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Glucuronidation of fenamates: Kinetic studies using human kidney cortical microsomes and recombinant UDP-glucuronosyltransferase (UGT) 1A9 and 2B7

Paraskevi Gaganis, John O. Miners, Kathleen M. Knights *

Department of Clinical Pharmacology, Flinders University and Flinders Medical Centre, Bedford Park, Adelaide 5042, Australia

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ABSTRACT

Mefenamic acid, a non-steroidal anti-inflammatory drug (NSAID), is used commonly to treat menorrhagia. This study investigated the glucuronidation kinetics of flufenamic, mefenamic and niflumic acid using human kidney cortical microsomes (HKCM) and recombinant UGT1A9 and UGT2B7. Using HKCM Michaelis–Menten (MM) kinetics were observed for mefenamic (K_m^{app} 23 μM) and niflumic acid (K_m^{app} 123 μM) glucuronidation, while flufenamic acid exhibited non-hyperbolic (atypical) glucuronidation kinetics. Notably, the intrinsic renal clearance of mefenamic acid (CL_{int} 17 ± 5.5 μL/min mg protein) was fifteen fold higher than that of niflumic acid (CL_{int} 1.1 ± 0.8 μL/min mg protein). These data suggest that renal glucuronidation of mefenamic acid may result in high intrarenal exposure to mefenamic acyl-glucuronide and subsequent binding to renal proteins. Diverse kinetics were observed for fenamate glucuronidation by UGT2B7 and UGT1A9. Using UGT2B7 MM kinetics were observed for flufenamic (K_m^{app} 48 μM) and niflumic acid (K_m^{app} 135 μM) glucuronidation and atypical kinetics with mefenamic acid. Similarity in K_m^{app} between HKCM and UGT2B7 suggests that UGT2B7 may be the predominant renal UGT isoform catalysing niflumic acid glucuronidation. In contrast, UGT1A9 glucuronidation kinetics were characterised by negative cooperativity with mefenamic (S_{50} 449 μM, *h* 0.4) and niflumic acid (S_{50} 7344 μM, *h* 0.4) while atypical kinetics were observed with flufenamic acid. Additionally, potent inhibition of the renal glucuronidation of the UGT substrate 'probe' 4-methylumbelliferone by flufenamic, mefenamic and niflumic acid was observed. These data suggest that inhibitory metabolic interactions may occur between fenamates and other substrates metabolised by UGT2B7 and UGT1A9 in human kidney.

1. Introduction

The continued and widespread use of prescribed and over-the-counter (OTC) non-steroidal anti-inflammatory drugs (NSAIDs) remains an important cause of morbidity and mortality, and represents a significant and costly health problem. In the USA, NSAIDs alone account for approximately 70 million prescriptions and 30 billion OTC sales [1].

* *Corresponding author at*: Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, Adelaide 5042, Australia. Tel.: +61 8 8204 4331; fax: +61 8 8204 5114.
E-mail address: kathie.knights@flinders.edu.au (K.M. Knights).

Abbreviations: 4-MU, 4-methylumbelliferone; CL_{int}, intrinsic clearance; *h*, Hill coefficient; HKCM, human kidney cortical microsomes; IC_{50}, concentration of inhibitor causing 50% inhibition; K_m^{app}, apparent Michaelis constant; MM, Michaelis–Menten; NSAIDs, non-steroidal anti-inflammatory drugs; OTC, over-the-counter; RPN, renal papillary necrosis; S_{50}, substrate concentration at half-maximal velocity; UDP, uridine diphosphate; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; V_{max}, maximal velocity

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Substantial use of prescribed and OTC NSAIDs occurs in the treatment of heavy menstrual bleeding (menorrhagia). NSAIDs commonly used for this condition include aspirin, diclofenac, flurbiprofen, ibuprofen, indomethacin, meclofenamic acid, mefenamic acid and naproxen. Although, it is generally assumed that clinical efficacy is similar between NSAIDs, mefenamic acid is one of the most effective first-line drugs with demonstrated benefit in comparison to placebo in the treatment of menorrhagia [2]. Additionally, on a cost-benefit scale, mefenamic acid is considered an economical choice [3].

In the year 2000, mefenamic acid was listed in the top twenty five most used prescription pharmaceuticals in the English National Health Service by weight accounting for 14,522 kg and 544,000 prescriptions [4]. A comparable number of prescriptions (424,900) were dispensed for mefenamic acid in the UK in 2005 [5]. Interestingly, detection of mefenamic acid in the influent and effluent from wastewater treatment plants in Switzerland (17,275 kg sold/year) [6], southern England [7] and Japan [8] suggests substantial human consumption.

