Urinary Proton Magnetic Resonance Studies of Early Ifosfamide-induced Nephrotoxicity and Encephalopathy


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ABSTRACT

Ifosfamide is an oxazaphosphorine widely used in the treatment of cancer in children and adults. Nephrotoxicity and neurotoxicity are major side effects. The aim of this study was to use high-resolution proton nuclear magnetic resonance (1H NMR) spectroscopy of urine to identify novel biochemical markers of ifosfamide-induced toxicity. Urine samples were collected from 10 nonencephalopathic patients (who had not previously received nephrotoxic chemotherapy) immediately prior to the first ifosfamide dose and at timed intervals for up to four treatment cycles. The findings were compared with those for urine samples collected from five patients during acute encephalopathic episodes. 1H NMR urinalysis identified a series of characteristic time-related changes in the excretion profiles of low molecular weight endogenous metabolites during ifosfamide therapy. These changes included a decreased excretion of hippurate and an increased excretion of glycine, histidine, glucose, lactate, and trimethylamine-N-oxide. Two nonencephalopathic patients had marked but transient glutaric or adipic aciduria during the second cycle of ifosfamide treatment. Urinary retinol-binding protein rose acutely after each cycle. The ratio of the urinary excretion of the uroprotectant for urinary aciduria, and i.v. methylene blue did not reverse neurotoxicity in the two patients who received it. The results suggest that ifosfamide nephrotoxicity involves both cortical and medullary regions of the nephron and that the urinary mesna:dimesna ratio may be important in assessing the degree of cytoprotection. This study demonstrates that 1H NMR can provide novel biochemical information on ifosfamide-induced toxicity and will be of value in the optimization of ifosfamide therapy.

INTRODUCTION

The alkylating agent ifosfamide has significant clinical activity against a wide variety of sarcomas and carcinomas in adults and children (1–4). Two of the major clinical side effects are nephrotoxicity and neurotoxicity. Evaluation of renal function is essential during ifosfamide treatment, especially in children, to minimize the risk of severe chronic morbidity, such as that caused by Fanconi’s syndrome (3, 4). Proximal tubule dysfunction is common; distal tubule and collecting duct injury may also develop, but a reduction in the GFR of more than 20% is rare. Considerable patient variation in nephrotoxic response to ifosfamide has been reported. In most studies, however, renal function was measured at least 6 months after ifosfamide chemotherapy, and many patients had also received cisplatin (1–4). Assessment of nephrotoxicity is routinely based on conventional tests of renal function, such as blood urea, plasma electrolytes, and creatinine, and the urinary excretion of low MW proteins and renal tubular enzymes (3, 4). In general, these tests require a range of analytical methods and provide limited information on the site of the renal lesion, which is important in understanding the pathophysiology of ifosfamide-induced nephrotoxicity.

A significant percentage of patients also develop neurotoxic side effects (1), but the underlying biochemical processes remain unknown. The risk factors identified as being associated with ifosfamide-induced encephalopathy include low serum albumin, high serum creatinine, and the presence of pelvic disease (5). Typical symptoms are somnolence, mental confusion, paranoid hallucinations, and, rarely, fits and coma (1, 6). A fatal outcome is unusual. Kupfer et al. (7) have suggested that ifosfamide-induced encephalopathy may be due to a defect of fatty acid metabolism at the mitochondrial level, which is reversed by administration of the electron acceptor methylene blue. This hypothesis, however, is based on one encephalopathic patient who had high urinary concentrations of glutaric acid and sarcosine following an overdose of ifosfamide (25 g i.v. over 24 h).

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Methylene blue was subsequently used for the prophylaxis and reversal of ifosfamide encephalopathy in two other patients, although urinary glutaric acid concentrations were not measured (7).

