# $^1$H NMR spectroscopic studies on the characterization of renal cell lines and identification of novel potential markers of *in vitro* nephrotoxicity

Maria L. Anthony, Peter C. R. McDowell, Tim J. B. Gray, Melanie Blackmore and Jeremy K. Nicholson

**Cell cultures are increasingly used in the evaluation of chemically-induced nephrotoxicity. The utility of renal cell culture systems in toxicology would be improved, however, if better characterized and more specific markers of toxicity were available. High resolution proton nuclear magnetic resonance ($^1$H NMR) spectroscopy is well suited to the study of toxicological events and has identified many novel markers of nephrotoxicity *in vivo*. In this study, $^1$H NMR spectroscopy has been used to characterize the biochemical composition of two renal cell lines of different nephronal origin, LLC-PK$_1$ (pig proximal tubule) and Madin–Darby canine kidney (MDCK, distal tubule). The early biochemical responses of these cell lines to the model proximal tubular toxin *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) and the renal medullary toxin 2-chloroethanamine (CEA) have also been investigated. For each line, 500 MHz $^1$H NMR spectra of protein-free acetone extracts of cells and culture medium gave characteristic and reproducible profiles of low MW constituents, including amino and organic acids, glucose and soluble membrane precursors, such as choline and *myo*-inositol. Treatment-related changes in several low MW compounds not routinely measured in toxicological studies were revealed by NMR spectroscopy before marked cytotoxicity was observed by phase contrast microscopy. For example, LLC-PK$_1$ cells treated with 60 μM DCVC showed a marked decrease in intracellular choline levels within 3 h which suggests an effect on the balance of choline synthesis and utilization. Within 9 h of treatment with DCVC there were decreases in intracellular acetate and alanine concentrations which may be indicative of a decrease in fatty acid oxidation and triglyceride metabolism accompanied by an increase in gluconeogenesis. In MDCK cells, 1 h post treatment with 5 mM CEA, intracellular glycine was decreased. This study indicates the potential power and applicability of $^1$H NMR spectroscopy for evaluating the biochemical and metabolic effects of toxins in cell culture systems and provides a novel approach to identifying new markers of tissue damage.**

***Maria L. Anthony** (author for correspondence), **Peter C. R. McDowell**, **Jeremy K. Nicholson** are in the Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H OPP, UK; **Tim J. B. Gray** and **Melanie Blackmore** are in the Department of Toxicology, Sanofi Research Division, Willowburn Avenue, Alnwick, Northumberland NE66 2JH, UK.*

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Abbreviations: *CEA, 2-chloroethanamine monohydrochloride; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; FID, free induction decay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MDCK, Madin-Darby canine kidney; PBS, phosphate buffered saline; TSP, 3-trimethylsilyl-[2,2,3,3-$^2$H$_4$]-1-propionic acid.*

## Introduction

The kidney tubular epithelium is a common target for chemically-induced toxicity in animals and man (Commandeur and Vermeulen 1990). Given the frequency and complexity of drug- and toxin-related injury, there is a need to investigate the biochemical mechanisms underlying renal epithelial cell damage. Many *in vitro* nephrotoxicity studies have been conducted using irreversibly immortalized cell lines derived from different parts of the nephron, such as LLC-PK$_1$ (pig proximal tubule) and Madin–Darby canine kidney (MDCK, distal tubule) cells (Bohets *et al.* 1995). These cell lines have undergone extensive biochemical and physiological characterization; however it is recognized that many differentiated cellular functions that may play an important role in the development of nephrotoxicity are lost after long-term passages in culture (Bohets *et al.* 1995). The partial de-differentiation of the renal cells in culture has made it difficult to identify reliable and relevant markers of nephrotoxicity *in vitro*.

High resolution $^1$H NMR spectroscopy has been shown to be an effective technique in biochemical toxicology studies, particularly when combined with complementary enzyme and histopathological assessments (Nicholson and Wilson 1989, Anthony *et al.* 1992, 1994a). The unique exploratory nature of $^1$H NMR spectroscopy enables the measurement of a wide range of biologically important low molecular weight (MW) metabolites, allowing the simultaneous monitoring of many biochemical processes (Nicholson and Wilson 1989). Much of the biochemical information necessary to classify both the site and severity of damage induced *in vivo* by a variety of nephrotoxic drugs and chemicals has been shown to be conveyed in the urinary excretion patterns of low MW metabolites. For instance, $^1$H NMR profiles of urine collected from rats following acute exposure to toxins affecting the proximal tubule, appear markedly different from those obtained after administration of renal medullary toxins. Proximal tubular-directing nephrotoxins, including cephaloridine (Anthony *et al.* 1992, Murgatroyd *et al.* 1992), mercury (II) chloride (Nicholson *et al.* 1985), uranyl nitrate (Anthony *et al.* 1994a) and the halogenated alkenes *S*-(1,2-dichlorovinyl)-L-homocysteine and 1,1,2-trichloro-3,3,3-trifluoro-1-propene (Anthony *et al.* 1994b), reproducibly altered the urinary excretion of glucose, amino acids, lactate and other organic acids over a 48 h time-course following toxin administration. In contrast, the renal medullary nephrotoxins propylene imine and 2-bromoethanamine caused elevations in urinary dimethylamine and trimethylamine *N*-oxide (TMAO) excretion within 8 h of dosing, followed by a

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sustained decrease in TMAO excretion and an elevation in urinary *N,N*-dimethylglycine, acetate and succinate at later time-points (Gartland *et al.* 1989, Holmes *et al.* 1995). Additionally, within groups of nephrotoxins that affect the same region of the kidney, there is variation in the NMR profile that can be related to mechanisms of toxic injury, as exemplified with studies investigating the nephrotoxicity of 4-aminophenol (Gartland *et al.* 1990, Anthony *et al.* 1993).

