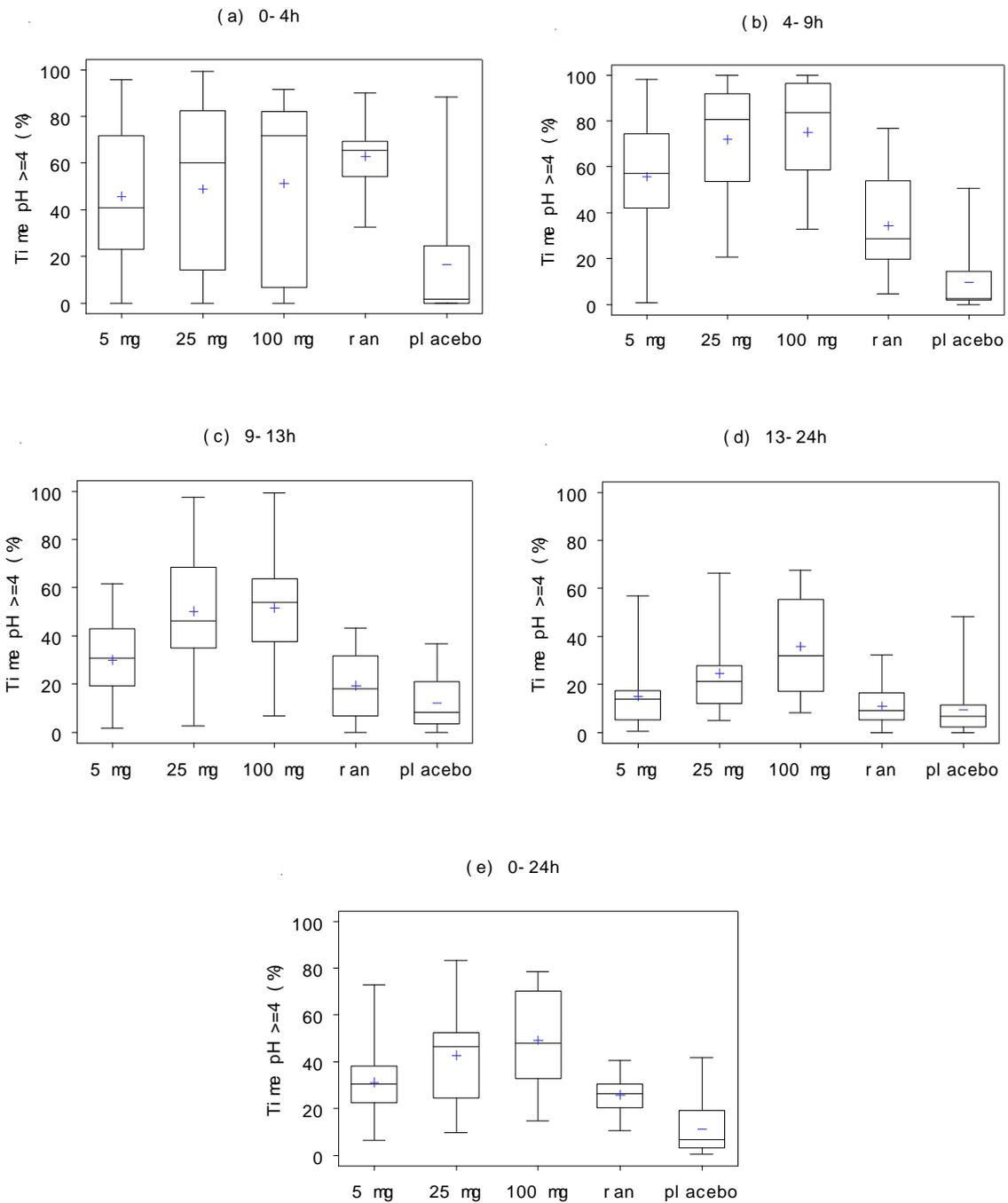


Figure 6. Fourth-order polynomial trendlines for the increase in pH induced by netazepide 5, 25 & 100 mg, calculated as the median differences between netazepide and placebo

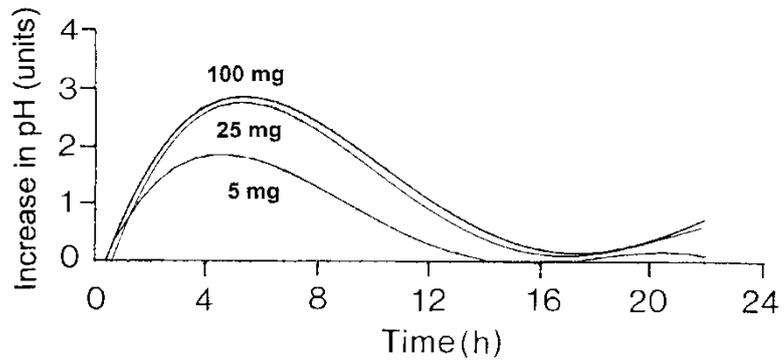


Figure 7. Modelled plasma concentrations of netazepide compared with placebo-adjusted increase in pH after netazepide 100 mg

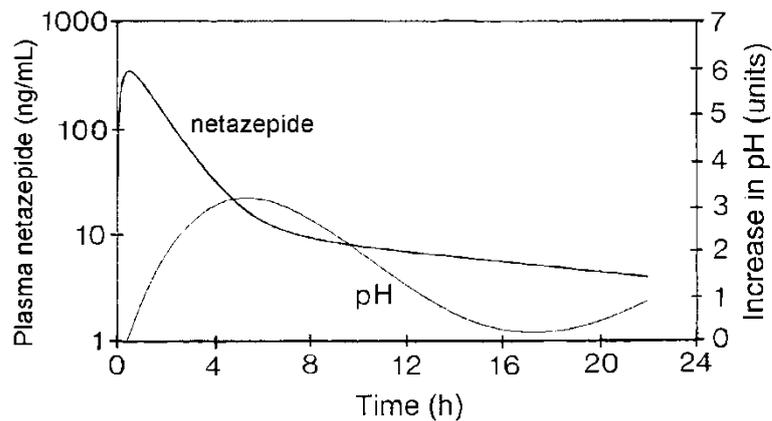
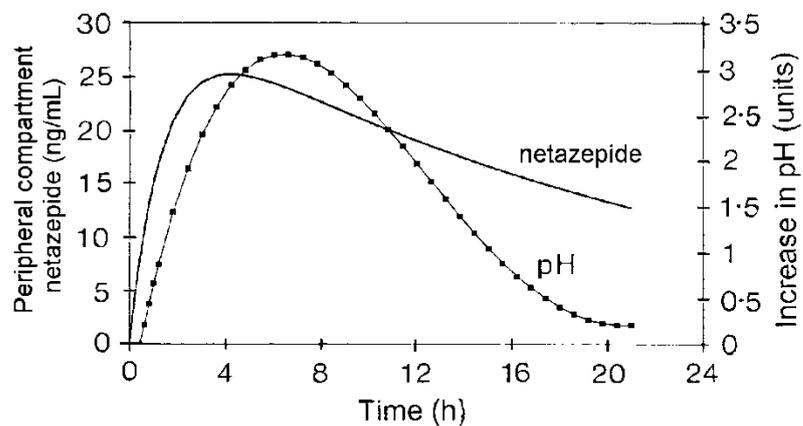


Figure 8. Modelled concentrations of netazepide in the peripheral compartment compared with the placebo-adjusted increase in pH after netazepide 100 mg



Chapter 4

Studies 3 and 4. Safety, tolerability, pharmacokinetics and effect on 24-h gastric pH and serum gastrin of repeated doses of netazepide, and comparison with placebo and omeprazole, a proton pump inhibitor, in healthy subjects

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Effect of repeated doses of netazepide, a gastrin receptor antagonist, omeprazole and placebo on 24 h gastric acidity and gastrin in healthy subjects.

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ABSTRACT

Aims

To administer repeated oral doses of netazepide to healthy subjects for the first time, to assess safety, tolerability, pharmacokinetics, and effect on 24-h gastric pH and plasma gastrin.

Methods

We did two randomised, double-blind, parallel-group studies. The first compared netazepide 25 and 100 mg 12-hourly, omeprazole 20 mg once daily, and placebo for 7 days. On Day 7 only, we measured pH and assayed plasma gastrin. The second study compared netazepide 5, 10 and 25 mg and placebo once daily for 14 days. We measured pH on Days 1, 7 and 14, and assayed plasma gastrin on Days 1 and 14. We compared treatments by time gastric pH ≥ 4 during 0–4, 4–9, 9–13 and 13–24h after the morning dose, and by plasma gastrin. $P < 0.05$ was significant.

Results

Netazepide was well tolerated. On Day 7 of the first study, netazepide increased pH significantly only during 9–13h after the 100 mg dose, whereas omeprazole raised pH significantly during all periods. But, both netazepide and omeprazole increased plasma gastrin significantly. Netazepide had linear pharmacokinetics. In the second study, netazepide caused dose-dependent, sustained increases in pH on Day 1, but as in the first study, netazepide had little effect on pH on Days 7 and 14. Again, netazepide increased plasma gastrin significantly.

Conclusion

Although repeated doses of netazepide led to tolerance to its effect on pH, the accompanying increase in plasma gastrin is consistent with continued inhibition of acid secretion, via gastrin receptor antagonism and gene up-regulation.

What is already known about this subject

- Non-clinical studies have shown that netazepide (YF476) is a potent, highly selective and orally-active gastrin receptor antagonist. Activity, including suppression of gastric acid secretion, persists during repeated dosing.
- In healthy subjects, single oral doses of netazepide causes dose-dependent increases in 24h gastric pH. Onset of activity is as fast as ranitidine but much longer lasting.

What this study adds

- A single oral dose of netazepide again caused dose-dependent, sustained increases in 24h gastric pH in healthy subjects, but tolerance developed during repeated dosing, whereas the effect of omeprazole persisted.
- Despite tolerance to the effect of netazepide on pH, netazepide increased plasma gastrin, as did omeprazole, which is consistent with persistent acid suppression by netazepide.
- pH is not the best way to assess acid suppression by netazepide.

Introduction

The hormone gastrin [1] causes gastric acid secretion via stimulation of gastrin receptors (CCK-B or CCK-2 receptors) on enterochromaffin-like (ECL) gastric mucosal cells, which release histamine. That in turn stimulates histamine H₂ receptors on parietal cells in the gastric mucosa [2], and thereby secretion of acid into the lumen of the stomach. Acid production is mediated by the proton pump on parietal cells, which also express gastrin receptors [3], but the role of those receptors in acid secretion is uncertain [4]. Gastrin also stimulates growth of ECL and parietal cells [1, 5, 6].

Non-clinical studies have shown that netazepide (YF476) is a potent, selective, competitive and orally active antagonist of gastrin receptors [7–9]. In our double-blind, placebo-controlled, crossover study of single doses of netazepide 5, 25 or 100 mg compared with ranitidine 150 mg in healthy subjects, netazepide was well tolerated and caused dose-dependent, long-lasting increases in basal and food-stimulated 24-h gastric pH, consistent with antagonism of gastrin receptors [10]. Onset of activity of netazepide and ranitidine was similarly rapid. However, activity of ranitidine lasted about 12 h, whereas that of netazepide exceeded 24 h. Compared with ranitidine 150 mg, netazepide 5 mg was as effective, and netazepide 25 and 100 mg were much more effective, over the 24 h after dosing. Median t_{\max} and $t_{1/2}$ after the 100 mg dose were about 1 and 7 h, respectively, and the pharmacokinetics were dose-proportional.

Those encouraging results justified further studies in healthy subjects, and we now report the first administration of repeated doses of netazepide. We did two parallel-group studies. The first compared netazepide 25 and 100 mg twice daily, omeprazole 20 mg once daily, and placebo for 7 days. The aim was to assess the safety, tolerability and pharmacokinetics of netazepide, and to compare its effects on gastric pH and plasma gastrin with those of omeprazole. The second study compared netazepide 5, 10 and 25 mg and placebo once daily for 14 days, to find out whether lower doses of netazepide would give results similar to those of the first study.

We have presented these studies at meetings of the Clinical Section of the British Pharmacological Society [11–12].

Methods

We did the studies at the Central Middlesex Hospital, London, England in accordance with the ICH Guideline for GCP and Declaration of Helsinki. Brent REC approved the studies. Subjects gave written informed consent. ClinicalTrials.gov NCT01599858 and NCT01597674.

Treatments

Yamanouchi Pharmaceutical Co, Japan, supplied netazepide 5 and 25 mg capsules and matching placebo. The hospital pharmacy: supplied encapsulated omeprazole 20 mg tablets (Losec; AstraZeneca) and matching placebo capsules; packed and labelled treatments; and randomised subjects to treatments.

Study design

First study

This study was double-blind, double-dummy and parallel-group in design, and required 48 healthy men or women. Women not using a reliable method of contraception were excluded. Subjects were randomised to one of 4 treatments by mouth for 7 days: netazepide 25 mg twice daily; netazepide 100 mg 12-hourly; omeprazole 20 mg once daily; and placebo. They took the morning dose after fasting overnight. Subjects were resident for 8 nights, and returned for follow-up 5–10 days after the last dose. On Day 7, they fasted overnight and had standard meals and drinks at 4, 9, 13 and 22 h after dosing. Meals and drinks were at usual times on all other days. We recorded intragastric pH every 6 seconds for 24 h on Day 7, using a nasogastric pH electrode [10]. We took blood samples before each morning dose on Days 2–7 and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 h after the second dose on Day 7, for assay of plasma netazepide. We also took blood samples at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 20, 22 and 24 h after the morning dose on Day 7, for assay of plasma gastrin. We assessed safety and tolerability by adverse events, vital signs, ECG, and safety tests of blood and urine.

Second study

This study was double-blind and parallel-group in design, and required 48 men. Subjects were randomised to one of 4 treatments by mouth once daily for 14 days: netazepide 5, 10 and 25 mg, and placebo, which they took in the morning after fasting overnight. On Days 1, 7 and 14, they ate standard meals at 4, 9, 13 and 22 h after dosing. On Days 3–6 and 9–13, they did not eat until at least 30 min after dosing, but were allowed water as required. All other meals and drinks were at usual times on those days. Subjects were resident for 2 nights on each of 3 occasions: from the evening before until the morning after dosing on Days 1, 7 and 14. On Days 3–6 and 9–13, subjects attended each morning, for dosing only. On Days 1, 7 and 14, we recorded ambulatory gastric pH every 6 seconds from 0.5 h before until 24 h after dosing, and we took frequent blood samples from 0 to 24 h after dosing, for assay of plasma gastrin, as in the first study. We also assessed safety and tolerability as in the first study.

Gastric pH

We prepared subjects and measured 24-h gastric pH, as described previously [10].

Plasma gastrin

We put 4 mL blood into lithium-heparin tubes containing 0.2 mL aprotinin 10,000 units/mL (Trasylol; Bayer), and centrifuged them (4°C; 800 G for 10 min) within 15 min of collection. We stored plasma in polypropylene tubes at -20 °C until assay by ASI, St George's Hospital, London using a validated ¹²⁵I-radioimmunoassay (GammaDab[®], DiaSorin, Stillwater, Minnesota, USA). The calibration line was linear over the concentration range 40 to 1000 pg/L. The sensitivity was 6 pg/L and the coefficient of variation was ~4%.

Plasma netazepide

We assayed plasma netazepide by HPLC-MS [13], as described previously [10].

Statistics

24-h gastric pH

From the results of our study of single doses of netazepide [10], we calculated 12 subjects per group to be sufficient for both studies to detect a 70% increase in area-under-the-curve (AUC) for gastric pH versus time, with at least 80% power, assuming a between-subject coefficient of variation of 0.46 and significance of 5%. We analysed pH data in 4 intervals: 0–4, 4–9, 9–13 and 13–24 h after the morning dose. For each interval, we calculated AUC, using the trapezoidal rule, and time pH ≥4.

We tested for differences among treatments by analysis of variance (ANOVA), after log-transformation of variables, where appropriate. All tests were two-sided, and the significance level was $\alpha = 0.05$. If treatment effect was significant in the overall model, we made pair wise comparisons between each active treatment and placebo, using the Wilcoxon rank sum test. The primary response variable was time pH ≥4.

To illustrate pH results, for the first study we calculated median values for every 1 h from 0 to 14 h after dosing and every 2 h from 14 to 24 h after dosing, and for the second study we calculated median values for every 2 min from 0 to 24 h after dosing. We calculated median values because the data were not normally distributed.

Plasma gastrin

For both studies, we calculated plasma gastrin AUC_{0–24 h} by the trapezoidal rule. In the first study, we compared treatments by ANOVA. In the second study, we measured plasma gastrin only for the netazepide 5 and 25 mg groups on Days 1 and 14. So, in a post-hoc analysis, we

used a Kruskal Wallis test to compare the results for $AUC_{0-24\text{ h}}$ of plasma gastrin for netazepide 5 and 25 mg with those of placebo from the first study. We used a Wilcoxon rank sum test to do pairwise comparisons between netazepide dose levels and placebo only if there was an overall significant difference among treatments.

Pharmacokinetics

We used WinNonlin to derive pharmacokinetic parameters for plasma netazepide concentrations after the second dose on Day 7 of the first study: C_{\max} ; T_{\max} ; AUC_t ; $AUC_{0-\infty}$; and $t_{1/2}$, as described previously [10]. We used netazepide concentrations before dosing on Days 2–7 to assess when steady state had been reached and whether netazepide had accumulated after repeated doses.

Results

Subjects

49 subjects, 25 women and 24 men, entered the first study. Their mean age and body mass index (BMI) were 24.1 y (range 19–33 y) and 23.3 kg/m^2 (range 19.5–29.6 kg/m^2), respectively. All subjects were Europid. We withdrew one woman whose pH electrode failed. 4 groups of 12 subjects completed the study.

49 men entered the second study. Their mean age and BMI were 24.9 y (range 18–40 y) and 23.86 kg/m^2 (range 19.5–28.4 kg/m^2), respectively. 47 subjects were Europid, 1 was Negroid and 1 was Asian/Indian. We withdrew 1 subject after 5 doses of what proved to be netazepide 25 mg, because of nausea, abdominal discomfort and diarrhoea. Another subject had a high basal gastric pH and plasma gastrin, consistent with achlorhydria, so we excluded his results from the statistical analysis. 3 groups of 12 subjects and 1 group of 11 (netazepide 5 mg) subjects completed the study.

Tolerability and safety

Netazepide was well tolerated in both studies. Subsequent re-challenge of the subject who had gastro-intestinal symptoms after 5 doses of netazepide 25 mg was uneventful. Otherwise, any adverse events were minor, transient and occurred across the treatments. There were no clinically relevant changes in any of the safety assessments.

Pharmacodynamics

First study

Median times gastric $\text{pH} \geq 4$ are in Table 1, and median gastric pH values versus time are illustrated in Figure 1a. Median $AUC_{0-24\text{ h}}$ of plasma gastrin concentrations are in Table 2, and median plasma gastrin concentrations versus time are illustrated in Figure 1b.

There were characteristic and predictable variations in gastric pH during placebo treatment, corresponding to the times at which subjects ate and drank. Compared with placebo, gastric pH tended to be higher in the post-prandial periods after netazepide, but the time $\text{pH} \geq 4$ was significant ($p < 0.05$) only for netazepide 100 mg during the period 9–13 h after the morning dose. Omeprazole increased the time gastric $\text{pH} \geq 4$ significantly ($p < 0.05$) compared with either placebo, netazepide 25 or 100 mg, during all periods except 9–13 h after netazepide 100 mg. Compared with placebo, netazepide 25 mg ($p = 0.02$), netazepide 100 mg ($p = 0.01$) and omeprazole ($p = 0.001$) all significantly increased $\text{AUC}_{0-24 \text{ h}}$ of plasma gastrin. Gastrin concentrations after omeprazole were higher than those after netazepide 25 and 100 mg, especially after food, but the differences were not significant.

Second study

Median times gastric $\text{pH} \geq 4$ are in Table 3 and median gastric pH values versus time are illustrated in Figure 2. Median $\text{AUC}_{0-24 \text{ h}}$ of plasma gastrin concentrations are in Table 4, and median plasma gastrin concentrations versus time are illustrated in Figure 3. Again, there were characteristic and predictable variations in median gastric pH during placebo treatment, corresponding to the times at which the subjects ate and drank.

On Day 1, gastric pH increased quickly after dosing with netazepide. After all meals, gastric pH fell more slowly after netazepide than after placebo. Even after breakfast, at 24 h after dosing, pH was higher after netazepide than after placebo. Trends were similar for all dose levels of netazepide. On Day 1, compared with placebo: netazepide 5 mg significantly increased the time $\text{pH} \geq 4$ only during the 0–4 h period; netazepide 10 mg significantly increased the time $\text{pH} \geq 4$ during the 0–4, 4–9 and 9–13 h periods; and netazepide 25 mg significantly increased the time $\text{pH} \geq 4$ during all time periods up to 24 h after dosing. In contrast, on Days 7 and 14, compared with placebo, gastric pH tended to be higher in the post-prandial periods after netazepide, but the time $\text{pH} \geq 4$ was significantly higher only during 9–13 h after netazepide 10 or 25 mg.

Compared with placebo, plasma gastrin concentrations were higher after netazepide 5 and 25 mg on Days 1 and 14. On Day 1, there were no significant differences among netazepide 5 mg and 25 mg and placebo for $\text{AUC}_{0-24 \text{ h}}$ of plasma gastrin ($p = 0.26$). However, there was a significant difference on Day 14 ($p = 0.04$). Therefore, we used a Wilcoxon rank-sum test to do pairwise comparisons for Day 14. There was no significant difference between netazepide 5 mg and placebo ($p = 0.37$), but there was a significant difference between netazepide 25 mg and placebo

($p=0.01$). There was no significant difference among netazepide doses for AUC_{0-24h} of plasma gastrin on Day 1, nor on Day 14.

Pharmacokinetics

Mean pharmacokinetic parameters for plasma netazepide concentrations are in Table 5, and trough concentrations of YF476 are in Table 6. Netazepide was rapidly absorbed; mean T_{max} for 25 and 100 mg after the second dose on Day 7 were 0.75 and 1.0 h, respectively. Trough concentrations of netazepide indicate that steady state was reached by Day 3 and that there was little or no accumulation by Day 7. AUC_t and C_{max} increased in the ratio 4.8:1 and 4.7:1, respectively, while the dose increased in the ratio 4:1. Thus, the pharmacokinetics of netazepide were dose-proportional in the range studied.

Discussion

We were surprised by the trivial effect on 24-h gastric pH of netazepide on Day 7 of 25 and 100 mg 12-hourly in the first study, given that single doses of 5, 25 and 100 mg had caused dose-dependent, sustained increases in 24-h gastric pH in our previous study in healthy subjects [10]. The discordant results cannot be attributed to deficiencies in our methods, because omeprazole increased gastric pH on Day 7, as expected. Furthermore, netazepide 25 and 100 mg and omeprazole all increased 24-h plasma gastrin on Day 7. Proton pump inhibitors (PPI) such as omeprazole increase circulating gastrin via inhibition of gastric acid production and up-regulation of the gastrin gene [14]. However, tolerance to the acid-suppressant effect of omeprazole does not develop after repeated doses despite the increase in gastrin, because PPI act directly on the proton pump – the final stage in acid secretion. Histamine H_2 -receptor antagonists (H_2RA), such as cimetidine and ranitidine, which are competitive antagonists, also increase circulating gastrin, via inhibition of acid secretion and up-regulation of histamine H_2 -receptors and adenylate cyclase in the parietal cell [15]. But, unlike the increase in circulating gastrin induced by PPI, the increase induced by H_2RA does lead to tolerance [16 17], because the increase in circulating gastrin by H_2RA stimulates release of histamine from ECL cells, which reduces antagonism by the H_2RA of histamine H_2 -receptors on the parietal cell. Indeed, lupitidine (SK&F 93479), a potent, long-acting and irreversible H_2RA , caused hypergastrinaemia profound enough to overcome suppression of gastric acid production [18, 19]. PPI replaced H_2RA as the preferred treatment for acid-related conditions when it was discovered that tolerance to H_2RA after repeated dosing reduces their efficacy [20].

Initially, we wondered whether tolerance to netazepide in the first study might have been due to the observed increase in plasma gastrin, similar to the mechanism of tolerance to an H_2RA .

Therefore, we did a second study using netazepide 5, 10 and 25 mg once daily for 14 days, to assess whether lower and longer exposure to netazepide might prevent or reduce tolerance. We also improved the study design, by measuring 24-h gastric pH and collecting plasma samples for gastrin assay on Days 1 and 14 as well as on Day 7. On Day 1, the first dose of netazepide 5, 10 and 25 mg caused dose-dependent, sustained increases in gastric pH, as did single doses of netazepide 5, 25 and 100 mg in our previous study [10]. However, the effect of netazepide on gastric pH was small on Day 7, and even smaller on Day 14. Because of those confirmatory results, the study sponsor decided to limit the number of plasma samples assayed for gastrin to those collected after netazepide 5 and 25 mg on Days 1 and 14. Therefore, for comparison, we used the results for placebo from the first study; the comparison was valid because both studies were parallel group in design and used similar subjects and methods. Despite the limited data from the second study, netazepide clearly increased circulating gastrin, as in the first study.

Thus, overall, the results of these two repeated-dose studies of netazepide were similar.

Tolerance to the effect of netazepide on gastric pH occurred throughout the dose range studied.

Studies in rats subsequent to our studies in healthy subjects have also shown that netazepide increases circulating gastrin [14, 21–29]. The response is secondary to acid suppression by netazepide, which like omeprazole increases gastrin gene expression [14]. Furthermore, activity of netazepide persisted in rats dosed for up to 6 months, whether assessed by its ability to suppress gastric acid production or to prevent the growth-promoting effects of hypergastrinaemia on ECL cells [14, 21–29]. In all the aforementioned studies in rats, gastric acid production was assessed by measurement of H^+ secretion, not by pH. Studies of other gastrin receptor antagonists in rats have also shown that activity persists after chronic dosing [30, 31]. The increase in circulating gastrin induced by netazepide does not lead to tolerance to its ability to inhibit H^+ secretion, because netazepide blocks gastrin receptors on ECL cells thereby reducing or preventing release of histamine. Any reduction in gastric acid production – such as by PPI, H_2RA , vagotomy [32, 33], or chronic atrophic gastritis [34] – leads to an increase in circulating gastrin. Thus, the increase in circulating gastrin induced by repeated doses of netazepide in our healthy subjects is consistent with persistent suppression of gastric acid production. Indeed, we have since confirmed that netazepide does cause persistent suppression of gastric acid production, by showing that repeated doses of 100 mg daily inhibit the increase in pentagastrin-stimulated volume and H^+ content of gastric aspirate, despite tolerance to the effect on pH [35].

Gastric pH is easy to measure continuously with a nasogastric electrode, and has been widely used in clinical trials as a surrogate measure of acid suppression by H₂RA and PPI [36]. A substantial increase in time pH \geq 4 is regarded as essential to heal acid-related conditions. Because repeated doses of netazepide failed to achieve that goal, the development of netazepide for its original target disease of gastro-oesophageal reflux was abandoned for several years. pH is a logarithmic scale, so gastric pH may change little despite a large change in H⁺ secretion [4, 37] Furthermore, measurement of gastric pH alone ignores changes in volume of secretion. Therefore, the total amount of H⁺ collected per unit time would be a better test of acid suppression than pH.

Plasma concentrations of netazepide after repeated doses were similar to those after single doses in our previous study [10], which excludes a pharmacokinetic explanation for tolerance to the effect of netazepide on gastric pH. As in our single-dose study, netazepide appeared to be eliminated biphasically, the terminal elimination phase beginning at 4–12 h after dosing. Since the interval between T_{max} and the last sampling time was only 11 h, and the last sample was at 12 h after dosing, the calculated t_{1/2} is unlikely to represent only the terminal elimination phase. The mean half-lives of about 2–4 h quoted for many subjects are probably influenced by the more rapid first phase of elimination, whereas the higher values are likely to represent a more accurate estimate of the true terminal elimination half-life. Also, the mean values of λ_z , t_{1/2}, and AUC should be regarded only as estimates, because of the variability of the data and the short measurement period relative to the calculated terminal half-life.

Pre-dose plasma netazepide concentrations on Days 2–7 suggest that little if any accumulation of netazepide occurred during repeated dosing. The trough values show that steady state had been reached by Day 3, 48 h after the first dose, which is consistent with an elimination half-life of netazepide of 10 h or less, based on the assumption that steady state is typically reached within 5 half-lives after the start of dosing.

Conclusions

Netazepide was well tolerated. Single doses caused dose-dependent, sustained increases in gastric pH, as in our previous study. Although tolerance to the effect on gastric pH developed during repeated doses of netazepide, the increase in circulating gastrin is consistent with persistent suppression of gastric acid, via gastrin receptor antagonism and up-regulation of the gastrin gene. Further studies are required to find out the mechanism for tolerance, and whether it might matter therapeutically.

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Table 1. First study: median time (h) gastric pH ≥ 4 on Day 7

Time after morning dose	Placebo n=12	Netazepide 25 mg twice daily n=12	Netazepide 100 mg twice daily n=12	Omeprazole 20 mg once daily n=12
0–4 h	0.0 (B)	0.0 (B)	0.0 (B)	2.1 (A)
4–9 h	0.9 (B)	0.6 (B)	0.6 (B)	2.6 (A)
9–13 h	0.5 (C)	0.7 (BC)	1.2 (AB)	1.8 (A)
13–24 h	1.1 (B)	0.2 (B)	1.0 (B)	2.9 (A)

In each time period, treatments with the same letter in parentheses are not significantly different from each other ($p < 0.05$)

Table 2. First study: median (range) $AUC_{0-24\text{ h}}$ of plasma gastrin (ng.h/L) on Day 7

Gastrin $AUC_{0-24\text{ h}}$ (ng.h/L)	Placebo (n = 12)	Netazepide 25 mg twice daily (n = 12)	Netazepide 100 mg twice daily (n = 12)	Omeprazole 20 mg once daily (n = 12)
Median	985	1701*	1556**	2140***
Range	295–1620	390–3086	858–3743	1144–3211

Compared with placebo: * $p=0.02$ ** $p=0.01$ *** $p=0.001$

Table 3. Second study: median time (h) gastric pH ≥ 4 on Days 1, 7 & 14

Day	Day, and time interval (h) after morning dose	Placebo n=12	Netazepide		
			5 mg n=11	10 mg n=12	25 mg n=12
1	0–4	0.0	1.5*	1.7*	2.3*
	4–9	0.1	1.0	1.2*	3.6*
	9–13	0.3	0.5	1.2*	1.5*
	13–24	0.3	0.4	1.0	2.0*
7	0–4	0.1	0.0	0.1	0.1
	4–9	0.5	0.7	0.9	1.4
	9–13	0.4	0.9	1.2*	1.4*
	13–24	0.7	0.8	0.9	1.2
14	0–4	0.0	0.0	0.0	0.0
	4–9	0.6	0.7	0.6	1.0
	9–13	0.8	0.6	0.9	1.5
	13–24	1.1	0.9	0.3	1.1

* Compared with placebo, $p < 0.05$

Table 4. Second study: median AUC_{0-24 h} of plasma gastrin concentrations (ng.h/L)

	Placebo (n=12)	Netazepide 5 mg (n=11)		Netazepide 25 mg (n=12)	
		Day 1	Day 14	Day 1	Day 14
Median	985	1093	1093	1111	1315*
Range	295–1620	772–1998	738–2046	748–3304	981–5296

Placebo data from Day 7 of Study 1

* Compared with placebo, p=0.01

Table 5. First study: mean (range; n=12) pharmacokinetic parameters of plasma netazepide after the second dose on Day 7

Parameter	Netazepide 25 mg 12-hourly	Netazepide 100 mg 12-hourly
C _{max} (ng/mL)	120 (76–188)	569 (270–958)
T _{max} (h)	0.75 (0.5–4.0)	1.0 (0.5–2.0)
AUC _t (ng h/mL)	196 (160–300)	933 (686–1379)
AUC _{0-∞} (ng h/mL)	205 (177–307)	981 (754–1415)
λ _z (h ⁻¹)	0.20 (0.10–0.34)	0.17 (0.11–0.25)
t _{1/2} (h)	3.4 (2.0–7.3)	4.1 (2.8–6.3)

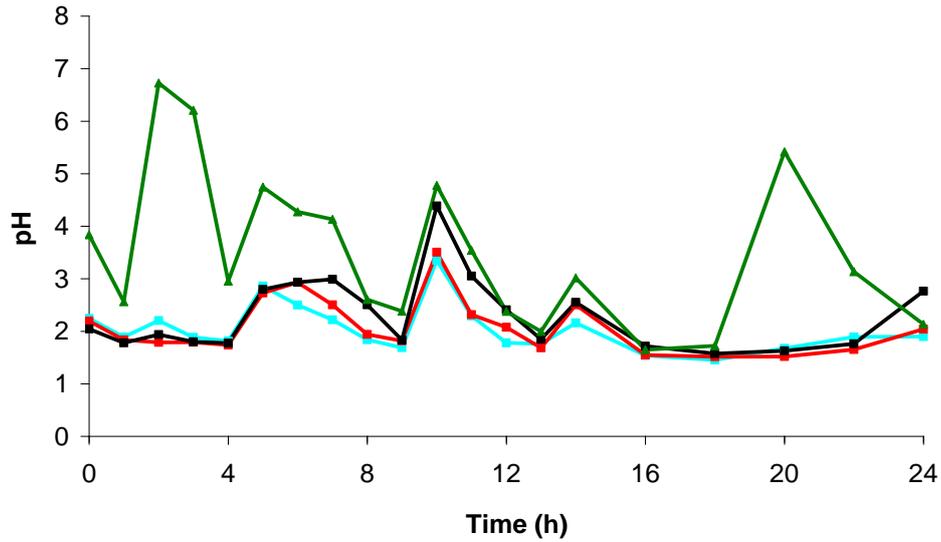
Table 6. First study: mean (sd; n=12) plasma netazepide concentrations (ng/mL) before the morning dose on Days 2–7

Day	Netazepide 25 mg 12-hourly	Netazepide 100 mg 12-hourly
2	1.66 (0.81)	5.41 (3.00)
3	1.97 (1.20)	8.43 (5.34)
4	1.96 (1.33)	6.59 (2.34)
5	1.88 (0.69)	6.83 (3.55)
6	1.80 (0.77)	6.79 (2.96)
7	1.50 (0.73)	7.66 (3.06)

Figure 1. First study: median (a) 24-h gastric pH and (b) 24-h plasma gastrin (ng/L) on Day 7. n=12 per group

placebo — omeprazole — netazepide 25 mg — netazepide 100 mg —

(a)



(b)

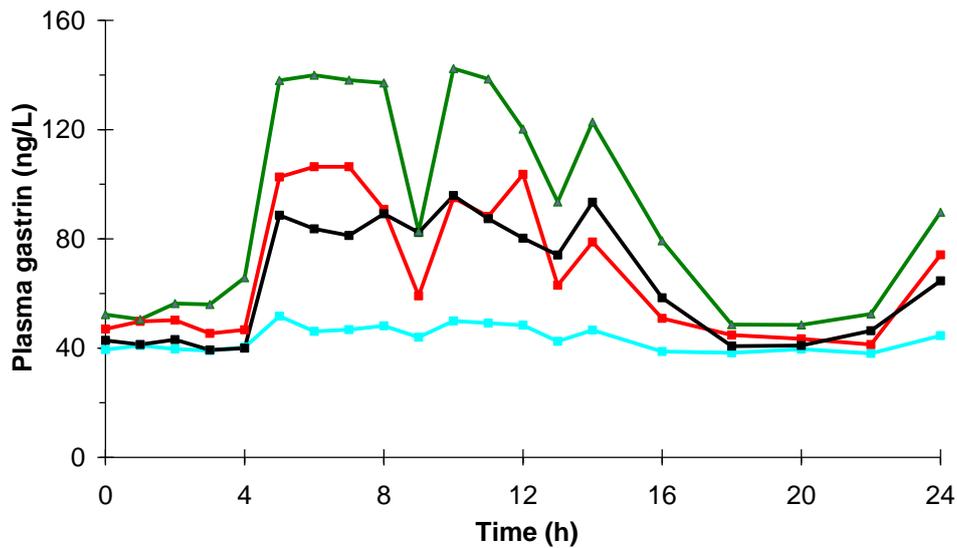
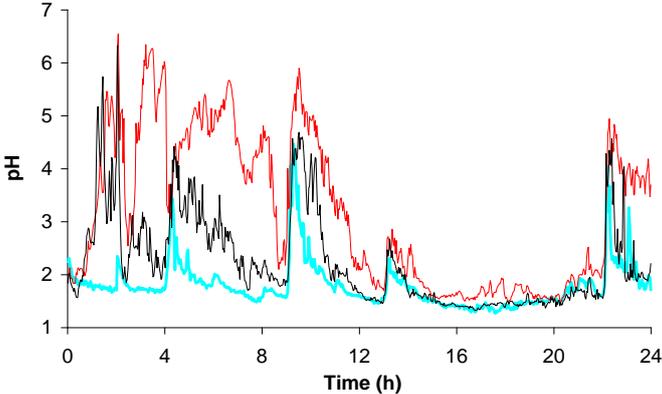


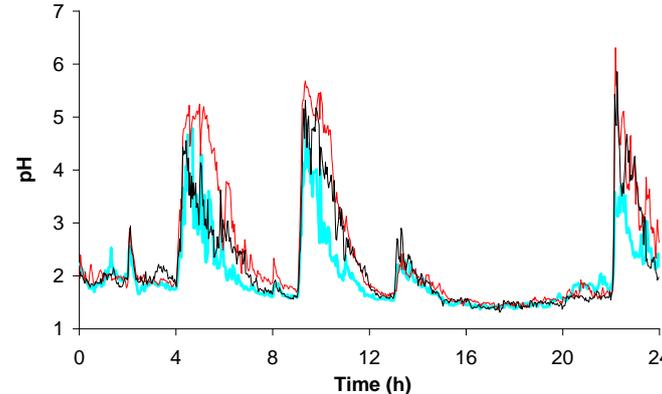
Figure 2. Second study: median gastric pH on Days 1, 7 and 14 of netazepide 5 mg (—), netazepide 25 mg (—) and placebo (—).

n=12 per group, except netazepide 5 mg where n=11.
Netazepide 10 mg omitted for clarity

Day 1



Day 7



Day 14

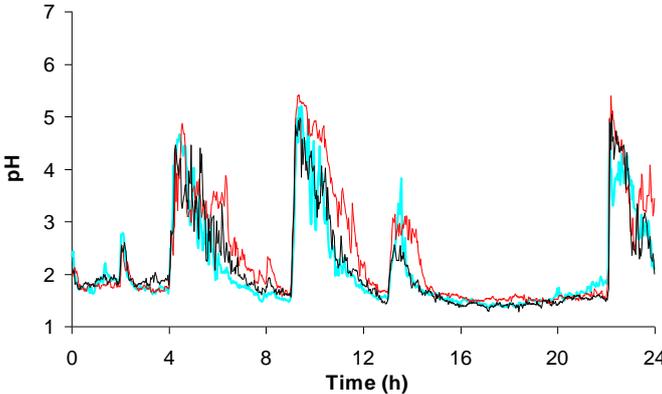
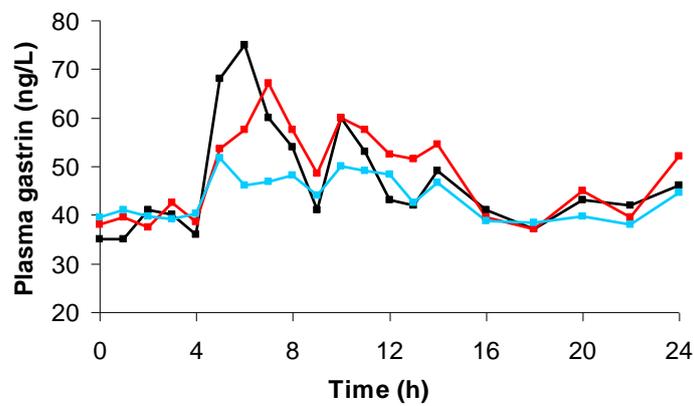
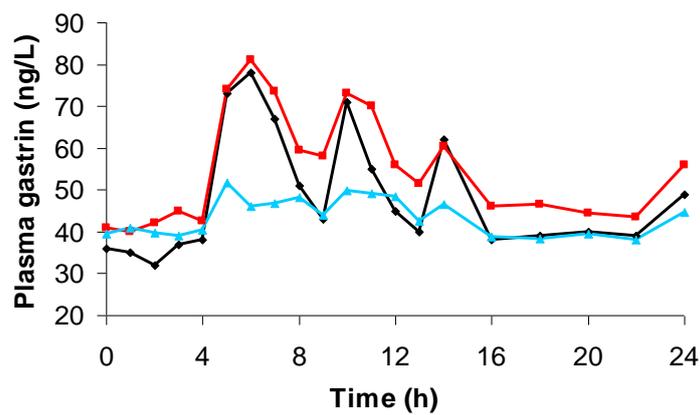


Figure 3. Second study: median plasma gastrin concentrations (ng/L) on Days 1 and 14 of netazepide 5 mg (n=11) and 25 mg (n=12) once daily. Placebo data (n=12) from Day 7 of the first study.
netazepide 5 mg (—), netazepide 25 mg (—) and placebo (—)

Day 1



Day 14



Chapter 5

Studies 5 and 6. Netazepide, a gastrin receptor antagonist, causes dose-dependent, persistent inhibition of pentagastrin-induced gastric acid secretion in healthy subjects

Boyce M, Warrington S, Black JW.

Netazepide, a gastrin/CCK₂ receptor antagonist, causes dose-dependent, persistent inhibition of the responses to pentagastrin in healthy subjects.

Br J Clin Pharmacol 2013; 76: 689–698.

Abstract

Aims

To confirm by means of pentagastrin, a synthetic gastrin agonist, that netazepide is a gastrin receptor antagonist in healthy subjects, and that antagonism persists during repeated dosing.

Methods

We did two studies in which we infused pentagastrin 0.6 µg/kg/h intravenously and measured volume, H⁺ secretion rate and pH of gastric aspirate. First, we did a double-blind, 5-way crossover study (n=10) to assess the effect of single oral doses of netazepide 1, 5, 25 and 100 mg and placebo on the response to pentagastrin. Then, we did a single-blind, placebo-controlled study (n=8) to assess the effect of the first and last oral doses of netazepide 100 mg twice daily for 13 doses on the response to pentagastrin.

Results

Netazepide was well tolerated. Pentagastrin increased volume and H⁺ secretion rate, and reduced pH, of gastric aspirate after placebo. Compared with placebo, single doses of netazepide caused dose-dependent inhibition of the pentagastrin response (p<0.05); netazepide 100 mg abolished the response. After 13 doses, the reduction in volume and H⁺ secretion rate persisted (p<0.05), but the effect on pH was mostly lost.

Conclusions

Netazepide is an orally active, potent, competitive antagonist of human gastrin receptors. Antagonism is dose-dependent and persists during repeated dosing, despite tolerance to the effect on pH. Further studies are required to explain that tolerance. Netazepide is a tool to study the physiology and pharmacology of gastrin, and merits studies in patients, to assess its potential to treat gastric acid-related conditions and the trophic effects of hypergastrinaemia.

What is already known about this subject

- Gastrin controls gastric acid secretion and mucosal cell growth, especially ECL and parietal cells.
- Hypergastrinaemia can be harmful, and there is an unmet clinical need for a gastrin receptor antagonist.
- Non-clinical studies have shown that netazepide is a potent, highly selective and orally-active gastrin receptor antagonist.
- In healthy subjects, single oral doses of netazepide caused sustained dose-dependent increases in 24-h gastric pH, but that effect was mostly lost after repeated dosing, whereas the effect of omeprazole on 24-h gastric pH persisted. However, like omeprazole, netazepide increased 24-h circulating gastrin, consistent with persistent suppression of gastric acid secretion.

What this study adds

- Despite tolerance to its effect on pH, during repeated dosing netazepide causes persistent inhibition of H⁺ secretion.
- H⁺ secretion rate is a better indicator of acid production than is pH.
- Netazepide is a potential new treatment for acid-related conditions and the trophic effects of hypergastrinaemia.

Introduction

Non-clinical studies have shown that netazepide (YF476) is a potent, selective, competitive and orally-active antagonist of gastrin receptors (CCK-B or CCK-2 receptors) [1–3]. We have shown in healthy subjects that single oral doses of netazepide cause dose-dependent, sustained increases in basal and food-stimulated 24-h gastric pH, consistent with suppression of gastric acid production via antagonism of gastrin receptors [4]. We have also shown that repeated doses of netazepide lead to tolerance to that effect on gastric pH; however, repeated doses increased plasma gastrin, suggesting persistent suppression of gastric acid secretion [5, 6]. Subsequent studies in rodents have also shown that netazepide increases circulating gastrin, via gastric acid suppression and up-regulation of the gastrin gene [7–16].

Here we report two more studies in healthy subjects, a single-dose study and a repeated-dose study. The objectives were to confirm that netazepide causes dose-dependent antagonism of gastrin receptors, and that antagonism persists during repeated dosing. We used intravenous pentagastrin – a synthetic pentapeptide consisting of β -alanine and the C-terminal tetrapeptide of gastrin – as an agonist to stimulate gastrin receptors [17].

We presented the results of the second study at a meeting of the Clinical Section of the British Pharmacological Society [18].

Methods

We did the studies at our facilities in the Central Middlesex Hospital, London, England, in accordance with the ICH Guideline for Good Clinical Practice and the Declaration of Helsinki. Brent Ethics Committee approved the studies. Subjects gave written informed consent. The trial was registered as ClinicalTrials.gov NCT01601418 and NCT01601405.

Materials

Ferring A/S, Indertofte 10, DK-2720 Vanlose, Denmark, supplied capsules of netazepide (1, 5, 25 and 100 mg) and matching placebo. The hospital pharmacy packed and labelled treatments, and randomised subjects to treatment in the single-dose study using sequentially numbered containers. Pentagastrin was supplied by Cambridge Laboratories Ltd (Wallsend, Tyne and Wear, England) as ampoules of 500 μ g/2 mL.

Study design

Single-dose study

The study was double-blind, randomised, single-dose, and 5-way complete crossover in design. There was one week between successive treatments. The protocol required 10 healthy, *H. pylori*

negative (by ^{13}C -urea breath test), non-smoking men or women not at risk of pregnancy to complete the study. Subjects fasted from midnight, then at about 0800 h we passed a nasogastric tube (14 G Salem sump tube), to collect gastric aspirate by continuous suction while the subject was semi-recumbent. We confirmed the correct position of the tube by the water recovery test [19].

First, we collected basal gastric aspirate continuously in 15-min epochs for 30 min. Immediately afterwards, the subject took a single dose of netazepide 1, 5, 25 or 100 mg or placebo by mouth with 150 mL water. We allowed 55 min for absorption of netazepide, after which we collected gastric aspirate for 5 min, to empty the stomach. Then, we began an intravenous infusion of pentagastrin 0.6 $\mu\text{g}/\text{kg}/\text{h}$ for 2 h, via a syringe pump. We collected gastric aspirate continuously every 15 min during the infusion, to measure the volume, titratable acidity (H^+ secretion rate) and pH of each sample. Subjects continued to fast until we removed the nasogastric tube, at the end of the infusion. We assessed tolerability and safety of treatments by vital signs, ECG, safety tests of blood and urine, and adverse events.

Repeated-dose study

The study was single blind and placebo controlled. The protocol required 8 healthy men or women, defined as in the single-dose study, to complete the study. Subjects were resident from Day 0 to Day 7, and on Day 14. They took the following treatments by mouth: a single dose of placebo on Day 0; netazepide 100 mg twice daily on Days 1–6; a single dose of netazepide 100 mg on Day 7; and a single dose of placebo on Day 14. We told all subjects that they would receive placebo on some days, but we did not tell them which days. On Days 0, 1, 7 and 14, we passed a nasogastric tube, collected and analysed gastric aspirate, dosed the subjects, and infused pentagastrin, as in the single-dose study. Subjects fasted on dosing days, as in the single-dose study. We collected blood for plasma netazepide assay before and 1 h and 3 h after the morning dose on Days 0, 1, 7 and 14. We assessed tolerability and safety of treatments as in the single-dose study.

Gastric aspirate

In both studies, we collected gastric aspirate via the nasogastric tube into conical flasks, primed with a few drops of a silicone anti-foaming agent. We used a suction pump to apply continuous negative pressure of 100 mm Hg to the nasogastric tube. If necessary, we increased the negative pressure to a maximum of 600 mm Hg to clear blockage of the tube by thick mucus. We measured the volume of each collection. We also measured titratable acidity and pH with a pH meter and automatic titrator (Radiometer), which we calibrated with standard buffers before and

after each batch of samples. We used 0.1 M sodium hydroxide as the base, and calculated H⁺ secretion rate in $\mu\text{mol}/\text{min}$.

Plasma netazepide

We collected blood and separated and stored plasma for netazepide assay, by a validated HPLC-MS method [20], as described previously [4].

Sample size

Single-dose study

In our previous single-dose, complete crossover study, 10 healthy subjects were enough to show significant differences in 24-h ambulatory gastric pH between netazepide 5, 25 or 100 mg and placebo [4]. Therefore, we judged that 10 subjects would be enough to detect differences in the response to pentagastrin between single doses of netazepide 1, 5, 25 or 100 mg and placebo.

Repeated-dose study

The number of subjects was determined by feasibility, rather than a power calculation. The results of the single-dose study indicated that 8 subjects would be enough to show a significant effect of 13 doses of netazepide 100 mg versus placebo on the response to pentagastrin.

Statistics

Single-dose study

We calculated mean and standard deviation of volume, H⁺ secretion rate, and pH of gastric aspirate in each interval for each treatment. We judged the efficacy of netazepide by its effect on pentagastrin-induced H⁺ secretion rate of gastric aspirate 60–180 min after dosing, compared with placebo. At times when pH ≥ 7.0 , we set titratable acidity to 0. We analysed the results by analysis of covariance (ANCOVA), using the average of the two basal measurements (0–15 and 15–30 min after the start of aspiration) as a covariate.

Repeated-dose study

We calculated mean and standard deviation and expressed efficacy of netazepide similarly to the single-dose study, except that we also expressed efficacy of netazepide in terms of its effect on volume and pH of gastric aspirate 60–180 min after dosing. We analysed the results for placebo (Day 0) and the first dose (Day 1) and last dose (Day 7) of netazepide by ANCOVA, using the average of basal measurements on Day 0 as a covariate.

Results

Single-dose study

Subjects

12 subjects entered the study; one withdrew for personal reasons and another could not tolerate the nasogastric tube. 10 Europid subjects, 6 women and 4 men, completed the study according to the protocol. Their mean age and body mass index were 27.6 y (range 20–42 y) and 24.6 kg/m² (range 19.9–27.9), respectively.

Basal

Before each treatment, H⁺ secretion rate was similarly low (Table 1), and volume and pH differed little (Figure 1).

Placebo

After placebo, pentagastrin increased the volume and H⁺ secretion rate, and reduced the pH, of gastric aspirate (Table 1; Figure 1). Compared with basal measurements, mean volume and H⁺ secretion rate increased 2.4-fold and 8-fold, respectively, and mean pH decreased from 2.90 to 1.29, in the epoch 60–75 min after dosing.

Netazepide

Single doses of netazepide 1, 5, 25 and 100 mg inhibited all three measures of the response to pentagastrin in a dose-dependent manner (Table 1; Figure 1). Netazepide 100 mg abolished the response. Indeed, netazepide 100 mg not only reversed the fall in pH, it also increased pH substantially above basal levels. Compared with placebo, all doses of netazepide significantly ($p < 0.05$) reduced the mean H⁺ secretion rate (Table 1; Figure 2).

Safety and tolerability

All doses of netazepide were well tolerated. Any adverse events were minor, transient and unlikely to be dose-related. There were no clinically relevant changes in any of the safety assessments.

Repeated-dose study

12 subjects entered the study. None had participated in the single-dose study. 4 withdrew or were withdrawn, for reasons unrelated to their treatment. 8 subjects (4 men and 4 women, 7 Europid and 1 Oriental) completed the study according to the protocol. Their mean age and body mass index were 25.8 y (range 20–44 y) and 21.9 kg/m² (range 18.8–26.1), respectively.

Basal

On Days 0, 1, 7 and 14, basal H⁺ secretion rates were similarly low, and volume and pH differed little (Table 2; Figure 3).

Day 0 (placebo)

After placebo, pentagastrin increased the volume and H⁺ secretion rate, and reduced the pH (Figure 3), of gastric aspirate. Compared with basal measurements, mean volume and H⁺ secretion rate increased 1.7-fold and 4.2-fold, respectively, and mean pH decreased from 2.0 to 1.36, in the epoch 60–75 min after dosing.

Day 1 (first dose of netazepide)

Compared with placebo on Day 0, the first dose of netazepide 100 mg abolished all three measures of the response to pentagastrin (Figure 3). The reductions in mean volume and H⁺ secretion rate, and the increase in pH, of gastric aspirate (Table 2; Figure 4) were all significant ($p < 0.05$).

Day 7 (last dose of netazepide)

Compared with placebo on Day 0, the last dose of netazepide 100 mg on Day 7 abolished the increases in mean volume (Figure 3a) and H⁺ secretion rate (Figure 3b) of aspirate induced by pentagastrin, as did the first dose on Day 1, but failed to achieve the increase in pH that netazepide caused on Day 1 (Table 2; Figure 3c). The reductions in volume and H⁺ secretion rate (Table 2; Figure 4) were significant ($p < 0.05$), whereas the effect on pH did not differ significantly from placebo.

Day 14 (placebo)

On Day 14, 7 days after the last dose of netazepide, the three measures of the response to pentagastrin after placebo were similar to those after placebo on Day 0 (Table 2; Figure 3).

Plasma netazepide

Plasma netazepide concentrations at 60 and 180 min after dosing on Days 1 and 7 are in Table 3.

Safety and tolerability

The results were similar to those from the single-dose study.

Discussion

In both studies, which were done in different groups of subjects, basal volume, H⁺ secretion rate and pH of gastric aspirate were low on all study days, as would be expected after an overnight fast. Also in both studies, pentagastrin increased the volume and H⁺ secretion rate of gastric

aspirate, and reduced pH, after every dose of placebo. Pentagastrin 0.6 µg/kg/h, which is a submaximal dose, was well tolerated.

In both studies, netazepide was well tolerated, and a single dose inhibited all three measures of the response to pentagastrin. In the single-dose study, netazepide 1, 5, 25 and 100 mg caused dose-dependent inhibition of the pentagastrin-stimulated increases in the volume and H⁺ secretion rate, and the reduction in pH, of gastric aspirate. Netazepide 100 mg abolished all three measures of the response to pentagastrin. In the repeated-dose study, the effects of the first of the 13 doses of netazepide 100 mg were very similar to those of netazepide 100 mg in the single-dose study. Also, the effects of the last of the 13 doses of netazepide 100 mg on pentagastrin-stimulated increases in volume and H⁺ secretion rate were similar to those of the first dose. But, compared with the first dose, the last dose had much less effect on the pH response to pentagastrin, as in our previous studies, in which we measured pH by the 24-h ambulatory method [5, 6].

Dose-dependent inhibition of the response to pentagastrin by single doses of netazepide is consistent with antagonism of gastrin receptors. Suppression of pentagastrin-induced increases in volume and H⁺ secretion rate of gastric aspirate by repeated doses of netazepide is consistent with persistent antagonism. Those findings support our conclusion that the increase in plasma gastrin after repeated doses of netazepide in our previous studies [5, 6] reflects suppression of gastric acid secretion via antagonism of gastrin receptors and up-regulation of the gastrin gene [7–16]. All our findings thus far are in accord with those of non-clinical studies, which have shown netazepide to be an orally active, highly selective and competitive antagonist of gastrin receptors [1–3] whose action persists after chronic dosing [8, 9].

We allowed 60 min after netazepide dosing before starting infusion of pentagastrin, because that was about the time of maximum plasma concentration in our previous studies [4, 5]. Netazepide concentrations at 60 and 90 min after dosing on Days 1 and 7 in the repeated-dose study were variable, and were only 10–15% of those measured at similar times after dosing in our previous studies [4, 5]. The reason is that for those studies the sponsor supplied what we now know to be a much more bioavailable formulation. Clearly, netazepide is an effective gastrin receptor antagonist in man, even at low plasma concentrations.

After only 7 days' of netazepide 100 mg twice daily, nearly complete tolerance had developed. That phenomenon resembles the development of tolerance to the effect of netazepide on 24-h

ambulatory gastric pH after repeated doses in our previous studies [5, 6]. What might be the mechanism of that tolerance?

Tolerance might reflect a change in a constituent of gastric juice which takes longer to occur than does the immediate effect on acid production, and which reduces the buffering capacity of gastric juice. Gastric juice contains many substances apart from hydrochloric acid, including pepsin, mucus, peptides, amino acids, and electrolytes such as bicarbonate (HCO_3^-) and phosphate. Also, it usually contains saliva and sometimes bile. In man, the buffers of H^+ in gastric juice, which prevent large changes in gastric pH, are mainly HCO_3^- [21], non-pepsin protein [22–24] and phosphate. Pepsin degradation products of non-pepsin protein may also add to the buffering capacity of gastric juice [26]. Non-protein buffers, especially HCO_3^- , probably play a more important role than do the other buffers [24]. Mucin, the protein of mucus, has little if any buffering activity [22].

Various studies have been done to assess the mechanisms that control gastric HCO_3^- secretion in humans. HCO_3^- secretion was increased by cholinergic stimulation and reduced by prostaglandin E_2 [27] but was unaffected by pentagastrin [27, 28]. Sham feeding stimulated gastric HCO_3^- secretion, confirming its central nervous system control via the vagus [29–31]. HCO_3^- is difficult to measure in the presence of acid, and must first be unmasked by suppressing acid production with a histamine H_2 -receptor antagonist (H_2RA) or a proton pump inhibitor (PPI) [32]. Using that method, the rate of secretion of HCO_3^- by the human stomach *in vivo* was equivalent to 10–20% of basal acid secretion [32]. Repeated doses of neither an H_2RA nor a PPI affected HCO_3^- secretion [33]. However, the different methods by which gastric HCO_3^- secretion has been measured in humans have produced discordant results [34]. Nevertheless, the available evidence suggests that gastrin is not directly involved in control of gastric HCO_3^- secretion.

Gastrin, a hormone secreted by G cells in the gastric antrum [17], stimulates gastrin receptors on enterochromaffin-like (ECL) cells in the gastric mucosa to secrete histamine, which in turn stimulates adjacent gastric parietal cells to secrete acid into the lumen of the stomach. Secretion of gastric acid is mediated by the action of (H^+ , K^+)-ATPase (the proton pump) in response to stimulation of histamine H_2 -receptors or muscarinic M_3 -receptors. There is evidence that parietal cells also express gastrin receptors [35] although their role in acid secretion is uncertain [36]. Gastrin also acts as a growth factor on normal gastric oxyntic mucosa, stimulating ECL and parietal cell growth [17, 37, 38]. Recent studies using knockout mice that either lack the gastrin gene or overexpress it, and genomic methods, have revealed that the physiological role

of gastrin is far more than just regulation of acid secretion [39–41]. Gastrin is pivotal in organising and maintaining the structure of gastric epithelium. It upregulates various genes, such as histidine decarboxylase, vesicular monoamine transporter type 2, chromogranin A, and protein Reg 1A [37], and stimulates paracrine cascades [38], including cytokines, growth factors such as trefoil factor [42, 43], and prostanoids. Gastric acid secretion is regulated by endocrine, paracrine, and neurocrine mechanisms via at least three signalling pathways: gastrin-histamine (stimulation), CCKA-somatostatin (inhibition) and neural network (both stimulation and inhibition). Studies in gene-knockout mice have shown that there is complex interplay among those pathways [36, 44]. Different pathways are suppressed or dominate, depending on the circumstances. Therefore, it is possible that antagonism of gastrin receptors by repeated doses of netazepide led to a switch in control of the buffering capacity of gastric juice in our study.

What might be the impact of the tolerance to gastric pH on the potential therapeutic uses of netazepide? Gastric pH is easy to measure continuously with a nasogastric electrode, and has been widely used as a surrogate clinical endpoint in trials of the acid suppressants H₂RA and PPI [45]. A substantial increase in the time pH \geq 4 is regarded as essential to heal peptic ulcers or erosive oesophagitis. pH is a logarithmic scale, so gastric pH may change little despite a large change in H⁺ secretion [36, 46]. Furthermore, measurement of gastric pH alone ignores changes in volume. Therefore, the amount of H⁺ secreted per unit time is a more sensitive and reliable test of acid suppression than is pH. Repeated doses of netazepide cause persistent blockade of gastrin receptors and could prove a useful treatment for patients with acid-related conditions, such as gastro-oesophageal reflux disease, despite tolerance to the effect on pH. Likewise, netazepide should inhibit the trophic effects of gastrin on parietal and/or ECL cells in the gastric mucosa [17, 37, 38], for example in patients with hypergastrinaemia caused by autoimmune chronic atrophic gastritis (CAG) [47], Zollinger-Ellison syndrome [47], and prolonged PPI [48–51] or H₂RA treatment [52]. Indeed, in pilot studies in patients with CAG and multiple gastric carcinoids secondary to hypergastrinaemia, netazepide reduced the number and size of the tumours and normalised plasma chromogranin A (CgA), which increased to pre-treatment levels after stopping netazepide [53, 54]. CgA is a validated biomarker of increased ECL cells from which the gastric carcinoids originate.

Many gastrin receptor antagonists have been described [55, 56], but most have had problems with selectivity, potency or bioavailability. Several have been tested in man [57–61], but none has been developed as a medicine. Whether tolerance to the effect on gastric pH is specific to netazepide or a class effect must await studies of other gastrin receptor antagonists.

The design of the single-dose study was ideal: randomised, double-blind, placebo-controlled, complete-crossover and a range of doses. The design of the repeated-dose study – single-blind, fixed dose and no randomisation – was a compromise, but the methods are robust and the results have face validity.

Conclusions

In healthy subjects, netazepide is an orally active, potent, competitive antagonist of gastrin receptors in the stomach. Antagonism is dose-dependent, and persists during repeated dosing, despite the reduced effect on pH. Further studies are required to find the mechanism of that reduced effect. Netazepide is a tool to study the physiology and pharmacology of gastrin, and merits studies in patients, to assess its potential to treat gastric acid-related conditions and the trophic effects of hypergastrinaemia.

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Table 1. Single-dose study: mean (sd; n=10) basal and pentagastrin-induced H⁺ secretion rate of gastric aspirate before and after netazepide 1, 5, 25 or 100 mg and placebo

Dose	Basal (0–30 min before dosing)	Pentagastrin (60–180 min after dosing)
	Mean (sd)	Mean (sd)
Placebo	37 (34)	291 (103)
Netazepide 1mg	31 (30)	180* (141)
Netazepide 5 mg	22 (16)	140* (130)
Netazepide 25 mg	22 (34)	67* (66)
Netazepide 100 mg	20 (21)	10* (14)

* Significantly different from placebo (p<0.05, by ANCOVA with basal value as covariate).

Table 2. Repeated-dose study: mean (sd; n=8) basal and pentagastrin-induced volume, H⁺ secretion rate and pH of gastric aspirate before and after placebo (Days 0 & 14) and before and after the first dose (Day 1) and last dose (Day 7) of 13 doses of netazepide 100 mg twice daily

Parameter	Day	Basal (0–30 min before dosing)	Pentagastrin (60–180 min after dosing)
		Mean (sd)	Mean (sd)
Volume (mL/min)	0	2.4 (1.7)	3.8 (1.3)
	1	1.3 (0.7)	0.8* (0.5)
	7	1.0 (0.5)	1.3* (0.5)
	14	1.6 (1.2)	3.2 (0.8)
H ⁺ secretion rate (μ mol/min)	0	105 (73)	429 (250)
	1	59 (43)	30* (53)
	7	41 (38)	51* (40)
	14	108 (142)	405 (119)
pH	0	2.0 (1.3)	1.4 (0.5)
	1	1.9 (0.5)	4.3* (2.1)
	7	2.2 (1.2)	1.9 (0.4)
	14	2.0 (1.0)	1.3 (0.4)

*Significantly different from Day 0 (p <0.05 by ANCOVA with basal value as covariate).

Table 3. Mean (range; n=8) plasma netazepide concentrations after the first dose (Day 1) and last dose (Day 7) of 13 doses of netazepide 100 mg twice daily

Day	Mean (range) netazepide concentration ng/mL
1 (60 min)	118.2 (1.4–302.9)
1 (180 min)	18.5 (2.7–34.2)
7 (60 min)	48.2 (22.3–94.6)
7 (180 min)	25.0 (9.2–78.3)

Samples collected at 60 and 180 min after netazepide dosing, corresponding to start and end of pentagastrin infusion, respectively.

Figure 1. Single-dose study: mean (sd; n=10) basal and pentagastrin-induced: (a) volume; (b) H⁺ secretion rate; and (c) pH of gastric aspirate before and after netazepide 1 mg [■], 5 mg [▲], 25 mg [✧] or 100 mg [✱] and placebo [◆]

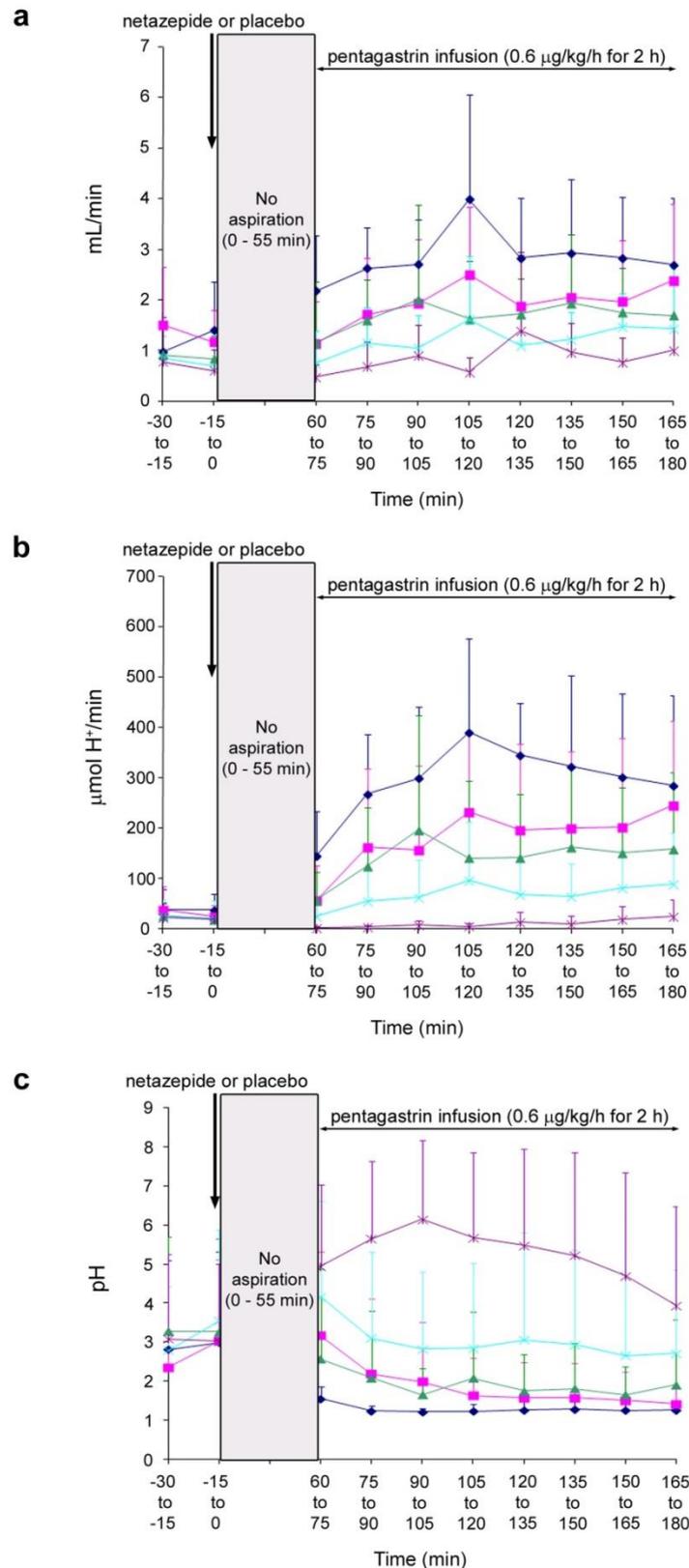


Figure 3. Repeated-dose study: mean (sd; n=8) basal and pentagastrin-induced: (a) volume; (b) H⁺ secretion rate; and (c) pH of gastric aspirate after placebo on Day 0 [—○—] and Day 14 [—×—], and after the first dose on Day 1 [—■—] and last dose on Day 7 [—▲—] of 13 doses of 100 mg netazepide twice daily

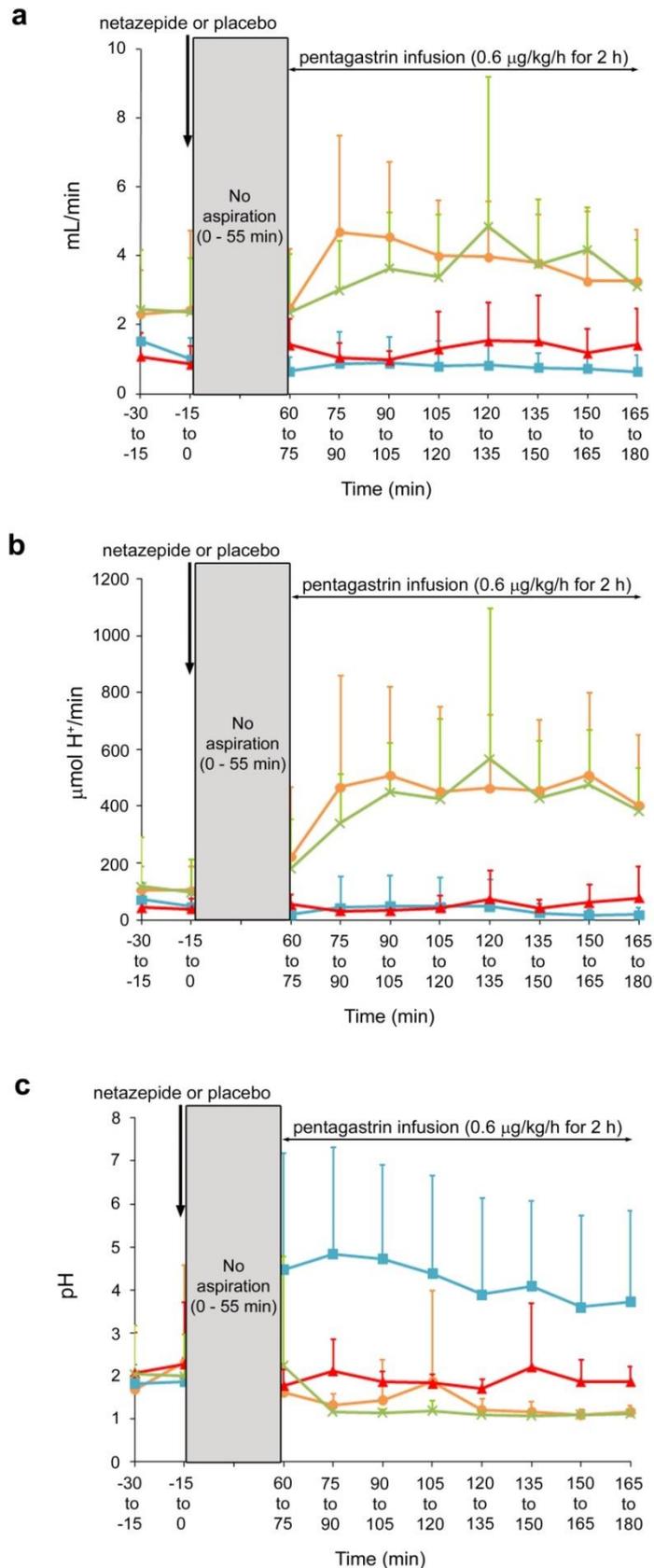
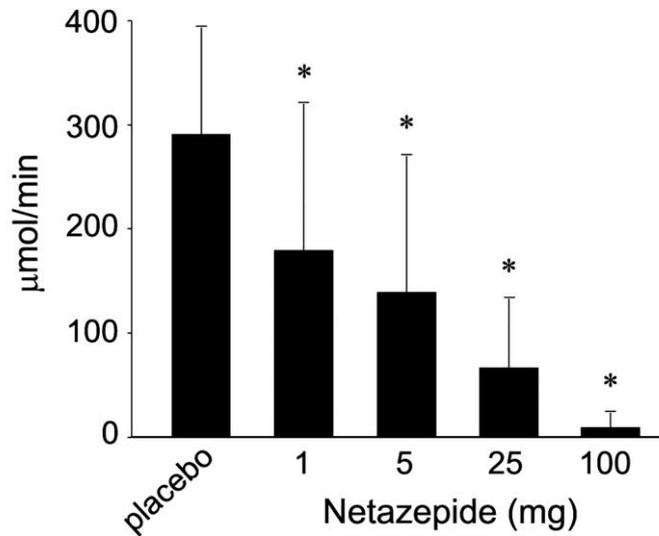
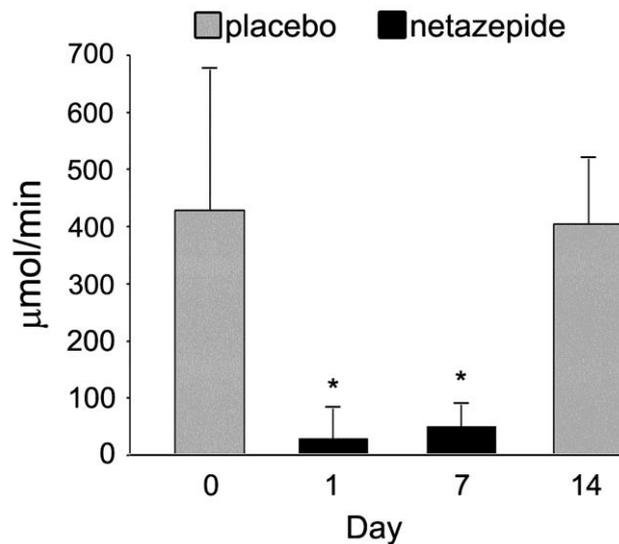


Figure 2. Single-dose study: effect of netazepide dose on mean (sd; n=10) pentagastrin-induced H⁺ secretion rate



* Significantly different from Day 0 (p<0.05, by ANCOVA, with basal measurements as covariate)

Figure 4. Repeated-dose study: mean (sd; n=8) pentagastrin-induced H⁺ secretion rate after placebo (Days 0 & 14) and the first dose (Day 1) and last dose (Day 7) of 13 doses of netazepide 100 mg twice daily



* Significantly different from Day 0 (p<0.05, by ANCOVA with basal measurements as covariate)

Chapter 6

Study 7. Effect of netazepide, a gastrin/CCK₂ receptor antagonist, on gastric acid secretion and rabeprazole-induced hypergastrinaemia in healthy subjects

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Effect of netazepide, a gastrin/CCK₂ receptor antagonist, on gastric acid secretion and rabeprazole-induced hypergastrinaemia in healthy subjects.

