# Potentiation of Ifosfamide Toxicity by Chlordiazepoxide, Diazepam and Oxazepam

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**The effects of chlordiazepoxide, diazepam and oxazepam on the lethal toxicity and metabolic activation of ifosfamide were investigated in mice. Ifosfamide was administered 24 h after the final injection of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, i.p.). The prior administration of chlordiazepoxide, diazepam or oxazepam enhanced the toxicity of ifosfamide (778 mg/kg, i.p.) during observation for 6 d after the administration of ifosfamide. In chlordiazepoxide-, diazepam- or oxazepam-treated mice, a higher concentration of active metabolite in the plasma after the administration of ifosfamide (200 or 600 mg/kg, i.p.) was observed as compared with that in mice treated with ifosfamide alone. On the other hand, chlordiazepoxide, diazepam or oxazepam markedly enhanced the activity of ifosfamide oxidase in the liver microsomes. These results suggest that the potentiation of ifosfamide toxicity is due to stimulation of the metabolic activation of ifosfamide by chlordiazepoxide, diazepam and oxazepam.**

**Keywords** ifosfamide; chlordiazepoxide; diazepam; oxazepam; toxicity; mice; active metabolite; ifosfamide oxidase

Ifosfamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide] is an analog of cyclophosphamide differing from the parent compound in that the 2 functional chloroethyl groups are not attached to the same nitrogen.[1] This alkylating agent has activity against many solid tumors.[2] The clinical toxicity of ifosfamide is similar to that of cyclophosphamide. However, the most serious and dose-limiting toxicity of ifosfamide is that on the genitourinary system.[3] Ifosfamide requires metabolic activation by the hepatic microsomal cytochrome P-450-dependent mixed-function oxidases before demonstrating antitumor activity *in vivo*.[4]

It has been shown that microsomal enzyme induction with phenobarbital leads to enhanced cyclophosphamide antitumor activity and toxicity.[5,6] Chlordiazepoxide, diazepam and oxazepam are known to induce hepatic microsomal drug-metabolizing enzymes in rats.[7,8] We have reported that the activity of masked compounds such as tegafur and cyclophosphamide was increased by several injections of chlordiazepoxide, diazepam or oxazepam.[9,10] This suggested that minor tranquilizers might alter the therapeutic activity or toxicity of ifosfamide *in vivo*.

The work reported here was undertaken to elucidate the effects of chlordiazepoxide, diazepam and oxazepam on toxic activity of ifosfamide and the mechanism of the drug interaction in mice.

## Experimental

**Chemicals** The chemicals used were as follows: ifosfamide (Ifomide, Shionogi Seiyaku Co.), chlordiazepoxide (Takeda Yakuhin Kogyo Co.), diazepam (Maruko Seiyaku Co.), and oxazepam (extracted from the commercial product, Banyu Seiyaku Co.). Ifosfamide for injection was dissolved in physiological saline. Chlordiazepoxide, diazepam and oxazepam were suspended in 0.5% carboxymethylcellulose just before use. All chemicals were administered intraperitoneally.

**Animals** Male ddY mice, weighing 22—24 g, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice were caged in groups of 5 and maintained on a standard diet and water *ad libitum*.

**Acute Toxicity of Ifosfamide** The lethal toxicity was estimated at 3 doses for each schedule, and each dose was evaluated in 10 animals. The observation period was 6 d.

**Assay of the Active Metabolites of Ifosfamide in Plasma** Chlordiazepoxide, diazepam or oxazepam (100 mg/kg) was injected intraperitoneally once a day for 3 consecutive days, the last injection being given 24 h prior to administration of ifosfamide. Normal mice were given ifosfamide (648, 778 and 933 mg/kg, i.p.) with or without chlordiazepoxide, diazepam or oxazepam. At serial times up to 150 min after ifosfamide, mice ($n=3$ per time point) were killed and blood was collected for separation of the plasma fraction. The γ-(*p*-nitrobenzyl)pyridine (NBP) assay used to quantitate active species is a modification of the original Friedman and Boger method[11] which reacts alkylating species with NBP. The acetone-extractable fraction containing alkylating species forms an intense blue color with NBP which is read spectrophotometrically at 540 nm (Hitachi type 124). Standard curves were established using normustard [bis-(2-chloroethyl)amine hydrochloride, Nakarai Chemical Co., Ltd.].

**Assay of Ifosfamide Oxidase** Liver enzymes were prepared from tranquilizer-treated or untreated mice by homogenization of the liver in 0.15 M KCl solution, followed by centrifugation at 9000 × *g* for 20 min. The incubation mixture consisted of 9000 × *g* supernatant fluid (about 125 mg liver), 2 mM ifosfamide, 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 5 mM magnesium chloride, 0.05 mM ethylenediaminetetraacetic acid (EDTA), and 1 unit of glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 7.4. The mixture was incubated at 37 °C for 30 min by shaking. The formation of alkylating products (active ifosfamide) was determined spectrophotometrically using normustard as a standard.