In view of the success with which the $^1$H NMR approach has provided mechanistic information and identified low MW biochemical markers of nephrotoxicity *in vivo*, the purpose of the present work has been to explore the potential of using NMR to examine renal injury *in vitro*. The specific aims of this study were to use $^1$H NMR spectroscopy to study the low MW metabolite composition of MDCK and LLC-PK$_1$ cells and to investigate the early biochemical responses of these cells to injury by a model proximal tubular nephrotoxin, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC; Elfarra *et al.* 1986) and the renal medullary toxin, 2-chloroethanamine (CEA; Powell *et al.* 1991). In this way it will be possible to evaluate the utility of NMR spectroscopy as a tool in *in vitro* nephrotoxicity studies.



## METHODS

### Cell culture conditions

Routinely, cultures were grown in 175 cm$^2$ flasks, incubated at 37°C in a humidified atmosphere containing 5% $CO_2$/95% air and subcultured using trypsin/EDTA. The MDCK and LLC-PK$_1$ cell lines (Flow Laboratories, Ricksmansworth, UK and American Type Culture Collection respectively) were used between passage numbers 69–71 and 205–213 respectively. MDCK cells were maintained in HEPES-free Eagle's Minimal Essential Medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% nonessential amino acids. LLC-PK$_1$ cells were maintained in HEPES-free Medium 199 supplemented with 5% fetal calf serum and 2 mM L-glutamine.

### Toxicity studies and sample preparation

For NMR toxicity studies, LLC-PK$_1$ and MDCK cells were seeded at a density of 2 × 10$^6$ cells/flask and maintained for 72 h when they formed confluent monolayers. Confluent LLC-PK$_1$ and MDCK cell cultures were exposed to solutions of DCVC and CEA respectively in culture medium corresponding to doses of 60 μM DCVC and 5 mM CEA for up to 24 h. Control cells were incubated with culture medium alone. These levels of DCVC and CEA were the 24 h $LC_{50}$ concentrations determined in preliminary dose-range experiments using the neutral red (NR) uptake assay (Borenfreund and Puerner 1985) essentially as follows. LLC-PK$_1$ and MDCK cells were seeded onto 96-well tissue culture plates at a density of 5.7 × 10$^4$ cells/well and maintained in a humidified atmosphere of 5% $CO_2$/95% air at 37°C. Serial doses of DCVC and CEA were added 24 h after seeding (when cells formed confluent monolayers) for a total incubation period of 24 h. After this time, monolayers were washed with phosphate buffered saline (PBS) containing $Ca^{2+}$ and $Mg^{2+}$ and incubated for 1 h at 37°C with medium containing NR. Monolayers were again washed with PBS and NR was extracted from the cells by incubation with destain solution [1% (v/v) glacial acetic acid, 50% (v/v) ethanol and 49% (v/v) filtered distilled water] for 1 h at room temperature. Optical absorbance was determined at 540 nm. Blank wells with no cells were used to correct for non-specific NR binding.

For NMR studies, aliquots of culture medium (3 ml) were removed from flasks after 1, 3, 6, 9 and 24 h. The remaining medium was discarded and cell monolayers were washed with PBS (3 × 10 ml). Cells were physically scraped into PBS (2 × 2.5 ml) and the resulting suspensions lysed by sonication for 30 s with an exponential microprobe (Soniprep 150) set to oscillate ultrasonically at an amplitude of 10 microns to cause maximal release of intracellular contents. Cells and culture medium were extracted into acetone (5 and 3 ml respectively) and precipitated proteins and other cellular debris removed by centrifugation at 3000 rpm for 10 min at 4°C. Acetone was removed with a stream of dry nitrogen, the pH of each extract was adjusted to 7.0–7.4 (with NaOD) and samples were maintained at −20°C prior to NMR analysis.

### $^1$H NMR analysis of extracts of cells and culture medium

$^1$H NMR spectra were measured on a JEOL GSX500 spectrometer operating at 500.14 MHz $^1$H resonance frequency at ambient probe temperature (298 K). Cell and culture medium extracts (5 and 3 ml respectively) were analysed by NMR after lyophilization and reconstitution in $^2H_2O$ (750 μl). For culture medium, 64 free induction decays (FIDs) were collected into 32 K computer points using a 45° pulse width, a spectral width of 5000 Hz, an acquisition time of 2.73 s and an additional $T_1$ relaxation delay of 2.27 s. For cell extracts, 512 FIDs were accumulated. Selected cell extracts were also analysed using a Varian VXR600s spectrometer operating at 599.95 MHz $^1$H resonance frequency. In all cases, water suppression was achieved by gated secondary irradiation at the water resonance frequency (off during acquisition). Exponential weighting functions, corresponding to line broadenings of 0.37 and 1.0 Hz for culture medium and cell extracts respectively, were applied to the FIDs prior to Fourier transformation. Chemical shifts were referenced to internal sodium 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-1-propionate (TSP; δ 0) present at final concentrations of 0.1 and 1 mM for cell extracts and medium respectively. Concentration data in mmol were determined from the measurement of the NMR peak intensities of selected cellular and culture medium constituents that were expressed relative to the corresponding peak intensity of TSP (taking account of the known TSP concentrations and the number of protons contributing to each resonance). Resonances were assigned by consideration of chemical shifts, literature values and, where necessary, spiking with authentic compounds (Nicholson and Wilson 1989).