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Abstract

Aims

To compare gastric acid suppression by netazepide, a gastrin/CCK₂ receptor antagonist, with that by a proton pump inhibitor (PPI), and to determine if netazepide can prevent the trophic effects of PPI-induced hypergastrinaemia.

Methods

Thirty healthy subjects completed a double-blind, randomised, parallel-group trial of oral netazepide and rabeprazole, alone and combined, once daily for 6 weeks. Primary endpoints were: basal and pentagastrin-stimulated gastric acid and 24-h circulating gastrin and chromogranin A (CgA) at baseline, start and end of treatment; gastric biopsies at baseline and end of treatment; and basal and pentagastrin-stimulated gastric acid and dyspepsia questionnaire after treatment withdrawal.

Results

All treatments similarly inhibited pentagastrin-stimulated gastric acid secretion. All treatments increased serum gastrin, but the combination and rabeprazole did so more than netazepide alone. The combination also reduced basal acid secretion.

Rabeprazole increased plasma CgA, whereas netazepide and the combination reduced it. None of the biopsies showed ECL-cell hyperplasia. Withdrawal of treatments led neither to rebound hyperacidity nor dyspepsia.

Conclusions

Netazepide suppressed pentagastrin-stimulated gastric acid secretion as effectively as did rabeprazole; the reduction in basal acid secretion and greater increase in serum gastrin by the combination is consistent with more effective acid suppression. Despite our failure to show rabeprazole-induced ECL-cell hyperplasia and rebound hyperacidity, the increase in plasma CgA after rabeprazole is consistent with a trophic effect on ECL cells, which netazepide prevented. Thus, netazepide is a potential treatment for the trophic effects of hypergastrinaemia and, with or without a PPI, is a potential treatment for acid-related conditions.

What is already known about this subject

- ✓ Gastrin controls gastric acid secretion and mucosal cell growth, especially of ECL cells, which express gastrin/CCK₂ receptors and release CgA into the circulation when stimulated by gastrin.
- ✓ In non-clinical studies, acid suppression by a PPI causes hypergastrinaemia which results in ECL-cell growth and, after PPI withdrawal, rebound hyperacidity. Netazepide is an orally-active, selective gastrin/CCK₂ receptor antagonist, which suppresses acid production and prevents the trophic effects of PPI-induced hypergastrinaemia.
- ✓ In healthy subjects, oral netazepide causes dose-dependent, persistent inhibition of gastric acid secretion, which leads to increased serum gastrin.

What this study adds

- ✓ In healthy subjects, netazepide and the PPI rabeprazole were similarly effective at suppressing pentagastrin-stimulated gastric acid secretion and increasing serum gastrin.
- ✓ Rabeprazole increased plasma CgA – a sign of ECL-cell hyperactivity – whereas netazepide reduced plasma CgA – a sign of ECL-cell hypoactivity. Netazepide also prevented the increase in CgA resulting from rabeprazole-induced hypergastrinaemia, probably because netazepide blocks gastrin/CCK₂ receptors on ECL cells.

Introduction

Gastrin, a hormone produced by G cells in the gastric antrum, stimulates gastric acid secretion and the proliferation, migration, differentiation and anti-apoptosis of gastric epithelial cells [1, 2]. Gastrin activates gastrin (CCK₂) receptors on enterochromaffin-like (ECL) cells in the oxyntic mucosa to secrete histamine, which in turn stimulates adjacent parietal cells to secrete acid. Acid secretion is mediated by the proton pump via histamine H₂- and muscarinic M₃-receptors on parietal cells [3].

Acid suppression by a proton pump inhibitor (PPI) induces hypergastrinaemia [4]. Prolonged PPI-induced hypergastrinaemia may cause ECL-cell [5] and parietal-cell hyperplasia [6]. PPI withdrawal may result in rebound hyperacidity [7, 8] and dyspepsia [9, 10]. Other causes of hypergastrinaemia include chronic autoimmune gastritis (CAG), Zollinger-Ellison syndrome (ZES), and *H. pylori*-induced gastritis [11, 12].

There are several potential clinical indications for a gastrin/CCK₂ receptor antagonist. Many have been described [13], but none has been developed into a medicine, mainly because of problems with potency, selectivity for CCK₁ or CCK₂ receptors, agonist activity, and oral bioavailability. In non-clinical studies, netazepide (YF476) is a potent gastrin/CCK₂ receptor antagonist, is highly selective for the CCK₂ receptor, has good oral bioavailability, and suppresses gastric acid secretion [14-17]. In animal models, netazepide prevents ECL-cell hyperplasia and the rebound hyperacidity that follows PPI withdrawal [18], and also prevents development [19, 20] and causes regression [20] of ECL cell-tumours. Acid suppression by netazepide leads to hypergastrinaemia in animals [19, 21-24], but causes neither ECL-cell growth nor rebound hyperacidity [18], because netazepide blocks the gastrin/CCK₂ receptors on ECL cells.

We have characterised the clinical pharmacology of netazepide as a gastric acid suppressant, in healthy subjects [25-27]. Single oral doses caused dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion, which persisted after repeated doses. Repeated doses also caused hypergastrinaemia, which is consistent with persistent acid suppression, in spite of partial tolerance to the effect of netazepide on gastric pH [26].

Aims

We studied healthy subjects to: (i) compare acid suppression by netazepide and the PPI rabeprazole, alone and in combination; (ii) determine whether netazepide can prevent the effects of PPI-induced hypergastrinaemia, namely ECL-cell hyperplasia and rebound hyperacidity and dyspepsia; and (iii) show that hypergastrinaemia induced by netazepide causes neither ECL-cell hyperplasia nor rebound hyperacidity and dyspepsia.

Methods

We complied with the ICH Guideline for Good Clinical Practice. The Medicines and Healthcare products Regulatory Agency, UK, and Ravenscourt Ethics Committee approved the study. Subjects gave written, informed consent. We did the study during 2007–2010, and registered it as ClinicalTrials.gov NCT01699113.

Materials

Trio Medicines Ltd, England, supplied netazepide 50 mg capsules, matching placebo, and rabeprazole (Pariet[®]; Eisai) 20 mg delayed-release, enteric-coated tablets. Over-encapsulation ensured that treatments looked identical. Cambridge Laboratories (Wallsend, England) supplied pentagastrin 500 µg/2 mL. Subjects were supplied with an antacid/alginate (Gaviscon[®]; Reckitt Benckiser) that they could take after treatment withdrawal, if needed.

Study design

The study was randomised, double-blind and parallel-group, and required 3 cohorts of 10 healthy, *H. pylori* negative (¹³C-urea breath test), non-smoking, men or women not at risk of pregnancy, with normal gastroscopy and no history of dyspepsia, and taking no medicines. We deemed subjects healthy by: medical history and examination; ECG; tests for drugs of abuse, hepatitis B & C, and HIV 1 & 2; and blood and urine tests. We allocated subject numbers sequentially, using separate randomisation schedules for men and women. Treatments consisted of netazepide 100 mg and rabeprazole 20 mg, alone and in combination, once daily by mouth for 6 weeks.

We studied subjects on: 3 days (Days –14, –13 and –12) before starting treatment (baseline); the first 2 days (Days 1 and 2) and last 3 days (Days 40, 41 and 42) of treatment; and 2 days (Days 57 and 58) starting 15 days after the end of treatment. Subjects were resident on study days, and had clinic visits after 2 and 4 weeks' treatment and 1 week after withdrawal, for safety checks. Table 1 shows the procedures during residence.

We gave subjects a diary card to record adverse events and compliance during treatment, and a validated questionnaire [28] and a diary card to record dyspepsia symptoms and antacid/alginate usage, respectively, during 3 weeks after treatment withdrawal.

We assessed safety and tolerability by medical examination, ECG, blood and urine tests, and adverse events.

Ambulatory gastric pH

At baseline and on Days 1, 40 and 57, we measured gastric pH continuously for 0.5 h before and 24 h after dosing, as described previously [25]. Subjects fasted overnight before treatment,

which they took at the same time (0900–1000) in each session. They rested for 6 h after dosing and were ambulant thereafter. After dosing, they consumed standard meals and water at standard times.

Pentagastrin tests

At baseline and on Days 2, 41 and 58, subjects fasted overnight. At 1000–1100, we passed a nasogastric tube, to collect gastric aspirate by continuous suction while the subject was semi-recumbent, as described previously [27]. First, we collected basal aspirate every 15 min for 30 min. We allowed 55 min for absorption of treatment [27], after which we collected aspirate for 5 min, to empty the stomach. Then, we gave pentagastrin intravenously 0.6 µg/kg/h for 2 h by infusion pump, and collected aspirate continuously in 15-min epochs. We measured volume, and then titratable acidity, of each collection by pH meter and automatic titrator (Radiometer) calibrated regularly with buffers. We used NaOH (0.1 M) as base and calculated H⁺ secretion rate in µmol/min.

Gastroscopy and mucosal biopsies

At baseline and on Day 40, we took four biopsies of the gastric oxyntic mucosa from the anterior and posterior wall, and prepared and coded specimens for blinded assessment of ECL cells by: histology; immuno-histochemistry of CgA, histidine decarboxylase (HDC), vesicular monoamine transfer (VMAT2), and Ki67; and electron microscopy (EM) of ECL-cell profile area, nuclear area and volume density, cytoplasmic area, numbers and volume densities of granules, secretory vesicles, microvesicles, secondary lysosomes, vacuoles, lipofuscin bodies, and volume densities of rough endoplasmic reticulum and Golgi complex. Preparation of specimens for assessment and methods are described elsewhere [18].

Serum gastrin and plasma CgA

At baseline and on Days 1 and 40, we collected blood before and frequently for 24 h after dosing, separated serum or plasma, and stored samples at –20°C until assay by ELISA (Gastrin: Immulite 2000, DPC. CV = 6.9%; CgA: DAKO. CV = 7.2%).

Plasma netazepide and rabeprazole

On Days 1 and 40, we collected blood before and frequently for 24 h after dosing, separated plasma, and stored samples at –20°C until assay by validated HPLC/MS methods [29]. The lower limit of quantification was 0.5 and 0.25 ng/mL for netazepide and rabeprazole, respectively.

Dyspepsia

Subjects completed the dyspepsia questionnaire and recorded antacid/alginate usage during

each week of the 3-week post-treatment period. The questionnaire uses 4- or 5-point Likert scales to measure frequency and severity of 15 upper gastrointestinal symptoms, and the bother they cause [28].

Assessment of compliance

We assessed compliance by diary card and capsule counts. Subjects wore a wristwatch with an alarm, and we telephoned them weekly, to remind them to take their treatment.

Statistics

Sample size

The study was exploratory in nature. We chose H^+ secretion rate in pentagastrin-stimulated gastric aspirate as the primary endpoint, and calculated sample size and power ($n = 10$; $\alpha = 0.05$; power at least 80%), using data from a previous study [27]. We opted not to adjust p values for multiple comparisons because of the exploratory nature of the study.

Pharmacodynamics

For AUC_{0-24h} of 24-h gastric pH, pentagastrin-stimulated volume and H^+ secretion rate of gastric aspirate (60–180 min after dosing), we summarised changes from baseline (mean and 95% confidence intervals on Days –14, –13 and –12) at the start (Days 1 and 2), end (Days 40, 41 and 42), and after withdrawal (Days 57 and 58) of each treatment, and used ANOVA to test for significant differences ($p < 0.05$). For basal H^+ secretion rate of gastric aspirate (0–30 min before dosing) and AUC_{0-24h} of serum gastrin and plasma CgA, before carrying out the analyses, we log-transformed the data because it was not normally distributed.

For immunoreactive cells and ECL-cell ultrastructure we used the estimates from ANOVA to make pairwise comparisons among subject groups at the start and end of treatment, and a two-tailed t test to compare each treatment.

Pharmacokinetics

We used WinNonlin to derive pharmacokinetic parameters by standard non-compartmental analysis, and SAS for Windows for statistical analysis, as described previously [25].

Dyspepsia

We summarised the number of subjects per treatment group with symptoms during each week of the withdrawal period, added the scores for the three groups of symptoms to obtain a dyspepsia score, and compared treatment groups informally. We also summarised antacid/alginate usage and compared groups in terms of number of subjects and doses per treatment group.

Results

Subjects

We entered 32 subjects (17 men, 15 women; 28 white, 4 black), of whom 30 completed the study, as required. One woman and one man (both on combination treatment) withdrew on Day 2 and 42, respectively, for reasons unrelated to treatment. We used data from both for safety assessments, and pharmacokinetic data from the subject who withdrew on Day 42. Mean (range) age and body mass index of subjects receiving netazepide, rabeprazole and combination treatments were: 38.7 (21–66) y and 25 kg/m²; 30.4 (21–67) y and 24.4 kg/m²; and 35.9 (21–57) y and 23.7 kg/m², respectively. There were similar numbers of men and women on each regimen.

Safety, tolerability and compliance

All treatments were well tolerated. Adverse events were minor, transient and occurred across treatments. There were no clinically relevant changes in safety assessments. Treatment compliance based on capsule counts and diary cards was 99%.

Pharmacokinetics (Tables S1 and S2)

Plasma netazepide concentrations were low: about 10–15% of those in our previous studies [25, 26], and more variable. Mean AUC_{0–24 h} of rabeprazole was higher when combined with netazepide than after rabeprazole alone, but not significantly so.

Pharmacodynamics

24-h ambulatory gastric pH (Table S3; Figure 1)

Baseline: AUC_{0–24 h} of pH was similar across groups.

Day 1: Compared with baseline, netazepide (mean change 18.7; CI = 5.9 to 31.5; p = 0.009) and rabeprazole (mean change 22.4; CI = 8.3 to 36.5; p = 0.006) were equally effective at increasing AUC_{0–24 h} of pH; the combination was more effective than netazepide (mean difference –17.8; CI = –33.2 to –2.5; p = 0.03), but not rabeprazole (mean difference –14.1; CI = –30.4 to 2.2; p = 0.09).

Day 40: Netazepide was about as effective as it was on Day 1 (mean change from baseline 12.0; CI = 1.6 to 22.4; p = 0.03), whereas rabeprazole (mean change 57.7; CI = 46.4 to 69.0; p = <0.0001) and the combination (mean change 59.8; CI = 51.5 to 68.1; p = <0.0001) were much more effective. Rabeprazole (mean difference 45.7; CI = 31.5 to 60.0; p = <0.0001) and the combination (mean difference –47.9; CI = –60.2 to –35.5; p = <0.0001) were more effective than netazepide, but there was no significant difference between the combination and rabeprazole (mean difference –2.1; CI = –15.2 to 10.9; p = 0.73).

Day 57: There were no significant changes from baseline, and no significant differences among treatments.

Pentagastrin stimulation test (Tables 2 and 3; Figure 2)

Baseline: The groups were not well matched for basal H⁺ secretion rate (μmol/min) before treatment, but mean secretion rate in all groups was low compared with secretion after pentagastrin.

Day 2: There were no significant differences in basal H⁺ secretion rate among treatments. Netazepide (mean change -1.9; CI = -2.88 to -0.97), rabeprazole (mean change -2.6; CI = -3.52 to -1.74), and the combination (mean change -1.3; CI = -2.66 to 0.04) all reduced pentagastrin-stimulated volume (mL/min). Also, netazepide (mean change -233; CI = -330 to -137), rabeprazole (mean change -252; CI = -349 to -154), and the combination (mean change -253; CI = -355 to -151) all reduced pentagastrin-stimulated H⁺ secretion rate. There were no significant differences among treatments for pentagastrin-stimulated volume or for H⁺ secretion rate.

Day 41: The combination significantly reduced basal H⁺ secretion rate compared with baseline. There was no significant difference between netazepide and rabeprazole (p = 0.5083) or rabeprazole and the combination (p = 0.2176). However, the combination (p = 0.0273) reduced basal H⁺ secretion rate more than netazepide alone. Netazepide (mean change -1.8; CI = -2.64 to -0.98), rabeprazole (mean change -1.9; CI = -2.91 to -0.93), and the combination (mean change -2.2; CI = -3.49 to -0.99) all reduced pentagastrin-stimulated volume. And, netazepide (mean change -205; CI = -273 to -138), rabeprazole (mean change -238; CI = -333 to -143), and the combination (mean change -263; CI = -364 to -162) all reduced H⁺ secretion rate. There were no significant differences among treatments for pentagastrin-stimulated volume or for H⁺ secretion rate.

Day 58: Compared with baseline, basal H⁺ secretion rate was higher after rabeprazole withdrawal, albeit not significantly so. There were no significant differences among treatments with respect to basal H⁺ secretion rate or to pentagastrin-stimulated responses, although again the H⁺ secretion rate response to pentagastrin was highest after rabeprazole withdrawal.

Serum gastrin (Table 4; Figure 3)

Baseline: AUC_{0-24h} of serum gastrin (pmol.h/L) was similar across groups.

Day 1: Compared with baseline, netazepide, rabeprazole, and the combination all increased AUC_{0-24h} of gastrin. There were no significant differences among treatments.

Day 40: Netazepide, rabeprazole, and the combination all increased $AUC_{0-24\text{h}}$ of gastrin significantly compared with baseline. There was no significant difference between rabeprazole and the combination ($p = 0.1368$). However, compared with netazepide, rabeprazole ($p = 0.0243$) and the combination ($p = 0.0006$) increased $AUC_{0-24\text{h}}$ of gastrin.

Plasma chromogranin A (Table 5; Figure 4)

Baseline: $AUC_{0-24\text{h}}$ of plasma CgA (U.h/L) was similar across groups.

Day 1: Compared with baseline, netazepide and the combination reduced $AUC_{0-24\text{h}}$ of CgA, whereas rabeprazole tended to increase it, albeit not significantly. Also on Day 1, compared with rabeprazole, netazepide ($p = 0.0022$) and the combination ($p = 0.0060$) reduced $AUC_{0-24\text{h}}$ of CgA. There was no difference between netazepide and the combination ($p = 0.7183$).

Day 40: Netazepide significantly reduced $AUC_{0-24\text{h}}$ of CgA, whereas rabeprazole significantly increased it. Also, compared with either netazepide ($p = 0.0001$) or the combination ($p = 0.0002$), rabeprazole increased $AUC_{0-24\text{h}}$ of CgA significantly. Again, there was no difference between netazepide and the combination ($p = 0.2961$).

Gastric biopsies (Tables S4 and S5)

Despite the increase in $AUC_{0-24\text{h}}$ of CgA on Day 40 by rabeprazole, overall there were no significant differences between baseline and Day 42 biopsies for any treatment. There was a significant reduction in VMAT2 in the netazepide group. Biopsy results varied a lot within and among subjects.

Dyspepsia

The numbers of subjects per group with symptoms during withdrawal weeks 1, 2 and 3, respectively, were: netazepide 2, 3 and 2; rabeprazole 3, 1 and 2; and combination 2, 2 and 2. Out of a possible total score of 1,950 per group of 10 subjects, scores per group for weeks 1, 2 and 3, respectively, were: netazepide 58, 30 and 8; rabeprazole 45, 19 and 44; and combination 52, 9 and 11. Symptoms occurred on 1–3 days, were mainly mild to moderate in severity, and bothered subjects only a little or moderately.

In the netazepide group, one subject took one dose of antacid/alginate on Day 43, and another subject took one dose on Day 50. In the rabeprazole group, one subject took one dose on Days 45 and 49. No subject in the combination group used antacid/alginate.

Discussion

The groups were matched at baseline in all respects, apart from basal H^+ secretion rate in the pentagastrin test. All treatments were well tolerated and safe. Treatment compliance was

remarkably high, probably because subjects were prompted by the daily wristwatch alarm and weekly calls, and because of good tolerability of the treatments.

Pharmacodynamics

Rabeprazole alone

As expected, rabeprazole increased $AUC_{0-24\text{ h}}$ of ambulatory intragastric pH, inhibited the effects of pentagastrin on gastric acid secretion, and increased circulating concentrations of gastrin and CgA. The effects on pH, gastrin and CgA were greater after repeated doses. Overall, those results are consistent with suppression of gastric acid production, which leads to hypergastrinaemia and in turn stimulation of ECL cells and release into the circulation of CgA, a biomarker of ECL-cell activity and mass [30-32].

Gastric biopsies showed no evidence of rabeprazole-induced ECL-cell hyperplasia, whereas the same methods in rats given omeprazole and netazepide, alone and in combination, showed unequivocal ECL-cell hyperplasia after omeprazole, but not after netazepide or the combination [18]. Possible reasons why we found no evidence of ECL-cell hyperplasia are: (1) the short duration of and lower exposure to hypergastrinaemia; (2) marked variability of biopsy findings within and among subjects; (3) small numbers of subjects; and (4) rigour of blinded assessments. In a study of patients on PPI treatment for up to 15 years, only a few had biopsy evidence of ECL-cell hyperplasia [33]. Transformation of ECL cells by hypergastrinaemia through a sequence of hyperplasia, dysplasia and metaplasia to gastric carcinoids in rats given high-dose PPI seems to need prolonged and high exposure to gastrin [34]. Likewise, the ECL cells of CAG and ZES patients take years of exposure to hypergastrinaemia to develop into gastric carcinoids [11, 35, 36].

We failed to show that rabeprazole withdrawal leads to significant rebound hyperacidity. However, even though there were no statistically significant differences among treatments, the fact that basal and pentagastrin-stimulated H^+ secretion rates were highest after withdrawal of rabeprazole alone is at least consistent with the concept of PPI-induced rebound hyperacidity. Studies in animal models have shown that withdrawal of large doses of PPI, but not netazepide or a combination, results in rebound hyperacidity [18]. However, a review of studies in healthy subjects and patients concluded that the evidence for rebound hyperacidity after PPI withdrawal is weak [37].

Despite our selecting subjects with no history of dyspepsia, several subjects in each group reported symptoms during the withdrawal period. However, we failed to show any significant differences among treatments with respect to dyspepsia after withdrawal, whereas others have reported an incidence of 40% after PPI withdrawal in healthy subjects [9, 10].

Netazepide alone

The results in the netazepide group were similar to the rabeprazole group, with two exceptions. First, netazepide reduced rather than increased the $AUC_{0-24\text{ h}}$ of CgA. Non-clinical studies have shown that large, repeated doses of gastrin/CCK₂ receptor antagonists such as netazepide cause hypotrophy of ECL cells and a reduction in the weight and thickness of the oxyntic mucosa reminiscent of changes seen after fasting [18], so our finding of a reduction in $AUC_{0-24\text{ h}}$ of CgA is most likely real. Second, as reported previously [26, 27], compared with a single dose, the effect of netazepide on $AUC_{0-24\text{ h}}$ of pH in healthy subjects tended to wane with repeated doses, whereas repeated doses of rabeprazole caused a greater increase in $AUC_{0-24\text{ h}}$ of pH than did a single dose. We have discussed elsewhere the possible reasons for the apparent attenuation of the effect of netazepide on ambulatory intragastric pH [26]. However, whatever the reason, the results of the pentagastrin stimulation tests clearly show that the efficacy of netazepide as a gastrin/CCK₂ receptor antagonist is fully maintained during repeated dosing.

That netazepide did not increase CgA shows that netazepide-induced hypergastrinaemia, which is secondary to acid suppression, does not stimulate ECL cells, because netazepide blocks the gastrin receptors on ECL cells. The reduction in VMAT2 in gastric biopsy after netazepide is probably a false positive. Netazepide-induced hypergastrinaemia, and its lack of effect on ECL cells, is in accord with studies in animal models [18-24].

Combination of netazepide and rabeprazole

All three treatments inhibited pentagastrin-stimulated gastric acid secretion, but there were no differences among treatments. The combination reduced basal H⁺ secretion rate after repeated doses. This reduction was greater than with netazepide alone. Also, the combination increased $AUC_{0-24\text{ h}}$ of gastrin at steady state more than did netazepide alone. Some studies have shown that serum gastrin concentrations tend to be inversely related to the degree of acid suppression by a PPI [38]. So, the results of the $AUC_{0-24\text{ h}}$ of gastrin, together with the reduced basal H⁺ secretion rate, lends support to the concept that the combination suppressed gastric acid secretion more than either treatment alone, but that would require confirmation in further trials.

Netazepide and loxidine, an insurmountable histamine H₂-receptor antagonist, were synergistic and caused nearly complete suppression of acid in an animal model [39].

The addition of netazepide to rabeprazole abolished the increase in $AUC_{0-24\text{ h}}$ of CgA induced by rabeprazole alone, consistent with netazepide blocking the trophic effect of rabeprazole-induced hypergastrinaemia on ECL cells. Also, after repeated doses, the combination was much more effective at reducing the basal H⁺ secretion increasing $AUC_{0-24\text{ h}}$ of pH than netazepide, and as

effective as rabeprazole. In previous studies [26, 27], and to a lesser extent in this one, repeated doses of netazepide alone led to partial tolerance to its effect on gastric pH. However, there was no such tolerance when netazepide and rabeprazole were combined in this study.

Pharmacokinetics

Plasma concentrations of netazepide in both the netazepide and combination groups were much lower and more variable among subjects than in our previous studies [25, 26]. Mean C_{\max} and $AUC_{0-24\text{ h}}$ after one dose of netazepide 100 mg were 39 ng/mL and 86 ng.h/mL, respectively, whereas mean C_{\max} and $AUC_{0-24\text{ h}}$ after one dose of netazepide 100 mg in our previous study were 397 ng/mL and 658 ng.h/mL, respectively [25]. Thus, in comparison, mean C_{\max} and $AUC_{0-24\text{ h}}$ in the current study were 10-fold and 8-fold lower, respectively. That was because the formulation in this study contained crystalline netazepide, which proved to have low bioavailability, whereas the previous formulation contained amorphous netazepide, which was much more bioavailable. The present study shows that netazepide is very effective even at low plasma concentrations, but the results cannot be compared directly with those obtained with 100 mg doses of a superior formulation [25].

Rabeprazole pharmacokinetics were consistent with published data [40], apart from $t_{1/2}$, which was about 3 h in our study compared with 1.5 h, possibly an effect of over-encapsulation. Unlike other PPI, rabeprazole is metabolised mainly via non-enzymatic pathways, with minor CYP2C19 and CYP3A4 involvement, and its activity is little affected by CYP2C19 genotype [41], whereas netazepide is metabolised mainly via CYP3A4 and is a weak CYP3A4 inhibitor [42]. Neither netazepide nor rabeprazole significantly affected the pharmacokinetics of the other. However, a formal study [43] would be required to confirm that finding.

Study design limitations

The omission of a placebo, short duration of the treatments, and small groups of subjects are limitations of the study design. We decided against a placebo because the study was exploratory, demanding of subjects, the methods were mostly objective, and baseline measurements could serve as controls where necessary, albeit less satisfactory than placebo values. Likewise, we decided against measuring circulating gastrin and CgA after treatment withdrawal, because the study was already demanding enough. Completion of the dyspepsia questionnaire during a run-in period before the start of treatment might have helped to assess the relevance of symptoms reported by some subjects after treatment withdrawal; a placebo treatment would have been even better.

Conclusions

Netazepide suppressed gastric acid secretion as effectively as did rabeprazole. The reduction in basal acid secretion and the greater increase in serum gastrin after the combination are consistent with more effective acid suppression. The fact that basal and pentagastrin-stimulated H^+ secretion rates were highest after rabeprazole withdrawal, albeit not significantly so, is also consistent with the concept of PPI-induced rebound hyperacidity. Further studies are required to clarify whether those assumptions are correct.

Hypergastrinaemia resulting from acid suppression by rabeprazole caused ECL-cell hyperactivity, whereas hypergastrinaemia resulting from acid suppression by netazepide was associated with ECL-cell hypoactivity, probably because netazepide blocked the gastrin/CCK₂ receptors on ECL cells. For the same reason, netazepide prevented ECL-cell hyperactivity resulting from rabeprazole-induced hypergastrinaemia.

Thus, netazepide is a potential treatment for the trophic effects of gastrin and, with or without a PPI, netazepide is a potential treatment for acid-related conditions.

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Table 1. Study plan for procedures during residence

Procedure	TREATMENT*									
	Baseline			Start		End			Withdrawal	
	Day			Day		Day			Day	
	-14	-13	-12	1	2	40	41	42	57	58
24-h gastric pH	•			•		•			•	
Serum gastrin profile	•			•		•				
Plasma CgA profile	•			•		•				
Pharmacokinetics				•		•				
Pentagastrin infusion		•			•		•			•
Gastric biopsies			•					•		

* 42 days of netazepide and rabeprazole, alone and combined

Table 2. Basal H⁺ secretion rate (µmol/min) 0–30 min before dosing: (a) mean (SD) and geometric mean (SD) basal H⁺ secretion rate (µmol/min) at baseline, on Days 2 and 41 of treatment, and Day 58 (withdrawal); (b) ratio of treatment means adjusted for baseline for basal H⁺ secretion rate on Days 2, 41 and 58

a

Day	Treatment	Mean H ⁺ secretion rate (SD) (µmol/min)	Geometric mean H ⁺ secretion rate (µmol/min)	Geometric mean of ratio to baseline	95% confidence intervals	
Baseline	netazepide	81.4 (77.0)	49.9	–	–	–
	rabeprazole	63.9 (68.4)	29.3	–	–	–
	combination	36.4 (39.2)	24.4	–	–	–
2	netazepide	44.7 (35.0)	34.3	0.69	0.23	2.01
	rabeprazole	55.1 (83.9)	73.7	1.59	0.16	15.61
	combination	8.4 (8.6)	8.8	0.29	0.06	1.30
41	netazepide	59.8 (36.6)	45.9	0.92	0.33	2.57
	rabeprazole	28.3 (31.8)	15.9	0.54	0.11	2.53
	combination	6.1 (10.4)	5.9	0.16	0.05	0.58
58	netazepide	72.4 (48.7)	57.8	1.16	0.37	3.65
	rabeprazole	135.9 (132.3)	54.7	1.87	0.58	5.98
	combination	60.4 (83.1)	19.7	0.77	0.16	3.75

b

Day	Treatment comparison	Ratio of geometric treatment means adjusted for baseline	95% confidence intervals		P values
2	rabeprazole v netazepide	2.31	0.34	15.65	0.3601
	rabeprazole v combination	5.51	0.59	51.57	0.1198
	netazepide v combination	2.38	0.47	12.18	0.2750
41	rabeprazole v netazepide	0.58	0.11	3.14	0.5083
	rabeprazole v combination	3.31	0.45	24.33	0.2176
	netazepide v combination	5.67	1.25	25.71	0.0273
58	rabeprazole v netazepide	1.61	0.35	7.35	0.5173
	rabeprazole v combination	2.43	0.42	14.11	0.3007
	netazepide v combination	1.51	0.26	8.65	0.6250

Table 3. Pentagastrin-induced gastric aspirate 60–180 min after dosing at baseline, on Days 2 and 41 of treatment, and Day 58 (withdrawal): (a) mean (SD) volume (mL/min); (b) mean (SD) H⁺ secretion rate (μmol/min) at baseline, on Days 2 and 41 of treatment, and Day 58 (withdrawal), and mean change from baseline on Days 2, 41 and 58; (c) estimated differences between mean change from baseline for volume and H⁺ secretion rate on Days 2, 41 and 58

a

Day	Treatment	Mean volume (mL/min)	Mean change from baseline	95% confidence intervals	
Baseline	netazepide	4.0 (0.7)	–	–	–
	rabeprazole	4.2 (1.5)	–	–	–
	combination	3.9 (1.2)	–	–	–
2	netazepide	2.1 (1.0)	–1.9	–2.88	–0.97
	rabeprazole	1.8 (0.7)	–2.6	–3.52	–1.74
	combination	2.6 (1.4)	–1.3	–2.66	0.04
41	netazepide	2.3 (0.7)	–1.8	–2.64	–0.98
	rabeprazole	2.2 (1.1)	–1.9	–2.91	–0.93
	combination	1.9 (0.8)	–2.2	–3.49	–0.99
58	netazepide	4.4 (1.0)	0.45	–0.57	1.48
	rabeprazole	4.6 (1.7)	0.43	–0.49	1.34
	combination	3.9 (1.4)	–0.26	–1.19	0.66

b

Day	Treatment	Mean H ⁺ secretion rate (μmol/min)	Mean change from baseline	95% confidence intervals	
Baseline	netazepide	317.0 (123.7)	–	–	–
	rabeprazole	348.3 (166.2)	–	–	–
	combination	286.5 (151.8)	–	–	–
2	netazepide	83.6 (61.6)	–233	–330	–137
	rabeprazole	96.8 (85.3)	–252	–349	–154
	combination	33.6 (39.6)	–253	–355	–151
41	netazepide	111.6 (55.1)	–205	–273	–138
	rabeprazole	110.4 (52.6)	–238	–333	–143
	combination	21.1 (24.3)	–263	–364	–162
58	netazepide	389.8 (72.0)	72.8	–3.9	149
	rabeprazole	466.4 (167.1)	118	–10.1	246
	combination	304.3 (134.4)	12.5	–59.7	84.6

c

Day	Treatments compared	Gastric aspirate	Difference between treatment means adjusted for baseline	95% confidence intervals	
2	rabeprazole v netazepide	Volume	-0.71	-1.94	0.52
		H ⁺ secretion rate	-18.1	-146	110
	rabeprazole v combination	Volume	-1.32	-3.03	0.39
		H ⁺ secretion rate	1.5	-133	136
	netazepide v combination	Volume	-0.61	-2.23	1.00
		H ⁺ secretion rate	19.5	-115	154
41	rabeprazole v netazepide	Volume	-0.11	-1.32	1.09
		H ⁺ secretion rate	-32.5	-140	75
	rabeprazole v combination	Volume	0.32	-1.15	1.79
		H ⁺ secretion rate	25.3	-105	155
	netazepide v combination	Volume	0.43	-0.99	1.85
		H ⁺ secretion rate	57.8	-58	174
58	rabeprazole v netazepide	Volume	-0.03	-1.29	1.24
		H ⁺ secretion rate	45.3	-93.4	184
	rabeprazole v combination	Volume	0.69	-0.52	1.90
		H ⁺ secretion rate	106	-30.9	242
	netazepide v combination	Volume	0.72	-0.55	1.99
		H ⁺ secretion rate	60.3	-37.4	158

Table 4. Serum gastrin: (a) mean (SD) and geometric mean AUC_{0-24 h} at baseline and on Days 1 and 40 of treatment, and geometric mean of ratio to baseline on Days 1 and 40; (b) ratio of geometric means adjusted for baseline for AUC_{0-24 h} of gastrin on Days 1 and 40

a

Day	Treatment	Mean AUC _{0-24 h} (pmol.h/L)	Geometric mean (pmol.h/L)	Geometric mean of ratio to baseline	95% confidence intervals	
Baseline	netazepide	581 (419)	505	–	–	–
	rabeprazole	449 (168)	414	–	–	–
	combination	629 (365)	559	–	–	–
1	netazepide	1236 (1347)	913	1.81	1.47	2.23
	rabeprazole	920 (528)	785	1.90	1.43	2.51
	combination	1584 (1263)	1252	2.24	1.74	2.88
40	netazepide	1552 (1504)	1177	2.33	1.75	3.10
	rabeprazole	1841 (1030)	1594	3.86	2.67	5.56
	combination	3874 (2612)	3081	5.52	3.81	7.98

b

Day	Treatments compared	Ratio of geometric treatment means adjusted for baseline	95% confidence intervals		P values
1	rabeprazole v netazepide	1.05	0.76	1.45	0.7561
	rabeprazole v combination	0.85	0.60	1.20	0.3340
	netazepide v combination	0.81	0.60	1.09	0.1553
40	rabeprazole v netazepide	1.66	1.08	2.55	0.0243
	rabeprazole v combination	0.70	0.43	1.13	0.1368
	netazepide v combination	0.42	0.27	0.65	0.0006

Table 5. Plasma CgA: (a) mean (SD) and geometric mean AUC_{0-24 h} at baseline and on Days 1 and 40 of treatment, and geometric mean of ratio to baseline on Days 1 and 40; (b) ratio of geometric means adjusted for baseline for AUC_{0-24 h} of CgA on Days 1 and 40

a

Day	Treatment	Mean AUC _{0-24 h} (U.h/L)	Geometric mean (U.h/L)	Geometric mean of ratio to baseline	95% confidence intervals	
Baseline	netazepide	241 (55)	235	–	–	–
	rabeprazole	218 (69)	209	–	–	–
	combination	261 (49)	257	–	–	–
1	netazepide	203 (51)	198	0.84	0.76	0.93
	rabeprazole	241 (96)	226	1.09	0.96	1.23
	combination	230 (68)	221	0.86	0.77	0.97
40	netazepide	211 (55)	205	0.87	0.77	0.99
	rabeprazole	718 (789)	494	2.37	1.54	3.66
	combination	249 (55)	242	0.94	0.85	1.05

b

Day	Treatments compared	Ratio of geometric treatment means adjusted for baseline	95% confidence intervals		P values
1	rabeprazole v netazepide	1.29	1.11	1.50	0.0022
	rabeprazole v combination	1.26	1.08	1.47	0.0060
	netazepide v combination	0.98	0.84	1.13	0.7183
40	rabeprazole v netazepide	2.72	1.79	4.14	0.0001
	rabeprazole v combination	2.52	1.66	3.81	0.0002
	netazepide v combination	0.92	0.79	1.08	0.2961

Supplementary tables

Table S1. Mean (SD) pharmacokinetic parameters of netazepide on Days 1 and 40

	Day 1		Day 40	
	Netazepide (n=10)	Netazepide and rabeprazole (n=10)	Netazepide (n=10)	Netazepide and rabeprazole (n=11)
C_{max} (ng/mL)	38.9 (38.7)	49.9 (60.0)	47.9 (73.6)	37.1 (97.8)
AUC_{0-t} (ng.h/mL)	79.9 (80.7)	72.5 (72.1)	119.4 (120.1)	90.2 (189.4)
AUC_{0-∞} (ng.h/mL)	85.6 (80.2)	76.5 (71.1)	126.6 (116.8)	101.7 (187.8)
t_{max} (h)*	0.5 (0.5–2.0)	1.0 (0.5–1.0)	1.5 (0.5–8.0)	2.5 (0.5–4.0)
t_{1/2} (h)	6.5 (7.1)	4.8 (4.8)	8.5 (7.2)	9.6 (10.1)

* Median (range)

Table S2. Mean (SD) pharmacokinetic parameters of rabeprazole on Days 1 and 40

	Day 1		Day 40	
	Rabeprazole (n=9 or 10**)	Rabeprazole and netazepide (n=11)	Rabeprazole (n=9 or 10**)	Rabeprazole and netazepide (n=11)
C_{max} (ng/mL)	380.0 (287.1)	429.0 (210.0)	369.4 (279.0)	472.7 (304.6)
AUC_{0-t} (ng.h/mL)	595.6 (394.8)	987.2 (522.9)	718.7 (502.6)	1125.7 (796.2)
AUC_{0-∞} (ng.h/mL)	658.5 (364.5)	1001.6 (522.0)	798.5 (470.6)	1132.8 (801.3)
t_{max} (h)*	2.8 (2.0–8.0)	3.0 (2.0–8.0)	2.8 (0.5–8.0)	4.0 (2.0–4.0)
t_{1/2} (h)	3.0 (1.1)	5.0 (1.4)	3.8 (1.4)	4.4 (1.3)

* Median (range)

** C_{max}, AUC_{0-t} and t_{max}: n=10; AUC_{0-∞} and t_{1/2}: n=9

Table S3. 24 h ambulatory pH: (a) mean (SD) AUC_{0-24 h} of pH at baseline and on Days 1 and 40 (treatment) and Day 57 (withdrawal), and mean change from baseline on Days 1, 40 and 57; (b) estimated differences between the mean change from baseline for AUC_{0-24 h} of pH on Days 1, 40 and 57

a

Day	Treatment	Mean AUC _{0-24 h}	Mean change from baseline	95% confidence intervals		P value
Baseline	netazepide	40.2 (12.2)	–	–	–	–
	rabeprazole	44.2 (11.2)	–	–	–	–
	combination	40.7 (12.1)	–	–	–	–
1	netazepide	58.9 (8.1)	18.7	5.9	31.5	0.0091
	rabeprazole	66.6 (18.0)	22.4	8.3	36.5	0.0058
	combination	79.7 (14.2)	36.5	26.1	47.0	<0.0001
40	netazepide	52.1 (11.4)	12.0	1.6	22.4	0.0285
	rabeprazole	101.9 (18.6)	57.7	46.4	69.0	<0.0001
	combination	103.4 (12.4)	59.8	51.5	68.1	<0.0001
57	netazepide	44.6 (10.8)	4.4	–6.1	14.9	0.3683
	rabeprazole	61.2 (38.2)	17.0	–10.8	44.8	0.2005
	combination	57.4 (42.5)	17.1	–12.8	46.9	0.2281

b

Day	Treatment comparison	Difference between treatment means adjusted for baseline	95% confidence intervals		P value
1	rabeprazole v netazepide	3.7	–14.0	21.4	0.6671
	rabeprazole v combination	–14.1	–30.4	2.2	0.0850
	netazepide v combination	–17.8	–33.2	–2.5	0.0253
40	rabeprazole v netazepide	45.7	31.5	60.0	<0.0001
	rabeprazole v combination	–2.1	–15.2	10.9	0.7346
	netazepide v combination	–47.9	–60.2	–35.5	<0.0001
57	rabeprazole v netazepide	12.581	–15.0	40.2	0.3511
	rabeprazole v combination	–0.071	–37.9	37.8	0.9969
	netazepide v combination	–12.652	–42.0	16.7	0.3775

Table S4. ECL-cell ultrastructure

	Treatment	Baseline Mean (SD)	End of treatment Mean (SD)	% change (p value)
Cell area (μm^2)	rabeprazole	41.8 (5.4)	48.8 (15.2)	16.7 (0.21)
	netazepide	45.0 (10.6)	39.6 (12.2)	-12.0 (0.34)
	combination	47.0 (13.7)	45.9 (15.4)	-2.3 (0.86)
Nuclear area (μm^2)	rabeprazole	18.2 (2.8)	19.2 (6.5)	5.4 (0.68)
	netazepide	18.9 (3.3)	15.8 (5.1)	-16.1 (0.16)
	combination	18.6 (6.0)	18.7 (6.6)	0.9 (0.95)
Nuclear volume density (% cell area)	rabeprazole	44.2 (3.6)	39.5 (11.6)	-10.6 (0.26)
	netazepide	42.5 (7.3)	40.3 (11.0)	-5.3 (0.63)
	combination	40.6 (7.9)	41.5 (6.4)	2.3 (0.76)
Cytoplasmic area (μm^2)	rabeprazole	23.0 (3.6)	30.7 (13.2)	29.0 (0.14)
	netazepide	25.9 (8.3)	23.6 (9.3)	-8.8 (0.60)
	combination	27.9 (9.0)	27.4 (10.1)	-1.8 (0.90)
Microvesicles (number/cell)	rabeprazole	22.5 (7.7)	24.4 (7.5)	8.3 (0.62)
	netazepide	20.8 (6.4)	21.0 (7.0)	1.3 (0.93)
	combination	21.8 (5.5)	26.7 (11.2)	22.1 (0.24)
Microvesicle volume density (% cytoplasm)	rabeprazole	1.9 (0.5)	1.9 (0.6)	-0.9 (0.95)
	netazepide	2.2 (0.8)	1.8 (0.9)	-16.7 (0.39)
	combination	1.5 (0.4)	2.6 (2.4)	73.3 (0.18)
Granules (number/cell)	rabeprazole	25.9 (9.8)	20.2 (12.8)	-21.9 (0.32)
	netazepide	10.3 (6.3)	16.8 (14.2)	63.0 (0.24)
	combination	12.6 (5.7)	25.8 (19.2)	105.2 (0.06)
Granule volume density (% cytoplasm)	rabeprazole	4.2 (1.4)	3.3 (2.0)	-20.7 (0.30)
	netazepide	1.9 (0.9)	4.1 (4.2)	119.7 (0.15)
	combination	2.3 (1.1)	4.2 (2.4)	80.5 (0.04)
Secretory vesicles (number/cell)	rabeprazole	19.9 (6.9)	23.8 (25.9)	19.6 (0.66)
	netazepide	16.9 (4.3)	33.9 (43.1)	100.9 (0.27)
	combination	23.7 (15.4)	35.1 (36.0)	48.1 (0.37)
Secretory vesicle volume density (% cytoplasm)	rabeprazole	6.1 (1.0)	5.3 (3.8)	-12.6 (0.56)
	netazepide	5.7 (2.1)	8.6 (8.2)	50.3 (0.33)
	combination	6.7 (4.7)	7.8 (7.5)	17.4 (0.68)
Vacuoles (number/cell)	rabeprazole	0.1 (0.4)	0.3 (0.5)	93.2 (0.55)
	netazepide	0.4 (0.7)	0.3 (0.5)	-22.4 (0.78)
	combination	0.8 (1.6)	0.6 (1.3)	-27.4 (0.74)
Vacuole volume density (% cytoplasm)	rabeprazole	0.1 (0.2)	0.2 (0.3)	134.3 (0.45)
	netazepide	0.4 (0.7)	0.5 (0.8)	32.1 (0.75)
	combination	0.5 (1.0)	0.7 (1.7)	36.2 (0.76)
Lipofuscin body density (% cytoplasm)	rabeprazole	3.1 (4.6)	1.6 (1.6)	-48.4 (0.43)
	netazepide	1.7 (2.1)	1.5 (1.5)	-11.3 (0.83)
	combination	1.3 (1.3)	0.5 (0.6)	-59.2 (0.09)
Rough endoplasmic reticulum volume density (% cytoplasm)	rabeprazole	1.2 (0.6)	1.6 (1.5)	34.2 (0.46)
	netazepide	1.4 (0.4)	1.2 (0.7)	-13.8 (0.48)
	combination	1.6 (0.9)	1.3 (0.8)	-18.7 (0.44)
Golgi volume density (% cytoplasm)	rabeprazole	0.3 (0.41)	0.3 (0.6)	-6.3 (0.94)
	netazepide	1.0 (1.0)	0.6 (0.7)	-36.3 (0.44)
	combination	0.7 (1.5)	0.7 (1.2)	-11.6 (0.88)

Table S5. Mucosal HDC, CgA, VMAT2 and Ki67 immunoreactive cells

	Treatment	Mean cells/mm ² mucosa (SD)		% change (p-value)
		Baseline	End of treatment	
HDC	rabeprazole	7.5 (4.7)	61.1 (104.2)	712.9 (0.14)
	netazepide	14.7 (19.3)	37.5 (79.5)	155.5 (0.27)
	combination	22.4 (34.3)	6.9 (6.6)	-69.1 (0.20)
CgA	rabeprazole	130.7 (45.1)	149.4 (74.4)	14.3 (0.60)
	netazepide	192.5 (90.5)	180.3 (67.4)	-6.3 (0.78)
	combination	176.4 (106.8)	146.5 (84.8)	-17.0 (0.37)
VMAT2	rabeprazole	47.5 (31.6)	41.4 (28.6)	-13.0 (0.64)
	netazepide	70.4 (50.6)	19.5 (22.3)	-72.4 (0.01)
	combination	34.4 (53.7)	54.3 (67.0)	58.0 (0.24)
Ki67	rabeprazole	158.9 (123.0)	113.6 (70.9)	-28.5 (0.37)
	netazepide	226.8 (247.9)	115.6 (87.1)	-49.1 (0.16)
	combination	111.5 (100.3)	93.5 (66.0)	-16.2 (0.72)

Figure 1. 24-h ambulatory pH: mean (SD) AUC_{0-24 h} of pH at baseline, on Days 1 and 40 (treatment), and on Day 57 (withdrawal). * p <0.05 * p ≤0.0001
netazepide (■) rabeprazole (■) combination (■)**

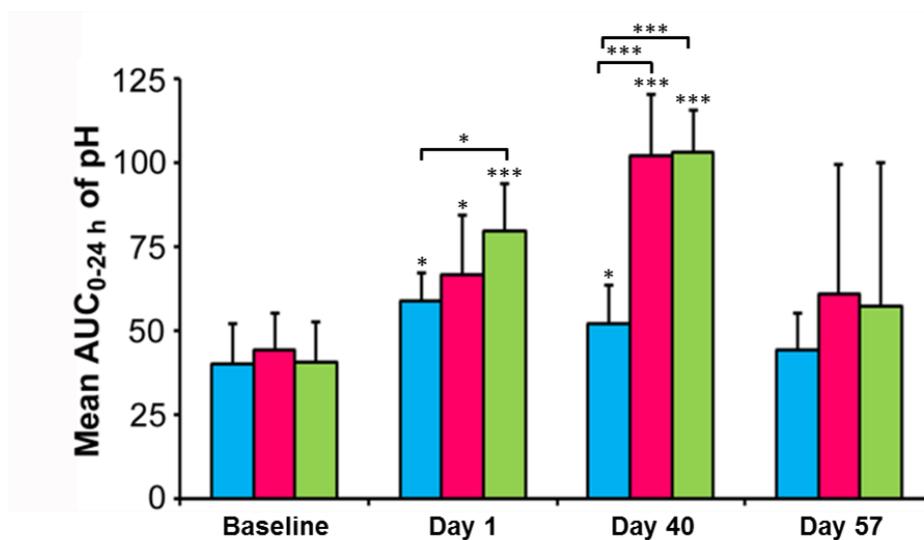


Figure 2. Gastric aspirate 60–180 min after dosing at baseline, on Days 2 and 41 (treatment), and on Day 58 (withdrawal). (a) mean (SD) pentagastrin-induced volume (mL/min), (b) mean (SD) pentagastrin-induced H⁺ secretion rate (μmol/min) and (c) geometric mean of basal H⁺ secretion rate (μmol/min).

* significant ratio to baseline.

netazepide (■) rabeprazole (■) combination (■)

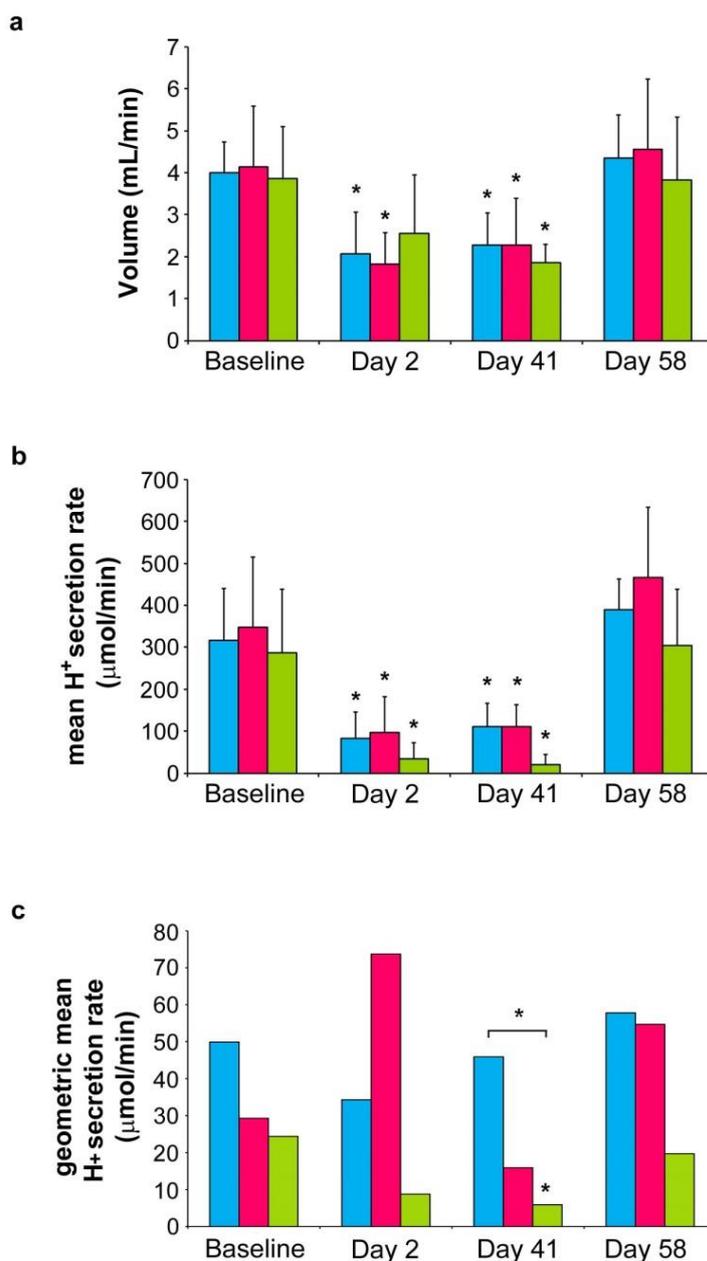


Figure 3. Serum gastrin: (a) mean (SD) concentration vs time on Day 40 of treatment. (b) geometric mean $AUC_{0-24\text{ h}}$ at baseline and on Days 1 and 40 of treatment. * significant ratio to baseline.

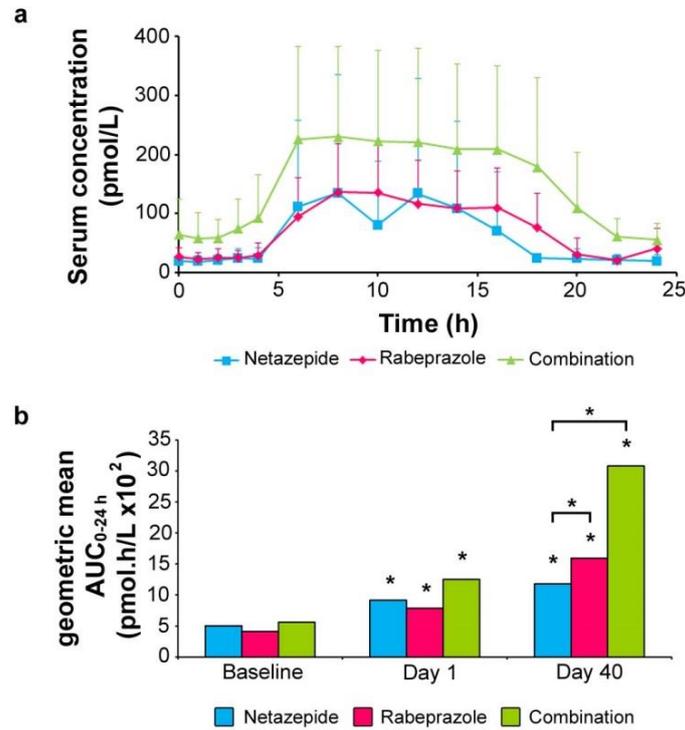
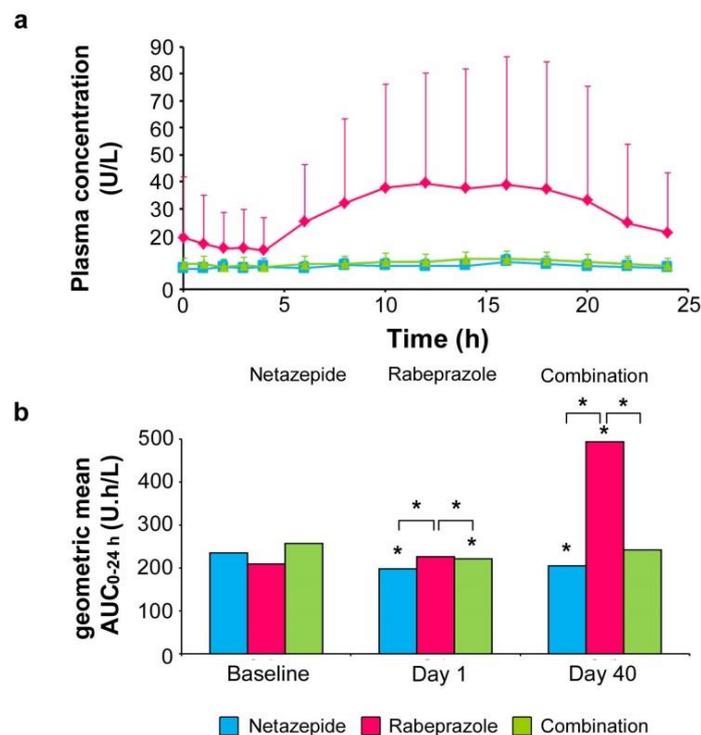


Figure 4. Plasma CgA: (a) mean (SD) concentration vs time on Day 40 of treatment. (b) geometric mean $AUC_{0-24\text{ h}}$ at baseline and on Days 1 and 40 of treatment. * significant ratio to baseline



Chapter 7

Study 8. Pharmacokinetics of a single dose of a formulation of crystalline netazepide taken after an overnight fast and after a high-fat breakfast

Introduction

Before the start of this study (Study 8), we had completed Studies 1–6. We had also completed the clinical part of Study 7, which comprised netazepide and rabeprazole, alone and in combination, as described in the previous chapter, but measurement of the plasma concentrations of netazepide and rabeprazole was still in progress, and the study had not yet been unblinded. We studied the pharmacokinetics of single and repeated doses of netazepide in Studies 1, 2, 3 and 6 after an overnight fast, but not after food. Food can alter the bioavailability of a drug substance by various means, including delaying gastric emptying, stimulating bile flow, changing gastrointestinal pH, increasing splanchnic blood flow, changing luminal metabolism, and physically or chemically interacting with the dosage form. Food affects bioavailability most when the drug substance is administered shortly after a meal. The nutrient and caloric content of the meal and its volume and temperature can cause physiological changes in the gastrointestinal tract that affect transit time, luminal dissolution, permeability and bioavailability of a drug substance. In general, meals high in calories and fat are more likely to affect bioavailability. The relative direction and magnitude of the effect of food on the bioavailability of a formulation are difficult, if not impossible, to predict without doing a study in humans.

The ‘industry standard’ to assess the effect of food on the bioavailability of an investigational medicinal product is to do a two-way crossover study in healthy subjects that compares the pharmacokinetics of the product after a high-fat, high-calorie test meal and after an overnight fast (FDA 2002). The meal should provide about 150, 250, and 500-600 calories from protein, carbohydrate and fat, respectively. The FDA test meal is two eggs fried in butter, two strips of bacon, two slices of toast with butter, four ounces of hash brown potatoes and eight ounces of whole milk. Substitutions can be made providing the meal has a similar amount of calories from protein, carbohydrate, and fat and has comparable meal volume and viscosity. A food-effect bioavailability study should be done early in a drug development programme, to guide and select formulations for further development. The highest expected therapeutic dose should be tested.

Objective

The purpose of this study (Study 8) was to assess the effect of food on the pharmacokinetics of the formulation of netazepide used in Study 7.

Study design

The study followed the FDA Guidance on Food-effect Bioavailability Studies (FDA 2002). 12 healthy subjects took part in a randomized, open, two-way crossover study of netazepide 100 mg after an overnight fast and a high-fat breakfast. The MHRA and Brent Research Ethics Committee approved the study, which was done during July–August 2007. Subjects were

deemed healthy as in previous studies, and gave informed, written consent. They were resident for two nights on two occasions separated by at least one week. On one occasion, they ate the FDA high-fat breakfast in the 30 min before dosing. They had to eat all of the meal. On the other occasion, they fasted overnight before dosing. Their other meals were standard and eaten at standard times. No food was allowed for 4 hours after dosing. We took blood for measurement of plasma netazepide concentrations before and frequently up to 24 h after dosing, as described previously.

Netazepide

HMR pharmacy prepared capsules containing netazepide API 25 mg mixed with HPMC and CCNa ('2006 formulation'). Subjects took netazepide 100 mg (4 capsules) with 240 mL water.

Statistics

We calculated the sample size using data from Study 1 and showed that 12 subjects would provide enough power to show bioequivalence if the 90% confidence intervals of the fed:fasted ratio were compared against the standard limits 0.8–1.25.

C_{\max} , $AUC_{0-\infty}$, and AUC_{0-t} of netazepide taken with and without food were logarithmically transformed and subjected to ANOVA, with subject as a random effect. For each of those parameters, the 90% confidence intervals of the least-square geometric means of netazepide with and without food were calculated

Results

Subjects

Six men and six women of mean age 28 (range 19–43) years and mean BMI 23 (range 19–27) started and completed the study. As in previous studies, netazepide was safe and well tolerated.

Pharmacokinetics

Individual and mean plasma concentrations of netazepide are listed in Tables and 2, respectively. Mean plasma concentrations plotted against time are shown in Figure 1. Selected mean pharmacokinetic parameters are summarised in Table 3.

Plasma concentrations of netazepide varied among subjects, especially after fasting. Mean C_{\max} varied by 26-fold after fasting, and by 5-fold after food. Mean $AUC_{0-\infty}$ varied by 24-fold after fasting and by 4-fold after food. Mean $AUC_{0-\infty}$ was 93% higher whereas C_{\max} was 50% lower after food compared with fasting. C_{\max} was delayed after food: median t_{\max} was: 0.75 h after fasting and 3.50 h after food. Mean $t_{1/2}$ was longer after food: $t_{1/2}$ was 5.39 h after food and 3.9 h after fasting. However, $t_{1/2}$ after fasting might be an underestimate, because plasma concentrations in many subjects at 8–24 h after dosing were near the LLQ or were BLQ.

Mean apparent clearance (CL/f) and mean apparent volume of distribution (V/f) were higher in after fasting than after food, because both are calculated using AUC, which was lower after fasting. $CL/f = \text{Dose}/AUC$ and $V/f = \text{Dose}/(\lambda_z \cdot AUC)$.

Plasma concentrations of netazepide were considerably lower than those in Studies 1–4. In Study 1, after an overnight fast, mean C_{\max} and AUC_{0-24h} of netazepide 100 mg were 397 ng/mL and 658 ng.h/mL, respectively, whereas in the current study mean C_{\max} and AUC_{0-24} of netazepide 100 mg after fasting were 83 ng/mL and 44 ng.h/mL, respectively. Thus, mean C_{\max} and AUC_{0-24h} after fasting were 8–9 fold lower in the current study.

The 90% CI of the log ratio fed:fasted did not fall within the range 80–125%, so there was a statistically significant effect of food on C_{\max} , $AUC_{0-\infty}$ and AUC_{0-t} of netazepide. Food delayed absorption and reduced C_{\max} of netazepide, although the overall impact was to increase its exposure.

Discussion

While the current study was in progress, we were surprised to learn that the bioavailability of netazepide in Study 7, which used the same formulation as the current study (Study 8), was poor given the favourable pharmacodynamic results. However, the bioavailability of the netazepide formulation used in the current study turned out to be similarly poor, and substantially less than that of the formulation used in Studies 1–4.

Conclusion

We decided to suspend formal clinical studies of netazepide while we investigated the reasons for the differences. The next two chapters describe our findings.

Reference

FDA Guidance. Food-effect and bioavailability and fed bioequivalence studies. Dec 2002.

Table 1. Mean (SD, n = 12) plasma concentrations of netazepide (ng/mL) after a single dose of netazepide 100 mg taken after a high-fat breakfast and an overnight fast

Time (h)	After a high-fat breakfast	After an overnight fast
0.00	BLQ	BLQ
0.25	0.00 (0.00)	0.49 (1.50)
0.50	0.97 (3.03)	18.7 (22.1)
0.75	3.82 (7.36)	30.0 (45.0)
1.00	5.41 (9.52)	25.7 (31.0)
1.25	6.65 (8.79)	20.2 (25.2)
1.50	7.82 (8.48)	16.9 (20.1)
2.00	10.6 (7.24)	14.2 (17.3)
3.00	16.2 (14.5)	10.5 (13.1)
4.00	14.0 (15.1)	7.40 (8.32)
8.00	9.33 (7.38)	2.69 (3.64)
12.00	4.29 (5.07)	0.88 (1.00)
16.00	1.44 (1.59)	0.34 (0.46)
24.00	0.69 (0.73)	0.16 (0.30)

BLQ = below limit of quantification.

Table 2. Mean (SD) pharmacokinetic parameters of netazepide after a single dose of netazepide 100 mg taken after a fatty breakfast and an overnight fast

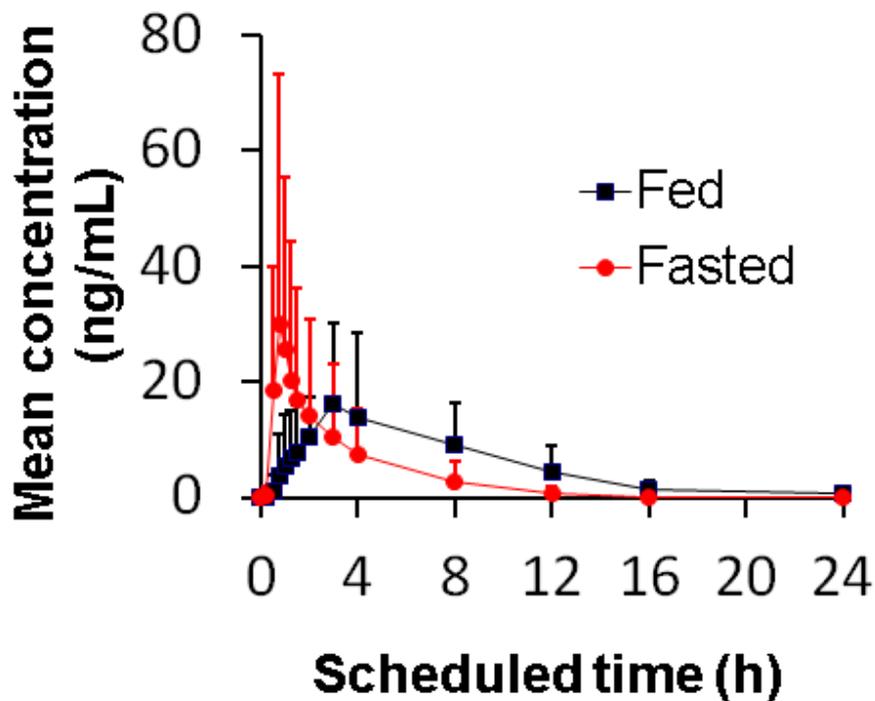
Parameter	After a fatty breakfast	After an overnight fast
C_{max} (ng/mL)	23.0 (13.0)	44.4 (44.4)
t_{max} (h)*	3.50 (1.00–8.0)	0.75 (0.50–4.0)
t_{1/2} (h)	5.39 (2.5)	3.81 (1.5)
AUC_{0-t} (ng.h/mL)	124 (60.5)	82.8 (71.2)
AUC_{0-∞} (ng.h/mL)	130 (64.7)	87.9 (74.3)
CL/f (L/h)	971 (490)	2901 (3055)
V/f (L)	6608 (3116)	15329 (16045)

* Median (range) t_{max}

Table 3. Bioequivalence analysis of pharmacokinetic parameters after a fatty breakfast and an overnight fast

Parameter	Ratio of least-square geometric means	90% confidence intervals
C_{max} (ng/mL)	72.4	47.5–110
AUC_{0-t} (ng.h/mL)	204	135–309
$AUC_{0-\infty}$ (ng.h/mL)	201	131–310

Figure 1. Mean (SD, n = 12) plasma netazepide concentrations (ng/mL) after a single dose of netazepide 100 mg taken after an overnight fast and a fatty breakfast



Chapter 8

**Self experiments with single doses of
various netazepide formulations**

Background

Although the pharmacodynamic results from the study of netazepide and rabeprazole, alone and in combination, in healthy subjects (Study 7) sponsored by Trio in 2006–2007 were favourable, the plasma concentrations of netazepide were very low compared to those in the netazepide studies in healthy subjects (Studies 1–4) sponsored by Ferring in 1996–1997. Mean C_{\max} and AUC_{0-24h} for a single dose of netazepide 100 mg were 39 ng/mL and 86 ng.h/mL, respectively, for Study 7, and 251 ng/mL and 612 ng.h/mL, respectively, for Study 2. Thus, bioavailability of the 1996 formulation was about seven-fold higher than that of the 2006 formulation. So, I investigated the problem.

Huntingdon Life Sciences (HLS) had supplied the netazepide 0.5, 5 and 20 mg capsules for the Ferring studies in 1996–1997, but at the time we were given no information about the method of manufacture other than netazepide was mixed with the excipients hydroxypropylmethyl-cellulose (HPMC) and croscarmellose (CCNa). HLS also supplied netazepide 1, 5 and 25 mg capsules for the next two studies in healthy subjects (Studies 5 and 6), which were sponsored by the James Black Foundation (JBF), in 2001. HLS told us that they had used a ‘coffee grinder’ to grind netazepide active pharmaceutical ingredient (API), and then mixed the ground netazepide with HPMC and CCNa in a Turbula 2 mixer. We collected blood at 2 h after dosing in Study 6 for assay of plasma concentrations of netazepide, but the samples were not assayed until weeks after we had completed the study, and we did not review the results at the time.

When in 2006 Trio did its first netazepide study in healthy subjects (Study 7), namely the study of rabeprazole and netazepide, alone and in combination, HMR pharmacy manufactured the netazepide capsules. They used a mortar and pestle rather than a ‘coffee grinder’ to grind netazepide API, mixed it with HPMC and CCNa, and spread the mix into capsules using a ProFill 100 capsule filling machine (Torpac, Fairfield, NJ07004, USA). The process of making capsules is illustrated in Figure 1. As stated above, the plasma concentrations of netazepide in Study 7 were very low compared with those from the Ferring studies of 1996–1997. When I checked the results of the JBF studies of 2001–2002, I discovered that the mean plasma netazepide concentration at 2 h after dosing in Study 6 was similarly low, about 25% of that in Study 2, although once again the pharmacodynamics results were favourable despite the low bioavailability. Mean C_{\max} and AUC_{0-24h} results from the Ferring, JBF and Trio studies are listed in Table 1. The 1996–1997 formulation was clearly much more bioavailable than either the 2001 or the 2006 formulation.

To help solve the problem of the marked differences in bioavailability, we used Pharmaterials Ltd, Reading, Berkshire, a contract research organisation specialising in the optimisation of the

physical properties of drug substances, to analyse and compare three samples of netazepide: (1) API from batch 55593, the remainder of which Ferring had donated to Trio in 2006; (2) netazepide in capsules for the rabeprazole/netazepide study (Study 7), which were made from batch 55593; and (3) netazepide in capsules from the 1996–1997 studies, which were made from netazepide batch 48388. HMR pharmacy had retained some of the netazepide capsules from both the 1996–1997 and the 2006 studies, but not from the 2001 studies. Pharmaterials used hyper-differential scanning calorimetry, Raman spectroscopy, X-ray diffraction and polarised optical microscopy to make the comparisons. Netazepide in the 2001 and 2006 formulations proved to be identical with respect to particle size, crystalline content, dissolution rate, melting point and thermal events, but the particle size and shape of netazepide in the 1996–1997 formulation indicated it was amorphous (Figure 2). It wasn't until circa 2010 that we learnt from Yamanouchi, Japan, that the netazepide capsules for the 1996–1997 studies had been manufactured by Yamato Sciences Company, Japan, in 1995. They mixed netazepide with HPMC, to improve the solubility of netazepide, dissolved the mix in methanol and then spray dried it. After spray drying the mix, they added croscarmellose (CCNa), to improve dispensability of the formulation. Spray drying is a method of producing a dry powder from a liquid or slurry by rapidly drying with a hot gas, and is a common method of increasing exposure to molecules with low solubility. Spray drying has been successfully applied to many compounds with low solubility (Paudel *et al* 2013; Bohr *et al* 2014). Spray drying would have reduced the particle size of netazepide API, which is crystalline and poorly soluble, and made it amorphous with increased surface area and thereby increased bioavailability.

