EFFECTS OF AMIFOSTINE ON LIVER OXIDATIVE STRESS CAUSED BY CYCLOPHOSPHAMIDE ADMINISTRATION TO RATS

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SUMMARY

Cyclophosphamide is an inactive cytostatic, which is metabolised into active metabolites mainly in the liver. During bioactivation, reactive oxygen species (ROS) are also formed, which can modify the components of both healthy and neoplastic cells leading to decreased antioxidative capacity. Amifostine is a drug that can inactivate ROS. The aim of the present study was to evaluate the influence of amifostine on the antioxidative system of the liver of rats exposed to cyclophosphamide. Intraperitoneal administration of cyclophosphamide was found to decrease the activity of liver antioxidative enzymes, i.e. superoxide dismutase, glutathione peroxidase and glutathione reductase, and to increase catalase activity. Amifostine slightly influenced antioxidative enzyme activity, causing a significant increase only in superoxide dismutase activity. Co-administration of cyclophosphamide and amifostine nearly prevented changes in activities of superoxide dismutase, glutathione reductase and catalase, as well as to a high degree of glutathione peroxidase. Cyclophosphamide also evoked a decrease in the level of non-enzymatic antioxidants, such as reduced glutathione and vitamins C, E and A, as well as total antioxidant status. Administration of amifostine alone caused a significant increase in non-enzymatic antioxidant level that

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resulted in an increase in total antioxidant status. Administration of amifostine together with cyclophosphamide to a large extent prevented changes in the evaluated non-enzymatic antioxidative parameters, decreasing values of their concentration to the values of control group. Changes of liver antioxidative abilities during detoxification of cyclophosphamide were accompanied by intensified lipid peroxidation, manifested by an increase in concentration of products such as malondialdehyde and 4-hydroxynonenal. Amifostine caused the inhibition of lipid peroxidation in the liver of both control and cyclophosphamide-treated rats. In conclusion, our results suggest that amifostine significantly protects liver antioxidant properties from changes caused by cyclophosphamide treatment and in consequence prevents oxidative stress and phospholipid peroxidative damage.

KEY WORDS

cyclophosphamide, amifostine, liver, antioxidants, lipid peroxidation

INTRODUCTION

An oxidative-reductive balance, determined by reactive oxygen species (ROS) generation and cell antioxidative properties, exists in living organisms in physiological conditions. Nevertheless, many xenobiotics, including drugs, intensify the production of ROS and decrease the antioxidative abilities of the organism, leading to disturbances in redox properties. Such drugs include alkylating anticancer agents, e.g. cyclophosphamide, which is used in the treat ment of many tumours, such as breast carcinoma /1/, lymphoma /2/, multiple myeloma /3/, carcinoma of lung /4/ and some sarcomas /5/. Cyclophosphamide is a prodrug requiring bioactivation before exerting its cytotoxicity. This activation by microsomal mixed-function oxidase takes place predominantly in the liver /6/. Cyclophosphamide is metabolised (Fig. 1) into phosphoramide mustard, a potent alkylating agent, and acrolein, which is the most reactive and toxic product /7/. The main target molecules involved in the anti-tumour effect of alkylating agents are DNA molecules; however, other compounds including membrane lipids and proteins may also interact with phosphoramide mustard and acrolein, resulting in the production

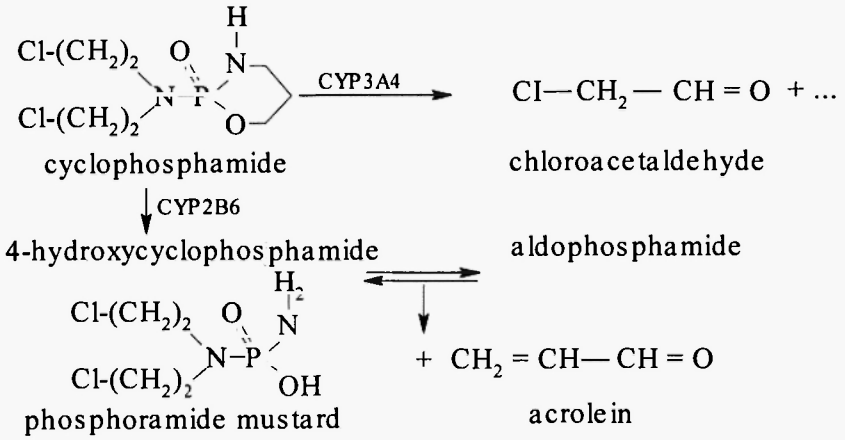

Fig. 1: Metabolism of cyclophosphamide.

of ROS /8/. One of the major alterations in cell components after cyclophopshamide administration is lipid peroxidation, which causes an increase in generation of hydroxyl radicals, highly reactive oxygen species /9/.

