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Ifosfamide induced depletion of glutathione in human peripheral blood lymphocytes and protection by mesna

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We studied the effects of ifosfamide and major metabolites on intracellular glutathione (GSH) levels in human peripheral blood lymphocytes (PBL). *In vitro* exposure of PBL to 4-hydroperoxyifosfamide (4-OOH-IF), acrolein or chloroacetaldehyde at 37°C for 60 min led to a concentration dependent depletion of intracellular GSH. The concentration of the three metabolites to cause a 50% depletion of GSH in PBL was in the micromolar range (acrolein: 16 ± 4 μM; 4-OOH-IF: 22 ± 9 μM; chloroacetaldehyde: 30 ± 7 μM). Exposure to ifosfamide, the non-activated drug, had no effects on the intracellular GSH levels. Pretreatment with 4-OOH-IF suppressed dose-dependently the interleukin-2-induced proliferation of PBL. Incubation of PBL together with 2-mercaptoethanesulfonate (mesna) and 4-OOH-IF, acrolein or chloroacetaldehyde prevented the GSH depletion. The protecting effect of mesna in combination with 4-OOH-IF was independent of GSH biosynthesis, because addition of buthionine sulfoximine had no significant influence on this effect. These findings indicate a novel protective mechanism of mesna against intracellular GSH depletion of PBL during exposure to metabolites of ifosfamide.

Key words: Acrolein, chloroacetaldehyde, glutathione, ifosfamide, lymphocytes, mesna.

Introduction

Ifosfamide is an effective alkylating cytostatic drug with a broad spectrum of antitumor activity. Ifosfamide is a isomer of cyclophosphamide but has its own profile of pharmacology and action.[1] Both agents are prodrugs which need a metabolic activation by hepatic mixed-function oxidases. The initial activation involves hydroxylation of the 4-carbon in the oxazaphosphorine ring system. The unstable 4-hydroxyifosfamide (4-OH-IF) exists in equilibrium with aldoifosfamide which spontaneously decomposes to the alkylating agent ifosfamide mustard and acrolein, as shown in Figure 1. In a side reaction, chloroacetaldehyde is generated from ifosfamide. The metabolites ketoifosfamide, carboxyifosfamide, 2- and 3-dechloroethylifosfamide do not contribute to the cytostatic action of ifosfamide.[2] For investigation of activated ifosfamide *in vitro*, 4-hydroperoxyifosfamide (4-OOH-IF) can be used. 4-OOH-IF rapidly decomposes to 4-OH-IF following dissolution without enzymic involvement.[3]

During chemotherapy, the levels of ifosfamide and its metabolites can be measured in the blood for several hours.[4,5] During this time the peripheral blood lymphocytes (PBL) are exposed to this agents. It is well known that cells of the immune system are important for the treatment of tumor cells *in vivo*. To study the effects of the different ifosfamide metabolites on PBL we used an *in vitro* system. There is considerable evidence from the literature that intracellular GSH plays a key role in the toxic action of cyclophosphamide.[6] For metabolites of cyclophosphamide, an intracellular depletion of GSH was demonstrated for several cell types.[6,7] Therefore, exposure to the ifosfamide metabolites 4-OH-IF, acrolein and chloroacetaldehyde may also influence the GSH status of PBL.

The thiol mesna is simultaneously administered in clinical trials investigating ifosfamide as a chemotherapeutic agent.[8–10] Mesna allows regional detoxification of ifosfamide metabolites in the kidney and urinary tract.[11] Mesna is systemically administered