And so began my series of 18 self-experiments, from 2007 to 2012, to assess the bioavailability of single doses of many different formulations of netazepide, and to help identify new formulations with better bioavailability. During that period, a physician colleague also did a self-experiment with two of the netazepide formulations, to confirm and compare with my results, making a total of 20 tests, which I refer to as 'n = 1 tests'.

The Royal College of Physicians report on Research in Healthy Volunteers (1986) states that: "In Britain there is a long tradition of investigators doing research on themselves. We would not wish to discourage this, but anyone who desires to experiment on himself should seek guidance of the Ethics Committee before doing so". Three physicians, Verner Forssmann (1956), Barry Marshall (2005) and Ralph Steinman (2011), won Nobel Prizes on the basis of self-experiments (Collins 2013; Ghani 2011). Marshall showed that self-administration of *H. pylori* caused acute gastritis and proposed that chronic colonisation with *H. pylori* leads directly to peptic ulceration (Marshall *et al* 1985).

Methods

Subjects

Throughout the series of tests, I had several medical examinations, including *H. pylori* (negative breath test), ECG recordings and safety tests of blood and urine to confirm eligibility. I fasted overnight before dosing the next morning. Before dosing, a physician colleague inserted a cannula into a forearm vein to collect blood samples for assay of plasma netazepide, mostly up to 12 h after dosing, but occasionally up to 24 h after dosing, as described in previous chapters.

Netazepide

HMR pharmacy supplied the single doses of netazepide. I tested various formulations: the 1996 and 2006 formulations; netazepide API alone; netazepide ground by mortar and pestle; spray dried netazepide with or without excipients; ball milled netazepide with or without excipients; nanoparticulate netazepide; and nano-milled formulations ('Zydis'). The date of each of the twenty $n = 1$ tests, the source of the netazepide, and details of the formulation and dose are in Tables 2A and 2B.

Results

The results of the plasma netazepide concentrations and the C_{\max} and AUC from each of the 20 tests are listed in Tables 3A and 3B. Netazepide concentrations *versus* time for the 20 tests are illustrated in Figures 4A and 4B. C_{\max} and $AUC_{0-12\text{ h}}$ for each test are shown in Figure 5. The main findings of the tests were as follows.

- Netazepide from batches 48388 and 55593, made in 1995 and 2001, respectively, is very stable. We are still using batch 55593 in ongoing studies.
- The capsule of netazepide made from spray-dried mix in 1995 that I tested in 2007 (Test 1) was less bioavailable than capsules from the same batch used in Studies 1–4 in 1996–1997. That isn't surprising given the 12-year period of storage.
- The capsule of netazepide in Test 2, which came from a batch of capsules made by HMR pharmacy from crystalline netazepide ground by mortar and pestle in 2006 for Studies 5 and 6, was substantially less bioavailable than the capsule from the batch of capsules made from amorphous netazepide in 1995 (Test 1) and used in Studies 1–4 in 1996–1997.
- Netazepide API alone in a capsule made by HMR pharmacy without HPMC and CCNa and without grinding in a mortar and pestle (Test 3) improved its bioavailability compared with Test 2. Grinding netazepide API by mortar and pestle appears to reduce its bioavailability.
- Ball milling crystalline netazepide API by Pharmaterials and by HMR pharmacy, to reduce the particle size (Tests 4 and 5, respectively), increased bioavailability compared with Test 3.

- The capsule of netazepide prepared by HMR pharmacy from natazepide API ground in a ‘coffee grinder’ (Test 6) had low bioavailability, similar to that of netazepide API ground by mortar and pestle. It transpired that HLS had used a ‘coffee grinder’ to prepare netazepide for their non-clinical studies, not for the capsules they made in 2001 for Studies 5 and 6.
- Micronised netazepide (Test 7), prepared by Pharmaterials, had increased bioavailability.
- Scale-up ball-milled netazepide (Test 8), made by Pharmaterials, had bioavailability as good as if not better than the spray-dried netazepide made in 1995 and used in Studies 1–4.
- Ball milling crystalline netazepide for about 5 h with balls of 10 mm (Test 8) was the best of the methods used to increase bioavailability by ball milling, and gave results similar to those for some of the spray-dried formulations.
- Bioavailability of HMR pharmacy’s first capsule made from R5 spray-dried netazepide was disappointing (Test 9), but bioavailability of subsequent capsules made from R5 spray-dried netazepide was much better (Tests 10–16). Bioavailability varied among tests, number of capsules and subject. Did the solvent used to dissolve netazepide influence particle size?
- Omitting starch as an excipient from a spray-dried formulation (Test 12) did not appear to affect bioavailability compared with the same formulations with starch (Tests 10 and 11).
- Bioavailability of spray-dried netazepide (Tests 17 and 18) made by Bend, a USA company, was no better than that made by R5.
- The two Zydys formulations, which were made from nano-milled netazepide, fish gelatin and mannitol, gave the best results (Tests 19 and 20).
- My C_{\max} and AUC results were similar to the mean values for the same strength dose in formal studies of the same formulation of netazepide used either in previous or subsequent formal studies in healthy subjects. My physician colleague, who did two $n = 1$ tests (Tests 11 and 13), had higher plasma concentrations of netazepide than me (Tests 10 and 14), illustrating the variability among subjects that we have seen in formal studies. Overall, the results of the $n = 1$ tests have face validity.
- $N = 1$ tests of recent new batches of spray-dried netazepide (Tests 15 and 16) confirmed their suitability for formal studies in healthy subjects or patients.

Discussion

These $n = 1$ tests highlight the importance of formulation development and the need to test the bioavailability of new formulations before embarking on pharmacodynamic studies in healthy subjects or patients. We didn’t test the formulations for the 2001 and 2006 studies before we started the studies; we were fortunate that low plasma concentrations of netazepide proved effective at blocking responses mediated via gastrin/CCK₂ receptors.

The $n = 1$ results clearly show that reducing the particle size of netazepide renders it more bioavailable than crystalline netazepide. Ball-milling, spray-drying and nano-milling all produced netazepide with increased bioavailability. A ball mill consists of a hollow cylindrical shell partially filled with balls and rotating about its axis. The grinding medium is the balls, which are usually made of chrome or stainless steel (Ball milling 2015). When the first ball-milled netazepide formulation, made by Pharmaterials, showed improved bioavailability, we bought our own ball-mill machine. However, when we ball-milled netazepide, it became electrostatic and adhered to all the equipment, so we abandoned the method lest we contaminate the pharmacy. Eventually we settled for spray drying as the method of reducing the particle size of netazepide (Paudel *et al* 2013; Bohr *et al* 2014). However, spray drying is expensive and wasteful of material. Nano-milled Zydis[®] formulations gave the best results, although that method is also expensive. The Zydis[®] ODT fast-dissolve formulation is a freeze-dried oral solid dosage form that disperses instantly in the mouth – no water is required. Fish gelatin supports nanoparticle formation of poorly water soluble drugs during wet milling and maintains stable nanoparticulates manufactured by freeze-drying. 20 Zydis[®] products are available worldwide (Bahl *et al* 2015).

We stopped searching for the 'ideal' netazepide formulation when we subsequently discovered that the main metabolite of netazepide, TR2, is also a potent gastrin/CCK₂ receptor antagonist, and that TR2-A, the acetyl derivative of TR2, is a prodrug of and more bioavailable than TR2 (see Chapters 16–20).

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Figure 1. HMR pharmacy using a ProFill 100 capsule filling machine to make capsules of netazepide for Trio's studies

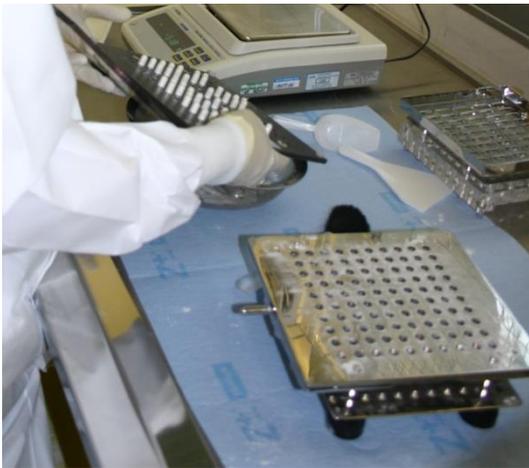
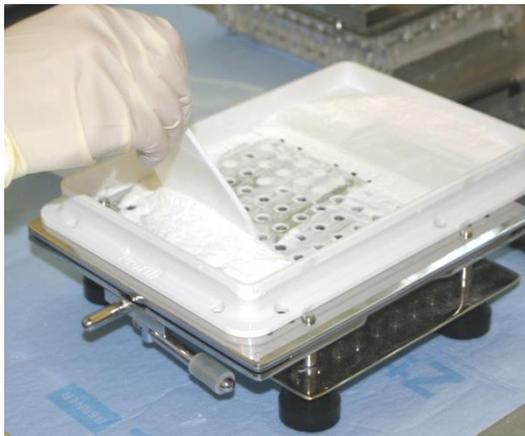


Figure 2. Raman spectroscopy of netazepide.
A. 1995 formulation (spray dried and amorphous). B. API batch 55593 (crystalline).
C. 2006 formulation (crystalline)

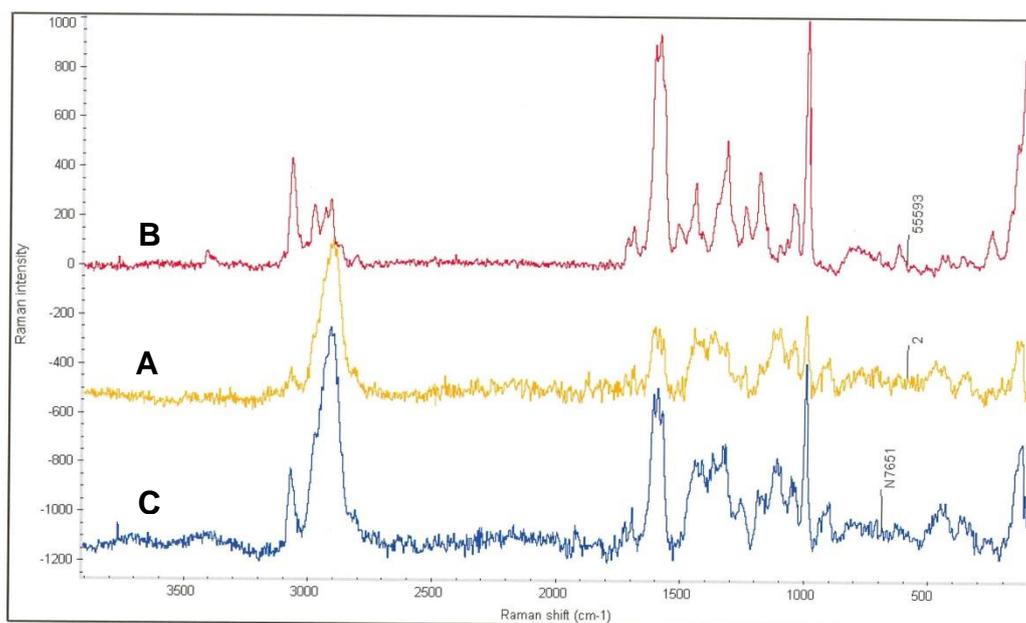


Figure 3. Polarised optical microscopy x 400 of netazepide.
A. 1995 formulation (spray dried and amorphous). B. API batch 55593 (crystalline).
C. 2006 formulation (crystalline)

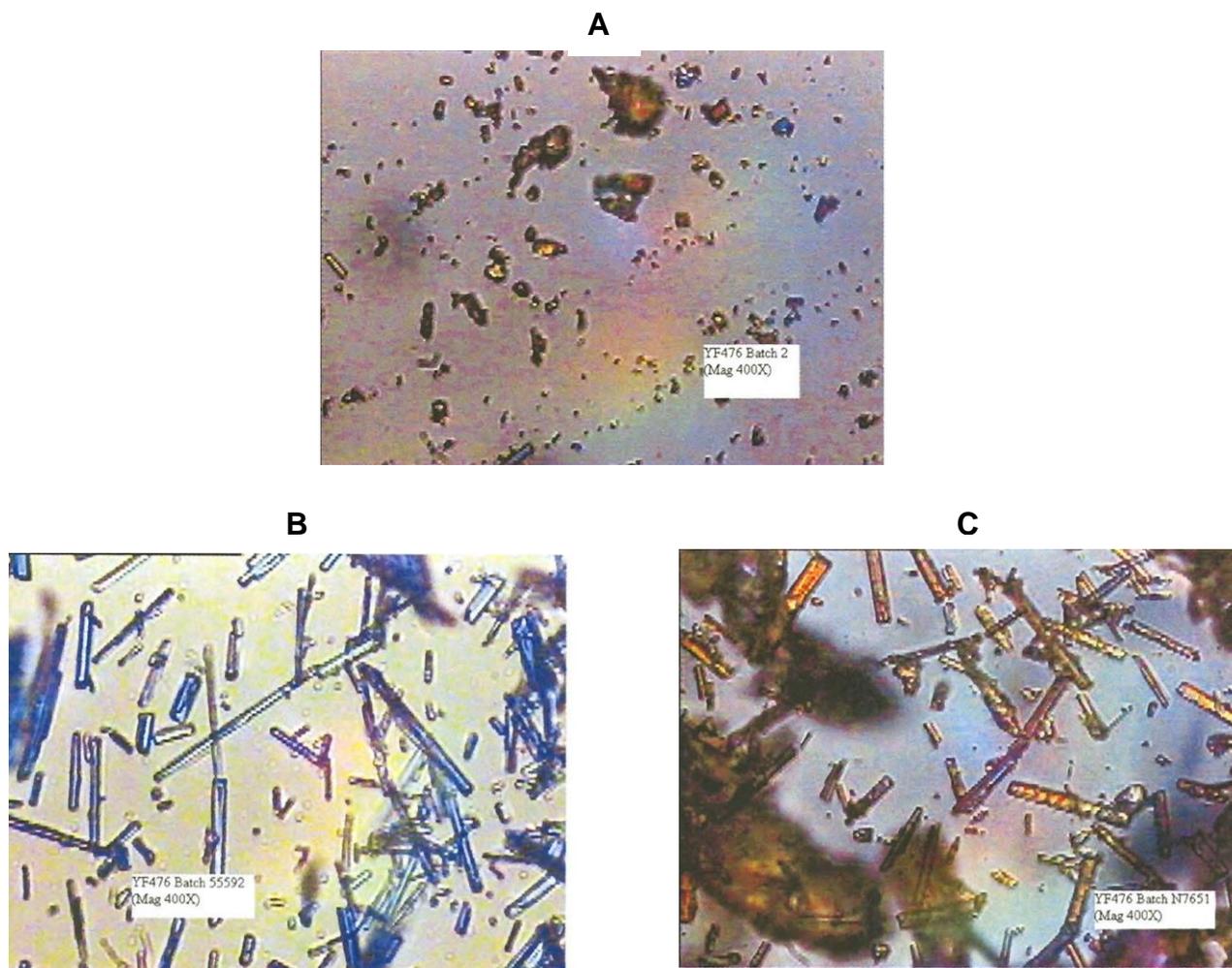


Figure 4A. N = 1 tests 1-10

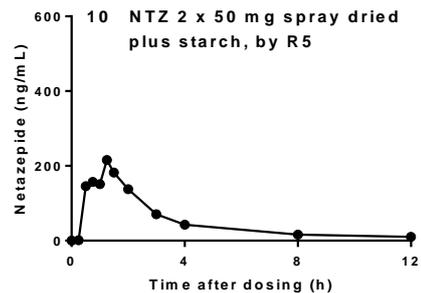
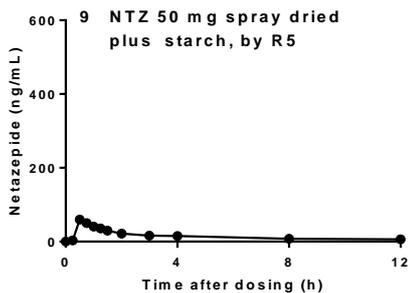
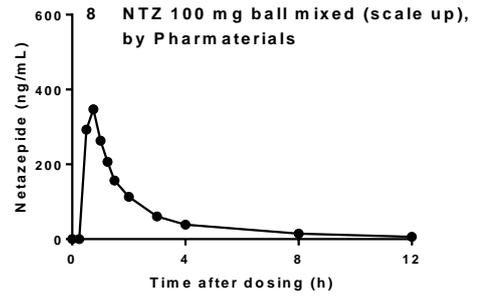
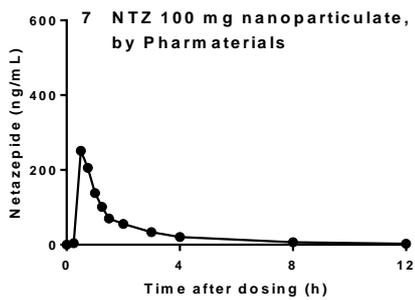
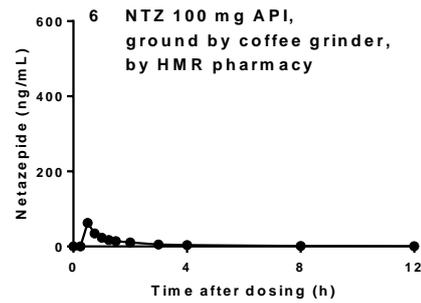
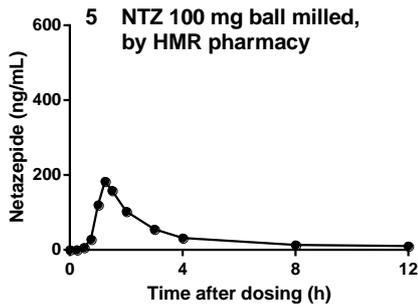
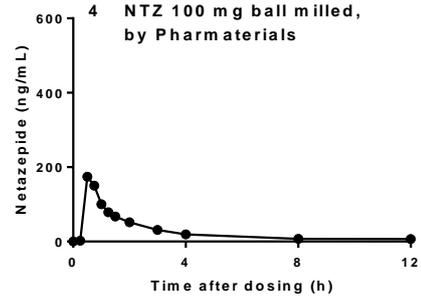
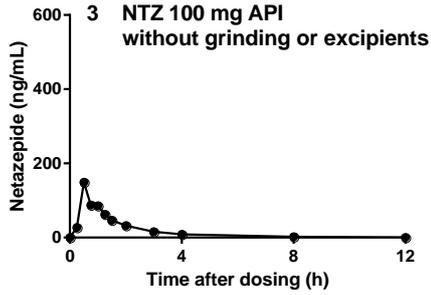
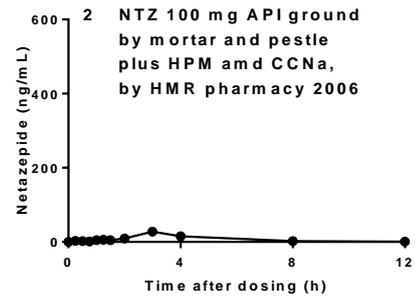
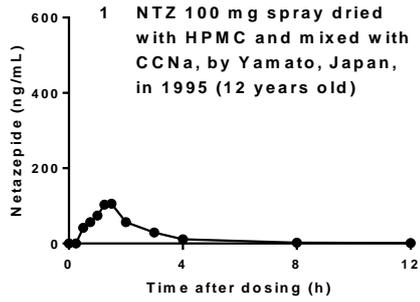


Figure 4B. N = 1 tests 11–20

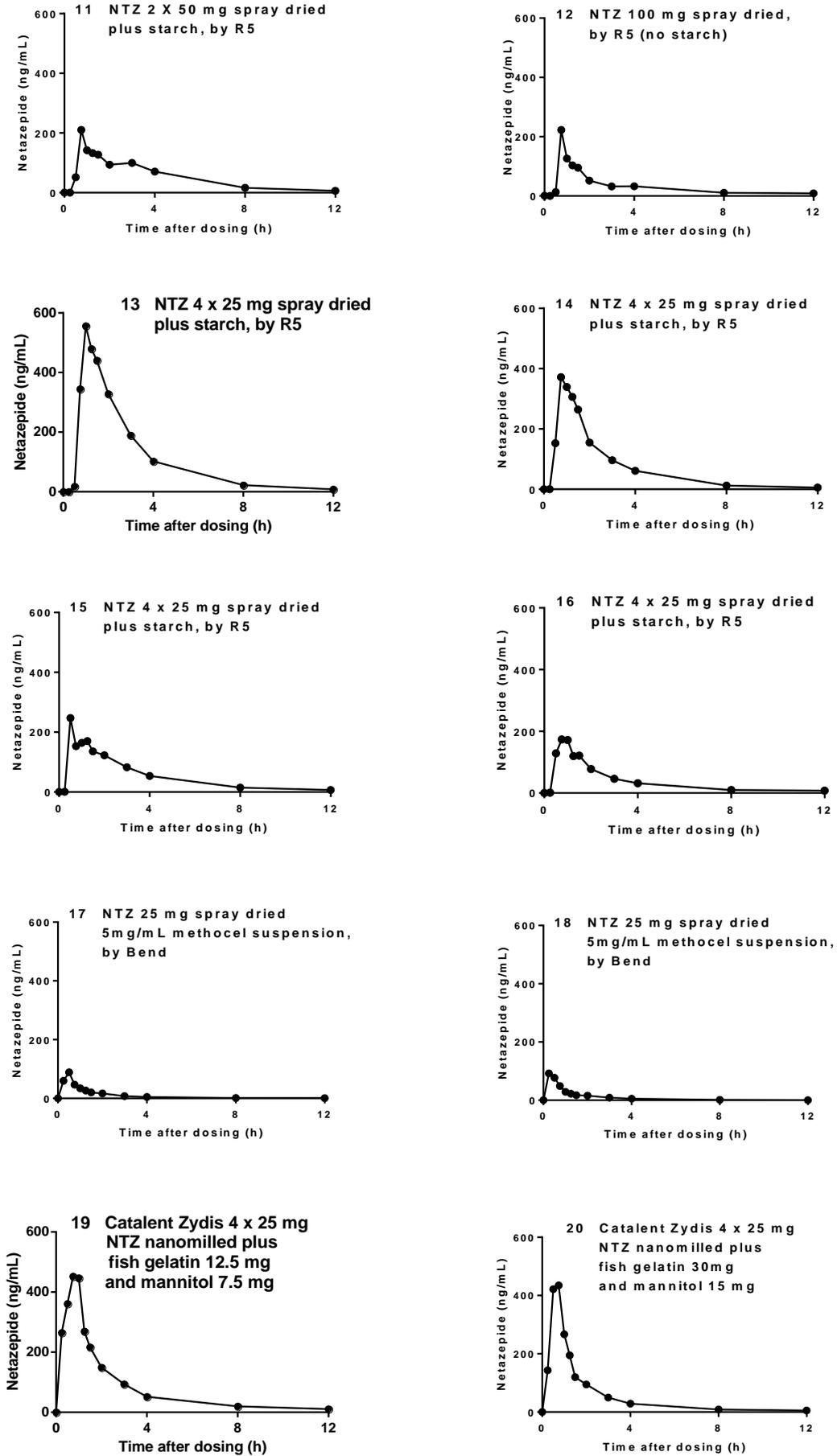
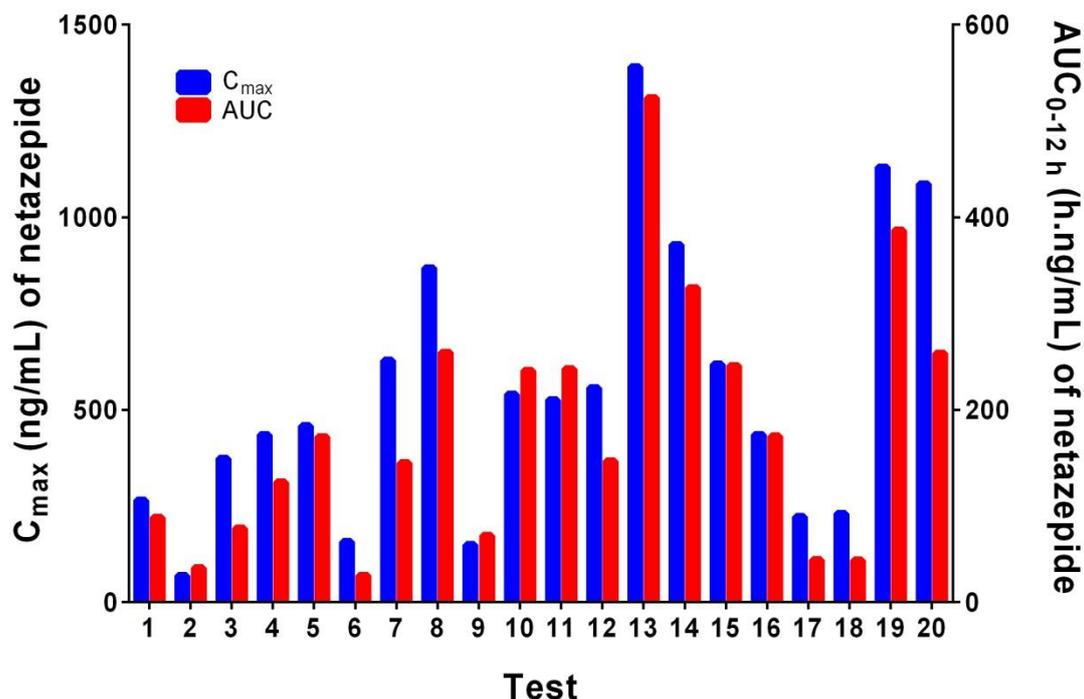


Figure 5. C_{max} and $AUC_{0-12 h}$ of netazepide. n = 1 tests 1–20



Legend to Figure 5. Tests 1–20

Test	Subject	Dose (mg)	Source	Formulation
1	MB	100	Yamato, Japan, 1995	SD (MeOH) with HPMC plus CCNa
2	MB	100	HMR, 2006	Ground API plus HPMC and CCNa
3	MB	100	HMR	API w/o grinding or excipients
4	MB	100	Pharmaterials	Ball milled for 24h
5	MB	100	HMR	Ball milled for 24h; 5 mm balls
6	MB	100	HMR	Ground API w/o excipients
7	MB	100	Pharmaterials	Micronised
8	MB	100	Pharmaterials	Ball milled (scale-up) for 5h; 10 mm balls
9	MB	50	R5	SD (MeOH and DCM) plus starch
10	MB	2 x 50	R5	SD (MeOH and DCM) plus starch
11	SW	2 x 50	R5	SS (MeOH and DCM) plus starch
12	MB	100	R5	SD (MeOH and DCM) w/o starch
13	SW	4 x 25	R5	SD (MeOH and DCM) plus starch
14	MB	4 x 25	R5	SD (MeOH and DCM) plus starch
15	MB	4 x 25	R5	SD (DCM and IPA) plus starch
16	MB	4 x 25	R5	SD (DCM and IPA) plus starch
17	MB	25	Bend	SD; methocel suspension
18	MB	25	Bend	SD; methocel suspension
19	MB	4 x 25	Catalent	Nanomilled plus fish gelatin 12.5 mg and mannitol 7.5 mg
20	MB	4 x 25	Catalent	Nanomilled plus fish gelatin 30 mg and mannitol 15 mg

SD = spray dried; API = active pharmaceutical ingredient; HPMC = hydroxypropylmethyl cellulose; CCNa = croscarmellose sodium; MeOH = methanol; DCM = dichloromethane; IPA = isopropyl alcohol

Table 1. Summary of mean C_{max} and AUC_{0–24h} of different formulations of netazepide 100 mg taken by healthy subjects after an overnight fast

Study	Capsule formulation	Year made	No. of subjects	Dose regimen	Mean C _{max} (ng/mL)	Mean AUC _{0–24h} (ng.h/mL)
1	SD netazepide (with HPMC) and excipient CCNa	1996	8	Single	397	658
2	SD netazepide (with HPMC) and excipient CCNa	1996	21	Single	251	612
3	SD netazepide (with HPMC) and excipient CCNa	1996	13	7 days bd	569 ¹	981 ¹
6	Netazepide API with excipients HPMC and CCNa	2001	8	6.5 days bd	118 ²	–
7	Netazepide API with excipients HPMC and CCNa	2006	10	6 weeks od	39 ¹	86 ¹
8	Netazepide API with excipients HPMC and CCNa	2006	12	Single	44	83

API = active pharmaceutical ingredient

HPMC = hydroxypropylmethylcellulose

CCNa = croscarmellose sodium

SD = spray-dried dispersion

¹ After last dose

² At 1 h after last dose

Netazepide not assayed during Studies 4 and 5

Table 2A. N = 1 tests. Single doses of netazepide formulations 1–9

Test	Date	Subject	Netazepide source	Batch	Formulation	Dose	Fillers	Studies	C _{max}	AUC _{0-12h}
1	06 Sep 07	MJB	Yamato Sciences Co, Japan, made capsules from spray-dried netazepide in 1995.	48338* (6505.7)	HLS did stability tests on Yamato's capsules before supplying them to HMR for studies sponsored by Ferring in 1996–1997. Some of the capsules were retained by HMR pharmacy.	100 mg	HPMC CCNa	96-001* 96-016* 96-018* 97-010*	105.8	220.0
2	10 Sep 07	MJB	Batch made by Clausson Kaas, Denmark, in 2001, when JNJ licensed netazepide for JBF.	55593*	Ferring gave Trio remainder of batch 55593 in 2006. HMR pharmacy ground API by mortar and pestle for the netazepide/ rabeprazole study in 2006. Some capsules were retained by HMR pharmacy.	100 mg capsule	HPMC CCNa	05-021*	27.6	83.4
3	11 Oct 07	MJB	Trio	55593	Netazepide API alone.	100 mg capsule	—	—	149.3	188.3
4	19 Oct 07	MJB	Trio	55593	Pharmaterials ball milled API for 24 h.	100 mg capsule	—	—	174.0	491.9
5	22 Oct 07	MJB	Trio	55593	Pharmaterials ball milled netazepide API + fillers for 24 h. (80 mL stainless steel grinding bowl, 5 mm balls, 8 grams API).	100 mg capsule	HPMC CCNa	—	183.1	349.9
6	24 Oct 07	MJB	Trio	55593	HMR pharmacy ground netazepide API with a coffee grinder ('HLS recipe').	100 mg capsule	HPMC CCNa	—	62.7	72.6
7	30 Oct 07	MJB	Trio	55593	Pharmaterials made nanoparticulate netazepide.	100 mg capsule	—	—	251.3	364.4
8	14 Nov 07	MJB	Trio	55593	Pharmaterials ball milled netazepide API for 5 h. (Scale-up. 250 mL stainless steel grinding bowl, 10 mm balls, 70 grams API).	100 mg capsule	Starch	—	347.3	633.3
9	22 Sep 08	MJB	Trio	55593	R5 made 97 g spray-dried netazepide (batch RX50265/Upp1).	50 mg capsule	Starch	—	59.7	171.0

* In 2007, Pharmaterials showed that: netazepide in capsules made from batch 48338 for studies in 1996–1997 was **amorphous**; netazepide in capsules made for studies in 2006 was **crystalline**; and netazepide in batch 55593 was **crystalline**. HPMC = hydroxypropylmethyl cellulose. CCNa = croscarmellose sodium.

Table 2B. N = 1 tests. Single doses of netazepide formulations 10–20

Test	Date	Subject	Netazepide source	Batch	Formulation	Dose	Fillers	Studies	C _{max}	AUC _{0-12h}
10	25 Sep 08	MJB	Trio	55593	R5 made spray-dried netazepide RX50265/Upp1 HMR pharmacy made a capsule.	2 x 50 mg capsules	Starch	—	215.9	587.2
11	26 Sep 08	SJW	Trio	55593	Spray-dried netazepide by R5. RX50265/Upp1	2 x 50 mg capsules	Starch	—	210.2	575.5
12	29 Sep 08	MJB	Trio.	55593	Spray-dried netazepide by R5. RX50265/Upp1	100 mg capsule	—	—	222.8	354.6
13	03 Oct 08	SJW	Trio	55593	Spray-dried netazepide by R5. RX50265/Upp1	4 x 25 mg capsules	Starch	—	556.0	1254.0
14	06 Oct 08	MJB	Trio	55593	Spray-dried netazepide by R5. RX50265/Upp1	4 x 25 mg capsules	Starch	—	371.5	783.6
15	09 Feb 09	MJB	Trio	55593	Spray-dried netazepide by R5. RX50420.002	4 x 25 mg capsules	Starch	07-024; 05-029; 09-013; 07-012; 07-505; 10-501; 07-504	347.2	592.2
16	20 Sep 09	MJB	Trio	55593	Spray-dried netazepide by R5. RX50377.03 plus RX50377.04	4 x 25 mg capsules	Starch	—	173.9	419.1
17	24 Nov 09	MJB	Trio	55593	Spray-dried netazepide by Bend Research. 5 mg/mL methocel suspension.	25 mg	—	—	88.8	106.1
18	03 Dec 09	MJB	Trio	55593	Spray-dried netazepide by Bend Research. 5 mg/mL methocel suspension.	25 mg	—	—	92.2	107.3
19	21 Aug 12	MJB	Trio	55593	Catalent 'Zydis'. Small chalky tablet. (nano-milled netazepide 25 mg, fish gelatin 12.5 mg and mannitol 7.5 mg). Lot # CAE-J003-120522-B	4 x 25 mg tablets	—	—	452.0	1011.0*
20	29 Aug 12	MJB	Trio	55593	Catalent 'Zydis'. Larger tablet. (nano-milled netazepide 25 mg, fish gelatin 30 mg and mannitol 15 mg). Lot # CAE-J0003-120613-A	4 x 25 mg tablets	—	—	434.5	660.6*

Tests 11 and 13 were done by a physician colleague (SW). All other tests were done by the author.

*AUC_{0-24h}

Table 3A. Plasma netazepide (ng/mL) after single doses. Tests 1–10

Time (h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
0.25	0	2.8	27.1	2.1	0	0	4.2	0	3.0	1.4
0.5	41.6	2.0	149.3	174.0	6.2	62.7	251.2	292.9	59.7	145.7
0.75	56.6	1.4	87.9	150.0	28.0	34.7	205.6	347.3	50.1	157.1
1	74.4	4.7	85.7	100.4	120.1	23.0	138.0	263.0	40.8	151.3
1.25	103.3	5.7	62.5	78.9	183.1	17.0	101.4	206.7	35.6	215.9
1.5	105.8	4.6	46.7	67.4	158.9	14.0	70.4	156.5	29.7	182.4
2	56.6	9.1	32.3	51.9	102.9	10.6	55.9	113.0	21.9	137.7
3	29.3	27.6	15.6	31.3	55.0	5.3	33.5	60.9	16.2	70.7
4	11.0	15.2	8.9	19.1	31.9	3.6	20.7	38.7	15.4	42.8
8	2.3	2.3	2.3	7.2	13.2	1.4	6.8	14.5	7.9	16.1
12	1.4	0.9	0	6.5	10.4	0.8	2.8	6.0	6.0	10.2
24	0	0	—	—	—	—	—	—	—	—
C_{max}	105.8	27.6	149.3	174.0	183.1	62.7	251.2	347.3	59.7	215.9
AUC_{0-12h}	220.0	83.4	188.3	491.9	350.0	72.6	364.4	633.3	171.0	587.2

Table 3B. Plasma netazepide (ng/mL) after single doses. Tests 11–20

Time (h)	11	12	13	14	15	16	17	18	19	20
0	0	0.7	0	0	0	0	0	0	0	0
0.25	0	0.3	0	0	0.5	1.1	59.9	92.2	264.8	143.4
0.5	51.9	13.4	17.5	152.5	247.2	128.8	88.8	77.2	361.4	421.5
0.75	210.2	222.8	344.6	371.5	153.2	173.9	46.8	48.5	452.0	434.5
1	142.1	126.1	556.0	339.1	164.0	171.5	34.1	28.9	446.5	266.5
1.25	132.9	103.2	479.8	306.1	170.3	119.7	26.8	22.	269.0	194.9
1.5	127.3	95.2	440.8	264.1	135.6	121.4	20.2	17.3	216.5	119.7
2	93.9	51.5	328.1	154.8	122.5	77.8	16.9	15.6	149.1	94.9
3	99.8	32.4	189.4	96.4	82.8	46.3	7.9	8.5	93.6	49.7
4	70.8	32.8	102.2	61.1	53.5	31.5	4.8	5.2	51.9	28.7
8	16.3	10.9	22.1	12.3	14.5	9.5	1.1	1.0	19.9	8.5
12	6.0	8.6	8.1	5.5	6.6	7.6	0.5	NS	10.8	5.0
24	0	—	—	—	—	—	—	—	2.1	0.9
C_{max}	210.2	222.8	556.0	371.5	247.2	173.9	88.8	92.2	452.0	434.5
AUC_{0-12h}	575.5	354.6	1254	783.6	592.2	419.1	106.1	107.3	1011	660.6

NS = no sample

Chapter 9

Study 9. Pharmacokinetics of a new spray-dried formulation of netazepide in healthy subjects

Introduction

Three different capsule formulations of netazepide were used in Studies 1–8 described in previous chapters. Details are summarised in Tables 1 and 2. The first formulation was an amorphous spray-dried dispersion (SDD) of netazepide with hydroxypropylmethylcellulose (HPMC) encapsulated with the excipient crosscarmellose sodium (CCNa), and used in Studies 1–4 that were sponsored by Ferring and done in 1996–1997. We call that the 1996 formulation. The second formulation contained crystalline netazepide blended with HPMC and CCNa, and was used in Studies 5 & 6 that were sponsored by the James Black Foundation (JBF) and done in 2001–2002. We refer to that as the 2001 formulation. The third formulation contained crystalline netazepide blended with HPMC and CCNa, and was made by HMR pharmacy for the study of netazepide and rabeprazole, alone and in combination (Study 7) and the fed/fasting study (Study 8), which were sponsored by Trio. We call that the 2007 formulation. The formulation of spray-dried netazepide used in Studies 1–4 gave plasma concentrations of netazepide about seven-fold higher than those of the formulation of crystalline netazepide used in Studies 5 & 6.

The $n = 1$ studies described in Chapter 7 confirmed that spray-drying increases the bioavailability of netazepide. Spray drying reduces the particle size of crystalline netazepide and makes it amorphous. Amorphous material usually has a higher intrinsic dissolution rate (IDR) than crystalline material, which is usually indicative of increased bioavailability. Indeed, amorphous netazepide had a 10-fold higher IDR than crystalline netazepide at pH 1.99 (Pharmorphix 2008).

So, we had R5 Pharmaceuticals, Nottingham, make a batch of 105 grams of amorphous netazepide by spray-drying a solution of netazepide with HPMC. This chapter describes a study (Study 9) of a formulation of netazepide made from that batch. We call that the 2009 formulation. Details of the formulation, and earlier formulations for comparison, are summarised in Tables 1 and 2.

During the period of about 18–24 months that we investigated the reasons for the differences among the netazepide formulations, we did no clinical studies of netazepide

Objectives

The aims of this study were to assess in healthy subjects: (1) the bioavailability of single, escalating doses of the new formulation of spray-dried netazepide before re-starting clinical studies; (2) the effect of food on the pharmacokinetics of the new formulation, and (3) the effect of the new formulation on serum gastrin and 24-h ambulatory gastric pH.

Study design

The study was in two parts. 10 subjects were required to complete both parts. The first part (Periods 1–5) was a randomised, five-way crossover assessment of the pharmacokinetics, safety and tolerability of single, rising doses of netazepide 50, 100, 200 and 400 mg, and placebo. The second part (Period 6) was an open assessment of the effect of food on the pharmacokinetics of a single dose of netazepide 100 mg. In each of Periods 1–5, eight subjects received netazepide and two received matching placebo, after an overnight fast. In Period 6, all subjects took netazepide after a standard, high-fat breakfast. There was a washout of seven days between doses. The study was approved by the MHRA and the Independent Research Ethics Committee, Plymouth, and was done during March–June 2009.

Method

10 subjects, deemed healthy as described in previous chapters, took part in the study. We collected blood frequently 0–24 h after each dose, to measure plasma concentrations of netazepide, as described in Chapter 3. On days when subjects fasted overnight, we also measured serum gastrin in the blood samples collected 0–24 h after dosing. We measured ambulatory 24-h gastric pH in some subjects. And we assessed safety and tolerability of netazepide. The methods are described in previous chapters.

Materials

R5 Pharmaceuticals Ltd, Nottingham, England (now part of Aesica Pharmaceuticals Ltd) used a Buchi B191 Spray Dryer to spray dry to cGMP standards a solution of netazepide (batch 55593) and HPMC (ratio of 1:3.5) dissolved in dichloromethane/isopropanol (7:2 v/v), to give a final solute concentration of 4%. The formulated intermediate was a white, amorphous powder. During production, the solution and spray-dried powder were shielded from light (IMP dossier).

HMR pharmacy prepared capsules containing the co-formulation of spray-dried netazepide 25 mg (118.6 mg formulated intermediate) and HPMC blended with starch, and matching placebo capsules containing starch only.

Statistics

Sample size

This was a pilot study and a formal sample size determination was deemed inappropriate.

Pharmacokinetic analysis

The following pharmacokinetic parameters of netazepide were derived using the measured plasma concentration of netazepide and the actual sampling times: peak concentration (C_{\max}); time to peak concentration (t_{\max}); terminal half-life calculated from the terminal slope of the log

concentration-time curve ($t_{1/2}$); area under the concentration-time and moment curves (AUC_{0-24h} , AUC_{0-t_n} , $AUC_{0-\infty}$ and AUMC); total body clearance from plasma (CL/f); apparent volume of distribution (V/f); and mean residence time (MRT).

Dose proportionality

AUC_{0-24} , $AUC_{0-\infty}$ and C_{max} were tested for dose-proportionality by regression analysis of the value of the log-transformed parameter versus the log-transformed dose, with subject as a random effect. Dose proportionality was concluded if the 95% CI for the regression parameter included the value 1.0. The significance of the regression model was tested using the F -test, and the lack-of-fit of the model was calculated and tabulated.

Effect of food

To compare the pharmacokinetics of netazepide taken after an overnight fast and after a fatty breakfast, a bioequivalence analysis was done, as follows. C_{max} , $AUC_{0-\infty}$, and AUC_{0-t_n} were logarithmically transformed and subjected to analysis of variance (ANOVA). Using the residual variation of ANOVA, 90% confidence intervals (CI) for the ratio food:fasting of geometric means of pharmacokinetic parameters of netazepide were calculated. If the 90% CI of log-ratio food:fasting did not fall entirely within the range 80–125%, a statistically significant effect of food was concluded. The median and 90% confidence intervals of the difference test-reference for t_{max} were calculated from the Wilcoxon signed rank test.

Serum gastrin

For the comparison of each dose of netazepide and placebo, AUC_{0-24h} of serum gastrin was analysed using an ANOVA model with a term for dose and a random effect for subject. A point estimate and 95% confidence intervals for the difference of the netazepide dose to placebo was calculated.

Results

Subjects

Eight men and two women of mean age 30.4 (range 23–40) years and mean body mass index 25.1 (range 19.2–27.9) completed the study. There were no drop-outs.

Pharmacokinetics

Mean concentrations of plasma netazepide from the first part of the study are listed in Table 3 and illustrated in Figure 1, and mean pharmacokinetic parameters are listed in Table 4. Plasma concentrations varied among subjects: C_{max} varied up to about 6-fold after netazepide 50–200 mg, and up to 3.7-fold after 400 mg. The value for t_{max} was consistently 0.8–1 h across the dose range, whereas $t_{1/2}$ increased with dose. The $t_{1/2}$ values for netazepide 50 and 100 mg are

probably underestimates because in many of the subjects netazepide concentrations from 12–24 h after dosing were either LLQ or BLQ. The $t_{1/2}$ of netazepide 400 mg is probably an overestimate because dose-proportionality was lost at netazepide 400 mg (see below). The $t_{1/2}$ 11.1 h of netazepide 200 mg is probably the most reliable. However, a reliable estimate of $t_{1/2}$ would require blood sampling up to ~36 h after dosing. Mean apparent clearance (CL/f) and mean apparent volume of distribution (V/f) increased with dose, because both are calculated using AUC parameters (CL/f = Dose/AUC; and V/f = Dose/(λ_z ·AUC), and AUC was not proportional to dose.

Dose proportionality

The mean slopes of the lines for log C_{max} and log AUC_{0-24h} were 0.76 and 0.75, respectively. Thus, the 95% confidence intervals of the mean slopes did not encompass 1.0, so neither C_{max} nor AUC_{0-24h} was deemed proportional to dose. However, mean log C_{max} and log AUC_{0-24h} of netazepide 50–200 mg were 2.5–3.2 ng/mL/mg and 5.2–6.9 ng.h/mL/mg (Figure 3), respectively, which would suggest that C_{max} and AUC_{0-24h} of netazepide doses up to 200 mg *do* increase approximately in proportion. But, mean log C_{max} and log AUC_{0-24h} of netazepide 400 mg were 1.76 ng/mL/mg and 3.89 ng.h/mL/mg, respectively, which would suggest that at doses >200 mg, netazepide concentrations do *not* increase in proportion to dose.

Effect of food

Mean concentrations of plasma netazepide 100 mg taken after a high-fat breakfast and an overnight fast are compared in Table 6 and Figure 2. Mean pharmacokinetic parameters of netazepide 100 mg taken with and without food are summarised in Table 7. Results of the bioequivalence analysis are in Table 8.

Netazepide was more bioavailable when taken after food compared with fasting. The 90% confidence intervals of the log ratio food : fasting did not fall within the range 80–125%, so food significantly increased C_{max} , $AUC_{0-\infty}$, and AUC_{0-24h} . C_{max} and AUC_{0-24h} were 1.9-fold greater ($p = 0.021$) and 1.6-fold greater ($p = 0.006$), respectively, when netazepide was taken after food. Median t_{max} of netazepide 100 mg taken after food was delayed: after food t_{max} was 1.5 h compared with 0.8 h after fasting.

Serum gastrin

Mean concentrations of serum gastrin for netazepide 50–400 mg taken after an overnight fast are listed in Table 9 and illustrated in Figure 4. Mean values for AUC_{0-24h} of serum gastrin are summarised in Table 10 and illustrated in Figure 5. All doses of netazepide increased the AUC_{0-24h} of serum gastrin ($p < 0.0001$), but the response was similar across the dose range.

24-h ambulatory gastric pH

The protocol was amended during the study to include 24-h gastric pH monitoring after dosing in periods 3–5. Only 4 of the 10 subjects consented to the procedure. Individual values for AUC_{0-24h} of gastric pH are listed in Table 11. The available data show that netazepide doses 100–400 mg all increased AUC_{0-24h} of gastric pH, but the response was similar across the dose range.

Safety and tolerability

All doses of netazepide were safe and well tolerated. Adverse events were reported after active and placebo treatments, and were not dose-related. There were no clinically relevant changes in vital signs, ECG recordings and safety tests of blood and urine.

Discussion

The new 2009 formulation made from spray-dried netazepide was much more bioavailable than the 2001 and 2006 formulations made from crystalline netazepide (Tables 1 and 2). Although the 2009 formulation was about 20% less bioavailable than the 1996 formulation, which was also made from spray-dried netazepide, that difference is as much likely to reflect the differences in populations of healthy subjects studied rather than differences in the manufacture of the spray-dried netazepide. The results of my $n = 1$ study of the 2009 formulation (Test 10; Chapter 8) are within the range of results obtained from 10 healthy subjects in the study reported here, confirming the value of my studies.

Plasma concentrations of the new formulation varied among subjects, as they have done in previous studies. Although dose-proportionality for C_{max} and AUC was lost at netazepide 400 mg, C_{max} and AUC were dose-proportional for the lower netazepide doses of 50–200 mg, as they were for single doses of netazepide 5–100 mg in the first studies in healthy subjects (Studies 1 and 2).

All doses of the new formulation increased serum gastrin, but there were no difference between doses. The increases in serum gastrin are consistent with antagonism of gastrin/CCK₂ receptors and thereby suppression of gastric acid production, as in Study 7. Anything that reduces gastric acid production, such as a PPI, H₂RA or CAG, results in a secondary increase in circulating gastrin. There is evidence for and against a direct relationship between serum gastrin concentration and inhibition of gastric acid secretion (Ligumsky *et al* 2001; Bonaface *et al* 2000). Although few subjects had their 24-h gastric pH measured, the results are also consistent with suppression of gastric acid production. Again, there were no apparent differences among doses of netazepide.

The serum gastrin and gastric pH results together indicate that increasing the dose of netazepide beyond 50 mg does not increase efficacy. Indeed, pharmacokinetic modelling of the results from Study 2 showed that 25 mg of the 1996 spray-dried netazepide formulation was already at the top of the dose-response curve for acid suppression.

Food increased the bioavailability of the new spray-dried formulation of netazepide, as it did for the crystalline formulation in Study 8. The nutrient and caloric contents of the meal, the meal volume, meal temperature, transit time, luminal dissolution, drug permeability and systemic availability can cause physiological changes in the gastro-intestinal tract in a way that affects the drug product (FDA Guidance 2002).

The main limitation of the study is that it was done in only 10 subjects. However, the results seem valid despite the small number of subjects.

Conclusions

We decided to restart the clinical development of netazepide using a spray-dried formulation of netazepide taken after food, to increase its bioavailability.

Acknowledgements

My colleagues Thomas Kumke, Head of Statistics and Data Management, did the statistical analyses, Kate Hanrott, Clinical Project Manager, wrote the clinical study report, and Kirsten Heukelbach, Head of Pharmacy Production, prepared the netazepide and placebo capsules.

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Table 1. Summary of netazepide capsule formulations

Date	Study	Source of netazepide/ capsule manufacturer	Capsule formulation
1996–1997	1, 2, 3 & 4	Yamato SDD API batch 48338/ Yamanouchi	Netazepide (SDD with HPMC in methanol solution) with excipient CCNa
2001–2002	5, 6	API batch 55593/HLS	Netazepide API mixed with excipients HPMC and CCNa
2006–2007	7, 8	API batch 55593/HLS	Netazepide API mixed with excipients HPMC and CCNa
2009– onwards	9 (Current study)	R5 SDD API batch 55593/HMR	Netazepide (SDD with HPMC in IPA/DCM solution) with excipient partially pre-gelatinised starch

API = active pharmaceutical ingredient HPMC = hydroxypropyl methyl cellulose
CCNa = croscarmellose sodium

Table 2. Summary of mean C_{max} and AUC_{0–24h} of different formulations of netazepide 100 mg taken by healthy subjects after an overnight fast

Study	Capsule formulation	Year made	No. of subjects	Dose regimen	Mean C _{max} (ng/mL)	Mean AUC _{0–24h} (ng.h/mL)
1	SDD netazepide (with HPMC) and excipient CCNa	1996	8	Single	397	658
2	SDD netazepide (with HPMC) and excipient CCNa ¹	1996	21	Single	251	612
3	SDD netazepide (with HPMC) and excipient CCNa	1996	13	7 days bd	569 ¹	981 ¹
6	Netazepide API with excipients HPMC and CCNa	2001	8	6.5 days bd	118 ²	–
7	Netazepide API with excipients HPMC and CCNa	2006	10	6 weeks od	39 ¹	86 ¹
8	Netazepide API with excipients HPMC and CCNa	2006	12	Single	44	83
9	SDD netazepide (with HPMC) and excipient starch	2009	10	Single	265	527

API = active pharmaceutical ingredient. HPMC = hydroxypropylmethylcellulose.

CCNa = croscarmellose sodium. SDD = spray-dried dispersion.

¹ After last dose. ² At 1 h after last dose.

Table 3. Mean (SD, n = 10) plasma netazepide concentrations (ng/mL) of single doses of netazepide 50–400 mg taken after an overnight fast

Time (h)	Netazepide dose (mg)			
	50	100	200	400
0.25	4.8 (7.2)	12.6 (27.7)	8.5 (17.0)	49.3 (68.1)
0.50	82.1 (48.6)	135.5 (77.2)	333.9 (285.6)	473.2 (333.1)
0.75	111.3 (75.4)	223.8 (113.1)	429.7 (304.7)	524.1 (226.1)
1.00	110.0 (68.6)	209.6 (141.1)	400.9 (212.9)	485.3 (209.4)
1.25	126.8 (99.4)	169.5 (111.1)	380.7 (145.4)	493.4 (226.6)
1.50	107.0 (85.3)	131.4 (72.7)	346.0 (149.8)	464.8 (294.7)
2.00	65.7 (42.8)	90.4 (38.0)	204.5 (69.8)	315.3 (175.9)
3.00	38.1 (20.2)	52.8 (23.6)	110.5 (43.5)	167.8 (81.5)
0.00	0.2 (0.5)	BLQ	BLQ	BLQ
4.00	22.0 (9.5)	35.6 (20.9)	67.1 (32.3)	101.5 (50.9)
6.00	15.0 (6.6)	17.0 (4.0)	33.1 (13.8)	47.0 (19.3)
8.00	8.6 (4.6)	11.2 (3.2)	19.7 (8.1)	27.4 (12.1)
12.00	3.8 (2.0)	6.8 (2.5)	13.5 (7.0)	17.2 (7.2)
16.00	1.7 (1.0)	3.5 (1.7)	7.6 (3.9)	10.3 (4.4)
24.00	1.1 (0.9)	2.8 (1.5)	6.0 (3.5)	9.2 (4.8)

BLQ = below limit of quantification

Table 4. Mean (SD, n = 10) pharmacokinetic parameters of plasma netazepide concentrations for single doses of 50–400 mg taken after an overnight fast

Parameter	Netazepide dose (mg)			
	50	100	200	400
C_{max} (ng/mL)	162 (89)	265 (139)	513 (262)	704 (295)
t_{max} (h)*	0.8 (0.5–1.5)	0.8 (0.5–1.3)	1.0 (0.5–1.5)	0.8 (0.5–2.0)
$t_{1/2}$ (h)	6.1 (3.6)	7.9 (3.2)	11.1 (3.3)	15.3 (7.8)
AUC_{0-24h} (ng.h/mL)	346 (108)	527 (154)	1113 (415)	1557 (484)
AUC_{0-t} (ng.h/mL)	344 (107)	526 (152)	1113 (415)	1557 (484)
$AUC_{0-\infty}$ (ng.h/mL)	356 (102)	557 (149)	1204 (453)	1738 (530)
CL/f (L/h)	150 (387)	192 (55.6)	202 (118.9)	250 (82.3)
V/f (L)	1402 (985)	2247 (1051.8)	3257 (2386.3)	6061 (6038.2)

* Median (range) t_{max}

Table 5. Mean (n = 10) C_{max} and AUC_{0-24h} of single doses of netazepide, taken after an overnight fast, dose-normalised to 1 mg

Parameter	Netazepide dose normalised to 1 mg			
	50 mg	100 mg	200 mg	400mg
C_{max} (ng/mL/mg)	3.23	2.65	2.56	1.76
AUC_{0-24} (ng.h/mL/mg)	6.92	5.27	5.57	3.89

Table 6. Mean (SD, n = 10) plasma netazepide concentrations (ng/mL) for a single dose of netazepide 100 mg taken after an overnight fast and a fatty breakfast

Time (h)	Netazepide 100 mg	
	Overnight fast	Breakfast
0.00	BLQ	BLQ
0.25	12.6 (27.7)	1.2 (2.5)
0.50	135.5 (77.2)	53.1 (111.3)
0.75	223.8 (113.1)	136.2 (192.4)
1.00	209.6 (141.1)	311.9 (347.2)
1.25	169.5 (111.1)	359.6 (300.9)
1.50	131.4 (72.7)	377.4 (277.2)
2.00	90.4 (38.0)	257.0 (118.8)
3.00	52.8 (23.6)	141.4 (61.0)
4.00	35.6 (20.9)	71.2 (29.0)
6.00	17.0 (4.0)	23.2 (5.6)
8.00	11.2 (3.2)	10.5 (3.2)
12.00	6.8 (2.5)	3.7 (1.1)
16.00	3.5 (1.7)	1.5 (0.5)
24.00	2.8 (1.5)	1.0 (0.4)

BLQ = below limit of quantification

Table 7. Mean (SD, n = 10) pharmacokinetic parameters for a single dose of netazepide 100 mg taken after an overnight fast and a fatty breakfast

Parameter	Netazepide 100mg	
	Overnight fast	Breakfast
C_{max} (ng/mL)	265 (139)	515 (276)
t_{max} (h)*	0.8 (0.5–1.3)	1.5 (0.8–3.0)
t_{1/2} (h)	7.9 (3.2)	6.9 (3.1)
AUC_{0-24h} (ng.h/mL)	527 (154)	869 (303)
AUC_{0-t} (ng.h/mL)	526 (152)	868 (303)
AUC_{0-∞} (ng.h/mL)	557 (149)	879 (306)
CL/f (L/h)	192 (56)	129 (55)
V/f (L)	2247 (1052)	1148(443)

* Median (range) t_{max}

Table 8. Bioequivalence analysis of netazepide 100 mg taken after an overnight fast and a fatty breakfast

Parameter	Ratio of least-square geometric means	90% confidence intervals
C_{max} (ng/mL)	193.2	173.2–215.4
AUC_{0-24h} (ng.h/mL)	161.6	132.0–198.0
AUC_{0-∞} (ng.h/mL)	153.6	126.2–187.0

Table 9. Mean (SD, n = 10) serum gastrin concentrations (pmol/L) after single doses of netazepide 50–400 mg or placebo taken after an overnight fast

Time (h)	Placebo	Netazepide dose (mg)			
		50	100	200	400
0	14.2 (9.3)	11.9 (5.8)	12.4 (5.7)	13.5 (7.0)	12.1 (6.0)
1	12.8 (7.6)	18.6 (13.7)	18.2 (17.4)	19.3 (15.3)	23.5 (20.9)
2	13.9 (8.6)	29.8 (35.6)	31.4 (36.8)	31.90 (35.0)	39.5 (52.8)
3	13.9 (7.8)	31.8 (36.6)	32.1 (38.8)	39.1 (29.8)	39.2 (53.8)
4	13.7 (8.1)	35.7 (46.6)	32.6 (43.7)	39.5 (41.7)	48.1 (68.1)
6	45.0 (62.2)	73.8 (56.6)	84.4 (100.9)	95.1 (108.2)	87.9 (76.5)
8	29.4 (15.9)	98.5 (94.8)	79.0 (70.4)	86.9 (83.9)	84.0 (61.6)
10	23.9 (13.8)	105.7 (121.6)	63.9 (54.5)	71.8 (73.8)	99.4 (119.5)
12	57.8 (67.8)	111.5 (135.2)	101.9 (101.4)	106.4 (113.4)	101.5 (91.8)
14	33.3 (23.2)	78.7 (67.4)	94.9 (93.2)	87.8 (93.7)	94.3 (112.5)
16	17.5 (7.2)	68.6 (79.5)	74.1 (94.3)	56.4 (67.8)	58.1 (88.1)
18	14.3 (6.5)	26.7 (13.4)	31.4 (25.4)	28.6 (23.0)	23.9 (18.4)
20	14.1 (8.08)	20.3 (16.0)	21.2 (21.0)	20.9 (11.90)	20.7 (16.6)
22	15.0 (9.9)	19.2 (10.1)	16.9 (8.1)	26.0 (23.4)	28.9 (38.1)
24	13.4 (7.5)	26.2 (20.2)	31.5 (39.3)	34.7 (32.5)	42.8 (54.2)

Table 10. Mean serum gastrin AUC_{0–24h} (pmol.h/L) (SD) after single doses of netazepide 50–400 mg YF476 or placebo taken after an overnight fast

Placebo	Netazepide dose (mg)			
	50	100	200	400
582.2 (438.9)	1337.4 (1228.8)	1303.9 (1312.0)	1351.5 (1305.1)	1422.0 (1454.5)

Table 11. AUC_{0–24h} of gastric pH after single doses of netazepide 100–400 mg or placebo taken after an overnight fast by individual subjects

Subject	Placebo	Netazepide dose (mg)		
		100	200	400
2	—	73	74	74
3	45	—	57	61
6	61	—	108	—
10	42	—	127	140

Figure 1. Mean (n = 10) concentrations of plasma netazepide after single doses of 50, 100, 200 and 400 mg taken after an overnight fast

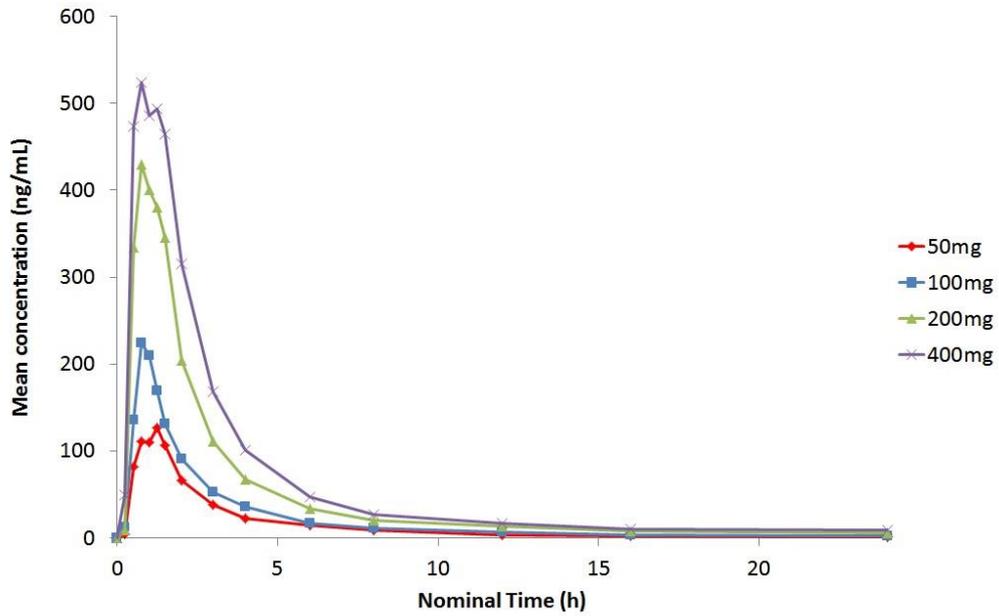


Figure 2. Mean (n = 10, SD) concentrations of plasma netazepide after a single dose of netazepide 100 mg, taken after an overnight fast or a fatty breakfast

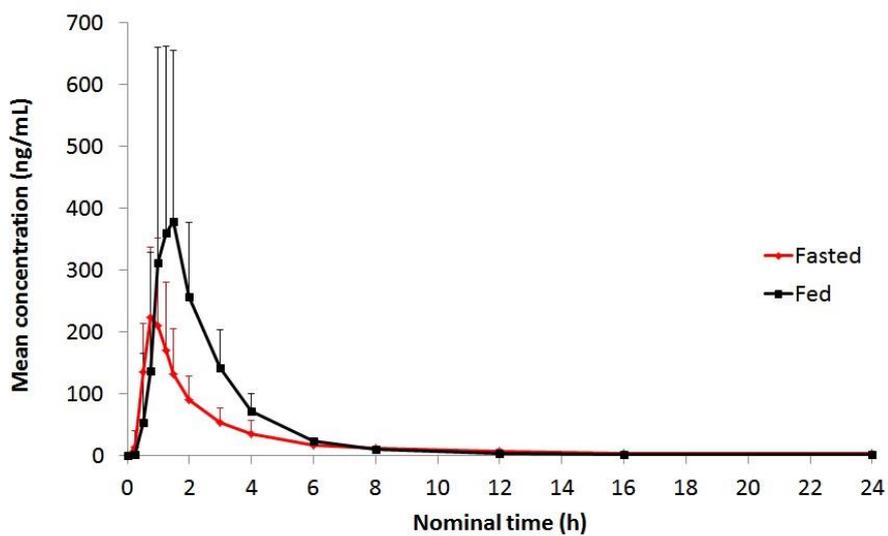


Figure 3. Fitted regression line for individual C_{max} (ng/mL) and AUC_{0-24h} (ng.h/mL) values versus netazepide dose 50–400 mg normalised to 1mg

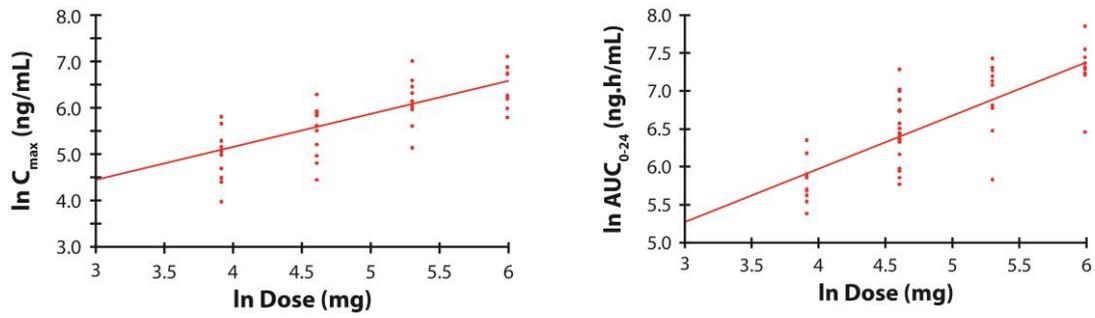


Figure 4. Mean (n = 10) serum gastrin concentrations after netazepide 50–400 mg or placebo taken after an overnight fast

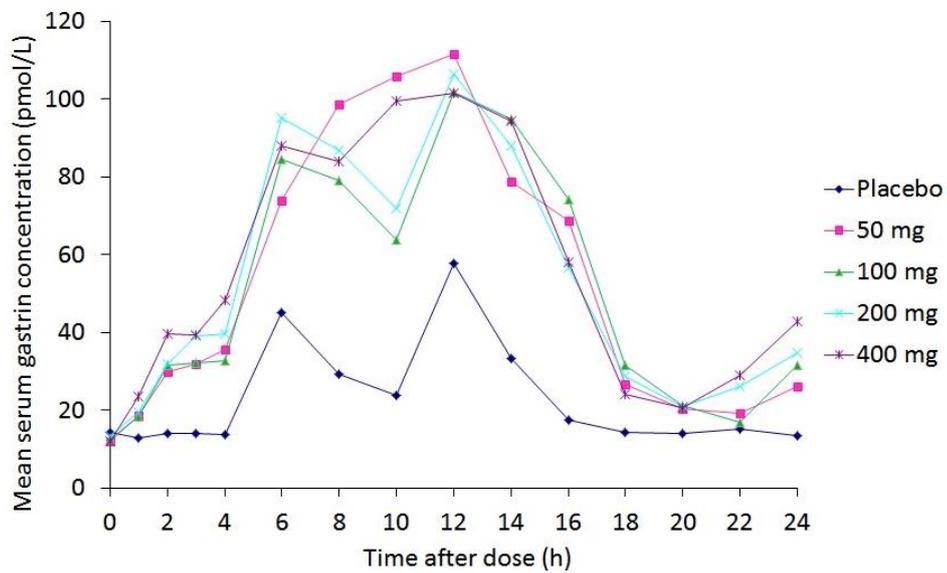
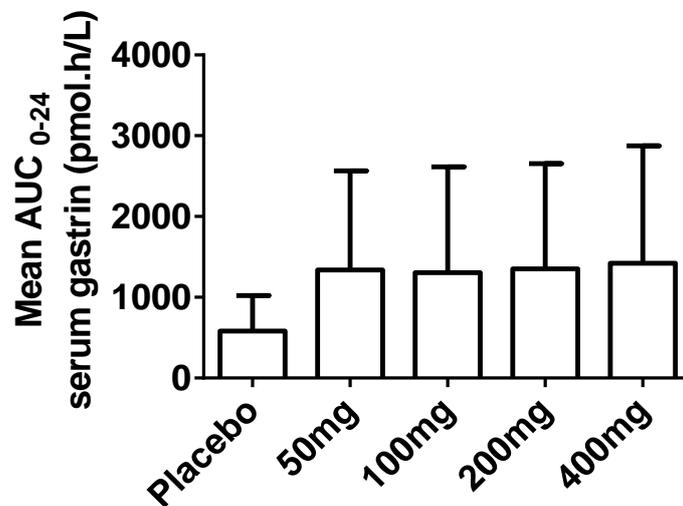


Figure 5. Mean (n = 10; SD) AUC_{0-24h} of serum gastrin concentration (pmol.h/L) after single doses of netazepide 50–400 mg or placebo taken after an overnight fast



Chapter 10

**Study 10. Absolute bioavailability of netazepide
in healthy subjects using a microdose of ^{14}C -netazepide
and accelerator mass spectrometry**

Introduction

Formulations made from spray-dried netazepide have so far given the most favourable pharmacokinetics and pharmacodynamics in healthy subjects (Studies 2 and 7). However, we do not know the absolute bioavailability of any of the formulations tested. Absolute bioavailability is the amount of netazepide from a formulation that reaches the systemic circulation relative to an intravenous dose, which is assumed to be 100% bioavailable. Unfortunately, the poor solubility of netazepide prevents preparation of an intravenous formulation for a conventional absolute bioavailability study. Furthermore, there have been no toxicology studies to support administration of intravenous netazepide to humans. However, the regulations (ICH guideline M3 2009) allow a microdose (defined as less than 100µg) of an investigational medicinal product (IMP) to be given intravenously if it is intended for oral administration and an oral non-clinical toxicology package already exists, as is the case for netazepide (see Chapter 2).

Accelerator mass spectrometry technology (AMS) is an extremely sensitive method for measuring small amounts of an IMP in biological samples if the IMP is labelled with a tiny dose of ^{14}C (typically <10 kBq). Only 1000 atoms are required for a valid measurement (Lappin and Garner 2004; Sarapa *et al* 2005; Lappin and Stevens 2008). The technique is ideal for absolute bioavailability studies because coadministration of an oral therapeutic dose and an intravenous radiolabelled microdose allows simultaneous assessment of plasma concentrations of orally and intravenously administered investigational medicinal product (IMP). Plasma concentrations of the ^{14}C -labelled IMP (from the intravenous dose) can be measured using HPLC-AMS, while concentrations of IMP derived from the oral dose can be measured using conventional quantitative analytical techniques. The dose of IMP given via the intravenous route is too small to affect measurement of plasma concentrations of unlabelled, orally administered IMP.

Objectives

The aims of this study in healthy subjects (Study 10) were to assess: (1) the absolute bioavailability of the 2009 formulation of netazepide made from spray-dried material (Study 9); and (2) the pharmacokinetics of intravenous netazepide.

Study design

The study was an open-label, non-randomised trial of an oral dose of netazepide 100 mg and an intravenous microdose of ^{14}C -labelled netazepide in six men or women deemed healthy as in previous studies. Eligible subjects were admitted to the ward on the afternoon before the dosing day. They fasted overnight and took a single oral dose of netazepide 100 mg in the morning. One hour later, they were given an intravenous dose of ^{14}C -netazepide 15 µg. They left the ward on the following morning, after completion of all the tests, and were followed up 7–14 days later.

Subjects took a single oral dose of ‘cold’ netazepide 100 mg (4 x 25 mg capsules) and 1 h afterwards they were given a single intravenous injection of ‘hot’ ^{14}C -netazepide 15 μg (9.9 kBq; 267.6 nCi). ‘Cold’ and netazepide were measured by LCMS/MS and AMS, respectively. The intravenous dose was administered 1 h after the oral dose, to coincide with the approximate t_{max} after oral dosing.

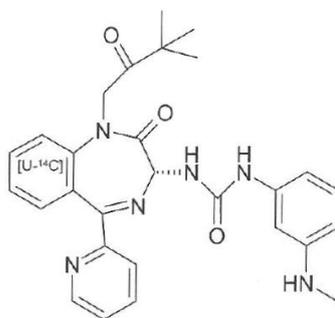
Methods

‘Cold’ netazepide

R5 Pharmaceuticals Ltd, Nottingham, England (now part of Aesica Pharmaceuticals Ltd) used a Buchi B191 Spray Dryer to spray dry to cGMP standards a solution of netazepide (batch 55593) and HPMC (ratio of 1:3.5) dissolved in dichloromethane/isopropanol (7:2 v/v), to give a final solute concentration of 4%. The formulated intermediate was a white, amorphous powder. At all stages of production, the solution and resulting spray-dried powder were shielded from light. HMR pharmacy prepared capsules containing the co-formulation of spray-dried netazepide 25 mg (118.6 mg formulated intermediate) and HPMC blended with starch.

