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ORIGINAL ARTICLE

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Urinary excretion of ifosfamide, 4-hydroxyifosfamide, 3- and 2-dechloroethylifosfamide, mesna, and dimesna in patients on fractionated intravenous ifosfamide and concomitant mesna therapy

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Abstract The oxazaphosphorine antineoplastic ifosfamide (IF) is metabolized by two different initial pathways: ring oxidation ("activation"), forming 4-OH-IF ("activated IF"), and side-chain oxidation with liberation of chloroacetaldehyde (CAA), forming the inactive metabolites 3-dechloroethylifosfamide or 2-dechloroethylifosfamide (3-DCE-IF, 2-DCE-IF). 4-OH-IF and 4-OH-IF-derived acrolein are thought to be responsible for IF-induced urotoxicity (hemorrhagic cystitis), whereas CAA may be involved in IF-associated nephrotoxicity (renal tubular damage). The thiol compound 2-mercaptoethane sulfonate sodium (mesna) has proved to inactivate sufficiently the urotoxic metabolites of oxazaphosphorine cytostatics and is therefore routinely given to patients receiving IF chemotherapy. The cumulative urinary excretion of IF, 4-OH-IF, 3-DCE-IF, 2-DCE-IF, mesna, and its disulfide dimesna was studied in 11 patients with bronchogenic carcinoma receiving IF on a 5-day divided-dose schedule (1.5 g/m^2 daily) with concomitant application of mesna (0.3 g/m^2 at 0, 4, and 8 h after IF infusion). On day 1 the mean cumulative 24-h urinary recoveries (percentage of the IF dose) recorded for IF, 4-OH-IF, 3-DCE-IF, and 2-DCE-IF were 13.9%, 0.52%, 4.8%, and 1.5%, respectively. On day 5 the corresponding values were 12.2%, 0.74%, 9.9%, and 3.6%, respectively. This time-dependent increase in urinary excretion of IF metabolites, which is caused by rapid autoinduction of hepatic oxidative metabolism, may result in a higher probability for the development of urotoxic and nephrotoxic side effects during prolonged IF application. The mean 24-h urinary recoveries (percentage of the daily mesna dose) recorded for mesna/dimesna on day 1 (day 5) were 23.8%/45.2% (21.2%/39.8%), respectively. The mean molar excess of urinary reduced ("free") mesna over 4-OH-IF ranged from 11 to 72 on day 1 and from 6 to 40 on day 5. This indicates that although urinary excretion of 4-OH-IF rises with repeated IF application, mesna in standard doses should sufficiently inactivate the urotoxic IF metabolites.

Key words Fractionated ifosfamide therapy · Urinary ifosfamide metabolites · Urinary mesna excretion

Abbreviations *IF* Ifosfamide · *4-OH-IF* 4-hydroxyifosfamide ("activated ifosfamide") · *aldo-IF* aldo-ifosfamide · *3-DCE-IF* 3-dechloroethylifosfamide · *2-DCE-IF* 2-dechloroethylifosfamide · *CAA* chloroacetaldehyde · *mesna* 2-mercaptoethanesulfonate sodium · *dimesna* disodium bis-2-mercaptoethane sulfonate (disulfide of mesna) · *HPLC* high-pressure (performance) liquid chromatography · *NEM N*-ethylmaleimide

Introduction

The alkylating oxazaphosphorine antineoplastic ifosfamide (IF) is a prodrug that requires activation by the hepatic microsomal mixed-function oxygenase system before it can exert its cancerotoxic effect [39]. The initial step of IF activation (ring oxidation) results in the generation of 4-hydroxyifosfamide (4-OH-IF), which is in equilibrium with its acyclic tautomer aldo-ifosfamide (aldo-IF). Aldo-IF splits spontaneously into the alkylating agent isophosphoramide mustard and acrolein [20]. In addition to this toxification metabolism for IF the competitive initial metabolic pathway of side-chain oxidation at the cyclic (position 3) and the extracyclic (position 2) nitrogen with liberation of chloroacetaldehyde (CAA) has been decribed [33, 39], resulting in the formation of the cytostatically inactive

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metabolites 3-dechloroethylifosfamide (3-DCE-IF) and 2-dechloroethylifosfamide (2-DCE-IF), respectively.

In clinical and experimental studies it has been shown that fractionated application of IF has a better therapeutic index than a single bolus injection [24, 32]. Therefore, IF is usually given in divided-dose schedules over a period of several days [6]. It has repeatedly been reported that fractionation of the IF dose over several days results in a time-dependent decrease in the IF half-life without producing an increase in the renal clearance of the mother compound (reviewed in [40]). This is paralleled by an increase in the urinary excretion of some IF metabolites [28, 30] and by higher AUCs of plasma alkylating activity [27, 28]. We recently showed that during a 5-day observation period with daily intravenous administration of IF, plasma peak concentrations (C_{max}) and AUC values recorded for 4-OH-IF, CAA, 2-DCE-IF, and 3-DCE-IF had nearly doubled [25], revealing that the extent of the increase in metabolism was virtually the same for both of the initial metabolic pathways of IF (ring oxidation and side-chain oxidation).