All cyclophosphamide metabolites, ROS in particular, are very reactive compounds which can modify components of malignant as well as normal cells /10/. Therefore, drugs which protect healthy cells should be applied during cyclophosphamide therapy. One such drug could be amifostine - an organic thiophosphate compound that has been shown to protect normal tissues from the cytotoxic damage induced by chemotherapy and radiation therapy /11/. It has been demonstrated that pretreating animals with amifostine provides protection against the cytotoxic effects of alkylating drugs, such as organoplatinums or anthracyclines /11,12,13/. Amifostine is dephosphorylated by cell membrane-bound alkaline phosphatase and transported into normal tissues where its metabolite (WR-1065) exerts its cytoprotective property (Fig. 2) /11/. Cytoprotective levels of amifostine or its metabolites have been found in many organs, including the liver /14/. Amifostine as a thiol compound possesses antioxidant properties and protects cells against damage, mainly by scavenging free radicals, competing with oxygen to prevent oxygen radicals interactions with DNA, and promoting cell repair through hydrogen donation to free radicals /15/.

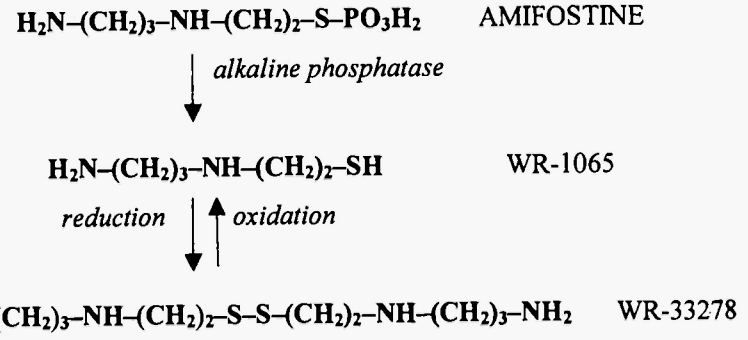

Fig. 2: Metabolic conversion of amifostine to WR-1065 and WR-33278.

The aim of this study was to evaluate the influence of amifostine on antioxidative properties and lipid peroxidation processes in the liver of rats exposed to cyclophosphamide.

MATERIALS AND METHODS

Male Wistar rats (approximately 230 g body wt.) fed on a standard diet were used for the experiment. The experimental protocol was approved by the Ethics Committee on Human and Animal Experimentation of our Medical Academy.

The rats were divided into four groups:

A. Control group (n = 18).

B. Cyclophosphamide group. The rats were treated intraperitoneally with cyclophosphamide at a dose of 150 mg/kg body wt. (n = 18).

C. Amifostine group. The rats received amifostine intraperitoneally at a dose of 200 mg/kg body wt. (n = 18).

D. Cyclophosphamide and amifostine group. The rats were treated intraperitoneally with amifostine at a dose of 200 mg/kg body wt. and subsequently with cyclophosphamide at a dose of 150 mg/kg body wt. (n = 18).

The rats were sacrificed 1, 5, and 14 days after drug treatments following ether anaesthesia (six animals in each group). The liver was removed, perfused with 0.15 M NaCl solution to remove blood cells,

weighed and homogenised in 0.25 M sucrose with the addition of 250 μM butylated hydroxytoluene in ethanol, to prevent formation of new peroxides during the assay. The homogenisation procedure was performed under standardised conditions. 10% homogenates were centrifuged at 10,000 *g* for 15 min at 4°C and the supernatant was kept on ice until assayed.

Superoxide dismutase (Cu,Zn-SOD; EC.1.15.1.1) activity was determined by the method of Misra and Fridovich /16/ modified by Sykes /17/. Catalase (CAT; EC.1.11.1.9) activity was measured spectrophotometrically by determination of H_2O_2 decomposition /18/. Glutathione peroxidase (GSH-Px; EC.1.11.1.6) activity was measured spectrophotometrically using a technique based on Paglia and Valentine /19/. Glutathione reductase (GSSG-R; EC.1.6.4.2) activity was measured by monitoring the oxidation of NADPH at 340 nm /20/. Reduced glutathione (GSH) concentration was measured using the Bioxytech GSH-400 test (Bioxytech S.A., Bonneuil/Marne Cedex, France). High-performance liquid chromatography (HPLC) methods were used to determine the level of vitamins C /21/, E and A /22/. Total antioxidant status (TAS) was measured with 2,2′-azino-di-[3-ethylbenzthiazoline] sulphonate (ABTS) using the Randox test.