### Homonuclear 2-dimensional J-resolved $^1$H NMR spectroscopy (JRES) of cell extracts

To help confirm the signal assignments of some of the low MW intracellular components, selected cell extracts were analysed at 600 MHz using 2-dimensional JRES spectroscopy (Foxall *et al.* 1993). $^1$H JRES spectra were obtained by sequential repetition of the pulse sequence:

$[D - 90° - t_1 - 180° - t_1 - \text{collected FID for time } t_2]$

where $D$ = 2.7 s, and $t_1$ was an incremented variable delay. A secondary irradiation field was applied at the water resonance frequency during delay $D$ which was gated-off during the application of the pulse sequence and acquisition. The $F_2$ (chemical shift) domain was collected into 8192 computer points with a spectral width of 6000 Hz and the $F_1$ (J-coupling) domain covered 30 Hz with 64 increments of $t_1$. Typically 32 transients were collected for each $t_1$ increment in the JRES experiments. Prior to double FT, the data were apodized by means of a sine-bell function in $t_1$ and $t_2$. The spectra were tilted by 45° to provide orthogonality of the chemical shift and coupling constant axes. Following a magnitude calculation, spectra were displayed both in the form of contour plots of the $F_1$ and $F_2$ domains and skyline $F_2$ projections.

## Results

### $^1$H NMR analysis of control cell extracts and culture media

Representative partial 500 MHz $^1$H NMR spectra of acetone extracts of untreated LLC-PK$_1$ and MDCK cells and their

respective culture media (after a 1 h incubation) are illustrated in Figure 1. Each cell line (A,B) and medium (C,D), expressed qualitatively similar $^1$H NMR spectral profiles of the more abundant low MW metabolites. Metabolite signals most readily assigned in both matrices at this field strength included amino and organic acids (e.g. alanine, valine, lactate and tyrosine), glucose, certain nucleotides and related compounds (ATP, NAD, GTP, UTP; in the range δ 7.8–8.7). Additional resonances from creatine, glycine and substances involved in membrane biosynthesis such as choline and *myo*-inositol were also observed in the $^1$H NMR spectra of cell extracts. Peaks that were well-resolved and identified unambiguously at 500 MHz were quantitated relative to the added TSP standard. Although a full statistical analysis of these data was not undertaken, each cell line displayed characteristically different $^1$H NMR spectral patterns (Figure 1 (A and B)). For example, typically, in confluent MDCK cells, concentrations of choline were approximately four-fold higher than in confluent LLC-PK$_1$ cells (0.2 versus 0.05 mM), whereas concentrations of alanine and glycine were two- to four-fold lower (0.06 versus 0.21 mM and 0.12 versus 0.25 mM respectively). A variation in the level of glutamine was observed between the cell lines, consistently being present in relatively higher amounts in LLC-PK$_1$ than in MDCK cells. However, the concentration of glutamine was not quantified with respect to TSP as the glutamine proton signals were incompletely resolved in the 500 MHz single pulse $^1$H NMR spectra (Figure 1).

The 600 MHz $^1$H NMR spectrum of an acetone extract of untreated MDCK cells (after 3 h incubation is illustrated in Figure 2 and highlights a significant improvement in signal resolution in certain regions of the spectrum at this field strength. For example, resonances that were overlapped at 500 MHz, particularly in the region δ 0.5–4.6, such as protons from valine, isoleucine and leucine (in the range δ 0.9–1.05) choline (δ 3.23), *myo*-inositol (δ 3.28), taurine (δ 3.27) and glutamine (δ 2.37), were more clearly distinguished at 600 MHz (Figure 2(A)).

Additionally an improved separation of signals from nucleotides was observed in the chemical shift range δ 5.0–9.0, accompanied by an enhanced resolution of the aromatic resonances from tyrosine and phenylalanine (Figure 2(B)). However, the high complexity of the spectra was retained at 600 MHz, notably in the $^1$H chemical shift range δ 3.0–4.0, consistent with the presence of sugars and α-CH resonances of amino acids. Application of the 2-D JRES $^1$H NMR experiment at 600 MHz to the cell extract illustrated in Figure 2 further simplified the spectrum, by the dispersion of the chemical shift