Here, a new NMR spectroscopic approach to the investigation of ifosfamide-induced toxicity is described. Videen and Ross (8) recently reviewed the clinical advantages of $^1$H NMR spectroscopic urinalysis in investigating human disease processes. $^1$H NMR provides extensive information on both the structure and composition of low MW metabolites (including amino acids, organic acids, amines, and sugars) in body fluids such as urine, blood plasma, and cerebrospinal fluid and provides a wealth of biochemical information that is not available with any other single analytical technique. As such, NMR spectroscopy has become a powerful research tool with which to study inherited metabolic diseases, renal function, including urinary RBP, a sensitive marker of proximal renal tubule damage. Furthermore, we have used the unique exploratory nature of $^1$H NMR spectroscopy to investigate further the reported association between ifosfamide-induced encephalopathy and glutaric aciduria.

**PATIENTS AND METHODS**

Informed patient consent was obtained before commencement of the study, which had local ethics committee approval.

**Patients.** Two groups of patients were studied. Details of the patients’ characteristics and ifosfamide regimens are given in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (nonencephalopathic)</th>
<th>Group 2 (encephalopathic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Age range (yr)</td>
<td>26–71</td>
<td>34–65</td>
</tr>
<tr>
<td>No. of males/no. of females</td>
<td>3/7</td>
<td>3/2</td>
</tr>
<tr>
<td>Tumor types (no.)</td>
<td>Ewing’s sarcoma (1); soft tissue sarcoma (9)</td>
<td>Ewing’s sarcoma (2); soft tissue sarcoma (3)</td>
</tr>
<tr>
<td>Ifosfamide dose</td>
<td>6–9 g/m² in 2–3 days</td>
<td>5–15 g/m² in 1–5 days</td>
</tr>
<tr>
<td>Schedule</td>
<td>1-h bolus or 24-h infusion</td>
<td>1-h bolus or 24-h infusion</td>
</tr>
<tr>
<td>Other drugs</td>
<td>Mesna, doxorubicin, vincristine, etoposide, actinomycin D</td>
<td>Mesna, doxorubicin, vincristine, etoposide, actinomycin D</td>
</tr>
<tr>
<td>Urine collection</td>
<td>Prior to each cycle of treatment and at six hourly timed intervals during ifosfamide infusion</td>
<td>Timed collections during encephalopathic symptoms</td>
</tr>
</tbody>
</table>

Group 1 consisted of 10 adults, who had not previously received nephrotoxic chemotherapy, with no clinical evidence of encephalopathy during ifosfamide treatment. All patients had normal renal function before starting chemotherapy. Volume loading and a diuresis were established with saline prior to and throughout chemotherapy. Each patient received 2–4 cycles of ifosfamide and mesna (sodium 2-mercaptoethane sulfonate). The cycles alternated between 1-h bolus and continuous 24-h i.v. infusions, as part of a randomized comparison of these methods of administration (17). The administrations of saline and mesna were kept constant throughout.

Group 2 consisted of five adults, also receiving i.v. ifosfamide, who developed typical signs and symptoms of encephalopathy. Symptoms consisted of mental confusion, paranoia, and drowsiness (in the absence of sedative drugs), which led to coma in two patients. Methylene blue (50 mg in 2% aqueous solution) was administered i.v. to two encephalopathic patients (not in coma), and an infusion of 5% dextrose was also given to these patients.

**Urine Collection.** Urine samples were collected from patients in group 1 immediately prior to ifosfamide treatment and for six hourly periods over the next 3–5 days, as long as the patients were willing to remain in the hospital. For patients in group 2, timed urine collections were only obtained at the onset of encephalopathic symptoms and for a further 48 h. All urine samples were tested for the presence of blood, glucose, ketones, and protein using dry multireagent strips (Ames Multistix) and stored frozen in 3-ml aliquots at −20°C until analysis.

