医薬品相互作用研究 18(2) 77 (1994)

Beneficial Effect of Disulfiram on Cyclophosphamide-Induced Urotoxicity in Mice

MASAAKI ISHIKAWA, RYOKO SEKIMOTO, TOMOKO AOKI, YOSHIO TAKAYANAGI and KEN-ICHI SASAKI

Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku College of Pharmacy*

Summary

The use of disulfiram (DSF) to prevent cyclophosphamide (CPA)-induced urotoxicity was studied in mice. A single dose of CPA (100-400 mg/kg, *i.p.*) produced a significant increase in urinaary bladder weight in a dose dependent manner within 48 hr of treatment. DSF prevented CPA-induced bladder damage in a dose-dependent manner in mice when orally administered simultaneously with antitumor agent. The protective effect of DSF on the bladder was critically dependent on the timing of administration. Oral administration of DSF between 60 min before and 60 min after the injection of CPA was found to be effective. The optimum time was simultaneous administration of both drugs. DSF potentiated slightly the antitumor activity of CPA against EL-4 leukemia in vivo when administered simultaneoulsy with CPA. From these preliminary studies, DSF appears to be a like candidate for protecting against CPA-induced urotoxicity without compromising the therapeutic utility of the alkylating agent.

Introduction

Cyclophosphamide (CPA), an alkylating agent, is the most commonly prescribed chemotherapeutic agent in clinical medicine. CPA is also used as an immunosuppressive agent in preparation for organ transplantation procedures and in treatment of disease states thought to be of autoimmune etiology. Unfortunately, its clinical use has been compromised by the dose-dependent/dose limiting toxicity to the bone marrow and bladder (1). CPA must be metabolically activated by components of the microsomal mixed-function oxidase (MFO) system to generate cytotoxic species. Of these, acrolein and chloroacetaldehyde are considered to be the prime candidates responsible for the potent urotoxicity of CPA (1). The phosphoramide mustard also liberated during CPA metabolism is not thought to contribute to the bladder damage, but rather responsible for the antitumor activity of CPA (2).

* 〒981 仙台市青葉区小松島 4-4-1 ; 4-4-1 Komatsushima Aoba-ku, Sendai 981, Japan

Glutathione is known to be important in the detoxification of oxazaphosphorine metabolites (1). Also, the protective role that thiol compounds have against toxic effects of alkylating agents has been recognized. In particular, glutathione has been shown to prevent CPA-induced bladder damage (1). Therefore, much work has centered on relievintg the toxicity of CPA by using thiol-containing compounds such as N-acetylcystein (3), 2-mercaptoethane sulfonate (4) and dimercaptosuccinic acid (5) that may achieve "sulfhydryl" rescue.

Recently, antitumor drugs in combination with various detoxifying agents have been studied, as well as derivatives with weaker side-effects of CPA. We have carried out a variety of studies on the combined use of routine drug (e.g, biological response modifiers, imipramine, caffeine, cysteamine, cysteine, disulfiram, etc.) with antitumor drugs (6-12). Up to now, we investigated the effects of several routine drugs used in combination with CPA (6, 7, 9, 10) and to determine whether or not their combined use could promote antitumor activity without compromising antitumor activity.

We observed, during the course of another investigation, that mice treated with disulfiram (DSF, Antabuse) and CPA for cancer chemotherapy diminished urotoxicity with compared to CPA alone. This observation suggested a possible interaction of two drugs on the combined use of routine drug for detoxifying of CPA. DSF has been used as an alcohol adverse drug in the treatment of alcoholism (13). We set out to determine whether DSF could function as a protective agent against CPA urotoxicity without compromising its useful therapeutic activity.

Materials and methods

Animals Male ddY and C_{57} BL/6 mice (Japan SLC, Hamamatsu, Japan) at the age of 5 weeks and weighing of 23-24 g and 18-19 g, respectively, were used. Mice were caged in groups of 8-10 and maintained identically on a 12 hr light/dark cycle in a humidity- and temperature-controlled facilities, and received commercially available mouse diet (CE-2, Japan Clea Co., Tokyo, Japan) and water *ad libitum* until the day throughout the experiments. All animal used were performed in compliance with guidelines established in "Guideline for Animal Experimentation for Tohoku College of Pharmacy". Animals were housed in facilities accredited by the Japan Association of Laboratory Animals Care, and the research protocols were approved by the Institutional Animal Care and Use Committee of the Tohoku College of Pharmacy.