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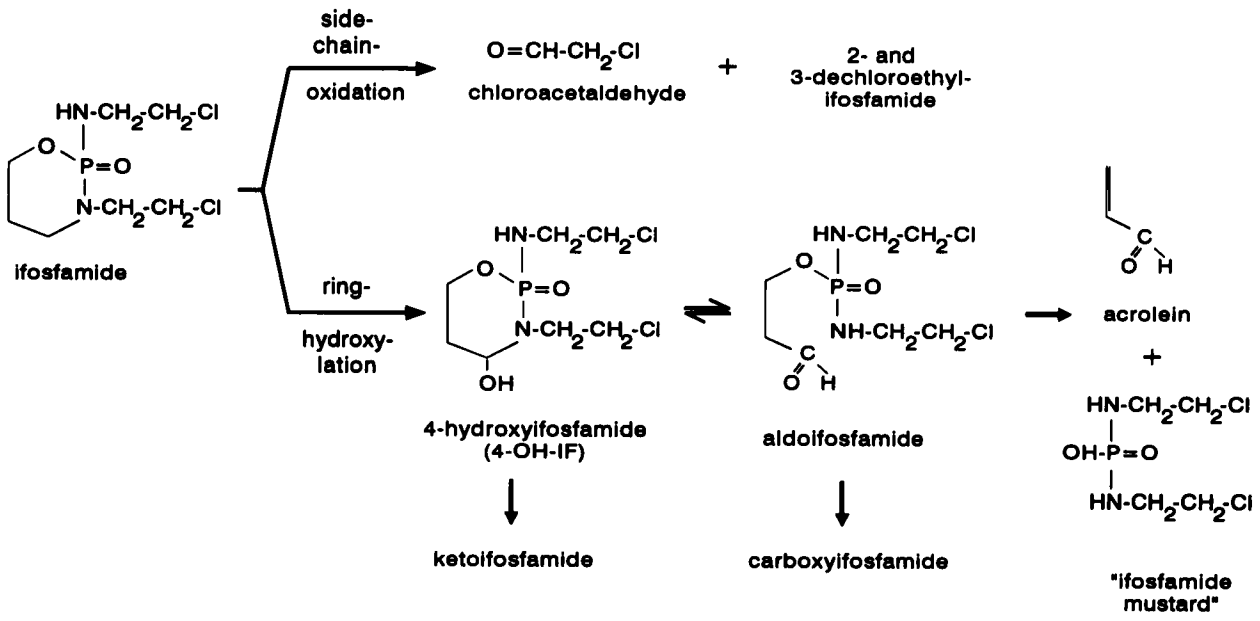

Figure 1. Metabolism of ifosfamide.

and may also show effects on PBL during exposure to metabolites of ifosfamide. The present study was designed to: (i) investigate the effects of ifosfamide and major metabolites on GSH status of PBL *in vitro*, and (ii) determine whether mesna can protect PBL under these conditions.

Materials and methods

4-OOH-IF was a generous gift from ASTA-Pharma (Frankfurt, Germany). 4-OOH-IF is rapidly decomposed to 4-OH-IF following aqueous dissolution. 4-OOH-IF was desiccated and stored at −30°C. The agent was dissolved in phosphate buffered saline (PBS), pH 7.4, immediately before adding to the cells. Then, 100 µl of the stock solution was added to 5 ml of culture medium containing the cell suspension to obtain the final concentration. Mesna was also obtained from ASTA-Pharma. Human recombinant interleukin 2 (rIL-2) was a gift from Euro Cetus (Frankfurt, Germany). L-buthionine-(*S,R*)-sulfoximine (BSO) was obtained from Sigma (Deisenhofen, Germany), acrolein from Aldrich (Steinheim, Germany) and chloroacetaldehyde from Merck (Darmstadt, Germany).

Cell culture

Chinese hamster ovary (CHO) cells were routinely grown and subcultured in McCoy's 5A medium supplemented with 10% newborn and 5% fetal calf serum (FCS) with glutamine and antibiotics. The human 5838 Ewing's sarcoma (ES) cell line was kindly provided by Dr ML Meltz (University of Texas, San Antonio, TX) and subcultered in RPMI 1640 supplemented with 20% FCS, glutamine and antibiotics. Human PBL were obtained from healthy blood donors. The blood was diluted with RPMI 1640 medium (Gibco, Eggenfelden, Germany) and the PBL were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation. The cells in the interface were washed twice with RPMI 1640 medium and resuspended in RPMI 1640 containing 10% heat inactivated FCS, 25 mM HEPES, pH 7.4, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). The adherent monocytes were removed by incubation of the cells in plastic culture flasks (Falcon, New Jersey, USA) at 37°C for approximately 2 h. The resulting non-adherent PBL were used for the experiments as described. Cell viability was determined by the Trypan blue exclusion method.