Flufenamic acid (Fig. 1), one of a series of *N*-phenylanthranilic acid derivatives synthesised during the early 1960s, was the first fenamate to undergo clinical trial as an anti-inflammatory drug. It was followed closely by mefenamic acid (Fig. 1), meclofenamic acid and niflumic acid (Fig. 1) [9]. Within ten years of marketing, cases of non-oliguric renal failure were reported with mefenamic acid [10–13]. Histological examination provided evidence of renal papillary necrosis (RPN) in some individuals [10] while in others nephrotoxicity was consistent with allergic interstitial nephritis [11,13]. The latter is often characteristic of an immune mediated response to irreversible binding of either a drug or its metabolites to various cell proteins [14].

RPN was described originally in diabetic humans but it is also commonly associated with analgesic abuse [15]. Early reports implicated chronic consumption of OTC compound analgesics containing phenacetin as a major factor in RPN. Population surveys reported wide geographic variation in analgesic abuse [16] with Australia having both the highest consumption of phenacetin (40 g/year in 1976) and the highest incidence of RPN at autopsy (3.7–21.4%) [17]. Investigations into the mechanism of analgesic induced RPN led to the development of animal models of RPN using either *N*-phenylanthranilic acid (a close structural analogue of mefenamic acid) [18] or mefenamic acid [19].

Subsequently, clinical studies implicated a range of NSAIDs in the development of RPN including alclofenac, amidopyrine, antipyrine, aspirin, benoxaprofen, fenoprofen, flufenamic acid, ibuprofen, indomethacin, mefenamic acid, naproxen, phenazone and phenylbutazone [17,20–22]. The exact mechanism of NSAID induced RPN is unknown but theories have centred on either a direct toxic effect on medullary interstitial cells from local generation of reactive drug metabolites or ischaemic injury through loss of production of renal protective vasodilatory prostaglandins [9,20]. It is probable that more than one mechanism prevails.

Metabolism is the principal route of NSAID elimination in vivo and only a small fraction of the administered dose is excreted unchanged in urine [23]. Like many other carboxylic

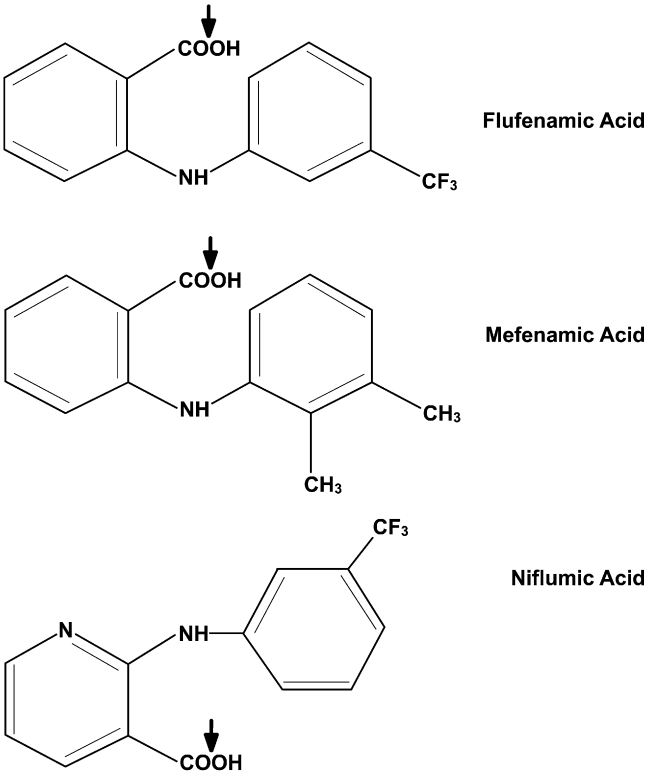

Fig. 1 – Structures of *N*-phenylanthranilic acid derivatives showing site of glucuronidation.

acid NSAIDs, the fenamates are metabolised extensively by glucuronidation as either the parent drug or oxidative metabolites. Various members of the UDP-glucuronosyltransferase (UGT) superfamily of enzymes catalyse the glucuronidation of NSAIDs. To date, of the nine human UGTs (UGT 1A3, 1A6, 1A9, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17) identified in the kidney, UGT 1A9 and 2B7, and to a lesser extent UGT1A3 are the predominant NSAID glucuronidating forms [24,25]. Unlike other glucuronides, however, NSAID acyl-glucuronides are electrophilic and bind covalently to plasma and tissue proteins both in vitro and in vivo [26]. Covalent modification of macromolecules by acyl-glucuronides (the hapten theory) results in production of circulating antibodies, which cause an immune response and drug hypersensitivity. In the case of mefenamic acid, formation of an acyl-glucuronide is known to result in irreversible binding of mefenamic acid glucuronide to proteins in vitro and ex vivo [27]. The question arises whether there is any basis for considering intrarenal formation of fenamate acyl-glucuronides as a contributing factor in the development of RPN in humans. However, fenamate glucuronidation by human kidney has not been investigated fully to date, hence any relationship to fenamate-induced RPN is unknown. This study sought to investigate the glucuronidation kinetics of flufenamic, mefenamic and niflumic acid using human kidney cortical microsomes (HKCM) and recombinant UGT1A9 and UGT2B7.