Materials Cyclophosphamide (CPA) for injection and disulfiram were purchased from Shionogi Pharmaceutical Co. (Osaka ; Endoxan®) and Tokyo Tanabe Pharmaceutical Co. (Tokyo ; Nocbin®), respectively. Drugs were dissolved in saline and injected into mice intraperitoneally or orally. All other chemicals used were of the highest purity available.

Urotoxicity studies The assay of bladder toxicity, based on the drug-induced increase in bladder weight was essentially similar to that described previously (9). Organ weight was expressed in milligrams of bladder weight per 100 g body weight. Mice were divided into experimental groups of 8-12 animals and were injected i.p. with CPA at various doses. DSF was administered *p.o.* according to different treatment schedules as indicated. DSF dosage

and treatment schedule, as were selected after preliminary examination of the protective effect of DSF on the urotoxicity of CPA.

Mice received CPA *i.p.* alone or in combination with DSF administered *p.o.* simultaneously with CPA, unless otherwise indicated, and killed by cervical dislocation 48 hr later. Control animals were treated with identical volumes of solvent solution for drug. To correlate bladder weight changes with the extent of CPA-induced bladder damage, the bladders were fixed in 10% neutral phosphate buffered formalin and prepared for histological examination.

Antitumor activity studies To determine the effect of DSF on the therapeutic efficacy of CPA, the following experimental models and treatment schedules were used. Groups of 10 mice received CPA *i.p.* alone, or in combination with DSF administered *p.o.* simultaneous with CPA. CPA was administered intraperitoneally to tumor-bearing mice in a volume of 10 ml/kg on 1 day after tumor cell inoculation. (A) The experiments with transplantable EL-4 leukemia were carried out in male C_{57}BL/6 mice inoculated subcutaneously with 3×10^4 cells/mouse into the flank of mice, and the long and short diameters of the tumor were measured after the transplantation. The tumor size was calculated according to the following equation: Tumor size (cm^3) $=3\pi/4\times$(long diameter/2)$\times$(short diameter/2)^2. (B) Experiments with Sarcoma 180 were initiated by inoculating male ddY mice intraperitoneally with 3×10^6 cells. Animals were observed daily and antitumor activity was determined on the basis of two parameters: i) the fraction of long-term survivors (>60 days=cured mice), and ii) the % T/C, which was defined as the median survival time (MST) of mice dying in the drug-treated group (T) divided by the MST of the untreated control group (C), $\times100$.

Acute toxicity test Mice were divided into experimental groups of 10 animals and were injected intraperitoneally with CPA at various doses. The mortality rate of the animals was observed for 21 days, during which the survivors were observed daily.

Statistical analysis Data were analyzed for statistical significance using Students's *t*-test, and $P<0.05$ was considered significantly.

Results

Effect of DSF on CPA-induced bladder toxicity

The effect of DSF on the bladder toxicity of CPA was studied at various dose levels of both antitumor agent and DSF. As shown in Fig. 1, the administration of CPA caused a dose-dependent increase in the bladder weight coupled with a decrease in body weight. Macroscopic evaluation of the bladder showed dramatic inflammation and hemorrhage in the organ. Profound pathological changes in bladder tissue were also visible by light microscopic examination of tissue slides. DSF administered simultaneously with CPA prevented the CPA-induced increase in bladder weight (at doses of DSF as low as 100 mg/kg). The uroprotection was accompanied by a reduction in body weight loss. It also reduced the bladder inflammation but did not bring bladder weights back to levels recorded for untreated animals. Visual evaluation revealed a decrease in tissue changes following the administration of DSF but, again, did not show complete reversal of these changes. Histological sections examined at this point showed that damage had been done to the bladder tissue in