**Tests of Renal Function and RBP Analysis.** Blood samples were collected prior to each cycle of ifosfamide administration for the measurement of blood urea nitrogen, glucose, plasma creatinine, and electrolytes. For all patients in group 1,
TMAO
dimesna-
creatinine
P-
N
mesna
DMA
histidine
glycine citrate
taurine

Fig. 1 \(^{1}\)H NMR spectra [500 MHz; \(\delta 0.5-4.5\) and \(\delta 6.4-8.4\) (\(\times 2\) vertical height expansion)] of urine collected from a nonencephalopathic patient, prior to the first cycle of ifosfamide treatment (A) and at 6–12 h post-ifosfamide administration (B). ifos, ifosfamide parent drug and/or metabolites.

GFR was determined by \(^{51}\)Cr EDTA clearance measurements prior to the first cycle of chemotherapy. Subsequent GFR estimations were calculated from timed creatinine clearance measurements. Urinary RBP analysis was measured using an ELISA (20). Urine testing positively for blood was not used for RBP measurement. Results were expressed as \(\mu\)g RBP/mM creatinine.

\(^{1}\)H NMR Spectroscopy of Urine. A 3-ml volume from each patient’s timed urine collection was lyophilized and reconstituted in 0.75 ml of \(D_2O\) containing 1 \(mM\) TSP as a chemical shift reference (\(\delta 0.0\)) and an internal standard for quantitation. Single-pulse \(^{1}\)H NMR measurements were made on a JEOL GSX500 spectrometer operating at 500.13-MHz \(^{1}\)H resonance frequency. For each urine sample, 64 FIDs were collected into 32,768 computer points with a spectral width of 6000 Hz. The data acquisition time per FID was 2.73 s, and a further delay of 2.27 s was added between the 40° pulses to permit full \(T_1\) relaxation. The residual water signal was suppressed by application of a secondary irradiation field (gated off during acquisition) at the water resonance frequency. Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 0.4 Hz. Assignment of resonances was made by consideration of chemical shifts relative to TSP, spin-spin coupling patterns, the pH dependence of the chemical shifts, and comparison with literature values (9–17). Confirmation of analyte identification was ultimately by addition of the standard material to samples and remeasuring spectra. All spectra were scaled to the same signal:noise ratio so that resonance intensities were directly comparable between urine samples. Quantitation of selected metabolite resonances was by peak height measurement relative to that of TSP and consideration of the number of protons contributing to the signals.

Statistical Analysis. Statistical analysis was performed with Instat for Macintosh (version 2.03) statistical software. For each ifosfamide cycle, the urinary excretion, expressed as a ratio to urinary creatinine concentration, of glycine, histidine, lactate, hippurate, and RBP was compared with pretreatment values using the Wilcoxon signed rank sum test. The significance level was set at \(P \leq 0.05\).

RESULTS
All patients in group 1 received the full prescribed dose of chemotherapy for up to four cycles of ifosfamide treatment. The
Fig. 2 Pretreatment (open bars) and peak (patterned bars) urinary excretion of glycine, histidine, lactate, and hippurate, expressed as molar ratios to urine creatinine, during the first two cycles of ifosfamide treatment. Columns, means for urine samples collected for 48-72 h during each cycle; bars, SE; n = 10 for all metabolites. a, P < 0.05; b, P < 0.01, compared to baseline values.

Assessment of Renal Function in Nonencephalopathic Patients

Conventional measurements of renal function (plasma creatinine and blood urea nitrogen) were within established normal ranges prior to commencing chemotherapy, as was the GFR. The pretreatment GFR of one patient was 74 ml/min/1.73 m² and was considered below the lower limit of normal for a male of that age (80 ml/min/1.73 m²). For all patients, plasma creatinine remained stable throughout the cycles of treatment, but GFR values decreased.

