The Role of SLC4A4 in the Pathogenesis of NSAID-induced Upper Gastro-intestinal Injury

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By

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Declaration

This thesis is the result of my own work; and the material contained herein has not been presented, previously or currently, either wholly or in part for any other degree or qualification. I declare that appropriate credit is given where the ideas or expressions of others have been used.

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed analgesic and anti-inflammatory agents. However, they are often associated with serious adverse events, including upper gastrointestinal (GI) injuries caused by acid-back diffusion into the GI mucosa. The electrogenic sodium bicarbonate co-transporter (NBCe1) protein, encoded by the solute carrier family, member 4 (SLC4A4) gene mediates transportation of bicarbonate ions and contributes to regulation of intracellular pH. It was hypothesised that impaired function of the SLC4A4 gene may have a role in the pathogenesis of gastric injury resulting from NSAID exposure. This study aimed to investigate the role of SLC4A4 in NSAID-related GI ulceration.

Initial analysis from a genome-wide association study identified genetic variants within the SLC4A4 locus on chromosome 4 as putative risk factors for aspirin-induced upper GI ulceration (185 cases, 217 controls). Native and SLC4A4-transfected human gastric adenocarcinoma cell lines (AGS) were used as in vitro models to study NBCe1 transporter activity and the mechanisms of cell death by aspirin and other NSAIDs. SLC4A4 expression in AGS cells was determined using TaqMan real time-polymerase chain reaction (PCR) and western blotting. The effects of S0859, a sodium bicarbonate transport inhibitor, and 4 NSAIDs (aspirin, ketoprofen, diclofenac and naproxen) on accumulation of bicarbonate in AGS cells were assessed using 14C-labelled $NaHCO_3$.

In *H. Pylori* negative aspirin-induced gastric ulceration, rs4521314, an intronic SNP within the SLC4A4 locus was associated with ulcer risk (p=7.5x10-5; heterozygous OR (95% CI) = 2.33 (1.44-3.80). RNA sequencing data (n = 10 cases, 10 controls) showed that the SLC4A4 AA variant was associated with higher transcript levels in gastric biopsy taken from the antrum of aspirin tolerant controls (AA vs. AC allele; p = 0.01). This study was therefore also carried out in a larger cohort of control gastric corpus biopsies (n=93), but did not show a difference in expression of SLC4A4 mRNA between the groups with different genotypes.

A concentration-dependent (0-50mM) fall in cell viability was recorded in aspirin-exposed cells (p = 0.006), with ~50% reduction at 50mM. Further investigation revealed that this does not occur solely via the caspase (3/7)-dependent pathway as lower amounts of caspases were detected with increasing aspirin concentrations. Transport studies showed that in transfected AGS cells, treatment with S0859 [100μM], as well as with all the NSAIDs studied, significantly inhibited bicarbonate uptake at 30 minutes (p < 0.05) compared to untreated transfected cells, suggesting that NSAIDs inhibit the influx of bicarbonate which may have a potential role in GI injury pathogenesis.

Taken collectively, the data suggest that reduced bicarbonate transport, which could be due to genetic variants and inhibition by concomitantly administered NSAIDs may have a role in GI injury. Further work should evaluate the role of genetic variations in the SLC4A4 locus, and other bicarbonate transporters, in the pathogenesis of NSAID-induced upper GI ulceration. The work should also encompass class-specific differences in the interactions of different NSAIDs on the bicarbonate transporters.

Abbreviations

Acronym	Meaning
ABC	ATP-binding cassette transporter
ADME	Absorption, distribution, metabolism, excretion
ADR	Adverse drug reaction
AE	Anion exchanger
AGS	Atypical gastrin secreting cells
ANOVA	Analysis of variance
bp	Base pair
BSA	Bovine serum albumin
CA	Carbonic anhydrase
cDNA	Complementary DNA
CEU	Northern Europeans from Utah
CI	Confidence interval
COX	Cyclooxygenase
CYP	Cytochrome P450 enzyme
DEVD	Asp-Glu-Val-Asp amino acid sequence, caspase cleavage site
DID	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid
DILI	Drug-induced liver injury
DME	Drug metabolising enzyme
DMEM	Dulbecco Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide triphosphate
DPM	Disintegrations per minute
DRESS	Drug rash with eosinophilia and systemic symptoms
FAM	6-carboxyfluorescein
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
GI	Gastro-intestinal
GWAS	Genome wide association study
HBSS	Hank's Balanced Salt Solution
HWE	Hardy-Weinberg equilibrium

Acronym	Meaning
IL	Interleukin
LB	Lysogeny broth
LD	Linkage disequilibrium
LDA	Low dose aspirin
M	Molar
MAF	Minor allele frequency
mg	Milli grams
ml	Milli litre
mM	Milli molar
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBC	Sodium bicarbonate co-transporter
$NaHCO_3$	Sodium bicarbonate
NFQ	Non-fluorescence quencher
NSAID	Non-steroidal anti-inflammatory
NTC	No template control
OR	Odds ratio
ORF	Open reading frame
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PUD	Peptic ulcer disease
pMol	Pico moles
qPCR	Quantitative (real time) polymerase chain reaction
RIPA	Radio-immuno-precipitation assay
RNA	Ribonucleic acid
RLU	Relative luminescence
RT PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of mean
SJS	Stevens Johnson syndrome
SLC	Solute carrier family
SNP	Single-nucleotide polymorphism
TEER	Trans-epithelial electrical resistance

Acronym	Meaning
TEN	Toxic epidermal necrolysis
TM	Transmembrane
UGIB	Upper gastro-intestinal bleeding
Z-VAD-FMK	Carbobenzoxy-Val-Ala-Asp-[O-Me]-fluoromethylketone
μCi	Micro curie
μM	Micro molar
μl	Micro litre

Chapter 1   **General Introduction**

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1.1.1   Definition of Adverse Drug Reactions

The term adverse drug reaction (ADR) is often defined as 'an appreciably harmful or unpleasant reaction resulting from an intervention related to the use of a medicinal product; adverse effects usually predict hazard from future administration and warrant prevention, or specific treatment, or alteration of the dosage regimen, or withdrawal of the product' (Edwards and Aronson, 2000). However, as from the year 2012, the definition was modified by the European Parliament and Council of the European Union to include reactions and unintended effects that happen as a result of any error, abuse or misuse, and to reactions from unlicensed medicines that are being used outside the marketing authorisation, as well as to the authorised use of a medicinal product in normal doses (EU, 2010).

There have been attempts by some authorities to make a distinction between ADR and adverse drug event (ADE), which are defined as damage connected with ADR in addition to hazardous consequences from human mistake (Edwards and Aronson, 2000). In general, the term ADR shows a presumption of the drug as causal, while ADE could be associated with any incident occurring in connection with the medicine or treatment process, regardless of origin.

1.1.2   Classification of Adverse Drug Reactions

It has been reported that adverse drug reactions account for 6.5% of healthcare admissions in the United Kingdom (Pirmohamed et al., 2004), based on a retrospective

study of 18,820 patients admitted in the NHS, and are the 6th leading cause of death reported in the United States (Lazarou et al., 1998). The estimated amount of inter-individual variation in drug response and safety as a result of genetic variation is reportedly between 20-50% (Evans and McLeod, 2003).

ADRs have generally been categorized on the basis of how severe or unpleasant the effects are. This might be grouped on a scale as mild, moderate or severe. The Naranjo probability scale (Naranjo et al., 1981) is a commonly utilized and also very easy to use scale consisting of 10 sets of questions (table 1.1), which was established in an attempt to create a standard method of assessing the causality of ADRs, as well as for application in clinical trials and registration of newly licensed drugs. However, there are other scales and tools such as the Liverpool causality tool (Gallagher et al., 2011). The Hartwig Severity scale (Petrova et al., 2017) grades ADR severity based on numbers from 1 (least severe) to 7 (most severe). In addition, there are other more specific scales which focus on the particular area being adversely affected by the drug.

Table 1.1 – The Naranjo Adverse Drug Reaction Causality Algorithm
(Derived from Naranjo et al., 1981; Zaki, 2011)

Question	Yes	No	Don't Know / NA	Score
1. Are there previous conclusive reports on this reaction?	1	0	0	
2. Did the adverse event appear after the suspected drug was administered?	2	-1	0	
3. Did the adverse event improve when the drug was discontinued or a specific antagonist was administered?	1	0	0	
4. Did the adverse event reappear when the drug was re-administered?	2	-1	0	
5. Are there alternative causes that could on their own have caused the reaction?	-1	2	0	
6. Did the reaction reappear when a placebo was given?	-1	1	0	
7. Was the drug detected in blood or other fluids in concentrations known to be toxic?	1	0	0	
8. Was the reaction more severe when the dose was increased or less severe when the dose was decreased?	1	0	0	
9. Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	1	0	0	
10. Was the adverse event confirmed by any objective evidence?	1	0	0	
			Overall Score	

The Naranjo scale: Score of *≥ 9 = definite ADR, *5-8 = probable ADR, *1-4 = possible ADR and *0 = doubtful ADR

Adverse drug reactions can be classified into 2 broad categories - Type A (dose-dependent) and Type B (non-dose-dependent) responses. Later on, 4 other categories were added to describe the ADRs not covered by the A/B classification (Edwards and Aronson, 2000). These 6 classifications of ADRs are summarised in Table 1.2.

Various factors can increase the occurrence and seriousness of type A ADRs, including age, gender, drug-drug interactions, and pharmacogenetic variation (Meyer, 2000). Type A reactions are the most commonly occurring type of ADR and represent up to 80% of ADRs (Pirmohamed and Park, 2001). They are typically predictable due to being related to the recognized primary pharmacologic effects of the drug. A few examples include renal problems from NSAID use, and bleeding from warfarin use (Patrono and Dunn, 1987; Srinivasan et al., 2004).

Type B ADR responses are typically rare and more severe reactions. They are a significant cause of post-marketing drug withdrawal and attrition during the drug-development process. The B type responses are idiosyncratic in nature (Pirmohamed et al., 1998; Phillips and Mallal, 2010) and not easily predictable as they are not linked to the pharmacology of the drug.

Table 1.2 – Classification of Adverse Drug Reaction
(modified from Edwards and Aronson, 2000)

Type of reaction	Description	Features	Examples	Management
A: Dose related	Augmented	Common, Related to pharmacological action of the drug. Predictable, low mortality	Digoxin toxicity, serotonin syndrome with SSRIs, Anticholinergic effects of tricyclic antidepressants	Reduce dose or withhold. Consider effects of concomitant therapy
B: Non-dose-related	Bizarre	Uncommon. Not related to a known pharmacological action of the drug, Unpredictable. High mortality	Immunological reactions: Penicillin hypersensitivity. Idiosyncratic reactions: acute porphyria, malignant hyperthermia, Pseudo allergy (e.g., ampicillin rash)	Withhold and avoid in future
C: Dose and time related	Chronic	Uncommon. May be related to the cumulative dose	Hypothalamic-pituitary-adrenal axis suppression by corticosteroids	Reduce dose or withhold; withdrawal may have to be
D: Time-related	Delayed	Uncommon. Usually dose-related. Occurs or becomes apparent sometime after the use of the drug	Teratogenesis (e.g., vaginal adenocarcinoma with diethylstilbestrol). Carcinogenesis. Tardive dyskinesia with dopamine agonists	Often intractable
E: Withdrawal	End of use	Uncommon. Occurs soon after withdrawal of the drug	Opiate withdrawal syndrome. Myocardial ischemia(β-blocker withdrawal)	Reintroduce and withdraw slowly
F: Unexpected failure of therapy	Failure	Common. Dose related. Often caused by drug interactions	Inadequate dosage of an oral contraceptive, particularly when used with specific enzyme	Increase dosage. Consider effect of concomitant therapy

1.1.3   Characteristics of Adverse Drug Reactions Type B

Type B reactions are characterised by a delayed onset which can vary from a couple of minutes to many hours or even months after initial exposure to the drug. Adverse reactions to drugs also occur much more quickly if there is a re-challenge in people who have formerly been exposed to a particular drug (Pirmohamed, 2010). In addition, it is characteristic that these types of reactions do not show a clear dose-response relationship, i.e. the risk from the drug does not necessarily increase in proportion with the dose (Uetrecht, 2007; Coleman and Pontefract, 2016).

Practically any kind of organ or system in the body can be affected by Type B reactions (Table 1.3). Each particular drug is related to its own unique series of responses, impacting single organs or numerous systems. Nevertheless, amongst patients, there are variable manifestations in clinical symptoms brought on by a drug (Uetrecht and Naisbitt, 2013).

Table 1.3 – Various ADRs Affecting Different Body Organs
(Adapted from Oshikoya et al., 2011)

System/Organ	ADR effects	Drug
Gastro-intestinal	Nausea Abdominal pain Abdominal distension Constipation Stomach ulcer Vomiting Diarrhoea Ileus	Ferrous supplements, NSAIDs, Metronidazole, Amoxicillin, Frusemide
Generalised	Fatigue Malaise Anaphylactic shock Hypothermia	Ceftriaxone, Artesunate, Amodiaquine
Nervous system	Dizziness Headache Seizure Muscle spasms Dystonia Tremor	Artesunate, Cefixime, Amodiaquine
Skin	Rash Pruritus Angioedema Steven-Johnson syndrome Peripheral oedema Alopecia	Phenytoin, Amlodipine, Ampicillin, vancomycin, Amodiaquine
Cardiovascular	Tachycardia	Salbutamol
Ocular	Loss of vision	Quinine

1.1.4   Factors influencing Adverse Drug Reactions

1.1.4.1   Age and Gender

There are higher risks of ADRs during the period considered to be the extremes of life (in children and the elderly). The reason for this is due to the fact that at these times of life, the body's organ development or function is not very efficient. In neonates, many enzymes associated with the metabolism of drugs are premature (Court, 2010); for this reason, medicines which are removed by these enzymes could build up, leading to raised levels of toxins in the body, while a decrease in functionality of body organs as seen in older people could trigger a reduction in the clearance of drugs.

It has also been shown that females tend to have a greater risk of experiencing ADRs compared to males. In addition to gender-specific variations in the expression of some enzymes responsible for drug metabolism, for instance CYP3A4 (Ajayi et al., 2000), the distinction between both genders could possibly be due to the tendency of women to be more inclined to seek out healthcare early when problems occur. In addition, the unsuitable use of medicines, as well as the understanding and emphasis placed on health and disease between genders could also be an explanation for the differences.

1.1.4.2 Drug-Drug Interactions

Drug-drug interactions are a prominent aspect connected with the manifestation of clinically important ADRs. Inhibition of enzymes may occur if the two drugs share a common metabolic pathway, and compete for the same enzyme binding site. The drug that is more potent will predominate, resulting in reduced metabolism of the other competing drug (Ogu and Maxa, 2000). The pharmacological activity of any drug, from the standpoint of toxicity or effectiveness, is in one way or another determined either by systemic exposure to the parent drug and/or to its metabolites. Some drugs go through first pass metabolism with the metabolites which are much more potent than the parent drug. Examples consist of one of the metabolites of a selective H1-histamine antagonist, loratadine, called descarboethoxyloratadine which is up to 4 times more active than the original parent drug (Friedman et al., 1999). Furthermore, E3174 the 5- carboxylic acid derivative of Losartan, is 10 to 40 times more effective than Losartan (Stearns et al., 1995; Sica et al., 2005).

Also, inhibiting the metabolic process of certain drugs can often cause serious ADRs. Terfenadine is one such drug whose metabolism by CYP3A4 is hindered by drugs such as ketoconazole and clarithromycin. Co-administration of one of these drugs with terfenadine leads to accumulation of the drug, and a subsequent increase in QT interval, development of arrhythmias and death in some cases (Ajayi et al., 2000). Furthermore, the metabolism of many drugs can be affected by rifampicin which induces the CYP450 enzymes, especially CYP3A4, responsible for catalysing the metabolism and elimination of 50% of drugs. This can result in inadequate efficacy of the drug (Yamashita et al., 2013).

1.1.5   Pharmacogenetics of Adverse Drug Reactions

Pharmacogenomics and pharmacogenetics aims to optimise medicines on the basis of the genetic profile of each person, thereby reducing as much as possible, the chance of any adverse reaction and improve efficacy. Xenobiotic metabolism is the main pathway for the removal of toxic substances, pharmaceutical substances and carcinogens. Overall, how compounds are absorbed, distributed, metabolised and excreted (ADME) determines how bioavailable they are, as well as their effectiveness and safety. Drug metabolising enzymes (DMEs) help with the metabolic process and the biotransformation of compounds. These enzymes were first divided into 2 groups: Phase I and Phase II DMEs. In 1992, the removal of products of detoxification by the drug transporter class of proteins was categorised as Phase III drug metabolism (Ishikawa, 1992). The liver is the primary organ for drug metabolism, but other organs such as the kidney, lung and intestines also contain reduced levels of enzyme activities. Enzymes and transporters have also been reportedly discovered in some white blood cells (Park et al., 1995).

Furthermore, genetic variation can add to the variability seen in the effectiveness and safety of clinical compounds (see Table 1.4). Variability can involve drug transporters, genes encoding the DMEs and also immunogenic elements (Ma and Lu, 2011). Apart from genetic variation, epigenetic factors and physiological differences such as age, existing poor health, alcohol and smoking can bring about individual differences in how drugs are tolerated by a person's body.

Table 1.4 – Genes Affecting Metabolism of some Therapeutic Agents
(PharmGKB, 2015)

Gene	Drug
CYP2C19	Clopidogrel, Tricyclic antidepressant
VKORC1	Warfarin
HLA-B	Carbamazepine, Allopurinol
CYP3A5	Tacrolimus
TPMT	Azathioprine, Mercaptopurine

1.2   Non-Steroidal Anti-Inflammatory Drugs (NSAIDS)

1.2.1   Background and Classification

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs recognised mainly for their analgesic, anti-inflammatory and anti-pyretic effects, and are used largely in the treatment of rheumatoid arthritis and other inflammatory diseases. NSAIDs are among one of the most used drug classes in the world, with an approximated use of over 30 million pills each day (Singh and Triadafilopoulos, 1999). It is a well-known fact that although NSAIDs are beneficial for treating a number of different ailments, they often possess some serious side effects. Research has documented a growing number of ADRs including the ability to induce hypertension and other cardiovascular diseases, renal failure, and, perhaps one of the mostly widely known effects, upper gastro-intestinal injury (Gambaro and Perazella, 2003). There are two classes of NSAIDs – the non-selective NSAIDs, which inhibit both COX-1 and COX-2 enzymes, and the selective NSAIDs,

which inhibit only the COX-2 enzyme, called COX-2 inhibitors (Coxibs for short). NSAID

classification is summarised in Table 1.5.

Table 1.5 – Classification of Non-Steroidal Anti-Inflammatory Drugs
(Derived from Sánchez-Borges, 2008)

NSAID Group	Name of Drug	Chemical Classification	COX-Selectivity
Salicylates	• Aspirin • Salsalate • Diflunisal • Trilisate	Carboxylic acids	Non-selective COX-inhibitors
Acetic Acids	• Ketorolac • Diclofenac • Aceclofenac • Indomethacin		
Propionic Acids	• Naproxen • Ibuprofen • Ketoprofen • Fenoprofen		
Fenamates (Anthranilic Acids)	• Mefenamic Acid • Flufenamic Acid • Tolfenamic Acid • Meclofenamic Acid		
Oxicams (Enolic Acids)	• Meloxicam • Piroxicam • Droxicam • Tenoxicam	Enolic acids	
COX-2 inhibitors (Coxibs)	• Celecoxib • Valdecoxib* • Rofecoxib* • Parecoxib*	Other	COX-2 inhibitors

This is an abridged list of drugs in the NSAID classification. * = drugs which have been discontinued.

1.2.2   General Structure of NSAIDs

Structurally, NSAIDs comprise of an acidic group (carboxylic acid or enols) attached to an aromatic ring moiety. Various examples of this structure are represented by NSAIDs used in this study (figure 1.1). Most NSAIDs are weak acids with pKa between the ranges of 3– 5. The acidic group is important for a number of functions, including for the purpose of COX inhibition, ionic binding with plasma proteins as well as functioning as a main site of metabolism by conjugation (Asirvatham et al., 2016). However, NSAIDs differ in their lipophilic tendencies based on their aryl groups and additional lipophilic moieties. NSAIDS have been shown to cause GI toxicity, and one hypothesis is that they destabilise intracellular pH by inhibiting the neutralising actions of bicarbonate, amongst other mechanisms.
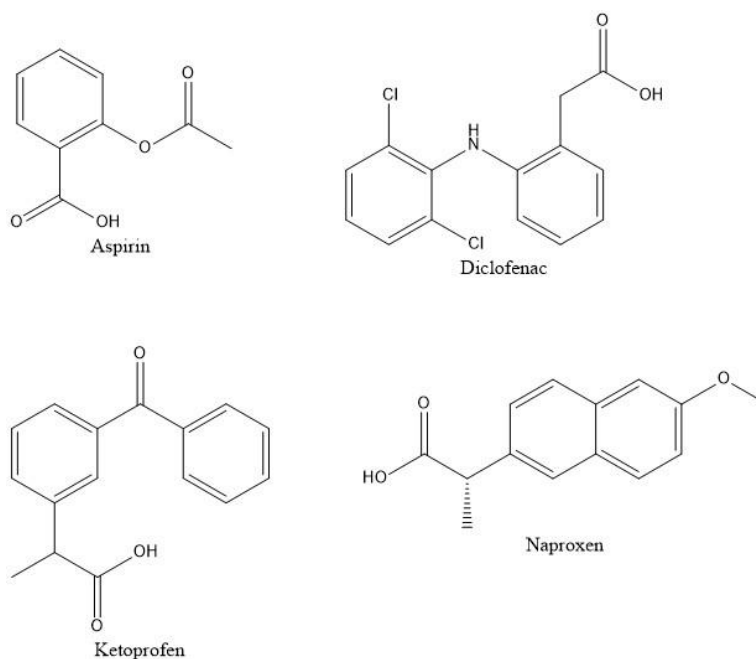

Figure 1.1 – Structures of Common NSAIDs used in this Study

1.2.3   Healthcare Burden of NSAIDs

Approximately 15-35% of people develop some form of ulcer lesion which can be established via an endoscopy, within 3 months of beginning an NSAID treatment (Bjarnason, 2013). It is estimated that NSAIDs trigger about 3,500 admissions to hospitals and up to 400 fatalities caused by ulcer-related blood loss every year in the UK in people who are aged 60 years and above (Langman, 2001). The United States figure for NSAID-related events is more than 100,000 hospitalisations and up to 17,000 fatalities each year (Wolfe et al., 1999).

Pirmohamed et al. (2004) conducted a study on the fatalities resulting from adverse drug reactions in a group of 1,225 individuals in the United Kingdom, and found that over 60% of these deaths were connected to NSAIDs, making NSAID-related ADRs a significant cause of death directly connected to the use of medicines. In Germany, NSAID-related gastrointestinal ADRs is considered as more dangerous, than road traffic accidents due to there being an estimate of 1 in 1,220 people dying from NSAID-related gastrointestinal ADRs yearly (in the UK, the figure is 1 in 2,000 people) (Cryer, 2005). According to an independent estimate reported in the NHS Community pharmacy Non-Steroidal Anti-Inflammatory Drug safety audit in 2014, the estimated expenditure for managing NSAID related complications from stomach ulcers in the UK was up to £6825 for each individual (NHS Specialist Pharmacy Service, 2014).

1.2.4   Pathophysiology of Cyclooxygenase (COX) Inhibition

Cyclooxygenase enzymes consist of two isoforms, COX-1 and COX-2 which are differentially expressed and coded by different genes (Cryer and Feldman, 1998; Smith et

al., 2000). Usually in many tissues, the COX-1 enzyme is constitutively expressed as it is important for maintaining normal physiological function of the tissues. These include reproductive and cardiovascular function, control of blood flow in the renal system, pulmonary function (Antman et al., 2005; Grosser et al., 2006), as well as gastro-intestinal protection (Musumba et al., 2009). On the other hand, COX-2 is often expressed during inflammation (described as induced) sometimes provoked by endotoxins, growth factors and cytokines. This process leads to the release of various prostaglandins via the arachidonic acid pathway (Vane and Botting, 1998).

In order to understand the mechanisms by which NSAIDs might induce an adverse effect, it is important to first understand how they produce their desired effects as anti-pyretic, analgesic and anti-inflammatories. The enzyme phospholipase A2 (PLA2) mediates the formation of arachidonic acid by acting on phospholipids (figure 1.2). Formation of prostaglandin H2 (PGH2) then occurs as a result of the activity of PGH2 synthase and cyclooxygenase enzymes. From the PGH2, the effects of other acting enzymes, including thromboxane, prostacyclin, prostaglandin E and prostaglandin D synthases gives rise to the production and release of thromboxane, prostacyclin (PGI2), prostaglandin E2 and prostaglandin D2 respectively (Batlouni, 2010).

All of these compounds exert different effects in many parts of the body, with some being anti-inflammatory, and others pro-inflammatory, mediating body changes in response to fever. PGE2, for example acts on the prostaglandin E receptor 3 (EP3) located on neurons in the preoptic area (POA) of the hypothalamus. This triggers the body's set-point temperature to an elevated level and remains so until PGE2 is no longer detected

(Boulant, 2000). When the sympathetic system is stimulated, thermogenesis occurs, causing the body to produce heat.

Generally, NSAIDs act on the COX-1 and COX-2 enzymes, thereby preventing the production of PGH2 ¬and all its metabolites, together with their corresponding effects on various tissues, a summary of which is shown in Table 1.6.
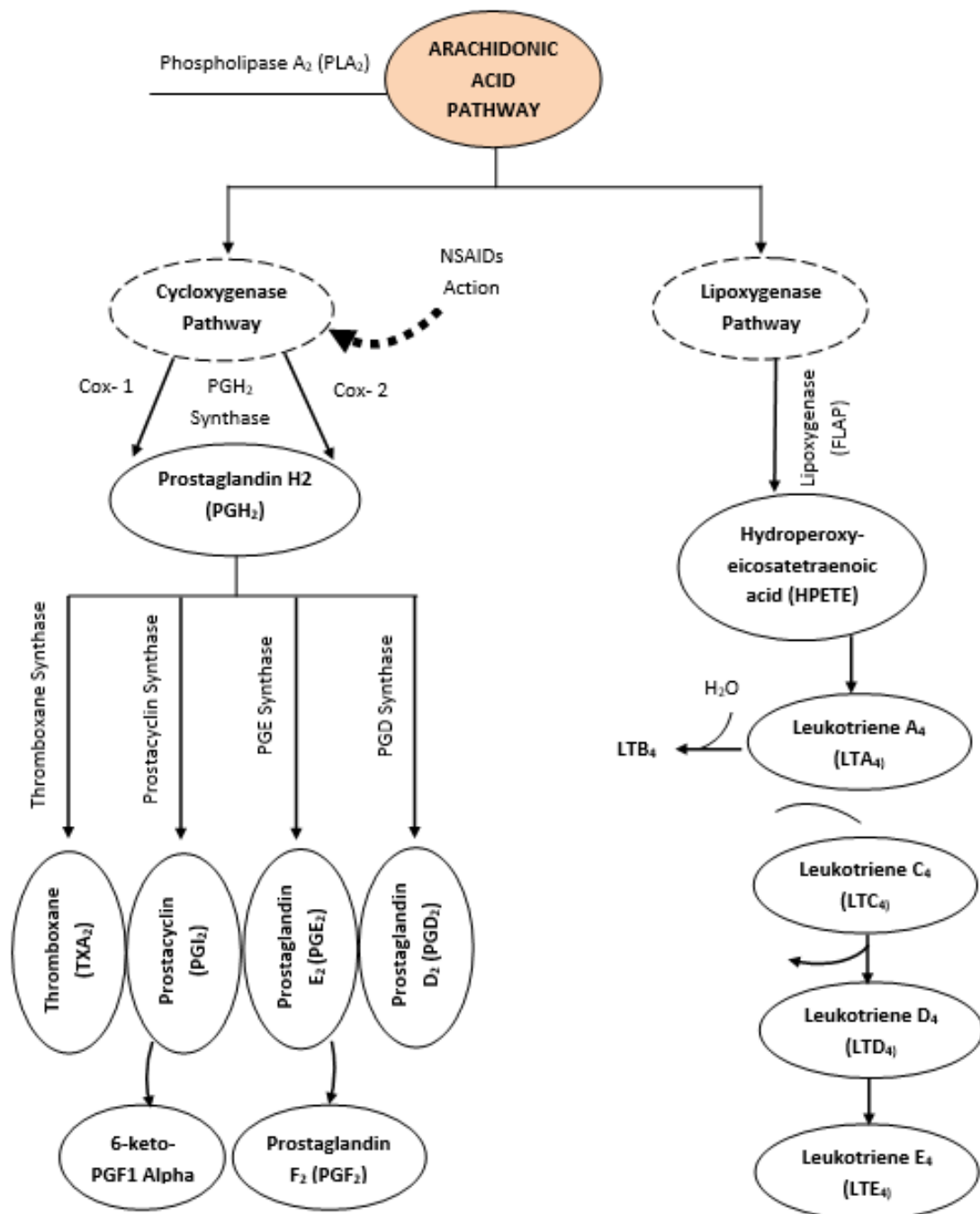
Figure 1.2 – Schematic Illustration summarising the Arachidonic Acid Pathway
Note the prostanoids resulting from the action of the cyclooxygenase enzymes.
Source: Derived from Martel-Pelletier et al., 2003.

Table 1.6 – Summary of the Effects of Prostanoids on Tissue Systems

Type of Prostanoid	Tissue system	Effects/Function	Reference	COX isoform
PGD_2	Lungs, gastro-intestinal	Bronchoconstriction Modulates fibroblast/collagen gel contraction in scar formation	(Kohyama et al., 2002)	COX-2
PGI_2	Cardiovascular Lungs	Inhibition of Platelet aggregation Bronchodilation Vasodilatation Pain Modulator	(Noda et al., 2007), (Pulichino et al., 2006)	COX-1, COX-2
PGE_2	Renal Gastro-intestinal	Vasodilation Increase in mucus secretion Decrease in acid secretion Encourages secretion of bicarbonate	(Baumgartner et al., 2004), (Aoi et al., 2004)	COX-1
PGF_2 6-keto-PGF1-α	Cardiovascular Gastro-intestinal, Reproductive	Tachycardia, blood pressure increase Smooth muscle contraction & Initiation of parturition	(Takayama et al., 2005), (Yu et al., 2012), (Sugimoto et al., 1997)	COX-1
TXA_2	Cardiovascular, general tissue Gastro-intestinal, renal	Platelet activation, Vasoconstriction Promotes angiogenesis, stimulates Epidermal growth factors	(FitzGerald, 1991), (Daniel et al., 1999), (Rodrigues et al., 2001)	COX-1, COX-2

1.2.5   Effects on the Renal System

As a result of their apparent efficiency, NSAIDs are the most common root cause of drug-induced kidney injury. Results from nephrotoxicity studies (Whelton, 1999) suggest that prostaglandins E2 which is produced in the kidneys by COX-1 action causes the vascular resistance of the renal system to decrease due to dilation of the vasculature. This leads to increased organ perfusion and blood flow into the intra-medullary region from the cortex under normal situations. When these mechanisms are inhibited by NSAIDs, there is vasoconstriction and a decrease in total renal perfusion, as blood flow is returned to the cortex area, resulting in medullary ischemia and kidney failure. In an attempt to accommodate the reduced blood flow, the kidney responds partly by inciting a process which promotes fluid and sodium retention, as well as increased vascular constriction. This system, called the renin-angiotensin-aldosterone system (RAAS), works to maintain a balance of fluid and blood pressure as shown in figure 1.3. Pro-renin is activated by the kidney juxtaglomerular cells, in the event of low blood volume, thereby secreting renin into the blood stream. The renin then converts angiotensinogen produced by the liver into angiotensin I. Subsequent conversion of angiotensin 1 to angiotensin II occurs (Paul et al., 2006), a step mediated by the angiotensin converting enzyme (ACE). Not only is angiotensin II a potent vasoconstrictor, it also stimulates production of aldosterone, which is secreted from the adrenal cortex. In high risk individuals already with decreased renal capability, these regulatory mechanisms of prostaglandins are ways in which NSAIDs further impair renal function.
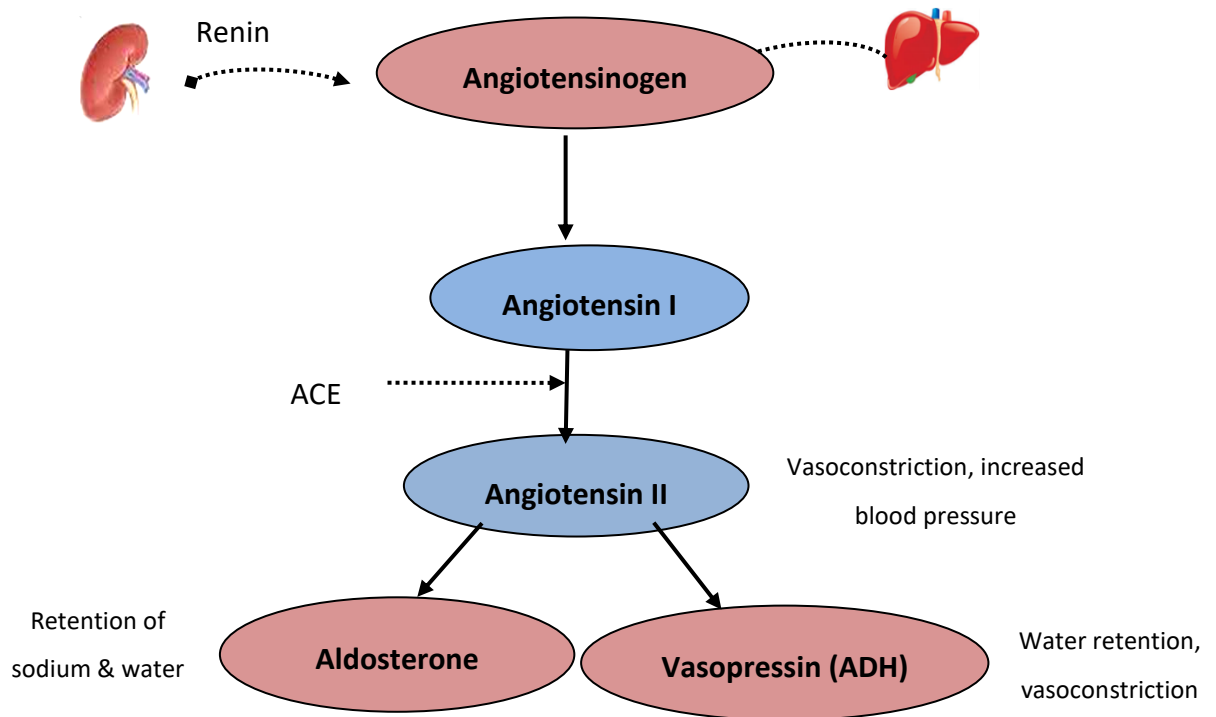
Figure 1.3 – Schematic Illustration summarising the Renin-angiotensin-aldosterone System (RAAS), and Important Effects of End Products.

