BIOMARKERS, VOL. 8, NOS. 3–4 (MAY–AUGUST 2003), 272–286

Taylor & Francis
healthsciences

A potential biomarker of kidney damage identified by proteomics: preliminary findings

LASANTHA R. BANDARA[1]*, MIKE D. KELLY[1], EDWARD A. LOCK[2] and SANDY KENNEDY[1,3]

[1] Oxford GlycoSciences (UK) Ltd, The Forum, 86 Milton Park, Abingdon, Oxon, OX14 4RY, UK
[2] Syngenta CTL, Alderley Park, Cheshire, SK10 4TJ, UK
[3] GlaxoSmithKline, Park Road, Ware, Hertfordshire, SG12 0DP, UK

Received 22 November 2002, revised form accepted 24 February 2003

4-Aminophenol (4-AP) and D-serine are established rodent nephrotoxins that selectively damage renal proximal tubules. In an attempt to understand the mechanism of action of these toxicants in greater detail, a high throughput proteomics approach was used to profile protein changes in the plasma of animals treated with these compounds. Male Fischer 344 and Alderley Park rats were treated with increasing doses of 4-AP or D-serine and plasma samples were collected over time. Control groups received either saline or the non-toxic enantiomer, L-serine. Using high throughput two-dimensional gel analysis, a number of plasma proteins showing dose- and time-dependent regulation were identified. One toxicity-associated plasma protein was identified as the cellular enzyme fumarylacetoacetate hydrolase (FAH), which is known to be required for tyrosine metabolism. The FAH gene is mutated in the human genetic disorder type I tyrosinaemia, which is associated with liver and kidney abnormalities and neurological disorders. FAH was elevated in the plasma of animals treated with 4-AP and D-serine at early time points and returned to baseline levels after 3 weeks. The protein was not elevated in the plasma of control animals or those treated with L-serine. The presence of FAH in plasma is intriguing as it is normally a cellular enzyme with no known function in plasma. It is possible that 4-AP and D-serine may work through a previously unknown mechanism in the kidney via regulation of tyrosine metabolism or FAH activity. Therefore, FAH may function in a fashion analogous to the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes that are used to measure liver injury. The link between kidney toxicants and inherited tyrosinaemia also raises the possibility that FAH may be a marker of kidney toxicity in humans. These observations highlight the value of proteomics in identifying new biomarkers and providing new unprecedented insights into complex biological mechanisms.

Keywords: kidney, toxicity, proteomics, tyrosinaemia, biomarker, two-dimensional polyacrylamide gel electrophoresis, protein profiling, metabolism.

Introduction

The paracetamol (acetaminophen) metabolite, 4-aminophenol (4-AP), is an established nephrotoxin that produces severe necrosis of the pars recta of the proximal tubule in the rat (Green *et al*. 1969, Davis *et al*. 1983, Gartland *et al*. 1989). The mechanism by which 4-AP exerts its nephrotoxic action

* Corresponding author: Lasantha R. Bandara, Oxford GlycoSciences (UK) Ltd, The Forum, 86 Milton Park, Abingdon, Oxon, OX14 4RY, UK. Tel: (+44) 1235 208000; fax: (+44) 1235 207626; e-mail: lan.bandara@ogs.co.uk

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/1354750041233133297

is not completely understood, although it is thought to be mediated by the formation of a toxic metabolite 1,4-benzoquinoneimine, which may bind to thiol groups in proteins (Calder *et al.* 1979, Crowe *et al*. 1979). In support of this concept, studies performed using radiolabelled 4-AP demonstrated binding to renal proteins and a corresponding decrease in renal glutathione levels (Eckert *et al.* 1990). Recent studies have shown that the glutathione conjugate of 4-AP is targeted to the kidney, where further processing of the conjugate and oxidation may produce the nephrotoxic metabolite (Fowler *et al.* 1991, 1994). Several groups have also reported rat strain differences in sensitivity to 4-AP toxicity, with the Fischer 344 rat being more sensitive to renal injury than the Sprague-Dawley rat (Newton *et al.* 1983). D-Serine is the enantiomer of L-serine, which is also known to selectively damage the pars recta of proximal tubules in the kidney (Ganote *et al*. 1974, Kaltenbach *et al.* 1982). The basis for the selective toxicity of D-serine is not fully understood, although the amino acid has been shown to concentrate in the region of the nephron that is damaged (Imai *et al*. 1998). D-Serine enters cells in the pars recta by reabsorption (Silbernagl *et al*. 1999), where it is a substrate for D-aminoacid oxidase, which may lead to the formation of toxic oxidative metabolites. Thus, although both compounds damage the same part of the nephron, there is little evidence to suggest they act via a similar mechanism or pathway.

Recently a range of genomic and proteomic technologies have been highlighted as tools that may be of help in addressing certain issues in toxicology (Smith 2001, Aardema and MacGregor 2002). Proteomics based on two-dimensional (2D) technologies are already showing promise in the identification of novel mechanistic pathways and biomarkers of toxicity (Kennedy 2001, Bandara and Kennedy 2002). Several proteomic toxicity studies have already been performed on rat renal tissue in an attempt to gain new mechanistic insights. For example, Witzmann *et al.* (1999) identified a number of proteins associated with oxidative stress and mitochondrial function that were regulated in the rat kidney in response to treatment with lead acetate. Studies using the aminoglycoside antibiotic gentamicin reported changes in a variety of proteins responsible for stress response, glucose metabolism and lipid biosynthesis (Charlwood *et al*. 2002, Kennedy 2002). Several other kidney proteome studies investigating glomerular injury have also been reported (Cutler *et al*. 1999, Steiner *et al.* 1996). Surprisingly, few proteomic studies have been published to date on analysing changes in blood following a toxic insult. Serum or plasma toxicity markers may be particularly attractive as a clinical tool to monitor patient health, since, unlike kidney tissue, blood samples can be readily obtained with minimal risk to the patient. We therefore chose to examine the nephrotoxicity of 4-AP and D-serine in greater detail by monitoring protein changes in the plasma of treated rats using the high throughput 2D gel proteomics technology developed at Oxford GlycoSciences (OGS) (Page *et al*. 1999, Harris *et al.* 2002). Here we report the results of this work, focusing in particular on the plasma levels of the enzyme fumarylacetoacetate hydrolase, which correlated with the extent of nephrotoxicity despite having no previously reported role in the plasma.

Materials and methods

Compounds

4-AP hydrochloride was purchased from the Aldrich Chemical Company (Gillingham, UK) and dissolved in isotonic saline for dosing. Both D- and L-serine were purchased from Sigma-Aldrich Company Ltd (Gillingham, UK).

