METABOLIC BIOMARKERS OF AMINOGLYCOSIDE-INDUCED KIDNEY INJURY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

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CONTENTS

ABSTRACT

Drug-induced nephrotoxicity is a limiting factor to the efficacy and safety of various therapeutics including the aminoglycoside antibiotics. Aminoglycosides, such as gentamicin, cause proximal tubule injury in a significant proportion of individuals they are given to. The onset of this adverse drug reaction is currently managed by the monitoring of serum peak and trough levels and measurement of the classic renal functional markers serum creatinine and blood urea nitrogen. The limitations of these biomarkers are well established but novel, sensitive proximal tubule-specific biomarkers, such as kidney injury molecule-1, are gradually coming to the fore. Still, management of aminoglycoside nephrotoxicity is lacking a personalised strategy whereby the risk of a patient developing proximal tubule injury can be established at the individual level before exposure.

Prior studies of gentamicin nephrotoxicity pin-pointed that HMG-CoA reductase inhibitors, also known as statins, could inhibit the accumulation of gentamicin *in vitro* and therefore reduce the cytotoxicity of the drug. In order to study HMG-CoA reductase and its relationship to aminoglycoside accumulation further, an LC-MS/MS based assay was developed and validated to measure the product of the enzyme, mevalonic acid, in the urine of rats and humans. Urinary mevalonic acid was converted to mevalonolactone at pH 2, extracted alongside a deuterated internal standard using ethyl acetate and quantified by reversed-phase LC-MS/MS. The assay had a broad dynamic range of 0.0156–10 μg/mL with precision <15% CV and accuracy 85–115% to suit the natural variation within species and between non-clinical and clinical samples.

To demonstrate the utility of the assay and to ascertain the natural diurnal oscillations in HMG-CoA reductase activity, mevalonic acid was quantified in the urine of rats, mice and healthy children. In rats the excretion of mevalonic acid was significantly greater in urine collected during 22:00–10:00 h (mean 9.7 ± 2.3 μg/mg UCr) compared with 10:00–22:00 h (mean 3.4 ± 1.3 μg/mg UCr). In a human paired urine study, in 60% of individuals, morning collections had significantly greater concentrations of mevalonic acid than evening collections where the morning excretion was, on average, 105% greater.

The diurnal rhythm of HMG-CoA reductase activity was investigated in relation to aminoglycoside nephrotoxicity in a repeat-dose gentamicin rat model. A strong positive relationship between pre-dose mevalonate excretion, gentamicin accumulation and kidney injury in the renal cortex was observed. Animals administered gentamicin at 10:00 h experienced greater gentamicin accumulation and kidney injury, compared to animals on the 22:00 h dosing schedule which corresponded to greater mevalonate excretion in the hours prior to 10:00 h compared to 22:00 h. These data support the idea that there is a contributory relationship between HMG-CoA reductase activity, the uptake of gentamicin and subsequent nephrotoxicity. Investigations with the aminoglycoside tobramycin did not reach the same conclusion as no clear relationship was observed.

In contrast to the HMG-CoA reductase focussed research, a non-targeted metabonomic approach to understanding gentamicin nephrotoxicity was undertaken. Multivariate analyses of ^{1}H-NMR spectra from gentamicin-exposed rats revealed multiple major perturbations in the urine and serum metabolome, prior to kidney injury molecule-1 elevation (urine OPLS-DA model 12 h post-dose $Q^2Y=0.93$, $p=0.007$). Depletion of metabolites related to energy production and elevation of metabolites implicated in oxidative stress suggests gentamicin had a profound effect on the mitochondria of the proximal tubule epithelial cells. Quantification of metabolites such as the TCA cycle intermediates could be a non-invasive alternative to monitoring the toxicity of aminoglycosides prior to overt renal functional changes.

Multivariate analyses of ^{1}H-NMR urine spectra were also subjected to a pharmacometabonomic approach whereby the pre-dose or early post-dose metabolome

was integrated with post-dose kidney injury molecule-1 measurements in order to group individuals based on their differential response to gentamicin. Early-intervention metabolite signatures were identified to have an inverse relationship to kim-1 excretion, providing further evidence that the TCA cycle intermediates could be useful prognostic biomarkers of gentamicin nephrotoxicity. Analysis of pre-dose profiles identified gut-microbial metabolite 3-HPPA as correlated to the post-dose toxicity of gentamicin; follow up studies demonstrated that 3-HPPA excretion also had a positive relationship to urinary mevalonic acid. Hence, the pharmacometabonomic analyses implicated gut microbial *and* host HMG-CoA reductase activity as related to the extent of gentamicin nephrotoxicity, which certainly warrants additional investigations.

The adoption of targeted and non-targeted biomarker identification techniques has proven successful in this research. Mevalonic acid and HMG-CoA reductase are promising mechanistic factors which may affect susceptibility to aminoglycoside nephrotoxicity in man and research will be facilitated by the development of the LC-MS/MS assay described herein. Certainly, the use of statins as a prophylactic measure against aminoglycoside nephrotoxicity will be explored. Comprehensive analysis of the metabolome has identified the importance of the perturbation of energy metabolism and oxidative stress in the onset and development of gentamicin nephrotoxicity and in addition, integration of these vast data sets with the novel biomarker kidney injury molecule-1 has revealed that the gut microbiome could also influence an individual's susceptibility to this adverse drug reaction.

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PUBLICATIONS

THESIS

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ABBREVIATIONS

1D	One dimensional
^{1}H-NMR	Proton nuclear magnetic resonance
ADR	Adverse drug reaction
AG	Aminoglycoside
AKI	Acute kidney injury
AMP	Adenosine monophosphate
ATN	Acute tubular necrosis
BCAAs	Branched-chain amino acids
BUN	Blood urea nitrogen
BW	Body weight
D_2O	Deuterium oxide
d_4-MVL	4, 4, 5, 5-Tetradeuteromevalonolactone
EID	Extended interval dosing
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FT	Fourier transformation
GC	Gas chromatography
GFR	Glomerular filtration rate
gp330	Glycoprotein 330 or megalin or LRP2
GTPase	Guanosine triphosphatase
H_2O	Water
HMGR	3-hydroxy-3-methyl-glutaryl CoA reductase
Hz	Hertz
IC50	Half maximal inhibitory concentration

IS	Internal standard
K	Kelvin
Kim-1/KIM-1	Kidney injury molecule 1
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LRP2	Low density lipoprotein-related protein 2/megalin/gp330
MeOH	Methanol
mg	Milligrams
MHz	Megahertz
µg	Micrograms
mL	Millilitres
µL	Microlitres
mM	Millimolar
mRNA	Messenger ribonucleic acid
MVA	Mevalonic acid
MVL	Mevalonolactone
NAG	*N*-acetyl-β-D-glucosaminidase
NaCl	Sodium chloride
NaN_3	Sodium azide
ng	Nanograms
NGAL	Neutrophil gelatinase-associated lipocalin
NSAID	Non-steroidal anti-inflammatory drug
OPLS-DA	Orthogonal partial least squares discriminant analysis
PC	Principal component
PCA	Principal component analysis

PFA	Paraformaldehyde
ppm	Parts per million
Q^2	Predictive capacity of a model
R^2Y	Goodness of fit of *Y* data
RDA	Representational differential analysis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SCr	Serum creatinine
Tcv	Predictive score vector
TFEC	S-(1,1,2,2-tetrafluoroethyl)-l-cystein
TMAO	Trimethylamine N-oxide
TSP	Trimethylsilyl propanoic acid
TY(osc)	Orthogonal score vector
UCr	Urinary creatinine
UPR	Unfolded protein response

1. GENERAL INTRODUCTION

Contents

1.1 Adverse drug reactions

An adverse drug reaction (ADR) is an undesirable side effect caused by exposure to a drug. ADRs are diverse and a classification system has been developed to divide ADRs into groups (Park et al. 1992). Type 'A' ADRs are reactions which can be attributed to the pharmacological action of the drug and they are typically dose-dependent, accounting for approximately 80% of ADRs (Kalgutkar and Didiuk 2009). Type 'B' ADRs are also known as idiosyncratic ADRs and have complicated dose-response relationships with effects unrelated to the known pharmacology of a drug. Type 'C' ADRs refer to therapeutics that have chronic adverse effects, whilst Type 'D' ADRs have a delayed adverse effect such as teratogens or carcinogens (Edwards and Aronson 2000).

As a leading cause of drug attrition and a growing public health issue, ADRs are a significant concern to the pharmaceutical industry, medical professionals, governing authorities and the general public. Between 1999 and 2005 the number of adverse event reports increased 2.6-fold, with serious adverse event reports increasing 2.7-fold (Moore et al. 2007). Serious ADRs were estimated to be the cause of 106,000 deaths in 1994 in the US, ranking ADRs as the fourth to sixth largest cause of death that year (Lazarou et al. 1998).

Not only do ADRs increase the duration of hospitalisation, alongside increased patient morbidity and mortality, but they are a great financial burden. A prospective analysis of hospital admissions in two Merseyside National Health Service hospitals between November 2001 and April 2002 revealed that 6.5% of admissions were caused by ADRs (93% of the ADRs were Type A) at a projected cost to the National Health Service of £466 million (Pirmohamed et al. 2004). ADRs are also responsible for a significant number of drug withdrawals; between 1975 and 2000 10% of FDA-approved drugs were either withdrawn or given black-box warnings as result of unanticipated ADRs (Lasser et al. 2002). Although some of the epidemiological data available on ADRs are more than a decade old, the prevalence and importance of ADRs to drug development and public health still remains.

ADRs can affect any organ system but some organs are more susceptible than others. Hepato- and cardiotoxicity are the most widely cited reasons for drug attrition during development and post-marketing (Lasser et al. 2002; Stevens and Baker 2009), yet other organ-specific ADRs such as nephrotoxicity are of major concern pre- and post-marketing, limiting the development, efficacy and prescription of treatments.

1.2 The kidney

A single mammalian kidney is a bean-shaped organ with a concave renal hilum, where blood vessels connect and the ureter exits, and a convex outer surface also known as the cortex (Deshmukh 2009). The kidney has distinct zones that form a cone shaped renal lobe comprising the cortex at the widest region, adjoined to the medulla and the papilla at the tip (Seldin 2008).

The functional unit of the kidney, the nephron, spans the cortex and medulla. The physiological function of the nephron is to regulate blood entering the kidney by adjustment of water and salt content, volume and pH and removal of toxins and unwanted substances (Seldin 2008). A nephron is composed of several structures with specific functions – the glomeruli and Bowman's capsule, proximal tubule, loop of Henle and the distal tubule which adjoins the collecting duct (Deshmukh 2009), see Figure 1.1.

The glomerulus is a tuft of capillaries in the cortex which receives and filters blood from the afferent arteriole. The fenestrated capillary wall allows passage of molecules up to 70 kDa (Deshmukh 2009) and pressure is created by the difference in diameter between the afferent arteriole and the narrower efferent arteriole (although this can also be hormonally regulated). Collectively, the glomerulus, basement membrane and podocytes of Bowman's capsule selectively filter small molecules such as water, ions, low molecular weight proteins, glucose and amino acids whilst excluding blood cells, platelets and high molecular weight and anionic proteins (Seldin 2008) producing an ultrafiltrate.

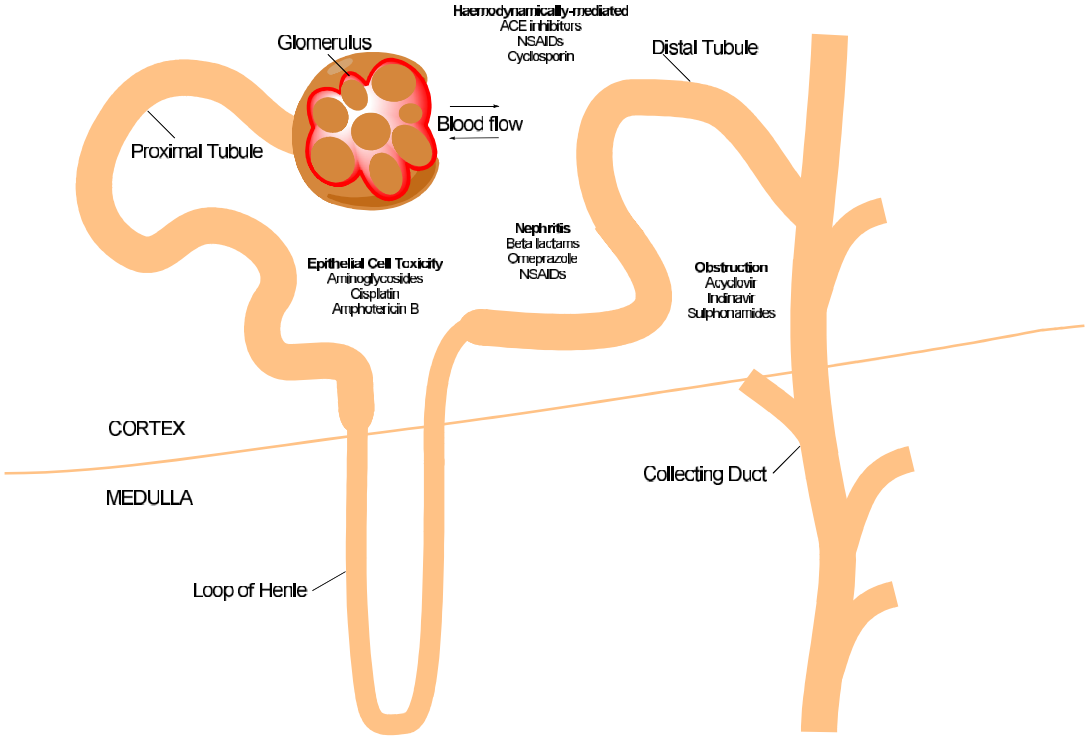


Figure 1.1 Drug-induced toxicity in the nephron. The nephron consists of distinct functional parts which are susceptible to injury as a result of exposure to a variety of therapeutic agents.

Ultrafiltrate is processed further to form urine entering the proximal tubule in the cortex, which has resorptive (glucose, salt, water, proteins) and secretive (creatinine) functions (Deshmukh 2009). Proximal tubule epithelial cells are characterised by their dense microvilli on the apical surface which increases their surface area to facilitate reabsorption. The loop of Henle extends into the medulla from the proximal tubule and facilitates water and salt exchange. The remaining filtrate enters the distal tubule which mediates the uptake of calcium, phosphate, sodium and potassium from the filtrate (Deshmukh 2009) before it reaches the collecting duct, draining towards the ureter.

1.2.1 Acute kidney injury

Acute kidney injury (AKI), previously known as acute renal failure, is a term used to describe the acute loss of kidney function which manifests from a variety of clinical scenarios and can present as mild increases in serum creatinine to complete anuria (Mehta et al. 2007).

The causes of AKI can be classified into pre-renal, intrinsic and post-renal; pre-renal causes affect blood flow, post-renal causes are obstructions of the urinary tract and intrinsic causes damage the kidney tissue, usually by cellular toxicity or nephritis.

1.2.1.1 Drug-induced nephrotoxicity

Drug-induced nephrotoxicity is a type of kidney injury where functional or structural damage to the organ, pre-renal, intrinsic or post-renal, is caused by exposure to one or several therapeutic agents. It is a common clinical adverse event and although the true incidence is difficult to determine, US hospital data suggests that drug-induced kidney injury accounts for 18-27% of all acute kidney injury cases in the US (Taber and Pasko 2008). Therapeutics can alter renal perfusion or directly injure glomerular, tubular, vascular or interstitial cells (Choudhury and Ahmed 2006). Pre-renal kidney injury, observed in the use of non-steroidal anti-inflammatory drugs (NSAIDs) and angiotensin-converting enzyme (ACE) inhibitors, arises from a reduction in intraglomerular pressure which is normally autoregulated by the organ in order to preserve the glomerular filtration rate (GFR) and urine production (Naughton 2008). The aminoglycosides cause toxicity in the proximal tubule cells and the beta-lactam antibiotics and the NSAIDs provoke nephritis of the interstitial cells (D'Agati et al. 1989), see Figure 1.1.

1.2.1.2 Susceptibility to toxicity

The functional characteristics of the kidney render it susceptible to drug exposure and therefore toxicity. Approximately ¼ of cardiac output is filtered by the kidney and 90-95% of this blood is in the cortex with the remainder in the medulla. The liver transforms many drugs to more polar hydrophilic metabolites which will inevitably be filtered from the systemic circulation at the glomerulus and pass through the tubules for elimination. The exchange of solutes and reabsorption of low molecular weight proteins and amino acids in the renal tubules is essential in salt, water and protein homeostasis. However the large number of transporters and endocytic receptors in the tubules confers a concentrating

ability which means that although a drug may be in low abundance in the systemic circulation and the ultrafiltrate, the selective transport of drugs and metabolites into the tubular cells will cause higher cytosolic concentrations that may be toxic. Furthermore the progressive concentration of the ultrafiltrate along the nephron could cause precipitation of compounds resulting in tubular obstruction. In addition, due to the high metabolic rates involved in the Na+-K+-ATPase active transport of solutes in and out of renal cells, the cells have a largely hypoxic environment, rendering them more sensitive to injury. Whilst the GI tract and liver are major sites of metabolism, the kidney also expresses a large number of drug metabolising enzymes including the cytochrome P450 family, which are implicated in various drug-induced toxicities (Perazella 2009).

1.2.1.3 Detection of nephrotoxicity

Whilst animal studies can use histopathology to confirm AKI, clinicians rely on the 'gold standard' established biomarkers serum creatinine and blood urea nitrogen as indicators of renal function. However, their inherent characteristics limit the timely detection of AKI and stratification of patient regimens; as such there is a strong research effort towards identifying and qualifying novel sensitive and specific biomarkers of kidney injury.

1.2.1.3.1 Serum creatinine

Serum creatinine (SCr) is routinely measured in clinical practice as an indicator of renal function. As a non-specific biomarker of renal function it is relatively uninformative of kidney injury. The reciprocal relationship of SCr and creatinine clearance is widely used to estimate the GFR; however, concern over the overestimation of GFR and therefore the clinical value of this 'gold standard' renal biomarker is widespread (Slocum et al. 2012).

Creatinine is the nonenzymatic product of muscle creatine and muscle mass determines the size of the creatinine pool in an individual, with age and sex being major determinants. As a low molecular weight cation, creatinine is freely filtered by the glomerulus but it is also secreted by the renal tubules. In states of renal insufficiency, as much as 60% of urinary

creatinine is derived from the tubules (Perrone et al. 1992). As a result creatinine clearance can exceed the GFR and in health and disease this could lead to overestimations of GFR and overlooking kidney injury.

Extrarenal clearance of creatinine *via* enterohepatic routes can also lead to overestimation of GFR (Jones and Burnett 1974). In individuals with normal renal function enterohepatic creatinine clearance is not observed, however in individuals with a compromised GFR and theoretically a raised SCr, degradation of creatinine by intestinal microbiota counters the serum rise causing an overestimation of creatinine clearance.

A major limitation to the use of serum creatinine to detect kidney injury is manifested in the delayed onset in serum changes. Typically a delay of 24-72 h is observed post-insult, the so-called 'accumulation phase', where serum creatinine reaches a new higher steady state after kidney injury. The new GFR, extent of tubular secretion, balance between creatine formation and turnover and the volume of distribution contribute to this delay, which impedes the early detection of kidney injury in the clinic (Waring and Moonie 2011).

Of further concern to the clinical utility of this biomarker is the sensitivity of serum creatinine to detect a reduction in GFR, owing to the inter-individual variability in baseline concentrations. Clinical guidelines suggest ≥1.69 mg/dL or ≥50% increase in baseline serum creatinine with reduced urine output is indicative of kidney injury (Waring and Moonie 2011). However a wide physiological dynamic range is observed, influenced by age, sex, muscle mass and consumption of dietary creatine. Vegetarians have been observed to have a significantly lower creatinine clearance compared to individuals with a greater protein intake (Bosch et al. 1983). In patients with low muscle mass and low baseline serum creatinine a modest increase after kidney injury would bring the measurement into the 'normal' reference range and without baseline measurements, kidney injury may go undetected.

Although AKI may reduce the number of functional nephrons, the remaining nephrons compensate for this loss by hyperfiltration (Bosch et al. 1983). A patient may have AKI but it could be subclinical if measured only by GFR and serum creatinine (Ronco et al. 2012). The renal functional reserve contributes to delayed serum creatinine elevation and compromises early detection of kidney injury and the prognosis.

1.2.1.3.2 Blood urea nitrogen

Blood urea nitrogen (BUN) is the measure of the nitrogen content of the urea present in serum and the normal range is 5 to 20 mg/dL. Urea is a physiological waste product of protein turnover and a large proportion of urea is excreted *via* the kidney. Although 40 to 60% of urea filtered by the glomerulus is reabsorbed, BUN is also a measure of GFR (Hosten 1990). Similar to creatinine, urea production is affected by high-protein diets, and as urea is predominantly produced by the liver, blood urea concentrations are affected by liver function and high catabolic states. BUN can also be modified by processes such as gastrointestinal bleeding and heart failure (Fuchs and Hewitt 2011). Due to the drawbacks of these traditional biomarkers, clinical measurements of BUN and SCr are best interpreted as ratios and can indicate whether disease is pre-renal, post-renal or extra-renal (Hosten 1990).

1.2.1.4 Emerging biomarkers of acute kidney injury

The limitations of SCr and BUN as renal biomarkers have prompted research into novel biomarkers of AKI. The ideal biomarker of acute kidney injury would be tissue and site-specific, sensitive, with changes from baseline proportional to the degree of injury allowing early detection with a wide diagnostic window. Unlike SCr and BUN, the biomarker should not be influenced by environmental factors and from a practical and analytical perspective, can be obtained by non-invasive sampling.

1.2.1.4.1 Neutrophil-associated gelatinase lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, has been proposed as an early diagnostic biomarker of AKI that could contend with serum creatinine. NGAL is a protein expressed in various cell types including epithelial cells and neutrophils but in the kidney it has been associated with renal development, recovery and regeneration (Schmidt-Ott et al. 2007). NGAL mRNA and protein are upregulated in the post-ischemic mouse kidney (Mishra et al. 2003) and it has been validated as a biomarker of AKI in ischemic and nephrotoxic animal models (Mishra et al. 2004).

Despite the pre-clinical success, the clinical validation of NGAL has been less definitive. There is large variability in baseline concentrations of serum NGAL in patients who do not have AKI, complicating the determination of reference ranges. Furthermore, the sensitivity and specificity of NGAL varies between patient populations, for instance in paediatric versus adult populations the predictability of AKI by NGAL is age-dependent. Mishra *et al* studied serum and urine NGAL changes to predict AKI in children undergoing cardiopulmonary bypass surgery and observed significant increases in NGAL in both biofluids just 2 h post-surgery, with sensitivity of 100% and specificity of 98% (Mishra et al. 2005). In contrast, studies of urinary NGAL after adult cardiac surgery revealed less convincing specificity and sensitivity 1 h post-surgery, at 59% and 80%, respectively (Wagener et al. 2006). Moreover, NGAL is not specific to the kidney, as exemplified in the difference between NGAL in septic versus non-septic AKI (Bagshaw et al. 2010); co-morbidities such as the presence of infection or inflammation modulate NGAL levels and contribute to the low specificity, which could ultimately lead to false positive diagnosis of AKI. These drawbacks mean it is unclear what measurement of urinary or serum NGAL adds to the clinical decision process.

1.2.1.4.2 N-acetyl-β-D-glucosaminidase

N-acetyl-β-D-glucosaminidase (NAG) is a lysosomal enzyme found in the proximal tubule epithelia in abundance. Increased urinary excretion of NAG suggests tubular damage as its high molecular weight (130-140 kDa) precludes it from glomerular filtration. Urinary NAG levels are found to remain elevated throughout renal disease and as such, NAG has been measured experimentally for several decades and could serve as a prognostic biomarker (Price 1992).

Notwithstanding these attributes, urinary NAG is also observed to increase with lysosomal activity (Bosomworth et al. 1999) and in glomerular diseases (e.g. diabetic nephropathy) (Koh et al. 1993). Therefore NAG may lack the specificity for diagnosis of AKI and clinical decision making.

1.2.1.4.3 Kidney injury molecule-1

Kidney injury molecule-1 (kim-1) is a protein that was recognised as a potential biomarker of kidney injury in a 48 h adult rat post-ischemic unilateral kidney model by representational differential analysis (RDA) in which mRNA populations in normal and regenerating kidneys were compared (Ichimura et al. 1998). The mRNA transcript of kim-1 identified by RDA was present at low levels in sham-operated control kidneys and embryonic kidneys but was substantially upregulated in the post-ischemic model of kidney injury. Expression of the kim-1 transcript was co-localised with the apical end of regenerating (proliferating and dedifferentiated) proximal tubule epithelial cells and cell debris in the post-ischemic rat kidney as determined by RNA in situ hybridisation and immunohistochemistry. Importantly, kim-1 was not found in other parts of the nephron, or in the normal or embryonic kidney.

Further characterisation of rat kim-1 and human KIM-1 indicated that it is a Type 1 transmembrane protein. The cytoplasmic tail is relatively short and contains a phosphorylation site, indicative of a role for kim-1 in cell signalling, whereas the N-termini

ectodomain, which characteristically protrudes into the luminal space, contains a six-cysteine immunoglobulin super family domain (Ichimura et al. 1998) and mucin domain which confer protein-protein interactions at the cell surface and cell adhesion properties. The protein has been shown to be cleaved by metalloproteinase and excreted in the urine (Bailly et al. 2002; Han et al. 2002) which is important to its development as a non-invasive renal biomarker of injury.

1.2.1.4.3.1 Physiological function of kim-1

KIM-1 is a member of a large family of proteins also referred to as T-cell immunoglobulin mucin proteins (TIMs) which are extracellular sensors or receptors for cell adhesion and cell signalling. KIM-1 is also expressed on the T-cell surface and is involved in differentiation of T helper cells (Meyers et al. 2005), however in renal injury KIM-1 is not localised to the basal surface (rather, the apical surface) of the proximal tubule epithelial cells where it would have the potential to interact with infiltrating macrophages. Therefore it is unlikely that KIM-1 plays a role in the immune reaction to proximal tubule injury.

After nephron damage, the segments can be remodelled and repaired to regain function; a critical part of this recovery is removal of apoptotic and necrotic cell debris by phagocytosis. During early characterisation of Kim-1 it was found to be co-localised with markers of cell proliferation (Ichimura et al. 1998), hence it was postulated that kim-1 plays a role in cell regeneration. Since then kim-1 has been localised to the phagocytic cup (the site of apoptotic cell internalisation) on epithelial cells in the tubular lumen of rats with ischemia-induced proximal tubule injury and has been demonstrated as a phosphatidylserine receptor to recognise the apoptotic cell surface (Ichimura et al. 2008). The phagocytic phenotype which kim-1 confers to epithelial cells in the damaged renal tubule is considered important to enhancing the remodelling process and dampening the inflammatory response after injury and therefore critical to functional recovery.

1.2.1.4.3.2 Non-clinical investigations into the utility of kim-1

The establishment of kim-1 as a biomarker of ischemia-induced proximal tubule injury prompted investigations with model nephrotoxicants. Kim-1 upregulation in S-(1,1,2,2-tetrafluoroethyl)-l-cystein (TFEC), cisplatin, and high-dose folic acid induced proximal tubule injury was examined in male rats (Ichimura et al. 2004). Kim-1 protein levels were elevated in the kidney tissue of TFEC-treated rats 1 day after exposure and persisted for 14 days; in contrast, serum creatinine was elevated 24 hours post exposure, peaked at day 2 and returned to baseline levels by day 5. Similarly, kim-1 was detected in the urine of the same animals from day 1 after TFEC treatment and persisted to day 7. Upregulation of kim-1 was also demonstrated in the folic acid model (without a rise in serum creatinine) and of cisplatin (Ichimura et al. 2004), cadmium (Prozialeck et al. 2007), mercury, chromium and gentamicin (Zhou et al. 2008) nephrotoxicity, where uKim-1 outperformed serum creatinine through earlier and more sensitive increases. Of particular interest, administration of gentamicin (subcutaneous 25-400mg gentamicin/kg) in male Sprague-Dawley rats led to dose and time-dependent increases in urinary kim-1 excretion at doses ≥100 mg/kg which correlated with renal histopathological severity, whereas significant rises in BUN, SCr and urinary NAG excretion were only seen at the highest dose of 400 mg/kg (Zhou et al. 2008).

A multi-centre academic and industrial initiative (the Predictive Safety Testing Consortium) thoroughly investigated kim-1 by replicating the kidney injury specific 100-fold induction of kim-1 gene expression, induction of protein expression and urinary excretion, and direct comparison of kim-1 measurements with SCr, BUN, NAG and histopathology in nephrotoxicant-dosed animals (Vaidya et al. 2010). As a result urinary kim-1 has been qualified by the US Food and Drug Administration as a biomarker of renal injury in the pre-clinical development of pharmacologic agents (Dieterle et al. 2010) .

1.2.1.4.3.3 Clinical evaluation of KIM-1

KIM-1 was evaluated in kidney tissue biopsies from six patients diagnosed with acute tubular necrosis caused by ischemia or allograft dysfunction (Han et al 2002). Akin to the localisation seen in the rat, KIM-1 was localised to the apical membrane of human proximal tubule epithelial cells whereas it was not detected in the glomeruli. Urine analyses demonstrated that a soluble released form of KIM-1 was detected at higher levels in patients with confirmed acute tubular necrosis compared to patients with chronic renal diseases and those without renal disease. Indicative of the early changes in the presence of urinary KIM-1, in two patients who had experienced an ischemic insult urinary KIM-1 was raised within 12 hours following injury and notably before any urinary casts were identified. Likewise in paediatric patients undergoing cardiac pulmonary bypass who developed AKI, urinary KIM-1 was significantly elevated 12 h after surgery and in adults significance was reached immediately after cardiac surgery (Han et al. 2009) and at 2 h post-cardiac surgery (Liangos et al. 2009).

The surge in functional genomics studies has aided the identification and characterisation of novel biomarkers of kidney injury. Although there has been a promising start to the qualification of KIM-1/kim-1 as a renal biomarker in clinical/non-clinical research, it is likely that a panel of biomarkers will make the greatest impact to clinical decisions in the future. Until then, the qualification of these emerging biomarkers for pre-clinical use will facilitate the investigation of mechanisms of drug-induced nephrotoxicity for new and existing chemical entities.

1.3 Methods for identifying putative biomarkers

There are two major approaches that can be chosen to identify potential biomarkers of health and disease. The first is a targeted approach whereby research focusses on a target that has been linked to the biological process and the relationship of this putative biomarker and the disease state is further probed with specific research questions. The

second approach is a non-biased, comprehensive analysis of the biological system when challenged with the disease. In this way, novel targets can be identified which can be further investigated by the targeted approach. Both approaches can be conducted using human samples if available but it is more common to initiate targeted and non-targeted biomarker identification using an animal model of the disease and if successful, follow-up with investigations in humans to ascertain whether the biomarker is translational.

1.3.1 Analytical technologies in biomarker identification

Targeted and non-targeted 'omics approaches to biomarker identification and investigation rely heavily on analytical technology, for example, a non-targeted comprehensive analysis of kidney mRNA by PCR, identified kim-1 as a putative biomarker of kidney injury (Ichimura et al. 1998). Whilst analysis of nucleic acids is enabled by PCR-based technology, analysis of the proteome and metabolome is permitted by analytical technologies such as chromatography coupled to mass spectrometry (LC-MS^n) and/or nuclear magnetic resonance (NMR) spectroscopy.

1.3.1.1 Chromatography

There are a wide range of chromatographic options that can be used to enhance the separation of complex biological mixtures. The selection of gas (GC) or liquid chromatography (LC), column chemistry and temperature and mobile phase or carrier gas can tailor molecular separation to maximise the number of analytes or select for a specific target, making gas or liquid chromatography useful techniques in non-targeted and targeted biomarker analysis. LC or GC are typically coupled to detection instruments such as mass spectrometers or NMR instruments.

1.3.1.2 Mass spectrometry

Mass spectrometry measures the abundance of individual charged molecules according to their mass-to-charge ratio (m/z). At the ion source, analytes are delivered to the instrument and ionised to positively charged ions, denoted [M+H]+ or negatively charged ions, denoted

[M-H]-. Using electrodes and a magnetic field, the charged analytes are directed and accelerated to the mass analyser where they are sorted based on their m/z. As with chromatography, instrument and method options, such as charge and magnetic field, can be optimised to analyse a specific target (i.e. a metabolite of interest) which is termed selective reaction monitoring (SRM), or, the instrument and method can be selected to encompass a wide range of analytes (i.e. analysis of a metabolome). Charged analytes can be fragmented to smaller analytes, or product ions, providing a highly specific molecular pattern. Ions of interest are selected at the first mass analyser and fragmented in a collision cell before the product ions are measured in the second mass analyser. This technique, termed 'tandem mass spectrometry', permits structural identification of putative biomarkers from a non-targeted biomarker approach but also confers the molecular fidelity of an analytical method when used in a targeted biomarker investigation.

Despite its uses, mass spectrometry is plagued by specific limitations. At the ion source, components of the matrix can suppress or enhance the ionisation of analytes, which can skew the abundance of an analyte in the MS spectra, and unless matrix effects are proven to be eliminated or uniform between samples or absent, the quantitative use of MS for certain analytes is limited. In addition if a clinically useful biomarker is expected to be quantified in multiple laboratories the lack of inter-laboratory reproducibility of LC-MS methods poses an issue.

1.3.1.3 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) is a phenomenon whereby atomic nuclei that have rotational momentum in an external magnetic field, such as a proton (^{1}H) or carbon-13 (^{13}C), absorb a specific electromagnetic frequency to align against the external magnetic field; the re-emission of this energy, typically a radiofrequency, causes fluctuations in the magnetic field which is measured as a resonance. In NMR spectroscopy, these fluctuations generate a current in a surrounding sample coil which is measured electronically and

converted to an NMR spectrum. The atomic nuclei are naturally present in alignment or opposition to the magnetic field and it is the nuclei naturally in alignment that are exploited by the technique (James 1998). At room temperature the number of nuclei in alignment, or 'low energy state' outnumbers the number of nuclei in opposition, also known as the 'high energy state'. This can be described by the Boltzmann equation which relates the nuclei population ratio to the energy difference at an absolute temperature:

$$\frac{N\ higher}{N\ lower} = e\frac{-\Delta E}{kT} = e\frac{-h\upsilon}{kT}$$

N = number of nuclei, E= energy difference between the two spin states, k = Boltzmann constant, T= absolute temperature (Kelvin)

Atomic nuclei that are surrounded by different structural environments will experience the external magnetic field to different extents. When the external magnetic field is applied, the electrons of the neighbouring atoms generate a secondary magnetic field which shields or deshields the nuclei from the external field, thus affecting the radiofrequency required to align the nuclei against it. NMR spectra are not reported in frequency units, due to between-instrument differences, but rather parts per million (ppm). Frequency and ppm are directly proportional such that a proton requiring a lower radio frequency will be positioned lower on the ppm scale in a spectrum and conversely protons that require a higher radio frequency to align against the magnetic field will be positioned at the higher end of the ppm scale. Peaks in NMR spectra appear as singlets, doublets, triplets and multiplets, with the number of peaks relative to the number of adjacent surrounding nuclei (James 1998).

NMR spectroscopy has emerged as a fundamental tool in metabolic profiling, circumventing the inherent issues associated with mass spectrometry such as reproducibility and ionisation suppression. Whilst the reproducibility of NMR spectroscopy is a strong attribute, the lower sensitivity of the technique is a long-standing drawback. The sensitivity of NMR

spectroscopy lies in the population size of nuclei that are aligned with the external magnetic field (and can therefore be excited by the radiofrequency), compared to the population size that are already in opposition. It is the difference between these two populations, only 128 nuclei out of 2,000,128 nuclei for ^{1}H NMR, which delivers sensitivity at the low micromolar range (James 1998; Robertson et al. 2011).

1.4 Aminoglycoside-induced nephrotoxicity

The aminoglycosides are a class of antibiotics used to treat severe Gram negative bacterial infections such as those caused by *Pseudomonas aeruginosa* and also Gram positive bacterial infections, particularly *Mycobacterium tuberculosis*. The aminoglycoside class began as a group of natural compounds; the first aminoglycoside, streptomycin, and the second, neomycin, were isolated from strains of *Streptomyces* bacteria in the 1940s and were found to inhibit bacterial growth (Jones et al. 1944; Waksman and Lechevalier 1949). Gentamicin (Weinstein et al. 1963) and tobramycin (Stark et al. 1967) were identified in the following decades alongside the semi-synthetic aminoglycosides amikacin (Price et al. 1974) and netilmicin (Kabins et al. 1976).

Structurally, the aminoglycosides consist of two or more amino sugars with a glycosidic linkage to an aminocyclitol ring, see Figure 1.2. Minor differences in substitutions on these molecules mean that an aminoglycoside can have several conformations, such as for gentamicin. Multiple hydroxyl groups on the sugar moieties make aminoglycosides hydrophilic with poor lipid solubility and their amino groups confer a positive charge at physiological pH.

1.4.1 Mechanism of antibiotic action

The polycationic nature of aminoglycosides allows for high affinity interactions with nucleic acids and it is this characteristic which affords the antibiotic mechanism of the drug class. Binding studies of the aminoglycosides with ribosomal RNA (rRNA) of *Escherichia coli* demonstrated that the compounds bind to the A site of 16s rRNA within the 30S ribosomal

subunit (Fourmy et al. 1996), which, with close proximity to the codon recognition site, interferes with physiological ribosome-RNA interactions and transfer RNA specificity during translation. As the viability of a bacterial cell is inversely related to the number of mis-translated proteins, the aminoglycosides have a bactericidal (as opposed to bacteriostatic) effect.

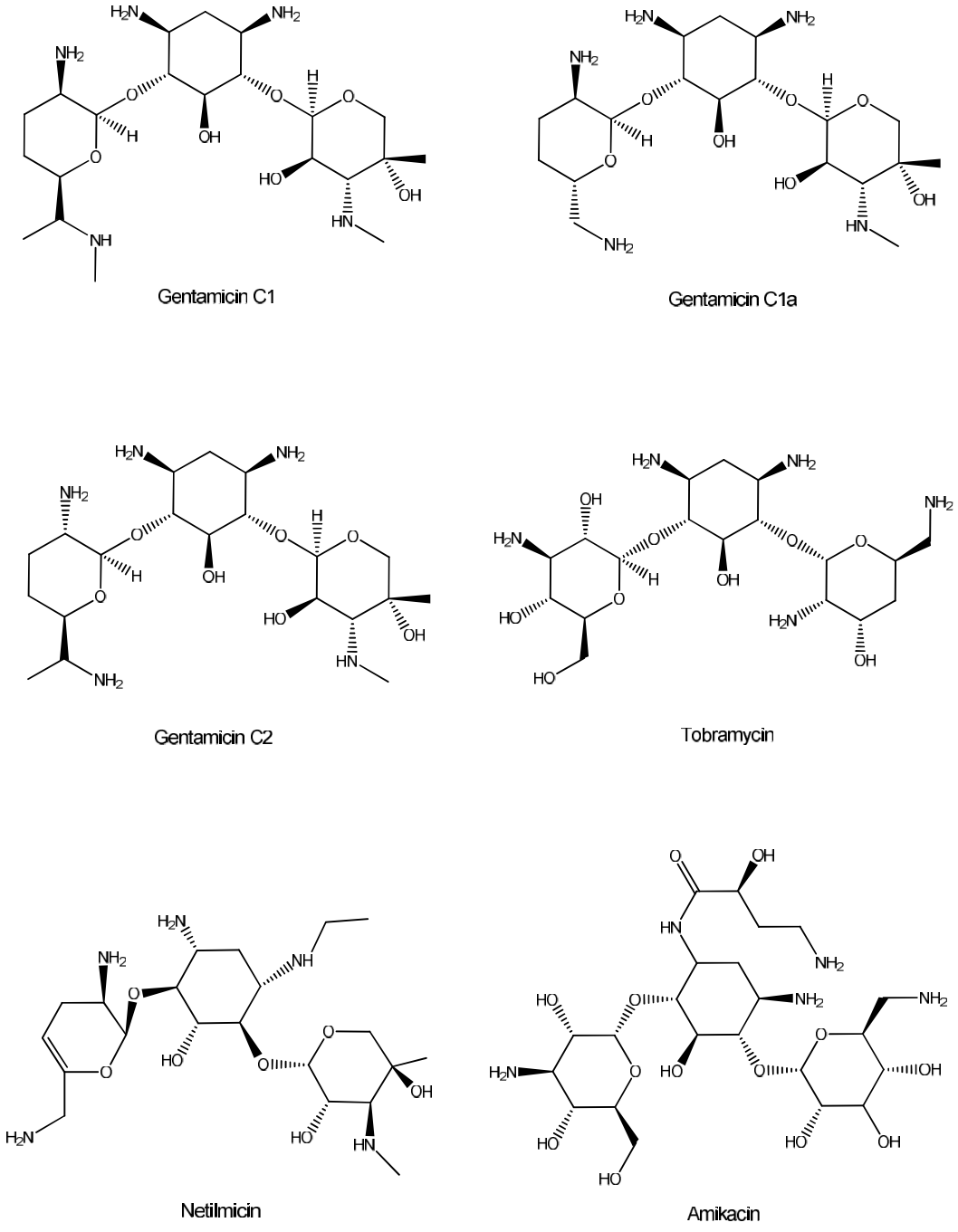


Figure 1.2 Structural similarities, a central aminocyclitol ring, and differences, modified amino sugars, of commonly studied and clinically used aminoglycoside antibiotics.

1.4.2 Pharmacokinetics

The intrinsic hydrophilic nature of the aminoglycosides results in poor oral bioavailability and they are typically administered parenterally. Once in the systemic circulation, their poor lipid solubility prevents the aminoglycosides being absorbed into most tissues and the majority of the drug passes through glomerular filtration and is renally excreted in an unchanged form. However a significant proportion, typically 5% (Mingeot-Leclercq and Tulkens 1999), accumulates within the proximal tubule epithelial cells (Vandewalle et al. 1981).

1.4.3 Proximal tubule injury

Nephrotoxicity is one of the most significant side effects and therapeutic limitations of the aminoglycoside class. The site-specific accumulation of the aminoglycosides within the epithelial cells of the proximal tubule is a key step in the onset of aminoglycoside-induced nephrotoxicity.

1.4.4 Uptake

Autoradiography experiments in isolated tubules of rabbits (Vandewalle et al. 1981) and rats (Wedeen et al. 1983) administered ^{3}H-gentamicin illustrated that increasing amounts of drug were incorporated into the S1 and S2 tubule segments but not in the S3 segment, distal tubule or collecting ducts. In addition, radiolabeled gentamicin experimentation in rats charted the movement of the drug from the apical surface of the proximal tubule cells to the interior of the cells by endocytosis (Silverblatt and Kuehn 1979). Ligand binding (Moestrup et al. 1995), mouse knock-out (Schmitz et al. 2002) and competitive ligand experiments (Nagai et al. 2001) have pin-pointed the endocytic receptor megalin as a major driver in the endocytosis and accumulation of these drugs within this tissue.

1.4.5 Intracellular trafficking

Once inside the epithelial cells gentamicin is localised initially to the lysosomal compartment as a result of endocytosis (Wedeen et al. 1983). Intracellular trafficking investigations of gentamicin utilised the conjugation of gentamicin to the fluorescent dye

Texas Red and confocal microscopy to track the rapid movement of conjugated gentamicin in LLC-PK_1 cells from the endocytic compartment to the Golgi body (Sandoval et al. 1998) and to the endoplasmic reticulum (ER) before being released into the cytosol (Sandoval and Molitoris 2004). A decrease in mitochondrial membrane potential coincided with the cytosolic release of gentamicin and as a result it is accepted that the presence of high concentrations of drug within the cytosol causes the cytotoxicity.

1.4.6 Membrane destabilisation

A critical component of the release of high concentrations of the aminoglycosides into the cell cytosol is the destabilisation of their transporting lysosomes. The polycationic nature of the aminoglycosides aids their electrostatic interaction with anionic phospholipids of intracellular membranes, disrupting membrane structure, function and phospholipid turnover (Josepovitz et al. 1985; Ramsammy et al. 1989). Aberrant accumulation of phospholipids, also known as phospholipidosis, is a chief manifestation of the effects of aminoglycosides in the rat (Giuliano et al. 1984) and in man (De Broe et al. 1984). Although disruption of membrane homeostasis may be contributory and an obligate step in releasing a concentrated amount of aminoglycoside into the cell cytoplasm, it is not necessarily the decisive trigger that leads to cell death processes.

1.4.7 Induction of apoptosis

Rupture of lysosomes not only releases high concentrations of drug into the cytosol but also releases proteolytic enzymes which have been demonstrated to cleave the BH3-interacting domain death agonist (Bid), a pro-apoptotic member of the Bcl-2 family, releasing cytochrome C and initiating apoptosis (Stoka et al. 2001). Whilst the initiation of cell death by the aminoglycosides could be indirect through the release of proteolytic cathepsins from destabilised lysosomes, much evidence indicates that the aminoglycosides exert a direct effect on the mitochondria; gentamicin inhibited oxidative phosphorylation *in vitro* and in isolated renal cortical mitochondria from rats exposed to the drug causing a

reduction in ATP levels (Simmons et al. 1980). Gentamicin has also been shown to increase the production of reactive oxygen species (ROS) in isolated rat mitochondria (Walker and Shah 1987). As such, numerous attempts to ameliorate the renal toxicity of the aminoglycosides have involved co-administration of anti-oxidants.

The localisation of aminoglycosides within the endoplasmic reticulum may provide another route to initiating apoptosis. The mechanism of aminoglycoside antibiotic action relies on its affinity for nucleic acids, particularly prokaryotic ribosomal RNA; hence an interaction with eukaryotic ribosomal RNA could provoke disruption to mammalian protein translation. A study of isolated renal microsomal fractions and cortical homogenates from gentamicin-treated rats exhibited an inhibition of protein synthesis ascertained by a reduction in ^{3}H-leucine incorporation (Bennett et al. 1988; Sundin et al. 2001). In addition, disruption to protein synthesis has been demonstrated for aminoglycoside ototoxicity, another adverse effect of the drug class, whereby binding to rRNA and cessation of protein translation results in the activation of the JNK pathway and apoptosis (Francis et al. 2013). However the affinity of aminoglycosides for prokaryotic rRNA is greater than for human rRNA as demonstrated by the aminoglycosides paromomycin and G418 (Kaul et al. 2005) with the adenosine nucleotide at position 1408 conveying prokaryotic specificity; gentamicin and tobramycin have an IC50 of 1.8 μM and 1.9 μM for the prokaryotic wild-type 16S RNA whereas mutation of position 1408 to the eukaryotic phenotype (guanosine) gives an IC50 of 230 μM and 240 μM.