1.2.6   Effects on the Cardiovascular System

A number of cardiovascular effects have been observed with NSAIDs. Research by Bavry et al. (2011) demonstrated that patients with symptoms of coronary artery disease and hypertension had a 47% increase in risk of occurrence of death, non-fatal stroke or nonfatal myocardial infarction if they had been using NSAID for over a period of 2.7 years, on average. A meta-analysis of NSAID users (Morrison et al., 2007) over a shorter period reported a significant increase in blood pressure due to ibuprofen. The harmful effects of NSAIDs in the cardiovascular system have been suggested to be due to its ability to interfere with PGI2. As this prostanoid is responsible for dilation of blood vessels and inhibition of platelet aggregation, suppressing its release causes the effect of TXA2 to be more pronounced, leading to thrombosis. As shown from the results of a

recent experiment (Yu et al., 2012), suppression of the cardio-protective effects of COX-2 or PGI2 by NSAID sufficiently increased the risk of hypertension, thrombosis and heart failure, as this also led to a depression of nitric oxide release. It was observed from the work of this group that the amount of metabolites of PGI2 in the urine was inversely proportional to the blood pressure of the mice studied.

1.2.7   Effects on Hepatic system

Most NSAIDs are eliminated via hepatic metabolic processes, through oxidation and conjugation. A rise of approximately 3.5% in the level of transaminase, which is a known marker for hepato-cellular injury, has actually been observed for the majority of NSAIDs, including diclofenac (Bessone, 2010). A number of NSAIDs such as benoxaprofen and bromfenac have actually been withdrawn or prevented from reaching the marketplace as a result of their propensity to cause hepatotoxicity (Goldkind and Laine, 2006). The toxicity of a lot of NSAIDs to the liver has been reported to be low, in a population based study. A US-based drug induced liver injury (DILI) study reported that of 133 instances triggered by single drugs, just 4 were linked to bromfenac while other NSAIDs caused at most one or two cases (Reuben et al., 2010). In chronic NSAID users, it is suggested that the levels of bilirubin and transaminase are routinely checked, though the usefulness of this method is still uncertain. However, a threefold elevation of the level of transaminase is a necessary reason for cessation of the NSAID (Wehling, 2014). According to Hy's law, a rise in the level of serum bilirubin should occasion immediate discontinuation of the NSAID despite of the level of transaminase (Temple, 2006). Hepatocellular injury is the

most usual pattern observed in NSAID hepatotoxicity, and the most regularly implicated

agent is diclofenac, which accounts for 53% of cases (Schmeltzer et al., 2016).

1.2.8 Effects on the Gastro-Intestinal System

Prostaglandins also play a major role in the protection of gastro-intestinal epithelium by

regulating mucous and bicarbonate secretion as well as decreasing the secretion of

gastric acid (Aoi et al., 2004). As potent vasodilators, PGE2 and PG12 sustain the flow of

blood and fluid in the mucus layer (see table 1.7). They are thus important in managing

nearly all areas of gastro-intestinal mucosal defence mechanisms. As reported previously

(Tanaka et al., 2001), mice lacking in either COX-1 or COX-2 did not show signs of gastric

injury, though there was little prostaglandin synthesis, but had gastric injury when both

COX-1 and COX-2 were inhibited concurrently, e.g. by a NSAID. Their findings suggest

that each enzyme isoform is able to compensate, to some extent for the other in terms of

providing protection. Therefore, as COX-1 is constitutive and important for most

physiological functions, it seemed logical that the less expressed, inducible COX-2 isoform

is selectively inhibited by the newer models of NSAIDs.

This however, presents a concern as COX-2 has been shown to play an important role in

gastro-intestinal protection. It has been demonstrated that following inflammation and

PGE2 stimulation, there is an increase in the release of growth factors at the site of

ulcers. These facilitate gastro-intestinal repair and confer protection by promoting

angiogenesis and formation of tissue granulation (Daniel et al., 1999; Szabo and Vincze,

2000). These effects have been shown to be mediated through the action of COX-2 and

thromboxane, which plays a role in mobilising trefoil peptides, thereby preventing

apoptosis, stabilising mucous gel layers (Rodrigues et al., 2001; Hoffmann, 2005) and increasing production of nitric oxide by nitric oxide synthetase (Sasaki et al., 2003). NSAIDs therefore may hinder the repair of the gastrointestinal mucosal layer by repressing production of growth factors.

In addition, NSAIDs cause injury directly by irritating the upper part of the mucosa in the gastrointestinal epithelium, thus leading to a breach in the mucosal barrier. As a result, diffusion of acid back into the gastro-intestinal mucosa has been described as one of the ways NSAIDs may cause harm. Acid increases the permeability of the gastric mucosa and enhances the absorption of the NSAID, causing blood vessels to rupture and may cause minor lesions to deepen when the membrane becomes damaged (Fromm, 1987). In a recent study on rats, Funatsu et al. (2007) demonstrated the important role of acid in the development and advancement of gastro-intestinal lesions when they reported that even a reduction in mucosal blood flow caused by NSAID takes place in an acid environment. The ability of the tissue to rebalance its pH in such situations, is thus of high importance. The effects and mechanisms of NSIADs action on the GI system is detailed later in section 1.4.

Table 1.7 – Prostaglandin Effects on the GI System

Type of PG	Receptor	GI Effect
PGE2	EP_1	contraction of GI smooth muscles
	EP_2	Relaxation of GI smooth muscles
	EP_3	Increased gastric mucus secretion
		Decreased gastric acid secretion
		Contraction of GI smooth muscles

1.2.9   Allergy-like Hypersensitivity Effects

According to Kowalski et al. (2013), a range of allergy-like hypersensitivity responses are associated with the consumption of NSAIDs. These hypersensitivity responses are distinctive responses and also vary from the various other adverse effects in organs which were noted earlier. Some hypersensitivity responses caused by NSAIDs are allergic in nature and include: 1) recurring IgE-mediated urticarial skin rash, angioedema, as well as anaphylactic reactions beginning instantaneously or several hours after consuming a NSAID; 2) T cell-mediated delayed onset reactions (usually starting after 24 hours) which are relatively mild or moderate, skin responses such as maculopapular exanthemas, drug eruptions, urticaria, contact dermatitis, as well as photosensitivity responses; or 3) much more serious and potentially fatal T-cell-mediated delayed systemic responses such as the drug reaction with eosinophilia and systemic symptoms (DRESS), severe generalised

exanthematous reactions, the Stevens-Johnson syndrome (SJS), and toxic epidermal

necrolysis (TEN).

People who are affected could be unusually sensitive to these metabolites or produce

them in excess, and are at risk with a vast array of structurally different NSAIDs,

especially those that tend to inhibit COX1. Signs and symptoms, which may begin right

away or several hours after consuming any one of the different NSAIDs that are COX-1

inhibitors include: 1) worsening of rhinitis and asthmatic symptoms in people who have a

history of bronchial asthma or rhinitis as well as 2) development or worsening of wheals

or angioedema in people who may or may not have a history of persistent urticarial rash

or angioedema (Kowalski et al., 2013).

1.3   Risk Factors for NSAID-induced Gastrointestinal Injury

1.3.1   Age and Gender

Research has shown that the gastric mucosa which is aging tends to have reduced

mucosal defence, such as decreased production of prostaglandins, and reduced secretion

of mucus and bicarbonate (Lee and Feldman, 1997). Furthermore, Cryer et al. (1992)

established that in humans, aging minimizes the concentrations of duodenal and gastric

prostaglandins. Other research showed that in the aging gastric mucosa, there is

decreased level of the activity of NO synthase and impaired response to luminal acid. In

addition, Goto et al. (2001) showed in their experiment that aging raises the vulnerability

of the gastric mucosa to injury by a range of agents, including ethanol, hypertonic

solutions, and NSAIDs and impairs recovery of both intense injury and persistent gastric ulcers.

Recent research (Tarnawski et al., 2007) in a group of aging rats revealed that there was a 60% reduction in gastric mucosal blood flow, together with hypoxia and the presence of atrophy of the glandular cells. Additionally, caspases-3 and -9 activity was amplified, while survivin was lowered, leading to an increase in apoptosis. Moreover, this research study established an increase in the level of susceptibility to ethanol injury in the mucosa of the aging stomach, which down-regulated the raised phosphatase and tensin (PTEN) homologue in gastric mucosa of aging rats completely overturning the increased vulnerability. These observations may well explain the rise in cases of NSAID-related gastro-intestinal ulcers in the elderly population.

1.3.2   Drug-Drug Interactions from Multidrug Use

Selective serotonin reuptake inhibitors (SSRIs), including the often recommended antidepressants fluoxetine are believed to increase the overall risk of gastrointestinal incidences by obstructing the reuptake of serotonin in the thrombocytes (Alderman et al., 1992).

Up until lately there were no reports of the possible gastric adverse effects brought on by combining the use of NSAIDs and SSRIs. In 1999, a case-control study was carried out (de Abajo et al., 1999), and it reported that the concomitant use of SSRIs in patients taking NSAIDs resulted in an increased risk of UGIB much greater than the sum of the GI effects from each independent drug. In a parallel editorial, it was concluded that the combination of both drugs results in a higher risk far greater than a basic effect (Po,

1999). Also, in another cohort study, the combination of NSAIDs and SSRIs led to a big boost in the gastrointestinal incidence rate (which took into consideration the number of first peptic ulcer drug prescriptions), from 0.051 to 0.634 as well as an increase in the incidence ratio to 12.4 in the cohort with concomitant drug use of SSRI and NSAID, compared to 0.051 in the control group, who took non-selective antidepressants only (de Jong et al., 2003).

Bisphosphonates are thought of as a primary treatment for the prevention of osteoporosis. It was discovered that oral bisphosphonates do not increase the danger of upper GI bleeding. The simultaneous use of NSAIDS and bisphosphonates raises the risk of upper GI bleeding (Etminan et al., 2009).

Proton pump inhibitors (PPIs) which are useful for treating acid-related upper gastro-intestinal diseases, including prevention of NSAID-induced upper GI bleeding have been reported to cause possible exacerbation of lower GI bleeding, i.e. in the small intestines (Lue and Lanas, 2016). This could be because NSAID-related gastropathy is pH-dependent, while NSAID enteropathy has been suggested to be mainly due to PG depletion (Lichtenstein et al., 1995). Using PPIs may therefore lead to destabilisation of the normal intestinal microbiome, and susceptibility to lower GI bleeds.

1.3.3   Helicobacter Pylori Infection

It is accepted that the risk of NSAID-induced ulcers differs in accordance with the presence of certain risk factors, including previous peptic ulcers, as well as *H. pylori* infection. It has been established that the use of NSAID in the presence of an infection with *H. pylori* enhances the danger of peptic ulcer disease, PUD (Chan et al., 2002; Lanas

et al., 2002). In 2002, an interesting meta-analysis was published (Huang et al., 2002) that consisted of data from 25 observational studies which evaluated these risks in terms of PUD development. They demonstrated that the incidence of PUD was considerably more common in NSAID users who also had *H. pylori* infection compared to those without infection. They assessed the occurrence of *H. pylori* infection and also NSAIDs usage in a total of 893 individuals with bleeding ulcers and 1,002 controls without bleeding. The use of NSAIDs or presence of *H. pylori* infection increased the susceptibility to ulcer bleeding by 4.85 fold and 1.79 fold respectively. When both factors prevailed, the risk was increased to 6.13. Consequently, with this proof, it could be concluded that the use of NSAIDs and *H. pylori* infection are separate factors, with possible synergistic effects, when considering the risks for the development of PUD.

Current evidence recommends that, in patients newly taking NSAID, eradication of *H. pylori* can efficiently reduce the risk of ulcer. Nevertheless, in those patients that have been long-term users of NDAIDs, there is no clear advantage (Sostres et al., 2014). In addition, *H. pylori* elimination does not appear adequate for ulcer prevention in NSAID users. PPI co-therapy has been suggested for suitable avoidance in this group of patients. Treatment and eradication of infection as well as PPI therapy, is most likely the better option for protection.

1.3.4 Co-morbidities

Experimental research has shown that people with portal hypertension have damaged or reduced gastric mucosal defence, minimal oxygenation ability, raised susceptibility to injury, as well as impaired capacity to recover (Auroux et al., 2003) just like what has

been observed in the aging mucosa. A study by Tomikawa et al. (2000) found that capillary endothelial irregularities in portal hypertensive gastropathy cause a 3.5-fold decrease in the diameter of the mucosal capillary lumen, triggering reduced capillary blood flow as well as decreased mucosal oxygenation. Activation of TNF-α, as well as increased endothelial expression of the enzyme NO synthase take place with a boost in the production of toxic peroxynitrate (Kawanaka et al., 2002). Moreover, a remarkable 10-fold decrease in stomach angiogenesis takes place as a result of alcohol injury in mucosa of the portal hypertensive patients (Ichikawa et al., 1994). The contribution of this impairment in gastric mucosal defence to the blood loss seen in some individuals with portal hypertensive gastropathy has not yet been specified. Patients with diabetes who were taking NSAIDs had a substantially greater risk of upper gastrointestinal bleeding (Kim et al., 2015).

Table 1.8 – Risk Factors for NSAID-induced Gastrointestinal Complications

Risk Factors	Identified Risk due to NSAIDs	Identified Risk due to aspirin	References
Patient – Specific:			
Concomitant use of NSAIDs and coxibs	Yes	Definite	(Lanas et al., 2006)
History of GI bleeding or PUD	Yes	Definite	(Lanas et al., 2000)
Old age	Yes	Controversial	(Rodríguez et al., 2001)
Concomitant use of anticoagulant (e.g. warfarin)	Yes	Probable	(Gulløv et al., 1999)
Co-morbidities	Yes	Definite (hypertension, diabetes)	(Sirois et al., 2014)
Male gender	No	No	(Okada et al., 2009)
Concurrent corticosteroids	Yes	Controversial	(Lanas et al., 2002)
H. pylori infection	Yes	Definite	(Lanas et al., 2002)
History of dyspepsia	No	Probable	(Cea Soriano and Rodríguez, 2010)
Aspirin-related:			
Regular aspirin duration	No	Probable	(Kelly et al., 1996)
Short aspirin duration	No	Definite	(Lanas et al., 2006)
High dose aspirin	Yes	Probable	(Serebruany et al., 2005)

1.3.5   Pharmacogenetics

A number of studies have evaluated the contribution of genetics to NSAID-induced gastrointestinal problems and the results from these investigations have shown the essential roles of polymorphisms affecting various genes associated with enzymes that are involved in the conjugation and oxidation of drug compounds, as well as genetic mutations of drug receptors and their target sites, proteins involved in drug transport and also ion channels. There have been some interesting results reported about the relationship between polymorphisms in genes which metabolise enzymes in the pathogenesis of gastroduodenal injuries related to the use of NSAIDs and also aspirin. As a matter of fact, there have been a couple of studies that have investigated the possible ways in which GI ulcer susceptibility could be influenced by a variation in the genes which have a role in cell repair, leading to suggestions for a broadened search of plausible genes involving diverse targets with emphasis on tissue injury repair genes and the NSAID-induced gastrointestinal problems (Pirmohamed and Park, 2001).

An investigation of the relationship between CYP2C19 gene polymorphism and peptic ulcers was conducted in a population of 1,239 Caucasian individuals with or without peptic ulcer, many of whom were on NSAIDs. It was reported that a gain of function polymorphism in CYP2C19*17 was significantly associated with peptic ulcer disease, and that this association was regardless of NSAID use. Also, there were no relationship between peptic ulcer disease and the various other CYP2C SNPs (Musumba et al., 2013). In addition, an independent team of researchers (van Oijen et al., 2005) has reported an absence of any connection between upper GI ulcer symptoms and CYP2C9 or UGT1A6

polymorphisms in a study involving 160 individuals who used aspirin in a coronary unit. A summary of the studies undertaken evaluating genetic factors involved in NSAID-induced gastrointestinal toxicities is presented in Table 1.9.

Table 1.9 – Summary of Studies on the Genetic Associations of NSAID-induced GI Complications

Gene studied	Study Design	Outcome measured	Summary of findings	Reference
AGT	Prospective (n = 211)	Endoscopic UGIB	AGT A-20C variant allele increases risk	(Negovan et al., 2015)
CYP2C9	Case-control (n = 78)	Endoscopic UGIB	CYP2C9*1/*3, CYP2C9*1/*2↑risk. Higher risk among CYP2C9*3 carriers	(Pilotto et al., 2007)
UGT1A6	Prospective (n =160)	Symptomatology	No association	(van Oijen et al, 2005)
CYP2C family	Case-control (n = 1239)	Endoscopic UGIB	CYP2C19*17 associated with PUD	(Musumba et al., 2013)
COX-1	Retrospective (n = 480)	PUD	c.-842A>G/c.50G>T no association seen; 1676T allele increases risk	(Arisawa et al., 2007)
COX-1	Retrospective (n = 194)	PUD	c.-842A>G/c.50G>T showed inverse association	(van Oijen et al., 2005)

1.4   Gastrointestinal Physiology

1.4.1   Gastrointestinal Defence Mechanisms

The mucus-bicarbonate-phospholipid layer makes up the first line of mucosal protection through a barrier function (Allen and Flemstrom, 2005).   This layer consists of phospholipid surfactants, bicarbonate and mucous gel, which serves as protection for the surface of the mucosal area.   This undisturbed layer of coating keeps intact the bicarbonate produced by the epithelial cells so as to keep the environment neutral, with a pH of approximately 7.0, and stops proteolytic digestion of the surface cells due to the infiltration of pepsin (Allen and Flemstrom, 2005).   It has been shown that the mucus gel also consists of phospholipids, and that its luminal area is covered with phospholipids having hydrophobic characteristics (Lichtenberger, 1999).

Bicarbonate secretion into a secure, adherent layer of mucus gel causes a pH gradient to be maintained in the epithelial area of the gastroduodenal regions affording the initial line of mucosal protection against luminal acid (Allen and Flemstrom, 2005).   Research has revealed that sodium and bicarbonate cotransport taking place at the basolateral area of the membrane is the main mechanism by which HCO_3- is imported.   Experiments on the gastric mucosa of rabbit and rat showed that in the gut, prostaglandins enhance HCO_3- secretion via the expression of PGE1 receptors, as well as expression of the $Cl-/HCO_3-$ anion exchanger in the apical membrane of the gastric epithelial cell surface (Rossmann et al., 1999).

The next avenue for mucosal protection is afforded by an unbroken layer of epithelial cells, producing bicarbonate, mucus, heat shock proteins and prostaglandins. These cells are hydrophobic as a result of the surface phospholipids which they have on them, and are thus able to repel water-soluble harmful compounds (Lichtenberger, 1999). Heat shock proteins are produced by epithelial cells in the gastric mucosa as a response to varying forms of stressful situations, such as the presence of cytotoxic materials, raised temperature, and oxidative stress (Tanaka et al., 2007). The surface epithelial cells create an obstacle as they are linked by intercellular tight junctions, preventing the diffusion of pepsin and acid back into the cells (Allen and Flemstrom, 2005).

Additionally, the microcirculation in the gastroduodenal mucosa is vital for oxygen and nutrient supply, as well as for the elimination of poisonous materials. Powerful vasodilators like prostacyclin (PGI2) and nitric oxide (NO) are produced by the endothelial cells which line the blood vessels. These vasodilators shield the mucosa from injury and the destructive actions which vasoconstrictors such as thromboxane A2 and leukotriene C4 may have. According to Guth (1992), PGI2 and NO preserve endothelial cell viability, preventing the adherence of platelets and leukocytes, and hence the mucosal microcirculation is not compromised. Renewal of the mucosal cells ensures that the integrity of the mucosa is structurally sound. Proliferation of progenitor cells (a process regulated by growth factors) enables the consistent renewal of the epithelium with cells which replace impaired or older cells. Epithelial restitution after minor injury takes place within a few minutes by cell migration. The epidermal growth factor receptor (EGF-R) is the main growth factor receptor found in the progenitor cells of the gastric region, while

the main mitogenic growth factor that activates the EGF-R is transforming growth factor-α (TGF-α), as well as the insulin-like growth factor-1 (Nguyen et al., 2007).

Finally, the constant production of PGI2 and PGE2 by the gastric mucosa is vital for maintaining the integrity of the mucosa against necrotizing and ulcerogenic agents (Kobayashi and Arakawa, 1995; Takeuchi et al., 1997). Generally, a majority of the mechanisms for gastroduodenal mucosal protection are regulated in one form or another by prostaglandins. PGs enhance mucosal blood flow, hinder acid secretion; promote mucus production, bicarbonate and lipid secretion, and also increase mucosal epithelial restitution and recovery.
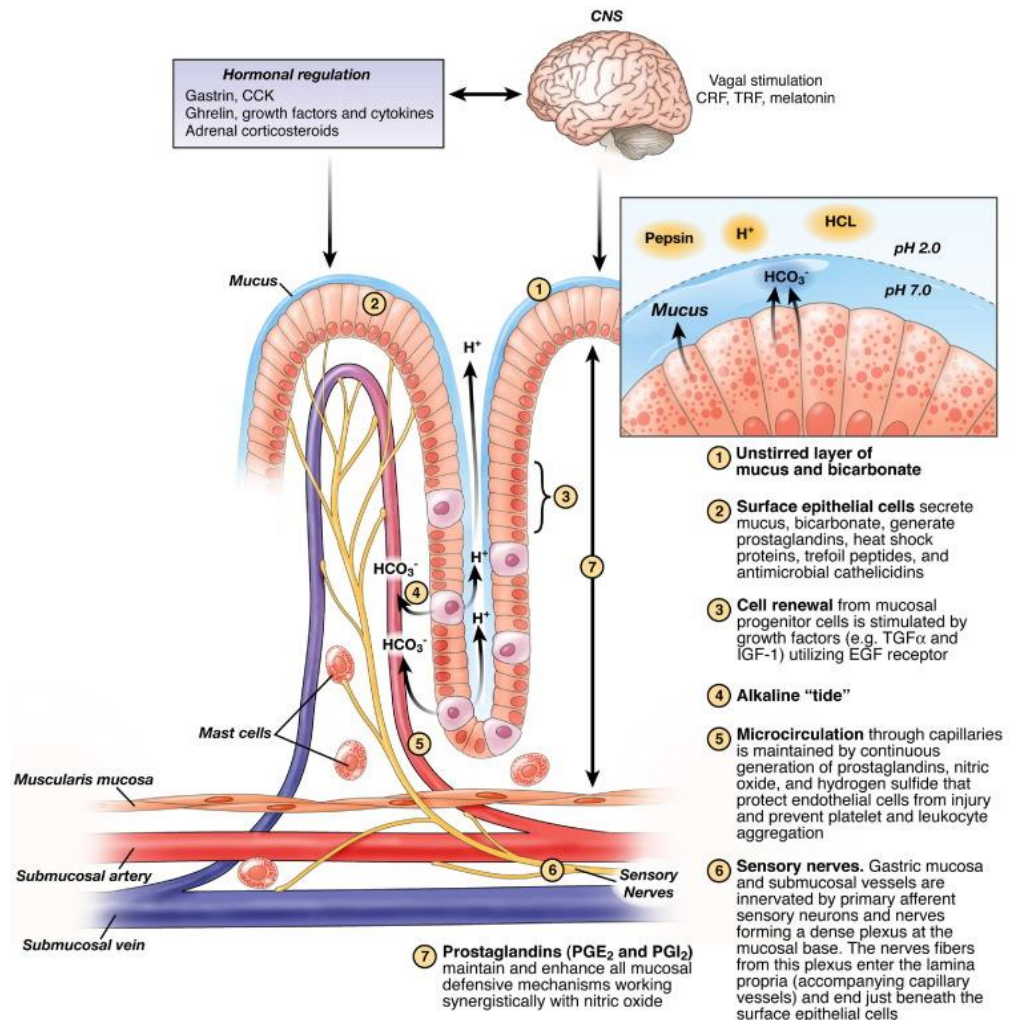
Figure 1.4 – Gastric Mucosal Defence
Note the various mechanisms by which protection is achieved by the gastric mucosal cells
(Laine et al., 2008)

1.4.2   Mechanisms of NSAID-induced GI Injury

1.4.2.1   Inhibition of the COX Enzymes

The role of the COX-1 and COX-2 enzymes in the synthesis of prostaglandins which mediate a host of important protective effects in the GI mucosa, as well as their subsequent inhibition by NSAIDs has already been discussed in details in section 1.2.3.

1.4.2.2   Reduction of Hydrogen Sulphide and Nitric Oxide

Two endogenously produced gases, hydrogen sulphide and nitric oxide are essential to maintaining the homeostasis of the GI mucosa and its stability by replicating a majority of the biological effects of PGs (Oh et al., 2006). Both gases induce the expression of a powerful anti-oxidant called heme oxygenase 1 (HO-1), as well as anti-inflammatory and anti-apoptotic proteins; and directly cause anti-apoptotic and anti-necrotic effects on the mucosal cells (Li et al., 2007).

Hydrogen sulphide (H_2S) has actually been shown to have anti-inflammatory properties based on the observation that leukocyte adherence to the blood vessel endothelium was attenuated by H_2S-donors and triggered a decrease in leucocyte migration to inflammatory locations, preventing the development of oedema, while when endogenous H_2S was inhibited, a contrasting effect was triggered (Zanardo et al., 2006). More importantly, after the use of a NSAID, there was a reduction in the amount of endogenous H_2S synthesised. This was shown to be due to the expression of a crucial enzyme, cystathione-y-lyase which is essential for the conversion of L-cysteine to H_2S

being inhibited (Fiorucci and Santucci, 2011). Hence mucosal H_2S reduction might be a likely mechanism by which NSAIDs cause GI injury.

Nitric oxide is produced by means of the action of nitric oxide synthase on the amino acid L- arginine (Chung et al., 2001), and this crucial enzyme exists in 3 isoforms – one, an inducible isoform (iNOS), and the other 2 are constitutive enzymes (endothelial NOS and neuronal NOS). Depending upon the kind of tissue, its characteristics (i.e. oxidation-reduction state), and the expression level of iNOS, iNOS might potentially increase mucosal blood circulation resulting in improved healing of ulcers, or they could revitalize pro-apoptotic mechanisms and thereby cause tissue ulcer and mucosal injury (Calatayud et al., 2001). NO can be created in several ways via non-enzymatic systems consisting of the use of NO donors. These NO donors have been combined with COX inhibitors, to form a substance referred to as COX-inhibitor nitric oxide donor (CINODs), with the possibility of enhancing the GI safety of the existing COX-inhibitors. This was however, rejected by the US food and drug agency (FDA), for the absence of long term clinical data (Fiorucci and Santucci, 2011).

1.4.2.3   Growth Factor Inhibition

One of the ways by which NSAIDs might trigger a delay in GI injury repair is by reducing PGE2-mediated production of growth factors. The polyamines such as putrescine, spermine, and spermidine, which are sufficiently expressed in the majority of eukaryotic cells play important roles towards cell development and expansion and are likewise important for the purpose of various processes of restitution after NSAID-induced GI injury or injury resulting from other mucosal irritants. Since intracellular amounts of

polyamines are vital for identifying exactly what effects they may have - a high amount would promote increasing cell proliferation, while a low level would trigger apoptosis, polyamine levels are effectively regulated by the enzyme ornithine decarboxylase (ODC), and the catabolic enzyme spermine/spermidine N1-acetyltransferase (SSAT).

Furthermore, in an in vitro study, a group (Hughes et al., 2003) has shown an increase in cell death in cancer cells due to the reduction in intracellular levels of polyamines brought on by the repressive actions of NSAIDs on ODC. Readily available data show that there might be a role for the deficiency of polyamines in NSAID-related ulcers. This is in line with the discovery that in rats treated with indomethacin, there was an increased level of secretion of gastric acid, although considerably minimized levels of gastric ulcer index, mucin content, and decrease in the neutrophil infiltration rates in rats that were given exogenous spermine (Motawi et al., 2007).

It has further been demonstrated that Indomethacin affects the levels and activity of matrix metalloproteinases (MMPs) which has an essential function in the breaking down of the extracellular matrix and its re-structuring due to growth factor activation (Swarnakar et al., 2005; Singh et al., 2011).

1.4.2.4   Direct Cell Injury and Topical Effects

Just like aspirin, most other NSAIDs are a weak organic acid that have a pKa of 3-5. This acidic nature that NSAIDs possess begins and perpetuates the preliminary mucosal injury to the gastrointestinal mucosa by interfering with the barriers of the epithelial cells of the stomach. The carboxylic acid moiety of NSAIDs improves considerably their water solubility and this enhances their interaction with cell membrane phospholipids,

therefore boosting their capacity to enter into the stomach epithelial cells (Bjarnason et al., 2007). After they get into the intracellular area which has a neutral pH of about 7, being weak acids, they quickly dissociate into their ionised forms, consequently triggering intracellular ion trapping.

The tendency of aspirin to trigger injury through the topical impacts has been described by experiments which have actually reported that the animals develop gastric lesions following the administration of aspirin orally, while other NSAIDs caused gastric injury regardless of how they were administered (Mashita et al., 2006). There have been reports about the contribution of oxidative stress to ulcers caused by indomethacin, and it has been proposed that in rats, inactivation of the gastric peroxidase accompanies indomethacin treatment (Chattopadhyay et al., 2006).

Numerous practical studies have revealed that when there is accumulation of NSAIDs in the gastric cells, the uncoupling of mitochondrial oxidative phosphorylation and the inhibition of the electron transport chain, thus causes a substantial usage of intracellular ATP, increased production of reactive oxygen species (ROS), increased intracellular calcium and cellular toxicity (Musumba et al., 2009).

1.4.2.5   **Acid Back-Diffusion**

It is thought that endogenously produced prostaglandins act as mediators of adaptive cyto-protection. It has been reported that the protective function of regional spinal afferent nerves takes place in practically all areas of the gastrointestinal system however those which involve the gastro-duodenal area have been the most thoroughly studied (Sharkey and Savidge, 2014). There is substantial evidence which shows that these

afferent nerves stemming from the dorsal root ganglia have other crucial functions in gastrointestinal blood circulation, mucosal homeostasis, motility, secretion, acid sensing, and repair (Holzer, 2011).

Acid back-diffusion from the lumen to the mucosal cells interrupts the mucosal barrier and this elevation in the intracellular level of acidity sets off the spinal afferent nerves to release calcitonin gene-related peptide (CGRP) and nitric oxide (NO), both of which trigger instant hyperaemia of the gastric mucosa, as well as call into action other defence mechanisms of the gastric mucosa by means of secretion of bicarbonate and reduction of gastric acid secretion (Musumba et al., 2009). There is an intrinsic potential of prostaglandins to preserve the GI mucosa against damage and this takes place even at concentrations that would not normally interfere with the secretion of gastric acid. There have been reports of the extremely important and early consequences of microvascular damage in NSAID-associated ulcers, which lends credibility to the evidence that the mucosal vasculature response is usually the most vital element of gastric mucosal defence from injury (Musumba et al., 2009).

1.4.2.6 Stimulation of Leukotrienes

Another group of arbitrators which may add to the increase in the adherence of neutrophils are leukotrienes. They can lead to mucosal injury which takes place after the administration of NSAIDs. Similarly to prostaglandins, leukotrienes are produced from arachidonic acid, and have been reported to increase the vulnerability of the gastro-intestinal mucosa to injury, as a result of the neutrophil stimulatory effects and adherence to vascular endothelium (Asako et al., 1992). There is likewise proof of raised

production of leukotriene B4 (LTB4) following the administration of NSAIDs to both

human beings and animals (Asako et al., 1992; Hudson et al., 1993). Inhibitors of

leukotriene synthesis and an antagonist of LTB4 receptor have been reported to have

protective effects in NSAID-gastropathy research, and reduce NSAID-induced neutrophil

adherence to vascular endothelium in vivo (Asako et al., 1992; Wallace, 2001).

1.5   Aims of this Research

In an earlier investigation, a genome-wide association study (GWAS) of NSAID-induced intestinal complications was carried out at the University of Liverpool (details of participants and recruitment to follow in subsequent chapters). This identified a putative association between NSAID-related peptic ulcer disease and variants in the *SLC4A4* gene locus on chromosome 4. *SLC4A4* is an essential solute carrier, and plays an important role in the transport of molecules into the cells to avoid an imbalance in intracellular pH. The ability of the cells to attain this balance would be dependent on whether the *SLC4A4* gene is present and functional or not. Previous studies that have been done on the pharmacogenetics of NSAID-related gastrointestinal issues have focused largely on polymorphisms affecting drug metabolism, ion channels and drug transport proteins. There are however, currently no research studies which have taken a look at the impacts of genes associated with the prevention and repair of intracellular acidity in relation to NSAID-induced GI injury and more importantly, no studies have previously examined the function of *SLC4A4* in NSAID-related intestinal injuries. Since SLC4A4 helps to rebalance the pH of the GI mucosa thus preventing acid back-diffusion, the hypothesis of this research is that poor expression could play a role in NSAID-induced upper GI complications. Using an *in vitro* gastric adenocarcinoma (AGS) cell line model, the main objective of this research was to investigate i) the functional impact of *SLC4A4* gene variants in the pathogenesis of NSAID-related gastrointestinal injury, and ii) the functional effect of NSAID interaction with *SLC4A4* on bicarbonate flux.