If it is assumed that the antineoplastic activity as well as possible side effects are related to the concentrations of the IF metabolites, it can be concluded that fractionated IF therapy would lead to a time-dependent increase in both desired and undesired drug activity. The main dose-limiting side effect of IF is urotoxicity [6], which is thought to be caused by the highly reactive metabolite acrolein [7, 13] or by 4-OH-IF [40, 41]. In addition to bladder toxicity, IF may also cause nephrotoxicity (renal tubular damage) that (in contrast to IF) is not observed with the use of the oxazaphosphorine cyclophosphamide [10, 18, 38]. It might well be that IF-induced nephrotoxicity is caused by CAA, since this metabolite substantially depletes intracellular glutathione stores and, therefore, may predispose to cell damage [29, 40].

An important advance in the prevention of oxazaphosphorine-induced urotoxicity was the introduction of the thiol compound 2-mercaptoethane sulfonate sodium (mesna) given concomitantly with cytostatics such as cyclophosphamide or IF [9, 36]. Mesna serves as a regional detoxificant that has proved to inactivate sufficiently the urotoxic oxazaphosphorine metabolites. The dose of mesna required for successful uroprotection is about 60% of the IF dose (corresponding to a molar IF-to-mesna ratio of about 1), which, due to the rapid renal elimination of mesna, has to be given repeatedly at 4-h intervals for at least 8 h after IF bolus injection or short-term infusion [8, 40].

Despite the widespread clinical use of IF, only limited data exist concerning the urinary excretion of IF metabolites that might contribute to IF-associated uro- and nephrotoxicity [5, 16, 17, 31, 41]. The present study was designed to investigate the urinary excretion of IF, of its active metabolite 4-OH-IF (as the possible cause for IF-associated bladder toxicity), and of 2-DCE-IF and 3-DCE-IF (as determinants of IF side-chain oxidation that also generates CAA as the possible cause for IF-associated renal tubular damage). By intraindividual comparison of the metabolic pattern seen on day 1 versus day 5 of a 5-day divided-dose schedule of IF application, the extent of autoinduction of the hepatic metabolism of IF should be elucidated. In addition, the urinary excretion of mesna and its inactive disulfide dimesna should be determined and compared with urinary 4-OH-IF levels with respect to a possibly limited detoxification capacity of the delivered dose of mesna that might result from rising 4-OH-IF concentrations due to accelerated IF metabolism.

Patients and methods

Patients and study design

After approval of the local ethics committee had become available, 11 patients with bronchogenic carcinoma (Table 1) were studied. All patients gave their written consent and did not reveal substantial hepatic or renal dysfunction as measured by standard laboratory parameters (creatinine < 150 μmol/l, bilirubin < 20 μmol/l, serum cholinesterase > 2.5 kU/l, GOT/GPT < 40 U/l). The Karnofsky performance status of all patients was $> 70\%$. No cytostatic was

Table 1 Patients' data and absolute IF and mesna doses (*Pat. #* Patient number)

Pat. #	Age (years)	Sex (M/F)	Body surface (m^2)	Histology of bronchogenic carcinoma	Daily IF dose		Single mesna dose		% of IF dose (mol/mol)
					mg	μmol	mg	μmol	
1	59	M	1.77	Small-cell	2,650	10,150	600	3,660	36.1
2	71	M	1.80	Non-small-cell	2,700	10,350	550	3,350	32.4
3	60	M	1.87	Small-cell	2,806	10,730	600	3,660	34.1
4	47	F	1.80	Small-cell	2,700	10,350	550	3,350	32.4
5	64	M	1.80	Non-small-cell	2,700	10,350	600	3,660	35.4
6	69	M	1.70	Small-cell	2,550	9,770	500	3,050	31.2
7	43	F	1.80	Small-cell	2,700	10,350	600	3,660	35.4
8	55	F	1.80	Small-cell	2,700	10,350	600	3,660	35.4
9	62	M	1.93	Small-cell	2,900	11,110	600	3,660	32.9
10	55	M	2.07	Small-cell	3,100	11,880	600	3,660	30.8
11	52	M	1.84	Small-cell	2,760	10,580	550	3,660	34.6

given within at least 4 weeks prior to the commencement of the study therapy. The chemotherapeutic regimen consisted of a combination of IF and etoposide that has been proven to be effective in small-cell and non-small-cell lung cancer [14, 42]. IF was given as a brief i.v. infusion (60 min) on days 1–5 at a daily dose of 1.5 g/m^2. Etoposide was also applied as a brief i.v. infusion (30 min) on days 2–4 at a daily dose of 120 mg/m^2.

Mesna was given concomitantly with IF therapy on days 1–5 at 0, 4, and 8 h after the start of IF infusion. The single mesna dose was approximately 0.3 g/m^2 (20% of the daily IF dose), with little deviation due to adjustment of the injected drug amount to the nearest 50 mg. Details concerning the patients' data and absolute IF and mesna doses are given in Table 1. During the entire 5-day study interval, all patients received standard antiemetic prophylaxis with metoclopamide. Sufficient diuresis was achieved by continuous infusion of Ringer's solution at a rate of 125 ml/h.

Urine sampling

On days 1 and 5, patients were instructed to void immediately prior to the start of IF infusion. The blank urine obtained on day 1 was collected and frozen for the preparation of individual standards and for the determination of individual pH values (see below). Urinary fractions were sampled into vessels containing 2 ml 1 *N* HCl and 200 mg ethylenediaminetetraacetic acid (EDTA) during six 4-h intervals for up to 24 h after IF administration. To assure complete urine collection, patients were again asked to void at the conclusion of each sampling interval. The resulting urinary volumes were measured to the nearest 5 ml. For the assay of IF, 3- and 2-DCE-IF, 4-OH-IF, mesna, and dimesna, aliquots obtained during each sampling interval were immediately frozen and stored at $-70\,^{\circ}$C.