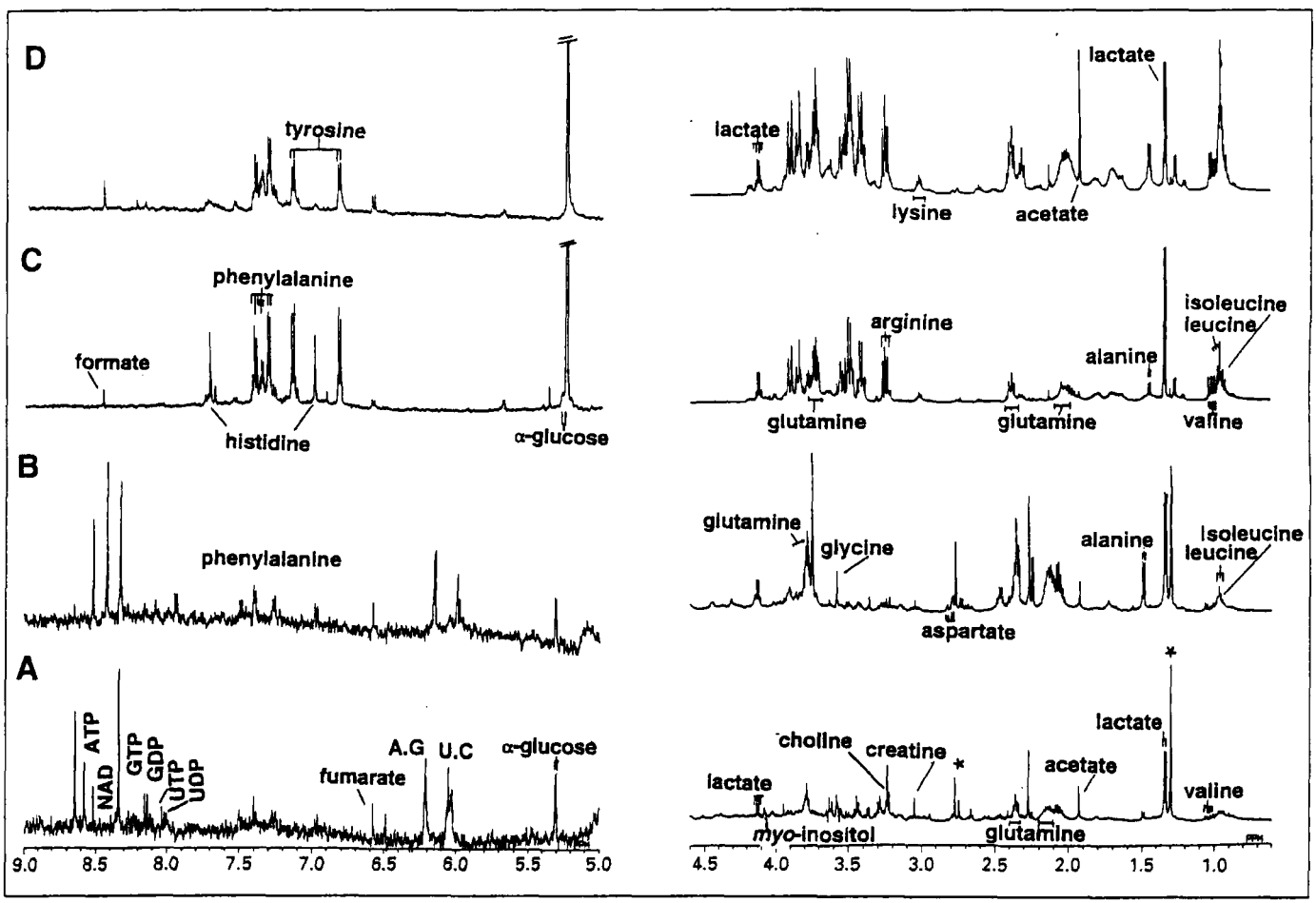


**Figure 1.** Partial 500 $^1$H NMR MHz spectra (δ 0.6–4.6) and (δ 5.0–9.0) of extracts of untreated MDCK (A) and LLC-PK$_1$ (B) cells and Eagle's minimal essential (C) and M199 culture medium (D) harvested after 1 h of incubation. A, Adenosine, G, guanosine, U, uridine, C, cytidine; * indicates signals for 3-hydroxyisovaleric acid, an impurity from sample preparation procedure (also illustrated in Figures 2–5).