Determination of intracellular GSH

Total soluble intracellular GSH (reduced and oxidized form) was quantified using the modified method by Reed[12] as previous described.[13] Briefly, the cell pellet was treated with 1 N perchloric acid and γ-glutamylglutamate was added as an internal standard. After derivatization of the free thiol

groups with iodoacetic acid and of the amino groups with 1-fluoro-2,4-dinitrobenzene, the reaction mixtures were separated by high performance liquid chromatography (HPLC). Aliquots were injected onto a μBondapak amine column (4 × 250 mm; Waters, Eschborn, Germany) and eluted with a sodium acetate gradient (flow rate 2 ml/min) in a water : methanol : acetic acid solvent at pH 4.5. The dinitrophenyl derivatives were detected at 360 nm. GSH was quantified in relation to the internal standard. Protein was determined according to the method of Lowry.

Proliferation assay

Treated PBL were washed twice with PBS, counted, checked for >95% viability by Trypan blue exclusion and seeded ($1–2 \times 10^{-5}$ in 200 μl media) in a 96-microwell plate (Greiner, Frickenhausen, Germany). For stimulation, human rIL-2 (100 U/ml) was added to the medium. After stimulation for 3 days, [^{3}H]-thymidine (Amersham, Braunschweig, Germany) was added (1 μCi/well) and incorporation was determined after 18 h using a liquid scintillation counter (Beckmann Instruments, München Germany).

Results

Incubation of PBL with 4-OOH-IF, acrolein or chloroacetaldehyde led to a concentration-dependent depletion of intracellular GSH (Figure 2). Exposure of PBL to ifosfamide, the non-activated form which is applied during chemotherapy *in vivo*, did not deplete the GSH levels even in a concentration range up to 1000 μM.

The depletion of cellular GSH following treatment with metabolites of ifosfamide was not caused by leakage to the medium as a result of a loss of membrane integrity. As determined by the Trypan blue exclusion technique, no change in the ability of the cells to exclude the vital dye was observed under either condition (data not shown). The tested metabolites of ifosfamide depleted the GSH in a micromolar concentration range. To quantify the effects of the metabolites, the I_{50} values were calculated from the data shown in Figure 2. The I_{50} value represents the drug concentration that causes a 50% depletion of intracellular GSH (37°C, 1 h). In PBL, acrolein was the most effective drug ($I_{50} = 16 \pm 4$ μM) compared with 4-OOH-IF ($I_{50} = 22 \pm 9$ μM) and chloroacetaldehyde ($I_{50} = 30 \pm 7$ μM).

The potency of 4-OOH-IF to deplete the intracellular GSH level is not cell type specific. As shown in Table 1, the GSH depletion of 4-OOH-IF is also observed in CHO and ES cell lines. The I_{50} values show further that PBL *in vitro* are more sensitive than CHO and ES cells.

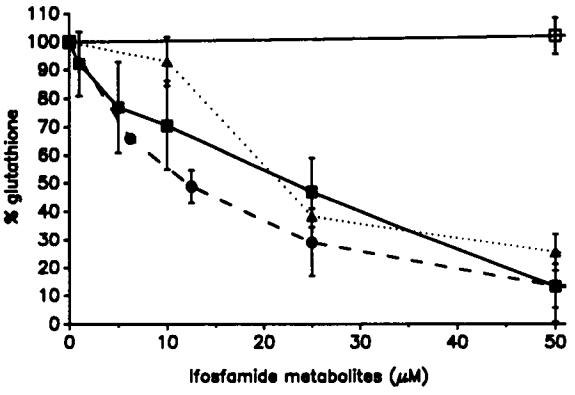

Figure 2. The effect of ifosfamide and major metabolites of ifosfamide on cellular GSH of PBL. PBL were exposed to different concentrations of ifosfamide, 4-OOH-IF, acrolein and chloroacetaldehyde at 37°C for 1 h. After this incubation time the total intracellular GSH was determined. Means ± SD from at least three independent experiments. The total GSH content of untreated PBL was 35.8 ± 14.5 nmol/mg protein ($n = 18$). □□, Ifosfamide; ■■, 4-OOH-IF; ●●, acrolein; ▲▲, chloroacetaldehyde.