Assay for IF, 3-DCE-IF, and 2-DCE-IF

IF and its dechloroethyl metabolites were simultaneously quantified by means of N/P flame-ionization gas chromatography. Details of this assay have been given in a previous publication from our laboratory [25]. In brief, IF, 3-DCE-IF, and 2-DCE-IF were extracted from the thawed urine samples into dichloromethane using the oxazaphosphorine derivative trofosfamide as the internal standard. The organic layer was evaporated and the residue was redissolved in ethyl acetate, an aliquot of which was injected onto a fused-silica capillary column under isothermal conditions using N_2 as the carrier gas. Standards were prepared by the addition of known amounts of IF, 3-DCE-IF, and 2-DCE-IF (which were kindly supplied by Asta Medica, Frankfurt, Germany) to urine samples obtained from a healthy volunteer. If necessary, patients' urine samples were diluted to achieve concentrations of IF and its metabolites that were within the linear peak area versus concentration curve as calculated for the calibration standards (5–100 nmol ml^{-1} for IF, 1–30 nmol ml^{-1} for 3-DCE-IF and 2-DCE-IF).

Assay for 4-OH-IF

Principle of the assay, recovery, and limit of detection

The term 4-OH-IF ("activated IF") denotes the sum of all IF metabolites that give rise to the liberation of acrolein. These include 4-OH-IF, its acyclic tautomer aldo-IF, and (since the formation of 4-S,R-sulfido-oxazaphosphorines is a reversible reaction [13]) the 4-S,R-sulfido-IF deactivation metabolites with thiols, such as the adducts of 4-OH-IF with mesna, with glutathione, or with cysteine [25, 41]. The principle of the assay is based on the realease of acrolein from the activated IF metabolites by acid hydrolysis and the subsequent derivatization of acrolein with 3-aminophenol to form the fluorescent compound 7-OH-quinoline [1]. For the elimination of interfering fluorescence, 7-OH-quinoline is extracted from the derivatization mixture into an organic solvent under alkaline conditions and then quantified by HPLC using a fluorometric detector. Mesna, which is present in the urine of IF-treated patients, interferes with the assay, as it forms irreversible and chemically stable inactivation metabolites with acrolein [8]. Therefore, acrolein, which (due to spontaneous decomposition of activated IF metabolites) might be present in the urine prior to the acid hydrolysis procedure, is not measured by this method. To prevent the binding of mesna to that proportion of acrolein that (during the assay) is released from activated IF metabolites by the acid hydrolysis procedure, *N*-ethylmaleimide (NEM), which can block free sulfhydryl groups, has to be added to the urine samples. As compared with the assay procedure without NEM, the addition of NEM improved the recovery rates of 4-OH-IF by a factor of 3–5, depending on the concentration of mesna present in the urine. The amount of NEM chosen for the assay (see below) does sufficiently prevent the interference of urinary mesna with the determination of 4-OH-IF up to mesna concentrations, which range 1 order of magnitude higher than the peak levels measured in our study patients. The overall recovery rates of the assay (including the acid hydrolysis step, the derivatization procedure, and the extraction) were determined by the comparison of detected versus calculated amounts of fluorescent 7-OH-quinoline using acrolein and the cyclohexylamine salt of the oxazaphosphorine derivative mafosfamide (ASTA Z 7557, a kind gift from ASTA Medica, Frankfurt, Germany). The mean recovery rates obtained for acrolein and ASTA Z 7557, with concentrations of between 0.5 and 5.0 nmol/ml being added to mesna- and NEM-spiked urine, were 69% and 74%, respectively. The lower limit of detection for the assay at a signal-to-baseline-noise ratio of 3:1 in the HPLC chromatogram was 0.1 nmol/ml.

Procedure

Immediately after thawing, 500 µl of urine was mixed with 200 µl of a freshly prepared NEM solution (120 m*M* in double-distilled water). After 3 min, 600 µl of the freshly prepared and light-protected reagent solution containing both 3-aminophenol (2.5 mg/ml) and hydroxyl ammonium chloride (3.0 mg/ml) resolved in 1 *N* HCl was added. After vigorous shaking, the mixture was heated for 20 min at 95 °C and subsequently centrifuged for 1 min. Then, 1 ml of the supernatant was transferred to a round-bottom glass vial and 110 µl 5 *N* NaOH, 200 µl saturated carbonate buffer (pH 9.8), and 6 ml of a chloroform/isopropanol mixture (90/10, v/v) were added. After extraction on a reciprocal shaker for 15 min and centrifugation for 10 min, 5 ml of the organic layer was transferred to a conical glass vial and evaporated to dryness under a gentle stream of air. The residue was redissolved in 200 µl mobile phase of the HPLC procedure, of which 40 µl were injected into the HPLC system. The HPLC method has been described in a previous publication from our laboratory [26]. All patients' samples as well as the standards (see below) were run in duplicate.

Standards

Standards were prepared by the addition of known amounts of the cyclohexylamine salt of the oxazaphosphorine derivative mafosfamide (ASTA Z 7557) to the individual blank urine obtained from each patient prior to the start of IF infusion. The procedure described for the patients' urine samples resulted for ASTA Z 7557 in the release of 4-OH-cyclophosphamide and further liberation of acrolein that could be derivatized as 4-OH-IF-derived acrolein [26]. The concentration range used for calibration was 0.2–5.0 nmol/ml and generated a linear peak area versus concentration curve.