Animals and dosing

Four groups of 20 male Fischer 344 (F344) rats (7–8 weeks old, weighing 150–200 g on arrival) (Charles River Ltd, Margate, UK) were given a single intraperitoneal injection of either isotonic saline or 4-AP in isotonic saline at a dose of 20, 50 or 80 mg kg^{-1} at a dose volume of 2.5 ml kg^{-1} bodyweight. In addition, two groups of 20 male Alderley Park (Alpk) rats (7–8 weeks old, weighing 201–230 g on arrival) (Rodent Breeding Unit, Alderley Park, Macclesfield, UK) were dosed via the same route with either isotonic saline or 4-AP at a dose of 80 mg kg^{-1}. Five groups of 20 male Alpk rats were also given a single intraperitoneal injection of either isotonic saline, D-serine in isotonic saline at a dose of 75, 250 or 750 mg kg^{-1}, or L-serine at a dose of 750 mg kg^{-1}, at a dose volume of 4.0 ml kg^{-1} bodyweight. An additional group of Alpk rats were placed in metabolism cages, and urine collected over solid carbon dioxide before and then 12, 24 and 36 h after a single intraperitoneal injection of 250 mg kg^{-1} D-serine.

Assessment of renal function

Animals were sacrificed by inhalation of an overdose of halothane 4, 8 or 24 h post-dosing or following a 3 week treatment-free period. Blood samples were collected via cardiac puncture in heparinized tubes for clinical chemistry assessment and in ethylene diamine tetra-acetic acid (EDTA) tubes for proteomic analysis. The blood was then centrifuged at 1500 *g* at 4°C for 10 min and the plasma removed. For the proteomic samples, protease inhibitors were also added to plasma and all samples were snap frozen in liquid nitrogen prior to analysis. Blood urea nitrogen and plasma creatinine were measured with a Kone automated analyser using standard procedures. A macroscopic examination was carried out post mortem for signs of toxicity. In addition, the left kidney and a sample of liver and heart were removed and fixed in 10% neutral buffered formol saline. Following processing into wax, 5 μm sections were stained with haematoxylin and eosin and examined by light microscopy.

Proteome analysis

Plasma samples were analysed using the high throughput 2D gel proteomics technology developed at OGS (Page *et al*. 1999, Harris *et al*. 2002). Briefly, samples were initially enriched to remove the high abundance proteins, immunoglobulins, haptoglobin, transferrins and albumin (Kennedy 2001, Bandara *et al.* 2003). Enriched samples were then separated by isoelectric focusing in the first dimension and by apparent molecular weight in the second dimension. Gels were subsequently fixed, stained with a fluorescent dye and scanned using a digital scanner (Page *et al*. 1999). The resulting images were compiled into a database (mastergroup) containing all the protein data points from each sample with the associated clinical chemistry data. Further data analysis was performed using the Rosetta™ software developed at OGS (Page *et al.* 1999).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

Enriched plasma samples from D-serine-treated rats taken 4 h after 75 mg kg^{-1} or 4 or 8 h after 750 mg kg^{-1} and from L-serine-treated rats taken 8 h after 750 mg kg^{-1} were used. Urine samples from rats before and then 0–12 h, 12–24 h and 24–36 h after 250 mg kg^{-1} of D-serine were also analysed. Approximately 3–5 μg of plasma protein or 15 μg of urinary protein were mixed with sample buffer consisting of 0.125 M Tris-HCl, pH 6.8, containing 8% sodium dodecyl sulphate (w/v), 20% glycerol (v/v), 0.002% bromophenol blue (w/v) and dithiothreitol (6 mg ml^{-1}), and boiled for 5 min. Aliquots were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-RAD Criterion minigels with 12.5% acrylamide in the resolving gel for 120 min at 120 V (urine) or 180 min at 80 V (plasma). The protein was transferred overnight at 0.2 mA at 4°C onto Hybond-P membranes (Amersham). The membranes were incubated for over 60 min at room temperature in a blocking solution consisting of Tris buffered saline (TBS), Tween-20 (0.1% v/v) and fat-free dry milk (5% w/v). Anti-fumarylacetoacetate hydrolase antibody, a generous gift from Professor R. Berger, Department of Metabolic Diseases, Wilhelmina Children's Hospital, Holland, was used at a dilution of 1:1000 in 20 mM Tris-HCl and 0.9% sodium chloride, pH 8.2 (i.e. TBS), containing Tween-20 (0.1% v/v) for 60 min, after which the blots were thoroughly washed with TBS-Tween-20 and incubated for 60 min with donkey anti-rabbit horseradish peroxidase (HRP) conjugate at a dilution of 1:1500 in TBS-Tween. The membranes were washed again in TBS-Tween prior to the antibody reactivity being visualized using enhanced chemiluminescence HRP (ECL Plus, Amersham Life Sciences).

Table 1. BUN (mmol l^{-1}) data from F344 rats treated with 4-AP. 4-AP treatment resulted in a dose-dependent increase in BUN, peaking at 24 h post-dosing.

	4-AP dose (mg kg^{-1})			
Time post-dose	0	20	50	80
4 h	1.85 ± 0.24	2.07 ± 0.17	3.15 ± 0.26**	3.29 ± 0.64**
8 h	1.95 ± 0.26	1.87 ± 0.30	3.61 ± 0.83**	4.36 ± 1.91**
24 h	2.76 ± 0.22	2.28 ± 0.22*	5.11 ± 2.06*	6.60 ± 2.62*
3 weeks	2.89 ± 0.35	2.91 ± 0.36	2.89 ± 0.19	2.88 ± 0.32

Values represent the mean ± SD of five animals. *$p < 0.05$ and **$p < 0.01$ compared with concurrent control (0 mg kg^{-1}) group using the Student's t-test.

Results

Blood urea nitrogen (BUN) levels in F344 animals treated with 4-AP indicated the presence of marked kidney damage in both the 50 and 80 mg kg^{-1} groups between 4 and 24 h post-dosing (Table 1). The BUN levels peaked at 24 h and returned to control levels by the end of the 3 week recovery period, indicating repair of the lesion. The Alpk, Wistar-derived rat strain also showed a marked increase in BUN following treatment with 80 mg kg^{-1} 4-AP (Table 2). The increase in BUN in Alpk rats indicated an equivalent level of renal damage to that seen in F344 rats. The plasma creatinine level paralleled the elevation in BUN readings in all groups in the 4-AP study (data not shown). Microscopic examination of kidney sections from rats receiving doses of 50 and 80 mg kg^{-1} revealed necrosis to the pars recta of proximal tubular epithelial cells in the corticomedullary region. This was visible as an early degenerative change at 4 h, progressing by 8 and 24 h to widespread coagulative necrosis, with tissue repair evident after 3 weeks, consistent with the BUN and plasma creatinine data (Figure 1a–e). At a dose of 20 mg kg^{-1} 4-AP, mild reversible vacuolation of some tubular epithelial cells was seen at 4 and 8 h but not at later time points (data not shown). A very similar pattern of toxicity was also observed following D-serine treatment, with elevated BUN and creatinine evident in the 250 and 750 mg kg^{-1} groups between 4 and 24 h, followed by recovery after 3 weeks (Table 3). The pattern of tubular necrosis to the pars recta of the proximal tubules extending into the medullary rays in the D-serine group was very similar to that observed in the 4-AP-treated groups (Figure 1f). Therefore both toxicants elicited a similar severity of toxicity in the corticomedullary region, peaking at 24 h

Table 2. BUN (mmol l^{-1}) data from Alpk rats treated with 4-AP. 4-AP treatment resulted in a dose-dependent increase in BUN, peaking at 24 h post-dosing.

