EFFECTS OF CHLORDIAZEPOXIDE, DIAZEPAM AND OXAZEPAM ON THE ANTITUMOR ACTIVITY, THE LETHALITY AND THE BLOOD LEVEL OF ACTIVE METABOLITES OF CYCLOPHOSPHAMIDE AND CYCLOPHOSPHAMIDE OXIDASE ACTIVITY IN MICE

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Effects of chlordiazepoxide, diazepam and oxazepam on the antitumor activity and acute toxicity of cyclophosphamide and the level of its active metabolites in the plasma were investigated in mice. Cyclophosphamide was administered 24 h after the final injection of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, *i.p.*). Pretreatment with these drugs increased the acute toxicity of cyclophosphamide (300 or 450 mg/kg, *i.p.*), whereas drugs had no effect on the antitumor activity of cyclophosphamide (100 mg/kg, *i.p.*) against Ehrlich solid carcinoma. A high level of active metabolites of cyclophosphamide in the plasma after the administration of cyclophosphamide (300 or 450 mg/kg, *i.p.*) was observed in chlordiazepoxide-, diazepam- or oxazepam-treated mice. On the other hand, chlordiazepoxide, diazepam or oxazepam enhanced significantly the activity of cyclophosphamide oxidase in hepatic microsomes. It is concluded that potentiation of the acute toxicity at a high dose of cyclophosphamide by chlordiazepoxide, diazepam and oxazepam is due to an induction of microsomal drug-metabolizing enzyme which are responsible for the *in vivo* activation of cyclophosphamide.

Keywords—chlordiazepoxide; diazepam; oxazepam; antitumor activity; acute toxicity; active metabolite; cyclophosphamide; mice

2-[Bis(2-chloroethyl) amino] tetrahydro-2H-1, 3, 2-oxazophosphorine 2-oxide hydrate (cyclophosphamide) is a latent alkylating agent, which is almost inactive *in vitro*, and becomes biologically active *in vivo* only on appropriate activation, which is thought to be accomplished by the hepatic microsomal cytochrome P-450 system.^{1–3)} A number of routine drugs may increase or decrease hepatic microsomal enzyme activity and thereby alter cyclophosphamide metabolism. Phenobarbital, a hepatic microsomal cytochrome P-450 inducer, has been extensively studied by a number of investigators concerning its potential interaction effects on cyclophosphamide metabolism.^{4,5)}

Jori *et al.*⁶⁾ and Jablonska *et al.*⁷⁾ reported that chlordiazepoxide, diazepam and oxazepam

induced hepatic microsomal drug-metabolizing enzymes in rats. Recently, we demonstrated that the antitumor activity of masked compound such as 1-(2'-tetrahydrofuryl)-5-fluorouracil (tegafur) in Sarcoma 180-bearing mice was increased by the injection of chlordiazepoxide, diazepam and oxazepam.⁸⁾ The results implied that chlordiazepoxide, diazepam and oxazepam promote the conversion from tegafur to active metabolite 5-fluorouracil (5-FU) mainly according to the induction of the hepatic drug-metabolism. These facts suggest that these minor tranquilizers promote metabolic conversion of cyclophosphamide to alkylating products.

This paper reports the effects of chlordiazepoxide, diazepam and oxazepam on the antitumor activity, the acute toxicity and the blood level of

active metabolites of cyclophosphamide and cyclophosphamide oxidase activity in mice.

MATERIALS AND METHODS

Animals — Male ddY mice weighing 20–22 g were used. The mice were fed with a commercial diet (Nippon Clea Co., CE-2) and allowed access to water *ad libitum*.

Drugs — The drugs used were as follows: cyclophosphamide (Endoxan, Shionogi Seiyaku Co.), chlordiazepoxide (Takada Yakuhin Kogyo Co.), diazepam (Maruko Seiyaku Co.) and oxazepam (extracted from commercial product, Banyu Seiyaku Co.). Cyclophosphamide for injection was dissolved in physiological saline. Chlordiazepoxide, diazepam or oxazepam was suspended in 0.5% carboxymethylcellulose just before use. All drugs were administered intraperitoneally.

Tumor Cells — Ehrlich ascites tumor cells that have been maintained in ddY mice by weekly passage in our laboratory were used.