¹H NMR Spectroscopic Analysis of Urine from Nonencephalopathic Patients

Initial Changes in Urinary Metabolite Excretion Patterns. The standard one-dimensional 500-MHz ¹H NMR spectrum of urine collected from a nonencephalopathic patient following initiation of diuresis and prior to ifosfamide treatment is shown in Fig. 1A. Typically, a good signal:noise ratio was obtained from 64 scans, requiring about 5 min of acquisition time. The NMR analysis of human urine and resonance assignment are well documented (10-13, 21, 22) and will not be elaborated further here. The spectrum shows a urinary biochemical profile for low MW metabolites typical for humans with good renal function (23) and is representative of urine collected.
Partial 500-MHz $^1$H NMR spectra ($\delta$ 0.5–4.5) of urine collected from a nonencephalopathic patient at the completion of cycles 1 (A) and 2 (B) and at 6–12 h during cycle 3 (C) of ifosfamide treatment. Region A, the complex $\delta$ 3.2–3.9 region of the spectrum where there is extensive overlap of signals from mesna, dimesna, amino acids ($\alpha$-CH resonances), and glucose (present in high concentrations in C).

From all nonencephalopathic patients before commencement of chemotherapy. Both the aliphatic ($\delta$ 0.5–4.5) and aromatic ($\delta$ 6.4–8.4) regions of the spectrum are shown, including assignment of the major metabolites creatinine, hippurate, glycine, taurine, TMAO, DMA, citrate, and alanine. The 500-MHz $^1$H NMR spectrum of urine collected from the same patient 6–12 h following ifosfamide and mesna administration is shown in Fig. 1B. Ifosfamide treatment produced marked changes in the excretion patterns of several low MW metabolites, principally increased glycine and histidine and decreased hippurate, within 12 h of administration. Resonances from ifosfamide (parent drug and/or metabolites) were also observed (Fig. 1B), but identification of individual ifosfamide metabolites in the one-dimensional $^1$H NMR spectra of urine was difficult because of resonance overlap with those from endogenous metabolites that are generally present at much higher concentrations. Here, the ifosfamide metabolites were measured using an established chromatographic method (24), and the results will be described elsewhere. Compared with the urine collected pre-ifosfamide treatment (Fig. 1A), strong resonances were observed in the...

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region δ 2.8–3.4, which we have assigned to the reduced forms of the uroprotectant mesna (as administered) and the oxidized form (dimesna), which consists of two mesna molecules joined by a disulfide bridge (25). In general, a low urinary excretion of the uroprotectant mesna (active form), relative to that of the oxidized inactive form, was observed, a pattern that was consistent throughout all treatment cycles.

Quantitation of Urinary Biochemical Markers of Early Ifosfamide Toxicity. All nonencephalopathic patients demonstrated an ifosfamide-induced change in the excretion pattern of glycine, histidine, hippurate, and lactate that occurred as early as the first treatment cycle. Urinary glycine, histidine, and lactate levels were elevated during ifosfamide administration, whereas hippurate excretion decreased. Maximum effect, compared to baseline values, was observed between 48 and 72 h (P < 0.05), as shown in Fig. 2. There was no statistical difference between the baseline levels prior to cycles 1 and 2 or between the peak values, which suggested that the nephrotoxicity was acute and reversible at this stage of treatment.

Ifosfamide-induced Proximal Tubular Dysfunction. Four nonencephalopathic patients were monitored by $^1$H NMR for four consecutive treatment cycles, to determine whether there was a cumulative nephrotoxic response to ifosfamide treatment. Two of the patients showed marked changes in the excretion patterns of endogenous organic compounds that were consistent with increasing proximal tubule damage. This nephrotoxic effect is shown in the 500-MHz $^1$H NMR spectra of urine collected from one patient at the completion of cycles 1