‘Hot’ netazepide

Selcia, Fyfield Road, Ongar, Essex CM5 0GS, prepared ^{14}C -labelled netazepide. The specific activity and radiochemical purity were 329.3 MBq/mmol (8.90 mCi/mmol) and 99.65%, respectively. The structural formula is below.



dispatch to ASI, The Medical School, St George's Hospital, Cranmer Terrace, London SW17 0RE, for assay by HPLC with MS/MS detection.

Processing of blood samples for HPLC-AMS

Blood samples (5 mL) were taken into foil-wrapped or amber lithium heparin tubes, and immediately placed on ice. Samples were centrifuged at 700–1500 G for 10 min at 4°C. Plasma was transferred to foil-wrapped or amber polypropylene storage tubes. Plasma samples were placed at –20°C within 90 min after collection, and stored in darkness until dispatch to Xceleron Ltd, York Biocentre, Innovation Way, Heslington, York YO10 5NY, for analysis by HPLC-AMS. Samples were obtained and processed in ward and laboratory areas that had not been used in trials involving doses of radioactivity that were quantifiable by liquid scintillation counting.

Assay of netazepide concentrations

Netazepide assays were done by the ASI using a validated high performance liquid chromatography / mass spectroscopy method. Test samples were analysed alongside quality control samples at low (2.0 ng/mL), medium (40.6 ng/mL) and high (304.5 ng/mL) concentrations over the calibration range of the assay (1–500 ng/mL). The lower limit of quantification (LLQ) for the assay was 1 ng/mL.

Total ¹⁴C and ¹⁴C- netazepide assays were done by Xceleron Ltd using AMS and HPLC-AMS, respectively. Total ¹⁴C was measured in selected samples; ¹⁴C- netazepide was measured in all samples. The LLQ was 0.0020 ng equivalent/mL for the total ¹⁴C assay, and 0.0009–0.0047 ng/mL for the ¹⁴C- netazepide assay. Four QC samples (high, medium and 2 x low dpm/mL) were run in duplicate with the clinical samples.

Pharmacokinetics

The following pharmacokinetic parameters of netazepide were derived by WinNonlin 4.1: C_{max}, t_{max}, C₀, AUC_{0–tn}, AUC_{0–24h}, AUC_{0–∞}, t_{1/2}, CL/f, V/f, CL and V after oral and intravenous doses; and absolute bioavailability. C₀ is the concentration of ¹⁴C-YF476 at time 0 derived by extrapolation.

The oral bioavailability (*f*) of netazepide was derived by comparing the AUC after oral and intravenous doses, as follows:
$$f = 100 * \frac{(AUC_{oral} / D_{oral})}{(AUC_{IV} / D_{IV})}$$

Results

Subjects

Four men and two women of median age 31 (range 26–50) years and median body mass index 24.2 (range 21.3–29.8) entered and completed the study, which we did during Sept–Nov 2009.

Pharmacokinetics of netazepide and ¹⁴C-netazepide in plasma

The plasma concentrations of netazepide and ¹⁴C-netazepide in all pre-dose samples were below the limit of quantification (BLQ). The lower limit of quantification (LLQ) was 1 ng/mL for netazepide and 0.0009–0.0047 ng/mL for ¹⁴C-netazepide.

Mean plasma concentrations and mean parameters of netazepide and ¹⁴C-netazepide are listed in Tables 1 and 2, respectively. Mean plasma netazepide concentrations after oral netazepide 100 mg and intravenous ¹⁴C-netazepide 15 µg, with ¹⁴C-netazepide concentrations scaled to a 100 mg dose, are illustrated in Figure 1. Mean concentrations of *total* ¹⁴C (netazepide ng equivalent/mL) and mean concentrations of *unchanged* ¹⁴C-netazepide (ng/mL) after intravenous ¹⁴C-netazepide 15 µg are illustrated in Figure 2. The total concentration of radioactivity was measured in selected plasma samples after intravenous ¹⁴C-netazepide. Estimates of bioavailability of netazepide, assessed by AUC₀₋₂₄ and AUC_{0-∞}, are in Table 3.

After oral netazepide 100 mg, plasma concentrations of netazepide varied moderately among subjects, and C_{max} varied about 4-fold. Plasma concentrations rose rapidly after dosing; median t_{max} was 0.9 h. They remained above the LLQ of the assay during the 24 h after dosing. Elimination was biphasic. Overall exposure, assessed by mean AUC_{0-∞}, was 695.5 ng.h/mL. Mean elimination t_{1/2} was 9.9 h, mean CL/f was 165.7 L/h and mean V/f was 2392.8 L.

After intravenous ¹⁴C-netazepide 15 µg, plasma concentrations of ¹⁴C-netazepide varied little among Subjects 1–5, but were substantially lower in Subject 6. Elimination was biphasic. Mean t_{1/2} was 2.7 h. Mean C₀ was 0.884 ng/mL. Overall exposure, assessed by mean AUC_{0-∞}, was 0.795 ng.h/mL. Mean CL was 30.6 L/h, and mean V was 95.7 L.

Mean concentrations of total radioactivity were derived by assay of selected plasma samples (Figure 2). The apparent peak in mean concentration at 7 h after the intravenous dose represents total ¹⁴C concentration in a single sample, from Subject 3, who had the highest plasma concentrations of ¹⁴C-netazepide. Thus, the apparent increase in plasma concentration of total radioactivity at that time point is spurious.

Mean concentrations of total radioactivity and ¹⁴C-netazepide were similar at the first time-point, 0.5 h after the intravenous dose. Thereafter, the proportion of *total* radioactivity that was accounted for by *unchanged* ¹⁴C-netazepide declined, reaching only about 10% by 7 h after dosing. If the LLQ values are taken as zero, the mean unchanged ¹⁴C-netazepide declined even further, to about 2% of total radioactivity by 16h. At 24h after dosing, total radioactivity greatly exceeded radioactivity of unchanged ¹⁴C-netazepide. The plots of total and unchanged radioactivity versus time after dosing with ¹⁴C-netazepide were both curvilinear suggesting that

netazepide exhibits a rapid but prolonged distribution phase before elimination becomes the dominant factor.

Discussion

Mean pharmacokinetic parameters of oral netazepide 100 mg dose taken after an overnight fast, were very similar to those of Study 8 of the same formulation. Netazepide was rapidly absorbed, and underwent biphasic elimination, with a mean $t_{1/2}$ of about 10 h.

Whereas almost all the radioactivity in plasma was in the form of unchanged netazepide at 0.5 h after the intravenous dose, the proportion of total radioactivity in the form of ^{14}C -netazepide declined over time, reaching about 10% by 7 h after the intravenous dose. That is consistent with metabolism being a significant route of clearance of netazepide. Netazepide is metabolised by human hepatocytes *in vitro* to 10 potential metabolites (report HMU0005/063553). Given the extensive metabolism, netazepide probably undergoes first pass metabolism after an oral dose.

After an intravenous dose, mean elimination $t_{1/2}$ of ^{14}C -netazepide was 2.7 h, whereas after the oral mean $t_{1/2}$ was 9.9 h. That discrepancy occurred because ^{14}C -netazepide could not be measured for long enough after the intravenous dose to allow estimation of terminal elimination $t_{1/2}$: the mean value of 2.7 h reflects the first phase of elimination rather than the terminal phase. In only one subject did the estimated $t_{1/2}$ reflect the terminal phase: $t_{1/2}$ in that subject was 5.6 h, which is similar to the minimum $t_{1/2}$ derived from plasma concentrations after oral netazepide.

The proportion of total radioactivity accounted for by unchanged ^{14}C -netazepide started to decline soon after dosing and was only about 10% by 7 h after dosing. It was even lower if <LLQ values were taken as zero. The decline in unchanged ^{14}C -netazepide is consistent with metabolism being a significant route of clearance of netazepide. That *total* radioactivity greatly exceeded radioactivity of *unchanged* ^{14}C -netazepide at 24h after dosing is consistent with the presence of a metabolite or metabolites with slower clearance than parent netazepide.

Because most of the estimates of λ_z reflect the first rather than the terminal phase of elimination of ^{14}C -netazepide, AUC_{0-24} and $\text{AUC}_{0-\infty}$ after the intravenous dose are underestimates. As a result, absolute oral bioavailability of netazepide must be less than the calculated value of about 15%. A higher dose of ^{14}C -netazepide is required for an accurate estimate of bioavailability.

Netazepide is poorly soluble in water. The oral formulation used in this study consisted of amorphous spray-dried netazepide stabilised by an HPMC polymer matrix. Amorphous netazepide has a higher intrinsic dissolution rate than crystalline material. Study 8 showed that the same formulation is more bioavailable than previous formulations made from crystalline

netazepide. Nevertheless, the absolute bioavailability of the new formulation is low. That is likely to be a result of both poor solubility and first pass metabolism. The variability in concentrations of netazepide among subjects reflects low oral bioavailability.

Underestimation of AUC also means that clearance and volume of distribution are overestimates. However, we can conclude that CL does not exceed 31 L/h and V does not exceed 96 L.

The low concentrations of ^{14}C -netazepide in Subject 6 cannot be easily explained. The concentrations of total radioactivity in that subject at 0.5 h after the intravenous dose were similar to those in the other subjects, so Subject 6 must have received the correct dose.

However, at 0.5 h, concentrations of ^{14}C -netazepide in that subject were about one-sixth of those in the other five subjects. It is implausible that the subject metabolised the majority of the dose in only 30 min, particularly as he subsequently eliminated ^{14}C -netazepide at a similar rate to the other subjects; moreover, he did not eliminate unlabelled netazepide more rapidly than did the other subjects. It is possible that the discordant results are due to a technical error.

Conclusions

- Oral netazepide has an absolute bioavailability of less than 15%. The dose of intravenous ^{14}C -netazepide was too low to give an accurate value.
- Low bioavailability of oral netazepide probably reflects both poor solubility of netazepide and first pass metabolism.
- Metabolism is a significant route of clearance of netazepide.
- Bioavailability of oral netazepide in healthy subjects (<15%) is lower than that in rats (25%) and dogs (25–50%), assessed by comparing the pharmacodynamic effects of intravenous and oral doses (Chapter 2).
- The bioavailability of the formulations made from crystalline netazepide for Studies 5–8 was probably as low as a few percent, yet the pharmacodynamic results were favourable. Thus, netazepide blocks gastrin/CCK₂ receptors at quite low plasma concentrations.
- The much slower decline of *total* radioactivity after dosing with intravenous ^{14}C -netazepide compared with the decline in radioactivity of unchanged ^{14}C -netazepide is consistent with the presence of a metabolite or metabolites of netazepide. An active metabolite would explain the effect of oral netazepide on gastric pH beyond 24h in Studies 2–4.

Acknowledgements

Jeremy Hague, Xceleron, facilitated the AMS part of the study, and his Xceleron colleague, Mike Seymour, analysed the plasma samples and interpreted the results. Thomas Kumke, Head

of Statistics, HMR, did the WinNonlin analyses. Kate Hanrott wrote the clinical study report, which I reviewed.

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Table 1. Mean (SD, n = 6) plasma netazepide concentrations (ng/mL) after oral netazepide 100 mg and intravenous ¹⁴C-netazepide 15 µg

Time (h)*	Netazepide (ng/mL)	¹⁴ C-Netrazepide (ng/mL)
0	BLQ	BLQ
0.25	0.9 (1.1)	—
0.50	151.7 (158.0)	0.3986 (0.1989)
1	176.6 (126.3)	0.1868 (0.1088)
1.5	140.4 (108.4)	—
2	121.4 (65.8)	0.0835 (0.0542)
3	89.1 (39.1)	0.0539 (0.0312)
4	55.9 (23.7)	—
5	—	0.0196 (0.0126)
6	30.3 (21.0)	—
7	—	0.0132 (0.0106)
8	15.0 (9.8)	—
11	—	0.0040 (0.0061)
12	8.7 (4.2)	—
15	—	0.0009 (0.0013)
16	4.9 (3.0)	—
24	3.2 (1.8)	0.0002 (0.0005)

* Relative to oral or intravenous dose of netazepide, as appropriate

Table 2. Mean (SD, n = 6) of pharmacokinetic parameters of netazepide after oral netazepide 100 mg and intravenous ¹⁴C-netazepide 15 µg

Parameter	Netazepide	¹⁴ C-Netazepide
C ₀ (ng/mL)	—	0.884 (0.438)
C _{max} (ng/mL)	220.5 (114.7)	—
t _{max} (h)*	0.9 (0.5–3.0)	—
t _{1/2} (h)	9.9 (3.1)	2.7 (1.5)
AUC _{0-t} (ng.h/mL)	649.4 (334.1)	0.793 (0.434)
AUC ₀₋₂₄ (ng.h/mL)	649.4 (334.1)	0.794 (0.434)
AUC _{0-∞} (ng.h/mL)	695.5 (354.7)	0.795 (0.437)
CL/f (L/h)	165.7 (54.5)	—
CL (L/h)	—	30.6 (32.0)
V/f (L)	2392.8 (1155.8)	—
V (L)	—	95.7 (78.2)

* Median (range) t_{max}

Table 3. Mean (SD, n = 6) oral bioavailability (%) of netazepide

Parameter	Mean (SD)
f [AUC ₀₋₂₄]	15.5 (10.7)
f [AUC _{0-∞}]	16.7 (11.8)

Absolute bioavailability was less than 15%.

Figure 1. Mean (SD, n = 6) plasma netazepide concentrations (ng/mL) after oral netazepide 100 mg and after intravenous ¹⁴C-netazepide 15 µg scaled to a 100 mg dose

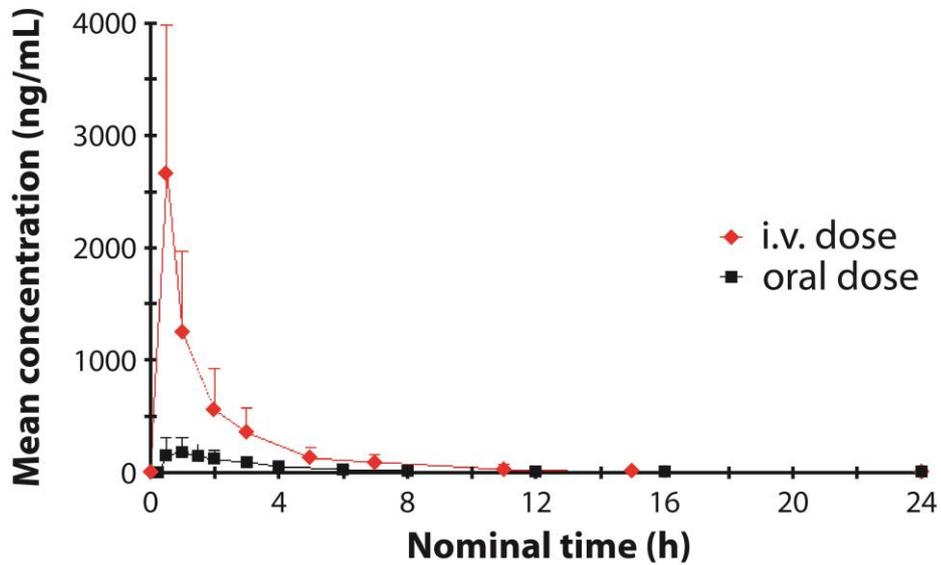
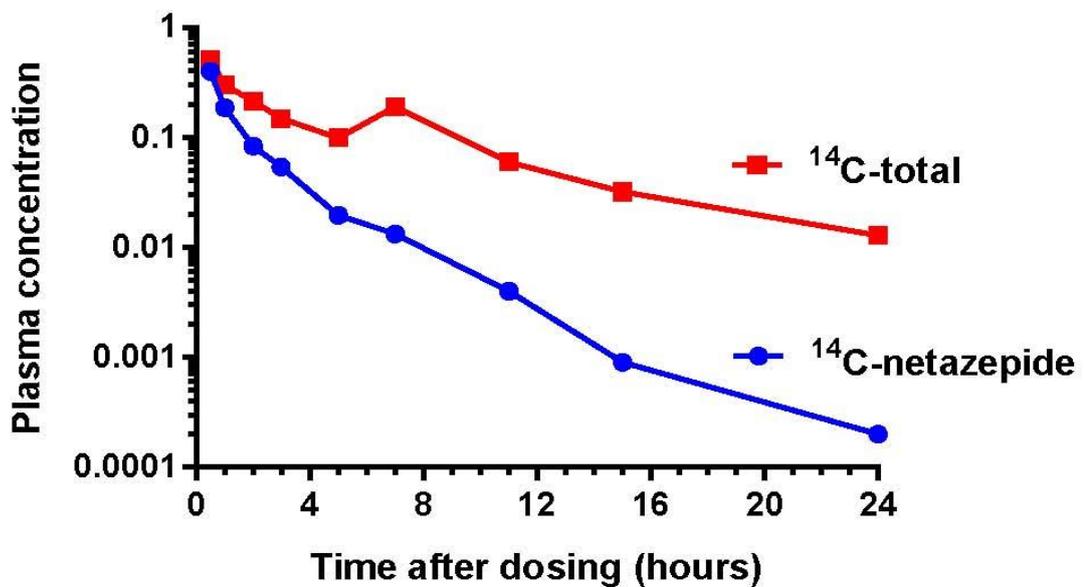


Figure 2. Mean log concentrations of *total* ¹⁴C-netazepide related material (netazepide ng equivalent/mL) and mean log concentrations of *only* ¹⁴C-netazepide (ng/mL) after administration of intravenous ¹⁴C-netazepide 15 µg



Chapter 11

Study 11. Randomised trial of the effect of a gastrin/CCK₂ receptor antagonist on esomeprazole-induced hypergastrinaemia: evidence against rebound hyperacidity

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ABSTRACT

Background

Hypergastrinaemia induced by proton pump inhibitor (PPI) therapy may cause ECL-cell and parietal-cell hyperplasia, and rebound hyperacidity and dyspepsia after PPI withdrawal.

Objectives

To assess the effect of different dosage-regimens of netazepide, a gastrin/CCK₂ receptor antagonist, on PPI-induced hypergastrinaemia and elevated chromogranin A (CgA).

Methods

Six groups of 8 healthy subjects participated in a randomised, double-blind study of esomeprazole 40 mg daily for 28 days, in combination with netazepide 1, 5 or 25 mg or placebo, daily, during the last 14 days of esomeprazole or during 14 days after treatment withdrawal. Fasting serum gastrin and plasma CgA were measured during treatment and after withdrawal, as biomarkers of acid suppression and ECL-cell activity, respectively. Dyspepsia was monitored throughout the study.

Results

Esomeprazole increased gastrin and CgA. Netazepide increased gastrin, but not CgA, and inhibited dose-dependently the CgA response to esomeprazole. Gastrin and CgA returned to baseline within 2–3 days of esomeprazole withdrawal; netazepide did not shorten that time. There was no rebound dyspepsia after esomeprazole withdrawal.

Conclusions

Esomeprazole and netazepide each increase gastrin, consistent with a secondary effect of gastric acid suppression, but by different mechanisms. Esomeprazole-induced hypergastrinaemia stimulates ECL cells and thereby increases CgA. Netazepide-induced hypergastrinaemia does not increase CgA, because netazepide blocks gastrin/CCK₂ receptors on ECL cells. Co-administration of netazepide 5 mg abolishes the effect of esomeprazole-induced hypergastrinaemia on ECL cells. The quick return to baseline of gastrin and CgA and absence of dyspepsia after esomeprazole withdrawal do not support the concept of rebound hyperacidity.

STUDY HIGHLIGHTS

1. What is current knowledge

- PPIs increase circulating gastrin and CgA, which reflect reduced acid secretion and increased ECL activity, respectively.
- Gastrin and CgA responses continue after PPI withdrawal, and can lead to rebound hyperacidity and dyspepsia ~2 weeks after withdrawal.

2. What is new here

- Gastrin and CgA responses to esomeprazole return to baseline within 2–3 days of withdrawal of esomeprazole after 28 days' treatment, which does not support the concept of rebound hyperacidity.
- Netazepide, a gastrin antagonist, prevents the CgA increase induced by esomeprazole.

Introduction

Gastrin controls gastric acid secretion and has a trophic effect on cells in the gastric mucosa, especially enterochromaffin-like (ECL) cells, which possess gastrin (CCK₂) receptors [1–3]. Stimulation of CCK₂ receptors by gastrin causes release of histamine, which in turn stimulates histamine H₂ receptors on parietal cells and secretion of acid via H⁺/K⁺-ATPase (proton pump) into the stomach lumen. CCK₂ receptors are expressed on parietal cells [4], but are not thought to be involved in acid secretion [5]. Reduced acid production by disease or an acid suppressant, such as a proton pump inhibitor (PPI), causes secondary hypergastrinaemia [6].

In rats, omeprazole reduced acid secretion and induced hypergastrinaemia, but one week after omeprazole withdrawal, acid production had increased above baseline [7]. The effect lasted ≥70 days [8]. YM022, a gastrin/CCK₂ receptor antagonist, prevented the rebound hyperacidity response to omeprazole withdrawal in rats, whereas in control animals rebound hyperacidity lasted for ≥56 days [9].

Withdrawal of omeprazole 40 mg daily after 12 and 8 weeks in patients with gastro-oesophageal reflux disease (GORD) and healthy subjects, respectively, also resulted in increased basal and pentagastrin-stimulated acid output 2 weeks afterwards [10, 11]. However, a literature review concluded that clinical evidence for PPI withdrawal causing rebound hyperacidity was weak, mainly because the studies were done in few subjects and were not well controlled [12]. In two subsequent studies in healthy subjects, both of which were placebo-controlled and involved many more subjects, PPI withdrawal was followed by dyspepsia. In one study, 60 subjects received esomeprazole 40 mg daily for 8 weeks followed by placebo daily for 4 weeks, and 60 subjects received placebo daily for 12 weeks. During the last 3 weeks of placebo treatment, there was a significant difference between esomeprazole and placebo (44% vs 15%) with respect to symptoms of heartburn, acid reflux or dyspepsia after treatment withdrawal [13]. In the other study [14], of 48 subjects who took pantoprazole 40 mg or placebo daily for 4 weeks, significantly more developed dyspepsia after pantoprazole withdrawal than after placebo (44% vs 9%).

The main indications for treatment with a proton pump inhibitor are peptic ulcer disease or GORD [15]. After *H. pylori* eradication was introduced, the need for maintenance therapy for peptic ulcer disease was largely eliminated, and GORD became the main indication for prolonged gastric acid inhibition. PPIs are also used empirically for treatment of non-ulcer dyspepsia. The prevalence of long-term PPI treatment is rising, and there is increasing concern that PPIs are overprescribed [16]. The above studies in healthy subjects and patients suggest that attempts to withdraw PPI treatment in patients who originally responded might fail because of

rebound dyspepsia. In other words, PPI withdrawal might cause the very symptoms for which the PPI was first prescribed [17].

Non-clinical studies have shown that netazepide (YF476) is a potent, highly-selective, competitive and orally active gastrin/CCK₂ receptor antagonist [18]. We have characterised the clinical pharmacology of netazepide in healthy subjects. Single oral doses caused dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion, which persisted after repeated doses [19]. Rabeprazole alone and netazepide alone for 6 weeks were similarly effective in reducing acid and increasing serum gastrin. A combination of rabeprazole and netazepide increased serum gastrin and reduced basal acid secretion more than did either treatment alone, suggesting more effective acid suppression. Rabeprazole alone increased plasma CgA – a sign of ECL-cell hyperactivity – whereas netazepide alone reduced plasma CgA – a sign of ECL-cell hypoactivity. When combined with rabeprazole, netazepide prevented the increase in CgA resulting from rabeprazole-induced hypergastrinaemia, which is consistent with blockade of gastrin/CCK₂ receptors on ECL cells [20]. Circulating gastrin is a biomarker for gastric acid secretion [21, 22] and CgA is a biomarker for ECL-cell activity [23–25]. However, stopping rabeprazole after 6 weeks led neither to rebound hyperacidity nor dyspepsia. Two limitations of the study were that the number of subjects (10 per treatment group) was small, and we measured neither serum gastrin nor plasma CgA during the withdrawal period. We concluded that PPI withdrawal should be studied in an adequately powered trial in patients on long-term PPI treatment. If PPI withdrawal really can lead to rebound hyperacidity, a gastrin/CCK₂ receptor antagonist, such as netazepide, should prevent it. But, first a study was required to establish a suitable dose regimen of netazepide, and the time-course of the response to PPI withdrawal. Hence the study we report here.

Objectives

The primary aims were: to assess the effect of different dose-regimens of netazepide on esomeprazole-induced increases in circulating gastrin and CgA in healthy subjects; and to choose a dose-regimen for future studies of PPI withdrawal in patients.

The secondary aims were: to assess if esomeprazole withdrawal leads to dyspepsia, and if so whether netazepide can prevent it; and to assess the likelihood of an interaction between esomeprazole and netazepide.

Methods

We complied with the ICH Guideline for Good Clinical Practice. The Medicines and Healthcare products Regulatory Agency and Brent Medical Ethics Committee (REC reference

09/H0717/73) approved the study on 2 November 2009 and 13 November 2009, respectively, and a protocol amendment on 9 June 2010 and 18 May 2010, respectively. Subjects gave written, informed consent. We did the study during Nov 2009–Sept 2010, and registered it with EudraCT (2009-016201-42) and ClinicalTrials.gov (NCT02620696).

Materials

The sponsor, Trio Medicines Ltd, London, England, provided: capsules of netazepide 1, 5 and 25 mg, and matching placebo; esomeprazole (Nexium[®]; Astra Zeneca) 40 mg tablets; and an antacid (Gastrocote tablets, Actavis Group) for subjects to take after treatment withdrawal, if needed.

Study design

The study was randomised, double-blind and parallel-group in design, and in two parts. The protocol required 48 healthy adults (6 groups of 8; 3 groups in each part), who were *H. pylori* negative (¹³C-urea breath test), non-smoking men or women, with normal serum gastrin and no history of dyspepsia, and were taking no medicines. Pre-menopausal women at risk of pregnancy were excluded. Subjects were deemed healthy by: medical history and examination; ECG; tests for drugs of abuse, hepatitis B & C, and HIV 1 & 2; and blood and urine safety tests. A statistician generated the random allocation sequence.

Part 1

In Part 1, we assessed the impact of the timing of netazepide 25 mg on the response to esomeprazole. Eligible subjects were randomised to one of 3 treatment groups (Figure 1).

Group 1

- Esomeprazole 40 mg for 28 days (Days 1–28); and
- Netazepide placebo for 42 days (Days 1–42)

Group 2

- Esomeprazole 40 mg for 28 days (Days 1–28);
- Netazepide 25 mg for 14 days (Days 15–28); and
- Netazepide placebo for 28 days (Days 1–14 and Days 29–42)

Group 3

- Esomeprazole 40 mg for 28 days (Days 1–28);
- Netazepide 25 mg for 14 days (Days 29–42); and
- Netazepide placebo for 28 days (Days 1–28)

Subjects took their treatment by mouth once daily for 42 days, and attended the clinic at weekly intervals, on Days -1, 7, 14, 21, 28, 35, 42, 49 and 56. They were resident overnight on Day -1 only. At visits when they were taking treatment, we dosed them *circa* 09.00 h, after an overnight fast. They took all other doses at home, with breakfast. At 0, 2 and 4 h after dosing in the clinic, or at equivalent times when there was no dosing, we drew blood for measurement of serum gastrin and plasma CgA. At 0, 2 and 4 h after dosing on Days 21, 28, 35 and 42 only, we also drew blood for measurement of plasma netazepide.

On Days 1–56, subjects used a diary card to record adverse events and treatment compliance, and completed the short form of the validated Nepean questionnaire, which uses 4- or 5-point Likert scales to measure frequency and severity of 15 upper gastrointestinal symptoms, and the bother they cause [26]. On Days 29–56, subjects recorded antacid usage in the diary card.

We assessed safety and tolerability by medical examination, ECG, blood and urine tests, and adverse events throughout the study.

Part 2

After reviewing the data from Part 1, we amended the protocol to test lower doses of netazepide, 1 and 5 mg, and to change the study design in Part 2. Eligible subjects were randomised to one of 3 treatment groups (Figure 1).

Group 4

- Esomeprazole 40 mg for 28 days (Days 1–28); and
- Netazepide placebo for 28 days (Days 1–28)

Group 5

- Esomeprazole 40 mg for 28 days (Days 1–28);
- Netazepide 1 mg for 14 days (Days 15–28); and
- Netazepide placebo (Days 1–14)

Group 6

- Esomeprazole 40 mg for 28 days (Days 1–28);
- Netazepide 5 mg for 14 days (Days 15–28); and
- Netazepide placebo (Days 1–14)

Subjects took their treatment by mouth once daily for 28 days. They attended the clinic on Days -1, 7, 14, 21 and 28. They were resident overnight on Day -1 only. After completion of dosing, they attended the clinic on Days 29, 30, 31, 32, 33, 34 and 35. At visits when they were taking treatment, we dosed them *circa* 09.00 h, after an overnight fast. They took all other doses at home, with breakfast. At 0, 2 and 4 h after dosing in the clinic, or at equivalent times when there

was no dosing, we drew blood for measurement of serum gastrin and plasma CgA. At 0, 2 and 4 h after dosing on Days 21 and 28 only, we also drew blood for measurement of plasma netazepide. On Days 1–35, subjects recorded adverse events, treatment compliance and dyspepsia symptoms, as in Part 1. On Days 29–35, they recorded any antacid usage, as in Part 1. We assessed safety and tolerability, as in Part 1.

Measurement of gastrin, CgA and netazepide

We separated serum or plasma from blood, and stored samples at -20°C until assay by ELISA (serum gastrin: Immulite 2000, DPC. $\text{CV} \leq 3.1\%$; plasma CgA: DAKO. $\text{CV} \leq 10.8\%$) and validated HPLC/MS method (plasma netazepide: lower limit of quantification 0.5 ng/mL) [27].

Assessment of compliance

We assessed compliance by diary card and capsule counts. Subjects wore a wristwatch with an alarm, and we telephoned them weekly, to remind them to take their treatment.

Statistics

Sample size

The study was exploratory in nature. The sample size was based on feasibility and data from our previous study which showed significant suppression by netazepide of rabeprazole-induced increases in circulating CgA [20]. The sample size was not expected to be big enough to show significant differences between treatment groups for dyspepsia symptoms.

Fasting serum gastrin and plasma CgA

We compared treatments by analysis of covariance, with baseline (Day -1) values as co-variates, treatment and visit as fixed effects, time-point as a repeated variable, and the interaction term treatment*visit. We transformed data before analysis, as appropriate.

Plasma netazepide

We plotted plasma netazepide concentrations versus time for all subjects who took netazepide in Groups 2, 3, 5 and 6, and who completed the study.

Dyspepsia scores and antacid usage

We summarised the number of subjects per group with dyspepsia symptoms during each week, and compared groups informally. Likewise, we summarised the number of doses of antacid taken by each subject in each week after esomeprazole withdrawal and compared treatment groups informally.

Results

Demography

Of 53 subjects who entered the study, 5 (3 in Part 1; and 2 in Part 2) withdrew for reasons unrelated to treatment. As required by the protocol, 48 subjects (Groups 1–6; 8 subjects per group) completed the study. Mean (range) age, weight, and height of subjects who completed the study in Groups 1–3 in Part 1 were 33 (21–74) years, 67 (49–88) kg, and 167 (156–179) cm, respectively. Mean (range) age, weight, and height of Groups 4–6 in Part 2 were 31 (20–69) years, 68 (49–94) kg, and 168 (151–184) cm, respectively. Treatment compliance at home was 96%.

Statistical analyses

Analysis of data from Parts 1 and 2 showed a significant ($p < 0.05$) treatment by visit interaction, indicating that the time-profiles of the different treatments were non-parallel, making it impossible to compare the effects of netazepide 1, 5 and 25 mg on the esomeprazole-induced changes in gastrin and CgA. Nor was it possible to explore the effect of esomeprazole withdrawal on gastrin and CgA, or whether that effect was altered by previous dosing with netazepide. Therefore, we did a post-hoc analysis.

To compare the effect of co-administration of netazepide 1, 5 and 25 mg on plasma CgA with that of placebo, we analysed the percentage changes on Days 21–28 for Groups 1, 2, 4, 5 and 6 relative to the pre-dose time-point on Day 14, before the start of netazepide treatment. A repeated measures analysis was done on percentage change from pre-dose on Day 14 in plasma CgA concentration, with terms fitted for treatment, patient within treatment, study day and time within study day plus interactions with treatment. Interaction terms which were not significant were removed from the model. There were no significant interactions with treatment, i.e. the effect of treatment was approximately the same at each time, so treatment least squares means were calculated and the differences from placebo calculated (Table 1). To assess the effect of netazepide 1 and 5 mg on CgA after treatment withdrawal, we used data from Part 2 (Groups 4, 5 and 6) at 0 h on each of Days 28–35. One-way ANOVA was used to analyse the data, and, as there was a statistically significant treatment difference, two-sample t-tests were used to compare pairs of treatments at the 0 h time-points from Day 28 onwards, until there was no longer a statistically significant difference between treatments (Table 2). Also, we plotted mean concentrations of serum gastrin and plasma CgA with respect to day and time-of-day for each group at each visit. Given the many data points, we assessed the effect of treatments from the plots and did not do a formal analysis. However, we did explore the relationship between gastrin and CgA concentrations in individual subjects during esomeprazole dosing. We used gastrin and

CgA concentration data on days when subjects had received esomeprazole alone (Days 1–28 of Treatments 1, 3 and 4, and Days 1–14 of Treatments 2, 5 and 6), and used linear regression to select an appropriate model. In each model, subject and day were factors, CgA was the dependent variable and serum gastrin was a covariate. The model that best fitted the data ($R^2 = 0.9534$) had one line per subject per day with individual slopes and intercepts.

Fasting serum gastrin and plasma CgA

The results are illustrated in Figures 2–5, each of which has a caption describing the effect of treatment and interpretation of the results, so only a summary follows.

Effect of esomeprazole alone on gastrin and CgA

Groups 1–3 and **Groups 4–6** were evenly matched for gastrin and CgA at baseline (Day –1). Esomeprazole increased gastrin and CgA on Days 7, 14, 21 and 28 in Groups 1, 3 and 4, and Days 7 and 14 in Groups 2, 5 and 6. Gastrin increased with time at 0, 2 and 4 h after dosing, whereas CgA was similar at each time-point. Gastrin and CgA responses to esomeprazole varied among subjects and groups.

In **Group 1**, gastrin and CgA returned to baseline (Day –1) by Day 35, the first sampling day after withdrawal of esomeprazole.

Effect of netazepide on esomeprazole-induced increases in gastrin and CgA

In **Groups 5, 6 and 2**, co-administration of netazepide 1, 5 and 25 mg, respectively, with esomeprazole on Days 15–28 suppressed the CgA response in a dose-dependent manner. Netazepide 5 and 25 mg both abolished the CgA response to esomeprazole. Netazepide 1 mg suppressed it only partially. Table 1 shows that, on Days 21 and 28, netazepide significantly reduced the high circulating CgA concentrations resulting from esomeprazole. The effect was dose-dependent: 5 mg and 25 mg doses reduced CgA to a similar extent; 1 mg was less effective.

In **Group 2**, after treatment withdrawal, the gastrin responses returned to baseline by Day 35, as they did in Group 1 subjects, who received esomeprazole alone.

In **Groups 4, 5 and 6**, daily measurements after treatment withdrawal showed that gastrin and CgA returned to baseline within 2–3 days in all groups, regardless of whether they had received esomeprazole alone or combined with netazepide.

Effect of netazepide alone on gastrin and CgA

In **Group 3**, netazepide 25 mg alone on Days 29–42 increased gastrin, but not in a time-dependent manner, and did not increase CgA. Indeed, CgA returned to baseline by Day 35, the first sampling day after the start of netazepide alone.

Relationship between of gastrin and CgA concentrations in individual subjects

There was no evidence of a linear relationship between gastrin and CgA within subjects: the slope term in the model was not significant ($p > 0.05$).

Dyspepsia questionnaire

No subject recorded dyspepsia symptoms in the questionnaire on Day -1, before starting treatment. However, 29 subjects (17 in Part 1 and 12 in Part 2) reported dyspepsia symptoms in their questionnaires on one or more visits thereafter. The most common symptom was discomfort in stomach, followed by pain/ache in stomach, nausea, and bloating. Subjects in Part 1 (Groups 1–3) reported dyspepsia symptoms 45 times during Days 1–28, when esomeprazole was administered, and 23 times during Days 29–56, the period after withdrawal of esomeprazole. Subjects in Part 2 (Groups 4–6) reported dyspepsia symptoms 41 times during Days 1–28 and 6 times during Days 29–35. Thus, there were about twice as many reports per week during esomeprazole treatment than after its withdrawal.

Dyspepsia symptoms were reported during all treatments: esomeprazole, netazepide and placebo. There was no obvious relationship to treatment; dyspepsia symptoms were reported after Day 42 in Part 1, and after Day 28 in Part 2, when all treatments had stopped.

Antacid usage

Three subjects took the antacid for dyspepsia: one subject in Group 2 took a total of 11 doses during the weeks before Day 35 and Day 42 visits, while taking placebo and after he had finished taking esomeprazole and netazepide; one subject in Group 3 took one dose on Day 31, after finishing esomeprazole but while taking netazepide; and one subject in Group 6 took 18 doses between Days 30 and 34, after she had finished taking esomeprazole and netazepide.

Netazepide concentrations

Mean plasma concentrations of netazepide in Groups 2, 3, 5 and 6 at 0, 2 and 4 h after dosing are listed in Table 3. Figures 6a and 6b show mean concentrations at 0, 2 and 4 h after dosing with netazepide for 7 and 14 days, respectively. The concentrations at 0 h (pre-dose) were below the limit of quantification. Concentrations at 2 h and 4 h after dosing were dose proportional and similar for the common dose of 25 mg.

Adverse events

Of 53 subjects who entered the study, 42 reported one or more adverse events, such as headache and upper respiratory tract infection, which were all minor and resolved spontaneously. Fewer subjects reported adverse events when taking esomeprazole plus netazepide or netazepide alone than when taking esomeprazole alone.

Discussion

Despite the finding of a significant treatment by visit interaction in Parts 1 and 2, and the variability of the fasting gastrin and CgA responses to esomeprazole in our subjects, it was still possible to interpret the results.

Esomeprazole alone increased circulating gastrin and CgA in all subjects in Groups 1–6. The increase in gastrin is consistent with inhibition of gastric acid secretion [20–22].

Co-administration of netazepide suppressed the CgA response to esomeprazole in a dose-dependent manner. Those results, which are in accord with the results from our previous study of rabeprazole and netazepide, alone and in combination [20], are consistent with esomeprazole-induced hypergastrinaemia stimulating gastrin/CCK₂ receptors on ECL cells and releasing CgA into the circulation, and with netazepide inhibiting gastrin/CCK₂ receptors and thereby blocking the CgA response. In 1,920 patients from 16 studies of patients on long-term PPI therapy, mean gastrin levels increased by one to three times the upper limit of the normal range (~100 pg/mL) [28].

Others have shown a positive correlation between gastrin and CgA in single blood samples taken from patients during short, medium and long-term acid suppression [23, 24]. In our previous study, 24-h serum gastrin increased after a single dose of rabeprazole, whereas 24-h plasma CgA required longer, which supports the concept that gastrin drives the increase in CgA [20]. In this study, we explored the relationship between gastrin and CgA in individual subjects during 2–4 weeks' esomeprazole treatment, using concentration data obtained at 3 time points (0, 2 and 4 h) on several study days. In general, CgA and gastrin were substantially higher in individual subjects during esomeprazole treatment than at baseline; however, there was no direct relationship between their concentrations within individual subjects.

We were surprised that in Part 1 both gastrin and CgA had returned to baseline by 7 days, the first sampling point, after esomeprazole withdrawal in Groups 1–3, regardless of whether or not netazepide 25 mg had been taken in the period before esomeprazole withdrawal. That finding is contrary to that of Reimer *et al*, who reported that CgA was still significantly raised 4 weeks after esomeprazole withdrawal in healthy subjects, whereas gastrin was back to normal at that time [13]. However, an earlier study showed that serum gastrin and CgA had decreased significantly at 5 days after withdrawal of 6 months' PPI therapy in GORD patients [29], which is in accord with our results in healthy subjects.

After reviewing the results from Part 1, we amended the protocol for Part 2, to measure fasting gastrin and CgA daily after esomeprazole withdrawal, and to test the effect of lower doses of netazepide 1 and 5 mg compared with placebo on the gastrin and CgA responses to

esomeprazole (Figure 1). Like netazepide 25 mg daily, netazepide 5 mg daily completely suppressed the CgA response to esomeprazole, whereas the 1 mg regimen had only a modest effect. After esomeprazole withdrawal, both gastrin and CgA returned to baseline within 2–3 days, whether or not esomeprazole had been co-administered with netazepide, confirming the finding in Part 1 that both gastrin and CgA returned to baseline within 7 days of esomeprazole withdrawal.

Although no subject reported a history of dyspepsia before entry to the study, 29 of the 48 who completed the study reported dyspepsia symptoms one or more times during treatment. However, there was no increase in incidence of dyspepsia following esomeprazole withdrawal – indeed, dyspepsia symptoms were more frequent during esomeprazole treatment than after its withdrawal. Thus, esomeprazole withdrawal did not lead to dyspepsia. That finding, plus the return to baseline of circulating gastrin and CgA within 2–3 days of esomeprazole withdrawal, excludes the possibility of rebound hyperacidity in this study. We also failed to demonstrate rebound hyperacidity and dyspepsia after withdrawal of rabeprazole treatment for 6 weeks in a previous study in healthy subjects [20]. Retrospective analysis of data from 287 patients with erosive oesophagitis who participated in a trial of dexlansoprazole therapy for 4–8 weeks also revealed no worsening of heartburn symptoms after treatment withdrawal [29].

The results of our study of esomeprazole withdrawal after 4 weeks' dosing, and our previous study of rabeprazole withdrawal after 6 weeks' dosing [20], are contrary to reports of rebound hyperacidity after withdrawal of 12 weeks of omeprazole therapy in GORD patients [10] and 8 weeks of omeprazole in healthy subjects [11], and dyspepsia after withdrawal of 8 weeks of esomeprazole [13] and 4 weeks of pantoprazole [14] in healthy subjects.

All treatments were safe and well tolerated, and there was no relationship between treatments or the number and type of adverse events. Esomeprazole did not affect plasma netazepide concentrations. A formal study is required to assess whether there is a drug-drug interaction between the two treatments.

The limitations of the study were: the small groups of subjects, the short course of esomeprazole, and the use of biomarkers rather than measurements of gastric acid secretion and histology of gastric biopsies. However, the results were consistent across the groups and have face validity. The proportion of healthy subjects who developed dyspepsia after PPI withdrawal in the above two studies [13, 14] was the same (44%), despite the difference in duration of dosing. Therefore, we chose to study the effect of esomeprazole for the shorter period of 4 weeks [14].

Conclusions

- Esomeprazole and netazepide each increase fasting serum gastrin, which is consistent with a secondary response to suppression of gastric acid secretion via inhibition of the proton pump on parietal cells and inhibition of gastrin/CCK₂ receptors on ECL cells, respectively.
- Esomeprazole-induced hypergastrinaemia leads to hyperactivity of ECL cells and an increase in plasma CgA. Gastrin causes the increase in CgA. Netazepide does not increase CgA, and suppresses the increase in CgA induced by esomeprazole, because netazepide blocks gastrin/CCK₂ receptors on ECL cells.
- The return to baseline of circulating gastrin and CgA within 2–3 days of esomeprazole withdrawal, and the absence of dyspepsia symptoms after esomeprazole withdrawal in the control groups, are contrary to the findings of others, and show that a standard 28-day course of esomeprazole, and probably all other PPIs, is unlikely to lead to rebound hyperacidity and dyspepsia symptoms. A study in many patients on long-term PPI therapy, randomised to netazepide 5 mg or placebo, is required to establish whether PPI withdrawal really can lead to rebound hyperacidity, and if so its importance.

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Conflict of interest

MB designed and, together with FvdB and SW, carried out the study. TM did the statistical analyses. MB wrote the manuscript. SW and KD contributed to the final version. All authors approved the final version. The study was funded by Trio Medicines, London, England (Trio), a subsidiary of Hammersmith Medicines Research (HMR). MB owns HMR and Trio. SW, FvdB, KD and TM are HMR employees.

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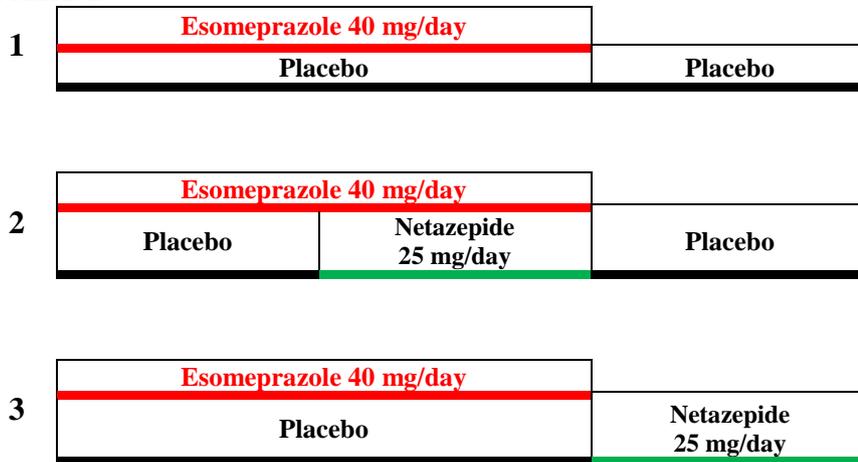
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Figure 1. Dosing schedule: Groups 1–6

PART 1



PART 2

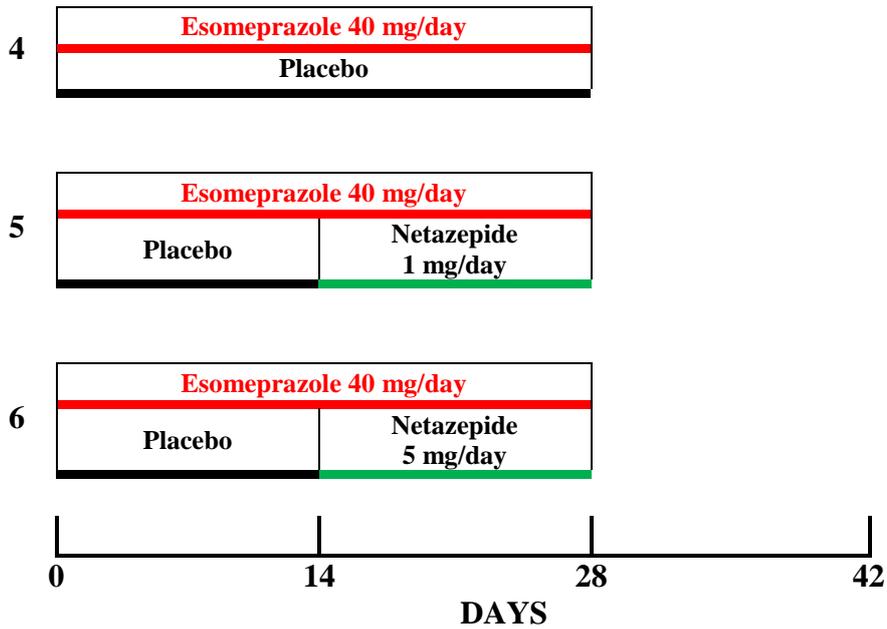
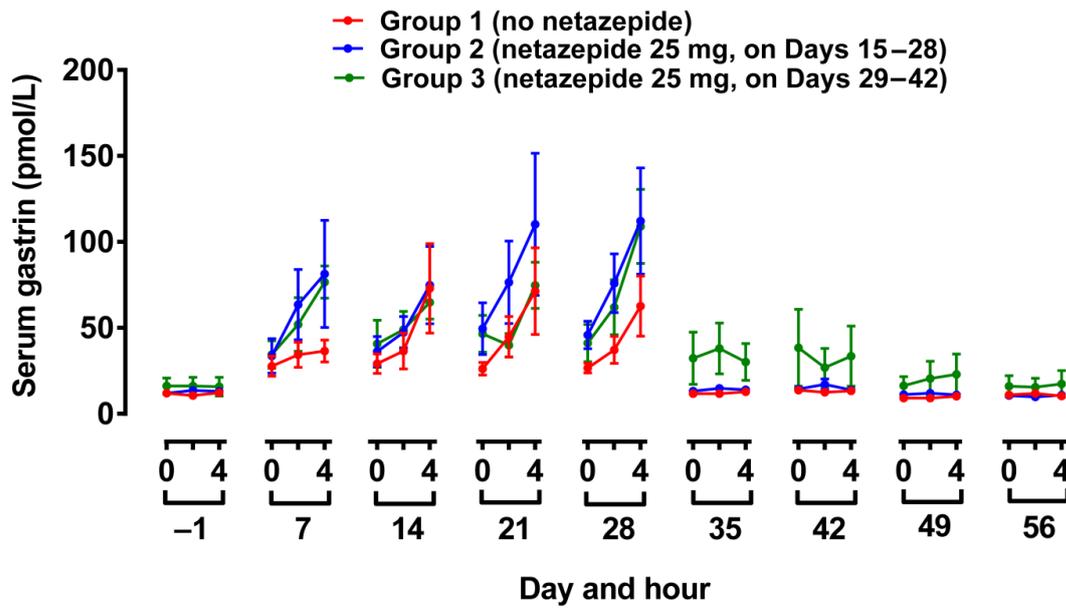


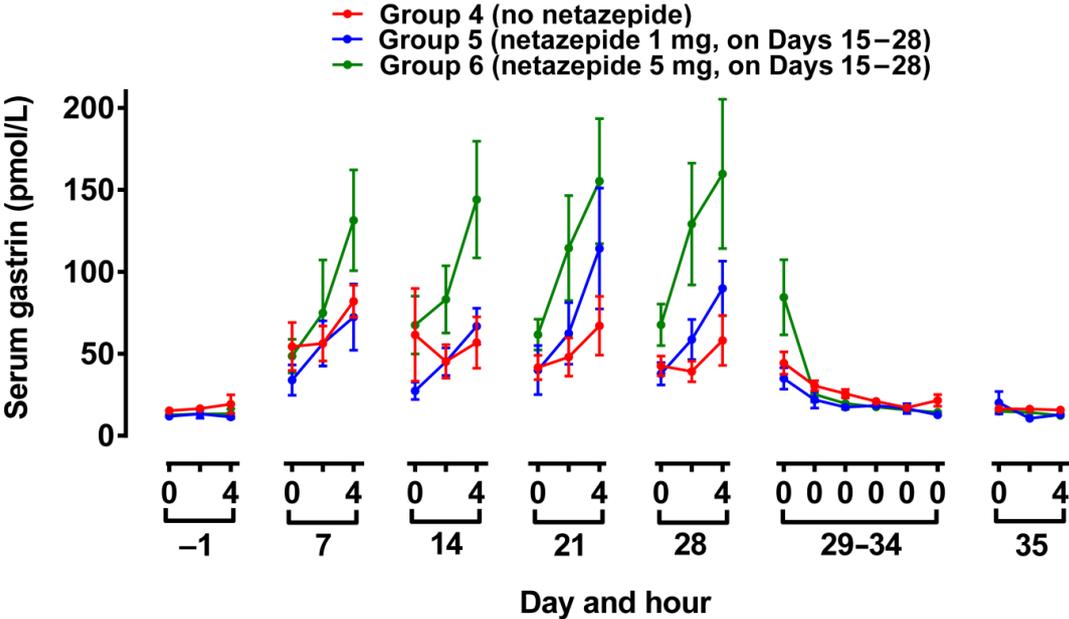
Figure 2(a). Part 1. Mean (n = 8; ± sem) fasting serum gastrin (pmol/L) before (0 h) and after (2 & 4 h) treatment of Groups 1, 2 & 3 on Days -1 to 56
All groups took esomeprazole 40 mg daily on Days 1 to 28.



All treatments increase fasting gastrin on Days 1–28, consistent with a secondary response to gastric acid suppression; the response to esomeprazole alone resolves within 7 days of its withdrawal whether or not netazepide 25 mg is co-administered with esomeprazole on Days 15–28.

Netazepide 25 mg alone on Days 29–42 (Group 3), after withdrawal of esomeprazole, increases fasting gastrin, consistent with a secondary response to acid suppression via antagonism of gastrin/CCK₂ receptors on ECL cells.

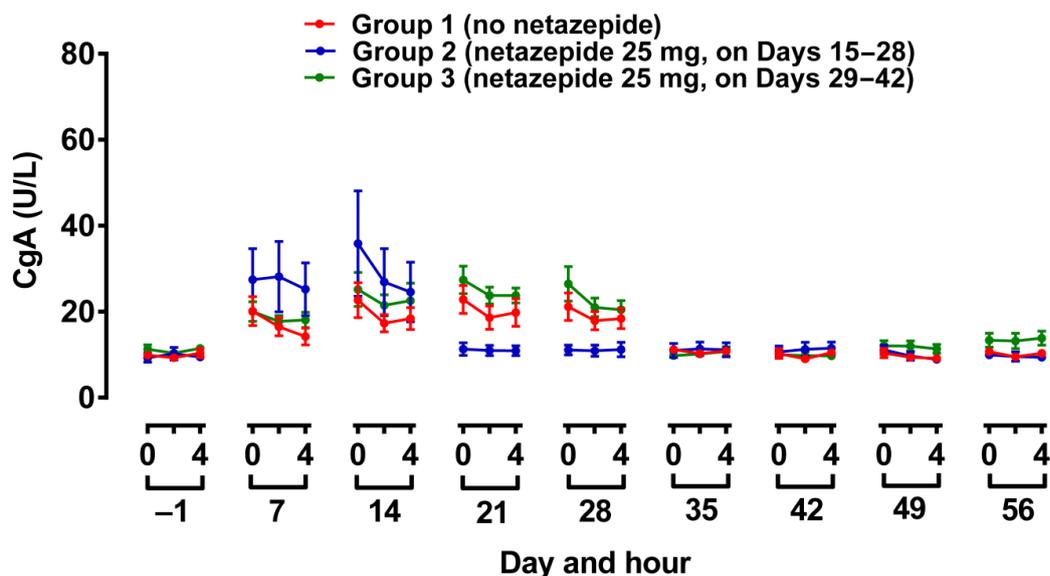
Figure 2(b). Part 2. Mean (n = 8; ± sem) fasting serum gastrin (pmol/L) before (0 h) and after (2 & 4 h) treatment of Groups 4, 5 & 6 on Days -1 to 35
All groups took esomeprazole 40 mg daily on Days 1 to 28.



Esomeprazole alone on Days 1–28 increases fasting gastrin; the response resolves within 2–3 days of esomeprazole withdrawal, whether or not netazepide 1 or 5 mg is co-administered with esomeprazole on Days 15–28.

The disproportionate increase in gastrin in Group 6 reflects a large effect of esomeprazole in 3 of the 8 subjects.

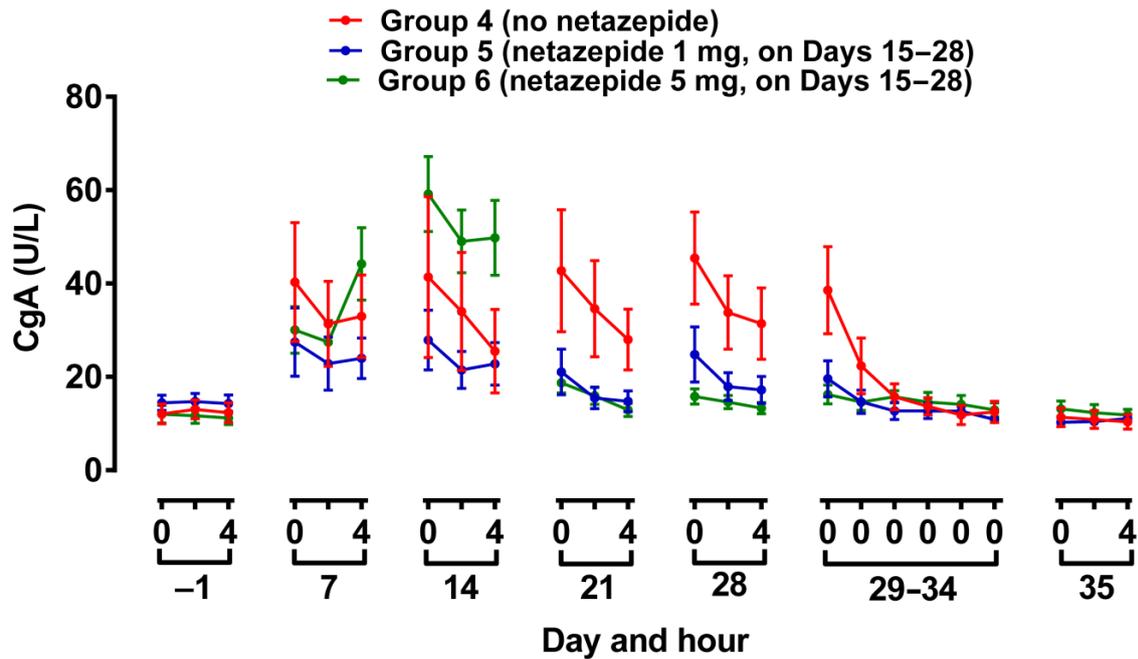
Figure 3(a). Part 1. Mean (n = 8; ± sem) fasting plasma CgA (U/L) before (0 h) and after (2 & 4 h) treatment of Groups 1, 2 & 3 on Days -1 to 56
All groups took esomeprazole 40 mg daily on Days 1 to 28.



Hypergastrinaemia induced by esomeprazole alone increases fasting CgA. In Groups 1 and 3, the response resolves within 7 days of esomeprazole withdrawal, whether or not netazepide 25 mg is administered after esomeprazole withdrawal. However, co-administration of netazepide with esomeprazole on Days 15–28 (Group 2) abolishes the increase in fasting CgA.

Netazepide 25 mg alone on Days 29–42 (Group 3) does not increase CgA, despite increasing gastrin during that time (Figure 2a), because netazepide blocks gastrin/CCK₂ receptors on ECL cells.

Figure 3(b). Part 2. Mean (n = 8; ± sem) fasting plasma CgA (U/L) before (0 h) and after (2 & 4 h) treatment of Groups 4, 5 & 6 on Days -1 to 35. All groups took esomeprazole 40 mg daily on Days 1 to 28.



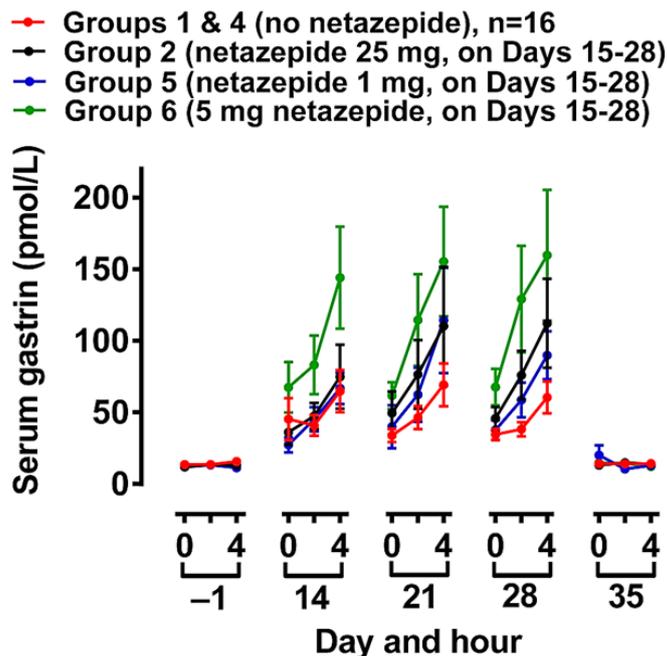
Hypergastrinaemia induced by esomeprazole alone (Group 4) increases fasting CgA; the response resolves within 2–3 days of esomeprazole withdrawal.

Co-administration of netazepide 1 mg (Group 5) and 5 mg (Group 6) on Days 15–28 inhibits the CgA response, which is consistent with antagonism of gastrin/CCK₂ receptors on ECL cells.

The disproportionate increase in CgA in Group 6 reflects the marked esomeprazole-induced hypergastrinaemia in 3 of the 8 subjects.

Figure 4(a). Parts 1 and 2. Mean (n = 8 or 16; ± sem) fasting serum gastrin (pmol/L) before (0 h) and after (2 & 4 h) treatment of Groups 1, 2, 4, 5 & 6 on Day -1 (baseline) and Days 14 to 35

All groups took esomeprazole 40 mg daily on Days 1 to 28.



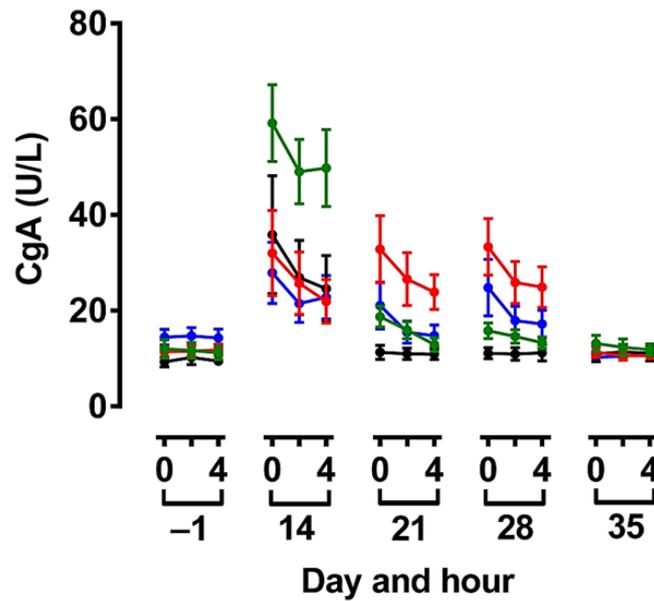
All treatments increase serum gastrin; the response returns to baseline (Day -1) within 7 days of the end of all treatments.

The disproportionate increase in gastrin in Group 6 reflects the large effect of esomeprazole in 3 of the 8 subjects (as evidenced by mean serum gastrin concentration on Day 7 of esomeprazole treatment; see Figure 2(b)).

Figure 4(b). Parts 1 and 2. Mean (n = 8 or 16; ± sem) fasting plasma CgA (U/L) before (0 h) and after (2 & 4 h) treatment of Groups 1, 2, 5 & 6 on Day -1 (baseline) and Days 14 to 35

All groups took esomeprazole 40 mg daily on Days 1 to 28.

- Groups 1 & 4 (no netazepide), n=16
- Group 2 (netazepide 25 mg, on Days 15-28)
- Group 5 (netazepide 1 mg, on Days 15-28)
- Group 6 (netazepide 5 mg, on Days 15-28)

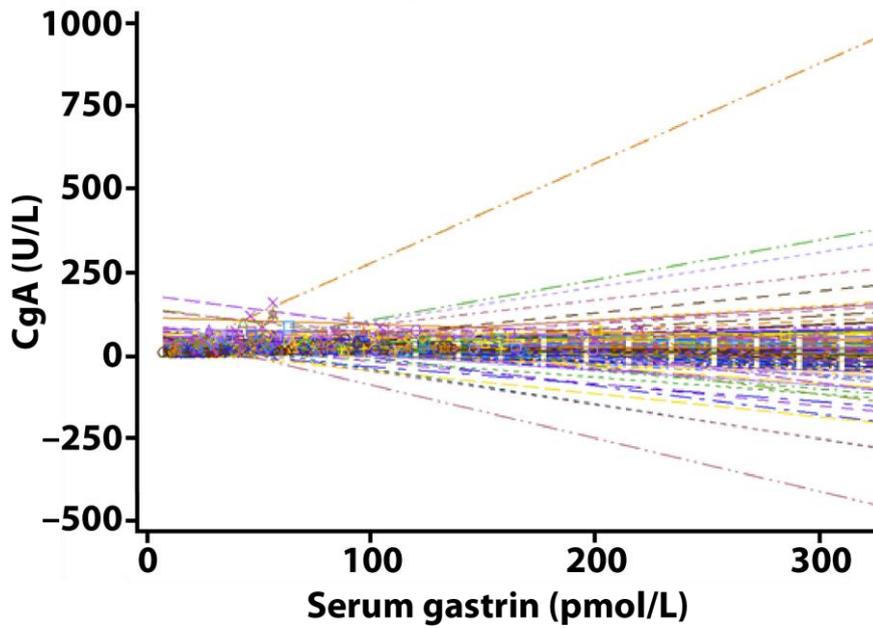


Co-administration of netazepide 1, 5 and 25 mg on Days 15–28 inhibits in a dose-dependent manner the increase in CgA by esomeprazole-induced hypergastrinaemia, which is consistent with gastrin/CCK₂ receptor antagonism.

CgA returns to baseline (Day -1) within 7 days of the end of all treatments.

The disproportionate increase in CgA in Group 6 reflects the marked esomeprazole-induced hypergastrinaemia in 3 of the 8 subjects (as evidenced by mean CgA concentration on Day 7 and at 0 h on Day 14 of esomeprazole treatment; see Figure 3(b)).

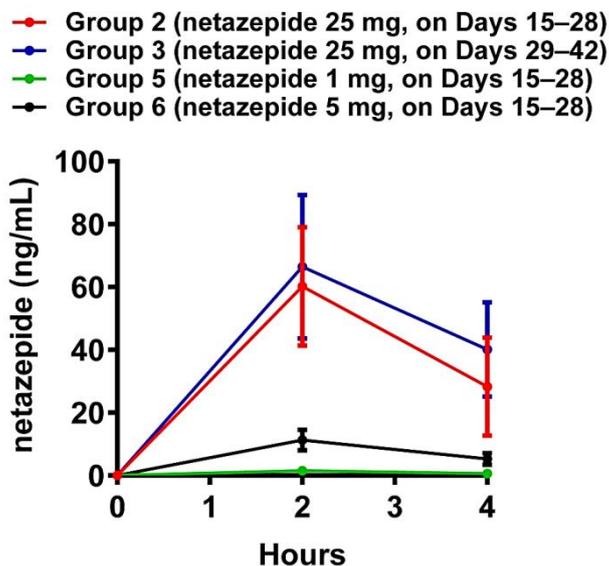
Figure 5. Analysis of covariance of fasting gastrin and fasting CgA concentrations within subjects in Groups 1–6 during periods of treatment with esomeprazole alone



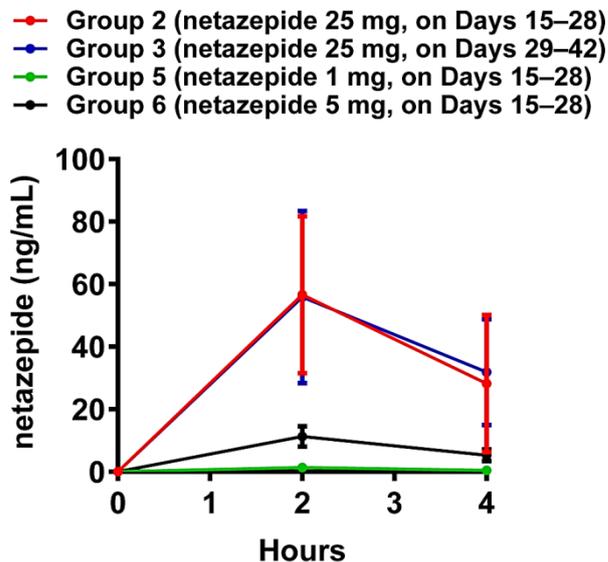
Using our statistical model, there was no evidence of a linear relationship between fasting gastrin and CgA concentrations within individual subjects (based on 425 observations from 47 subjects).

Figure 6. Mean (\pm sd) plasma netazepide concentrations (ng/mL) by time-point

(a). Mean (\pm sd) plasma netazepide concentrations (ng/mL) on Day 21 (Groups 2, 5 and 6) or Day 35 (Group 3), 7 days after starting netazepide



(b). Mean (\pm sd) plasma netazepide concentrations (ng/mL) on Day 28 (Groups 2, 5 & 6) or Day 42 (Group 3), 14 days after starting netazepide



Netazepide concentrations at 2 and 4 h after dosing are dose-dependent. Co-administration with esomeprazole does not affect netazepide concentrations.

Table 1. Change in plasma CgA relative to Day 14 (0 h) in Groups 1, 2, 4, 5, and 6

Treatment	LS mean ¹ (%)	Treatment – Placebo	
		Estimate	95% confidence intervals
Placebo (n=16)	1.39	-	-
Netazepide 1 mg (n=8)	-22.40	-23.79	-30.15, -17.43
Netazepide 5 mg (n=8)	-67.87	-69.26	-75.39, -63.14
Netazepide 25 mg (n=8)	-55.73	-57.12	-63.47, -50.76

¹ LS means of all measurements on Days 21 and 28

Table 2. Comparison of netazepide 1 and 5 mg and placebo with respect to plasma CgA in Groups 4, 5 and 6 at 0 h, on Day 28 and after treatment withdrawal (Days 29–35) (n=8 per group)

Day	Mean (U/L)			p-value	
	NTZ Placebo	NTZ 1 mg	NTZ 5 mg	1mg – Placebo	5mg – Placebo
28	45.45	27.33	15.81	0.09	0.01
29	38.56	21.21	16.21	-	0.02
30	22.38	15.73	14.58	-	0.26
31	15.74	13.53	15.73	-	-
32	13.69	13.47	14.60	-	-
33	11.84	12.64	14.14	-	-
34	12.52	11.63	12.94	-	-
35	11.33	10.69	13.16	-	-

p <0.05 is significant

NTZ = netazepide

Table 3. Plasma concentrations (ng/mL) of netazepide by day and time-point

Group	Dose (mg)	Visit	Time (h)	N	Mean	SD
2	25 mg on Days 15–28	Day 21 ¹	0	8	0.00	0.0
			2	8	60.24	18.9
			4	8	28.26	15.6
2	25 mg on Days 15–28	Day 28 ²	0	8	0.00	0.00
			2	8	56.60	25.1
			4	8	28.21	21.9
3	25 mg on Days 29–42	Day 35 ¹	0	8	0.00	0.0
			2	8	66.46	22.8
			4	8	40.09	15.0
3	25 mg on Days 29–42	Day 42 ²	0	8	0.00	0.0
			2	8	55.88	27.5
			4	8	31.88	16.9
5	1 mg on Days 15–28	Day 21 ¹	0	8	0.00	0.0
			2	8	1.55	0.5
			4	5	0.64	0.2
5	1 mg on Days 15–28	Day 28 ²	0	8	0.00	0.0
			2	8	1.34	0.9
			4	4	0.43	0.3
6	5 mg on Days 15–28	Day 21 ¹	0	8	0.00	0.0
			2	8	11.26	3.3
			4	8	5.26	1.9
6	5 mg on Days 15–28	Day 28 ²	0	8	0.00	0.0
			2	8	10.80	2.7
			4	8	4.89	2.4

¹ 7 days after the start of netazepide dosing

² 14 days after the start of netazepide dosing

Treatment was taken after an overnight fast on these days

Chapter 12

**Study 12. Interaction study of netazepide and midazolam,
a CYP3A4 substrate, in healthy subjects**

Introduction

Netazepide inhibits cytochrome P450 enzyme CYP3A4 in human liver microsomes *in vitro* (Chapter 2). Over 50% of available medicines are metabolised by CYP3A4 (Dresser *et al* 2000; Zhou 2008), which is found mainly in the liver and intestine. Exposure to medicines metabolised by CYP3A4 is increased by coadministered medicines that inhibit CYP3A4, such as macrolide antibiotics and azole antifungals, and some fruits, such as grapefruit. Increased exposure may result in toxicity, depending on many factors associated with the enzyme, medicine and patient. The possibility of such an interaction needs to be considered when a new medicine is under development and subsequently marketed.

Midazolam, a benzodiazepine used as a hypnotic or sedative (Crevoisier *et al* 1983; Bornemann *et al* 1985; Hypnovel 2015), is a substrate for CYP3A4 and a suitable probe to assess the relevance of the inhibition of CYP3A4 by netazepide in human liver microsomes *in vitro*.

Objectives

The primary objective was to assess the pharmacokinetics of a single dose of midazolam and steady-state netazepide, when taken alone and in combination. The secondary objective was to assess the tolerability and safety of midazolam coadministered with netazepide at steady state.

Methods

We complied with the ICH Guideline for GCP and the EU Clinical Trials Directive. The MHRA and Brent REC approved the study. Subjects gave written, informed consent.

Materials

R5 Pharmaceuticals prepared netazepide (batch NS7650) by spray drying a solution of netazepide API (batch 55593) and HPMC (ratio 1:3.5) dissolved in dichloromethane/isopropanol (7:2 v/v). HMR pharmacy made netazepide 25 mg capsules, as described for Study 9 and supplied oral doses of midazolam 5 mg solution prepared from vials of midazolam (Hypnovel[®], Roche) 10mg/2mL solution.