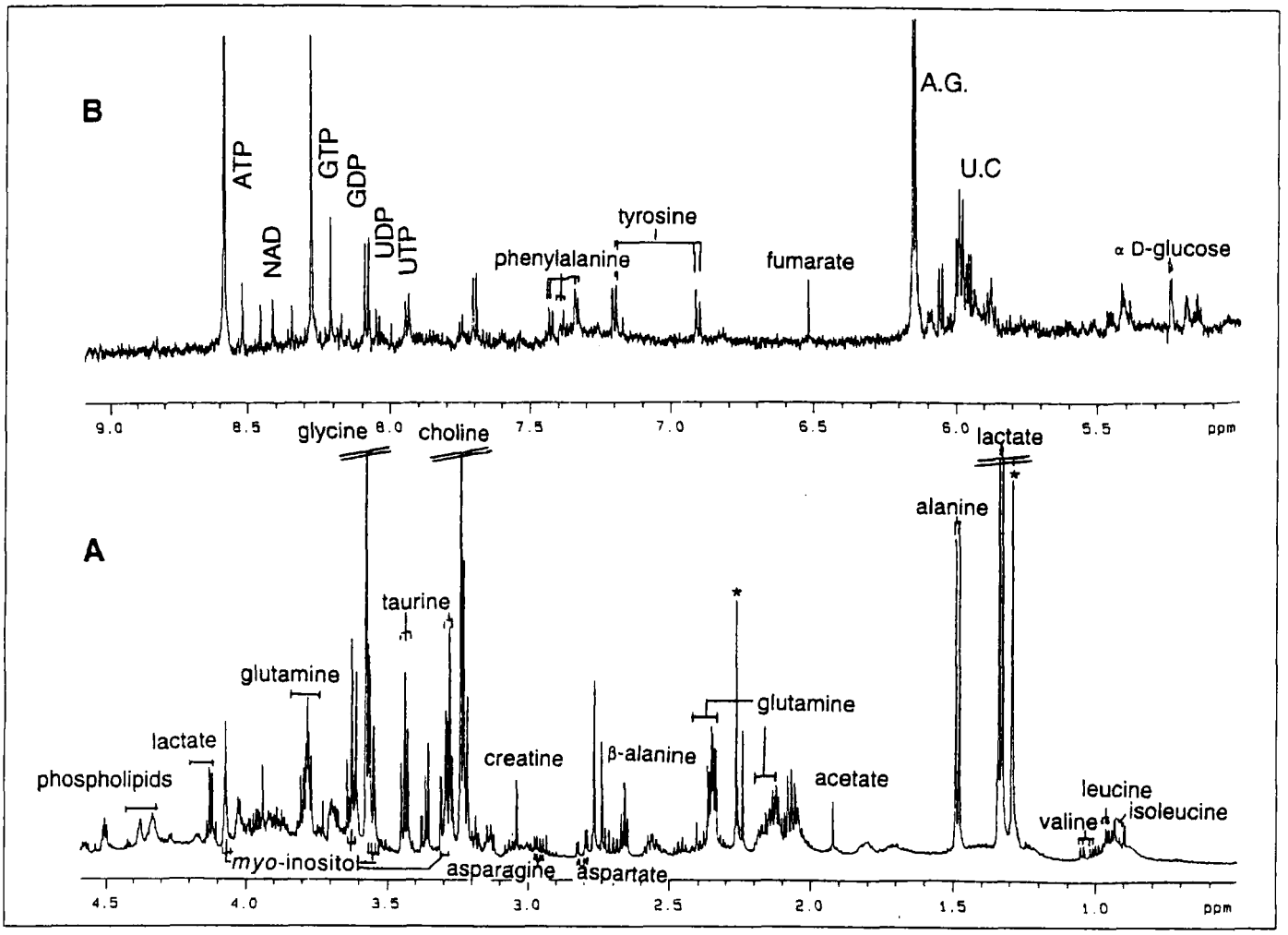


**Figure 2.** Partial 600 $^1$H NMR MHz spectra (δ 0.5–4.6, A) and (δ 5.0–9.0, B) of an untreated MDCK cell extract harvested after 3 h of incubation.



and coupling constant data into two orthogonal frequency domains (Figure 3). The skyline projection of the spectrum through $F_2$ (the chemical shift axis) gives a high resolution $^1$H-decoupled $^1$H spectrum in which all peaks are presented as singlets (Foxall *et al.* 1993). The dispersion of the signals into two frequency domains allowed a much greater proportion of the biochemical information present in the chemical shift range δ 0.5–4.5 to be accessed in both the contour plot and the *J*-coupling free skyline $F_2$ projection, thereby aiding signal assignment (Figure 3). Numerous intracellular metabolites, including several amino acids, choline and *myo*-inositol, were resolved more completely in the JRES spectrum. Additionally, signals from dimethylamine, methylamine and *N,N*-dimethylglycine, which had overlapped with signals from other metabolites in the single pulse spectrum, were revealed.

### *Alterations in $^1$NMR profiles of cell extracts following toxic insult*

Typical 500 MHz $^1$H NMR profiles and graphs showing changes in the levels of various intracellular constituents during a 24 h exposure of LLC-PK$_1$ cells to the proximal tubular toxin DCVC and MDCK cells to the renal medullary toxin CEA are illustrated in Figures 4 and 5, respectively. Selected NMR resonances were quantitated relative to the added TSP standard. These $^1$H NMR data highlight treatment- and time-related changes in many cellular constituents, not routinely measured in toxicity studies. Many of these changes were detected prior to overt cytotoxicity observed by phase contrast microscopy. For example, in LLC-PK$_1$ cells, 60 μM DCVC decreased choline levels (to 24% of control) within 3 h, while by 9 h minor decreases in acetate and alanine (to 55 and 78% of control respectively) were apparent (Figure 4). A reduction in the intracellular level of aspartate was also detected in $^1$H NMR spectra measured at the 9 h time-point (Figure 4), accompanied by an increase in lactate (to 178 and 108% of control at 6 and 9 h respectively) and decrease in glucose (98% of control at 9 h). The concentration of aspartate was not quantified with respect to TSP, however, owing to the complexity of the aspartate proton signals at 500 MHz. The most pronounced change following incubation of cells with DCVC was the increase in glutamine observed at the 9 h time-point, however, again NMR concentration data were not calculated for glutamine because of its complex NMR spectrum. Morphological