Chapter 2   **Characterisation and Quantification of the SLC4A4 Gene and Protein**

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2.1   Introduction

2.1.1   Association of SLC4A4 Variants with NSAID-induced Peptic Ulcer Disease

A genome-wide association study (GWAS) undertaken by colleagues within the Department of Molecular and Clinical Pharmacology, University of Liverpool was undertaken on a cohort of 676 individuals (217 cases with endoscopically-confirmed gastroduodenal ulcer and 459 controls with no gastroduodenal ulcer) to identify genetic associations with NSAID-induced peptic ulcer disease. A biologically plausible (albeit not genome-wide significant ($p > 5 \times 10^{-8}$)) signal was identified within the *SLC4A4* gene locus on chromosome 4 (figure 2.1). Thus, the putative role of *SLC4A4* in the pathogenesis of NSAID-induced peptic ulcer disease was investigated.
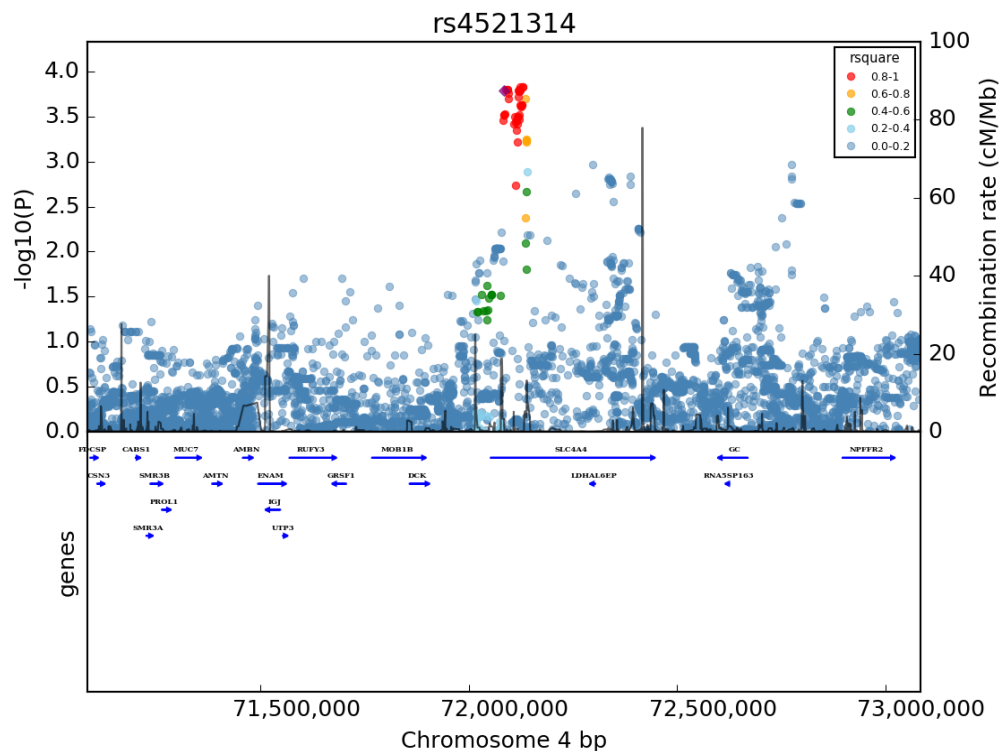
Figure 2.1 – Zoom Plot from GWAS Study of Association Signal Located within the *SLC4A4* Gene Locus
The plot is centred around the rs4521314 SNP which indicated the highest statistical significance with the signal. SNPs in Linkage

2.1.2 Location and Expression of SLC4A4 Gene

The *SLC4A4* gene is located on chromosome 4q21, between the 72,053,002 to 72,437,803 base pairs (figure 2.2; Genetic Home Reference, 2013). Schmitt et al. (1999) reported this gene in basolateral membranes of the renal proximal tubule. Other areas where it has been found include the epididymis (Jensen et al., 1999), ocular cells (Bok et al., 2001; Usui et al., 2001), pancreatic ducts (Marino et al., 1999), cardiac blood vessels (Williams et al., 2003) and brain neurons (Schmitt et al., 2000). Expression in the eye appears to be in a cell-specific manner, with NBCe1-B being detected in the cornea, lens epithelium, retina and surface cells of the conjunctiva in rat eye; and NBCe1-A is expressed in basal cells of the conjunctiva (Bok et al., 2001). NBCe1 was also found expressed in the

epithelial cells of rabbit gastric mucosa, including in the mucous and parietal cells
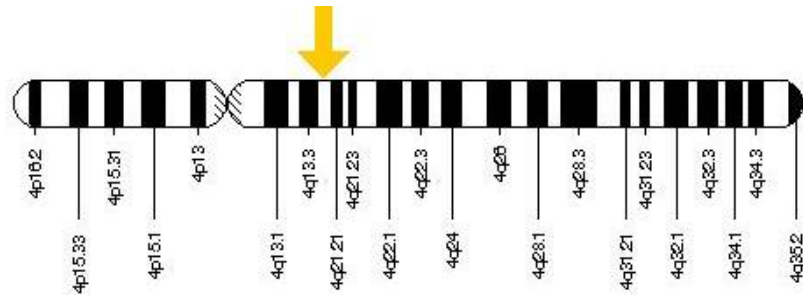
(Rossmann et al., 1999).

Figure 2.2 – Molecular Location of *SLC4A4* Gene
The gene is located on chromosome 4, base pairs 72,053,002 to 72,437,803 (Source:
http://ghr.nlm.nih.gov/gene/SLC4A4#location).

2.1.3   Structure of the SLC4A4 Protein

The SLC4A4 protein, also referred to as electrogenic sodium bicarbonate cotransporter 1

(NBCe1) was first described by Boron and Boulpaep (1983). Romero et al. (1997) were

successful in obtaining the first complementary DNA which encodes a sodium-coupled

bicarbonate transporter (called the renal electrogenic Na/HCO_3 co-transporter). It

possesses a long N-terminal domain which is hydrophilic, just like other members of the

SLC4 family, and a shorter C-terminal domain, which is also hydrophilic, and both of these

domains are intracellular (Kuma et al., 2002). The N and C termini are separated by

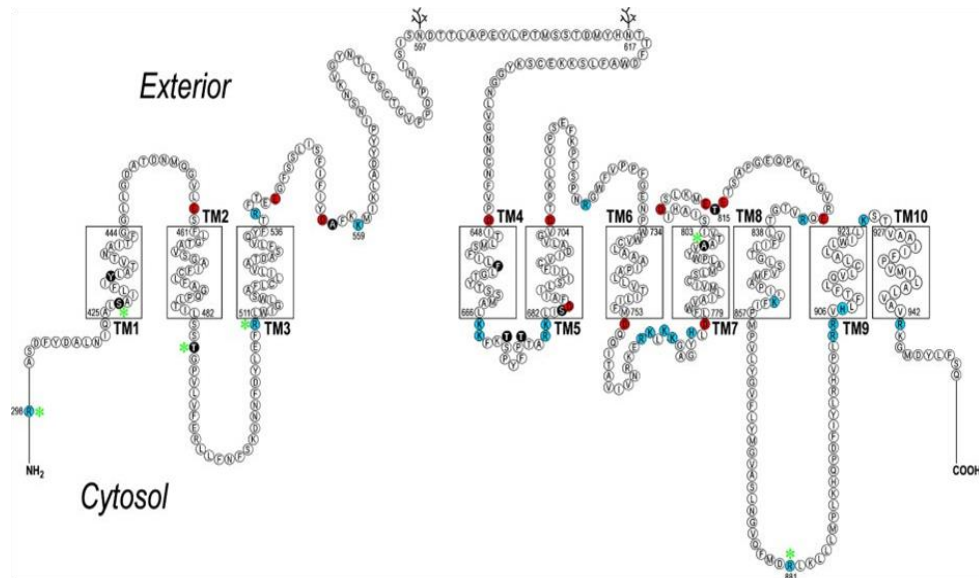between 10 to 14 trans-membrane segments (figure 2.3).

Figure 2.3 – Topological Model of the Human SLC4A4 Protein (NBCe1)
The red, blue and black colours depict important amino acids for NBCe1-mediated transport (acidic, basic and others respectively). Green stars represent residues
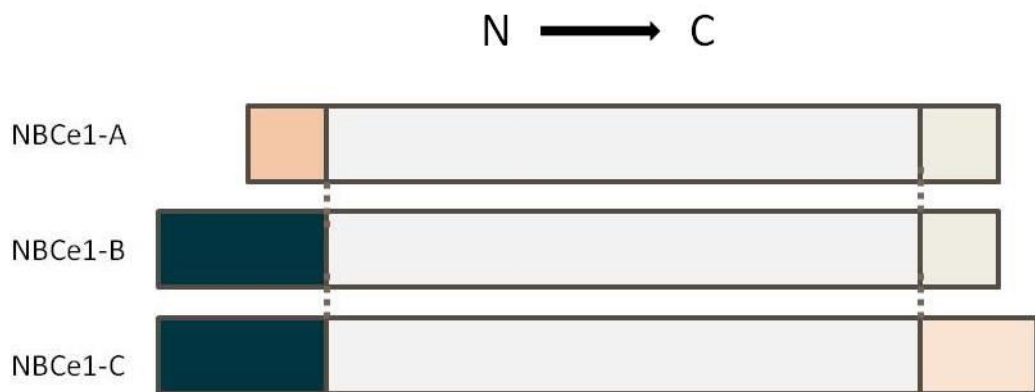

Figure 2.4 – Variant Isoforms of the SLC4A4 Protein
Differently coloured parts indicate existing differences in the isoforms. Modified from Suzuki et al., 2012

This clone, found in the kidney was called NBCe1-A ('e' describes its electrogenic nature, '1' identifies it as the first gene that encodes sodium bicarbonate co-transporters and 'A' connotes the first splice variant of the gene identified, the second being the one identified in the pancreas – NBCe1-B). This gene was later cloned in humans, as

described by Burnham et al. (1997). NBCe1-A and NBCe1-B are identical, except at the N terminal. NBCe1-A encodes 1035 amino acids, while NBCe1-B has 85 amino acids substituting the first 41 amino acids, hence having a total of 1079 amino acids, and an expected protein of 120 kDa. NBCe1-B is found in more tissues (Bok et al., 2001; Suzuki et al., 2012). The third isoform (NBCe1-C) is more commonly found in the brain (Suzuki et al., 2012), and is identical to NBCe1-B, except at the C terminal, where 61 unique amino acids replace the last 46, therefore having 1094 amino acids (figure 2.4).

2.1.4   Role of SLC4A4 in Human Disorders

A number of diseases and disorders have been associated with the loss of function of the *SLC4A4* gene. Mutations in the *SLC4A4* gene of two unrelated patients were first reported in 1999 (Igarashi et al., 1999). The individuals showed signs of mental retardation, severe proximal renal tubular acidosis (pRTA), short stature, hypokalemia, raised serum amylase levels and thyroid abnormalities. They also had several ocular abnormalities which included cataracts, band keratopathy, bilateral calcification of the basal ganglia and glaucoma. A mis-sense p.R298S mutation in the NBCe1-A was identified in the first patient, while there was p.R510H substitution in the second patient. Both NBCe1-A and NBCe1-B variants are affected by these mutations. A different study reported a case of homozygous p.S427L mis-sense mutation in the *SLC4A4* gene in an individual (Dinour et al., 2004). This patient also showed a serious case of pRTA with abnormal dentition, corneal opacities and glaucoma, but showed no signs of mental retardation, or calcification of the basal ganglia.

In the kidney, complete reabsorption of HCO_3^- from lumen to blood is facilitated by NBCe1-A which mediates the transportation of HCO_3^- from proximal tubule to blood. Romero et al. (2004) reported that the principal step in the secretion of HCO_3^- in exocrine ducts is the process of accumulation of HCO_3^- from the cytoplasm.

2.1.5   Role of SLC4A4 in the Upper Gastrointestinal Tract

In the gastrointestinal tract, NBCe1-B plays a vital role in accumulating intracellular bicarbonate ions within the pancreas (Marino et al., 1999). Furthermore, in the pancreas, it contributes to nutrient-based insulin secretion (Soyfoo et al., 2009). The sodium/bicarbonate co-transporter also contributes to the regulation of intracellular pH in non-epithelial cells, by responding to acid loads (figure 2.5). In the duodenum, SLC4A4 has been found expressed in the form of NBCe1-B, and it plays a role in bicarbonate secretion and protects the duodenal cells against injury from acids (Alpern et al., 2013).
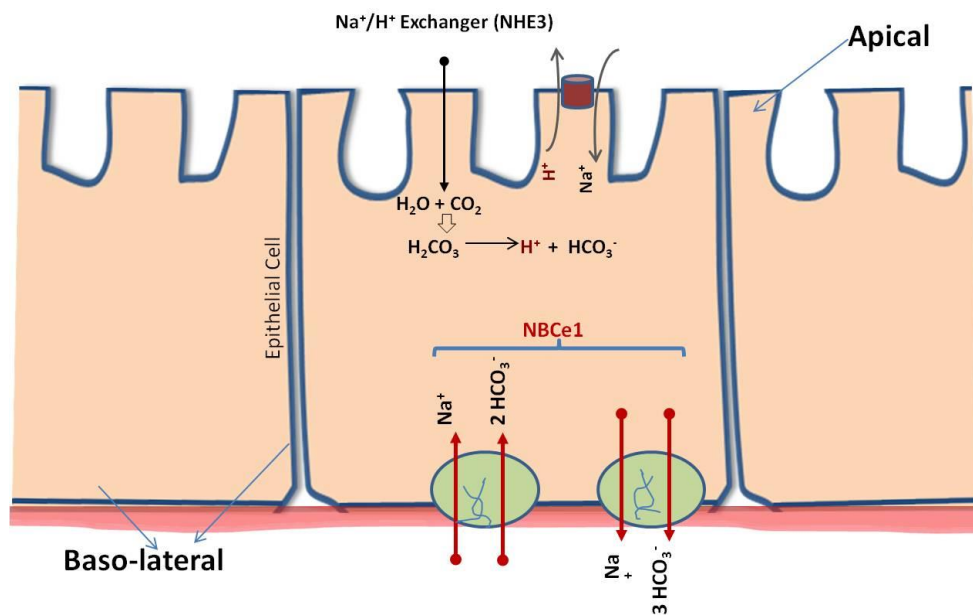
Figure 2.5 – Schematic Illustration of the Mechanism of Transport of Sodium and Bicarbonate (Na$^+$ & HCO$_3^-$) Molecules
This mechanism is effected by the electrogenic Sodium bicarbonate co-transporter (NBCe1) via expressed, functionally normal *SLC4A4* gene.

2.1.6   Aims and Objectives

Using an *in vitro* gastric adenocarcinoma (AGS) cell line model, the aims of this chapter are:

a)   Characterise the presence of the *SLC4A4* gene in gastric epithelial cells

b)   Quantify and identify expression of SLC4A4 protein and its cellular localisation.

c)   Create a stably transfected AGS cell-line with increased expression of the SLC4A4 protein.

2.2 METHODS

2.2.1 Cell Culture

Human gastric adenocarcinoma cells (**a**typical **g**astrin **s**ecreting / AGS cell line) were a kind gift from Prof Andrea Varro, (University of Liverpool). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 1% antibiotic/antimycotic and 1% penicillin/streptomycin (all from Sigma Aldrich, UK), at 37°C, 5% CO_2. All experiments were done using cells that were at least 95% viable.

2.2.2 SLC4A4 Gene Expression in AGS Cells

2.2.2.1 RNA Extraction and Purification

Pelleted AGS cells were disrupted and homogenised using QIAzol lysis reagent according to the manufacturer's protocol (Qiagen, UK). The homogenate was stored in 1.8mLs of RNAlater stabilising solution (Applied Biosystems, UK) in the -80°C freezer. Purification of RNA was carried using the RNeasy mini kit (Qiagen, UK) as detailed in the manufacturer's protocol.

2.2.2.2   TaqMan Gene Expression Assay

Concentration of the RNA sample was determined using the Nanodrop 8000 Spectrophotometer (Thermo scientific, UK). The RNA sample was normalised to 50ng/μl - 100ng/μl concentrations and reverse transcribed by RT-PCR to cDNA using the high capacity reverse transcription kit and Thermal cycler (Life Technologies, UK). For each concentration, a 20 μl reaction volume consisted of: 2 μl 10x RT buffer, 2 μl 10x RT random primer, 0.8 μl 25x dNTP mix, 1 μl Multiscribe Reverse Transcriptase, and 1 μl RNAase inhibitor, 3.2 μl nuclease-free water and 10 μl of the RNA sample (50ng/μl - 100ng/μl). The thermal cycler protocol was 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C, before being cooled to 4°C using the Veriti 96-well Thermal cycler (Life Technologies, UK).

Real Time PCR was then carried out on the resulting cDNA samples with the gene expression master mix kit using a 384-well plate in the PCR machine (Applied Biosystems 7900HT PCR System). cDNA from human renal cortical epithelial cells (RCE) was used as a positive control. The TaqMan gene expression assay (Applied Biosystems, UK) consists of specific probes and primers designed to yield quantitative measurements of specific PCR products. Fluorophores and quenchers are bound to the 5' and 3' ends of the probe (bound to single-stranded DNA), respectively. The polymerase (Taq) amplifies the cDNA primers, causing the fluorophore to fluoresce in accordance to the amount of DNA.

A 20 μl reaction consisted of 1 μl 20x Taqman gene expression assay (4331182) for target (SLC4A4) and endogenous control (beta-actin) (Life Technologies, UK), 10 μl gene expression master-mix (life Technologies), 6 μl of RNase-free water and 3 μl of cDNA into

each well of a 384-well plate. PCR amplification was performed for 45 cycles using the Applied Biosystems 7900HT PCR system as follows: AmpliTaq polymerase activation at $95^{\circ}C$ for 10 minutes, 45 cycles of denaturing at $95^{\circ}C$ for 15 seconds, 45 cycles of annealing at $60^{\circ}C$ for 1 minute. All reactions were carried out in triplicate with appropriate negative controls.

Table 2.1 – TaqMan Gene Expression Assays

Gene symbol	Gene name	Fluorophore/Quencher	Assay ID
SLC4A4	Solute carrier family 4, member 4	FAM/NFQ	Hs00186798_m1
ACTB	Beta actin	FAM/NFQ	Hs01060665_g1

Source: Applied Biosystems (UK). *FAM* 6-carboxyfluorescein, *NFQ* non-fluorescent quencher

2.2.2.3 Analysis of Gene Expression Data

Analysis of the data was performed using the SDS software, version 2.2 (Life Technologies, UK) using the comparative C_T method. Cycle time (C_T) for all samples was determined and the level of *SLC4A4* expression was determined using the ΔC_T method as previously described by Livak and Schmittgen (2001).

$$\Delta C_T = C_T \text{ (target gene)} - C_T \text{ (reference gene)}$$

Normalised expression for the sample was determined by using $2^{-\Delta C_T}$, while fold change between sample groups was compared by $2^{-\Delta C_T}$ (AGS cells) / $2^{-\Delta C_T}$ (RCE cells). Unpaired t

test was used to determine the significance of the difference between the two samples using the StatsDirect software (version 3).

2.2.3   Detection of SLC4A4 Protein in AGS Cells by the Western Blot

2.2.3.1   Preparation of AGS Cells Protein Lysates

Cells were washed with 10ml PBS twice to remove residual growth media; 1ml of PBS was added to the flask and a cell scraper was used to detach the cells. Cell pellet was then centrifuged at 2000rpm for 2 minutes. RIPA buffer (70µl) of and 2µl Protease inhibitor cocktail were added to the pellet and sonicated. The suspension was centrifuged at 4°C at 13,000rpm for 20 minutes. The resulting protein supernatant was transferred to a new tube and stored at -80°C.

2.2.3.2   Quantification of Protein Lysate

The Pierce BCA assay kit (Thermo Scientific, UK) was used to quantify the amount of protein in cell lysate according to the manufacturer's protocol. Briefly, a set of protein standards at concentrations between 0 µg/ml – 2000 µg/ml was made from bovine serum albumin (BSA), using RIPA buffer (Sigma Aldrich, UK) as diluent. A working reagent was prepared, by mixing 50 parts of the BCA reagent A to 1 part of BCA reagent B. 10 µl per well of each BSA protein standard was added in triplicate to wells of a 96-well plate. 10 µl of unknown concentration of protein lysate sample was added to separate wells of the 96-well plate. To all standards and lysate samples, 200 µl of the working reagent was added and mixed on a plate shaker. The plate was then covered and incubated at 37°C

for 1 hour, and thereafter cooled to room temperature for 10 minutes. The absorbance at 595nm was measured in a plate reader (Beckman Coulter DTX 880 Multimode detector). A standard curve was constructed and used to determine concentrations of all unknown protein samples.

2.2.3.3 Western Blot Assay

Based on the BCA assay, 15μg/mL and 20μg/mL concentration of protein lysates from AGS cells as well as 20μg/mL concentration from conditionally immortalised proximal tubule epithelial cells (ciPTEC, used as control) were mixed with 5μl loading dye (30μl reducing agent and 70μl sample buffer) and heated for 5 minutes at 85°C, then cooled on ice for 5 minutes. All samples were loaded, together with a molecular weight marker into separate wells in a 10-well precast SDS-PAGE gel plate (Invitrogen, UK) to undergo separation by electrophoresis at 170V for 1hour using 1x running buffer (50mL of Invitrogen 20x MOPS solution, 950mL of distilled water). Protein was transferred to nitrocellulose membrane (Invitrogen, UK) in 1x transfer buffer (50mL of NuPAGE 20x stock solution, 200mL of methanol, 750mL of distilled water, 1mL of NuPAGE antioxidant) at 80V for 1 hour. Protein bands on the membrane were visible upon addition of Ponceau S stain, which was subsequently washed off using TBS-Tween20 solution - TBST (TBS composition: 80g NaCl, 2g KCl, 30.3 Trizma base, 800ml distilled water). The membrane was then blocked overnight to prevent non-specific binding at 4°C using 5% non-fat milk.

The membrane was incubated with 1:500 dilution of rabbit polyclonal primary antibody to SLC4A4 protein (Abcam, UK) for 1 hour at room temperature. After washing four

times with TBST, the HRP-conjugated goat-anti-rabbit secondary antibody (Abcam, UK) was added at a 1:10,000 dilution and allowed to also incubate for 1 hour at room temperature. The membrane was again rinsed in TBST, excess solution removed by patting dry, and the chemiluminescence reagent (Abcam, UK) added for 1 minute prior to photographic film exposure and development.

2.2.4   **Immunocytochemistry and Fluorescent Microscopy**

Immunofluorescent staining was carried out on the cells to determine the localisation of protein. The AGS cells were plated onto coverslips in 12-well plates at a density of 1 X 10^5 cells per well and cultured overnight at 37°C. At ~80% confluence, media was aspirated and cells were washed twice in PBS and fixed with 2% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. The PFA was aspirated and fixation quenched with fresh 50mM Ammonium Chloride solution for 10 minutes. After washing with PBS, cells were permeabilised with 0.2% Triton-X 100 in PBS for 10 minutes at room temperature, and rinsed in TBS.

Blocking was done with TBS containing 0.1% Tween-20, 1% BSA and 5% goat serum for 1 hour, after which cells were incubated with the primary antibody – rabbit polyclonal anti-SLC4A4 (Abcam, UK) overnight at a dilution of 1:100. Cells were rinsed with TBST and the fluorescent-labelled secondary antibody, Dylight 488-goat anti-rabbit IgG (Abcam, UK) at a 1:250 dilution was added for 1 hour at room temperature. Phalloidin (1:250 dilution, Abcam, UK) was used to stain for Actin filament, while Hoechst stain (1:5000 dilution, Abcam, UK) was used to stain the nuclei for 10 minutes. Coverslips were mounted face down onto glass slides with 10 µl Prolong Gold mounting reagent (Life Technologies, UK)

and allowed to set overnight in the dark. Fluorescent Images were obtained using the Zeiss Axio Observer Z1 fluorescent microscope.

2.2.5 Transfection of SLC4A4 Clone in AGS Cells

2.2.5.1   Selection of SLC4A4 Clone

The SLC4A4 clone, obtained from Origene, USA was the myc-ddk- tagged human SLC4A4 transcript variant 1 (RC217660) with a pCMV6-entry and expressing the complete ORF. This variant was selected because it encodes the longest isoform of the gene and is expressed across multiple human body tissues (Bok et al., 2001).
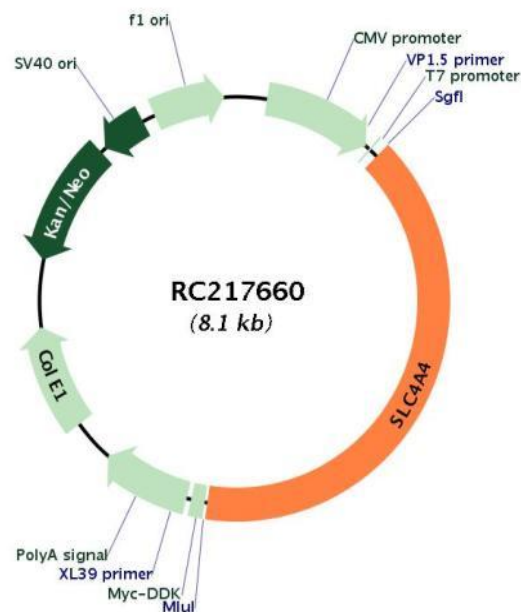
Figure 2.6 – Molecular Structure of myc-ddk-tagged Human *SLC4A4* Transcript Variant 1 - RC217660
(Source: Origene, USA)

To determine the sequence of the DNA clone, 100ng/µl of the sample was sent off for SANGER sequencing (Source Bioscience, UK).

2.2.5.2   Preparation of LB Agar and Broth

For the LB broth, 5g of LB broth powder (Sigma-Aldrich), UK) was added to 250mls of distilled water in a sterile bottle and mixed by swirling. The LB Agar media was prepared by adding 6.4g of Agar (Sigma-Aldrich UK) to 200mls of distilled water in a sterile bottle and mixing. Both solutions were sterilised by autoclaving at 121°C for 15 minutes.

Agar solution was allowed to slowly cool to 50°C, before 25µg/ml of kanamycin antibiotic was added. Twenty-five mls of the warm media was added to each 25mm sterile round culture dish and allowed to cool before using.

2.2.5.3   Culture and Transformation of E. coli Bacteria with SLC4A4 Clone

Five microlitres of the *SLC4A4* cDNA clone was added to 50µl of *E. coli* suspension, and incubated for 30 minutes on ice. Cells were heat-shocked for 30 seconds at 42°C, and put on ice for 2 minutes. S.O.C medium (350µl) was added and the mixture was incubated on a horizontal shaker at 37°C for 1 hour, then 20µl - 200µl from the transformed *E. coli* suspension was placed in pre-warmed selective agar plates and spread with a sterile glass rod. Plates were then inverted and incubated at 37°C for 24 hours.

A single colony of *E. coli* from each of three culture plates was added to three bottles containing 10mls LB broth (containing 25µg/ml kanamycin) and incubated at 37°C for 8 hours on a shaker. Once the culture became turbid in appearance, 200 µl was added to

100mls of fresh broth (with 250 µl of kanamycin) and incubated at 37°C on a shaker overnight.

2.2.5.4 Isolation of Plasmid DNA from Recombinant E. coli Culture

Plasmid DNA isolation from recombinant bacteria was done using the GeneJET Plasmid Maxiprep kit (Thermo Scientific, UK) according to manufacturer's protocol. DNA concentration was determined using a Nanodrop 8000 Spectrophotometer (Thermo scientific, UK).

2.2.5.5 Agarose Gel Electrophoresis

Four microliters (4µl) of a 1kb ladder (New England Biolabs, UK) was additionally used. Samples were run on a 1% agarose gel (1g of agarose – Sigma Aldrich, UK, 100mls of Tris-borate EDTA (TBE) and 60 µl of ethidium bromide) at 100V for 1 hour. Separation was captured using a UV-based gel Transilluminator with a camera system.

2.2.5.6 Transient Transfection of SLC4A4 Clone

Cells were seeded at a density of 400,000 cells per well in a 6-well plate and allowed to incubate overnight at 37°C. The next day, a transfection master-mix containing 6 µl Lipofectamine 2000 (Invitrogen, UK) and 100 µl of Optimem media (Life Technologies, UK) per well was prepared in a tube. A mixture containing either 1µg or 2µg of *SLC4A4* plasmid DNA, 100µl of Optimem and 200ng of enhanced green fluorescent protein (EGFP) (gifted by Dr Shankar Varadarajan, University of Liverpool) was used to determine the best conditions and amount of *SLC4A4* to use in subsequent experiments. EGFP was utilised to determine transfection efficiency.

200μl of mastermix solution, combined with the DNA mix (1:1 volume) was incubated at room temperature for 20 minutes. Two hundred microlitres of the DNA-mastermix solution was then added to the reaction wells. An extra well which contained the EGFP, but not *SLC4A4* was added as a control. The cells were incubated at 37°C and checked with a fluorescent microscope after 24 hours. HEK293 cells were used as controls in parallel to compare transfection.

2.2.5.7   Development of Stable Pool of SLC4A4-Expressing Cells

Cells were seeded and transfected as described in section 2.2.5.6 using 1μg of *SLC4A4* for 24 hours. The solution was removed from the wells and replaced with fresh media for 24 hours to allow the cells to recover. Antibiotic selection was then performed using 0.5μg/mL and 0.75μg/mL of puromycin in media for AGS and HEK293 cells respectively, for 48 hours recurrently until cells began to show increased resistance to the antibiotic. The antibiotic solution, including dead detached cells was removed and replaced with conditioned media. Conditioned media was prepared by filtering previously used growth media (containing cell secretomes and proteins) through a 0.2μm pore syringe and fortifying with 50% v/v of fresh FBS whole media to replace glucose and other essential media components. When cells became confluent in the wells, they were passaged as described in section 2.2.1 and re-plated into new wells.

To determine suitable puromycin dose for the antibiotic selection step above, a dose-response experiment was initially performed with the MTT assay on untransfected cells using puromycin concentrations between 0μg/mL - 2μg/mL for 48 hours. The chemical compound used was 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (or

MTT). This assay is used to detect live and metabolically active cells which are able to convert the yellow tetrazolium salt into an insoluble purple formazan product via the action of reducing enzymes. The purple precipitate is then solubilised and quantified (Maioli et al., 2009).

After the cells had been treated with the drug, the drug-media was removed and 20 µl of the 0.5mg/ml MTT working solution, made by diluting the MTT stock (5mg of thiazolyl blue tetrazolium bromide reagent, 1ml of Hank's balanced salt solution) in media was added to the wells. Thereafter, cells were incubated further at 37°C for 3 hours in the dark, after which 100 µl of lysis buffer (50mls of dimethylformamide, 50mls of distilled water, 20g of sodium dodecyl sulphate, SDS) was added to each well to dissolve the purple formazan. The plates were then incubated overnight at 37°C and the absorbance was measured at a wavelength of 595 nm using a micro-plate reader (Beckman Coulter DTX 880 Multimode detector).

2.3   Results

2.3.1   SLC4A4 Gene Expression in AGS Cells

The level of endogenous expression of *SLC4A4* gene in native AGS cells was determined.

The result (figure 2.7) showed that the *SLC4A4* level was 31 times lower in AGS cells than

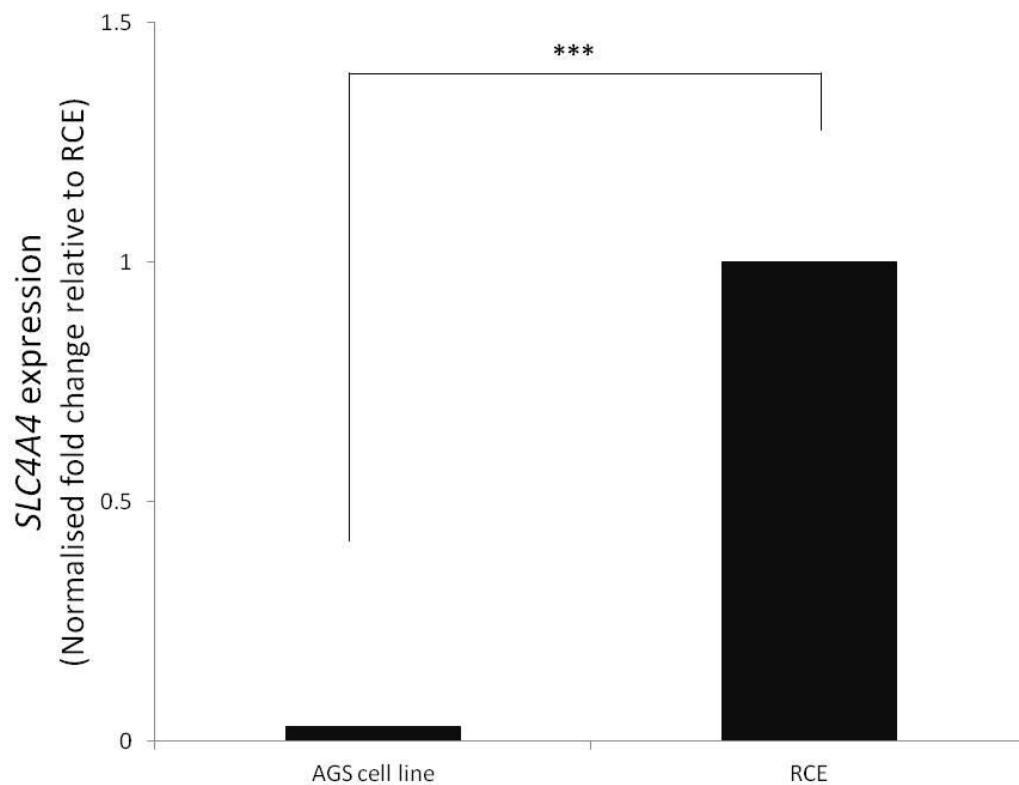in renal cortical epithelial cells (RCE); this was highly significant (p = 0.0001).

Figure 2.7 – Gene Expression Levels of *SLC4A4*
The relative level of *SLC4A4* expression in AGS cells was compared to RCE. Native AGS cells showed significantly lower levels of the gene compared to the RCE cells used as control. Fold change represents normalised gene expression levels of AGS to RCE. *** = *p* ≤ 0.001.