Lipid peroxidation was assayed by measuring malondialdehyde (MDA) as an MDA-thiobarbituric acid adduct by HPLC /23/ and 4-hydroxynonenal (4-HNE) as fluorimetric derivatives, also using HPLC /24/.

Statistical analysis

Data obtained in this study are expressed as means + SD. The data were analysed by the use of standard statistical analyses, one way ANOVA with Scheffe's F test for multiple comparisons to determine significance between different groups. A values of $p < 0.05$ was considered significant.

RESULTS

Cyclophosphamide caused a statistically significant decrease in liver cytosol superoxide dismutase activity during 5 days after its administration, while at the same time activity of this enzyme was significantly increased after amifostine treatment (Table 1). After

TABLE 1

The activity of antioxidant enzymes in the liver of control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide and amifostine (CP+A)

Enzyme	Time after injection (days)	Group			
		C	CP	A	CP+A
SOD	1	14.6 ± 1.0	13.2 ± 1.1[a]	20.5 ± 1.6[a]	14.2 ± 1.1[c]
U/mg protein	5	14.2 ± 1.0	12.3 ± 1.1[a]	19.3 ± 1.4[a]	13.8 ± 1.2[c]
	14	14.2 ± 1.2	13.1 ± 1.0[1]	14.9 ± 1.2	13.5 ± 1.1
GSH-Px	1	154 ± 11	95 ± 5[a]	149 ± 0	124 ± 14[a, b, c]
U/mg protein	5	157 ± 10	73 ± 6[a]	154 ± 12	115 ± 10[a, b, c]
	14	151 ± 11	131 ± 12[1]	152 ±12	134 ± 11[a, c]
GSSG-R	1	32.5 ± 2.5	15.2 ± 1.2[1]	35.7 ± 2.8	29.5 ± 2.5[b, c]
U/mg protein	5	31.4 ± 2.3	22.8 ± 2.8[1]	34.6 ± 2.7[c]	27.6 ± 2.4[a, b, c]
	14	31.7 ± 2.3	29.7 ± 2.3	33.2 ± 2.3	30.5 ± 2.5
CAT	1	27.2 ± 2.0	29.3 ± 2.5	29.3 ± 2.4	28.9 ± 2.5
U/mg protein	5	26.9 ± 2.0	33.5 ± 2.8[1]	30.3 ± 2.5[c]	32.9 ± 2.8[1]
	14	28.5 ± 1.9	40.1 ± 3.4[a]	28.8 ± 2.3	33.7 ± 2.7[a, b, c]

Data points represent means ± SD; n = 6.

[a] $p < 0.05$ in comparison with control group;

[b] $p < 0.05$ in comparison with cyclophosphamide group;

[c] $p < 0.05$ in comparison with amifostine group.

SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSSG-R = glutathione reductase; CAT = catalase.

cyclophosphamide and amifostine injection, the activity of Cu,Zn-SOD was similar to the value in the control group, but was significantly decreased in comparison with the amifostine group. The activity of GSH-Px was significantly decreased throughout the experiment, whereas it was similar to controls in the amifostine group. After injection of the two drugs, the examined enzyme activity was decreased in comparison with the control and amifostine groups but enhanced in comparison with the cyclophosphamide group. The activity of glutathione reductase was significantly decreased during 5 days after cyclophosphamide administration, and after injection of the two drugs the activity of this enzyme was also decreased in comparison with controls. Catalase activity increased gradually during 2 weeks after alkylating drug injection, while cyclophosphamide and amifostine administration caused similar changes but of smaller intensity.

Administration of cyclophosphamide caused a significant decrease in GSH level throughout the experiment, while amifostine gradually enhanced the level of this tripeptide (Table 2). After injection of cyclophosphamide and amifostine, the level of GSH was similar to that in the control group. Cyclophosphamide caused a significant decrease in vitamin C level, while administration of amifostine significantly increased the level of this vitamin. Cyclophosphamide given with amifostine did not significantly change the level of this vitamin compared to the control group. The levels of vitamins E and A changed similarly: after cyclophosphamide administration the levels of these parameters were significantly lower than in the controls, whereas amifostine caused a significant increase. After injection of cyclophosphamide and amifostine, the levels of these vitamins were similar to those in the control group. Total antioxidant status was significantly reduced after cyclophosphamide administration, enhanced after amifostine injection, and increased after treatment with the two drugs.

Administration of cyclophosphamide caused a significant increase in the level of lipid peroxidation products - MDA and 4-hydroxynonenal - throughout the experiment, while amifostine decreased the level of these compounds (Figs. 3, 4). After injection of cyclophosphamide and amifostine, the levels of MDA and 4-HNE were significantly lower than in the cyclophosphamide group, and became gradually enhanced during the experiment in comparison with the control group.