Chemotherapy — Groups of ten mice were used. Solid type tumor of Ehrlich carcinoma was obtained by the intramuscular injection of Ehrlich ascites cells (1×10^6 cells) into the right thigh of mice. Cyclophosphamide (50 or 100 mg/kg) was administered once intraperitoneally 24 h after tumor transplantation. Chlordiazepoxide, diazepam or oxazepam (100 mg/kg) was administered intraperitoneally once a day for 3 d, and cyclophosphamide was administered 24 h after the final injection of these drugs. On the 15th day after Ehrlich cell inoculation, mice were sacrificed, and the tumors were removed and weighed. The antitumor activity was evaluated in terms of the mean weight of tumors in treated mice as a percentage of that in control mice.

Acute Toxicity — Groups of ten mice were used. Cyclophosphamide (300 or 450 mg/kg) was injected 24 h after the final injection of minor tranquilizers. The survival of mice was observed continuously for 20 d.

Assay of the Active Metabolites of Cyclophosphamide in Plasma — Chlordiazepoxide, diazepam or oxazepam (100 mg/kg) was injected

intraperitoneally once a day for 3 consecutive days and the last injection was given 24 h prior to intraperitoneal administration of cyclophosphamide (100, 300 or 450 mg/kg). The mice were decapitated at 5, 15, 30, 45, 60, 90, 120 and 150 min after the injection of cyclophosphamide, respectively. The mouse plasma samples were collected for the assay of active metabolites of cyclophosphamide by the method of Friedman and Boger⁹⁾ and Morita *et al.*¹⁰⁾ Normustard [bis-(2-chloroethyl) amine hydrochloride, Nakarai Kagaku Yakuhin Co.] was used as a standard. Alkylating activity in the plasma was expressed as μg of alkylating products (normustard equivalents) formed/ml plasma.

Enzyme Source and Assay of Cyclophosphamide Oxidase — The livers of mice were homogenized in 4 volumes of 1.15 % KCl-10 mM Na, K-phosphate buffer (pH 7.4) and the homogenate was centrifuged at $9000 \times g$ for 20 min to remove cell debris, nuclei and mitochondria. The microsomal fraction of liver was prepared by centrifuging the $9000 \times g$ supernatant at $105000 \times g$ for 60 min. The activity of cyclophosphamide oxidase was determined according to the method of Kato *et al.*¹¹⁾ The incubation mixture for the assay of cyclophosphamide oxidase contained 100 mM Na, K-phosphate (pH 7.4), 0.05 mM EDTA, 5 mM MgCl_2 , 0.5 mM NADP, 5.0 mM glucose-6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase, microsomes (about 1 mg protein) and 2.0 mM cyclophosphamide as a substrate in a total volume of 1 ml. The mixture was incubated aerobically at 37°C for 15 min.

Protein Assay — Protein concentration was determined by the method of Lowry *et al.*,¹²⁾ using bovine serum albumin as a standard.

Statistical Evaluation — Student's *t*-test was used to determine the statistical significance.

RESULTS

Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Antitumor Activity of Cyclophosphamide

The effects of chlordiazepoxide, diazepam and

oxazepam treatment on the antitumor activity of cyclophosphamide against Ehrlich solid tumor in mice were examined, and the results are shown in Fig. 1. The solid tumor weight (mean $\pm$ S.E.) in

FIG. 1. Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Antitumor Activity of Cyclophosphamide

FIG. 2. Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Lethality of Cyclophosphamide
a) All mice were alive.

control mice on the 15th day after the implantation of tumor cells was 6.12 ± 0.34 g. When cyclophosphamide 50 or 100 mg/kg was injected once intraperitoneally 24 h after the cell implantation, the solid tumor weight (mean $\pm$ S.E.) of cyclophosphamide-treated mice was 5.08 ± 0.43 g or 3.23 ± 0.42 g, the inhibition being about 17 % or 47 %. When cyclophosphamide was injected 24 h after the final injection of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, *i.p.*), the antitumor activity of cyclophosphamide

FIG. 3. Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Level of Normustard Expressed as Active Metabolites of Cyclophosphamide in the Plasma after the Administration of Cyclophosphamide

Each point represents the mean of three values obtained from pooled plasma of three mice. ○: saline (control). ●: chlordiazepoxide. □: diazepam. ■: oxazepam.

was not altered by pretreatment.

Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Acute Toxicity due to Cyclophosphamide Administration

Toxic effects of cyclophosphamide, alone and in combination with chlordiazepoxide, diazepam or oxazepam, were observed for 20 d, and the results are shown in Fig. 2. Pretreatment with chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, *i.p.*) increased the lethality of cyclophosphamide (300 or 450 mg/kg, *i.p.*).

Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Plasma Level of Active Metabolites of Cyclophosphamide

Figure 3 shows time course of the level of active metabolites (normustard-like substances) in the plasma of mice after the administration of cyclophosphamide (100, 300 or 450 mg/kg, *i.p.*). The level of active metabolites in the plasma after the administration of cyclophosphamide at a dose of 100 mg/kg was not markedly changed by the injection of chlordiazepoxide, diazepam and oxazepam (100 mg/kg/d for 3 d, *i.p.*). In this case, the plasma level of normustard-like substances liberated from cyclophosphamide reached a maximum in 15 min and then rapidly decreased. In contrast, in a large dose of cyclophosphamide, 300 or 450 mg/kg, the level of active metabolites liberated from cyclophosphamide in the plasma was higher in chlordiazepoxide-, diazepam- or oxazepam-treated mice.

Effects of Chlordiazepoxide, Diazepam and

Oxazepam on Cyclophosphamide Oxidase

The effects of chlordiazepoxide, diazepam and oxazepam on the activity of cyclophosphamide oxidase were examined in the hepatic microsomal fraction of mice as shown in Table I. The activity of cyclophosphamide oxidase significantly increased 24 h after the final injection of chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, *i.p.*). Chlordiazepoxide among the three drugs showed the greatest inducing effect on the activity of cyclophosphamide oxidase in hepatic microsomes.

DISCUSSION

The potent anticancer drug cyclophosphamide requires activation *in vivo* by the microsomal cytochrome P-450 system in order to act as an alkylating agent. It is considered that the active metabolites of cyclophosphamide are aldophosphamide³⁾ and 4-hydroxycyclophosphamide¹³⁾ which have a higher antitumor activity. However, the exact mechanism of activation of cyclophosphamide still remains obscure. The enzyme system which activates cyclophosphamide has been demonstrated to be affected by a number of factors. For example, phenobarbital⁵⁾ induces microsomal enzymes and SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate)¹⁴⁾ inhibits microsomal enzymes.

Previously, we found that the antitumor activity of masked compounds such as tegafur on Sarcoma 180 solid tumor in mice was decreased

TABLE I. *Effects of Chlordiazepoxide, Diazepam and Oxazepam on the Activity of Cyclophosphamide Oxidase in Hepatic Microsomes of Mice*

Drug (mg/kg/d for 3 d)	Cyclophosphamide oxidase			
	(nmol/mg protein/15 min)	(%)	(nmol/g liver/15 min)	(%)
Saline (Control)	27.3 ± 2.3	100	824 ± 65	100
Chlordiazepoxide 100	95.5 ± 6.1 ^{a)}	350	4374 ± 173 ^{a)}	531
Diazepam 100	71.5 ± 5.1 ^{a)}	262	2859 ± 159 ^{a)}	347
Oxazepam 100	83.6 ± 4.7 ^{a)}	307	3050 ± 200 ^{a)}	370

Each analytical value represents the mean value ± S.E. from seven mice. Percentage shows the value to levels of control mice. Significant differences from the control value are indicated as a) ($p < 0.01$).

by the intravenous injection of lipopolysaccharide (obtained from *Escherichia coli*, LPS).¹⁵⁾ We also found that the antitumor activity and toxicity of tegafur were increased by the consecutive injection of chlordiazepoxide, diazepam or oxazepam.⁸⁾ These findings suggest that the activation of tegafur is depressed or stimulated by the administration of LPS or minor tranquilizers.