As well as perturbing protein translation, the aminoglycosides have been suggested to initiate apoptosis through the unfolded protein response (UPR) and the binding of gentamicin to various chaperone proteins has been reported (Horibe et al. 2004; Horibe et al. 2002). To date, the relative contribution of these intracellular events in initiating cell death has not been resolved and it may be the case that one mechanism presides over another or it is that multiple catastrophic events culminate in the cell inducing apoptosis.

1.4.8 Aminoglycoside-induced nephrotoxicity in the clinic

The incidence of aminoglycoside-induced nephrotoxicity varies in the literature; between 5 and 26% of aminoglycoside courses result in nephrotoxicity (Bailie and Mathews 1987; Kahlmeter and Dahlager 1984; Laurent et al. 1990) despite the employment of strategies to minimize risk. This wide range is likely to be caused by differences in the criteria used to confirm renal injury, the use of different aminoglycosides and demographic factors which increase the risk such as gender and age. Signs of aminoglycoside-induced renal failure usually present after 5-7 days following the start of treatment. Patients will typically experience nonoliguria (>0.5 L urine/day) or polyuria (>2.5 L/day) producing low quality hypoosmolar urine, which is reflective of the inability of the kidneys to filter blood efficiently, with appreciable proteinuria, amino aciduria, electrolyte changes, hyaline and granular casts; gradual increases in serum creatinine are also observed (Mingeot-Leclercq and Tulkens 1999).

The development and clinical use of any therapeutic agent is a continual balancing act of risk/benefit assessment. For the aminoglycosides, their clinical importance, even 70 years after their discovery, in treating serious life threatening infections frequently outweighs the risk of patients developing nephrotoxicity. The major drivers in the first or second line use of aminoglycosides in the clinic are the low rates of resistance by prominent pathogens, their synergism with other antibiotics, their bactericidal efficacy, chemical stability and low cost (Begg and Barclay 1995). However the onset of nephrotoxicity and management of this adverse reaction is still an important limiting factor in the successful treatment of bacterial infections by the aminoglycosides.

1.4.9 Strategies to reduce the incidence of nephrotoxicity

The strategies employed to manage aminoglycoside induced nephrotoxicity include those which are still in the experimental phase, i.e. molecular intervention, and those which have been adopted into clinical practice, i.e. optimised dosing regimens.

1.4.9.1 Extended interval dosing

Traditional dosing regimens for aminoglycosides typically involve multiple intravenous infusions of the antibiotic every 8-12 h over a 24 h period. The concept of a once-daily high dose was popularised because of pharmacodynamic advantages, namely the concentration-dependent killing of bacteria, post-antibiotic effect and low adaptive resistance. Incidentally once-daily or 'extended interval' dosing had favourable toxicokinetics. A 1998 survey of US acute care hospitals found that 75% had adopted extended interval aminoglycoside dosing (Chuck et al. 2000) which was a 4-fold increase from 1993. This approach is now widely accepted in clinical practice in a wide variety of patient subpopulations.

The evidence for the use of once-daily administration of aminoglycosides to reduce the incidence of nephrotoxicity is closely related to the endocytic uptake mechanism of the drug into the proximal tubule epithelial cells. Binding and internalisation of some aminoglycosides at the apical surface is a non-linear saturable process (Giuliano et al. 1986), hence a less frequent but larger aminoglycoside dose reduces the exposure of the proximal tubule epithelial cells to the drug since more of it is excreted. Studies in animal models and humans support this hypothesis; adult male F344 rats given a single 40 mg/kg gentamicin dose had a renal gentamicin concentration of 269 ± 99 μg/g whereas in animals given the same 40 mg/kg gentamicin dose split into three subcutaneous injections the renal accumulation reached 820 ± 29 μg/g (Bennett et al. 1979). Similarly, short-term infusion of netilmicin and gentamicin administration in humans, yielded significantly lower tissue accumulation of the drugs compared to a 24 h infusion of the same dose (Verpooten et al. 1989). This result was also replicated for tobramycin and amikacin (De Broe et al. 1991). A prospective clinical trial of extended interval versus multiple daily dosing regimens of aminoglycosides in adults receiving treatment for ≥72 h found that 15.4% of patients receiving aminoglycosides twice daily experienced nephrotoxicity whilst none of the 35

patients on the extended interval regimen experienced nephrotoxicity (Rybak et al. 1999). This research suggests that the prolonged serum concentration of aminoglycosides leads to greater renal accumulation and potential for nephrotoxicity.

1.4.9.2 Therapeutic drug monitoring

Active therapeutic drug monitoring is important for both efficacy and safety when using the traditional dosing regimen. Clinical evidence indicates that frequent patient evaluation of aminoglycoside serum concentrations, as opposed to measurements at the clinician's discretion, resulted in a shorter duration of therapy, a shorter hospital stay, increased efficacy and a 10.5% lower incidence of nephrotoxicity as well as a lower cost of treatment (van Lent-Evers et al. 1999). For extended interval dosing, Nicolau et al reported a 40% decrease in requests for serum drug measurements for gentamicin and tobramycin treatment compared to traditional dosing, which, in combination with a reduction in nephrotoxicity, related to an annual saving of more than US $>100,000 for a 600-bed occupancy (Nicolau et al. 1996).

1.4.9.3 Molecular intervention

Molecular approaches to reduce and prevent aminoglycoside-induced nephrotoxicity have focussed on current mechanistic knowledge and are mainly experimental investigations in animal models of gentamicin exposure rather than clinical-based studies on the whole drug class. Nonetheless, the approaches to ameliorate experimental gentamicin nephrotoxicity cover a broad area of pharmacology including anti-oxidants, calcium-channel blockers and antibiotics. Whilst some of these experimental investigations may one day be clinically useful in the interim they offer supporting information on the underlying mechanisms by which the aminoglycosides elicit toxicity.

Fosfomycin, a broad spectrum antibiotic, has been considered as a therapeutic that can be used to reduce the nephrotoxicity observed in the aminoglycoside class. Research in rat renal mitochondria indicates that fosfomycin inhibits aberrant iron mobilisation from the

mitochondria that is caused by gentamicin-induced hydrogen peroxide formation, therefore preventing lipid peroxidation (Yanagida et al. 2004). Preventing iron release and toxicity has also been successfully investigated in gentamicin-induced ototoxicity using iron chelators such as deferoxamine (Song et al. 1997). Whilst fosfomycin appears to reduced toxicity through limiting the intracellular effects of gentamicin, other antibiotics such as fleroxacin (Beauchamp et al. 1997) and ceftriaxone (Beauchamp et al. 1994) are thought to reduce the renal cortical accumulation and toxicity of gentamicin and tobramycin, respectively.

The dihydropyridine calcium channel antagonists are another drug class which has been demonstrated to have applications in renal disease. Initially, calcium channel antagonists became of interest in this field because gentamicin was found to increase intracellular calcium load causing contraction of the mesangial cells which surround renal blood vessels (Martinez-Salgado et al. 2000). Co-administration of nifedipine, nitrendipine, and amlodipine with gentamicin in a rat model of nephrotoxicity showed that nifedipine and amlodipine had a renoprotective effect, which was actually shown to be through their anti-oxidant activity rather than their anti-hypertensive pharmacology (Li et al. 2009). However nitrendipine exacerbated the toxicity of gentamicin as did another calcium channel antagonist verapamil (Ali et al. 2002). The discrepancies between the effect on gentamicin nephrotoxicity of drugs of the same pharmacological class highlights that although calcium channel blockers have potential as prophylactic treatments, more research is required to understand the precise mechanisms by which they offer protection or worsen injury.

The prominent role of oxidative and mitochondrial related stress in the mechanism of aminoglycoside nephrotoxicity has mean that a vast number of anti-oxidant based agents have been employed to attenuate the adverse drug reaction in animal models. These include vitamins C (Stojiljkovic et al. 2012) and E (Abdel-Naim et al. 1999), plant and herbal extracts such as curcumin (Ali et al. 2005), trans-resveratrol (Morales et al. 2002) and diallyl sulfide (Pedraza-Chaverri et al. 2003) and hormones such as melatonin (Shifow et al. 2000).

The vast majority of these experimental efforts have concentrated on limiting the intracellular effects of the aminoglycosides on the proximal tubule cell. Research indicates that the uptake of the aminoglycosides is a key factor in the onset of toxicity hence some research has attempted to prevent the uptake of the drugs before they can alter cell physiology.

1.4.10 Megalin as a target to prevent aminoglycoside nephrotoxicity

Numerous investigations have pinpointed megalin as the endocytic receptor of the aminoglycosides in the proximal tubule epithelial cells. Megalin, also known as gp330 in rodents, is a 600-kDa transmembrane glycoprotein that, after cloning, was found to be a part of the low density lipoprotein (LDL) receptor gene family (Raychowdhury et al. 1989). The protein is localised to the surface of a select number of absorptive epithelia, notably the apical surface of proximal tubule epithelial cells and, relevant to the mechanism of ototoxicity, the inner ear epithelium (Kounnas et al. 1994). The expression of megalin on the apical surface of both the renal tubule and the inner ear implicated it as a likely receptor for the aminoglycosides and polymyxin B, which is another cationic antibiotic that causes nephrotoxicity (Moestrup et al. 1995). Direct evidence for megalin-mediated uptake of the aminoglycosides was achieved in a key piece of research that included competitive binding experiments of the aminoglycosides to purified rabbit megalin, equilibrium dialysis experiments with tritiated gentamicin, and combined binding and uptake studies using rat proximal tubules and a megalin-expressing rat carcinoma cell line (Moestrup et al. 1995). Since then there has been a raft of strong evidence that agrees with this research; Nagai et al demonstrated that in normal male Wistar rats, amikacin accumulated in the renal cortex but in rats administered maleate, a compound that causes shedding of megalin, amikacin accumulation was reduced; in addition administration of gentamicin led to increased urinary concentrations of megalin ligands, calcium and vitamin D binding protein (Nagai et al. 2001). Crucially, a megalin genetic knock-out mouse was developed to show that

without the receptor, accumulation of tritiated gentamicin was negligible (Schmitz et al. 2002).

1.4.10.1 Regulation of megalin-mediated endocytosis by G proteins

G proteins are hydrolases that act on guanosine triphosphate and are critical in a wide range of biological processes including the regulation of endocytosis, from ligand binding to vesicular budding and intracellular trafficking (Watson et al. 2006). They have also been shown to regulate the localisation and efficiency of megalin. A large quantity of G proteins are present on the apical surface of the proximal tubule cells, including heterotrimeric G protein alpha subunit (Gαi3), GAIP and GIPC (regulatory components of the G protein pathway) (Brunskill et al. 1991; Lou et al. 2002) and also Arf1 and Arf6 which are small GTPases that are co-localised with megalin in rat proximal tubule cells (El-Annan et al. 2004; Maranda et al. 2001). In addition Arf6 played a role in the internalisation of megalin ligands in a rat proximal tubule immortalised cell line (Wolff et al. 2008) and a study using the G protein stimulators mastoparan and aluminimum fluoride in LLC-PK1 cells showed they increased gentamicin endocytosis (Decorti et al. 1999).

The cellular localisation and activity of a G protein is regulated by post-translational modifications. G proteins are heterotrimers composed of Gα, Gβ and Gγ subunits; the Gγ subunit is post-translationally modified by lipid moieties, also known as prenylation, in the form of farnesyl or geranylgeranyl isoprenoids at a cysteine residue located at the C-terminus of the protein (Choudhury and Ahmed 2006). The properties conferred to the Gγ subunit by prenylation include higher affinity interaction and stability between the Gβγ complex and the Gα subunit, greater G protein to receptor recognition (Higgins and Casey 1996) and increased membrane association (Muntz et al. 1992; Simonds et al. 1991).

The isoprenoids, farnesyl and geranylgeranyl pyrophosphate, are metabolites derived from the mevalonate pathway, more commonly studied in the *de novo* biosynthesis of cholesterol. The rate limiting step of this pathway originates with the enzyme 3-hydroxy-3-

methyl-glutaryl-CoA reductase (HMGR) which catalyses the conversion of HMG-CoA to mevalonic acid (Mishra et al. 2003). Mevalonic acid is then further processed in a series of reactions to generate either sterols, or the non-sterols farnesyl pyrosphosphate and geranylgeranyl pyrosphosphate which are used to prenylate the Gγ subunit of G proteins.

Studies of lovastatin and compactin, inhibitors of HMGR, helped to identify the incorporation of tritiated mevalonic acid into prenylated protein (Schmidt et al. 1984) and also demonstrated that upon HMGR inhibition there was an increase in cytosolic Gβγ G protein subunits, whilst addition of mevalonate to the system restored their membrane association (Muntz et al. 1992). Therefore the mevalonate pathway and agents which modify its activity are linked to G protein prenylation and consequently the physiological processes, such as megalin-mediated endocytosis, that the G proteins regulate.

1.4.10.2 The mevalonate pathway and aminoglycoside accumulation

The mevalonate pathway and isoprenoid synthesis have been linked to endocytosis in the proximal tubule. A key study in an opossum kidney cell line used HMGR inhibitors to confirm the link between the mevalonate pathway and the megalin-mediated endocytic uptake of megalin ligands albumin and β2-microglobulin (Sidaway et al. 2004). Rosuvastatin, simvastatin, pravastatin, atorvastatin and fluvastatin inhibited the uptake of FITC-albumin in a concentration dependent manner and further investigation with simvastatin demonstrated the inhibition of FITC-β2-microglobulin uptake; the inhibition was counteracted by addition of mevalonate and geranylgeranylpyrophosphate but not cholesterol. This relationship was replicated in primary human proximal tubule cells by a separate research group who investigated simvastatin, rosuvastatin and pravastatin (Verhulst et al. 2004).

More recently, Antoine *et al* investigated the potential for HMGR inhibitors to reduce the megalin-mediated uptake and accumulation of aminoglycosides in an opossum kidney cell line (Antoine et al. 2010). Statins caused a reduction in cholesterol biosynthesis, as

determined by incorporation of tritiated acetate, and an increase in unprenylated Rap1A which is a G protein. Co-administration of simvastatin, pravastatin or rosuvastatin with gentamicin not only reduced cholesterol biosynthesis and prenylation but reduced the accumulation and cytotoxicity of gentamicin. To significantly reduce accumulation and cytotoxicity of gentamicin, cholesterol synthesis had to be reduced by 60% and 70%, respectively. Again, addition of mevalonate and geranylgeranyl pyrophosphate nullified the inhibitory effects of the statins on gentamicin accumulation and cytotoxicity.

Whilst these studies show promise that statins could be a prophylactic treatment for aminoglycoside-induced nephrotoxicity, they also highlight the role of the mevalonate pathway in the mechanism of accumulation which is vital to the development of proximal tubule injury, see Figure 1.3. The activity of this pathway and in particular the activity of HMGR and its relationship to aminoglycoside-induced nephrotoxicity is something that certainly merits further exploration.

1.4.11 Susceptibility to aminoglycoside nephrotoxicity

Historical research into susceptibility to aminoglycoside-induced nephrotoxicity is limited. In terms of clinical aminoglycoside nephrotoxicity, risk factors are largely related to differential pharmacokinetics between demographics such as age (or 'renal age'), dose and comorbidities. Hormonal and gender differences have been the major lines of investigation in animal models of gentamicin nephrotoxicity but there has been little published in the last decade. In these studies it was identified that susceptibility in the rodent models was species and strain specific.

Studies of aminoglycoside-induced ototoxicity, however, have identified a maternal inheritance pattern and a predisposing mutation in mitochondrial DNA (Fischel-Ghodsian 1999). The mutation was identified as an A to G transition at position 1555 of the 12S rRNA (Fischel-Ghodsian et al. 1997). In individuals with the mutation who have not been exposed to aminoglycosides, the mutation can lead to deafness. The mutation is considered to allow

for a novel high affinity binding site for aminoglycosides to the 12S rRNA which is not present in the wild type (Hamasaki and Rando 1997). The doubling time and translation rate of cells isolated from patients with this mutation in the presence of paromomycin was, in comparison to wild type cells, significantly increased and decreased, respectively, which suggests an inhibitory action of aminoglycoside binding to 12S RNA (Guan et al. 2000). It has been estimated that 1 in 40000 people have this mutation in the UK but due to incomplete gene penetrance and variable exposure the figure is likely to be much higher (Bitner-Glindzicz and Rahman 2007).

Other familial cases have been identified, for example in a family where 5 individuals became deaf following exposure to aminoglycosides, a mutation around nucleotide 961 of the mitochondrial DNA was identified (Casano et al. 1999). In addition, the A827G mutant was identified in the mitochondrial 12S rRNA in related and non-related patients who developed hearing loss after exposure to aminoglycosides (Xing et al. 2006). Identification of predisposing mutations can be used in clinical decision making through genetic screening or assessment of family history and importantly, to inform maternal relatives of patients with the mutant to avoid exposure to aminoglycosides.

Whilst there has been progress in identifying genetic susceptibility factors for aminoglycoside ototoxicity, the incidence of aminoglycoside nephrotoxicity could be improved by identifying patient characteristics which predispose an individual to the adverse drug reaction. The establishment of the role of megalin-mediated endocytosis in the mechanism of nephrotoxicity has widened the possibilities of identifying the inter-individual variation which predisposes a patient to nephrotoxicity. Variation in the activity of the mevalonate pathway could leave some individuals more or less susceptible to aminoglycoside nephrotoxicity and warrants thorough investigation.

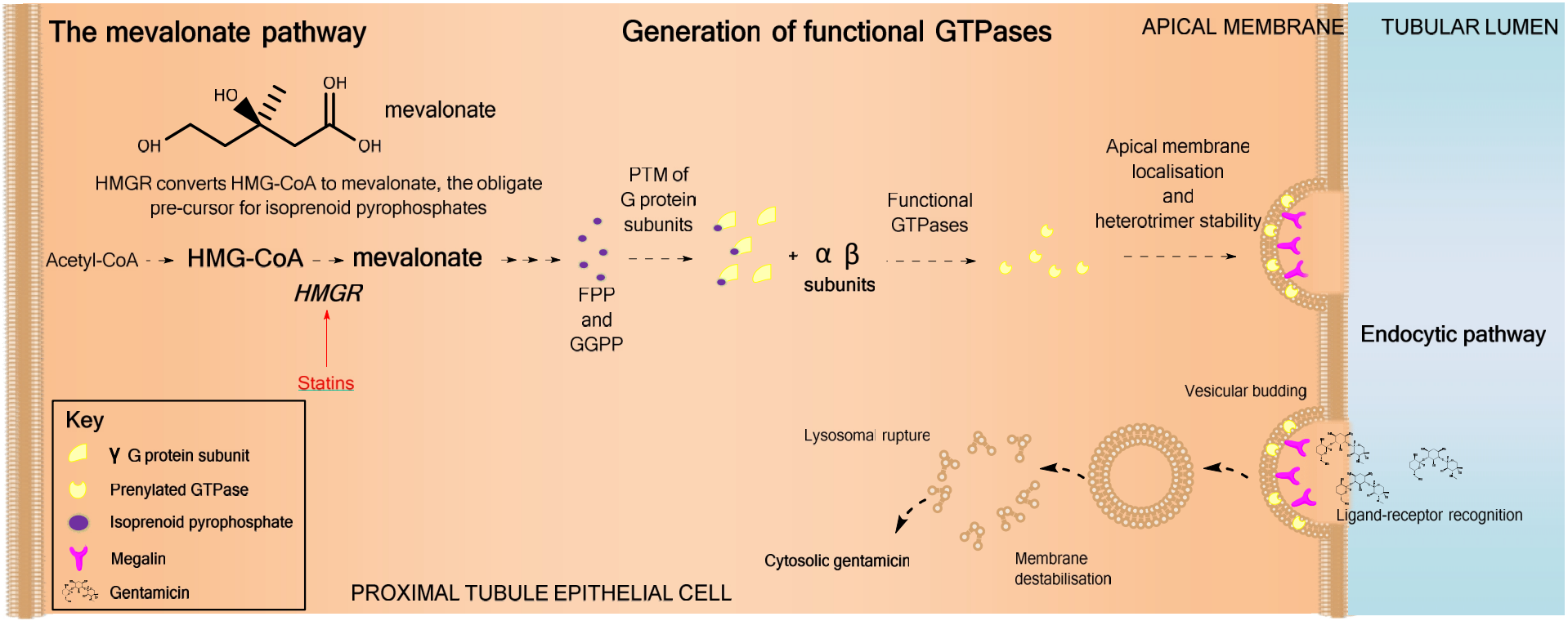


Figure 1.3 Renal uptake of the aminoglycosides is dependent on the presence of functional GTPases. G protein subunits are post-translationally modified by isoprenoids pyrophosphates of the mevalonate pathway.

1.5 Aims & Objectives

The aim of this research was to advance mechanistic knowledge of aminoglycoside nephrotoxicity and identify factors which affect the susceptibility of individuals to this adverse event. To achieve these aims a rat model of aminoglycoside nephrotoxicity, targeted and non-targeted biomarker identification approaches were employed with the following objectives:

- Targeted biomarker identification
  - To develop and validate an assay to measure the activity of the mevalonate pathway.
  - To determine the role of HMG-CoA reductase in aminoglycoside nephrotoxicity.
- Non-targeted biomarker identification
  - To determine perturbations in the metabolome upon exposure to the model aminoglycoside gentamicin.
  - To identify pre-disposing factors in aminoglycoside nephrotoxicity using pharmacometabonomics.

2. DEVELOPMENT AND VALIDATION OF A QUANTIFICATION METHOD FOR HMG-CO A REDUCTASE ACTIVITY

Contents

2.1 Introduction

The successful treatment of bacterial infections with aminoglycoside antibiotics is hindered by the nephrotoxic potential of the drug class. The main management strategy for aminoglycoside nephrotoxicity is the choice of dosing regimen, therapeutic drug monitoring and measurement of serum creatinine as an estimate of kidney function. In the early 1990s once-daily dosing of aminoglycosides became the recommended treatment regimen as it maintained efficacy and it was suggested that the incidence of toxicity was reduced (Barclay et al. 1994).

Despite the refinement of the dosing strategy, a significant proportion of individuals still experience nephrotoxicity when exposed to aminoglycosides, which suggests that patient characteristics may play a role in susceptibility. Patient factors such as pre-existing medical conditions (such as liver or renal disease) and concomitant medications can inform clinical decisions but there is no other method of stratifying individuals based on their risk of developing nephrotoxicity before the aminoglycoside regime begins. However, susceptibility factors have been identified for aminoglycoside-induced ototoxicity. Research into the mechanism of hearing loss led to identification of a susceptibility factor, namely a genetic predisposition caused by the A1555G mutation in the mitochondrial 12S ribosomal RNA gene (Fischel-Ghodsian et al 1997, Casano et al 1999). In order to improve antibiotic efficacy and safety, it is equally important that susceptibility biomarkers are identified for aminoglycoside-induced nephrotoxicity as has been achieved for ototoxicity.

2.1.1 Approaches to identifying biomarker candidates

Two molecular approaches can be taken to identify a susceptibility biomarker for aminoglycoside-induced nephrotoxicity. Firstly, an ‘omics approach uses comprehensive analytical techniques to identify potential biomarkers from a large pool of candidates, such as in a genome-wide association study, proteomic analysis or metabolomics. Secondly, a targeted examination of the mechanism of toxicity can be undertaken to select a potential

biomarker, which, although minimising the number of potentials, increases the likelihood that the candidates are directly linked to the disease. In this and the following chapter, a targeted approach is undertaken, whilst in Chapter 4 and Chapter 5 an untargeted approach is employed.

2.1.2 Targeted biomarker identification

2.1.2.1 HMG-CoA reductase and the mevalonate pathway

Recently, HMG-CoA reductase inhibitors, or 'statins' have been shown to inhibit the accumulation of gentamicin into opossum kidney cells and reduce cytotoxicity (Antoine et al. 2010). The close relationship of HMG-CoA reductase, megalin-mediated endocytosis and aminoglycoside accumulation and cytotoxicity suggest a contributory link between HMG-CoA reductase activity and susceptibility to aminoglycoside nephrotoxicity. HMG-CoA reductase (HMGR) is the rate limiting enzyme of the biosynthetic mevalonate pathway. This biosynthetic pathway feeds isoprenoid pyrophosphates into non-sterol and sterol metabolic fates (Goldstein and Brown 1990). The metabolites of the mevalonate pathway are therefore potential biomarkers of susceptibility to aminoglycoside nephrotoxicity.

In order to test the hypothetical link between HMGR and aminoglycoside nephrotoxicity it was essential to select a biomarker candidate and to develop an accurate, precise and robust quantification method.

2.1.3 Candidate biomarkers in the mevalonate pathway

The metabolites of the mevalonate pathway are illustrated in Figure 2.1. Acetyl-CoA is condensed with acetoacetyl-CoA to HMG-CoA by HMG-CoA synthase. The rate limiting step of the pathway is catalysed by HMGR, where HMG-CoA is reduced to mevalonic acid. Mevalonic acid is phosphorylated by mevalonate kinase to 5-phosphomevalonate, which is further phosphorylated to 5-pyrophosphomevalonate by phosphomevalonate kinase. Mevalonate 5-pyrophosphate decarboxylase converts 5-pyrophosphatemevalonate to isopentenyl pyrophosphate, which can be isomerised to dimethylallyl pyrophosphate, or,

converted to geranyl-pyrophosphate and then farnesyl-pyrophosphate by geranyl-pyrophosphate synthase and farnesyl-phosphate synthase, respectively (Goldstein and Brown 1990).

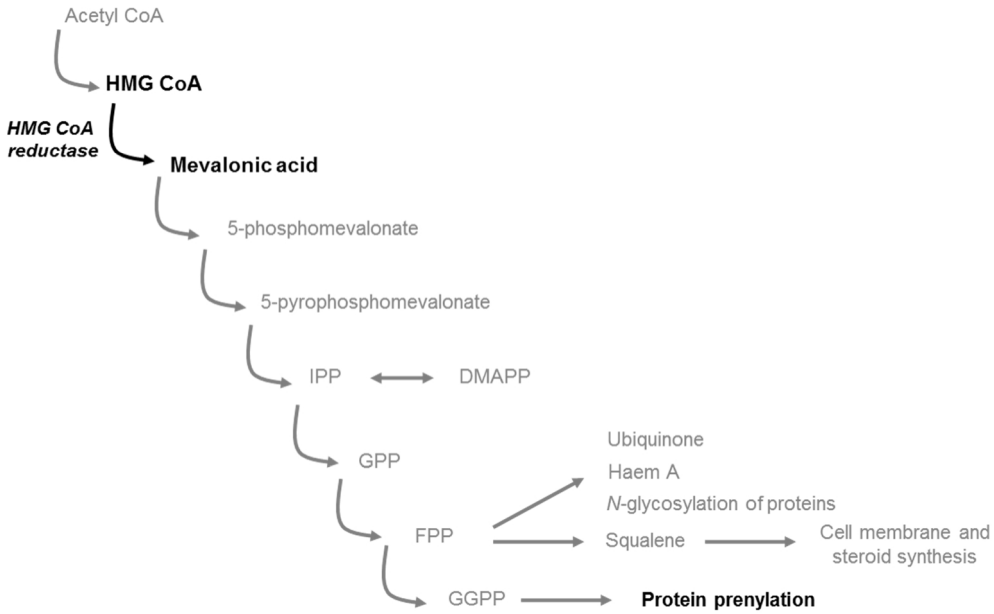


Figure 2.1 Metabolites of the mevalonate pathway. The rate limiting step is the conversion of HMG CoA to mevalonic acid. End-metabolites of the mevalonate pathway, the isoprenoid pyrophosphates are used for sterol and non-sterol products. IPP - isopentenylpyrophosphate, DMAPP - dimethylallyl pyrophosphate, GPP - geranyl pyrophosphate, FPP - farnesyl pyrophosphate, GGPP - geranylgeranyl pyrophosphate.

2.1.3.1 Mevalonic acid

Following consideration of the major metabolites of the mevalonate pathway, mevalonic acid was selected as a potential biomarker candidate for its physiological and practical characteristics. The other intermediates of the mevalonate pathway were excluded from consideration due to the impracticalities of quantification and exogenous influences.

2.1.3.1.1 Physiological properties of mevalonic acid

Mevalonic acid (3, 5-dihydroxy-3-methylpentanoic acid, MVA) is a metabolic intermediate that is present in human and rat plasma, and excreted into urine (Kopito and Brunengraber 1980; Kopito et al. 1982). It is produced from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) by HMGR (Friesen and Rodwell 2004). MVA is synthesized predominantly in the liver (Hellstrom et al. 1973) and most of the metabolite in plasma is taken up by the

kidneys, where it is incorporated into squalene and sterols, catabolized or excreted in urine (Hellstrom et al. 1973; McNamara et al. 1985; Parker et al. 1982). Urinary output accounts for 29% of the total renal flux of MVA (Kopito and Brunengraber 1980).

Although MVA has no known physiological function other than as a metabolic intermediate, MVA production is the rate-limiting obligatory step in the mevalonate pathway. As the only precursor of isoprenyl intermediates that is committed to the pathway and present in accessible biofluids, MVA is effectively the only metabolite that is sensory of HMGR activity and mevalonate pathway flux.

2.1.3.1.1.1 Regulation and diurnal variation

The mevalonate pathway is tightly regulated by negative feedback inhibition by downstream intermediates (the isoprenoids pyrophosphates) and sterol-mediated inhibition of enzyme expression. The HMGR gene promoter region contains sterol regulatory element (SRE-1) which suppresses transcription of the gene in the presence of sterols (Goldstein and Brown 1990). Conversely the sterol regulatory-element binding protein (SREBP) can translocate to the nucleus to initiate HMGR transcription (Goldstein et al. 2006). At a post-translational level, the rate of translation of HMGR mRNA is governed by non-sterol isoprenoids and the degradation of HMGR protein is accelerated by the presence of sterols and non-sterol isoprenoids (Goldstein et al. 2006). HMGR activity is also regulated at serine residue (S872) near the active site whereby phosphorylation by AMP-activated protein kinase (AMPK) reversibly reduces enzyme efficiency (Omkumar et al. 1994).

The plasma concentration and urinary excretion of MVA display parallel diurnal variations that track oscillations of cholesterol synthesis (Parker et al. 1982; Parker et al. 1984; Popjak et al. 1979), in man (Parker et al. 1984) and in preclinical models (Clarke et al. 1984; Higgins et al. 1971). Diurnal variations in HMGR activity have been attributed to food intake, with early investigations noting that cholesterol synthesis in mice was inversely proportionate to

dietary intake (Schoenheimer 1933). Subsequent studies demonstrate the peak of HMGR activity occurred 3–6 h post feeding (Carlson et al. 1978; Gregory et al. 1972; Kopito et al. 1982). Likewise, Kopito *et al.* observed a fourfold, feeding-induced rise in serum MVA concentration (Kopito et al. 1982).

2.1.3.1.2 Chemical properties of mevalonic acid

MVA is a low molecular weight ($C_6H_{12}O_4$; MW 148.16) dihydroxy-monocarboxylic acid. It is a polar molecule and at low pH undergoes spontaneous cyclisation to its lactone form (mevalonolactone, MVL, $C_6H_{10}O_3$, MW 130.14), see Figure 2.2

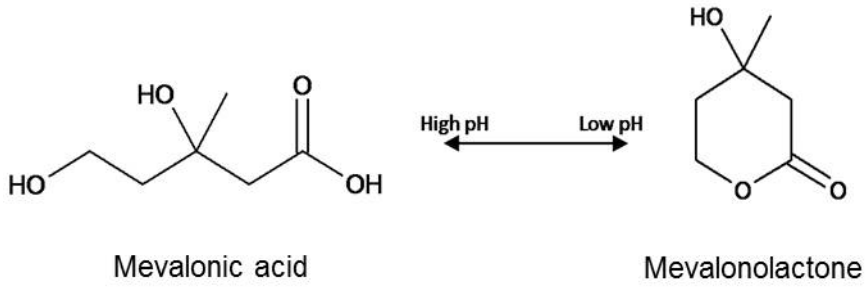


Figure 2.2 The chemical structure of mevalonic acid and mevalonolactone. Mevalonic acid spontaneously converts to mevalonolactone form at low pH.

2.1.4 Fit-for-purpose analytical method validation for biomarkers

Biomarker assays are distinct from pharmacokinetic bioanalysis and therefore long-established paradigms of drug bioanalysis and bioanalytical method validation do not wholly apply to the quantification of endogenous molecules such as MVA. Assays for novel biomarkers frequently pose unique analytical challenges (i.e. the lack of a true blank matrix) and adherence to validation parameters is often impractical. As a result, biomarker method development and validation guidelines have been established (Chau et al. 2008; Lee et al. 2006), which include the routine assessment of dynamic range, analytical precision and accuracy, relative accuracy, but also incorporate the validation of calibration standards made in surrogate matrices, see Figure 2.3. Traditional regulatory bioanalysis guidelines are considered as a framework for biomarker assay validation and the process accommodates

the characteristics of the analyte, the limitations of the analytical platform and the intended use of the assay with a 'fit-for-purpose' approach.

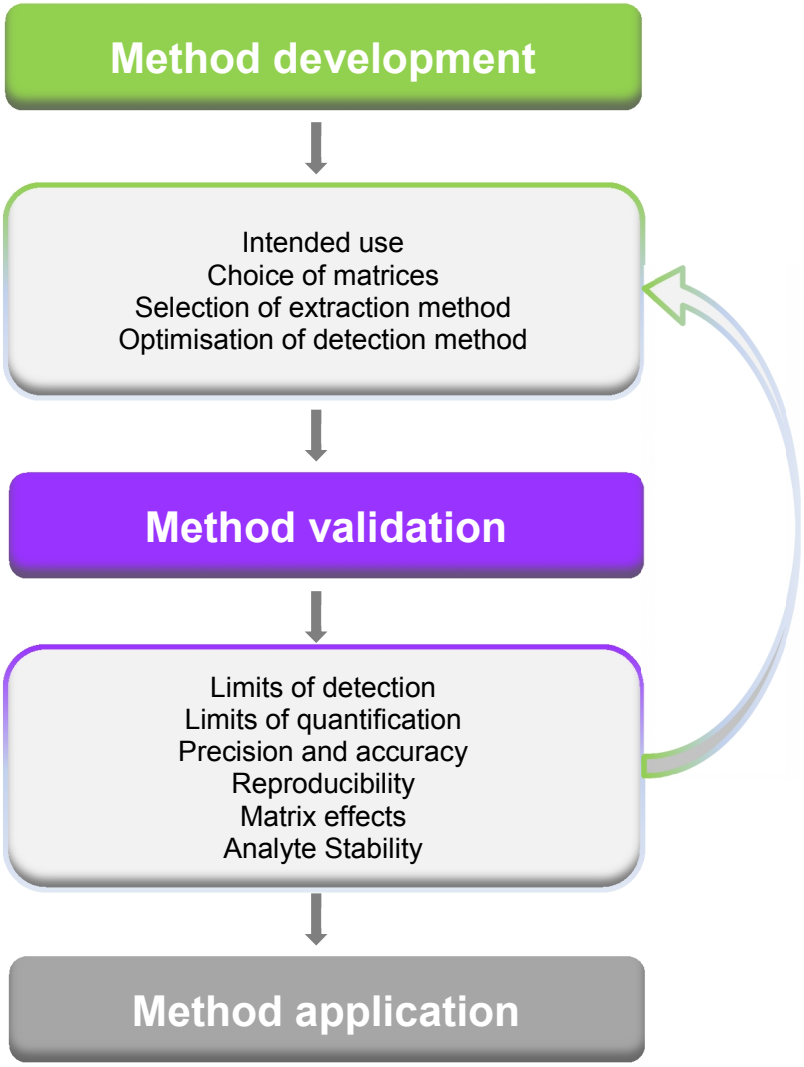


Figure 2.3 Schematic of the iterative fit-for-purpose biomarker method development and validation process.

2.2 Aims

- Develop an LC-MS/MS based quantification method for urinary mevalonic acid that can be used for rat and human urine.
- Validate the method according to fit-for-purpose biomarker guidelines.

2.3 Materials and Methods

2.3.1 Materials

(*R*)-MVA and mevalonolactone (MVL) were obtained from Sigma-Aldrich (St Louis, MO, USA). 4, 4, 5, 5-Tetradeuteromevalonolactone (d_4-MVL) was obtained from CDN isotopes (Pointe-Clare, Quebec, Canada). Methanol, ethyl acetate and formic acid were of analytical grade and were obtained from Fisher Scientific (Loughborough, UK). Human urine for method development was obtained by spot collection from five healthy human volunteers (two male, three female, mean age 23.2±1.3 yrs) not subject to dietary or beverage constraints and from adult male Sprague-Dawley rats (150-250g, 12 donors) (Alderley Park, AstraZeneca). Protocols were undertaken in accordance with a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee.

2.3.2 Method development

2.3.2.1 Optimisation of the LC-MS/MS method

LC-MS/MS was chosen as the analytical platform as it would provide the sensitivity, specificity and rapid analysis that the method requires. MS/MS conditions were optimised using automatic and manual tuning methods (Analyst software) by direct infusion (10 µL/minute) of a 1 µg/mL solution of MVL dissolved in methanol (positive ion mode) and a 1 µg/mL solution of MVA dissolved in methanol (negative ion mode) into an API 4000 Qtrap instrument.

The following columns were screened for suitability to retain either MVL or MVA detected by either positive or negative ion mode, respectively: Luna 3µ HILIC (50 x 2.00 mm), Gemini 5µ C18 (50 x 2.00 mm), Aqua 3µ C18 (100 x 2.00 mm), Synergi Hydro-RP 2.5µ (100 x 2.00 mm) (all Phenomenex). Chromatographic separation was achieved on an Acquity UPLC® (Waters, Millford, MA, USA) and a range of solvents were tested in isocratic or gradient conditions: 100 mM ammonium formate and acetonitrile, 10 mM ammonium formate 0.1%

formic acid and methanol, 20 mM ammonium formate pH 8, 0.5 mM formic acid in water/methanol 70:30, 0.1% formic acid in water and 0.1% formic acid in methanol.

2.3.2.2 Optimisation of the pH dependent conversion of MVA to MVL

The lactone of mevalonic acid, mevalonolactone, was selected as the analyte for the method as it is less polar than the acid and therefore more amenable to simple extraction procedures and reversed-phase chromatography.

Under acidic conditions MVA is known to convert to a less polar lactone, mevalonolactone (MVL). The optimal pH was selected by incubation of MVA standards (0.01, 0.1 and 0.5 µg/mL in water-methanol 9:1 v/v) adjusted to pH 2 to pH 6 with formic acid. Following incubation at ambient temperature for 90 min 10 µL aliquots of each standard were injected on to the LC-MS/MS method. The peak area of MVA and MVL was calculated (Analyst software) and the optimal pH (pH 2) for the production of MVL was selected for further development.

The rate of conversion of MVA (0.1 µg/mL in water-methanol 9:1 v/v) to MVL was determined by LC-MS/MS analysis of 10 µL aliquots of each standard incubated at pH 2 and ambient temperature, 37°C or 60°C for 180 min. The temperature at which the conversion to MVL equilibrated in the shortest time was selected for the method. MVL yield was also determined by overnight incubation at ambient temperature and pH 2. The optimal temperature (60°C) and incubation period (minimum of 15 min) for the extent of the reaction were chosen. Aliquots of MVL (0.1 µg/mL in water-methanol 9:1 v/v at pH 2) were incubated at 60°C for 120 min to establish analyte stability.

2.3.2.3 Selection of extraction method

Extraction of MVA and MVL from urine was investigated using solid-phase and liquid-liquid extraction methods. The following SPE sorbents were investigated for retention of MVA and MVL: Oasis MAX, Oasis HLB (Waters) Strata-XCW, Strata-XC (Phenomenex), Hypersep

VERIFY XC (ThermoScientific), EVOLUTE-CX (Biotage). SPE cartridges were used in accordance to the manufacturer's instructions.

A LLE procedure using ethyl acetate was also tested for recovery of MVL. 800 µL of ethyl acetate was added to 200 µL of 0.1 µg/mL MVL (prepared in pH 2 buffer) and shaken vigorously for 10 minutes. The ethyl acetate layer was recovered (600 µL), dried down under oxygen-free-nitrogen, and reconstituted in water-methanol 9:1 v/v (0.1% formic acid) before analysis by LC-MS/MS. To correct for between sample variation in extraction efficiency, an internal standard d_4-MVL was spiked into the MVL aliquot at a concentration of 0.15 µg/mL, prior to the addition of ethyl acetate.

2.3.3 Method validation

2.3.3.1 Determining % conversion of MVA to MVL under selected conditions

The percentage of MVA converted to MVL was calculated from the concentration of MVL in MVA standards (4 and 6 µg/mL in distilled water) and in rat (n = 3) and human (n = 3) donors, that had been acidified (pH 2) and incubated at 60°C for a minimum of 15 min. Intra-day (n = 3 analyses per standard) and inter-day (n = 18, representing three analyses per standard, two standards and analyses on 3 days) variation of the conversion was also determined. The endogenous presence of MVL in human and rat urine was determined by analysis of 10 µL aliquots of unprocessed urine (from three rat and three human donors) using LC–MS/MS.

2.3.3.2 Determining the % extraction of MVL and IS by ethyl acetate

Recovery of MVL from urine (50 µL) was achieved by LLE with ethyl acetate (1:4, v/v). The recoveries of MVL (0.4–6.0 µg/mL) and the IS, d_4-MVL (0.15 µg/mL), were determined by spiking MVL and IS (dissolved in acidified methanol 3% formic acid) into buffer (0.1 M potassium phosphate, adjusted to pH 2 with orthophosphoric acid) or into acidified rat (n = 3) or human (n = 3) urine. Samples were shaken vigorously for 10 min at ambient temperature and centrifuged at 8700 *g* for 10 min. Approximately 600 µL of the organic

layer was taken and evaporated under oxygen-free nitrogen. The samples were reconstituted in 200 µL of water-methanol (9:1, v/v) containing 0.1% formic acid (pH 2.4) before injection of 10 µL of solution on to the HPLC column. The extracted compounds were assayed by comparison with standards (water-methanol 9:1, v/v, 0.1% formic acid) of the same concentration. For the urine extraction recovery, the endogenous concentration of MVL was factored into the calculation.

2.3.3.3 Assay performance

To determine the sensitivity, range, precision and accuracy of the LC–MS/MS method, MVL standards (0.078–10 µg/mL in pH 2 buffer; n = 3) were spiked with IS (final concentration, 0.15 µg/mL), extracted with ethyl acetate, reconstituted in water-methanol-formic acid (9:1:0.1, v/v) and quantified by LC–MS/MS. Independently prepared QC MVL standards and a validation sample (VS, a small population sample, in this case pooled human or rat urine, that is representative of the individual analytical samples) were prepared at the beginning of the validation, and analysed alongside analytical samples and calibration standards to confirm batch acceptability and between-batch reproducibility of precision, accuracy and stability. Pooled human urine was prepared by mixing equal volumes of urine from five donors.

The LOD was designated to be the concentration of extracted MVL with a peak height greater than three-times the background signal. The LLOQ and ULOQ were, respectively, the lowest and highest concentrations of extracted MVL with acceptable accuracy and precision. The acceptance criterion of precision was 15% coefficient of variation, or 20% for the low QC and LLOQ, while the acceptance criterion of accuracy was 85–115% or 80–120% for the low QC and LLOQ, which was determined by extraction of each of the nine calibration standards (three replicates) or three QC (six replicates) in three independent batches. A batch was deemed acceptable if more than four of the six QC replicates were within 85–115% of the set value, in accordance with biomarker assay validation guidelines (Lee et al.

2006). LC–MS/MS carryover was determined by repeat injection of 10 µL of a 10 µg/mL MVL standard followed by injection and analysis of 10 µL of a blank reconstitution solution.

2.3.3.4 Validation of the surrogate matrix

Rat and human urine (50 µL aliquots from five donors) was acidified, extracted and reconstituted, and spiked with MVL and IS (final concentration 5 µg/mL) and serially diluted (water-methanol 9:1 v/v, 0.1% formic acid). The effect of dilution on the analyte/IS ratio was measured by the LC–MS/MS method. An ionization suppression/enhancement experiment was conducted using the post-column infusion method. Due to the endogenous presence of the analyte in acidified urine, a solution of d_4-MVL (0.1 µg/mL) in water-methanol containing formic acid (9:1 v/v, 0.1%) was infused directly into the MS source at a constant rate of 10 µL/min (HPLC eluate entered the source at 280 µL/min) and 10 µL of a reconstituted ethyl acetate extract of an acidified human or rat urine sample (10 µL) was injected on to the HPLC column. The continuous MS/MS response to the d_4-MVL (*m/z* 135→75) was monitored.

Human or rat urine was spiked with a high concentration of MVA (in order to minimize the contributory effect of endogenous MVA) and the percentage recovery was used to evaluate relative accuracy. MVA in spiked (0.1, 0.5 or 4 µg/mL) and non-spiked human and rat urine (multiple donors) was converted to MVL, extracted, reconstituted and quantified by the LC–MS/MS method. The concentration of MVA in non-spiked urine was subtracted from the concentration determined in the aliquot of spiked urine and the percentage recovery was calculated.

2.3.3.5 Stability

Stability of MVL in the QC standards was determined by storage at room temperature, 4°C and at -20°C for 14 days. Freeze–thaw cycles of QC standards at -20°C and room temperature were also carried out. The stability of MVL in reconstituted extracts of urine samples at 4°C in the autosampler was also determined.

2.3.4 Validated method

Calibration standards were prepared on the day of analysis from 0.0156 to 10 µg/mL at pH 2 with IS (0.15 µg/mL). Aqueous QC standards (0.045, 2 and 6 µg/mL at pH 2) and a pooled rat urine or human urine VS were prepared at the start of the validation process and stored at -20°C. Urine or MVL calibration standards (50 µL) were incubated with 148 µL of buffer (0.1 M potassium phosphate, pH 2) at 60°C for 25 min. After cooling to ambient temperature, samples and standards were spiked with 2 µL of d_4-MVL solution (15 µg/mL acidified aqueous stock, final concentration 0.15 µg/mL). The analytes were extracted with 800 µL of ethyl acetate: the samples were shaken vigorously for a minimum of 10 min at ambient temperature before centrifuging at 8700 *g* for 10 min. Approximately 600 µL of the organic layer were removed, evaporated to dry residue under oxygen-free nitrogen and reconstituted in 200 µL of water-methanol (9:1, v/v) containing 0.1% formic acid before injection of 10 µL of solution on to the HPLC column.