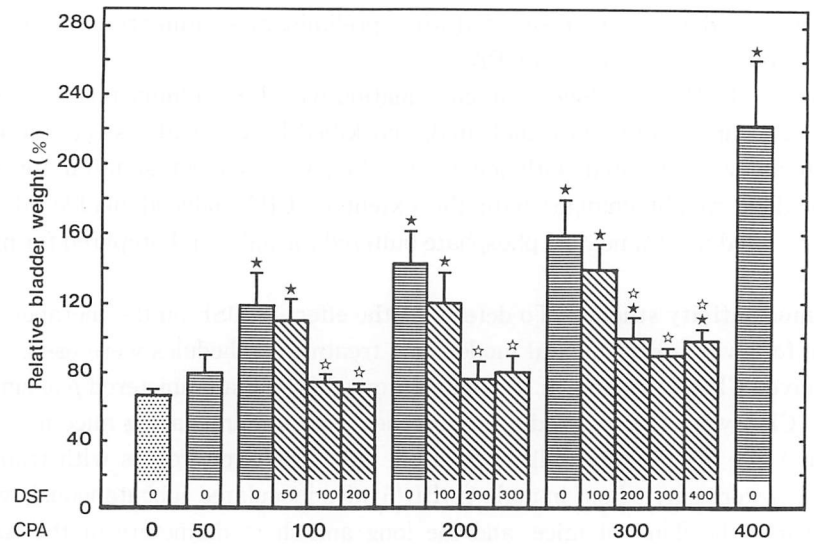

Fig. 1 Effect of disulfiram on cyclophosphamide-induced increase in bladder weight of mice
Mice were given various doses of cyclophosphamide (CPA, intraperitoneally) simultaneously with various doses of disulfiram (DSF, orally), and 48 hr later bladder wet weights and body weights were measured. Data are represented as the mean±S.E. of the relative bladder weights (mg/100 g body weight) of 8-10 animals in a previous report (10). Closed and open starts indicate significant differences form the control and CPA alone, respectively ($P<0.05$).

some animals even in the presence of DSF. DSF itself exhibited no toxicity to the bladder.

Since 200 mg/kg of CPA consistently increased urinary bladder weight without life-threatening toxicity, this dose was administered in the subsequent toxicity studies.

Since the uroprotection efficacy of thiol compounds is critically depedent on the timing of thiol administration (1), further experiments were designed to determine whether a similar schedule-dependence also occurred for the uroprotective effect of DSF. When given simultaneously with CPA, DSF prevented an increase in bladder weights at doses lower than 200 mg/kg ; in the subsequent studies, 200 mg/kg of DSF was used. Fig. 2 shows that DSF was effective if administered within 60 min before or 60 min after CPA. Administration of DSF earlier, or later, diminished the protective efficacy.

Interference by DSF on the antitumor activity of CPA

The interference by DSF with CPA antitumor activity was examined against EL-4 leukemia cells transplanted into mice in two experimental models. EL-4-bearing mice were given DSF, using the dosing schedule that most effectively prevented CPA-induced bladder damage (Fig. 1). As seen in Fig. 2, suppression of the tumor growth and prolongation of the survival time were obviously observed after the treatment with CPA (90 mg/kg, *i.p.*). DSF (200 mg/kg, *p.o.*) alone did not alter the antitumor activity of tumor-bearing mice, but further increase the antitumor activity of mice treated with CPA (90 mg/kg, *i.p.*). To confirm the increase in the antitumor activity of CPA in DSF-coadministered mice, another study was undertaken, employing a range of CPA doses (Table 1). DSF produced an increase in the