	4-AP dose (mg kg^{-1})	
Time post-dose	0	80
4 h	2.09 ± 0.17	2.66 ± 0.23**
8 h	2.03 ± 0.41	3.33 ± 0.97*
24 h	2.44 ± 0.27	6.43 ± 3.29*
3 weeks	2.68 ± 0.27[a]	2.77 ± 0.47

Values represent the mean ± SD of five animals. *$p < 0.05$ and **$p < 0.01$ compared with concurrent control (0 mg kg^{-1}) group using the Student's t-test.

[a]$n = 4$.

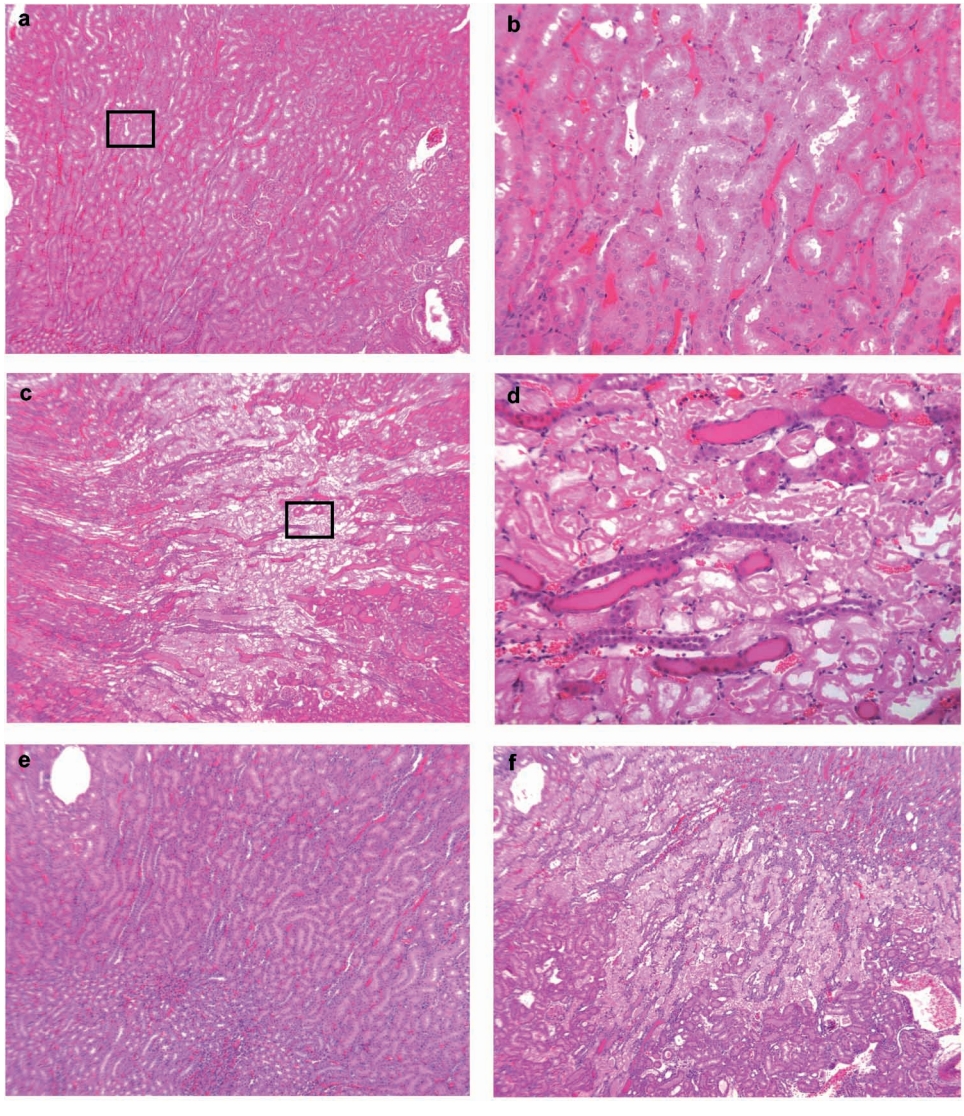

Figure 1. Morphology of rat kidneys following 4-AP- and D-serine-induced kidney damage. (a) Section of the renal cortex from control F344 rats 24 h post-dosing with saline. The boxed region is shown at high power in (b). (c) Typical section from an F344 rat treated with 80 mg kg^{-1} 4-AP 24 h post-dosing showing tubular degeneration/necrosis. The boxed region is shown at high power in (d). (e) Section from F344 rats treated with 80 mg kg^{-1} 4-AP following the 3 week treatment-free period, showing recovery of the cortical lesions. (f) Similar representative region from the kidney of Alpk rats treated with 750 mg kg^{-1} D-serine 24 h post-dosing, displaying a conserved pattern of tubular degeneration. Haematoxylin and eosin.

and recovering after 3 weeks. No toxicity was observed in the L-serine treated group or the in the saline-treated controls; the slight elevation in BUN found at the 4 h time point in the L-serine group (Table 3) was considered spurious as the creatinine values were not elevated at this time point. In addition, microscopic examination of kidney sections from this group did not support evidence of damage (data not shown). Further microscopic analysis of liver and heart sections from all animals in

Table 3. BUN (mmol l^{-1}) data from Alkp rats treated with D- or L-serine. D-serine treatment resulted in a dose-dependent increase in BUN, peaking at 24 h post-dosing.

		Dose			
		D-Serine (mg kg^{-1})			
Time post-dose	Control	75	250	750	L-Serine (750 mg kg^{-1})
4 h	1.89 ± 0.25	2.02 ± 0.32	2.69 ± 0.39**	3.68 ± 0.61**	3.06 ± 0.50**
8 h	2.03 ± 0.15	1.98 ± 0.10	3.67 ± 0.34**	5.12 ± 0.57**	2.21 ± 0.29
24 h	2.38 ± 0.27	2.25 ± 0.28	8.23 ± 1.13**	8.43 ± 1.54**	2.03 ± 0.34
3 weeks	2.52 ± 0.40	2.57 ± 0.22	2.52 ± 0.26	2.64 ± 0.17	2.61 ± 0.35

Values represent the mean ± SD of five animals. **$p < 0.01$ compared with concurrent control (0 mg kg^{-1}) group using the Student's t-test.

this study showed no evidence of toxicity in these tissues (data not shown). This is consistent with previous reports highlighting kidney toxicity as the most prominent effect of 4-AP and D-serine at these doses.