2.3.2   SLC4A4 Protein Expression in AGS Cells

After confirming the presence of the gene in AGS cells, it was important to determine if it was being translated into protein, and hence subsequent experiments focused mainly on assessing protein levels.   Firstly, the endogenous levels of SLC4A4 protein were determined in the native AGS cells by Western blotting (figure 2.8).   The SLC4A4 protein bands can be seen in the films exposed to the nitrocellulose membrane after the protein transfer.   The bands appeared around the 120 kDa mark as expected, and showed an increase in density corresponding to the amount of protein that was loaded.   The ciPTEC cells, used as control, showed higher expression of the protein.
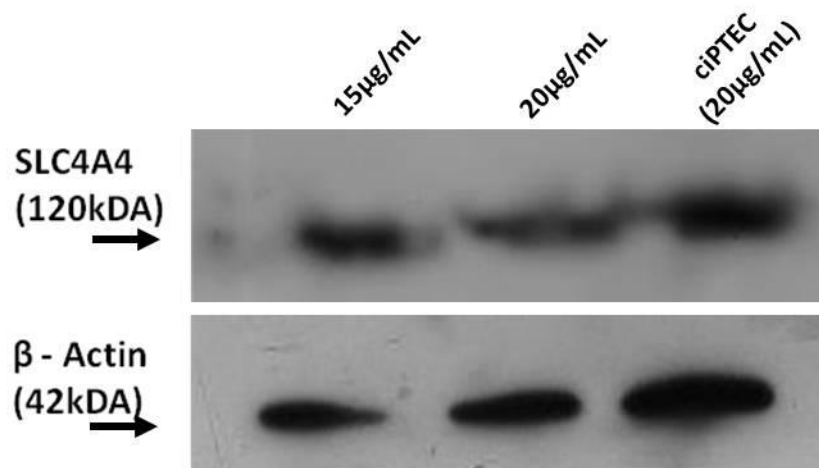
Figure 2.8 – Western Blot Analysis of Native AGS Cells showing SLC4A4 Bands
15µg/ml and 20µg/ml of protein from AGS, and 20µg/ml of protein from ciPTEC was loaded into the gel wells, and resolved by electrophoresis.   Visible protein bands can be seen corresponding to SLC4A4 and β-Actin.   ciPTEC: conditionally immortalised proximal Tubule epithelial cell line (used as positive control)

2.3.3 Cellular Localisation of SLC4A4 by Immunocytochemistry

Cellular localisation of SLC4A4 protein in native AGS cells was determined by immunocytochemistry. The results from the staining (figure 2.9) showed that modest amounts of SLC4A4 protein was localised to the cell cytosol, as well as to the plasma membrane of native AGS cells.
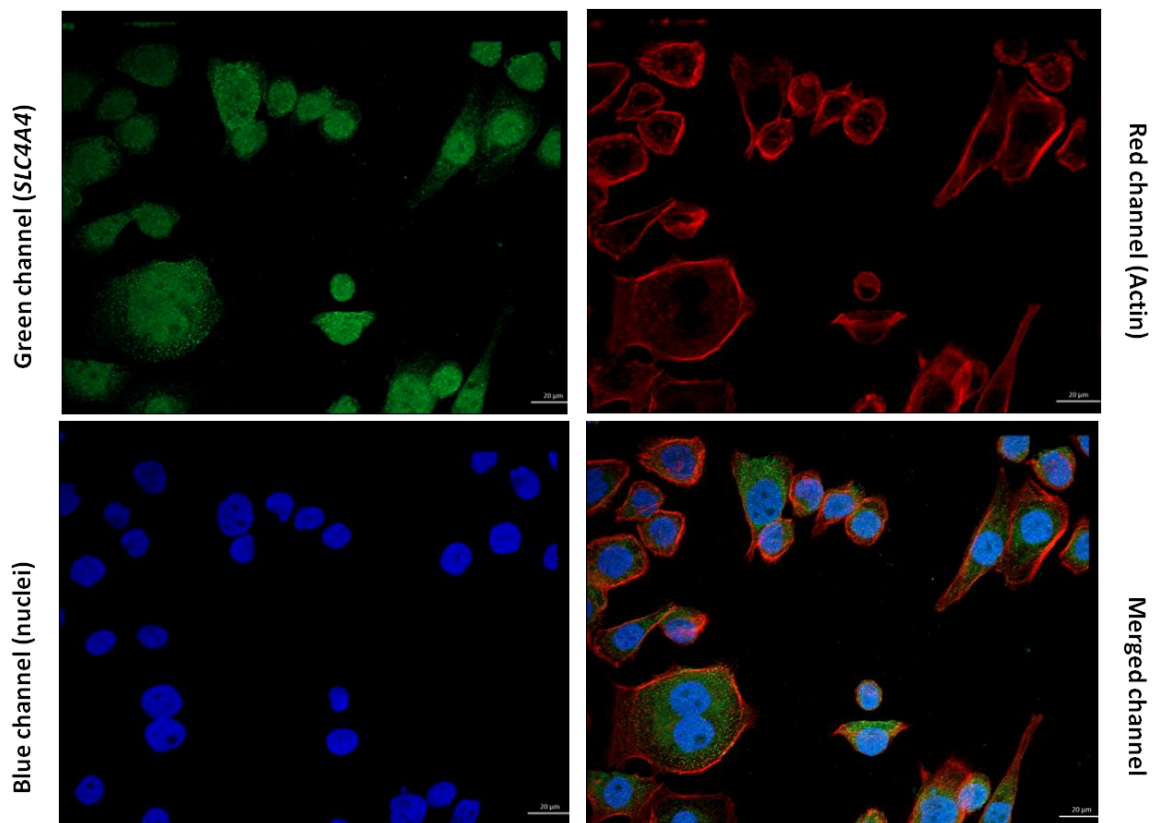

Figure 2.9 – Fluorescent Images showing Localisation of SLC4A4 Protein in Native AGS Cells
Cells were treated with the primary antibody (rabbit anti-SLC4A4, ab56215) for 1 hour, at a dilution of 1:100, after which the fluorescent secondary antibody (Dylight 488 goat anti-rabbit IgG - Green) was used at a dilution of 1:250. Phalloidin (red) was used to show the distribution of actin filaments, and Hoechst was used to stain the nuclei (blue). The SLC4A4 protein, stained green, can be seen in the cytosol. Scale bar: 20μm

2.3.4 Characterisation of SLC4A4 Plasmid DNA Sequence

2.3.4.1 Matching Sequences between Plasmid DNA and Reference Sequence

To determine if the plasmid DNA which was obtained was authentic, it was decided that it should be sent it for sequence review, so as to compare it with the gene reference sequence from NCBI (Accession number: NM_001098484). Once the plasmid sequence was obtained from SANGER sequencing (Source Bioscience, UK)), it was compared with the reference using an online tool (diff-online.com). The DNA base sequences was then translated into the amino acid sequences in the protein using the ExPASy translate tool (Swiss Institute of Bioinformatics). Table 2.2 shows the comparison of the amino acid sequences in the SLC4A4 reference protein and those in our prepared plasmid.

It was observed that the SLC4A4 clone used in this study was 100% homologous with the NCBI reference sequence with the exception of the insertion of 19 additional amino acids right at the carboxyl terminus, prior to the stop codon thus elongating the expected peptide from 1,079 to 1,098 amino acids. This extra sequence of amino acids however corresponds to the cloning region and the tag that was used in our clone, as the myc tag amino acid sequence is comprised of 10 amino acids, EQKLISEEDL (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu).

Table 2.2 – Comparison of SLC4A4 Plasmid and Reference Sequences

Reference protein sequence (Accession: NM_001098484, Protein ID: NP_001091954.1)

MEDEAVLDRGASFLKHVCDEEEVEGHHTIYIGVHVPKSYRRRRRHKRKTGHKEKKEKERISENYSDKSDIENADESSSSILKPL

ISPAAERIRFILGEEDDSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEEKVEQGGERWSKPHVATLSLHSLFELRTCME

KGSIMLDREASSLPQLVEMIVDHQIETGLLKPELKDKVTYTLLRKHRHQTKKSNLRSLADIGKTVSSASRMFTNPDNGSPAM

THRNLTSSSLNDISDKPEKDQLKNKFMKKLPRDAEASNVLVGEVDFLDTPFIAFVRLQQAVMLGALTEVPVPTRFLFILLGPK

GKAKSYHEIGRAIATLMSDEVFHDIAYKAKDRHDLIAGIDEFLDEVIVLPPGEWDPAIRIEPPKSLPSSDKRKNMYSGGENVQ

MNGDTPHDGGHGGGGHGDCEELQRTGRFCGGLIKDIKRKAPFFASDFYDALNIQALSAILFIYLATVTNAITFGGLLGDATD

NMQGVLESFLGTAVSGAIFCLFAGQPLTILSSTGPVLVFERLLFNFSKDNNFDYLEFRLWIGLWSAFLCLILVATDASFLVQYF

TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINSNFKVGYNTLFSCTCVPPDPANISISNDTTLAPEYLPTMSSTDMYHNTTFD

WAFLSKKECSKYGGNLVGNNCNFVPDITLMSFILFLGTYTSSMALKKFKTSPYFPTTARKLISDFAIILSILIFCVIDALVGVDTP

KLIVPSEFKPTSPNRGWFVPPFGENPWWVCLAAAIPALLVTILIFMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILMVIC

SLMALPWYVAATVISIAHIDSLKMETETSAPGEQPKFLGVREQRVTGTLVFILTGLSVFMAPILKFIPMPVLYGVFLYMGVAS

LNGVQFMDRLKLLLMPLKHQPDFIYLRHVPLRRVHLFTFLQVLCLALLWILKSTVAAIIFPVMILALVAVRKGMDYLFSQHDL

SFLDDVIPEKDKKKKEDEKKKKKKKKGSLDSDNDDSDCPYSEKVPSIKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC-Stop

Plasmid protein sequence

MEDEAVLDRGASFLKHVCDEEEVEGHHTIYIGVHVPKSYRRRRRHKRKTGHKEKKEKERISENYSDKSDIENADESSSSILKPL

ISPAAERIRFILGEEDDSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEEKVEQGGERWSKPHVATLSLHSLFELRTCME

KGSIMLDREASSLPQLVEMIVDHQIETGLLKPELKDKVTYTLLRKHRHQTKKSNLRSLADIGKTVSSASRMFTNPDNGSPAM

THRNLTSSSLNDISDKPEKDQLKNKFMKKLPRDAEASNVLVGEVDFLDTPFIAFVRLQQAVMLGALTEVPVPTRFLFILLGPK

GKAKSYHEIGRAIATLMSDEVFHDIAYKAKDRHDLIAGIDEFLDEVIVLPPGEWDPAIRIEPPKSLPSSDKRKNMYSGGENVQ

MNGDTPHDGGHGGGGHGDCEELQRTGRFCGGLIKDIKRKAPFFASDFYDALNIQALSAILFIYLATVTNAITFGGLLGDATD

NMQGVLESFLGTAVSGAIFCLFAGQPLTILSSTGPVLVFERLLFNFSKDNNFDYLEFRLWIGLWSAFLCLILVATDASFLVQYF

TRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINSNFKVGYNTLFSCTCVPPDPANISISNDTTLAPEYLPTMSSTDMYHNTTFD

WAFLSKKECSKYGGNLVGNNCNFVPDITLMSFILFLGTYTSSMALKKFKTSPYFPTTARKLISDFAIILSILIFCVIDALVGVDTP

KLIVPSEFKPTSPNRGWFVPPFGENPWWVCLAAAIPALLVTILIFMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILMVIC

SLMALPWYVAATVISIAHIDSLKMETETSAPGEQPKFLGVREQRVTGTLVFILTGLSVFMAPILKFIPMPVLYGVFLYMGVAS

LNGVQFMDRLKLLLMPLKHQPDFIYLRHVPLRRVHLFTFLQVLCLALLWILKSTVAAIIFPVMILALVAVRKGMDYLFSQHDL

SFLDDVIPEKDKKKKEDEKKKKKKKKGSLDSDNDDSDCPYSEKVPSIKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC-

TRTRPLEQKLISEEDLAAN -Stop

The plasmid sequence was checked by SANGER sequencing (Source Bioscience, UK)), and compared with the reference sequence using an online tool (diff-online.com). The DNA base sequences was then translated into the amino acid sequences in the protein using the ExPASy translate tool (Swiss Institute of Bioinformatics).

2.3.4.2 Analysis of Plasmid DNA Purification by Agarose Gel Electrophoresis

In order to determine the successful isolation of plasmid DNA after the maxi-prep, electrophoresis was carried out in a 1% agarose gel. The ethidium bromide which was added during the gel preparation intercalates and binds between the basepairs of the DNA, allowing the bands to be visible when the gel was exposed to UV light. In figure 2.10, the DNA bands in lanes 2 and 3 are clearly seen, besides the molecular ladder in lane 1.
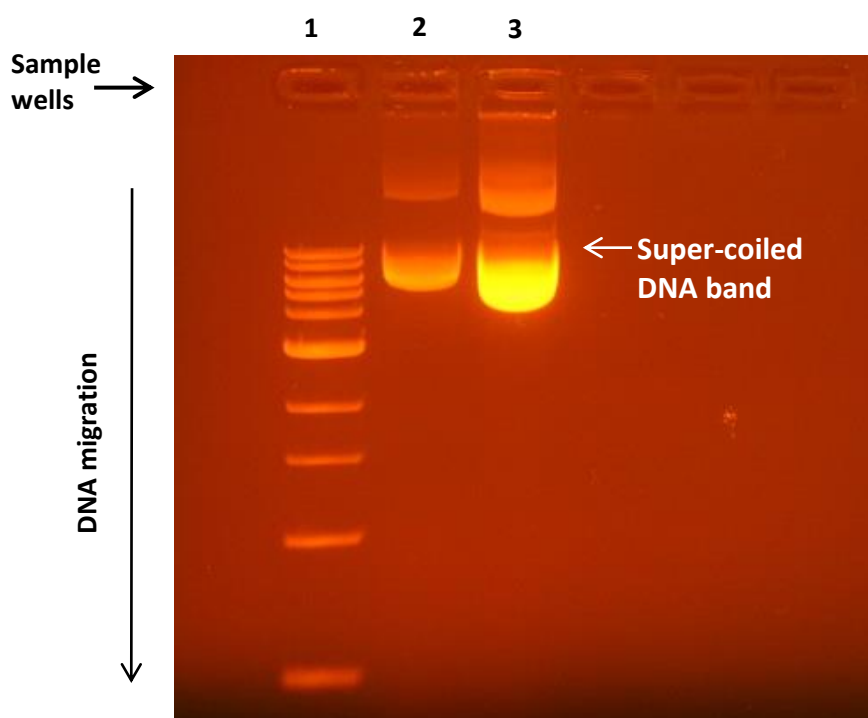

Figure 2.10 – Digital Image of DNA Bands on Agarose Gel after Plasmid Purification
Samples were diluted with the loading buffer to a 1:20 (lane 2) and 1:4 (lane 3) concentration, and run on a 1% agarose gel, alongside a 1kb DNA ladder (lane 1). Direction of migration of the DNA is indicated. Notice the DNA bands in lanes 2 and 3 which confirm the successful purification of the plasmid.

2.3.5   Transient Transfection of SLC4A4 Clone

1µg and 2µg of *SLC4A4* plasmid DNA was transiently transfected into cells, with 200ng of EGFP to observe transfection efficiency. The results obtained from fluorescent images (figure 2.11) confirmed that in both AGS and HEK293 cells, transfection yield was better with the 1µg of DNA compared with 2µg. Hence 1µg DNA was used for later experiments.
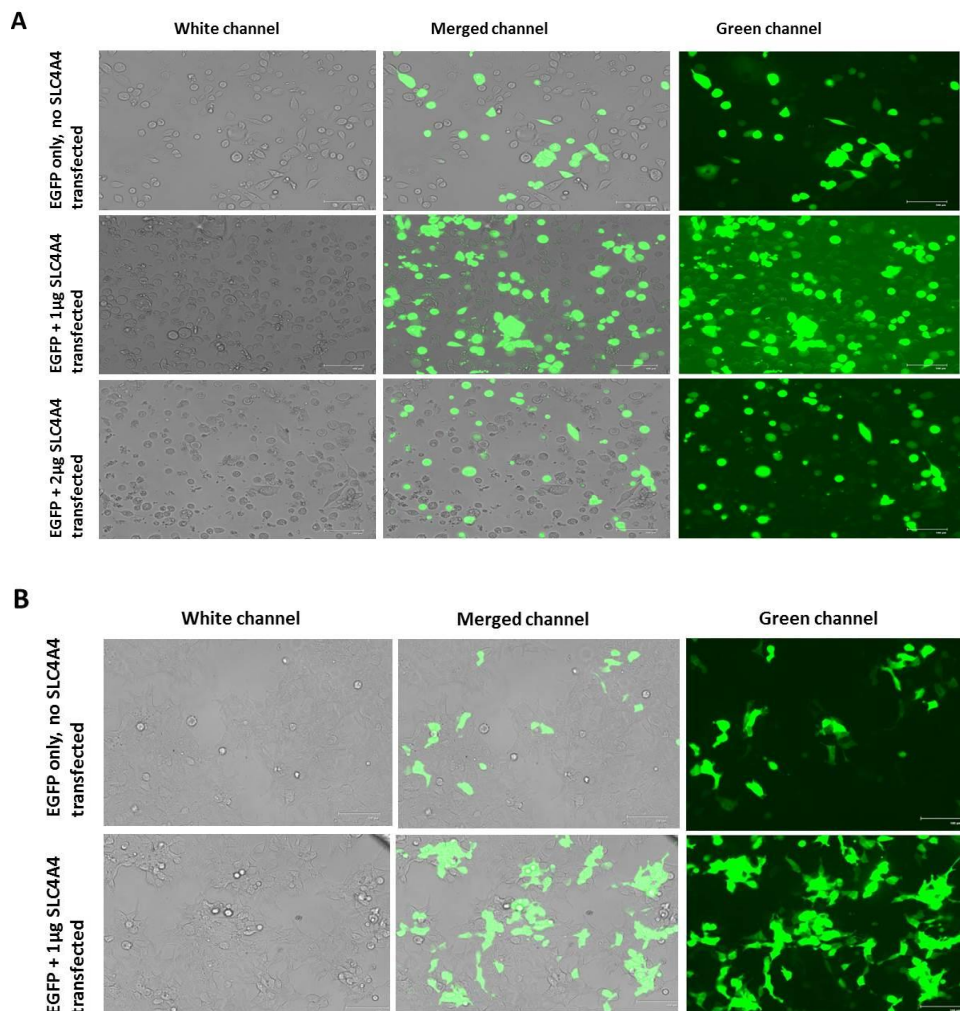

Figure 2.11 – Transfection of *SLC4A4*, with EGFP to Monitor Transfection Efficiency
(A) AGS cells and **(B)** HEK293 cells after 24 hours transfection with *SLC4A4*. Enhanced green fluorescent protein (EGFP) was used to monitor transfection efficiency between 1µg and 2µg of *SLC4A4* DNA under a fluorescent microscope. Cells showed a better uptake with 1µg DNA. Layout of cells: white channel; cells which took up transfected material: green channel. Scale bar: 100µm

Immunofluorescent staining was again performed on the transiently transfected AGS cells, concurrently with untransfected cells. There was a clear localisation of SLC4A4 protein in the cytoplasm of the transfected cells as well as in the untransfected cells (figure 2.12).
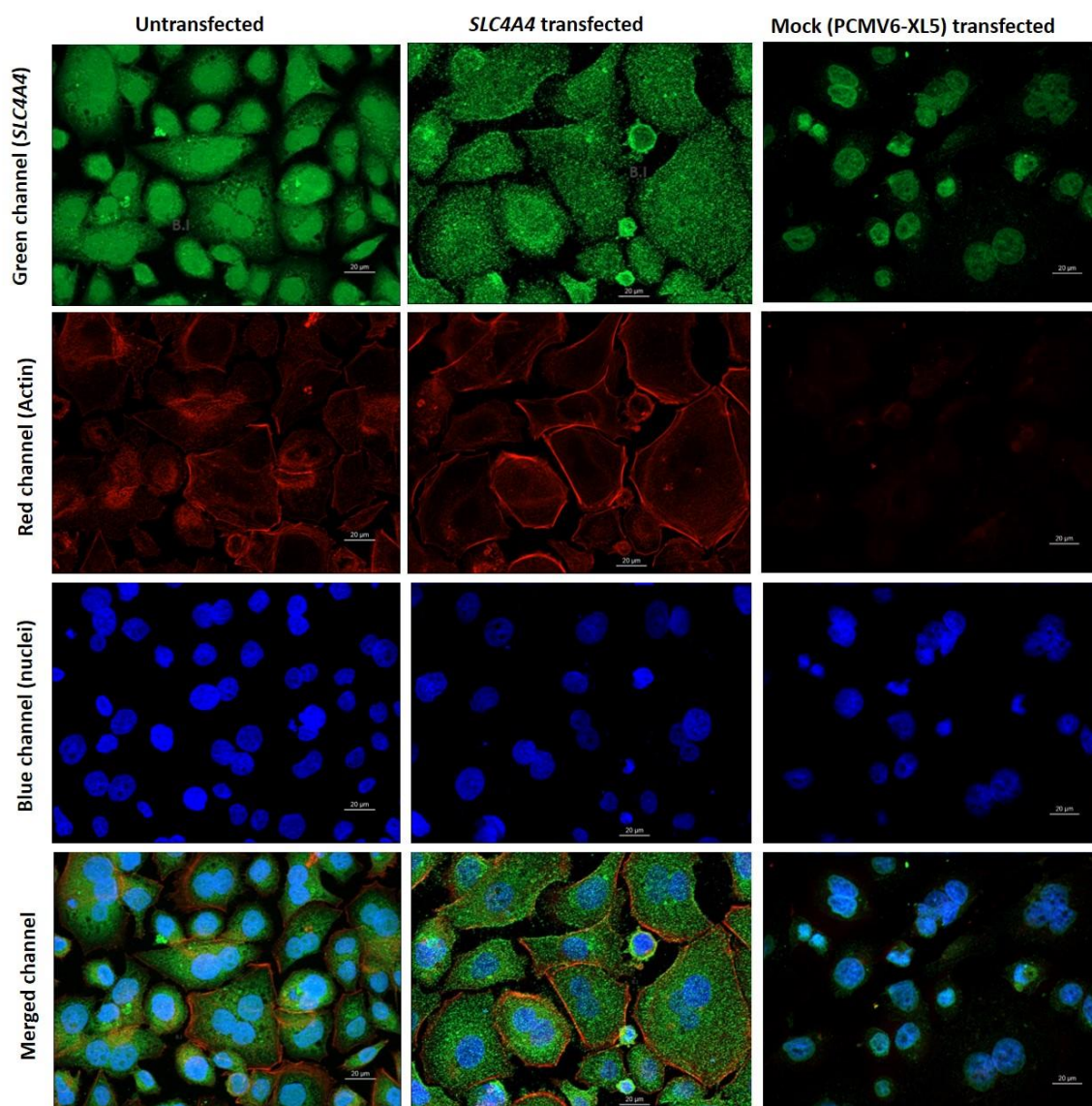

Figure 2.12 – Fluorescent Images showing Localisation of SLC4A4 Protein in Transiently Transfected AGS Cells

After 24 hours Cells were treated with primary antibody (rabbit anti-SLC4A4, ab56215) for 1 hour, at a dilution of 1:100, after which fluorescent secondary antibody (Dylight 488 goat anti-rabbit IgG - Green) was used at a dilution of 1:250. Phalloidin (red) was used to show the distribution of actin filaments, and Hoechst was used to stain the nuclei (blue). The SLC4A4 protein, stained green, can be seen in the cytosol and membrane. Scale bar: 20μm

2.3.6   Development of Stable Pool of SLC4A4-Expressing Cells

2.3.6.1   Puromycin Sensitivity in AGS and HEK293 Cells

Prior to creating a stable pool of cells expressing *SLC4A4,* the selection of cells was carried out using a puromycin dose that was sufficient to kill the cells and reduce viability by about 50%. To determine the amount of resistance to puromycin, a dose-response curve was done with increasing doses of puromycin. Figure 2.13 shows the dose-response curves for puromycin exposed AGS and HEK293 cells. From the results, it was observed that AGS cells showed a higher sensitivity to puromycin, compared to HEK293 cells. At 1µg/mL of the drug, only about 12% of AGS cells remained viable, while for HEK293 cells, 35.5% of cells showed continued viability at this dose.

At the maximum dose used (2µg/mL), the viability of HEK293 cells was over twice that of AGS at 25% and 11.7% respectively. The suitable dose chosen, which caused close to 50% response in the cells, was 0.5µg in AGS cells with 39% viability and 0.75µg in HEK293 cells with 46% viability. These doses were used to perform the antibiotic selection of resistant cells.
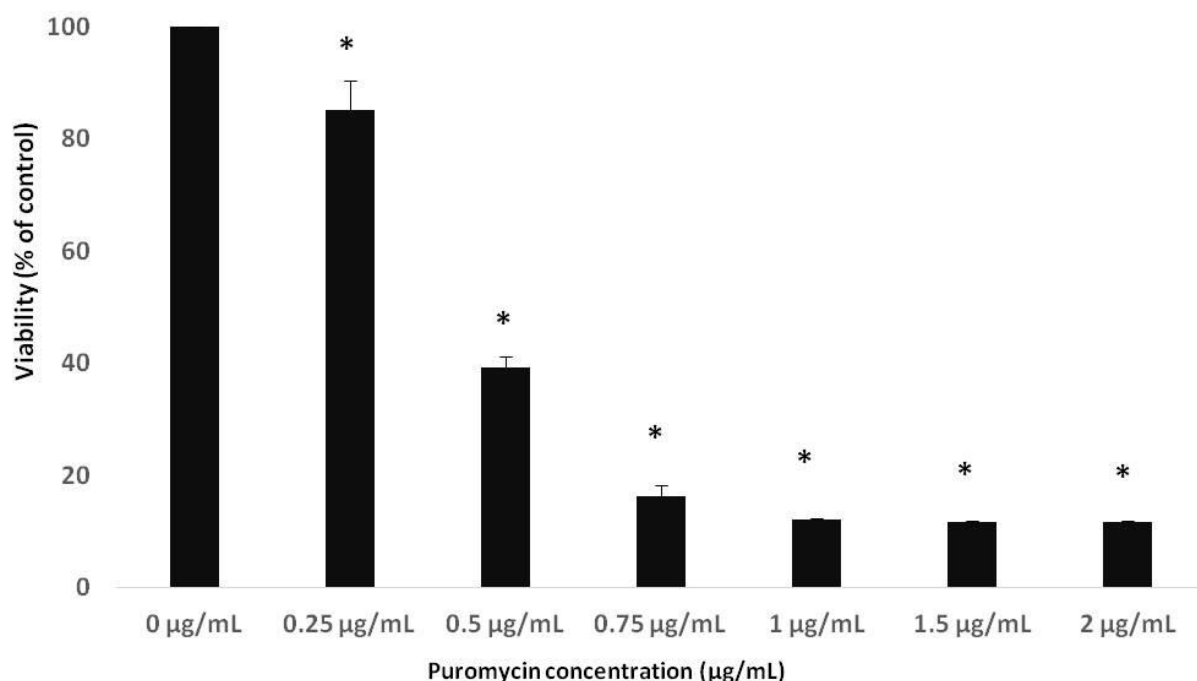
Figure 2.13 – Dose-Response Graph showing Puromycin Effect
AGS cells showed sensitivity to puromycin. The dose, which caused close to 50% response in the AGS cells was 0.5µg, with 39% viability. *= p ≤ 0.05

2.3.6.2   Confirmation of Protein Expression Post-Transfection

After transfecting the cells, another western blot assay was performed to confirm whether there was over-expression of the SLC4A4 protein. As the cells had been transfected with an SLC4A4 plasmid which was tagged with the myc epitope sequence, protein analysis was performed using a HRP-conjugated myc antibody (Thermo Scientific, UK) to detect the presence of the tagged protein of interest. Cells with the various treatments were processed as described in 2.2.3.1 to extract the protein, and about 20µg/mL from each treatment were loaded into a 15-well gel and analysed. The results showed visible bands detected at 57 kDa in all the lanes where the protein was loaded

(figure 2.14).   In native AGS cells, the endogenous myc protein was also detected,

showing a visible but faint band compared to cells in which the protein had been over-
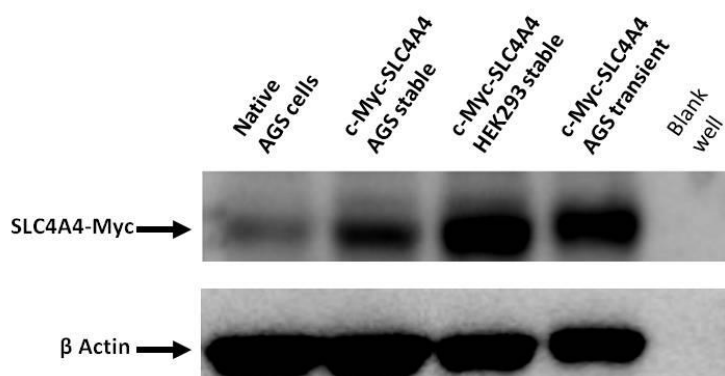
expressed, along with the myc.

Figure 2.14 – Western Blot Analysis of Cells after Transfection
Myc-tagged SLC4A4 plasmid was used. 20μg/mL of protein was loaded into the gel wells,
and resolved by electrophoresis before being transferred to a nitrocellulose membrane.
The membrane was blocked, and probed with the HRP-conjugated myc antibody at a
dilution of 1:1000. Chemiluminescent detection was performed and protein bands were
detected using the ChemiDOC system.

2.4 Discussion

Several studies have shown the presence of *SLC4A4* gene in a range of tissues, including cerebrum, cerebellum, choroid plexus, renal cortex and medulla, pancreas, duodenum, ileum, colon, and stomach (Damkier et al., 2007; Danielsson et al., 2015). It is thought to play a vital role in the influx/efflux of sodium and bicarbonate ions during active transport, thereby helping to maintain pH balance. Some studies have identified *SLC4A4* as a gene of interest in blood pressure (Jin and Eom, 2012), hypertension (Yang et al., 2012), ulcerative colitis, and colorectal cancer (Zhu et al., 2015).

This chapter has focused on the characterisation and quantification of *SLC4A4* in AGS cells. The results presented have been able to confirm the presence of the gene in AGS cells, albeit in small quantities. The data supplements the sparse data currently available on expression of SLC4A4 in AGS cells. Similar result to what was obtained in this study, comparing SLC4A4 expression levels in AGS and RCE cells has been described by Rossman et al. (1999) using rabbit gastric cells. Moreover, the results in this chapter are in harmony with other studies which have shown low *SLC4A4* levels in carcinoma cells. In studies of papillary thyroid cancers, the level of expression of *SLC4A4* mRNA was reduced compared to the surrounding normal tissue (Galeza-Kulik et al., 2006; Kim et al., 2010). Furthermore, analysis of colon adenocarcinoma cells revealed that the levels of mRNA of several members of the SLC4 family, including *SLC4A4*, were diminished, compared to normal tissue (Gorbatenko et al., 2014). This could explain the low bicarbonate levels, and low pH, acidic environment in tumour cells. In future experiments, however, studies

carried in this chapter could be improved by using cells from similar organs/tissues chosen as positive control in both gene and protein studies.

It has also been shown in this chapter that in AGS cells, the SLC4A4 protein (NBCe1) was localised in the cytosol, as well as in the plasma membrane as shown previously (Toye et al., 2006; Perry et al., 2008).

The detection of myc in the myc-tagged SLC4A4-transfected clone was a more suitable option to adequately monitor the protein expression after transfection. An epitope tag sequence of 10 amino acids derived from the c-myc protein, the myc tag sequence is comprised of EQKLISEEDL (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) and helps to distinguish the wild type protein from the over-expressed protein bearing the tag. The tag is considered too small to cause any significant changes in the function of the original *SLC4A4* gene.

These results presented in this study show for the first time, a successful characterisation of *SLC4A4* in AGS cells. AGS cells are validated models for studying GI toxicity as they constitutively express cyclooxygenase-1 and cyclooxygenase-2 which play a role in responding to gastric injury (Hall et al., 2006). These cells shall subsequently be utilised in upcoming experiments to study the functional aspects of the gene with regard to accumulation of drugs and bicarbonate.

Chapter 3 Correlation between SLC4A4 Genetic Variants and SLC4A4 Gene Expression in Gastric Biopsy Tissue

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3.1   Introduction

The bicarbonate-transporter family which can also be called the SLC4 family of transporters consist of up to ten different genes in the human species (Burnham et al., 1997). Each of these genes are grouped into three and named according to their function. Among these, there are the anion exchangers (AEs) which transport chloride/bicarbonates (Cl^-/HCO_3^-) ions; the Sodium-driven Chloride-bicarbonate exchangers (NDCBE), which usually transport Sodium or chloride ions within the cells, and the Sodium-bicarbonate (Na^+/HCO_3^-) co-transporters (NBCs), which transport sodium and bicarbonate across the cells. Some members of the SLC4 family are inhibited by disulfonic stilbene derivatives such as 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, DIDs (Jessen et al., 1986; Lane et al., 1999). The extracellular part of position 5 of the trans-membrane (TM5) in sodium/bicarbonate co-transporters group 1 (NBC1) have been shown to react with drugs of this nature (Heyer et al., 1999).

In addition, it has been found that members of the SLC4 family exhibit glycosylation. The electrogenic NBC1, the AE1, AE2, and AE3 are N-glycosylated. In AE1, the N-glycosylation occurs on extracellular loop 4 only, while in the other anion exchangers, AE2 and AE3; and in NBC1, the N-glycosylation occurs in extracellular loop 3 only. The other members of the SLC4 family possess up to two positions of N-glycosylation on extracellular loop 3, and no glycation on extracellular loop 4. In addition, all of the family members with well defined functions are integral membrane proteins which carry no less than one

monoatomic ion and bicarbonate (HCO_3^-) and/or carbonate (CO_3^{2-}) ion through the plasma membrane. Table 3.1 shows a summary of the SLC4 family (Romero et al., 2004).

In spite of all the similarities above, there are important differences between the subgroups of the SLC4 family. Apart from differing in their structure and function, one of the most remarkable differences between them is their transport activities. For instance, the sodium-driven chloride-bicarbonate exchanger (NDCBE) more likely to function as an exchanger and co-transporter which co-transports Na^+ and either two HCO_3^-, or one CO_3^-, or only one pair of one $NaCO_3^-$ ion through the cells in return for one chloride ion (Cl^-). On the other hand, the anion exchangers AE1, AE2 and AE3 exchange monovalent anions from one side of the membrane to another and the sodium bicarbonate (Na^+/HCO_3^-) co-transporters transports Na^+ and HCO_3^- from one membrane point to another.

Also, another main difference between the SLC4 members is the kind of ion that is carried in addition to the bicarbonates, i.e. either a cation or an anion. Some members such as the AE1, AE2 and AE3 carry the chloride ion, Cl^-; others such as the NBCn1, NBCe1 and NBCe2 carry the sodium ion, Na^+ and yet another, the NDCBE carries both the chloride and sodium ions, Cl^- and Na^+ respectively.

Table 3.1 – The SLC4 family

Human gene	chromoso	Protein	Function	pH regulation
SLC4A1	17q21	AE1	Cl^-/HCO_3^- exchange	Acid loader
SLC4A2	7q35	AE2	Cl^-/HCO_3^- exchange	Acid loader
SLC4A3	2q36	AE3	Cl^-/HCO_3^- exchange	Acid loader
SLC4A4	4q21	NBCe1 (NBC1)	Na^+/HCO_3^-	Acid extruder
SLC4A5	2p13	NBCe2 (NBC4)	Na^+/HCO_3^-	Acid extruder
SLC4A7	3p22	NBCn1 (NBC3)	Na^+/HCO_3^-	Acid extruder
SLC4A8	12q13	NDCBE	Na^+/HCO_3^-	Acid extruder
SLC4A9	5q31	AE4	Cl^-/HCO_3^- exchange	Acid loader
SLC4A10	2q23	NBCn2 (NBCE)	Na^+/HCO_3^-	Acid extruder
SLC4A11	20p12	NaBC (BTR1)	$Na^+/B(OH)_4^-$	Acid extruder for

Another unique difference is the nature of the transporter, i.e. whether it is electrogenic or electro-neutral. In electrogenic transporters, one cycle of transport would result in the movement of at least one net negative charge through the membrane (Dinour et al., 2004; Romero et al., 2004). This leads to a shift in membrane potential (Vm). NBCe1 and NBCe2 are electrogenic, whereas the other members are electro neutral; i.e. no net movement of electrical charges result from one cycle of transport through the membrane, and consequently there is no change in the membrane potential (Sciortino and Romero, 1999; Romero et al., 2004).

A recent genome-wide association study (GWAS) undertaken by colleagues within the department of molecular and clinical pharmacology, University of Liverpool was undertaken on a cohort (see section 2.2.1) to identify genetic associations with NSAID-induced peptic ulcer disease. The study identified a notional association of aspirin induced gastric ulcerations with a signal in the *SLC4A4* gene locus on chromosome 4 region matching SNP. The top SNP in this signal was rs4521314 which is positioned on Chr4: 72084653 and is an intronic variant, encoding C > A substitution. In the HapMap CEU population of Northern and Western European ancestry, the minor allele frequency, MAF for this SNP is A=0.09, that is 9% of the total population sampled (The International HapMap, 2003).

Little is known as to the effect of *SLC4A4* variation and expression in human tissues or in disease. However, a few studies (Jin and Eom, 2012; Guo et al., 2016; Yang, et al., 2012) have described the possible role of certain other SNPs of *SLC4A4* in blood pressure and

hypertension. Other studies (Igarashi, et al., 1999; Dinour, et al., 2004) have reported a

link between the gene and renal tubular acidosis.

The aims of this chapter were therefore, to: i) assess the relationship between *SLC4A4*

genotypes and gene expression in gastric biopsy tissues, and ii) determine the

relationship between NSAID-induced ulceration and *SLC4A4* expression.