The drug-metabolizing enzymes play a central role on activation of masked compounds. Stimulating effects of chlordiazepoxide, diazepam and oxazepam on hepatic microsomal drug-metabolizing enzymes in rats have been reported.^{6,7)} The changes in hepatic microsomal metabolism provoked by the drugs are important from the viewpoint of the drug interaction. However, little work has been done on the drug interaction between minor tranquilizers and anti-tumor drugs.

In the present study, we investigated the effects of chlordiazepoxide, diazepam and oxazepam on the antitumor activity and acute toxicity of cyclophosphamide, plasma concentration of normustard expressed as the active metabolites of cyclophosphamide, and hepatic cyclophosphamide oxidase activity in mice.

The antitumor activity of a single administration of cyclophosphamide (50 or 100 mg/kg, *i.p.*) against mice bearing Ehrlich solid tumor was not changed by pretreatment with chlordiazepoxide, diazepam or oxazepam (100 mg/kg/d for 3 d, *i.p.*) when cyclophosphamide was administered 24 h after the final injection of these drugs. These results indicated that chlordiazepoxide, diazepam and oxazepam did not affect the antitumor effect of cyclophosphamide (50 or 100 mg/kg, *i.p.*) and a sufficient amount of alkylating products converted from cyclophosphamide was not observed at this dose.

In toxicity studies, it has become apparent that the prior administration of chlordiazepoxide, diazepam or oxazepam enhanced the toxicity of cyclophosphamide (300 or 450 mg/kg, *i.p.*) during the observation for 20 d after the administration of cyclophosphamide. Dixon¹⁶⁾ and Cohen and Jao¹⁷⁾ have reported that chloramphenicol

reduced the metabolism and toxicity of cyclophosphamide in rats and phenobarbital enhanced those of cyclophosphamide and that the biologically active metabolites of cyclophosphamide were decreased by the administration of chloramphenicol and increased by the administration of phenobarbital. Consequently, it is considered that the increase in the toxicity of a high dose of cyclophosphamide (300 or 450 mg/kg, *i.p.*) induced by chlordiazepoxide, diazepam or oxazepam is perhaps due to increase activation of cyclophosphamide. On the relationship between the activity and the toxicity of cyclophosphamide, Hart and Adamson¹⁴⁾ and Dixon¹⁶⁾ suggested that the acute toxicity as well as the antitumor activity of cyclophosphamide are primarily due to the active products from cyclophosphamide.

In the metabolic study of cyclophosphamide, we determined the time course of plasma level of active metabolites of cyclophosphamide in mice after the administration of cyclophosphamide. Pretreatment with chlordiazepoxide, diazepam or oxazepam considerably affected the conversion of cyclophosphamide to active alkylating substances. The level of active metabolites released from cyclophosphamide in the plasma was maximum at 15 min after the administration of cyclophosphamide (100 mg/kg, *i.p.*). In chlordiazepoxide-, diazepam- or oxazepam-treated mice, a higher concentration of active metabolites in the plasma after the administration of cyclophosphamide (300 or 450 mg/kg, *i.p.*) was observed as compared with that in mice treated with cyclophosphamide alone. However, in the case of administration of cyclophosphamide at a dose of 100 mg/kg, *i.p.*, pretreatment with these drugs did not show a significant effect on the formation of active metabolites of cyclophosphamide. These findings indicate that the activation of cyclophosphamide by chlordiazepoxide, diazepam or oxazepam occurs in the case of cyclophosphamide overdosage in the treatment. Ohira *et al.*¹⁸⁾ reported that pretreatment with inducer of drug-metabolizing enzymes caused a marked increase in plasma levels of alkylating substances after the

administration of cyclophosphamide at a large dose. Sladek⁴⁾ showed, using Walker 256 carcinosarcoma, that inducer had no effect on cyclophosphamide activity when cyclophosphamide was used at a low dose. Above description agreed closely with the present results obtained by minor tranquilizers.

The influence of pretreatment with chlordiazepoxide, diazepam or oxazepam on the activity of cyclophosphamide oxidase in hepatic microsomes of mice was examined. Chlordiazeoxide, diazepam or oxazepam markedly enhanced the activity of cyclophosphamide oxidase in microsomes. From the above results it is considered that the potentiation of acute toxicity of cyclophosphamide may be due to stimulated cyclophosphamide oxidase by chlordiazeoxide, diazepam and oxazepam.