Proteome analysis was performed using the Rosetta™ software on the 2D gel images of plasma samples taken from control and treated animals (Page *et al*. 1999). For the 4-AP study, protein expression maps (PEMs) containing the 2D gel digital image data for the plasma samples from the control F344 animals (4–24 h) were complied together to create a group containing 15 images. The PEMs from the 80 mg kg^{-1} 4-AP-treated F344 animals (4–24 h) were also grouped together. The 80 mg kg^{-1} group was specifically chosen for analysis as this treatment produced the greatest toxicity at all time points (Table 1). A binary comparison was performed between the control and treated group to identify proteins that were increased or decreased based on a statistical difference using the Student's t-test ($p \leq 0.05$). Using this analysis, 8509 protein features or molecular cluster indexes were reduced to 163 significantly altered features (0.02% of the total number of features). The Rosetta™ software was used to plot the profile (percentage volume) of each protein to ascertain if a dose–response and temporal relationship was evident. The percentage volume represents the fluorescence of a protein feature as a percentage of the total gel fluorescence for each image (Page *et al*. 1999). In this analysis only proteins that increased with the dose of 4-AP and returned to baseline levels after the recovery period were selected for identification. For the D-serine study, PEMs from the control plasma samples (4–24 h) were grouped together, as were the 250 and 750 mg kg^{-1} D-serine-treated groups (4–24 h). A second binary comparison was performed between the control and the D-serine-treated group as described above, resulting in the selection of 120 protein features (0.02% of the total number of features) from an initial set of 5022 molecular cluster indexes. As before, a further filtering of proteins based on the dose and temporal response was used to identify a subset of protein features with appropriate expression patterns. Although the L-serine-treated group was not included in the binary comparison, the abundance of selected proteins was confirmed in this groups using Rosetta™. Only proteins that were altered in the D-serine group but unaffected in the L-serine group were evaluated further. A total of 20 proteins, nine from the 4-AP study and

11 from the D-serine study were selected for identification by mass spectrometry annotation (Page *et al.* 1999). Of the proteins identified, several isoforms of the rat-specific T-kininogen protein were noted (Bandara *et al.* 2003). In addition, a number of proteins were shown to be isoforms of the inter-α-inhibitor H4P heavy chain. The remaining proteins were identified as complement C3, fibrinogen α/β and retinal-binding protein (Bandara *et al*. 2003).

One protein of interest from both studies was subsequently identified as the enzyme fumarylacetoacetate hydrolase (fumarylacetoacetase; FAH). The identification of FAH was particularly interesting as the other proteins identified in our analysis were all established plasma proteins, whereas FAH is an intracellular protein with no known function in the plasma. It is also noteworthy that we did not observe any other intracellular proteins in the plasma during our analysis. The migration of the FAH protein on 2D gels suggested an apparent molecular weight of approximately 41 kDa (Figure 2), which is similar to the published predicted

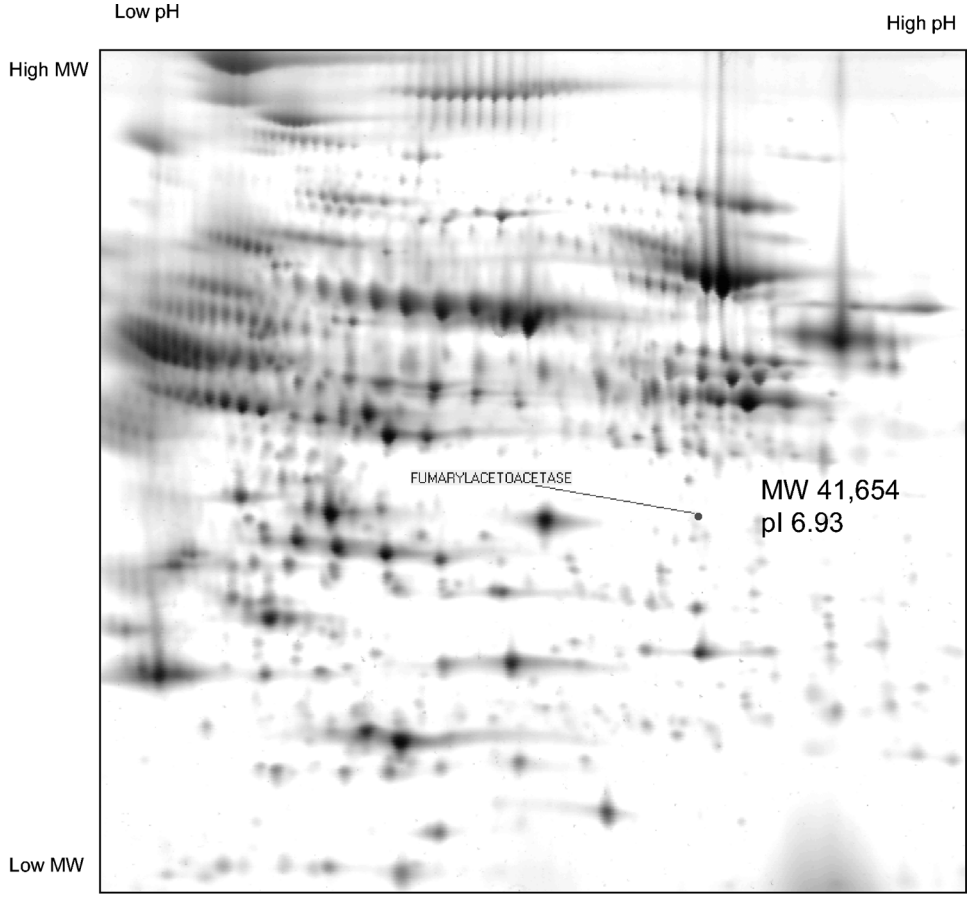

Figure 2. Digital image of the master gel from the 4-AP study. The composite (master) gel represents all the 2D gels in the 4-AP study. pH (3–10) increases from left to right and the molecular weight (MW) increases from bottom to top. The position of FAH on the master gel is indicated and the predicted MW and pI based on the Rosetta™ software analysis are shown.

molecular weight of 45 964 for the rat protein (Labelle *et al.* 1991). We could not determine whether the protein has undergone a post-translational modification since only a portion of the protein was sequenced (data not shown). Consequently, we also could not confirm whether the FAH protein released into the blood following kidney damage is enzymatically active. In the 4-AP study, FAH concentrations increased in the blood in a dose-dependent fashion in F344 plasma samples, the effect being most prominent at 4 and 8 h and disappearing after 24 h, indicating a rapid response (Figure 3a,b). Levels of FAH remained low in the 3 week recovery group but were upregulated in treated Alpk rats, which was consistent with the observed renal damage (data not shown). These findings suggest that FAH may be a common marker of 4-AP-induced renal toxicity in at least two different rat strains. Furthermore, FAH was also detected in the plasma of D-serine-treated but not L-serine-treated animals, indicating that FAH release may be a general marker of renal proximal tubular toxicity. The pattern of protein expression in the D-serine study was very similar to that observed for 4-AP, with FAH being present at early time points and disappearing by 24 h (Figure 3c). Again a dose-dependent increase in FAH was apparent at the 4 h time point (Figure 3d) The plasma creatinine data obtained at 4 h (Figure 4a,b) confirmed that kidney damage was present at this time point even though the peak of damage was at 24 h