3.2   Methods

3.2.1   NSAID Ulceration Patient Cohort

The study involved the recruitment of 1239 patients from hospitals within the United Kingdom, 835 (67.4%) of whom had a history of Peptic ulcer disease (PUD). Among those with a history of PUD, 485 (58.1%) were NSAID users, 109 of whom had upper GI bleeding (UGIB), and 376 of whom had no UGIB. The other 350 PUD patients (41.9%) were non-NSAID users (49 of them having UGIB, and 299 having no UGIB). Of the PUD patients, 318 (38.1%) were on low dose aspirin (LDA). The remaining 404 patients (32.6%) in the study were without any PUD history; 123 of whom were NSAID users, and 281 non-NSAID users. Figure 3.1 summarises the entire study and the details of the demographics of the cohorts used

cDNA and genomic DNA samples from gastric corpus biopsies from a total of 100 healthy control patients with non-specific upper abdominal pain were a kind gift from Professor Andrea Varro, University of Liverpool. Gastric pathological conditions were excluded via endoscopy and gastric biopsy examination.

Ethical approval was obtained from the Liverpool Research Ethics Committee, and informed consent was given by the patients prior to commencement of the study.
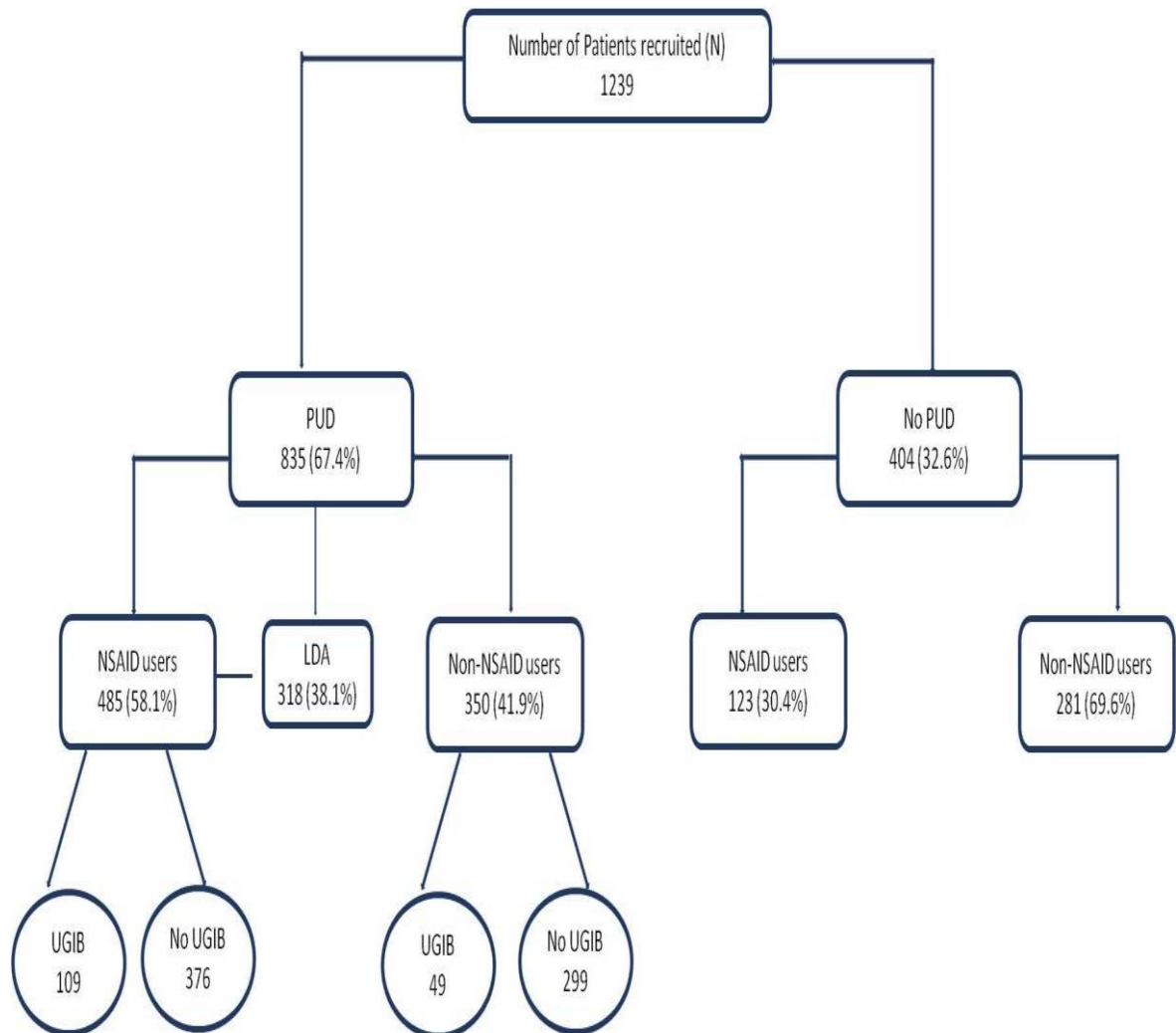
Number of Patients recruited (N)
1239

PUD
835 (67.4%)

No PUD
404 (32.6%)

NSAID users
485 (58.1%)

LDA
318 (38.1%)

Non-NSAID users
350 (41.9%)

NSAID users
123 (30.4%)

Non-NSAID users
281 (69.6%)

UGIB
109

No UGIB
376

UGIB
49

No UGIB
299

Figure 3.1 – Schematic Summary of Patients' Demographics and Cohorts.
The study involved the case cohort (patients with a history of PUD) vs. the control cohort (patients without PUD history). Both cohorts were irrespective of NSAID use. The case cohort was further categorised into patients with or without UGIB among the NSAID and non-NSAID users. UGIB status was not known for two of the patients. *UGIB*, upper GI bleeding.

3.2.2 RNA Sequencing

RNA sequencing analysis was carried out on 30 gastric biopsy samples taken from ulcerated and healthy stomach tissue (antrum) of study cohorts, adjusting for the absence of clinical covariates including *H. pylori* infection, use of proton pump inhibitors (PPI) and previous GI diseases. The samples were from the antrum of 10 healthy control

patients, and 10 ulcer patients (ulcer edge and antrum). The 10 ulcer patients were categorised based on their NSAID use as follows: 3 non-aspirin NSAID users, 3 aspirin only users, and 4 users of aspirin and other NSAIDs.

RNA extraction from the samples was carried out using the RNeasy mini kit (Qiagen, UK) following the manufacturer's protocol. The Agilent 2100 Bioanalyser was then used with the RNA Nano 6000 kit (Agilent Technologies, USA) to determine the RNA integrity number (RIN) following the manufacturer's protocol, with a cut-off of 7 applied. The RNA samples were subsequently selected using Dynabeads (Invitrogen, UK), and the Epicentre ScriptSeq, version 2 RNA-seq library preparation kit (Illumina, USA) was used to prepare RNA-seq libraries from 50ng poly-A RNA. The 10-cycle amplification was followed by purification of the libraries using Ampure XP beads (Beckman Coulter, USA), and then quantified with the Invitrogen Qubit fluorometer (Thermofisher Scientific, USA).

Six libraries per pool were multiplexed, in a total of 5 separate pools, and sequenced using HiSeq (Illumina, USA). TopHat 2.0.8 and Bowtie 2.1.0 software (CCB, Johns Hopkins University) were then used to subsequently map the sequences to human genome (hg)-19 reference sequence. Counts were finally reported at the gene level.

3.2.3   Assessing the Correlation between SLC4A4 SNP rs4521314 and SLC4A4 Gene Expression in Healthy Gastric Biopsy Samples

3.2.3.1   SLC4A4 Gene Expression Analysis in Gastric Biopsy Samples

SLC4A4 gene expression was determined in the cDNA samples by RT-PCR using the Taqman gene expression assay (Applied Biosystems, UK), as previously described in section 2.2.2.2.

3.2.3.2   Genotyping and Allelic Discrimination for SLC4A4 SNP rs4521314

Genotyping of rs4521314 SNP was performed on 20ng/µl of the genomic DNA samples from healthy patients using the Taqman SNP genotyping assays (Applied Biosystems, UK) according to the manufacturer's instructions. This assay is PCR-based and utilises the 5' nuclease activity of Taq DNA polymerase to differentiate between alleles. Each assay contains both a VIC and a FAM labelled probe that detects one of the SNP-specific alleles by the emission of an allele-specific fluorescent signal after the probe has been degraded.

Fluorescence and allelic discrimination were determined by the use of the SDS software, version 2.2 (Applied Biosystems, UK). Each 5µl reaction comprised of 1 x Taqman genotyping master mix, 20 x Taqman genotyping assay containing the primers and fluorescent probes, nuclease-free water, and 20ng/µl of genomic DNA. Amplification of DNA was carried out according to the manufacturer's protocol, in the Applied Biosystems HT 7900 fast Real-Time PCR System using a 384-well plate after activation of the enzyme at 95°C for 10 minutes. The program was 40 cycles of 15 sec at 95°C and 60 sec at 50°C.

Genotypes were determined by automatic calling on the VIC/FAM signal cluster plots in the SDS software (Applied Biosystems, UK).

3.2.4   Statistical Analysis

Analysis of the gene expression data was done as described in section 2.2.5, using the SDS software, version 2.2 (Life Technologies, UK). Patients with missing genotype or gene expression data were excluded from the data analysis. The frequencies of genotypes were tested for Hardy-Weinberg equilibrium (HWE), with a p value of ≤ 0.01 taken to indicate a deviation. Mann Whitney test was used to determine the difference in gene expression levels between the genotypes, with a p value of ≤ 0.05 considered significant.

3.3   Results

3.3.1   Association SLC4A4 SNP rs4521314 with SLC4A4 mRNA Expression in Healthy Gastric Tissue

A genome-wide association study (GWAS) identified an association between an intronic *SLC4A4* SNP, (rs4521314) and aspirin-induced ulcer risk ($p=7.5 \times 10^{-5}$; heterozygous OR (95% CI) = 2.33 (1.44-3.80) (Carr et al., unpublished data).

Out of 100 DNA samples obtained from the gastric corpus of controls, seven of them were excluded from the final data analysis due to incomplete data. Among the 93 healthy control samples analysed, 75.3% (n = 70) were homozygous for the allele C, 19.3% (n = 18) were heterozygous, and 5.4% (n = 5) were homozygous for the variant allele, A.

The statistics for all 3 groups were calculated using the 1-way ANOVA, and mean expressions by group were found to be 0.022, 0.02 and 0.035 for the AA, AC and CC genotypes, respectively. The maximum expressions recorded for the different genotypes were more varied at 0.051, 0.12, and 0.38 for AA, AC, and CC respectively. Overall, most of the samples had a similar level of expression, regardless of genotype, as the median expression was 0.015, 0.017, and 0.017 for AA, AC, and CC respectively. Of the total samples, there was no statistically significant difference between genotype groups in terms of association between genotype and *SLC4A4* gene expression in biopsies from the gastric corpus (figure 3.2).
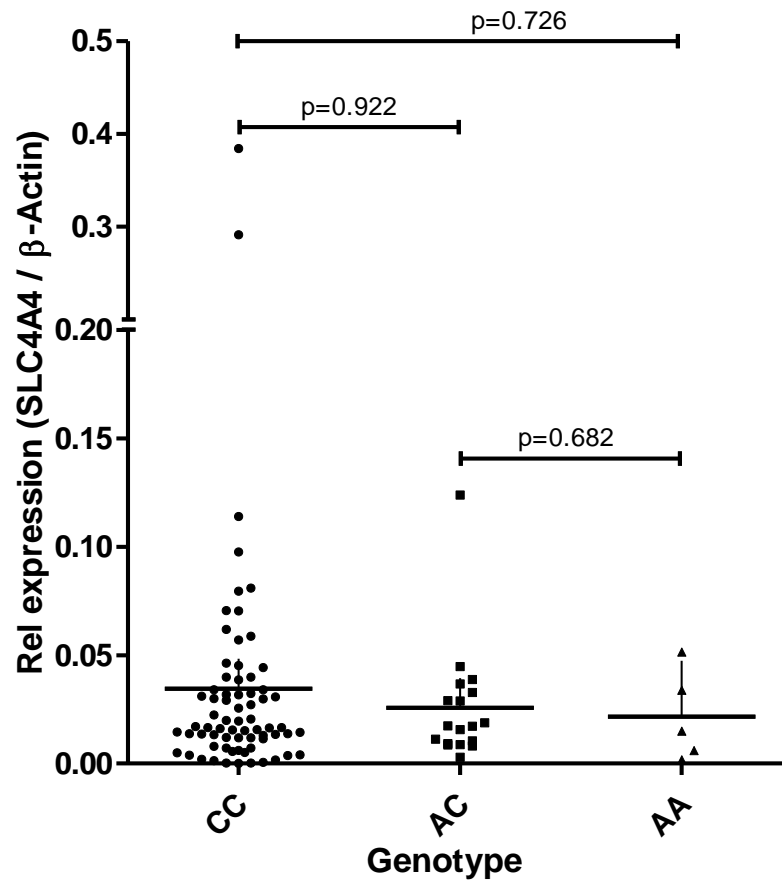
Figure 3.2 – *SLC4A4* **gene expression by genotype of rs4521314 in gastric corpus biopsy tissue from healthy controls**

About 75.3% (n = 70) of the samples were homozygous for the allele C, 19.3% (n = 18) were heterozygous, and 5.4% (n = 5) were homozygous for the A (variant) allele. No significant difference was observed in the *SLC4A4* gene expression between all three genotypes.

3.3.2 Correlation of rs4521314 with SLC4A4 mRNA Expression in Case and Antrum Biopsy Tissue of Control and Ulcer Patients using RNA Sequencing Data

RNA sequencing suggested that carriage of the variant 'A' allele generally correlated with *SLC4A4* transcript levels. In the unstratified data (figure 3.3), no significant difference was observed between the control and case antrum biopsies. However, stratified according to genotype, the individuals who were AA homozygotes (AA) had significantly higher transcript levels of *SLC4A4* in gastric biopsy taken from the antrum of patients without PUD (p = 0.01) (figure 3.4).
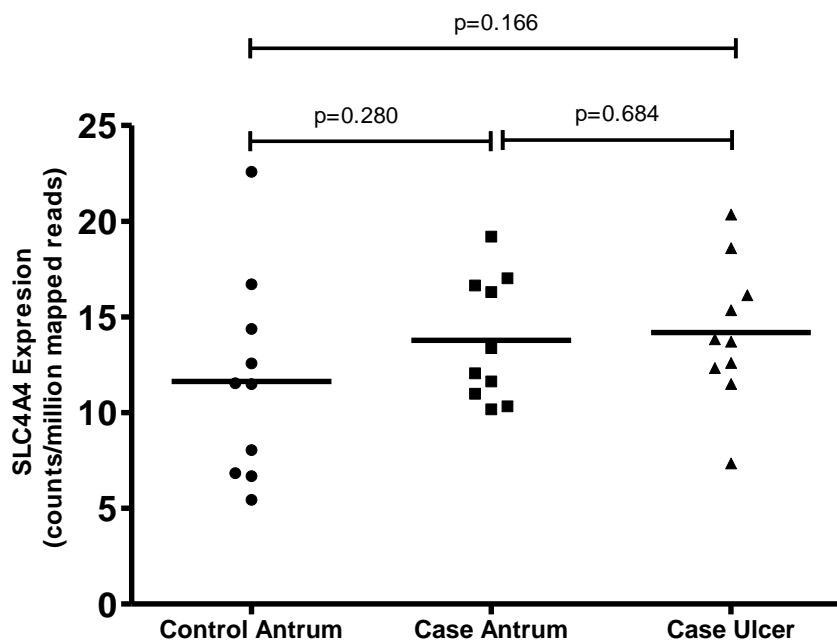
Figure 3.3 – SLC4A4 expression determined by RNASeq in gastric biopsy samples
Samples were from 10 cases (ulcer and antrum) and 10 healthy controls. There was no significant difference observed in the *SLC4A4* gene expression between the cases and controls.
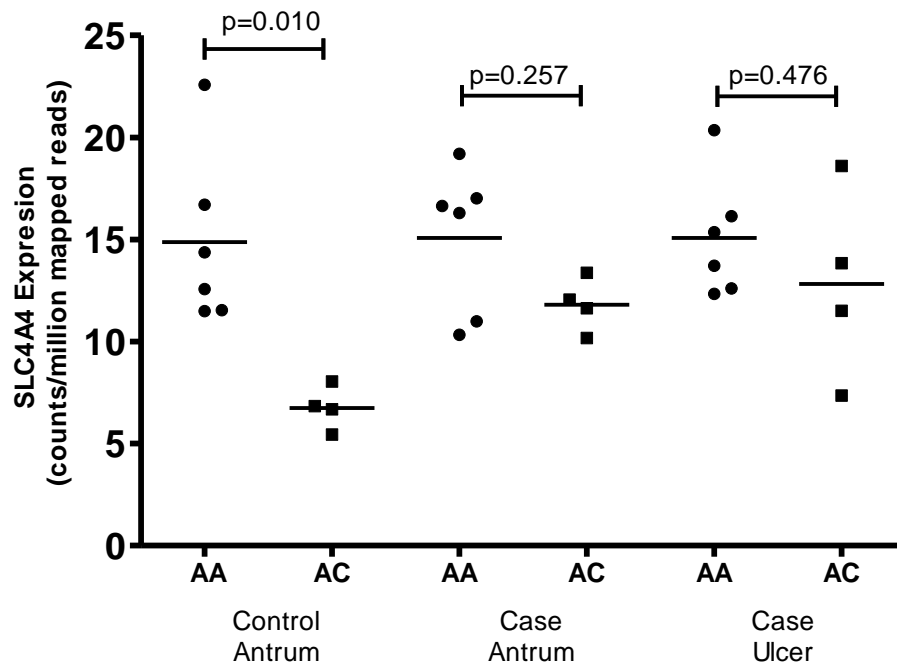
Figure 3.4 – SLC4A4 expression determined by RNASeq for *SLC4A4* in gastric biopsy samples stratified by genotype.

Biopsy samples from antrum of 10 cases (ulcer and antrum) and 10 controls were stratified according to rs4521314 genotypes, for *SLC4A4* gene expression. A statistically significant difference (p=0.01) was observed in the *SLC4A4* gene expression between the homozygous variant (AA) and the heterozygous in the controls.

3.4   Discussion

In this chapter, the correlation between *SLC4A4* variants and the expression of this transcript in gastric control and ulcer tissue using mRNA samples has been investigated. Little research has been undertaken looking at *SLC4A4* expression in gastric tissues, and the effect of genetic variation on its expression. These results are novel in the sense that this work is the first to report a trend between the *SLC4A4* genotypes and gene expression in gastric biopsies.

The data from RNA sequencing of the samples from gastric antrum did not directly correlate with those from the gene expression study in the gastric corpus, in terms of significant differences between the genotypes. This may be due to different sites of biopsy (antrum vs. corpus) or due to a difference between RNA sequencing and RT-PCR in identifying alternatively spliced variants. It has been suggested that phosphorylation may have a role in RNA splicing (Misteli, 1999), modulating protein–RNA and protein–protein interactions during formation of spliceosome. Other studies have shown that *SLC4A4* activity is modulated by phosphorylation of serine (Gross et al., 2001; Gross et al., 2003).

In gastric corpus biopsies from controls there was no statistically significant difference in the expression of *SLC4A4* mRNA between subjects with different genotypes. However, only a few samples (25%) carried the associated variant, and it is possible there was not adequate power as the total number of samples was small. Of course, it is also possible there may be other variants with exons which are more important, and which was not genotyped in the present study.

In the gastric antrum taken from healthy stomach, carriage of the 'A' variant allele generally increased *SLC4A4* transcript levels. It is possible that having the variant allele for rs4521314 as demonstrated in the present data affected the transcriptional activity of *SLC4A4* gene, enhancing its expression in healthy tissue. However, the data shown for control tissue differs from that in tissue taken from cases (figure 3.4). It is possible that this may be due to disease having an effect on expression. Alternatively, this may be a false positive.

Parks and Pouyssegur (2015) reported that *SLC4A4* mRNA expression was induced by hypoxia in the colon adenocarcinoma cell line, LS174, via the hypoxia inducible factor 1 alpha - HIF1α. In addition, earlier research (Syam et al., 2011) linked exposure to hypoxic conditions to the development of gastric ulceration, while wound healing was found to be regulated by HIF1α (Ruthenborg et al., 2014). Furthermore, McIntyre et al. (2016) observed that in certain cancer cells, pharmacologically (e.g. via the use of S0859 as in their study) or genetically disrupting bicarbonate uptake by *SLC4A4,* resulted in the acidification of intracellular pH, an increase in apoptosis, and a net effect of reduction in cell growth. Thus increase in *SLC4A4* expression is a result of the affected tissue trying to stabilise the imbalance in pH_i of the compromised cells by promoting bicarbonate influx in order to restore healing. This may be good news for ulcer healing and highlights the helpfulness of *SLC4A4* in the process. However, in hypoxic cancer cells HIF1α genes lead to sustaining the vicious cycle of cancer proliferation as demonstrated by several studies (Wigerup et al., 2016; Shay et al., 2014; Walsh et al., 2014). Whether changes in expression due to disease overwhelm the effect of any genotype is unclear, but seems

likely and much larger sample sizes will be needed to distinguish between genotype and disease-related changed in gene expression.

This study has some limitations. Only one SNP in the *SLC4A4* gene was evaluated, and it is likely that several polymorphisms in the *SLC4A4* gene that were not included in this study could have an influence. This is an intronic SNP and may be in linkage disequilibrium with the causal variant. Also, the sample size for the RNA sequencing studies was small and thus the study lacked some power. It is also possible that the SNP identified in the GWAS represents a false positive signal since it did not reach genome-wide significance, but there is good biological plausibility (and other evidence) that it is involved in the pathogenesis of gastric ulceration.

The linkage disequilibrium (LD) pattern between the top hit rs4521314 and other SNPs in the literature and public database were assessed to ascertain whether any other SNP was directly affecting its function. A number of SNPs were identified that were in LD, but none of these SNPs are known to have a functional effect. Also there were no functional SNPs affecting expression levels for this gene within the GTex (Genotype-tissue expression) website domain.

It can be concluded from the data that there is little influence of the SNP rs4521314 on the transcription levels of *SLC4A4.* Further research to assess the functional significance of genetic variation in *SLC4A4* in the GI region is required.
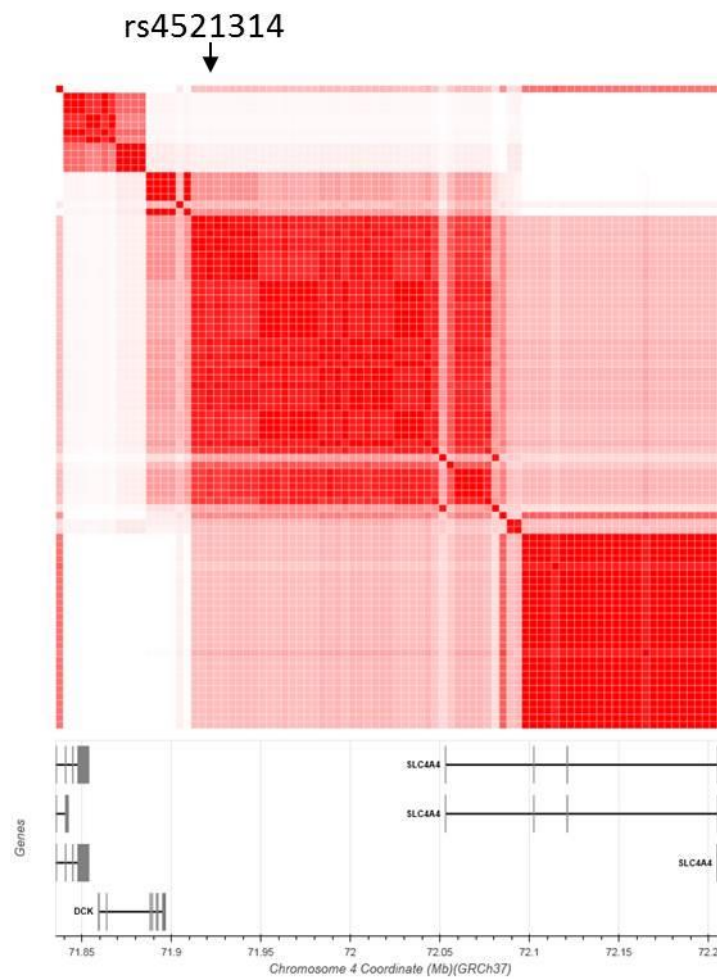
Figure 3.5 – LD plot for rs4521314 produced using LDLink
(Machiela and Chanock, 2015) from data R2 < 0.5 in pairwise comparison
with rs4521314 obtained from the 1000 Genomes Pilot dataset using SNAP
(Pers et al., 2015).

Chapter 4 **Characterisation of NSAID-induced Gastric Epithelial Cell Death**

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4.1   Introduction

4.1.1 Defense Mechanisms in Gastric Epithelial Cells

Besides prostaglandins, the defense mechanisms of gastric cells can be broadly grouped into three categories (Del Valle, 2008). These include pre-epithelial, epithelial and post-epithelial defense mechanisms (table 4.1).

The first and earliest aspect of mucosal defense is the pre-epithelial mechanisms encompassing secretion of mucous and bicarbonate in the mucosa as well as surface phospholipids present in the mucous layer. These mechanisms prevent epithelial cell exposure to toxic insults. In particular, secretion of bicarbonate by the gastric epithelial cells helps to maintain local pH at neutral when the gastric luminal pH is acidic (Matsui et al., 2011).

The second layer of mucosal defense is the epithelial mechanisms consisting of tight junction complexes of cells which minimize the diffusion of acidic ions into the cells. The cells also proliferate and migrate into areas of the mucosa that have been injured, thus assisting with healing of lesions (Grant Maxie, 2015).

The third layer of mucosal defense is the post-epithelial mechanisms which consist of mucosal blood flow, which helps in supplying adequate oxygen and nutrients and removing toxic metabolic by-products (Kvietys et al., 2015). This is important in aiding recovery and minimizing damage in the event that earlier mechanisms are breached.

Additionally, prostaglandins are important in gastric epithelial defense through regulation of mucous and bicarbonate secretion, improved blood flow to mucosal cells, reduction of gastric acid secretion and restitution in epithelial cells (Wallace, 2008).

Table 4.1 – Mechanisms of Gastric Mucosal Defence

Category	Mechanisms
• Pre-epithelial mechanisms	Bicarbonate Mucous secretion Surface active phospholipids
• Epithelial mechanisms	Tight junction complex Bicarbonate Growth factors Cell proliferation Restitution
• Post-epithelial mechanisms	Microcirculation of blood Bicarbonate Leukocytes
• Prostaglandins	Reduction of gastric acid secretion Improved blood flow

4.1.2   NSAIDs and Cell Death in Gastric Epithelial Cells

NSAIDs have long been involved in gastric epithelial toxicity associated with ulcerogenesis and upper GI bleeding. Direct cellular toxicity in the upper part of the mucosa has been shown to be one of the ways by which NSAIDs injure the gastrointestinal epithelium (DeLisa et al., 2005). This breach in the barrier causes diffusion of acid back into the mucosa, leading to rupture of the blood vessels.

4.1.3   Mechanisms of NSAID-induced Gastric Injury

4.1.3.1   Inhibition of Prostaglandins (PG)

Prostaglandins play a major role in the defense of gastric mucosal cells. Hence the inhibition of prostaglandins via cyclooxygenase (COX) inhibition is considered an important mechanism of NSAID-induced gastric injury. Most NSAIDs inhibit COX-1 and COX-2 in a reversible manner; however aspirin irreversibly inhibits COX-1 by acetylating the serine residue of the enzyme (Flower, 2003; Baigent and Patrono, 2003). Studies (Wallace et al., 2000) have shown that COX-1 and COX-2 inhibition is required to elicit gastric ulceration. This is due to the fact that both COX-1 and COX-2 play crucial roles in maintaining the integrity of gastric mucosal cells through the synthesis of prostaglandins, with COX-2 functioning in a supportive role when COX-1 is inhibited (Laine et al., 2008).

4.1.3.2   Ion Trapping

NSAIDs are weak acids - with aspirin having a pKa of 3.5 (NCBI, 2017) and as such they are non-ionized in the mucosa and in gastric juice which is highly acidic. They then penetrate

into the epithelial cells, which have neutral cytoplasmic pH, and become converted into ionized forms. The NSAIDs, with their acidic ions become trapped and may accumulate within the cells, causing injury (Laine, 1996).

4.1.3.3   Mitochondria Damage and Apoptosis

It has been hypothesised that NSAIDs, including aspirin could induce upper GI epithelial toxicity by uncoupling of oxidative phosphorylation (Mahmud et al., 1996), leading to reduced ATP, dissipation of mitochondrial trans-membrane potential (MTP), and a decrease in cellular ability to maintain normal function (such as regulation of intracellular pH). Aspirin has been shown to induce cell death in AGS cells by effecting cytochrome C release in the mitochondria, leading to the activation of caspase dependent apoptosis (Redlak et al., 2005). Moreover, some studies (Somasundram et al., 2000) have demonstrated a requirement for both mitochondrial damage and PG inhibition in gastric epithelial injury.

The aims of this chapter were to (i) determine the response of gastric epithelial (AGS) cells to selected NSAIDs, and (ii) characterise the mechanisms of NSAID-induced injury in AGS cells.

4.2   Methods

4.2.1   Chemicals and Drugs

Thiazolyl blue tetrazolium bromide (M2128) was procured from Sigma Aldrich, UK. Acetylsalicylic acid, diclofenac, ketoprofen and naproxen (Sigma Aldrich, UK) were

prepared freshly prior to use in DMSO. Staurosporine and z-VAD-fmk were purchased from Calbiochem, and Caspase Glo 3/7 reagent was purchased from Promega (UK).

4.2.2   Experimental Techniques

4.2.2.1   Seeding and Dosing of AGS Cells

Confluent AGS cells were trypsinised and retrieved from monolayer culture. Based on the cell count, complete media was used to dilute the cell suspension to 15,000 cells/well in 50µl volume in a 96-well flat-bottom plate. Cells were incubated at 37°C, in 5% CO_2 overnight. Culture media was carefully aspirated and 50µl of drug-containing media (0.5% DMSO) was added to each well, in triplicate. Cells were then incubated at 37°C for 12 hours. Staurosporine (10µM) was utilised as a toxicity positive control.

The following concentration ranges were used: aspirin, 0 - 50mM; diclofenac, 0 - 500µM; while for ketoprofen and naproxen, the concentrations were 0 - 100µM. All drugs (besides aspirin) were prepared in 0.1% DMSO-containing media. DMSO-containing media only was used as the vehicle control.

4.2.2.2   MTT Cell Viability Assay

Viability of drug-exposed AGS cells was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The assay is used to detect live and metabolically active cells which are able to convert the yellow tetrazolium salt into an insoluble purple formazan product via the action of reducing enzymes. The purple precipitate is then solubilised and quantified (Maioli et al., 2009).

MTT stock solution was prepared by dissolving 5mg of thiazolyl blue tetrazolium bromide reagent in 1ml of Hank's balanced salt solution (HBSS). Lysis buffer was made by adding 50ml dimethylformamide to 50ml of distilled water (50% v/v) and 20g of sodium dodecyl sulphate, SDS (20% w/v). The lysis buffer was warmed at 37°C and mixed thoroughly to ensure complete solubilisation.

Following 12-hour NSAID exposure, drug-media was removed by aspiration and 20 μl of MTT working solution (0.5mg/ml concentration,) was added to wells. Thereafter, cells were incubated further at 37°C for 3 hours in darkness, and 100 μl of lysis buffer was added to solubilise the purple formazan. Plates were incubated overnight at 37°C before shaking on a micro-plate shaker (Grant Bio PMS 1000) for 15 minutes. Absorbance at 595 nm was measured using a micro-plate reader (Beckman Coulter DTX 880 Multimode detector). Negative controls had no cells with only culture medium utilised.

4.2.2.3   Caspase 3/7 Assay

Caspase 3 and 7 activity was measured using the Caspase Glo 3/7 assay. The reagent contains the tetrapeptide sequence DEVD that provides the pro-luminescent substrate which is cleaved, releasing aminoluciferin useful in light production. This aminoluciferin is acted upon by luciferase, producing a luminescent glow signal proportional to the amount of caspase activity detected (Promega, UK).

Caspase Glo 3/7 reagent was constituted according to the manufacturer's instructions. Cells were seeded and treated as previously described in section 4.2.2.1, and after 12 hours, 50μl of the prepared Caspase-Glo 3/7 reagent was added to an equal volume of

media in each well. The solution was mixed gently with a plate shaker for 30 seconds and incubated at 37°C for 1 hour in the dark. Thereafter the luminescence was read with a Beckman Coulter DTX 880 Multimode detector reader.

4.2.3   Assessment of Aspirin-induced AGS Cell Death and Mechanisms

Cells were seeded and dosed with aspirin as previously described in section 4.2.2.1. In further experiments, cells were dosed with 0, 10, 20 and 50mM of aspirin, with and without pre-treatment with 20µM of the pan-caspase inhibitor, z-VAD-fmk for 1 hour in order to assess the contribution of caspase-mediated cell death to the decrease in cell viability. MTT and Caspase Glo 3/7 assays were carried out as already described in sections 4.2.2.2 and 4.2.2.3 respectively.

The concentration of aspirin that would be present in the gastric juice of a low dose aspirin user was calculated using its known molar mass in order to determine the concentration range of aspirin that would be needed in the experiment. Low dose aspirin (LDA) is below 300 mg daily, but typically prescribed between 75 – 150 mg daily (Lloyd and Bochner, 1996). The stomach usually contains between 20 – 100 mls of gastric juice (Steingoetter et al., 2015). Therefore this volume range was used in calculating the required aspirin concentration as below:

Concentration (C) = <u>number of moles of solute</u>
                        Volume of solution (in litres)

Molar mass of aspirin ($C_9H_8O_4$) = 180g/mol

For 75mg of aspirin (0.075g),

No of moles = <u>Mass </u>   = <u>0.075g</u>   = 4.166×10^{-4} M = 0.416 mmoles
              Molar mass $180gmol^{-1}$

Therefore, for 20mls of gastric juice, C = <u>0.416mM</u>   = 21mM
                                              0.02L

For 100mls of gastric juice, C = <u>0.416mM</u>   = 4.2mM
                                    0.1L

For 150mg of aspirin (0.15g),

No of moles =   <u>Mass </u>   = <u>0.15g</u> = 8.33×10^{-4} M = 0.83 mmoles
                Molar mass $180gmol^{-1}$

Therefore, for 20mls of gastric juice, C = <u>0.83mM</u>   = 41.5mM
                                              0.02L

   For 100mls of gastric juice, C = <u>0.83mM</u>   = 8.3mM
                                       0.1L

The above calculations informed the decision to use aspirin concentration ranges of up to

50mM to determine its effects on gastric (AGS) cell viability.

4.2.4   Assessment of AGS Cell Death by Other NSAIDs

Cells were seeded and dosed with diclofenac, ketoprofen or naproxen in the manner previously described in section 4.2.2.1., with 10µM staurosporine used as a positive control. All drugs were suspended in 0.1% DMSO and the MTT assay was carried out for 12 hours as already described in section 4.2.2.2.

4.2.5   Statistical Analysis

All the assays were conducted in triplicate for each experiment and then the results from the mean ± SE of three independent experiments were taken. The unpaired t test was used to determine the differences between each concentration compared to the control, using the Statsdirect software. A p value of < 0.05 was considered significant. All the results from the Caspase Glo experiments were expressed as relative luminescence (RLU). The means ± SEM from 3 experiments were graphically represented.