Study design

Ten men and women, deemed healthy as described previously, were allocated to one of two groups of five: Groups 1 and 2. There were two sessions: Sessions A and B. In Session A, subjects were resident for two nights (Days –1 and 1), and took a single dose of midazolam 5 mg by mouth on Day 1. In Session B, subjects took netazepide 4 x 25 mg capsules by mouth once daily for 10 days, and on Day 10 took a single dose of midazolam 5 mg by mouth. During Session B, subjects had out-patient visits on Days 1, 3 and 6, and were resident for three nights (Days 8–11). On Days 2, 4, 5 and 7, subjects took netazepide at home. Group 1 did Session A

first and, after a 5-day washout, then did Session B. Group 2 did Session B first and, after an 11-day washout, then did Session A. All subjects were followed up 5 days after their last dose of treatment.

Subjects had to fast overnight until 4 h after dosing with midazolam on Day 1 of Session A, netazepide on Day 9 of Session B, and netazepide and midazolam on Day 10 of Session B. They had standard meals and drinks at 4, 10 and 25 h after dosing on those days. On home dosing days (Days 2, 4, 5, 7 and 8), subjects were instructed to take netazepide 1 h before breakfast, after an overnight fast. No food or drink containing grapefruit was allowed from 7 days before the start of or during the study. No alcoholic or caffeinated drinks or exercise were allowed from 24 h before each admission to the ward until discharge.

During Session B, subjects recorded each dose of netazepide taken at home in a diary card. To ensure compliance, we telephoned subjects daily to remind them, checked their diary card and treatment container at each outpatient visit, and took blood samples on the morning of Days 3 and 6, to assay netazepide.

We collected blood samples for assay of: plasma midazolam before and frequently up to 24 h after each single dose; plasma netazepide before dosing on Days 3 and 6 of Session B, to assess compliance; and plasma netazepide before and frequently up to 24 h after dosing on Day 9 (netazepide alone) and Day 10 (midazolam coadministered with netazepide) of Session B. Analytical Services International Ltd did the assays using validated mass spectrometry and liquid chromatography (LC-MS/MS) methods. The lower limit of quantification (LLQ) was 1 ng/mL. Any values below the LLQ were reported as BLQ (below the limit of quantification). We assessed safety and tolerability as described previously. Subjects had to use a reliable method of contraception, as follows. Pre-menopausal women had to use an intrauterine device or their partner had to use a condom and spermicide. Steroid contraceptives such as 'the Pill' were not allowed. Men had to use a condom and spermicide.

Statistics

Sample size

Ten subjects were estimated to be adequate to detect any important interaction between netazepide and midazolam in this pilot trial. A sample size calculation was done to justify that number. Based on a sample size of 10 and published data (Crevoisier *et al* 1983; Bornemann *et al* 1985), the power calculation showed that, at the 5% significance level, we would have 79% power to detect a 50% increase in C_{max} and 96% power to detect a 50% increase in AUC of midazolam. For netazepide, we would have a power of 93% to detect an increase of 50% in C_{max} and 96% power to detect an increase of 30% in AUC.

Pharmacokinetic analysis

The following pharmacokinetic parameters were derived from plasma concentrations of netazepide and midazolam using non-compartmental analysis in WinNonLin 6.2: peak concentration (C_{\max}); time of peak concentration (T_{\max}); trough plasma concentration of netazepide (C_{τ}); half-life ($t_{1/2}$); area under the concentration-time and moment curves (AUC and AUMC, respectively) up to the time (t_n) at which the last non-zero level was recorded (AUC_{0-t_n} and $AUMC_{0-t_n}$) and infinity ($AUC_{0-\infty}$ and $AUMC_{0-\infty}$); apparent plasma clearance (CL/f); volume of distribution during terminal phase (V/f); and mean residence time (MRT).

Data for C_{\max} , $AUC_{0-\infty}$, and AUC_{0-t_n} were log-transformed and subjected to a linear mixed-effect model with treatment (alone/combination), sequence and period as fixed effects, and subject as a random effect for each treatment. The point estimate and 90% confidence intervals (CI) for the ratio of the geometric means between the two treatments (Test:Reference) were generated. Median and 90% CI of the difference Test:Reference for T_{\max} were calculated by Wilcoxon signed rank test. For netazepide: Reference = Session B, Day 9; Test = Session B, Day 10. For midazolam: Reference = Session A, Day 1 and Test = Session B, Day 10.

Results

Subjects

Ten subjects, 6 men and 4 women, median age 30.5 (range 19–44) years and median body mass index 21.1 (range 18.3–28.0) entered and completed the study, which was done during November 2011 to January 2012.

Pharmacokinetics

Mean plasma concentrations of netazepide and midazolam are listed in Tables 1 and 2, respectively. Mean pharmacokinetic parameters of netazepide and midazolam are listed in Tables 3 and 4, respectively. Mean plasma concentrations of netazepide and midazolam plotted against time are shown in Figures 1 and 2, respectively.

Plasma concentrations of midazolam in all pre-dose samples were BLQ. On Days 3, 6, 9 and 10 of Session B, plasma concentrations of netazepide in all pre-dose samples were above LLQ, confirming compliance. Plasma concentrations of netazepide when taken alone were similar to those in Study 9, which used the same formulation.

C_{\max} of netazepide failed to show equivalence between netazepide alone and in combination with midazolam, whereas $AUC_{0-\infty}$ and AUC_{0-t} of netazepide were similar for the test and reference treatments, and the 90% CI lay within the typical range accepted for bioequivalence, 80–125%. $AUC_{0-\infty}$, AUC_{0-t} and C_{\max} of midazolam were similar for the test and reference treatments, but

the 90% CI all suggest that they may have increased when midazolam was taken in combination with netazepide.

Safety and tolerability

The treatments proved safe and well tolerated. Any adverse events were mild and resolved spontaneously.

Discussion

$AUC_{0-\infty}$ and AUC_{0-t} of netazepide were similar for the test and reference treatments, and the 90% CI lay within the typical range accepted for bioequivalence, 80–125%. Although C_{max} of netazepide failed to show equivalence between netazepide alone and in combination with midazolam, equivalence cannot be ruled out because the 90% confidence interval (CI) 58–121 was broad, possibly because of the highly variable plasma concentrations of netazepide.

$AUC_{0-\infty}$, AUC_{0-t} and C_{max} of midazolam were similar for the test and reference treatments, but the 90% CI all suggest that they may have increased when midazolam was taken in combination with netazepide. In other words, non-equivalence cannot be ruled out.

Plasma concentrations of netazepide after the 100 mg dose was taken alone after an overnight fast were similar to those obtained for a 100 mg dose of the same spray-dried formulation taken after an overnight fast in Study 9.

Many established medicines are substrates, inducers and/or inhibitors of CYP3A4. Their ability to act as an inducer, inhibitor, or substrate for CYP3A is predictive of whether their coadministration with a known CYP3A substrate might lead to altered exposure and thereby reduced efficacy or increased toxicity. To date, the main clinically important CYP3A4 inhibitors include macrolide antibiotics (e.g., clarithromycin, and erythromycin), anti-HIV agents (e.g. ritonavir and delavirdine), antidepressants (e.g. fluoxetine and fluvoxamine), calcium channel blockers (e.g. verapamil and diltiazem), steroids and their modulators (e.g. gestodene and mifepristone), and dietary components (e.g. grapefruit products). Some medicines and herbal products induce rather than inhibit CYP3A4. To date, the main clinically important CYP3A4 inducers includeazole antifungals (e.g. ketoconazole), other medicines (e.g. rifampicin, phenytoin and ritonavir), and the herbal remedy, St John's wort.

CYP3A4 inhibitors can be classified by their potency (Flockhart 2007). Strong inhibitors cause at least a 5-fold increase in AUC, or more than 80% decrease in clearance. Moderate inhibitors cause at least a 2-fold increase in AUC, or 50–80% decrease in clearance. Weak inhibitors cause at least a 1.25-fold but <2-fold increase in AUC, or 20–50% decrease in clearance.

Netazepide also inhibits CYP2C8 in human liver microsomes *in vitro* (Chapter 2). However, very few medicines, such as montelukast, are metabolised via CYP2C8. They are best avoided until an interaction study with netazepide shows otherwise.

Conclusions

Overall, the results indicate that neither treatment affects the pharmacokinetics of the other. A larger sample size might strengthen that conclusion. If there is any interaction, it is likely to be small and not affect the recommended dose regimen of either treatment, especially as the therapeutic dose of netazepide is likely to be less than the 100 mg used in this study. Netazepide can be coadministered with existing medicines that are substrates of CYP3A4, apart from steroid contraceptives until evidence of their safe coadministration has been obtained.

It is reasonable to assume that netazepide is also a substrate for CYP3A4. Although netazepide appears safe even at high doses (Study 9), it should not be coadministered with a CYP3A4 inhibitor or inducer until an interaction study shows otherwise.

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Table 1. Mean (n = 10, SD) netazepide concentrations

Time (h)	Netazepide		Netazepide + midazolam	
	Mean	SD	Mean	SD
0	4.3	3.2	6.8	3.2
0.5	243	244	120	207
1	416	232	207	229
1.5	257	89.1	308	234
2	238	76.9	277	125
4	102	30.9	121	59.0
6	54.6	19.9	59.8	21.3
12	23.0	7.5	24.3	7.7
17	10.4	3.7	11.4	3.1
24	6.8	3.2	7.3	3.9

Table 2. Mean (n = 10, SD) midazolam concentrations

Time (h)	Midazolam		Netazepide + midazolam	
	Mean	SD	Mean	SD
0	0	0	0	0
0.5	30.3	9.8	33.7	8.6
1	19.9	8.7	20.9	4.6
1.5	14.3	3.50	15.8	3.9
2	11.4	3.5	12.8	3.9
4	6.1	1.9	6.1	1.7
6	3.1	1.4	3.4	1.3
12	0.8	1.1	0.7	0.8
17	0.2	0.5	0.3	0.5
24	0	0	0	0

Table 3. Pharmacokinetic parameters for netazepide (n = 10)

Time (h)	Netazepide		Netazepide + midazolam	
	Geometrical mean	SD (logs)	Geometrical mean	SD (logs)
AUC_{0-∞} (h.ng/mL)	1363	0.160	1305	0.34
C_{max} (ng/mL)	457	0.155	381	0.54
t_{1/2} (h)	5.2	0.3	5.9	0.3
T_{max} (h)*	1.00	0.5–2.0	1.50	0.5–4.0

* Median and range

Table 4. Pharmacokinetic parameters for midazolam (n = 10)

Parameter	Midazolam		Netazepide + midazolam	
	Geometrical mean	SD (logs)	Geometrical mean	SD (logs)
AUC_{0-∞} (h.ng/mL)	71.9	0.41	77.2	0.32
C_{max} (ng/mL)	28.9	0.31	32.7	0.26
t_{1/2} (h)	2.75	0.56	2.54	0.44
T_{max} (h)*	0.50	0.5–0.5	0.50	0.5–0.5

* Median and range

Table 5. Statistical comparisons of netazepide pharmacokinetic parameters

Parameter	Geometric fitted means		Ratio: test/reference (%)	90% CI
	Netazepide (reference)	Netazepide + midazolam (test)		
AUC_{0-∞} (h.ng/mL)	1363	1305	96	82–111
AUC_(0-t) (h.ng/mL)	1319	1247	95	80–111
C_{max} (ng/mL)	457	381	83	58–121

Table 6. Statistical comparisons of midazolam pharmacokinetic parameters

Parameter	Geometric fitted means		Ratio: test/reference (%)	90% CI
	Midazolam (reference)	Netazepide + midazolam (test)		
AUC_{0-∞} (h.ng/mL)	71.9	77.2	107	92–125
AUC_(0-t) (h.ng/mL)	64.3	71.6	111	96–129
C_{max} (ng/mL)	28.9	32.7	113	97–134

Figure 1. Linear and semi-logarithmic mean (n=10) plasma concentrations (ng/mL) of netazepide (YF476), alone and in combination with midazolam

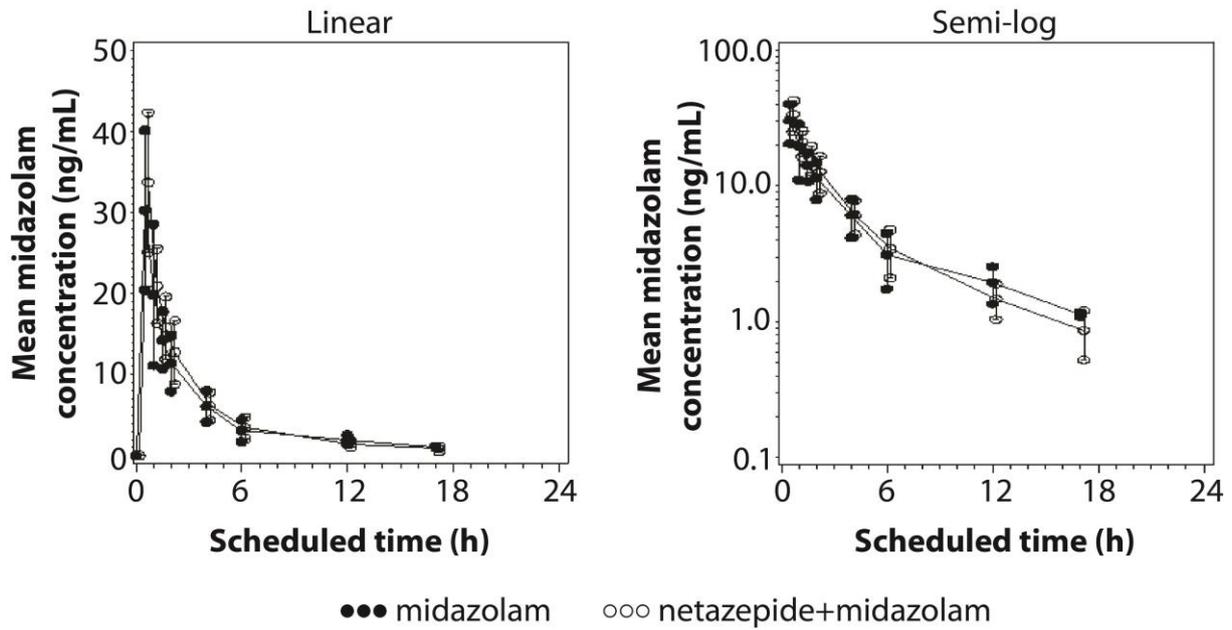
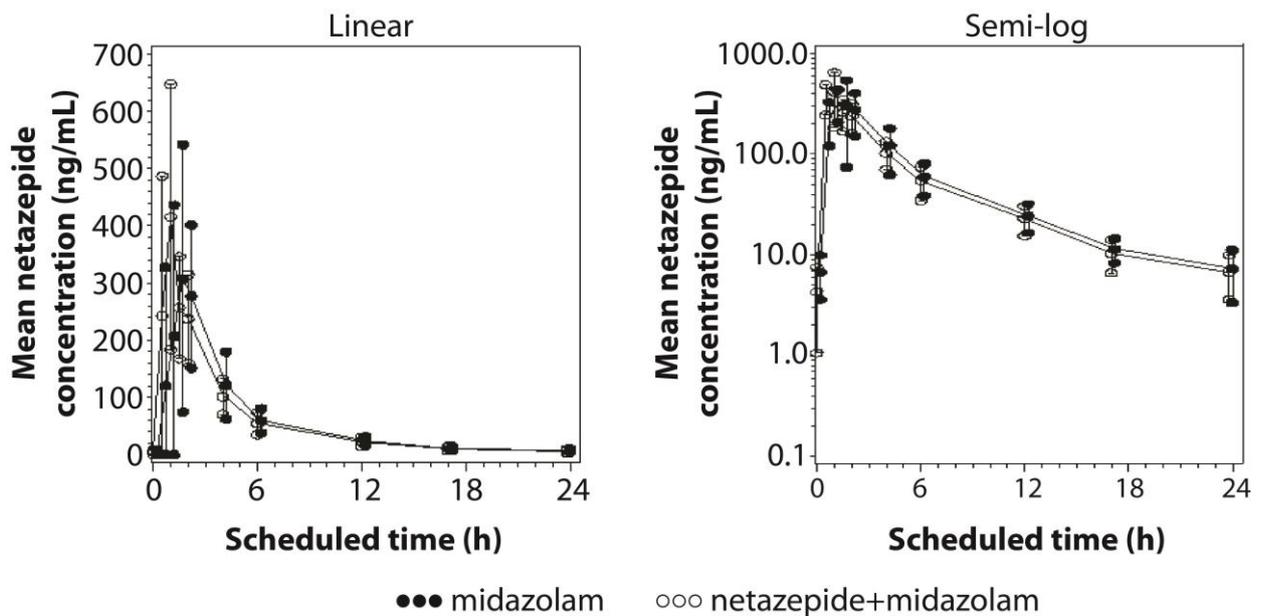


Figure 2. Linear and semi-logarithmic mean (n=10) plasma concentrations (ng/mL) of midazolam, alone and in combination with netazepide (YF476)



Chapter 13

Gastric neuroendocrine tumours: prevalence in Europe and the USA, and rationale for treatment with netazepide, a gastrin/CCK₂ receptor antagonist

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Gastric neuroendocrine tumors: prevalence in Europe, USA, and Japan, and rationale for treatment with a gastrin/CCK₂ receptor antagonist. *Scand J Gastroenterol* 2015; 50: 550–559.

ABSTRACT

Objective

Gastric carcinoids (neuroendocrine tumours) arise from enterochromaffin-like cells in the gastric mucosa. Most are caused by hypergastrinaemia. The objectives were to determine if their prevalence in Europe, USA and Japan meets the criteria for an orphan disease, and to justify treatment with a gastrin/CCK₂ receptor antagonist.

Method

We obtained data from European and USA cancer registries, and searched PubMed.

Results

Prevalence per 10,000 population obtained from cancer registries was: median 0.32 (range 0.09–0.92) for Europe; and 0.17 for the USA, equivalent to 4,812 for the whole population.

A PubMed search for gastric carcinoids yielded prevalence for Japan only, which was 0.05 per 10,000 population, equivalent to 665 for the entire population. A further search for gastric carcinoids in patients with pernicious anaemia (PA) or autoimmune chronic atrophic gastritis (CAG), two presentations of about 80% of gastric carcinoids, produced prevalence rates of 5.2–11%. Prevalence of PA itself was 0.12–1.9%. Data on CAG epidemiology were sparse.

Conclusions

Prevalence of gastric carcinoids varied widely. All sources probably underestimate prevalence. However, prevalence was below the limits required for recognition by drug regulatory authorities as an orphan disease: 5 per 10,000 population of Europe; 200,000 for the whole population of the USA; and 50,000 for the whole population of Japan. Because gastric carcinoids are an orphan disease, and non-clinical and healthy volunteer studies support treatment with netazepide, a gastrin/CCK₂ antagonist, netazepide has been designated an orphan medicinal product in Europe and the USA for development as targeted treatment for gastric carcinoids.

Introduction

Gastric carcinoids, which in recent years have been classified as gastric neuroendocrine tumours (NETs) [1,2], arise from enterochromaffin-like (ECL) cells in the gastric mucosa. ECL cells express gastrin receptors (also called CCK₂ receptors) [3], which are stimulated by gastrin, a hormone secreted by G cells in the gastric antrum, causing release of histamine. Histamine stimulates histamine H₂-receptors on parietal cells causing secretion of acid into the lumen of the stomach via the proton pump [4]. But, gastrin does more than just regulate gastric acid production. At physiological concentrations, gastrin organises and maintains the structure of normal gastric epithelium [5], whereas hypergastrinaemia disrupts epithelial structure, causing up-regulation of a variety of genes [6-9], such as histidine decarboxylase (HDC), chromogranin A (CgA), matrix metalloproteinase-7 (MMP-7), plasminogen activator inhibitor (PAI)-1 and 2, vesicular monoamine transporter type 2, and protein Reg 1A, as well as stimulating paracrine cascades [10], including cytokines, growth factors such as trefoil factor [11,12], and prostanoids. Overall, the responses to hypergastrinaemia lead to cell division, invasion, angiogenesis and anti-apoptotic activity, all of which can increase malignant potential [13,14].

Gastric carcinoids (Table 1) can be separated into three types [15,16]. Type 1 occur in patients with autoimmune chronic atrophic gastritis (CAG), are usually multiple and <1 cm in diameter, and comprise about 80% of gastric carcinoids. The features of autoimmune CAG are: chronic inflammation and atrophy of the gastric corpus; autoantibodies against parietal cells, leading to hypochlorhydria and in turn hypergastrinaemia and ECL-cell growth; autoantibodies against intrinsic factor, leading to malabsorption of vitamin B₁₂ and in turn pernicious anaemia and neurological signs [17,18]. *H. pylori* infection also induces chronic atrophic gastritis, but the distribution and histology of the mucosal changes differ from those of autoimmune CAG, and *H. pylori*-induced CAG predisposes to gastric adenocarcinoma rather than carcinoids [19]. However, there is evidence that *H. pylori* infection can start or worsen autoimmune CAG [20]. The gastrin/CCK₂ receptor antagonist, netazepide, prevented *H. pylori*-induced mucosal changes in an animal model, which suggests that gastrin has an important role in the inflammatory response to *H. pylori* infection [21]. Gastric carcinoids type 2 occur in patients with Zollinger-Ellison syndrome (ZES) – a gastrinoma, hypergastrinaemia and severe peptic ulcer disease [22] – are usually multiple and <1 cm in diameter, and comprise about 5% of gastric carcinoids. Hypergastrinaemia causes excessive gastric acid secretion. About 20% of ZES patients have the multiple endocrine neoplasia-1 gene (MEN-1), which increases their risk of developing gastric carcinoids. ECL-cell tumours are estimated to be over 70 times more common in MEN-1/ZES patients than in sporadic ZES patients. The MEN-1 gene probably renders ECL cells more sensitive to the trophic effects of hypergastrinaemia [23]. Gastric carcinoids type 3 are sporadic,

arise from otherwise normal gastric mucosa, and are usually 2–5 cm in diameter. Serum gastrin is normal. The potential for malignancy varies among the types: type 1 rarely metastasise, type 3 often metastasise, and type 2 are intermediate in their metastatic potential.

In animal models, sustained hypergastrinaemia – whether induced by prolonged treatment with intravenous gastrin or by inhibition of gastric acid secretion by a proton pump inhibitor (PPI) or loxidine, an insurmountable histamine H₂-receptor antagonist – increases ECL-cell density and causes diffuse, micronodular hyperplasia of ECL cells, followed by dysplasia and ultimately a carcinoid, which may become invasive [24]. Gastric carcinoids types 1 and 2 evolve in a similar manner in patients [25]. Gastric antrectomy is effective treatment for some but not all patients with gastric carcinoids type 1; it leads to a reduction in circulating gastrin and regression of the tumours, by removing the source of the hypergastrinaemia [26].

Many gastrin/CCK₂ receptor antagonists have been described [27], but none has been developed into a medicine, mainly because of problems with potency, selectivity at the gastrin/CCK₂ receptor, agonist activity, and oral bioavailability. Netazepide (YF476) has been called the ‘gold standard’ of gastrin/CCK₂ receptor antagonists [28], because in non-clinical studies it is potent, highly selective for the gastrin/CCK₂ receptor, and has good oral bioavailability [29-31]. In non-clinical studies, netazepide prevented mobilisation of ECL-cell histamine [32], and prevented increased ECL-cell activity and density and increased oxyntic mucosal thickness [33] resulting from PPI-induced hypergastrinaemia. Netazepide also reduced substantially the incidence of ECL-cell carcinomas in a strain of female cotton rats that develop such tumours spontaneously as a result of hypergastrinaemia secondary to gastric hypoacidity [34]. Furthermore, netazepide not only prevented formation of gastric carcinoids accelerated by loxidine-induced hypergastrinaemia in *Mastomys* rodents – which have a genetic predisposition to gastric carcinoids – but also caused shrinkage of formed lesions [35]. Other gastrin/CCK₂ receptor antagonists [27] have given similar results in animal models of hypergastrinaemia.

Netazepide is also an orally-active gastrin/CCK₂ receptor antagonist in healthy volunteers [36-39]. It caused dose-dependent inhibition of pentagastrin-induced gastric acid secretion, and prevented the increase in plasma CgA – a biomarker of ECL-cell hyperactivity – resulting from PPI-induced hypergastrinaemia. Furthermore, netazepide reduced baseline plasma CgA – a sign of ECL-cell hypoactivity.

Thus, there is evidence from non-clinical models and healthy subjects to justify developing a gastrin/CCK₂ receptor antagonist, such as netazepide, as a treatment for patients with gastric carcinoids types 1 and 2, which are gastrin dependent and comprise about 85% of all gastric carcinoids [15,16].

Gastric carcinoids are rare tumours [16]. Therefore, a gastrin/CCK₂ receptor antagonist is a potential orphan medicinal product (OMP) – a pharmaceutical agent for treatment of an orphan disease, a rare medical condition. The purpose of OMP designation is to encourage development of treatments for rare diseases for which there is either no treatment or the OMP offers significant benefit over existing treatments. An applicant to a drug regulatory agency for OMP designation must search the literature and obtain all information available on the prevalence of the condition to prove its rarity, and must provide convincing scientific and medical evidence to justify its treatment with the OMP [40]. The European Medicines Agency (EMA) requires the prevalence to be <5 per 10,000 population in Europe [41]. The USA Food and Drug Administration (FDA) requires the prevalence to be <200,000 for the whole population of the USA [42]. The Japanese Pharmaceutical and Food Safety Bureau (JPFSB) requires the prevalence to be <50,000 for the whole population of Japan [43]. If and when the OMP is eventually marketed, the developer is granted 10 years' marketing exclusivity in Europe and Japan, and 7 years in USA.

Objectives

Our objectives were to find out if the prevalence of gastric carcinoids in Europe, the USA and Japan meets the criteria for an orphan disease, and if netazepide would be accepted as an OMP for development as a potential treatment for gastric carcinoids.

Methods

We sought information from the following three sources in 2007. In July 2014, we repeated and extended the PubMed search for this review.

(a) Registries for Europe and USA

For Europe, we contacted over 100 cancer registries in the European Network of Cancer Registries [44], requesting information about the current prevalence of gastric carcinoids of morphology codes 8240–8246 and 8249. If a registry could not provide prevalence data, we requested incidence data, from which we calculated prevalence by the method described below. We estimated the total number of cases of gastric carcinoids in the 27 countries of the European Union (EU) and the 3 countries of the European Economic Area (EEA) using known populations of those countries [45].

We calculated prevalence from incidence by the following method. In a review of 562 patients with gastric carcinoids covering 50 years, the 5-year survival was 63% [46]. Therefore, mean survival must be >5 years but <15 years (15 years is the estimated life expectancy at age 65, the average age of diagnosis). We used 12 years for the mean duration of the condition, and calculated prevalence from the product of incidence and estimated duration. Incidence is the

number of new cases arising within a period of time, whereas prevalence is the proportion or percentage of cases alive at a point in time. The higher the incidence and the longer the duration of the condition, the higher is the prevalence.

For the USA, we searched the National Cancer Institute's (NCI) Surveillance, Epidemiology and End Results (SEER) programme to obtain the prevalence of gastric carcinoids [47]. We used geographic areas SEER 9 and histology codes 8240–8246 and 8249 to produce a 28-year limited duration prevalence by age and sex. We estimated the prevalence of gastric carcinoids for the whole of the USA using a frequency matrix [48].

(b) Registry for England alone

We searched the National Cancer Registry for England for adults (aged ≥ 15 years) diagnosed with primary neoplasms of the stomach for which morphology is carcinoid, for 1971–2003, to assess if there was an increase in prevalence during that period. The morphology codes for carcinoid tumours are 8240–8244 inclusive (ICD-O-1, ICD-O-2, and ICD-O-3). To estimate prevalence, we divided the number of patients who were alive – that is, not known by the National Cancer Registry to be dead – at the end of 2003 by the estimated mid-year adult population of England [49].

(c) PubMed

We searched articles in English to find the prevalence or incidence of gastric carcinoids in Europe, the USA and Japan. We used the terms 'population prevalence gastric carcinoids,' 'study population carcinoids or review population carcinoids,' 'incidence gastric carcinoids,' and 'population incidence carcinoid gastric'. The search yielded 298 articles, which we screened by title and found 52 whose content might be relevant. We read abstracts of those 52 articles and the full text of 29 of them. We found prevalence data for Japan [50,51], but only incidence data for Europe and the USA. We cite data from 5 articles about Europe and the USA among our results [46,52–55].

Because we found few articles reporting prevalence of gastric carcinoids, we also searched for prevalence or incidence of gastric carcinoids in patients with pernicious anaemia (PA) or autoimmune chronic atrophic gastritis (CAG). Those are two of the possible presentations of type 1 tumours, which comprise about 80% of gastric carcinoids [15,16]. We used the terms 'pernicious anaemia and prevalence or incidence gastric carcinoids,' and 'chronic atrophic gastritis and prevalence or incidence gastric carcinoids'. The search yielded a total of 1,899 hits, which we screened by title and found 15 whose content might be relevant. We read the abstracts of those articles and the full text of 9 of them. Eight yielded useful information [56–63].

Results

(a) Registries for Europe and USA

Seven European registries provided prevalence data for gastric carcinoids. Three other European countries supplied incidence data, from which we calculated prevalence. The year was up to 2006. The prevalence per 10,000 population in the 10 European countries that supplied data was: mean 0.34; median 0.32; range 0.09–0.92 (Table 2). The USA prevalence was 0.17 per 10,000 population (Table 3), which gives 4,919 for the entire population of 289,365,098, obtained by averaging numbers for 2002 and 2003. The total number of cases for the entire population of 491,586,372 for all 30 European countries (27 EU plus 3 EEA), estimated from prevalence values for the 10 countries that supplied data, was: mean 16,713; median 15,730; and range 2,457–45,225 (Table 4).

(b) Registry for England alone

The prevalence per 10,000 population of England in 2003 was 0.05 for men, 0.08 for women, and 0.07 for men and women combined (Table 5). The total number of incident cases increased about 25-fold over the period 1971–2003; most of the increase was over the last 10 years (Figure 1). The increase occurred in men and women in a similar manner (Figure 2). Peak frequency was age 65–75 years in men and 70–80 years in women (Figure 3).

(c) PubMed

We found prevalence data only for Japan. The Neuroendocrine Tumour Workshop Japan did a preliminary study in 2002–2004 to find out more about the epidemiology of neuroendocrine tumours in Japan [50], and a full study in 2005, for which a nationwide stratified random sampling method was used [51]. The estimated number of patients treated for gastrointestinal carcinoid tumours in Japan during 2005 was 4,406, of which 665 (15.1% of gastrointestinal NETs) were in the stomach. That gives a prevalence of 0.05 per 10,000 people, using the 2005 population census of 127,756,815. The result is similar to that of the Niigata Registry for Gut-Pancreatic Endocrinomas in Japan, which estimated that 18.4% were gastric in origin [64], and to the finding that an estimated 15% of reported and autopsy cases in Japan were gastric in origin [65]. In an evaluation of 588 patients with gastric carcinoids in Japan collected from the literature, there was about a 20-fold increase in cases in 1975–1995 [66].

We found only incidence data for Europe and the USA. Five examples follow. First, the Norwegian Cancer Registry incidence for 2004 was 0.18 per 100,000 population [52]. Second, a 1-year (2004–2005) prospective survey using the WHO classification [1] and the European Neuroendocrine Tumour Society staging and grading [2] of all newly diagnosed gastrointestinal neuroendocrine tumours recorded by 40 out of 41 possible sources for the whole of Austria

reported 285 tumours, of which 65 (23%) were gastric, giving an annual age-adjusted incidence of 0.54 per 100,000 population [53]. Of those 65 gastric carcinoids, 13 (20%) were described as malignant. Third, of a total of 13,715 carcinoids identified from the USA SEER program for 1973–1999, and two earlier SEER programmes, 562 (4.1%) were gastric carcinoids. The incidence ranged from 0.12 to 0.25 per 100,000 population per year [46]. Fourth, the Florida Cancer Data System (FCDS) and the SEER program were used to identify all gastric carcinoids in 1981–2000 [54]. Over the 20-year period, the age-adjusted incidence per 100,000 population increased from 0.04 to 0.18 for FCDS, and from 0.03 to 0.25 for SEER. Fifth, a search of the SEER 9, 13 and 17 databases for 1973–2003 gave an age-adjusted incidence of 0.3 per 100,000 population of the USA for 2003, a 10-fold increase since 1973 [55].

The prevalence of gastric carcinoids for the USA in 2003, estimated from the highest incidence of 0.25 per 100,000 population from two of the above sources [46,54], was 3 per 100,000 population, and 8,681 for the whole USA population.

We found prevalence data for PA and for gastric carcinoids in patients with PA. The prevalence of PA itself was 0.12% of 16 million people in Great Britain [56] and 0.13% of 265,000 people in part of Denmark [57]. Of 726 people aged over 60 years screened for PA in California, USA, 14 (1.9%) had undiagnosed PA [58]. When 123 patients with PA in Sweden were screened by gastroscopy and biopsy, 5 (4.1%) had gastric carcinoids, one of which had metastasised [59]. Of 71 patients with PA in Finland who underwent gastroscopy and biopsy every 3 years for a mean of 5.8 years, 8 (11%) developed gastric carcinoids [60]. In a similar study in Finland, 5 (7%) of 70 patients with PA had gastric carcinoids at initial screening; when 56 were re-scoped 3 years later, a further 2 (3.6%) had gastric carcinoids [61]. All of the aforementioned studies involving gastroscopy and gastric biopsy of PA patients also reported gastric cancer in some patients, either at screening or follow-up.

We found little information about the epidemiology of autoimmune CAG and gastric carcinoids. Of 742 patients of mean age 53 years undergoing outpatient gastroscopy and biopsy in France, 206 (28%) had CAG, and 8 (3.9%) of them had autoimmune CAG [62], which gives a prevalence of 1.2% for autoimmune CAG. In a study of 367 patients with autoimmune CAG in Italy, 9 had gastric carcinoids type 1 at initial diagnosis by gastroscopy and biopsy, giving a prevalence of 2.4%; when 214 were re-scoped after at least 2 years, a further 6 (2.8%) had developed type 1 tumours [63].

Discussion

The prevalence of gastric carcinoids varied widely from 0.05 to 0.92 per 10,000 population in 10 European countries, the USA and Japan. The prevalence rates of 4,919 and 665 for the whole of

the USA and Japan, respectively, were also very different, even allowing for size of their populations.

Weaknesses of our study are: we could not obtain prevalence data from the cancer registries of all European countries, especially the large ones, despite reminders – registries in some of the countries had only recently been established at the time; we had to estimate prevalence from incidence for some countries; and we could not separate the types of gastric carcinoids. Also, cancer registries are more likely to record troublesome tumours that require treatment, than benign tumours. Therefore, our results from cancer registries probably underestimate the true prevalence of gastric carcinoids.

Published incidence rates per 100,000 population of Norway [52] Austria [53] and the USA [46,54,55] also varied widely, from 0.03–0.54, depending on the year. Prevalence for the whole USA population estimated from published incidence data was 8,681, which is higher than the value of 4,919 that we obtained from SEER. The prospective survey in Austria provided the most comprehensive and reliable incidence rate of 0.54 per 100,000 population [53].

The National Cancer Registry of England, which holds up-to-date information on the dates of birth, diagnosis, and either emigration or death, of all patients since 1971 registered with malignant and most benign neoplasms [67], provides a reliable estimated point of prevalence of gastric carcinoids in England. The prevalence of 0.07 per 10,000 population was lower than that for all the other registries.

Estimating the prevalence of gastric carcinoids from data for PA or autoimmune CAG also has its limitations: the diagnosis of PA must be well substantiated [18,63]; autoimmune CAG overlaps with *H. pylori*-induced gastritis, which might be a precursor of autoimmune CAG [20,68]; and gastric carcinoids type 1 account for only about 80% of all gastric carcinoids [15,16]. However, the available data suggest that gastric carcinoids are less rare than other sources indicate. The prevalence of PA in England [56] and Denmark [57] was 0.12 and 0.13%, respectively, whereas the prevalence of undiagnosed PA in people over 60 in the USA was 1.9%, which is much higher [58]. 4–11% of patients with PA [58–60] and 5.2% of patients with autoimmune CAG [62] had gastric carcinoids at gastroscopy and biopsy. Furthermore, the prevalence of autoimmune CAG in a population in France undergoing gastroscopy and biopsy was 1.2% [62], and the prevalence in patients with autoimmune CAG screened by gastroscopy and biopsy was overall 5.2%.

But, whatever the source of the data, and however derived, the prevalence of gastric carcinoids was below the limits set by the regulatory agencies for Europe, USA, and Japan. Because of the

low prevalence of gastric carcinoids and the favourable results of non-clinical and healthy volunteer studies of netazepide, the EMA and FDA designated netazepide an OMP for their treatment in 30 European countries in 2007 and in the USA in 2009 [69,70]. An application to the JPFBS is in progress.

Since 2007, when we first searched PubMed, the preferred term has changed from ‘gastric carcinoids’ to ‘gastric neuroendocrine tumours’ [1,2]. Therefore, we repeated our search using the new preferred term. The search yielded 58 articles, 17 of which were published before the end of 2007. We found most of those 17 articles in our earlier search, and none was relevant to this review. Furthermore, of the articles published after 2007, we have already cited the relevant ones in this review.

The 25-fold increase in prevalence of gastric carcinoids in England during 1971–2003 is higher than: the 8- to 9-fold increase in the incidence of gastric carcinoids in the FCDS and SEER databases for the USA during 1981–2000 [54]; the 10-fold increase in the SEER databases for the USA during 1973–2003 [55]; and the increase in Japan since the 1970s [66]. The reasons for the increases are unknown, but they may include improvements in clinical practice, such as diagnostic gastroscopy and biopsies, and greater awareness of the condition [16,71]. However, the increase in gastric carcinoids has coincided with the increase in PPI use, and it has been suggested that the two may be directly linked [46,54]. Most patients taking long-term PPI treatment develop hypergastrinaemia [72], but opinions differ about its importance. One view is that although long-term PPI treatment leads to ECL-cell hyperplasia in some patients [73–75], such changes do not progress to ECL-cell tumours [74,76]. Another view is that long-term PPI-induced hypergastrinaemia is potentially harmful, and can result in tumours that originate from ECL cells [77–79]. Patients with PA have a nearly 7-fold increased risk of gastric cancer [80], for which hypergastrinaemia might be at least a contributing factor.

A nationwide survey in Japan reported that the frequency of type 1 tumours (called type A gastritis in Japan) was 87% [51] of all gastric carcinoids, similar to Western countries. Type 2 tumours also occur in Japanese people [81]. But, there are some differences between Japan and Western countries [65]. First, gastric carcinoids accounted for 15.1% of gastrointestinal NETs in Japan [51] compared with only about 4% in Western countries [82]. Second, about 20% of ZES patients in Western countries have the MEN-1 gene [23], whereas only 1% of patients with gastrointestinal NETs in Japan have it [51]. Third, another type of gastric carcinoid has been described in Japanese people. It arises from ECL cells and is associated with gastric mucosal atrophy and hypergastrinaemia in the absence of circulating parietal cell antibody found in autoimmune CAG patients. All patients were infected with *H pylori*, the prevalence of which is

high in Japanese people [83]. Those patients support the concept that *H. pylori*-induced gastritis is a precursor of autoimmune CAG [20,68].

Gastric carcinoids are managed by endoscopic surveillance, medical treatment (somatostatin analogues) or surgery (endoscopic resection, antrectomy or gastrectomy), according to the findings at endoscopy, the histology of the lesion, and the cause of the hypergastrinaemia [16,84–86]. Management of type 3 tumours is usually partial or total gastrectomy. Regular endoscopic surveillance is appropriate in some patients with types 1 and 2; endoscopic resection of multiple tumours may be difficult, and recurrence occurs in most patients [87]. Somatostatin analogues [88], which require injection, are not recommended [84–86]. Antrectomy can be effective [26,89,90], but carries the risk of morbidity and mortality.

However, the logical treatment of types 1 and 2, which are gastrin dependent, is surely a gastrin/CCK₂ receptor antagonist. Indeed, in two exploratory studies in patients with autoimmune CAG, achlorhydria, hypergastrinaemia, multiple gastric carcinoids, and raised circulating CgA, netazepide given for 12 weeks reduced the number of tumours and the size of the largest one, and normalised plasma CgA, which returned to pre-treatment levels after stopping netazepide [91,92]. In one of those studies, netazepide also normalised raised mRNA abundancies of CgA, HDC and MMP-7 in tumour biopsies [92]. Again, they returned to pre-treatment levels after stopping netazepide. Longer term studies are in progress.

The treatment of choice for ZES patients is high-dose PPI, to control their gastric hyperacidity. But prolonged PPI usage itself causes hypergastrinaemia, which might further stimulate growth of ECL cells. In healthy subjects, when netazepide was combined with a PPI, the combination suppressed gastric acid production more than either treatment alone, and netazepide prevented the increase in circulating CgA induced by the PPI alone [39]. CgA is a biomarker of ECL growth. Also, a gastrin/CCK₂ antagonist prevented the increased ECL-cell activity and density and increased oxyntic mucosal thickness resulting from PPI-induced hypergastrinaemia in an animal model [33]. Thus, a combination of a gastrin/CCK₂ receptor antagonist and a PPI seems a more logical choice of treatment for ZES patients.

Conclusions

The prevalence of gastric carcinoids among cancer registries was below the limits of 5 per 10,000 population in Europe, 200,000 for the whole population of the USA, and 50,000 for the whole population of Japan required for recognition by the drug regulatory authorities in those territories as an orphan disease. Prevalence of gastric carcinoids in England increased in parallel with the increase in incidence for other countries. The true prevalence of gastric carcinoids is higher than our searches would suggest, although still within the limits for designation as an

orphan disease. Netazepide has been designated an OMP in Europe and the USA for development as a targeted medical treatment for gastric carcinoids because: they are rare; most are caused by hypergastrinaemia; and results from non-clinical and healthy volunteer studies suggest that the tumours will respond to a gastrin/CCK₂ receptor antagonist. Results from early studies of netazepide in patients with gastric carcinoids type 1 are encouraging.

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Table 1. Main characteristics of the types of gastric carcinoid

Characteristic	Type 1	Type 2	Type 3
Associated disease	Chronic atrophic gastritis and pernicious anaemia	Zollinger-Ellison syndrome and MEN type I	Sporadic
Percentage (%)	80	5	15
Site	Fundus	Fundus (sometimes antrum)	Antrum or fundus
Number	Multiple	Multiple	Single
Size of tumour	<1 cm	<1cm	2–5cm
Serum gastrin	High	High	Normal
Gastric acid	Low	High	Normal
Prognosis	Good; some metastasise	Mostly good; 30% metastasise	Poor; >50% metastasise

Table 2. Prevalence data for gastric carcinoids obtained from cancer registries in Europe and USA

Country	Population		Prevalence per 10,000	
	Number	Year(s)	Value	Year(s)
Northern Ireland	1,710,322	2004	0.11	2004
Ireland	4,043,700	2004	0.40	2004
South West England	6,755,578	2004	0.17 (C)	2000–2004
Modena, Italy	No data provided		0.36 (C)	2001–2005
Malta	391,415	2000	0.92	2006
Latvia	No data provided		0.49	2003–2005
Lithuania	3,462,552	2003	0.32 (C)	2001–2005
Norway	4,640,219	2005	0.16	2005
Iceland	299,404	2005	0.70	2005
Netherlands	No data provided		0.09	2006
USA	289,365,098	2003	0.17	2003

C = calculated from incidence x duration (estimated 12 years)

Table 3. Prevalence for USA estimated from incidence data

Source	Year	Prevalence (10,000 people)	Prevalence (whole of USA)*
Modlin <i>et al</i> 2004	NDP	0.3**(C)	8,681
Hodgson <i>et al</i> 2005	2000	0.3**	8,681

NDP = No data provided

C = calculated from incidence x duration (estimated 12 years)

* calculated from: prevalence per 10,000 x $\frac{\text{USA population in 2003}}{10,000}$

** calculated from the highest incidence reported

Table 4. Total number of cases of gastric carcinoids in 30 European countries

Countries	Population	Total number of cases		
		Mean	Median	Range
EU (27) + EEA (3)	491,586,372	16,713	15,730	2,457–45,225

Table 5: Incident cases of gastric carcinoids in England during 1971–2003, and their point prevalence by sex in 2003

Sex	Total number of incident cases 1971–2003	Total number of cases alive at end 2003	Prevalence per 10,000 population
Men	210	97	$97/19,728,000 \times 10,000 = 0.05$
Women	267	166	$166/20,979,700 \times 10,000 = 0.08$
Both sexes	477	263	$263/40,707,800 \times 10,000 = 0.07$

Figure 1. Total number of incident cases of gastric carcinoids in England by year of diagnosis during 1971–2003

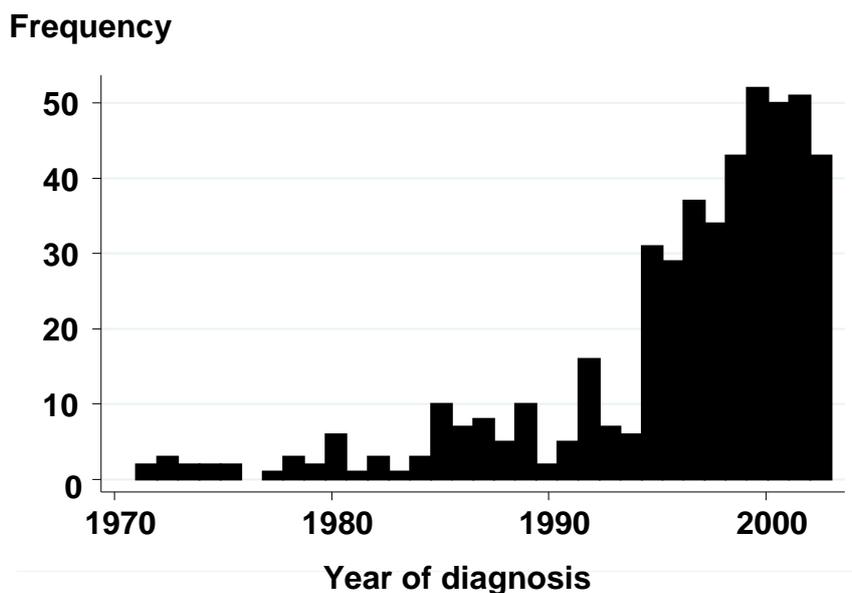


Figure 2. Total number of incident cases of gastric carcinoids in England by year of diagnosis and by sex during 1971–2003

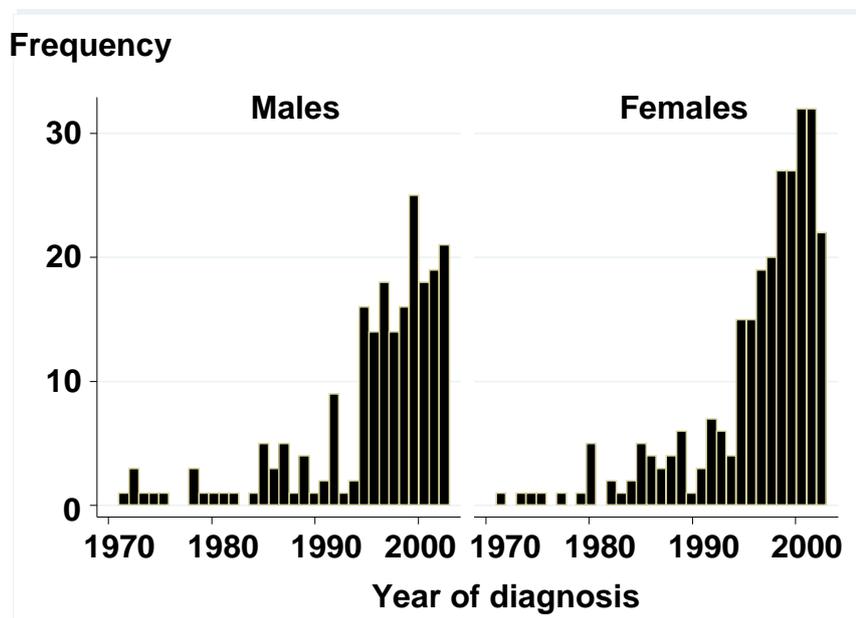
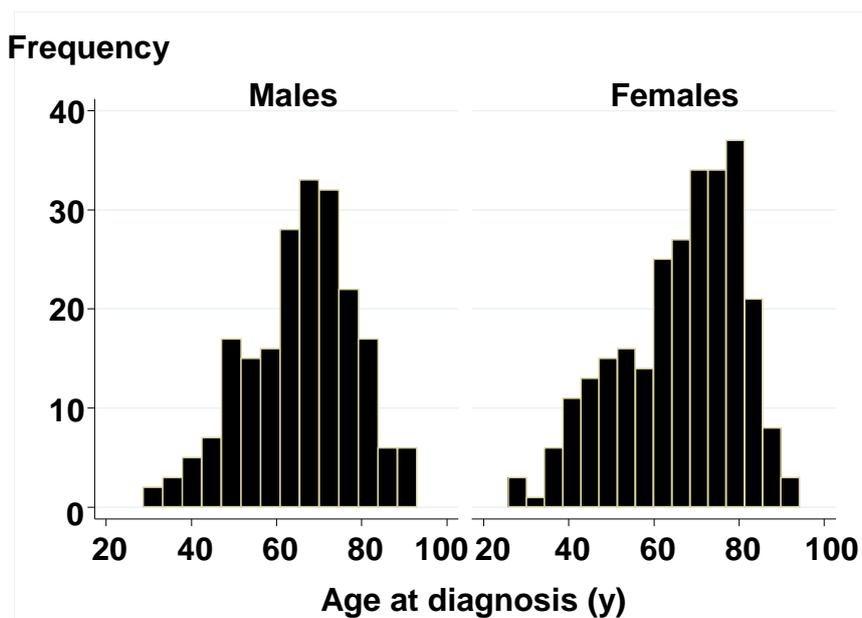


Figure 3. Total number of incident cases of gastric carcinoids in England by age of diagnosis and by sex during 1971–2003



Chapter 14

Studies of netazepide in patients with hypergastrinaemia and gastric neuroendocrine tumours

(Boyce M, Moore AR, Sagatun L, Parsons BN, Varro A, Campbell F, Fossmark R, Waldum HL, Pritchard DM. Netazepide, a gastrin/CCK₂ receptor antagonist, can eradicate gastric neuroendocrine tumours in patients with autoimmune chronic atrophic gastritis.

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Introduction

The main clinical indication for a gastrin/CCK₂ receptor antagonist such as netazepide has to be hypergastrinaemia, of which there are several causes (Chapter 1). Patients with CAG and ZES can develop gastric NETs types 1 and 2, respectively, as a result of excessive stimulation of their ECL cells by hypergastrinaemia. Both types of tumour have the potential to metastasise, especially type 2. Therefore, patients usually undergo gastroscopy every 6 or 12 months, to find out if there have been any changes in tumours that might require their surgical removal (Kulke *et al* 2010; Ozao-Choy *et al* 2010; Merola *et al* 2012). Gastric NETs types 1 and 2 are both gastrin driven, so patients with such tumours seemed an ideal population for a ‘proof-of-principle’ trial of netazepide.

Trio sponsored two trials of netazepide in patients with CAG and type 1 tumours by: supplying capsules containing a spray-dried formulation of netazepide (Chapter 9); assaying CgA, gastrin and/or netazepide in blood samples; monitoring the studies; preparing the clinical reports; and funding one of the centres. Trio also sponsored a trial in patients with ZES and type 2 tumours by supplying netazepide capsules and monitoring the study.

I set up the trials, wrote or contributed to the writing of the protocols and applications to the regulatory authorities and RECs for most of them, and was the medical monitor for all of them. I report the main results in this chapter. I am a co-author of papers based on the trials of type 1 tumours (Fossmark *et al* 2012; Moore *et al* 2013).

Gastric NETs type 1

12-week studies of netazepide

Two centres, one in Trondheim, Norway, and another in Liverpool, England, each did an open study in which *H. pylori* negative patients with autoimmune CAG, hypergastrinaemia, multiple gastric NETs type 1 and raised circulating CgA were treated with netazepide 50 mg once daily for 12 weeks, with 12-week follow-up. The current toxicology studies allow patients to be treated with netazepide for a maximum of 13 weeks (Chapter 2).

The same protocol was used for both studies. There were 7 outpatient visits. Visits 1 and 2 were to assess eligibility and to obtain consent. Visits 3, 4, 5 and 6 were at 3, 6, 9 and 12 weeks after starting treatment, respectively. Visit 7 was at 12 weeks after stopping treatment. Gastroscopy was done at visits 2, 4, 6 and 7, during which the number of tumours was counted, the largest tumour was measured, and the tumours and flat gastric corpus mucosa were biopsied for assessment of morphology. Liverpool also assayed corpus mucosal biopsies for real-time PCR abundances of CgA, histidine decarboxylase (HDC), matrix metalloproteinase-7 (MMP-7), plasminogen activator inhibitor (PAI)-1 and 2, and for microRNA analysis. Blood was collected

at Visits 2, 3, 4, 5, 6 and 7 for assay of fasting serum gastrin, plasma or serum CgA and plasma netazepide. Safety and tolerability of netazepide were assessed throughout the study. Medicines that inhibit or induce CYP3A4 or CYP2C8 were not allowed. Medicines that are metabolised via CYP3A4 and have a wide therapeutic window were allowed.

Trondheim: netazepide for 12 weeks

This study was done by the Departments of Gastroenterology and Hepatology, St. Olavs Hospital, and the Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway. The principal investigator was Professor Helge Waldum. The study was approved by the Norwegian Medicines Agency (NoMA) and the regional (REC), and was done during June 2011–May 2012.

Eight patients (5 women, 3 men; mean age 60, range 50–69 years) entered and completed the study. Three of them were receiving vitamin B12 treatment for treatment of pernicious anaemia (PA). Figure 1 shows representative photographs taken at gastroscopy, before and at the end of treatment. Tables 1 and 3 list individual and mean results. Figure 3 illustrates individual results. Results at baseline and during treatment were analysed by Wilcoxon signed rank test. Fossmark *et al* 2012 have published the results of the study.

At baseline, the mean number of tumours was 11.9 (range 4–24) and the mean diameter of the largest one was 5.8 mm (range 4–9). At 12 weeks, there was a significant reduction in the number of tumours ($11.8 \pm \text{SEM } 2.4$ vs 6.9 ± 2.5 ; $p=0.01$) and the size of the largest one (5.8 ± 0.6 vs 3.5 ± 0.7 mm; $p=0.01$). One patient's tumours were eradicated. The remaining tumours were deemed no longer hyperaemic in appearance. However, the histology of the tumours and the hyperplasia and dysplasia of ECL-cells in the flat mucosa appeared unchanged. There was a reduction in serum CgA at 3 weeks (7.8 ± 1.0 vs 4.3 ± 0.4 nmol/L; $p=0.02$), which was sustained until 12 weeks (4.2 ± 0.2 ; normal ≤ 5 nmol/L).

After 12 weeks off treatment, the number of tumours and the diameter of the largest one tended to increase, and CgA increased again (4.2 ± 0.2 vs 6.6 ± 0.8 nmol/L; $p=0.02$). The high serum gastrin at baseline (normal range ≤ 40 pM/L) was unaffected by 12 weeks' treatment (379 ± 88 vs 301 ± 61 pM/L; $p=0.2$).

Plasma netazepide concentrations before and 1 h after dosing ranged from 6.5 to 19.9 and 127.4 to 239.7 ng/mL, respectively. Netazepide was safe and well tolerated.

Liverpool: netazepide for 12 weeks

This study was done by the Departments of Gastroenterology and Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, and the Department of

Pathology, Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, England. The principal investigator was Professor Mark Pritchard. The study was approved by the MHRA and Cambridge East REC, and was done during January 2011 to July 2012.

Eight patients (4 women, 4 men; mean age 62, range 55–76 years) entered and completed the study. All of them were receiving vitamin B12 treatment for treatment of PA. Figure 2 shows photographs taken at gastroscopy, before and at the end of treatment. Tables 2 and 4 list individual and mean results. Figure 4 illustrates individual results. Moore *et al* 2013 have published the results, which were analysed by Wilcoxon signed rank test.

At baseline, the mean number of tumours was 11 (range 4–30) and the mean diameter of the largest one was 8 (range 3–13 mm). After 12 weeks' treatment, 5 patients had fewer tumours, 2 had the same number and one had one more tumour. Compared with baseline, there was a reduction in the number of tumours (means 11 vs 7; $p=0.046$). All but one patient had a reduction in the diameter of their largest tumour relative to baseline (means 8 vs 6; $p=0.02$). None of the tumours had increased in size after 12 weeks' off treatment.

At baseline, tumour biopsies showed that 7 patients had low grade NETs and one had micronodular ECL-cell hyperplasia, and gastric corpus mucosal biopsies showed that all patients had ECL-cell hyperplasia. After 12 weeks' treatment, 4 patients had low grade NETs and 4 had micronodular ECL-cell hyperplasia, and the histology of flat mucosa was unchanged (Table 7).

There was a reduction in plasma CgA in all patients at 3 weeks (means 63.2 vs 19.0 U/L; $p=0.01$), which was sustained up to 12 weeks (means 63.2 vs 19.6 U/L; $p=0.01$); the normal range is ≤ 40 U/L. After 12 weeks off treatment, CgA was increased again in all subjects (means 19.6 vs 51.6 U/L). The high serum gastrin at baseline (normal range ≤ 40 pM/L) was unaffected by 12 weeks' treatment (555 vs 613 pM/L). Real-time PCR abundances of the biomarkers CgA, HDC and MMP-7 (normalised to GAPDH) in biopsies at 12 weeks were reduced by 35% ($p=0.01$), 59% ($p=0.03$) and 56% ($p=0.02$), respectively, relative to baseline (Figure 10). They had almost returned to pre-treatment levels 12 weeks after the end of treatment. PAI-1 and PAI-2 did not change significantly. Netazepide also suppressed miR-222, which was overexpressed in samples before treatment (Lloyd *et al* 2015).

Plasma netazepide concentrations before and 1 h after dosing ranged from 4.6–7.0 and 87–220 ng/mL, respectively. Netazepide was safe and well tolerated.

52-week studies of netazepide

Because of the favourable results from the Liverpool study, the MHRA approved a protocol amendment for the eight patients to receive netazepide 50 mg once daily for another 52 weeks,

without Trio first completing long-term toxicology studies. The frequency of gastroscopy was reduced to 6-monthly, to make the extension to the study less demanding. NoMA would not agree to a similar protocol amendment for the Trondheim study. However, they did agree to long-term treatment under the Norwegian 'named patient' scheme at a lower dose of netazepide 25 mg once daily and with gastroscopy still at 3-monthly intervals. The RECs that approved the 12-week studies also approved the extensions to the studies.

Trondheim: netazepide for 52 weeks

Six of the eight patients consented to a further 12 months' treatment with netazepide. One withdrew soon after, for personal reasons. The interval between the end of netazepide treatment in the 12-week study and the start of 52 weeks' treatment ranged from 9 to 14 months. The study was done during February 2013 to April 2014. Tables 1 and 3 list individual and mean results. Figure 5 illustrates individual results.

While the five patients were off treatment between studies: the number of tumours increased slightly (means 6.0 vs 7.2); the size of the largest tumour increased (means 4.2 vs 7.6 mm), and serum CgA remained high (means 6.5 vs 6.8 nmol/L). The one patient who was free of tumours at the end of the 12-week study regrew two tumours in the interval off treatment. During treatment, three patients became free of tumours, including the one who became free in the 12-week study, and the other two had far fewer tumours (means 13.5 vs 4.5) and the largest one became smaller (means 8 vs 6 mm). CgA was within the normal range again in all 5 patients at the first visit after the start of treatment (means 6.8 vs 4.0 nmol/L), and continued to fall up to 52 weeks (2.5 nmol/L). Gastrin varied among patients before and during treatment, as in the 12-week study. Netazepide was safe and well tolerated.

Liverpool: netazepide for 52 weeks

All eight patients from the Liverpool study consented to and completed a further 12 months' treatment with netazepide. The interval between the end of netazepide treatment in the 12-week study and the start of 52 weeks' treatment ranged from 8 to 19 months. The study was done during October 2012 to February 2014. While the eight Liverpool patients were off treatment, the number of tumours increased (means 7 vs 10) and the size of the largest one also increased (means 3.5 vs 4.9 mm). Tables 2 and 4 list individual and mean results. Figure 6 illustrates individual results.

During treatment, two patients became free of tumours and one patient finished with only one tumour. At the end of 52 weeks' treatment, there were fewer tumours (means 8.6 vs 10) and the size of the largest one was halved (means 3.0 vs 6.4 mm). CgA stayed raised in the interval between studies (mean 45.3 U/L), but was reduced again at the first visit after the restart of

treatment (16.2 U/L), and remained so thereafter. Again, serum gastrin varied among patients before and during treatment but there was no evidence of a further increase. Netazepide was safe and well tolerated.

Liverpool continued to assess the real-time PCR abundances of CgA, HDC and MMP-7 in gastric mucosal biopsies during the 52-week study. Relative to GAPDH, there were significant reductions in CgA and HDC, but not MMP-7, after 26 and 52 weeks' netazepide treatment (Figure 11) (Moore *et al* 2015). Liverpool also used microarray analysis to assess the effect of netazepide on several other biomarkers in gastric mucosal biopsies (Moore *et al* 2015).

Compared with the first or second baselines, netazepide reduced the expression of pappalysin 2 (PAPPA2), endoplasmic reticulum protein (ERP27), eosinophil lysophospholipase (CLC), peptidyl-glycine alpha-amidating monooxygenase (PAM), secretogranin II (SCG2) and monoamine oxidase B (MAOB) (Figure 12). In contrast, netazepide increased claudin 10 (CLDN10).

Pooled results

Overall, the results from the two centres were similar, so I pooled common values for the 16 and 13 patients, respectively, who completed the 12-week and 52-week studies, and analysed them by non-parametric Wilcoxon signed rank test. Tables 5–7 list results for the 12- and 52-week studies, respectively. Figures 7 and 8 show box whisker plots for median, inter-quartile range, and range of results for the 12- and 52-week studies, respectively.

12-week studies

Netazepide 50 mg once daily for 12 weeks reduced significantly the number of tumours ($p < 0.001$), the size of the largest tumour ($p < 0.001$), and plasma CgA to within normal limits ($p < 0.001$). The response was evident at the first assessment. One patient's tumours were eradicated. Serum gastrin was unaffected. At 12 weeks after stopping treatment, the number of tumours, the size of the largest tumour, and plasma CgA had increased again, but the effect of netazepide was still significant. Plasma netazepide concentrations were 139 (s.d. 138), 225 (145), 178 (190) and 166 (234) ng/mL at 3, 6, 9 and 12 weeks, respectively. Netazepide was safe and well tolerated. There were few reported adverse events, and none was deemed treatment related.

52-week studies

Netazepide 25mg (Trondheim) or 50 mg (Liverpool) once daily for 52 weeks reduced significantly the number of tumours ($p < 0.01$), the size of the largest tumour ($p < 0.001$), and plasma CgA to within normal limits ($p < 0.001$). At 52 weeks, only 3 patients had histologically confirmed NETs (Table 7). Netazepide eradicated all tumours in 5 of the 13 patients. Serum

gastrin was unaffected. Again, netazepide was safe and well tolerated; there were few reported adverse events, and none was deemed related to treatment.

Interval off treatment between 12- and 52-week studies

The mean interval between the end of 12 weeks' netazepide treatment and the start of 52 weeks' treatment was 14 (range 8–19) months. Figure 9 shows that during that time, the number of tumours ($p < 0.01$), the size of the largest tumour ($p < 0.05$), and plasma CgA ($p < 0.001$) all increased significantly.

Netazepide treatment beyond 12 months

Like NoMA, the MHRA agreed to netazepide treatment on a 'named patient' basis. To date, six patients, five from the Trondheim study, and one from the Liverpool study, have taken netazepide continuously for up to 30 months. Once again, there have been few reported adverse events, and none was deemed related to treatment.

Gastric NETs type 2

The protocol for this study, which is being done by Professor Steve Wank, NIDDK, NIH, Maryland, USA, requires 30 patients with Zollinger-Ellison syndrome (ZES; a gastrinoma, hypergastrinaemia and hyperacidity) and gastric NETs type 2 to be treated with netazepide 50 mg once daily for 12 weeks. The dose can be increased to 75 or 100 mg daily only after treating six patients safely with netazepide 50 mg daily. The protocol is similar in design to that of gastric NETs type 1, except: there are no blood samples after cessation of treatment; patients take a PPI for treatment of hyperacidity; and the effect of netazepide on basal acid output after 7 days of PPI withdrawal and replacement for a day by ranitidine is assessed at the beginning of the study. The study was approved by the FDA and the NIDDK Institutional Review Board, and started in April 2011. Recruitment has been slow. To date, only 3 patients have entered and completed the study, despite the centre having the largest number of patients with such tumours worldwide (Jensen and Fraker 1994).

At first, the FDA regarded netazepide as a cytotoxic treatment and asked for a separate dose-rising study to assess safety and tolerability of netazepide before assessing its efficacy.

Eventually they accepted that netazepide is a hormone-targeted treatment, but they still required dose increments to be based on an algorithm similar to one used for studies of cytotoxic therapy.

At entry to the study, all three patients had multiple tumours and markedly raised serum CgA (mean 7,737 pg/mL; range 2,430–17,300; normal < 225 pg/mL). The mean (sd) of the mean size (width x height) of three tumours, identified by injection of a dye into the mucosa at baseline in each patient, was 50 ± 36 mm² at baseline and 41 ± 28 mm² after 12 weeks' netazepide

treatment. After 12 weeks off treatment, the mean size had increased again, to $57 \pm 42 \text{ mm}^2$. Netazepide reduced serum CgA in all three patients. Mean serum CgA after treatment was 6,060 pg/mL compared with 7,737 pg/mL at baseline. CgA was reduced to within the normal range in one patient after 6 weeks' treatment.

Those findings are consistent with a treatment effect. The investigator also had the impression that after netazepide treatment the tumours were umbilicated in appearance, suggesting a reduction in volume. The results of the basal acid measurements are difficult to interpret.

Discussion

Gastric NETs type 1

The limitations of the studies in patients with gastric NETs type 1 were: the small numbers of patients; subjective measurements of the tumours; and the open design, which might have resulted in investigator bias. Gastric NETs are rare tumours (Boyce and Thomsen 2015), the number of eligible patients was limited, and the studies were demanding of patients and investigators, so placebo controls seemed unreasonable for what were essentially exploratory studies. Furthermore, the gastroscopies were done by only one or two experienced physicians per centre, and they counted and measured the tumours carefully. All eight patients who participated in the initial Liverpool study consented to take part in the study extension whereas only five patients from the Trondheim study did so. The difference may reflect the reduced number of visits and gastroscopies in the Liverpool study.

Despite the limitations of the study design, the complete eradication of tumours in one patient after 12 weeks' treatment with netazepide and five of 13 patients during 52 weeks' treatment is convincing evidence of efficacy. One patient's tumours were eradicated twice. Overall, the reduction in number of tumours and/or the size of the largest one in the other patients are supportive evidence of efficacy, although those findings alone might not be considered enough. During the interval patients were off treatment for a mean of 14 (range 8–19) months, the number of tumours and the size of the largest one increased, and plasma CgA increased again. CgA is a valid biomarker of ECL-cell activity in healthy subjects (Boyce *et al* 2015) and patients with type 1 tumours (Sanduleanu *et al* 2001; Peracchi *et al* 2005). After 52 weeks' treatment, only 3 of 8 patients had histologically confirmed NETs, which is also consistent with a treatment effect. Some of the remaining tumours may have been sessile polyps (Goddard *et al* 2010) and not responsive to netazepide.

During the interval between studies, we were able to obtain regulatory approval for extensions to the studies without first doing long-term toxicology studies (ICH M3). Reduction of the dose of netazepide from 50 mg to 25 mg once daily for the extended study in Trondheim provides

evidence that the lower dose is just as effective. 25 mg of spray-dried netazepide was top of the dose-response curve for increasing gastric pH (Study 2), and 5 mg was enough to inhibit the increase in CgA induced by esomeprazole (Study 11), both of which support the concept that spray-dried netazepide 25 mg daily is enough to treat type 1 tumours.

Gastric NETs type 1 are derived from ECL cells, which are also the source of the increased circulating CgA in CAG patients (Peracchi *et al* 2005). The sustained reduction in raised CgA to within normal limits during both the initial and extended studies is consistent with netazepide inhibiting ECL-cell growth via antagonism of gastrin/CCK₂ receptors on the ECL cells. CgA increased again during the interval off treatment, when gastrin/CCK₂ receptors would not have been blocked.

In healthy subjects, acid suppression by netazepide leads to a secondary increase in circulating gastrin (Boyce *et al* 2015). Serum gastrin was unaffected by netazepide in the CAG patients confirming that they had achlorhydria as a result of atrophy of their parietal cells.

In patients with type 1 tumours treated for 12 weeks in the Liverpool study, netazepide normalised raised mRNA abundances of CgA, HDC and MMP-7, which returned to pre-treatment levels after stopping netazepide (Moore *et al* 2013), and suppressed miR-222 overexpression (Lloyd *et al* 2015). Netazepide also suppressed miR-222 overexpression in the gastric mucosa of transgenic mice with hypergastrinaemia and infected with *H. felis* (Lloyd *et al* 2015). miR-222 targets the tumour suppressor and oncogene p27, which increases cell proliferation, migration and angiogenesis. P27 loss is associated with a poor prognosis in patients with GEP NETs (Kim *et al* 2014). Netazepide treatment for 52 weeks continued to suppress abundances of CgA and HDC, and also suppressed abundances of several other biomarkers compared with baseline values (Moore *et al* 2015). In contrast, netazepide increased CLDN10, a membrane bound protein found in tight junctions. Disruption of these tight junctions by downregulation of CLDN10 may allow cancer cells to spread (Gao *et al* 2013).

Patients with pernicious anaemia (PA), which is one of the possible clinical presentations of atrophic gastritis, have a nearly seven-fold increased risk of gastric adenocarcinoma (Vannella *et al* 2013). Children with hypergastrinaemia caused by genetic mutations of *KCNQ1* or *KCNE1* (Rice *et al* 2011; Winbo *et al* 2013) or *ATP4A* (Calvete *et al* 2015), genes which control acid secretion by the parietal cell, not only develop gastric NETs, but also have a high risk of gastric adenocarcinoma. Some patients with those genetic mutations have needed gastrectomy. These findings support the concept that excessive gastrin has malignant potential. ENET guidelines (Delle Fave *et al* 2012) recognise that every NET has the potential to metastasise.

Although gastric NETs are rare tumours – prevalence per 10,000 population of Europe, USA and Japan was 0.32, 0.17 and 0.05, respectively – our survey of the literature revealed that the prevalence of PA and CAG, two possible presentations of about 80% of gastric NETs, was 5.2-11% (Boyce and Thomsen 2015). Prevalence of PA itself was 0.12-1.9%. Patients with CAG and PA have hypergastrinaemia and are at risk of developing gastric NETs.

The management of gastric NETs is controversial. Patients with types 1 and 2 tumours usually undergo once or twice yearly gastroscopy. If there are concerns about the morphology and histology of the tumours, they are removed surgically by polypectomy, antrectomy (removal of the part of the stomach that secretes gastrin) or gastrectomy (removal of the whole stomach). Type 3 tumours are usually removed surgically when first diagnosed, because they have the highest risk of malignancy (Burkitt and Pritchard 2006). Although they are not gastrin driven, they might still respond to treatment with a gastrin/CCK₂ receptor antagonist.

Somatostatin (SST) analogues, such as octreotide and lanreotide, are licensed for treatment of acromegaly and for severe diarrhoea and other symptoms (carcinoid syndrome) that occur with gastroenteropancreatic neoplasia. They are sometimes used off-label to treat patients with gastric NETs (Li *et al* 2014). SST analogues, which have to be given by injection, treat hypergastrinaemia indirectly by reducing secretion of gastrin by G cells, whereas an oral gastrin/CCK₂ receptor antagonist, such as netazepide, inhibits gastrin/CCK₂ receptors on ECL cells directly, and so is a more logical treatment. Although some studies have shown that SST analogues can cause regression of type 1 tumours (Fyske *et al* 2004 and 2005; Massironi *et al* 2015) and are well tolerated, SST analogues inhibit release of various hormones, such as insulin, glucagon, thyroid stimulating hormone and cholecystokinin, and cause side effects, including: diarrhoea, bradycardia, hyper- and hypoglycaemia, pancreatic inflammation, gall stones (Plockinger *et al* 1990), gall bladder sludging (Trendle *et al* 1997), increase in gut-transit time, and impaired gallbladder emptying (Hussaini *et al* 1996). Neither ENETS nor NANETS recommend SST analogues for treatment of gastric NETs (Delle Fave *et al* 2012 and Kulke *et al* 2010). In contrast, netazepide has so far been safe and well tolerated in clinical trials (Chapter 15). To date, about 220 healthy subjects have taken netazepide by mouth for up to 6 weeks, and 27 patients have taken it from 3 to 34 months. Adverse events have been minor, transient, independent of netazepide dose and as common in placebo or comparator groups.