Chromatographic separation was achieved on an Acquity UPLC® (Waters, Millford, MA, USA) (laboratory 1) with a Synergi™ 2.5-µm Hydro RP column (100 × 2.0 mm; Phenomenex, Macclesfield, UK). The LC method employed solvent A (water-0.1% formic acid) and solvent B (methanol-0.1% formic acid), see Table 2.2. The LC–MS/MS analysis time was 5 min per sample. A MRM method was optimized on an AB Sciex 4000 QTRAP® instrument (Foster City, CA, USA) in positive-ion mode using automatic and manual tuning with aqueous standards of MVL. The precursor ion for MVL was *m/z* 131, and the transition *m/z* 131→69.4 was used for the quantification of MVL with reporter transitions at *m/z* 131→113 and *m/z* 131→71. The reporter transitions and retention time of MVL conferred specificity. The IS d_4-MVL was monitored at *m/z* 135→75. The following conditions were used for LC–MS/MS acquisition: curtain gas setting, 10.0; collision gas, 4.0; IonSpray volt-

age, 5500 V; temperature, 450°C; ion source gas 1, 90; ion source gas 2, 20; interface heater, on; dwell time, 150 ms; declustering potential, 50 V; Q1 and Q3 resolution, high. Individual transition parameters are given in Table 2.1

2.4 Results

2.4.1 Method development

2.4.1.1 LC-MS/MS method

Mevalonolactone (MVL) and d_4-MVL MS settings were automatically tuned then adjusted manually using Analyst Software. Declustering potential, curtain gas, collision gas, collision energy and collision exit potential were optimised to maximise the MS response, see Table 2.1. The precursor ion chosen was m/z 130.956 and the product ions selected were m/z 69.4, m/z 71.0 and m/z 113.1. Figure 2.4 depicts a typical ion chromatogram from the API Qtrap 4000 of the product ions of MVL from which the MS/MS method transitions were selected.

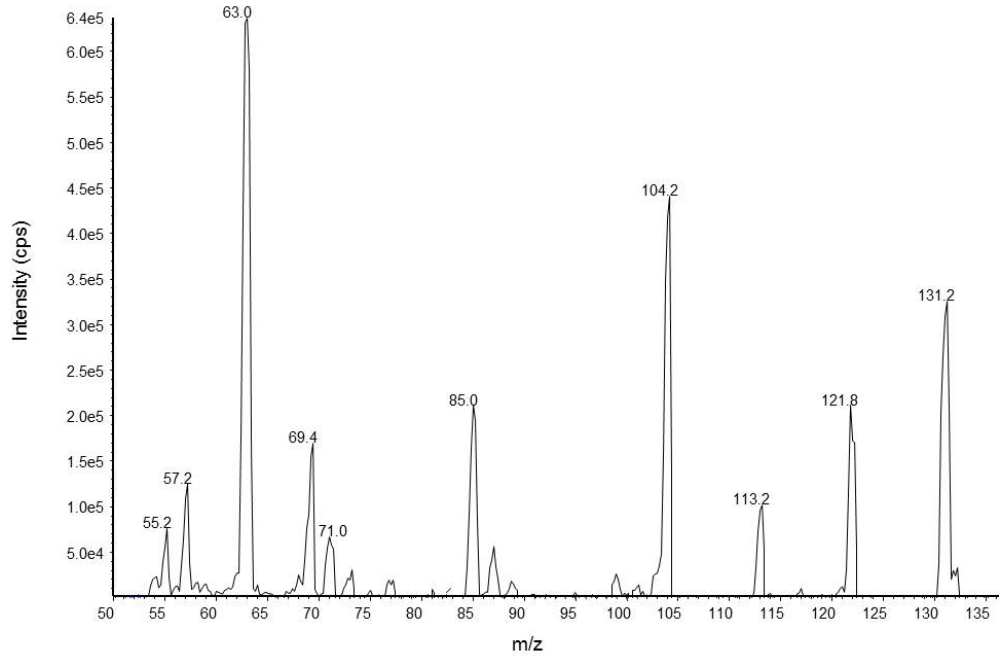


Figure 2.4 Ion chromatogram of product ions of MVL, m/z 131, on the API Qtrap 4000 instrument.

Table 2.1 MS/MS parameters for each transition in the MRM method

	Q1 mass	Q3 mass	collision energy	collision exit potential
MVL	130.956	113.1	13	8
	130.956	71.0	20	12
	130.956	69.4	15	10
d_4-MVL	134.945	75.0	20	12

Following the optimisation of the MS/MS method, several HPLC columns were tested for retention of MVL. The Luna HILIC and Gemini C18 columns did not retain MVL (or MVA) and the analyte eluted with the solvent front. The Aqua C18 and Synergi Hydro-RP columns retained MVL, with the Synergi Hydro-RP column providing the best peak shape and retention. HPLC conditions were optimised with the Synergi Hydro-RP column, see Table 2.2. An example chromatogram of MVL eluting can be seen in Figure 2.5.

Table 2.2 HPLC conditions for the analysis of extracted urine samples, standards and QCs.

Time	%A	%B	Flow rate (mL/min)
Initial	95	5	0.300
2.00	95	5	0.300
2.01	90	10	0.300
2.70	90	10	0.300
2.80	95	5	0.300
5.00	95	5	0.300
Phenomenex Synergi Hydro-RP column (2.5µ 100A 100 x 2.00 mm) linked to an Acquity UPLC and the API4000 Qtrap.			

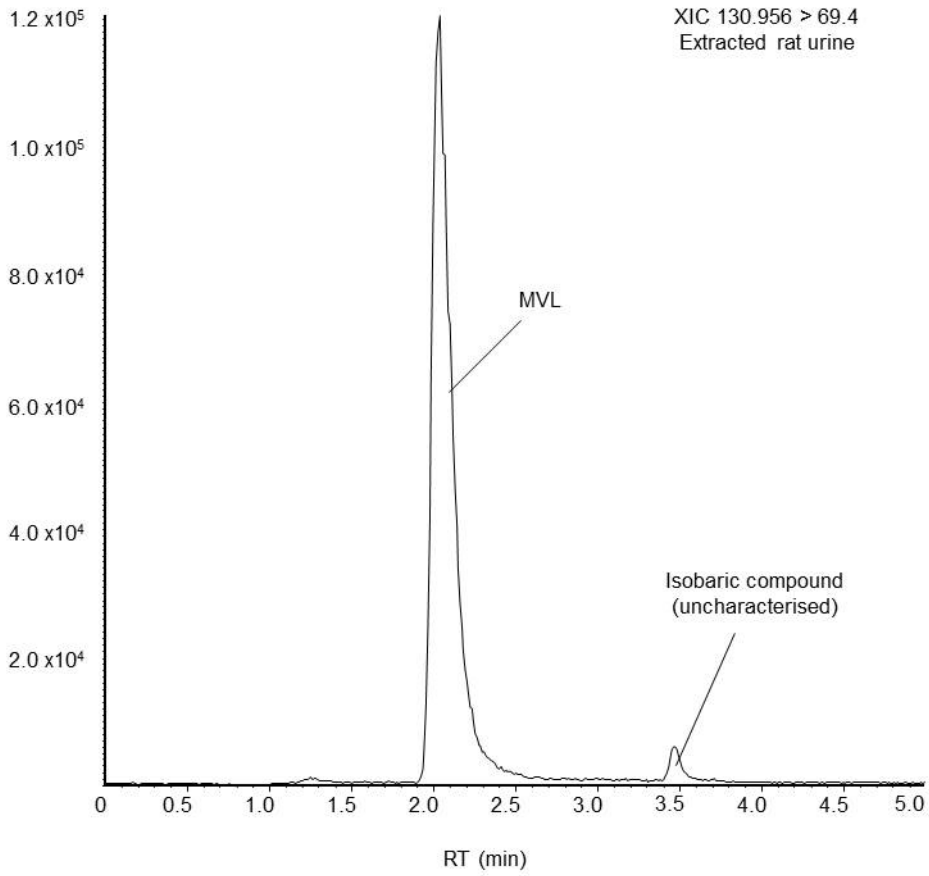


Figure 2.5 Extracted ion chromatogram of mevalonolactone (MVL) extracted from rat urine and analysed by the optimised LC-MS/MS method. An isobaric compound elutes after MVL.

2.4.1.2 Optimisation of the conversion of mevalonic acid to mevalonolactone

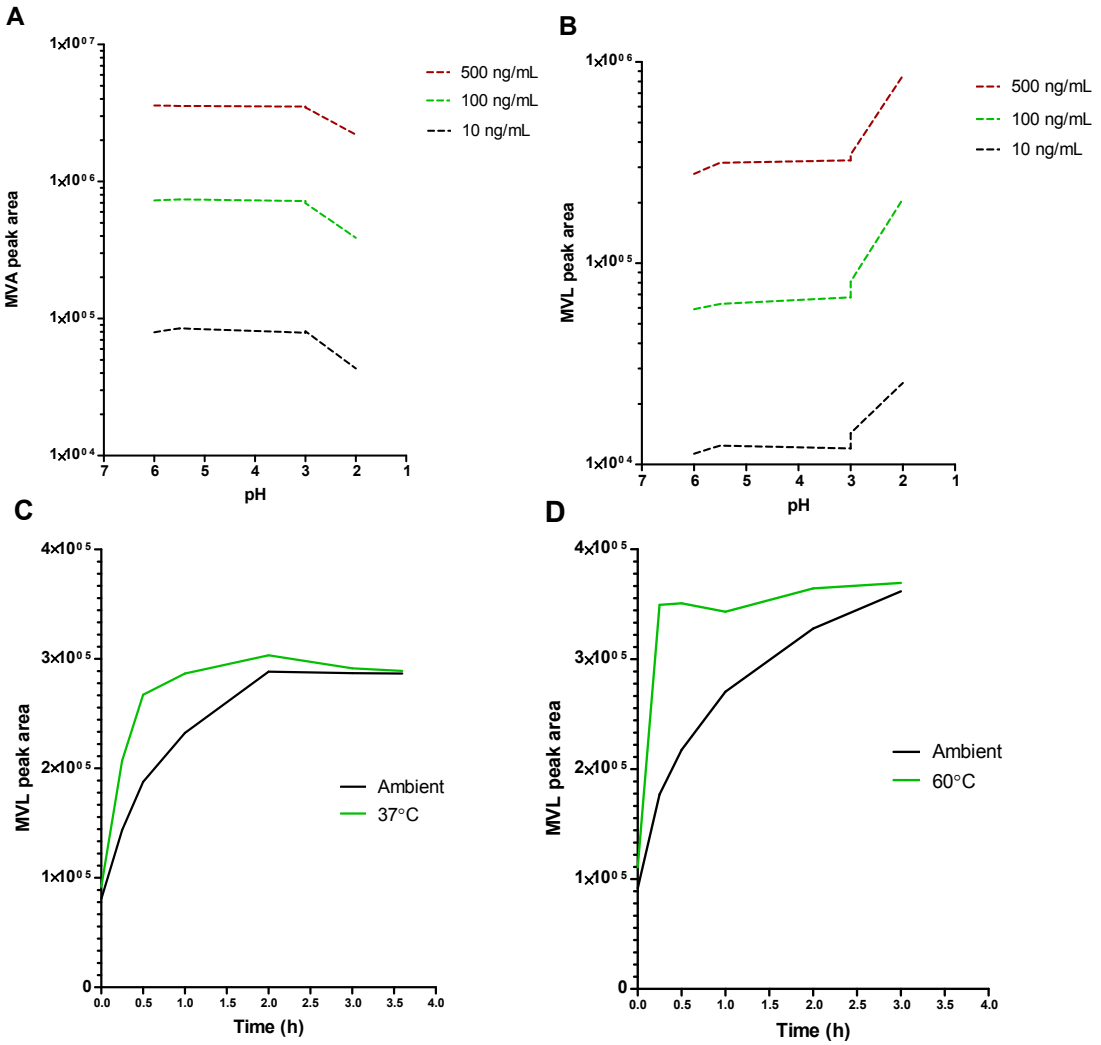


Figure 2.6 pH and temperature-dependent conversion of mevalonic acid to mevalonolactone. Aqueous standards of mevalonic acid were incubated at pH 2-6 and the amount of (A) mevalonic acid and (B) mevalonolactone was measured by LC-MS/MS peak area. Aqueous standard of mevalonic acid was incubated at pH 2 and the increase in mevalonolactone was measured over 180 minutes at (A) ambient and 37°C and (B) ambient and 60°C.

Mevalonic acid can be converted to its lactone form, mevalonolactone, in a pH-dependent manner. The acid-catalysed formation of MVL from MVA was optimised by incubating aqueous standards at a range of pH values. Below pH 3 the amounts of MVA and MVL decreased and increased, respectively, see Figure 2.6A and 2.6B. Based on these results, pH 2 was selected as the optimal condition to promote the production of MVL from MVA.

The production of MVL at pH 2 was examined over 180 min at ambient temperature, 37°C and 60°C. No significant difference was observed between the rate of MVL production at ambient and physiological temperature, see Figure 2.6C; incubations at 60°C for 25 min had a greater rate of MVL production Figure 2.6D. The percentage of MVA converted to MVL overnight at ambient temperature was not significantly different from the yield of MVL after 25 min at 60°C: 70.8± 3.9% (n=6) versus 67.5± 1.3% (n=6), respectively; therefore an overnight incubation offered no advantage.

Incubation of mevalonic acid at pH 2 for 25 minutes was selected as the optimal conditions. The percentage conversion of MVA to MVL in aqueous standards under the optimal conditions was 69.5± 4.4% (day 1; n=6), 66.0± 3.9% (day 2; n=6) and 67.5± 1.3% (day 3; n=6). In three rat urine donors the percent conversion of MVA to MVL was 73.8±6.5% and in three human donors 70.6 ±2.7%; aqueous standards in this experiment gave an equivalent conversion of 71.1±2.5%. Data are given as mean ± SD. Inter-day variation of 3.6% was observed and, as a result, aqueous MVA standards were converted at the same time as study samples to determine percentage conversion for each batch. The presence of MVL in human and rat urine was measured by the analysis of 10 µL of unprocessed rat (n=3) and human (n=3) urine by the LC-MS/MS method and MVL was not detected.

2.4.1.3 Extraction method

SPE and LLE were considered for the extraction of MVL (and MVA) from processed urine. Various sorbents (strong and weak cation exchange, strong anion exchange, mixed mode) were tested for their suitability for the assay. The Oasis MAX cartridges gave the best recovery of MVA from an aqueous standard, see Table 2.3. However the analyte was not recovered from the Oasis MAX sorbent when the recovery experiments were performed with rat urine. Similarly, MVL was not detectable when rat urine was processed with the STRATA XCW sorbent. Due to the lack of reproducibility between aqueous and urine analyte recovery, solid phase extraction was not pursued further.

Table 2.3 Recovery of MVA or MVL from various SPE cartridges

Sorbent	Type	Analyte	Recovery of analyte (%)
Oasis MAX (10 ng/mL analyte)	Mixed mode RP and SAX	**MVA**	86.85
Oasis MAX (100 ng/mL analyte)	as above	**MVA**	83.29
Strata-XCW	Mixed mode RP and WCX	**MVL**	37.15
Strata-XC	Mixed mode RP and SCX	**MVL**	18.44
Hypersep VERIFY XC	Mixed mode RP and CX	**MVL**	10.74
EVOLUTE-CX	Mixed mode RP and SCX	**MVL**	10.29
Oasis HLB	Hydrophilic and Lipophilic	**MVA MVL**	ND

RP - reversed phase, SAX - strong anion exchange, WCX - weak cation exchange, SCX - strong cation exchange, CX - cation exchange.

An alternative to SPE of mevalonolactone is LLE. Published methods have successfully used ethyl acetate to extract mevalonolactone (Kindt et al. 2010) and an internal standard, d_4-mevalonolactone (IS), was employed to correct for between-sample variation in extraction recovery. During method development, the LLE and IS approach was investigated for MVL.

MVL and the IS were not extracted by ethyl acetate to the same extent, with 72.7±2.1% (n=6) of the analyte recovered compared to 34.4±3.5% IS, but the analyte-IS peak area relationship was still reproducible, linear and accurately quantifiable by LC-MS/MS. The extraction recovery of the analyte and IS was assessed in human (n=3) and rat (n=3) donors. The mean extraction recovery of 0.4, 1.0 and 6.0 µg/mL MVL from three rat urine donors was 64.0±8.1%, 62.5±4.8% and 54.3±5.0%, respectively, and the mean recovery of 0.15 µg/mL IS was 27.9±3.3%. The mean extraction recovery of 0.4, 1.0 and 6.0 µg/mL MVL from

three human donors was 72.8±16.1%, 62.1±4.4% and 64.7±8.7% and the mean recovery of 0.15 µg/mL IS was 31.5±3.3%.

2.4.2 Method validation

2.4.2.1 Assay performance

Several weighted regression models (1/x, $1/x^2$, $1/x^3$) were tested to improve the residuals in the calibration model. The best model was the linear and forced through the origin, with acceptable precision and accuracy across the analytical range with R^2>0.99 without the need to weight the data. The LOD of extracted MVL was 0.0078 µg/mL. The LLOQ was 0.0156 µg/mL; the ULOQ was 10 µg/mL. Independently prepared QC standards also reached acceptable precision and accuracy; individual data are shown in Table 2.4. Carryover of the analyte to subsequent blank injections on the LC-MS/MS was not detected.

2.4.2.2 Internal standard

The peak area ratio of analyte to internal standard corrected for the variability in the extraction recovery between samples, enabling the accurate quantification of MVL. The peak areas of MVL and the IS in calibration standards and samples of equivalent concentration were similar. As an example the mean peak area of MVL in one rat study sample was determined to be 2.5 $x10^6$±5.2 x 10^4 and, using the analyte/internal standard peak area ratio, was calculated to be 5.9 µg/mL MVL. The closest standard, a QC at 6 µg/mL had a similar MVL peak area of 2.8$x10^6$± 2.7$x10^5$. The internal standard peak area for the rat urine sample was 1.2$x10^5$±3.2$x10^3$ and for the QC was 1.3$x10^5$±7.0$x10^3$, giving ratios of 20.8 and 21.5, respectively. The extracted internal standard peak areas varied by 7.9% across the rat urine study and by 16.0% across the human urine study.

Table 2.4 Intra-day validation of the extraction, reconstitution and LC-MS^2 analysis of MVL calibration and QC standards across three batches. Accuracy is within 85-115% and precision is within 15% coefficient of variation (CV)

	Batch 1					Batch 2				Batch 3			
QC	Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy	Precision (%CV)	n=	Calculated Concentration (ng/mL)	Accuracy	Precision (%CV)	n=	Calculated Concentration (ng/mL)	Accuracy	Precision (%CV)	n=
LOW	45	44.0	97.8%	5.3%	6	45.2	100.4%	3.2%	6	41.1	91.4%	2.7%	6
MID	2000	1991.0	99.5%	2.1%	6	2048.1	102.4%	4.9%	5	1855.7	92.8%	3.4%	6
HIGH	6000	5881.7	98.0%	4.2%	6	6199.3	103.3%	6.7%	5	5427.7	90.5%	4.1%	6
STD													
1	15.6	16.6	106.0%	8.3%	3	16.2	103.9%	5.6%	3	16.3	104.4%	3.8%	2
2	31.3	31.0	99.3%	2.5%	3	33.9	108.5%	4.1%	3	29.6	94.8%	1.3%	2
3	62.5	65.8	105.3%	10.8%	3	62.3	99.7%	5.2%	3	58.5	93.6%	4.3%	3
4	625	610.5	97.7%	1.6%	3	644.9	103.2%	10.5%	3	629.4	100.7%	4.1%	3
5	1250	1221.4	97.7%	1.9%	3	1255.5	100.4%	5.2%	3	1130.2	90.4%	0.9%	3
6	2500	2475.9	99.0%	3.1%	3	2589.0	103.6%	9.8%	3	2338.4	93.5%	0.6%	3
7	5000	5416.7	108.3%	5.1%	3	4930.5	98.6%	6.6%	3	4535.7	90.7%	4.2%	3
8	8000	7914.6	98.9%	2.7%	3	8149.9	101.9%	5.4%	3	7830.4	97.9%	6.4%	3
9	10000	10129.3	101.3%	5.2%	3	10093.6	100.9%	1.5%	3	10489.1	104.9%	7.8%	3

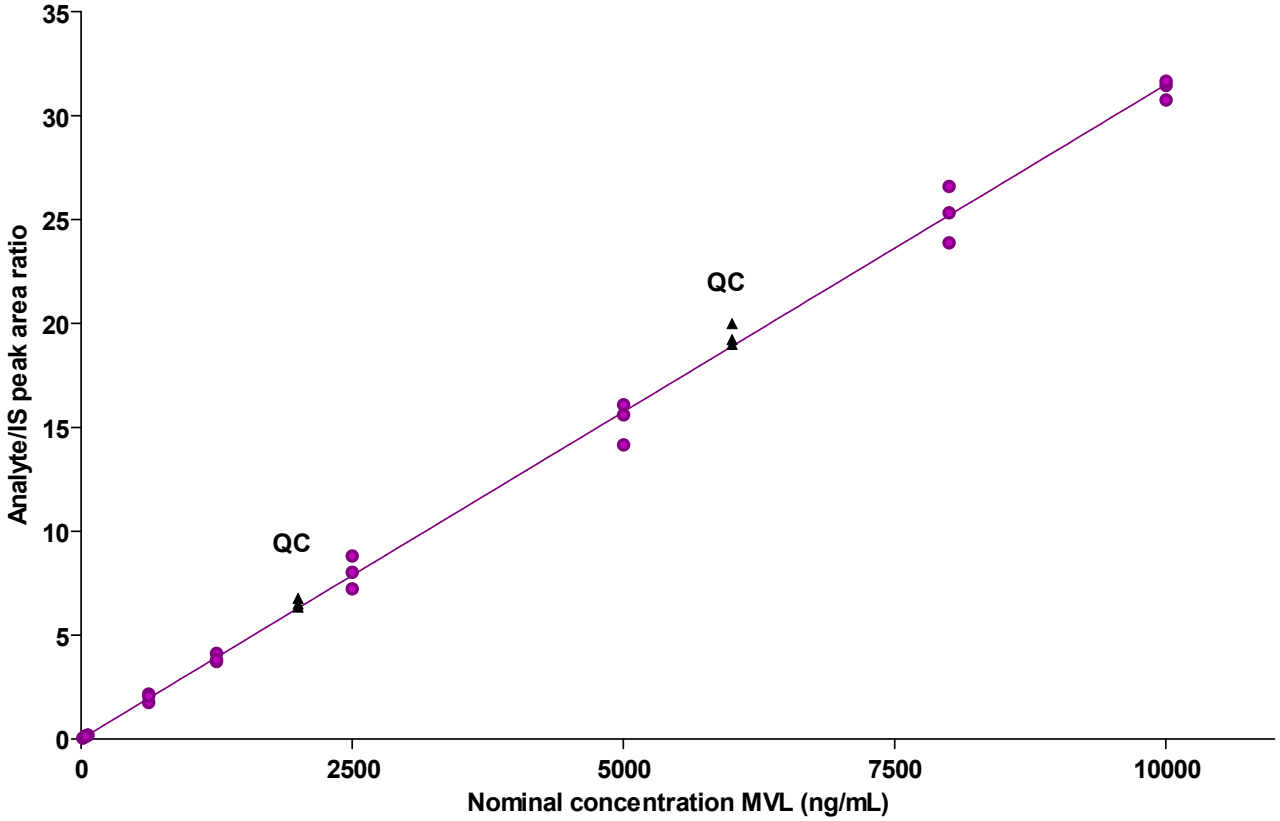


Figure 2.7 Linear relationship between nominal concentration and the analyte/IS peak area ratio. Independently prepared QC standards are within ±15% of the nominal concentration; data shown are mean values of three batches (n=3 for each batch).

2.4.2.3 Parallelism of rat urine to surrogate matrix

To validate the utility of a surrogate matrix for the calibration standards rat (n=5) and human (n=5) urine which had been acidified, extracted and reconstituted was serially diluted and the analyte and IS response was determined by LC-MS/MS. The observed analyte response remained within 20% of what was expected in both rat and human urine up to a dilution of 1 in 128, see Figure 2.8A and 2.8B. The observed IS response only remained within 20% of the expected response in both rat and human urine up to a dilution of 1 in 16, as further dilution reduced the IS response below the limits of detection. These data suggest that the rat and human urine matrix has no significant effect on the MS detection of MVL and the IS.

2.4.2.4 Matrix effects

Matrix effects were further investigated by post-column infusion of d_4-MVL and injection of 10 µL of a reconstituted ethyl acetate extract of acidified human or rat urine. The MS/MS response of the IS was monitored throughout the elution and there was no significant suppression or enhancement of the signal for d_4-MVL at or around the retention time of the analyte, see Figure 2.8C and 2.8D.

2.4.2.5 Relative accuracy

Rat and human urine spiked (0.1, 0.5 or 4 µg/mL MVA) and non-spiked was extracted and assayed to determine relative accuracy. The mean concentration of MVL in the non-spiked urine (human 222.6± 5.7 ng/mL, rat 3532.4± 171.9 ng/mL) was subtracted from that of the spiked urine. The recovery of the 0.1, 0.5 or 0.4 µg/mL spike in rat urine was 96.0±7.6%, 111.0±6.9% and 105.9±2.3%, respectively. Recovery of the 0.1, 0.5 or 0.4 µg/mL spike in human urine was calculated to be 96.0±4.8%, 118.2±3.5 and 107.3±0.4%, respectively. Data are mean ± SD. Full recovery of the spiked analyte from multiple donors suggests that this method can be used for the accurate quantification of MVA in both human and rat urine.

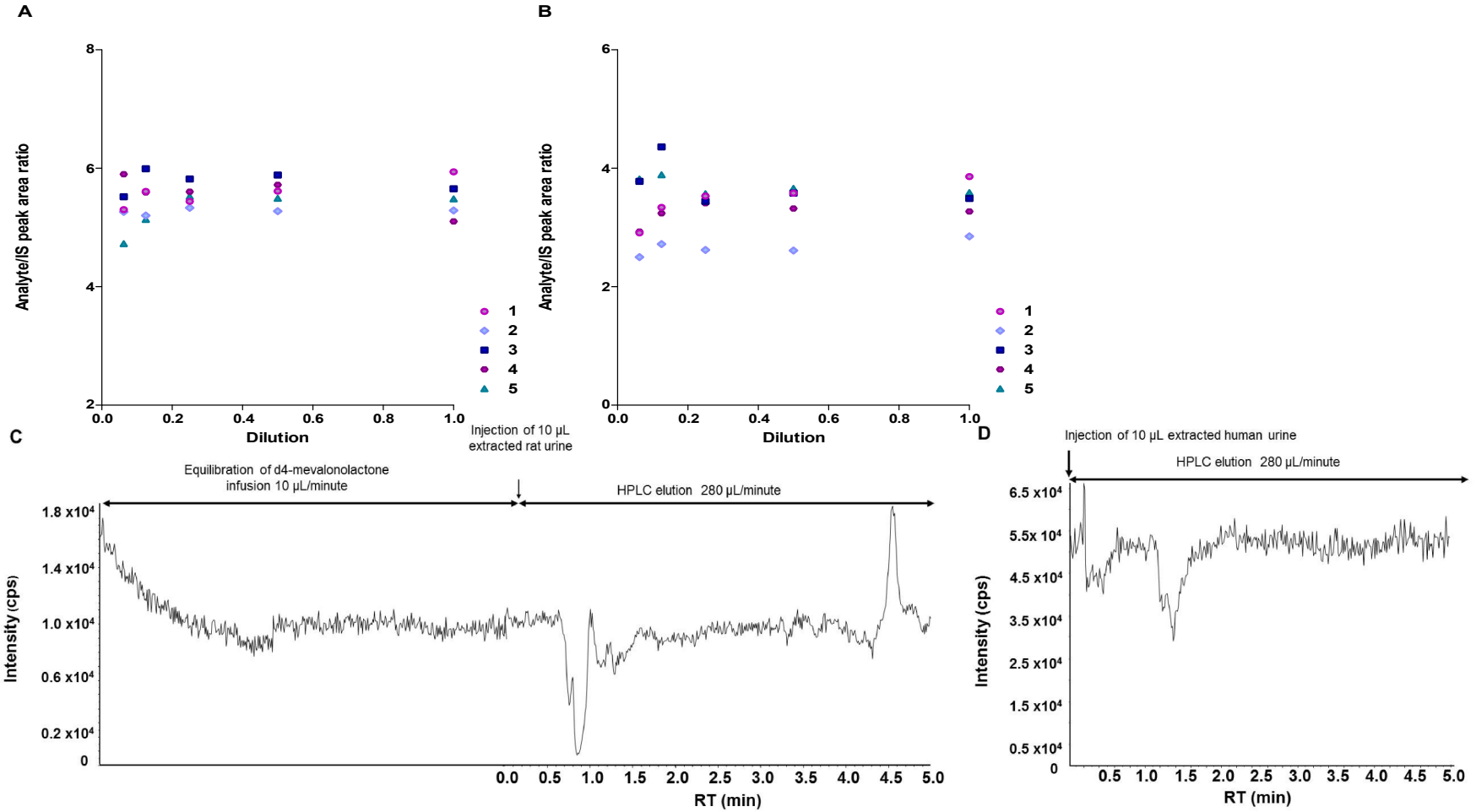


Figure 2.8 Validation of the surrogate matrix. Dilutional linearity of MVL extracted from (A) rat urine (n=5 donors) and (B) human urine (n=5 donors) Dilutional linearity of the analyte/IS peak area ratio confirms the parallelism of the surrogate matrix. The absence of ionisation suppression/enhancement of the IS signal (m/z 135 → 75 for d_4-MVL; retention time (RT), 2.05 min) by co-eluting materials extracted from the acidified rat (C) and human (D) urine matrix with ethyl acetate.

2.4.2.6 Reproducibility

Reproducibility of the full extraction and analysis method was determined by quantifying MVA in rat and human urine (VS) divided into three batches stored at -20°C. MVA was quantified on three separate occasions from each of these batches (n=3 per analysis) over 20 days. Within-batch variation was 2.01-4.58% coefficient of variation; while between-batch variation was 6.15%.

2.4.2.7 Stability

MVL QC standards were stable at room temperature, 4°C and at -20°C across 14 days, and at 60°C for 120 min. Freeze-thawing did not affect the integrity of aqueous standards.

2.5 Discussion

A contributory link between HMG-CoA reductase (HMGR) activity and the accumulation and cytotoxicity of aminoglycosides has been made recently (Antoine et al. 2010). As such, the physiological variation in HMGR between individuals could render them more or less susceptible to aminoglycoside accumulation and nephrotoxicity. In order to investigate this hypothesis, a reliable quantification method for HMGR activity in both non-clinical and clinical samples was required.

A literature search of methods of quantification and physiological properties of key metabolites of the mevalonate pathway and related products was undertaken in order to select the best candidate for quantification. The end-products of the mevalonate pathway, the isoprenoid pyrophosphates, are derived from isopentenyl-5-pyrophosphate and dimethylallyl-pyrophosphate and include farnesyl pyrophosphate (FPP), geranylpyrophosphate (GPP) and geranylgeranylpyrophosphate (GGPP). These metabolites are responsible for the production of ubiquinone (electron transport chain), squalene (precursor for cholesterol synthesis), dolichol (*N*-glycosylation of protein) (Goldstein and Brown 1990) and, important to aminoglycoside nephrotoxicity, the prenylation of multiple proteins including the small GTPases which regulate megalin localisation and activity.

FPP, GPP and GGPP are attractive biomarker candidates for mevalonate pathway activity and aminoglycoside uptake due to their metabolic proximity to prenylated small GTPases that affect megalin-mediated endocytosis of the aminoglycosides. However quantification of the isoprenoids pyrophosphates is difficult as they have a rapid turnover and are intracellular, requiring a complex extraction and analytical procedure and furthermore, their rapid degradation by phosphatases does not lend to rapid analysis (Holstein et al. 2009; Tong et al. 2008).

Another metabolite that was considered was squalene. Squalene is produced by condensation of two FPP units and is the precursor for steroid synthesis, most notably

squalene is a close precursor to cholesterol. Although HPLC methods for quantifying squalene are available (Lu et al. 2004), accurate quantification of endogenous squalene is hampered by significant contributions of dietary squalene and it is also present in cosmetics. Mevalonic acid was chosen as the most suitable candidate because of its direct relationship to HMGR and it is an existing biomarker of cholesterol synthesis, indicating its sensory potential. In addition, mevalonic acid is present in the serum and is excreted into the urine, allowing ease of sample collection for research purposes.

A simple, cross-species quantification method for urinary mevalonic acid (MVA) was not present in the literature. The only published LC-MS/MS assay of urinary MVA has a short dynamic range of 20 ng/mL-1000 ng/mL validated in human urine; in addition the protocol requires an initial sample volume of 125 µL (Jemal et al. 2003). In this chapter, the development and validation of an assay for MVA by LC-MS/MS that can be used for the analysis of rat and human urine is described.

The assay has a substantial dynamic range (0.0156 µg/mL-10 µg/mL) sufficient to determine normal biological and perturbed concentrations of MVA in both rat and human urine. Administration of statins is known to reduce the production of mevalonic acid by 30-40% (Beil et al. 1990; Naoumova et al. 1996; Woollen et al. 2001a). Given that the physiological urinary concentration of mevalonic acid in humans ranges between 116-1227 ng/mL the validated dynamic range of this assay will comfortably address perturbed mevalonic acid biosynthesis. The assay also benefits from requiring only a sample volume of 50 µL of urine, making the assay applicable where sample volumes are limited (e.g. rodent studies), and a short analytical run-time of 5 min.

This method builds upon published methods, incorporating acid-catalysed conversion of MVA to its lactone form MVL (Abrar and Martin 2002; Jemal et al. 2003; Kindt et al. 2010), extraction of the lactone and the IS with ethyl acetate (Kindt et al. 2010), and analysis by positive-ion LC-MS/MS (Abrar and Martin 2002; Kindt et al. 2010), but it is validated to a

large dynamic range to encompass MVA quantification in human and rat urine, has an optimised MVA → MVL conversion step and is without matrix effects.

The IS (internal standard) was chosen to correct for between sample inconsistencies. Ideally, the IS should behave in the same manner as the analyte throughout the analytical method. Our data have shown that the extraction recovery of the d_4-MVL was approximately 50% lower than MVL, and had a fractionally earlier elution time of (2.05 min) relative to the analyte (2.10 min) in the polar reversed-phase HPLC method which could suggest that d_4-MVL is more polar than MVL. Similar observations of reversed-phase HPLC resolution and different extraction recoveries between a low molecular weight analyte and its d_4-IS have been reported (Tranfo et al. 2008; Weiling 2002). Despite this, the analyte-IS relationship was consistent and linear, as shown by the validation. Therefore, these differences in behaviour between analyte and IS are not limiting for the quantification of MVA, and d_4-MVL, which was also used successfully in another published method (Kindt et al. 2010) is a suitable IS.

The use of MS/MS to assay MVL derived from MVA in urine encountered a significant selectivity challenge. In extracts of rat urine single-reaction monitoring with m/z 131 → 69.4 detected an uncharacterised isobaric compound which differed from MVL in retention time (Figure 2.5). This compound was left uncharacterised as its presence did not interfere with the quantification of the MVL. However, we used a combination of chromatographic retention time and MRM reporter transitions to confer enhanced specificity on our assay. MVA is a very polar compound, and was found to be incompatible with a number of widely used reversed-phase columns. Screening of several column chemistries identified a reversed-phase polar end-capped column which retained MVA and MVL satisfactorily.

The high endogenous concentrations of MVA in rat and human urine negate the use of 'blank' matrix for calibration standards. Calibration standards of MVL were prepared in pH 2 buffer, extracted and reconstituted in the same manner as urine. The use of a surrogate

matrix was validated by demonstrating that the presence of co-extracted components of the human and rat urine matrix did not affect the percentage of MVA converted to MVL, the extraction recovery of MVL and the IS, and the MS response of the analyte and IS, a phenomenon termed “parallelism”. By analysing the effect of matrix compared to aqueous standards at each stage of the assay, we found that dilution of the urine matrix had no effect on the analyte-IS ratio, indicating that the matrix has no appreciable bearing on the processing, detection and quantification of MVA.

Matrix effects are commonly encountered in electrospray ionisation when a matrix component which co-elutes with the analyte(s), but may not be detected in the MRM channel, attenuates or enhances the MS signal, and consequently compromises accuracy. Jemal et al. experienced extensive suppressive matrix effects when they used negative-ion detection of MVA regenerated from MVL to quantify MVA in human urine. In the current method, the [M+H]+ precursor ion generated abundantly by an eluent containing formic acid was exploited, instead of the [M+NH4]+ precursor generated by ammonium formate eluents (Abrar and Martin 2002; Kindt et al. 2010). There was no suppression or enhancement of the positive-ion d_4-MVL signal in the post-column infusion experiment and the addition recovery experiment gave near 100% recovery from rat and human urine.

The stability of MVA in various matrices, including urine, has been widely cited in the literature. In human plasma there was <6.7% change from baseline over 16 months over various storage conditions (Abrar and Martin 2002). Similarly in water, urine and plasma there was <8.2% change from baseline for at least 7 weeks (Jemal et al. 2003). Interim stability data from storage and freeze-thaw experiments for our QC standards composed of MVL are included herein and the method has a safeguard against potential analyte instability by analysis of a urine validation sample in each batch. However additional long-term studies should be carried out to confirm previous reports of analyte stability.

The development of a validated analytical method for MVA in rat and human urine allows for the investigation of HMGR activity as a susceptibility factor in the accumulation and cytotoxicity of aminoglycosides and in particular, it permits for the testing of MVA's biomarker potential in rats and humans. In the following chapter, this method will be employed to demonstrate the diurnal variation of MVA excretion and explore the role of HMGR activity in aminoglycoside nephrotoxicity.

3. URINARY MEVALONATE AS A POTENTIAL SUSCEPTIBILITY BIOMARKER OF AMINOGLYCOSIDE-INDUCED PROXIMAL TUBULE INJURY

Contents

3.1 Introduction

Aminoglycosides such as gentamicin and tobramycin are clinically effective antibiotics used to treat life threatening bacterial infections. The inherent nephrotoxic potential of this drug class limits their clinical efficacy and as such, mechanistic research has focussed with the impetus of improving aminoglycoside safety. It is widely accepted that the aminoglycosides accumulate within the proximal tubule epithelial cells (PTEC) through receptor mediated endocytosis. Knock-out studies (Schmitz et al. 2002), competitive ligand experiments (Nagai et al. 2006) and structural investigations (Dagil et al. 2013) have demonstrated the critical role of the multi-ligand endocytic receptor megalin (LRP2, gp330) to this process.

Megalin is known to be regulated by small GTPases (El-Annan et al. 2004; Lou et al. 2002) which have been prenylated by metabolites of the mevalonate pathway. The plausibility of reducing megalin-mediated aminoglycoside uptake by chemical inhibition of the rate limiting enzyme of the mevalonate pathway, HMG-CoA reductase (HMGR), was investigated *in vitro* (Antoine et al. 2010). In this study, opossum kidney (OK) cells primed with the HMGR inhibitors simvastatin, rosuvastatin and pravastatin, and then incubated with gentamicin, had lower levels of prenylated proteins in conjunction with a reduced gentamicin accumulation and cytotoxicity in comparison to OK cells with basal HMGR activity.

If such a metabolic relationship were to translate *in vivo* and between animal models and man, patients could be stratified according to their risk of developing aminoglycoside-induced kidney injury and furthermore, statins could be utilised as a prophylactic strategy. In order to translate the findings of the *in vitro* study, the relationship of basal mevalonate pathway activity to aminoglycoside nephrotoxicity must be investigated.

In Chapter 2 the development and validation of an accurate and precise analytical method for urinary mevalonate, the product of HMGR, was described and published recently (Rodrigues et al. 2014). In the following experiments, this method is employed to explore

the known diurnal rhythm of HMG-CoA reductase activity (Higgins et al. 1971; Parker et al. 1984) in rats, mice and healthy human volunteers and to investigate the relationship of HMGR activity to aminoglycoside nephrotoxicity in a repeat-dose rat model. The extent of gentamicin-induced kidney can be measured with established biomarkers of renal function serum creatinine and blood urea nitrogen and also the novel biomarker kidney injury molecule 1 (kim-1), which is a sensitive and specific marker of proximal tubule injury and its excretion correlates with the extent of renal injury (Zhou et al. 2008).

3.2 Aims

- To establish whether or not the diurnal rhythm of HMG-CoA reductase activity can be monitored *via* mevalonate excretion in rat, mouse and human urine using the validated LC-MS/MS method described in Chapter 2.
- Investigate the quantitative and diurnal relationship between HMG-CoA reductase activity and the extent of nephrotoxicity induced by aminoglycosides *in vivo*.
- Investigate mevalonate as a predictive mechanism-based biomarker of susceptibility to nephrotoxicity pre-aminoglycoside exposure *in vivo*.

3.3 Materials and methods

Gentamicin sulphate, (R)-MVA (purity ~96%) and MVL (purity~97%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Tobramycin sulphate was obtained from Discovery Fine Chemicals (Bournemouth, UK). 4,4,5,5-Tetradeutero-MVL (d4-MVL, purity 97%) was obtained from CDN Isotopes (Pointe-Clare, Quebec, Canada). Methanol, ethyl acetate and formic acid were of analytical grade and were obtained from Fisher Scientific (Loughborough,UK).

3.3.1 Diurnal rhythm of mevalonate excretion

Urine was collected from 16 adult male Sprague-Dawley rats (age approx. 8 weeks) *via* single housing in metabolic cages for 12 h between 10.00-22.00 h (n=8) or 22.00-10.00 h (n=8) and stored at -80°C until analysis. Urine was also collected from 5 adult male CD-1 mice (aged 8-10 weeks) in 4 h intervals over a 24 h period starting at 10:00 h. The animals had free access to food and water throughout the collections. The phases of the illumination cycle in the animal accommodation were 08:00-20:00 h (light) and 20:00-08:00 h (dark). Protocols were undertaken in accordance with a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee. Urine for the human investigation was obtained by spot collection in the morning (range 05.15- 08.50 h) and evening (range 17.30-22.30 h) from 25 healthy children (mean age 9.10 yrs, range 0.9 to 16.7 years). Children were recruited from schools and a nursery with informed written consent obtained from carers or guardians on the behalf of the participating children. The study was conducted with the approval of the Liverpool East Research Ethics Committee. Urine samples were collected from each child by an age-appropriate method, normally a clean catch urine sample into a sterile container. Samples were centrifuged, aliquoted into smaller volumes, then frozen and stored at -80˚C. Mevalonic acid and urine creatinine were quantified from the urine samples.

3.3.2 Gentamicin study

Sixteen adult male Sprague-Dawley rats were randomly assigned to either gentamicin or 0.9% saline dosing groups and further divided into AM or PM dosing schedules. Animals were housed individually in metabolic cages for 12 h either between 10.00-22.00 h (PM groups n=8 per group) or 22.00-10.00 h (AM groups, n=8 per group) where urine was collected and subsequently stored at -80°C until analysis. Following the pre-dose urine collection animals were administered 200 mg/kg gentamicin or 0.9% saline I.P according to their dosing group; the PM groups were dosed at 22:00 h and the AM groups at 10:00 h. The dosing schedule was repeated for 9 days. Urine was collected over 12 h following the final dose and animals were culled humanely by a rising concentration of CO_2 and exsanguination.

3.3.3 Tobramycin study

Thirty adult male Sprague-Dawley rats (age approx. 8 weeks) were randomly assigned to either tobramycin (n=16), gentamicin (n=8) or 0.9% saline (n=6) treatment groups. Animals were housed individually overnight prior to the first dose of tobramycin sulphate (120 mg/kg I.P) or gentamicin sulphate (120 mg/kg I.P) or 0.9% saline at approximately 10.00 h. The dosing schedule was repeated for 7 days and urine was collected overnight following the final dose. Animals were culled humanely using a rising concentration of CO_2 and exsanguination.

Protocols were undertaken in accordance with a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee.

3.3.4 Histology

Tissues were collected into a solution of 10% formalin before slicing and processing for H&E staining. Kidney tissue was scored for damage according to a system used previously (Zhang et al. 2009). All organs were removed and rinsed in ice cold saline. Renal tissue samples

were fixed in 10% neutral buffered formalin or snap frozen. For histological assessment of toxicity and immunohistological examination, formalin-fixed tissue sections were routinely paraffin wax embedded. 3-5µm sections were prepared and stained with hematoxylin and eosin (H&E) or used for immunohistology.

Immunohistology was performed to identify cells undergoing apoptosis (staining for cleaved caspase-3) as well as cells in the cell cycle as a tool to identify regenerative attempts (staining for PCNA) as previously described (Antoine et al. 2010; Piron et al. 1998). Briefly, H&E stained sections were examined for any histopathological features and sections stained for caspase-3 and PCNA for semi-quantitative analysis blind and independently by a pathologist (A.Kipar).

3.3.5 Biomarkers

3.3.5.1 Quantification of urinary mevalonic acid

Mevalonic acid was quantified according to the method described in Chapter 2. In brief, 50 µL of urine was acidified with a pH 2 buffer to lactonize mevalonic acid. An internal standard, tetra-deuterated mevalonolactone was added to samples and standards, and both compounds were extracted with ethyl acetate. The extract was dried down and reconstituted in 90% water 10% methanol 0.1% formic acid before 10 µL was injected on to an LC-MS/MS instrument. Samples were analysed and quantified in relation to calibration standards. The concentration of mevalonic acid was normalised to urine creatinine.

3.3.5.2 Quantification of urinary creatinine

Urine (25 µL of urine diluted 1 in 25) was incubated with 125 µL of alkaline picrate solution (0.14 M NaOH, 10 mM Na_2HPO_4, 90 mM sodium borate, 10 mM sodium dodecyl sulphate, 25% v/v picric acid, 2.7% v/v DMSO) for 2 min at room temperature. An acid solution (5 µL of 10:2:88 v/v acetic acid: concentrated H_2SO_4:distilled water) was added and incubated at room temperature for a further 10 min with shaking. Plates were read at 490 nm and creatinine was estimated using creatinine calibration standards.