4.3   Results

4.3.1   Effect of Aspirin in AGS Cells

A range of aspirin concentrations was used to initially assess its effect on the AGS cells after 12 hours. Cells showed a statistically significant reduction in viability at a 2mM concentration, up to 50mM, where the viability was reduced to ~50% as presented in figure 4.1.
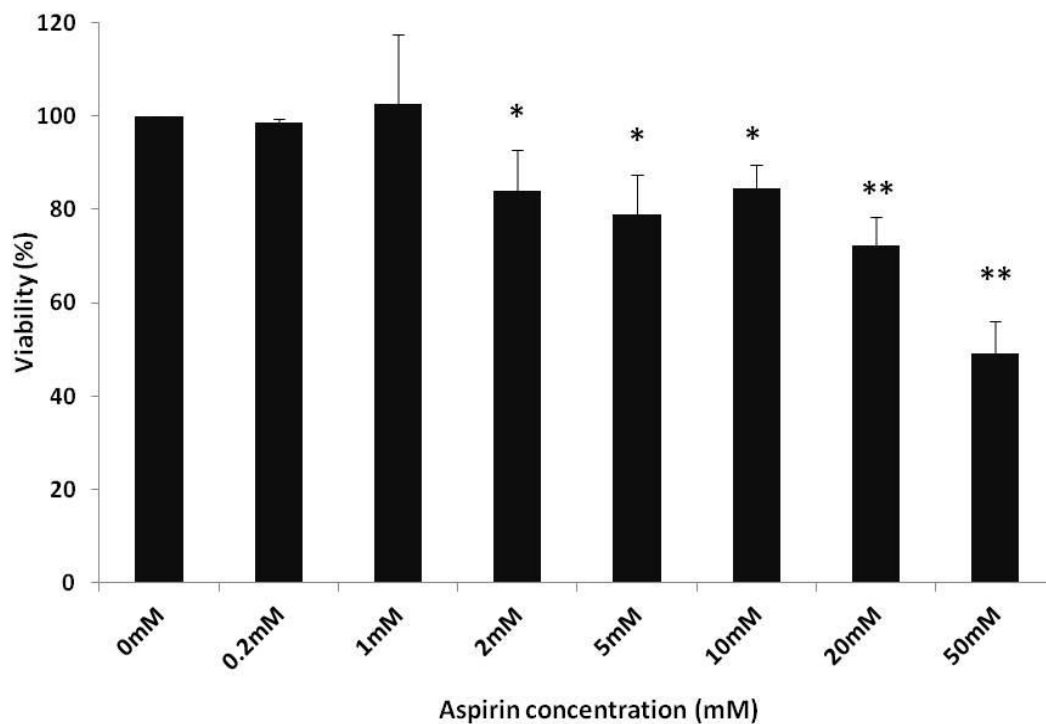

Figure 4.1 – Aspirin showed a Dose-dependent Effect in AGS Cell Viability
After 12 hours, the effect of aspirin on the cells was observed beginning with 2mM. This reduction in viability continued as aspirin concentrations were increased. The data expressed here are the means ± SE from three experiments. All data are compared with the 0μM control, and a *p value* of ≤ 0.05 was accepted as statistically significant. * = $p \le$ 0.05; ** = $p \le$ 0.01.
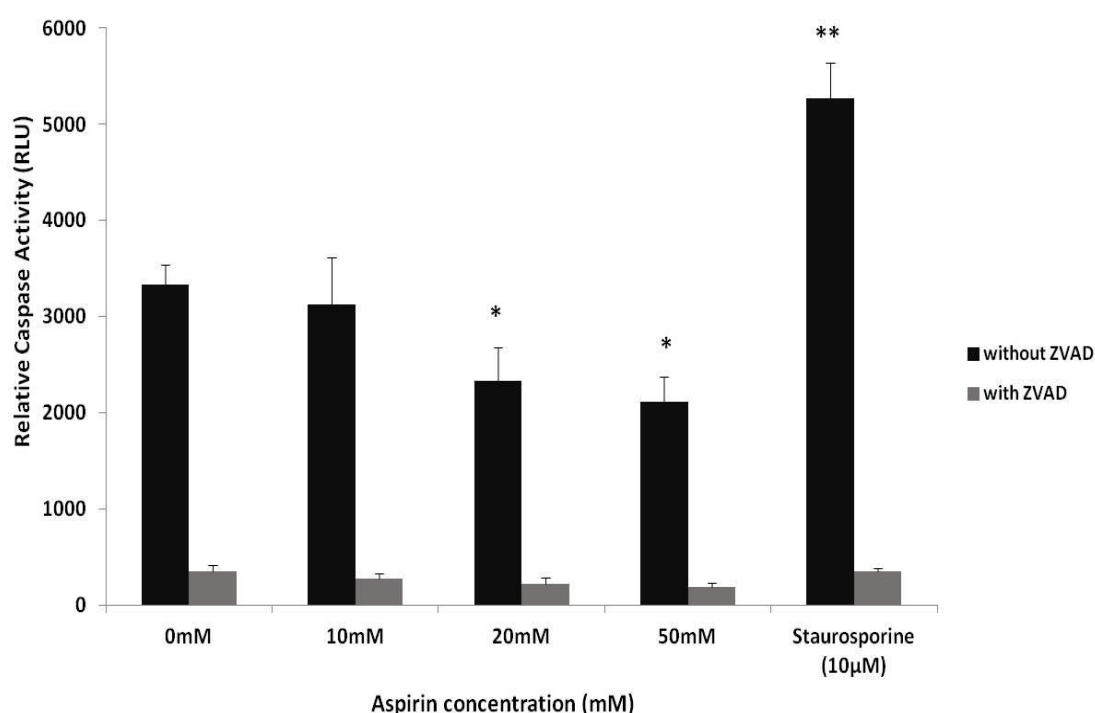
Figure 4.2 – Caspase Activity in Aspirin treated AGS Cells without and with Z-VAD
All cells were treated with aspirin for 12 hours. Some cells received prior treatment with
20µM of Z-VAD for 1 hour. Staurosporine was used as a positive control in both
treatment groups. Cells showed reduced caspase activity at increased doses of aspirin.
The data are expressed as means ± SE from three experiments and are compared with
the 0µM without Z-VAD. $* = p \leq 0.05$; $** = p \leq 0.01$.

Furthermore, the possible cell death mechanism of aspirin in AGS cells was characterised.

Caspase 3 and 7 activities were assessed with the Caspase Glo assay following 12 hours of

selected concentrations of aspirin. It was observed that as aspirin concentration

increased, the caspase activity reduced. In contrast, cells treated with the positive

control, staurosporine (a known caspase inducer) showed significantly higher caspase

activity, more than 2 times the level seen in the 50mM aspirin treatment (figure 4.2).

However, when cells were pre-treated with the pan-caspase inhibitor, Z-VAD prior to the

12-hour aspirin treatment, caspase activity was almost abolished. This effect of Z-VAD

was seen even in cells treated with staurosporine. The pan-caspase inhibitor reduced

caspase activity to a level comparable across all doses of aspirin and staurosporine used.

4.3.2   Effect of Caspase Inhibition on the Viability of Aspirin-treated AGS Cells

Following the significant effect Z-VAD had on reducing the caspase activity of aspirin-treated cells, the correlation between the modulated caspase activity and cell viability was assessed. An MTT assay was set up to determine Z-VAD modulation of aspirin-induced cell death. Data (figure 4.3) showed that pre-treatment with Z-VAD did not halt or completely reverse aspirin's effect on the cells, as there was still a reduction in viability, equivalent to without Z-VAD pre-treatment. At all aspirin concentrations, there was no significant difference in cell viability between groups without and with Z-VAD.
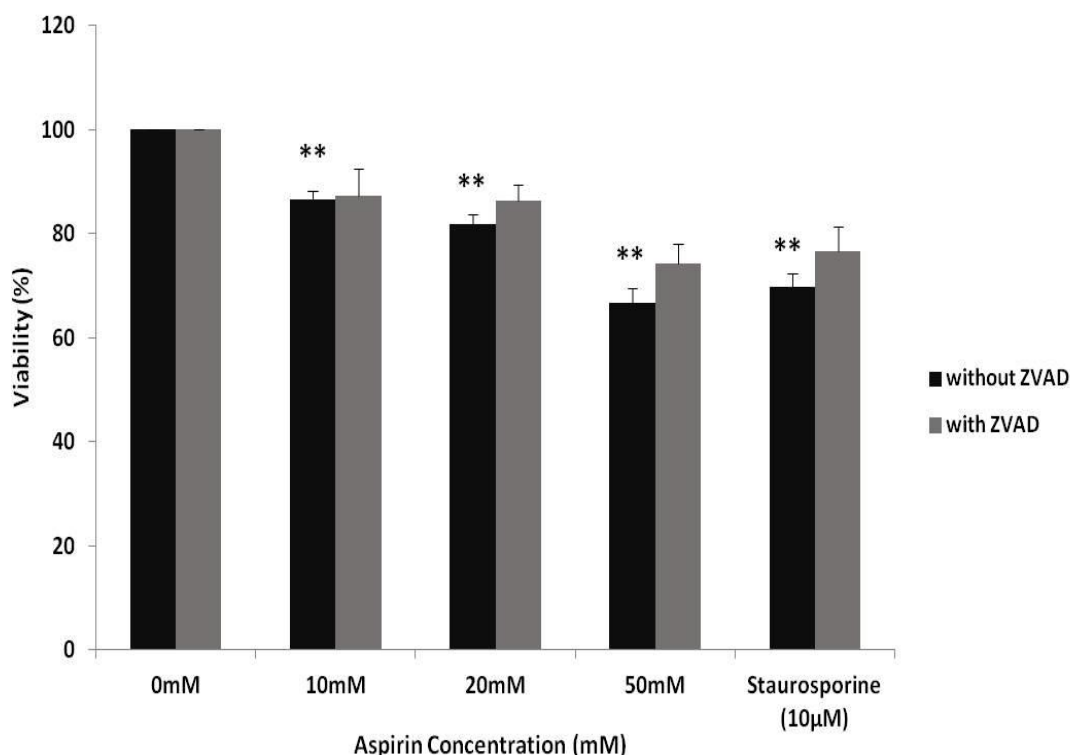

Figure 4.3 – Effect of Caspase Inhibition on Viability of Aspirin-treated Cells
The viability of AGS cells was reduced in a dose-dependent manner in response to aspirin treatment without and with Z-VAD after 12 hours. The statistically significant data are compared between the 0µM control and doses of aspirin-treated cells without Z-VAD. ** = $p \leq 0.01$.

4.3.3   Reduced Viability in Diclofenac-treated AGS Cells

Diclofenac toxicity was tested in AGS cells. Cells were treated with diclofenac from 0μM to 500μM.   Cells showed a downward trend of reduced viability with increasing concentrations of diclofenac. Significant reduction in cell viability was recorded at all concentrations of the drug after 12 hours, as shown in figure 4.4. Diclofenac, at 500μM, reduced cell viability to 59%. The positive control, staurosporine also elicited a significant cytotoxic effect at 46%.
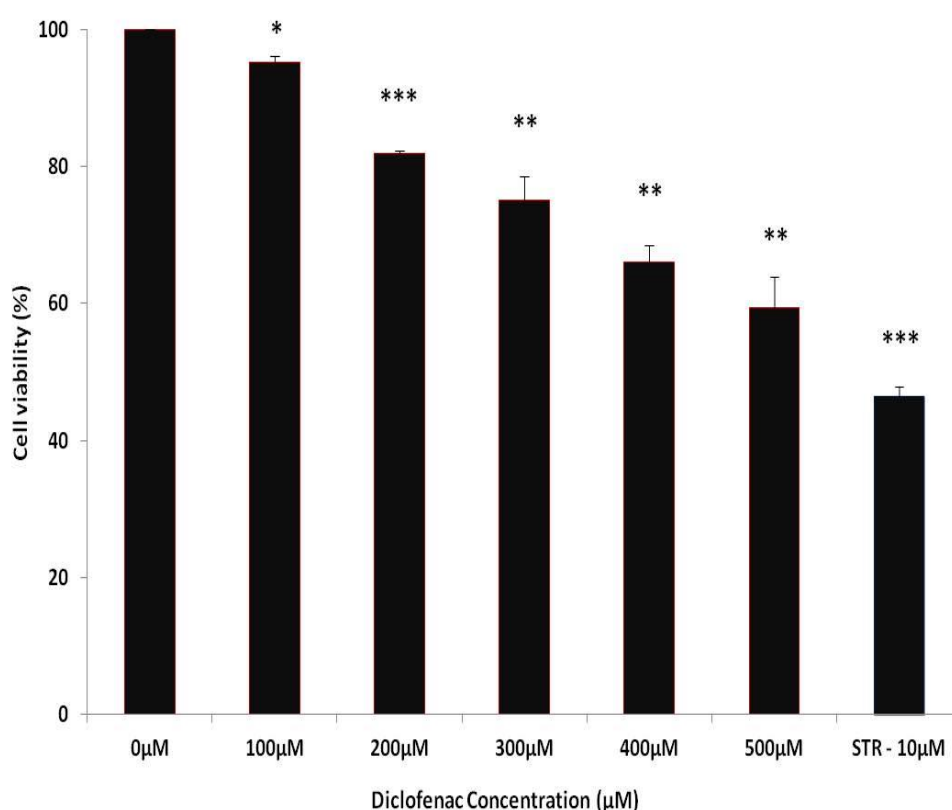

Figure 4.4 – AGS Cells showed Reduced Viability with Diclofenac Treatment
After 12 hours treatment, diclofenac significantly reduced AGS cell viability, starting from 100μM. Staurosporine was used as a positive control. The data are expressed as means ± SE from three experiments. All data are compared with the control (0μM) for statistical significance. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

4.3.4   AGS Cell Death Induced by Ketoprofen and Naproxen Treatments

AGS cells were treated with a range of concentrations of ketoprofen and naproxen for 12 hours and the effect of the drugs on viability of the cells was measured by the MTT assay. As seen in figure 4.5, AGS cells showed a significant response to ketoprofen in a dose dependent manner. Cell viability was significantly reduced by as little as 2µM of ketoprofen.
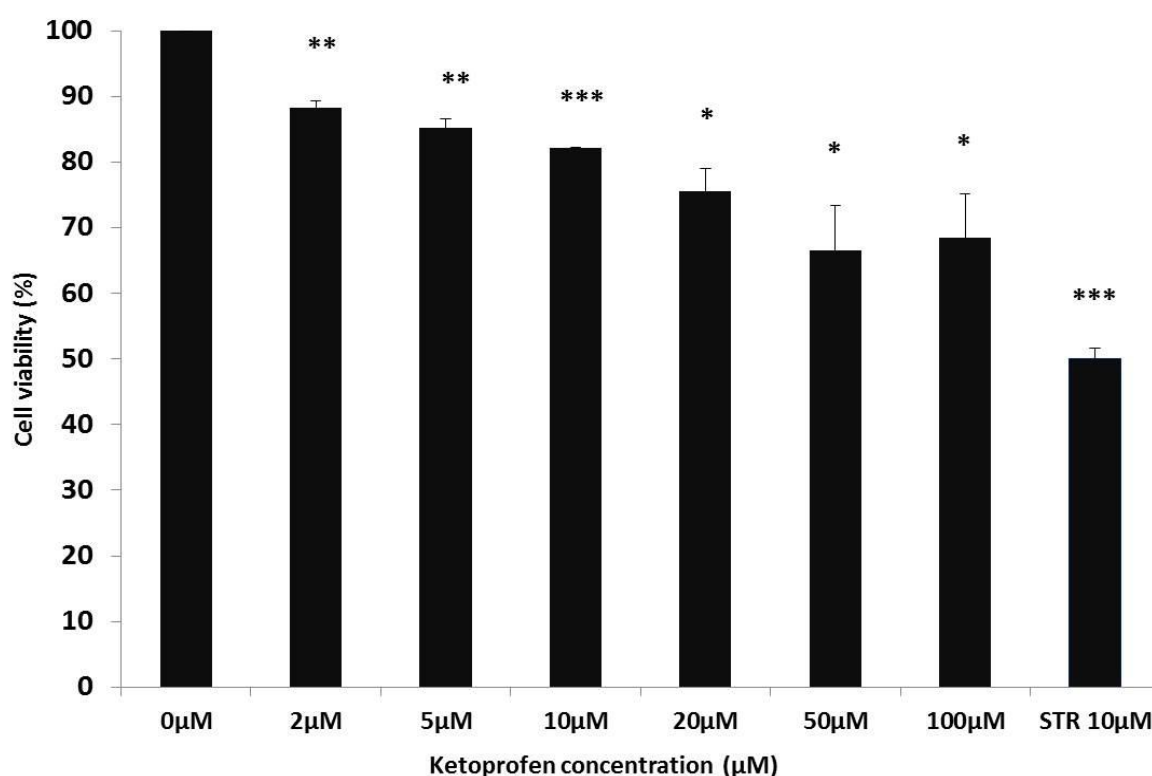

Figure 4.5 – Dose-dependent Reduction in AGS Cells treated with Ketoprofen
Cytotoxicity assay showing percentage viability of AGS cells with increasing concentrations of ketoprofen after 12 Hrs. Staurosporine (10µM) was used as a positive control. The data are expressed as means ± SE from three experiments. All data are compared with the control (0µM) for statistical significance. $*$ = $p \leq 0.05$; $**$ = $p \leq 0.01$; $***$ = $p \leq 0.001$.

Likewise, cells treated with naproxen, also showed some sensitivity to the drug, however not to the same extent as with ketoprofen. An obvious decrease in cell viability was noticed at the 20μM dose of naproxen, although there was a significant difference at 5μM. This can be seen in figure 4.6.
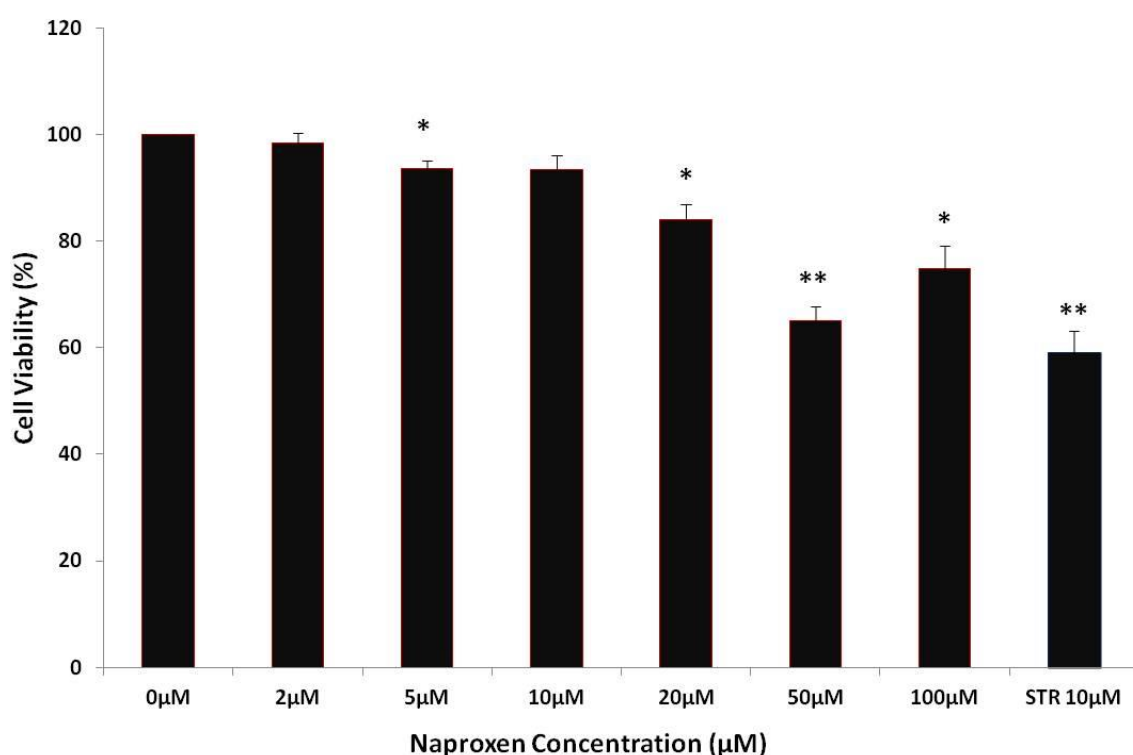

Figure 4.6 – Dose-dependent Reduction in AGS Cells Treated with Naproxen.
Cytotoxicity assay showing percentage viability of AGS cells treated with Naproxen, after 12 Hrs. 10μM of Staurosporine was used as a positive control. The data represented as means ±SE from three experiments. * = $p \leq 0.05$; ** = $p \leq 0.01$.

4.4   Discussion

This work has looked at the effects of four commonly used NSAIDs on the viability of AGS cells, and attempted to characterise the mechanisms by which cell death occurs in response to NSAID treatment, focusing mainly on aspirin. After 12 hours, all four NSAIDs tested dose-dependently reduced the viability of the cells, with aspirin having the least effect and ketoprofen having the most effect. A study done to rank NSAIDs based on their general systemic side effects and overall risk found that ketoprofen ranked higher, compared to diclofenac and naproxen, in that order (Moldoveanu et al., 2012). Preliminary experiments done in the present study on aspirin-induced AGS cell death at 3 hours and 6- hours drug exposure (data not shown), revealed that the toxicity seen in the data presented is drug-specific, and was the consequence of drug action over time.

The majority of the NSAIDs are classed as weak acids, having a pKa value in the range of 3 - 5 (Deruiter, 2012); they exist in non-ionized forms in the gastric acid juice, and are lipid soluble. When they have been absorbed by the gastric cells, and have diffused through the mucosal cell membrane barrier into the cytoplasm, the acidic moiety of the NSAIDs dissociates and becomes ionized (Laine, 1996). This is because the intracellular pH of the cells is usually neutral, in the range of 6.9 - 7.5 (Machen & Paradiso, 1987). The ions accumulate in the cells, and become trapped resulting in the cells becoming injured over time as they become acidic (Matsui et al., 2011). *In vivo*, and in a well-functioning cellular system, the cells should be able to adapt to neutralize the changes in pH, via naturally occurring internal mechanisms such as bicarbonate transport (Boku et al., 2001; Walter et al., 2009). However, the lack of such mechanisms in the *in vitro* cells could cause them

to be more susceptible to damage caused by NSAID ion trapping. Hence, there is a possibility that although statistically, the data presented in this chapter shows significant levels of cell death with NSAID treatment, this may not necessarily translate into biologically significant cell death.

In an earlier study, Sato et al. (1993) reported that gastric mucosal cytotoxity due to a reduction in the intracellular pH of aspirin-treated cells occurred only in the presence of acidic extracellular pH. In a study to specifically rank NSAID risks for GI toxicity, ketoprofen again ranked higher followed by naproxen, diclofenac and aspirin (Henry et al., 1996). Furthermore, an earlier work by Mahmud et al (1996) on a range of NSAIDs demonstrated an inverse correlation between pKa and concentration of NSAID required to cause a maximum uncoupling of mitochondrial cells, which has also been shown in the present study. The pKa values for the NSAIDs used in this thesis are 3.49, 4.15, 4.15 and 4.45 for aspirin, diclofenac, naproxen and ketoprofen, respectively (PubChem Compound Database). The pKa may also affect the COX-inhibitory properties, including selectivity for COX-2 inhibition, and thus the potential for upper GI injury. This is because selective inhibitors of COX-2, such as celecoxib which reduces NSAID GI toxicity risk seem to have a higher pKa value – pKa for celecoxib is 11.2 (Paulson et al., 2001).

To determine the contribution of caspase-mediated cell death in the decrease in AGS cell viability in this study, the presence of caspase 3 and 7 was determined in the aspirin treated cells, and it was discovered that caspase 3 and 7 activities were present and inversely proportional to the aspirin concentration. Thus, lower amount of the caspases were detected with increasing aspirin concentrations. The study done by Power et al.

(2004) corroborated our findings in this work regarding the presence of caspases 3 and 7 in aspirin-induced gastric cell death. However, the results from this study showed that treatment with ZVAD visibly reduced the amount of caspase 3 and 7 activities at all concentrations of aspirin-treated cells (figure 4.2). And although there was almost no activity of caspase 3 and 7 remaining after ZVAD treatment, aspirin-treated cells were still being affected at almost the same rate as without ZVAD (figure 4.3). This trend most likely suggests that there is no evidence for apoptosis-mediated cell death in aspirin-treated cells, although Gu et al. (2005) have described a role for caspase 8 in aspirin-induced apoptosis in gastric cells. The data is also supported by other research (Budihardjo et al., 1999; Perfettini and Kroemer, 2003) which have shown that cell death in general, is not prevented by the inhibition of caspase, but that inhibition of caspase causes the cells to undergo a switch into other mechanisms which are non-caspase dependent, such as necrosis. Furthermore, Leung et al. (2009) has suggested a mechanism which is not dependent on caspase for cell death caused by NSAIDs, but instead depends on the mitochondrial pathway, in a manner which is suggestive of apoptosis-like programmed cell death (necroptosis).

The present study on NSAID GI toxicity has shown that in the absence of an adequate repair system in the cellular environment, and with broken cellular barriers, even physiological concentrations and low doses of NSAIDs may result in damage which could worsen over time. The data from this study is strengthened because a validated *in vitro* model for gastric cells, the AGS (Hall et al., 2006) was used to perform all of the experiments. This would enable easy and acceptable interpretation of the results in a

clinical setting *in vivo*. In addition, valid techniques have been used to characterise the effects of several NSAIDs as well as the mechanism of aspirin toxicity on AGS cells.

Some of the limitations of this study include the fact that the caspase activities of AGS cells treated with the other NSAIDs used was not characterised, and hence, although it could be inferred from the aspirin results, it is not possible to be certain about the cell death pathways for these drugs. Also, besides caspases 3 and 7, the experiments did not check for the presence of the activation of other pathways associated with cell death, e.g. caspase 9. In addition, cell death by necrosis could also be determined by assessing the presence of necrotic markers such as lactic acid dehydrogenase activity (Redlak, 2003). Furthermore, as the MTT assay is subject to interference and could be affected by other factors that could inhibit the reduction of the tetrazolium salt by mitochondrial reducing enzymes, it can sometimes lead to results which falsely indicate cell death. Also, cells may still be alive and replicating, but only at a much slower rate. To improve this limitation, it will be necessary to utilise other known markers of cell death to validate these findings.

In conclusion, the data obtained in this chapter, considered with research from other studies is suggestive of the existence of other non-caspase mechanisms in the death of cells treated with aspirin NSAID. Further experiments are required to fully elucidate the mechanisms of action for NSAID-induced gastric cell death. In the meantime, efficient management of NSAID-induced upper GI injury would entail targeting multiple pathways, including the caspase-mediated and mitochondrial pathways.

Chapter 5   **Functional Assessment of NSAID Inhibition of Bicarbonate Ion Accumulation in a Gastric Epithelial Cell-Line**

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5.1   Introduction

5.1.1   Bicarbonate Transport in Human Cells

In humans, there are presently 15 genes involved in bicarbonate transport. Besides the SLC26A family - *SLC26A3* (Shcheynikov et al., 2006), *SLC26A4* (Soleimani et al., 2001), *SLC26A6* (Waldegger et al., 2001), *SLC26A7* (Xu et al., 2009; Vincourt et al., 2002), and *SLC26A9* (Avella et al., 2011; Dorwart et al., 2007) which are chloride/bicarbonate exchangers (Cl^-/HCO_3^-), the remaining 10 other genes belong to the SLC4A family (earlier summarised in section 3.1).

5.1.2   Physiology of Bicarbonate Transport and Protective Mechanism

As mentioned earlier in chapter 1, the secretion of HCO_3- into an adherent mucus gel layer is the initial line of mucosal protection against lumenal acid, and results in a pH gradient at the epithelial surface area in the stomach (Allen and Flemstrom, 2005). Mucous gel reduces lumenal loss of HCO_3- adequately to keep a neutral pH at the apical cell surfaces. Na+/ HCO_3- cotransport at the basolateral membrane layer is the major system for import of HCO_3-. Research studies in rat, as well as rabbit, stomach mucosa showed expression of a $Cl -/ HCO_3$ – anion exchanger in the apical membrane layers of gastric surface area epithelial cells (Rossmann et al., 1999). In the gastric mucosa, PGs promote HCO_3- secretion by means of EP1 receptors. Figure 5.1 shows a representation of the transport of bicarbonate across the gastric cells.
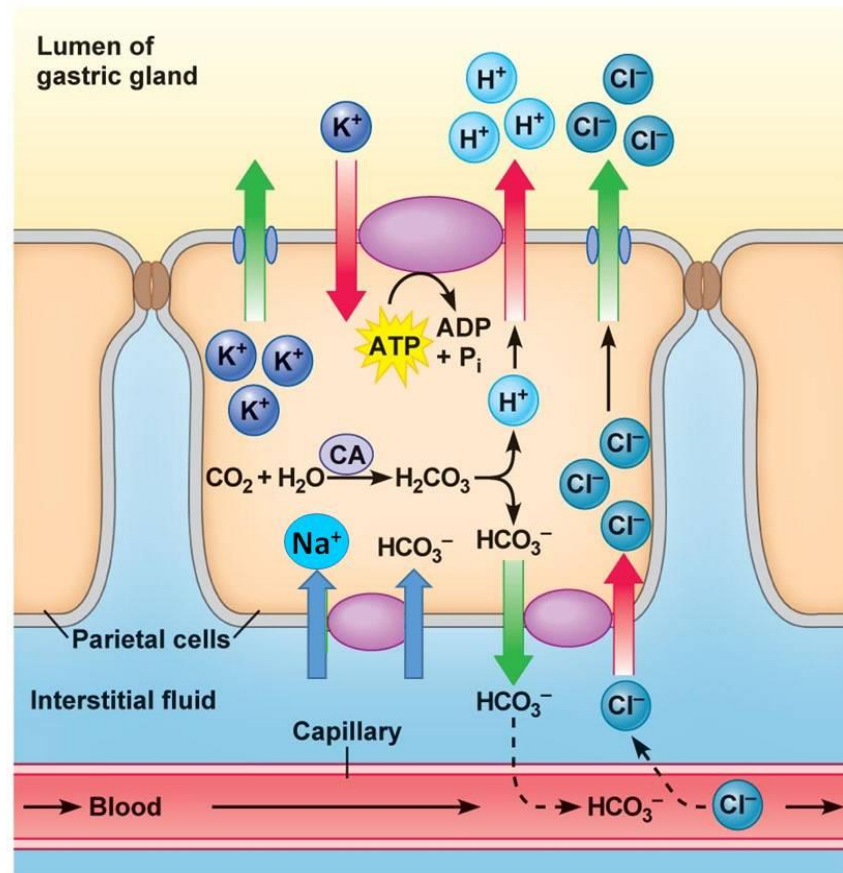
Figure 5.1 – Bicarbonate Transport and Flux in the Gastric Lumen
Bicarbonate transport is facilitated by the sodium bicarbonate cotransporter protein (SLC4A4). As can be noticed in the diagram, other channels and processes are also involved in balancing the amount of various other ions in the cells. Source: Modified from Pearson Education Inc. (2011).

It has been reported that carbonic anhydrase has an effect on bicarbonate transport rate

(Becker et al., 2014), given that carbonic anhydrase indirectly utilises, as well as produces

HCO_3^-, which is the bicarbonate transporter molecule. One study by Loiselle et al. (2004)

reported an interaction between the cytoplasmic C terminal region of the electroneutral

sodium bicarbonate co-transporter (NBCn1) with carbonic anhydrase. In another study

with *X. laevis* oocytes (Schueler et al., 2011), there was higher transport activity by the

electrogenic sodium bicarbonate co-transporter (NBCe1) when it was co-expressed with

carbonic anhydrase isoforms. Furthermore, Alvarez et al. (2003) showed that carbonic anhydrase IV interacted with extracellular loop 4 of NBCe1, enhancing its effect.

$$CO_{2\,(g)} + H_2O \overset{\boxed{CA}}{\rightleftharpoons} H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Figure 5.2 – Basic Equation of pH balancing by Bicarbonate
Bicarbonate (HCO_3^-) usually exists in equilibrium with carbonic acid (H_2CO^3), carbon dioxide (CO_2) and water (H_2O), in order to maintain a neutral pH. CA: Carbonic anhydrase

5.1.3 In Vitro Models for Monitoring Transporter Activities

A number of models exist by which the presence or activities of transporters, like the Na^+/HCO_3^- co-transporter can be measured. Cell-based transporter assays are a common model which involves the use of the transporter-expressing cells to study influx and/or efflux transporters. The barrier method requires cells seeded on a permeable membrane to form a polarized, intact monolayer through which the molecules diffuse (Brouwer et al., 2013). Trans-epithelial electrical resistance (TEER), which measures the resistance of a cell layer to the flux of ions, is a widely used technique to conveniently monitor cell layer integrity and the presence of tight junctions (Ferrell et al., 2010). In addition, cell uptake studies are also a valuable way to determine overall influx of the target molecules into the cells (Brouwer et al., 2013).

5.1.4 Inhibitors of Bicarbonate Transport

Certain members of the SLC4 family are inhibited by disulfonic stilbene derivatives such as 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid, DIDS (Lane et al., 1999). The extracellular part of position 5 of the trans-membrane (TM5) in sodium/bicarbonate co-

transporters, group 1 (NBC1) and anion exchangers, group 1 (AE1) have been shown to react with drugs of this nature (Heyer et al., 1999).

In addition, some studies have utilised tenidap, a cytokine-modulating drug to inhibit the activity of the bicarbonate transporter. It was reported that tenidap caused an alteration in pH via the inhibition of the Cl^-/HCO_3^- exchangers (McNiff et al., 1994). The Substance S0859 has also been shown to be a putative inhibitor of sodium and bicarbonate transport, having been used to successfully inhibit sodium bicarbonate co-transporter (NBC) activity in numerous cell lines, including canine kidney epithelial cells (Schwab et al., 2005) and ventricular myocytes (Ch'en et al., 2008). The structure of S0859 is shown in figure 5.3.
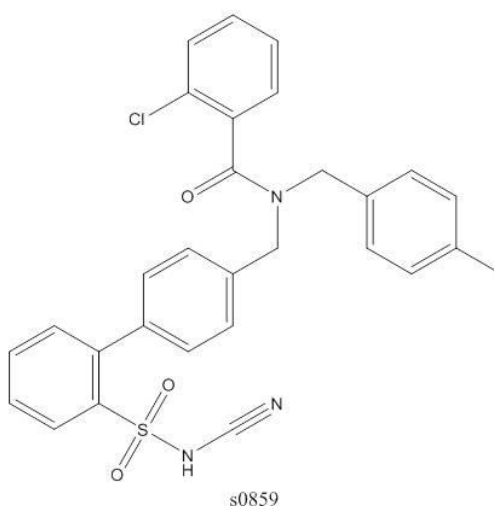
Figure 5.3 – Chemical Structure of the NBCe1 Inhibitor, S0859

In breast cancer cells, S0859 has been shown to prevent a great deal of acid extrusion which is mediated by sodium-driven bicarbonate transport (Larsen et al., 2012). It has

been suggested that S0859 inhibits NBC via a mechanism likely to be largely independent of carbonic anhydrase (CA) activity. In a study by Ch'en et al., (2008), inhibition of CA activity with acetazolamide (ATZ) did not affect the ability of NBC to mediate intracellular pH recovery from an induced acid load in cardiac cells. Table 5.1 shows a summary of some studies in which S0859 was used to inhibit bicarbonate transport.