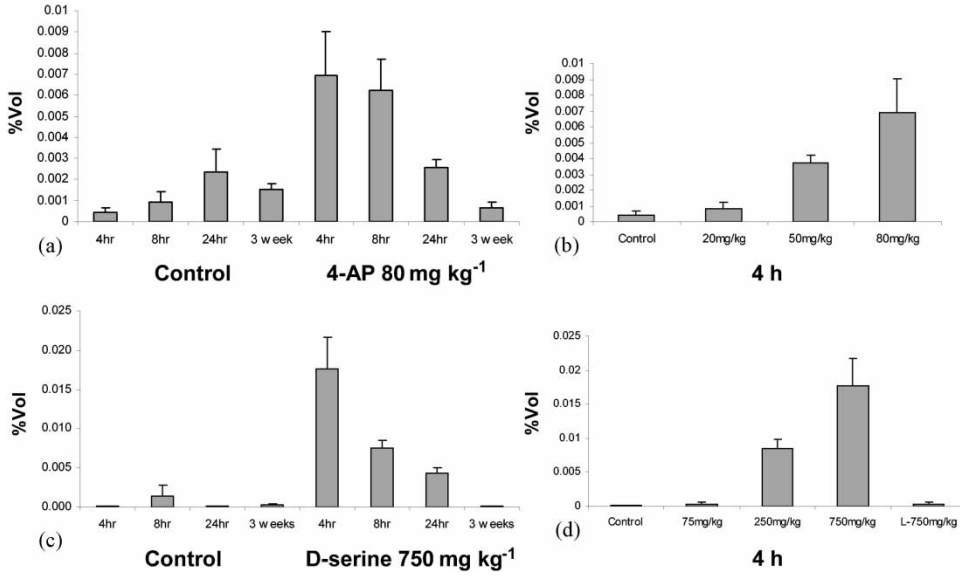

Figure 3. Plasma FAH expression levels in the 4-AP and D-serine studies. The relative expression of FAH in plasma samples is displayed as a percentage of the total gel fluorescence (%Vol). The histogram represents the average spot volume ($n = 5$) $\pm$SEM. (a) FAH levels were increased in plasma in the 4-AP groups, with the most prominent expression at 4 and 8 h and decreasing to control levels by 24 h. (b) A clear dose-dependent increase in expression is apparent with increasing doses of 4-AP at the 4 h time point. (c) FAH levels showed a similar temporal regulation in the D-serine groups, with maximal expression at 4 and 8 h and disappearing by later time points. (d) Dose-dependent regulation was also seen in the D-serine treatment groups at 4 h, with no significant expression in the L-serine (L-) or control groups.

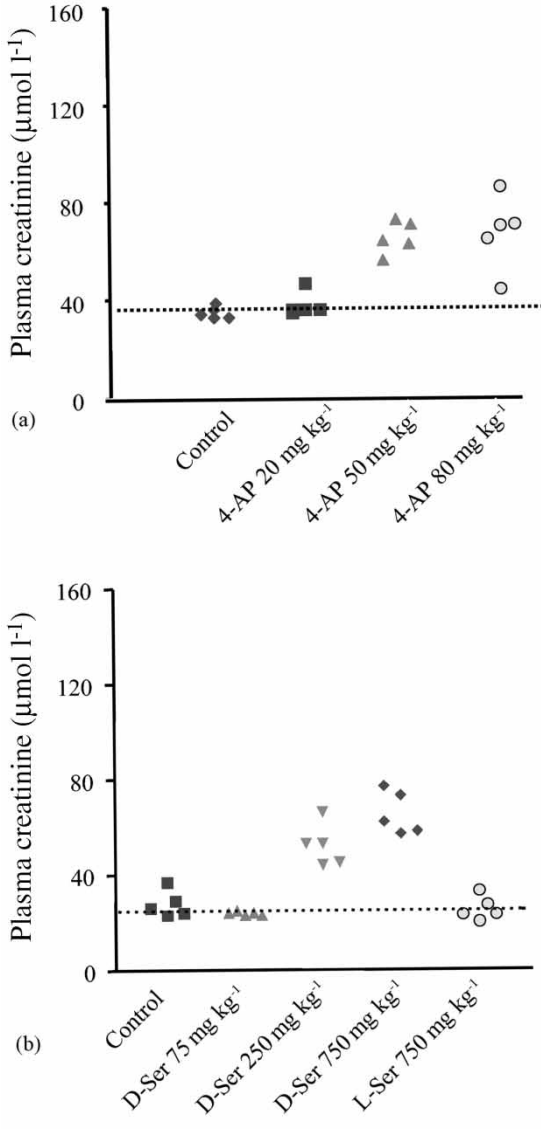

Figure 4. 4-AP and D-serine plasma creatinine levels (μmol l^{-1}) at the 4 h time point. Scatter plots of individual animal data revealed similar levels of kidney damage in both the 4-AP (a) and the D-serine (b) studies. Note the similarity between the dose response profile of FAH expression

(Table 2). This was in contrast to the change in FAH levels, which peaked between 4 and 8 h in both studies. FAH could also be detected by Western blotting in both the plasma and urine of rats treated with D-serine but not in rats treated with L-serine. The antibody, which was generated against human liver FAH, was shown to cross-react with mouse liver protein (data not shown) and rat liver protein, giving a band with the expected molecular weight of approximately 41 kDa (Figure 5a,b). Protein obtained from rat plasma 4 h post-doing with 75 mg kg^{-1} D-serine showed

Figure 5. Western blot of FAH in rat plasma (a) and rat urine (b) from control rats and rats treated with D- or L-serine. (a) Lane 1, rat liver cytosol (0.05 μg); lanes 2–4, rat plasma 4 h after 75 mg kg^{-1} D-serine (3.68–5.16 μg protein); lanes 5–7, rat plasma 4 h after 750 mg kg^{-1} D-serine (4.62–5.89 μg protein); lanes 8–10, rat plasma 8 h after 750 mg kg^{-1} D-serine (3.39–4.57 μg protein); lanes 11–13, rat plasma 8 h after 750 mg kg^{-1} L-serine (3.16–4.23 μg protein). (b) Lanes 1–3, rat liver cytosol (0.05 μg); lanes 4–7, control rat urinary protein (15 μg) pre-dose and 12, 24, and 36 h post-dose with water, respectively; lanes 8–11, rat urinary protein (15 μg) pre-dose and 12, 24, and 36 h post-dose with 250 mg kg^{-1} D-serine, respectively; lanes 12–15, as for lanes 8–11.

very faint bands in all three animals (Figure 5a), while 4 and 8 h after 750 mg kg^{-1} D-serine there was a more intensely staining protein band in all animals (Figure 5a). In contrast, no protein bands were detected in three rats 8 h after receiving L-serine (Figure 5a). Protein obtained from rat urine pre-dosing and post-dosing in controls given water showed no protein banding (Figure 5b), while in two rats given D-serine there was the appearance of a protein band 12 h and 12–24 h after dosing and in one animal 24–36 h after dosing (Figure 5b).

The FAH protein is normally located intracellularly; therefore its presence in the plasma of treated animals is surprising. FAH is required for the metabolism of tyrosine and the gene is also mutated in the inherited autosomal recessive genetic disorder type I tyrosinaemia. Since we did not detect other cellular proteins in our analysis, the presence of FAH in plasma appears be a specific cellular response to the toxicants used. These findings also imply that FAH may be a marker of kidney toxicity, which may be conserved in humans.