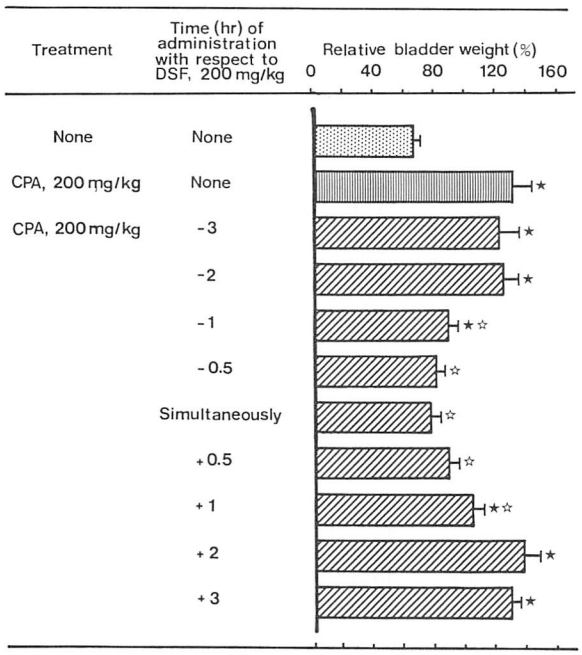

Fig. 2 Influence of the treatment schedule on the protective effect of disulfiram on cyclophosphamide-induced bladder toxicity in mice
Mice were given cyclophosphamide (CPA, 200 mg/kg, intraperitoneally) and disulfiram (DSF, 200 mg/kg, orally) at various times before or after CPA administration. The experimental condition analysis are as in Fig. 1.

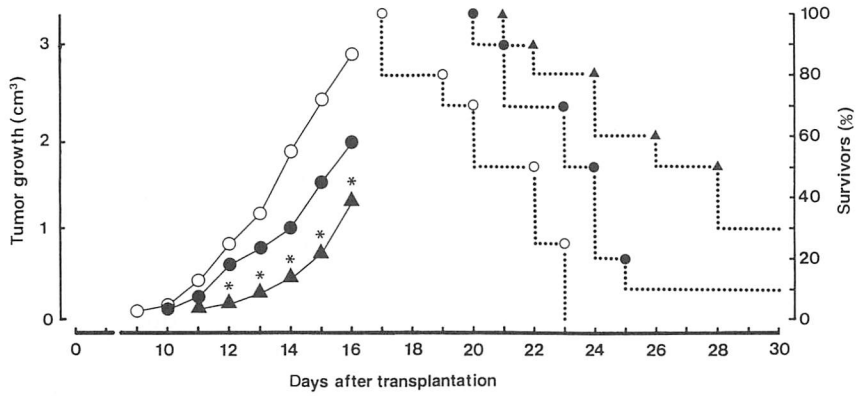

Fig. 3 Interference by disulfiram with the antitumor activity of cyclophosphamide against EL-4 leukemia
Groups of 10 mice were inoculated with 3×10^4 EL-4 leukemia cells, and 24 hr later were treatd with saline (control, ○), cyclophosphamide (CPA, 90 mg/kg, ●), or CPA together with disulfiram (▲). Animals were observed daily for 30 days. Antitumor activity are expressed as the tumor growth and survivours. * indicate significant differences from the CPA alone ($P<0.05$).

Table 1 Interference of disulfiram on the antitumor activity of cyclophosphamide against Sarcoma 180 in mice

Treatment		MST (mean±S.E.)	ILS (%)	Survivous (>60 days)
CPA	DSF			
0	0	18.5±1.05	100	0/10
60	0	20.7±1.95	112	0/10
60	200	22.9±2.23[a)]	124	1/10
90	0	28.3±2.98[a)]	155	1/10
90	200	35.5±3.01[a,b)]	192	2/10
120	0	32.9±3.23[a)]	189	2/10
120	200	40.8±3.64[a,b)]	221	3/10

Groups of 10 mice were inoculated with 3×10^6 Sarcoma 180 cells, and 24 hr later were treated with saline (control), cyclophosphamide (CPA, 60 to 120 mg/kg, intrapertitoneally), disulfiram (DSF, 200 mg/kg, orally), or CPA together with DSF. Animals were observed daily for 60 days. Antitumor activity was determined by calculations of % T/C, which is defined as the median survival time (MST) in the treatment group (T) divided by that of the control group (C), multiplied by 100. MST was determined only using dying animals. The study was terminated at 60 days. [a)] and [b)] indicate significant differences from the control and CPA alone, respectively ($P<0.05$).

antitumor activity of CPA, which was statistically significant at CPA doses of 90 and 120 mg/kg. At a dose of CPA of 60 mg/kg the increase in % ILS was not statistically significant, but there was increase in 60-day survivor.

The effect of DSF on the acute lethal toxicity of CPA was studied at various dose levels of both the antitumor agent and the thiol compound. CPA doses were chosen in the range which allowed determination of LD_{50} values. The results suggest a slightly increased lethality but there was not statistically significant when mice were given CPA simultaneous with DSF (data not shown).