An intracellular depletion of GSH in PBL may alter their physiological functions. We preincubated PBL with 4-OOH-IF and determined the incorporation of [^{3}H]thymidine as a marker for proliferation. The incorporation of [^{3}H]thymidine was dose-dependently reduced after 3 days of rIL-2 stimulation (Figure 3). The lymphocyte proliferation in response to rIL-2 correlates with the initial GSH content of the cells.

The depletion of intracellular GSH induced by metabolites of ifosfamide was reduced with the addition of the low molecular weight thiol mesna (Figure 4). Different concentrations of mesna (0–400 μM) were added immediately before each metabolite (50 μM) to the cell suspension. The reduced

Table 1. Determination of I_{50} values of GSH depletion for different cell types

Cells	I_{50} (μM)	SD (μM)	n^a
PBL	22	9	8
CHO	53	15	4
ES	100	79	4

The cells were incubated with different concentrations of 4-OOH-IF (0-200 μM) at 37°C for 1 h.
[a] Number of independent experiments.

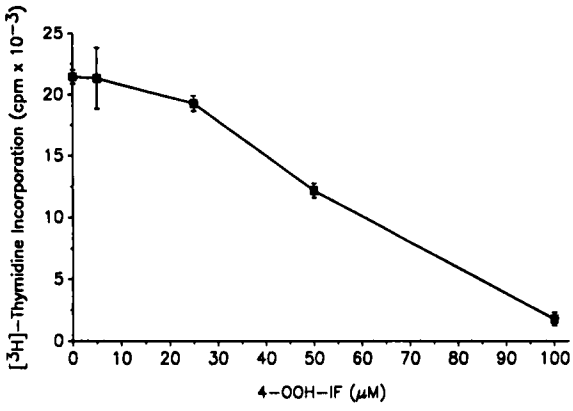

Figure 3. The effect of 4-OOH-IF on the [^{3}H]thymidine incorporation of PBL after IL-2 stimulation. PBL were exposed to different concentrations of 4-OOH-IF at 37°C for 1 h. Then the cells were washed and incubated with 100 U/ml human rIL-2 for 3 days. After labeling with [^{3}H]thymidine for 18 h, the incorporation of radioactivity was determined. Each data point represents the mean ± SD of four determinations, two repeated experiments gave similar results.

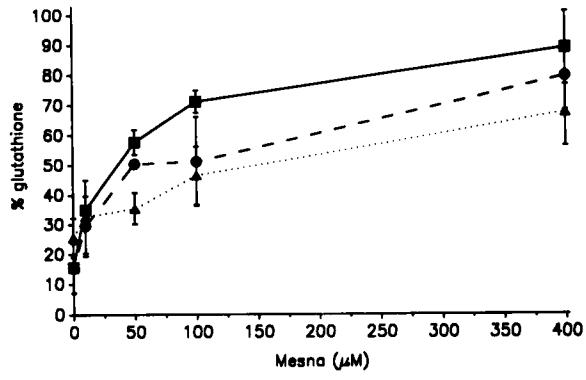

Figure 4. The effect of mesna and metabolites of ifosfamide on cellular GSH of PBL. Different concentrations of mesna were incubated together with 50 μM 4-OOH-IF, 50 μM acrolein and 50 μM chloroacetaldehyde at 37°C for 1 h. Means ± SD are from at least three independent experiments. ■■, 4-OOH-IF; ●●, acrolein; ▲▲, chloroacetaldehyde.