As fenamates have been shown to inhibit human liver phenol sulfotransferases [28,29], CYP2E1 [30] and mycophenolic acid glucuronidation by human liver and kidney [31] additional studies were undertaken to determine the uni-

versality of fenamates as potential inhibitors of renal glucuronidation using the UGT substrate 'probe' 4-methylumbelliferone (4-MU).

2. Materials and methods

2.1. Materials

The following compounds were purchased from Sigma–Aldrich (Sydney, Australia): uridine diphosphate glucuronic acid (UDPGA), 4-MU, and 4-MU-β-D-glucuronide. Flufenamic and mefenamic acid were obtained from Parke-Davis & Co. (Sydney, Australia), and niflumic acid from E.R. Squibb & Sons Inc. (Princeton, NJ, USA) all as the free acid. Other chemicals and reagents were of the highest analytical grade available.

2.2. Human kidney tissue

Human kidney tissue from six male subjects (K1, K4-K7, and K11) undergoing radical nephrectomy for malignant disease was obtained from the joint Flinders Medical Centre/Repatriation General Hospital Tissue Bank, South Australia. Approval for tissue collection and use for in vitro xenobiotic metabolism studies was obtained from the Research and Ethics Committee of the Repatriation General Hospital and the Flinders Clinical Research Ethics Committee, Flinders Medical Centre, South Australia. Renal cortical tissue distant to the primary tumour was isolated from fresh kidneys immediately following surgery and specimens were either: (1) placed in 10% neutral phosphate buffered formalin for routine histology; (2) used immediately for the preparation of microsomes; or (3) cryopreserved (−70°) for later studies.

Representative formalin-fixed and paraffin-embedded tissue samples were stained with haematoxylin and eosin, and examined by a specialist histopathologist. Typical for the age of the donors (43–83 years) only age related benign nephrosclerosis and, hypertensive-like vascular changes were reported for all sections. Full details of tissue histology and donor details for kidneys K1–K7 have been published previously [32]. Tissue (K11) was obtained from a male donor (65 years) with a drug history of docusate, irbesartan, nifedipine, oxycodone, ranitidine, and salbutamol. Histological examination of K11 tissue confirmed that the renal cortex was free of medullary tissue and revealed minimal glomerular obsolescence and focal interstitial fibrosis, and interstitial inflammation.

2.3. Preparation of human kidney cortical microsomes (HKCM) and expression of recombinant UGT1A9 and UGT2B7

HKCM were prepared using a standard differential ultracentrifugation technique as described previously [32]. The microsomal pellet was resuspended in 0.1 M Na_2HPO_4 (pH 7.4)/20% (w/v) glycerol, analysed for protein concentration [33] and stored at −70°. UGT1A9 and UGT2B7 were stably expressed in human embryonic kidney (HEK293) cells [32]. Cell lysates were prepared by probe sonication using four 1 s bursts interspersed by 3 min cooling on ice. The lysate was centrifuged (12,000 $g \times 5$ min) and the supernatant fraction stored at −70° until required for kinetic studies.

2.4. Quantification of fenamate glucuronides

The glucuronidation of flufenamic, mefenamic and niflumic acid by HKCM and recombinant UGT1A9 and UGT2B7 was determined using a modified radiometric thin-layer chromatography (TLC) assay [34]. Preliminary studies established linearity of the reaction with respect to time and protein concentration, and less than 10% consumption of each fenamate occurred during the course of the incubation. Final reaction conditions were as follows. Incubations (total volume 0.075 mL,) contained either flufenamic, mefenamic or niflumic acid (20–1000 μM, 0.5% v/v DMSO), phosphate buffer (0.1 M, pH 6.8), $MgCl_2$ (4 mM), UDPGA (2 mM) containing [^{14}C]-UDPGA (0.2 μCi, 0.01 mL) and either HKCM from kidneys K4, K7, or K11 (0.007 mg for studies with flufenamic and mefenamic acid or 0.025 mg for niflumic acid), or lysate from HEK293 cells expressing UGT1A9 or UGT2B7 (0.15 mg). Incubation duration was 180 min at 37° with HKCM and UGT2B7, and 60 min with UGT1A9. Reactions were terminated by the addition of HCl (0.005 mL, 0.18 M) and absolute ethanol (0.15 mL). Samples were vortex-mixed, centrifuged (6100 $g \times 5$ min) and aliquots (0.2 mL) of the ethanolic incubation mixture evaporated to dryness under a stream of nitrogen gas and reconstituted in acetone:water (1:1, 0.025 mL) prior to application to TLC plates (JT Baker, Si250F-(19C) silica gel) using a series III Nanomat (CAMAG-Muttenz, Switzerland).