Discussion

Proteomics is one of many new technologies that can aid toxicologists in defining new insights into mechanisms of toxicity. In addition to mechanistic studies, proteomics may support the identification of new biomarkers that are more sensitive or predictive than existing markers (Bandara and Kennedy 2002). In an attempt to understand the nephrotoxicity of 4-AP and D-serine in greater detail, we conducted a proteomic analysis of plasma samples from treated animals. A single intraperitoneal administration of 4-AP or D-serine has been reported to produce kidney toxicity in rodents (Green *et al.* 1969, Ganote *et al.* 1974, Kaltenbach *et al.* 1982, Davis *et al*. 1983). In this study three different single doses of these compounds were administered and plasma collected at a variety of time points after dosing for proteome analysis.

One of the plasma proteins identified that correlated with nephrotoxicity was the enzyme FAH, which is normally expressed intracellularly and is required for the

metabolism of tyrosine. FAH participates in a critical step of tyrosine metabolism involving the conversion of 4-fumarylacetoacetate (FAA) to acetoacetate and fumarate (Figure 6). Interestingly, the FAA substrate is highly toxic and has been shown to induce apoptosis of cells *in vitro* (Jorquera and Tanguay 1999). The FAH gene is mutated in type I hereditary tyrosinaemia (HT1). Patients with tyrosinaemia suffer from kidney damage, although liver dysfunction is the most prominent symptom associated with this defect (Kvittingen *et al.* 1991, Forget *et al.* 1999). This is consistent with the expression of FAH, which is enriched in both the kidney and liver but with the highest level evident in hepatocytes (Labelle *et al.* 1991). Of the multiple kidney defects reported in HT1 patients, renal proximal tubular damage is a common occurrence, highlighting the link with 4-AP- and D-serine-induced damage (Forget *et al.* 1999).

A genetic mouse model for tyrosinaemia has been developed through homologous recombination to knockout the murine *fah* gene. Knockout mice die after birth due to extensive liver and kidney damage (Ruppert *et al.* 1992, Kelsey *et al.* 1993, Grompe *et al.* 1993). This lethal phenotype can be rescued by a second

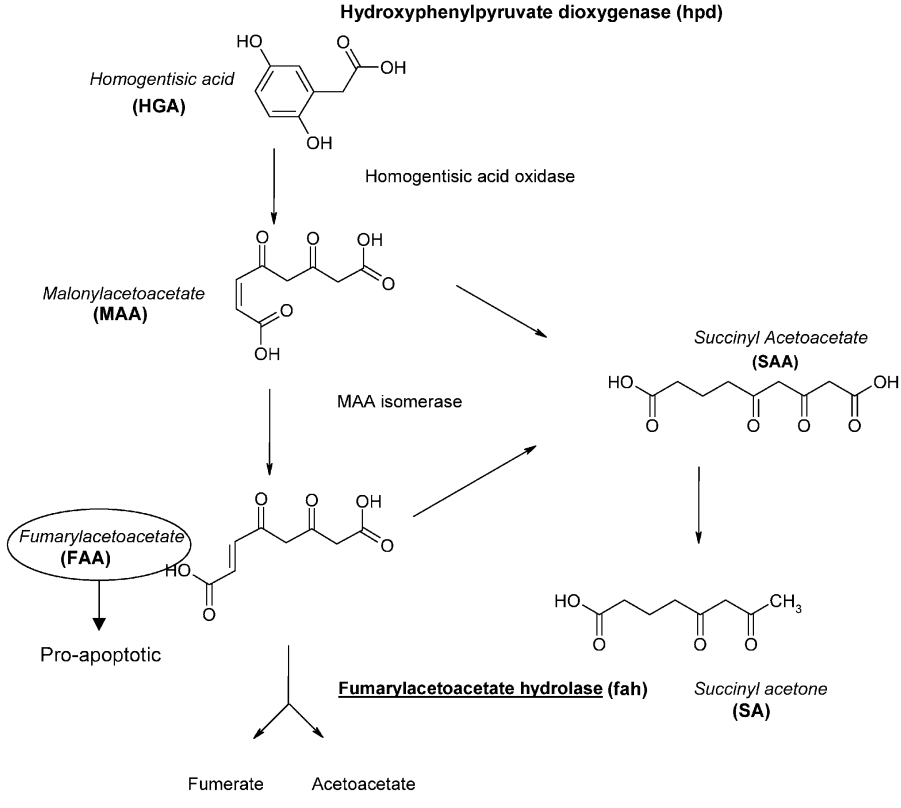

Figure 6. Summary of tyrosine metabolic pathway. Tyrosine is converted to fumarate and acetoacetate via a series of enzyme-catalysed reactions. The enzymes in the pathway are indicated; metabolites are shown in italics. The dotted arrows indicate the potential conversion of MAA and FAA to SAA and SA, which can be detected in urine and blood from patients with HT1. The enzymes hydroxyphenylpyruvate dioxygenase (HPD) and fumarylacetoacetate hydrolase (FAH), which are deleted in the mouse model of HT1, are shown in bold.

genetic alteration to remove the *hpd1* gene, which functions upstream of FAH in the tyrosine metabolic pathway (Figure 6) (Endo *et al.* 1997) or by pharmacological intervention with the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (Lindstedt *et al*. 1992, Grompe *et al*. 1995). Deletion of *hpd1* prevents the accumulation of FAA, although tissue damage can be restored by treatment with homogentisate (homogentisic acid) (Sun *et al.* 2000). In this double knockout mouse the introduction of homogentisate results in kidney damage, specifically in the proximal tubules, resulting in an elevation in BUN (Sun *et al.* 2000). The kidney damage observed was thought to be caused by FAA, which can induce apoptosis in the proximal tubular cells. In support of this concept, pretreatment of mice with caspase inhibitors before homogentisate treatment prevents apoptotic cell death (Kubo *et al.* 1998, Sun *et al*. 2000).

It is clear therefore that the FAH gene plays an important role in humans and rodents in both the kidney and the liver. The observation of FAH in the plasma of 4-AP- and D-serine-treated animals, confirmed by two separate methods – protein identification by mass spectral analysis from 2D PAGE gels and cross-reactivity with an antibody to FAH – is suggestive of a novel link between nephrotoxicity and tyrosine metabolism. However, the most interesting aspect of this observation is the detection of FAH in the blood compartment. The exact mechanism by which FAH appears in the plasma is not clear, nor is it clear if the source of the enzyme is from the liver or the kidney. One possibility is that the increased FAH in the blood is derived from the liver, although this would need to be selective as aminotransferase enzymes such as ALT and AST are not increased in the plasma after exposure to these doses of 4-AP (Fowler *et al*. 1991). Furthermore, this model seems inconsistent with the known mechanism of action of 4-AP and D-serine and the rapid response of FAH to both toxicants. In addition, microscopic examination of liver sections from treated animals revealed no apparent liver toxicity.