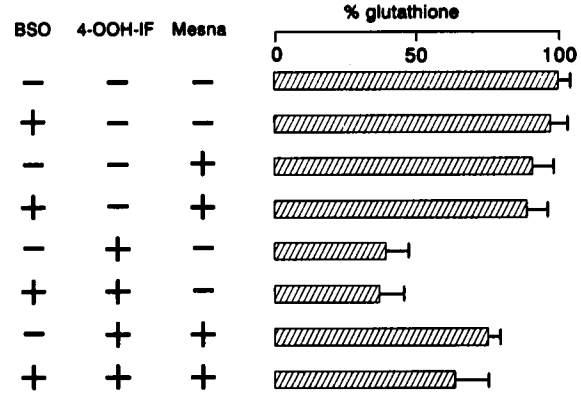

Figure 5. The effect of mesna, 4-OOH-IF and BSO on cellular GSH of PBL. PBL were exposed to 4-OOH-IF (50 μM), mesna (100 μM) and BSO (1000 μM) for 1 h at 37°C as described. Means ± SD are from at least three independent experiments.

depletion of GSH was dependent on the concentration of mesna, e.g. 400 μM mesna nearly completely prevented the GSH depletion of 4-OOH-IF. The GSH depletion induced by chloroacetaldehyde was less effectively reduced by mesna compared with the metabolites acrolein and 4-OOH-IF.

A depletion of GSH might be avoided by the ability of cells to increase the GSH biosynthesis. In a series of experiments we could demonstrate that in PBL the new biosynthesis of GSH did not significantly contribute to the effects of 4-OOH-IF and mesna on intracellular GSH levels (Figure 5). BSO is a specific inhibitor of γ-glutamylcysteinesynthetase, the enzyme responsible for the first and rate-limiting step of GSH synthesis. GSH levels of PBL are not affected by incubation with BSO (1 mM) for 1 h, which is in accordance with published data that BSO depletes intracellular GSH of resting lymphocytes very slowly.[14] Mesna alone (100 μM) or in combination with BSO did not significantly change the GSH levels of PBL. The GSH depletion of PBL induced by 4-OOH-IF is not enhanced in combination with BSO. Mesna (100 μM) partially prevented the 4-OOH-IF induced GSH depletion of PBL, but addition of BSO reduced this effect of mesna. However, the statistical analysis of both values showed no significant difference ($p > 0.05$; Student's paired *t*-test).

Discussion

The present data show that metabolites of ifosfamide are able to deplete the intracellular GSH of human PBL *in vitro*. As demonstrated for 4-OOH-IF, the depletion of GSH was not cell type specific and occurs at concentrations which were measured for 4-OH-IF in blood during ifosfamide chemotherapy. *In vivo* blood concentrations of 4-OH-IF are achieved up to 5 μM for several hours.[4] Intracellular depletion of GSH by activated cyclophosphamide is well known *in vitro*[15] and *in vivo*.[16] For

ifosfamide, less data are available, a depletion of GSH in mouse P388 cells by 4-OOH-IF was reported by Lind *et al.*[17] The described I_{50} value of 4-OOH-IF after treatment of P388 cells for 1 h was approximately 1 mM. This is about a factor of 10–50 more than calculated from our experiments for several cell types (Table 1). For comparison, the I_{50} values of the isomer 4-hydroperoxycyclophosphamide (incubation for 3 h at 37°C) in several tumor cell lines ranged from 1 to 25 μM.[6] A reaction between GSH and 4-hydroxycyclophosphamide at the 4-carbon position *in vitro* was described.[18] A similar reaction may occur between GSH and 4-OH-IF and may in part be responsible for the GSH depletion induced by 4-OOH-IF in PBL.

Chloroacetaldehyde, a ifosfamide metabolite which results from a deactivation reaction of ifosfamide, is generated during ifosfamide chemotherapy *in vivo*. Concentrations of chloroacetaldehyde up to 40 μM were determined in the plasma .[4,19] Chloroacetaldehyde is the candidate for the CNS toxicity of ifosfamide,[20] but the situation is not completely understood.[21] A GSH depletion induced by chloroacetaldehyde *in vitro* was reported by Lind in P388 cells.[17] A conjugation reaction between
chloroacetaldehyde and GSH to form a thioether conjugate was postulated,[17,22] and may be the molecular mechanism for the observed depletion of GSH in PBL.

Acrolein, a major factor for the urotoxicity of ifosfamide,[23] is spontaneously generated from 4-OH-IF. Acrolein can deplete intracellular GSH very efficiently,[15] whereas ifosfamide mustard, the probable primary alkylating metabolite of ifosfamide, has no influence on GSH levels of cells.[17] Therefore, part of the GSH depletion in PBL caused by 4-OOH-IF is probably attributable to the production of acrolein.