Stability of urinary 4-OH-IF

The stability of 4-OH-IF in an aqueous solution is pH-dependent, the stability optimum ranging in the weakly acidic region of pH 3.0–4.5 [21, 41]. Therefore, a small amount of hydrochloric acid was put into the sampling vessels prior to urine collection (see above). Repeated determination of 4-OH-IF in the HCl-acidified urine samples at room temperature revealed a mean recovery of 93.8% (range 89–97%, $n = 11$) for up to 4 h. The stability of 4-OH-IF in the HCl-acidified and deep-frozen urine samples was assayed by repeated 4-OH-IF measurements calibrated against freshly prepared standards; by this method, after 6 months of storage at $-70\,^{\circ}C$ a mean recovery rate of 84.8% (range 78–92%, $n = 11$) was determined. The most crucial period for the stability of urinary 4-OH-IF is the time during which (at body temperature and without the possibility of pH adjustment) the urine is in the bladder. To determine the extent of underestimation of 4-OH-IF excretion given in this study (which might be due to intravesical metabolite deterioration influenced by temperature and individual pH conditions), individual pH values for each patient were measured in the urine samples obtained prior to IF infusion on day 1 (mean pH 5.0, range 3.4–6.2, $n = 11$) and on day 5 (mean pH 5.2, range 3.6–6.0, $n = 11$). In addition, the stability of 4-OH-IF at $37\,^{\circ}C$ was calculated from urine samples from other IF-treated patients whose pH values had been adjusted to 3.5, 5.5, and 7.4 by the addtion of 100 m*M* (end concentration) citric acid or phosphate buffers, yielding deterioration half-lives of 3.4, 2.2, and 1.4 h, respectively (mean values from six experiments at each pH value).

Assay for mesna and dimesna

Mesna was analyzed as the concentration of directly measurable ("free") urinary thiol according to the Ellman method [15]. Since the binding of mesna to 4-OH-IF is reversible [13], the assay also measures mesna derived from the 4-OH-IF-mesna deactivation adduct. This proportion of mesna is directly measurable as "free" thiol, since the sulfhydryl group of mesna bound to 4-OH-IF is not oxidized and, hence, does not require reduction prior to determination. Mesna, which is bound to acrolein to form an irreversible and chemically stable adduct [8], is not quantified by the method. The term "dimesna" denotes the sum of all reducible urinary disulfides that give rise to mesna and includes a large proportion of the mesna-mesna disulfide and a minor proportion of mixed disulfides, e.g., mesna-glutathione or mesna-cysteine (reviewed in [40]). Dimesna was estimated by reduction of urinary disulfides with sodium borohydride, subsequent determination of total thiol compounds, and subtraction of the previously determined corresponding concentrations of free thiol. Details of this assay have been described elsewhere [19, 37]. The determination of free thiols and reducible disulfides is not specific for mesna and dimesna but includes negligibly low concentrations of physiologically present thiol compounds and disulfides. To reduce even this error, photometric readings at a wavelength of 412 nm were performed against blanks that had been derived from the individual patients' urine samples obtained directly prior to the application of IF and mesna. The standard calibration curves (urine samples from a healthy volunteer spiked with mesna) were linear within a range of 0.2–2.0 mg/ml. If necessary, patients' urine samples and the corresponding individual blanks were diluted up to 400-fold to meet this range.

Calculation and data presentation

Total drug amounts of IF, 3-DCE-IF, 2-DCE-IF, 4-OH-IF, mesna, and dimesna as excreted in the urine were calculated for each 4-h sampling period using the measured concentrations and the respective urinary volumes. From these data the cumulative urinary recovery was calculated for IF and its metabolites at the end of each sampling interval for up to 24 h after the start of IF infusion. In addition, the 24-h urinary recovery of each drug was calculated as a percentage of the delivered dose of IF and mesna, respectively. For purposes of comparison (calculation of the relative proportions of metabolites versus unchanged drug and delivered dose) the urinary amounts of IF and its metabolites 3-DCE-IF, 2-DCE-IF, and 4-OH-IF are expressed in moles. To avoid confusion (1 mol of the disulfide can be reduced to 2 mol of free thiol), in the case of mesna and dimesna, drug amounts are given in milligrams. For direct comparison with 4-OH-IF ("activated IF"), the excreted amount of mesna also had to be given in moles. For intraindividual comparison of the corresponding drug amounts recorded on day 1 versus day 5 of the study interval, the Wilcoxon test for paired data ($P < 0.05$) was used.

Results

Urinary excretion of IF and its metabolites

The absolute cumulative urinary excretion of IF, 4-OH-IF, 3-DCE-IF, and 2-DCE-IF as measured on days 1 and 5 of the 5-day divided-dose schedule (1.5 g/m^2 IF daily) is shown in Fig. 1. The corresponding values recorded for the 24-h urinary recoveries (expressed as percentages of the delivered IF dose) and the mean ratios obtained by intraindividual comparison of these parameters of day 5 versus day 1 are summarized in Table 2. Whereas the IF excretion on day 5 appeared slightly but not significantly lower than that on day 1, a substantial increase in excretion was observed for 4-OH-IF (as generated by ring oxidation of the mother compound) and for 3- and 2-DCE-IF (as derived from side-chain oxidation of IF), respectively (Fig. 1). The recovery of all metabolites appeared to have significantly increased by a factor of 1.6 to nearly 4 over the 5-day period of IF adminstration (Table 2, intraindividual comparison). The mean overall urinary recovery of the mother compound and all of its metabolites examined in this study (i.e., the sum of IF, 4-OH-IF, 3-DCE-IF, and 2-DCE-IF) accounted for 20.3% of the delivered IF dose on day 1 and for 25.9% of the IF dose on day 5, respectively. As can be seen, wide interindividual variability in urinary recovery was found for IF as well as for its oxidized metabolites (Table 2, range). Interindividually, the excretion of unchanged drug as well as the sum of 3-DCE-IF and 2-DCE-IF excretion (indicating the relative amount of side-chain oxidation) accounted for a range from only minor proportions up to one-third of the infused IF dose, respectively. As compared with the recovery of IF, 3-DCE-IF, or 2-DCE-IF, the relative amount of 4-OH-IF measured in the urine was found to be rather low and accounted in the majority of our patients for less than 1% of the delivered IF dose. In all patients the excretion of 3-DCE-IF (as formed by side-chain oxidation at the cyclic nitrogen) predominated over that of 2-DCE-IF (derived from side-chain oxidation at the extracyclic nitrogen), with the former reaching urinary recovery values that were about 3- to 4-fold those of the latter.