Plasma netazepide levels at 1 h after 25 or 50 mg doses in the 12-week studies varied widely but were within the range seen with those doses at that time in studies of spray-dried netazepide in healthy subjects (Study 9). Thus, achlorhydria does not impair the bioavailability of netazepide.

Gastric NETs type 2

Although to date only 3 patients with type 2 tumours have been treated, there are signs that netazepide reduces tumour size and plasma CgA. Compared with CAG patients, ZES patients have much higher concentrations of circulating gastrin and CgA. The gastrinoma is the source of the hypergastrinaemia and probably much of the increase in CgA. Netazepide is a competitive antagonist and the dose may need to be higher for some patients with type 2 tumours. We have just obtained FDA and IRB approval for a protocol amendment to allow the dose to be increased sooner to 100 mg once daily, and to increase the dose to 150 mg or 200 mg once daily after six patients have been treated safely. The acid suppressant and growth inhibiting properties of a gastrin/CCK₂ receptor plus a PPI should be an ideal treatment for ZES patients.

Conclusions

- A gastrin/CCK₂ receptor antagonist such as netazepide is a potential non-surgical, targeted treatment for gastric NETs type 1, and its use should avoid the need for regular gastroscopy and biopsy of the tumours. But that would need confirmation in a placebo-controlled study of a range of doses in a large number of patients. Given the rarity of the tumours, that would need an international, multicentre trial.
- Treatment of type 1 tumours with a gastrin/CCK₂ antagonist should be continuous and lifelong, because they will eventually regrow even if eradicated. In that respect, netazepide is a well-tolerated treatment. No adverse event has yet been attributed to netazepide treatment.
- Regular measurement of plasma CgA is a simple way to monitor the success of treatment.
- Netazepide may not be capable of eradicating large type 1 tumours that have been present for many years, but should reduce the risk of metastases. The capacity of the atrophic gastric mucosa to remodel itself may be limited. However, it might reduce the risk of invasion or metastasis.
- In patients with PA, a gastrin/CCK₂ receptor antagonist might prevent formation of gastric NETs type 1 and reduce the seven-fold increased risk of developing a gastric adenocarcinoma. Chronic hypergastrinaemia might have at least a contributory role in its development.
- Overexpression of miR-222 by excessive gastrin targets the tumour suppressor and oncogene, p27, which may be the mechanism for the harmful effect of hypergastrinaemia, which netazepide prevents. Patients with mutation of genes *MEN-1*, *KCNQ1*, *KCNE1* and *ATP4A* are at much greater risk.
- Patients with gastric NETs type 2 probably require a higher daily dose of netazepide than patients with gastric NETs type 1, because serum gastrin levels are higher in patients with type 2 tumours and netazepide is a competitive gastrin/CCK₂ receptor antagonist.

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Table 1. Trondheim study. (a) number of type 1 tumours and (b) size of the largest tumour (mm) in each patient before, during and after netazepide 50 mg daily for 12 weeks, and then netazepide 25 mg daily for 52 weeks

a Patient	Week				Week				
	0	6	12	24	0	13	26	39	52
1	11	8	4	3	6	3	2	1	0
2	11	11	6	9					
3	24	26	19	19					
4	4	2	1	0	2	2	1	0	0
5	7	3	3	4					
6	6	4	0	3	1	0	0	0	0
7	14	13	6	8	13	8	7	5	4
8	18	16	16	16	14	11	9	–	5
Mean	11.9	10.4	6.9	7.7	7.2	4.8	3.8	2.0	1.8

b Patient	Week				Week				
	0	6	12	24	0	13	26	39	52
1	5	4	4	4	6	3	3	3.5	0
2	7	7	5	7					
3	5	2	4	8					
4	4	4	3	0	4	3	2	0	0
5	6	3	3	3					
6	4	5	0	4	12	0	0	–	0
7	9	8	7	7	12	8	2	3	8
8	6	4	2	6	4	3	4	–	4
Mean	5.8	4.6	3.5	4.9	7.6	3.4	2.2	2.2	2.4

Patients 2, 3 and 5 declined to take part in the extension study

Table 2. Liverpool study. (a) number of type 1 tumours and (b) size of the largest tumour (mm) in each patient before, during and after netazepide 50 mg daily for 12 weeks, and then for 52 weeks

a Patient	Week				Week				
	0	6	12	24	0	13	26	39	52
1	8	4	2	3	5		0		0
2	8	8	9	9	12		10		10
3	4	2	2	1	2		0		0
4	9	7	7	6	12		6		10
5	30	20	10	8	12		12		12
6	10	6	6	6	12		10		1
7	12	12	12	14	13		12		12
8	10	10	10	12	12		12		15
Mean	11	9	7	7	10		7.8		8.6

b Patient	Week				Week				
	0	6	12	24	0	13	26	39	52
1	6	4	4	2	2		0		0
2	15	10	10	10	12		6		6
3	3	2	2	2	4		0		0
4	5	4	3	2	4		3		2
5	7	5	3	3	4		4		2
6	8	7	5	5	7		3		2
7	10	10	10	10	11		10		6
8	10	10	7	7	7		3		6
Mean	8	7	6	5	6.4		3.6		3.0

No gastroscopy at 13 and 39 weeks

Table 3. Trondheim study. Individual (a) CgA (nmol/L) and (b) gastrin (pmol/L) before, during and after treatment of gastric NETs type 1 with netazepide 50 mg daily for 12 weeks, and then netazepide 25 mg daily for 52 weeks

a Patient	Week						Week				
	0	3	6	9	12	24	0	13	26	39	52
1	11.1	3.8	4.9	4.6	5.1	9.0	8.7	6.0	2.3	2.6	2.4
2	10.1	6.3	4.4	4.9	4.7	8.8					
3	6.2	3.0	3.9	4.1	3.5	4.0					
4	7.8	3.7	2.6	4.1	4.0	4.4	6.3	4.9	1.7	2.2	1.9
5	5.7	3.9	3.8	3.7	3.7	7.3					
6	5.2	4.5	5.0	4.1	4.2	5.6	6.3	2.4	2.8	2.8	3.1
7	12.1	5.2	4.9	4.4	4.7	9.5	8.9	4.9	2.9	3.2	3.1
8	4.5	3.9	5.0	3.7	3.4	4.2	3.7	1.6	1.6	1.6	2.0
Mean	7.8	4.3	4.3	4.2	4.2	6.6	6.8	4.0	2.3	2.5	2.5

b Patient	Week						Week				
	0	3	6	9	12	24	0	13	26	39	52
1	387	380	343	375	400	820	357	542	385	443	620
2	200	388	196	347	140	190					
3	349	387	330	201	338	420					
4	674	953	425	720	400	266	392	360	543	872	489
5	220	458	120	180	160	274					
6	840	703	531	740	600	411	598	494	973	888	1230
7	215	580	190	280	295	264	492	401	425	682	328
8	150	220	274	232	78	218	346	150	482	164	275
Mean	379	509	301	384	301	358	437	389	562	610	598

Patients 2, 3 and 5 declined to take part in the extension study

Table 4. Liverpool study. Individual (a) CgA (U/L) and (b) gastrin (pmol/L) before, during and after treatment of gastric NETs type 1 with netazepide 50 mg daily for 12 weeks, and then for 52 weeks

a Patient	Week						Week				
	0	3	6	9	12	24	0	13	26	39	52
1	25.2	9.31	9.14	8.45	9.08	31.0	27.3	8.6	8.2	6.2	10.5
2	52.6	12.6	15.3	13.6	13.7	61.6	60.3	12.4	13.7	16.3	15.9
3	54.0	14.8	13.7	12.2	14.3	41.5	39.0	15.7	15.9	16.4	16.6
4	32.7	15.8	14.1	14.7	12.9	24.3	22.9	12.3	12.0	16.5	16.7
5	92.9	40.9	68.8	45.4	48.6	69.7	46.5	32.0	31.3	31.4	37.3
6	55.9	14.4	18.6	15.2	17.6	50.8	43.3	15.3	15.8	17.6	25.6
7	128.0	29.1	26.5	24.0	26.6	98.0	91.4	24.2	22.7	28.1	28.9
8	64.0	14.7	13.1	15.1	14.0	35.9	28.0	9.4	10.5	17.9	15.0
Mean	63.2	19.0	22.4	18.6	19.6	51.6	45.3	16.2	16.3	18.8	20.9

b Patient	Week						Week				
	0	3	6	9	12	24	0	13	26	39	52
1	531	412	511	339	692	628	570	484	413	316	257
2	494	592	409	706	490	464	486	666	506	488	486
3	414	365	211	555	293	451	274	449	336	318	346
4	645	890	800	904	953	437	333	638	510	526	400
5	655	842	594	406	425	388	193	168	179	620	391
6	332	686	896	770	820	367	520	524	403	413	363
7	953	776	510	335	825	670	816	378	498	323	470
8	415	378	368	582	407	742	456	284	556	307	341
Mean	555	618	537	575	613	518	463	461	425	414	382

Table 5. Pooled data from 12-week studies: A. Number of tumours; B. Size of largest tumour (mm); C. CgA (% change from baseline); and D. gastrin (pmol/L)

Parameter	A				B			
	Weeks				Weeks			
	0	6	12	24	0	6	12	24
N	16	16	16	16	16	16	16	16
Mean	11.63	9.50	7.06	7.56	6.88	5.56	4.50	5.0
SD	7.05	6.75	5.38	5.46	3.01	2.76	2.78	2.99
SEM	1.76	1.69	1.35	1.37	0.75	0.69	0.70	0.75
Min	4	2	0	0	3	2	0	0
Median	10	8	6	7	6	4.5	4	4.5
Max	30	26	19	19	15	10	10	10

Parameter	C						D					
	Weeks						Weeks					
	0	3	6	9	12	24	0	3	6	9	12	24
N	16	16	16	16	16	16	16	16	16	16	16	16
Mean	209	79	87	79	79	172	467	563	419	480	457	438
SD	135	36	63	36	42	102	235	221	217	231	258	189
SEM	34	9	16	9	10	26	59	55	54	58	65	47
Min	75	42	42	38	41	67	150	220	120	180	78	190
Median	177	66	72	67	66	149	415	519	389	391	404	416
Max	582	186	313	206	221	445	953	953	896	904	953	820

Table 6. Pooled data from 52-week studies: A. Number of tumours; B. Size of largest tumour (mm); C. CgA (% change from baseline); and D. gastrin (pmol/L)

Parameter	A			B		
	Weeks			Weeks		
	0	26	52	0	26	52
N	13	13	13	13	13	13
Mean	8.92	6.23	5.31	6.85	3.08	2.77
SD	4.91	4.99	5.69	3.67	2.72	2.89
SEM	1.36	1.38	1.58	1.02	0.75	0.80
Min	1	0	0	2	0	0
Median	12	7	4	6	3	2
Max	14	12	15	12	10	8

Parameter	C					D				
	Weeks					Weeks				
	0	13	26	39	52	0	13	26	39	52
N	13	13	13	13	13	13	13	13	13	13
Mean	169	71	60	67	74	449	426	478	489	461
SD	93	33	32	34	41	161	159	180	223	251
SEM	26	9	9	9	11	45	44	50	62	70
Min	62	27	27	27	32	193	150	179	164	257
Median	145	70	48	64	68	456	449	482	443	391
Max	415	145	142	143	170	816	666	973	888	1230

Table 7. Histology of tumour biopsies (n = 8 patients).

NET = neuroendocrine tumour. ECL-L = linear ECL cell hyperplasia.

ECL-M = micronodular ECL-cell hyperplasia.

ECL-D = ECL-cell dysplasia.

Patient	12-week study				52-week study		
	Week 0	Week 6	Week 12	Week 24	Week 0	Week 24	Week 52
1	NET	ECL-M	ECL-M	ECL-M	NET	ECL-M	ECL-M
2	NET	NET	NET	ECL-M	ECL-D	ECL-M	ECL-M
3	ECL-M	ECL-M	ECL-M	ECL-M	ECL-M	ECL-M	ECL-L
4	NET	ECL-M	ECL-M	NET	ECL-M	ECL-M	ECL-M
5	NET	NET	NET	NET	NET	ECL-M	NET
6	NET	NET	ECL-M	NET	NET	NET	NET
7	NET	NET	NET	NET	NET	NET	NET
8	NET	NET	NET	ECL-M	nd	ECL-M	ECL-M

Nd = not done

Figure 1. Trondheim study. Representative photographs of tumours taken at gastroscopy before (a, c, e) and after netazepide for 12 weeks (b, d, f)
 Arrows = same polyp before and after treatment. Circles = same area. (Fossmark *et al* 2012)

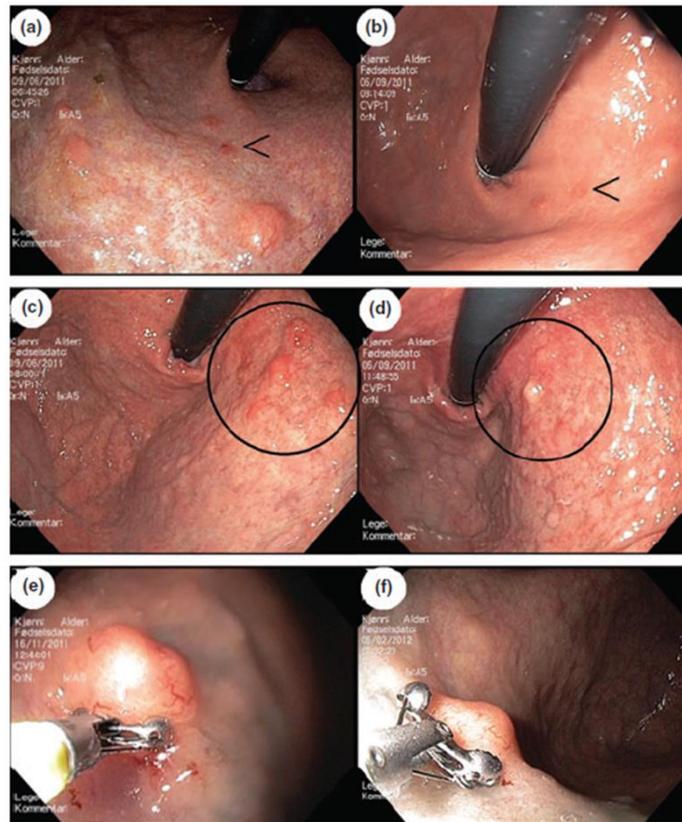


Figure 2. Liverpool study. Endoscopic photographs from the same area of stomach in patient 1 (a, b) and patient 2 (c, d) before (a, c) and after netazepide for 12 weeks (b, d). (Moore *et al* 2013)

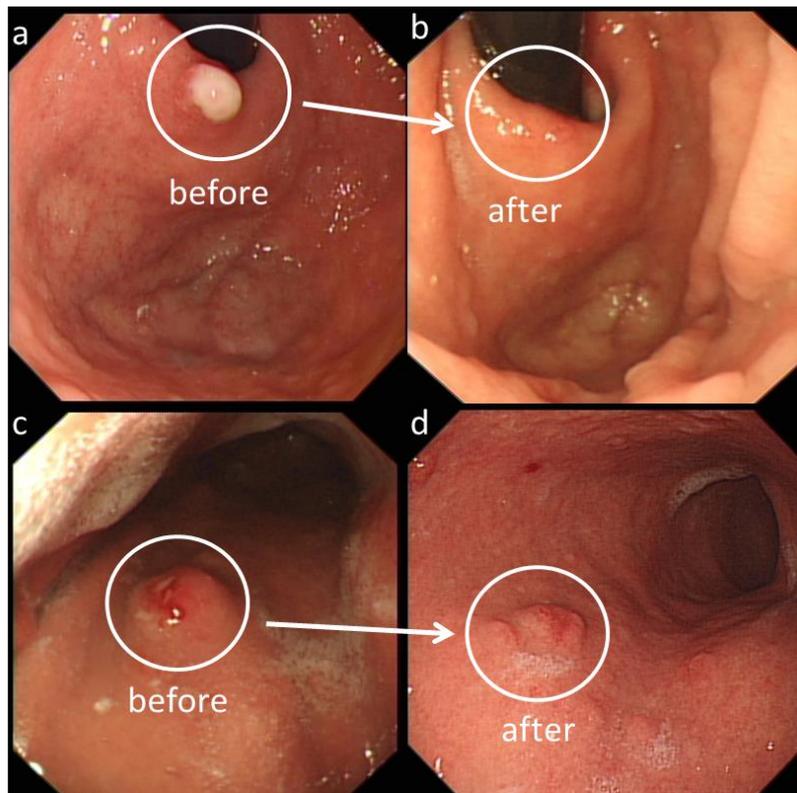


Figure 3. Trondheim study. Individual values (n = 8) for (a) number of tumours; (b) size of largest tumour; (c) serum CgA; and (d) serum gastrin before and during netazepide 50 mg once daily for 12 weeks, and at 12 weeks after stopping netazepide
 (Normal ranges: CgA ≤ 6 nmol/L; Gastrin = ≤ 40 pmol/L)

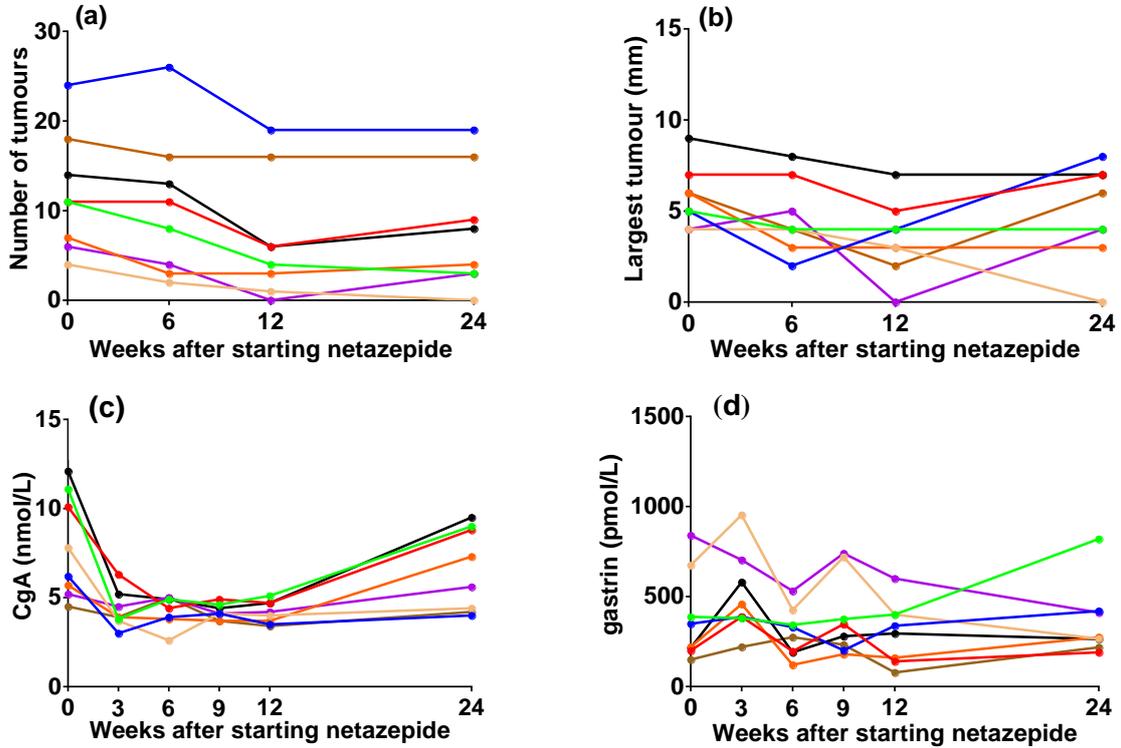


Figure 4. Liverpool study. Individual values (n = 8) for (a) number of tumours; (b) size of largest tumour; (c) plasma CgA; and (d) serum gastrin before and during netazepide 50 mg once daily for 12 weeks, and at 12 weeks after stopping netazepide
 (Normal ranges: CgA = ≤ 22 U/L; Gastrin = ≤ 40 pmol/L)

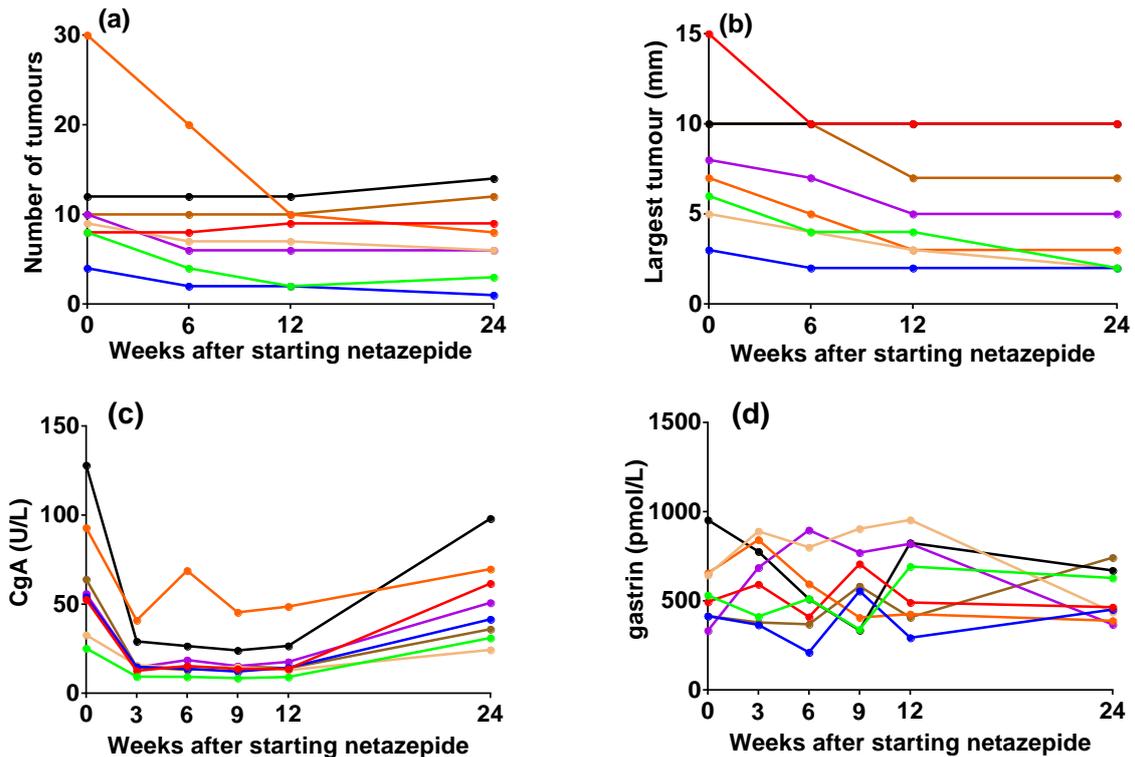


Figure 5. Trondheim study. Individual values (n = 5) for (a) number of tumours; (b) size of largest tumour; (c) plasma CgA; and (d) serum gastrin before and during netazepide 25 mg once daily for 52 weeks

(Normal ranges: CgA \leq 6 nmol/L; Gastrin = \leq 40 pmol/L)

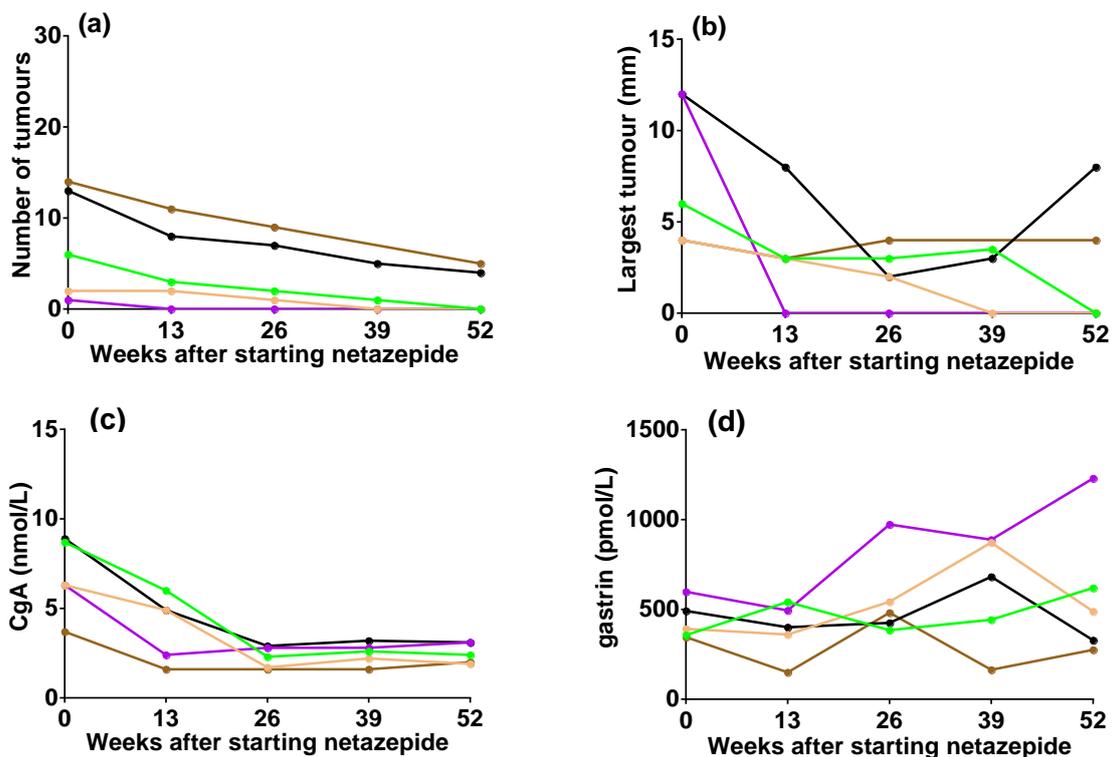


Figure 6. Liverpool study. Individual values (n = 8) for (a) number of tumours; (b) size of largest tumour; (c) plasma CgA; and (d) serum gastrin before and during netazepide 50 mg once daily for 52 weeks

(Normal ranges. CgA = \leq 22 U/L. Gastrin = \leq 40 pmol/L)

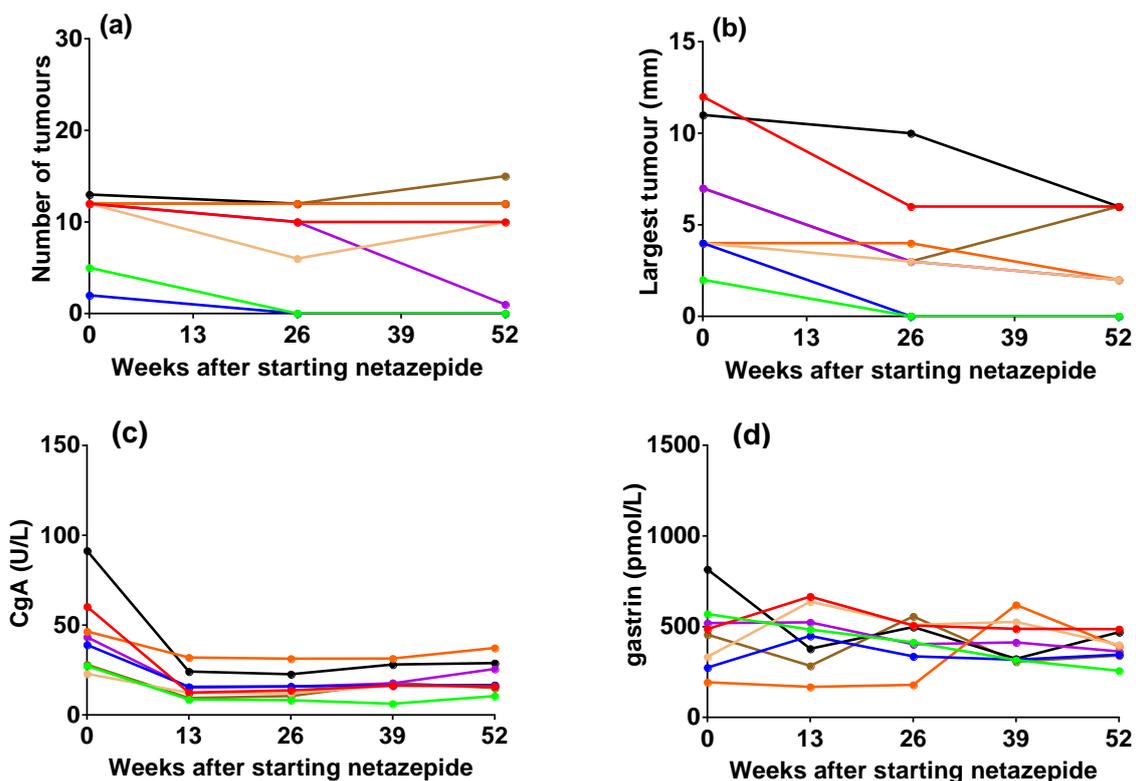


Figure 7. Pooled data from Trondheim and Liverpool studies. Effect (n = 16 patients) of netazepide 50 mg once daily for 12 weeks, and 12 weeks off treatment, on: (a) number of tumours; (b) size of largest tumour; (c) CgA (adjusted); and (d) gastrin. *p<0.05; **p<0.01; *p<0.001 (Normal ranges. CgA: Trondheim ≤6 nmol/L; Liverpool ≤22 U/L. Gastrin: <40 pmol/L)**

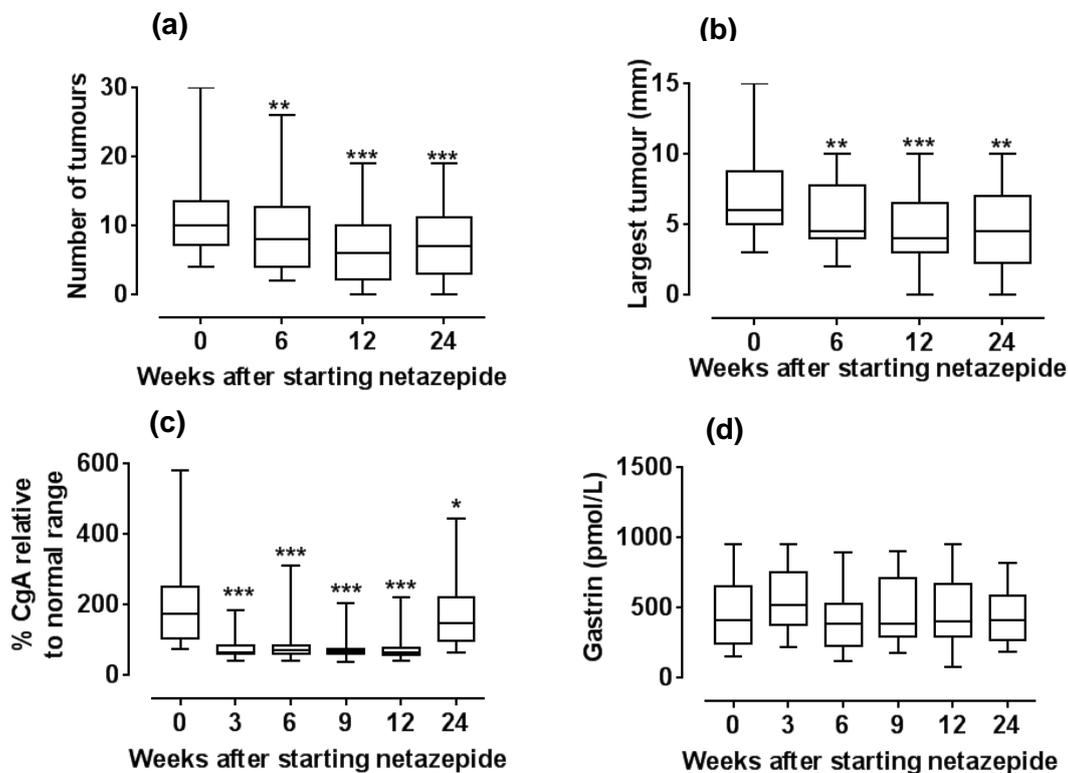


Figure 8. Pooled data from Trondheim and Liverpool studies. Effect (n = 13 patients) of netazepide 25 mg (n = 5) or 50 mg (n = 8) once daily for 52 weeks on: (a) number of tumours; (b) size of largest tumour; (c) CgA (adjusted); and (d) gastrin. **p<0.01; *p<0.001**

(Normal ranges. CgA: Trondheim ≤6 nmol/L; Liverpool ≤22 U/L; Gastrin: ≤40 pM)

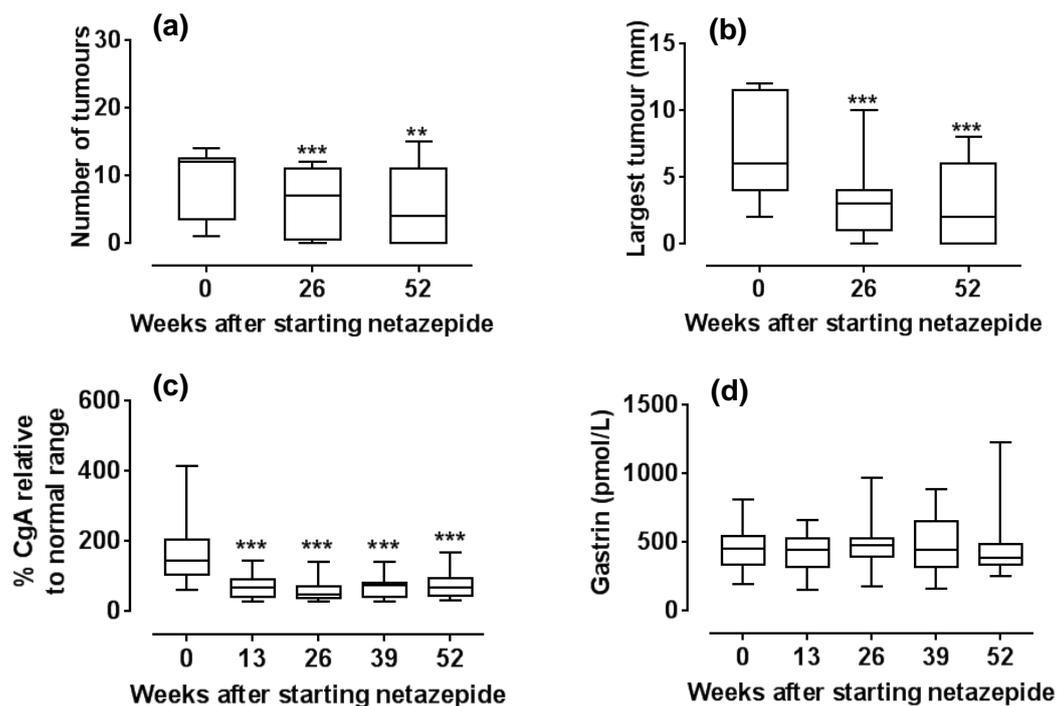


Figure 9. Pooled data from Trondheim and Liverpool studies. Changes (n = 13 patients) during the period off netazepide treatment in: (a). number of tumours; (b). size of the largest tumour; (c). CgA (adjusted); and (d). gastrin.

(Normal ranges. CgA: Trondheim ≤6 nmol/L; Liverpool ≤22 U/L; Gastrin: ≤40 pM)

*p<0.05; **p<0.01; ***p<0.001

B1 = end of 12-weeks' netazepide. B2 = start of 52 weeks' netazepide

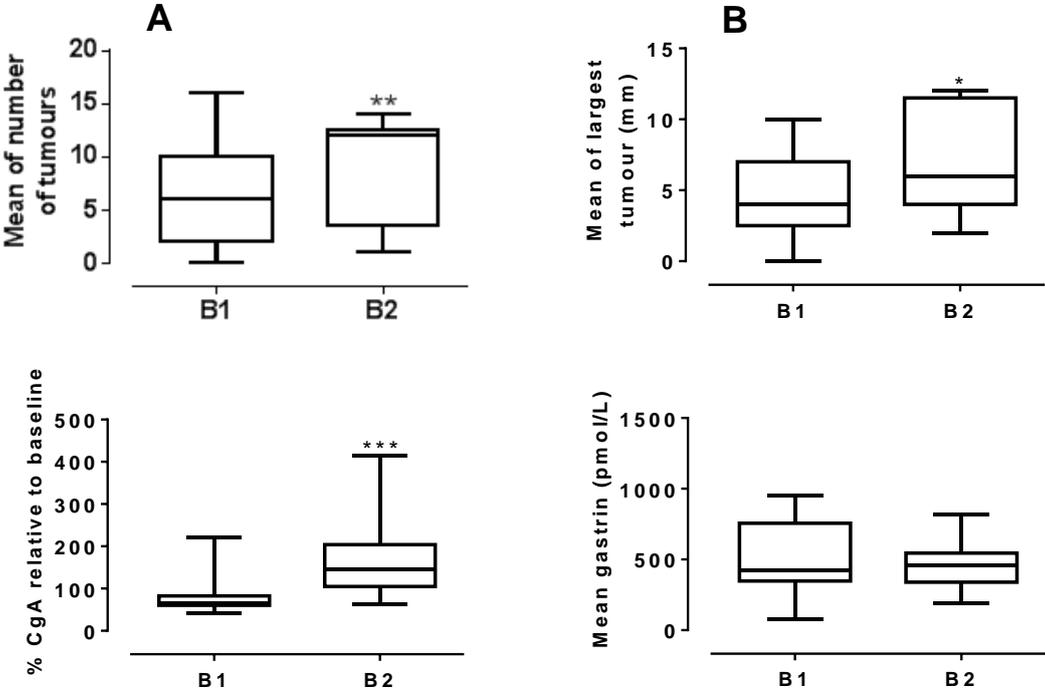


Figure 10. Liverpool study. Absolute quantities of abundances of biomarkers in gastric mucosal biopsies (n = 8) relative to GAPDH during 52 weeks of netazepide treatment (After Moore *et al* 2015)

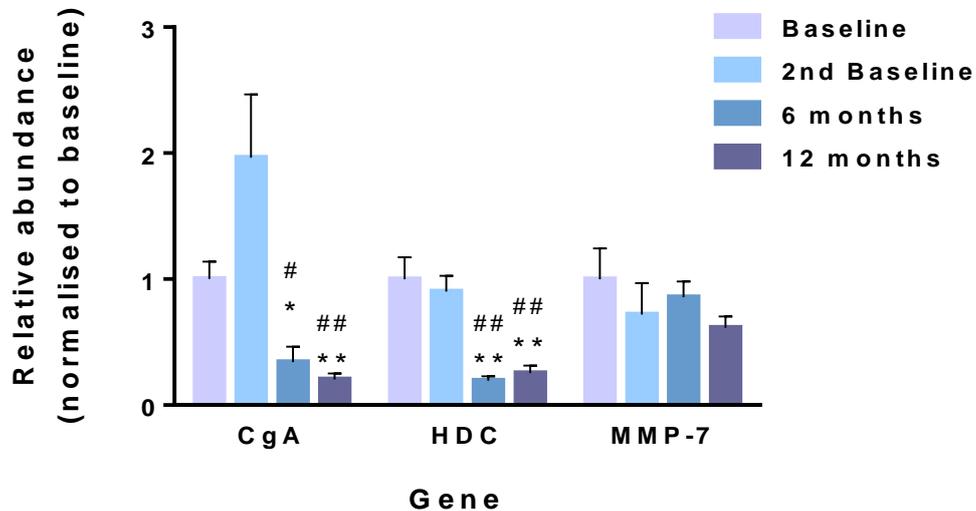
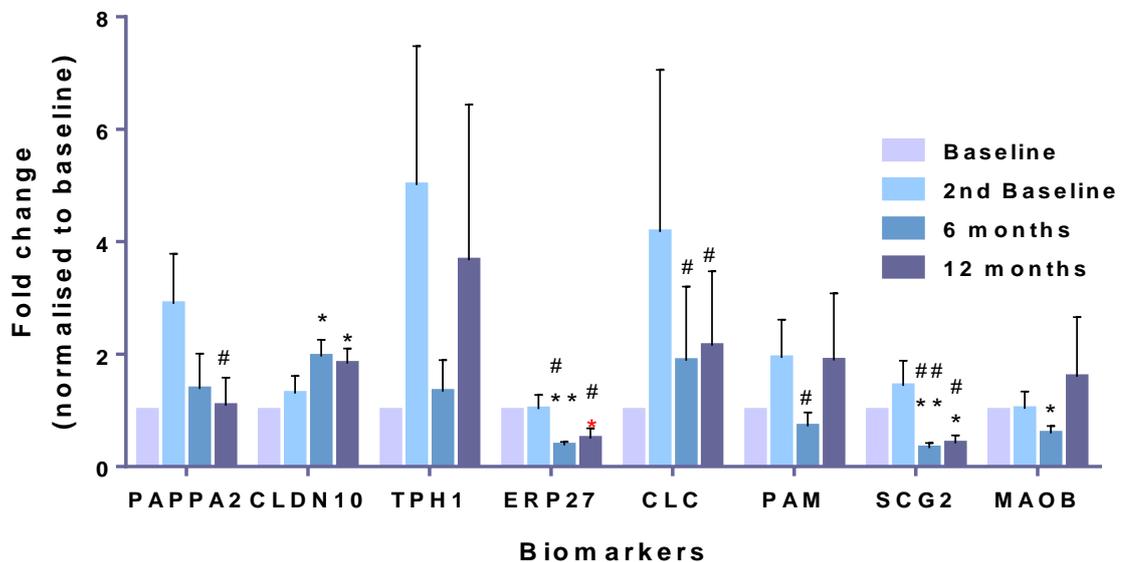


Figure 11. Liverpool study. Abundances of biomarkers in gastric mucosal biopsies (n = 8) relative to GAPDH during 52 weeks of netazepide treatment (After Moore *et al* 2015)



Roche Universal Probe Library. Wilcoxon signed rank tests. * p<0.05 compared to baseline.
 * p=0.055 compared to baseline. # p<0.05 and ## p<0.01 compared to 2nd baseline.
 pappalysin 2 (PAPPA2), claudin 10 (CLDN10), endoplasmic reticulum protein (ERP27),
 eosinophil lysophospholipase (CLC), peptidyl-glycine alpha-amidating monooxygenase (PAM),
 secretogranin II (SCG2) and monoamine oxidase B (MAOB)

Chapter 15

Safety and tolerability of netazepide

Safety and tolerability of netazepide

Subjects

To date, in 13 studies, 220 healthy men and women have received single doses of netazepide up to 400 mg and repeated doses up to 100 mg once or twice daily for up to 12 weeks. Netazepide has been co-administered with rabeprazole, esomeprazole, midazolam, clarithromycin and amoxicillin.

To date, 27 patients, in completed and ongoing clinical trials, and as 'named patients', have received netazepide 25-100 mg once or twice daily for up to 30 months. Many of them have taken concomitant medication allowed by the protocol.

Methods

During residence, healthy subjects are asked about symptoms that they may have experienced in association with their treatment. While at home, healthy subjects and patients record any adverse events in a diary card.

Results

Healthy subjects

No subject has had clinically significant changes in: haematology; clinical chemistry; urinalyses; vital signs; physical examination; or ECG, including QTc interval. Netazepide has been well tolerated. There have been no deaths and no serious adverse events. Adverse events (AEs) have been minor, transient, unrelated to dose, resolved spontaneously, and also occurred after placebo or active comparators. No subject has been withdrawn because of an AE. Headache and gastrointestinal symptoms, such as nausea, abdominal discomfort, and diarrhoea have been the most common AEs (Table 1).

Patients

No patient has had clinically significant changes in: haematology; clinical chemistry; urinalyses; vital signs; physical examination; and ECG, including QTc interval. Netazepide has been well tolerated. There have been no deaths and no serious adverse events. Very few AEs have been reported. None has been deemed related to treatment. Headache and diarrhoea have been the most frequently reported AEs across treatment groups (Table 2). No subject has been withdrawn because of an AE.

Conclusions

To date, netazepide has proved safe and well tolerated, and no treatment-related adverse event has been identified. However, the number of subjects exposed to netazepide is small.

Table 1. Adverse events reported by healthy subjects across treatments¹

Preferred term	Pre-treatment (n= 279)	Placebo (n=61)	Netazepide² (n=166)	Netazepide + treatment³ (n=64)	Comparator⁴ (n=70)
Respiratory, thoracic and mediastinal disorders	5	4	25	23	18
Nasal congestion	0	0	2	5	2
Pharyngolaryngeal pain	2	1	12	6	6
Oropharyngeal pain	0	0	2	3	3
Rhinorrhoea	1	2	4	3	4
Sneezing	1	0	1	2	0
Cough	1	1	4	4	3
Nervous system disorders	8	13	43	56	54
Headache	6	12	41	46	45
Dizziness	2	1	2	6	5
Dysgeusia	0	0	0	4	4
Gastrointestinal disorders	5	8	67	92	80
Stomach discomfort	0	1	6	0	0
Flatulence	0	0	2	3	2
Dyspepsia	0	1	6	11	2
Toothache	0	0	1	3	0
Eructation	0	0	1	4	4
Abdominal pain	3	0	6	7	7
Abdominal pain upper	1	4	9	11	18
Abdominal distension	0	0	5	17	13
Abdominal discomfort	0	0	0	16	11
Nausea	1	1	11	12	15
Vomiting	0	1	8	2	1
Diarrhoea	0	0	12	6	7
Immune system disorders	1	1	12	4	5
Rhinitis	1	0	1	3	5
Nasopharyngitis	0	1	6	1	0
Influenza	0	0	5	0	0
Reproductive system and breast disorders	1	0	6	7	4
Dysmenorrhoea	1	0	6	7	4
Musculoskeletal and connective tissue disorders	0	0	4	5	2
Back pain	0	0	4	2	1
Myalgia	0	0	0	3	1

Table 1. Continued

Preferred term	Pre-treatment (n= 279)	Placebo (n=61)	Netazepide ² (n=166)	Netazepide + treatment ³ (n=64)	Comparator ⁴ (n=70)
Skin and subcutaneous tissue disorders	0	0	2	2	1
Rash	0	0	2	2	1
Ear and labyrinth disorders	0	0	1	2	0
Ear pain	0	0	1	2	0
Vascular disorders	0	0	3	1	0
Epistaxis	0	0	3	1	0
Metabolism and nutrition disorders	0	0	1	2	3
Decreased appetite	0	0	1	2	3
General disorders and administration site conditions	1	0	8	1	3
Fatigue	1	0	8	1	3

- 1 AEs are listed only if they were reported ≥ 3 times in completed trials by subjects treated with netazepide, alone and/or in combination with another treatment. AEs are listed whether they were deemed possibly related to the treatment or not.
- 2 Single doses of 0.5–500 mg netazepide and repeated doses of 5–100 mg netazepide
- 3 Netazepide was taken with single or repeated doses of rabeprazole 20 mg, midazolam 5 mg, ¹⁴C-netazepide or esomeprazole 40 mg.
- 4 Netazepide treatment was compared with single or repeat doses of omeprazole 20 mg, ranitidine 150 mg, rabeprazole 20 mg, or midazolam 5 mg.

Table 2. Adverse events reported by patients

Preferred term	Placebo + Comparator ¹ (n=1)	netazepide ² (n=8)	netazepide + treatment ³ (n=1)
Nervous system disorders	0	1 [1]	2 [1]
Headache	0	1 [1]	1 [1]
Dysgeusia	0	0	1 [1]
Gastrointestinal disorders	3 [1]	0	2 [1]
Diarrhoea	1 [1]	0	1 [1]
Abdominal pain	1 [1]	0	0
Nausea	1 [1]	0	0
Lip dry	0	0	1 [1]

- 1 Treatment with netazepide placebo, amoxicillin 1000 mg and clarithromycin 500 mg (T-004).
- 2 Patients were treated with netazepide 50 mg once daily for 12 weeks (T-015). 3 patients were treated with 50 mg once daily for 6 weeks and then 100 mg for 6 weeks.
- 3 Treatment with netazepide 100 mg, amoxicillin 1000 mg and clarithromycin 500 mg (T-004).

Chapter 16

Study 13: Separation and characterisation of metabolites of netazepide from blood and urine of healthy subjects

Introduction

Three pieces of evidence suggested that netazepide gives rise to an active metabolite in humans. First, in Study 2, a single dose of netazepide increased gastric pH in healthy subjects beyond 24 h when netazepide could not be measured in blood samples (Figure 1). Second, in the absolute bioavailability study of ¹⁴C-netazepide in healthy subjects (Study 10), radioactivity persisted after parent compound (half-life about 6 h) had more or less disappeared from the circulation (Figure 2). Third, in a study of ¹⁴C-labelled netazepide in human, rat and dog liver microsomes *in vitro*, one metabolite occurred in larger amounts in human than in the other species (HLS report 2006).

The netazepide patent, which Tio holds, will expire worldwide in 2016. I wondered whether a metabolite of netazepide might be responsible for the long duration of action of the parent compound, and might be patentable.

Objectives

The objectives of this study in healthy subjects (Study 13) were: (1) to identify and characterise the metabolites of netazepide; and (2) to determine their concentrations relative to netazepide.

Methods

Regulations

We complied with the ICH Guideline for GCP and EU Clinical Trials Directive. The MHRA and NW Manchester REC approved the study. Subjects gave written, informed consent.

Study design

This was an open, single- and repeated-dose study in men deemed healthy as in previous studies. There were three parts. In Parts A1 and A2, subjects took a single, high dose of netazepide 500 mg, and in Part B, subjects took a therapeutic dose of netazepide 50 mg once daily for three consecutive days. Subjects ate a high-fat breakfast, as in Study 7, before dosing in Parts A1 and A2, and before Days 1 and 3 in Part B, in order to increase exposure to netazepide. Up to four subjects were allowed for each part. A subject could do more than one part providing there was an adequate washout period between consecutive parts. In Parts A1 and A2, and on Days 1 and 3 of Part B, subjects were dosed on the ward. On Day 2 of Part B, subjects took netazepide at home in the morning.

Materials

HMR pharmacy filled gelatin capsules with spray dried netazepide 25 mg and hydroxypropyl-methylcellulose (HPMC) (netazepide:HPMC ratio 1:3.5) blended with pregelatinised starch, as described in Study 9.

Metabolism

Blood and urine samples

We collected blood as follows: 400 mL at about 7 h after dosing in Part A1; 4 mL before and frequently up to 30 h after dosing in Part A2; and 4 mL at 2 and 7 h after dosing in Part B. Subjects collected their urine for 24 h after dosing in Part A1 only. Blood and urine samples were processed and stored until analysis, as described previously.

Metabolite identification and characterisation (Parts A1 and B)

Huntingdon Life Sciences (HLS) used LC-MS and LC-MS/MS to determine the presence of netazepide and any metabolites in plasma samples from Parts A1 and B and in urine from Part A1. They isolated the main metabolites by solvent extraction, followed by semi-preparative HPLC-MS. Dr Ute Gerhard, University of Hertfordshire, used nuclear magnetic resonance spectroscopy (NMR) to identify the structure of the isolated main metabolites.

Assay of netazepide and main metabolite (Part A2)

ASI assayed plasma concentrations of netazepide and its main metabolite (now known as TR2) using validated LC-MS/MS methods. LLQ for plasma assays was 0.5 ng/mL for netazepide and 1.0 ng/mL for TR2. Values below LLQ were reported as BLQ.

Pharmacokinetics

We derived the pharmacokinetic parameters and calculated the proportion of the main metabolite to netazepide in plasma using data from Part A2, which we started after identifying the main metabolite of netazepide in Parts A1 and B of the study.

Results

Subjects

Four subjects entered and completed the study during June 2012– May 2014. Three subjects participated in Parts A1 and Part A2, and one in Part B. Median age and BMI were 37 (range 24–73) years and 27.6 (range 26.0–29.6), respectively. Netazepide was safe and well tolerated even at high dose. There were no reports of adverse events.

Pharmacokinetics

Mean plasma concentrations of netazepide and its main metabolite, TR2, are listed in Tables 1 and 2 and illustrated in Figure 3. Plasma concentrations of netazepide were within those of the NOAEL in rats and dogs (Chapter 2). Mean pharmacokinetic parameters of TR2 and netazepide are listed in Table 3.

Netazepide was metabolised quickly to TR2, which accounted for 10–29% of netazepide-related compounds in the circulation after netazepide 50 mg daily for 3 days (steady state) and >40%

after netazepide 500 mg. Exposure to TR2 (C_{\max} 488 ng/mL; AUC_t 2,498 ng.h/mL) was substantial and quite similar to that of netazepide (C_{\max} 714 ng/mL; AUC_t 2,773 ng.h./mL). Median T_{\max} and mean $t_{1/2}$ of netazepide were similar to those of TR2.

Metabolism

Four prominent metabolites were detected; two were major metabolites, 'A' and 'C'. Their structures are shown in Figure 4. The extent of netazepide metabolism was not affected by the dose: at 7 h after doses of 50 mg and 500 mg, 51.5 and 48.5%, respectively, of the total plasma exposure was unmetabolised netazepide. However, the relative plasma concentration of the most abundant metabolite, metabolite A, was affected by dose: at 7 h after doses of 50 mg and 500 mg, 29.4 and 40.9%, respectively, of the total plasma exposure was metabolite A. So, at 7 h after dosing, the metabolite A/netazepide ratio was 0.57 for 50 mg and 0.84 for 500 mg. But, the relative plasma concentration of the other major metabolite, metabolite C, was similar after doses of 50 mg and 500 mg: at 7 h after 50 mg and 500 mg, 8.9 and 7.0%, respectively, of the total plasma exposure was metabolite C.

Metabolite A is a hydroxylated form of netazepide: the hydroxyl group is bound to the *t*-butyl group. We named metabolite A as TR2. Metabolite C results from loss of a methyl group from the *t*-butyl group of netazepide and formation of an *i*-propyl derivative. Mass spectrometry indicates that metabolite C is formed from oxidation of metabolite A to metabolite B (a putative carboxylic acid) followed by de-carboxylation. So, the conversion of metabolite A to B (putative oxidation) is slowed by a high dose of netazepide.

Non-clinical studies of TR2

To characterise the pharmacology of TR2, the main metabolite of netazepide, we used contract research organisations (CROs) and academic centres to do a series of non-clinical studies of TR2 and the *S*-enantiomer of TR2, which we named TR3. ProSynth Ltd, Suffolk, England, synthesised TR1, the racemic mixture of TR2 and TR3 (Wood 2013) from which Peakdale Molecular, Chapel-en-le-Frith, High Peak, England, separated the isomers.

(a). Receptor binding properties of TR2 and TR3

Cerep, 86600 Celle-Lévescault, France, assessed the receptor binding properties of TR2 using a library of 80 G-protein coupled receptors and radiolabelled ligands for those receptors (Report T-NC03). TR2 bound most strongly to CCK_1 and CCK_2 receptors – gastrin binds to CCK_2 receptors – and had little or no activity at other receptors. When netazepide, TR2 and TR3 were compared with respect to CCK_2 receptor binding, the pIC_{50} ($-\log_{10}$ of half maximal inhibitory concentration) was about 9.3 M for netazepide and 8.6 M for TR2. So, netazepide and TR2 have quite similar binding affinities. In contrast, the pIC_{50} of TR3 was only 7.0 M. The affinity of

TR2 for the CCK₂ receptor (K_B 0.31 nM) was 480-fold higher than the affinity for the CCK₁ receptor (K_B 150 nM), whereas the difference for netazepide was 370-fold. Thus, TR2 is more selective for the CCK₂ receptor than netazepide.

(b). Intestinal permeability of TR2

Cyprotex, Macclesfield, Cheshire, England used Caco-2 cells as a model of the human intestinal epithelium *in vitro* to assess the intestinal permeability of potential medicines (Report T-NC02). TR2 was added to the apical side of a confluent monolayer of Caco-2 cells and permeability was measured by monitoring the appearance of TR2 on the basolateral side of the cell membrane (receiver compartment) using LC-MS/MS. Permeability was approximately 6.0×10^{-6} cm/s, which is poor. But, the concentration of TR2 in the receiver compartment fell well below the estimated concentration, so the results are of doubtful validity.

(c). TR2 and the trophic effects of gastrin on cells *in vitro*

The cell line AGS, stably transfected with gastrin/CCK₂ receptors to make AGS_{GR} cells, was provided by Professor Andrea Varro, Liverpool University, and was used to assess the activity of TR2 on the trophic effects of gastrin (Reports T-NC04 and T-NC07). Gastrin inhibited proliferation of AGS_{GR} cells. Both TR2 and netazepide 100 nM completely reversed the anti-proliferative effects of gastrin (Figure 5). As well as cell proliferation, gastrin also caused migration and branching of AGS_{GR} cells. The effects of TR1 (a racemic mixture of TR2, the *R*-enantiomer, and TR3 (the *S*-enantiomer), TR2 and TR3 are shown in Table 4. TR2 was more potent than TR1 and TR3 at inhibiting the anti-proliferative and migratory responses to gastrin G17, whereas TR2 was less potent at inhibiting the branching morphology response to gastrin G17.

(d). Effect of TR2 on gastric acid secretion in rats *in vivo*

Rats were first fitted with a gastric fistula in the laboratory of Professor Duan Chen, University of Science and Technology, Trondheim, Norway (Report T-NC05). Conscious rats were given subcutaneous pentagastrin to stimulate gastric acid production. Gastric juice was collected and H⁺ concentration was measured. TR2, TR3 and netazepide 0.002, 0.02, 0.2 and 2.0 μmol/kg, given by oral gavage, each dose-dependently inhibited pentagastrin-induced acid secretion. Order of potency was netazepide >TR2 >TR3 (Figure 6).

(e). Pharmacokinetics of TR2

Huntingdon Life Sciences (HLS) gave rats single doses of TR2 either intravenously (0.1 mg/kg) or by oral gavage (0.1, 0.3 and 1.0 mg/kg) (Report T-NC09). C_{max} and AUC_t increased with dose over the range. The increase appeared linear for AUC_t, but was more than dose proportional for C_{max}. Absolute bioavailability was 0.63–1.30%, which is surprisingly low given

that in a similar study in rats, oral bioavailability of netazepide was 26–28% (Chapter 2). However, animals are poor predictors of bioavailability in humans (Musther *et al* 2014).

(f) Metabolism of netazepide and of TR2

HLS compared the metabolism of ¹⁴C-netazepide by human, rat and dog liver microsomes *in vitro* (Report HMU0005). Metabolism of parent netazepide was most efficiently catalysed by human > dog > rat. Phase I and II metabolites were formed. There was no evidence for formation of unique human metabolites, although the major metabolite was different from that formed by rat and dog.

HLS did another study to compare the metabolism of netazepide by human hepatocytes and other species *in vitro* (Report T-NC01). The first part of the study repeated study HMU0005, and structurally identified the significant metabolites. Human hepatocytes formed four metabolites, of which TR2 was the major one. The second part of the study assessed metabolites of netazepide from human, rat and dog plasma and urine. One dog and two rats were given 100 mg/kg daily by mouth for seven days. The results are listed in Table 5 together with those from a healthy human subject given a single dose of netazepide 500 mg and another given daily doses of 50 mg for 3 days in study for comparison. TR2 accounted for 10.2% of netazepide-related exposure in rats after netazepide 100 mg/kg/day at steady state. Assuming a comparable mass spectroscopic response for all analytes, the results suggest that, in healthy subjects, TR2 accounted for 10–29% of the netazepide-related compounds in the systemic circulation after netazepide 50 mg daily for 3 days (steady state) and >40% of netazepide-related compounds after a single dose of 500 mg. Therefore, humans produce more TR2 after high doses of netazepide than after low doses. The percentage of TR2 (of total netazepide-related compounds) at steady state (29.4% on Day 3) was similar to that after a single dose (27.9% on Day 1).

WIL Research incubated netazepide with rabbit, minipig, monkey and human hepatocytes *in vitro* and incubated TR2 with mouse, Sprague Dawley rat, Fischer rat, Guinea pig, rabbit, dog, minipig, monkey and human hepatocytes *in vitro* (Report T-NC06), to assess metabolic profiles and stability. TR2 was metabolised much more slowly than netazepide; $t_{1/2}$ was 283 and 71 minutes, respectively. Like netazepide, TR2 was metabolised very differently by dogs and humans. But, of the 6 possible metabolites produced by human hepatocytes, monkey and minipig produced 4, the rat strains both produced 4, and the dog produced 3 (Table 6).

(g). Solubility of TR2

Cyprotex measured the solubility study of TR2 (Report CYP0751-R1), which we compared with that of netazepide. TR2 was about 10 times more soluble in aqueous solution than netazepide at pH 4–6 (Table 7), which is the pH range of the part of the small intestine where

most drug absorption takes place (Levitt 2013). So, TR2 may be more bioavailable than netazepide.

Conclusions

- In non-clinical studies, TR2 is more selective than netazepide for the gastrin/CCK₂ receptor over the CCK₁ receptor, but is slightly less potent than netazepide for the gastrin/CCK₂ receptor.
- TR2 is probably responsible for a substantial proportion of the activity, and perhaps the long duration of activity, of netazepide in humans.
- The greater solubility of TR2 compared with netazepide may avoid the need for spray drying TR2, which is wasteful of material and expensive.
- TR2 is not covered by existing patents, whereas netazepide will be off patent in 2016.
- Thus, TR2 appears to be a possible successor to netazepide and merits further studies.

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Table 1. Mean (n=3) plasma netazepide after one dose of netazepide 500 mg

Time (h)	Plasma netazepide (ng/mL)			
	Mean	SD	Range	95% CI
0.25	41.7	71.4	0.0–124.2	–135.7, 219.2
0.5	216.6	349.9	4.8–620.4	–652.6, 1085.7
0.75	301.8	462.3	22.1–835.4	–846.7, 1450.2
1	271.2	387.4	29.7–718.1	–691.2, 1233.6
1.5	292.2	306.3	45.8–635.1	–468.6, 1053.0
2	567.9	126.0	482.0–712.5	254.9, 880.8
4	569.4	150.1	402.9–694.2	196.6, 942.2
6	145.2	59.0	85.8–203.7	–1.3, 291.7
8	70.2	21.1	45.9–84.3	17.7, 122.7
11	27.6	3.5	23.6–29.8	19.0, 36.2
24	3.0	1.2	2.0–4.3	0.0, 5.9
30	1.6	0.5	1.2–2.1	0.5, 2.7

Table 2. Mean (n=3) plasma TR2 after one dose of netazepide 500 mg

Time (h)	Plasma netazepide (ng/mL)			
	Mean	SD	Range	95% CI
Pre-dose	0	0	0.0–0.0	
0.25	1.1	1.85	0.0–3.2	–3.5, 5.7
0.5	45.0	75.73	0.0–132.4	–143.2, 233.1
0.75	113.8	187.2	2.7–330.0	–351.3–578.9
1	122.0	193.0	5.4–344.7	–357.4–601.3
1.5	137.5	188.1	11.8–353.8	–329.9–604.9
2	266.2	186.9	125.5–478.2	–198.3, 730.4
4	454.2	69.2	376.0–507.4	282.4, 626.0
6	197.9	47.0	147.7–240.8	81.2, 314.6
8	135.2	26.5	110.2–163.0	69.3, 201.0
11	61.6	11.9	49.9–73.6	32.2, 91.0
24	5.8	1.6	4.3–7.5	1.9, 9.8
30	3.0	0.4	2.6–3.4	2.0, 4.0

Table 3. Pharmacokinetic parameters of netazepide and TR2 after one dose of netazepide 500 mg

Parameter	Netazepide	TR2
C_{max} (ng/mL) ¹	708 (479, 1046)	488 (449, 531)
t_{max} (h) ²	4.0	4.0
$t_{1/2}$ (h) ³	4.3	3.9
AUC_{0-t} (ng.h/mL) ¹	2765	2495
$AUC_{0-\infty}$ (ng.h/mL) ¹	2774	2509

1. Geometric mean (%CV)
2. Median (range)
3. Arithmetic mean (standard deviation).

Table 4. Amount of TR1, TR2 and TR3 required to achieve an effect on the responses to gastrin G17

Effect of gastrin G17	TR1 (nM)	TR2 (nM)	TR3 (nM)
Complete reversal of anti-proliferative effects of gastrin	500	100	500
Significant inhibition of gastrin-stimulated migration	10	1	10
Significant inhibition of gastrin-induced branching morphogenesis	10	50	10

Table 5. Proportions of TR2, netazepide, TR2/netazepide ratio, and TR2 mg/kg in human, dog and rat plasma after single or repeated oral doses of netazepide.

Dose	500 mg	50/mg day for 3 days				100 mg/kg/day for 7 days				
Species	Subject 1	Subject 2				Dog			Rat	
Time	7h	Day 1 2h	Day 1 7h	Day 3 2h	Day 3 7h	0.25h	2h	24h	0.25h	2h
TR2 (%)	40.9	10.1	27.9	15.2	29.4	0.6	<0.1	nd	6.9	10.2
Netazepide (%)	48.5	81.2	62.1	62.9	51.5	97.2	97.2	84.0	86.1	81.0
TR2/netazepide	0.84	0.12	0.45	0.24	0.57	0.006	0.001	-	0.08	0.12
TR2 mg/kg	2.87	0.07	0.20	0.11	0.20	nc	nc	nc	nc	10.2

For humans, a nominal bodyweight of 75 kg was used; nc = not calculable; nd = not detected

Table 6. Metabolites of TR2 formed by hepatocytes *in vitro* from nine species

<i>m/z</i>	Presence in incubation (% relative to parent compound in T=120 min incubation)								
	Human	Mouse	SD rat	F rat	Guinea pig	Rabbit	Dog	Minipig	Monkey
	0.1	nd	0.24						
	100	100	100	100	100	100	100	100	100
	1.22	3.04	1.88	3.78	6.37	0.26	nd	0.14	2.98
	0.29	0.36	0.44	1.1	0.22	0.08	0.02	0.03	0.22
	0.01	0.01	0.05	0.11	nd	nd	nd	nd	nd
	0.89	1.07	2.48	3.85	0.77	0.2	0.27	0.16	0.46
	0.03	nd	nd	nd	nd	0.05	0.04	0.17	nd

SD = Sprague-Dawley; F = Fischer

Table 7. Solubility of TR2 compared with netazepide

pH	Netazepide (µg/mL)	TR2 (µg/mL)	Solubility TR2/netazepide
2.01	2650	5000*	1.9
3.06	99.7	645	6.5
4.06	5.9	58.2	9.9
5.08	1.4	9.8	7
5.99	1.3	11.6	8.9
6.98	1.4	5.8	4.2
8.16	1.5	7.8	5.2

* Maximum solubility reported. Undissolved solid was observed at end of assay, so the accuracy of the result is questionable.

Figure 1. Gastric pH (median; n=20) and plasma netazepide concentrations (mean; n=20) up to 24 h after netazepide 100 mg by mouth in healthy subjects. Gastric pH after placebo is shown for comparison. The difference between netazepide and placebo at 20–24 h after dosing is significant. Note that subjects were fasting at 0 h and had breakfast the following morning, at 22 h after dosing

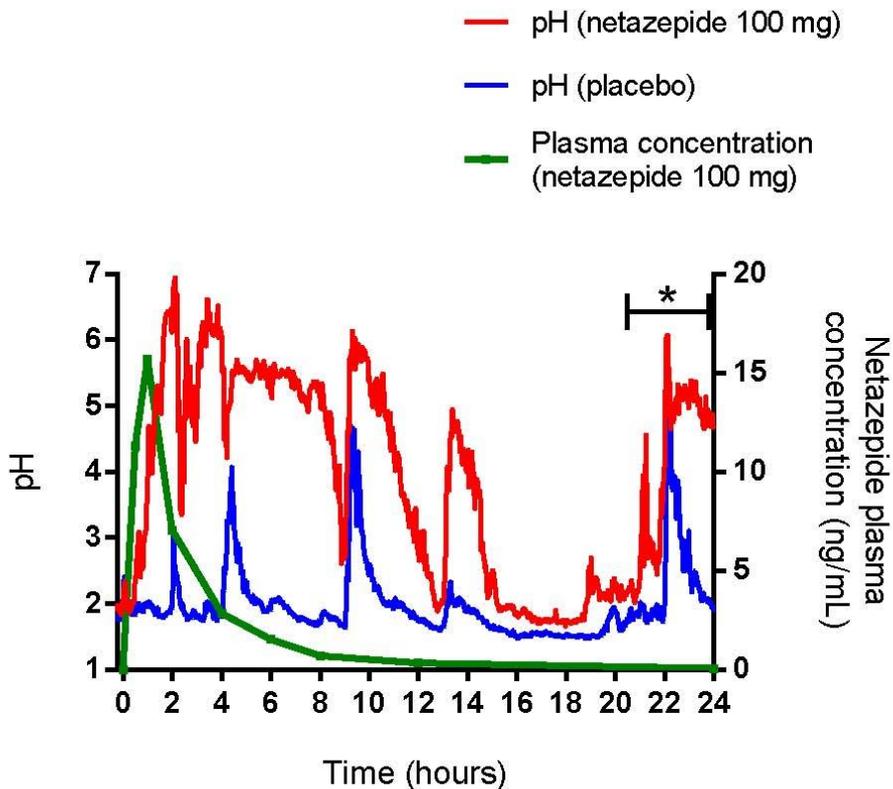


Figure 2. Total plasma ^{14}C concentrations (netazepide equivalent ng/mL) and ^{14}C -netazepide concentrations (ng/mL) versus time after 15 μg of ^{14}C -netazepide given intravenously

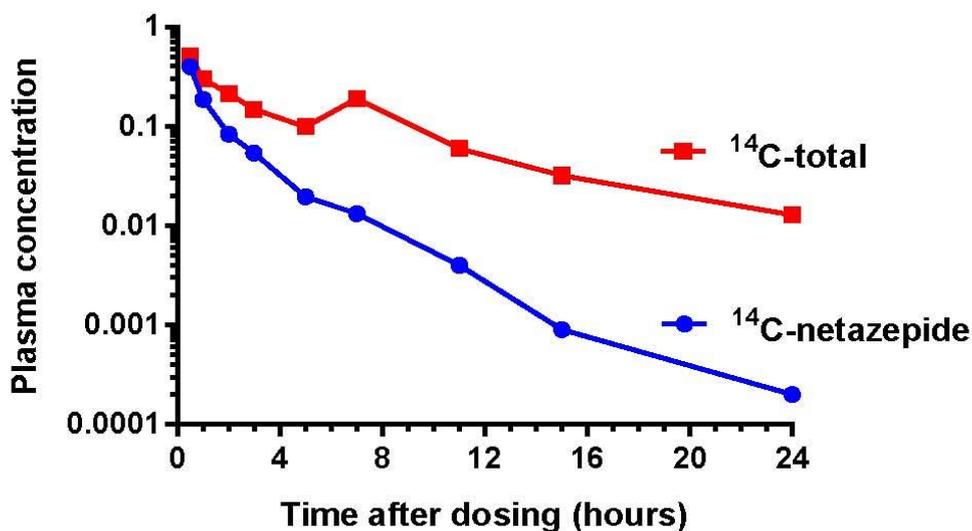


Figure 3. Plasma concentrations (means; n=3) of netazepide and TR2 after a single oral dose of netazepide 500 mg in healthy subjects

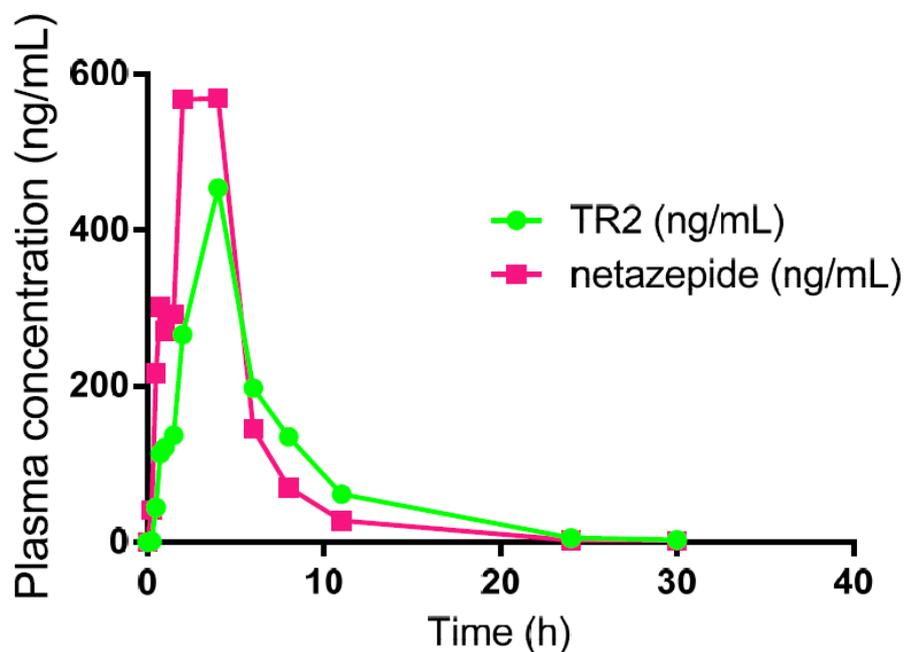


Figure 4. Structures of netazepide and its two main urinary metabolites

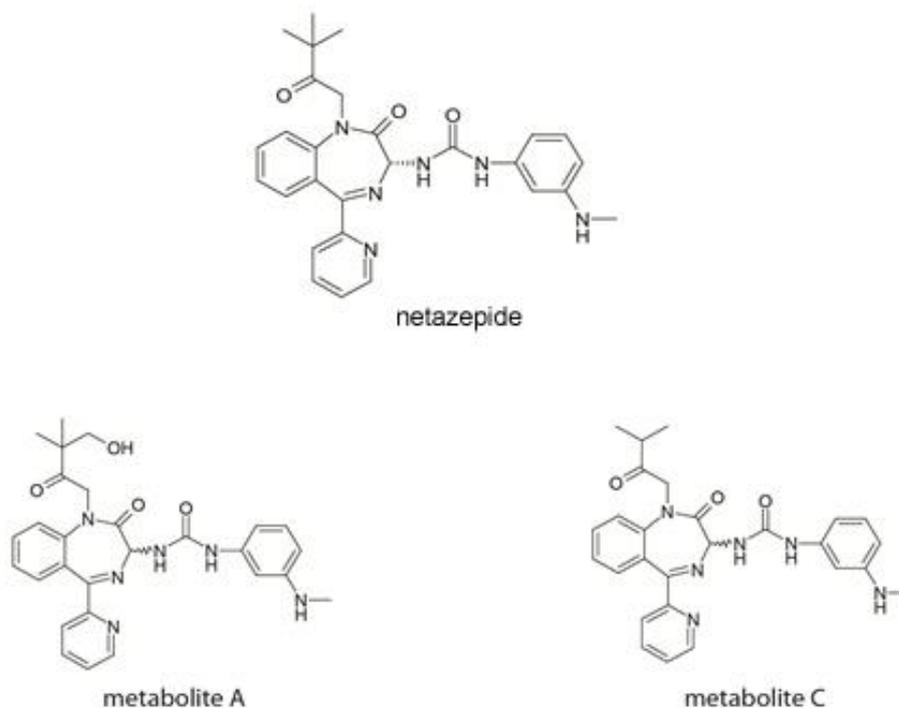


Figure 5. 100 nM concentrations of TR2, netazepide (YF476) and YM022 (another gastrin/CCK₂ antagonist) completely reversed the inhibition of AGS_{GR} cell proliferation by gastrin G17

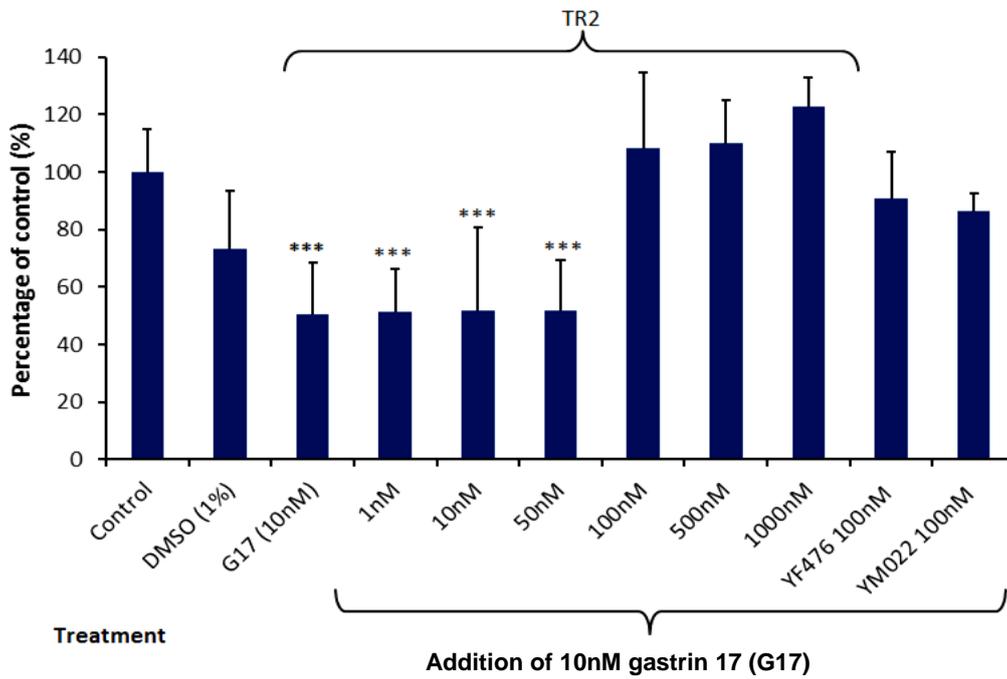
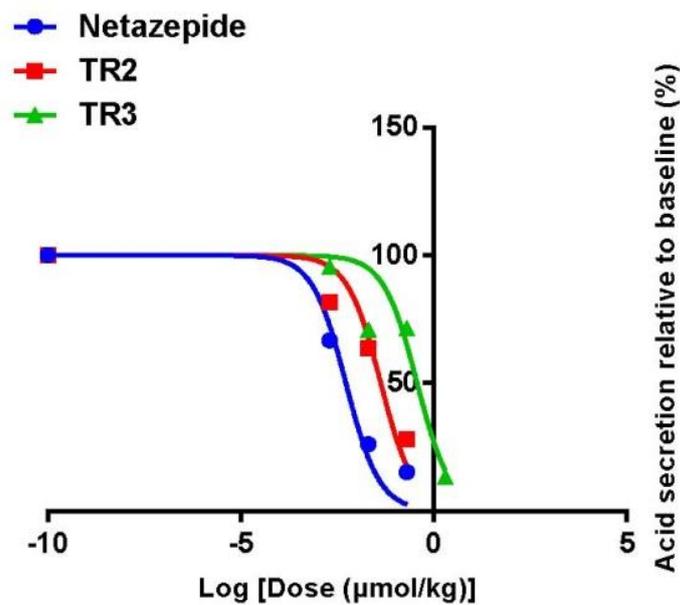


Figure 6. Gastric acid (H⁺) secretion at collection period 5.5–6 h expressed as a percentage of pentagastrin-induced gastric acid secretion (baseline collection period 3.5–4 h)



Chapter 17

**Self experiments with TR2, the main
metabolite of netazepide**

Introduction

Non-clinical studies showed that TR2 is a gastrin/CCK₂ receptor antagonist (Chapter 16). Although slightly less potent than netazepide, TR2 was more selective for the gastrin/CCK₂ receptor, 10 times more soluble, and not covered by existing patents. Netazepide will be off patent worldwide in 2016.