Discussion

The major problem in cancer chemotherapy is still the lack of selectivity of the available drugs. A promising approach to enhancement of the selective toxicity of anticancer drugs against tumor cells is represented by pharmacological attempts to reduce side effects (with particular reference to dose limiting) without interfering with antitumor properties of the cytotoxic agents (1). Since this approach may require administration of an antidote, the detoxifying interaction between the protective agent and the cytotoxic drug could also produce a partial loss of antitumor activity. Thus, the possibility of preventing toxic side manifestations without any adverse influence on therapeutic effectiveness occurs in two main situations: (a) when differences in the pharmacokinetic behavior between antitumor drug and protective agent are expected to afford a regional detoxification to prevent specific organotoxicity; and (b) when different mechanisms are responsible for antitumor effect and some organ-specific damage.

Haemorrhagic cystitis, which is produced by CPA and related compounds (1), is an example of the latter situation. Indeed, metabolic products of CPA, including acrolein, which is characterized by weak cytotoxic properties, and chloroacetaldehyde have been implicated in this characteristic toxic effect (5, 14, 15), which is limiting factor in the therapeutic use of the drug, particularly in high-dose chemotherapy regimens (16).

It is well known that glutathione serves many roles in the cell : it can act as a reductant ; as a transport and store form of cysteine ; and as a protective agent against free radicals, reactive oxygen species, and toxic compounds. Several studies have suggested that an increase in cellular glutathione may protect aganist CPA cytotoxicity, whereas a decrease may potentiate the effect of the drug (1). Also, the addition of thiol compounds (cysteine, N-acetylcysteine, sodium 2-mercaptoethanesulfonate, glutathione or glutathione esters) to in vivo or in vitro systems seems to protect cell against the toxicity of CPA and its metabolites (1). Furthermore, recently, we have attempted to assess the effect of both a depletion of and an increase in glutathione on CPA toxicity in mice. We showed that the depletion of glutathione by injection of buthionine sulfoximine potentiated the acute toxicity and urotoxicity of CPA, whereas the injection of cysteine increased glutathione levels and protected these effect against CPA (9, 10).

Collectively, these studies suggest that thiol-containg compounds protect against CPA toxicity ; however, the role of cellular glutathione in the metabolism and efficacy of the drug can be better understood only by studying the effect of careful modulation of cellular glutathione levels on the toxicity of CPA in a given system. Therefore, much work has centered on relieving the toxicity of CPA by using thiol-containing compounds that may achieve "sulfhydryl" rescue. Recently, parenteral administration of DSF has been reported to prevent haemorrhagic cystitis due to ifosfamide (IFA) (11). Many of the histological lesions caused by IFA are similar to those observation in CPA-induced haemorrhagic cystitis (1), and DSF has also been reported to decrease the toxicity of several alkylating agents (11, 17) as well as to inhibit the carcinogenicity of various N-nitroso compounds (18). In the present study, we investigated the effect of DSF on the bladder toxicity of CPA in mice.

Appropriate sulfhydryl-containing compounds have been reported to provide effective protection against CPA-induced urotoxic effects, probably through deactivation of toxic metabolites, without interfering with the chemotherapeutic activity of the antitumor drug (1, 19, 20). In the mechanism of the protective action of these thiol compounds, a central role of endogenous glutathione has been generally recognized (20, 21). In particular, a preferential interaction of the endogenous glutathione with the electrophilic metabolite, acrolein, has been proposed as a mechanism for protection against some toxic effects of CPA (20).

DSF has been used widely as a major adjunctive agent for the treatment of alcoholism (22). In vivo, DSF is metabolized to diethyldithiocarbamate methyl ester, carbon disulfide, and diethylamine, and these metabolites have been found in biological fluids and tissues.

The actual mechanism by which DSF protects against CPA urotoxicty is not known. Certainly, CPA and three of its metabolites have been shown to react with thiol compounds such as cysteine, N-acetylcysteine, sodium 2-mercaptoethanesulfonate, or glutathione (1). The reversible formation of 4-thioderivatives by substitution of the hydroxyl group of 4-hydroxy CPA has been reviewed (23). However, since several synthetic representatives of 4-

thio CPA have been shown to undergo rapid hydrolysis (24), the role of the reversible formation of thiol adducts as a mode of deactivation of the drug has been questioned (23). Upon the opening of the oxazaphosphorine ring and the release of phosphormide mustard, the chloroethyl groups react readily with nucleophilic centers, and substitution of the chlorides of phosphoramide mustard by thiol compounds has been described (14, 23). Another metabolite of CPA, acrolein, which has been suggested to be responsible for the urotoxicity of the drug, can also react with sulfhydryl groups (25, 26). Such reaction is explained in clinical treatments by the administration of sodium 2-mercaptoethanesulfonate to patients receiving CPA therapy so as to protect them against CPA-induced haemorrhagic cystitis (26).