TABLE 2

The level of non-enzymatic antioxidants in the liver of control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide with amifostine (CP+A)

	Time after inject on (days)	Groups of rats			
		C	CP	A	CP+A
GSH	1	13.5 ± 1.3	10.6 ± 1.4	16.9 ± 1.2[a]	13.7 ± 1.2[b, c]
nmol/g tissue	5	13.2 ± 1.2	10.2 ±1.3	17.8 ± 1.5[a]	11.8 ± 1.3[c]
	14	13.7 ± 1.3	10.9 ± 1.4[a]	18.3 ± 1.3[a]	13.5 ±1.3[b, c]
Vitamin C	1	72 ± 5	40 ±4[a]	90 ± 5[a]	65 ±4[a, b, c]
µg/g tissue	5	75 ± 4	37 ± 3[a]	92 ± 3[a]	70 ± 3[a, b, c]
	14	73 ± 5	33 ± 2[a]	120 ± 6[a]	82 ± 4[a, b, c]
Vitamin E	1	20.0 ±1.6	16.0 ± 1.9[a]	26.5 ± 1.5[a]	22.0 ± 1.9[c]
nmol/g tissue	5	17.3 ± 2.1	14.2 ± 1.8[a]	24.2 ± 1.3[a]	19.3 ± 1.8[b, c]
	14	21.2 ± 1.2	16.8 ±2.0[a]	23.8 ± 1.0[a]	19.5 ± 1.5[a, b, c]
Vitamin A	1	2.30 ± 0.11	1.52 ± 0.20[a]	3.32 ± 0.11[a]	2.11 ± 0.10[a, b, c]
nmol/g tissue	5	2.25 ± 0.11	1.65 ± 0.10[a]	2.81 ± 0.11[a]	2.10 ± 0.21[a, b, c]
	14	2.31 ± 0.12	1.44 ± 0.11[a]	3.10 ± 0.21[a]	2.41 ± 0.12[b, c]
TAS	1	1.41 ± 0.12	1.24 ± 0.11[a]	1.63 ± 0.11[a]	1.92 ± 0.12[a, b, c]
nmol/g tissue	5	1.31 ± 0.12	1.10 ± 0.11	1.70 ± 0.09[a]	1.70 ± 0.12[a, b]
	14	1.35 ± 0.11	1.25 ± 0.12[a]	1.93 ± 0.12[a]	1.77 ± 0.11[a, b, c]

Data points represent means ± SD; n = 6. GSH = reduced glutathione; TAS = total antioxidant status.
[a] $p < 0.05$ in comparison with control group; [b] $p < 0.05$ in comparison with cyclophosphamide group;
[c] $p < 0.05$ in comparison with amifostine group.

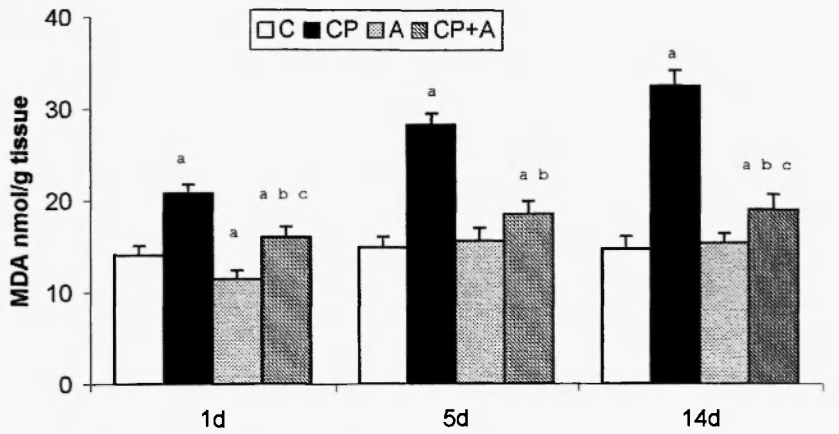

Fig. 3: The liver content of malondialdehyde (MDA) in control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide with amifostine (CP+A), 1 day (1d), 5 days (5d) and 14 days (14d) after administration of drugs. Data points represent means ± SD; n = 6. [a] $p < 0.05$ in comparison with control group; [b] $p < 0.05$ in comparison with cyclophosphamide group; [c] $p < 0.05$ in comparison with amifostine group.