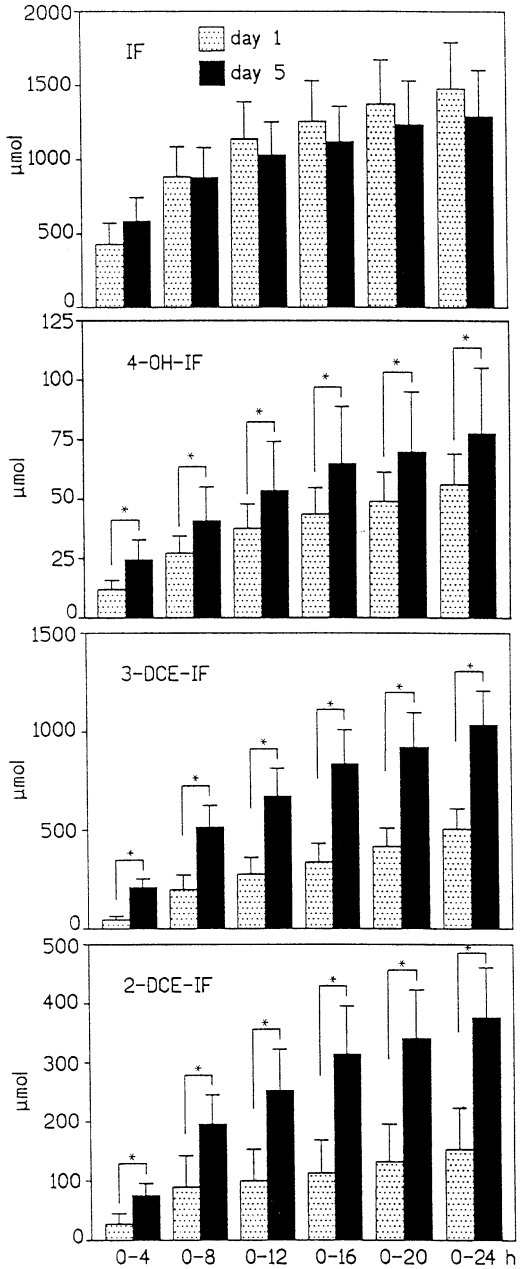

Fig. 1 Comparative cumulative urinary excretion of IF, 4-OH-IF, 3-DCE-IF, and 2-DCE-IF on day 1 (*dotted bars*) and day 5 (*black bars*) as determined in patients under fractionated i.v. IF therapy (1.5 g/m^2 given daily for 5 days). Time zero indicates the start of IF infusion. Data represent arithmetic mean values for the total drug amount ± SEM ($n = 11$, $^*P < 0.05$; Wilcoxon test for paired data)

Urinary excretion of mesna and dimesna

The urinary excretion of mesna, of dimesna, and of both as determined for each of the six 4-h sampling intervals on days 1 and 5 of the 5-day period of IF and concomitant mesna treatment (three daily mesna doses of 0.3 g/m^2 given at 0, 4, and 8 h after application of IF) is shown in Fig. 2. The corresponding 24-h recovery rates (expressed as percentages of the injected mesna dose) and the mean ratios derived from intraindividual comparison (day 5 versus day 1) are listed in Table 3. In addition to mesna excretion, Fig. 2 shows the mean urinary volumes on days 1 and 5 as measured for each sampling interval. No significant difference in the absolute excretion of mesna or dimesna or in urinary flow was observed on day 5 as compared with day 1 (Fig. 2). The same applies to the intraindividual comparison of drug excretion on day 5 versus day 1 (Table 3). Mesna was found to be subject to rapid renal elimination. The mean recovery of the sum of mesna and dimesna noted within the 4-h sampling interval following the first daily

Table 2 Urinary recoveries at 24 h of IF, 4-OH-IF, 3-DCE-IF, and 2-DCE-IF as determined in patients under 5-day fractionated i.v. IF therapy and intraindividual comparison of values recorded on day 5 versus day 1

Drug	Day	% of daily IF dose[a]		Intraindividual comparison (day 5 : day 1) x ± SEM[b]
		x ± SEM[b]	Range	
IF	1	13.9 ± 2.9	1.4–32.2	0.98 ± 0.37
	5	12.2 ± 2.9	2.0–34.4	
4-OH-IF	1	0.52 ± 0.11	0.11–1.21	1.59 ± 0.29*
	5	0.74 ± 0.27	0.09–3.10	
3-DCE-IF	1	4.8 ± 1.0	2.1–13.4	2.46 ± 0.47*
	5	9.9 ± 1.7	2.4–23.2	
2-DCE-IF	1	1.5 ± 0.7	0.6–8.3	3.77 ± 0.79*
	5	3.6 ± 0.8	0.5–9.7	
3-DCE-IF plus 2-DCE-IF	1	6.3 ± 1.6	2.7–21.7	2.67 ± 0.52*
	5	13.5 ± 2.4	3.6–32.9	