It is well known that GSH has central functions in cellular metabolism. A depletion of GSH can lead to different dysfunctions of cells.[24] In PBL, a GSH depletion induced by BSO was shown to cause a decreased proliferating activity of these cells.[25–27] In agreement with data from other groups, we found a decreased proliferation of 4-OOH-IF treated PBL after stimulation with IL-2 as shown in Figure 3. The decreased proliferation rate correlates with the initial GSH content of the cells after the treatment with 4-OOH-IF.

Mesna given together with metabolites of ifosfamide prevented the GSH depletion of PBL as shown in Figure 4. *In vivo* plasma concentrations of mesna up to 500 μM were measured for a short time after intravenous administration.[29] The reason for giving mesna during ifosfamide treatment *in vivo* is to avoid the urotoxicity of the drug. An interference of mesna with the therapeutic efficacy of ifosfamide has not been observed.[30,31] Mesna shows effective chemoprotection during oxazaphosphorine chemotherapy in the urinary bladder which is caused by direct interaction of mesna with metabolites of oxazaphosphorines. For metabolites of cyclophosphamide, some of these detoxification reactions have been described and are also relevant for metabolites of ifosfamide. The most important detoxification step is an addition reaction of mesna with the double bond of acrolein. A stable thio-ether product of mesna and acrolein was detected *in vivo*.[23] Further mesna was shown to reduce the breakdown rate of 4-hydroxycyclophosphamide. A deactivation product of mesna and 4-hydroxycyclophosphamide has been detected *in vivo*.[11] A reduced breakdown rate for the ifosfamide metabolite 4-OH-IF caused by a similar stabilization reaction with mesna seems probable and was proposed as an inactivation mechanism of 4-OH-IF.[32]

Mesna also prevents the GSH depletion induced by chloroacetaldehyde in PBL. As a simple explanation of this effect, we suggest an addition reaction of mesna with chloroacetaldehyde, a reaction based on a mechanism which may be similar to the addition reaction of GSH with chloroacetaldehyde.[22] Indeed, from our data this mechanism remains hypothetical and needs further investigation.

Mesna itself may influence the GSH status of cells. A stimulation of GSH synthesis by exogenous thiols was observed, e.g. in CHO cells.[33] Addition of different thiols like N-acetylcysteine, cysteamine or WR-2721 to the medium increased the GSH content of CHO cells.[34] The biochemical mechanism for this effect is a disulfide exchange reaction of the added thiol with the cystine present in the medium and the release of cysteine. The cysteine is taken up from the cells and promotes the GSH synthesis, because cysteine is in most cases the limiting factor for GSH synthesis.[35] Also, mesna can react with free thiols and disulfides in aqueous solution, e.g. a resulting decrease of plasma cysteine and cystine level after administration of mesna was observed *in vivo*.[29]

Our data showed that mesna alone had no influence on the GSH levels of PBL under the described conditions (Figure 5). To examine the possibility of an increased GSH synthesis after 4-OOH treatment by mesna we used BSO as specific inhibitor of GSH synthesis. As shown in Figure 5, the addition of BSO had no significant influence on the GSH depletion of

4-OOH-IF under either conditions. Therefore, we conclude that mesna prevents the depletion of GSH induced by 4-OOH-IF not by a promotion of intracellular GSH synthesis. Based on our experiments and the discussed data from the literature, we suggest that one GSH protecting effect of mesna in PBL is mediated by direct interaction of mesna with the metabolites of ifosfamide. In most cell types mesna cannot be taken up.[36] Therefore, mesna may be an extracellular GSH protecting drug which reacts with metabolites of ifosfamide instead of GSH. Further mechanisms, e.g. the possibility that other thiol compounds contribute to the GSH protection of PBL, should also be considered, e.g. the intracellular levels of cysteine were increased after exposure of lymphocytes to mesna.[29]

Conclusion

Metabolites of ifosfamide deplete the GSH of PBL *in vitro* at concentrations which are achieved during chemotherapy *in vivo*. Mesna protects the GSH levels of PBL which provides further arguments for the combination of ifosfamide and mesna *in vivo*.

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