Fenamate glucuronides were separated by development of TLC plates using *n*-butanol:acetone:glacial acetic acid:ammonia (35%):water (35:25:9:0.7:30.3) and quantified following exposure in a Storage Phosphor Screen Cassette (Molecular Dynamics Inc., CA, USA). Quantitative densitometry of glucuronides was based on a UDP-[U-^{14}C]glucuronic acid standard curve (0.6 pmol to 6 pmol) using a scanning laser PhosphorImager and associated ImageQuant software (Molecular Dynamics Inc., CA, USA).

2.5. Inhibition of 4-MU glucuronidation by NSAIDs

The fluorescence assay of Miners et al. [35], with modifications [32], was used for quantification of 4-MU glucuronide formation by HKCM, UGT1A9 and UGT2B7. Preliminary studies established linearity of the reaction with respect to time and protein concentration, and less than 10% substrate consumption occurred during the course of the incubation.

Incubations (total volume 0.6 mL) contained: 5 mM $MgCl_2$, UDPGA (2.5 mM with HKCM as the enzyme source; 5 mM with UGT1A9 and UGT2B7 as the enzyme sources); 4-MU (concentration corresponding to the apparent K_m (K_m^{app}) or S_{50}; viz. 20 μM for HKCM, 10 μM for UGT1A9 and 450 μM for UGT2B7 [32]; protein (HKCM and UGT1A9 0.017 mg/mL and UGT2B7 0.25 mg/mL); and flufenamic, mefenamic and niflumic acid (HKCM 20, 100 and 500 μM; UGT1A9 and UGT2B7 500 μM) dissolved in DMSO (final concentration 0.5% v/v). Control incubations contained DMSO (0.5% v/v) minus NSAID. Incubation duration at 37° was 20 min for HKCM and 45 min for UGT1A9 and UGT2B7. Reactions were terminated by the addition of 0.6 M glycine–0.4 M trichloroacetic acid (0.14 mL) and cooling on ice. Samples were extracted with chloroform and fluorescence of 4-MU glucuronide in the aqueous phase determined at 365 nm using an excitation wavelength of

Table 1 – Kinetic parameters for flufenamic, mefenamic and niflumic acid glucuronidation

Substrate	Enzyme source	Kinetic model[b]	Kinetic parameters			
			K_m^{app}, S_{50} (μM)	h[c]	V_{max} (pmol/min mg protein)	CL_{int}[d] (μL/min mg protein)
Flufenamic acid	HKCM[a]	Atypical*				
	UGT1A9	Atypical*				
	UGT2B7	MM	48 ± 0.15		81 ± 0.09	
Mefenamic acid	HKCM[a]	MM	23 ± 1.8		411 ± 6.8	17 ± 5.5
	UGT1A9	Hill	449 ± 201	0.4 ± 0.03	365 ± 32	
	UGT2B7	Atypical*				
Niflumic acid	HKCM[a]	MM	123 ± 0.65		134 ± 0.22	1.1 ± 0.8
	UGT1A9	Hill	7344 ± 61	0.4 ± 0.001	57 ± 0.15	
	UGT2B7	MM	135 ± 9.9		19 ± 0.6	

[a] Composite fit of HKCM data represent mean ± S.E. of the parameter estimate from three kidneys.
[b] MM (Michaelis–Menten: K_m^{app}); Hill (Hill equation: S_{50}).
[c] h denotes the Hill coefficient.
[d] mean ± S.D., $n = 3$ kidneys.
* Kinetic parameters not determined due to atypical kinetics.

315 nm. Quantification was undertaken by reference to standard curves prepared using authentic 4-MU-β-D-glucuronide (0.1–10 μM).

2.6. Kinetic and statistical analyses

The kinetic parameters S_{50}, K_m^{app} and V_{max} were derived from fitting untransformed data to the Michaelis–Menten and Hill equations using an extended least squares modelling program (EnzFitter, Biosoft 2004, v2.0.18.0). The goodness of fit was determined by comparison of statistical parameters (95% confidence intervals for the curve fit, and F-statistic) between the various models [36]. Kinetic data are reported (Table 1) as mean ± S.E. of the parameter estimate. In vitro intrinsic clearance (CL_{int}) was calculated as V_{max}/K_m^{app} for those reactions exhibiting Michaelis–Menten kinetics. Statistical significance of the effect of individual NSAIDs on 4-MU glucuronidation by HKCM was determined by a univariate Analysis of Variance (ANOVA; SPSS for Windows, Rel. 12.0, 2004, Chicago: SPSS. Inc.) and Dunnett's post-hoc analyses. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Kinetics of flufenamic, mefenamic and niflumic acid glucuronidation by HKCM