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**Fig. 4** The effect of increasing cycles of ifosfamide on the urinary excretion of glycine, histidine, lactate, and RBP for the same patient as in Fig. 3. Metabolites are expressed as molar ratios to urinary creatinine. □, cycle 1; ●, cycle 2; ○, cycle 3.
and 2 (Fig. 3, A and B, respectively) and at the beginning (6–12 h) of cycle 3 (Fig. 3C). Initially, glycine and histidine excretion increased, and hippurate excretion decreased, as described previously. In addition, the NMR profile of urine collected at the beginning of cycle 3 showed elevated levels of glucose, alanine, and TMAO. For this patient, the observed progression of ifosfamide-induced nephrotoxicity is supported by the quantitative data of glycine, histidine, lactate, and RBP excretion, expressed as a molar ratio to urine creatinine and shown in Fig. 4. The excretion of these metabolites increased, not only during ifosfamide infusion but also with each cycle of ifosfamide treatment. This indicated a cumulative ifosfamide dose-dependent nephrotoxicity. Similarly, for the second patient, significant glycosuria and amino aciduria (predominantly glycine, histidine, lysine, alanine, valine, tyrosine, and phenylalanine) had developed by the end of the fourth treatment cycle (Fig. 5). Of note, both patients had consistently higher excretions of inactive dimesna than of the active cytoprotective mesna during cycles 2 and 3 of treatment.

**Dicarboxylic Aciduria during Ifosfamide Treatment.**

Two other nonencephalopathic patients had transient dicarboxylic aciduria during ifosfamide chemotherapy (Fig. 6). One patient had high levels of glutaric acid (~6 mM) in the urine collected at 66–72 h (Fig. 6A) and 72–78 h during cycle 2. This patient had suffered severe nausea and vomiting and subsequent ketosis during cycle 1 but was comparatively well during cycle 2. Glutaric acid was not detected by $^1$H NMR spectroscopy in the corresponding plasma samples, and this suggests that the urinary glutaric acid was of renal origin. The other patient had high levels of adipic acid in the urine collected 6–12 h during cycle 2 (Fig. 6B) but, in contrast to the other patient, had a healthy appetite throughout the treatment period.

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**Fig. 5** $^1$H NMR spectrum (500 MHz) of urine, δ 5.0–9.0 (top) and δ 0.5–4.5 (bottom), to show the metabolite profile characteristic of ifosfamide-induced proximal tubule damage after four cycles of treatment.
Ifosfamide-induced Increase in RBP Excretion

For all patients in group 1, the NMR-generated data correlated well with the RBP measurements. The effects of increasing cycles of ifosfamide on urinary RBP excretion are shown in Fig. 7. Prehydration urinary RBP levels were normal for all patients (upper limit, 17 μg/mM creatinine for adults; Ref. 26) and remained unchanged following saline hydration prior to chemotherapy. For all patients, each cycle of ifosfamide adm-
istration produced significant elevations in urinary RBP excretion compared to baseline values. As for the low MW metabolites, the most pronounced rise in RBP occurred at 48–72 h during chemotherapy ($P < 0.05$ for cycle 1 and $P < 0.01$ for cycles 2–4), and levels returned to baseline values after cessation of ifosfamide administration (Fig. 7). These results confirmed our NMR-derived observations that ifosfamide treatment results in acute renal tubule dysfunction and that the majority of patients had complete recovery before commencing the next cycle of chemotherapy 21 days later.

**1H NMR Analysis of Urine Collected from Encephalopathic Patients**

For patients in group 2, urine samples were not collected prior to ifosfamide treatment but were obtained only when there was clinical evidence of neurotoxicity (see “Materials and Methods”). In general, the NMR-detected changes in the urinary low MW metabolite profile of patients during acute encephalopathic episodes were similar to those for the non-encephalopathic group during ifosfamide treatment and included elevated acetate, succinate, alanine, and lactate and decreased hippurate. These observations suggest that ifosfamide-induced renal damage was also present in these patients, although conventional tests of renal function were not available for comparison. Glutaric acid was not detected in any of the urine samples collected from the five encephalopathic patients. Methylene blue did not improve the neurological symptoms in the two patients who received it, although both patients recovered with time.