I wanted to know if TR2 has a similar pharmacological profile in humans, and if it might be a potential successor to netazepide. However, the prospect of having to undertake costly and time consuming toxicology studies in two species to find that out seemed pointless when animals, healthy subjects and patients had already been exposed safely to the compound. So, first I did several self experiments. A negative result would spare the animals and save money, whereas a positive result would lead to a formal study.

Objectives

The aim was to find out if TR2 is a gastrin/CCK₂ receptor antagonist in humans and whether it might merit clinical development instead of netazepide.

Methods

The experiments were open and exploratory in design. I took a range of single doses of TR2 to assess their effect on the response to intravenous infusion of pentagastrin 0.6 µg/kg/h for 2 h compared with control, as described previously. I collected blood samples for measurement of plasma TR2 before and at frequent intervals after dosing. ASI, St George's London University, measured plasma TR2 using a validated LC/MS/MS method.

Materials

ProSynth, Acton, Suffolk, synthesised the supply of TR2 active pharmaceutical ingredient (API), which turned out to be amorphous, unlike netazepide. HMR pharmacy supplied capsules hand-filled with API only for each dose of TR2, and pentagastrin for intravenous infusion. The control for the pentagastrin infusions was no treatment ('placebo').

Results

I took a single dose of 5, 50 and 100 mg of TR2 and 'placebo' 1 h before pentagastrin infusion on four separate occasions, and a single dose of 100 mg just for measurement of plasma concentrations of TR2 on a fifth occasion. The starting dose was 100 mg. A physician colleague also took a single dose of 100 mg just for measurement of plasma concentrations. Another physician colleague helped with the tests. The six doses and the dates that they were taken are listed in Table 1.

TR2 was first detected in the circulation at 0. T_{max} was mostly 0.h C_{max} and $AUC_{0-24 h}$ were dose proportional (Table 2 and Figure 1). Plasma concentrations of TR2 after all the doses were low. There was very little TR2 in the circulation beyond 24 h after dosing.

The H^+ response to pentagastrin after no treatment and after 5 mg TR2 was erratic, and there was no evidence that 5 mg was an active dose. However, the 25 mg and 100 mg doses of TR2 completely suppressed the H^+ response to pentagastrin (Figures 2 and 3). There were no adverse events.

Discussion

Bioavailability of TR2 API was low and similar in magnitude to that of single doses of netazepide API in my $n = 1$ tests of the parent compound (Chapter 8). However, 25 and 100 mg of TR2 still abolished the response to pentagastrin, which is consistent with gastrin/ CCK_2 receptor antagonism. Potency of TR2 seems similar to that of single doses of netazepide API in Study 5 (Figure 3).

TR2 in batch 8608-B1, from whence came all the doses, was amorphous, unlike netazepide API, which is crystalline. Amorphous material is usually more bioavailable. Aspiration of gastric juice to measure its H^+ content at the first time point, 1 h 15 min after TR2 dosing, may have removed any TR2 remaining in the stomach. However, $AUC_{0-24 h}$ of 100 mg TR2 (510 ng.h/mL) taken before pentagastrin infusion was higher than that of 100 mg TR2 (381 ng.h/mL) taken just to assess its pharmacokinetics.

Conclusions

- TR2 is a gastrin/ CCK_2 receptor antagonist that may be as potent as netazepide.
- TR2 probably contributes substantially to the activity of netazepide as a gastrin/ CCK_2 receptor antagonist. However, its pharmacokinetic profile does not easily explain the pharmacodynamic activity of single doses of netazepide on gastric pH beyond 24 h.
- TR2 has low bioavailability despite its amorphous state, which is surprising given that amorphous netazepide is more bioavailable than the crystalline form.
- TR2 is a potential successor to netazepide, but that requires confirmation in formal studies in a larger number of subjects. Its bioavailability would need improving.

Reference

Wood 2013. YF476: metabolite synthesis report.

Table 1. Summary of n = 1 tests of single doses of TR2

Test	Date	Subject	Dose (mg)	Procedure
21	14 Feb 2014	MJB	2 x 50	Dose taken 1 h before 2-h pentagastrin infusion. Plasma TR2 assays 0–72 h after dosing.
22	27 Feb 2014	MJB	25	Dose taken 1 h before 2-h pentagastrin infusion. Plasma TR2 assays 0–48 h after dosing.
23	7 Mar 2014	MJB	100	Plasma TR2 assays 0–48 h after dosing.
24	19 Mar 2014	MJB	5	Dose taken 1 h before 2-h pentagastrin infusion. Plasma TR2 assays 0–48 h after dosing.
25	27 Mar 2014	MJB	0	No treatment (control) taken 1 h before 2-h pentagastrin infusion.
26	24 Apr 2014	SJW	100	Plasma TR2 assays 0–48 h after dosing.

All doses were amorphous active pharmaceutical ingredient from batch 8608-B1.

Table 2. Plasma concentrations and C_{max} and AUC_{0–24 h} of TR2 for Tests 21, 22, 23, 24 and 26

Time (h)	Test				
	21	22	23	24	26
0	0.0	0.0	0.0	0.0	0.0
0.25	0.0	0.0	0.0	0.0	0.0
0.5	1.1	13.5	31.9	2.2	0.5
0.75	11.5	32.1	71.9	6.6	25.9
1.0	55.7	27.6	66.8	5.4	30.2
1.25	55.1	18	59.9	4.0	42.9
1.5	48.3	15.1	43.3	2.9	48.9
2.0	35.5	13.4	35.7	2.0	32.3
3.0	64.8	10.2	33.0	1.8	18.4
4.0	36.2	7.4	20.9	1.3	10.3
6.0	nd	nd	nd	nd	5.8
8.0	16.8	4.1	8.4	0.5	2.4
12.0	5.3	1.5	nd	0.0	nd
24	nd	nd	3.2	nd	nd
36	nd	nd	nd	0.0	nd
48	1.7	0.9	1.9	nd	1.3
72	0.8	nd	nd	nd	0.0
T_{max} (h)	3.0	0.75	0.75	0.75	1.5
C_{max} (ng/mL)	64.8	32.1	71.9	6.6	48.9
AUC_{0–24 h} (ng.h/mL)	510	140	381	21	201

Figure 1. N = 1 tests 21–24 and 26. Plasma concentrations of TR2 (ng/mL) after single doses of TR2

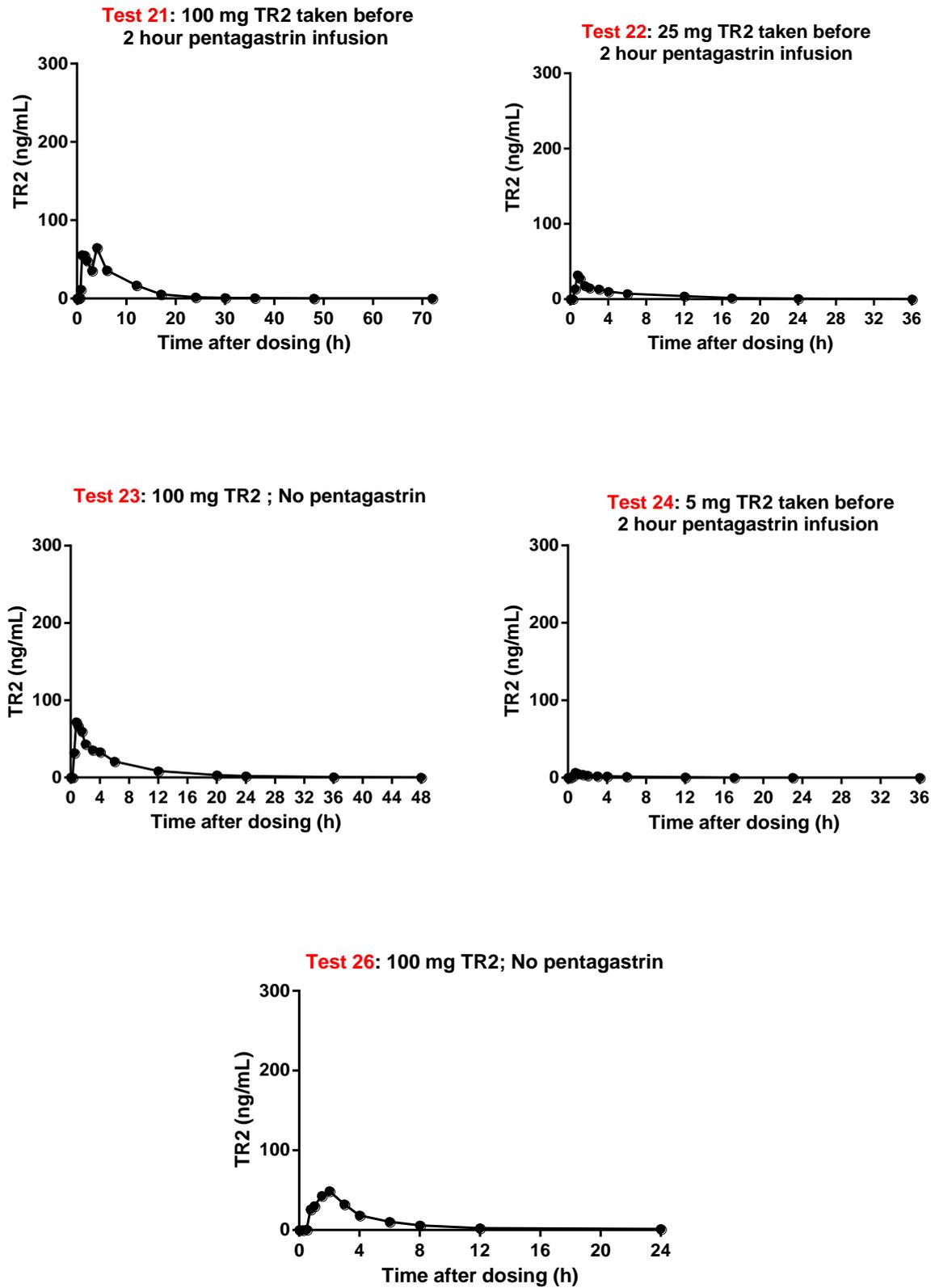


Figure 2. Effect of single doses of (a) TR2 (n=1) and (b) netazepide (means, n=10) on the increase in H⁺ content of gastric aspirate induced by intravenous infusion of pentagastrin 0.6µg/kg/h for 2 h

Pentagastrin was started 1 h after dosing

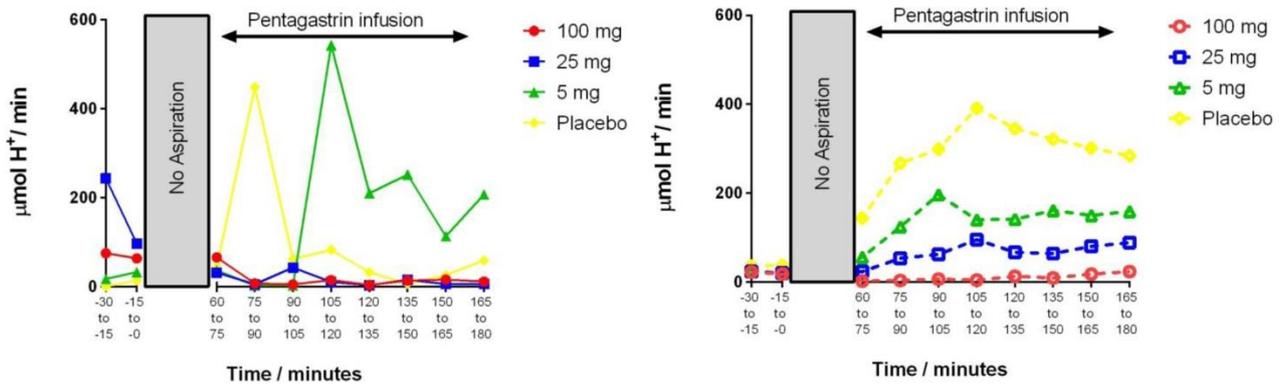
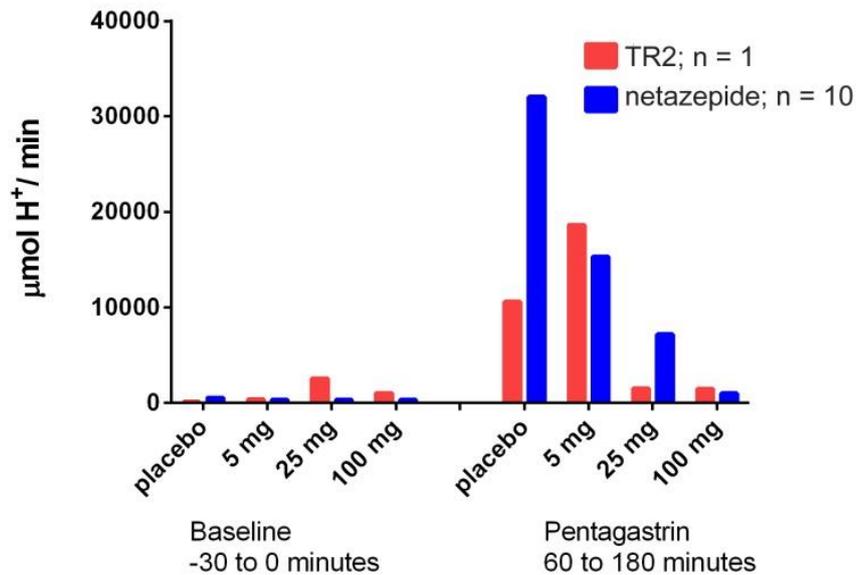


Figure 3. Comparison of the effect of single doses of (a) TR2 (n=1) and (b) netazepide (means, n=10) on the AUC_{60–180 min} of the increase in H⁺ content of gastric aspirate induced by intravenous infusion of pentagastrin 0.6µg/kg/h for 2 h

Netazepide data from Study 5



Chapter 18

Study 14: Single-dose study of TR2, the main metabolite of netazepide, in healthy subjects

Introduction

Non-clinical studies showed that TR2, the main metabolite of netazepide, is a more selective but slightly less potent gastrin/CCK₂ receptor than netazepide (Chapter 16), whereas self experiments suggested that TR2 is as potent as netazepide (Chapter 17). So, we decided to do a formal study of TR2 in healthy subjects. We reflected on the need for specific toxicology studies to support such a study, but deemed them unnecessary for the following reasons.

- In healthy subjects, TR2 accounted for 10–29% of netazepide-related compounds in the circulation after netazepide 50 mg daily for 3 days (steady state), and >40% after a large, single dose of netazepide 500 mg. Netazepide 500 mg was metabolised quickly to TR2; exposure to TR2 (C_{\max} 488 ng/mL; AUC_t 2,498 ng.h/mL) was substantial and quite similar to that of netazepide (C_{\max} 714 ng/mL; AUC_t 2,773 ng.h/mL).
- Netazepide has been well tolerated and safe in a total of 220 healthy subjects in 13 studies of single doses up to 500 mg and repeated doses up to 100 mg daily for 6 weeks, and by a total of 20 patients in studies of repeated doses up to 50 mg daily, currently for 3–30 months. All subjects would have been exposed to TR2 during those studies.
- Recent studies of netazepide metabolism in rats *in vitro* and *in vivo* have shown that TR2 is one of the main metabolites of netazepide. Therefore, rats must have been exposed to TR2 in the original 13-week toxicity study of netazepide 100, 300 and 1,000 mg/kg/day in that species. Although the NOAEL was strictly 100 mg/kg/day, findings at the higher doses, such as white stools and pink urine, were deemed of no toxicological significance. See Chapter 2 for C_{\max} and $AUC_{0-24\text{ h}}$ data. Estimated exposure to TR2, based on netazepide studies in rats *in vitro* and *in vivo*, was about 12% of netazepide dose. The dog makes very little TR2.

Given that animals, healthy subjects and patients had all been exposed safely to TR2 during netazepide studies, it seemed reasonable to assess the safety, tolerability, pharmacokinetics, metabolism and pharmacodynamics of TR2 in a formal study in healthy subjects without first doing specific toxicity studies. The MHRA agreed with that rationale. We assumed that the MHRA would regard such a study as a first-time-in-human (FTIH) study and would want it to be dose-rising in design, despite existing safety data on high exposure to TR2.

So, we selected the starting and maximum doses for a FTIH study of TR2 on the basis of data from non-clinical and healthy volunteer studies of netazepide, the parent compound. We assumed TR2 exposure to be 12% of netazepide dose, as in studies of rats *in vitro* and *in vivo*, in which case male and female rats were exposed safely to plasma TR2 concentrations up to an average mean C_{\max} of 430 ng/mL at 1,000 mg/kg/day in the original

13-week toxicity study. The dog *in vitro* produces little TR2, so the 13-week toxicity study in dogs was no guide to dose selection in humans. Healthy subjects given a single 500 mg dose of netazepide by mouth were exposed safely to plasma TR2 concentrations of mean C_{\max} 488 ng/mL and AUC_t 2,498 ng.h/mL. Thus, C_{\max} in rats and healthy subjects were similar, so it seemed reasonable to use C_{\max} and AUC_t results from healthy subjects to define the maximum dose for a first-time-in-human study of TR2. Earlier studies in healthy subjects of netazepide by mouth also provide useful information towards selection of doses for a FTIH study. In Study 1 of single doses of a spray-dried formulation of netazepide 5, 25 and 100 mg in 20 healthy subjects, the pharmacokinetics of netazepide were linear, and netazepide increased 24-h gastric pH in a dose-dependent manner. Modelling showed that netazepide 25 mg was almost at the top of the dose-response curve for the increase in gastric pH. Spray-dried netazepide was about 15% bioavailable in an absolute bioavailability study in healthy subjects (Study 10).

Single doses of netazepide 1, 5, 25 and 100 mg caused dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion in healthy subjects. 100 mg caused almost complete inhibition. However, netazepide was crystalline and only an estimated 2–3% bioavailable compared with about 15% for spray-dried netazepide. Thus, we expected a single dose of 5 mg TR2 to have pharmacological activity, and to be a safe starting dose for a FTIH study for several reasons. First, the current formulation of TR2 is API, which, if it behaves like netazepide API in healthy subjects, is likely to have low bioavailability. In that respect, the absolute bioavailability of TR2 in rats was only 0.63–1.30%. Second, if we assume that netazepide API has linear pharmacokinetics, and that in healthy subjects the bioavailability of TR2 API is similar to that of netazepide API, namely only 2–3%, the C_{\max} and AUC_t of 5 mg of TR2 should be very low – only about 2 ng/mL and 4 ng.h/mL, respectively. Therefore, even if TR2 API has 100% bioavailability in healthy subjects, exposure from a single dose of 5 mg should be more than covered by the estimated maximum exposure in rats (C_{\max} 430 ng.h/mL) and healthy subjects (C_{\max} 488 ng/mL and AUC_t 2,498 ng.h/mL). Third, TR2 is a benzodiazepine derivative, a well-studied and generally safe class of compounds, and the pharmacological effects of single doses of a gastrin/CCK₂ receptor antagonist are benign. The dose of TR2 should be increased or decreased on the basis of emerging safety, pharmacokinetic and pharmacodynamic data. Plasma concentrations should not exceed C_{\max} 488 ng/mL and AUC_t 2,498 ng.h/mL. The MHRA accepted that strategy.

Objectives

The objectives of this study (Study 14) in healthy subjects were: (1) to assess the safety and tolerability of single, rising doses of oral TR2; (2) to find out if TR2 is an orally active gastrin/CCK₂ receptor antagonist, and if so whether activity is of long duration; (3) to investigate the metabolism of TR2; and (4) to compare the results with previous, similar studies of netazepide.

Methods

Regulations

We followed the ICH Guideline for GCP and EU Clinical Trials Directive. The MHRA and London Brent REC approved the study. Subjects gave written, informed consent.

Study design

This was a double-blind, partial cross-over, single ascending-dose study in 10 men, deemed healthy as described previously. There were 4 study sessions. In each session, 8 subjects took active treatment and 2 took placebo. The starting dose in session 1 was 5 mg. On Day 1, subjects took a single dose of TR2 or placebo. Before and for up to 36 h after dosing, we measured ambulatory gastric pH continuously (Zephyr pH Monitoring System, Sandhill Scientific, USA), as described in Study 2, and collected frequent blood samples for assay of TR2. On Day 3, we collected gastric aspirate via a naso-gastric tube for 30 min before dosing and for 60–180 min after dosing, during which time we gave an intravenous infusion of pentagastrin 0.6 µg/kg/h for 2 h, as described in Study 2. We collected additional blood samples from 4 subjects at 2, 4 and 8 h after dosing on Day 1 of session 4, for analysis of TR2 metabolites. We assessed safety and tolerability as described previously.

Subjects were admitted to the ward on the evening of Day –1, and left after study activities were completed on Day 3. They fasted overnight on Days –1 and 2 and ate meals at standard times, as described in Chapter 3. Subjects had a follow-up visit 5–10 days after their last dose.

Materials

HMR pharmacy hand filled capsules with TR2 active pharmaceutical ingredient (API) 5 and 25 mg, which was amorphous. However, after further purification it proved to be crystalline. Empty capsules served as matching placebo. Anazao Health Corporation, Tampa, Florida, USA, supplied vials of pentagastrin 250 µg/vial (Boc-β-Ala-Trp-Met-Asp-Phe-NH₂). HMR pharmacy prepared pentagastrin for infusion.

Ambulatory gastric pH and pentagastrin infusion

We analysed gastric pH recordings and gastric aspirate as before (Study 2).

TR2 assay

We separated plasma from blood samples, as described previously. Analytical Services International, St George's University of London, measured plasma TR2 by a validated LC-MS/MS method. The lower limit of quantification (LLOQ) was 0.5 ng/mL. Any values below the LLOQ were reported as below the limit of quantification (BLQ).

Metabolites

We pooled plasma samples from 4 subjects on active treatment (100 mg) in session 4 for analysis of TR2 metabolites by WIL Research, Hertogenbosch, The Netherlands, by LC-PDA-MS. Plasma from a subject given placebo served as a control. MS data were screened for presence of m/z values of possible metabolites and for absence in the corresponding control samples.

Results

Subjects

10 men entered the study, which we did November and December 2014. Median age of the subjects and BMI were 36 (range 21–62) years and 24.6 (range 21.3–29.4), respectively. We withdrew one subject after session 3 (100 mg dose) because of severe abdominal pain, which lasted for several days. Central Middlesex Hospital initially diagnosed gall stones, but that diagnosis was excluded by investigations. Symptoms resolved spontaneously.

Pharmacokinetics

The escalating doses of TR2 were 5, 15, 50 and 100 mg. Mean plasma concentrations of TR2 are listed in Table 1 and illustrated in Figure 1. Mean pharmacokinetic parameters of TR2 are listed in Table 2. TR2 was rapidly absorbed; T_{max} was about 1 h. $t_{1/2}$ varied among doses; for the higher doses it was about 8 h, which is probably the most reliable value. Mean C_{max} and $AUC_{0-\infty}$ of TR2 plasma concentrations were both dose-proportional (Table 2; Figure 1). Mean C_{max} and $AUC_{0-\infty}$ of the 100 mg dose were 51 ng/mL and 229 ng.h/mL, respectively.

Pharmacodynamics

TR2 caused dose-dependent inhibition of the increase in H^+ content of gastric aspirate induced by intravenous pentagastrin (Table 3; Figures 2 and 3), and dose-dependent increases in gastric pH up to 36 h after dosing (Figure 5). 100 mg of TR2 completely inhibited the response to pentagastrin, and 50 mg almost did so.

Metabolites

Six metabolites of TR2 were found in the pooled human plasma samples at 2, 4 and 8 h after dosing (Table 4). Three of the metabolites detected were $\leq 1\%$ relative to TR2.

Safety and tolerability

The treatments proved safe and well tolerated. There were no clinically relevant changes in vital signs, ECG and safety tests of blood and urine. Adverse events were few, mild and resolved spontaneously. The serious adverse event in one subject was deemed unlikely to be related to TR2.

Discussion

We designed a dose-rising study of TR2 in the expectation that the MHRA would regard it as a FTIH study (MHRA 2012), which they did despite the history of animals, healthy subjects and patients having been exposed safely to TR2. The doses studied proved to be 5, 15, 50 and 100 mg, which are about what we predicted on the basis of the $n = 1$ tests described in Chapter 17. All doses proved safe and well tolerated. Abdominal pain in one of the subjects is of doubtful relevance to TR2.

Plasma concentrations of TR2 varied greatly among subjects (Table 1), as they do for netazepide. The pharmacodynamic profile of single doses of TR2 was very similar to that of netazepide: rapid absorption, with T_{\max} of about 1 h, and $t_{1/2}$ of about 8 h for the 50 and 100 mg doses. $t_{1/2}$ of the smaller doses of TR2 was only about 4 h, because plasma concentrations of TR2 after the smaller doses were too low to measure in some of the terminal samples. Therefore, 8 h is a more reliable value. The increase in $t_{1/2}$ with dose explains the increases in CL/F and Vd/F with dose, because calculation of both parameters involves $t_{1/2}$. Mean C_{\max} and $AUC_{0-\infty}$ of 100 mg TR2 API taken after an overnight fast were 51 ng/mL and 229 ng.h/mL, respectively. Mean C_{\max} and $AUC_{0-\infty}$ of a single dose of 100 mg netazepide API taken after an overnight fast were 38.9 ng/mL and 85.6 ng.h/mL, respectively, in Study 6, and 44.4 ng/mL and 87.9 ng.h/mL, respectively, in Study 7. Thus, TR2 appears to be slightly more bioavailable than a similar formulation of netazepide, possibly because TR2 is 10 times more soluble than netazepide.

TR2 inhibited the response to pentagastrin in a dose-dependent manner. 50mg was about as effective as 100 mg, which suggests that 50 mg is at the top of the dose-response curve. When we compared the effect of TR2 API on the response to pentagastrin with that of netazepide API in Study 5 (Figure 4), TR2 was at least as potent as netazepide.

TR2 also increased ambulatory gastric pH in a dose-dependent manner. Activity persisted up to 36 h after dosing. However, pH exceeded 4 for only short periods of time, a result similar to that for single doses of netazepide API in Study 7. Single doses of spray-dried netazepide in Study 1 gave a much better result.

The six metabolites of TR2 in plasma were present only in small amounts relative to the parent compound netazepide. Three of them were <1% in amount. According to the FDA Guidance for Industry Safety Testing of Drug Metabolites (FDA 2008) and the ICH guideline M3 (ICH 2009), metabolites are of interest only if plasma levels are greater than 10% of parent exposure. Thus, none of the metabolites of TR2 warrant further investigation.

Conclusions

- TR2 is at least as potent a gastrin/CCK₂ antagonist as netazepide in humans, and is probably responsible for much of the activity of netazepide, and possibly for its long duration of action. Note that in the pre-clinical studies, TR2 was not quite as potent as netazepide.
- Raising gastric pH ≥ 4 requires greater exposure to a single dose of TR2 than that required to abolish the response to an intravenous infusion of pentagastrin 0.6 μ g/kg/h for 2 h. Raising gastric pH ≥ 4 for a substantial part of the day is regarded by gastroenterologists as essential for successful treatment of acid-related conditions.
- TR2 appears to be more bioavailable than netazepide, possibly because of the greater solubility of TR2.
- The doses of TR2 required for clinical studies may be similar to those for netazepide. However, like netazepide API, TR2 API has low bioavailability, which needs to be improved for future studies.
- TR2 is a novel gastrin/CCK₂ antagonist, and has advantages over netazepide – better solubility and bioavailability – and is worthy of further study. An extensive search by us and by an experienced patent officer failed to show that TR2 is covered by existing patents. Netazepide will be off patent worldwide by 2016, so TR2 merits full clinical development rather than netazepide.

Acknowledgements

I thank Prosynth's chemists for their ingenuity in synthesising TR2, and Liv Thomsen, Trio Team Leader and medicinal chemist, for her commitment and substantial contribution to the TR2 project.

References

FDA guidance for industry. Safety testing of drug metabolites. 2008.

ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals. EMA/CPMP/ICH/286/1995. 2009.

MHRA guidelines on phase1 trials. TSO 2012.

Table 1. Mean (sd) plasma concentrations (ng/mL) after doses of TR2

Time (h)	Mean (sd) plasma concentrations (ng/mL) after doses of TR2							
	5 mg (n=8)		15 mg (n=8)		50 mg (n=8)		100 mg (n=7)	
0	0	(0)	0	(0)	0	(0)	0	(0)
0.25	0	(0)	0	(0)	0.16	(0.30)	1.23	(2.78)
0.5	1.37	(1.35)	3.16	(4.65)	5.31	(5.10)	21.56	(24.07)
0.75	2.71	(1.661)	9.49	(10.60)	13.19	(8.38)	39.23	(25.49)
1	3.44	(1.792)	11.23	(7.92)	26.15	(30.90)	44.91	(42.48)
1.25	3.69	(1.81)	13.36	(11.03)	28.09	(34.13)	51.23	(58.72)
1.5	3.94	(2.41)	12.40	(9.86)	28.39	(37.77)	55.03	(61.99)
2	3.56	(2.40)	9.10	(6.16)	25.04	(35.10)	40.61	(35.15)
3	2.41	(1.24)	6.94	(4.29)	20.63	(27.13)	28.30	(20.26)
4	2.03	(1.04)	5.69	(3.51)	14.59	(16.83)	17.78	(12.92)
6	1.50	(0.5)	3.76	(1.66)	9.85	(9.25)	13.51	(6.88)
8	0.97	(0.35)	2.60	(1.14)	7.24	(7.55)	8.96	(3.97)
12	0.40	(0.42)	1.29	(0.54)	3.40	(3.04)	4.47	(2.13)
16	0.07	(0.19)	0.51	(0.47)	1.78	(1.47)	2.89	(1.54)
24	0	(0)	0.06	(0.18)	0.99	(0.45)	1.66	(0.86)
30	0	(0)	0	(0)	0.68	(0.45)	1.00	(0.55)
36	0	(0)	0	(0)	0.30	(0.33)	0.67	(0.55)

Table 2. Mean pharmacokinetic parameters of TR2

Dose (mg)	C _{max} ¹ (ng/mL)	T _{max} ² (h)	AUC _{last} ¹ (ng.h/mL)	AUC _{0-∞} ¹ (ng.h/mL)	Lambda z ¹ (1/h)	t _{1/2} ³ (h)	MRT ³ (h)	CL/F ³ (L/h)	Vd/F ³ (L)
5	4.2 (2, 7)	1.0 (0.75-2)	17 (10, 27)	22 (14, 34)	0.17 (0.13, 0.23)	4.2 (1.4)	4.2 (0.9)	248 (105)	1297 (529)
15	14.1 (718, 28)	0.88 (0.5-1.5)	50 (302, 81)	56 (37, 84)	0.17 (0.12, 0.22)	4.4 (1.5)	4.9 (0.7)	298 (134)	1934 (1325)
50	20.2 (9, 47)	1.0 (0.75-6)	119 (62, 229)	127 (68, 238)	0.09 (0.07, 0.12)	8.2 (3.4)	7.7 (1.8)	478 (260)	5707 (3426)
100	51.1 (26, 101)	0.75 (0.5-2)	219 (128, 375)	229 (135, 389)	0.08 (0.06, 0.11)	8.7 (2.3)	7.1 (1.4)	501 (286)	5886 (2966)

1 = geometric mean (95% CI); 2 = median (range); 3 = arithmetic mean (standard deviation)

Table 3. Mean changes (CI) from baseline for AUC of gastric pH

Dose (mg)	AUC ₀₋₂₄ ¹	AUC ₀₋₃₆ ¹
Placebo	-7.2 (-20.8, 6.5)	-8.2 (-28.3, 12.0)
5	-11.8 (-35.7, 12.1)	-17.0 (-49.6, 15.7)
15	-25.2 (-67.3, 16.9)	-42.4 (-109.4, 24.7)
50	28.4 (17.8, 39.0)	38.5 (28.69, 48.34)
100	29.1 (15.3, 42.8)	40.4 (24.0, 56.8)

1 = arithmetic mean (95% CI)

Table 4. m/z values of possible metabolites of TR2 in pooled plasma

Retention time (min)	m/z of [M + H] ⁺	Mass shift	% relative to TR2		
			Pool 1 (T = 2 h)	Pool 2 (T = 4 h)	Pool 3 (T = 8 h)
9.2-9.3	278.178	237.061	1.2	2.2	2.6
13.8-13.9	531.235	+15.996	0.3	0.5	nd
14.5	501.225	-14.014	0.8	0.8	0.8
16.1-16.2	515.239	=TR2	100	100	100
18.0-18.1	529.219 ³⁾	+13.980	1.0	0.7	nd
18.2-18.3	485.230 ³⁾	-30.009	2.4	2.2	4.2
20.5	501.225 ³⁾	-14.014	0.5	0.7	2.0

Figure 1. Mean (n = 10) TR2 plasma concentrations (ng/mL) after single oral doses (range 5–100 mg) in healthy men

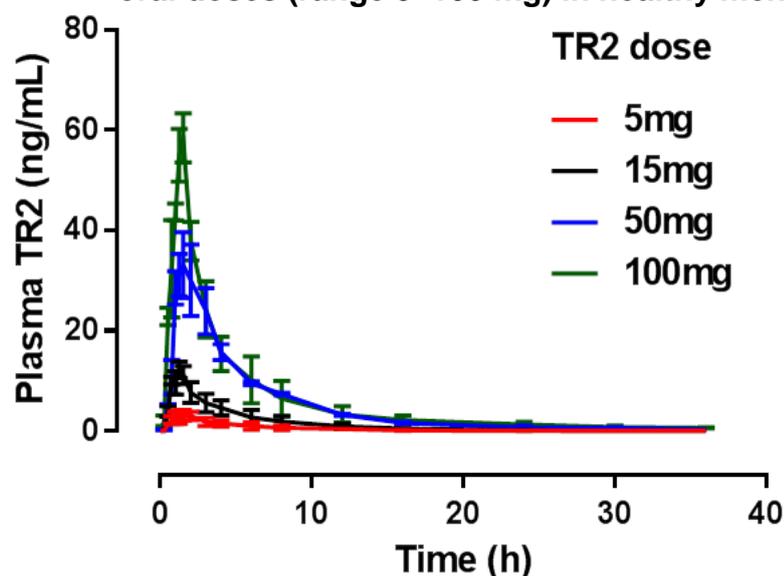


Figure 2. Mean (n=10) of H⁺ secretion rate for 30 min before dosing (from -30 to 0 min) and during pentagastrin infusion (from 60 to 180 min after dosing) in the presence of single doses of TR2* in healthy men

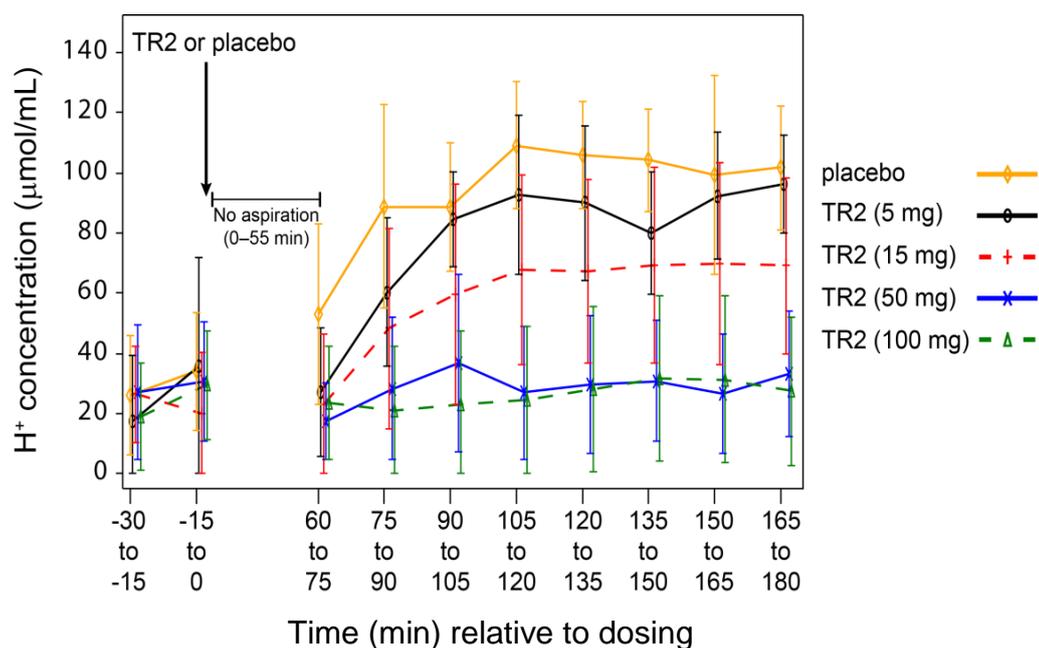
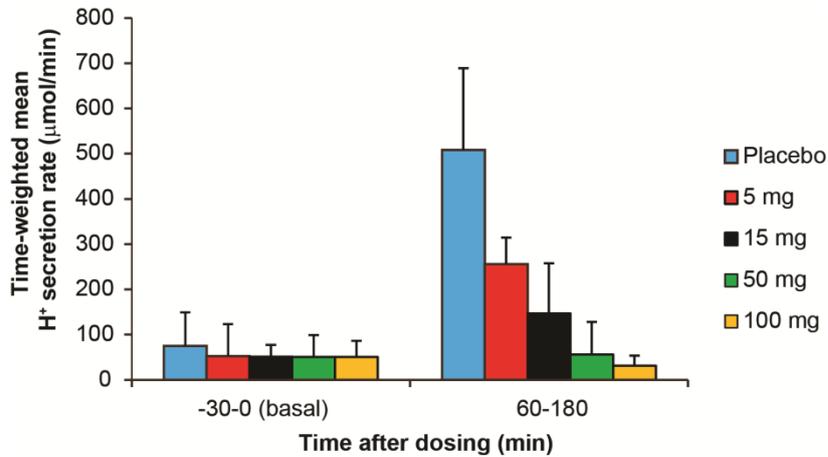
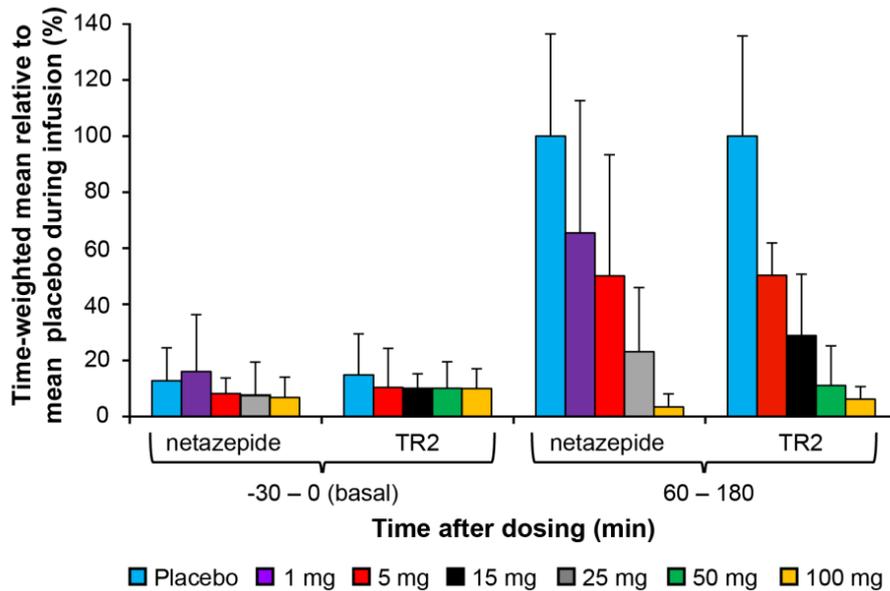


Figure 3. Time-weighted mean (n=10) of H⁺ secretion rate for 30 min before dosing (from -30 to 0 min) and during pentagastrin infusion (from 60 to 180 min after dosing) in the presence of single doses of TR2* in healthy men



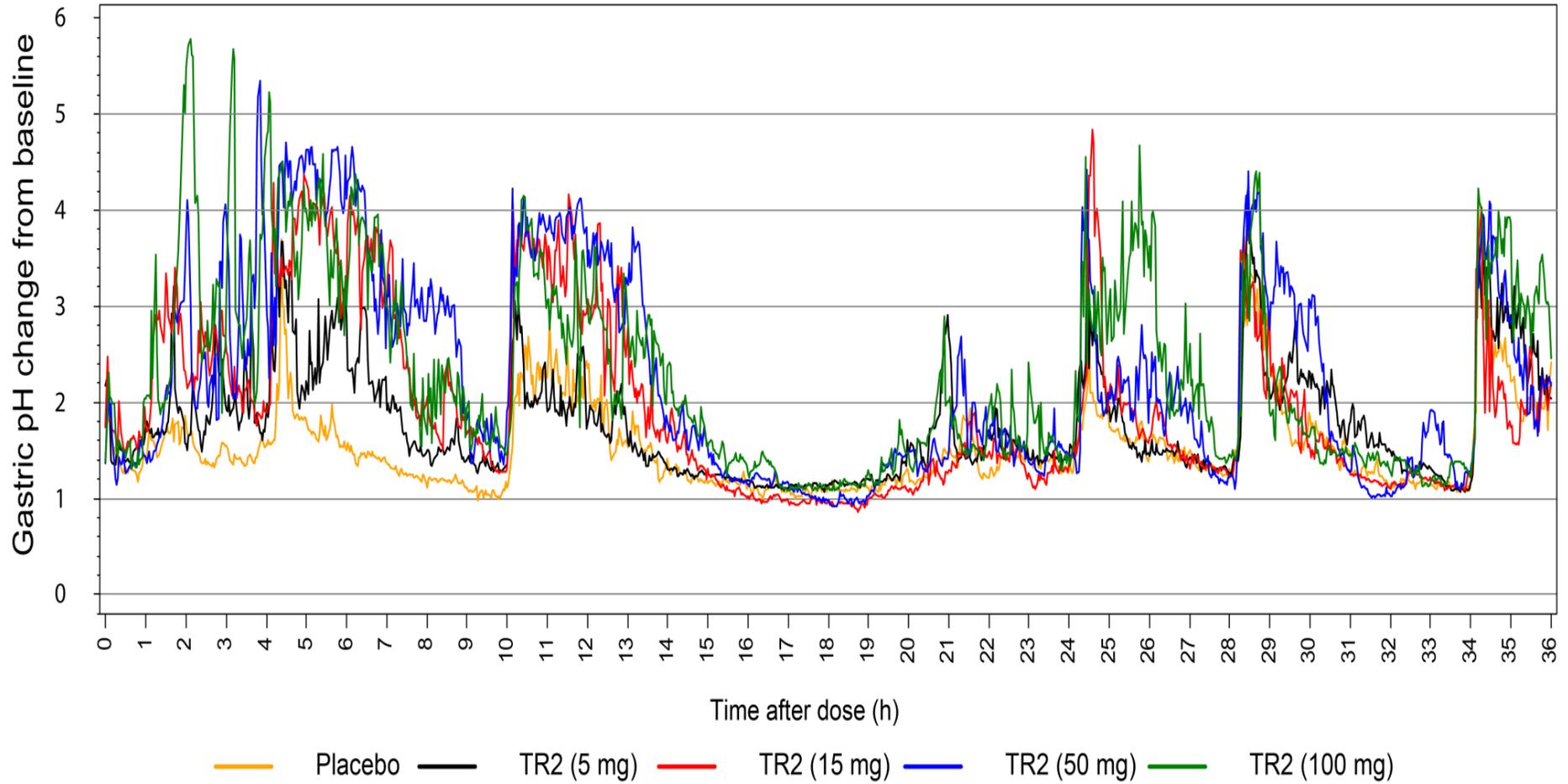
*TR2 active pharmaceutical ingredient (API) was administered at 0 min, after which 60 min were allowed for absorption of TR2 before starting aspiration of gastric juice

Figure 4. Time-weighted mean (n = 10 per study) of H⁺ secretion rate relative to placebo before dosing (-30 to 0 min) and during pentagastrin infusion (60 to 180 min after dosing) in the presence of netazepide* or TR2* in healthy men



*TR2 or netazepide active pharmaceutical ingredient (API) was administered at 0 min, after which 60 min were allowed for absorption of TR2 or netazepide before starting aspiration of gastric juice

Figure 5. Mean (n=10) ambulatory gastric pH before and for 36 h after TR2 on Day 1



Standard meals were eaten at 4, 10, 24, 28 and 34 h after dosing

Chapter 19

**Self experiments with TR2-A,
the acetyl derivative and prodrug of TR2**

Introduction

Study 14 showed that TR2 offers advantages over netazepide as a gastrin/CCK₂ receptor antagonist. But like netazepide, the bioavailability of TR2 is low despite it being 10 times more soluble than the parent compound (Table 7, Chapter 16). TR2-A – the acetyl derivative of TR2 and penultimate step in the synthesis of TR2 – is 90 times more soluble than netazepide. I wondered whether TR2-A might be more bioavailable than TR2. So, I decided to do some self-experiments with TR2-A for the same reason that I had done them with TR2. I describe those self experiments in this chapter.

Objectives

The aim was to find out if TR2-A is a gastrin/CCK₂ receptor antagonist in humans and whether it might be more bioavailable than TR2.

Methods

The experiments were open and exploratory in nature. I took single doses of TR2 to assess their pharmacokinetics, effect on 24-h ambulatory gastric pH, and effect on the response to intravenous infusion of pentagastrin 0.6 µg/kg/h for 2 h, as described previously. Blood samples were collected for measurement of plasma TR2 and TR2-A before and at frequent intervals after dosing. ASI, St George's London University, measured plasma TR2 and TR2-A using validated LC/MS/MS methods.

Materials

ProSynth, Acton, Suffolk, synthesised the supply of TR2-A active pharmaceutical ingredient (API). The early batches of TR2-A (8887 and 8921) used to prepare capsules for the first tests were amorphous, but the batch (8941) used to prepare capsules for the latter tests was crystalline. HMR pharmacy supplied capsules hand-filled with API for each dose of TR2-A, and supplied pentagastrin for intravenous infusion. Structures of netazepide, TR2 and TR2-A are shown in Figure 1.

Results

I took a single dose of 100 mg TR2-A on five occasions and a single dose of 15 mg on one occasion. I took the 100 mg doses to compare the pharmacokinetics of amorphous, crystalline and micronized TR2-A 24-h after an overnight fast and after a fatty breakfast, and to assess the effect of TR2-A on ambulatory 24-h gastric pH. I took the 15 mg dose to assess its effect on the response to pentagastrin infusion. A physician colleague inserted an intravenous cannula for blood sampling. The different tests are listed in Table 1.

Tests 27 and 30 enable comparison of the pharmacokinetics of amorphous and crystalline TR2-A after an overnight fast (Table 2; Figure 2). Crystalline TR2-A gave a lower C_{\max} and $AUC_{0-24\text{ h}}$ of plasma TR2 (C_{\max} 211.9 ng.mL; $AUC_{0-24\text{ h}}$ 594 ng.h/mL) compared with amorphous TR2-A (C_{\max} 267.3 ng.mL; $AUC_{0-24\text{ h}}$ 710.0 ng.h/mL) (Table 2; Figures 2 and 6). Micronised TR2-A in test 35 yielded both a slightly higher C_{\max} and a higher $AUC_{0-24\text{ h}}$ (223.0 ng.mL and 733.0 ng.h/mL, respectively) of plasma TR2 than crystalline TR2-A (211.9 ng.mL and $AUC_{0-24\text{ h}}$ 594 ng.h/mL, respectively) in test 30 (Table 2; Figure 2). I took tests 35 and 30 after an overnight fast. Compared to test 30, a fatty breakfast in test 31 delayed T_{\max} (1.5 vs 0.75 h), reduced C_{\max} (147.0 vs 267.3 ng.mL) and reduced $AUC_{0-24\text{ h}}$ (644.0 vs 710 ng.h/mL) (Table 2; Figures 2 and 4).

Unchanged TR2-A was detected in the circulation only in tiny amounts (<1% compared with TR2) after dosing (Table 2 and Figure 3). Unchanged TR2-A appeared transiently in the early plasma samples collected after the doses of TR2-A that I took after an overnight fast, whereas unchanged TR2-A was measured throughout the

Compared to results from n=1 studies of 100 mg TR2 (Figure 7), 100 mg TR2-A increased C_{\max} of TR2 by about 4-fold and $AUC_{0-24\text{ h}}$ by almost 2-fold.

A single dose of 100 mg TR2-A increased ambulatory gastric pH. However, 100 mg TR2-A did not increase $\text{pH} \geq 4$ as effectively as 100 mg of spray-dried netazepide in Study 2 (Figure 8). Likewise, a single dose of TR2-A did not suppress the H^+ content of gastric aspirate as effectively as TR2 in Study 14, although the values mostly lay within the standard deviations of the mean for TR2. There were no adverse events.

Discussion

Synthesis of TR2-A initially yielded amorphous material, but the final batch was crystalline, and stayed that way. That allowed comparison of amorphous and crystalline TR2-A in these n = 1 tests. Amorphous was more bioavailable than crystalline material. Micronisation of crystalline TR2-A appears to have increased its bioavailability slightly. Food slowed absorption of TR2 from TR2-A, and slightly reduced exposure to circulating TR2.

Very little unchanged TR2-A appeared in the circulation. Food reduced the conversion of TR2-A to TR2 slightly. When plasma concentrations of TR2 after a single dose of 100 mg TR2-A API are compared with those after the same dose of TR2 API in previous n = 1 tests, C_{\max} and $AUC_{0-24\text{ h}}$ of TR2 after TR2-A were increased by about 4-fold. Thus, TR2-A is a prodrug of and more bioavailable than TR2. Circulating TR2 raised gastric pH, which confirms that it is a gastrin/CCK₂ receptor antagonist and the active moiety of TR2-A.

Without a placebo control, it's not possible to say whether the dose of 15 mg of TR2-A affected the response to pentagastrin.

I stopped doing further n = 1 tests of TR2-A when the MHRA agreed to our carrying out a study of TR2-A in healthy subjects, providing we first show in a non-clinical study that TR2-A is converted to TR2, which we did (Chapter 20).

Conclusions

Although these are only n = 1 tests, the results indicate that in humans:

- TR2-A, the acetyl derivative of TR2 and penultimate molecule in its synthesis, is more bioavailable than TR2 itself. Very little unchanged TR2-A is found in the circulation. Circulating TR2 increases gastric pH and is therefore a gastrin/CCK₂ receptor antagonist and the active moiety of TR2-A. Thus, TR2-A is a prodrug of TR2.
- Making crystalline TR2-A amorphous might not increase its bioavailability.
- TR2-A is a more worthy successor to netazepide than TR2, but that requires confirmation in formal studies in larger numbers of subjects.

References

Huttenen K, Raunic H, Rautio J. Prodrugs – from serendipity to rational design. *Pharmacol Rev* 2011; 63: 750–771.

TR2: Investigator's Medicinal Product Dossier. October 2014.

TR2A: Investigator's Medicinal Product Dossier. March 2015.

TR2-A: Supplement to the Investigator's Brochure for netazepide. November 2015

Figure 1. Structures. A. Netazepide; B. TR2, the main metabolite of netazepide in humans; and C. TR2-A, the acetyl derivative and prodrug of TR2

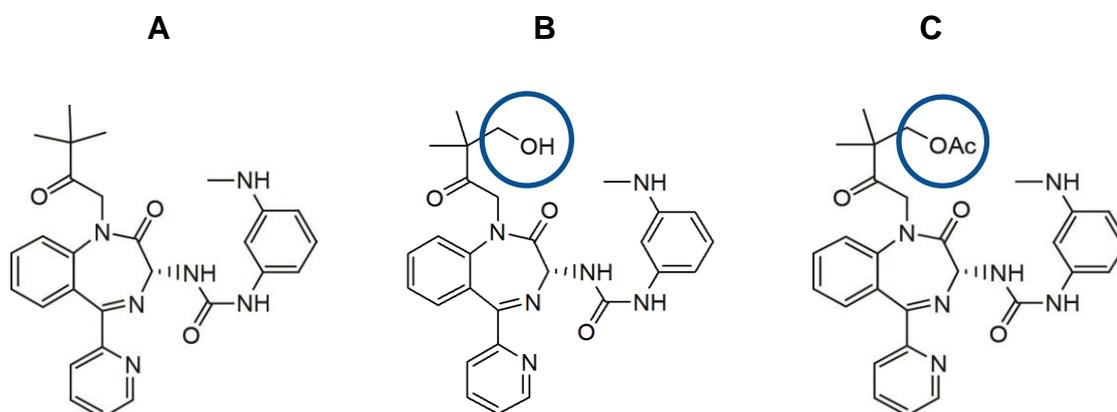


Table 1. Summary of n = 1 tests of single doses of TR2-A

Test	Date	Subject	Dose (mg)	Procedure
27	01 Sep 2014	MJB	100	TR2-A, batch 8887. Amorphous . Plasma TR2 and TR2-A assays 0– 24 h after dosing.
28	07 Oct 2014	MJB	100	TR2-A, batch 8887. Amorphous . Gastric pH recording 0– 24 h after dosing. No plasma assays.
29	15 Jan 2015	MJB	15	TR2-A, batch 8921. Amorphous . Taken 1 h before infusion of pentagastrin for 2-h. No plasma assays.
30	29 Jan 2015	MJB	100	TR2-A, batch 8941. Crystalline . Particle size 25 µm . Plasma TR2 and TR2-A assays 0–24 h after dosing.
31	11 Feb 2015	MJB	100	TR2-A, batch 8941. Crystalline . Taken after a fatty breakfast. Plasma TR2 and TR2-A assays 0–24 h after dosing.
35	14 Aug 2015	MJB	100	TR2-A, batch 8941M1. Micronised . Combined sample 4 & 5. Particle size 9–15 µm . Plasma TR2 and TR2-A assays 0–24 h after dosing.

Table 2. Plasma concentrations and pharmacokinetic parameters, C_{max} and AUC_{0–24} of TR2 and TR2-A for Tests 27, 30, 31 and 35

Time (h)	Test							
	27		30		31		35	
	TR2	TR2-A	TR2	TR2-A	TR2	TR2-A	TR2	TR2-A
0	<0.5	<0.5	<0.5	<0.1	<1.0	<0.25	0	0
0.25	<0.5	<0.5	<0.5	<0.1	<1.0	<0.25	0	0
0.5	47.2	0.9	34.7	2.12	4.4	1.27	0	2.1
0.75	211.9	1.3	267.3	2.06	79.9	5.55	13.3	12.7
1	159.8	<0.5	160.5	0.67	113.0	3.12	223.0	3.0
1.5	99.1	<0.5	113.9	0.28	147.0	2.04	192.3	0.9
2	85.6	<0.5	98.1	0.17	118.9	1.03	121.0	0.4
3	69.9	<0.5	76.2	<0.1	67.4	0.62	101.2	0
4	54.1	<0.5	59.8	<0.1	54.9	0.34	74.5	0
6	28.3	<0.5	35.4	<0.1	36.3	0.28	43.0	0
8	18.4	<0.5	28.6	<0.1	24.0	<0.25	22.2	0
12	10.1	<0.5	12.6	<0.1	13.9	<0.25	10.7	0
24	2.0	<0.5	3.3	<0.1	2.1	<0.25	1.4	0
T_{max}	0.75	0.75	0.75	0.75	1.5	0.75	1.0	0.75
C_{max}	211.9	1.3	267.3	2.12	147.0	5.55	223.0	12.7
AUC_{0-24h}	594	0.7	710	1.6	644	6.4	733	7.9

Figure 2. Plasma TR2 concentrations after 100 mg of TR2-A (n=1)

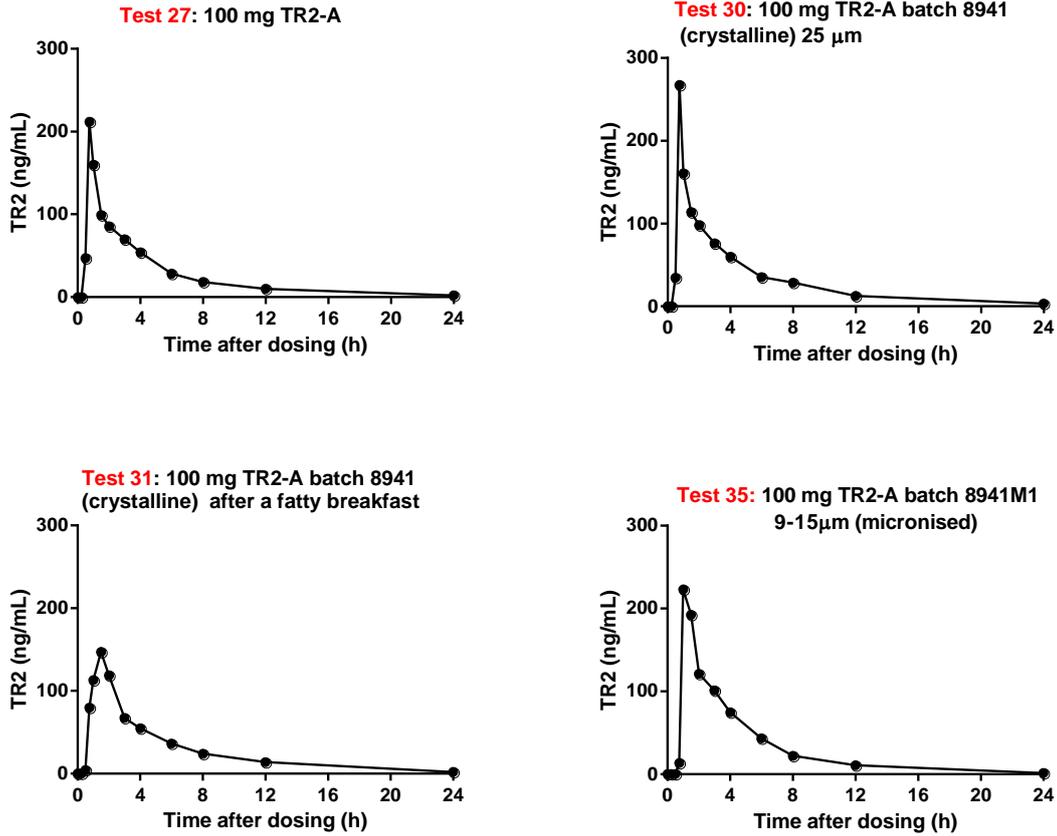


Figure 3. Plasma TR2-A concentrations after 100 mg of TR2-A (n=1)

Note the low concentrations of TR2-A

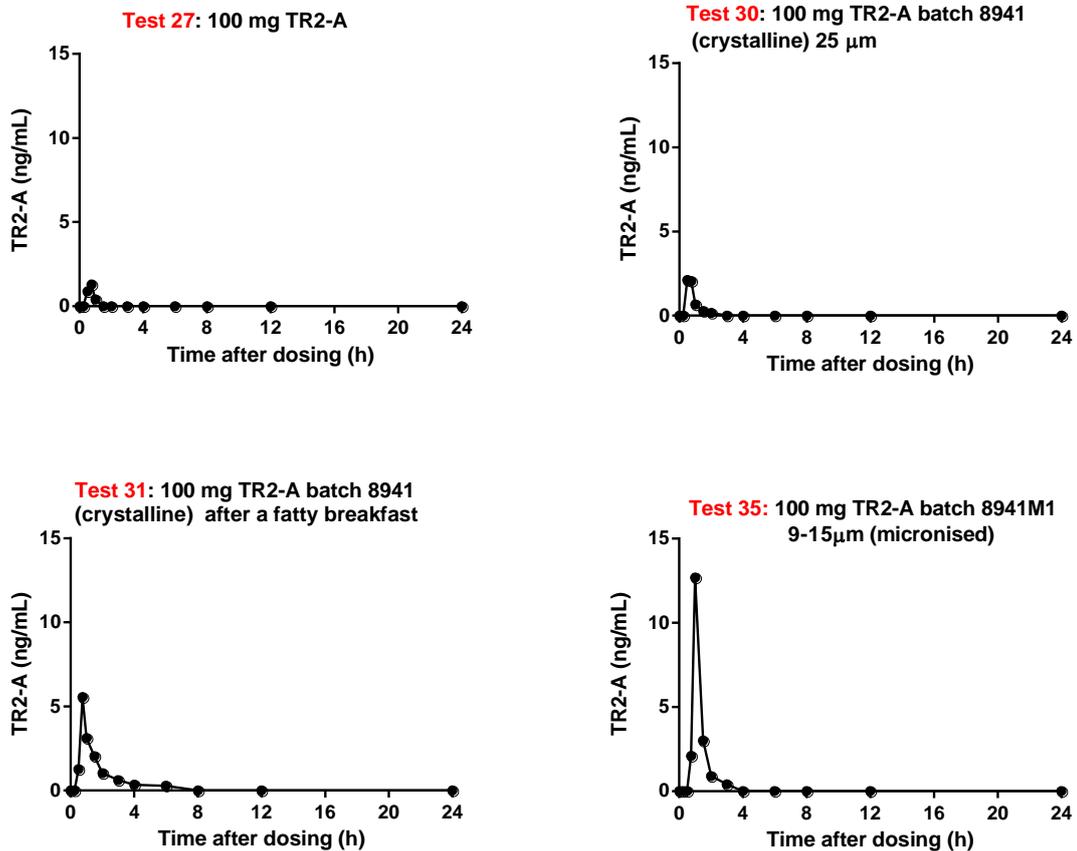


Figure 4. Effect of a fatty breakfast on plasma TR2 concentrations (n=1)

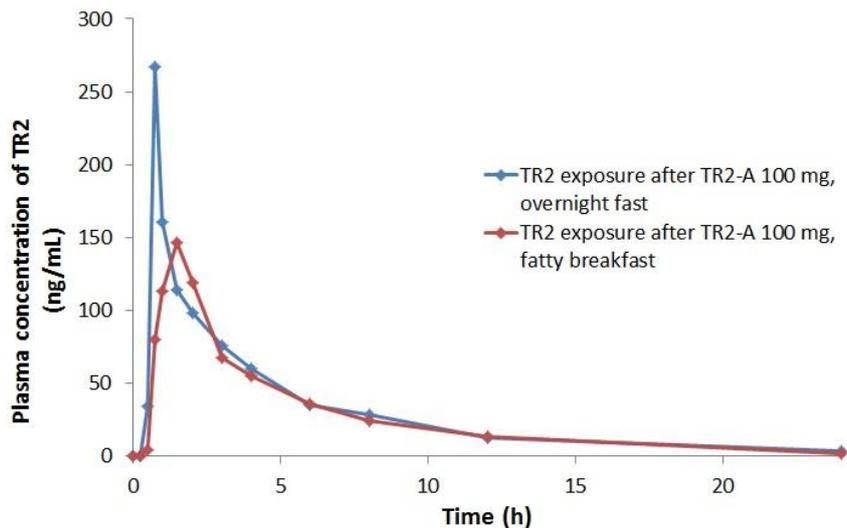
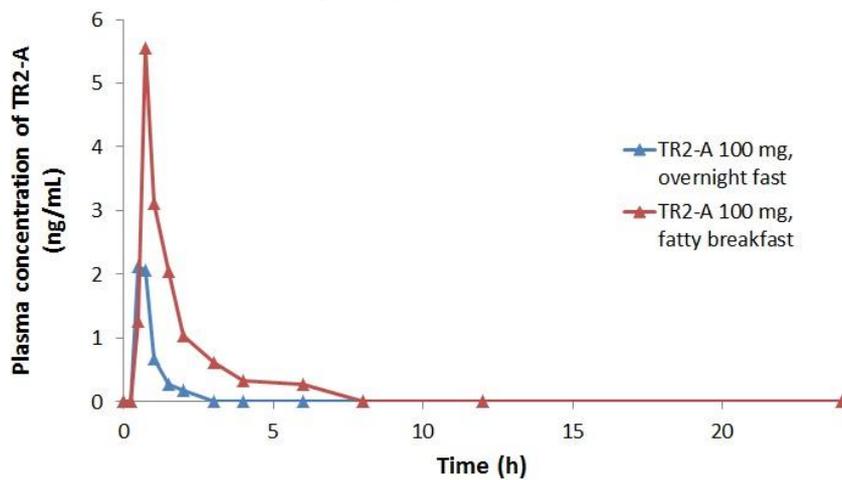


Figure 5. Effect of a fatty breakfast on plasma TR2-A concentrations (n=1)
Note the very low plasma concentrations.