3.3.5.3 Quantification of urinary kim-1

For the initial gentamicin study, urinary kim-1 was quantified using a sandwich ELISA as described previously (Vaidya et al. 2006) in collaboration with the Bonventre lab (Harvard University). In brief, the antibody monoclonal, anti-rat Kim-1 ectodomain, served as a primary antibody and coated the wells of the ELISA plate. The wells were blocked with 3% BSA/PBS with 0.02% sodium azide and washed with PBST. One hundred microliters of standard or sample were incubated in duplicate for 1.5 h at 37°C. Following three washes with PBST, the secondary antibody (biotinylated monoclonal anti-rat Kim-1 ectodomain) and HRP-conjugated streptavidin was added for 15 min. The reaction was stopped by addition of 1 N HCl and absorbance was read at 450 nm. Urinary kim-1 was calculated using a calibration curve and expressed as ng/mL.

For the tobramycin study, urinary kim-1 was quantified using the rat kim-1 singleplex assay kit (MesoScaleDiscovery, Rockville, MD). Urine was thawed, vortexed and centrifuged at 13,000 rpm for 5 min before an aliquot was diluted 1 in 5 with the diluent supplied in the kit. Plates were blocked for 1 h with shaking and rinsed three times with 300 µL of PBS-T. To each well, 25 µL of diluent and 25 µL of diluted sample were added; the plate was sealed and incubated at ambient temperature for 2 h with shaking. After the incubation the plate was washed again with PBS-T before addition of 25 µL of the detection antibody. The plate was incubated for a further 2 h with shaking, then washed with PBS-T before addition of 150 µL of read buffer. The 96-well plate was analysed using the SECTOR Imager (MesoScaleDiscovery, Rockville, MD). Calibration standards were also analysed with a dynamic range of 0.0137 – 10 ng/mL.

3.3.5.4 Serum creatinine

Serum creatinine was quantified by the Jaffe reaction according to a method optimised for serum (Sabbisetti et al. 2014) with collaborators in the Bonventre Lab (Harvard University).

3.3.6 Renal HMG-CoA reductase activity

HMG-CoA reductase was assayed in microsomal fractions derived from renal tissue. Rats were sacrificed by rising CO_2 and the kidneys were removed and immediately placed in an ice-cold homogenization medium (50 mM KH_2PO_4 (pH 7.0), 0.2M sucrose, 2 mM dithiothreitol (DTT)). The kidneys were homogenized in the medium. The homogenate was centrifuged at 15,000×g for 10 min and the supernatant obtained was centrifuged at 100,000×g for 75 min to obtain microsomal pellets, which were then resuspended in the homogenization medium containing 50 mM EDTA and centrifuged at 100,000×g for 60 min. The supernatant containing the microsome was used as an enzyme source.

The activity of HMG-CoA reductase was determined by employing ^{14}C-HMG-CoA (Shapiro et al. 1974) as follows. The enzyme in the supernatant containing the microsomes was activated at 37° C for 30 min. Added to a reaction tube were 20 µl of HMG-CoA reductase assay buffer (0.25M KH_2PO_4 (pH 7.0), 8.75 mM EDTA, 25 mM DTT, 0.45M KCl and 0.25 mg/ml BSA), 5 µl of 50 mM NADPH, 5 µl of ^{14}C-HMG-CoA (0.05 µCi/tube, final conc. 120 µM) and 10 µl of activated microsomal enzyme (0.03-0.04 mg), and the mixture was incubated at 37° C for 30 min. The reaction was terminated by adding 10 µl of 6 M HCl to the mixture and the mixture was incubated at 37° C for 15 min to allow complete lactonisation of the product (mevalonate). The precipitate was removed by centrifugation at 10,000×g for 1 min and the supernatant was applied to a Silica gel 60 G TLC plate (Altech Inc, Newark, USA) and then developed with benzene:acetone(1:1, v/v). A region having a Rf value ranging from 0.65 to 0.75 was removed by scraping with a disposable cover slips and assayed for radioactivity with 1450 Microbeta liquid scintillation counter (Wallacoy, Finland). Enzyme activities were calculated as picomoles mevalonic acid synthesised per min per mg protein (pmoles/min/mg protein). The assay was conducted by A.Srivastava (Department of Pharmacology and Therapeutics, University of Liverpool)

3.3.7 Gentamicin quantification

Gentamicin content in the renal cortex was determined by an LC-MS method as reported previously (Antoine et al. 2010) and was conducted by A.Srivastava (Department of Pharmacology and Therapeutics, University of Liverpool).

3.4 Results

3.4.1 Diurnal variation in mevalonate excretion

3.4.1.1 Rat

MVA was quantified in urine collected from adult male Sprague-Dawley rats during two 12 h collections, 10:00-22:00 h and 22:00-10:00 h, see Figure 3.1. The excretion of MVA was significantly greater (unpaired T test; $p<0.0001$) in urine collected during 22:00-10:00 h (6.2-13.0 µg/mg UCr; mean 9.7±2.3 µg/mg UCr) compared to 10:00-22:00 h (1.6-5.2 µg/mg UCr; mean 3.4 ±1.3 µg/mg UCr). When expressed as total MVA excretion by factoring in urine volume the diurnal differences remained and urine collected 22:00-10:00 h represented a mean excretion of 110.0±27.8 µg MVA/12 h (81.7-153.2 µg) compared to urine collected 10:00-22:00 h with a mean excretion of 21.5±6.6 µg MVA/12 h (12.8-33.1 µg).

3.4.1.2 Mouse

MVA was quantified in urine collected over 24 h in six 4 h intervals from 5 adult male CD-1 mice, see Figure 3.2. MVA excretion throughout the collections ranged from 4.90 - 25.05 µg/mg UCr with urine MVA output undergoing a mean 1.7-fold change (range 1.4 to 2.1-fold). The maximum MVA excretion was in either the 02:00-06:00 h or 06:00-10:00 h collections in the majority of the mice whilst the one animal had a reverse rhythm with peak and trough MVA excretion at 18:00 h and 06:00 h, respectively.

3.4.1.3 Human

MVA was also quantified in paired morning and evening urines obtained by spot collection from healthy children (n=25), see Figure 3.3. The excretion of MVA in evening spot collections had a median of 292.4 ng/mg UCr (n=25, inter quartile range 245.4ng/mg UCr), whereas the median concentration of MVA in morning spot collections was 427.0 ng/mg UCr (n=25, inter quartile range 230.1 ng/mg UCr). The paired and mean data are represented in Figure 3.3; in 15 out of 25 pairs, morning collections had significantly greater

concentrations of MVA than evening collections ($p<0.05$; T Test, paired, two-sided) where the morning excretion was, on average, 105% greater; however in four individuals the concentration of MVA was significantly greater in the evening collection ($p<0.005$; T Test, paired, two-sided). The remaining six morning and evening pairs had numerical differences but were not statistically significant.

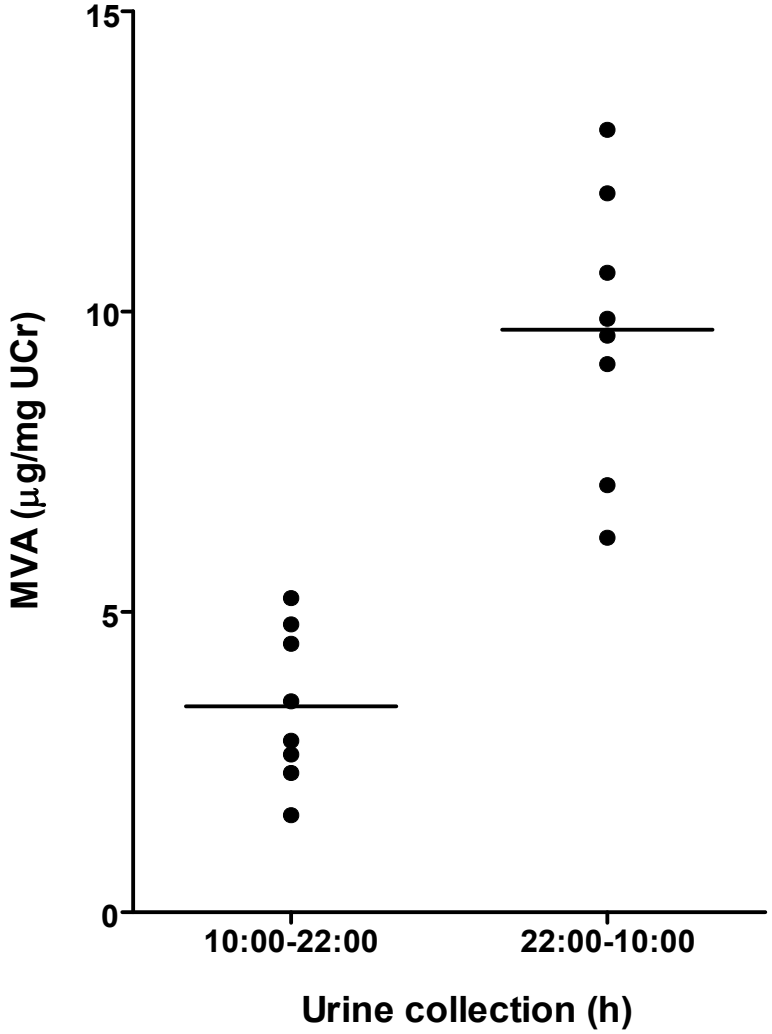


Figure 3.1 MVA excretion in adult male Sprague-Dawley rats in two 12 h collections (n=8 per group). Data are plotted as dot plots with the group mean (-). Individual data points are the mean of three analytical replicates.

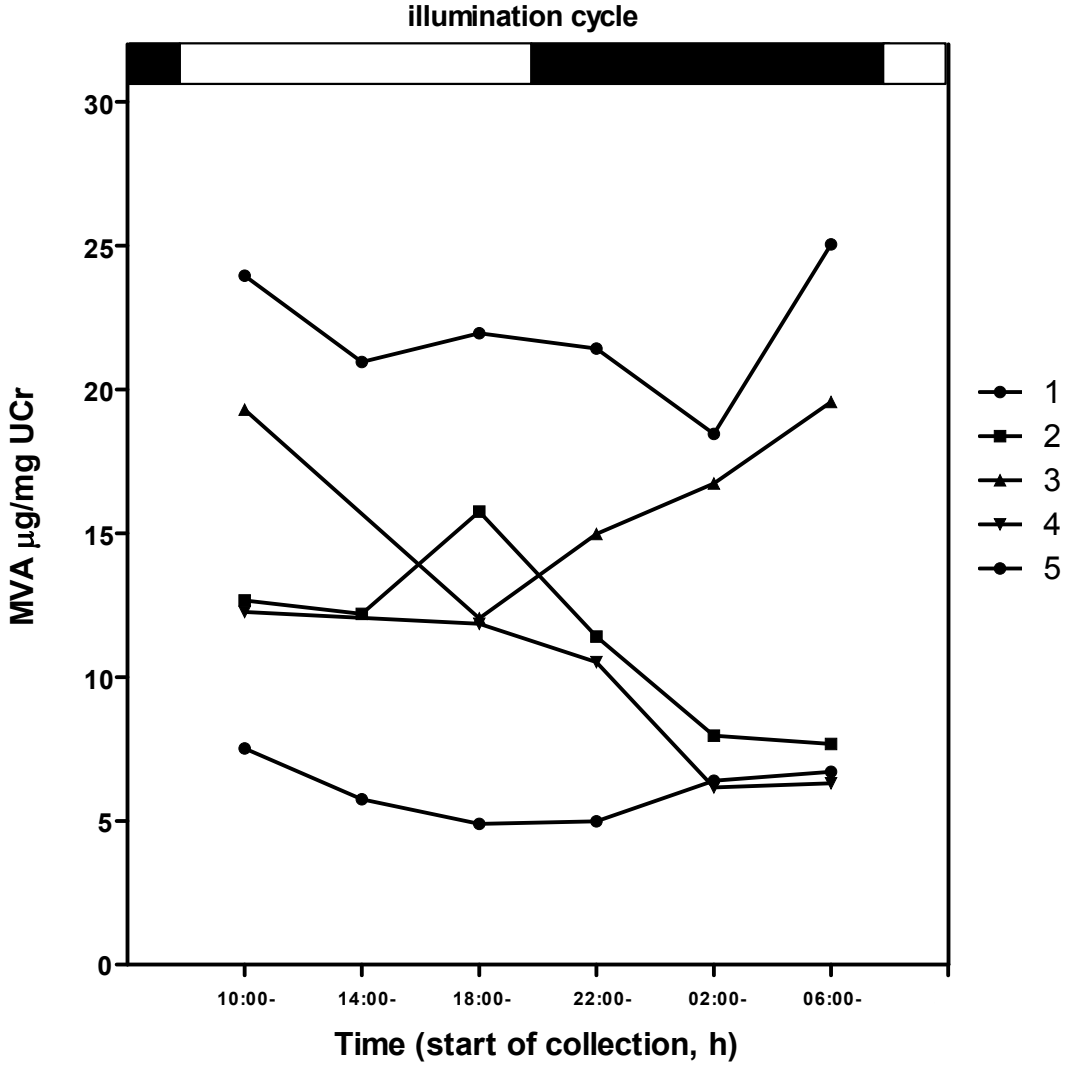


Figure 3.2 24 h diurnal variation in MVA excretion in adult male CD-1 mice. Data plotted are mean values of three analytical replicates from six 4 h urine collections (n=5).

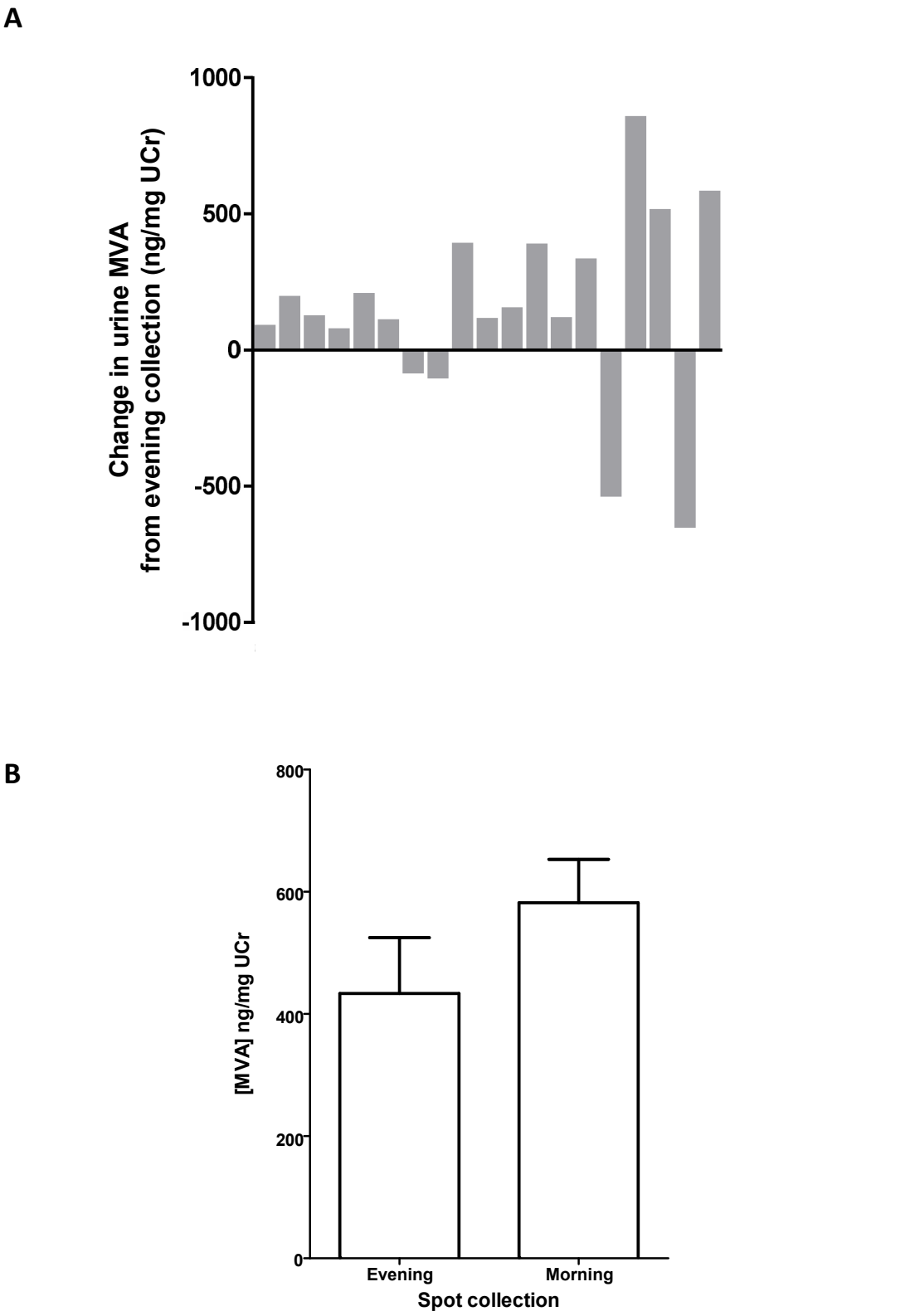


Figure 3.3 MVA excretion in human urine obtained by spot collection from healthy children (n=25). (A) Data are presented as change in urinary MVA from evening to morning per individual and (B) group means ± standard deviation.

3.4.2 Gentamicin study

3.4.2.1 Serum creatinine

Blood samples were obtained from 4 rats per dosing group. Creatinine was quantified in the sera of rats administered 200 mg/kg gentamicin daily at either 10:00 h or 22:00 h for 9 days. The mean serum creatinine concentration in the 22:00 h dosing group was 0.60±0.08 mg/dL and for the 10:00 h dosing group 2.13±0.34 mg/dL ($p<0.0001$, one-way ANOVA with Bonferroni correction). The mean serum creatinine in the saline animals dosed at 22:00 h was 0.30±0.14 mg/dL and for the 10:00 h group was 0.20±0.08 mg/dL, see Figure 3.4.

3.4.2.2 Histology

A dose of 200 mg/kg administered at 10:00 h for 9 days induced histological changes typical for gentamicin-induced nephrotoxicity (average score 3; Figure 3.5A). Immunohistology for cleaved caspase-3 demonstrated tubular epithelial cell death *via* apoptosis (Figure 3.5B) in association with toxic changes. When administered at 22:00 h, however, the same gentamicin doses did not lead to any histological evidence of nephrotoxicity; histological features were identical to those seen in untreated control animals (Figure 3.5C).

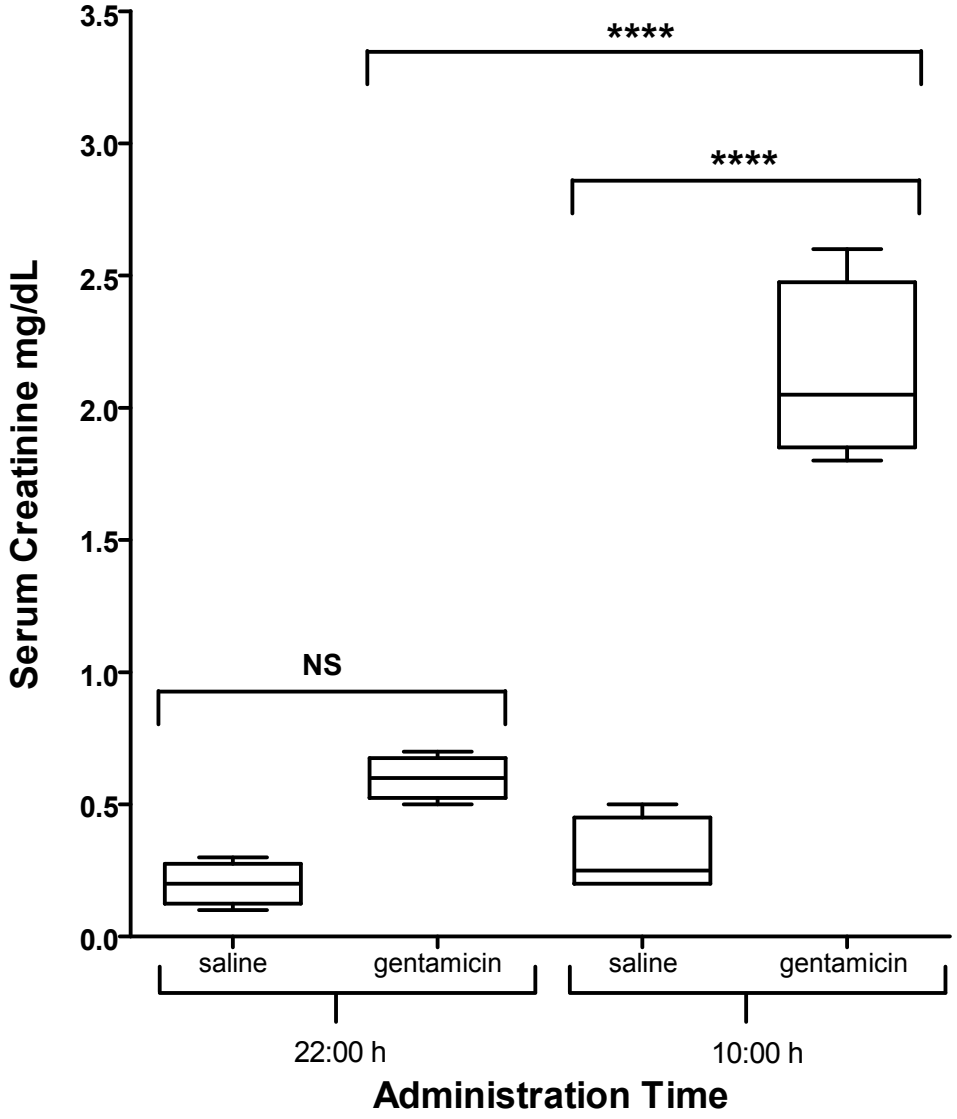


Figure 3.4 Serum creatinine levels after 9 days of I.P. 200 mg/kg gentamicin or 0.9% saline, administered once daily at either 22:00 h or 10:00 h. Data are presented as box plots with means, upper and lower quartiles and the minimum and maximum values (n=4). NS - not significant **** - $p<0.0001$ (One-way ANOVA with Bonferroni correction).

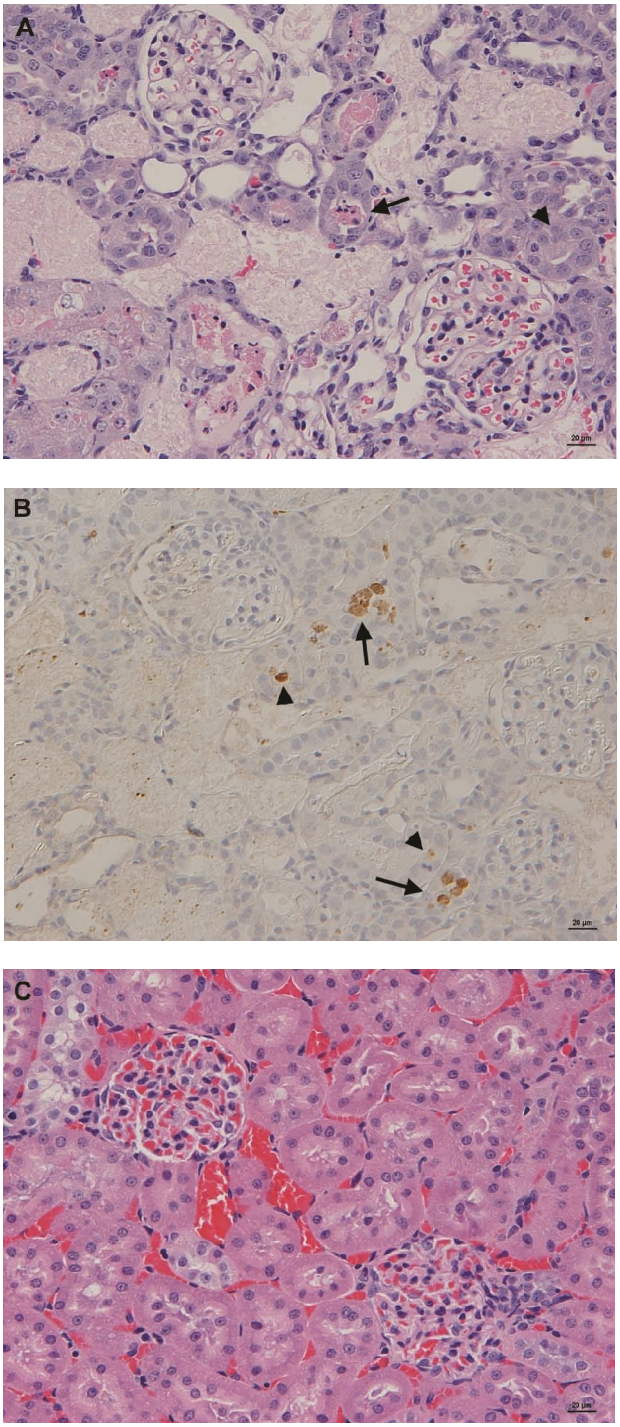


Figure 3.5 Histological changes in rats treated with gentamicin at 10:00 h or 22:00 h. (A) H&E stain of renal tissue from a rat treated with gentamicin (10:00 h group), there are numerous dying tubular epithelial cells (arrow). Other tubules show evidence of epithelial cell regeneration (arrowhead); score 4. (B) Tubular epithelial cell death is *via* apoptosis, as the expression of cleaved caspase-3 in small groups (arrows) or individual (arrowheads) tubular epithelial cells confirms. (C) H&E stain of renal tissue from a rat treated with gentamicin (22:00 h group), there are no significant pathological changes. B: Peroxidase anti-peroxidase method, Papanicolaou's hematoxylin counterstain. Bars = 20 µm.

3.4.2.3 Urine kim-1

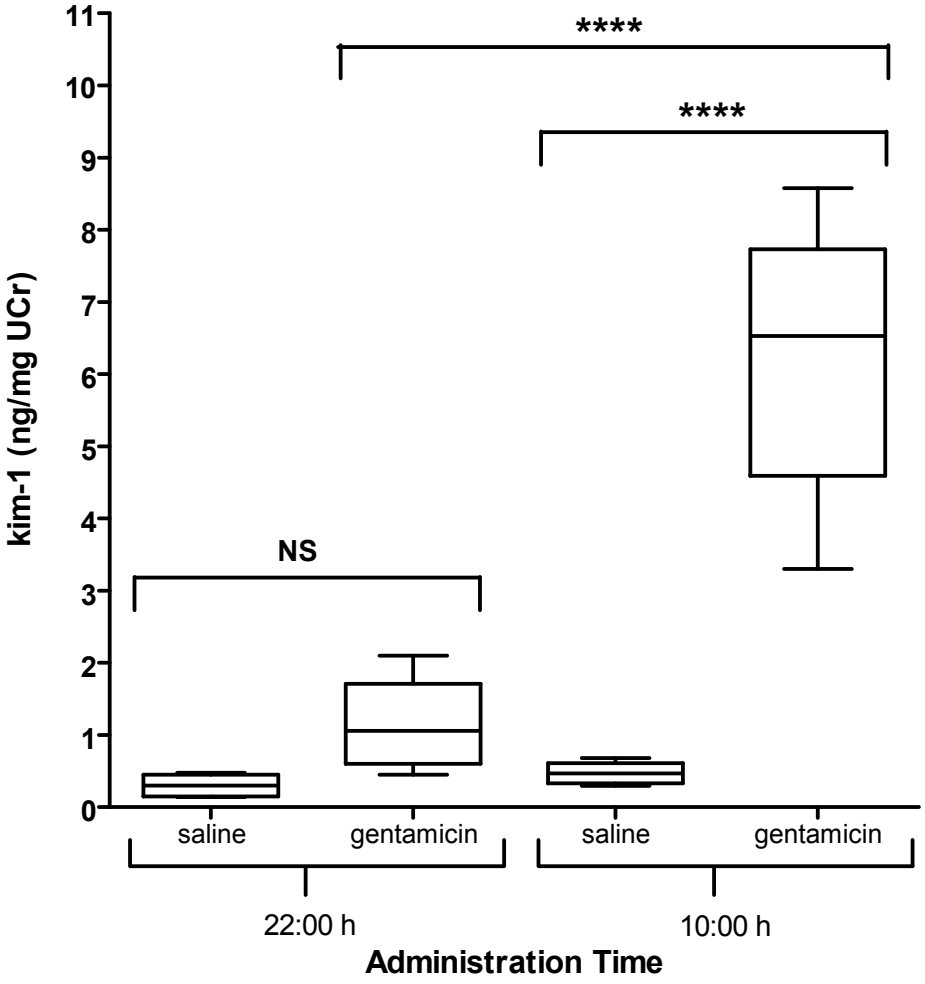


Figure 3.6 Urine kidney injury molecule 1 excretion after 9 days of I.P 200 mg/kg gentamicin administered daily at either 22:00 h or 10:00 h. Data are presented as box plots with means, upper and lower quartiles and the minimum and maximum values (n=8). NS - not significant **** - $p<0.0001$ (One-way ANOVA with Bonferroni correction).

The urinary excretion of kim-1 was significantly greater in animals dosed gentamicin at 10:00 h compared to the corresponding saline treated animals ($p<0.0001$, one-way ANOVA with Bonferroni correction). This difference was not seen in animals dosed saline or gentamicin at 22:00 h. Kim-1 excretion was significantly greater in animals administered 200 mg/kg gentamicin daily for 9 days at 10:00 h compared to the animals dosed at 22:00 h (mean 6.23±1.80 vs. 1.14±0.61 ng/mg UCr, respectively, $p<0.0001$ one-way ANOVA with Bonferroni correction), see Figure 3.6.

3.4.2.4 Renal HMG-CoA reductase activity

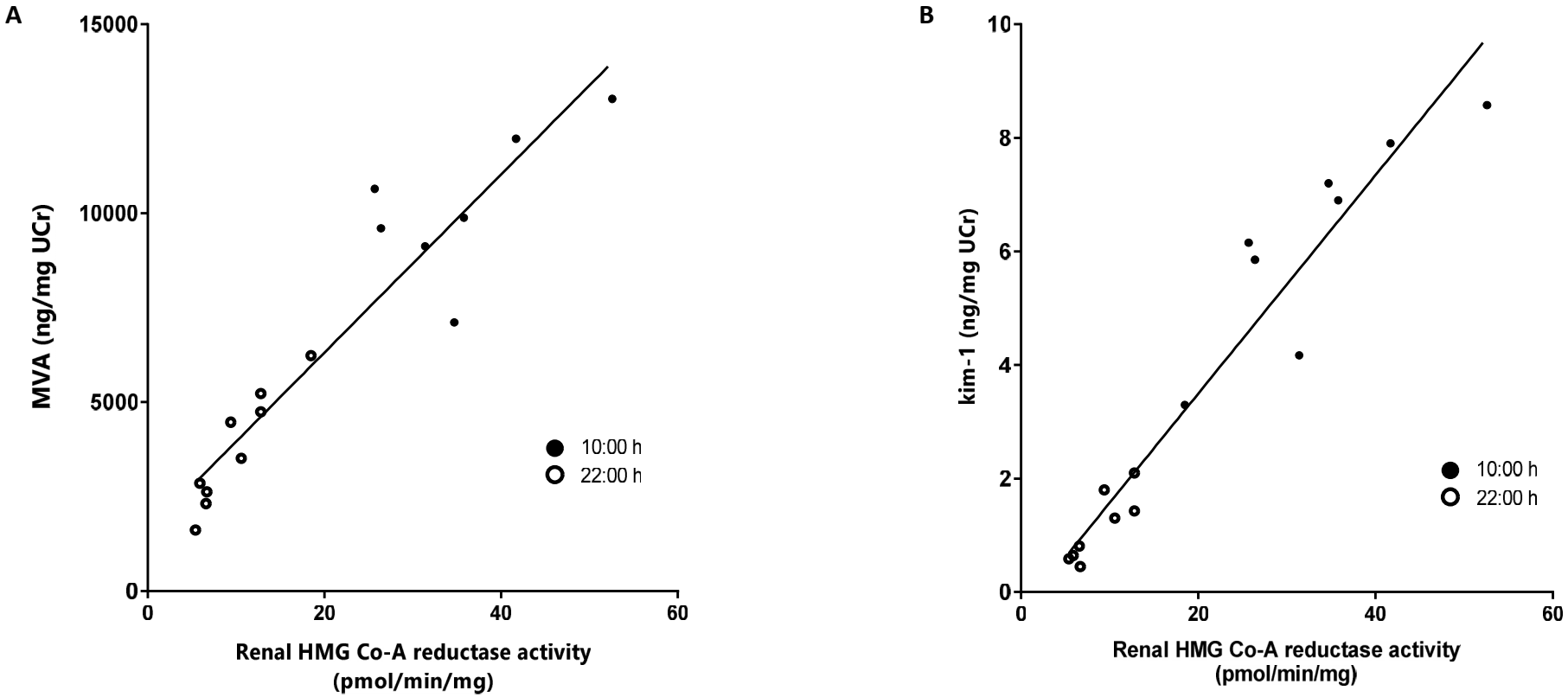


Figure 3.7 Regression of (A) pre-dose urinary mevalonic acid and (B) urinary kidney injury molecule 1 with renal HMG-CoA reductase activity from animals administered I.P 200 mg/kg gentamicin daily at either 10:00 h or 22:00 h. Data points are from individual animals (10:00 h n=8, 22:00 h n=8).

Renal activity of HMG-CoA reductase was measured and regressed against end-point urinary kim-1 excretion. A strong positive relationship was observed with R^2 =0.9277 ($p<0.0001$). The data points of the two dosing groups were clearly separated, see Figure 3.7.

3.4.2.5 Regression of urine kim-1 and pre-dose mevalonate

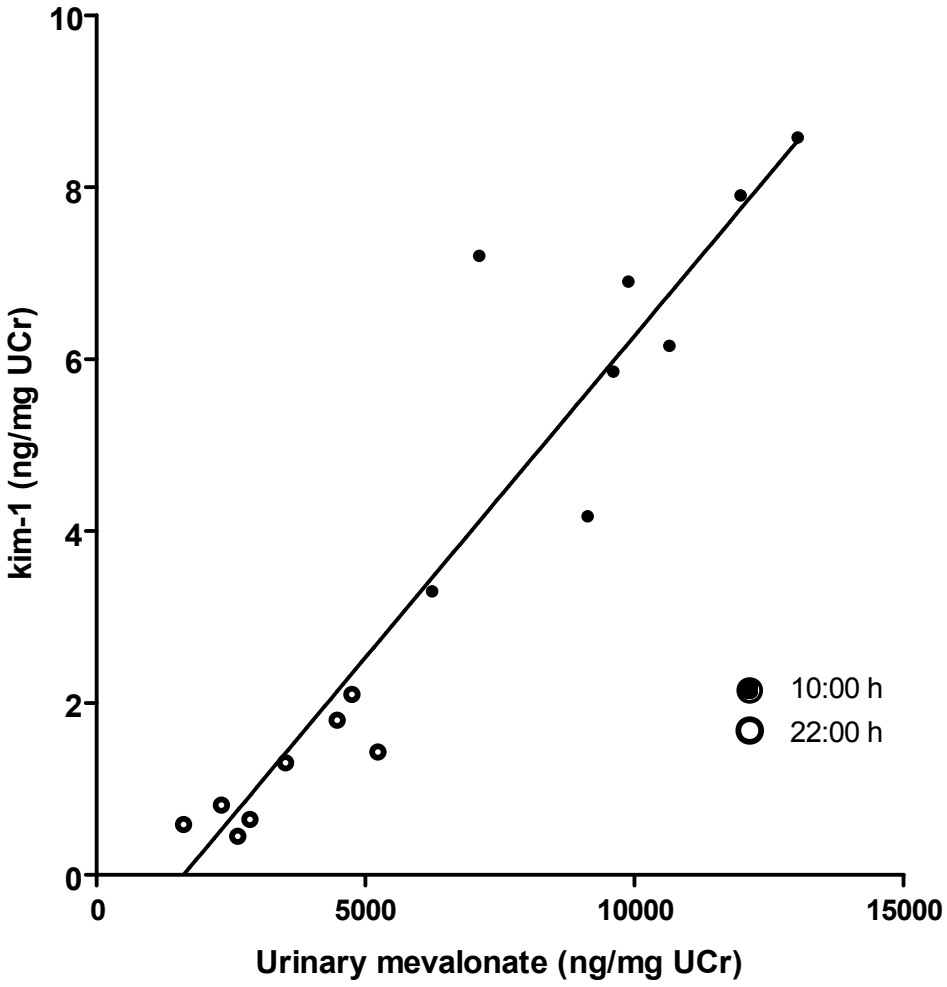


Figure 3.8 Regression of pre-dose mevalonate and end-point urine kidney injury molecule 1 from animals administered I.P 200 mg/kg gentamicin daily at either 22:00 h or 10:00 h. Data points are from individual animals (10:00 h n=8, 22:00 h n=8).

Pre-dose urinary mevalonate concentration was regressed against end-point urinary kim-1 excretion for each animal administered gentamicin. A significant positive relationship was

observed with a strong goodness of fit ($p<0.0001$, $R^2=0.8846$). Data points for animals administered gentamicin at 10:00 h were separated from those administered gentamicin at 22:00 h, see Figure 3.8.

3.4.2.6 Renal accumulation

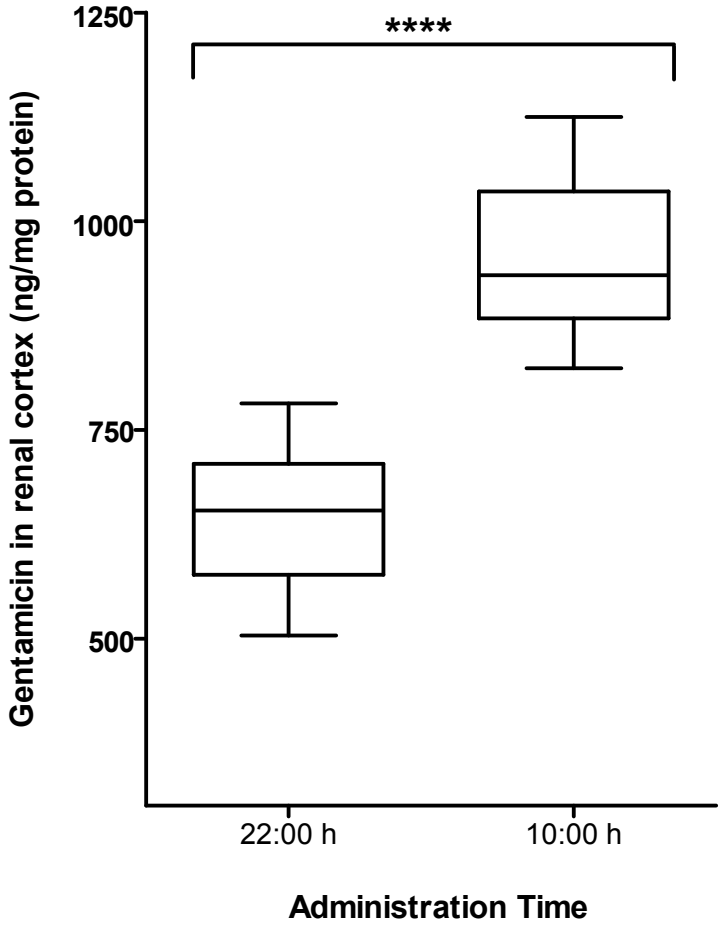


Figure 3.9 Accumulation of gentamicin in the renal cortex after repeat dose gentamicin at either 10:00 h or 22:00 h. Data are box plots of the means, upper and lower quartiles and the minimal and maximal values (n=8 per group), **** - $p<0.0001$.

The accumulation of gentamicin was measured by quantification of gentamicin in the renal cortex, see Figure 3.9. Animals administered gentamicin at 22:00 h had a mean renal cortex concentration of 647.3±89.7 ng/mg protein whereas animals administered gentamicin at 10:00 h had a mean renal cortex concentration of 954.0±97.6 ng/mg protein ($p<0.0001$, unpaired T test).

3.4.3 Tobramycin study

3.4.3.1 Urine Kim-1

Before administration of the aminoglycosides, kim-1 excretion was minimal ranging from 0.92 ng/mg UCr to 4.31 ng/mg UCr. After 7 days of repeat dose gentamicin or tobramycin (120 mg/kg) urine kim-1 significantly increased relative to pre-dose, reaching a mean of 37.82±19.54 ng/mg UCr and 24.77±20.73 ng/mg UCr for the gentamicin (p=0.0012) and tobramycin (p=0.0006) groups, respectively (paired, two-tailed T test). Although there was a numerical difference between the mean kim-1 excretion in gentamicin and tobramycin groups it was not statistically significant, see Figure 3.10.

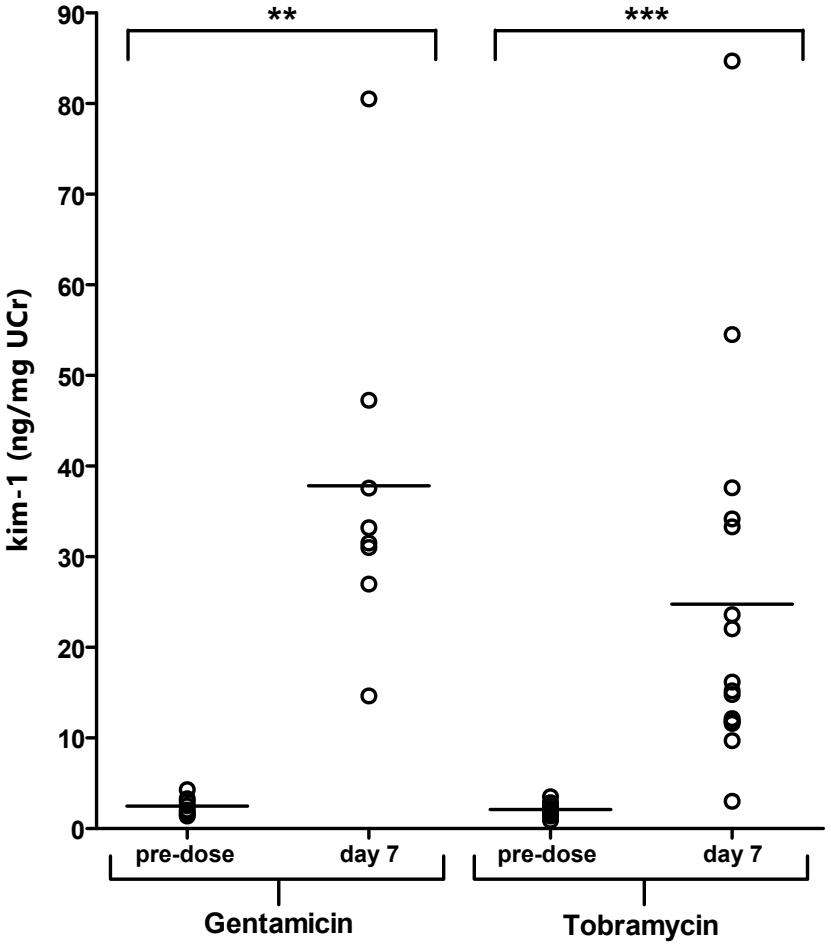


Figure 3.10 Urine kidney injury molecule 1 excretion pre-dose and after 7 daily injections of 120 mg/kg gentamicin or tobramycin. Individual data points are presented with a horizontal line to indicate the group mean (gentamicin n=8, tobramycin n=16, ** - p<0.005, *** - p<0.001).

3.4.3.2 Regression of kim-1 and pre-dose mevalonate

Tobramycin and gentamicin were administered at equal doses (120 mg/kg) once daily for 7 days. The positive relationship observed for gentamicin in the 200 mg/kg experiments was also observed at 120 mg/kg for 7 days but was not statistically significant (R^2=0.3839, p=0.19), see Figure 3.11. One animal was excluded from regression analysis because the histology score, BUN and kim-1 were not simultaneously elevated. An inverse relationship was observed for pre-dose mevalonate and tobramycin toxicity (as determined by kim-1 excretion) but it was not statistically significant, see Figure 3.12.

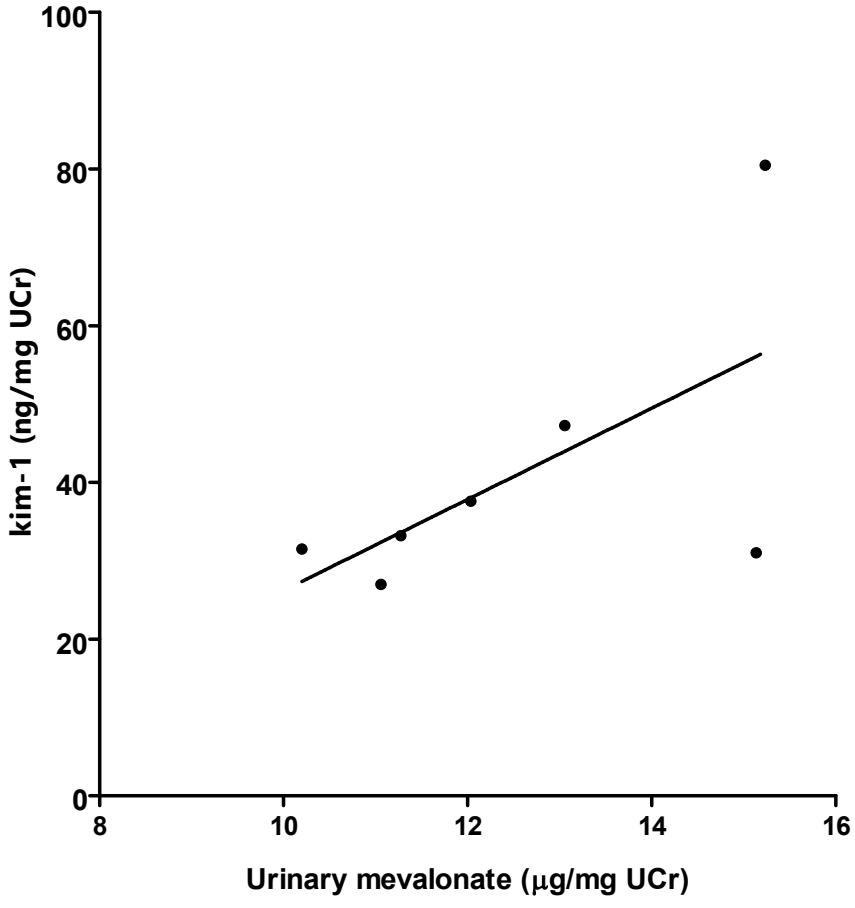


Figure 3.11 Linear regression of pre-dose mevalonate and end-point kidney injury molecule 1 from animals administered 7 daily injections of 120 mg/kg gentamicin at 10:00 h. Data points are from individual animals (n=7).