Flufenamic acid glucuronidation by HKCM ($n = 3$) exhibited atypical (i.e. non-hyperbolic) kinetics. Eadie-Hofstee plots were indicative of either the involvement of two enzymes or negative cooperativity (Fig. 2A). However, kinetic analyses were inconclusive; data were not adequately described by the kinetic models (2-enzyme Michaelis–Menten and Hill equations) normally used to describe these types of data. Although the goodness of fit was poor, the Hill coefficient was consistently <1 indicative of negative cooperativity. Mefenamic acid (Fig. 2B) and niflumic acid (Fig. 2C) glucuronidation by HKCM ($n = 3$) exhibited Michaelis–Menten kinetics. Mean kinetic data are summarised in Table 1. K_m^{app}, V_{max}, and CL_{int} values for mefenamic acid glucuronidation ranged from 23–26 μM, 255–568 pmol/min mg protein and 11–22 μL/min mg protein, respectively. The corresponding ranges of these parameters for niflumic acid glucuronidation were 100–210 μM, 98–201 pmol/min mg protein and 0.5–2 μL/min mg protein, respectively.

3.2. Kinetics of flufenamic, mefenamic and niflumic acid glucuronidation by UGT1A9 and UGT2B7

As observed with HKCM as the enzyme source, flufenamic acid glucuronidation by recombinant UGT1A9 exhibited non-hyperbolic kinetics (Fig. 2D), with Eadie-Hofstee plots indicative of either the involvement of 'two' enzymes or negative cooperativity. Again however, the data were poorly modelled by the 2-enzyme Michaelis–Menten and Hill equations normally used to describe these types of data. In contrast, UGT2B7 catalysed flufenamic acid glucuronidation exhibited Michaelis–Menten kinetics with a K_m^{app} of 48 μM (Fig. 2G and Table 1).

Mefenamic acid glucuronidation by UGT1A9 was well described by the Hill equation, with negative cooperativity (h 0.4 ± 0.03, Fig. 2E and Table 1). In contrast, UGT2B7 catalysed glucuronidation of mefenamic acid exhibited non-hyperbolic kinetics (Fig. 2H) that could not be modelled by the 2-enzyme Michaelis–Menten and Hill equations. Like mefenamic acid, niflumic acid glucuronidation by UGT1A9 (Fig. 2F) was characterised by negative cooperativity (Table 1, h 0.4 ± 0.001), whereas UGT2B7 catalysed niflumic acid glucuronidation (Fig. 2I) exhibited Michaelis–Menten kinetics with a K_m^{app} value of 135 μM similar to that observed with HKCM (123 μM) (Table 1).

3.3. Fenamate inhibition of 4-MU glucuronidation by HKCM, UGT1A9 and UGT2B7

At all concentrations studied (20–500 μM) flufenamic, mefenamic and niflumic acid significantly inhibited 4-MU