REFERENCES

- 1) N.Brock and H.J.Hohorts: Über die aktivierung von cyclophosphamid in vivo und in vitro, *Arzneim-Forsch.*, **13**, 1021–1031 (1963).
- 2) N.Brock, R.Gross, H.J.Hohorst, H.O.Klein and B.Schneider: Activation of cyclophosphamide in man and animals, *Cancer*, **27**, 1512–1529 (1971).
- 3) D.L.Hill, W.R.Laster, Jr. and R.F.Struck: Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite, *Cancer Res.*, **32**, 658–665 (1972).
- 4) N.E. Sladek: Therapeutic efficacy of cyclophosphamide as a function of its metabolism, *Cancer Res.*, **32**, 535–542 (1972).
- 5) S.Ohira, S.Maezawa, Y.Irinoda, K.Watanabe, K.Kitada and T.Saito: Combined cancer chemotherapy with cyclophosphamide and an inducer of microsomal drug-metabolizing enzymes (cytochrome P-450) in tumor-bearing rats, *Tohoku J. Exp. Med.*, **116**, 341–350 (1975).
- 6) A.Jori, P.E.Prestine and C.Pugliatti: Effect of diazepam and chlordiazeoxide on the metabolism of other drugs, *J.Pharm.Pharmacol.*, **21**, 387–390 (1969).
- 7) J.K.Jablonska, K.Knobloch, J.Majka and J.M.Wisniewska-Knypl: Stimulatory effects of chlordiazeoxide, diazepam and oxazepam on the drug-metabolizing enzymes in microsomes, *Toxicology*, **5**, 103–111 (1975).
- 8) K.Sasaki, S.Furusawa, K.Monma and G.Takayanagi: Potentiation of the antitumor activity of tegafur in Sarcoma 180-bearing mice by chlordiazeoxide, diazepam and oxazepam, *Chem. Pharm. Bull.*, **31**, 2451–2458 (1983).
- 9) O.M. Friedman and E.Boger: Colorimetric estimation of nitrogen mustards in aqueous media, *Anal. Chem.*, **33**, 906–910 (1961).
- 10) M.Morita, Y.Tochino, T.Iwata and T.Mineshita: Studies on cyclophosphamide, Part II. The separative determination of cyclophosphamide and nor-mustard in biological materials, *Shionogi Kenkyusho Nempo*, **17**, 114–121 (1967).
- 11) R.Kato, Y.Yamazoe, S.Mita, T.Kamataki, T.Kubota, Y.Hanatani and K.Mahejima: Decrease in the activity of hepatic microsomal drug-metabolizing enzymes in tumor-bearing nude mice, *Gann*, **73**, 907–991 (1982).
- 12) O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
- 13) N.Brock: Comparative pharmacologic study *in vitro* and *in vivo* with cyclophosphamide (NSC-26271), cyclophosphamide metabolites, and plain nitrogen mustard compounds, *Cancer Treat. Rep.*, **60**, 301–307 (1976).
- 14) L.G.Hart and R.H.Adamson: Effect of microsomal enzyme modifiers on toxicity and therapeutic activity of cyclophosphamide in mice, *Arch. Int. Pharmacodyn. Ther.*, **180**, 391–401 (1969).
- 15) K.Sasaki, S.Furusawa and G.Takayanagi: Drug interaction of antitumor drugs III, Antitumor activity of tegafur in lipopolysaccharide-treated mice, *Jpn. J. Pharmacol.*, **32**, 1135–1142 (1982).
- 16) R.L.Dixon: Effect of chloramphenicol on the metabolism and lethality of cyclophosphamide in rats, *Proc. Soc. Exp. Biol. Med.*, **127**, 1151–1155 (1968).
- 17) J.L.Cohen and J.Y.Jao: Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat, *J. Pharmacol. Exp. Ther.*, **174**, 206–210 (1970).
- 18) S.Ohira, S.Maezawa, Y.Irinoda, K.Watanabe, K.Kitada and T.Saito: Increased antitumor activity of cyclophosphamide (Endoxan) following pretreatment with inducer of drug-metabolizing enzyme (cytochrome P-450), *Tohoku J. Exp. Med.*, **114**, 55–60 (1974).