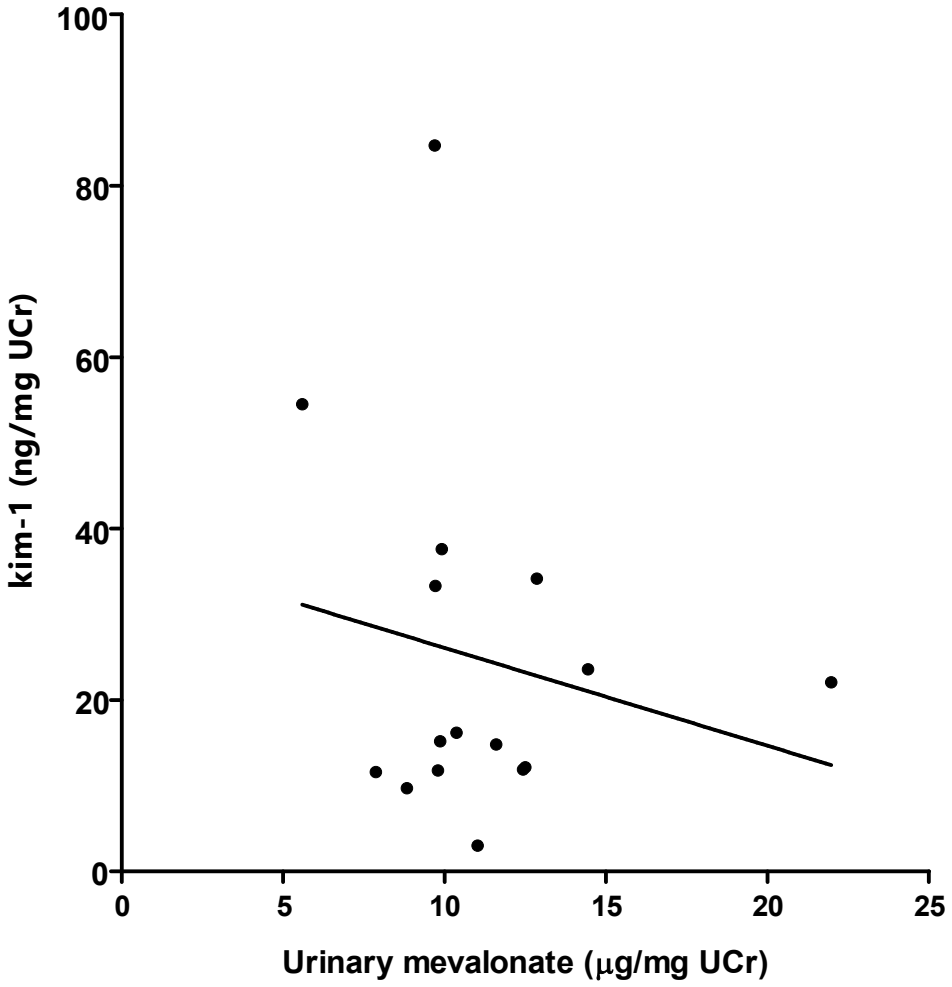


Figure 3.12 Regression of pre-dose mevalonate and end-point kidney injury molecule 1 from animals administered 7 daily injections of 120 mg/kg tobramycin at 10:00 h. Data points are from individual animals (n=16).

3.5 Discussion

Gentamicin nephrotoxicity demonstrates a strong relationship with megalin-mediated endocytosis and the mevalonate pathway. Reduction of megalin endocytosis *via* inhibition of the mevalonate pathway's rate limiting enzyme HMG-CoA reductase (HMGR) has been shown to reduce the accumulation and cytotoxicity of gentamicin (Antoine et al. 2010). As such, the diurnal and inter-individual variation in HMGR activity could influence the amount of aminoglycoside that accumulates within proximal tubule cells. The aims of this chapter were to establish urinary mevalonic acid as a biomarker of HMGR activity and to explore the relationship between the diurnal rhythm of HMGR, mevalonate excretion and aminoglycoside nephrotoxicity in a rat model.

The mevalonate assay developed in Chapter 2 was applied to the quantification of MVA in urine from rats, mice and healthy human children at various time points in a 24 h period. Each species demonstrated diurnal flux in urinary MVA consistent with the known diurnal activity of HMG-CoA reductase. In rats MVA excretion was significantly different between two 12 h rat urine collections, with 22:00–10:00 collections containing more than 3-fold the amount of MVA than 10:00–22:00 collections. The diurnal variation of HMGR activity is well established and has been previously examined in hepatic microsomal fractions of rats (Parker et al. 1982; Popjak et al. 1979) and plasma and urine MVA content in human subjects (Beil et al. 1990; Parker et al. 1982; Parker et al. 1984). However studies were unable to demonstrate statistically significant diurnal variation in plasma MVA in rats (Kopito et al. 1982). The lack of significance of the flux in rat plasma MVA in studies by Kopito et al. is likely to do with study design; in comparison to the present study, rats were allowed access to food between 09:00 h and 12:00 h and plasma mevalonate was measured across nine time points with different groups of animals at each time point (n = 5–6 per group). Given the inter-individual variation displayed between the animals in the present study, it is more likely that significant diurnal fluctuations would have been seen if

the concentration of plasma MVA over time had been compared in paired samples obtained from the same animals.

In the human data where the samples were paired, the trend was similar to that found in the rat study. In 60% of individuals the concentration of MVA was greater in the morning spot collection ranging from 27% to 469%. In four individuals a significantly lower urinary MVA content was observed in the morning compared with the evening collection. These human data conform to published urinary MVA data on a study of healthy volunteers which observed increases of up to 122% in MVA excretion in eight of 11 volunteers in 12 h urine collected between 19:00 and 07:00 h compared with 07:00 and 19:00 h; in the remaining three volunteers this trend was reversed (Lindenthal et al. 1996). Similarly, plasma MVA was observed to vary 5-fold in a 40 year old male with a maximum at 07:00 h and a minimum at 22:00 h (Beil et al. 1990).

CD-1 mice did not display a clear diurnal rhythm in urinary MVA excretion whereby a maximum 2.1-fold difference between the minimum and maximum MVA excretion in a 24 h period was measured. One animal demonstrated a reverse rhythm like that seen in the human volunteers, with peak excretion during 18:00-22:00 h. Although the diurnal activity of HMGR activity is widely accepted as a mammalian phenomenon, literature on HMGR activity and mevalonate excretion in the mouse is scarce with the majority of rodent studies focused on the manipulation of HMGR in rat models and the clinical measurements of mevalonate in plasma. Still, the quantification of mevalonate in non-clinical and clinical samples is vital to the investigation of the relationship of HMGR and the toxicity of aminoglycosides in animal models.

Whilst further investigations on HMGR and aminoglycoside nephrotoxicity were carried out in rats, studies were not pursued in mice due to negative results in a dose-ranging nephrotoxicity study; a repeat dosing regimen of once-daily intraperitoneal gentamicin

(200 mg/kg for 7 days) in C57BL/6 and CD-1 adult male mice did not cause kidney injury (see Appendix 1), hence a mouse model of gentamicin nephrotoxicity could not be established. The literature on aminoglycoside nephrotoxicity in mice is conflicting, with early studies suggesting that aminoglycosides accumulate within murine proximal tubule epithelial cells but the mice are resistant to toxicity (Suzuki et al. 1994; Suzuki et al. 1995), whereas multiple research groups demonstrate amelioration of aminoglycoside-nephrotoxicity by co-administering other compounds in Swiss albino background mice (Nabavi et al. 2012; Nitha and Janardhanan 2008; Whiting et al. 1981). The reason for this discrepancy is unclear but it could be that species differences between rats and mice and strain differences between C57BL/6 and albino background mice result in differential intracellular handling of the aminoglycosides. Although it appears that mice are not a reliable model for studying aminoglycoside nephrotoxicity, investigation into the mechanism behind their resistance to the intracellular adverse effects of aminoglycoside accumulation could offer new strategies and biomarkers for the stratification of patients based on risk.

Susceptibility to aminoglycoside-induced nephrotoxicity is long-established in rats (Luft et al. 1975). We investigated the relationship of pre-dose HMGR activity with the end-point toxicity of gentamicin in adult male Sprague-Dawley rats, by quantifying the product of HMGR, mevalonic acid, and quantifying a biomarker of proximal tubule injury, kidney injury molecule 1. Animals were assigned to one of two dosing regimens – either a 10:00 h or 22:00 h schedule to exploit the diurnal variation in HMGR activity. A strong positive relationship between pre-dose mevalonate excretion, kidney injury and gentamicin accumulation in the renal cortex was observed, with animals administered gentamicin at 10:00 h experiencing greater kidney injury and gentamicin accumulation, compared to animals on the 22:00 h schedule. Correspondingly, mevalonate excretion was greater in the hours prior to 10:00 h compared to 22:00 h supporting the idea that there is a contributory

relationship between HMGR activity, the uptake of gentamicin and subsequent nephrotoxicity.

The strong relationship between pre-dose mevalonate and gentamicin toxicity suggests that mevalonate could be a predictive biomarker of an individual's susceptibility to aminoglycoside nephrotoxicity and supports the further research into the use of statins as a prophylactic treatment (Antoine et al. 2010). In the study of 200 mg/kg gentamicin, animals with greater pre-dose mevalonate demonstrated the greatest extent of gentamicin nephrotoxicity; the trend was similar at 120 mg/kg gentamicin but the regression was not significant due to one animal with high pre-dose mevalonate and comparatively low urine kim-1 excretion. Although these data strongly suggest HMGR activity plays a significant part in gentamicin-induced nephrotoxicity, this anomaly illustrates that susceptibility is likely to be multifactorial. At present the management of this adverse drug reaction is based on monitoring trough serum levels and kidney function tests but, other than patient demographics and a history of renal disease, there is no method of predicting which patients are susceptible. Therefore it is important to examine whether the role of HMGR translates to a clinical setting, and if successful, measurement of an individual's HMGR activity could help stratify patients based on their risk for nephrotoxicity and suitability for prophylaxis.

The greater level of toxicity observed when gentamicin was administered once daily at 10:00 h may have wider implications for clinical dosing strategies. Guidance for clinical practice suggests that once-daily or extended interval dosing regimens reduce the incidence of aminoglycoside nephrotoxicity compared to the historical twice or thrice-daily regimens (Chuck et al. 2000; Rybak et al. 1999). Whilst a multiple-dosing strategy would circumvent the clinical issue of circadian toxicity, a once-daily regimen would not. There is no general consensus on the importance of the time of day at which it is best to administer aminoglycosides but a clinical study of 179 patients has identified the time of day as an

independent risk factor for toxicity; the incidence of nephrotoxicity was greatest when aminoglycosides were administered between midnight and 7 am (34.6%) whilst the incidence of nephrotoxicity when aminoglycosides were administered between 4.30 pm and 11.30 pm was 9.3% (Prins et al. 1997). The published data in conjunction with the data presented here indicates that administration of gentamicin when HMGR activity is at its peak has greater potential for nephrotoxicity than administration of gentamicin when HMGR activity is at its lowest. In addition, the data from our healthy volunteer children demonstrates that the diurnal rhythm of HMGR is not uniform between individuals (Rodrigues et al. 2014) with some children having an inverse rhythm to the majority. Although this information could be incorporated into clinical practice, it is recognised that it may not be efficacious to delay the start of an antibiotic regimen until HMGR activity falls.

Whilst a susceptibility relationship has been demonstrated between mevalonate and gentamicin, our investigations with the aminoglycoside tobramycin did not reach the same conclusion as no clear relationship was observed. Tobramycin nephrotoxicity has been studied in concert with gentamicin nephrotoxicity to show that they both accumulate within the renal cortex in non-clinical species (Nieminen et al. 1978; Sairio et al. 1978) and patients (Schentag et al. 1978), although tobramycin consistently accumulates to a lesser extent than gentamicin (Schentag et al. 1981). Later studies of the mechanism of proximal tubule uptake with gentamicin, netilmicin and amikacin established a role for megalin-mediated endocytosis (Decorti et al. 1999; Moestrup et al. 1995; Nagai et al. 2001; Schmitz et al. 2002), however a direct connection between tobramycin and megalin has not been published. Tobramycin also has different binding kinetics to gentamicin and netilmicin. Continuous infusion experiments with the aminoglycosides in conscious rats demonstrated that, while gentamicin and netilmicin had saturable Michaelis-Menten uptake kinetics, tobramycin demonstrated a linear relationship between serum level and cortical accumulation without saturation, suggesting that tobramycin has a different mechanism of

uptake to gentamicin and netilmicin (Giuliano et al. 1986). This is further supported by experiments that show netilmicin, when co-administered at an equi-molar concentration, inhibited the accumulation of gentamicin by >20% whereas a ratio of 2:1 tobramycin to gentamicin was required to cause a similar inhibition (Josepovitz et al. 1982). In light of the strong relationship between pre-dose mevalonate and gentamicin, the absence of a relationship with tobramycin and the different uptake kinetics of tobramycin compared to gentamicin, it is probable that the majority of tobramycin molecules are taken up into the proximal tubule by an alternative mechanism. Therefore although pre-dose mevalonate is a suitable susceptibility biomarker for gentamicin nephrotoxicity, these data suggest it is not suitable for tobramycin. Given the positive result with gentamicin and the indication that some aminoglycosides are taken up by alternative means, it would be prudent to investigate the mechanistic relationship of pre-dose mevalonate and nephrotoxicity with other clinically relevant aminoglycosides such as amikacin.

In order for urinary mevalonate measurement to be incorporated into clinical practice as a susceptibility biomarker for gentamicin nephrotoxicity, further research must be undertaken in order to satisfy biomarker qualification guidelines (Goodsaid and Frueh 2007). Qualification is the process of collecting evidence that supports the use of a biomarker in a specific context. Guidance for the active qualification of biomarkers is a relatively new process and much of the guidance refers to biomarkers which assist in the development of safer drugs by detecting adverse outcomes earlier than the gold standard biomarkers used clinically. Whilst mevalonic acid could be further investigated as a potential predictive biomarker, many of the concepts suggested for safety biomarkers apply.

Fundamental to the qualification process, the proposed context of use must be defined and non-clinical and clinical evidence is gathered within that framework. It may be the case that a wider context of use is identified as more research mounts. For mevalonic acid, the

demographic, population and drug regimen would need to be well-defined. In this research, time- and HMGR- dependent aminoglycoside nephrotoxicity was identified in rats and although the diurnal rhythm of HMGR in man has been shown herein, the translation of this relationship to human subjects exposed to the aminoglycosides needs to be explored.

Any data supporting the use of a novel biomarker in a disease area should be underpinned by existing biomarkers to demonstrate its performance. In terms of susceptibility biomarkers, demonstrating performance is more difficult than comparing two end-point biomarkers as was achieved in comparing urinary kim-1 with serum creatinine and blood urea nitrogen in its qualification as a pre-clinical biomarker of proximal tubule injury (Vaidya et al. 2010). The data collection supporting a susceptibility biomarker would be a lengthy process and is case-dependent and therefore may require a larger cohort of both animal and clinical data.

Underlying the value of any biomarker is its analytical method. The guidelines for qualification suggest that the evaluation of the biomarker should be considered conceptually independent to the assay which performs the measurements, that is to say the assay must be validated alone, and a biomarker cannot become qualified without an appropriate validated method of quantification. The assay developed and validated according to fit-for-purpose biomarker guidelines that is described in Chapter 2 can quantify mevalonic acid in urine of rodent and human urine. However it does take several hours to perform the assay which in the clinical context of a patient with a serious infection needing urgent treatment, may not be appropriate. Therefore a more rapid method of determining mevalonic acid and/or HMGR activity would be essential to its clinical implementation.

In summary, urinary mevalonic acid is a biomarker of HMGR activity *in vivo* and temporal changes in HMGR activity and mevalonic acid excretion are related to the extent of

gentamicin accumulation and nephrotoxicity. Although mevalonic acid needs to be investigated in more detail with regards susceptibility to aminoglycoside induced nephrotoxicity, the initial research presented here provides a framework for entering the in-depth biomarker qualification process. These data will be built upon in Chapter 4 and Chapter 5 by adopting an alternative approach to targeted biomarker identification - the comprehensive multivariate metabolite analyses of biofluids in animals exposed to gentamicin.

4. METABONOMIC ANALYSIS OF GENTAMICIN EXPOSURE

Contents

4.1 Introduction

Metabonomics is the study of metabolic profiles from complex biological samples, such as serum, urine or tissue, providing information on the perturbation of metabolites in response to pathophysiological conditions. These metabolic 'fingerprints' can be used to study drug toxicity. Analysis of the metabolome is complementary to the other 'omics approaches (genomics, transcriptomics, proteomics), giving a more accurate description of the phenotypic response to a chemical through direct measurements of the products of genes, transcripts and proteins (Nicholson et al. 2002). Another advantage of a metabonomics approach is that it enables the study of environmental influences, such as the diet or gut microbes, on a phenotype.

4.1.1 Analytical platforms for metabonomic analysis

Spectral methods provide the means to generate high-resolution metabolic profiles. Mass spectrometry (MS) coupled to Gas or Liquid Chromatography (GC or LC) and Nuclear Magnetic Resonance (NMR) spectroscopy are the most commonly employed, complementary, analytical tools for assessing metabolic changes. The hyphenation of chromatography and MS allows for partial sample clean-up by firstly separating metabolites chromatographically, followed by mass spectrometric ionisation and detection according to their mass to charge ratio (m/z). NMR spectroscopy exploits the property of atomic nuclei in a magnetic field where they absorb and re-emit electromagnetic radiation. The response of the nuclei, also known as the free induction decay (FID) is mathematically transformed by Fourier transformation to produce a spectrum, see Figure 4.1. Whilst NMR has the benefit of being non-destructive and provides information rich spectra (structural information and metabolite range) from complex mixtures, MS has greater analytical sensitivity, albeit plagued by metabolite-dependent ionisation suppression issues and general inter-laboratory reproducibility issues (Nicholson et al. 2002).

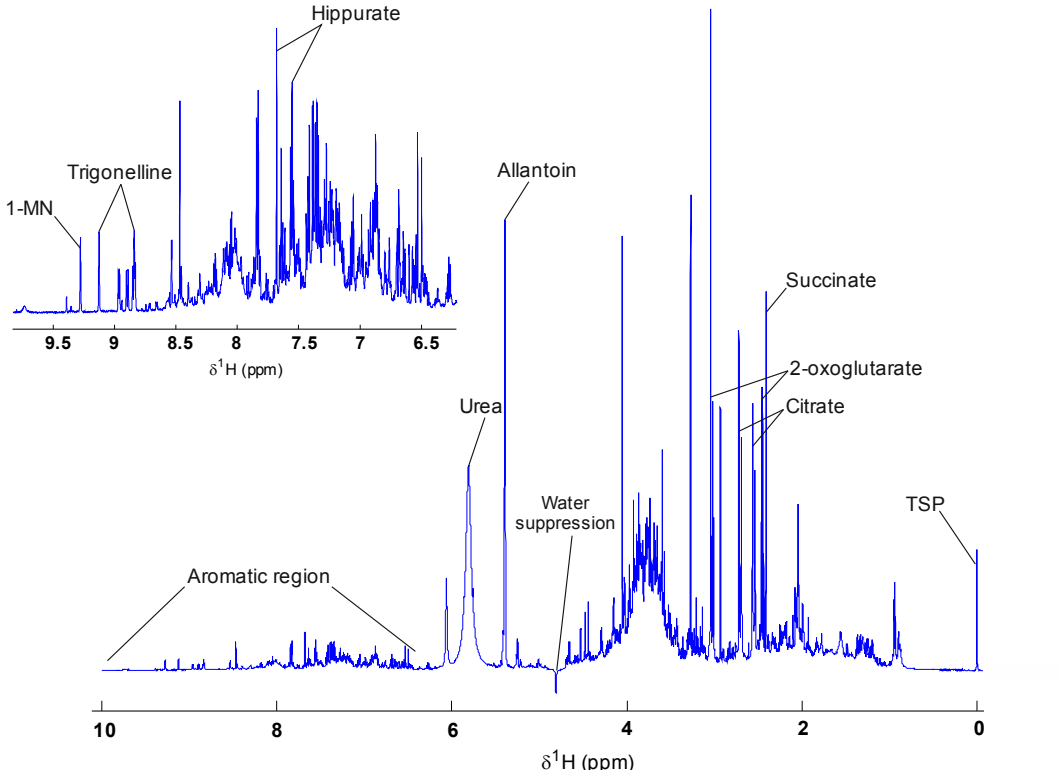


Figure 4.1 A typical 600 MHz ^{1}H-NMR spectrum of rat urine. Spectral peaks represent metabolites present in the urine and are calibrated to TSP (0 ppm). TSP - sodium 3-trimethylsilyl-[2,2,3,3,-^{2}H4]-1-propionate, 1-MN - 1-methylnicotinamide.

4.1.2 Multivariate analysis

A wealth of metabonomic data can be generated in a relatively short amount of time; whilst data analysis and pattern recognition is rapid, metabolite identification can be a rate-limiting step. The multidimensional data generated by metabolic profiling is commonly analysed using multivariate statistical methods including Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). PCA is an unsupervised statistical technique that is employed to find patterns and outliers in multivariate data (data with many dimensions) and it is useful when highlighting the similarities and differences within data sets. The data are compressed into new vectors, or principal components, which describe the relationships within the multivariate data, more specifically where the variance is large. PCA is an unsupervised method in that the origin of the data or prior information on class membership is not considered in the analysis. In contrast, OPLS-DA is a supervised method whereby data are categorised, the 'Y' variable, and the discriminating features of the data sets are identified, the 'X' matrix. Variation in

the data is dissected into two parts, variation in 'X' that is correlated with 'Y' also termed 'predictive' information and variation that is not, termed uncorrelated or 'orthogonal' information.

4.1.3 Metabonomics of gentamicin exposure

Various efforts have been made to characterise the mechanism of gentamicin nephrotoxicity using a metabonomic approach although a limited number of metabolites were identified as perturbed. An initial study used both LC-MS and ^{1}H-NMR technology to study urine from a twice-daily regimen of 120 mg/kg gentamicin for 7 days. Significant increases in urinary NAG and BUN were observed on day 3 and day 9, respectively, but, aside from a decrease in trimethylamineoxide (TMAO) and a rise in citrate, they did not detect major changes by ^{1}H-NMR until after the dosing ceased when glucosuria, raised lactate, and a reduction in urinary betaine was reported (Lenz et al. 2005). Complementary LC-MS analysis identified the depletion of xanthurenic acid and kynurenic acid. This study also focused on PCA as a tool for detecting metabolites responsible for separation from controls. Similarly, a study of age-related gentamicin nephrotoxicity in rats used PCA and found changes in glucose, hippurate and 6-hydroxymelatonin (Espandiari et al. 2007). Another study used a combined ^{1}H-NMR and GC-MS approach, analysing urine from rats given 60 and 120 mg/kg gentamicin, also dosed twice daily, for 7 days (Sieber et al. 2009). Dose-dependent significant increases NGAL were observed after 2 days and although kim-1 appeared to be raised after 1 day, it did not reach statistical significance. Pattern recognition by PCA and OPLS-DA picked out an increase in glucose excretion as the major driver between the metabolic separation of control and treated animals but the ^{1}H-NMR analysis also detected citrate, hippurate, trigonelline and 3-indoxylsulfate as having dose-dependent decreases.

It is likely that the limited number of metabolites detected as perturbed in these studies is largely caused by the dependence on PCA as a pattern recognition tool and/or the

restriction of analysing only the urine of these animals. Furthermore in order to detect perturbations, the metabolite profiles of treated animals were compared to control animals and although this is a good approach, inter-individual metabolic variation may dwarf more subtle drug-related changes. Therefore many other metabolite changes may be caused by exposure to gentamicin but go undetected.

4.2 Aims

To characterise the impact of once-daily exposure to gentamicin on the rat metabolome in order to gain insight into the mechanism of gentamicin nephrotoxicity. The methods employed will be:

- ^{1}H-NMR spectroscopy to profile pre-dose and post-dose rat urine, sera and aqueous faecal extracts

and

- Multivariate statistical analyses and metabolite identification.

4.3 Methods

4.3.1 Study protocol

Ten male 8-week old Sprague-Dawley rats were randomly divided into two dosing groups of 5 animals. Blood was obtained by sampling from the tail vein prior to the first injection. One group (n=5) received an intraperitoneal (IP) injection of 0.9% saline once daily for 9 consecutive days and served as healthy controls. The remaining 5 animals received 200 mg/kg gentamicin (dissolved in 0.9% saline) *via* I.P injection once daily for 9 days. Dosing occurred between 10.00 am and 12.00 pm. Animals were housed individually in metabolic cages 12 hours prior to the first injection and 12 hours after the first injection; animals were housed in metabolic cages again for the 24 hours after the fourth injection and finally for 24 hours after the final (ninth) injection, see Figure 4.2. 12 hour urine was collected into chilled vessels with a minimum of 0.1% wt/v sodium azide. Faeces were also collected into chilled containers and along with the urine aliquots were stored at -80°C until analysis. Body weight and overall condition were recorded daily. Animals were kept on a 12 h light/dark cycle throughout the study with free access to food and water. On day 10 of the study all animals were culled by an overdose of pentobarbitone followed by cardiac puncture. Blood was collected and allowed to clot at 4°C overnight and serum was collected and stored at -80°C. The kidneys and liver were removed and weighed; half of the right kidney and a section of the large lobe of the liver were snap frozen. The remainder of the kidney, liver and carcass was preserved in 4% paraformaldehyde for histological analysis.

4.3.2 Histology

The kidney that was tissue fixed in 4% paraformaldehyde was sagittally cut and divided into dorsal and ventral halves. The two tissue sections were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hemotoxylin-eosin. Renal lesions were assessed by microscopic examination, recording the frequency and severity using a semi-quantitative scoring scale of 0 to 5 as in Zhang et al (2009):

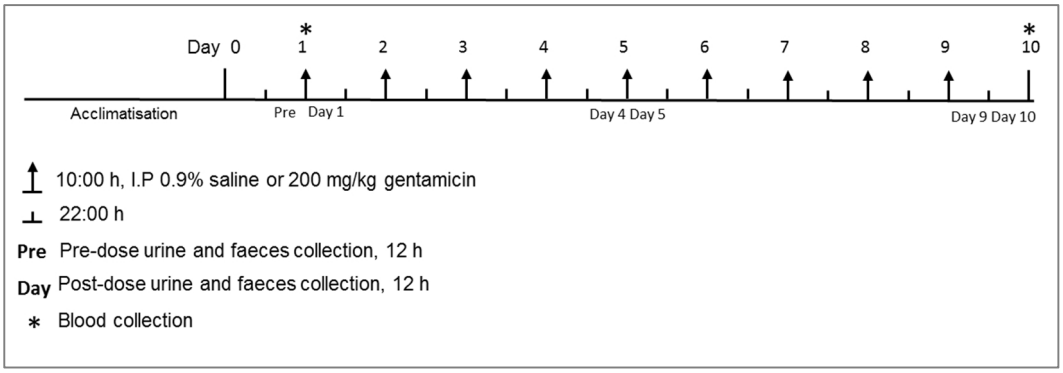


Figure 4.2 Schematic of the study protocol. Urine and faeces were collected in 12 h periods at the beginning, middle and end of the study where gentamicin or saline were administered daily.

The criteria of the scoring scale were as follows:

0 = Normal tubules, glomerulus, interstitium, and vessels.

1 = Scant number of tubular epithelial cells showing minimal degeneration, mild tubular dilatation, small number of proteinaceous casts, no regeneration, no definitely significant necrosis or apoptosis. No changes in the glomerulus, interstitium, and vessels.

2 = < 25% of tubular epithelial cells showing mild degeneration (large cytoplasmic vacuoles, a few hyaline droplets in the cytoplasm), mild degree of tubular dilation and proteinaceous casts, slight change in tubular brush border loss, acute tubular necrosis in individual cell or small group of cells, a few apoptotic cells, and no regeneration. No changes in the glomerulus, interstitium, and vessels.

3 = 25%-50% of tubular epithelial cells showing moderate degeneration (multiple large-sized vacuoles, multiple foci of hyaline droplets), mild regeneration, moderate tubular brush border loss, moderate acute tubular necrosis in small group of tubules, and increased number of apoptotic cells. Little involvement of mild glomerular vacuolization. No changes in the interstitium and vessels.

4 = 51%–75% of tubular epithelial cells showing extensive moderate degeneration; moderate regeneration; severe tubular brush border loss; severe acute tubular necrosis; and a large number of apoptotic cells, with apoptotic bodies in clusters of tubules. Little involvement of mild glomerular vacuolization and interstitial lymphocytic infiltration.

5 = > 75% of tubular epithelial cells showing severe degeneration, regeneration, severe tubular brush border loss, acute tubular necrosis, and a large number of apoptotic cells with numerous apoptotic bodies. Mild involvement of glomerular injury (vacuolization, mesangial cell proliferation, and increase in mesangial matrix) and interstitial lymphocytic infiltration. No significant changes in the vessels.

4.3.3 Biochemical measurements

4.3.3.1 Blood urea nitrogen

Blood urea nitrogen (BUN) was measured using the QuantiChrom Urea Assay kit (Bioassay Systems, Hayward, CA). Serum samples were diluted 1 in 2 with distilled water before running the colorimetric assay and quantified using urea calibration standards (0.78-50 mg/dL).

4.3.3.2 Urine creatinine

Urine (25 µL of urine diluted 1 in 25) was incubated with 125 µL of alkaline picrate solution (0.14 M NaOH, 10 mM Na_2HPO_4, 90 mM sodium borate, 10 mM sodium dodecyl sulphate, 25% v/v picric acid, 2.7% v/v DMSO) for 2 min at room temperature. An acid solution (5 µL of 10:2:88 v/v acetic acid: concentrated H_2SO_4:distilled water) was added and incubated at room temperature for a further 10 min with shaking. Plates were read at 490 nm and urine creatinine (UCr) was estimated using creatinine calibration standards (2.5-40 mg/dL).

4.3.3.3 N-acetyl-β-glucosaminidase

N-acetyl-β-glucosaminidase (NAG) was quantified using a colorimetic assay kit (Roche, West Sussex UK). Urine samples were diluted up to 1 in 5 with distilled water to fit within the

quantifiable range (Optical density <1). NAG concentration was quantified using the following equation:

$$\text{Concentration (U/mL)} = (\text{absolute-blank}) * 33.33/1000$$

4.3.3.4 Kidney injury molecule-1

Urinary kidney injury molecule-1 (kim-1) was quantified using a sandwich ELISA as described previously (Vaidya et al. 2006). In brief, the antibody monoclonal anti-rat Kim-1 ectodomain, served as a primary antibody and coated the wells of the ELISA plate. The wells were blocked with 3% BSA/PBS with 0.02% sodium azide and washed with PBST. One hundred microliters of standard or sample were incubated in duplicate for 1.5 h at 37°C. Following three washes with PBST, the secondary antibody (biotinylated monoclonal anti-rat Kim-1 ectodomain) and HRP-conjugated streptavidin was added for 15 min. The reaction was stopped by addition of 1 N HCl and absorbance was read at 450 nm. Urinary kim-1 was calculated using a calibration curve, expressed as ng/mL and normalised to UCr.

4.3.4 Metabolic profiling

4.3.4.1 Sample processing

Urine samples from each animal were thawed, vortexed and allowed to settle for 10 minutes before 400 µL of urine was mixed with 200 µL of 0.2 M phosphate buffer (with 3mM NaN_3, 1mM TSP in 100% D_2O). The mixtures were centrifuged at 13 000 rpm for 10 minutes at 4°C and 550 µL was transferred into 5 mm NMR tubes. Serum samples were processed in the same manner except for 200 µL of serum was mixed with 400 µL of saline (0.9% NaCl in 100% D_2O).

For faecal processing, approximately 50 mg of each faecal pellet was weighed, mixed with 750 µL of 0.2 M phosphate buffer and homogenised in a bead beater in two cycles (6500 Hz, 45 seconds per cycle). The homogenate was centrifuged at 13 000 rpm for 10 minutes at 4°C and 550 µL of supernatant was transferred to 5 mm NMR tubes.

4.3.4.2 Acquisition of ^{1}H NMR spectra

4.3.4.2.1 Urine and aqueous faecal extracts

One-dimensional (1D) ^{1}H-NMR spectra were acquired on a Bruker 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a temperature of 300 K using the standard 1D solvent suppression pulse sequence (relaxation delay, 90° pulse, 4 μs delay, 90° pulse, mixing time, 90° pulse, acquire FID) (Beckonert et al. 2007). 256 scans were collected into 64,000 data points using a spectral width of 12 MHz, a relaxation delay 4 s, an acquisition time of 2.72 s and a mixing time of 0.1 s. A line broadening factor of 0.3 Hz was applied to all spectra prior to Fourier transformation (FT).

4.3.4.2.2 Sera

The CPMG (Carr-Purcell-Meiboom-Gill) spin-echo pulse sequence (spin-spin relaxation delay, 2 n τ of 80 ms; n= 200, τ = 200 μs) with water presaturation was applied to profile sera in order to attenuate broad resonances from high molecular weight compounds (Beckonert et al. 2007). 384 scans were collected into 64,000 data points using a spectral width of 12 MHz with a relaxation delay 4 s, an acquisition time of 2.72 s and a mixing time of 0.1 s. Presaturation of the water signal was applied during the relaxation delay and mixing time. A line broadening factor of 0.3 Hz was applied to all spectra prior to Fourier transformation (FT).

4.3.4.3 Spectral processing

Spectra were manually phased and base-line corrected using TopSpin 3.2 software. Urine and faeces spectra were calibrated on the chemical shift scale using TSP (sodium 3-trimethylsilyl-[2,2,3,3,-^{2}H4]-1-propionate) set to 0 ppm. Spectra derived from sera were calibrated to the anomeric proton resonance of alpha-glucose (5.25 ppm). All spectra were imported into MATLAB 2013 using MetaSpectra 4.1.1. Regions containing urea and water were removed and in the urine and faeces spectra, TSP-related peaks were also removed. Several urinary spectra were excluded from further processing and analysis due to poor quality spectra dominated by a urea peak. Spectra were aligned to account for between

sample chemical shift variations caused by differences in pH by recursive segment-wise peak alignment (Veselkov et al. 2009) and normalised using probabilistic quotient normalisation (Dieterle et al. 2006) using in-house scripts.

4.3.4.4 Multivariate analysis

Urine spectra were subjected to unsupervised and supervised multivariate methods: Principal Component Analysis and Orthogonal Partial Least Squares-Discriminant Analysis.

4.3.4.4.1 Principal component analysis

PCA analysis was carried out on the control and gentamicin groups to identify inherent clustering and outliers. PCA was performed using SIMCA 13.0 software using unit variance (UV) scaling.

4.3.4.4.2 Orthogonal partial least squares - discriminant analysis

OPLS-DA analysis generated models by comparison of two groups of samples at a time. Models were generated using MATLAB 2013 and in-house scripts (Imperial College, London). Models were validated by permutation tests (1000 permutations) and calculation of the Q^2Y (the predictive capacity of the model) and the R^2Y (the goodness of fit to the Y data) values. Metabolites which contributed to significant models were identified using Chenomx Profiler software (Chenomx NMR Suite), 'spike-in' experiments with pure compounds and databases - the Human Metabolome Database (www.hmdb.ca/spectra/nmr_one_d/search), the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu/metabolomics).

Data are presented as OPLS-DA loadings coefficient plots (Cloarec et al. 2005b) where the loadings are back-scaled to highlight the urinary metabolites which are discriminant between the two groups and displays the associated correlation coefficient with a colour scale demonstrating the strength of the correlation; dark red indicates a very significant correlation between the groups and at the other end of the scale, dark blue indicates little variation between the groups.

4.3.4.5 Estimation of gentamicin excretion from ^{1}H-NMR spectra

The integral of the gentamicin singlet at 2.76 ppm in the urine ^{1}H-NMR spectra was adjusted according to the number of protons; excretion was estimated using the calculated creatinine excretion which was based on the proton-adjusted integral of the creatinine singlet at 4.04 ppm and the concentration of creatinine as measured by the Jaffe assay. Integration was achieved using MATLAB 2013 and in-house scripts (Imperial College, London) by selecting the upper and lower ppm scale boundaries of the region of interest.

4.4 Results

4.4.1 Body weight changes

Overall body weight (BW) increased from initial BW in all animals throughout the 10 day study except for animal 9 (gentamicin group) on day 10 where there was a decrease of 1.2%, see Figure 4.3. Body weight increases between groups were statistically different from day 4 of the study (T test, unpaired, p=0.019) where the control group had a mean BW gain of 12.6±3.7% (n=5) whereas the gentamicin group had a mean BW gain of 8.0±2.1% (n=5). This trend continued to the end of the study where on day 10 the mean BW gain of the control animals (mean BW on day 10, 336.2±17.0g, n=5) was 28.1±4.4% and in the gentamicin animals (mean BW on day 10, 286.9±12.3g, n=5) the mean BW gain was 7.7±4.7% (T test, unpaired, p<0.00005).

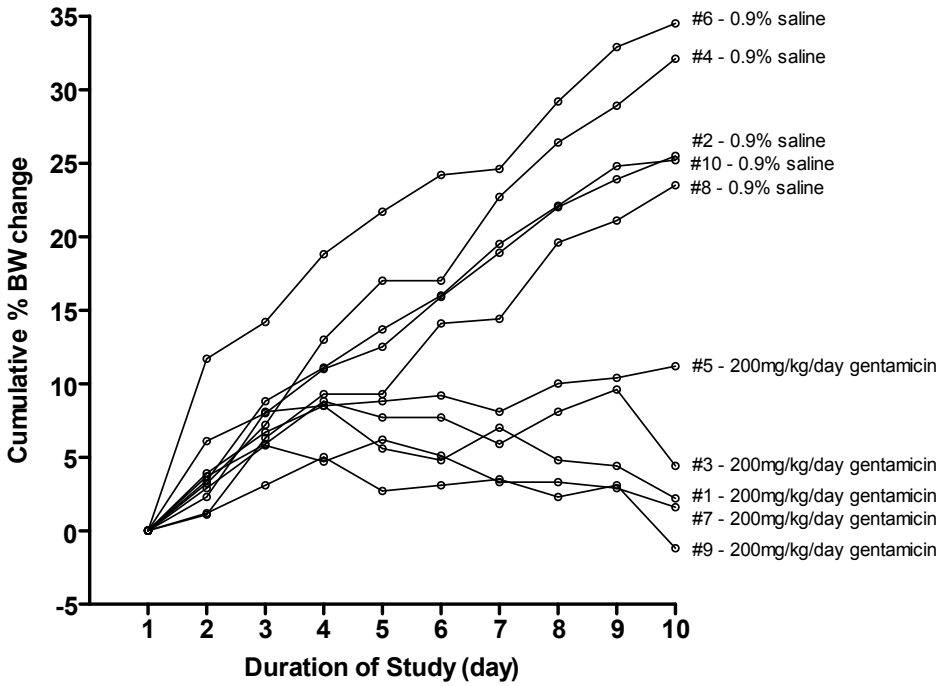


Figure 4.3 Body weight changes of male Sprague-Dawley rats given daily I.P injections of 0.9% saline or 200 mg/kg gentamicin for 9 days.

4.4.2 Kidney and liver observations

The mean weight of both kidneys at the end of the study in saline-treated animals (2.52±0.15g, n=5) was 0.75±0.06% of total BW whereas in the gentamicin-treated animals (3.18±0.31g, n=5) it was 1.15±0.11% of total BW ($p<0.001$), see

Figure 4.4A. During the necropsy the kidneys of the saline group were dark-reddish in colour whilst the kidneys of each animal in the gentamicin group appeared blanched and pale and were surrounded by an oedematous fluid filled sac. The mean weight of the liver of saline-treated animals was 10.03±1.68g (n=5), representing 4.16±0.32% of total BW; in the gentamicin group the mean liver weight was lower at 7.21±0.79g (n=5), which represented a smaller proportion of total BW, 3.45±0.38% ($p<0.05$), see

Figure 4.4B. Although there was a disparity in liver weight between the control and gentamicin groups, there were no lesions and they were a dark-red colour.

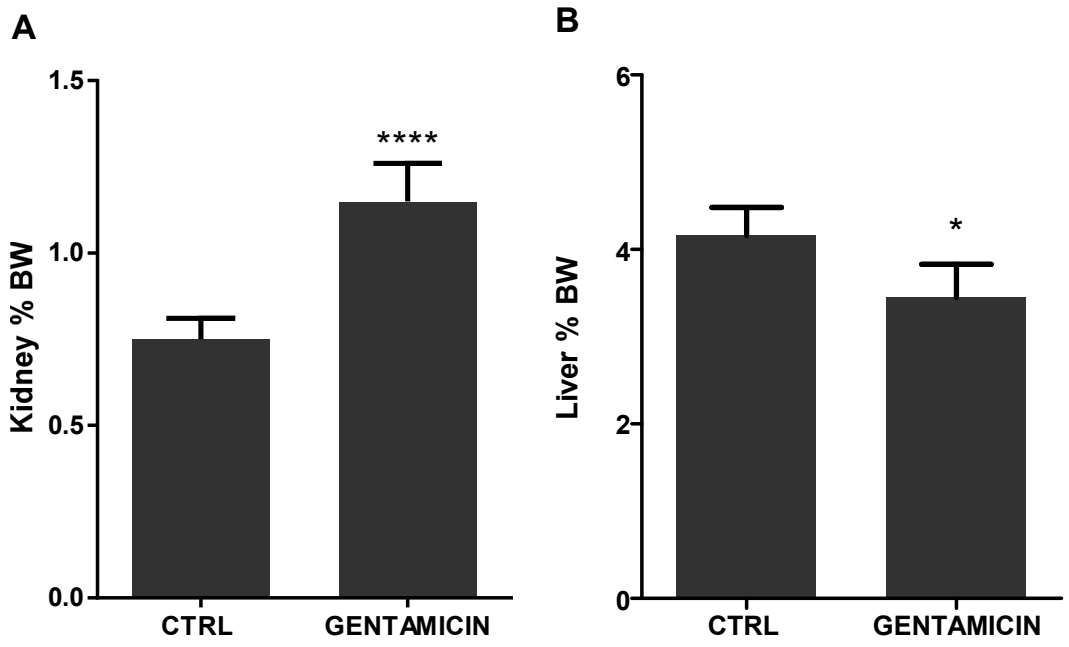


Figure 4.4 Kidney (A) and liver (B) weight as a percentage of total body weight in animals administered 0.9% saline or 200 mg/kg gentamicin for 9 days. Data are presented as the mean ± standard deviation (n=5). * $p<0.05$, **** $p<0.0005$ (Unpaired T Test).

4.4.3 Urine volume

Urine output was recorded in six 12 h collections prior to the first dose and throughout the study. Pre-dose animals (n=10) had a mean urine output of 3.25±1.32 mL over a 12 h overnight period (10pm-10am). In the 12 h period (10am-10pm) immediately after the first dose the mean urine output of the saline-treated animals was 2.04±0.00 mL (n=5) and in gentamicin treated animals 2.27±0.55 mL (n=5). On subsequent collections gentamicin-treated animals had a greater mean urine output than saline-treated animals in the equivalent 12 h collection. Despite the greater mean urine output in the gentamicin group in the day 10 collection (10pm-10am) there was also greater variation within the gentamicin group with a urine output range of 4.0-29.0 mL (n=5) compared to 3.5-6.0 mL (n=5) in the saline group. Animals 1 and 3 in the gentamicin group had a more than 7-fold greater urine output compared to animal 9, see Figure 4.5.

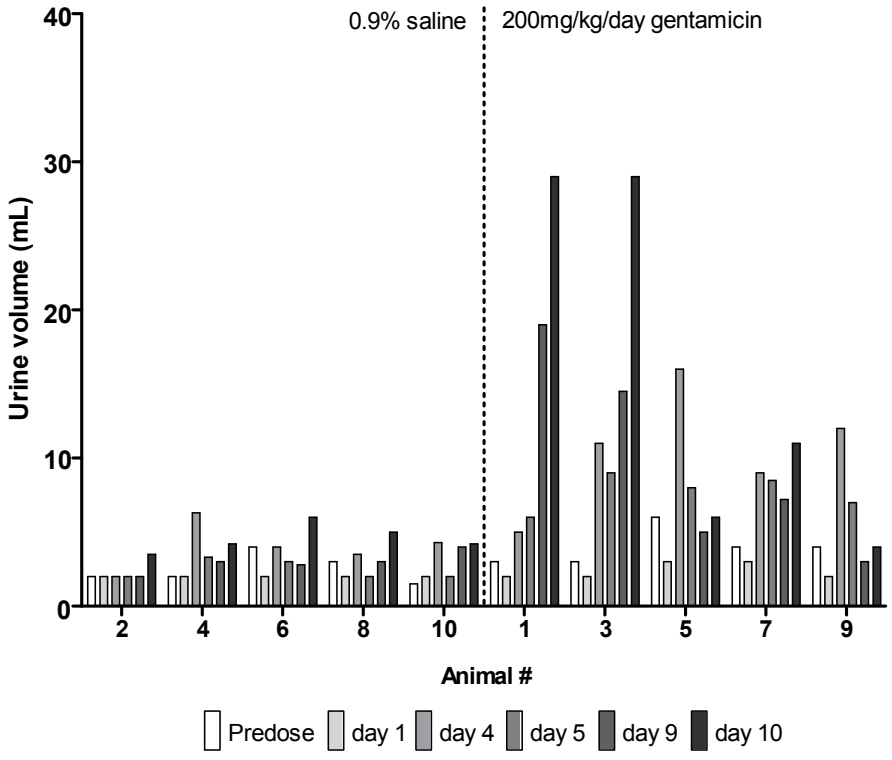


Figure 4.5 Recorded urine production throughout the study of rats administered either 0.9% saline or 200 mg/kg gentamicin daily for 9 days.