The aims of this chapter were (i) to assess the bicarbonate uptake ability of AGS cells, and (ii) to determine the effect of selected NSAIDS on bicarbonate accumulation in these cells.

Table 5.1 – Studies showing the Successful Use of S0859 as Inhibitor of Sodium Bicarbonate Transport

Authors	Title	S0859 Concentration	Cell line used
(Ch'en et al., 2008)	S0859, an N-cyanosulphonamide inhibitor of sodium-bicarbonate cotransport in the heart	0 – 30µM	Ventricular myocytes
(Schwab et al., 2005)	Functional role of Na$^+$-HCO3$^-$ cotransport in migration of transformed renal epithelial	50µM	Kidney cells, MDCK
(McIntyre et al., 2016)	Disrupting hypoxia-induced bicarbonate transport acidifies tumour cells and suppresses tumour growth	100µM	Human colon epithelial cell line, LS174T
(Yamamoto et al., 2005)	Functional diversity of electrogenic Na$^+$ - HCO3– cotransport in ventricular myocytes from rat, rabbit and guinea pig	10µM	Ventricular myocytes
(Heidtmann et al., 2015b)	Inhibition of monocarboxylate transporter by *N*-cyanosulphonamide S0859	0 - 15µM	*Xenopus* oocytes
(Larsen et al., 2012)	Gram-Scale Solution-Phase Synthesis of Selective Sodium Bicarbonate Co-transport Inhibitor S0859: in vitro Efficacy Studies in Breast Cancer Cells	0 – 50µM	Breast Cancer Cells

5.2   Methods

5.2.1   Chemicals and Drugs

S0859 (SML0638) was purchased from Sigma Aldrich, UK and stock solutions were made up in DMSO, and then stored at -20°C in aliquots. Acetylsalicylic acid, diclofenac, ketoprofen and naproxen (Sigma Aldrich, UK) were prepared freshly before use in DMSO. Scintillation fluid was purchased from Meridian, UK, while carbon-14 radiolabelled (14C) sodium bicarbonate molecule ($NaHCO_3$) was procured from Moravek Biochemicals, USA and stored in a special fridge. Use, disposal and monitoring of radioactive compound were done in accordance with health and safety requirements.

5.2.2   Cell Seeding and Dosing

For trans-epithelial electrical resistance (TEER) experiments, AGS cells were seeded into Corning 6-well culture plates containing permeable inserts (Fisher Scientific, UK). Cells were seeded in 1 ml of Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% foetal bovine serum (FBS) in the apical side, and 2 ml of DMEM in the basolateral side. They were maintained continually at 37°C, 5% CO_2 prior to measurements at periodic intervals. This method is called liquid covered culture (LCC).

For bicarbonate accumulation assays, AGS cells were seeded into 12-well plates at a density of 1.8×10^5 cells per well in 600µl volume of DMEM supplemented with 10%

foetal bovine serum (FBS).   They were left to incubate overnight at 37°C, 5% CO_2.

Addition of drugs to cells was carried out using plain DMEM media.

5.2.3   Measurement of Trans-epithelial Electrical Resistance (TEER)

TEER measurements were performed for monitoring the cell growth in the culture media,

as an increase in detected TEER is often a good indicator of cell confluence.   Each

electrode tip contains a silver pellet which measures the voltage and resistance of the

cells.   Measurements of TEER were carried out using the Millicell ERS-2 electrical

resistance system (Millipore, USA), according to the manufacturer's protocol.   The

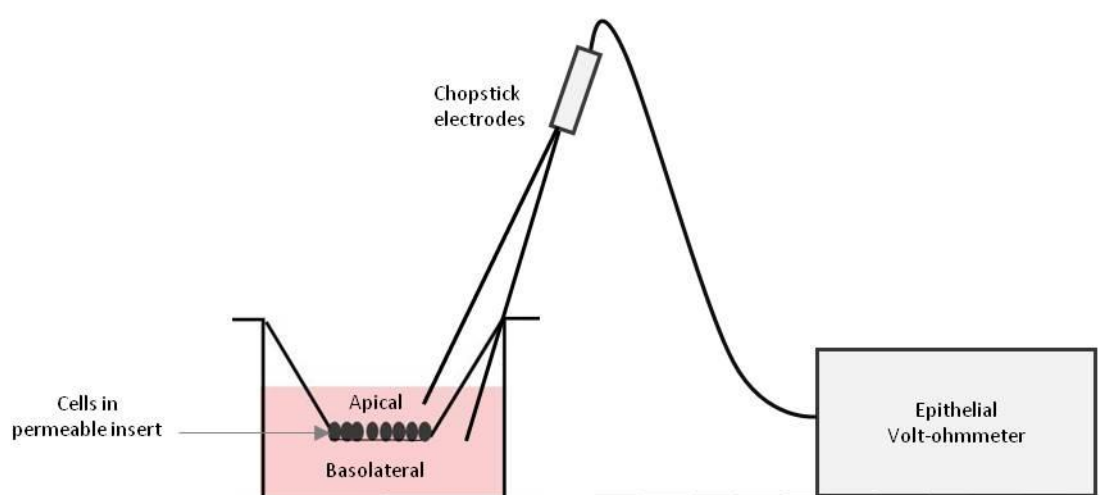apparatus was set up as illustrated in figure 5.4.

Figure 5.4 – Schematic Representation of Epithelial Volt-ohmmeter used in Measurement of TEER.

The cells were allowed to equilibrate for 15 minutes in the safety cabinet in order to

come to a steady room temperature, so as to reduce variability in TEER values caused by

temperature differences.   Blank resistance measurements were taken in well inserts

without cells, but with the media. Cell resistance was also measured in the wells with cells. Duplicate readings were taken for blank and cell wells, and the average values were recorded. The true cell resistance was obtained by subtracting the blank resistance from the sample resistance (R_{sample} - R_{blank}).

5.2.4 Analysis of Accumulation of Bicarbonate Ions in AGS Cells

5.2.4.1 Assessment of Bicarbonate Transporter Inhibitor (S0859) Toxicity in AGS Cells

The toxicity of the NBCe1 inhibitor, S0859 was assessed using the MTT assay, prior to the drug's use in further experiments. The method involved has already been described in section 4.2.2.2. The choice of concentration ranges used was informed by the amount successfully used in the literature (Table 5.1). Staurosporine was used as a positive control for cell death in the cells.

5.2.4.2 Bicarbonate Accumulation Assays

Functional bicarbonate uptake assays were performed on native and SLC4A4-transfected AGS cells for a time course in the absence or presence of pre-determined concentrations of S0859, aspirin, diclofenac, ketoprofen or naproxen. Preliminary experiments were timed between 1 minute and 90 minutes; however timing was subsequently switched to intervals of 1 minute to 30 minutes. Cells were pre-incubated with either the desired drug or with ordinary media for 1 hour, after which it was then aspirated. Carbon-14-labelled Sodium bicarbonate molecule ($[^{14}C]$ - $NaHCO_3$) was used as a tracer at a final concentration of 0.025μCi/ml in fresh serum-free media. The cells were then further

incubated at $37^{o}C$ for the required time periods. After the elapsed time, the radioactive solution was taken out and placed in Scintillation vials for analysis. The cells were washed twice with ice-cold phosphate buffered saline, PBS in order to stop accumulation. The cells were lysed with 200μl distilled water and a cell scraper, and the lysates were placed into Scintillation vials for analysis. 4 ml of Scintillation fluid (Meridian Gold Star) was added to the vials to measure the amount of radiation in the samples in disintegrations per minute (dpm) using a liquid scintillation counter (Packard Tri-Carb 1900TR). This was then used to calculate the amount of $NaHCO_3$ taken up in pMoles/10,000 cells. The concentration of sodium bicarbonate present was 43mM/L.

5.2.5 Statistical Analysis

All the assays were conducted in triplicates for each experiment and then the results from the mean ± SE of three independent experiments were taken and graphically represented. The unpaired t test was used to determine statistically significant differences between treatments, using the Statsdirect software version 3 (UK). Comparisons were made between the native cells without drugs versus the transfected cells with and without drugs; as well as transfected cells without drugs versus the transfected cells with the drugs. A p value of ≤ 0.05 was considered significant.

5.3   RESULTS

5.3.1   TEER Profiles of Cultured Native AGS Cells

TEER values in AGS cells were measured continually, daily for up to 10 days in 3 separate experiments. No significant increase in TEER measurement was obtained throughout the 10-day period. The results observed in the TEER experiments are however consistent with a number of other studies (Amieva et al., 2003; Jawhari et al., 1999), which have shown that the AGS cell line is unable to form tight junctions as a result of not expressing E-cadherin which is an essential tight junction protein. As a result of the inability of the cells to form tight junctions, further experiments with the 2-chamber transporter system was not utilised further.

5.3.2   NBCe1 Inhibitor, S0859 is Toxic to AGS Cells at Higher Concentrations

As S0859 was intended to be used as an inhibitor of bicarbonate transport in later parts of the study, it was necessary to determine its toxicity to AGS cells, to see if and how much the cells would be affected so as to select a concentration for use which would be safe and effective for the cells. The S0859 treatment was left for up to 12 hours before assessing viability in order to allow sufficient time for drug toxicity, if any. After 12 hours of treatment with S0859, cells were analysed for cell death responses to the drug. As shown in figure 5.5, cells did not show any significant AGS cell death at S0859 concentrations up to 150µM. However, there was significant and sudden response at 200µM ($p < 0.001$). A similar effect was elicited by the staurosporine control.
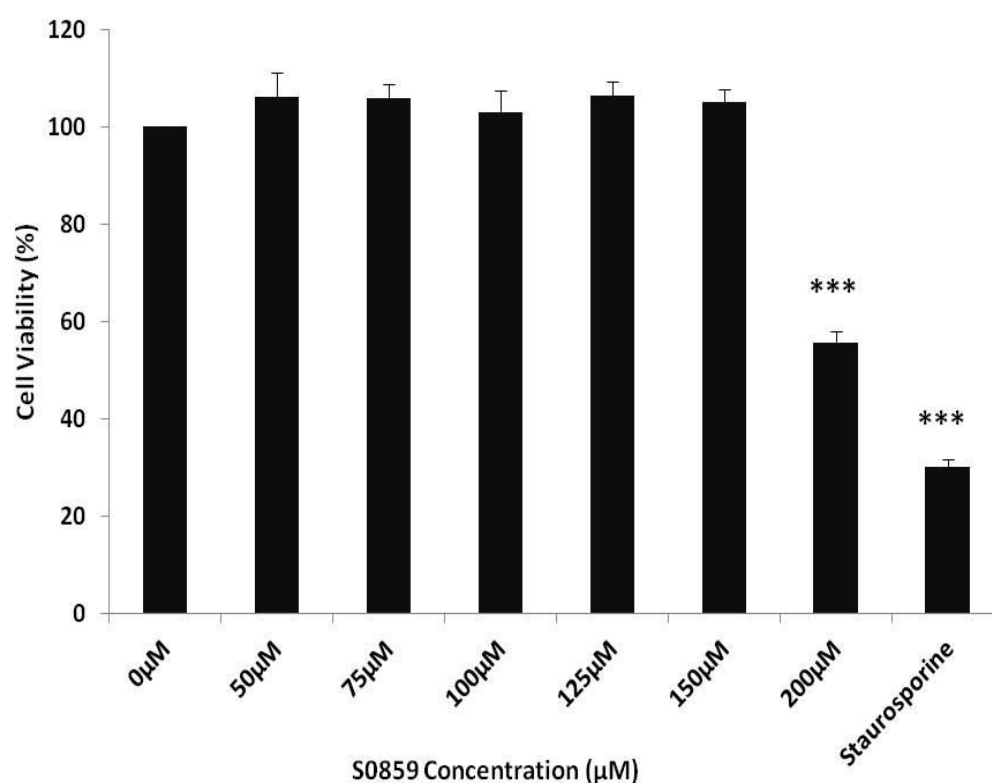
Figure 5.5 – Toxicity of S0859 to AGS Cells at Higher Doses
Percentage viability of AGS cells treated with the NBCe1 inhibitor, S0859 after 12 Hrs. S0859 treatment was left for up to 12 hours before assessing viability to allow sufficient time for drug toxicity, if any. Staurosporine was used as positive control for cell death. Cells showed a decrease in viability at a dose of 200µM. The data expressed are means ± SE from 3 experiments. Data are compared with control (0µM) for statistical significance, and p ≤ 0.05 was significant. *** = $p \leq 0.001$.

5.3.3 Optimisation Studies for Bicarbonate Accumulation Assay

Optimisation experiments were carried out on wild-type, untreated AGS cells to first determine if there was any accumulation of [^{14}C] - NaHCO$_3$ molecules with time. Accumulation was assessed for up to 90 minutes in these preliminary experiments. As shown in figure 5.6, cells showed a statistically significant increase in the amount of [^{14}C] - NaHCO$_3$ with time. At 1 minute, accumulation was 7.6 ± 0.3 pMol/10,000 cells, increasing to 9.1 ± 0.2 pMol/10,000 cells at 30 minutes (p = 0.02). After 90 minutes, the bicarbonate accumulation within the cells increased to 10.6 ± 0.4 pMol/10,000 cells (p = 0.005).

In addition, a trend showing a decrease in the amount of [^{14}C]-NaHCO$_3$ in the transport media was also observed. The amount of [^{14}C]-NaHCO$_3$ in the solution decreased significantly from 416.7 pMol/µl at 1 minute to 355.9 ± 13.8 pMol/µl at 30 minutes (p = 0.05), further decreasing to 239.2 ± 20.9 pMol/µl by the end of 90 minutes *(p* = 0.01).

While the amount of [^{14}C] - NaHCO$_3$ cannot exactly be compared between the cell lysates and media solution, as both are expressed in slightly different units, both graphs highlight the accumulation occurring in the cells when considered together.
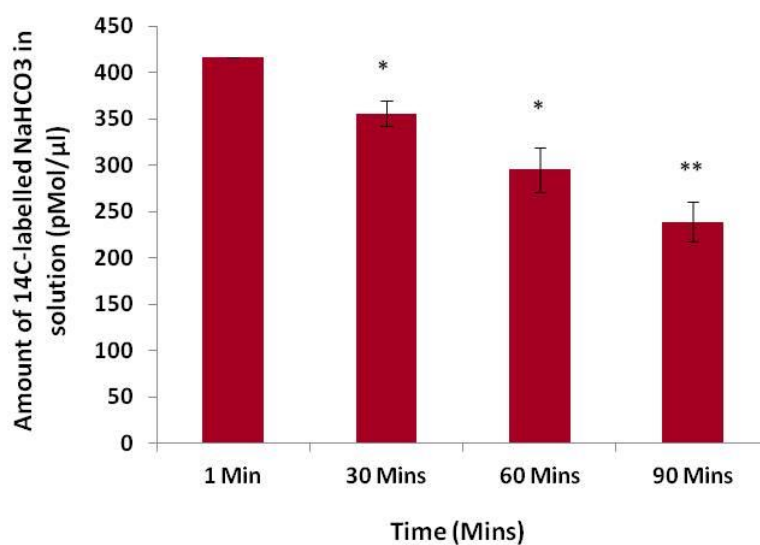
A
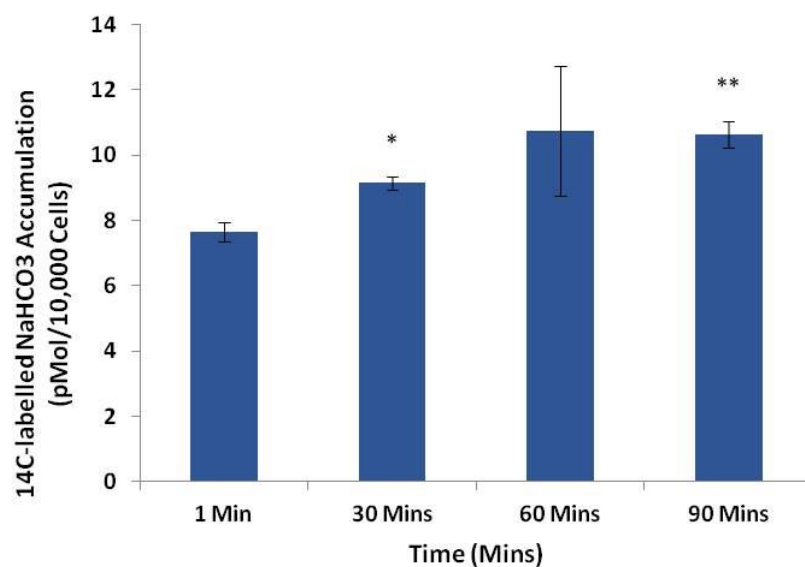
B

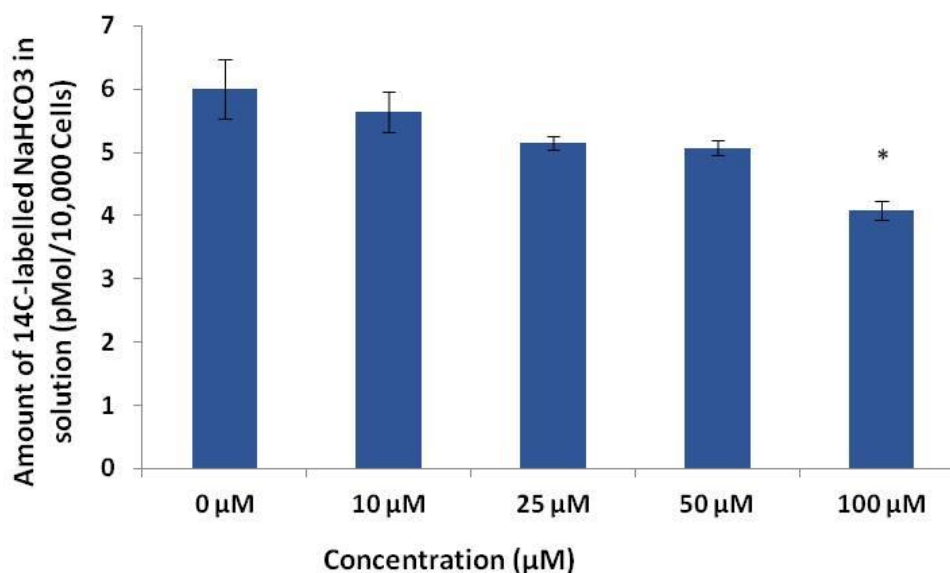**Figure 5.6 – Bicarbonate Accumulation in Untreated Native AGS Cells**
There was a significant increase in amount of bicarbonate with time in untreated AGS cell lysate (**A**); and a subsequent significant decrease in amount of bicarbonate with time in the transport media (**B**). The data represents the means ± SE from three experiments. * = $p \leq 0.05$; ** = $p \leq 0.01$.

5.3.4   S0859 Concentration and Time Experiments for Inhibition of Bicarbonate Transport

A range of S0859 concentrations between 0μM to 100μM was used to initially assess what concentration had the most inhibitory effect on bicarbonate accumulation by the AGS cells after 1 hour S8059 pre-incubation (figure 5.7). Labelled bicarbonate was then introduced and assessed after 1 hour.   At 100μM concentration, cells showed significantly reduced amount of accumulated $[^{14}C]$-$NaHCO_3$ compared to the untreated control. The amount was 6.0 ± 0.5 pMol/10,000 cells and 4.09 ± 0.2 pMol/10,000 cells at 0μM and 100μM respectively (p = 0.04).

The 100μM S0859 was thus further used in an accumulation time curve to determine its effect on the ability of native AGS cells to accumulate bicarbonate molecules over time. Figure 5.8 shows that with S0859 there was no increase in the amount of $[^{14}C]$-$NaHCO_3$ being accumulated into the cells with the passing of time. Accumulation levels remained similar across all time points with S0859 treatment, and significantly reduced when compared to levels in cells without S0859. In cells without S0859, accumulation levels increased to 6.52 ± 0.6 pMol/10,000 cells at 90 minutes, compared with 4.1 ± 0.5 pMol/10,000 cells with S0859 (p = 0.02).   There was no significant difference at 30 minutes between cells without S0859 and cells with S0859.

A

B
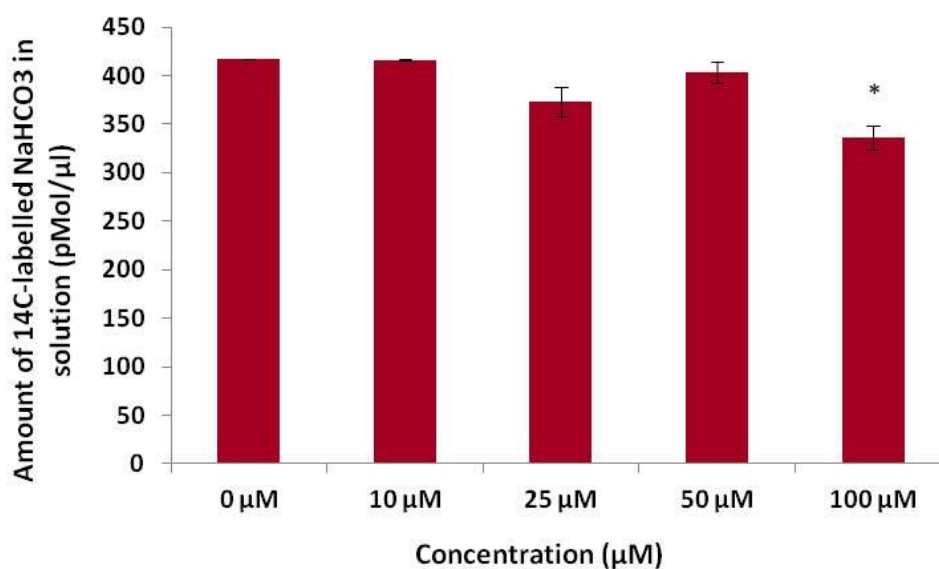
Figure 5.7 – S0859 Concentration Curve for Inhibition of Bicarbonate Transport
After 1 hour treatment with a range of S0859, bicarbonate accumulation in cell lysates **(A)** was significantly inhibited by the 100µM, compared to the untreated. Data are expressed as means ± SE from three experiments. The transport experiment was carried out for 1 hour. All data are compared with untreated (0µM) for statistical significance. * = $p \leq 0.05$. Graph **(B)** – transport solution.
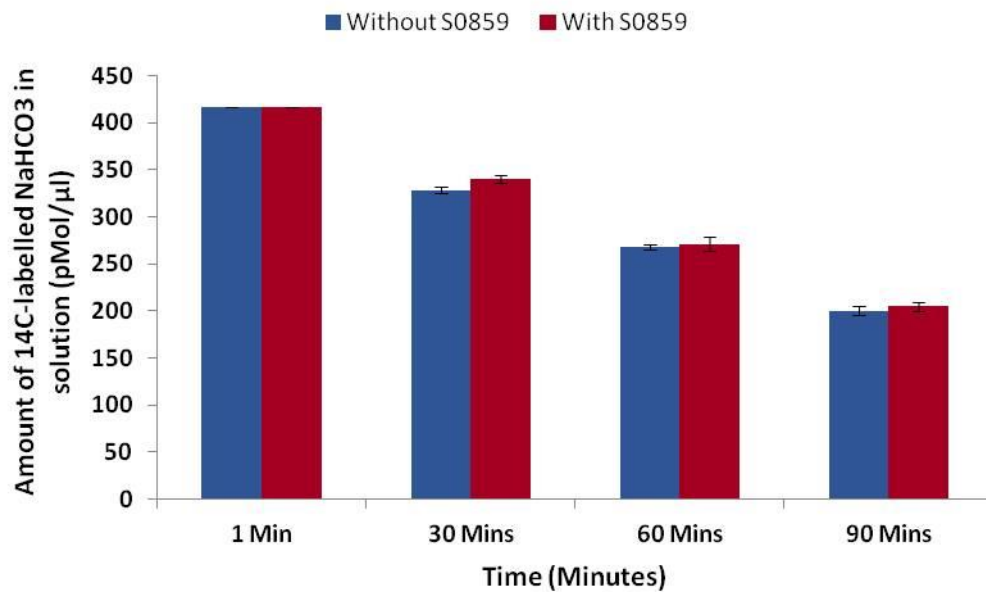
A
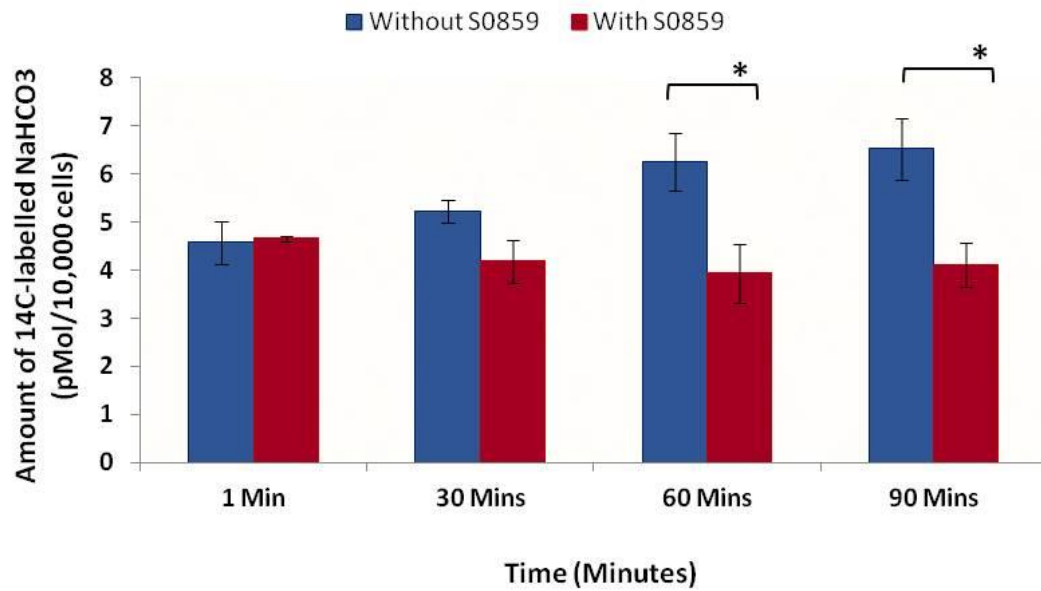
B

Figure 5.8 – S0859 time curve for inhibition of bicarbonate transport
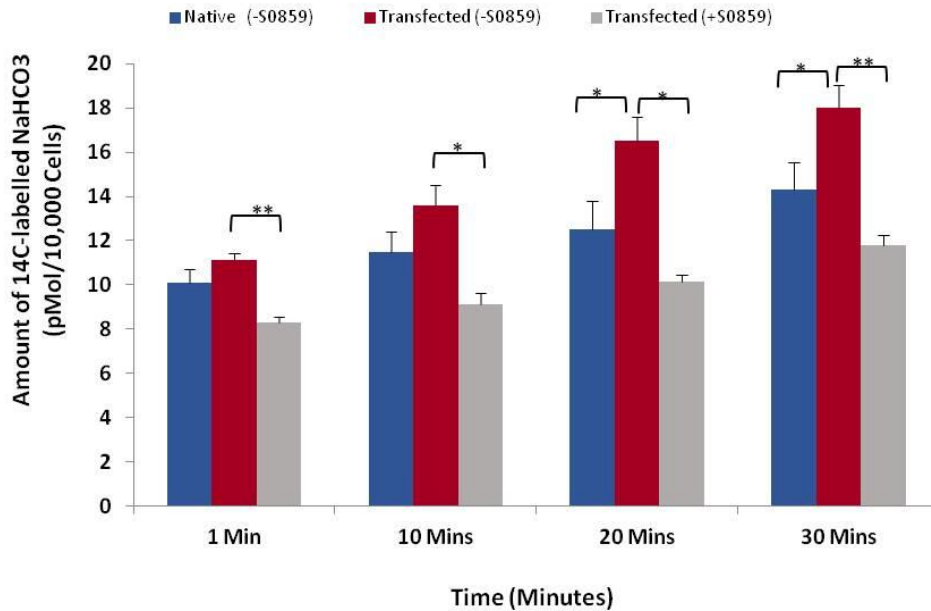100µM S0859 significantly reduced bicarbonate accumulation in AGS cell lysate (**A**) with time. (**B**) Shows a decrease in the amount also in the transport media. The data are expressed as means ± SE from three experiments. * = $p \leq 0.05$.

5.3.5 S0859 Inhibits Bicarbonate Accumulation in SLC4A4-transfected AGS Cells

Next, SLC4A4-transfected AGS cells were used in the functional accumulation studies to further investigate the effects of certain drugs on bicarbonate accumulation. Successful transfection of *SLC4A4* into the AGS cells has already been demonstrated and described in chapter 2. From here onward in the experiment, the accumulation time points were revised, to be assessed between periods of 1 minute to 30 minutes due to apparent instability of the labelled compound at longer times.

Stably-transfected cells were pre-treated with 100µM of S0859 for 1 hour and then the bicarbonate accumulation was monitored for up to 30 minutes. Transfected AGS cells showed a general increase in accumulation of bicarbonate molecules compared to native cells. At both 20 minutes and 30 minutes, untreated transfected cells showed a significant amount (p = 0.04) of accumulation compared to native cells. When the transfected cells were treated with S0859, however, their ability to accumulate bicarbonate molecules reduced significantly across all the time points (figure 5.9). At 1 minute, the amount of [^{14}C]-NaHCO$_3$ in the transfected untreated AGS cells was 11.1 ± 0.3 pMol/10,000 cells, while in transfected cells treated with S0859, it was 8.3 ± 0.2 pMol/10,000 cells (p = 0.002). At 30 minutes the amount of [^{14}C]-NaHCO$_3$ increased to 18.0 ± 1.0 pMol/10,000 cells in transfected untreated cells, and 11.8 ± 0.4 pMol/10,000 cells in transfected cells treated with S0859 *(p* = 0.01). There were no significant differences between the native cells and the S0859-treated transfected AGS cells across all time points.

A

B
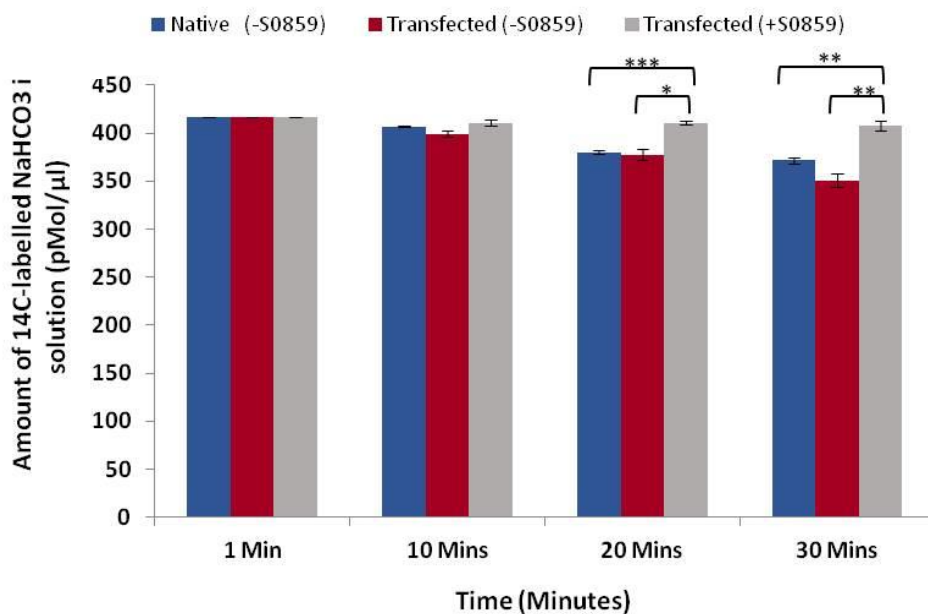
Figure 5.9 – S0859 inhibition of bicarbonate transport in transfected AGS cells
100µM S0859 significantly inhibited bicarbonate accumulation in (**A**) transfected AGS
cell lysates, compared to untreated cells. Conversely, the transport solution (**B**)
showed significantly higher amounts of $[^{14}C]$-NaHCO$_3$ remaining in wells treated with
S0859 compared to untreated. The data are expressed as means ± SE from three
experiments. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

5.3.6   Aspirin Inhibits Bicarbonate Accumulation in SLC4A4-transfected AGS Cells

When the transfected cells were treated with aspirin (20mM), their ability to accumulate bicarbonate molecules reduced significantly across all the time points (figure 5.10). Bicarbonate accumulation at 1 minute was similar across all three groups, however, from the 10-minute time point onwards, there was a significantly lower $[^{14}C]$-NaHCO$_3$ accumulation capacity in aspirin-treated transfected cells. Accumulation in untreated transfected AGS cells was 16.8 ± 0.2 pMol/10,000 cells at 10 minutes, while in aspirin-treated transfected cells, it was 15.0 ± 0.3 pMol/10,000 cells (p = 0.02). At 30 minutes the amount of $[^{14}C]$-NaHCO$_3$ increased to 21.2 ± 0.3 pMol/10,000 cells in untreated transfected cells, and 18.9 ± 0.3 pMol/10,000 cells in transfected cells treated with aspirin *(p* = 0.004). Transfected cells not treated with aspirin also showed a significant difference in increased bicarbonate accumulation compared to native cells at 10, 20 and 30 minutes; with *p* values of 0.003, 0.02, and 0.001 respectively.

A
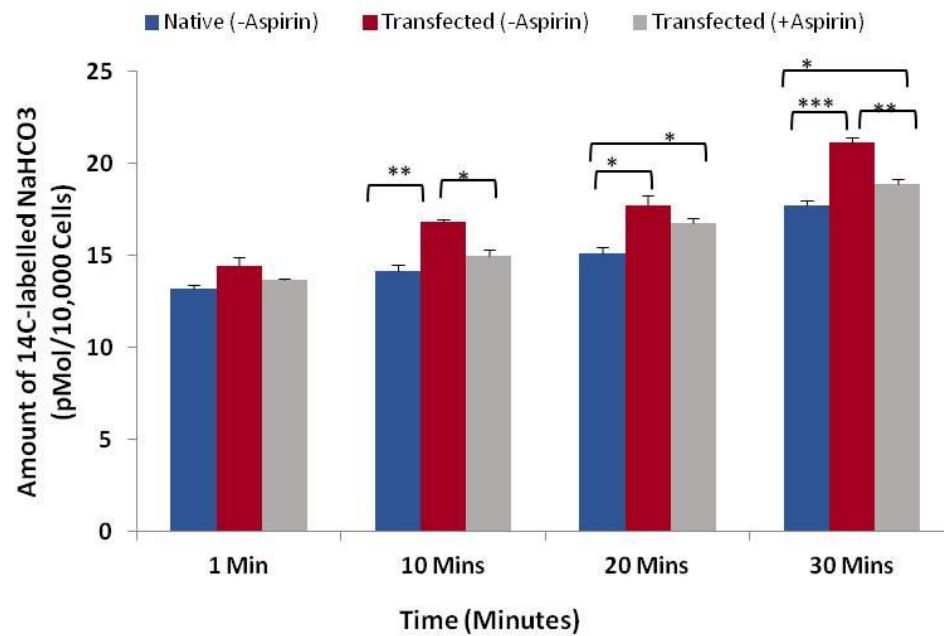
B

Figure 5.10 – Aspirin Inhibition of Bicarbonate Transport in Transfected AGS Cells
20mM aspirin treatment significantly inhibited bicarbonate accumulation in (**A**) transfected AGS cell lysates, compared to untreated cells. In the transport solution (**B**) there was a significantly higher level of $[^{14}C]$-NaHCO$_3$ remaining in wells treated with aspirin compared to untreated transfected wells.   * = $p \leq 0.05$; ** = $p \leq 0.01$.