The size of the FAH protein would suggest that it should be filtered at the glomerulus to appear in the tubular lumen and then be reabsorbed in the proximal tubule. Damage to the site of reabsorption, as occurs with 4-AP and D-serine, would presumably result in clearance into the urine. This was confirmed using an antibody to FAH, which showed the presence of the protein in urine 12 h and 12–24 h after dosing with D-serine but not L-serine (Figure 5b). We cannot exclude at this stage that some leakage of FAH may have occurred via the basolateral membranes of the proximal tubule, resulting in release of FAH into the plasma, although this seems the least plausible explanation at the present time. Such a mechanism is analogous to the regulation of the liver AST and ALT, suggesting that FAH could be used in a similar manner to measure kidney damage. We did not detect any other intracellular proteins in plasma during the course of our analysis, indicating that a general release of proteins due to kidney damage was not a likely explanation.

It is also tempting to speculate that 4-AP and D-serine function through a common pathway to activate the tyrosine metabolic pathway, resulting in an increase of the toxic metabolite FAA. An increase in the amount of FAH may be required in order to remove this highly toxic metabolite from the body. Kidney tissue samples were not analysed in this study; however, the FAH protein was found

to be upregulated in the renal cortex in a separate rat toxicity study using another kidney toxicant, gentamicin (Kennedy 2001). It is possible that FAH is actively secreted into the blood to remove an excess accumulation of FAA, which may ultimately be released from renal cells. Other metabolites of tyrosine degradation such as succinyl acetone can be monitored in the urine and blood of HT1 patients, supporting the possibility that FAA may also be released in a similar manner (Wyss *et al.* 1992). Thus, FAH may function in a highly novel manner to remove FAA from plasma to reduce any potential local tissue damage. The double knockout mouse model of tyrosinaemia provides a very useful tool for testing this hypothesis (Kubo *et al.* 1998, Sun *et al.* 2000). If 4-AP and D-serine function through the elevation of FAA, one might assume that the $fah^{-/-}$ $hpd^{-/-}$ mouse would be more resistant to renal toxicity. Alternatively, it may be possible to measure metabolites such as succinyl acetone in urine or blood from rats treated with renal toxicants since these metabolites may be generated during kidney damage. In further support of FAH as a kidney toxicity marker, we have detected FAH in the plasma of Wistar Kyoto rats treated with the compound PDE3 inhibitor SKF 95464 (Zhang *et al.* 2002). Again, a clear correlation between kidney damage and FAH expression in plasma was noted in this study (F. Sistare, unpublished data).

In this report, we have made a unique connection between kidney toxicants, FAH release and HT1, although the precise mechanism requires further study. It would be particularly interesting to examine a wider range of toxicants in future studies and to examine other compartments such as urine to confirm the specificity of the response and to investigate the regulation of FAH in more detail. Nevertheless, these data are intriguing and suggest that FAH may be a novel marker of kidney toxicity in humans. A mechanistic marker of this type may be very valuable in a clinical setting; since FAH is expressed before excessive kidney damage occurs, one might predict that kidneys may recover if treatment was stopped. We conclude that proteomics is a powerful tool for identifying new molecular pathways and biomarkers of disease.

Acknowledgements

We would like to thank Ken Twomey, Matt Jacobsen and Fiona Smith for conducting the studies at CTL-Syngenta, and Athula Herath and Thakor Patel at OGS for statistical analysis and mass spectrometry data. We would also like to thank other members of OGS for technical support and helpful comments, particularly Gordon Holt. We also wish to thank Professor R. Berger for the antibody to FAH and Guy Mainwaring, Sue Wiles and Rebecca Williams for help with the Western blotting studies.

References

Aardema, M. J. and MacGregor, J. T. 2002, Toxicology and genetic toxicology in the new era of 'toxicogenomics': impact of '-omics' technologies. *Mutattion Research*, **499**, 13–25.

Bandara, L. R. and Kennedy, S. 2002, Toxicoproteomics – a new preclinical tool. *Drug Discovery Today*, **7**, 411–418.

Bandara, L. R., Kelly, M. D., Lock, E. A. and Kennedy, S. 2003, A correlation between a proteomic evaluation and conventional measurements in the assessment of renal proximal tubular toxicity. *Toxicological Sciences*, **73**, 195–206.

Calder, I. C., Young, A. C., Woods, R. A., Crowe, C. A., Ham, K. N. and Tange, J. D. 1979, The nephrotoxicity of 4-aminophenol. II. The effect of metabolic inhibitors and inducers. *Chemico-biological interactions*, **27**, 245–254.

Charlwood, J., Skehel, M., King, N., Camilleri, P., Lord, P., Bugelski, P. and Atif, U. 2002, Proteomic analysis of rat kidney cortex following treatment with gentamicin. *Journal of Proteome Research*, **1**, 73–82.

Crowe, C. A., Yong, A. C., Calder, I. C., Ham, K. N. and Tange, J. D. 1979, The nephrotoxicity of 4-aminophenol. I. The effect on microsomal cytochromes, glutathione and covalent binding in kidney and liver. *Chemico-biological interactions*, **27**, 235–243.

Cutler, P., Bell, D. J., Birrell, H. C., Connelly, J. C., Connor, S. C., Holmes, E., Mitchell, B. C., Monte, S. Y., Neville, B. A., Pickford, R., Polley, S., Schneider, K. and Skehel, J. M. 1999, An integrated proteomic approach to studying glomerular nephrotoxicity. *Electrophoresis*, **20**, 3647–3658.

Davis, J. M., Emslie, K. R., Sweet, R. S., Walker, L. L., Naughton, R. J., Skinner, S. L. and Tange, J. D. 1983, Early functional and morphological changes in renal tubular necrosis due to 4-aminophenol. *Kidney International*, **24**, 740–747.

Eckert, K. G., Eyer, P., Sonnenbichler, J. and Zetl, I. 1990, II. Synthesis and structural elucidation of various thiol addition products of 1,4-benzoquinoneimine and *N*-acetyl-1,4-benzoquinoneimine. *Xenobiotica*, **20**, 333–350.

Endo, F., Kubo, S., Awata, H., Kiwaki, K., Katoh, H., Kanegae, Y., Saito, I., Miyazaki, J., Yamamoto, T., Jakobs, C., Hattori, S. and Matsuda, I. 1997, Complete rescue of lethal albino c14CoS mice by null mutation of 4-hydroxyphenylpyruvate dioxygenase and induction of apoptosis of hepatocytes in these mice by in vivo retrieval of the tyrosine catabolic pathway. *Journal of Biological Chemistry*, **272**, 24426–24432.

Forget, S., Patriquin, H. B., Dubois, J., Lafortune, M., Merouani, A., Paradis, K. and Russo, P. 1999, The kidney in children with tyrosinaemia: sonographic, CT and biochemical findings. *Pediatric Radiology*, **29**, 104–108.

Fowler, L. M., Moore, R. B., Foster, J. R. and Lock, E. A. 1991, Nephrotoxicity of 4-aminophenol glutathione conjugate. *Human and Experimental Toxicology*, **10**, 451–459.

Fowler, L. M., Foster, J. R. and Lock, E. A. 1994, Nephrotoxicity of 4-amino-3-*S*-glutathionylphenol and its modulation by metabolism or transport inhibitors. *Archives of Toxicology*, **68**, 15–23.