4.4.4 Histology

In control animals no histological abnormalities were observed in the liver and kidney tissue except for animal 4 which had random multifocal interstitial infiltration, see Appendix 2 for a detailed description of the liver and kidney tissue of each animal. In the gentamicin group apoptotic cells were observed. Vacuolation, hyaline (protein) droplets and tubular protein casts were present in all animals. Animals 3 and 5 had a tubular protein cast score of 2.5 whilst animals 1, 7 and 9 had a score of 3. In all animals receiving gentamicin, examination of the kidney tissue revealed multifocal mononuclear (lymphocytes or macrophages) interstitial infiltration. Animals 7 and 9 had occluded glomeruli. The liver tissue of animal 9 had a mild increase in leukocytes between hepatic chords and animals 5 and 9 had low liver glycogen. Animals 3 and 5 had a histology score of 3.5 whilst animals 1, 7 and 9 had a histology score of 4.

4.4.5 Biochemical measurements

A detailed description and discussion of the biochemical measurements is given in Chapter 5.

4.4.5.1 β-N-acetyl-glucosaminidase

Pre-dose urinary NAG excretion was 14.88±3.18 U/mg UCr (n=10). NAG excretion in the 12 h period immediately following the initial dose was statistically greater in animals administered 200 mg/kg gentamicin compared to 0.9% saline (T test, unpaired $p<0.0005$) and throughout the study where on day 10 an overnight urine collection gave a mean NAG excretion of 15.97±5.99 U/mg UCr in the saline group compared to 307.50±114.7 U/mg UCr in the gentamicin group.

4.4.5.2 Kidney-injury molecule 1

Pre-dose urinary excretion of kim-1 was measured as 0.39±0.22 ng/mg UCr. Throughout the study the excretion of kim-1 ranged from 0.25 – 1.73 ng/mg UCr in the saline group. Kim-1

excretion was significantly greater in the gentamicin group relative to the saline group from day 4 (p=0.044) to the end of the study on day 10 (T test, unpaired, p=0.001).

4.4.5.3 Blood urea nitrogen

The mean pre-dose BUN was determined to be 30.06±12.71 mg/dL (n=10). For the saline treated group, mean BUN at the end of the study was 20.97±2.82 mg/dL (n=5). After 9 doses of 200 mg/kg/day gentamicin, the mean BUN measured was 191.93±61.84 mg/dL (n=5). The animal with the largest change in BUN was animal 9 with a 15-fold increase whereas animal 7 had the smallest at 4.3-fold.

4.4.5.4 Gentamicin excretion

The urinary excretion of gentamicin was greatest in the 12 h collection following the initial dose (day 1) but varied between animals (range of 43.1-100.2 mg/h). In the subsequent daytime collection on day 4, urine gentamicin excretion was considerably lower with a range of 4.6-10.6 mg/h, see Figure 4.6. On day 9 gentamicin excretion in animals 1 and 7 increased relative to day 4 (37.8-71.7 mg/h), whereas gentamicin excretion for animals 3, 5 and 9 remained low (2.2-13.2 mg/h).

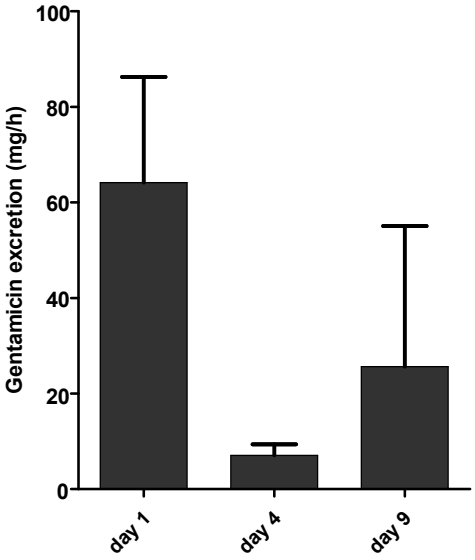


Figure 4.6 Urine gentamicin excretion in daytime collections on day 1, 4 and 9, calculated by the integral of 2.76 ppm and urine creatinine concentration and integral of 4.04 ppm.

4.4.6 Principal component analysis

4.4.6.1 Urine

4.4.6.1.1 Pre-dose and gentamicin-treated animals

Principal Component Analysis (PCA) was performed on the ^{1}H NMR urine spectra acquired from animals pre-gentamicin dose (n=5) and on day 1 (12 h), day 4 (120 h), day 5 (132 h), day 9 (228 h) and day 10 (240 h) of the study. A PCA scores plot for this model is shown in Figure 4.7 where 27.3% of the variance can be explained by the latent variable PC1 and 15.1% by PC2. Pre-dose, day 1, day 4 and day 5 animals form group clusters whilst the day 9 and day 10 samples show within group variation. The metabolic profiles shift away from the pre-dose profiles from day 1 (12 h after the first dose) and continue to shift throughout the study. Day 9 urine from animals 3 and 9 and day 10 urine from animals 3 and 7 are isolated from the metabolic space occupied by the other animals at the same time points and are closer to pre-dose profiles.

Diurnal variation is also evident between the two 12 h collections from the same 24 h period as exemplified by pre-dose and day 1, day 4 and day 5, and by day 9 and day 10. As such, PCA was performed on matching 12 h collections (10:00-22:00 h or 22:00-10:00 h). The PCA scores plot of pre-dose, day 4 and day 10 (22:00-10:00 h) is shown in Figure 4.8A. In this model 38.3% of the variance can be explained by the first latent variable PC1, and 14.2% can be explained by PC2. The separation between pre-dose and day 4 clusters is greater whilst the variation on day 10 is evident by the spread of animals 1, 5 and 9 and the clustering of animals 3 and 7 between the pre-dose and day 4 samples. In the PCA model of matched time points day 1, day 5 and day 9 (10:00-22:00 h) 27.4% of variance can be explained by the first latent variable PC1 whilst 19.8% can be explained by PC2, see Figure 4.8B. Day 1 urines (12 h after the first dose) are clustered whilst the metabolic space occupied by day 5 urines (5 doses) is greater. On day 9 (9 doses), animals 3 and 9 are

clustered near day 1 and animals 1 and 5 are separated, with animal 7 distinct from the others.

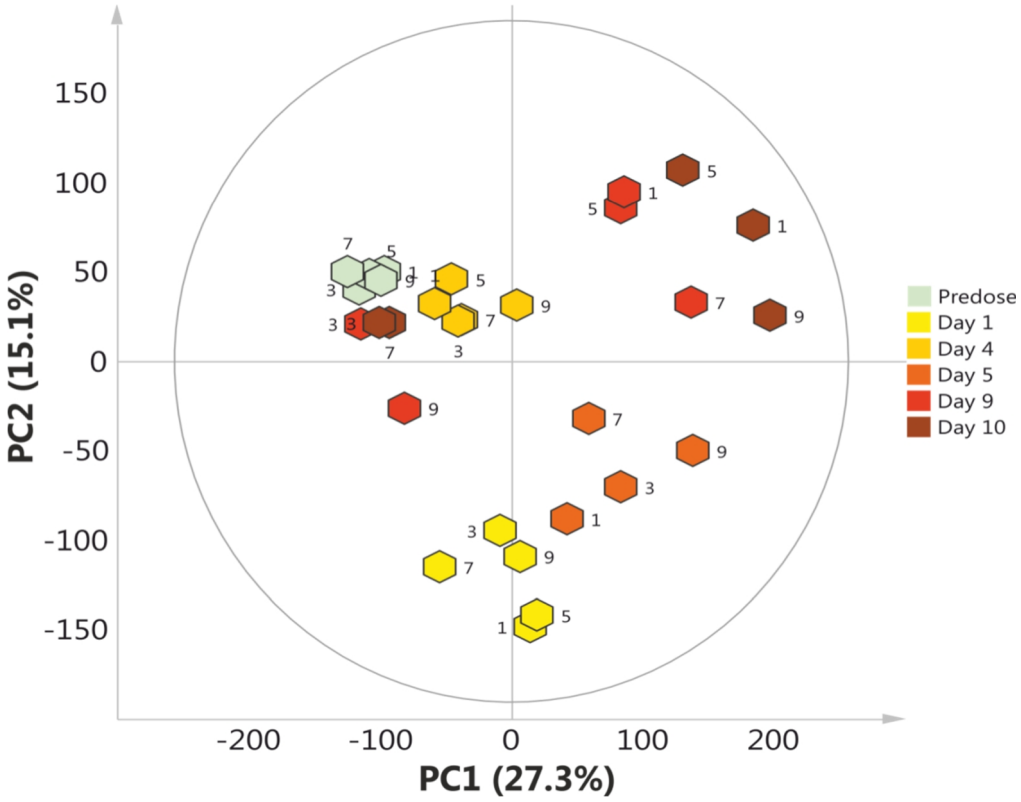


Figure 4.7 PCA scores plot of PC1 vs PC2 obtained from ^{1}H-NMR spectra from pre-dose urine and gentamicin treated animals; R^2=0.655, Q^2=0.405.

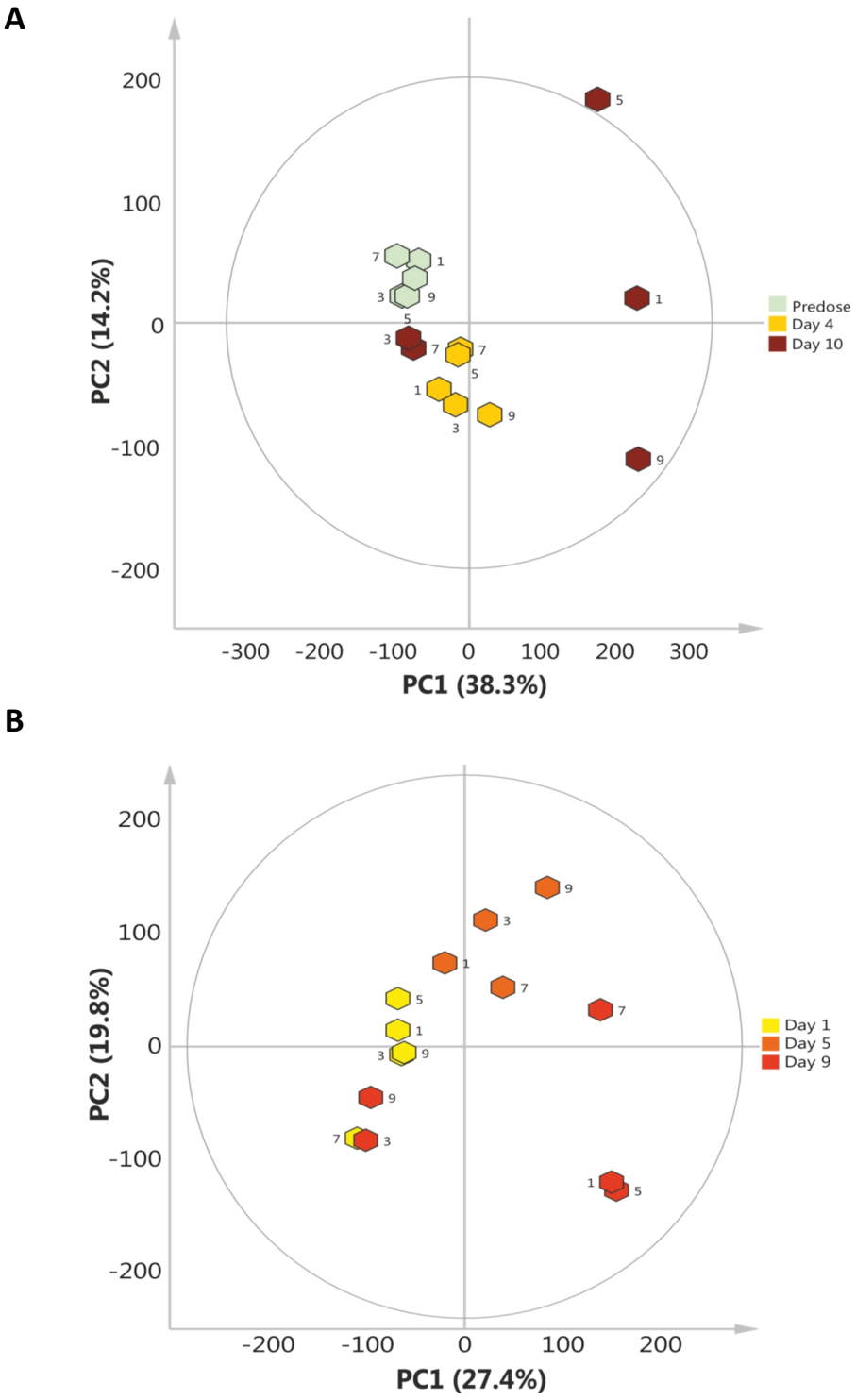
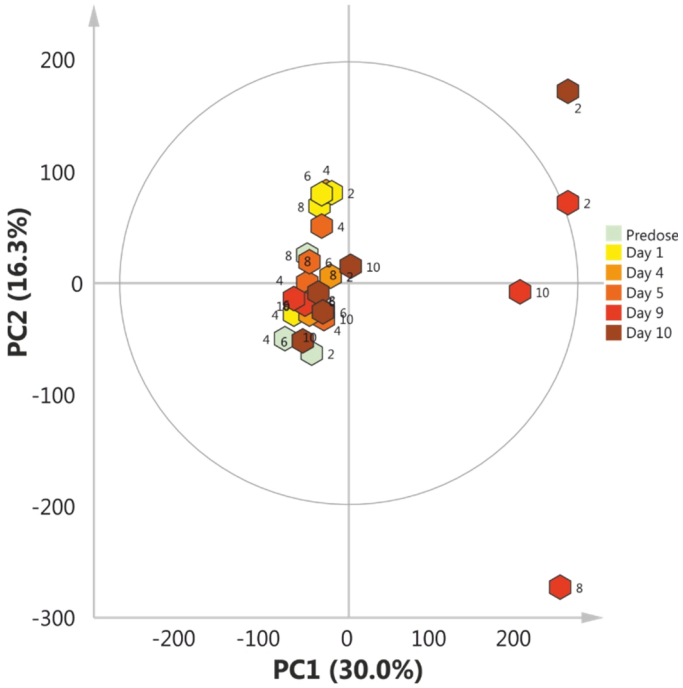


Figure 4.8 PCA scores plot of PC1 vs PC2 obtained from ^{1}H-NMR spectra from (A) pre-dose urine and urine from gentamicin treated animals on day 4 and day 10 of the study, R^2=0.525, Q^2=0.267. Samples were collected overnight, 22:00-10:00 h, and (B) urine from gentamicin treated animals on day 1, day 5 and day 9 of the study, R^2=0.639, Q^2=0.287. Samples were collected during the day, 10:00-22:00 h.

4.4.6.1.2 Saline control animals

PCA was performed on all ^{1}H NMR urine spectra obtained from the 5 animals dosed 0.9% saline throughout the study. In this model 30% of the variance could be explained by PC1 and 16.3% by PC2. The majority of the control animals clustered throughout the study, whilst animals 2, 8 and 10 on day 9 and animal 2 on day 10 were outliers, see Figure 4.9.

A



B

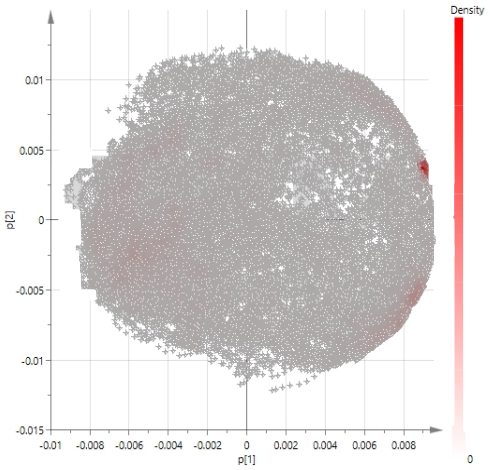


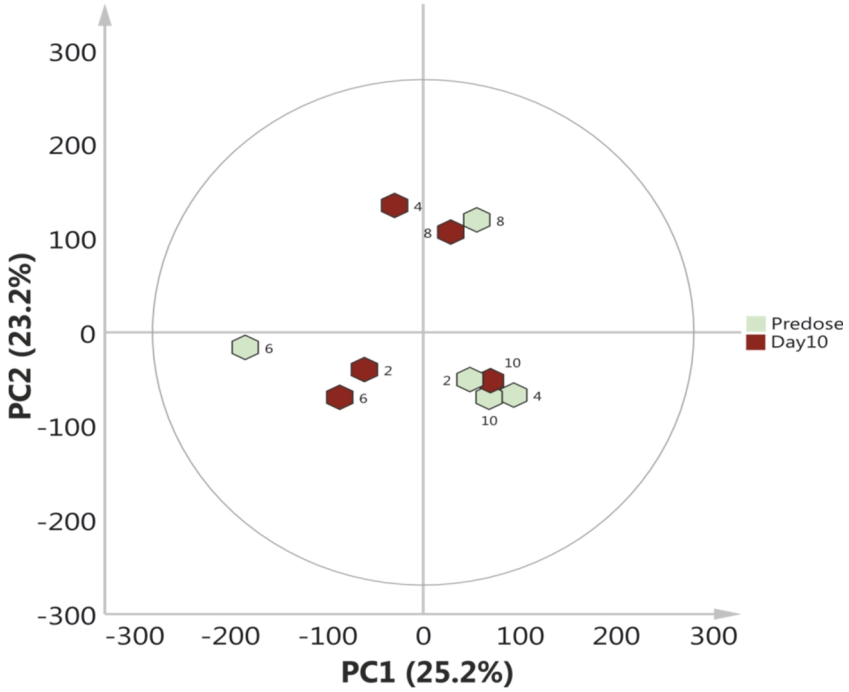
Figure 4.9 PCA scores (A) and loadings (B) plot of PC1 vs PC2 obtained from ^{1}H-NMR spectra from pre-dose urine and urine from saline treated animals, R^2=0.64, Q^2=0.311.

4.4.6.2 Serum

4.4.6.2.1 PCA models of pre-dose and day 10

PCA models of pre-dose and terminal time-points from saline-treated animals are displayed in a scores plot in Figure 4.10A, PC1 explains 25.2% of the variance and PC2 accounts for 23.2%. The pre-dose and day 10 metabolic profiles of animals 8 and 10 show little variation whereas animals 2, 4 and 6 occupy a different metabolic space compared to pre-dose. In a PCA model of pre-dose and day 10 sera from gentamicin-treated animals PC1 explained 31.3% of the variance and PC2 17.6%. Animals 3, 5, 7 and 9 cluster at pre-dose with animal 1 separated along PC1. On day 10 animals 1 and 9 are occupy a different metabolic space compared to pre-dose whilst animal 3, 5 and 7 are clustered with the pre-dose sera, see Figure 4.10B.

A



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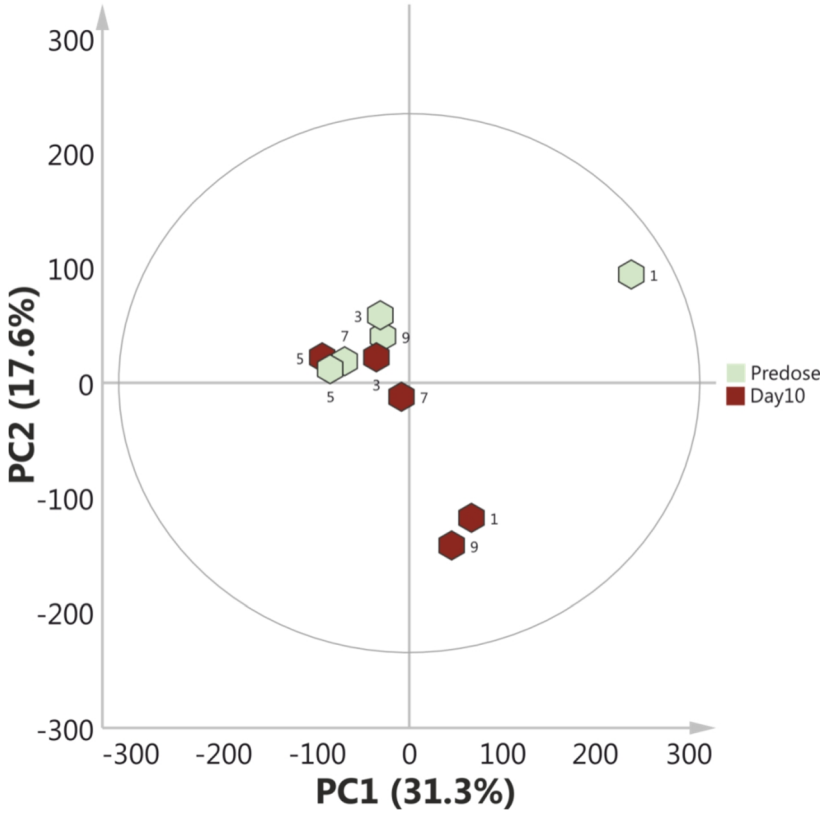


Figure 4.10 PCA scores plot of ^{1}H-NMR spectra of sera obtained (A) pre-dose and day 10 in the saline group, R^2=0.484, Q^2=0.044 and (B) from the gentamicin group, R^2=0.489, Q^2=0.-0.1.

4.4.6.3 Aqueous faecal extracts

4.4.6.3.1 Pre-dose and gentamicin-treated animals

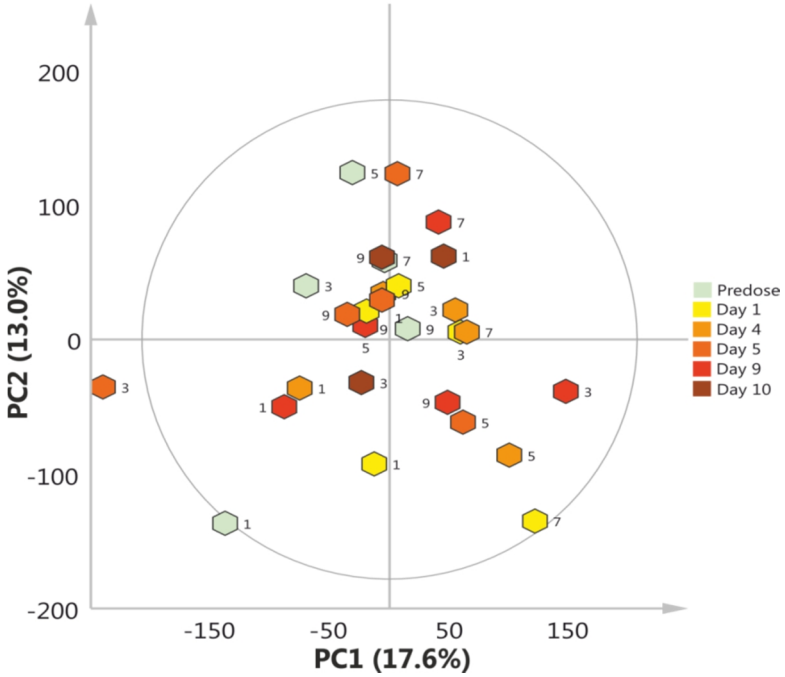
PCA was performed on all ^{1}H NMR data acquired from aqueous faecal extracts from animals pre-gentamicin dose (n=5) and on day 1 (12 h), day 4 (120 h), day 5 (132 h), day 9 (228 h) and day 10 (240 h) into the study. A PCA scores plot for this model is shown in Figure 4.11A where 17.6% of the variance can be explained by the latent variable PC1 and 13% by PC2. Clustering of animals at corresponding time-points was not achieved indicative of large metabolic variation in the sample matrix. Animals 5 and 7 did not produce faeces during the day 10 collection.

4.4.6.3.2 Saline-treated and gentamicin-treated animals

PCA was performed on the ^{1}H-NMR spectra of aqueous faecal extracts obtained from animals treated with 0.9% saline and with 200 mg/kg/day gentamicin. A PCA scores plot of control (n=5) and gentamicin-treated (n=5) animals on day 9 of the study is shown in Figure 4.11B. In this model 21.6% of the variance can be explained using PC1 and 18.9% in PC2. Saline-treated animals cluster with gentamicin-treated animal 7 whereas the remaining gentamicin-treated animals are dispersed along PC1.

The large amount of variability seen in the PCA models did not allow for meaningful interpretation of OPLS-DA models; therefore OPLS-DA models of faecal extract metabolites were not examined.

A



B

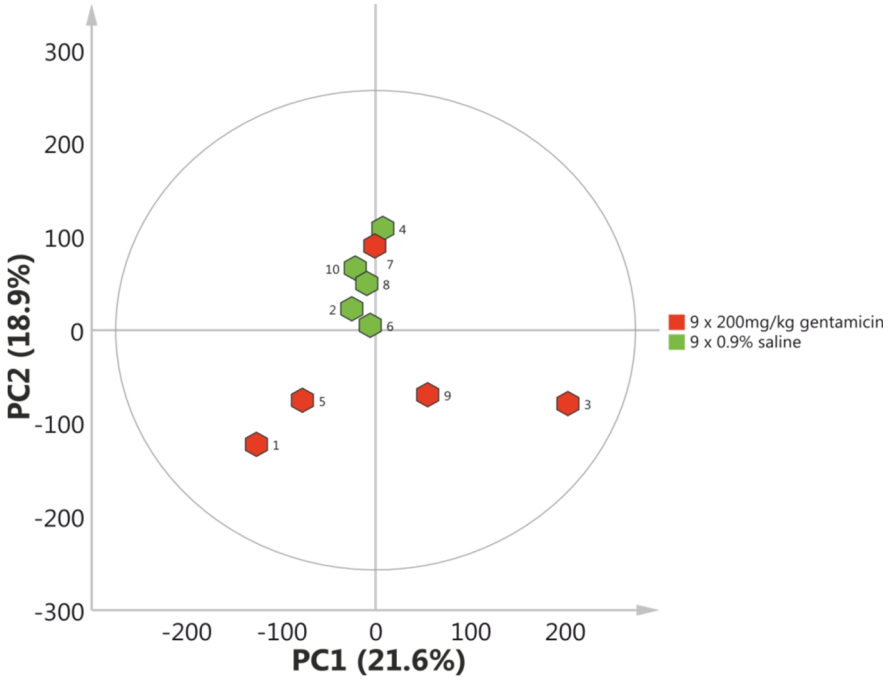


Figure 4.11 PCA scores plot of PC1 vs PC2 obtained from ^{1}H-NMR spectra from (A) pre-dose faeces and faeces from gentamicin treated animals, R^2=0.573, Q^2=0.183 and (B) day 9 faeces from gentamicin and saline treated animals, R^2=0.404, Q^2=-0.1.

4.4.7 OPLS-DA models

PCA indicated that substantial changes in urinary, serum and faecal metabolite composition had occurred due to the administration of gentamicin. OPLS-DA models of urine and sera were computed to determine which metabolites were most discriminatory by comparing the post-dose spectra to pre-dose spectra. Changes specific to gentamicin administration were determined by examining metabolic changes in saline treated animals at matched time-points.

4.4.7.1 Urine

4.4.7.1.1 Pre-dose and gentamicin-treated animals

OPLS-DA models of pre-dose and day 1 urine spectra were analysed in order to establish early changes in the 12 hours following the first 200 mg/kg dose of gentamicin. The Q^2 value, which describes the predictive capacity of the model, was high at 0.9346 and the R^2Y, indicating the goodness of fit to the *Y* data (pre-dose or day 1), was also high at 0.9978. Permutation analysis of this model with 1000 random permutations gave a significant model with p=0.007. Figure 4.12 displays an OPLS-DA loadings coefficient plot of pre-dose compared to day 1 spectra. Metabolites in greater abundance in the day 1 urine include glucose, 1-methylnicotinamide and allantoin. Gentamicin was also present in the urine at 12 h following the initial dose.

Metabolites found to be depleted on day 1 in comparison to pre-dose were leucine/isoleucine, 3-hydroxyisobutyrate, acetate, succinate, 2-oxoglutarate, citrate, cis-aconitate, choline, TMAO, trigonelline, fumarate, hippurate and formate. Several resonances that were highlighted as discriminant between the groups remain unassigned; 1.295 ppm (m), and 5.15 ppm were increased relative to pre-dose whereas 3.6 ppm (s), 3.65 ppm and 7.09 ppm (s) were less abundant. The OPLS-DA scores plot (Figure 4.12) for this model illustrates the separation between pre-dose and day 1 urine; the Tcv axis explains the major sources of biological variation in the data (predictive) where pre-dose

and day 1 form distinct clusters, whilst the TY(osc) axis explains the variation which is not related (uncorrelated) to discrimination of the classes pre-dose or day 1.

Models comparing pre-dose urine spectra with mid-study (day 4 and 5) samples were also performed to provide information on the progression of gentamicin-induced kidney injury. The OPLS-DA model of pre-dose compared to day 4 urine also had good predictive ability (Q^2 =0.771) and goodness of fit to the groups (R^2Y=0.9896), see Figure 4.13. In this model putrescine was increased relative to pre-dose as well as two unidentified resonances at 7.17 ppm and 7.23 ppm. Similarly to the pre-dose/day 1 model, 3-hydroxyisobutyrate, citrate, cis-aconitate, 3.6 ppm, 7.09 ppm and hippurate were reduced compared to pre-dose. Methylamine, 3-indoxylsulfate and unassigned resonances at 1.69 ppm, 3.16 ppm, 7.31 ppm were also reduced on day 4.

Analysis of the OPLS-DA model of pre-dose and day 5 urine spectra highlighted a reduction in leucine/isoleucine, 3-hydroxyisobutyrate, acetate, succinate, citrate, methylamine, 2-oxoglutarate, cis-aconitate, choline, TMAO, fumarate, 3-indoxylsulfate, hippurate, formate and trigonelline, relative to pre-dose, see Figure 4.14. Several unassigned metabolites were also found to be raised on day 5 relative to pre-dose at 1.295 ppm (m), 2.93 ppm (s) and 3.52 ppm (thought to be a sugar) and multiple unassigned resonances were found to be reduced relative to pre-dose at 3.16 ppm, 3.6 ppm, 3.73 ppm and 3.91 ppm.

OPLS-DA models comparing pre-dose and end-point urine spectra were analysed for metabolite changes following once-daily intraperitoneal injections of 200 mg/kg gentamicin for 9 days. The model statistics for pre-dose/day 9 were robust; Q^2 =0.8053 R^2Y =0.9701, permutation test p=0.007. Succinate, 2-oxoglutarate, citrate, methylamine, cis-aconitate, choline, TMAO, hippurate, allantoin, fumarate and unassigned resonances 1.69 ppm, 2.26 ppm, 3.16 ppm, 3.6 ppm were reduced compared to pre-dose. Putrescine was increased. The models statistics of pre-dose urine spectra compared to day 10 yielded similarly robust statistics; Q^2 =0.8666 R^2Y =0.9806, permutation test p=0.002. In this model putrescine was

found to be significantly increased compared to pre-dose whilst succinate, 2-oxoglutarate, citrate, hippurate, allantoin, fumurate, formate, trigonelline and the singlet at 3.6 ppm were reduced relative to pre-dose. Glucose was raised in two spectra.

4.4.7.1.2 Saline control animals

OPLS-DA models of the control animals at the same time points were analysed to identify changes in urine metabolites that were not attributable to daily administration of gentamicin. On day 1 there was an increase in 1.18 ppm (d) and a decrease at 3.6 ppm (s), 5.41 ppm and a small decrease in trigonelline (9.13 ppm (s)) compared to pre-dose. Small decreases in trigonelline were also observed on day 5 and day 9 in the control animals. On day 5 there was a decrease in the peak area of the unassigned singlets at 2.93 ppm and 3.91 ppm compared to pre-dose. On day 9, choline was decreased compared to pre-dose. Models of day 4, day 5 and day 10 highlighted the singlet at 3.16 ppm as significantly decreased compared to pre-dose; in the gentamicin animals 3.16 ppm was also shown to be depleted on days 4, 5 and 9. Hippurate was depleted compared to pre-dose on days 4, 5, 9 and 10, see Table 4.1 for a summary of these changes. Model statistics are summarised in Table 4.2

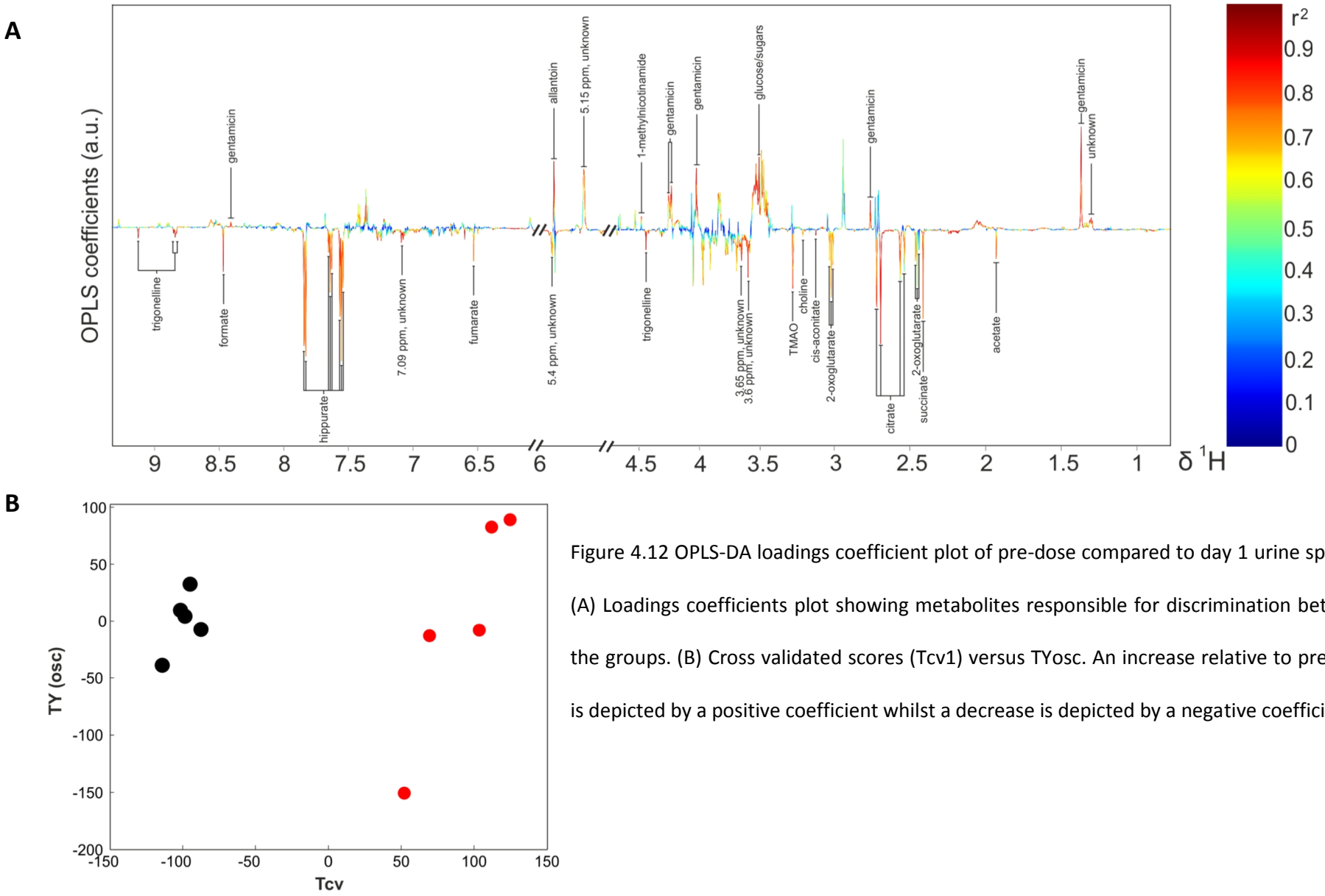


Figure 4.12 OPLS-DA loadings coefficient plot of pre-dose compared to day 1 urine spectra. (A) Loadings coefficients plot showing metabolites responsible for discrimination between the groups. (B) Cross validated scores (Tcv1) versus TYosc. An increase relative to pre-dose is depicted by a positive coefficient whilst a decrease is depicted by a negative coefficient.

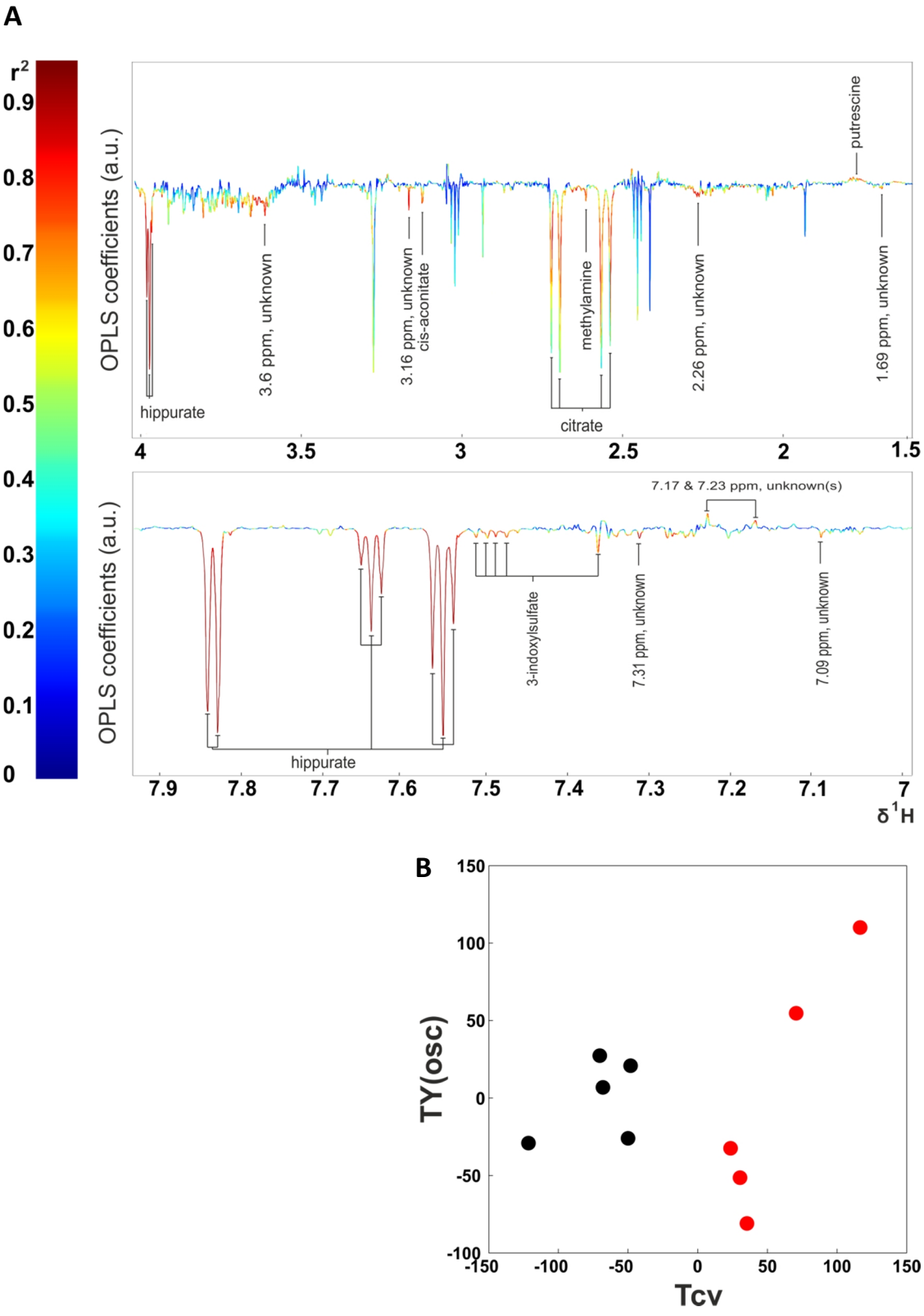


Figure 4.13 OPLS-DA loadings coefficient plot of pre-dose compared to day 4 urine spectra. (A) Loadings coefficients plot showing metabolites responsible for discrimination between the groups. (B) Cross validated scores (Tcv1) versus TYosc. An increase relative to pre-dose is depicted by a positive coefficient whilst a decrease is depicted by a negative coefficient.

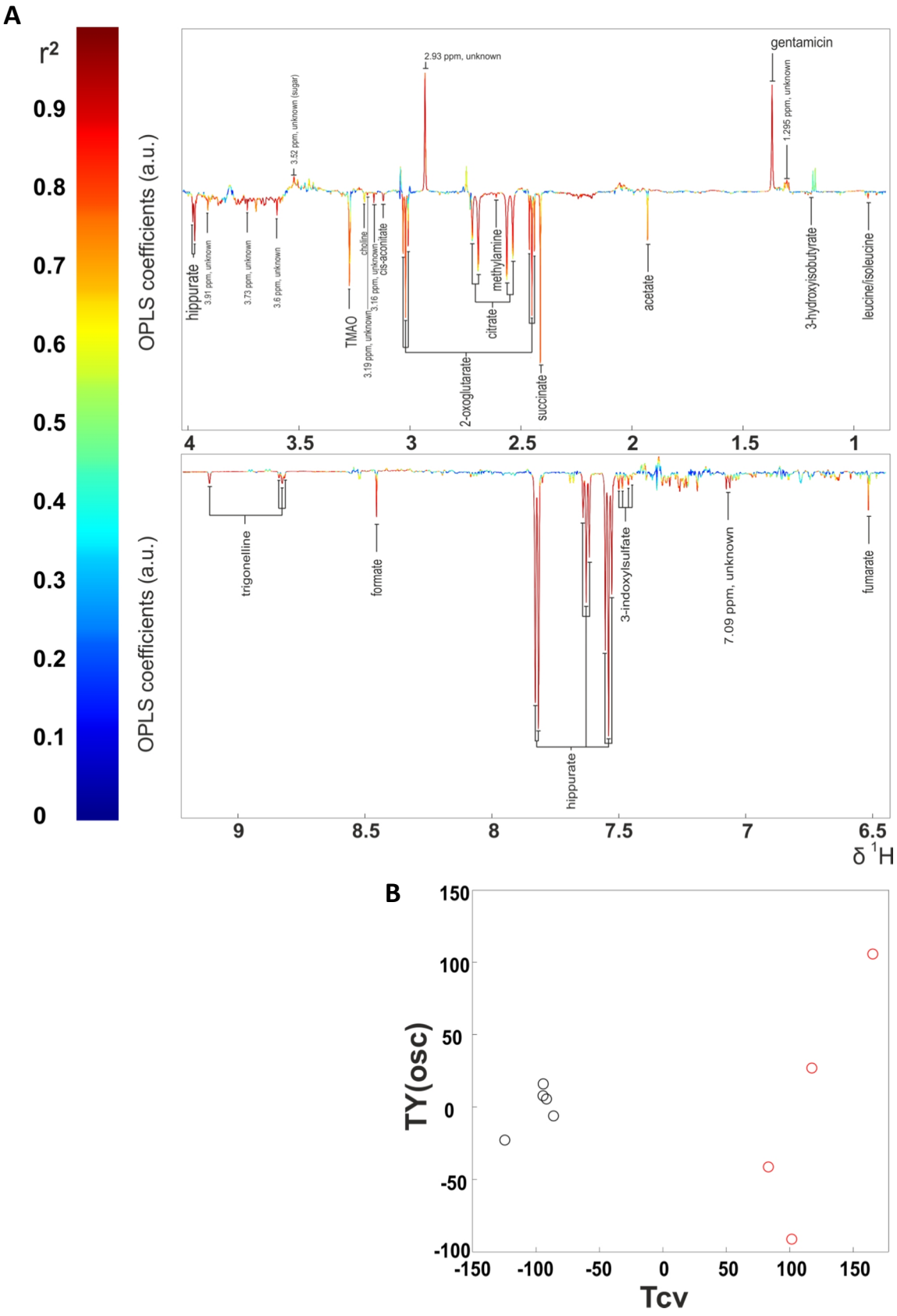


Figure 4.14 OPLS-DA loadings coefficient plot of pre-dose compared to day 5 urine spectra. (A) Loadings coefficients plot showing metabolites responsible for discrimination between the groups. (B) Cross validated scores (Tcv1) versus TYosc. An increase relative to pre-dose is depicted by a positive coefficient whilst a decrease is depicted by a negative coefficient.

Table 4.1 Summary of discriminant metabolites identified by OPLS-DA analysis of urine and sera from 0.9% saline treated animals during the course of the study.↑ indicates an increase and ↓a decrease relative to pr -dose spectra

Day of study	Urinary metabolites identified as discriminant between saline-treated and pre-dose cohorts from OPLS-DA models			
Day 1	Trigonelline↓ r^2=-0.78			1.18 ppm (d)↑ r^2= 0.84 3.6 ppm (s)↓ r^2= -0.71 5.41 ppm ↓ r^2= -0.88
Day 4		Hippurate↓ r^2= -0.63	3.16 ppm (s) ↓ r^2= -0.76	
Day 5	Trigonelline↓ r^2=-0.82	Hippurate↓ r^2=-0.84		2.93 ppm (s)↓ r^2=-0.90 3.16 ppm (s) ↓r^2=-0.83 3.91 ppm (s)↓ r^2=-0.82
Day 9	Trigonelline↓ r^2=-0.79	Hippurate↓ r^2=-0.69	Choline↓ r^2=-0.81	
Day 10		Hippurate↓ r^2=-0.58	3.16 ppm (s) ↓ r^2=-0.76	**Serum changes**: Acetate ↑r^2=0.74 Glucose ↑r^2=0.77

4.4.7.2 Serum

OPLS-DA models of pre-dose and day 10 sera were analysed for differences in metabolite composition. The model had robust statistics; Q^2 =0.7825, R^2Y=0.9753, p=0.009. Valine, leucine, isoleucine, alanine, lipids, glutamate, choline, TMAO, tyrosine, and formate were reduced compared to pre-dose. 1.05 ppm (d) (potentially 2-methylglutarate/3-hydroxyisobutyrate or isobutyrate), acetate, citrate, dimethylamine, creatinine, glucose, 3-hydroxyisobutyrate and allantoin were increased on day 10.

Table 4.2 Summary statistics of the urine and sera OPLS-DA models in gentamicin treated and saline treated animals. Models were performed comparing spectra from each day of the study with pre-dose.

Gentamicin treated		**Urine**			**Sera**		
		Q^2Y	R^2Y	Permutation test	Q^2Y	R^2Y	Permutation test
Pre-dose	**Day 1**	0.9346	0.9978	0.007			
	Day 4	0.7710	0.9896	0.009			
	Day 5	0.9431	0.9974	0.011			
	Day 9	0.8053	0.9701	0.007			
	Day 10	0.8666	0.9806	0.002	0.7825	0.9753	0.009
Saline-treated							
Pre-dose	**Day 1**	0.6236	0.9791	0.031			
	Day 4	0.4051	0.9641	0.090			
	Day 5	0.6375	0.9917	0.011			
	Day 9	0.6474	0.9744	0.027			
	Day 10	0.7278	0.9333	0.007	0.6021	0.9865	0.022

4.4.8 Summary heat map

Fluctuations in the urine and serum metabolomes over the course of the study are summarised in Figure 4.15 and Figure 4.16.