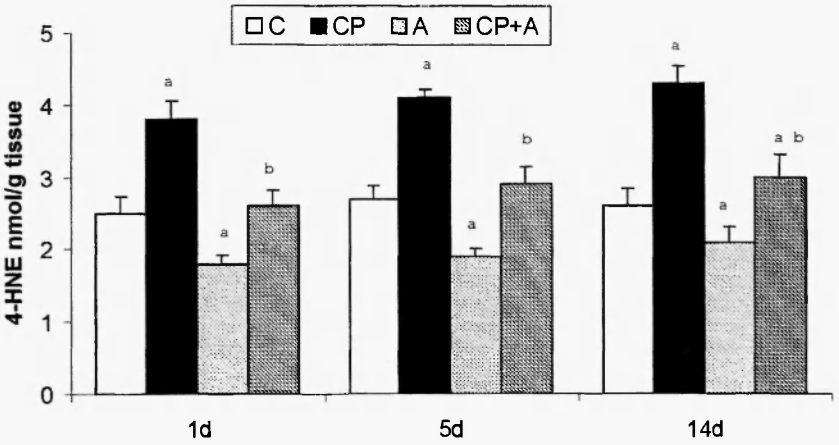

Fig. 4: The liver content of 4-hydroxynonenal (4-HNE) in control rats (C) and rats treated with cyclophosphamide (CP), amifostine (A) and cyclophosphamide with amifostine (CP+A), 1 day (1d), 5 days (5d) and 14 days (14d) after administration of drugs. Data points represent means ± SD; n = 6; [a] $p < 0.05$ in comparison with control group; [b] $p < 0.05$ in comparison with cyclophosphamide group; [c] $p < 0.05$ in comparison with amifostine group.

DISCUSSION

Metabolism of many drugs is accompanied by enhancement of ROS generation and changes in antioxidative status /25/, which may lead to oxidative stress. ROS may be produced at various steps of cyclophosphamide metabolism. This drug is metabolised by microsomal mixed-function oxidase and partially by peroxidase /6,26/. Hydroxycyclophosphamide, the bioactivated form of cyclophosphamide, induces lipid peroxidation in different tissues, reflecting the presence of free radicals /27,28/. It is also possible that superoxide anions are produced during activation of cyclophosphamide by cytochrome P450 /6,29/. Moreover, it has been suggested that products of cyclophosphamide metabolism may be degraded with free radical generation /30/. In such a situation it is difficult to evaluate which of these mechanisms actually occurs in cells and influences the increase in free radical concentration.

Whereas previous studies have shown that cyclophosphamide causes changes in lung antioxidant status /31/, the present paper indicates that liver antioxidant abilities are also changed after cyclophosphamide administration. First, a decrease in the activity of superoxide dismutase, glutathione peroxidase and glutathione reductase, as well as in the level of reduced glutathione, was observed. The reduction of antioxidant enzyme activities after cyclophosphamide administration is probably connected with damage to the structure of these enzymes caused by free radicals /32/. Modification of the amino acid residues that constitute the active centres of enzymes results in inhibition of their activity /33/. Reduced activity of Cu,Zn-SOD may also be due to inhibited biosynthesis of the enzyme by drug metabolites and/or to the effect of hydrogen peroxide, which may directly alter its catalytic centre /9/. The superoxide anion is the primary free radical species involved in various tissue injuries. It leads to the production of hydrogen peroxide, either spontaneously or catalysed by Cu,Zn-SOD; in the presence of iron ions, it produces hydroxyl radicals /34/. During oxidative stress, transition metal ions can be translocated from ceruloplasmin and iron-containing haem proteins, which can enhance free radical production. Generated hydroxyl radicals are the most widely proposed initiators of lipid peroxidation, evaluated by measurement of the level of malondialdehyde and 4-hydroxynonenal.

The key enzyme involved in maintaining the liver's redox state is GSSG-R, which catalyses the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH) using NADPH /35/. GSH, a powerful reducing agent, plays a major role in cellular defence against endogenous and exogenous oxidants /36/; it is also co-substrate of GSH-Px in removing hydrogen and lipid peroxides from cells. Furthermore, hepatic tissue is the main export organ of GSH, which is also necessary to ensure proliferation of its cells and their regeneration /37/. The combined deficiency of both intracellular GSH and GSH-Px may potentate cyclophosphamide toxicity by simultaneously increasing oxidative stress and decreasing oxidative defence.