Note the plasma concentration scale

Figure 6. Plasma TR2 concentrations after crystalline and amorphous TR2-A

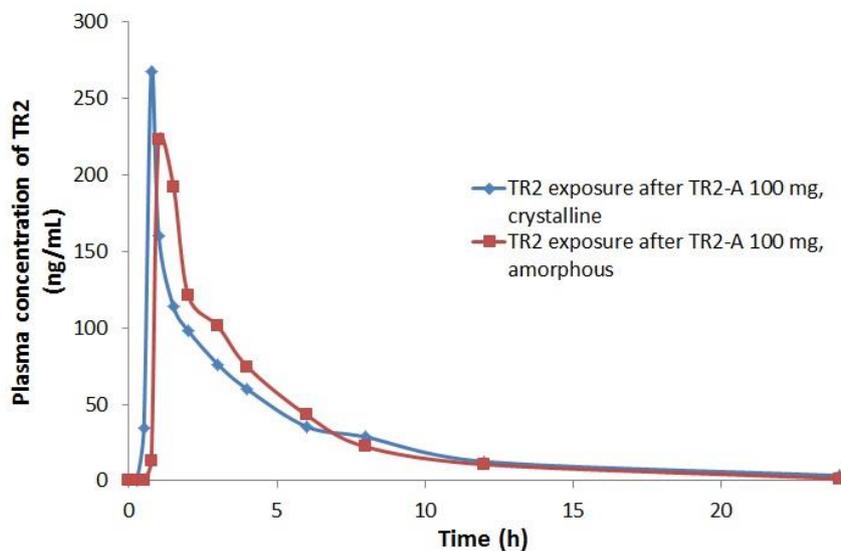


Figure 7. Comparison of plasma concentrations of TR2 after single doses of 100 mg TR2-A (n=1) and 100 mg TR2 (mean, n=2) taken after an overnight fast

Mean results for 100 mg TR2 are from n=1 tests described in Chapter 17

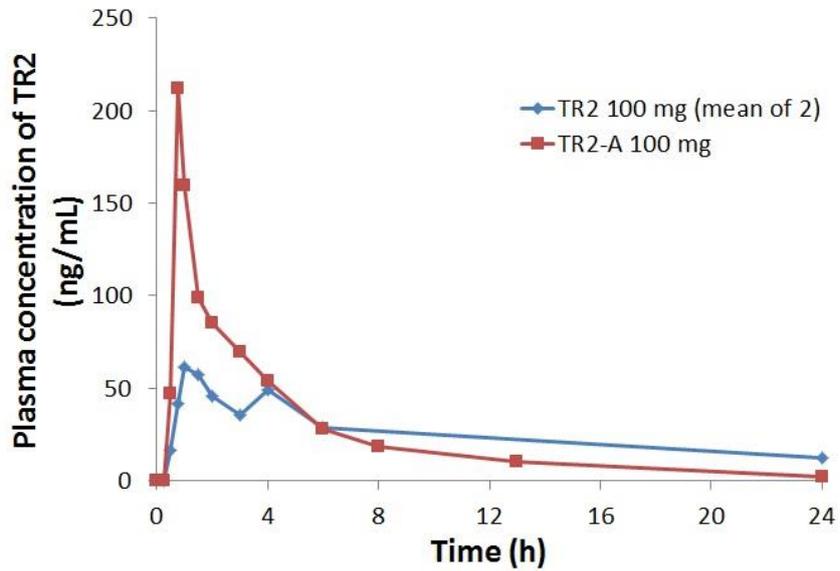
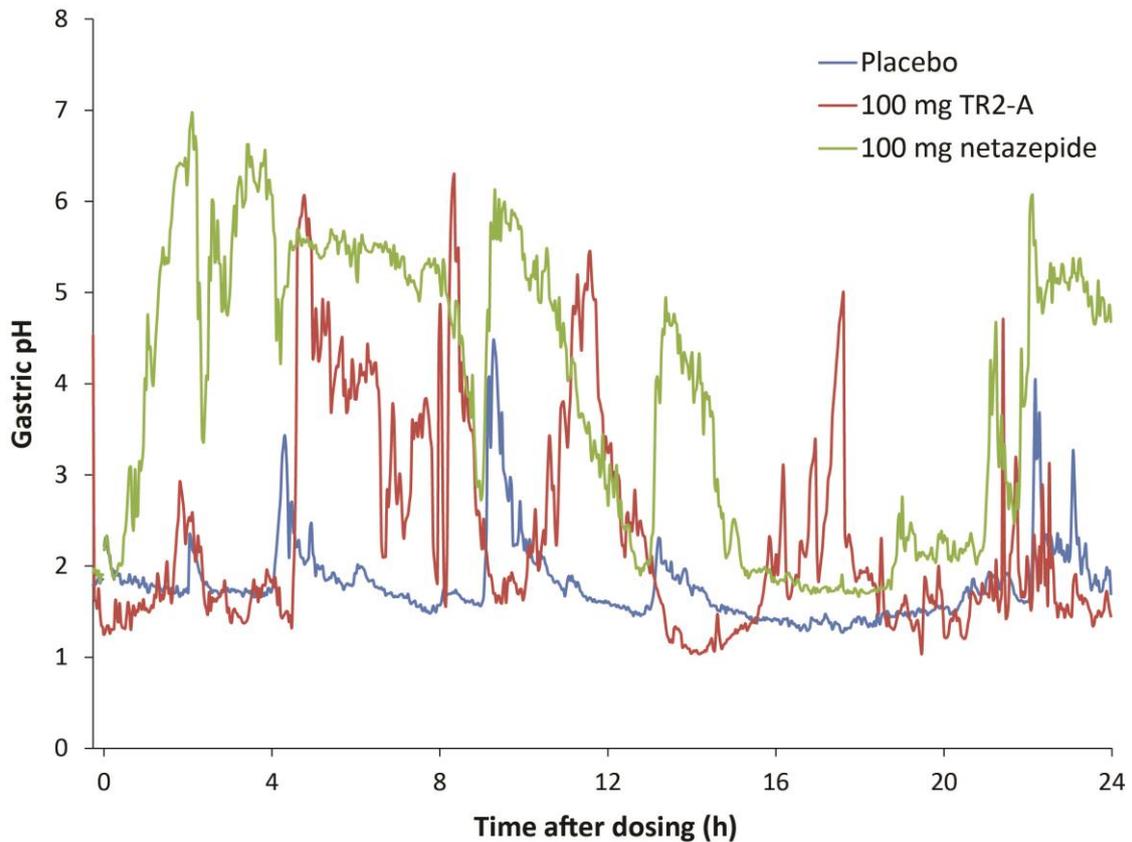


Figure 8. 24-h gastric pH after 100 mg TR2-A compared with Study 2 results for placebo and 100 mg spray-dried netazepide



Chapter 20

Study 15: Single-dose study of TR2-A, the acetyl derivative and prodrug of TR2, in healthy subjects

Introduction

Study 18 showed that TR2 is a gastrin/CCK₂ antagonist with potential advantages over netazepide. TR2-A, the acetyl derivative and penultimate compound in the synthesis of TR2, is up to 90-fold more soluble than netazepide at pH 4–6, the pH of the surface of small intestine epithelium where most drug absorption occurs. The self experiments described in Chapter 17 suggest that TR2-A is a prodrug of and more bioavailable than TR2.

As we did for TR2, we sought advice from the MHRA about the need for specific toxicology studies before doing the FTIH study of TR2-A. The MHRA agreed to such a study without specific toxicology studies providing we could first show in non-clinical studies that TR2-A is quickly metabolised to TR2. So, we organised three studies. In the first study (Table 1), after incubating TR2-A and human hepatocytes *in vitro* for 120 min, 83% had been metabolised to TR2, which was more than mouse, rat and minipig hepatocytes (Report T-NC12). In the second study, mice were given 1 mg/kg of TR2-A by gavage. It was not possible to quantify the amount of TR2-A in plasma, probably due to the high esterase activity typical of rodent plasma. However, when a method to screen but not quantify the presence of TR2-A was used, none was detected. T_{max} of TR2 was 0.5 h after TR2-A dosing (Figure 1), which suggests TR2-A is rapidly metabolised to TR2 by mice *in vivo* (Report T-NC142). In the third study, minipigs were given three daily oral doses of TR2-A on one occasion and one intravenous dose of TR2-A on another occasion. TR2-A was metabolised to TR2. Oral bioavailability of TR2 was an estimated 41–54%. (Report T-NC16). Thus, these three studies provide good evidence to support the concept that TR2-A would be quickly metabolised to TR2 in healthy subjects. Furthermore, the minipig is a suitable species for assessing the toxicity of TR2-A and TR2.

Objectives

The objectives of this study (Study 15) in healthy subjects were: (1) to assess the safety and tolerability of single, rising doses of oral TR2-A; (2) to find out if TR2 is an orally active gastrin/CCK₂ receptor antagonist; (3) to find out if TR2-A is more bioavailable than TR2; (4) to assess the effect of food on the pharmacokinetics of TR2-A; and (5) to compare the results with those from previous, similar studies of TR2 and netazepide.

Methods

Study design

This was an open, crossover study of single, rising doses of TR2-A. Eight men, deemed healthy as in previous studies, were required to complete the study, which was in 2 parts. The MHRA and Brent REC approved the study. Subjects gave written, informed consent.

In Part A, at each dose level, 8 subjects (2 groups of 4) took single doses of TR2-A on 2 days (Days 1 and 3), separated by at least 48 h. Up to 4 dose levels were permitted, with at least 7 days between consecutive dose levels. The starting dose was 15 mg. The dose was increased or reduced on the basis of a review of the results of the earlier dose(s). The maximum dose allowed was 100 mg. C_{\max} and $AUC_{0-24\text{ h}}$ should not exceed 488 ng/mL and 2,498 ng.h/mL, respectively, which was most unlikely given that C_{\max} and $AUC_{0-24\text{ h}}$ after 100 mg of TR2 were only 66 ng/mL and 241 ng.h/mL, respectively, in Study 14.

In Part B, subjects took one dose of TR2-A after eating a standard FDA high-fat breakfast. The dose was to be one of those taken in Part A. Part B took place at least 7 days after the last dose in the last session of Part A.

In Parts A and B, we collected blood samples for assay of TR2-A and TR2 before and frequently up to 36 h after dosing on Day 1. In Part A only, we recorded ambulatory gastric pH continuously for 0.5 h before and 36 h after dosing on Day 1. On Day 3, we measured the volume and H^+ content of 15-min epochs of gastric aspirate for up to 30 min before and during intravenous infusion of pentagastrin 0.6 $\mu\text{g}/\text{kg}/\text{h}$ for 2 h.

Subjects were admitted to the ward on the evening of Day -1, and left after study activities were completed on Day 3. They fasted overnight on Days -1 and 2 and ate meals at standard times, as in Study 14. There was a follow-up visit 5–10 days after the last dose.

Materials

TR2-A ((*R*)-1-[1-(4-Acetoxy-3,3-dimethyl-2-oxo-butyl)-2-oxo-5-(pyridin-2-yl)-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl]-3-(3-methylamino-phenyl)-urea) is a white crystalline powder with good stability at room temperature. It is acid-soluble and moderately water-soluble. HMR pharmacy hand filled capsules with 5 and 25 mg of TR2-A active pharmaceutical ingredient (API).

TR2 and TR2-A assays

We separated plasma from blood samples, as described previously. Analytical Services International, St George's University of London, measured plasma TR2 and TR2-A by validated LC-MS/MS methods. The lower limit of quantification (LLOQ) for the assay was 0.5 ng/mL. Values below LLOQ were reported as below the limit of quantification (BLQ).

Analysis of results

Pharmacokinetics

An HMR statistician calculated C_{\max} , t_{\max} , $AUC_{0-24\text{h}}$, AUC_{0-t} , $AUC_{0-\infty}$, $t_{1/2}$, CL/f , V/f and MRT in the absence and presence of food.

Pharmacodynamics

An HMR statistician also calculated (i) AUC_{0-24h} and AUC_{0-36h} of gastric pH; (ii) time-response curves of pentagastrin-induced increases in volume and H^+ content of gastric aspirate; and (iii) $AUC_{0-180\text{ min}}$ of pentagastrin-induced increases in volume and H^+ content of gastric aspirate, to assess the dose-response relationship, and to compare results with those for TR2 from Study 14 and for netazepide from a similar study in healthy subjects.

Results

Subjects

Eight men deemed healthy, as described previously, entered and completed the study during March to June 2015. Some of them took part in the study of TR2 (Study 14).

Safety and tolerability

TR2-A was safe and well tolerated. Any adverse events were minor and resolved spontaneously. There were no changes of clinical relevance in the safety assessments.

Pharmacokinetics

The geometric pharmacokinetic parameters of TR2-A are compared with those of TR2 from Study 14 in Table 2 and Figures 2 and 3. Mean C_{max} and $AUC_{0-24\text{ h}}$ of TR2 after 50 mg of TR2-A were 63 ng/mL and 169 ng.h/mL, respectively, compared with 20 ng/mL and 112 ng.h/mL, respectively, after 50 mg of TR2. Thus, TR2-A makes TR2 more bioavailable. Circulating concentrations of TR2-A were up to 160-fold lower than those of TR2. C_{max} and $AUC_{0-24\text{ h}}$ of TR2 when 50 mg TR2-A was taken after food were 64 ng/mL and 243 ng.h/mL, respectively, compared with 63 ng/mL and 168 ng.h/mL, respectively, when the same dose was taken an overnight fast. 90% confidence intervals for C_{max} and $AUC_{0-24\text{ h}}$ all exceeded the range 80–125%, so the active moiety TR2 after food is not bioequivalent to TR2 after fasting (see Investigator's Brochure for TR2-A).

Pharmacodynamics

TR2-A caused dose-dependent increases in gastric pH and dose-dependent inhibition of pentagastrin-stimulated H^+ concentration of gastric aspirate. 50 mg TR2-A completely inhibited the response to pentagastrin, and 25 mg almost did so (Figure 3). So, we stopped escalating the dose.

Discussion

The weakness of the study was the lack of a placebo. We decided against including a placebo and to use the results obtained with placebo in Study 14. That proved an unwise decision, because it wasn't possible to assess activity of the 5 mg dose. An open study with

measurement of the response to pentagastrin at baseline before escalating the dose would have been a better design, given that safety and tolerability were of minor importance.

Even after the 50 mg dose of TR2-A, pH ≥ 4 for short periods only. In Study 2, a single dose of netazepide 25 mg increased AUC_{0-24 h} of gastric pH ≥ 4 maximally. Mean AUC_{0-24 h} of netazepide after that dose was 193 ng.h/mL, whereas mean AUC_{0-24 h} of TR2 in Study 15 was 135 ng.h/mL. The difference in AUC_{0-24 h} reflects the differences in formulations: netazepide was spray-dried and amorphous and TR2-A was simple API and crystalline. The netazepide study used different pH recording equipment to that used in the TR2 and TR2-A studies. That may have contributed to the difference between results.

Conclusions

This study confirms that in healthy subjects:

- TR2-A, the acetyl derivative of TR2 and penultimate molecule in its synthesis, is more bioavailable than TR2. After dosing, TR2-A is quickly metabolised to TR2. Very little unchanged TR2-A is found in the circulation. TR2 is the active moiety of TR2-A and a gastrin/CCK₂ receptor antagonist.
- TR2-A administered as API is probably at least as potent as an equivalent dose of netazepide API at inhibiting the response to pentagastrin infusion, but is not as effective as an equivalent dose of spray-dried netazepide at raising ambulatory gastric pH.
- In retrospect, we should have assessed the effect of higher doses of TR2-A on gastric pH.
- Food slows absorption of TR2-A and its conversion to the active moiety TR2, but it's not clear whether or not food has a significant effect on the bioavailability of TR2.
- Thus, TR2-A is a prodrug of TR2 and a more worthy successor to netazepide than TR2. However, despite the favourable physicochemical properties of TR2-A and its increased oral bioavailability with respect to TR2 in humans, TR2-A will still need formulation development work in the interval between the start of the toxicology studies and the start of the clinical trial programme, in order to find the most bioavailable formulation possible.

References

Report T-NC12. Interspecies comparison of metabolism of TR2-A in different species of hepatocytes *in vitro*. WIL Research.

Report T-NC14. Single dose pharmacokinetics of TR2-A after oral administration in female CD-1 mice. WIL Research.

Report T-NC16. Single-dose pharmacokinetics of TR2-A and TR2 after oral and intravenous administration of TR2- in the male Göttingen minipig. WIL Research.

Table 1. Incubation of TR2-A with mouse, rat, minipig and human hepatocytes *in vitro*

m/z	Presence in T=120 min incubation (% relative to parent compound in T=1 min incubation)			
	Mouse	Rat	Minipig	Human
531.235	nd	nd	nd	0.48
501.224	3.28	2.88	3.77	0.21
501.224	nd	nd	nd	0.084
529.219	1.30	0.72	0.31	0.50
545.214	1.36	0.12	0.16	0.23
515.239 (TR2)	44.7	57.6	65.7	82.6
485.229	1.55	4.69	0.30	1.55
501.224	0.031	0.059	nd	0.015
557.251 (TR2-A)	100	100	100	100

Table 2. Geometric mean pharmacokinetic parameters of TR2 (n=10) and TR2-A (n=8) in healthy subjects after single doses of API in Studies 14 & 15

Dose (mg) of TR2 or TR2-A	C _{max} (ng/mL)			AUC ₀₋₂₄ (ng.h/mL)		
	Study 14	Study 15*		Study 14	Study 15*	
	TR2	TR2	TR2-A	TR2	TR2	TR2-A
5	4.2	5.0	BLQ	20.4	7.5	BLQ
15	14.1	15.7	1.3	53.9	36.2	1.9
25	–	27.1	1.4	–	92.8	0.8
50	20.2	62.7	2.3	112.4	169.4	1.4
50 (food)	–	63.8	1.2	–	242.6	1.7
100	51.0	–	–	208.1	–	–

BLQ = below level of quantification

* After TR2-A dosing in Study 15, there was: (1) very little TR2-A in plasma; and (2) more TR2 in plasma than after TR2 dosing in Study 14. Thus, TR2-A is a prodrug and makes TR2 more bioavailable.

Figure 1. Mean (n = 6) plasma concentration of TR2 (ng/mL) after a single oral dose of 1 mg/kg of TR2-A in mice

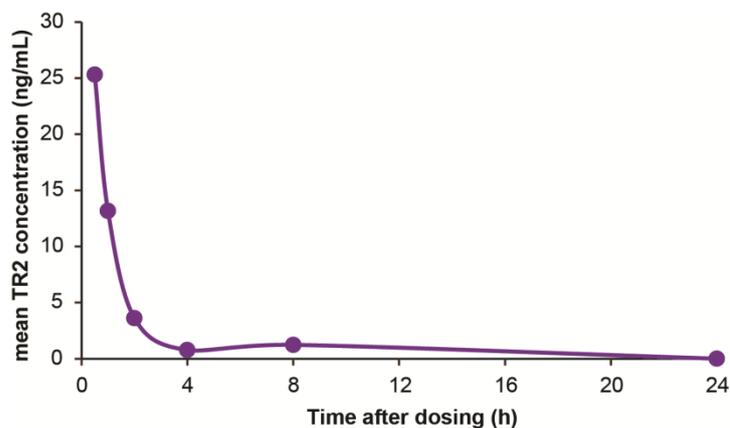


Table 3. Mean (sd) plasma concentrations (ng/mL) of TR2 after doses of TR2-A

Time (h)	Mean (sd) plasma concentrations (ng/mL) of TR2							
	5 mg (n=8)		15 mg (n=8)		25 mg (n=8)		50 mg (n=8)	
0	0	(0)	0	(0)	0	(0)	0	(0)
0.25	0	(0)	0.03	(0.09)	0.22	(0.63)	0	(0)
0.5	1.24	(1.75)	6.56	(8.21)	11.0	(14.3)	22.9	(36.9)
0.75	3.79	(3.17)	17.7	(16.5)	31.9	(26.6)	56.3	(56.5)
1	3.16	(2.00)	19.7	(19.7)	34.7	(26.4)	60.9	(47.1)
1.25	3.15	(2.47)	19.5	(19.6)	35.3	(32.7)	54.3	(44.2)
1.5	4.10	(2.38)	21.1	(19.5)	30.1	(31.8)	47.6	(32.7)
2	3.87	(2.64)	14.9	(13.3)	24.2	(30.3)	40.3	(31.2)
3	3.03	(1.50)	9.85	(8.54)	15.5	(15.8)	25.5	(20.0)
4	2.44	(0.11)	7.85	(6.90)	12.0	(12.8)	18.8	(14.3)
6	1.40	(0.29)	5.28	(3.95)	7.14	(4.90)	12.0	(8.37)
8	1.31	(0.37)	4.35	(2.75)	5.02	(3.35)	9.11	(5.35)
12	–	–	2.34	(0.97)	2.73	(1.38)	3.93	(2.39)
16	–	–	1.46	–	1.74	(0.65)	3.22	(1.53)
24	–	–	–	–	1.32	–	1.31	–

Table 4. Mean (sd) plasma concentrations (ng/mL) of TR2-A after doses of TR2-A

Time (h)	Mean (sd) plasma concentrations (ng/mL) of TR2-A							
	5 mg (n=8)		15 mg (n=8)		25 mg (n=8)		50 mg (n=8)	
0	–	–	0	0	0	0	0	0
-0.25	–	–	0	0	0	0	0	0
0.5	–	–	0.42	0.67	0.64	0.69	1.19	1.35
0.75	–	–	0.80	0.64	1.05	0.70	1.81	1.23
1	–	–	0.47	0.21	0.75	0.49	1.32	1.12
1.25	–	–	0.60	0.45	0.80	0.41	1.18	0.28
1.5	–	–	0.42	0.25	0.68	0.03	0.82	0.40
2	–	–	–	–	0.33	–	0.73	–
3	–	–	0.38	–	–	–	–	–
4	–	–	0.31	–	–	–	–	–
6	–	–	0.36	–	–	–	–	–
8	–	–	0.36	0.06	–	–	–	–
12	–	–	0.32	0.06	–	–	–	–
16	–	–	0.36	0.05	–	–	–	–
24	–	–	–	–	–	–	–	–

Table 5. Mean pharmacokinetic parameters of TR2 after dosing with TR2-A

Dose (mg)	C _{max} (ng/mL)	T _{max} (h)	AUC _{0-24 h} (ng.h/mL)	t _{1/2} (h)	MRT (h)	CL/F (L/h)	V/F (L)
5	4.07 (3.08)	0.84 (0.13)	8.41 (9.44)	2.12 (0.49)	3.51 (0.87)	350.0 (217.2)	979.5 (494.3)
15	24.22 (20.36)	0.91 (0.27)	73.07 (71.53)	4.87 (3.25)	7.25 (4.88)	276.3 (264.9)	2220.4 (2581.7)
25	46.59 (34.89)	1.00 (0.30)	130.8 (104.8)	6.59 (5.51)	9.28 (8.46)	269.3 (261.3)	3274.4 (4367.8)
50	79.54 (49.80)	1.03 (0.49)	212.45 (163.55)	3.43 (0.85)	(4.78) (0.96)	421.0 (507.5)	1631.8 (1291.4)

Figure 2. Comparison of C_{max} and AUC_{0-24 h} of plasma concentrations of TR2 after single doses of 5–50 mg of TR2-A and 5–100 mg of TR2 API, and of plasma netazepide after a single dose of 100 mg API

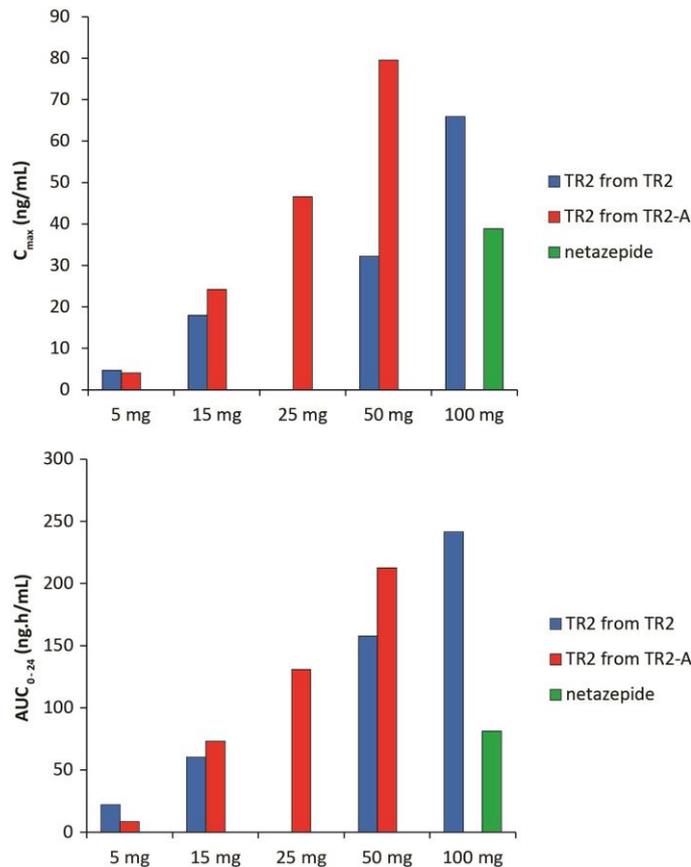


Figure 3. Mean (n = 8; sd) plasma concentrations of TR2 after single doses of (a) 5–50 mg of TR2-A; (b) 50 mg of TR2-A after fasting and food; and 5–100 mg of TR2, for comparison (data from Study 14)

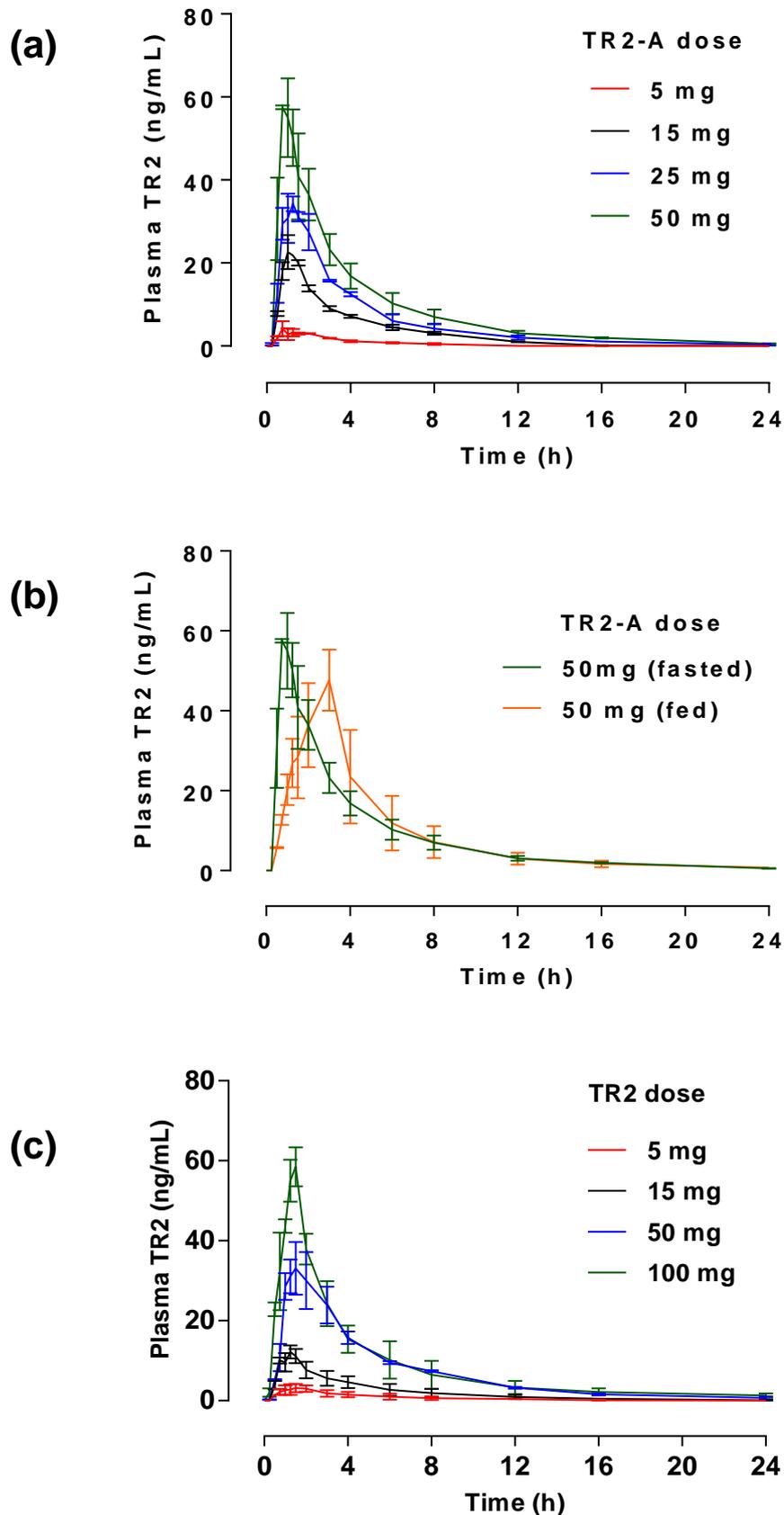
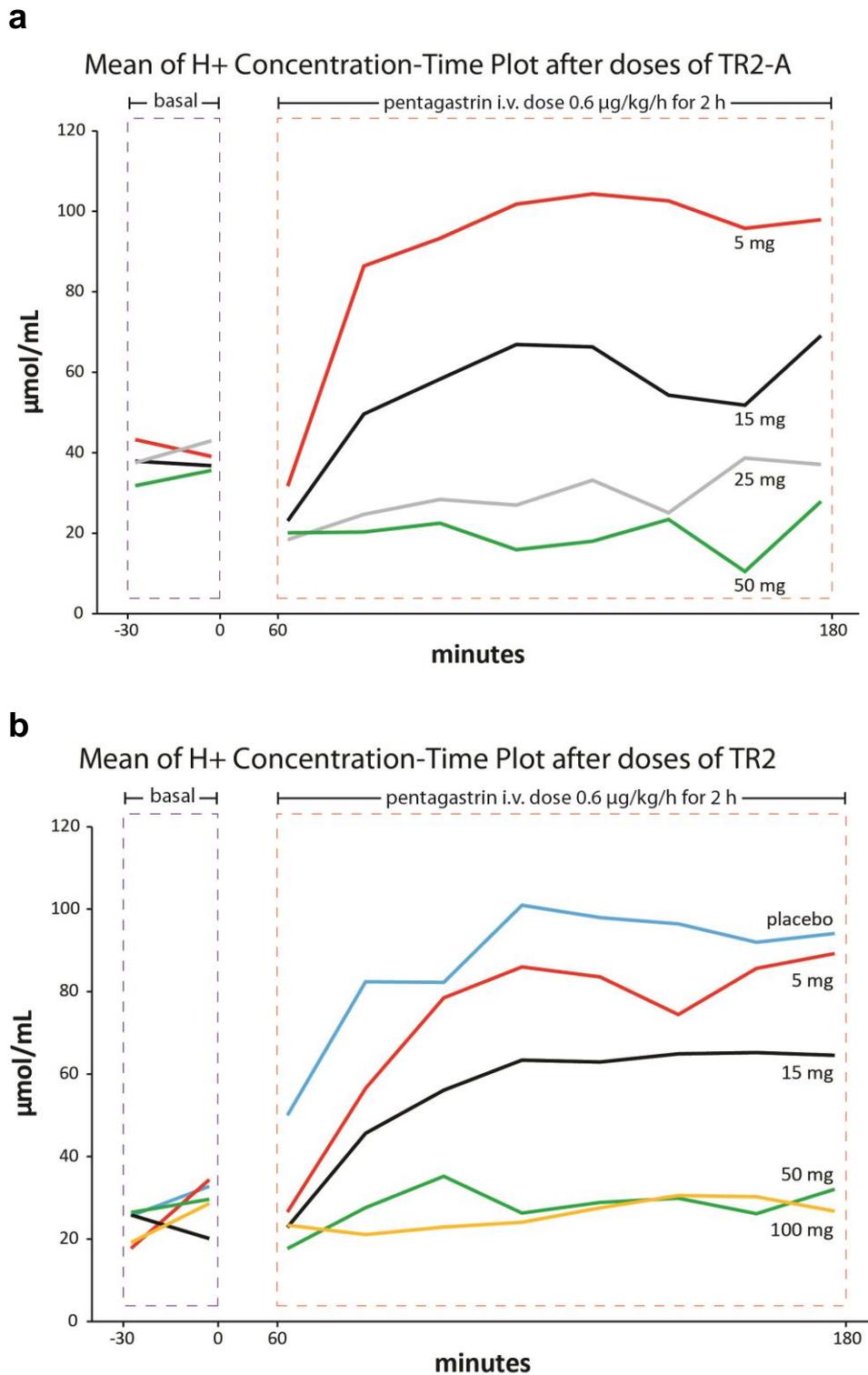


Figure 4. Mean pentagastrin-stimulated H⁺ concentration in healthy men before dosing (-30 to 0 min) and during pentagastrin infusion (60 to 180 min after dosing) in the presence of single doses of (a) 5, 15, 25 or 50 mg of TR2-A and (b) single doses of 5, 15, 50 or 100 mg of TR2 and placebo



API was administered at 0 min, after which 60 min were allowed for absorption before starting aspiration of gastric juice in 15-min epochs.

Figure 5. Time-weighted mean of H⁺ concentration (mmol/mL) before (-30-0 min) and during (60-180 min) pentagastrin infusion started 1 h after TR2-A dosing

Data for placebo from the study of TR2 (Study 14-022) is shown for comparison.

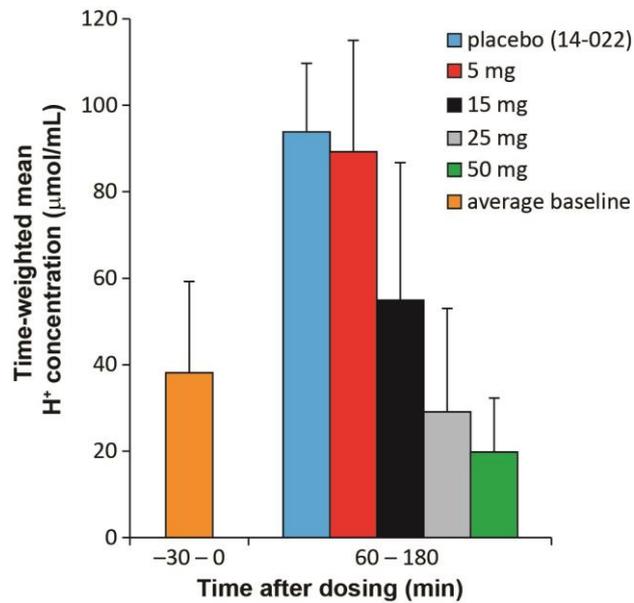
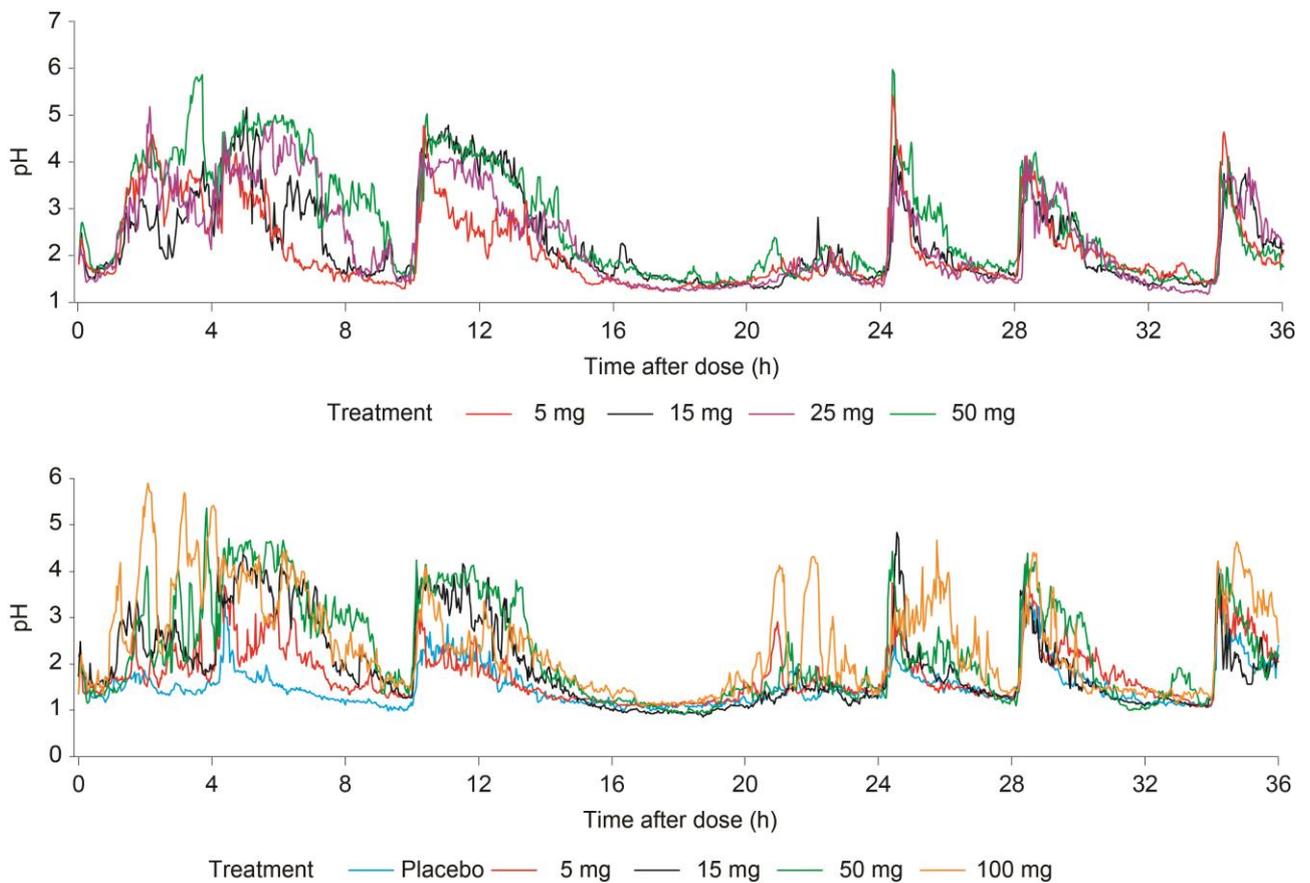


Figure 6. Comparison of gastric pH up to 36 h after single doses of TR2-A (upper plate) and TR2 (lower plate). Medians, n=8 per group



Chapter 21

Summing up

Ferring/Yamanouchi

Non-clinical studies done by Ferring, England, and Yamanouchi, Japan, showed that YF476 (netazepide) is a potent, highly selective and competitive gastrin/CCK₂ receptor antagonist (Chapter 2). Bioavailability of a simple oral formulation of active pharmaceutical ingredient mixed with methyl cellulose was 26% and 27–50% in rat and dog, respectively, when assessed by comparison of the pharmacodynamic responses to oral and intravenous doses. The 13-week toxicology studies of high doses of netazepide in rats and dogs, which were done to cover studies lasting up to 8 weeks in patients with GORD, the target disease, were unremarkable, as were the safety pharmacology studies.

The results of our first studies of netazepide in healthy subjects in 1996 were also very promising (Studies 1 and 2). Single doses caused dose-dependent, long-lasting increases in 24-h gastric pH, consistent with antagonism of gastrin/CCK₂ receptors. Plasma concentrations of netazepide were dose-proportional and 25 mg was at the top of the dose-response curve. Oral bioavailability seemed adequate. But to everyone's surprise, healthy subjects developed substantial tolerance to the effect of netazepide on gastric pH after twice daily dosing for seven days (Study 3). There was an associated increase in serum gastrin. Ferring consulted a panel of gastroenterologists who suggested testing lower doses of netazepide for longer, and that the mechanism for tolerance might be the associated increase in serum gastrin, similar to the mechanism for tolerance to repeated doses of an H₂RA (Wilder-Smith *et al* 1990). However, lower doses of netazepide for 14 days also resulted in tolerance and an associated increase in serum gastrin (Study 4). Consequently, the gastroenterologists advised Ferring against developing netazepide for GORD because, since the early studies of cimetidine in the 1970s, raising gastric pH >4 for a large part of the day has been regarded as essential for healing acid-related conditions.

Neither Ferring nor Yamanouchi did any pharmacology studies of repeated doses of netazepide in animals before starting clinical studies. Yamanouchi responded to the results from our repeated-dose studies in healthy subjects by doing repeated-dose studies in rats, which clearly showed that repeated doses of netazepide do not lead to tolerance to pentagastrin-stimulated gastric acid secretion in rats, despite an associated increase in serum gastrin (Chapter 2). Nevertheless, Yamanouchi also stopped clinical development of netazepide. And Ferring cancelled our planned study of pentagastrin in healthy subjects.

James Black Foundation

In 1999, I started to present our results at meetings of the Clinical Section of the British Pharmacological Society (Boyce *et al* 2000 and 2002). Unknown to me, the late Jim Black

was in the audience when I presented the results of the studies that showed tolerance to netazepide. We met and discussed the results. He was confident that the increase in serum gastrin was secondary to netazepide-induced hypoacidity and not the cause of tolerance. In 2001, he persuaded Johnson and Johnson (J&J), who funded research by the James Black Foundation (JBF), to license netazepide from Ferring. JBF carried out a repeated-dose study with pentagastrin in rats and, like Yamanouchi, did not observe tolerance to netazepide. So, JBF sponsored two studies in healthy subjects, which showed that single doses of netazepide cause dose-dependent inhibition of the increase in pentagastrin-stimulated acid secretion (Study 5), and that inhibition persists after repeated doses (Study 6). However, J&J decided not to fund further clinical development of netazepide, which Jim Black regarded as ‘the gold standard of gastrin/CCK₂ receptor antagonists’ (Black and Kalindjian 2002). In 2004, J&J returned netazepide to Ferring.

Yamanouchi again

In 2004, Yamanouchi licensed netazepide from Ferring again, for a spin-off company set up by former employees to develop netazepide for treatment of pancreatic cancer. JBF had shown in a small placebo-controlled trial that continuous intravenous infusion of their gastrin/CCK₂ receptor antagonist, JB95008, which had very poor oral bioavailability, prolonged the life of patients with pancreatic cancer (Black 2009). The spin-off company failed to raise money for the project and a year later returned netazepide to Ferring.

Trio

In 2006, J&J stopped funding JBF and it closed. Jim Black and I persuaded Ferring to license us netazepide. I founded Trio Medicines Ltd (Trio), to continue development of netazepide, and restarted clinical studies.

Healthy subjects

Ferring provided Trio with all available reports of non-clinical studies of netazepide and a supply of several kilos of API remaining from the batch of netazepide made for J&J when they licensed it in 2001. Regulatory requirements had changed since the early clinical studies of netazepide, and the MHRA asked for additional non-clinical studies before Trio could restart clinical studies (Chapter 2).

In the first study after licensing netazepide (Study 7), I aimed to show that it would not only inhibit the effect of gastrin on gastric acid production but would also inhibit the trophic effect of gastrin. Ferring and Yamanouchi had only ever studied its effect on acid secretion. However, by 2006 academia had published various non-clinical studies of the effect of a

GRA, mostly netazepide, on the trophic effects of gastrin (Chapter 1). Furthermore, clinicians were becoming interested in the associated effects of PPI-induced hypergastrinaemia, such as rebound hyperacidity. A PubMed search currently yields 31 papers on non-clinical studies of netazepide. All but two are about the trophic effects of gastrin.

Therefore, I designed a study (Study 7) of netazepide and rabeprazole, alone and in combination, to assess the effect of netazepide on pentagastrin-stimulated gastric acid production and the trophic effect of PPI-induced hypergastrinaemia, which showed that:

- netazepide suppresses pentagastrin-stimulated acid secretion as effectively as a PPI;
- the combination is more effective than either treatment alone at suppressing the response to pentagastrin;
- netazepide reduces plasma CgA – a sign of ECL-cell hypoactivity – whereas rabeprazole increases plasma CgA – a sign of ECL-cell hyperactivity; and
- netazepide prevents the increase in CgA resulting from PPI-induced hypergastrinaemia.

Thus, netazepide inhibited the dual effects of gastrin – stimulation of acid secretion and ECL-cell activity – although there was evidence neither of ECL-cell hyperplasia in gastric biopsies during rabeprazole treatment nor of rebound hyperacidity after its withdrawal.

However, despite the favourable pharmacodynamic effects, plasma concentrations of netazepide were very low compared with those in Studies 1–4. We confirmed the low bioavailability of netazepide in Study 8. In contrast, a similar formulation had had good bioavailability in the early non-clinical studies. Thereafter began a two-year period during which we investigated the cause of the low bioavailability in man. It turned out that netazepide used in Study 7, and in the preceding Studies 5 and 6 sponsored by JBF, was crystalline, whereas netazepide used in the early studies in healthy subjects (Studies 1 and 2) was spray dried, amorphous and more bioavailable. Plasma concentrations of netazepide in samples from Study 6, sponsored by JBF, were not assayed until long after the end of the study. At the time, we didn't spot that the concentrations were low compared with those from the early studies, possibly because the pharmacodynamic results were so good.

Finding out the cause of netazepide's low bioavailability would have taken even longer without my self experiments (Chapter 8). The Royal College of Physicians guidelines on studies in healthy volunteers recognise the long tradition of physicians experimenting on themselves, but advised that such experiments should be approved by an ethics committee (RCP 1986). In that respect, many netazepide studies in healthy volunteers have been approved by ethics committees. The Nobel prizes for medicine in 1956, 2005 and 2011 were awarded to physicians who experimented on themselves (Collins 2013).

Finally, after trying several methods of improving the bioavailability of netazepide, we settled for spray drying. Since we restarted clinical studies of netazepide in 2009, we have used spray-dried material for all of them. A spray-dried formulation:

- has dose-proportional and linear pharmacokinetics (Study 9);
- has an absolute bioavailability of <15% (Study 10);
- prevents the increase in plasma CgA caused by esomeprazole-induced hypergastrinaemia (Study 11);
- does not interact with midazolam (Study 12), a substrate for CYP3A4 – an enzyme for which netazepide is a weak inhibitor in human cells *in vitro* – thus allowing netazepide to be coadministered with existing medicines metabolised via that enzyme; and
- is metabolised mainly to its hydroxy metabolite, TR2, exposure to which is substantial and similar to that of netazepide (Study 13).

We have used several new batches of spray-dried netazepide since we restarted clinical studies. I did an n = 1 study of each batch before using it, to check its bioavailability (Chapter 8). There was variability even between batches of spray-dried material, and not all batches were as bioavailable as the netazepide used in the early studies.

Patients

Our studies in healthy subjects prepared the way for two studies in 8 patients per centre with CAG, achlorhydria, hypergastrinaemia, multiple gastric NETs, and raised circulating CgA (Chapter 14). Overall, netazepide 50 mg once daily by mouth for 12 weeks:

- reduced the number of tumours and size of the largest one;
- normalised serum CgA, which returned to pre-treatment levels after stopping netazepide, but did not increase serum gastrin further, confirming that all patients had achlorhydria;
- normalised raised mRNA abundancies of gastrin-dependent biomarkers in tumour biopsies, which returned to pre-treatment levels after stopping netazepide; and
- suppressed miR-222 overexpression in gastric biopsies of CAG patients with NETs.

In extensions of those two studies, which began after patients had been off treatment for a mean of 14 (range 8–19) months, 13 of the 16 patients consented to take netazepide 25 or 50 mg once daily for another 52 weeks. Tumours regrew and CgA increased again in the interval off treatment. During the study extensions, netazepide eradicated the tumours of 5 out of 13 patients and reduced the number and size of the largest one in the others. The effect on increased CgA and gastrin was the same as that in the 12-week studies. Some patients continue to take netazepide; to date, the duration is up to 36 months. Netazepide has been well tolerated, as it has been in all clinical studies so far (Chapter 15).

TR2 and TR2-A

The early studies of netazepide in 1996 showed that it increases gastric pH beyond 24 h after dosing, at a time when there is little or no netazepide in the circulation. I wondered then whether the long duration of activity of netazepide might be due to an active metabolite or covalent binding to the gastrin/CCK₂ receptor. In Study 10, the much slower decline of total radioactivity after dosing with intravenous ¹⁴C-netazepide compared with the decline in radioactivity of unchanged ¹⁴C-netazepide is consistent with a metabolite persisting after clearance of the parent compound. In a non-clinical study of ¹⁴C-labelled netazepide in human, rat and dog liver microsomes *in vitro*, one metabolite occurred in larger amounts in human than in the other species (Chapter 3). So, given that netazepide's patent expires worldwide in 2016, and that there are several other indications for GRA treatment apart from gastric NETs, together with two other healthy subjects I took part in a study of a large single dose of netazepide 500 mg, and a therapeutic dose of netazepide 50 mg once daily for three days (Study 13) with the aim of isolating and characterising the metabolites of netazepide, to find out if they were active and worthy of further development. We each ate a high-fat breakfast before dosing in order to increase exposure to netazepide. The study identified four metabolites. The hydroxy derivative of netazepide, which we called TR2, was the main metabolite. Non-clinical studies showed that TR2 is: more selective than netazepide for the gastrin/ CCK₂ receptor over the CCK₁ receptor, but slightly less potent than netazepide for the gastrin/ CCK₂ receptor; and 10 times more soluble than netazepide. And TR2 was not covered by existing patents.

Thus, TR2 appeared to be a possible successor to netazepide and to merit clinical studies. So, for a second time I did several self experiments (Chapter 17), which showed that TR2 is a gastrin/CCK₂ receptor antagonist and that it might be as potent as netazepide at similar exposures. However, despite TR2 being more soluble than netazepide, the bioavailability of TR2 was still low. Furthermore, its pharmacokinetic profile does not easily explain the pharmacodynamic activity of single doses of netazepide on gastric pH beyond 24 h in the early studies. We proceeded to a formal study in healthy subjects (Study 14), which confirmed my n = 1 findings. Thus, TR2 probably contributes substantially to the activity of netazepide as a gastrin/CCK₂ receptor antagonist.

The penultimate stage in the synthesis of TR2 is an acetyl derivative, TR2-A, which is 90 times more soluble than netazepide. So, I wondered whether TR2-A might be more bioavailable than TR2. To find out, for a third time I did several n = 1 experiments

(Chapter 19). The $AUC_{0-24\text{ h}}$ of TR2 after a single dose of TR2-A 100 mg was about twice that after a single dose of a similar formulation of TR2 100 mg. Only tiny amounts of TR2-A appeared in the circulation, so TR2-A is a prodrug of TR2. Again, we proceeded to a formal study in healthy subjects (Study 15), which confirmed my $n = 1$ findings. Further studies are required to characterise the clinical pharmacology of TR2-A, but those must await completion of toxicology studies.

Thus, TR2-A is a more worthy successor to netazepide than TR2. The advantages of TR2-A over TR2 and netazepide are summarised in Table 1. In August 2014, Trio applied for a patent for TR2-A and related compounds. In October 2015, the European Patent Office described our claim for TR2, TR2-A and related compounds as “novel, inventive and industrially competitive”. Unless there are unforeseen problems with TR2-A, I do not intend to develop netazepide further.

Potential indications for TR2-A

The potential indications for TR2-A are: prevention and treatment of hypergastrinaemia; treatment of acid-related conditions; and prevention and/or treatment of conditions in which gastrin/CCK₂ receptors are overexpressed, such as Barrett’s oesophagus.

The causes of hypergastrinaemia are:

- hypoacidity due to CAG;
- hypoacidity due to mutations in *KCNQ1*, *KCHE1* and *ATP4A* genes that control acid secretion;
- hypoacidity due to *H. pylori*-induced gastric atrophy;
- *H. pylori*-induced inflammation of the antrum without hypoacidity;
- hypoacidity due to H₂RA, PPI or potassium-competitive acid inhibitor, or vagotomy;
- incomplete antrectomy done for treatment of hypergastrinaemia;
- cysteamine therapy for children with cystinosis; and
- Zollinger-Ellison syndrome, which consists of a gastrinoma, hypergastrinaemia, hypersecretion of gastric acid and severe peptic ulceration.

1. CAG and ZES-induced hypergastrinaemia

CAG and ZES-induced hypergastrinaemia can lead to growth of ECL cells to form gastric NETs type 1 and 2, respectively. The risk of malignancy is higher for type 2 tumours, but all gastric NETs have the potential for malignancy (Chapter 13). Gastric NETs are rare tumours – the prevalence per 10,000 people in Europe, USA and Japan is 0.32, 0.17 and 0.05, respectively. On the basis of their rarity and life-threatening potential, the EMA and

FDA have designated netazepide an orphan medicinal product (OMP) for treatment of gastric NETs, which gives Trio marketing exclusivity in Europe for 10 years and in the USA for 7 years. The EMA designated TR2-A an OMP for treatment of gastric NETs in November 2015. The potential market is small, but pernicious anaemia (PA), which is one of the clinical presentations of CAG, has a higher prevalence, about 1 per 1,000 people. As well as being at risk of developing gastric NETs, patients with PA have a seven-fold increased risk of developing gastric adenocarcinoma. TR2-A might help prevent both types of tumour. There is increasing evidence, particularly from the children with mutations of *KCNQ1* or *KCNE1* and *ATP4A* genes that control acid secretion, that hypergastrinaemia has malignant potential, so tumour treatment or prevention in such patients is a worthwhile aim. Overexpression of miR-222 by excessive gastrin targets the tumour suppressor and oncogene, p27, which may be the mechanism for the harmful effect of hypergastrinaemia, which netazepide prevents (Lloyd *et al* 2015). P27 loss is associated with a poor prognosis in patients with GEP NETs (Kim *et al* 2014).

2. PPI-induced hypergastrinaemia

Although PPI are safe medicines, PPI-induced hypergastrinaemia has been reported to cause: ECL- and parietal-cell hyperplasia; fundic gland polyps; increased risk of bone fractures; rebound hyperacidity and dyspepsia after PPI withdrawal; and malignant ECL-cell tumours in case reports.

(i). ECL-cell hyperplasia

Lundell *et al* (2015) recently reviewed the effects of long-term PPI use, defined as >3 years, on serum gastrin and gastric histopathology in response to isolated case reports in which gastric NETs and gastric cancer were deemed related to long-term PPI-induced hypergastrinaemia (Jianu *et al* 2012 and 2012). In 1,920 patients from 16 studies of patients on long-term PPI therapy, mean gastrin levels increased by one to three times the upper limit of the normal range (~100 pg/mL), and the prevalence of ECL-cell hyperplasia increased by 7.8–52.0%. *H. pylori* positive patients had a much higher risk (OR 11.5) of ECL-cell hyperplasia and corpus atrophy. But, there was no evidence of neoplasia in gastric biopsies and no patient had gastric NETs or adenocarcinoma.

TR2-A treatment should not cause ECL-cell hyperplasia and should prevent the ECL-cell hyperplasia associated with long-term PPI therapy, but specific studies to demonstrate those effects would not be worthwhile initially.

(ii). Fundic gland polyps

Fundic gland polyps are found at gastroscopy and in the majority of cases are considered benign. They are regarded as a nuisance and not harmful. There is evidence that some cases are related to long-term PPI therapy (Jalving *et al* 2016), so they merit a trial of TR2-A.

(iii). Bone fractures

The association of PPI therapy with bone fractures was first reported in two large studies in 2006 (Yang *et al* 2006; Vestergaard *et al* 2006). Meta-analyses show that the risk of bone fractures is modestly but significantly raised (odds ratio 2.65) (Eom *et al* 2011). It has been known for many years that PPIs affect bone absorption, but the mechanism is still unknown. An effect of PPI-induced hypergastrinaemia on the vacuolar type H⁺-ATPase on osteoclasts that mediates acidification of intracellular organelles (Nishi and Forgac 2002) is among the various hypotheses (Ito and Jensen 2010). The risk of bone fractures is increased in patients with pernicious anaemia (Goerss *et al* 1992), and the risk of osteoporosis is increased in patients with chronic atrophic gastritis (Kim *et al* 2014), which would exclude a direct effect of PPIs on bone homeostasis as the cause, because both types of patient have hypoacidity and secondary hypergastrinaemia.

Gastrin/CCK₂ receptors are expressed only in scarce amounts outside of the gastric mucosa and brain, and have not been found in bone (Herrmann *et al* 2007). Aasarød *et al* 2015 showed that H⁺/K⁺-ATPase β-subunit knock-out mice with hypergastrinaemia develop bone loss and deterioration of bone quality, which was partly prevented by netazepide. Netazepide caused hypoacidity and secondary hypergastrinaemia in control animals, as expected, but no bone changes, which would suggest that hypergastrinaemia rather than hypoacidity is the mechanism for impaired bone quality in PPI users.

It has been known for many years that systemic mastocytosis, a condition with elevated histamine, predisposes patients to osteoporosis. A histamine H₁-receptor antagonist (H₁RA) can correct the bone changes in such patients (Graves *et al* 1990). Also, patients taking a PPI with an H₁RA for allergies had a lower risk of bone fractures than non-H₁RA users (Abrahansen and Vestergaard 2013). The effect of PPIs on calcium absorption is controversial (Ito and Jensen 2010). Several studies have shown that gastrin releases a peptide, named gastrocalcin but never characterised, from the acid-secreting part of the stomach that lowers serum calcium (Persson *et al* 1989).

Thus, combining TR2-A with a PPI might reduce the increased risk of fractures after long-term PPI therapy. A combination of TR2-A and an H₁RA might be even better at reducing the risk. However, to show a reduced risk would require a trial in many patients. A study of bone

biomarkers could be done in far fewer patients. The increased risk of fractures attracts more attention in the literature than any of the other associated effects of long-term PPI therapy.

(iv). Rebound hyperacidity

Rebound hyperacidity occurs in rats after PPI withdrawal (Nishida *et al* 1995), but whether it occurs in patients after withdrawal of long-term PPI therapy is still in doubt (Hunfield *et al* 2007). We did not observe rebound hyperacidity after withdrawal of rabeprazole in our healthy subjects (Study 7). Nor did our healthy subjects report dyspepsia after withdrawal of rabeprazole (Study 7) or esomeprazole (Study 11), unlike healthy subjects in published trials (Reimer *et al* 2009; Niklassen *et al* 2010). The increases in circulating gastrin and CgA induced by esomeprazole in healthy subjects in Study 11 settled within a few days of esomeprazole withdrawal, which is not consistent with rebound hyperacidity, because it is reported to occur around two weeks after PPI withdrawal. A study of TR2-A in patients withdrawn from long-term PPI therapy would help assess whether rebound dyspepsia is real, and if so, its clinical relevance.

3. Mutations in *KCNQ1*, *KCNE1* and *ATP4A* genes

There have been recent reports of gene mutations giving rise to type 1 gastric NETs. First, Winbo *et al* (2013) reported that patients with the rare Jervell and Lange-Nielsen (JLN) syndrome (congenital hearing loss, long QT interval and high risk of ventricular tachyarrhythmias) also have iron deficiency anaemia, gastric hyperplasia and hypergastrinaemia secondary to hypochlorhydria caused by a mutation of the *KCNQ1* (90% patients) or *KCNE1* (10%) potassium channel gene, which is essential for gastric acid secretion. Some patients had gastric cancer. Rice *et al* (2011) had earlier reported a patient with JLN syndrome who had achlorhydria, hypergastrinaemia and multiple ECL tumours, so the gastric hyperplasia reported by Winbo *et al* (2013) probably represents type 1 NETs too. Furthermore, hypergastrinaemia in such patients appears to be a risk factor for subsequent gastric cancer.

Second, exome sequencing of a family with consanguineous parents and ten children, five of whom had type 1 gastric NETs, identified a mutation of the *ATP4A* gene (Calvete *et al* 2015), which encodes the proton pump responsible for acid secretion by gastric parietal cells. All five children had hypergastrinaemia and three had nodal infiltration, one of whom had gastric adenocarcinoma. All five also had iron deficiency anaemia. These genetic mutations provide strong evidence that hypergastrinaemia has malignant potential. All type 1 tumours have the potential for malignancy especially ones that are >2 cm in size, infiltrate the muscularis propria, are angioinvasive and/or are G2 grade (Rappel *et al* 1995).

JLN syndrome is an autosomal recessive condition and rare; it affects an estimated 1.6 to 6 per million people worldwide. The prevalence is high in Denmark, Norway and Sweden, where it affects at least 1 in 200,000 people (Winbo *et al* 2014; Tranebjaerg *et al* 2015). JLN syndrome merits a trial of TR2-A in patients with gastric NETs, if only to test its effect on raised CgA, which surely these patients will have.

4. *H. pylori* infection

H. pylori infection is common and a major risk factor for peptic ulcer disease and gastric cancer (Helicobacter and Cancer Collaborative Group 2001). Netazepide completely prevented the inflammatory response of the gastric mucosa to *H. pylori* infection in Mongolian gerbils (Sørdal *et al* 2013). TR2-A, with or without a PPI, could be a useful addition to the regimen of antibiotics for eradication of *H. pylori* infection in patients. Trio started a trial of netazepide, clarythromycin and ampicillin in *H. pylori* positive healthy subjects in 2009, but stopped after treating only one subject when Study 7 revealed the low bioavailability of the netazepide formulation. Since then, Sørdal *et al* 2013 have published the results of their study of *H. pylori* infected Mongolian gerbils. A trial of TR2-A to assess its effect on the histology of gastric mucosal biopsies from healthy subjects who are *H. pylori* positive is a 'must do' trial.

5. Acid-related conditions

Although single doses of netazepide cause dose-dependent increases in gastric pH (Study 2), tolerance develops after repeated doses (Studies 3 and 4), despite netazepide causing dose dependent and persistent inhibition of basal and stimulated gastric acid secretion (Studies 5 and 6). We don't know the mechanism of the tolerance, and we don't know if it is a class effect or specific to netazepide, because Trio has studied neither repeated doses of TR2 nor TR2-A. And we don't know if tolerance matters therapeutically. Tolerance to the increase in gastric pH induced by netazepide developed neither in rats after 5 days' dosing (Webb *et al* 2013) nor in Mongolian gerbils after one year's dosing (Sordal *et al* 2013). A gastrin/CCK₂ receptor antagonist, such as TR2-A, might still prove a useful treatment for acid-related conditions. Recent studies with vonoprazan (TAK-438), a potassium-competitive acid inhibitor that binds selectively and reversibly to H⁺, K⁺-ATPase without acid activation (Scott *et al* 2015), would suggest that gastric pH>4 is not an essential determinant of healing rates in acid-related diseases. In healthy Japanese and UK men, vonoprazan 40 mg once daily for 7 days increased mean 24-h intragastric pH>4 holding time ratio (HTR) by a remarkable 100% and 93.2%, respectively, and mean night-time pH>4 HTR by 100% and 90.4%, respectively. Serum gastrin increased with dose (Jenkins

et al 2015). Thus, vonoprazan raises gastric pH>4 much more effectively than a PPI (see Study 3 for comparison). Nevertheless, in a multicentre study of vonoprazan versus lansoprazole in 732 patients with erosive oesophagitis (EO), Los Angeles (LA) grades A–D, vonoprazan was no better than the PPI. The proportion of healed EO patients after 4 weeks' treatment with vonoprazan 5, 10, 20 and 40 mg and lansoprazole 30 mg was 92.3, 92.5, 94.4, 97.0 and 93.2%, respectively. All vonoprazan doses were non-inferior to lansoprazole when adjusted for baseline LA grades A/B and C/D (Ashida *et al* 2015).

So, the best result that we could hope to achieve from a large trial of TR2-A versus a PPI in GORD patients is non-inferiority. However, a smaller trial of TR2-A and a PPI, alone and in combination, is worth doing. TR2-A alone should be free of the unwanted effects of PPI-induced hypergastrinaemia, and combining it with a PPI should prevent the unwanted effects of the PPI, and may improve acid control. If TR2-A treatment for 4 or 8 weeks were to heal oesophageal erosions without increasing pH≥4, which it might, that would cause gastroenterologists to take TR2-A seriously.

6. Cysteamine therapy for children with cystinosis

Cystinosis is a rare autosomal recessive condition in which the amino acid cystine accumulates in cells in the kidneys, liver, eyes, muscles and brain, causing early cell death. About 1 in 200,000 births are affected. About 500 people, mostly children, have the condition in the USA, and an estimated 2,000 have the condition worldwide (<http://www.cystinosisresearch.org>). Cysteamine (Cystagon[®]) slows the progression of cystinosis, by removing the cystine from cells, and is an orphan drug. Unfortunately, it has a very bad taste and smell and must be taken every six hours, every day. In laboratory animals, cysteamine increases serum gastrin and gastric acid production, and is ulcerogenic (Kirkegaard *et al* 1980). In children with cystinosis, cysteamine caused a 3-fold increase in gastric acid production and a 50% rise of serum gastrin levels above baseline (Dohil *et al* 2003). As a consequence, some patients with cystinosis on cysteamine therapy suffer gastrointestinal symptoms and are unable to take it regularly or at full dose. Some children are treated with a PPI (Dohil *et al* 2005). A new, delayed-release formulation of cysteamine (Procysbi[®]) can be taken twice daily, so patients can sleep through the night, and it reduces gastrointestinal side effects (Dohil *et al* 2010). However, some patients still require PPI therapy. TR2-A, with or without a PPI, is a potential therapy for such children.

7. Expression of gastrin/CCK₂ receptors on cells other than ECL cells

Gastrin/CCK₂ receptors or a splice variant are expressed on cells of some patients with pancreatic cancer (Smith *et al* 2015), Barrett's oesophagus (Haigh *et al* 2003), gastrointestinal stromal tumours (GIST) (Quattrone *et al* 2012), medullary thyroid cancer (Blaker *et al* 2002), colonic cancer (Watson *et al* 2002) and other types of cell. TR2-A provides a means to assess the role of those receptors in the disease process. Berna and Jensen 2007 reviewed the potential role of gastrin/CCK₂ receptors in gastrointestinal/metabolic diseases. Gastrin/CCK₂ receptors are found in the CNS and spinal cord. There is evidence that CCK₂ antagonists, such as L-365,260, enhance opioid analgesia in animal and human pain models (McCleane 2004).

(a). Cancer of the pancreas

Cancer of the pancreas is very difficult to treat. It rarely causes symptoms in its early stages, so it's often not detected until it's quite advanced. Not all patients are eligible for surgery, which isn't always successful anyway. The chemotherapy drugs used most often are gemcitabine and 5-fluorouracil (5-FU). They are not very effective. JB95008, a GRA from the James Black Foundation, prolonged life in a small placebo-controlled trial in patients with pancreatic cancer. A second trial showed no difference between JB95008 and 5-FU. JB95008 had to be given by continuous intravenous infusion because of its negligible bioavailability after oral dosing, so development was stopped (Black 2009). A trial of TR2-A in patients with pancreatic cancer would have to be add-on therapy to gemcitabine or 5-FU. If it's to be undertaken, it's best left until late phase 3 or phase 4 of development.

(b). Barrett's oesophagus

Cells of Barrett's oesophagus express gastrin/CCK₂ receptors (Haigh *et al* 2003). Netazepide prevented hypergastrinaemia-induced lesions in mice similar to those of patients with Barrett's oesophagus (Quante *et al* 2012). The main risk factor for Barrett's oesophagus is reflux of gastric juices into the oesophagus, and therefore the usual treatment is a PPI. PPI-induced hypergastrinaemia is associated with advanced oesophageal adenocarcinoma in patients with Barrett's oesophagus (Wang *et al* 2010). Combining TR2-A with a PPI should increase acid suppression by the PPI alone and inhibit any trophic effect of the associated hypergastrinaemia. A study, sponsored by Trio, is in progress in the USA and UK to assess the effect of netazepide on biomarkers in patients with Barrett's oesophagus treated with a PPI. A positive result would merit a trial of TR2-A as preventative treatment in patients with Barrett's oesophagus, but such a trial would have to be large and best done in late phase 4 of development. Oesophageal intestinal metaplasia

recurred in 33% of patients with Barrett's oesophagus who underwent endoscopic mucosal resection and radiofrequency ablation of their lesions (Gupta *et al* 2013). A trial in patients who have undergone such therapy, to assess the effect of TR2-A on recurrence of lesions, would require fewer patients and could be done in phase 3.

(c.) *Gastrointestinal stromal tumours*

Gastrointestinal stromal tumours (GISTs) occur mainly in the stomach wall. The majority are driven by oncogenic mutations. Tumours that cannot be removed surgically are treated with a tyrosine kinase inhibitor, such as imatinib. GISTs can 'escape' imatinib treatment. Quattrone *et al* 2012 showed that nude mice with human GIST xenografts *in vivo* express CCK₂ receptors. Gastrin increased tumour volume, mitotic index and Ki-67 index, and stimulated kinase pathways. CCK₂ receptors were involved in the hyperplasia and early stages of gastric GIST development. Thus, TR2-A may have a role in the management of patients with GIST.

(d.) *Gastric adenocarcinoma*

Gastric cancer usually presents at an advanced stage. Surgery can be curative for early tumours, but few patients present at an early stage. Many patients with gastric cancer have raised serum gastrin (Goetze *et al* 2013; Fossmark *et al* 2015). Alpha-amidated gastrins and their receptors are detectable in 80% of gastric adenocarcinomas (Goetze *et al* 2013). Hypergastrinaemia is associated with adenocarcinomas in the gastric corpus and shorter patient survival (Fossmark *et al* 2015). Netazepide is effective in animal models of gastric cancer (Cui *et al* 2006). However, whether there's a role for a TR2-A in the treatment of patients with gastric cancer is another matter. TR2-A has greater potential as a preventative medicine in patients with increased risk of gastric cancer, such as pernicious anaemia, atrophic gastritis and mutations of the genes that control gastric acid secretion.

Market potential for TR2-A

There is an unmet need and many potential indications for a gastrin/CCK₂ receptor antagonist, and the commercial potential is substantial. The short remaining patent life of netazepide was a disincentive to develop it for indications other than gastric NETs, for which it has marketing exclusivity as an OMP. TR2-A will have a 20-year patent, so it merits development for other indications as well as for treatment of gastric NETs. For example, prevention of gastric NETs and gastric cancer in at-risk patients is a bigger potential market. A combination of TR2-A and a PPI would give a new lease of life to the PPIs, which are now all off patent. An estimated 5% of people in western countries take a PPI; many do so long-term. The market for PPI, mainly for GORD, is huge: \$24 billion a

year. If TR2-A could steal at least some of that market, it would be a commercial success. If TR2-A were to behave in patients with *H. pylori* infection and Barrett's oesophagus, as it does in animal models, that would really expand the market. Trio will need a partner to take TR2-A through to the market.

Future plans for TR2-A

I plan to fast-track the development of TR2-A for treatment of gastric NETs on the basis of experience with netazepide (Figures 1 & 2). Phase 1 studies should start in 2016. A phase 2/3 study in patients with gastric NETs could start second quarter 2017. That trial would be a large, multicentre study of TR2-A and placebo (ratio 5:1) in ENETS Centres of Excellence (www.enets.org/center_list.html) in countries of Europe (Belgium, Denmark, France, Germany, Italy, The Netherlands, Norway, Sweden, Switzerland and UK), and in centres in the USA and Japan. The EMA designated TR2-A an OMP for treatment of gastric NETs in November 2015. They provide free advice about protocol design for OMP studies.

Prosynth, Suffolk, UK, is currently manufacturing a bulk supply of TR2-A for making a tablet formulation and for doing: toxicology studies for 4-weeks in rats and minipigs, to support phase 1 studies; toxicology studies for 6- and 9-months in rats and minipigs, respectively, to support phase 2/3 studies; and safety pharmacology studies, to support all studies. The minipig is the non-rodent species because dogs metabolise TR2 differently from humans (Chapter 2). Some of the studies have started.

There are exciting times ahead for TR2-A development.

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Table 1. Comparison of netazepide, TR2 and TR2-A

Characteristic	Netazepide	TR2	TR2-A
Human CCK ₂ R binding (pIC ₅₀)	9.3 M	8.6 M	8.6 M
Affinity for human CCK ₂ R vs CCK ₁ R	370 fold	480 fold	480 fold
Solubility at pH 4–6 vs netazepide	–	↑ 10 fold	↑ 90 fold
Bioavailability in HV vs netazepide*	–	↑	↑↑
Dose required to abolish response to PG infusion in HV*	100 mg	100 mg	50 mg
Dose required to increase 24-h gastric pH maximally	25 mg**	>100 mg*	>50 mg*
Patent	Ends 2016	Until 2035	Until 2035

HV = healthy volunteers. PG = i.v. pentagastrin 0.6 µg/kg/h for 2 h.

*Crystalline IMP. **Spray-dried IMP.

Figure 1. Expected dates of draft reports of non-clinical studies of TR2-A

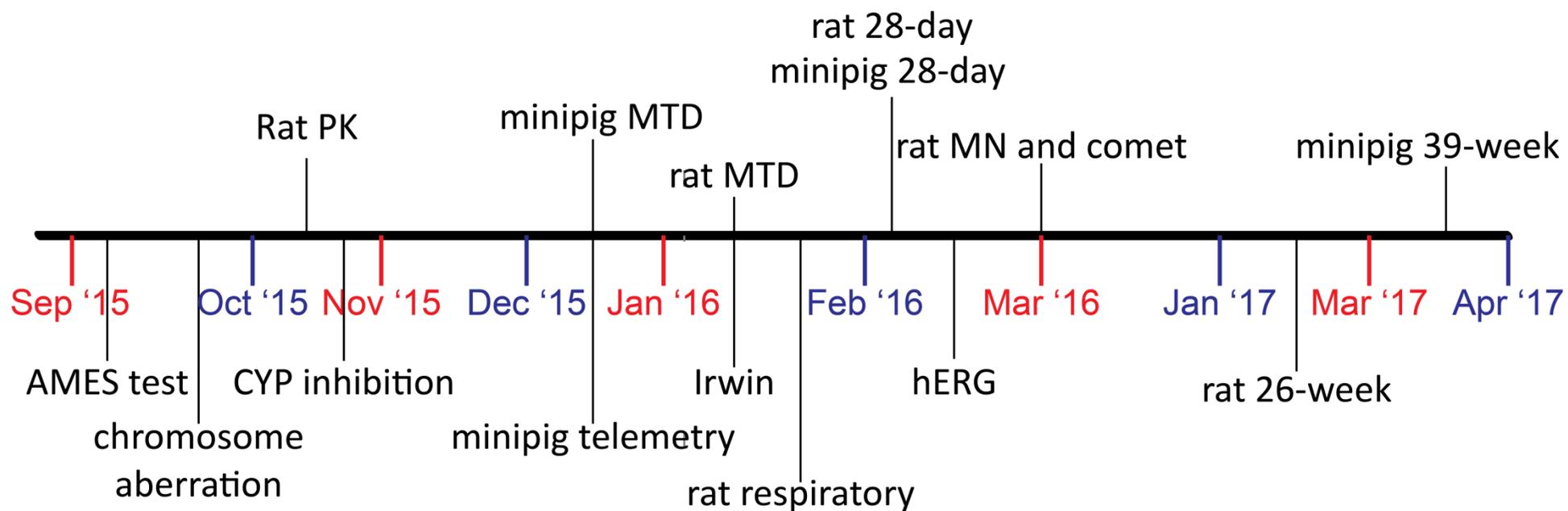


Figure 2. TR2-A and gastric NETs: clinical development plan