5.3.7 Diclofenac Inhibits Bicarbonate Accumulation in SLC4A4-transfected AGS Cells

The accumulation of $[^{14}C]$-NaHCO$_3$ over 30 minutes in AGS cells exposed to diclofenac was slightly different from that seen with other drugs as there was a drop at 20 minutes in the difference noticed between cell lysates across the three treatment groups, unlike what was observed with the other drugs at that time point.

Statistically, bicarbonate accumulation was similar across the 3 treatment groups at 1 minute and 20 minutes. The significant differences observed were at 10 minutes and 30 minutes. 100µM of Diclofenac treatment significantly (p = 0.01) reduced the amount of bicarbonate in AGS cells at both times compared to untreated transfected cells (figure 5.11). At 10 minutes, accumulation in untreated transfected AGS cells was 18.3 ± 0.5 pMol/10,000 cells, while in diclofenac-treated transfected cells, it was 15.2 ± 0.4 pMol/10,000 cells. At 30 minutes the amount of $[^{14}C]$-NaHCO$_3$ increased to 20.8 ± 0.4 pMol/10,000 cells in untreated transfected cells, and was 13.1 ± 0.8 pMol/10,000 cells in transfected cells treated with diclofenac. Also, there were significant differences in accumulation of bicarbonate in transfected cells not treated with diclofenac, which showed an increase compared to native cells at 10, 20 and 30 minutes; with p values of 0.04, 0.03, 0.04 respectively.
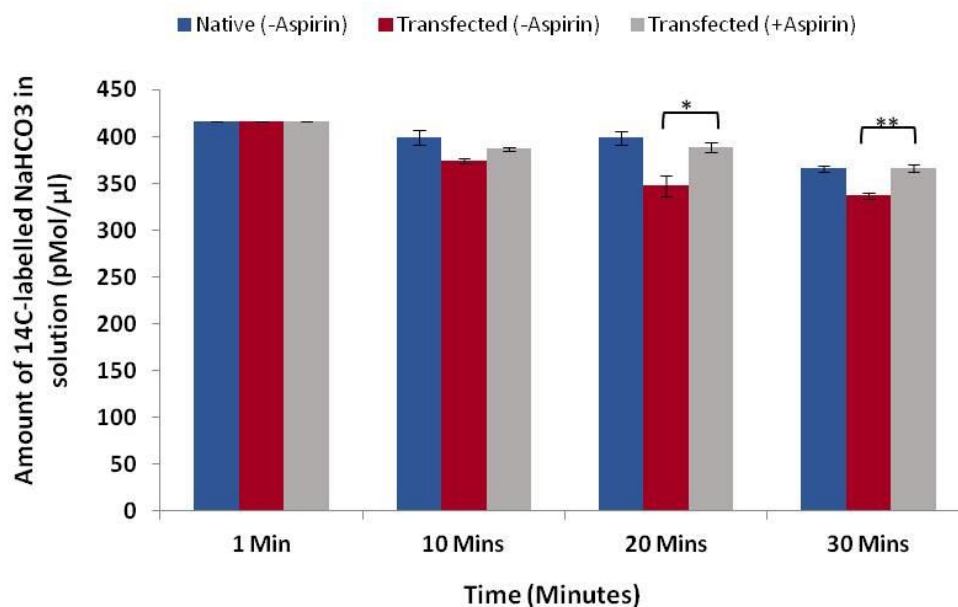
A

B

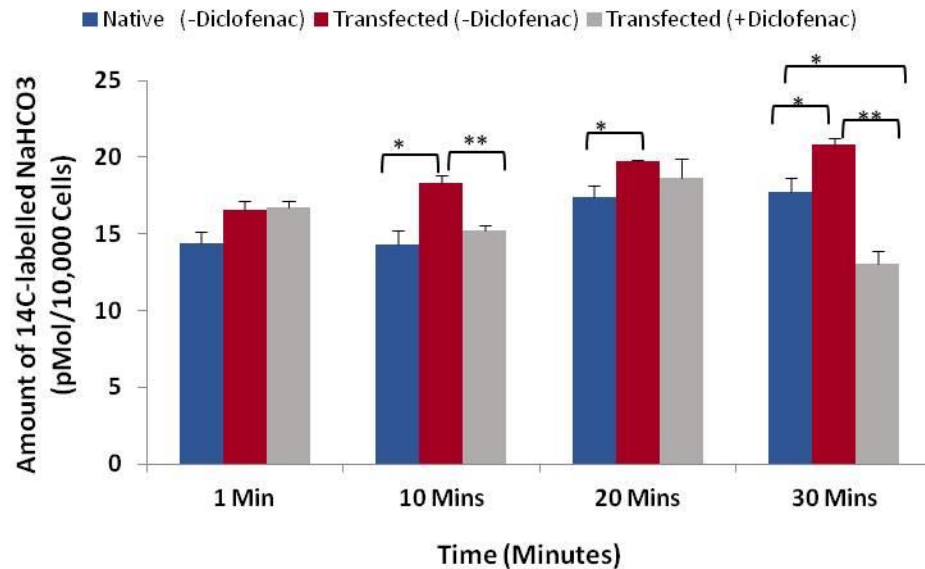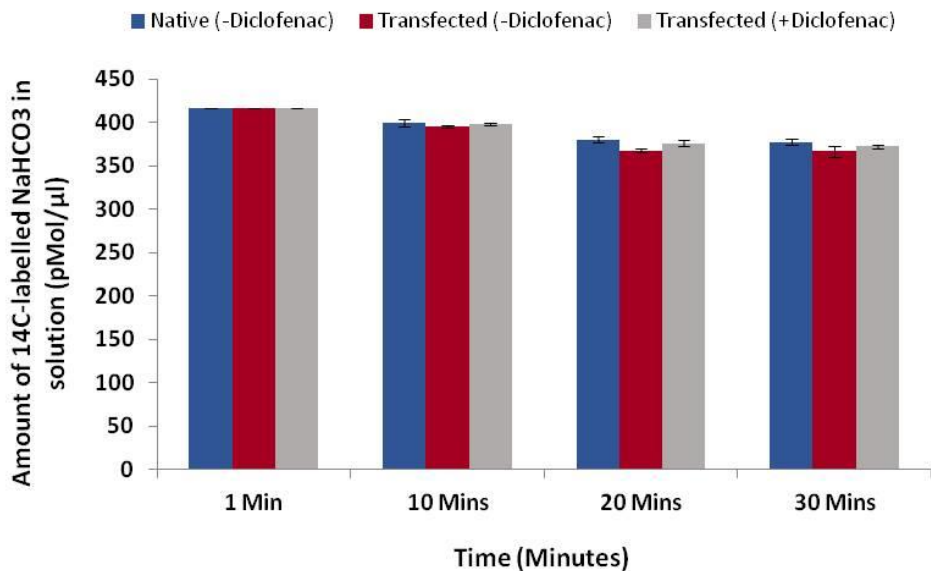**Figure 5.11 – Diclofenac Inhibition of Bicarbonate Transport in Transfected AGS Cells**
100µM diclofenac treatment significantly inhibited bicarbonate accumulation in (**A**) transfected AGS cell lysates, compared to the untreated cells. In the transport solution (**B**), no difference at each time point between groups. * = $p \leq 0.05$; ** = $p \leq 0.01$.

5.3.8 Bicarbonate Accumulation Inhibited in Ketoprofen-treated SLC4A4-Transfected AGS Cells

AGS cells were exposed to 2μM ketoprofen. The concentration of ketoprofen (or other NSAIDs) used for cell treatment was determined in earlier experiments (see section 4.2). Drug treatment was for 1 hour, prior to starting the timed accumulation experiments, to see if the drug had any inhibitory effect.

The capacity of the treated cells to accumulate bicarbonate molecules reduced significantly across all the time points from 10 minutes to 30 minutes (figure 5.12). Accumulation in untreated transfected AGS cells was 18.2 ± 0.2 pMol/10,000 cells at 10 minutes, while in transfected cells treated with ketoprofen, it was 15.7 ± 0.2 pMol/10,000 cells (p = 0.002). By the end of the experiment at 30 minutes the amount of [^{14}C]-NaHCO$_3$ increased to 20.6 ± 0.9 pMol/10,000 cells in untreated transfected cells, and 15.9 ± 0.4 pMol/10,000 cells in transfected cells treated with ketoprofen *(p = 0.02)*. There was also a significant reduction when compared with the untreated native cells at 10 minutes and 30 minutes (p = 0.03 and 0.02 respectively). Likewise, in untreated transfected cells, there was a significant difference in accumulation of bicarbonate compared to native cells at 20 minutes (p = 0.007).

A
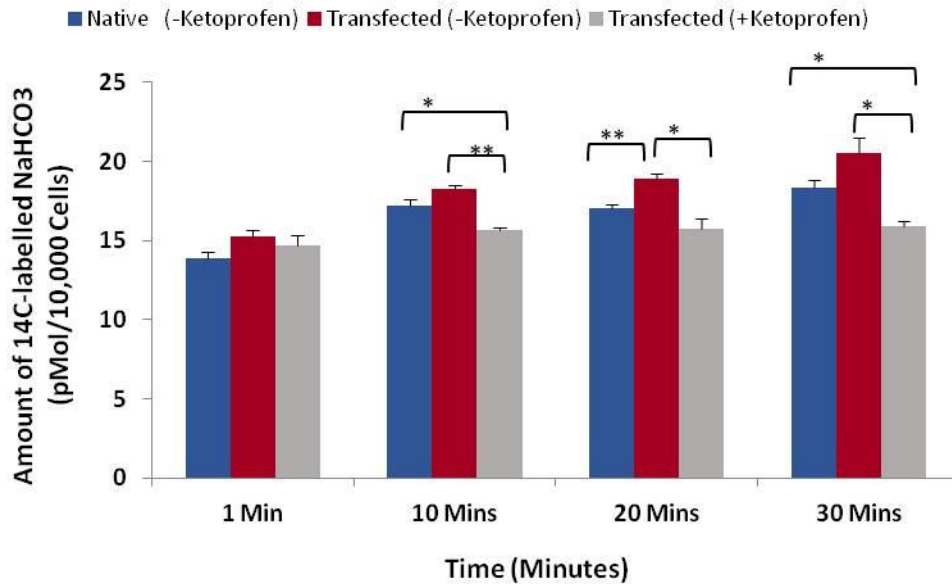
B

Figure 5.12 – Ketoprofen inhibition of bicarbonate transport in transfected AGS cells
Transfected cells were treated with 2μM of ketoprofen, which significantly inhibited the amount of bicarbonate detected in the cell lysates (**A**), compared to untreated cells.   The transport solution (**B**) conversely showed higher amounts in ketoprofen-treated solution. * = $p \leq 0.05$; ** = $p \leq 0.01$.

5.3.9   Bicarbonate Accumulation Inhibited in Naproxen-treated SLC4A4-Transfected AGS Cells

Furthermore, transfected AGS cells were also treated with 10µM naproxen, and showed a decreased ability to accumulate bicarbonate molecules, just like with the other drugs. The data in figure 5.13 show that there was a significantly reduced amount of $[^{14}C]$-NaHCO$_3$ across all the time points compared to untreated cells. At 10 minutes, the amount of $[^{14}C]$-NaHCO$_3$ in the untreated transfected AGS cells was 21.6 ± 0.5 pMol/10,000 cells, while in transfected cells treated with naproxen, it was 17.9 ± 0.3 pMol/10,000 cells (p = 0.004). At 30 minutes the amount of $[^{14}C]$-NaHCO$_3$ increased to 24.5 ± 1.0 pMol/10,000 cells in transfected untreated cells, and 19.7 ± 0.4 pMol/10,000 cells in treated transfected cell lysate $(p$ = 0.02). There were no significant differences between the native cells and the naproxen-treated AGS cells. However, a significant increase (p = 0.03) was seen at 30 minutes in untreated transfected cells, compared to the native cells.
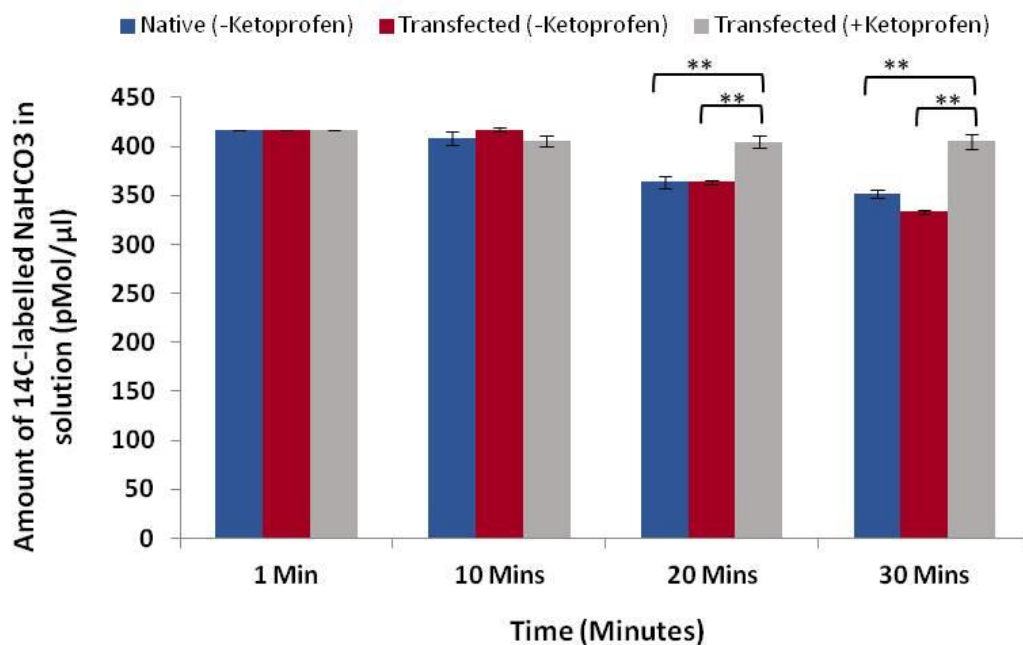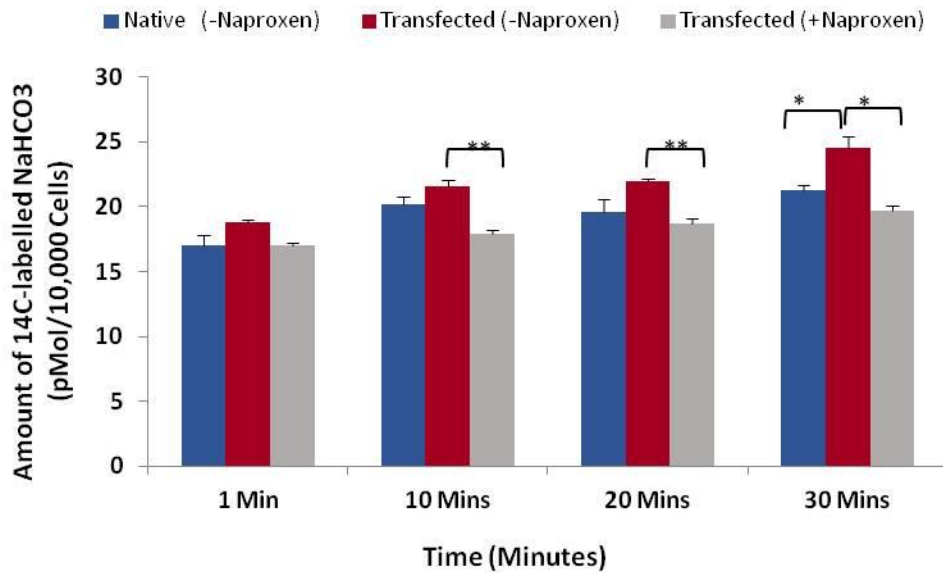
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B
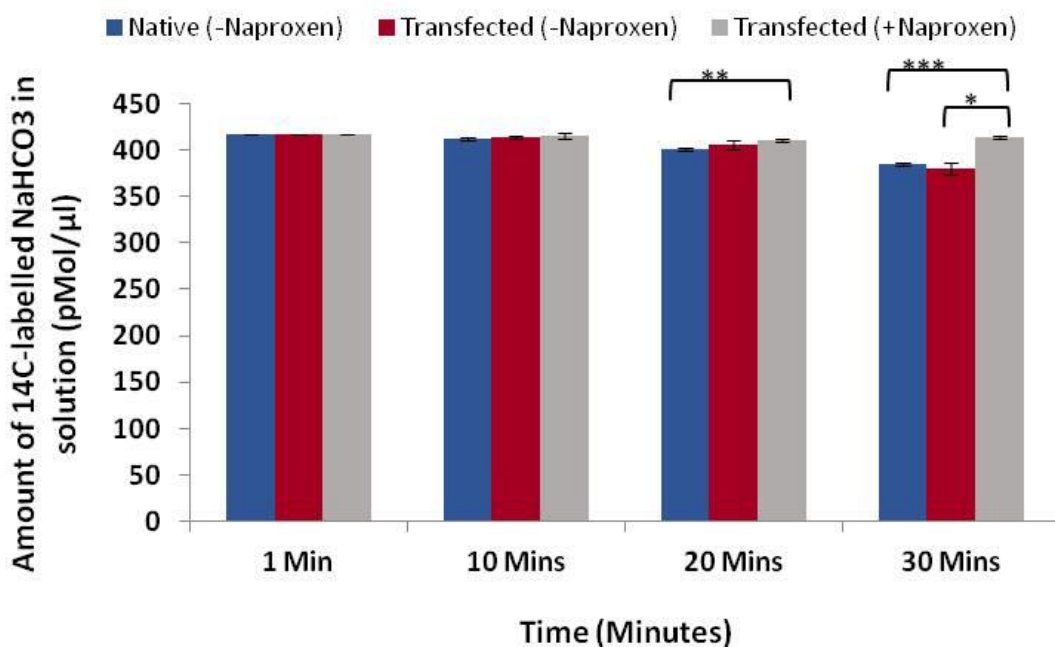
Figure 5.13 – Naproxen inhibition of bicarbonate transport in transfected AGS cells
Transfected cells were treated with 10µM of naproxen, which caused a significant inhibition of the amount of bicarbonate detected in the cell lysates (**A**), compared to untreated cells. (**B**) Shows the transport solution. * = $p \leq 0.05$; ** = $p \leq 0.01$.

5.4   Discussion

In this chapter, bicarbonate accumulation in AGS cell lines was assessed, with the aim of determining whether various NSAIDs had any effect, in terms of diminishing or inhibiting SLC4A4-mediated bicarbonate accumulation. Four different drugs belonging to various NSAID prototypes were studied, as well as a compound (S0859) known for its putative inhibition of bicarbonate transport. Accumulation was evaluated for up to 30 minutes because it was shown from our preliminary studies and other research (Bartholome et al., 2007) that measurement beyond the initial uptake range could lead to confounding factors in both influx and efflux processes.

From the results obtained, it was observed that S0859 inhibited the cellular accumulation of bicarbonate ions over a period of 30 minutes. It also corroborates earlier findings (Ch'en et al., 2008; Heidtmann et al., 2015a; Schwab et al., 2005 and Gorrieri et al., 2016) that have utilised the compound as an inhibitor though the specific mechanism by which S0859 inhibits bicarbonate transport is not yet fully established.

In a comparative study between two inhibitors –DIDS and 5-nitro-2-(3-phenylpropylamino) benzoic acid, NPPB, Akiba et al. (2001) discovered that bicarbonate secretion was abolished by both inhibitors after increasing lumenal acid, although through varying patterns. Apparently, DIDS, by inhibiting bicarbonate uptake across the membrane increased acid-induced epithelial injury, while NPPB by inhibiting bicarbonate exit, lessened the injurious effect of the acid.

Aspirin, diclofenac, ketoprofen and naproxen were used in the NSAID study. All four drugs diminished bicarbonate accumulation to varying degrees. Transfected, untreated AGS cells showed a higher level of bicarbonate accumulation compared to other cells. Accumulation in the drug-treated transfected AGS cells was significantly lower than in transfected cells which received no drug treatment. This reduction was similar to the levels found in the native (un-transfected) AGS cells. The reason for this is based on the fact that cells whose structure have been modified, either naturally or pharmacologically are not as metabolically active as they otherwise ought to be, and are not able to respond as they should to injury. From the data obtained from the NSAIDs in this experiment, the rank order of inhibition (from strongest to weakest) of bicarbonate transport in the cells was ketoprofen, naproxen, diclofenac and aspirin.

Cell exposure to NSAIDS reduces bicarbonate synthesis, via prostaglandin inhibition, causing the cells to be more permeable to external substances, including hydrochloric acid (HCl) from the lumen. In a recent study (Rodriguez-Stanley et al., 2006) in healthy volunteers, naproxen increased the concentration of gastric acid by reducing gastric fluid volume. In an earlier study, Tønnessen (1991) showed that Cl^-/HCO_3^- antiport was inhibited after cells were pre-incubated with therapeutic concentrations of NSAIDs and that this inhibition was related to their anti-inflammatory effects. The concentration of aspirin used in this study, although determined from the administrative dose and the average volume of gastric content (refer to chapter 4), may be considered high. However, it is important to take into account that the bioavailability of aspirin might lead

to higher concentrations of the drug in gastrointestinal contents *in vivo,* and thus may be comparable in an in vitro study.

Other research has suggested that the acidosis seen in NSAID toxicity is not related to COX or prostaglandin inhibition, but to acidic metabolites accumulating within the cells (Tønnessen, 1991; Hunter et al., 2011). This has been shown in our findings, as despite the availability of bicarbonate ions in the solution, the cells were unable to maintain uptake after NSAID treatment.

Some studies have established the importance of a mucus-bicarbonate barrier in gastric defence and recovery from toxic assault, pointing out that in peptic ulcer patients, the gastric mucus gel is defective (Nelis et al., 2012). Bicarbonate transport has also been shown to have an essential role in the mechanism of mucus release. Gorrieri et al. (2016) demonstrated that the mechanism by which cells released mucus when triggered with a purinergic agonist was inhibited significantly by keeping the cells in a bicarbonate-free solution. Also, a defective capacity to release mucus was shown in intestinal goblet cells of cystic fibrosis mice, having a deficient bicarbonate secretion (Liu et al., 2015).

In the experiments, measurement of the amount of $[^{14}C]$-NaHCO$_3$ remaining in the transport solution was also carried out. The results showed a decrease over time. Although loss of compound from the media solution has been suggested as an alternative for quantifying direct uptake by cells (Soars et al., 2007), this method alone does not adjust for discrimination between the compound being absorbed into plates used for incubation, or that being taken up into cells, and excludes any kinetic analysis. Furthermore, at the higher time points, some of the decrease in substance may be due to

the molecule being gradually disintegrated and lost as CO_2. For this reason, the amount of $[^{14}C]$ - $NaHCO_3$ cannot precisely be compared between the cell lysates and media solutions.

All around, our results showed a general trend of increased levels of accumulation in untreated transfected AGS cells compared to native cells, although in some instances, the difference was not significant. This may have been due to fluctuations which occur between periods in the stably transfected cells. Perhaps, using transiently transfected cells in future might show more differences.

Finally, it is important to take into account the fact that the NSAIDs were added for 1 hour prior to assessing cellular capacity for accumulation. However, in vivo, and for the purpose of pharmacological intervention in a diseased state, gastrointestinal cells are subjected to NSAID exposure continually over a longer time. It is therefore useful to recommend that in future studies, pre-incubation with NSAIDs could be carried out for longer periods before assessing bicarbonate accumulation. Overall, from this experiment, it can be concluded that the presence of the SLC4A4 protein causes gastric epithelial cells to show a differential effect in bicarbonate absorption, compared to cells where the protein is either absent or non-functional.

Chapter 6   **General Discussion**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most widely used classes of drug , with over 30 million pills used each day worldwide (Singh and Triadafilopoulos, 1999). It is well-recognised that although NSAIDs are beneficial in the treatment of many conditions associated with inflammation, such as arthritis, bursitis and fever they are often associated with some serious adverse events. There is growing evidence of this, including their ability to induce hypertension and other cardiovascular diseases, renal failure, and, the most common adverse event, upper gastro-intestinal injuries (Gambaro and Perazella, 2003).

 A study conducted in the UK (Pirmohamed et al., 2004) looking at fatalities resulting from adverse drug reactions in a group of 1,225 individuals found that over 60% of deaths were due to NSAIDs, making NSAID-related ADRs a significant cause of death directly connected to the use of medicines. In Germany, 1 in 1,220 people die from NSAID-related gastrointestinal ADRs each year (UK figures - 1 in 2,000 people) (Cryer, 2005) making them a bigger cause of fatalities than road traffic accidents. NSAIDs are responsible for about 400 fatalities caused by ulcer-related blood loss every year in the UK in people who are above the age of 60 years (Langman, 2001). Approximately 15-35% of people develop some form of ulcer lesion, which is discernible via endoscopy, within 3 months of beginning NSAID treatment (Bjarnason, 2013).

Pharmacogenomics aims to optimise medicines based on the genetic profile of each patient. There is thus the potential to identify at-risk individuals and thereby reduce the risk of adverse drug reactions. A number of studies have researched the contribution of genetics to NSAID-induced gastrointestinal problems (Pirmohamed and Park, 2001). For

example, the Liverpool group reported that a gain-of-function polymorphism in *CYP2C19*17* showed a significant association with peptic ulcer disease regardless of NSAID use. There was no relationship between peptic ulcer disease and the various other CYP2C SNPs (Musumba et al., 2013).

A genome-wide association study (GWAS) on the same patient group in Liverpool identified a biologically plausible signal with the variant in the vicinity of the *SLC4A4* gene. This thesis therefore has focused on the role of the *SLC4A4* gene, and its protein product in the predisposition to NSAID-induced PUD. *SLC4A4* encodes the sodium bicarbonate cotransporter protein. The aim of the work presented was to determine the putative interaction between NSAIDs and *SLC4A4* function.

In chapter 3, the linkage disequilibrium (LD) between the top associated SNP in *SLC4A4* (rs4521314) from the GWAS data and other SNPs in the public databases was assessed to ascertain which SNP was most likely to lead to a functional effect. However, it is still unclear which SNP (if any) within *SLC4A4* might be causative in altering *SLC4A4* expression or function.

It has been shown in the data presented (chapter 2) that in human gastric adenocarcinoma cell lines (AGS), the SLC4A4 protein (NBCe1) was localised in the cytosol, as well as in the plasma membrane, consistent with previous reports (Toye et al., 2006; Perry et al., 2007). Additionally, the presence of the *SLC4A4* mRNA transcript at low levels in native AGS cells has been confirmed in this chapter. The data obtained supplements the currently sparse data on expression and function of *SLC4A4* in AGS cells but does concur with studies, comparing *SLC4A4* expression levels in AGS and RCE using

rabbit gastric cells (Rossman et al., 1999). Although the native AGS cells were generally a good model for the present study, the low levels of *SLC4A4* expression meant that they were unlikely to be used in the functional studies of SLC4A4-mediated bicarbonate flux. The cells were therefore subsequently transfected with *SLC4A4* with the intention of increasing expression for use in the functional accumulation assays.

Additionally, cDNA samples from the gastric corpus of controls in a larger cohort (n = 93) was obtained, to determine if there was a correlation between *SLC4A4* gene expression and genotype (chapter 3) as had been observed in the stratified data from the gastric antrum. The data from the RNA sequencing of samples from gastric antrum did not directly concur with those from the gene expression of the gastric corpus samples in terms of significance between the genotypes. This may be due to different sites of biopsy (antrum vs. corpus) or due to a difference between RNA sequencing and RT-PCR in identifying alternatively spliced variants. It has been suggested that phosphorylation may have a role in RNA splicing (Misteli, 1999), modulating protein–RNA and protein–protein interactions during formation of spliceosome. Other studies have shown that *SLC4A4* activity is modulated by phosphorylation of serine (Gross, et al., 2001; Gross et al., 2003).

In chapter 4, this research looked at the effect of the SLC4A4 inhibitor, S0859 and four commonly used NSAIDs – aspirin, diclofenac, ketoprofen and naproxen on AGS cell viability, and attempted to broadly characterise cell death mechanisms. S0859 did not demonstrate cytotoxicity in AGS cells at concentrations up to 150µM. Thus it was suitable to be utilised for its SLC4A4 inhibitory properties at sub-toxic concentrations. All four NSAIDs tested reduced the viability of the cells in a dose-dependent manner, with aspirin

having the least effect and ketoprofen having the greatest effect. The IC50 (50% inhibitory concentration) values were calculated to be 12.2μM for ketoprofen, 20.1μM for naproxen, 391.7μM for diclofenac, and 52.5mM for aspirin (i.e. ketoprofen>naproxen>diclofenac>aspirin). In agreement with these data, a previous study based on the risk of NSAID-induced GI toxicity, specifically placed the ranking as ketoprofen> naproxen>diclofenac>aspirin (Henry et al., 1996). In their study on a range of NSAIDs, including aspirin, naproxen, ketoprofen and diclofenac, amongst others, Mahmud et al (1996) established that the relationship between pKa and NSAID concentration required for maximum uncoupling in mitochondrial cells is inversely correlated. They showed that higher pKa drugs require a smaller concentration to uncouple oxidative phosphorylation. This, implies that weakly acidic NSAIDs whose pKa are higher would tend to more easily cause GI damage, perhaps because they are more easily able to cross the membrane barrier into the cells' neutral environment where they then dissociate into their acidic moiety, and become ionised. However, with a highly acidic NSAID in an acidic environment, a greater part of the drug exists in an ionised state already so more of the acidic component stays outside of the cells and do not easily cross the barrier (Mahmud et al., 1996). This may also be one of the reasons that PPIs are effective at reducing NSAID upper GI effects, because they lower the acidity of the mucosa, hence allowing the dissociation to take place outside the mucosal cells (instead of inside).

It is also worth suggesting that subsequent research in the area of NSAID GI toxicity could include the implementation of *in vitro* systems which simulate the constant flow and

motion of systemic fluids *in vivo*, e.g. via intermittent and continual supply of drug-solutions and drug-free media.

The presence of caspase 3 and 7 activity was observed to be inversely proportional to aspirin concentrations which was in agreement with previous findings (Power et al., 2004), and suggests that cell death in aspirin-treated cells may involve several pathways. Caspase 3 and 7 activity was negligible after pre-treatment with the pan-caspase inhibitor Z-VAD. However, there was no significant rescue of cell viability, suggesting that the mode of cell death in aspirin-treated cells may not entirely be due to caspase-dependent apoptosis and may involve the activation of other caspases and indeed alternative cell-death pathways. The results from the present study are supported by other research (Budihardjo et al., 1999; Perfettini and Kroemer, 2003) which also showed that aspirin-induced cell death is not prevented by the inhibition of caspase, but that cells undergo a "switch" to other non-apoptotic mechanisms, such as necrosis. The caspase activities of AGS cells treated with the other NSAIDs used was not characterised, and hence, it is not possible to comment on the mode of AGS cell-death for these drugs.

Having created a SLC4A4 stably transfected AGS cell-line and ascertained appropriate concentration ranges for the 4 NSAIDs and positive control (S0859), cell transport assays was undertaken with a view to using existing methodologies which utilise a tight monolayer of cells seeded on a semi-permeable membrane. After seeding AGS in this manner, trans-epithelial electrical resistance (TEER) values in AGS cells was measured, but no significant increase in TEER measurement was observed throughout the 10-day culture period. The results however are consistent with a number of other studies

(Amieva et al., 2003; Jawhari et al., 1999), which have shown that the AGS cell line is unable to form tight junctions and is thus unsuitable for such transporter assays. Cellular radio-labelled compound accumulation assay was therefore utilised instead.

From the results obtained from the accumulation assays in chapter 5, it was observed that, as expected, S0859 greatly inhibited the cellular accumulation of bicarbonate ions over a period of 30 minutes   This concurs with previous findings (Ch'en et al., 2008; Heidtmann et al., 2015a; Gorrieri et al., 2016 and Schwab et al., 2005).

In addition, all 4 NSAIDs also diminished bicarbonate accumulation to varying degrees. Accumulation in the drug-treated transfected AGS cells was significantly lower than in transfected cells which received no drug treatment. From the data obtained, a proposed scale of inhibition (from strongest to weakest) of bicarbonate transport in the cells is suggested to be ketoprofen>naproxen>diclofenac>aspirin. To the best of our knowledge, this is the first study to highlight the direct inhibitory effect of NSAIDs on transporter mediated bicarbonate accumulation in AGS cells.

In a recent study (Rodriguez-Stanley et al., 2006) in healthy volunteers, naproxen was shown to increase the concentration of gastric acid. An earlier study (Tønnessen, 1991) also showed that Cl^-/HCO_3^- antiport was inhibited after cells were pre-incubated with therapeutic concentrations of NSAIDS (aspirin, indomethacin, piroxicam and salicylic acid) and that this inhibition was related to their anti-inflammatory effects. This, could, theoretically, be explained by NSAID-inhibition of SLC4A4 which has been demonstrated in this work.

Additionally, the quantification of $[^{14}C]$-NaHCO$_3$ in cell supernatant was carried out and a decrease was observed over time. However, the difference in amount of $[^{14}C]$ - NaHCO$_3$ between the cell lysates and media solutions could not be precisely compared since loss of bicarbonate from the media solution due to degradation has been suggested as a confounding factor for quantifying direct uptake by cells (Soars et al., 2007). The methodology excludes any kinetic analysis and does not adjust for discrimination between the compound being absorbed into plasticware, degrading, or being taken up into the cells. Essentially, it was not possible to account for all radiolabelled bicarbonate used in each incubation by measuring the cell lysate and supernatant which represents a limitation of this approach.

In conclusion, the data obtained has identified a novel putative mechanism by which NSAIDs may elicit an effect on gastric bicarbonate flux, an important protective mechanism against peptic ulceration. Bicarbonate secretion affords mucosal protection from lumenal acid (Allen and Flemstrom, 2005). In the gut, prostaglandins enhance secretion of HCO$_3^-$ via the expression of PGE1 receptors (Aoi et al., 2004) and NSAIDs action on COX enzymes thereby prevent the production of prostaglandins, such as PGE2, which has been shown to encourage bicarbonate secretion, while decreasing production of acid.

This study is the first to identify a putative inhibition of transporter protein-mediated bicarbonate flux by a range of NSAIDs. Further work needs to be done to provide understanding of how this *in vitro* observation may extrapolate to the clinical phenotype of NSAID-induced peptic ulcer disease, via the use of other cell models, primary cells or

whole organs. Overall, the findings presented also offer an opportunity to: i) better understand one component of the pathogenesis of a complex, yet common NSAID-induced adverse drug reaction; and ii) develop novel therapeutic interventions for the treatment/ prevention of NSAID-induced peptic ulcer disease, for example via tailor-made, patient-specific approach in provision of care in patients requiring NSAIDs, such as a screening program for the presence of *SLC4A4* mutations to identify 'at risk' groups. Also, a possibility for the development of therapeutic formulations containing the SLC4A4 protein is worth considering in the future for localised administration in compromised patients, which would be quite an exciting and challenging advancement.

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