Intravenous Ifosfamide/Mesna Is Associated with Depletion of Plasma Thiols without Depletion of Leukocyte Glutathione

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ABSTRACT

Depletion of cellular glutathione (GSH) enhances the efficacy of many anticancer agents in preclinical systems. Limited published data showing depletion of GSH in vitro and in patients by ifosfamide and/or mesna provided the rationale for a Phase I trial. Ifosfamide and mesna were infused over 24 and 36 h, respectively, at equal daily doses; carboplatin was given after ifosfamide to a target plasma area under the curve of 4 mg-min·ml⁻¹. Plasma and peripheral WBC thiols were quantitated by high-performance liquid chromatography. The dose of ifosfamide was escalated from 2 to 8 g/m²; the maximum tolerated dose was 6 g/m². Significant depletion in plasma cysteine and homocysteine, precursors for GSH synthesis, was observed (maximum, 95% to >99% at 8 g/m²). Plasma mesna and cysteine/homocysteine levels were inversely correlated; nadir levels of cysteine/homocysteine were maintained for several hours after ifosfamide infusion had stopped and while mesna infusion was continuing. In vitro coinubation experiments confirmed that mesna reduces these thiols from disulfides to sulfhydryls, which are readily cleared, as evidenced by the significantly increased rate of excretion of cysteine in urine. In contrast, ifosfamide/mesna treatment caused a moderate depletion of plasma GSH in only 60% of the patients, with a nadir at 24 h and recovery immediately after the end of ifosfamide infusion. The GSH depletion in these patients was not dose related. The profile of GSH recovery in plasma after ifosfamide and the fact that mesna could reduce GSH disulfides in vitro suggest that the observed GSH depletion in plasma in 60% of the patients may be related to direct reactions of GSH with ifosfamide metabolites and/or mesna. Our results indicate that mesna is a modulator of GSH precursors and that a prolonged infusion of mesna may be required to achieve GSH precursor starvation and the consequent GSH depletion in cells.

INTRODUCTION

GSH is a predominant intracellular thiol involved in several normal cellular processes including maintenance of the essential thiol status of proteins, reduction of ribonucleotides to deoxyribonucleotides, metabolism of leukotriene and prostaglandin, and conversion of methyglyoxal to lactate (1, 2). GSH is an antioxidant and a major detoxifying agent. Detoxification occurs through direct conjugation reactions or through GST-mediated reactions (1, 2).

Several studies have shown a relationship between GSH levels and drug resistance (3–5). In addition to detoxification, GSH may contribute to resistance indirectly and may enhance the repair of DNA damage caused by anticancer drugs (6–9). Increased efflux of GSH-Pt conjugates may contribute to decreased cellular cisplatin accumulation (10, 11). The multidrug-resistance-associated protein is either a GSH S-conjugate carrier (12) or requires GSH to pump the drugs out of cells (13). Recent studies indicate that GSH may be involved in Bcl-2 function (14).

Data from many experimental models suggest that depletion of cellular GSH can improve chemotherapy efficacy. Inhibition of the key biosynthetic enzyme α-glutamylcysteine synthetase by BSO depleted GSH and enhanced activity for several Pt and alkylating agents in drug resistance models (15–17). In Phase I trials, a significant reduction in GSH levels was seen in the PBLs of patients receiving BSO (18–20).

A metabolite of ifosfamide, chloroacetaldehyde, has been shown to deplete GSH in P388 cells (21). In one patient, ifosfamide infusion (5 g/m²/8 h) given with an equal dose of mesna depleted GSH in the PBLs to 30% of baseline in 4 h (21). In another clinical study (22), a 5-day continuous infusion of 2.4–3.2 g/m²/day ifosfamide given with mesna (80% of the ifosfamide dose for 5 days) depleted total GSH, total cysteine, and total homocysteine in plasma. The GSH levels in cells were not measured in this study, but a depletion of cellular GSH was predicted from the findings of thiols in plasma. On the basis of this study and on the basis of an earlier study in which mesna alone was given to healthy volunteers (23), it has been suggested that the effect on thiols in plasma is due to mesna. In a more

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recent study, Malik et al. (24) reported a maximal 26–93% decline of GSH in PBLs on the 3rd day of a 6-day continuous infusion of ifosfamide (1 g/m²/day) given with mesna at the same dose.