In summary, ifosfamide treatment results in changes in the excretion patterns of low MW endogenous metabolites, accompanied by low MW proteinuria. For all patients, the metabolite levels and the ratios of one to another changed during the cycles of ifosfamide treatment, demonstrating an intra- and interpatient variation in toxic response to the drug. To determine the renal significance of these observations, we have compared these data with our NMR-derived data base of experimental region-specific nephrotoxins (14–17). The identification of the NMR detected biochemical markers of ifosfamide-induced nephrotoxicity, in patients with and without encephalopathy, and the time of onset and suggested site of nephron damage are shown in Table 2.

### DISCUSSION

$^1$H NMR spectroscopic analysis of urine has allowed the identification of a series of characteristic time-related changes in the excretion profiles of low MW metabolites during ifosfamide therapy. We have related the novel biochemical information to the site of the ifosfamide-induced renal lesions (Table 2 and Fig. 8) and compared the data with those for patients with encephalopathic symptoms.

The reported onset and severity of ifosfamide-induced nephrotoxicity varies widely between studies and between individual patients, ranging from subclinical toxicity to chronic renal tubular impairment (1–4). In this study, maximum nephrotoxicity was observed by cycles 3 and 4. By this stage, the pattern of glycosuria, amino aciduria, and organic aciduria (Figs. 3C and 4) was characteristic of the generalized disturbance of proximal tubular function associated with Fanconi’s syndrome. Amino acid hyperexcretion is directly related to the degree of their normal physiological reabsorption in the proximal tubule (28), and the fractional excretion of glycine and histidine is significantly higher than that of other amino acids (29). We observed an initial increase in glycine and histidine

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Table 2  Time-related urinary biochemical markers of ifosfamide-induced nephrotoxicity, based on NMR-generated data

<table>
<thead>
<tr>
<th>Urinary metabolite</th>
<th>Frequency</th>
<th>Time of onset</th>
<th>Site of renal damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>10/10</td>
<td>Cycles 1 and 2</td>
<td>Cortex (S1/S2)</td>
</tr>
<tr>
<td>Histidine</td>
<td>10/10</td>
<td>Cycles 1 and 2</td>
<td>Cortex (S1/S2)</td>
</tr>
<tr>
<td>Lactate</td>
<td>10/10</td>
<td>Cycles 1 and 2</td>
<td>Cortex (S1/S2)</td>
</tr>
<tr>
<td>Hippurate</td>
<td>10/10</td>
<td>Cycles 1 and 2</td>
<td>Cortex (S1/S2)</td>
</tr>
<tr>
<td>Acetate</td>
<td>3/10</td>
<td>Cycles 1 and 2</td>
<td>Medulla</td>
</tr>
<tr>
<td>Succinate</td>
<td>3/10</td>
<td>Cycles 1 and 2</td>
<td>Medulla</td>
</tr>
<tr>
<td>TMAO</td>
<td>6/10</td>
<td>Cycles 1 and 2</td>
<td>Medulla</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>1/10</td>
<td>Cycle 2 only</td>
<td>Medulla</td>
</tr>
<tr>
<td>Glutaric acid</td>
<td>1/10</td>
<td>Medulla</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>4/5</td>
<td>Cycles 3 and 4</td>
<td>Cortex (S1/2/3)</td>
</tr>
<tr>
<td>Arginine</td>
<td>2/5</td>
<td>Cycles 3 and 4</td>
<td>Cortex (S1/2/3)</td>
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<tr>
<td>Glucose</td>
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<td>Cycles 3 and 4</td>
<td>Cortex (S1/2/3)</td>
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<td>Glutamine</td>
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<td>Lysine</td>
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<td>Phenylalanine</td>
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</tr>
<tr>
<td>Valine</td>
<td>2/5</td>
<td>Cycles 3 and 4</td>
<td>Cortex (S1/2/3)</td>
</tr>
</tbody>
</table>

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1. Refs. 11–17.
2. Number of patients in group 1 showing change in metabolite excretion, relative to the number of patients studied per cycle. Arrows, direction of change with respect to urinary creatinine to compensate for patient variation in urine output.
3. Refer to Fig. 7.
4. Indicates metabolite changes observed in the urine of the encephalopathic patients.
5. Indicates metabolites that exhibited a diurnal variation in excretion.