It is well known that an important role in reducing oxidative stress is played by ascorbic acid (vitamin C), which efficiently scavenges superoxides, hydrogen peroxide, hypochloride, hydroxyl and peroxyl radicals, and restores the antioxidant properties of fat-soluble alpha-tocopherol (vitamin E) /40/. The decrease in ascorbate and alpha-tocopherol concentrations, observed in our study, can diminish the cellular resistance of liver cells to oxidative damage. This phenomenon was confirmed by the parallel increase in lipid peroxidation products (MDA and 4-HNE) concentration observed in this paper. These data are in agreement with the results of other authors, who also showed the interrelationship between ascorbate and lipid peroxidation products /41/. It is known that administration of cyclophosphamide causes an increase in the formation of low molecular weight aldehydes generated during drug metabolism, e.g. acrolein, as well as during lipid peroxidation, e.g. malondialdehyde and 4-hydroxynonenal /39/. These reactive aldehydes show various biological activities, including cytotoxicity, genotoxicity, reduced chemotactic activity and effects on cell proliferation /40/. The most cytotoxic aldehydes are 4-hydroxy-nonenal and acrolein. They can react, for example, with thiol groups of different cell components, leading to reduction of GSH level and rapid loss of protein -SH groups /40/.

Administration of amifostine simultaneously with cyclophosphamide to rats resulted in restoration of the examined parameters. Amifostine is a prodrug that requires activation by membrane-bound alkaline phosphatase /41/. Following the formation of the active thiol form (WR-1065), the molecules can interact with other thiol compounds to form their symmetric disulphides or mixed disulphides /42/. A high concentration of symmetric disulphides in the liver has been

shown /43/. It has been demonstrated that the disulphides formed by thiol amifostine metabolites and reduced glutathione or sulphydryl groups of proteins protect GSH and proteins against oxidation /14/. It was found that about 50% of WR-1065 is bound to proteins in mouse plasma /14/. Moreover, WR-1065 is thought to exert a cytoprotective effect by direct scavenging of free radicals or depleting oxygen /44/. Amifostine and its metabolites can scavenge superoxide anions as well as hydroxyl radicals /45/, whose levels are increased after cyclophosphamide administration. The ability of amifostine to scavenge free radicals appears to be the key element in the efficacy of this drug, since ROS are extremely aggressive against cell components. The observed decrease in the amount of lipid peroxidation products (MDA and 4-HNE) after amifostine injection suggests that free radicals react first of all with the thiol group of the drug, converting it into a relatively innocuous thiol group, thus protecting lipid and protein molecules. Moreover, WR-1065, possessing free amino and sulphydryl groups, is a nucleophilic compound which might bind covalently directly to the carbonium ion of cyclophosphamide and react with active terminal cyclophosphamide metabolites such as acrolein and phosphoramide mustard, thus removing potentially dangerous compounds.

In addition to the above scavenging properties of amifostine metabolites, free thiol and disulphide forms can affect the intracellular redox state that in turn can also alter gene expression, synthesis of other intracellular thiols /42/ and enzyme activities (i.e. redox control of sensitive cysteine residues) /46/. Redox-based regulation of signal transduction and gene expression is recognised as an important mechanism in cell biology /47/. It has been shown that amifostine can activate the redox-sensitive transcription factor NFκB enhancing in this way manganese superoxide dismutase gene expression /48/. This is a possible mechanism for the enhancement of cytosolic superoxide dismutase activity observed in this paper.

It might also be suggested that amifostine's enhancement of the antioxidant potential of normal tissue, as well as its direct protection against ROS, could act indirectly to prevent metastasis. Up to now amifostine's antimetastatic properties were attributed mainly to its metal ion chelating abilities and thereby changes in metal-requiring metalloprotease activities /42/, but they may be also connected with its antioxidative properties. By shifting the redox balance in the direction

of reduction processes, amifostine could prevent oxidative modification of proteolytic enzyme inhibitors and, as a result, contribute to decrease in the activity of proteases secreted by tumour cells which can degrade the extracellular matrix and enhance cell adhesion /49/. This point of view seems to be very important in clinical practice, because both these drugs (cyclophosphamide and amifostine) are administered in anticancer therapy. The cyclophosphamide dose used in this study is equivalent to high dose therapy used clinically, and the dose of amifostine is just a little higher than the ideal doses recommended for different clinical conditions /50-52/. Amifostine's cytoprotective potential has been established for various tissues, including haematopoietic progenitor cells, renal cells, myocardium, and intestinal, epithelial and neuronal cells /15/. It is demonstrated here that this drug also protects liver cells against cyclophosphamide-induced oxidative stress.