Because mesna is an approved cytoprotective agent and is normally given with the anticancer agent ifosfamide, we evaluated the usefulness of the ifosfamide/mesna combination as a possible means of depleting GSH in leukocytes and on GSH and GSH precursors (cysteine and homocysteine) in plasma. The results of a Phase I study to determine the feasibility of GSH depletion by escalating doses of ifosfamide/mesna are reported here. Because there are extensive data that GSH depletion can help overcome resistance to Pt antitumor agents, carboplatin was included at a low-target area under the curve immediately after ifosfamide to take advantage of the expected nadir levels of GSH. The toxicity and response data from the clinical trial will be presented elsewhere.6

**MATERIALS AND METHODS**

**Drug Administration, Patients, and Sampling for Thiol Measurements.** Ifosfamide and mesna were given in escalating doses, with a starting dose of 2 g/m²/day for both. Ifosfamide was given by a 24-h infusion; mesna was started concurrent with ifosfamide and was given for 36 h. The ifosfamide dose was escalated in cohorts of three to six patients to 3, 4, 6, and 8 g/m², which was then reduced to 7 g/m². Carboplatin was given in a 30-min infusion immediately after ifosfamide, at a dose calculated to give a target plasma AUC of 4 mg-min/ml⁻¹, according to the formula of Calvert et al. (25) The study included 30 patients.

Thiols were measured in 24 patients. Plasma for thiol measurements was collected before treatment and at 1, 3, 6, 24, 25, 28, and 48 h after the start of the ifosfamide/mesna infusion. Pooled urine was collected before the treatment and again at 24 and 48 h after the beginning of ifosfamide/mesna infusion. The blood and urine were brought to the laboratory on ice. Plasma and cells were collected immediately. The WBCs were collected by dextran sedimentation. In three patients, both WBCs and PBLs were collected for comparison of GSH levels. PBLs were collected by Ficoll-Hypaque gradient centrifugation. Plasma samples stored for GSH measurements received 50 μl of L-serine/sodium borate to inhibit l-glutamyl transpeptidase activity, which would otherwise destroy GSH (26). Cells were stored frozen in 5% sulfosalicylic acid to prevent oxidation of GSH (27).

**Thiol Measurements.** The following thiols were measured in plasma: total cysteine, total homocysteine, total GSH and total mesna. In WBCs free and total GSH were measured. In urine total cysteine was measured. “Total” refers to the free sulfhydryl form plus mixed disulfides of each of the thiols.

**Fig. 1** Total cysteine in plasma at different doses of ifosfamide/mesna. Values are presented as a percentage of the pretreatment value. Reference values by dose level: 2 g/m², n = 5, 100% = 284 ± 30 μM; 3 g/m², n = 6, 100% = 283 ± 30 μM; 4 g/m², n = 3, 100% = 283 ± 58 μM; 6 g/m², n = 3, 100% = 216 ± 44 μM; 7 g/m², n = 1, 100% = 265 μM; and 8 g/m², n = 4, 100% = 283 ± 33 μM.

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pared analytical standards. The completeness of the reduction of disulfides to the free form by DTT was ensured by control experiments, and the entire HPLC procedure for the measurement of thiols in the clinical specimens was validated as described previously (29). The concentration of thiols was expressed on a per milliliter basis for plasma and on a per milligram of protein basis for the cells. Protein was measured by the Lowry assay (31).

Measurement of Chloroacetaldehyde in Plasma. Chloroacetaldehyde in plasma was measured by reverse-phase HPLC with UV detection after derivatizing chloroacetaldehyde in deproteinized ( perchloric acid) extracts of plasma with thiourea to 2-aminothiazole (32). The aminothiazole was isolated by solid-phase extraction (Bond-Elut Scx; Analytichem) and subjected to HPLC. Quantitation was based on identically derivatized chloroacetaldehyde analytical standards. For the measurements of chloroacetaldehyde, blood samples were stabilized with formaldehyde (32).

Reducing Property of Mesna. The reducing ability of mesna was evaluated after incubating mesna with cystine or homocysteine in PBS for 1 h at 37°C. The free cysteine and homocysteine resulting from the incubation were quantitated by thiolyte derivatization and HPLC, as described above. The mesna concentrations (5, 25, and 50 μg/ml) chosen were arbitrary to represent the low, mid-level, and high concentrations observed for mesna in plasma at different doses. The concentrations of the thiol disulfides used in these experiments reflect the physiological concentrations. DTT (2.5 mM), a typical reducing agent, was used for comparison.

Statistical Evaluation. All statistical analyses were carried out using the computer program Epistat. The decline of thiols in plasma from pretreatment to the nadir level was evaluated using a paired t test. The correlation between the dose of ifosfamide/mesna and the extent of the decline in thiols and of recovery at 48 h were evaluated using linear regression analysis with calculation of Pearson’s correlation coefficient. The correlation between the total mesna levels in plasma and those of cysteine or homocysteine in plasma was also evaluated using linear regression analysis.