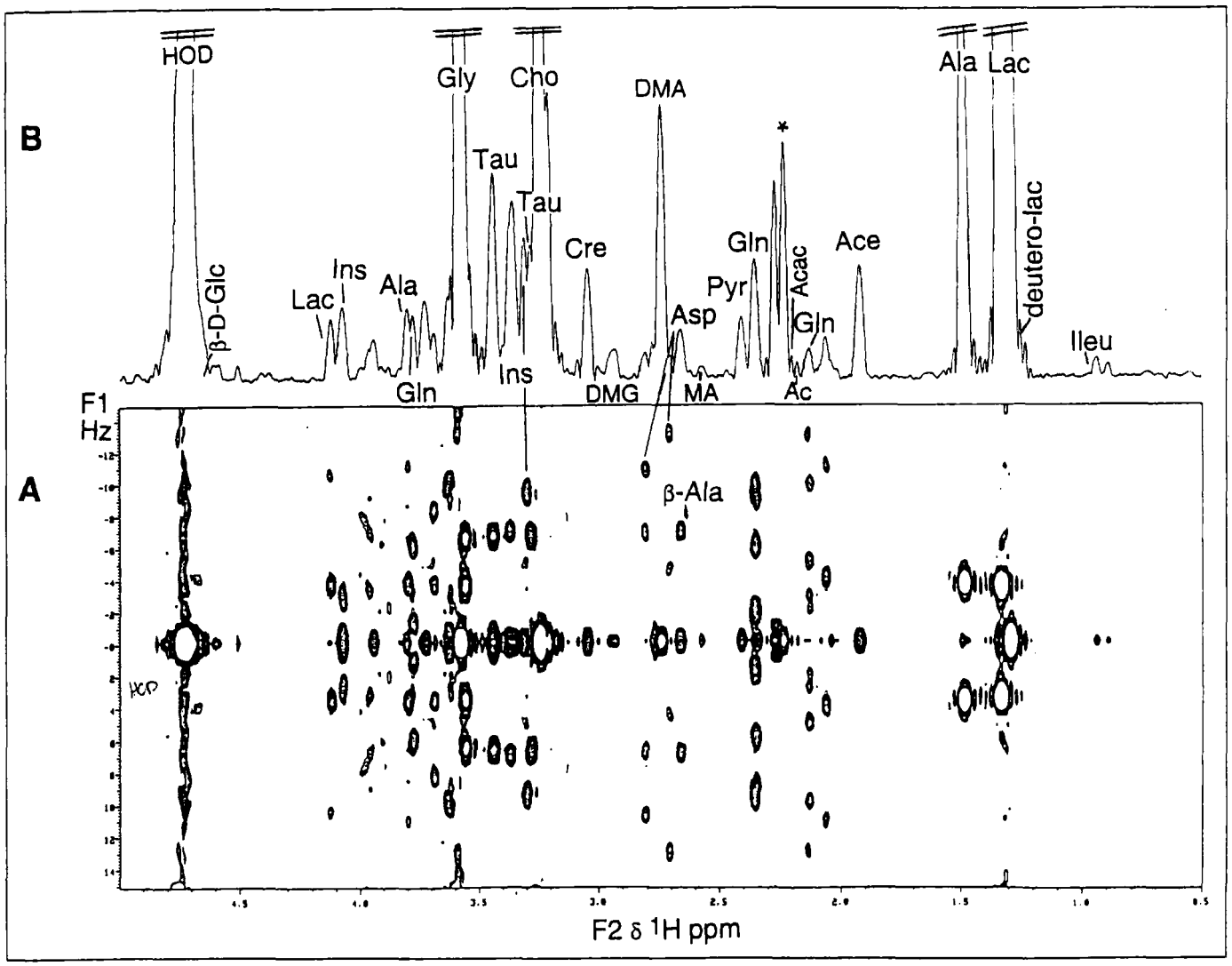


**Figure 3.** 600 MHz 2-D JRES $^1$H NMR spectrum of the untreated MDCK cell extract illustrated in Figure 2 harvested after 3 h of incubation from δ 0.5–5.0, showing contour plot (A) and skyline $F_2$ projection (B). Acac, Acetoacetate; Ac, acetone; Ace, acetate; Ala, alanine; β-Ala, β-alanine; Asp, aspartate; Cho, choline; Cre, creatine; deutero-lac, deuterated-lactate; DMA, dimethylamine; DMG, *N,N*-dimethylglycine; β-D-Glc, β-D-glucose; Gln, glutamine; Gly, glycine; Ins, *myo*-inositol; Lac, lactate; MA, methylamine; Pyr, pyruvate; Tau, taurine; Ileu, isoleucine.

changes were first observed 9 h following exposure of cells to DCVC when cells were more spherical and the cell monolayers contained fewer domes than in the control (data not shown). A comparison of partial 600 MHz $^1$H NMR spectra (δ 0.5–4.7) of MDCK cell extracts prepared at 3 and 9 h time-points during a 24 h exposure to 5 mM CEA is illustrated in Figure 5 which also shows selected quantitative data generated at 500 MHz for the time-points prior to 24 h. In MDCK cells, 5 mM CEA decreased levels of glycine at all time-points (Figure 5). In addition, CEA increased lactate (to 186 and 142% of control at 6 and 9 h respectively) and decreased glucose (to 93% of control at 9 h). There was also spectral evidence of a reduction in aspartate levels by 9 h, accompanied by a slight decrease in the intracellular level of alanine (at 9 h) and increased acetate.

## Discussion

### $^1$H NMR characterization of the LLC-PK$_1$ and MDCK renal cell lines

The present study used 500 and 600 MHz $^1$H NMR spectroscopy to analyse the biochemical composition of MDCK and LLC-PK$_1$ cell lines. The application of 600 MHz JRES $^1$H NMR spectroscopy simplified the cell spectra, and clarified the assignment of cell constituents in the highly complex spectral region covering the δ 3.0–4.0 range. Overall, many low MW constituents involved in important processes in cellular biochemistry were readily identifiable in spectra of protein-free cell extracts, including amino and organic acids, intermediary metabolites, soluble membrane components and nucleotides. Few other bioanalytical techniques can detect such a diversity of biochemical classes of compound without extensive sample preparation and possibly chromatographic separation. There