Ganote, C. E., Peterson, D. R. and Carone, F. A. 1974, The nature of D-serine-induced nephrotoxicity. *American Journal of Pathology*, **77**, 269–282.

Gartland, K. P., Bonner, F. W., Timbrell, J. A. and Nicholson, J. K. 1989, Biochemical characterisation of para-aminophenol-induced nephrotoxic lesions in the F344 rat. *Archives of Toxicology*, **63**, 97–106.

Green, C. R., Ham, K. N. and Tange, J. D. 1969, Kidney lesions induced in rats by 4-aminophenol. *British Medical Journal*, **i**, 162–164.

Grompe, M., Al-Dhalimy, M., Finegold, M., Ou, C. N., Burlingame, T., Kennaway, N. G and Soriano, P. 1993, Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes and Development*, **7**, 2298–2307.

Grompe, M., Lindstedt, S., Al-Dhalimy, M., Kennaway, N. G, Papaconstantinou, J., Torres-Ramos, C. A., Ou, C.-N. and Finegold, M. 1995, Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditiary tyrosinaemia type 1. *Nature Genetics*, **10**, 453–460.

Harris, R. A., Yang, A., Stein, R. C., Lucy, K., Brusten, L., Herath, A., Parekh, R., Waterfield, M. D., O'Hare, M. J., Neville, M. A., Page, M. J. and Zvelebil, M. J. 2002, Cluster analysis of an extensive human breast cancer cell line protein expression map database. *Proteomics*, **2**, 212–223.

Imai, K., Fukushima, T., Santa, T., Homma, H., Huang, Y., Shirao, M. and Miura, K. 1998, Whole body autoradiographic study on the distribution of ^{14}C-D-serine administered intravenously to rats. *Amino Acids*, **15**, 351–361.

Jorquera, R. and Tanguay, R. M. 1999, Cyclin B-dependent kinase and caspase-1 activation precedes mitochondrial dysfunction in fumarylacetoacetate-induced apoptosis. *FASEB Journal*, **13**, 2284–2298.

Kaltenbach, J. P., Carone, F. A. and Ganote, C. E. 1982, Renal tubular necrosis induced by compounds structurally related to D-serine. *Experimental and Molecular Pathology*, **37**, 225–234.

Kelsey, G., Ruppert, S., Beermann, F., Grund, C., Tanguay, R. M. and Schutz, G. 1993, Rescue of mice homozygous for lethal albino deletions: implications for an animal model for the human liver disease tyrosinaemia type 1. *Genes and Development*, **7**, 2285–2297.

Kennedy, S. 2001, Proteomic profiling from human samples: the body fluid alternative. *Toxicology Letters*, **120**, 379–384.

Kennedy, S. 2002, The role of proteomics in toxicology: identification of biomarkers of toxicity by protein expression analysis. *Biomarkers*, **7**, 1–22.

KUBO, S., SUN, M., MIYAHARA, M., UMEYAMA, K., URAKAMI, K., YAMAMOTO, T., JAKOBS, C., MATSUDA, I. and ENDO, F. 1998, Hepatocyte injury in tyrosinaemia type 1 is induced by fumarylacetoacetate and is inhibited by caspase inhibitors. *Proceedings of the National Academy of Sciences of the USA*, **95**, 9552–9557.

KVITTINGEN, E. A., TALSETH, T., HALVORSEN, S., JAKOBS, C., HOVIG, T. and FLATMARK, A. 1991, Renal failure in adult patients with hereditary tyrosinaemia type I. *Journal of Inherited Metabolic Disease*, **14**, 53–62.

LABELLE, Y., PHANEUF, D. and TANGUAY, R. M. 1991, Cloning and expression analysis of a cDNA encoding fumarylacetoacetate hydrolase: post-transcriptional modulation in rat liver and kidney. *Gene*, **104**, 197–202.

LINDSTEDT, S., HOLME, E., LOCK, E. A., HJALMARSON, O. and STRANDVIK, B. 1992, Treatment of hereditary tyrosinaemia type 1 by inhibition of 4-hydroxyphenylpyruvate dioxygenase. *Lancet*, **340**, 813–817.

NEWTON, J. F., YOSHIMOTO, M., BERNSTEIN, J., RUSH, G. F. and HOOK, J. B. 1983, Acetaminophen nephrotoxicity in the rat – II. Strain differences in nephrotoxicity and metabolism of *p*-aminophenol, a metabolite of acetaminophen. *Toxicology and Applied Pharmacology*, **69**, 307–318.

PAGE, M. J., AMESS, B., TOWNSEND, R. R., PAREKH, R., HERATH, A., BRUSTEN, L., ZVELEBIL, M. J., STEIN, R. C., WATERFIELD, M. D., DAVIES, S. C. and O'HARE, M. J. 1999, Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammoplasties. *Proceedings of the National Academy of Sciences of the USA*, **96**, 12589–12594.

RUPPERT, S., KELSEY, G., SCHEDL, A., SCHMID, E., THIES, E. and SCHUTZ, G. 1992, Deficiency of an enzyme of tyrosine metabolism underlies altered gene expression in newborn liver of lethal albino mice. *Genes and Development*, **6**, 1430–1443.

SILBERNAGL, S., VOLKER, K. and DANTZLER, W. H. 1999, D-Serine is reabsorbed in rat renal pars recta. *American Journal of Physiology*, **276**, F857–F863.

SMITH, L. L. 2001, Key challenges for toxicologists in the 21st century. *Trends in Pharmacological Sciences*, **22**, 281–285.

STEINER, S., AICHER, L., RAYMACKERS, J., MEHEUS, L., ESQUER-BLASCO, R, ANDERSON, N. L. and CORDIER, A. 1996, Cyclosporine A decreases the protein level of the calcium binding protein calbindin-D 28 kDa in rat kidney. *Biochemical Pharmacology*, **51**, 253–258.

SUN, M. S., HATTORI, S., KUBO, S., AWATA, H., MATSUDA, I. and ENDO, F. 2000, A mouse model of renal tubular injury of tyrosinaemia type 1: development of de Toni Fanconi syndrome and apoptosis of renal tubular cells in Fah/Hpd double mutant mice. *Journal of the American Society of Nephrology*, **11**, 291–300.

WITZMANN, F. A., FULTZ, C. D, GRANT, R. A., WRIGHT, L. S., KORNGUTH, S. E. and SIEGEL, F. L. 1999, Regional protein alterations in rat kidneys induced by lead exposure. *Electrophoresis*, **20**, 943–951.

WYSS, P. A., BOYNTON, S. B., CHU, J., SPENCER, R. F. and ROTH, K. S. 1992, Physiological basis for an animal model of the renal Fanconi syndrome: use of succinylacetone in the rat. *Clinical Science*, **83**, 81–87.

ZHANG, J., HERMAN, E. H., KNAPTON, A., CHADWICK, D. P., WHITEHURST, V. E., KOERNER, J. E., PAPOIAN, T., FERRANS, V. J. and SISTARE, F. D. 2002, SK&F 95654-induced acute cardiovascular toxicity in Sprague-Dawley rats – histopathologic, electron microscopic, and immunohistochemical studies. *Toxicological Pathology*, **30**, 28–40.