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Fig. 7  Pretreatment (□) and peak (■) urinary RBP levels, corrected for urine creatinine excretion, for each cycle of chemotherapy. Columns, means for urine samples collected for 48–72 h during each cycle; bars, SE. Cycle 1, n = 10; cycle 2, n = 10; cycle 3, n = 5; cycle 4, n = 4. a, P < 0.05; b, P < 0.01, compared to baseline values.
excretion (cycle 1) that preceded the onset of maximum tubular dysfunction (cycles 3 and 4). Nissim and Weinberg (30) recently reported that ifosfamide dosing in rats leads to a depletion of glycine in renal cortical tissue and that glycine supplementation can provide protection against ifosfamide-induced renal tubular dysfunction. The decreased excretion of hippurate, also observed in all patients from the first treatment cycle, suggests that ifosfamide or one of its metabolites inhibits the proximal tubule secretion of this anion (31).

Here, all patients had elevated urinary RBP levels during ifosfamide treatment which returned to baseline values at completion of therapy (Fig. 7). These results correlated well with the NMR-generated results, the data indicating the presence of a reversible proximal tubule lesion that was dependent on the cumulative dose of ifosfamide. RBP is a low MW protein ($M_r 21,400$) that crosses the glomerular barrier without restriction and is almost completely reabsorbed at the tubular level (20). Increased urinary excretion of RBP is a widely used marker of proximal tubule damage and has the advantage of being very stable in urine. Previous studies in ifosfamide-treated children have shown that urinary RBP excretion increases progressively in a dose-dependent manner but remains elevated after treatment has stopped (32).

The $^1$H NMR analysis of serial urine collections has revealed time-related metabolic changes that are related to the onset, progression, and reversal of renal toxicity. These observations are consistent with those for experimental models of nephrotoxicity (14–17) and highlight the limitations of inter-
interpreting data derived from single, random urine samples in clinical and toxicological studies. The biochemical markers of ifosfamide-induced nephrotoxicity in humans were similar to those following sodium chromate (a cortical S1 toxin), mercurochloride (a cortical S2/3 toxin), and 2-bromoethanamine (a papillary toxin) poisoning in rats (14, 17). These observations lead to the hypothesis that early ifosfamide-induced renal damage affects the S1, S2, and S3 sections of the renal cortex and the renal medulla (Table 2). Although the underlying mechanisms of ifosfamide renal damage remains unclear, an increased understanding of the site of ifosfamide-induced renal damage and correlation with the metabolism of the drug could lead to the development of novel cytoprotective strategies and have a significant impact on ifosfamide therapy (30).

The mechanism of the transient excretion of glutaric acid and adipic acid (Fig. 6) in two nonencephalopathic patients during ifosfamide treatment is unexplained. High urinary excretion of glutaric acid and associated encephalopathy following an overdose of ifosfamide has previously been reported (7). In contrast, the ifosfamide dose in our patients with dicarboxylic aciduria was within the recommended guidelines for ifosfamide administration, and there was no clinical evidence of encephalopathy (Table 1). Glutaric aciduria was not observed in any of the five encephalopathic patients. Methylen blue did not reverse the neurological symptoms in the two encephalopathic patients who received it. Short-chain dicarboxylic acids are not detectable by $^1$H NMR in normal human urine (23). Raised levels of dicarboxylic acids can be found in the urine under conditions of extreme stress and starvation, when carbohydrate reserves are depleted and there is generation of ketone bodies (33). Ketosis can occur during chemotherapy due to prolonged anorexia and vomiting. However, acetone, acetoacetate, and $\beta$-hydroxybutyrate are detected readily by $^1$H NMR, and we confirm that neither of our patients was ketotic during their episodes of dicarboxylic aciduria.