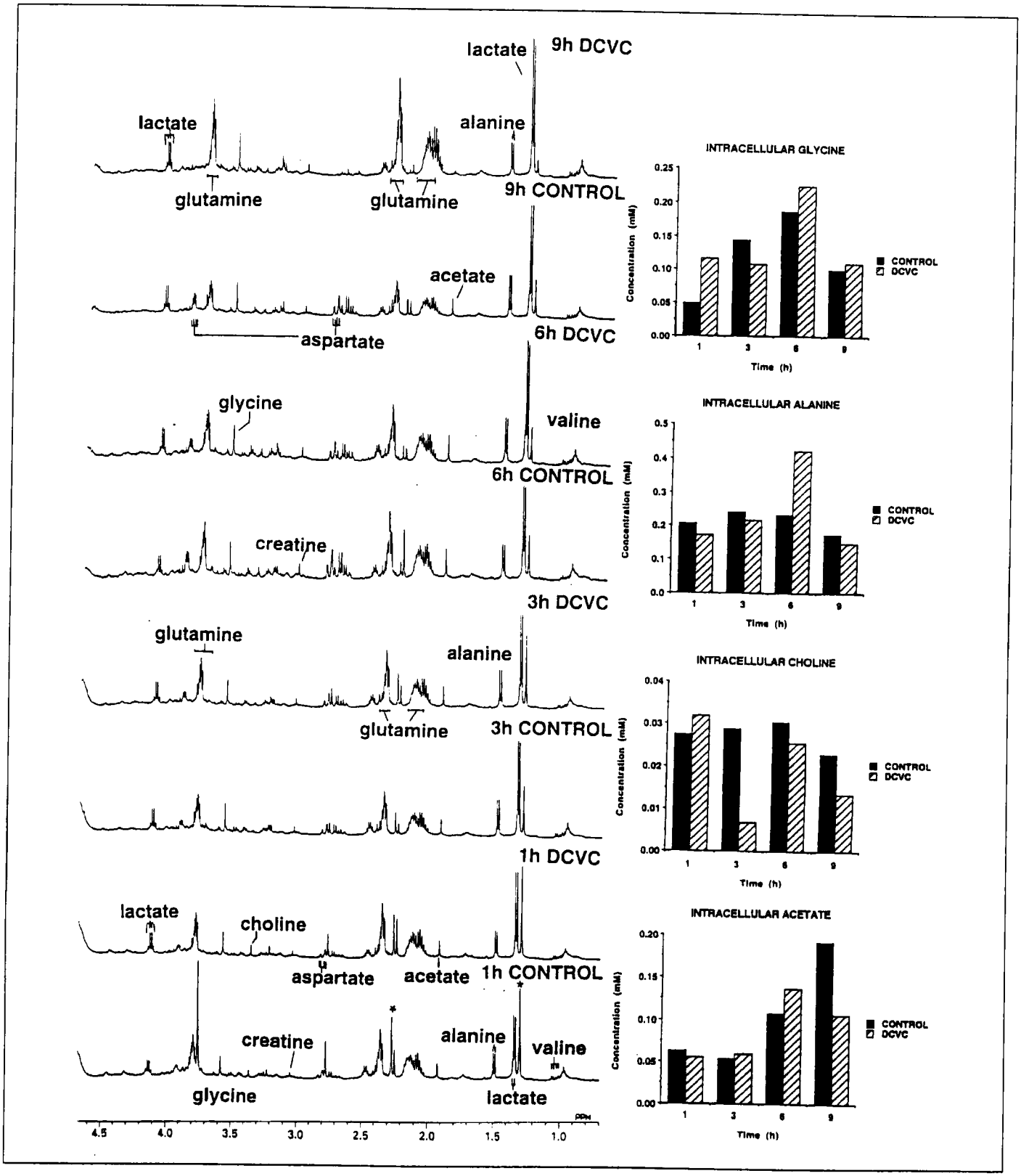


**Figure 4.** Representative partial 500 $^1$H NMR MHz spectra and graphs showing selected quantitative data from control LLC-PK$_1$ cell extracts and extracts of cells harvested at various time-points during a 24 h exposure to 60 μM DCVC ($n$ = 1 per time-point).