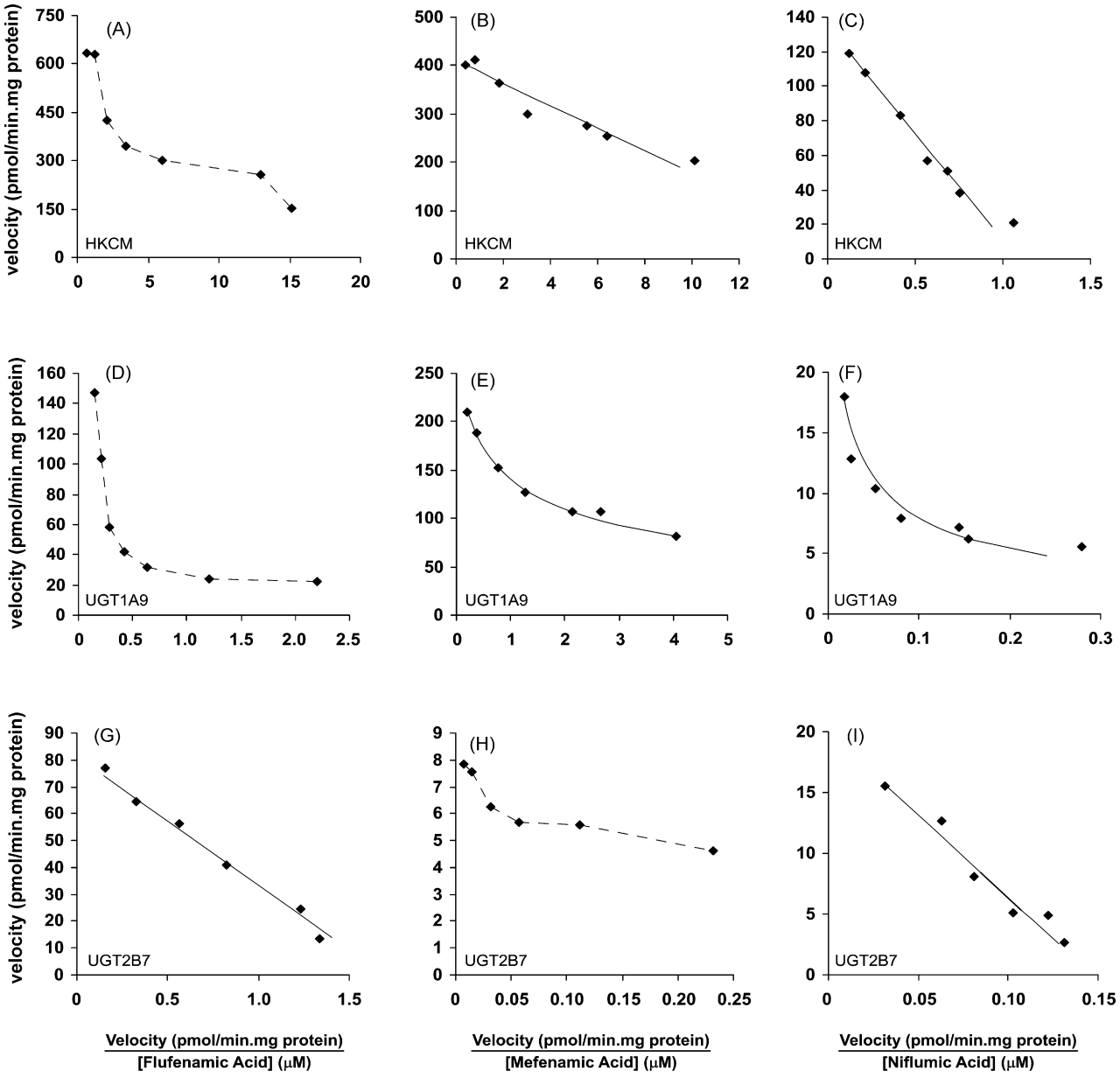

Fig. 2 – Flufenamic acid glucuronidation catalysed by (A) HKCM (non-hyperbolic kinetics), (D) UGT1A9 (non-hyperbolic kinetics), and (G) UGT2B7 (Michaelis–Menten kinetics); mefenamic acid glucuronidation catalysed by (B) HKCM (Michaelis–Menten kinetics), (E) UGT1A9 (negative cooperativity), and (H) UGT2B7 (non-hyperbolic kinetics); niflumic acid glucuronidation catalysed by (C) HKCM (Michaelis–Menten kinetics), (F) UGT1A9 (negative cooperativity), and (I) UGT2B7 (Michaelis–Menten kinetics). Concentration range 20–1000 μM for all fenamates. Data points are experimentally determined values (mean of three kidneys, single determinations), solid lines depict the composite model-fitted curve of best fit, dashed lines are for visualisation and do not depict model fitted curves.

glucuronidation by HKCM. Greater than 90% inhibition ($P < 0.001$) was observed with all fenamates at a concentration of 500 μM (Fig. 3). Similar to HKCM, the fenamates (500 μM) were potent inhibitors of 4-MU glucuronidation by UGT1A9 and UGT2B7 (Fig. 3). With UGT1A9 as the enzyme source, glucuronidation of 4-MU was inhibited (>99%) by each fenamate. Similarly, UGT2B7 catalysed 4-MU glucuronidation was inhibited by mefenamic acid and flufenamic acid (>95%), while niflumic acid inhibited UGT2B7 activity to a lesser extent (viz. 66%).

4. Discussion

A substantial number of clinically used NSAIDs from various chemical classes are metabolised by glucuronidation [37]. Although the majority of studies involve investigation of hepatic metabolism, the relative contribution of the kidney to the systemic clearance of NSAIDs is unknown. However, NSAIDs can cause renal dysfunction, ranging from electrolyte disturbances to acute renal failure and RPN. The latter is often associated with the use of the fenamate class of NSAIDs, in