**Statistical Analysis** The statistical significance of differences between groups was determined by means of the chi-square-test and Student's *t* test.

## Results

The influence of chlordiazepoxide, diazepam and oxazepam treatment on the lethality of ifosfamide in mice is shown in Fig. 1. The dose ranged from 648 to 933 mg/kg. Mice were observed for a period of 6 d. Pretreatment with chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, i.p.) significantly increased the mortality of ifosfamide in mice treated with 778 mg/kg of ifosfamide. However, the mortality of ifosfamide given at 648 mg/kg (i.p.) was not significantly altered by pretreatment with chlordiazepoxide, diazepam or oxazepam. Although in mice given ifosfamide at 933 mg/kg, i.p., the mortality was increased by pretreatment with chlordiazepoxide, diazepam or oxazepam, the difference between the ifosfamide-alone and minor tranquilizer-pretreated groups was not statistically significant.

Figure 2 illustrates time courses of active metabolites (normustard-like substances) in the plasma of mice after the administration of ifosfamide. At a dose of 200 mg/kg ifosfamide, i.p., produced a peak plasma level of alkylating metabolites was achieved approximately 15 min after injection. The maximum plasma concentrations with and without tranquilizer were markedly different. In the higher

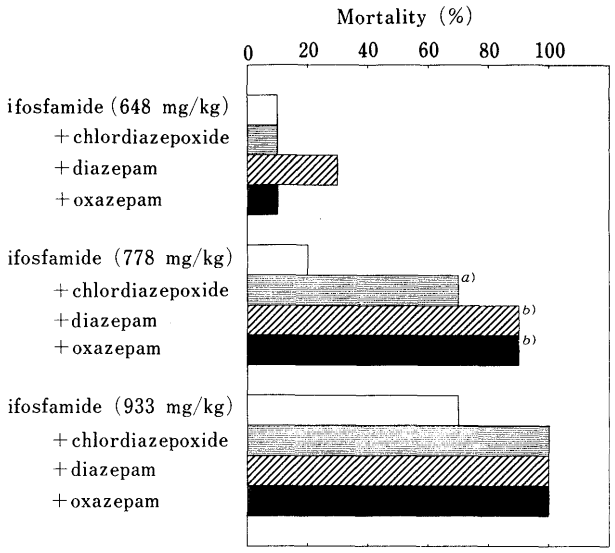


Fig. 1. Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Acute Toxicity of Ifosfamide

Animals were observed for 6d after the administration of ifosfamide. *a)* Significantly different from ifosfamide-alone group ($p<0.05$) in chi-square-test. *b)* Significantly different from ifosfamide-alone group ($p<0.01$) in chi-square-test.

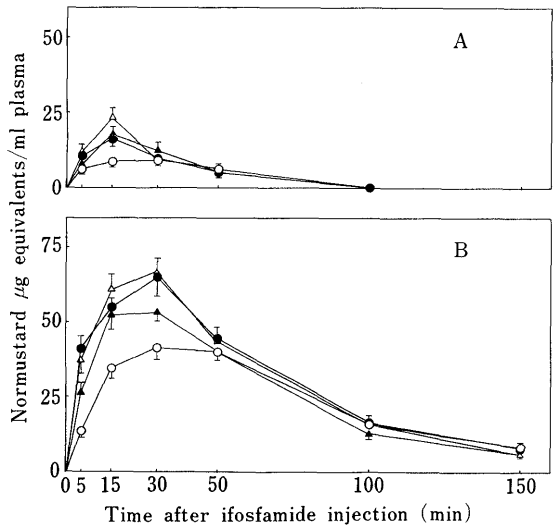


Fig. 2. Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Level of Normustard Expressed as Active Metabolites of Ifosfamide in the Plasma after the Administration of Ifosfamide

A: 200 mg/kg. B: 600 mg/kg. Each point and vertical bar represent the mean ± S.E. of three experiments. ○, saline (control); △, chlordiazepoxide; ●, diazepam; ▲, oxazepam.

TABLE I. *In Vivo* Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Activity of Ifosfamide Oxidase in the Hepatic 9000 × *g* Supernatant Fraction of Mice

| Drug | Ifosfamide oxidase | |
|---|---|---|
| | (nmol/g liver/30 min) | (%) |
| Saline (control) | 0.42 ± 0.03 | 100 |
| Chlordiazepoxide | 0.97 ± 0.03[a)] | 231 |
| Diazepam | 1.00 ± 0.04[a)] | 238 |
| Oxazepam | 0.97 ± 0.04[a)] | 231 |

Each analytical value represents the mean value ± S.E. from five mice. Percentages show the value compared with control mice. Significant differences from the control value are indicated as *a)* ($p<0.01$, Students' *t* test).

dose study (600 mg/kg, i.p.), the plasma level of active metabolites formed from ifosfamide was similarly higher in chlordiazepoxide-, diazepam- or oxazepam (100 mg/kg/d for 3 d, i.p.)-treated mice.