* $P < 0.05$ (significantly different from 1.0)
[a] Patients were given IF at a daily dose of 1.5 g/m^2
[b] Arithmetic mean values ± SEM ($n = 11$)

Table 3 Urinary recoveries at 24 h of mesna and dimesna as determined in patients under 5-day fractionated i.v. IF therapy with concomitant i.v. application of mesna and intraindividual comparison of values recorded on day 5 versus day 1

Drug	Day	% of daily mesna dose[a]		Intraindividual comparison (day 5 : day 1) x ± SEM[b]
		x ± SEM[b]	Range	
Mesna	1	23.8 ± 12.3	9.1–41.2	1.04 ± 0.17
	5	21.2 ± 10.3	6.4–42.3	
Dimesna	1	45.2 ± 16.2	18.8–64.2	0.97 ± 0.17
	5	39.8 ± 15.9	9.2–63.1	
Mesna plus dimesna	1	69.0 ± 25.6	32.6–97.7	0.98 ± 0.16
	5	61.1 ± 23.6	15.6–92.1	

[a] Patients were given mesna at a daily dose of 0.9 g/m^2 (consisting of 3 single doses of 0.3 g/m^2 given at 0, 4, and 8 h after application of 1.5 g/m^2 IF)
[b] Arithmetic mean values ± SEM ($n = 11$)

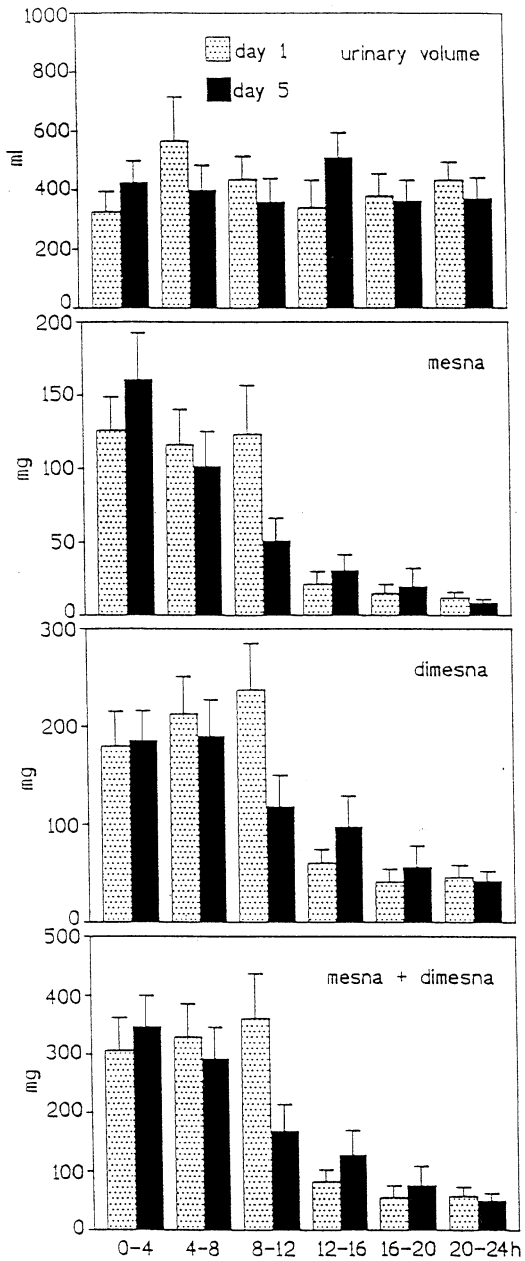

Fig. 2 Urinary volume and comparative urinary excretion of mesna, its disulfide dimesna, and the sum of mesna plus dimesna on day 1 (*dotted bars*) and day 5 (*black bars*) as determined in patients under 5-day fractionated i.v. IF therapy with concomitant i.v. application of mesna (consisting of 3 single daily doses of 0.3 g/m^2 given at 0, 4, and 8 h after IF infusion). The sampling periods indicate the time after the start of IF application. Data represent arithmetic mean values for the urinary volume or total excreted drug amount $\pm$ SEM ($n = 11$)

dose of mesna was more than 50% of the injected drug (Fig. 2, 0–4 h). The overall recovery of mesna plus dimesna recorded during the 24-h observation period accounted on both days for approximately two- thirds of the daily mesna dose, with substantial variations being noted (Table 3, range). In all patients and all sampling intervals the amount of dimesna was found to be 2-fold that of mesna.

Comparative urinary excretion of mesna and 4-OH-IF

The mean amounts of mesna ("free," i.e., nonoxidized mesna) and 4-OH-IF ("activated IF," the sum of all IF metabolites that can release acrolein) as measured in all 4-h sampling periods on day 1 day 5, respectively, are presented in Table 4. In addition, Table 4 shows the mean "molar excess" values for each sampling interval as calculated by the molar ratios of mesna to 4-OH-IF. Within the first 12 h after the brief IF infusion (and at 12, 8, and 4 h after the daily injections of mesna, respectively) the urinary concentrations of mesna ranged between 1 and 2 orders of magnitude higher than those of 4-OH-IF. Due to higher amounts of 4-OH-IF, the molar excess ratios recorded on day 5 appeared to be only 30–80% of the values calculated on day 1. In the urine samples obtained at 12–24 h after the application of IF (and at 8–16 h after the last daily dose of mesna, respectively) the molar excess values noted for mensa over 4-OH-IF became smaller, ranging at 1 order of magnitude. In contrast to the values recorded for the sampling intervals for up to 12 h after the IF infusion, the molar excess ratios calculated for the 12- to 24-h sampling periods on days 1 and 5, respectively, did not significantly differ from each other.