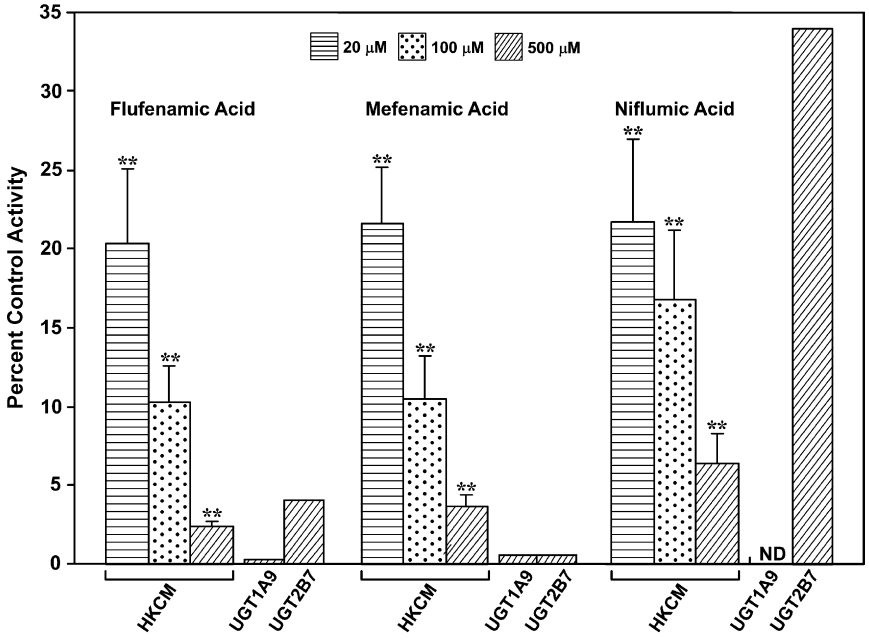

Fig. 3 – The effect of fenamates on 4-MU glucuronidation catalysed by HKCM, UGT1A9 and UGT2B7 was determined in the presence of 4-MU (20, 10 and 450 μM, respectively) and either 20, 100, or 500 μM flufenamic, mefenamic or niflumic acid. Results are presented as percent control activity (absence of fenamate); HKCM 3.9 ± 1.1 nmol/min mg protein, mean ± S.D. from five individual kidneys each studied in duplicate; UGT1A9 and UGT2B7, mean of duplicate determinations, control activity 3.8 and 0.5 nmol/min mg protein, respectively. Statistical significance is denoted as $^{**}P < 0.001$. ND = no detectable activity.

particular mefenamic acid. Currently there is no definitive explanation for NSAID induced RPN but it may be due in part to the effect of NSAIDs on renal haemodynamics and/or intrarenal formation of reactive NSAID acyl-glucuronides. This study has demonstrated that the *N*-phenylanthranilic acid derivatives flufenamic, mefenamic and niflumic acid are substrates for glucuronidation by human kidney cortical microsomes and recombinant human UGT1A9 and UGT2B7; NSAID-glucuronidating enzymes that are expressed in human kidney. Local formation of in particular mefenamic acid acyl-glucuronide, a reactive metabolite, may result in covalent binding to proteins within the renal cortex, potentially eliciting an immune response.

The kinetics of mefenamic and niflumic acid glucuronidation by human kidney cortical microsomes were described by the Michaelis–Menten (hyperbolic) equation, with K_m^{app} values of 23 and 123 μM, respectively. In comparison to niflumic acid, the intrinsic in vitro renal clearance of mefenamic acid via glucuronidation was approximately fifteen-fold greater indicating significant potential for intrarenal exposure to the highly reactive acyl glucuronide of mefenamic acid [26,27]. UGT1A9 catalysed mefenamic acid (S_{50} 449 μM) and niflumic acid glucuronidation kinetics (S_{50} 7344 μM) were characteristic of homotropic negative cooperativity (Hill equation, *h* 0.4). However, the lower affinity of UGT1A9 suggests that its involvement in renal clearance of niflumic acid via glucuronidation is likely to be minor. In addition, UGT1A3 ([24] and unpublished data) also catalyses niflumic acid glucuronidation and hence the kinetics observed with HKCM may represent the composite influence of at least three renal UGTs.

In contrast to UGT1A9, atypical (non-hyperbolic) kinetics were observed for mefenamic acid glucuronidation by UGT2B7. Although the Michaelis–Menten (one and two-enzyme) and Hill equations are most commonly used for the modelling of glucuronidation kinetics [38], in this study the UGT2B7 data for mefenamic acid glucuronidation were not modelled well by any of these models. Moreover, fitting data to a 2-site model [39] was unsuccessful (data not shown). Hence, the mechanism of the atypical glucuronidation kinetics reported here for mefenamic acid by UGT2B7 remains unknown. Interestingly, atypical kinetic profiles previously overlooked or ignored [40], are reported increasingly in the field of glucuronidation [39,41,42]. Non-specific binding of mefenamic acid was considered a source of atypical kinetics although measures were taken to avoid this by using the lowest possible concentration of protein as recommended [40]. In addition, acidic drugs exhibit relatively low non-specific protein binding [43]. Unlike mefenamic acid, niflumic acid glucuronidation by UGT2B7 followed Michaelis–Menten kinetics and the similarity in K_m^{app} 135 μM with that observed with HKCM (K_m^{app} 123 μM) suggests that in human kidney UGT2B7 may be the predominant UGT isoform catalysing niflumic acid glucuronidation.

In contrast to the Michaelis–Menten kinetics observed with mefenamic and niflumic acid, flufenamic acid glucuronidation by HKCM exhibited atypical kinetics characteristic of homotropic negative cooperativity (Hill coefficient <1). Negative cooperativity implicitly assumes the binding of more than one substrate molecule to the enzyme active site, which may occur within a single active site or at two distinct sites [44]. In

this regard, UGTs may act cooperatively as multimeric enzymes through dimerization and UGT1A9 has been shown to form homo- and hetero-dimers [45]. Interestingly the kinetics of UGT1A9 catalysed flufenamic acid glucuronidation exhibited negative cooperativity suggesting that UGT1A9 may be the predominant isoform influencing the HKCM profile. The kinetics of UGT2B7 flufenamic glucuronidation were modelled by the Michaelis–Menten equation (K_m^{app} 48 μM) indicating a role for UGT2B7 in the renal clearance of flufenamic acid.