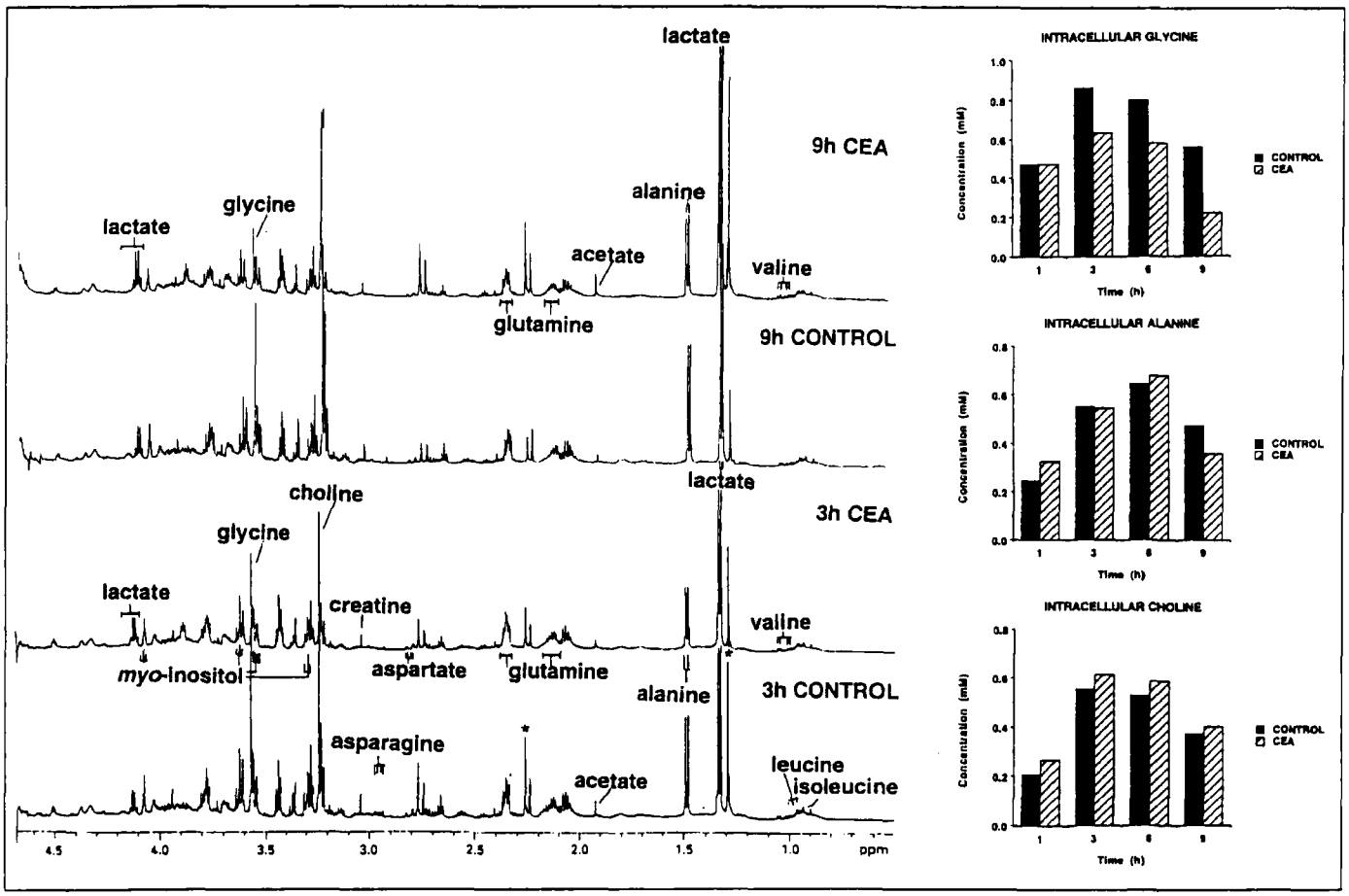


**Figure 5.** Representative partial 600 $^1$H NMR MHz spectra from control MDCK cell extracts and extracts of cells harvested at 3 and 9 h following incubation with 5 mM CEA and graphs showing selected quantitative data, generated at 500 MHz, during a 24 h exposure to 5 mM CEA ($n$ = 1 per time-point).



was evidence that $^1$H NMR spectroscopy can enable the recognition of biochemical differences between LLC-PK$_1$ and MDCK cells (the two lines originating from different parts of the nephron) on the basis of the types and amounts of the low MW metabolites observed. For instance, the distal tubule-derived MDCK cells generally contained greater amounts of compounds involved in membrane phospholipid biosynthesis, such as *myo*-inositol and choline (Figures 1(A) and 2). In contrast, LLC-PK$_1$ cells, of proximal tubular origin, were higher in amino acids (alanine and glycine) and lactate (Figure 1(B)). This is analogous to the different $^1$H NMR profiles of low MW metabolites observed in acid extracts of renal cortex and medulla (Gartland *et al.* 1989). Whereas the renal cortex was shown to contain predominantly amino acids and lactate, the medulla was high in inositol and cholines (Gartland *et al.* 1989). Previous workers have demonstrated that choline was confined to the renal inner medulla of rats and rabbits where it has been suggested to play a role in the maintenance of intracellular osmotic balance (Bagnasco *et al.* 1986). *Myo*-inositol has also been reported to be present in high concentrations in the inner medulla where it is an important organic osmolyte (Beck *et al.* 1992).

### *Effect of toxic insult on intracellular constituents*

The present studies investigated the potential of using NMR spectroscopy to study renal cell dysfunction caused by model nephrotoxins known to affect the proximal tubule (DCVC) or the renal medulla (CEA). Analysis of LLC-PK$_1$ and MDCK cell extracts following their respective exposure to DCVC and CEA revealed early changes in the levels of several low MW constituents (Table 1) which can be linked to general alterations in cellular metabolism. Both toxins decreased intracellular glucose and increased intracellular lactate, general non-cell specific changes that are consistent with glucose utilization and the accompanying production of lactate by the cells by glycolysis. Changes in the levels of glycine and choline were also noted following exposure of cells to both toxins (Figures 4 and 5; Table 1). The reason for the observed changes in choline is unclear, although this could implicate differences in phospholipid metabolism. The minor decreases in acetate and alanine detected 9 h following exposure of LLC-PK$_1$ cells to DCVC (Figure 4) suggest a shift from fatty acid metabolism to gluconeogenesis. In addition, DCVC decreased intracellular aspartate levels and increased

| Cell line (treatment) | Direction of metabolite change[a] | | | | | |
|---|---|---|---|---|---|---|
| | Glycine | Choline | Alanine | Acetate | Aspartate | Glutamine |
| MDCK (CEA) | – | + | – | + | – | = |
| LLC-PK$_1$ (DCVC) | + | – | – | – | – | + |

**Table 1**. Summary of the main metabolites changing in response to the proximal tubular nephrotoxin DCVC and the renal medullary toxin CEA.

[a] Direction of metabolite change 9 h following treatment (+, increase; –, decrease, =, no observed change from control).

glutamine suggesting the utilization of glutaminolysis by the culture system may have been affected.

The current findings with this limited number of model toxins indicate that $^1$H NMR monitoring of renal cell extracts can potentially provide a useful window onto the early biochemical activities of renal cells following toxic injury. An important question that arises from these observations, however, is whether the subtle biochemical changes detected are causally related to the toxicity expressed by DCVC and CEA. A more detailed analysis of this type of data is currently being undertaken to identify which changes are toxicologically and statistically significant. 'Cross over' experiments, in which MDCK cells are treated with proximal tubular toxins and vice versa, are also being conducted to evaluate the specificity of the trends observed. In this way, the feasibility of using NMR to search for new biochemical markers of cell-specific nephrotoxicity *in vitro* will be verified.

## Conclusions

In summary, the present studies show that $^1$H NMR spectroscopy can generate biochemical data on a wide range of low MW renal cell constituents and intermediary metabolites not routinely measured in toxicological studies. Further, changes were observed by NMR spectroscopy before the onset of overt cytotoxicity. This type of approach offers promise in providing novel end-points for use in *in vitro* toxicity studies and will ultimately assist in the elucidation of mechanisms of cell-specific toxicity in renal (and other) cell cultures.

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