In further investigations, the activity of ifosfamide oxidase in mouse liver was determined after pretreatment with chlordiazepoxide, diazepam or oxazepam (Table I). The tranquilizer treatments significantly raised the alkylating activity of ifosfamide in the liver. When the final administration of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, i.p.) was given, the activity of ifosfamide oxidase in the hepatic 9000 × *g* supernatant fraction of mice was increased 2.3- to 2.4-fold.

## Discussion

Like cyclophosphamide, ifosfamide is a masked compound, and its activation is initiated by hepatic mixed-function oxidase-catalyzed $C_4$-hydroxylation.[12] The resulting 4-hydroxyifosfamide undergoes ring opening to aldoifosfamide, followed by generation of ifosfamide mustard and acrolein by $\beta$-elimination.[13] The 4-hydroxyifosfamide is believed to be responsible for the potent cytotoxic activity.[14] Although the activation and metabolism of ifosfamide are similar to those of cyclophosphamide, important differences have been observed. Activation of ifosfamide proceeds more slowly than that of cyclophosphamide, perhaps because of the chloroethyl group on the adjacent nitrogen.[12]

As reported previously, we found that the antitumor activity and toxicity of masked compounds such as tegafur in Sarcoma 180-bearing mice were increased by the consecutive injection of chlordiazepoxide, diazepam or oxazepam.[9] We also found that the acute toxicity of cyclophosphamide was increased by these drugs.[10] These three agents also potentiated the liver injury induced by the toxic agents that require metabolic activation by the liver microsomal monooxygenase system.[15] These agents also affected the pharmacologic actions of other drugs, *e.g.* shortening of pentobarbital sleeping time and zoxazolamine paralysis time.[15] Inductive effects of chlordiazepoxide, diazepam and oxazepam on hepatic microsomal drug-metabolizing enzymes have been demonstrated by a number of investigators.[7,8] Thus, it is possible that the anitumor activity and toxicity of a masked compound could be altered by these three drugs.

The major object of present investigation was to determine the effects of chlordiazepoxide, diazepam and oxazepam on the acute toxicity of ifosfamide. The mechanism of toxicities of antitumor drugs has been studied in part in terms of toxic metabolites. Hart and Adamson[16] suggested that the host toxicity as well as the antitumor efficacy of a masked compound (cyclophosphamide) are primarily due to the activated products. Conversely, the inhibition of microsomal enzymes with chloramphenicol[17] or diethyldithiocarbamate[18] has led to a decrease in the activity of cyclophosphamide. In the present study, we showed that the prior administration of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, i.p.) significantly augmented the toxicity of ifosfamide (778 mg/kg, i.p.) during observation for 6 d after the injection of ifosfamide. In addition, the data (Fig. 1) indicated that the increase in the toxicity of ifosfamide by chlordiazepoxide,

diazepam or oxazepam is significantly associated with dosage of ifosfamide. It is considered that this augmentation of ifosfamide (778 mg/kg, i.p.) toxicity induced by chlordiazepoxide, diazepam or oxazepam is perhaps owing to increased activation of ifosfamide. The minor tranquilizer-mediated increase in ifosfamide toxicity correlated with the changes in the pharmacokinetics of ifosfamide alkylating metabolites. As shown in Fig. 2, ifosfamide (200 mg/kg, i.p.) injection caused an earlier and lower peak of alkylating metabolite concentration in the plasma, whereas ifosfamide given at 600 mg/kg (i.p.) caused a later and higher peak. The activation of ifosfamide in the liver of mice proceeds by ring hydroxylation, and this phenomenon clearly suggest that ifosfamide metabolism is due to direct interaction of ifosfamide with cytochrome P-450. The concentration of active metabolite (normustard-like substances) in the plasma after ifosfamide (200 or 600 mg/kg, i.p.) treatment is remarkably increased by chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, i.p.). Nevertheless, no difference was observed in metabolite level between minor tranquilizer-treated mice and untreated mice 50 min after the administration of ifosfamide (600 mg/kg, i.p.). As regards this mechanism, it is considered that a rapid disappearance of active metabolite of ifosfamide in the plasma of minor tranquilizer-treated mice is probably due to the enhancement of inactivation by dechloroethylation of ifosfamide. However, there is no direct evidence on this possibility. Ohira *et al.*[19] reported that a potent P-450 inducer (phenobarbital) caused a marked increase in plasma levels of alkylating metabolite after administration at a high dose. Their account agrees with our data obtained with chlordiazepoxide, diazepam or oxazepam. In metabolic enzyme studies, we observed that chlordiazepoxide, diazepam or oxazepam markedly enhanced the activity of ifosfamide oxidase in the $9000 \times g$ supernatant fraction of liver homogenates.

In conclusion, our results demonstrate that the *in vivo* combination of ifosfamide and minor tranquilizers will result in an increase in alkylating activity and toxicity in mice.

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