Mefenamic, flufenamic and niflumic acid are extensively glucuronidated (>50%) as either the parent drug or as oxidative metabolites [27,46,47]. These fenamates have previously been shown to inhibit mycophenolic acid glucuronidation by human kidney, with respective IC_{50} values 49 ± 4, 13 ± 2, and 8 ± 2 μM [31]. Further investigation of the three fenamates as inhibitors of glucuronidation was undertaken using the non-selective UGT substrate 4-methylumbelliferone with HKCM, UGT1A9 and UGT2B7 as the protein sources. Although the concentration range used spanned 20–500 μM in experiments with HKCM, it was evident by the extent of inhibition observed at 20 μM (>75%) that the fenamates were potent inhibitors of UGTs. The estimated IC_{50} values using HKCM were within the range 0.3–0.4 μM for all three fenamates, one to two orders of magnitude less than that reported for inhibition of renal mycophenolic acid glucuronidation by the same fenamates [31]. (However, since the fenamate concentrations used did not span the calculated IC_{50} range the values should be considered estimates only). Despite this caveat, the estimated IC_{50} values for mefenamic, flufenamic and niflumic acid inhibition of 4-MU glucuronidation by HKCM are comparable to the therapeutic unbound plasma drug concentrations in vivo (0.28, 0.4 and 0.68 μM, respectively) [23,48]. Similar to HKCM mefenamic and flufenamic acid (at a concentration of 500 μM) inhibited UGT1A9 and UGT2B7 4-MU glucuronidation by >95%. The exception was niflumic acid, which abolished 4-MU glucuronidation by UGT1A9 and inhibited UGT2B7 by 66%. The mechanism of the inhibitory interaction was not studied further because of the complexity of the glucuronidation kinetics of 4-MU by UGT1A9 (substrate inhibition) and UGT2B7 (sigmoid kinetics) [32]. However, these data suggest that the fenamates may inhibit in vivo renal glucuronidation of substrates for UGT1A9 and UGT2B7, both important in the renal metabolism of a variety of drugs and endogenous chemicals, including aldosterone [49]. Our data provides evidence that mefenamic, flufenamic and niflumic acid are potent inhibitors of renal UDP-glucuronosyltransferases (including UGT1A9 and UGT2B7) in addition to a number of other enzyme families, such as human liver catechol and phenol sulfotransferases [28,29], human CYP2E1 [30] and human aldo-keto reductase (AKR) isoforms, particularly the AKR1C family [50,51].

In conclusion, human kidney cortical microsomes catalyse the glucuronidation of mefenamic acid, flufenamic acid and niflumic acid. Arguably UGT2B7, which is expressed in kidney, is one of the most important UGTs in terms of xenobiotic metabolism and our data identifies a role for UGT2B7 in the renal glucuronidation of mefenamic, flufenamic acid and niflumic acid. In contrast to the predominant use of mefenamic acid in young women of reproductive age, widespread use of mefenamic acid (proposed in the treatment of Alzheimer's disease [52]) in the elderly may result in an increased incidence of NSAID-induced nephrotoxicity. Although the exact mechanism of NSAID-induced RPN remains unclear, it has been suggested that RPN may result from a combination of NSAID-induced decreased renal papillary perfusion and excessive concentrations of NSAID and/or NSAID metabolites [53]. Our study has shown that human kidney metabolises mefenamic acid to an acyl-glucuronide. Thus, in situations of renal function impairment (e.g. the elderly) hydrolysis of mefenamic acid acyl-glucuronide may occur, leading to release of parent drug. The high local intra renal concentration of mefenamic acid would provide an opportunity for continued suppression of vasodilatory prostaglandin synthesis through persistent inhibition of renal cyclo-oxygenases, thus further exacerbating a decline in renal function in at-risk individuals. This further reduction in renal function would reduce excretion of fenamate glucuronides increasing the likelihood of binding to macromolecules within the kidney. Occurrence of the latter situation may provide an explanation for the rare cases of mefenamic acid-induced RPN. Additionally, we have demonstrated that mefenamic, flufenamic and niflumic acid are potent inhibitors of renal UGTs. As glucuronidation, principally by UGT2B7, limits the duration of action of a multitude of endogenous molecules that contribute to regulation of renal function (e.g. arachidonic acid and PGE_2) the potential arises for significant inhibitory metabolic interactions in vivo between fenamates (and other drugs) and endogenous substrates metabolised in the kidney via glucuronidation [54].

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