Since DSF is currently in clinical use and has few undesirable side effects, its protective effect may be useful in cancer chemotherapy.

DSF has shown the ability to protect against the potent urotoxicity of CPA without adversely affecting its antitumor activity. Numerous studies concerning the dose response and the time of administration will assist in the evaluation of DSF as a sulfhydryl rescue agent for CPA. Thus it appears that the use of DSF in combination with CPA may be helpful to decrease the toxicity of CPA and thus increase the therapeutic index of CPA.

Acknowledgement

This work was partially supported by a grant from Science Reearch Promotion Fund (the Japan Private School Promotion Foundation).

References

1) T.A. Connor: Alkylating agents, nitrosoureas and alkyltrazenes. In: Cancer chemotherapy/6. Eds.: H.M. Pinedo and B.A. Chabner. Elsevier, Amsterdam, New York, Oxford, 1991, pp. 31-57.
2) D.E. Seitz, C.L. Katterjohn, S.M. Rinzel and H.L. Pearce: Thermodynamic analysis of the reaction of phosphamide mustard with protector thiols. *Cancer Res.*, **49**, 3525-3528 (1989).
3) J.A. Botta, L.W. Nelson and J.H. Weikel: Acetylcysteine in the prevention of cyclophosphamide-induced cystitis in rats. *J. Natl. Cancer Inst.*, **55**, 1051-1057 (1973).
4) A. Kedar, C.L. Simpson, P. Williams, R. Moore, G. Tritsch and G.P. Murphy: The prevention of cyclophosphamide induced bladder swelling in the rat by i.v. administration of sodium 2-mercaptoethane sulfonate. *Res. Commun. Chem. Pathol. Pharmacol.*, **29**, 339-348 (1980).
5) P.J. Cox and G. Abel: Cyclophosphamide cystitis studies aimed at its minimization. *Biochem. Pharmacol.*, **28**, 3499-502 (1979).
6) K. Sasaki, M. Saitoh and Y. Takayanagi: Drug interaction on antitumor drugs I. Antitumor activity of cyclophosphamide in mice consecutively administered aminopyinre, chlorpromazine, or morphine. *Jpn. J. Pharmacol.*, **75**, 543-549 (1979) (in Japanese, abstract in English).
7) M. Ishikawa, K. Sasaki and Y. Takayanagi: Drug interaction of antitumor drugs. (V). Enhancement of cyclophosphamide-induced antitumor activity with imipramine. *Annu. Rep. Tohoku Coll. Pharm.*, **32**, 251-262 (1985) (in Japanese, abstract in English).
8) K. Sasaki, M. Ishikawa, M. Satoh and Y. Takayanagi: Drug interaction on antitumor drugs. VII. Antitumor activity of cyclophosphamide and tegafur in sodium selenite-treated mice. *Annu. Rep. Tohoku Coll. Pharm.*, **34**, 251-261 (1987) (in Japanese, abstract in English).
9) M. Ishikawa, M. Mori, K. Sasaki and Y. Takayanagi: Study on the toxicity of cis-diamminedichloroplatinum. II. Effect of caffeine on the toxicity of cis-diamminedichloroplatinum in mice. *Annu. Rep. Tohoku Coll. Pharm.*, **35**, 261-272 (1988) (in Japanese, abstract in English).