Discussion

This paper focuses on the urinary excretion of IF and its metabolites as formed by ring oxidation (yielding 4-OH-IF) and by the competitive initial pathway of side-chain oxidation (liberation of CAA, yielding 3-DCE-IF or 2-DCE-IF, respectively) and on the change in excretion pattern occurring during fractionated IF therapy over a 5-day period. In addition, the urinary excretion of concomitantly applied mesna and its disulfides (dimesna) was monitored. The excretion of mesna, which is supposed to prevent IF-induced hemorrhagic cystitis by chemical neutralization and stabilization of urotoxic IF metabolites such as 4-OH-IF and acrolein, was compared with the excretion of 4-OH-IF (activated IF, i.e., the sum of all IF metabolites that give rise to the liberation of acrolein). The comparison of urinary amounts of mesna with those of 4-OH-IF should elucidate the question as to whether a possible loss in the detoxification capacity of mesna would occur over the 5-day period of IF treatment due to higher 4-OH-IF concentrations resulting from accelerated metabolism of IF.

The large interindividual variation in the urinary pattern of IF and its metabolites as determined in this

Table 4 Comparative 24-h urinary excretion of mesna and 4-OH-IF as determined during the 6 consecutive 4-h intervals on days 1 and 5 of 5-day fractionated i.v. IF therapy with concomitant i.v. application of mesna

Drug/parameter	Day	Urinary fraction, time (h) after start of IF infusion[b]					
		0–4	4–8	8–12	12–16	16–20	20–24
Mesna [μmol][a]	1	768	707	749	128	91	73
	5	975	621	311	183	116	49
4-OH-IF [μmol][a]	1	11.90	15.38	10.35	5.93	6.10	6.45
	5	24.38	16.43	12.75	10.51	4.79	7.75
Molar excess (ratio of mesna: 4-OH-IF)	1	65	46	72	22	15	11
	5	40	38	24	17	24	6

[a] Data represent arithmetic mean values for the total excreted drug amount ($n = 11$)
[b] Patients were given IF at a daily dose of 1.5 g/m^2 and mesna in 3 single daily doses of 0.3 g/m^2 (at 0, 4, and 8 h after application of IF)

study (Table 2, range) is in good agreement with previous findings of substantial variability in IF plasma half-life (reviewed in [40]), in plasma concentrations of IF metabolites [25, 26], and in plasma alkylating activity following IF treatment [27, 28]. The mean urinary excretion of unchanged drug by our patients (Fig. 1, Table 2; 24-h urinary recovery of IF as a percentage of the daily IF dose) corresponds well with data from other laboratories obtained from patients who had received comparable IF doses [3–5, 16, 17, 31, 33]. Only a few papers have reported somewhat higher [2] or lower [28] IF recoveries.

The major urinary metabolites of IF that have been detected in previous studies are the dechloroethylated compounds 3-DCE-IF and 2-DCE-IF. The recoveries of these metabolites measured in our patients on day 1 of the study interval (Table 2; 24-h urinary recovery of 3- and 2-DCE-IF as a percentage of the daily IF dose) ranged slightly lower than the values reported by most authors [3–5, 16, 17, 31]. However, similar [33] or substantially lower [28] recoveries have also been observed. The predominance of 3-DCE-IF over 2-DCE-IF, indicating the preference of side-chain oxidation of IF at the cyclic (position 3) rather than the extracyclic (position 2) nitrogen, has been described by all authors who have been methodologically capable of discriminating both compounds [3–5, 16, 17, 31]. The proportion of IF metabolized via side-chain oxidation and, thus, generating the presumably nephrotoxic and highly unstable agent CAA may be judged by monitoring of the urinary excretion of the stable dechloroethylated metabolites (Fig. 1, Table 2; 24-h urinary recovery of the sum of 3- and 2-DCE-IF as a percentage of the daily IF dose). The proportion of side-chain oxidation involved in IF metabolism in our patients varied widely (Table 2; range). The possible correlation of renal tubular damage caused by CAA that may deplete intracellular glutathione stores [29] with the individual extent of side-chain oxidation as indicated by urinary excretion of 3- and 2-DCE-IF should be the subject of further investigation. Since fecal excretion of IF and its metabolites is negligibly low [40], the sum of the urinary recovery values noted for IF and its stable dechloroethylated metabolites, which within the 24-h observation period did not exceed 25% of the infused IF dose (Table 2), reveals that the major proportion of the delivered amount of the parent drug is metabolized by ring oxidation and is thus "activated" to form metabolites that may give rise to alkylating compounds.