RESULTS

Thiols in Plasma. The changes in total cysteine, total homocysteine, and total GSH in plasma of patients over a 48-h period during and after ifosfamide/mesna/carboplatin administration are shown in Figs. 1–3.

Total cysteine in plasma declined from time 0 (start of the ifosfamide/mesna infusion) to a nadir at 24–28 h after the start of the infusion. This profile of decline was consistent for all of the patients at all of the doses. The decline of cysteine in plasma was dose dependent (r = 0.84; P = <10^{-6}), with more rapid and greater depletion evident at higher doses. At the highest dose of 8 g/m², the levels fell below detection limits (,<1 μM) in one patient and to 2% and 5% of the pretreatment level in two other patients. By 12 h after the end of the mesna infusion, the cysteine levels in plasma had recovered to 71±10%, 67±8%, 54±4%, 49±12%, and 38±7% of pretreatment levels at doses of 2, 3, 4, 6, and 8 g/m². In the patient treated at 7 g/m², the cysteine level showed a recovery to only 27%. The recovery of cysteine was inversely related to the dose (r = −0.78; P = 1.4 × 10^{-5}).

Total homocysteine in plasma was measured at doses of 4, 6, 7, and 8 g/m² (Fig. 2). The general profile of decline in the total homocysteine in plasma in these patients was similar to that of cysteine and was related to the ifosfamide/mesna dose (r = 0.9; P = 2 × 10^{-5}). At the highest dose of 8 g/m², the levels fell below detection limits (<1 μM) by 24 h in two of four patients. The one patient treated at 7 g/m²/day showed depletion to 0.5% of the pretreatment level. The mean recovery at 48 h in
these patients was 63 ± 17%, 60 ± 25%, and 29 ± 16% at the
doses of 4, 6, and 8 g/m²/day. The patient treated at 7 g/m²/day
showed a recovery only to 12% of the pretreatment level. The
recovery of homocysteine levels was inversely correlated to the
dose (r = 0.58; P = 3.8 × 10⁻²).

In contrast to cysteine and homocysteine, the total GSH
levels measured in plasma showed two different patterns
(Fig. 3). In 15 of 24 patients, there was a moderate depletion
of GSH in plasma (Fig. 3, pattern A). In these patients, no
clear relation was observed between the drug dose and the
decline in plasma GSH. The nadir GSH levels in this group
ranged from 22% to 67% of pretreatment levels (mean, 53 ±
16%) and were significantly different from the pretreatment
levels (P = 1.7 × 10⁻⁶). Unlike the levels of cysteine and
homocysteine, GSH levels started to recover soon after the
end of ifosfamide infusion. In 9 of 24 patients, the GSH
levels showed some random fluctuation, with a suggestion of
minor increases (Fig. 3, pattern B).

**GSH in WBCs.** Free GSH constituted >90% of the total
GSH in WBCs. The levels of free GSH in WBCs are shown in
Fig. 4. Total GSH in WBCs had an identical profile (data not
shown). In contrast to the observed depletion in thiols in plasma,
no decline in GSH was observed in these cells, even at the
highest dose. In three patients, GSH in PBLs was also measured,
and the profile was found to be similar to that in WBCs, with no
decline in the levels (data not shown).
Thiols during Ifosfamide/Mesna Treatment

In our trial, ifosfamide/mesna treatment produced a dose-dependent depletion of total plasma cysteine and homocysteine in all patients. At the highest dose levels (7 and 8 g/m²), these thiols declined to 0–5% of the pretreatment levels in most patients. The rate of recovery of these thiols was also dose dependent. Total plasma cysteine and homocysteine nadir occurred at 24 h after treatment, and minimal levels were maintained throughout mesna infusion.

Our in vitro data show that mesna can reduce cysteine and homocysteine to cysteine and homocysteine. The total cysteine and homocysteine levels in plasma were inversely related to mesna levels. These results suggest that mesna reduces mixed disulfides to free forms, which are readily cleared by renal excretion, reactions with mesna/ifosfamide metabolites, or tissue uptake (22, 23). The increased urinary total cysteine excretion seen during and after ifosfamide/mesna treatment supports this hypothesis.

In contrast, plasma GSH showed only a modest decline in only 60% of the patients, with no clear dose-response relationship. The GSH nadir occurred at 24 h after treatment, followed by immediate recovery. These data, plus the observation that mesna could not reduce GS-SG to GSH in vitro, suggest that GSH depletion may depend mostly on direct reactions with ifosfamide metabolites. This is possible because ~65% of total plasma GSH is in the free form as compared with <5% of total cysteine and homocysteine (22). Reactions between GSH and ifosfamide metabolites are shown to be catalyzed by GST-π (34, 35). Therefore, the modest depletion of GSH may reflect levels of GST-π in plasma and interindividual variation in the GST-π genotype/phenotype (36, 37). This hypothesis does not preclude GSH from also reacting with mesna.