10) M. Ishikawa, K. Sasaki and Y. Takayanagi: Injurious effect of buthionine sulfoximine, and inhibitor of glutathione biosynthesis, on the lethality and urotoxicity of cyclophosphamide in mice. *Jpn. J. Pharmacol.*, **51**, 146-149 (1989).
11) M. Ishikawa, Y. Takayanagi and K. Sasaki: Modification of cyclophosphamide-induced urotoxicity by buthionine sulfoximine and disulfiram in mice. *Res. Commun. Chem. Pathol. Pharmacol.*, **67**, 265-268 (1989).
12) M. Ishikawa, Y. Takayanagi and K. Sasaki: Drug interaction effects on antitumor drugs (VIII): Prevention of ifosfamide-induced urotoxicity by disulfiram and its effect on antitumor activity and acute toxicity of alkylating agent in mice. *Pharmacol. Toxicol.*, **68**, 21-25 (1991).
13) P.M. Friedman, A. Myles and M. Colvin: Cyclophosphamide and related phosphoramide mustards: current status and future prospects. *Adv. Cancer Chemother.*, **1**, 143-204 (1979).
14) M. Colvin and J. Hilton: Pharmacology of cyclophosphamide and metabolites. *Cancer Treat. Rep.*, **65**, 89-95 (1981).
15) I.C. Shaw, M.I. Graham and A.E.M. McLean: 2-Chloroacetaldehyde: a metabolite of cyclophosphamide in the rat. *Cancer Treat. Rev.*, **10**, 17-24 (1983).
16) R.L. Souhami, P.G. Harper, D. Linch, C. Trask, A. Goldstone, G.S. Tobias, S.G. Spiro, D.M. Geddes and J.D.M. Richards: High-dose cyclophosphamide with autologous marrow transplantation for small cell carcinoma of the bronchus. *Cancer Chemother. Pharmacol.*, **10**, 205-211 (1983).
17) R.F. Borch and M.E. Pleasants: Inhibition of cis-platinum nephrotoxicity by diethyldithiocarbamate rescue in a rat model. *Proc. Natl. Acad. Sci.*, **76**, 6611-6614 (1979).
18) H. Habs and M. Habs: Effect of pretreatment with disulfiram on the toxicity and antitumor activity of 1-(2-hydroxyethyl)-3-(2-chlorethyl)-nitrosourea in Sprague Dawley rats. *Cancer Lett.*, **13**, 63-69 (1981).
19) M.J. Berrigan, A.J. Marinello, Z. Pavellic, C.J. Williams, R.F. Struck and H.L. Gurtoo: Protective role of thiols in cyclophosphamide-induced urotoxicity and depression of hepatic drug metabolism. *Cancer Res.*, **42**, 3688-3692 (1982).
20) H.L. Gurtoo, J.H. Hipkens and S.D. Sharma: Role of glutathione in the metabolism-dependent toxicity and chemotherapy of cyclophosphamide. *Cancer Res.*, **41**, 3584-3592 (1981).
21) K. Ormstad, S. Orrenius, T. Lastbom, N. Uehara, J. Pohl, J. Stekar and N. Brock: Pharmacokinetics and metabolism of sodium 2-mercaptoethanesulfonate in the rat. *Cancer Res.* **43**, 333-340 (1983).
22) M.D. Faiman: Biochemical pharmacology of disulfiram. In: Biochemistry and Pharmacology of Ethanol (Eds. Majchrowicz, E and Noble, E.P.), Vol. 2, pp. 325-348. Plenum Press, New York, 1979.
23) G. Zon, S.M. Ludeman, J.A. Brandt, V.L. Boyd, G. Ozkan, W. Egan and K. Shao: NMR spectroscopic studies of intermediary metabolites of cyclophosphamide. A comprehensive kinetic analysis of the interconversion of cis- and trans-4-hydroxycyclophosphamide with aldophosphamide and the concomitant partitioning of aldophosphamide between irreversible fragmentation and reversible conjugation pathways. *J. Med. Chem.*, **27**, 466-475 (1984).
24) G. Peter and H.-J. Hohors: Synthesis and preliminary antitumor evaluation of 4-(SR)-sulfidocyclophosphamides. *Cancer Chemother Pharmacol.*, **3**, 181-186 (1979).
25) R.A. Alarcon: Studies on the in vivo formation of acrolein: 3-hydroxy-propylmercapturic acid as an index of cyclophosphamide activation. *Cancer Treat. Rep.*, **60**, 327-330 (1976).
26) N. Brock: Oxazaphosphorine cytostatics: past, present, fufure. *Cancer Res.*, **49**, 1-7 (1989).