REFERENCES

1. Hassan M, Svensson US, Ljungman PA. Mechanism-based pharmacokinetic-enzyme model for cyclophosphamide autoinduction in breast cancer patients. Br J Clin Pharmacol 1999; 48: 669-677.
2. Wang JY, Prorok G, Vaughan WP. Cytotoxicity, DNA cross-linking and DNA single strand breaks induced by cyclophosphamide in a rat leukemia in vivo. Cancer Chemother Pharmacol 1993; 31: 381-386.
3. Van der Wall E, Beijnen J, Rodenhuis S. High-dose chemotherapy for solid tumors. Cancer Treat Rev 1995; 21: 105-132.
4. Bunn PA Jr, Carney DN. Overview of chemotherapy for small cell lung cancer. Semin Oncol 1997; 24 (Suppl 7): S7-69–S7-74.
5. Murphy SB, Bowman WP, Abromowitch M, et al. Results of treatment of advanced stage Burkitts lymphomas and B-cell (SIg+) acute lymphoblastic leukaemia with high dose fractionated cyclophosphamide and co-ordinated high-dose methotrexate and cytarabine. J Clin Oncol 1986; 4: 1732-1739.
6. Schlezinger J, White RD, Stegeman J. Oxidative inactivation of cytochrome P450 1A (CYP1A) stimulated by 3,3′,4,4′-tetrachlorobiphenyl: production of reactive oxygen by vertebrate CYP1As. Mol Pharmacol 1999; 56: 588-597.
7. Brock N. Oxazophosphorine cytostatics: past, present, future. Cancer Res 1989; 49: 1-7.
8. Kanekal S, Kehrer J. Metabolism of cyclophosphoramide by lipooxygenases. Drug Metab Dispos 1993; 22: 74-78.
9. Dumontet C, Drai J, Thieblemont C, et al. The superoxide dismutase content in erythrocytes predicts short-term toxicity of high-dose cyclophosphamide. Br J Haematol 2001; 112: 405-409.

10. Fraiser L, Kanekal S, Kehrer JP. Cyclophosphamide toxicity: characterizing and avoiding the problem. Drugs 1991; 42: 781-795.
11. Capizzi RL. Amifostine: the preclinical basis for broad-spectrum selective cytoprotection of normal tissues from cytotoxic therapies. Semin Oncol 1996; 23: 2-17.
12. Dorr RT. Cytoprotective agents for antracyclines. Semin Oncol 1996; 23: 23-24.
13. Tannehill SP, Mehta MP. Amifostine and radiation therapy: past, present and future. Semin Oncol 1996; 23: 69-77.
14. Shaw LM, Bonner HS, Brown DQ. Metabolic pathways of WR-2721 (ethyol®, amifostine) in the BALB/c mouse. Drug Metab Dispos 1994; 22: 895-902.
15. Foster-Nora J, Siden R. Amifostine for protection from antineoplastic drug toxicity. Am J Health Syst Pharm 1997; 54: 787-800.
16. Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinefrine and a simple assay for superoxide dismutation. J Biol Chem 1972; 247: 3170-3174.
17. Sykes JA, McCormac FX, O'Breien TJ. Preliminary study of the superoxide dismutase content of some human tumors. Cancer Res 1978; 38: 2759-2762.
18. Aebi H. Catalase in vitro. Meth Enzymol 1984; 105: 121-126.
19. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70: 158-169.
20. Mize CE, Langdon RG: Hepatic glutathione reductase. I. Purification and general kinetic properties. J Biol Chem 1962; 237: 1589-1595.
21. Takeshita S, Nakata R, Tsukamoto I. Change in the level vitamin C and lipid peroxidation in tissues of the inherently scorbutic rat during ascorbate deficiency. J Agric Food Chem 1996; 44: 2748-2753.
22. Ivanovic D, Popovic A, Radulovic D. Reversed phase ion-pair HPLC determination of some water-soluble vitamins in pharmaceuticals. J Pharm Biomed Anal 1999; 18: 999-1004.
23. Londero D, Greco PL. Automated HPLC separation with spectrofluorometric detection of malondialdehyde-thiobarbituric acid adduct in plasma. J Chromatogr A 1996; 729: 207-210.
24. Yoshino K, Matsuura T, Sano M. Fluorometric liquid chromatographic determination of aliphatic aldehydes arising from lipid peroxides. Chem Pharm Bull 1986; 34: 1694-1700.
25. Mates JM, Perez-Gomez C, Nunez de Castro I. Antioxidant enzymes and human diseases. Clin Biochem 1999; 32: 595-603.
26. Kanekal S, Kehrer JP. Evidence for peroxidase-mediated metabolism of cyclophosphamide. Drug Metab Dispos 1993; 21: 37-42.
27. Haddad IY, Panoskaltsis-Mortair A, Ingbar DH, et al. High levels of peroxynitrite are generated in the lungs of irradiated mice given cyclophosphamide and allogeneic T cells. A potential mechanism of injury after marrow transplantation. Am J Respir Cell Mol Biol 1999; 20: 1125-1135.
28. Sulkowska M, Sulkowski S, Skrzydlewska E, Farbiszewski R. Cyclophosphamide-induced generation of reactive oxygen species. Comparison with