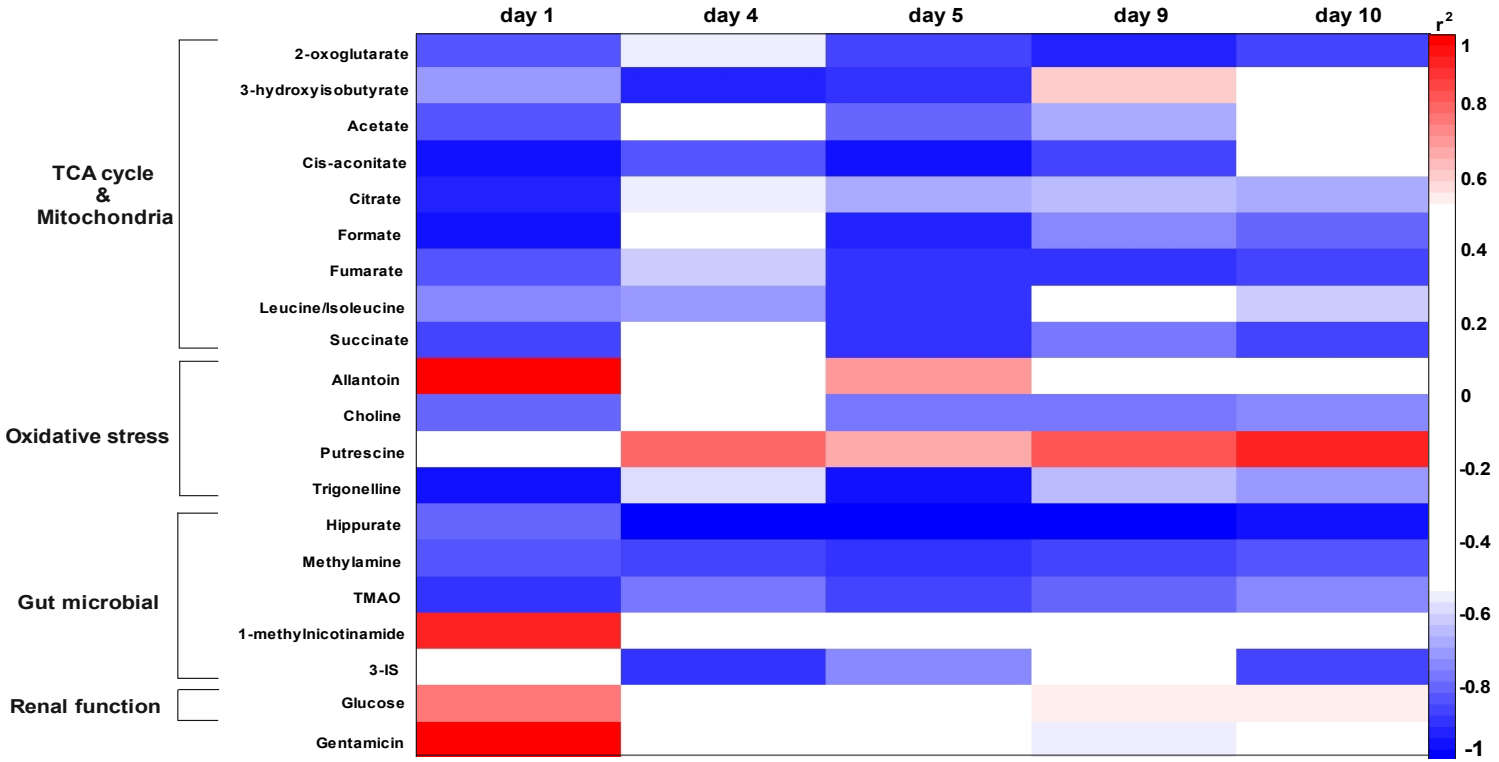


Figure 4.15 Heat map of the changes associated with discriminant metabolites from OPLS-DA models of pre-dose spectra and gentamicin treated animals. Data are loadings correlation coefficients (r^2) plotted by colour where red signifies a coefficient of +1 and blue signifies -1.

4.5 Discussion

Metabolic changes after exposure to the experimental model aminoglycoside gentamicin could give insight into the mechanism of proximal tubule injury and the progression of the toxicity. ^{1}H-NMR and multivariate analyses were performed on biofluids and aqueous faecal extracts from various time points in a study of rats treated with a high dose of gentamicin for 9 days. Gross metabolic disturbances were visualised by PCA of urine and sera from gentamicin treated rats. Unfortunately a large amount of metabolic variation within groups was identified in the PCA of faecal extracts. This may be owing to the inherent nature of the sample, and sample collection and processing methods employed. The intra-group variation did not favour further analyses using OPLS-DA.

During the study, metabolic disturbances were also identified in the control saline-treated rats. Subtle depletion of trigonelline, choline and hippurate were evident and although changes in these metabolites were also identified in the gentamicin group, it is likely that these are stress related changes as a result of repeat dosing and housing within metabolism cages which contributed to a reduction in feeding and subsequent decrease in these metabolites.

Nevertheless, widespread perturbations in the urine and sera metabolomes were identified by multivariate analyses which were symptomatic of the pharmacological activity of gentamicin, the off-target adverse effects associated with the mechanism of toxicity and the functional impairment of the kidneys. Depletion of metabolites associated with gut microbial metabolism demonstrates the antibiotic action of gentamicin whilst substantial depletion of metabolites involved in energy production and elevation of metabolites implicated in oxidative stress suggests gentamicin had a profound effect on the mitochondria of the proximal tubule epithelial cells.

4.5.1 TCA cycle metabolites and the mitochondria

The depletion of TCA cycle intermediates is an established phenomenon of gentamicin administration. Urinary citrate and 2-oxoglutarate excretion were identified as depleted (Sieber et al. 2009) after rats were exposed to gentamicin, whereas in another study citrate excretion was cited as increased (Lenz et al. 2005). OPLS-DA of urine spectra in the present study identified a more comprehensive number of TCA cycle intermediates, 2-oxoglutarate, citrate, fumarate, succinate, acetate, cis-aconitate and formate, as depleted 12 hours after the initial dose and at mid and late time points in the study. Analysis of the sera revealed elevations of acetate and citrate and a reduction in formate. The early and sustained disruption of TCA cycle intermediates indicates that gentamicin had a potent effect on the mitochondria and energy metabolism as a whole; furthermore the OPLS-DA urine and sera models highlighted a reduction and an increase in 3-hydroxyisobutyrate, respectively, which is a substrate of 3-hydroxyisobutyrate dehydrogenase, a mitochondrial enzyme involved in the degradation of branched-chain amino acids (BCAAs). After a series of reactions degradation of BCAAs produces acetyl CoA or succinyl CoA, which are fed into the TCA cycle. Accordingly we observed significant decreases in the presence of the branched-chain amino acids valine, leucine and isoleucine in the OPLS-DA models of pre-dose and day 1 urine and day 10 sera. Other amino acid changes were observed in the sera: tyrosine, glutamate and alanine were depleted on day 10 of the study. Glutamate and alanine are associated in the glucose-alanine cycle where pyruvate and glutamate form alanine and 2-oxoglutarate (also depleted in the sera) which feeds into gluconeogenesis and the TCA cycle, respectively. It is likely that the depletion of tyrosine is also associated with gluconeogenesis.

Gentamicin could also act indirectly on mitochondrial function to affect the TCA cycle, specifically through initiating apoptosis. Accumulation of gentamicin within and the subsequent rupture of lysosomes releases cathepsins into the cytosol (Servais et al. 2005),

causing cleavage of the pro-apoptotic protein BH3 interacting-domain death agonist (Bid), destabilisation of the mitochondrial membrane and the release of Cytochrome C (Mather and Rottenberg 2001), a obligate step for formation of the apoptosome and cell death. Retention of gentamicin was evident in the ^{1}H-NMR urine spectra of treated animals where excretion of gentamicin after the initial dose was high but measurements on day 4 showed lower excretion of the drug. Although from the NMR data alone it was not possible to determine which mechanism was responsible for the mitochondrial distress, the early changes in the TCA cycle intermediates seen here support findings in comparable studies in which mitochondrial disturbances occur before morphological changes and loss of renal function.

The metabolic disturbances observed in the OPLS-DA models coincided with a raised urinary level of NAG in the gentamicin-treated animals at the 12 h time-point. Conversely, kim-1, which is a more sensitive, proximal tubule injury-specific biomarker, was not raised at 12 h. Although increased NAG excretion is typically associated with proximal tubule injury, the excretion of NAG, which is a lysosomal enzyme, is known to increase with lysosomal activity too (Bosomworth et al. 1999). Given the adsorptive endocytosis of gentamicin and accumulation of the drug within lysosomes, the increase in NAG seen at this early time point is likely to be indicative of increased turnover of proximal tubular lysosomes driven by an increase in the activity of the endocytic system brought about by an influx of gentamicin in the tubular lumen, rather than the onset of proximal tubule injury.

4.5.2 Oxidative stress

Early studies on gentamicin exposure and the mitochondria were completed in isolated renal cortical mitochondria and clearly showed that gentamicin directly inhibits oxidative phosphorylation (Simmons et al. 1980) and increases production of reactive oxygen species (ROS) (Nakajima et al. 1994; Walker and Shah 1987); in addition many attempts to ameliorate gentamicin nephrotoxicity have involved co-administration of antioxidants

(Morales et al. 2002; Morales et al. 2010). Metabonomic analysis of post-dose urine spectra highlighted several endogenous metabolites which suggest an increase in oxidative stress after exposure to gentamicin. A significant rise in urine and serum allantoin was identified in OPLS-DA models of pre-dose and day 1 urine spectra and in day 10 sera. Allantoin is a non-enzymatic product of the oxidation of uric acid (derived from purine catabolism) and both uric acid and allantoin have been implicated in diseases with increased oxidative stress such as Down's syndrome (Zitnanova et al. 2004) and chronic renal failure (Kand'ar and Zakova 2008). In addition, perturbation of trigonelline, choline, glutamate and polyamine (putrescine) excretion suggests changes in glutathione (GSH) homeostasis, with each of these metabolites linked to the methionine cycle and the biosynthesis of cysteine, a precursor of GSH. Trigonelline is produced from the methylation of nicotinic acid during nicotinamide metabolism, generating by-products of s-adenosylhomocysteine (from consumption of s-adenosylmethionine). Depletion of trigonelline was noted by a previous study (Sieber et al. 2009) and could be linked to GSH depletion as suggested in the metabonomic analysis of acetaminophen-induced liver injury (Sun et al. 2008). However control animals also experienced perturbations in urine trigonelline so the significance of this metabolite to gentamicin exposure is uncertain.

Similarly, choline metabolism feeds into GSH synthesis *via* betaine and s-adenosylmethionine therefore the depletion of this metabolite in early and mid-point urine and day 10 sera could also reflect the disturbance in GSH homeostasis. Whilst choline catabolism has also been linked to gut microbiota (see section 4.5.3), the concomitant decrease in its metabolite trimethylamine N-oxide (TMAO) suggests that if the gut microbiota were responsible for the overall change in choline, then choline excretion should increase after exposure to an antibiotic. Therefore the observation that serum and urine choline has decreased in this study indicates that it is likely to be catabolised by the rat in a compensatory mechanism for the increased demand for GSH. However it should be

noted that GSH depletion is known to occur when feeding is reduced, i.e. during periods of stress. Therefore the interpretation of these changes related to GSH must be regarded with caution.

4.5.3 Gut microbial metabolites

The depletion of several metabolites in the urine and sera after gentamicin exposure can be attributed to the antibiotic pharmacological action of the drug. Hippurate, TMAO and methylamine are co-metabolites of the gut microbiome and mammalian metabolism and the depletion of hippurate and TMAO has been noted in previous studies of gentamicin exposure (Espandiari et al. 2007; Lenz et al. 2005; Sieber et al. 2009). Hippurate is a product of the biotransformation of dietary aromatics that are initially metabolised by gut bacteria to benzoate before being conjugated to glycine by mammalian mitochondrial enzymes in the liver and kidney to form hippurate (Gatley and Sherratt 1977). Likewise, TMAO is the product of the oxidation of trimethylamine, a product of gut microbial choline metabolism (Zeisel et al. 1989). Although methylamine production is largely *via* mammalian degradation of creatine and sarcosine (Davis and De Ropp 1961; Zeisel et al. 1985), studies of germ-free rats demonstrate reduced excretion of methylamine (Zeisel et al. 1985), suggesting that the depletion of methylamine identified in the OPLS-DA models of mid-study urine spectra is as a result of the antibiotic pharmacology of gentamicin.

4.5.4 Metabolites of renal tubule dysfunction

The OPLS-DA model comparing pre- and post-dose spectra of biofluids highlighted an increase in urinary 1-methylnicotinamide, urinary and serum glucose and serum creatinine. In previous metabolic studies of gentamicin nephrotoxicity glucosuria has been identified (Lenz et al. 2005; Sieber et al. 2009) but the elevation of 1-methylnicotinamide has not been noted. A product of nicotinamide metabolism, 1-methylnicotinamide is an organic cation generated by nicotinamide-*N*-methyltransferase. Its precursor nicotinamide is itself a precursor of NAD and NADP, whilst its co-product is s-adenosyl cysteine, a pre-cursor for

cysteine synthesis. The elevation in 1-methylnicotinamide represents an early biochemical change in nicotinamide metabolism or excretion which, apart from a single animal with raised 1-methylnicotinamide in day 4 and day 5 urine, is not seen in the mid and end-point urine spectra. Given the diverse physiological roles of nicotinamide as a precursor for the co-enzymes NAD and NADH, the biochemical significance of this perturbation is unclear. However, 1-methylnicotinamide has been investigated as a non-invasive biomarker of renal tubular function (Shim 1984, Maiza 1992) and therefore the increase in its excretion may be an indication of early renal tubular dysfunction following a single dose of gentamicin. Likewise, despite the wide-ranging physiological function of glucose, the increase in its excretion is likely to be linked to changes in its reabsorption in the proximal tubule. The multivariate analyses of urine spectra also highlighted urinary 3-indoxylsulfate (3-IS) as significantly depleted mid-study compared to pre-dose. 3-IS is a uremic toxin (a substance which is retained in the circulation as a result of renal failure) which is physiologically taken up by organic anion transporters at the basolateral membrane and secreted by the proximal tubule (Deguchi et al. 2002), thus the reduction in its excretion could suggest dysfunction in the uptake and/or secretion mechanisms. Although it would have been thorough to determine a concomitant increase in 3-IS in the sera, 3-IS is protein bound hence it was not visible in serum spectra. However 3-IS and 1-methylnicotinamide have also been shown to be gut-microbial metabolites therefore their depletion could be related to the antibiotic action of gentamicin. Nonetheless the perturbation in the excretion of glucose and creatinine charts the early perturbation in proximal tubule function with a prominent loss of renal function by day 4.

4.5.5 Metabonomics in drug toxicity

Many of the metabolites identified in this study are common to a number of types of toxicity and therefore, rather than biomarkers unique to gentamicin and proximal tubule injury, they may reflect the non-specific effects of toxicity as a whole (Robertson et al.

2011). It has been proposed that a reduction in food intake and body weight loss would account for the changes in energy-related and gut microbial metabolites (Connor et al. 2004) and pair-feeding could circumvent this limitation. Whilst a reduction in food intake and body weight loss could in part explain the reduction in several energy and gut-related metabolites, it may not account for metabolites which were identified as elevated after gentamicin exposure such as allantoin in the day 1 and day 10 urine. In addition, metabolic perturbations in the gentamicin group preceded body weight gain differences between gentamicin and saline-treated animals.

Whilst body weight changes and feeding are an explanation for some metabolic perturbations in toxicity studies, these metabolites still offer useful information about mechanisms of toxicity; certainly the commonalities in response to a toxin are just as interesting as the differences. It is important that these metabolites are not disregarded as they reflect the global changes in the metabolome in response to a toxin and it is this inclusive quality which is at the core of the metabonomic approach.

4.5.6 Concluding remarks

- The metabonomic analysis of a once-daily dosing schedule of gentamicin has identified major mitochondrial disturbances from as little as 12 hours following the first dose. These changes precede elevations in kim-1, a proximal tubule-specific biomarker of injury and were sustained until the end of the study and therefore demonstrate an early mechanistic response to gentamicin exposure.
- Given that the TCA cycle metabolites' excretion is elevated prior to kim-1 and certainly before serum creatinine or BUN, clinical quantification of these metabolites could be a valuable, non-invasive alternative to monitoring the toxicity of aminoglycosides, although this would require additional targeted investigations into specificity, sensitivity and the physiological levels in different demographics.

- After several days exposure, renal function was deteriorating as evidenced by glucosuria and polyuria. Certainly, day 4 appears to be the tipping-point of the study where the toxicity began to affect daily body weight gain and the gentamicin group no longer gained weight at the same rate as the saline control animals.
- Aside from toxicity, the unbiased ^{1}H-NMR analysis has enabled the measurement of changes in the mammalian-microbial co-metabolome, namely the reductions in co-metabolites hippurate, TMAO, methylamine and 3-IS. It is likely that the role of the gut microbiome and metabolome will become increasingly important to the study of drug efficacy and safety, which is something that is further explored in Chapter 5.

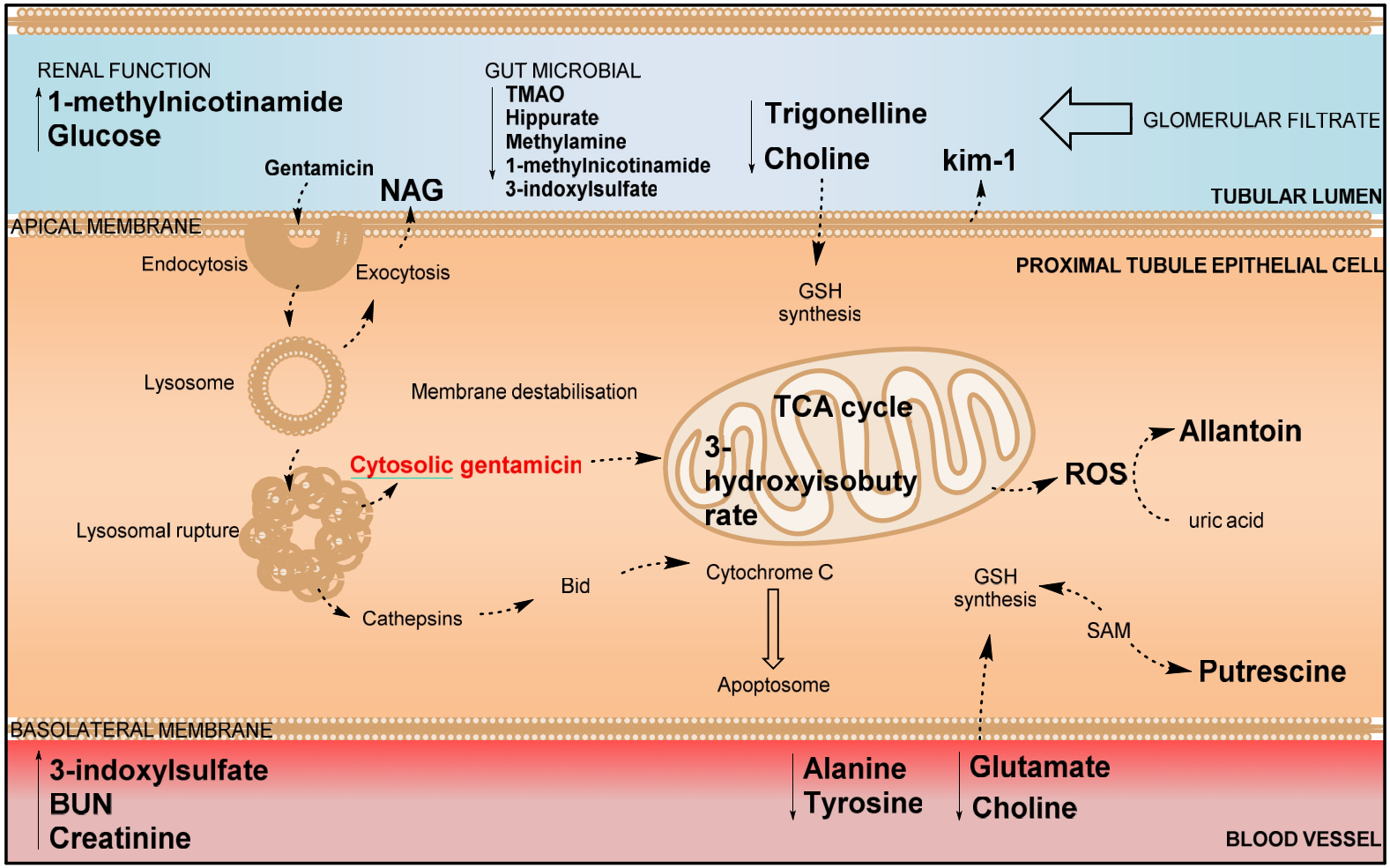


Figure 4.16 Metabolic perturbations in the urine and serum as a result of gentamicin exposure and accumulation within proximal tubule epithelial cells. TMAO – trimethylamineoxide, kim-1– kidney-injury molecule 1, NAG – N-acetyl-β-glucosaminidase, BUN – blood urea nitrogen, GSH – glutathione, SAM – s-adenosylmethionine, TCA – tricarboxylic acid.

5. INTEGRATED METABOLITE-BIOMARKER ANALYSIS OF GENTAMICIN-INDUCED PROXIMAL TUBULE INJURY

Contents

5.1 Introduction

Metabonomic studies of drug exposure, such as described in Chapter 4, can provide a comprehensive list of metabolites associated with toxicity. Establishing the metabolic perturbations caused by exposure to a toxin not only gives insight into the mechanism of toxicity but can also drive targeted candidate biomarker experiments. Early changes in metabolites due to toxicity could have a predictive capacity for the onset, progression and prognosis of renal injury, and may precede changes in renal functional biomarkers such as rises in BUN and serum creatinine. In addition, identification of metabolites prior to administration that predict differential responses to therapy could help stratify patients on a risk/benefit basis. This concept is known as pharmacometabonomics and is discussed below. By identifying panels of predictive metabolites from urinary pre-treatment or early intervention spectral profiles, clinicians could not only circumvent the requirement of therapeutic drug monitoring and renal function testing throughout the use of aminoglycosides, but also reduce the prevalence of aminoglycoside-induced nephrotoxicity altogether.

5.1.1 Pharmacometabonomics

Pharmacometabonomics is defined as, “the prediction of outcome of a drug or xenobiotic intervention in an individual based on a mathematical model of pre-intervention metabolite signatures,” and was coined after metabonomics-based studies into drug-induced liver injury (Clayton et al. 2006). The idea that the innate status of an individual can influence the outcome of exposure to a drug is akin to pharmacogenomics, whereby the genetic type of an individual determines the handling and response to a drug. In pharmacometabonomics, multivariate analysis of the pre-dose or early post-dose metabolome when integrated with post-dose outcomes (such as biomarkers of injury and histopathology score) can group individuals based on their differential response to a drug, i.e. through drug efficacy, safety, metabolism and pharmacokinetics.

An example of the pharmacometabonomic approach in practice is in drug-induced liver injury caused by acetaminophen (APAP). APAP research was conducted to determine if pre-dose metabolic profiles were predictors of APAP metabolism in humans (Clayton et al. 2009; Clayton et al. 2006). Multivariate analysis of ^{1}H-NMR spectra from baseline urine samples from patients given a single dose of APAP found that individuals with a greater level of para-cresol sulphate in their pre-dose urine had lower post-dose ratios of APAP sulphate to glucuronide, potentially indicative of metabolic competition between gut microbial para-cresol and APAP in the liver (Clayton et al. 2009). This finding highlights the importance of microbial influences on the handling of drugs which has the potential to explain the inter-individual variability in the response to aminoglycoside nephrotoxicity.

Another demonstration of the utility of pharmacometabonomics was in a series of proof-of-principle studies on liver injury induced by galactosamine hydrochloride, allyl alchohol and APAP in rats (Clayton et al. 2006). The APAP study identified the pre-dose amount of the metabolites TMAO, taurine and betaine as associated with the histology score after a single toxic-threshold dose of 600 mg/kg (Winnike et al. 2010). When integrated with other measurements of toxicity such as liver biomarkers, early intervention pharmacometabonomics identified that changes in endogenous metabolites such as glycine and creatine, gut microbial-related metabolites TMAO, betaine and hippurate, and drug-related metabolites separated responders from non-responders of APAP exposure.

After more than a decade since its inception, pharmacometabonomics has demonstrated its applicability and value across a variety of drugs in the study of their efficacy and toxicity (Everett et al. 2013). It is clear that in the future the pharmacometabonomic approach to understanding human health and disease will play a large role in the pursuit of personalised healthcare.

5.2 Aims

To identify pre-dose and early-intervention metabolite biomarkers that predict and chart the onset of gentamicin nephrotoxicity. This was achieved by;

- Analysing early-intervention metabolite signatures in gentamicin-induced nephrotoxicity by integrating a biomarker of kidney injury with ^{1}H-NMR spectra using OPLS-regression analysis.
- Examining pre-dose metabotypes in relation to kidney injury molecule-1 to predict the extent of gentamicin-induced nephrotoxicity.
- Characterising the discriminatory pre-dose metabolite(s) and investigating their relationship to aminoglycoside-induced nephrotoxicity.

5.3 Methods

5.3.1 Animals

5.3.1.1 Gentamicin study 1

The animals and samples used for the initial biomarker and metabolite investigations were also described in Chapter 4. Male 8-week old Sprague-Dawley rats were administered gentamicin (200 mg/kg I.P, n=5) or 0.9% saline (n=5) once daily at 10:00 h for 9 days. Blood was obtained by sampling from the tail vein prior to the first injection. Animals were housed individually in metabolic cages 12 hours prior to the first injection and 12 hours after the first injection; animals were housed in metabolic cages again for the 24 hours after the fourth injection and finally for 24 hours after the final injection. 12 hour urine was collected into chilled vessels with a minimum of 0.1% wt/v sodium azide. Animals were kept on a 12 h light/dark cycle throughout the study with free access to food and water. On day 10 of the study all animals were culled by an overdose of pentobarbitone followed by cardiac puncture.

5.3.1.2 Gentamicin study 2

The animals and samples used for the secondary study were collected and analysed as described in Chapter 3. In brief, urine was collected overnight from twenty male Sprague Dawley rats (aged approx. 8 weeks) prior to the first I.P injection of 120 mg/kg gentamicin (n=8) or 0.9% saline (n=4). The remaining eight animals were administered tobramycin but post-dose measurements were not required for the purposes of this chapter. Solutions were administered once daily at 10:00 h for 7 days, followed by overnight urine collection and on day 8, schedule 1 culling by a rising concentration of CO_2. Urine was stored at -80°C until analysis for biomarkers of kidney injury and the acquisition of ^{1}H-NMR urine spectra.

All animal protocols were undertaken in accordance with a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee.

5.3.2 Biochemical measurements

5.3.2.1 Urine creatinine

Urine (25 µL of urine diluted 1 in 25) was incubated with 125 µL of alkaline picrate solution (0.14 M NaOH, 10 mM Na_2HPO_4, 90 mM sodium borate, 10 mM sodium dodecyl sulphate, 25% v/v picric acid, 2.7% v/v DMSO) for 2 min at room temperature. An acid solution (5 µL of 10:2:88 v/v acetic acid: concentrated H_2SO_4:distilled water) was added and incubated at room temperature for a further 10 min with shaking. Plates were read at 490 nm and urine creatinine (UCr) was estimated using creatinine calibration standards (2.5-40 mg/dL).

5.3.2.2 Kidney injury molecule-1

In the gentamicin study urinary kidney injury molecule-1 (kim-1) was quantified using a sandwich ELISA (Vaidya et al. 2006). In gentamicin study 2 kim-1 was quantified using the rat kim-1 MesoScaleDiscovery assay, as described in Chapter 3.

5.3.3 Sample processing and acquisition of ^{1}H NMR spectra

Samples from the preliminary gentamicin study were processed and spectra were acquired as described in Chapter 4. Urine from gentamicin study 2 was processed in a slightly different manner. Urine was diluted with buffer (1.5M KH_2PO_4/D_2O, pH 7.4 (with D_2O), 2mM NaN_3, 0.1% TSP (3-(trimethyl-silyl)propionic acid-d4) 9:1 v/v before addition of 600 µL to a 5 mm NMR tube. Spectra were acquired on a 600 MHz Bruker spectrometer and automatically phased, baseline corrected and calibrated to TSP (0.0 ppm) using TopSpin 3.2 software. Spectra were imported into MATLAB 2013 using MetaSpectra 4.1.1 and were aligned as described in Chapter 4. Water, urea, the high ppm region and TSP-related peaks were excised from the spectra before probabilistic quotient normalisation (Dieterle et al. 2006) with in-house scripts.

5.3.4 Multivariate analysis

Urine spectra were subjected to supervised multivariate methods: Orthogonal Partial Least Squares-Regression Analysis. OPLS-regression analysis was performed by comparison of metabolic profiles time-matched with kim-1 excretion measurements.

Metabolite identification was performed using Chenomx Profiler software (Chenomx NMR Suite) and databases: the Human Metabolome Database (www.hmdb.ca/spectra/nmr_one_d/search), and the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu/metabolomics), statistical total correlation spectroscopy (STOCSY) analysis (Cloarec et al. 2005a) and spike-in experiments.

STOCSY is an analysis method that displays the correlation between the intensity of a given driver peak and all other peaks in a set of 1D NMR spectra. In this way, for an unknown metabolite of interest, molecular or biochemically associated (or correlated) peaks are highlighted and can aid in the metabolite's identification.

Data are presented as OPLS-DA loadings coefficient plots and OPLS-regression loadings coefficient plots (Cloarec et al. 2005a; Cloarec et al. 2005b) where the loadings are back-scaled to highlight the urinary metabolites which have a significant correlation (r^2) with kim-1 or in the case of STOCSY analysis, a significant correlation with the peak of interest. The plots display the covariance together with the associated correlation coefficient (r^2) with a colour scale demonstrating the strength of the correlation; dark red indicates a very significant correlation between the groups and at the other end of the scale, dark blue indicates little variation between the groups.

5.3.5 Spike-in experiment

The identification of 3-HPPA was confirmed using a spike-in experiment. A ^{1}H-NMR spectrum was acquired for a pre-dose urine sample; it was then spiked with 5 µL of a 3-HPPA standard (0.5 mg/mL in sample buffer) and a ^{1}H-NMR spectrum was acquired. A further 10 µL of standard was added to the sample before another ^{1}H-NMR spectrum was acquired. The process was repeated by adding 30 µL of 3-HPPA standard. Spectra were overlayed using TopSpin 3.2 software to compare the resonances in spectra from un-spiked and spiked urine.

5.3.6 Estimation of metabolite excretion

The integral of the metabolite of interest 3-HPPA in the urine ^{1}H-NMR spectra was adjusted according to the number of protons; excretion was estimated using the calculated creatinine excretion which was based on the proton-adjusted integral of the creatinine singlet at 4.04 ppm and the concentration of creatinine as measured by the Jaffe assay (see Chapter 4). Integration was achieved using MATLAB 2013 and in-house scripts (Imperial College, London) by selecting the upper and lower ppm scale boundaries of the region of interest.

5.4 Results

5.4.1 Biochemical measurements

The extent of nephrotoxicity was determined by measurement of kidney injury molecule-1 excretion in the urine of rats pre- and post-gentamicin exposure.

5.4.1.1 Gentamicin study

Pre-dose urinary excretion of kim-1 was measured as 0.39±0.22 ng/mg UCr. Throughout the study the excretion of kim-1 ranged from 0.25 – 1.73 ng/mg UCr in the saline group. Kim-1 excretion was significantly greater in the gentamicin group relative to the saline group from day 5 to the end of the study on day 10 (Kruskal-Wallis, Dunn's correction, p=0.0002), Figure 5.1. Variability in kim-1 excretion within the gentamicin group was evident; on day 4 the range was 1.93 – 23.98 ng/mg UCr with similar patterns seen on subsequent days. Animals 7 and 9 tended to have a lower, but significant from control, excretion of kim-1 relative to the other gentamicin animals peaking at 17.66 ng/mg UCr (day 9) and 17.11 ng/mg UCr (day 10), respectively, compared to animals 1, 3 and 5 with kim-1 excretion of 46.06, 32.37 and 30.87 ng/mg UCr on day 9, day 10 and day 9, respectively.

5.4.1.2 Gentamicin study 2

Pre-dose kim-1 excretion was 2.49±0.99 ng/mg UCr and at the end of the study the excretion of kim-1 in saline-treated rats was not significantly different at 2.10±0.64 ng/mg UCr. The excretion of kim-1 in the gentamicin group after 7 days of dosing was significantly greater than pre-dose ($p<0.001$, T test, paired) and relative to saline group ($p<0.001$, T test, unpaired) at 37.84±19.54 ng/mg UCr.

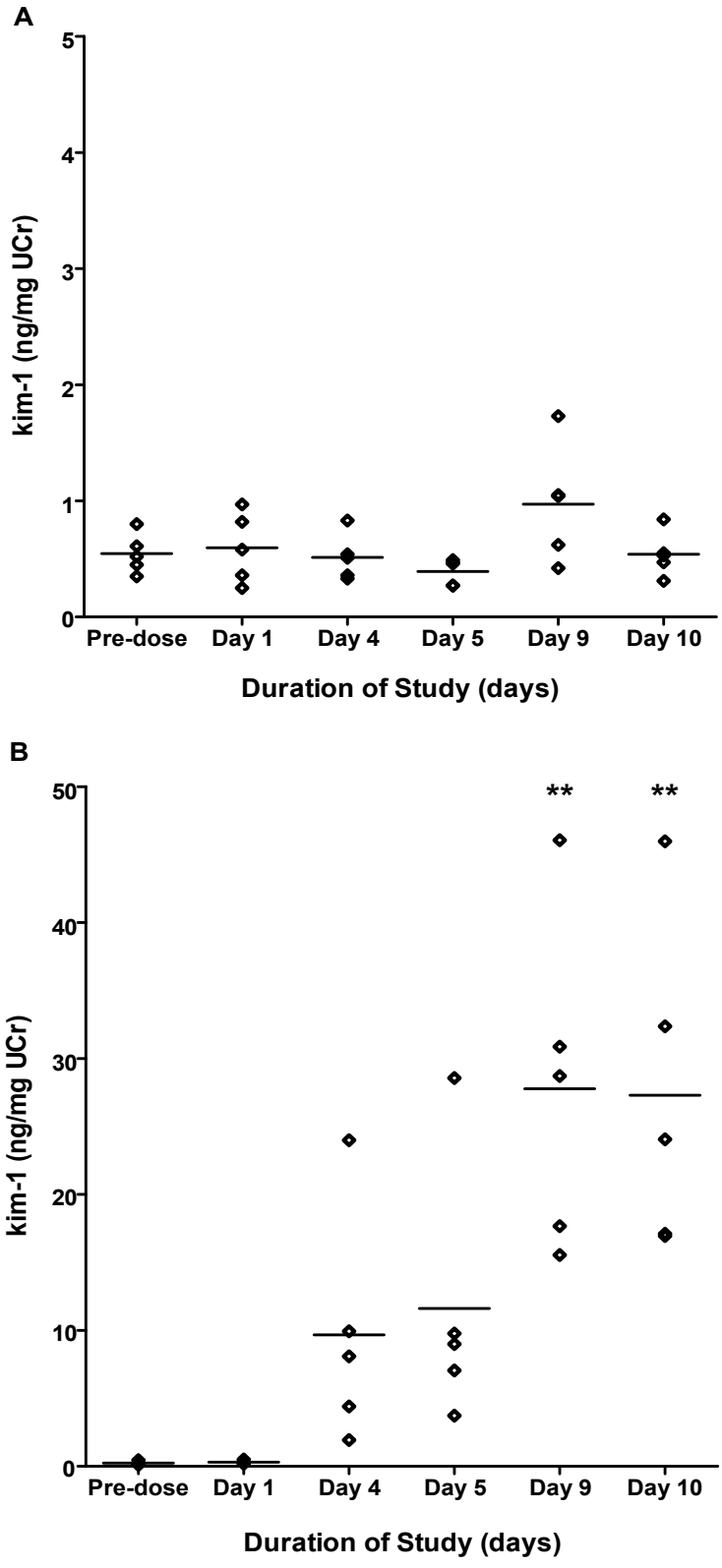


Figure 5.1 Urinary kim-1 excretion in rats administered (A) 0.9% saline and (B) 200 mg/kg gentamicin. Data are presented as aligned scatter plots with mean (-) and individual values (n=5).

5.4.2 OPLS-DA regression models

OPLS-regression models were generated using time-matched biomarker data and ^{1}H-NMR spectra. Firstly, the changes in urinary kim-1 excretion in relation to metabolic perturbations throughout the study were analysed. Secondly, the relationship between pre-dose metabotype and post-dose kim-1 excretion was investigated.

5.4.2.1 Kim-1 and metabolic changes

Models of time-matched urine spectra from gentamicin-treated animals at pre-dose, day 4 and day 10 against corresponding kim-1 data were analysed for correlating metabolites (Q^2Y=0.5784, R^2Y=0.7295, $p<0.0001$); the singlet at 3.6 ppm, 2-oxoglutarate, succinate, citrate, trigonelline, fumarate and formate had a negative relationship with increasing kim-1. These changes are illustrated in Figure 5.2 and the statistics of the significant metabolites are described in Table 5.1.

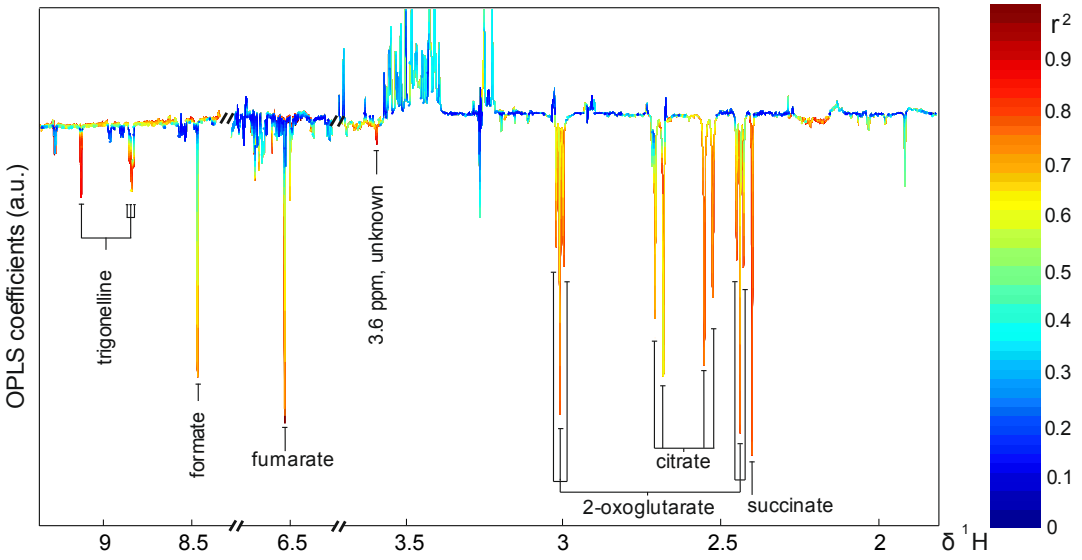


Figure 5.2 OPLS-DA loadings coefficient plot of pre-dose, day 4 and day 10 urine ^{1}H NMR spectra regressed against time-matched urinary kidney injury molecule -1 excretion.

Table 5.1 Urinary metabolites that were identified to have an inverse relationship with changes in kim-1 after daily exposure to 200 mg/kg gentamicin. r^2 denotes correlation value

Metabolite	**r^2**	**Classification**
2-oxoglutarate	-0.77	TCA cycle intermediate
Succinate	-0.84	
Citrate	-0.76	
Fumarate	-0.80	
Formate	-0.77	
Trigonelline	-0.86	Nicotinamide metabolism & GSH depletion
3.6 ppm (s)	-0.90	unknown

5.4.2.2 Pre-dose urine spectra and kim-1

Pre-dose urine spectra were modelled against urinary kim-1 values from day 4, 5, 9 or 10 of the study. Day 1 kim-1 was not used for modelling because the values were not raised from pre-dose. A negative relationship between an uncharacterised singlet at 2.04 ppm and kim-1 was identified in all models of pre-dose spectra and urinary kim-1 (e.g. r^2=-0.85 with day 10 kim-1). Pre-dose citrate (r^2=-0.84) and glucose (r^2=-0.86) levels also had a negative relationship with day 10 kim-1. Pre-dose 3-(3-hydroxyphenyl) propionic acid (3-HPPA) and a putative sugar (5.415 ppm, doublet) had a positive correlation with urinary kim-1 in day 4, 9 and 10 models, see Figure 5.3.

5.4.3 Identification of 3-HPPA

Assignment of 3-HPPA to the multiplet at 6.76 ppm was confirmed by STOCSY analysis, a review of the literature and a spike-in experiment of a synthetic 3-HPPA standard. STOCSY analysis of the resonance at 6.76 ppm found significant correlations with several other resonances in the urine spectra; multiplets at 2.48, 2.84, 6.8, 6.87 and 7.24 ppm were identified, see Figure 5.4, which are consistent with 3-HPPA as reported in the literature (Stanley et al. 2005). ^{1}H-NMR spectra of rat urine before and after the spike-in of 3-HPPA are shown in Figure 5.5. The intensity of the resonances at 6.76, 6.87 and 7.24 increased with each addition of the standard, further confirming that the metabolite of interest is 3-HPPA.

3-HPPA is a gut microbial-host co-metabolite that is produced from the dietary metabolites chlorogenic acid and caffeic acid. The strong correlation between pre-dose levels of 3-HPPA and kim-1 in multiple models in the preliminary study prompted further investigation of pre-dose 3-HPPA and its relationship to gentamicin nephrotoxicity in a separate animal cohort.

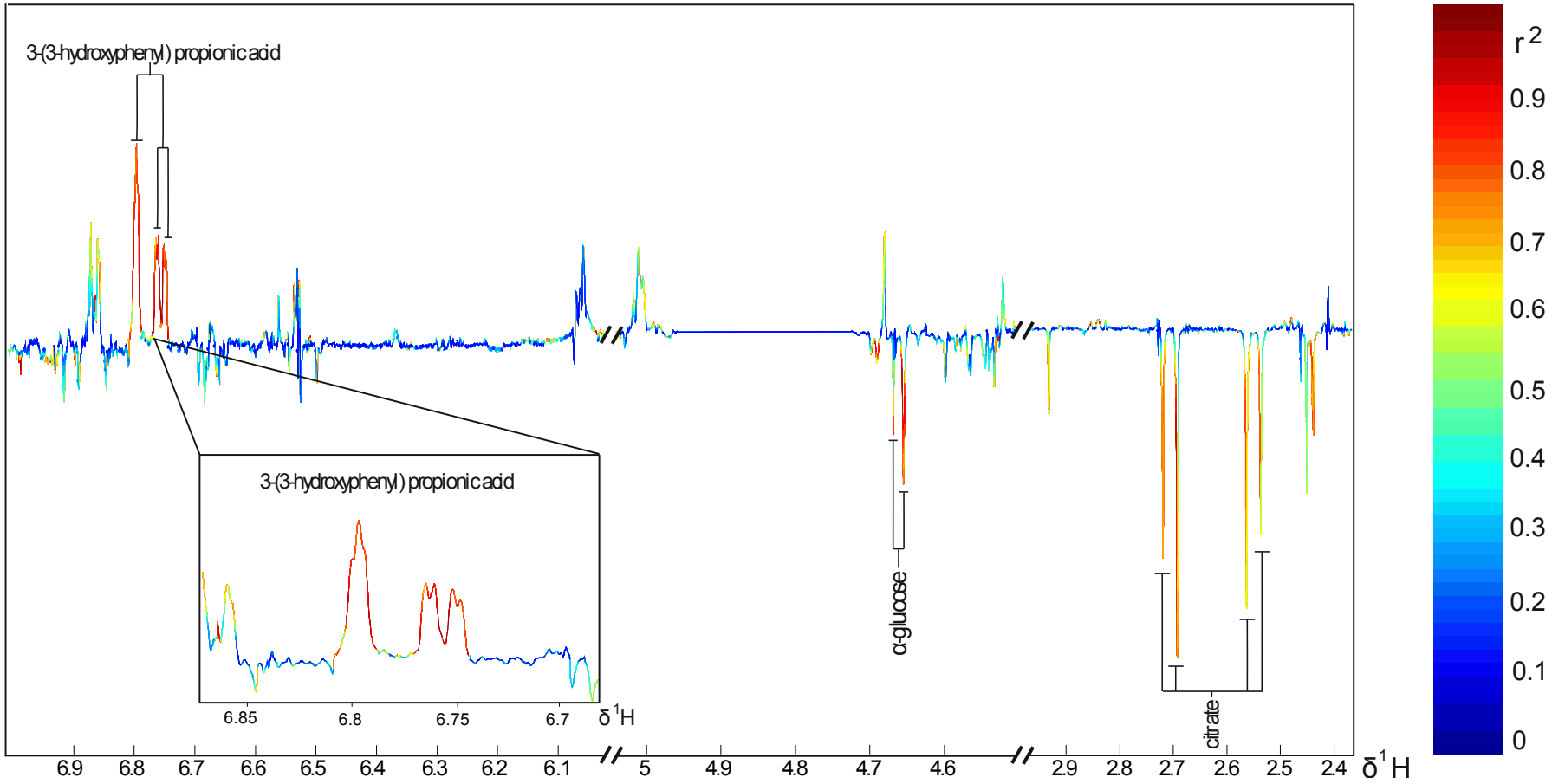


Figure 5.3 OPLS-regression coefficient plot of pre-dose urine ^{1}H NMR spectra and post-dose urinary kim-1 excretion on day 10 of the study. Pre-dose 3-HPPA had a positive relationship with kim-1 whereas pre-dose citrate and α-glucose had and inverse relationship with kim-1.