Dicarboxylic aciduria is also associated with Reye’s syndrome, sodium valproate therapy, and the inhibition of fatty acid oxidation (33, 34). Inherited disorders of fatty acid $\beta$-oxidation, such as glutaric aciduria type II and Jamaican vomiting sickness, are due to a deficiency or inhibition of acyl-CoA-dehydrogenases and impaired function of electron-transferring flavoproteins (33). In such cases, patients present with multiple dicarboxylic aciduria, whereas our patients excreted high levels of either glutaric or adipic acid alone. Due to the small number of reported cases, the biochemical similarities between the inborn error of metabolism and ifosfamide-induced encephalopathy are tenuous, and the use of methylene blue may not be appropriate in all cases.

However, there is an equal amount of evidence for alternative mechanisms for ifosfamide-induced dicarboxylic aciduria. First, our observations correlate with those for rats following exposure to the renal medullary toxin 2-bromoethanamine (17). This suggests that ifosfamide has a similar site and/or mechanism of toxic action that is unrelated to any encephalopathic side effects. Second, recent in vitro studies have demonstrated that glutaric acid is the end product of the oxidation of arachidonic acid, a long-chain unsaturated fatty acid that is present in membrane phospholipids (35). The formation of glutaric acid is a distinctive feature of the oxidative attack at the position of the double bond (C5=C6) closest to the carboxylic group of arachidonic acid. Glutaric aciduria may therefore serve as a novel biochemical marker of ifosfamide-induced oxidative attack on membrane phospholipids, a possible cytotoxic effect of the ifosfamide metabolite chloracetaldehyde (36). Thus, the origin of ifosfamide-induced dicarboxylic aciduria remains unclear, as does the biochemical basis of the encephalopathy associated with the drug.

Efficient protection against the ifosfamide metabolites chloracetaldehyde and acrolein, considered to be responsible for the drug-induced toxicity (1–4), is afforded by the coadministration of the thiol mesna (3, 4, 25). Following i.v. administration, mesna is rapidly oxidized to dimesa, which is chemically unreactive within the renal tubular cells, a proportion of dimesa is reduced back to mesna by reaction with intracellular glutathione. In its reduced state, mesna will detoxify the chemically reactive ifosfamide metabolites. Depletion of glutathione by the metabolites of ifosfamide or other coadministered drugs, such as acetaminophen, may lead to insufficient reduction of dimesa and increased vulnerability of the renal tubule cells to toxicity. We have shown that $^1$H NMR spectroscopy is a rapid and efficient analytical means of simultaneously measuring the two forms of mesna. Preliminary observations in this study suggest that a low urinary excretion of the uroprotectant mesna (active form), relative to that of the oxidized inactive form, was associated with increased renal toxicity. The ratio of the active mesna to the unreactive dimesa may therefore be important in assessing the degree of cytoprotection. However, the ratio measured in the urine may not reliably reflect that in the tubular filtrate, and further studies are required to determine whether there is a correlation between mesna excretion and site of renal damage.

In conclusion, $^1$H NMR urinalysis has provided novel biochemical information on the early nephrotoxic effects of ifosfamide in patients with and without encephalopathy. The advantages of using NMR spectroscopy in parallel with conventional tests of renal function to monitor ifosfamide chemotherapy have been demonstrated. For example, there is no other single analytical method that can simultaneously measure such a diverse range of compounds as glucose, TMAO, glutaric acid, and mesna. The application of NMR spectroscopy to complex clinical biochemical problems is developing rapidly but is currently restricted to research establishments with high-field instruments and NMR-trained scientists. However, the NMR identification of new urinary markers of ifosfamide-induced toxicity could lead to the development of specific assays, using other spectroscopic or chromatographic methodologies, for their use in the hospital environment. In the long term, the application of an NMR approach to the combined study of the toxicity and metabolism of ifosfamide may be of value in optimization of the clinical use of the drug.

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Urinary proton magnetic resonance studies of early ifosfamide-induced nephrotoxicity and encephalopathy.


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