In contrast to 3-DCE-IF and 2-DCE-IF, the urinary excretion of IF metabolites as generated by ring oxidation of IF has in most studies been reported to be rather low. For the inactive metabolites 4-ketoifosfamide (derived from oxidation of 4-OH-IF) and carboxyifosfamide (formed by oxidation of aldo-ifosfamide, the acyclic tautomer of 4-OH-IF) mean urinary recoveries ranging from 0 to 0.6% and from 1.3 to 8.9% of the delivered IF dose, respectively, have been reported [3, 4, 16, 28, 30, 31]. The urinary excretion of 4-OH-IF as monitored in this study is of special interest since this metabolite itself or acrolein derived from further metabolism of 4-OH-IF to isophosphoramide mustard (which is thought to be the ultimate alkylating agent formed after IF application) is incriminated as the putative cause of urotoxicity (hemorrhagic cystitis) observed in IF-treated patients [7, 12, 40, 41]. The problems associated with the quantitative detection of 4-OH-IF are due to the substantial instability of activated and, in consequence, highly reactive oxazaphosphorine compounds. The rapid degradation of 4-OH-IF requires immediate processing of all biological specimens such as blood or urine samples [25, 26, 41]. The rather small proportion of the infused IF dose that was excreted as 4-OH-IF in this study (Fig. 1, Table 2; 24-h urinary recovery of 4-OH-IF as a percentage of the daily IF dose) agrees with the value of 0.32% found in one of our previous investigations [41] and with the results of Norpoth [33], who has found that the amount of acrolein-releasing urinary metabolites does not exceed 1% of the delivered IF dose. Also in accordance with our results, by the use of thin-layer chromatography and phosphorus-31 nuclear magnetic resonance spectroscopy mean urinary recoveries of

isophosphoramide mustard ranging from 0 to 1.47% and from 0 to 0.5%, respectively, have been reported [16, 28].

In the present study we measured a substantial increase in the urinary excretion of all IF metabolites following fractionated IF therapy over 5 days (Fig. 1, Table 2; intraindividual comparison of values recorded on day 5 versus day 1). This is in accordance with the well-known phenomenon of a time-dependent decrease in the IF plasma half-life (reviewed in [40]), which is generally accepted to be caused by autoinduction of hepatic IF metabolism. The time-dependent increase in IF metabolism as judged by comparison of the urinary excretion of IF metabolites on day 5 versus day 1 (Fig. 1, Table 2) might to some extent have been overestimated due to a minor degree of metabolite excretion resulting from the IF dose given on day 4 of the study interval. This could particularly be true for the stable dechloroethylated compounds. However, the 1.6- to 4-fold increase in metabolite excretion noted in this study confirms our previous observation that fractionated IF therapy over 5 days results in a doubling of plasma AUC and C_{max} values for 4-OH-IF, 3-DCE-IF, 2-DCE-IF, and CAA, respectively [25].

If it is assumed that the urinary concentrations of 4-OH-IF reflect the urotoxic activity of a given IF dose, the possibility of hemorrhagic cystitis would increase over time in patients on prolonged fractionated IF therapy. The same should be true with respect to the proposed role of CAA, which is released by formation of 3- and 2-DCE-IF, in the development of IF-associated tubular damage.

Despite its well-established clinical use and its efficacy in the prevention of urotoxic side effects in patients on oxazaphosphorine treatment, the urinary excretion of mesna has thus far been the subject of relatively few investigations. The rapid renal elimination observed in our study (Fig. 2) confirms findings from previous investigations obtained both in animals and in humans [11, 19, 22, 23, 34, 35]. However, the existing data concerning the urinary recovery of the injected dose and the ratio of urinary mesna to its disulfide dimesna are not quite uniform. Whereas some authors report an almost complete urinary recovery of mesna/dimesna [19, 22, 34, 35], the mean percentage of the applied dose of mesna that was detectable as urinary mesna and dimesna in our patients (Table 3) ranged somewhat lower and was quantitatively similar to the recovery noted in another study [23]. The urinary excretion of dimesna measured in our study was quantitatively important and predominated over that of mesna by a factor of 2 (Fig. 2, Table 3). Similar findings have been reported in some previous studies [19, 22], whereas others have found urinary amounts of mesna equivalent to [23] or even higher than [11, 34] those of dimesna. Since the deactivation of urotoxic IF metabolites supposedly occurs by the free sulfhydryl groups of thiols that serve as nucleophilic partners and are thus preferentially alkylated [8, 40], only the urinary concentration of mesna, not that of dimesna, can be pharmacodynamically active in regional uroprotection.

In our study, for the first time the direct comparison of urinary 4-OH-IF (as the proposed causative factor of urotoxicity) and mesna (as the local detoxificant of urotoxic IF metabolites) was performed. In all consecutive sampling periods after IF infusion a substantial molar excess of mesna over measurable 4-OH-IF in the range of at least 1 order of magnitude was observed (Table 4). Due to instability [21, 41], the detectable concentrations of 4-OH-IF will to some extent be falsely low. From the mean time of 2 h of intravesical urine retention (due to the 4-h sampling period), from the stability experiments at different pH values, and from the mean urinary pH values measured in our patients prior to IF infusion (see Materials and methods, stability of urinary 4-OH-IF) it can be estimated that the real amount of 4-OH-IF excreted in the urine might range up to 2-fold that measured in our study. However, even in this case the urinary amounts of mesna given above yield reliable uroprotection, since detoxification occurs on a molar mesna-to-4-OH-IF ratio of 1. This applies not only to day 1 of the fractionated-dose schedule of IF but also to day 5, when substantially increased concentrations of 4-OH-IF can be detected as a consequence of accelerated IF metabolism. Thus, from this pharmacokinetic point of view, our results agree with the clinical observation that mesna given in well-established empirical standard regimens such as the one used in our study can provide successful prevention of IF-associated urotoxicity.

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