Peak chloroacetaldehyde levels in plasma ranged from 10–35 μM, well below the 1 mM concentration that significantly depleted GSH in P388 cells in vitro (21). Chloroacetaldehyde levels did not correlate with plasma GSH depletion.

Although significant depletion of GSH precursors was apparent in plasma, no depletion of GSH in was seen in WBCs, contrary to previous reports (24). This unexpected result was not an artifact of differing GSH assay methodologies: an enzymatic method (24) and our HPLC assay yielded similar results when tested in parallel in a nonselected subset of patients (data not shown). These observations suggest that: (a) mesna does not enter WBCs because this could deplete GSH by mesna-GSH conjugation; (b) free cysteine and homocysteine from reduction of mixed disulfides may enter WBCs and support new GSH synthesis (23); and (c) GST-π activity in WBCs may not be high enough to deplete GSH through conjugation to ifosfamide metabolites.

Our results suggest that prolonged infusion of mesna alone inhibited the key GSH biosynthetic enzyme γ-glutamylcysteine synthetase and depleted GSH in PBLs in clinical trials (18–20, 33). However, BSO is not yet widely available for clinical investigations. Limited clinical studies suggest that ifosfamide/mesna can deplete GSH and its precursors in plasma (22) and GSH in PBLs (21, 24). The aim of the current study was to confirm that ifosfamide/mesna can act as a potential alternative to BSO. Carboplatin was given immediately after ifosfamide, based on the enhanced cytotoxicity seen after GSH depletion in in vitro studies.

**DISCUSSION**

In vitro studies indicate that depleting cellular GSH can improve the cytotoxic potential of many drugs (15–17).
could potentially deplete cellular GSH by limiting precursors for GSH synthesis. If nadir levels of total plasma cysteine and homocysteine were maintained for a prolonged time, substrates for GSH synthesis would not be available. Along these lines, it is interesting to note that the results from one study with a more prolonged infusion schedule (a 6-day continuous ifosfamide/mesna infusion) than ours decreased PBL GSH levels by 25–93% in 9 of 14 patients with advanced ovarian cancer (24).

Clinical toxicity and response data from our study will be published separately. However, a brief comment on thiol depletion and clinical end points is appropriate. The maximum tolerated dose for ifosfamide was 6 g/m²; neutropenia and thrombocytopenia were dose-limiting. Cysteine and homocysteine depletion increased with ifosfamide/mesna dose; therefore, it is difficult to separate thiol effects from dose effects. Similarly, no relationship between GSH levels and toxicity was apparent. Only one objective response was observed (a partial response was seen in a patient with advanced colorectal cancer who was treated at the 8 g/m² dose level). Interestingly, this patient had the greatest plasma GSH depletion of all (78%, from baseline); he did not experience qualitatively or quantitatively greater toxicity than other patients at the same dose level. Our observation suggests that further examination of the relationships between plasma GSH depletion, response to chemotherapy, and toxicity is warranted.

In conclusion, our data suggest the following: (a) mesna
significantly depletes cysteine and homocysteine in plasma by reducing mixed disulfides to free forms, which are then cleared; (b) depletion of plasma GSH may be due to direct reactions between free GSH and ifosfamide metabolites and/or mesna; and (c) the lack of effect on WBC GSH may be related to the inability of mesna to enter these cells and to an inadequate duration of infusion. The individual effects of ifosfamide and mesna on plasma thiol modulation are unclear, and this relationship could be clarified by a randomized cross-over trial comparing the effects of a continuous infusion of mesna versus ifosfamide/mesna on thiol levels. In light of the extensive preclinical data implicating GSH in resistance to chemotherapy, it seems reasonable to perform additional investigations of clinically available, well-tolerated potential biochemical modulators.

REFERENCES


Fig. 8 The reducing property of mesna when incubated with cystine or homocysteine. The concentrations of cystine and homocysteine approximate the pretreatment levels. The mesna concentrations chosen were arbitrary to represent the low, midlevel, and high concentrations observed for mesna in plasma at different doses. Cystine or homocysteine were incubated with mesna at the final concentrations shown in PBS at 37°C for 1 h, and free cysteine and homocysteine were quantitated by means of thiolyte derivatization and HPLC. DTT was used as a reducing agent for comparison.
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