morphological changes in type II alveolar epithelial cells and lung capillaries. Exp Toxicol Pathol 1998; 50: 209-220.
29. Parke DV, Sapota A. Chemical toxicity and reactive oxygen species. Int J Occup Med Env Health 1996; 9: 331-340.
30. Benckhuysen C, van der Steen J, Spanjersberg EJ. Two stable Fenton oxidation products of cyclophosphamide (NSC-26271) as precursors of 4-hydroxycyclophosphamide (NSC-196562) under physiologic conditions. Cancer Treat Rep 1976; 60: 369-372.
31. Stankiewicz A, Skrzydlewska E, Sulkowska M, et al. Effect of amifostine on lung oxidative stress after cyclophosphamide therapy. Bull Vet Inst Pulawy 2002; 46: 87-94.
32. Davies KJA, Goldberg AL. Proteins damage and degradation by oxygen radicals. IV. Degradation and denaturation of protein. J Biol Chem 1987; 262: 9914-9920.
33. Salo DC, Pacifici RE, Lin SW, et al. Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. J Biol Chem 1990; 265: 11919-11927.
34. Southorn PA, Powis G. Free radicals in medicine. I. Chemical nature and biological reactions. Mayo Clin Proc 1988; 63: 381-389.
35. Meister A. Selective modification of glutathione metabolism. Science 1983; 220: 471-477.
36. Giles G, Tasker K, Jacob C. Hypothesis: the role of reactive sulfur species in oxidative stress. Free Radical Biol Med 2001; 10: 1279-1283.
37. Kretzschmar M. Regulation of hepatic glutathione metabolism and its role in hepatotoxictity. Exp Toxicol Pathol 1996; 48: 439-446.
38. Gebicki S, Gebicki JM. Formation of peroxides in amino acid and proteins exposed to oxygen free radicals. Biochem J 1993; 289: 743-749.
39. Berringan MJ, Struck RF, Gurtoo HL. Lipid peroxidation induced by cyclophosphamide. Cancer Treat Rep 1987; 9: 265-270.
40. Esterbauer H, Schaur RJ, Zollner J. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. Free Radical Biol Med 1991; 11: 81-128.
41. Bonner HS, Shaw LM. Measurement of both protein-bound and total S-2-(3-aminopropylamino)ethanethiol (WR-1065) in blood by high-performance liquid chromatography. J Chromatogr B 2000; 739: 357-362.
42. Grdina DJ, Kataoka Y, Murley JS. Amifostine: mechanisms of action underlying cytoprotection and chemoprevention. Drug Metab Drug Interact 2002; 16: 237-279.
43. Kretzschmar M, Franke H, Zimmermann T. Glutathione synthesis and export in experimental liver cirrhosis induced by thioacetamide: relation to ultrastructural changes. Exp Pathol 1989; 36: 113-122.
44. Marzatico F, Porta C, Moroni M, Bertorelli L. In vitro antioxidant properties of amifostine (WR-2721, Ethyol). Cancer Chemother Pharmacol 2000; 45: 172-176.

45. Ohnishi ST, Ohnishi T, Glick JH, Schein PS. In vitro study on the antioxidant activities of amifostine (WR-2721). Proc Am Assoc Cancer Res 1992; 33: 419a.
46. Gilbert H. Redox control of enzyme activities by thiol/disulfide exchange. Meth Enzymol 1984; 107: 330-351.
47. Das KC, Lewis-Molock Y, White CW. Activation of NFκB and elevation of MnSOD gene expression by thiol reducing agents in lung adenocarcinoma (A549) cells. Am J Physiol 1995; 269: L588-L602.
48. Murley J, Kataoka Y, Hallahan E. Activation of NFκB and MnSOD gene expression by free radical scavengers in human microvascular endothelial cell. Free Radical Biol Med 2001; 30: 1426-1439.
49. Kleiner DE, Stetler-Stevenson WG. Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol 1999; 43: S42-S51.
50. Huitema ADR, Smits KD, Mathot RAA, et al. The clinical pharmacology of alkylating agents in high-dose chemotherapy. Anti-Cancer Drugs 2000; 11: 515-533.
51. Vose JM, Reed EC, Pippert GC, et al. Mesna compared with continuous bladder irrigation as uroprotection during high-dose chemotherapy and transplantation: a randomized trial. J Clin Oncol 1993; 11: 1306-1310.
52. Yuhas JM. Active vs passive absorption kinetics as the basis for selective protection of normal tissues by S-2-(3-aminopropyloamino)-ethylphosphorohoic acid. Cancer Res 1980; 40: 1519-1524.