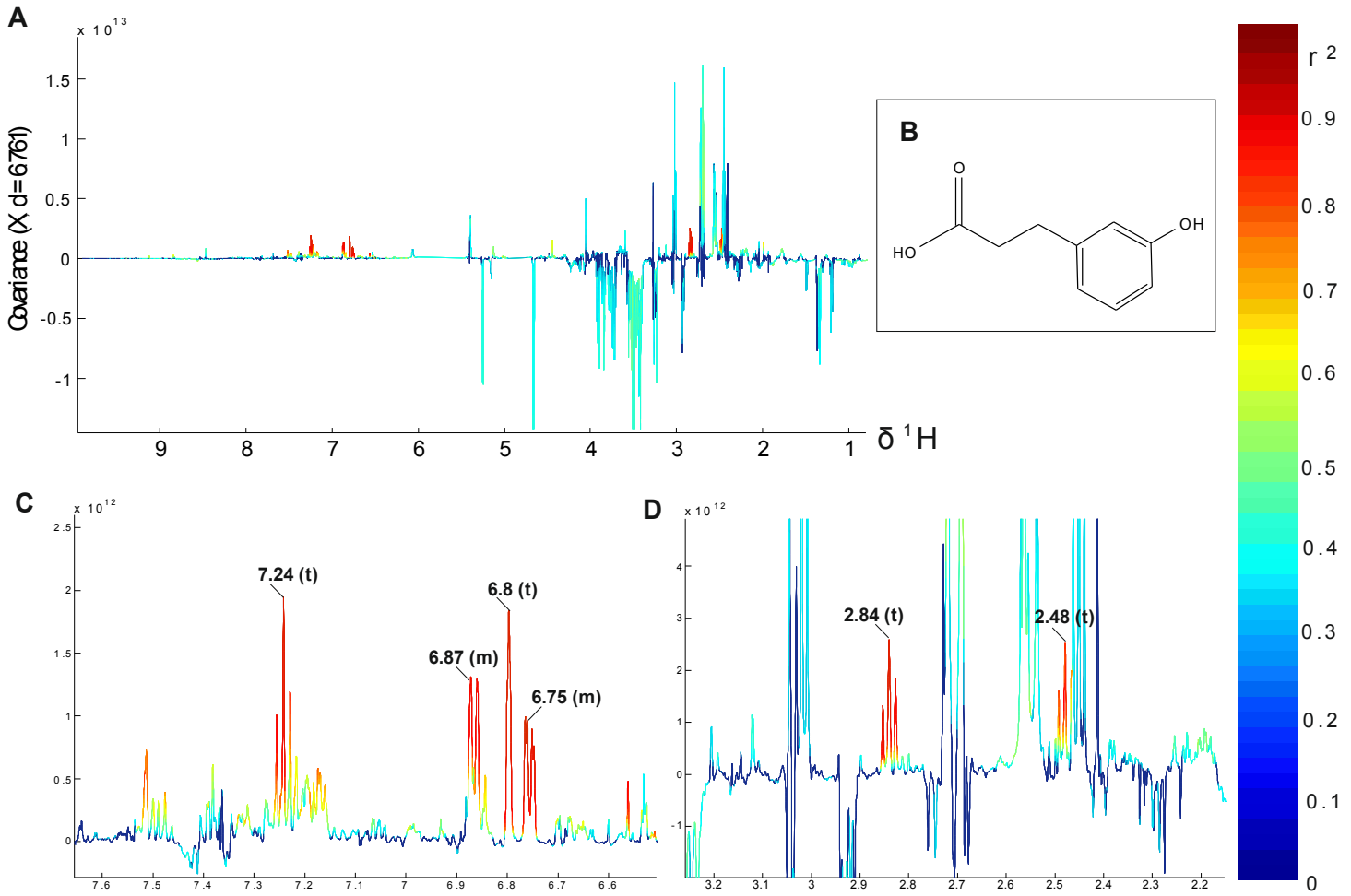


Figure 5.4 STOCSY analysis of the multiplet at 6.76 ppm identified in the pre-dose rat urine spectra to have a relationship with kim-1 excretion. (A) Correlation of 6.76 ppm with all resonances, (B) The metabolite was identified as 3-HPPA. Enlargement of the STOCSY analysis (C) 6.5-7.6 ppm and (D) 2.2-3.2 ppm labelled with the 3-HPPA resonances described in the literature (Stanley et al. 2005).

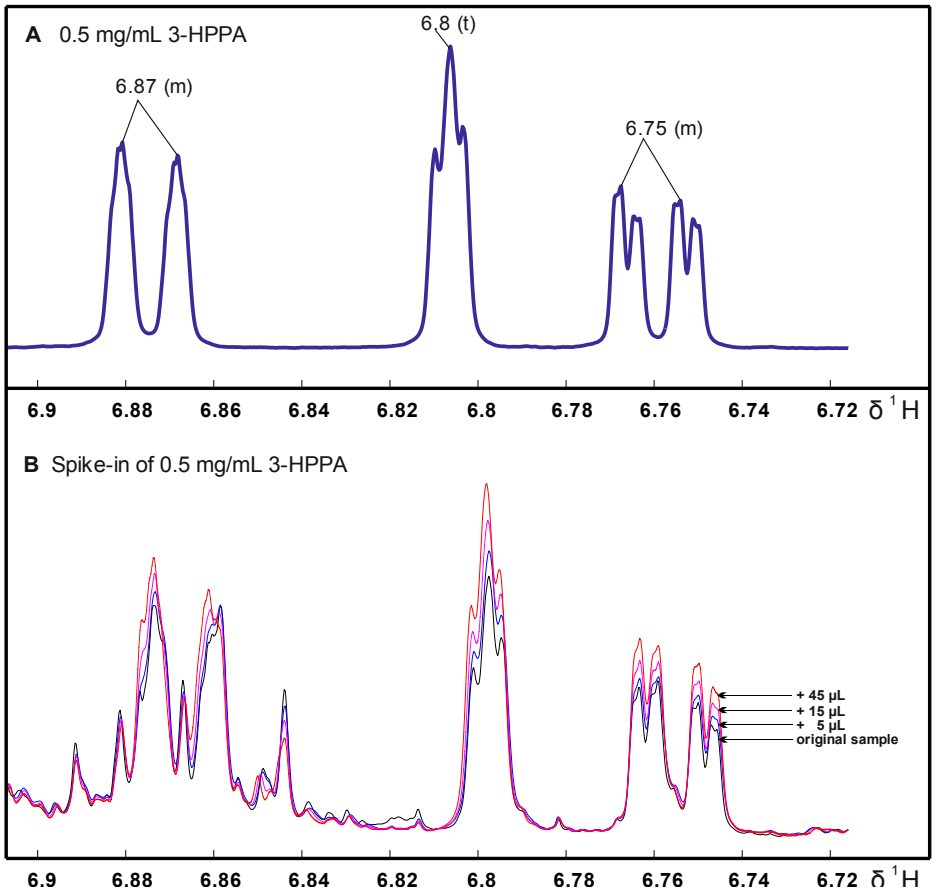


Figure 5.5 Spike-in ^{1}H-NMR experiments confirming identify of 3-HPPA. (A) ^{1}H-NMR spectra of high ppm region of 0.5 mg/mL 3-HPPA standard prepared in buffer and (B) Progressive spike-in of 0.5 mg/mL 3-HPPA standard into rat urine.

5.4.4 Analysis of 3-HPPA in separate gentamicin cohort

The strong relationship between pre-dose 3-HPPA and kim-1 in the initial study was investigated in pre-dose ^{1}H-NMR spectra of a separate group of animals which were administered either 0.9% saline or 120 mg/kg gentamicin for 7 days.

5.4.4.1 Variation in urinary biomarkers

Pre-dose kim-1 excretion ranged from 1.40 ng/mg UCr to 4.31 ng/mg UCr whereas post-dose day 7 kim-1 excretion ranged from 27.0-80.5 ng/mg UCr (n=7). Pre-dose urinary mevalonic acid ranged from 10.2-15.2 µg/mg UCr (n=7). As detailed in Chapter 3, there was a positive relationship between pre-dose mevalonic acid and post-dose kim-1 excretion.

5.4.4.2 OPLS-regression model

Pre-dose ^{1}H-NMR spectra were regressed against post-dose kim-1. However the model did not highlight any pre-dose urinary metabolites as significantly correlated to end-point urinary kim-1 excretion (data not shown).

5.4.4.3 Regression of 3-HPPA with biomarkers

As an alternative to the OPLS-regression model, pre-dose 3-HPPA was measured in the ^{1}H-NMR spectra and regressed against pre-dose mevalonic acid, see Figure 5.6A and B, and with end-point kim-1, see Figure 5.6C. A significant positive relationship was found between pre-dose mevalonic acid and the 3-HPPA integral ($p=0.0027$) and the calculated excretion of pre-dose 3-HPPA ($p=0.0127$). A positive trend was observed between the pre-dose excretion of 3-HPPA and toxicity of gentamicin (Figure 5.6C), although the linear regression was not statistically significant. There was a weak positive correlation between the 3-HPPA integral and the calculated excretion of 3-HPPA ($p=0.0279$), see Figure 5.6D.

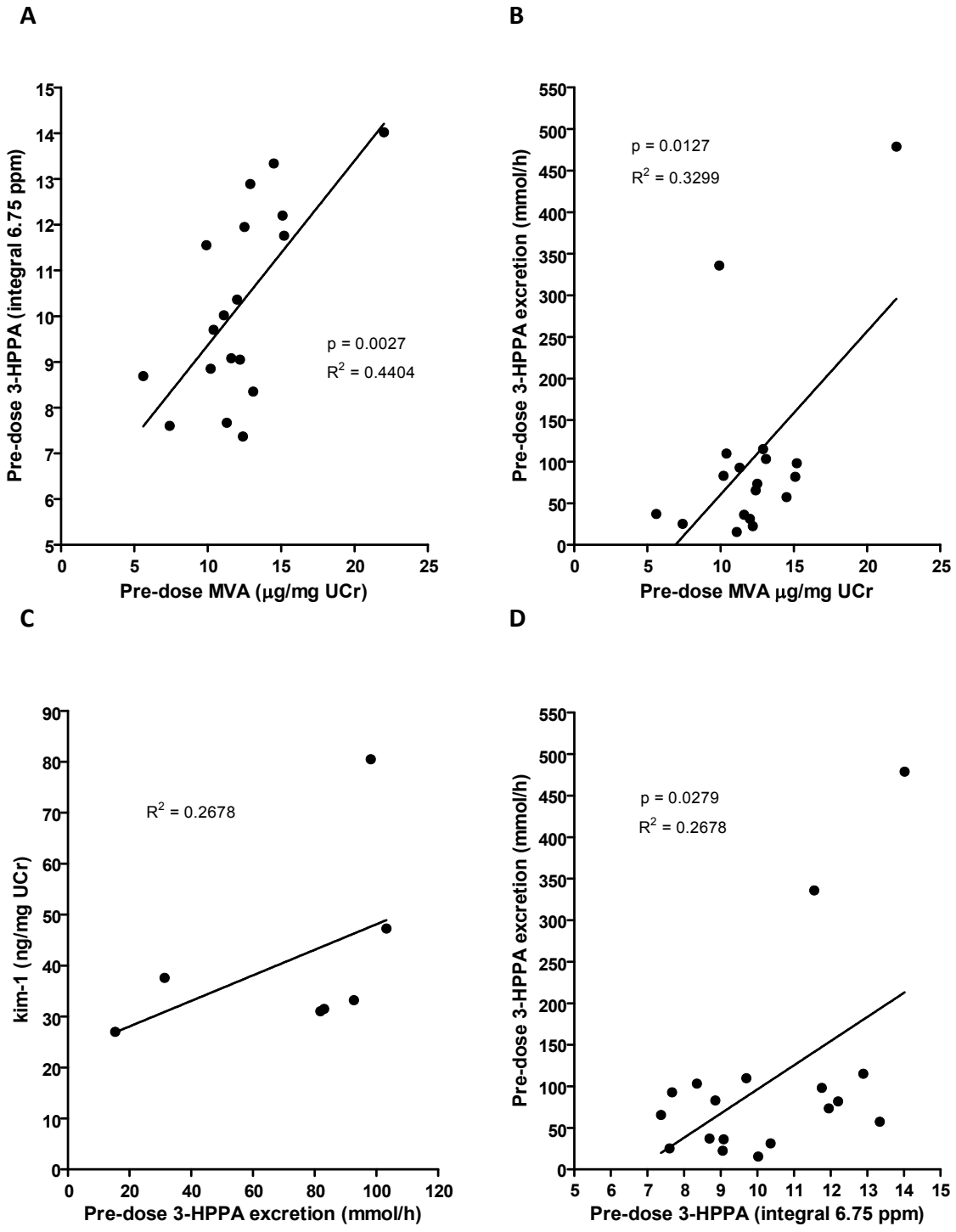


Figure 5.6 Regressions of pre-dose urinary 3-HPPA. (A) Pre-dose integral of 3-HPPA and pre-dose mevalonic acid (n=18), (B) pre-dose excretion of 3-HPPA and pre-dose mevalonic acid (n=18), (C) relationship between pre-dose excretion of 3-HPPA and end-point kim-1 in animals administered 120 mg/kg gentamicin (n=7), (D) correlation between pre-dose 3-HPPA ^{1}H-NMR integral and calculated excretion (n=18).

5.5 Discussion

Pharmacometabonomics aims to use metabolite signatures pre- and post-drug exposure and end-point measures of toxicity to predict the response to the drug (Clayton et al. 2006). The approach has been used to predict the extent of APAP-induced liver injury by identifying early changes in metabolites (Winnike et al. 2010) after drug exposure and pre-dose metabolites such as p-cresol sulphate (Clayton et al. 2009) and taurine (Clayton et al. 2006). Herein, a ^{1}H-NMR based methodology was used to probe the pre-dose and post-dose metabolic signatures of rats administered gentamicin and to analyse their relationship to kidney injury molecule-1 (kim-1), a biomarker of proximal tubule injury, with a view to identifying metabolites which predict the variability in gentamicin-induced nephrotoxicity.

The OPLS-regression analysis of ^{1}H-NMR urine spectra with matched urinary kim-1 data revealed multiple metabolites that had an inverse relationship to the increasing excretion of kim-1. The majority of the metabolites identified as having a negative relationship to kim-1 were TCA cycle intermediates (succinate, citrate, formate, fumurate and 2-oxoglutarate) which were identified in OPLS-DA models as depleted post-dose relative to pre-dose following administration of gentamicin (Chapter 4). Regression of the spectra against the biomarker data demonstrated that the depletion of the TCA cycle metabolites was correlated to kim-1 and therefore the extent of nephrotoxicity. Similarly, trigonelline, a metabolite implicated in GSH homeostasis (Sun et al. 2008), was also observed to have a negative relationship with increasing kim-1. Further characterisation of the relationship of these metabolites with kidney injury and with other aminoglycosides is warranted but certainly, changes in the TCA cycle intermediates and trigonelline could potentially serve on a panel of biomarkers to monitor the response to exposure to gentamicin.

The OPLS-regression analysis with kim-1 also highlighted that the singlet at 3.6 ppm had a strong negative relationship with kim-1 excretion. This ‘unknown’ was also highlighted in multiple OPLS-DA models in Chapter 4. Given its position at 3.6 ppm this metabolite could

be glycine which is typically observed as a singlet around 3.56 ppm. Glycine is a component of GSH and was reported to be elevated in the urine of responders to APAP hepatotoxicity (Winnike et al. 2010) and differential changes in glycine were suggested to be linked to inter-individual susceptibility to toxicity. Equally this metabolite could be malonate, which is a mitochondrial complex II inhibitor which can induce cell death through a loss of mitochondrial membrane potential, cytochrome C release and GSH depletion (Fernandez-Gomez. 2005). Perturbations in GSH homeostasis were identified in the metabonomic analysis in Chapter 4, therefore if the singlet at 3.6 ppm is malonate or glycine its depletion relative to increasing kim-1 supports the idea that oxidative stress is key player in the development of gentamicin-induced nephrotoxicity.

STOCSY analysis of this unknown and investigation of the literature did not lead to its identification; as it is a singlet resonance identification is more difficult as 2D experiments do not lead to spin-system coupling information. Although its identity has not been confirmed, this endogenous metabolite does appear to be important to gentamicin exposure and toxicity, and, whilst the multivariate methods used in this research are powerful tools for interrogating vast, complex sets of data, a limitation of the metabonomic approach, as demonstrated here, is our ability to identify unknowns in a complex mixture. Computational approaches have been developed that can aid in the assignment of metabolites of interest, such as STOCSY analysis and the collation of metabolites in the Human Metabolome Database, but these resources are finite and often further 2D NMR experiments are required to elucidate structures.

Pharmacometabonomics also considers baseline or pre-dose metabotypes and their relation to responses to drug exposure. In this preliminary investigation 3-(3-hydroxyphenyl)-propionic acid (3-HPPA) was highlighted by the OPLS-regression analysis of pre-dose ^{1}H-NMR urine spectra and post-gentamicin exposure kim-1 excretion. A strong positive relationship was observed between pre-dose 3-HPPA and kim-1 excretion on

multiple days of the study (e.g. r^2=0.93, day 10), which developed the hypothesis that there may be a mechanistic relationship between 3-HPPA metabolism and gentamicin nephrotoxicity.

3-HPPA is a metabolite of microbial origin which is assimilated by the MCT transporter (Konishi and Kobayashi 2004) and excreted in the urine. Along with hippurate, 3-HPPA is a major end product of the metabolism of dietary chlorogenic acid (Tomas-Barberan et al. 2014), see Figure 5.7 . It is estimated there are >1000 species of gut microbes accounting for approximately 1 kg of weight in an individual (Nicholson et al. 2005) and there is strong evidence of a mammalian-microbial 'co-metabolome', whereby the host and microbes share metabolites (Nicholson and Wilson 2003); Indeed microbial and host metabolites can exchange between the intestines and human circulation from the superior and inferior mesenteric veins which drain into the hepatic portal vein and the bile ducts, allowing direct interaction between gut microbe metabolism and human physiology. With such a substantial presence in the human body, the potential influence of gut microbial metabolism on the handling and toxicity of drugs cannot be overlooked.

Whilst the physiological or pathogenic effects of 3-HPPA have not been investigated, precursors of the metabolite, dietary chlorogenic acid and caffeic acid, have been studied with regards to their anti-hyperlipidemic properties (Cho et al. 2010; Karthikesan et al. 2010) which, given the role of HMG-CoA reductase (HMGR) in gentamicin accumulation in the proximal tubule (see Chapter 3), could have wider implications for susceptibility to aminoglycoside nephrotoxicity. A feeding study in streptozotocin-induced diabetic rats, which have greater HMGR activity than healthy rats, showed that chlorogenic acid and caffeic acid treatment lowered the activity of this enzyme (Karthikesan et al. 2010). Later studies identified that caffeic acid, not chlorogenic acid, is an activator of AMPK (Tsuda et al. 2012) which is an inhibitor of HMGR activity.

To examine the relationship of 3-HPPA, HMGR activity and gentamicin nephrotoxicity further, an independent set of urine samples from rats treated with gentamicin (120 mg/kg IP for 7 days) were analysed for pre-dose levels of 3-HPPA by ^{1}H-NMR and correlated to pre-dose mevalonic acid (MVA) and end-point kim-1; again, positive trends were observed between pre-dose 3-HPPA and MVA, the biomarker of HMGR activity, and pre-dose 3-HPPA and kim-1. Together, the relationship between pre-dose 3-HPPA and kim-1 excretion, pre-dose 3-HPPA and MVA and the inhibitory effect of chlorogenic and caffeic acid on HMGR *in vivo*, suggest that the gut microbial chlorogenic acid metabolites could influence the activity of host HMGR and therefore the toxicity of gentamicin.

Only 8% of chlorogenic acid and 19% of caffeic acid is absorbed from the gut in rats (Lafay et al. 2006b), with the bioavailability of chlorogenic acid and caffeic acid dependent on the composition and activity of the microbiome (Gonthier et al. 2003; Nicholson et al. 2005). A change in the gut metabolism of these phenolic compounds could affect systemic exposure and inhibition of the cholesterol biosynthetic pathway. Research into intestinal ecology has demonstrated that modulation of bacterial populations by the antibiotics penicillin and streptomycin had a profound effect on the co-metabolome, leading to a reduction in the degradation of typically metabolised metabolites thus increasing their bioavailability. In addition, the recolonisation conditions following a disruption in intestinal ecology plays a significant role in the re-establishment of intestinal microbiota and their inherent metabolic capacity (Swann et al. 2011). At a clinical level, patients repeatedly exposed to antibiotics throughout their lives could inadvertently develop health issues, such as susceptibility to adverse drug reactions, based on the recolonisation of their intestinal microbiota. In the context of gentamicin nephrotoxicity, differential systemic exposure to chlorogenic acid and caffeic acid could have a downstream effect on mevalonic acid production, megalin endocytosis and the accumulation and cytotoxicity of gentamicin.

Interestingly, metabonomic analysis has identified sex differences in 3-HPPA excretion in Han Wistar rats with larger amounts of 3-HPPA excreted by female rats in comparison to males (Stanley et al. 2005). Likewise, sex differences in the severity of aminoglycoside nephrotoxicity have been reported, but they are contradictory depending on the species, strain and choice of aminoglycoside (Goodrich and Hottendorf 1995; Sweileh 2009). Nonetheless if the relationship between 3-HPPA, HMG-CoA reductase and gentamicin nephrotoxicity is confirmed and translational, inter-individual and gender differences in susceptibility to gentamicin nephrotoxicity could be, in part, caused by the handling of chlorogenic acid by the gut microbes and its effect on HMGR activity.

Studies in chlorogenic acid-dosed rats indicate that it is rapidly hydrolysed to caffeic acid in the gut and the liver (Gonthier et al. 2003; Lafay et al. 2006a) whereas the first pass metabolism of caffeic acid is low (<7% metabolised) (Gumbinger et al. 1993); as such it is likely that chlorogenic acid is a poorly absorbed metabolic pre-cursor and caffeic acid is the effector on HMGR *via* AMPK. A preliminary study was carried out to determine whether HMGR activity could be lowered by daily intraperitoneal administration of caffeic acid, however in this experiment mevalonic acid excretion was unchanged after 7 days, data are shown in Appendix 3. It may be that the route of administration (I.P as opposed to oral or I.V), the dose (10 mg/kg) or the duration of the study requires adjustment to see the sustained effect on HMGR activity. There is also evidence that 3-HPPA precursors have antioxidant effects with investigations into ischemia (Sato et al. 2011), paraquat (Tsuchiya et al. 1996) , APAP and carbon tetrachloride toxicity (Janbaz et al. 2004). In addition, a study into iron overload demonstrated that caffeic acid supplemented diets reduced oxidative stress *and* hypercholesterolemia caused by high dose iron (Lafay et al. 2005). Therefore the relationship seen between 3-HPPA metabolism and gentamicin nephrotoxicity could be as a result of HMGR inhibition and/or antioxidant capacity.

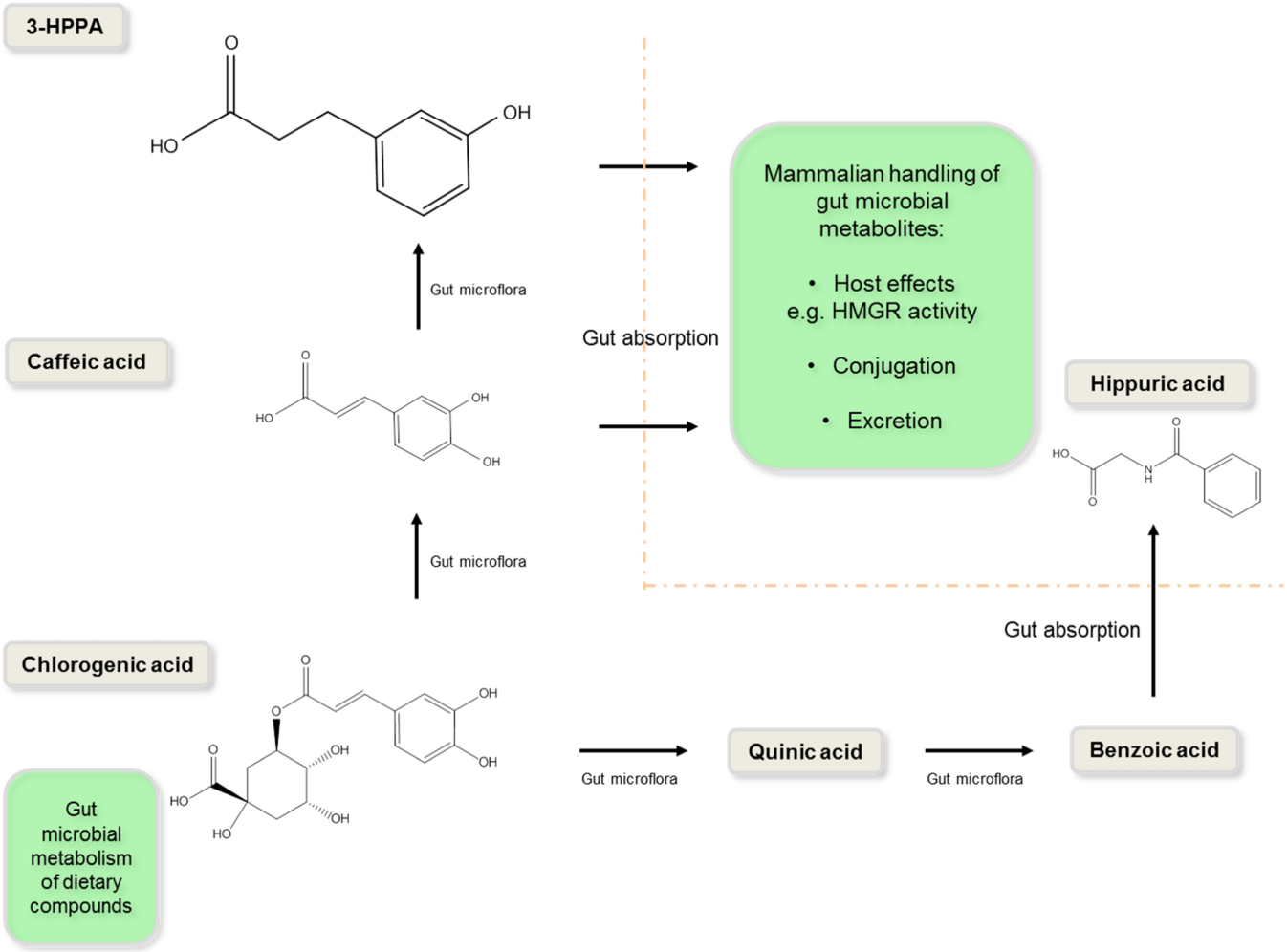


Figure 5.7 The metabolic fate of chlorogenic acid is the result of both gut microbial and mammalian metabolism. The end-products of chlorogenic acid metabolism, hippurate and 3-HPPA are competing pathways and the prevalence of one over the other is dependent on the composition of the microbiota.

Pharmacometabonomics, as demonstrated here, has enormous potential as a hypothesis-driving tool in not only the investigation of mechanisms of toxicity, but also the discovery of novel biomarkers related to therapeutic efficacy and the susceptibility to and progression of drug-induced organ damage. Even so, what is restrictive of this powerful technique is the management of the data, i.e. identifying every metabolite in a complex mixture, and the progression of these multivariate analyses into targeted experiments with specific questions, with the ultimate aim of changing those metabolite correlations into biochemical mechanisms.

6. GENERAL DISCUSSION

Contents

6.1 Introduction

The aim of this research was to develop a deeper understanding of the mechanism of aminoglycoside nephrotoxicity by pursuing molecular factors which influence the susceptibility of individuals to this adverse drug reaction. The current clinical strategy to manage aminoglycoside nephrotoxicity lacks the personalised stratification of patients based on risk and although mechanistic information is available it does not address the presence of susceptibility factors at the biochemical level. To address the aims of this research, targeted and non-targeted molecular approaches and a rat model of aminoglycoside nephrotoxicity were applied to detect biomarkers that can be used to understand the mechanism, to identify susceptibility factors and to guide therapeutic interventions of aminoglycoside nephrotoxicity.

The leading hypothesis of the targeted research was that the activity of the rate-limiting enzyme of the mevalonate pathway, HMG-CoA reductase, influences the accumulation and cytotoxicity of aminoglycosides in the proximal tubule. The primary hypothesis of the non-targeted studies was that comprehensive analysis of the metabolome, when integrated with biomarkers of toxicity, will reveal underlying molecular mechanisms and highlight specific metabolites related to the susceptibility and progression of aminoglycoside nephrotoxicity.

6.2 Measuring HMG-CoA reductase activity

In vitro investigations had established that inhibition of HMGR with statins could reduce megalin activity and the accumulation and toxicity of gentamicin (Antoine et al. 2010). It was postulated that the diurnal and inter-individual variation in HMGR activity could affect megalin-mediated endocytosis and therefore aminoglycoside nephrotoxicity. Before this could be investigated, it was necessary to develop and validate a LC-MS/MS method to quantify the product of HMGR, mevalonic acid, to serve as a biomarker of its activity. An LC-MS/MS method was validated to be accurate and precise over a biologically relevant range

for use in both rat and human urine. The method does have its limitations. The dynamic range is not suitable for the quantification of serum mevalonic acid which may be a more sensitive biomarker of systemic HMGR activity as only 30% of mevalonic acid filtered by the kidney is excreted in the urine. However these studies have demonstrated a good concordance between renal HMGR activity and urinary excretion and in addition, urine collection is less invasive than blood sampling and the sensitivity of the analytical method could be enhanced with a more sensitive MS instrument if deemed necessary.

The development and publishing of the assay is useful not only to studies of HMGR activity in relation to aminoglycoside nephrotoxicity but also to the multifarious health and disease states associated with the mevalonate pathway including, HMGR inhibitors administered for the treatment of hyperlipidemia (Woollen et al. 2001b), the diagnosis of mevalonate kinase deficiency (characterized by mevalonic aciduria) (Haas and Hoffmann 2006) and recently the treatment of cancer (Kato et al. 2010; Nilsson et al. 2011). Furthermore, HMGR activity has been observed to increase in a protective response to acute renal injury, causing cholesterol accumulation (Zager et al. 2002). The mevalonate pathway is clearly an important target in human pathology, toxicology and pharmacotherapy and the assay presented in herein will facilitate its investigation.

The biomarker method was employed to initially detect patterns of natural variation in rat, mouse and human HMGR activity and then to examine HMGR activity relative to aminoglycoside nephrotoxicity. Diurnal variation in mevalonic acid excretion was demonstrated in adult male Sprague-Dawley rats; this is the first demonstration of a significant diurnal rhythm of mevalonate production as previous attempts to quantify this variation in rats did not reach significance (Kopito et al. 1982). Oscillations in mevalonic acid excretion over a 24 h period in adult male CD-1 mice were also detected, which is another novel observation. The diurnal variation was translational, with paired samples from healthy volunteer children also showing contrasts between morning and evening urinary

mevalonic acid excretion. The diurnal activity of HMGR is clearly a conserved mammalian phenomenon and these translational findings add weight to the significance of the rat model of gentamicin nephrotoxicity used in these experiments.

6.3 HMG Co-A reductase activity and aminoglycoside nephrotoxicity

Measures of gentamicin accumulation and nephrotoxicity regressed against the pre-dose mevalonic acid data showed significant positive relationships with a distinct temporal component. These data are supportive of a causal relationship between HMGR activity and the accumulation and cytotoxicity of gentamicin. This is an important finding for aminoglycoside nephrotoxicity and certainly upholds the idea that the occurrence of this adverse drug reaction may be governed by susceptibility factors at a molecular level, i.e. the activity of HMGR in an individual.

The temporal component identified in these studies corroborates the concept that the time of administration is important in the development and extent of aminoglycoside nephrotoxicity. With the adoption of the once-daily dosing regimen into clinical practice (Chuck et al. 2000), circadian variation in the handling and toxicity of the aminoglycosides should be of concern. Previous studies suggested that the chronotoxicology of the aminoglycosides is related to the individual's activity period, whereby in rats aminoglycoside clearance and toxicity is greater and lesser, respectively, which was attributed to the circadian variation in glomerular filtration rate (Prins et al. 1997). However the temporal separation between the extent of gentamicin accumulation and nephrotoxicity could essentially be related to fluctuations in HMGR activity.

The study of urinary mevalonic acid in morning and evening samples from healthy volunteer children poses another issue related to temporal changes and HMGR activity. Whilst the majority of individuals had greater mevalonic acid excretion in the morning, several individuals had the opposite rhythm, which in clinical practice would complicate the formulation of dosing guidelines. Prior knowledge of an individual's HMGR status would be

useful in a dosing strategy. That being said, any influence this information might have on the clinical guidelines to aminoglycoside administration will be a result of balancing the risk of developing nephrotoxicity against optimising the efficacy of treating a life-threatening bacterial infection.

Unexpectedly, no relationship between HMGR activity and tobramycin nephrotoxicity was observed. With this result in mind, it is tempting to suggest that mevalonic acid excretion is a gentamicin-specific susceptibility biomarker. However, whilst gentamicin accumulation in the proximal tubule has been demonstrated to be megalin and HMGR-dependent, tobramycin proximal tubule accumulation has not been demonstrated to be caused by megalin, rather it has been presumed as a result of the definitive research with gentamicin, amikacin and netilmicin (Nagai et al. 2001). The result that tobramycin shows no relationship with HMGR activity is interesting as it is supporting evidence that tobramycin may have additional mechanisms of uptake into the proximal tubule aside from megalin. Indeed, there has been recent debate over endocytic-dependent and endocytic-independent uptake mechanisms regarding the aminoglycosides (Nagai and Takano 2014) with the suggestion that renal cation channels can also facilitate the influx of the aminoglycosides, although this research was conducted in cell lines with gentamicin rather than tobramycin, and some of the cation channels are distal tubule-specific.

It is clear that this is just the beginning of investigations into the differential uptake mechanisms of the aminoglycosides and it is likely that one mechanism of uptake may not fit every compound in the aminoglycoside class. Nevertheless, the observations with gentamicin and tobramycin have influenced the design of animal studies and a Phase IIb clinical trial in which statins are being investigated as prophylactic treatment for aminoglycoside nephrotoxicity. If statins prove to be a successful method of reducing aminoglycoside accumulation *via* megalin, statins could be utilised to augment the uptake of other megalin ligands such as the nephrotoxin polymyxin B (Abdelraouf et al. 2014).

6.4 A non-targeted approach to candidate biomarker identification

The non-targeted approach to identifying biomarkers of aminoglycoside nephrotoxicity detected numerous metabolic perturbations after exposure to gentamicin as early as 12 h after the first dose. Given the biochemical roles of the metabolites that were affected by gentamicin exposure, they are likely to be toxicity related changes and, prospectively, they could be biomarker candidates for the onset of adverse reactions to aminoglycoside exposure.

A major criticism of the technique is that the changes in metabolites that are deemed the 'usual suspects' appear in metabonomic studies independent of the type of toxin or its pharmacology and are a result of the physiological, unified response to stress. While such criticisms are valid and should be considered when interpreting the data, researchers should not overlook that metabonomics is a multivariate tool whereby the data set should be considered comprehensively. Just as kidney injury molecule-1, serum creatinine and blood urea nitrogen are individually useful, when used together they provide a greater depth of information. For metabonomics, it is the panel of metabolites and the patterns of change, rather than a metabolite in isolation, which paints a picture of the metabolic perturbation caused by drug exposure and will be useful in informing the diagnosis, prognosis and stratification of patients.

In Chapter 5 the pharmacometabonomic strategy identified that a pre-dose gut microbial metabolite, 3-HPPA, had a significant relationship to gentamicin nephrotoxicity and pre-dose mevalonic acid excretion, suggesting that not only could gut microbes affect the host response to gentamicin but that this could be biochemically connected to HMGR activity. The comprehensive analysis of the metabolome spurred new lines of investigation into 3-HPPA precursors and has addressed the notion that HMGR is influenced at multiple levels by the host, the gut microbiota and the environment.

Much attention has been focussed on the human genome for answers about human health, disease and the cause of inter-individual responses to drug efficacy and toxicity. While there have been some successes, many disease states and adverse drug reactions are likely to be as a consequence of host-environment interactions. There is a growing recognition of the role of gut microbes in human health and adverse drug reactions (Nicholson et al. 2005) and metabonomics permits the non-discriminant analysis necessary to encompass both human-derived and gut microbial metabolites, providing a comprehensive metabolic signature that can be assessed in relation to toxicity. However metabonomic studies are only as valuable as the samples used in the analysis; this research was limited by the number of animals that could be in experiment at any one time and thus the 'n' number used in the multivariate analyses was lower than optimum. As a result, stringent criteria were placed on the analysis (i.e. the strength of the correlation) and a follow up sample set was obtained to confirm the 3-HPPA result in the preliminary study.

There is no doubt that metabonomic approaches to understanding drug exposure and toxicity can rapidly yield a vast amount of information. The translation of 'big data' into useful targeted experiments that advance the field and influence clinical and pharmaceutical practice appears to be the drawback here. Since the inception of 'omics approaches we have not seen the surge in the number of drug approvals that was the promise of the 'omics era. However it is fair to say that metabonomics and its cousins, proteomics, transcriptomics and genomics, are still in their data-amassing infancy which with time will be converted to scientific understanding to inform drug development paradigm shifts and clinical practice.

6.5 Recommendations for future studies

Future experiments should be considered to further characterise the relationship of HMGR and gentamicin nephrotoxicity. Thresholds of HMGR activity should be examined, i.e. what is the tipping point between the minor and major accumulation and cytotoxicity of

gentamicin relative to mevalonic acid? Importantly, the relationship between pre-dose mevalonic acid and aminoglycoside toxicity should be examined in a clinical setting to examine whether this phenomenon is a translational finding. However it may be problematic to obtain baseline urine samples from patients that are in a critical condition and require immediate antibiotic treatment. Given the result with tobramycin, it is imperative that other aminoglycosides are studied; netilmicin and amikacin are proven ligands of megalin and might have a similar relationship with HMGR as observed with gentamicin.

Other factors which influence HMGR activity should also be explored. In addition to the known dietary influences on HMGR activity and cholesterol biosynthesis, HMGR activity is known to be influenced by hormones such as insulin, estrogens (Ness and Chambers 2000) and gender. In a clinical study 31.6% of females and only 6.7% of males experienced nephrotoxicity after amikacin treatment (Sweileh 2009) whilst in animal studies gender differences are dependent on strain and the aminoglycoside in question (Goodrich and Hottendorf 1995; Pai et al. 2013). The administration of testosterone to female rats did not exacerbate toxicity (Bennett et al. 1982) but estradiol worsened toxicity in both sexes (Carraro-Eduardo et al. 1993). There is a tendency to believe that most gender differences in physiology and disease are related to morphology and body size, but, it is important to consider differences at the molecular level that could influence an individual's response to drug exposure. Notably, sex differences in HMGR activity have been observed in rats (Carlson et al. 1978; De Marinis et al. 2008) which could contribute to inter-individual susceptibility to aminoglycoside nephrotoxicity. The issue of gender and susceptibility is complex and may vary depending on the species, strain and aminoglycoside in question but it is certainly an important avenue of investigation.

Finally, the role of 3-HPPA and chlorogenic acid metabolism on gentamicin nephrotoxicity is worth pursuing. An experiment which may be interesting to complete is a concerted

analysis of differential gut microbial populations relative to the chlorogenic acid metabolites, HMGR activity and aminoglycoside nephrotoxicity. The nature of gut microbial populations can be determined by fluorescent *in situ* hybridisation analysis of faeces, probing for specific species (Swann et al. 2011). Characterisation of the effect of gut microbes on drug toxicity is an exciting area of research that could prove very clinically useful in the reduction of adverse drug reactions and improvement of drug efficacy.

6.6 Contributions to toxicity research

In the research presented here, the overriding aim was to pursue susceptibility factors by using existing mechanistic knowledge to perform targeted investigations and to probe the mechanism of toxicity by employing non-targeted analytical techniques. A promising susceptibility biomarker of gentamicin nephrotoxicity, urinary mevalonic acid, has been identified and a robust analytical method has been developed and validated; multivariate analysis has highlighted the important intracellular mechanisms of gentamicin nephrotoxicity and revealed a relationship between pre-dose gut microbial metabolism and toxicity. What this research has also pin-pointed is that when it comes to mechanistic studies of drug toxicity, whilst there are unifying observations to be made between drugs in one class, one size does not fit all. Tempting as it is to assume a mechanism of toxicity fits a whole drug class and wide demographic, structurally related toxins may not have the same mechanism and more importantly, an individual's response to drug exposure may be influenced by an intricate endogenous-exogenous dynamic.

Toxicity continues to be a major concern to drug development and to the clinical efficacy and safety of therapeutics. Drug-induced kidney injury is just one of the types of adverse drug reaction that is encountered but the commonality between all adverse drug reactions is that they impede the approval and prescription of novel and existing therapies. Research is operating in an era where it has been accepted that biomarkers can aid in the detection and prognosis of disease but the search for and validation of these biomarkers is still a work

in progress. The techniques used in this research can be adapted to a wide range of diseases and adverse drug reactions and as presented here, significant advances in knowledge can be made, but it will be the continuation of this research that will effectively drive pharmaceutical and clinical change towards safer medicines.

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Appendix 1

Gentamicin dose-ranging study in mice

Strains: wild type C57BL/6, Nrf2-/- and wild type CD-1 mice

Dosing groups: 0.9% saline, 50, 100 or 200 mg/kg I.P gentamicin sulphate once daily (10:00-11:00 h) for 7 days or single I.P 20 mg/kg cisplatin. n= 2 animals per genotype per dosing group.

Free access to food and water.

Overnight urine collection day 3 to day 4.

Daily body weight change recorded, animals checked for signs of ill health before injections.

Overnight urine collection day 7 to day 8

Day 4 schedule 1 (rising CO_2 & cardiac puncture) of cisplatin group

Day 8 schedule 1 of gentamicin animals.

Outcome

The dose ranging study in wild type and Nrf2-/- mice (C57 background) and CD-1 albino mice indicated that gentamicin 50-200mg/kg once daily for 7 days did not cause nephrotoxicity, as determined by estimation of BUN, urinary excretion of NAG, body weight change and kidney weight ratio (no differences between gentamicin treated and saline treated animals). There we no overt visible signs of distress or ill health during the study and the kidneys were not visibly different to animals treated with 0.9% saline. Cisplatin-treated animals (positive kidney injury control) experienced significant body weight loss, raised BUN and urinary NAG.

Appendix 2

Histology results

Animal	Treatment	Organ	Histology
1	200 mg/kg gentamicin once daily	Liver (x2)	Diffuse glycogen (variable amount; PAS); NHAIR
		Kidney (x3)	- tubular protein casts +++ (PAS) - cortical tubules: dilated and devoid of epithelial cells, with individual or numerous apoptotic cells (**caspase-3**: pos.), with scattered mitotic cells, with hyaline droplets and vacuolation - multifocal, mononuclear (lymphocytes, macrophages) interstitial infiltration + Score: 4
2	0.9% saline once daily	Liver (x2)	Diffuse glycogen (variable amount; PAS); NHAIR
		Kidney (x3)	NHAIR; **caspase-3**: neg
3	200 mg/kg gentamicin once daily	Liver (x2)	Diffuse glycogen (variable amount; PAS) and slight mononuclear portal infiltration; NHAIR
		Kidney (x2)	- tubular protein casts ++/+++ (PAS) - cortical tubules: dilated and devoid of epithelial cells, with individual or numerous apoptotic cells (**caspase-3**: pos.), with scattered mitotic cells, with hyaline droplets and vacuolation - multifocal, mononuclear (lymphocytes, macrophages) interstitial infiltration ++ Score: 3-4
4	0.9% saline once daily	Liver (x2)	Diffuse glycogen (variable amount; PAS) and slight multifocal random mononuclear infiltration; NHAIR
		Kidney (x3)	NHAIR, apart from occ. tubular protein casts; **caspase-3**: neg
5	200 mg/kg gentamicin once daily	Liver (x2)	Patchy and generally low amount of glycogen (PAS); NHAIR
		Kidney (x3)	- tubular protein casts ++/+++ (PAS) - cortical tubules: dilated and devoid of epithelial cells, with individual or numerous apoptotic cells (**caspase-3**: pos.), with scattered mitotic cells, with hyaline droplets and vacuolation - multifocal, mononuclear (lymphocytes, macrophages) interstitial infiltration + Score: 3-4
6	0.9% saline once daily	Liver (x2)	Diffuse glycogen (PAS); NHAIR
		Kidney (x3)	NHAIR, apart from some tubular protein casts (PAS) ; **caspase-3**: neg
7	200 mg/kg	Liver (x2)	Diffuse glycogen; NHAIR

	gentamicin once daily	Kidney (x3)	- tubular protein casts +++ (PAS) - cortical tubules: dilated and devoid of epithelial cells, with individual or numerous apoptotic cells (**caspase-3**: pos.), with scattered mitotic cells, with hyaline droplets and vacuolation - glomeruli: some with swollen and hyaline appearance of glomerular tufts - multifocal, mononuclear (lymphocytes, macrophages) interstitial infiltration ++ Score: 4
8	0.9% saline once daily	Liver (x2)	Diffuse glycogen (PAS); NHAIR
		Kidney (x3)	NHAIR (PAS: ditto); **caspase-3**: neg
9	200 mg/kg gentamicin once daily	Liver (x2)	Diffuse glycogen (PAS); mild increase in leukocytes between hepatic cords
		Kidney (x3)	- tubular protein casts +++ (PAS) - cortical tubules: dilated and devoid of epithelial cells, with individual or numerous apoptotic cells (**caspase-3**: pos.), with scattered mitotic cells, with hyaline droplets and vacuolation - glomeruli: occ. with swollen and hyaline appearance of glomerular tufts - multifocal, mononuclear (lymphocytes, macrophages) interstitial infiltration (+) Score: 4
10	0.9% saline once daily	Liver (x2)	Diffuse glycogen (PAS); NHAIR
		Kidney (x2)	NHAIR; **caspase-3**: neg

Comments:
Histological analyses were completed by A. Kipar. The scoring system used is that previously published by Zhang J et al., 2009. Toxicol. Path. 37, 629-643.

Appendix 3

The effect of caffeic acid on HMGR activity

The effect of daily I.P injections of 6 mg/kg caffeic acid on HMGR activity was investigated. HMGR activity was measured on day 1, day 4 and day 7 by quantifying mevalonic acid in the urine. HMGR activity in rats was also monitored in animals given vehicle.

Study design

10 x male SD rats

2 dosing groups – vehicle or 6 mg/kg caffeic acid I.P

Vehicle – 1 x PBS

n=5 per group

I.P injection once daily (15:30 h)

Outcome

Caffeic acid 6 mg/kg per day I.P for 7 days did not decrease the excretion of mevalonate: