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RELATIVE MUTAGENICITY AND TERATOGENICITY OF CYCLOPHOSPHAMIDE AND TWO OF ITS STRUCTURAL ANALOGS

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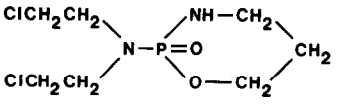
Abstract—In this report, cyclophosphamide was compared to two of its structural analogs, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide, with respect to mutagenic and teratogenic activities. Mutagenicity was assessed using *Salmonella typhimurium* TA 1535; teratogenicity was assessed in Sprague–Dawley rats on day 20 of gestation after intra-amniotic drug administration on day 13. After metabolic activation, cyclophosphamide caused base substitution mutations in *S. typhimurium* TA 1535 and major structural defects in both intra-amniotically injected and contralateral uninjected fetuses. 5,5-Dimethylcyclophosphamide was neither mutagenic nor teratogenic. Diethylcyclophosphamide was not mutagenic but was teratogenic. However, diethylcyclophosphamide was less potent as a teratogen than cyclophosphamide and, unlike cyclophosphamide, caused malformations only in the intra-amniotically injected fetuses. Diethylcyclophosphamide does liberate acrolein after metabolic activation. If acrolein is responsible for the teratogenic effects of diethylcyclophosphamide, the other major cytotoxic metabolite of cyclophosphamide, phosphoramide mustard, may account for the difference in teratogenic potency between cyclophosphamide and diethylcyclophosphamide. These results would suggest that acrolein, although apparently not mutagenic, mediates the teratogenicity of diethylcyclophosphamide and a significant proportion of the teratogenicity of cyclophosphamide.

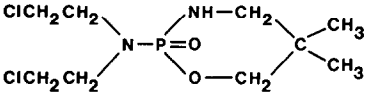
Cyclophosphamide is used extensively as both an anti-tumor drug and an immunosuppressive agent. This drug must be metabolized to be therapeutically active [1]. The first step in this activation, catalyzed by the cytochrome P-450 system, is 4-hydroxylation to form the unstable intermediate, 4-hydroxycyclophosphamide-aldophosphamide [2]. This intermediate decomposes by β-elimination to yield phosphoramide mustard and acrolein. Phosphoramide mustard is believed to account for most of the cytotoxicity of cyclophosphamide in tumor cells [3], whereas acrolein has been identified as the metabolite responsible for the urotoxicity of this drug [4, 5].

Although cyclophosphamide is not teratogenic *in vitro* in either limb bud or whole embryo culture systems without metabolic activation [6–9], the 'proximal' teratogen or teratogens have not yet been definitely identified. In embryos cultured prior to implantation [10] or during organogenesis [11], phosphoramide mustard is the most embryotoxic and teratogenic metabolite of cyclophosphamide. In post-implantation embryos, *in vitro* acrolein is embryolethal but not teratogenic [12]. *In vivo*, in rabbits or in rats after injection into the amniotic space during organogenesis, both phosphoramide mustard and acrolein are embryotoxic and teratogenic [13, 14]. Phosphoramide mustard is a potent base substitution mutagen whereas acrolein, although bacteriotoxic, does not appear to be mutagenic [14, 15].

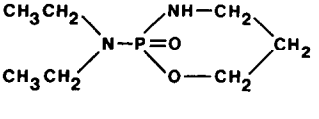
To further study the fetotoxicity and teratogenicity of metabolites of cyclophosphamide, and to attempt to relate these effects to mutagenicity, we have compared cyclophosphamide and two of its structural analogs, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide (Fig. 1), with respect to teratogenicity *in vivo* and mutagenicity toward *Salmonella typhimurium* TA 1535. 5,5-Dimethylcyclophosphamide is metabolized to the hydroxylated intermediate but the gem-dimethyl grouping pre-



Cyclophosphamide



5,5-Dimethylcyclophosphamide



Diethylcyclophosphamide

Fig. 1. Structures of cyclophosphamide and two of its analogs.

vents the liberation of phosphoramide mustard and acrolein by ring scission [16]. If hydroxylation to a 4-hydroxy compound is sufficient, this compound should be mutagenic with activation and teratogenic *in vivo*. Previous studies [17] have demonstrated that this metabolite is of low cytotoxicity to normal or tumor tissue. In diethylcyclophosphamide, the bis-(2-chloroethyl)amine group of cyclophosphamide is replaced by diethylamine. Following metabolism by 4-hydroxylation, acrolein is liberated along with phosphoric acid diamide [18]. Diethylcyclophosphamide is inactive as an antitumor agent [19, 20] but is urotoxic [4, 5]. Thus, these studies should help delineate the relative role of the primary hydroxylated intermediate and of acrolein in mediating *in vivo* the teratogenicity and mutagenicity of cyclophosphamide.

MATERIALS AND METHODS

Chemicals. Cyclophosphamide was purchased from Koch-Light Laboratories Ltd., Colnbrook, England. 5,5-Dimethylcyclophosphamide (ASTA-707) and diethylcyclophosphamide were gifts from Dr. N. Brock, Asta-Werke, Bielefeld, Germany. Solutions of these chemicals were prepared immediately prior to use. Phenobarbital was purchased from Allen & Hanburys, Toronto, Ontario. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Mutagenicity testing. Salmonella typhimurium strain TA 1535 (provided by Dr. Bruce Ames) was used to assay for mutagenicity activity. TA 1535 is a histidine-requiring auxotroph that is reverted to prototrophy by mutagens that cause DNA base-pair substitutions. The plate incorporation assay was done according to the procedure of Ames *et al.* [21] as previously described [22, 23]. This mutagenicity assay was linear with respect to the concentration of both the cyclophosphamide and the microsomal fraction [22]. Maximal activation of cyclophosphamide to mutagenic metabolites was obtained with 50 μl of the hepatic microsomal fraction from phenobarbital-pretreated rats; this is the enzyme preparation used in these experiments.

To evaluate bacteriotoxicity, tubes containing the designated drug concentration (in 0.1 ml), microsomal activation system (0.5 ml) and bacteria (0.1 ml) were incubated for 60 min at 37°. Aliquots (0.1 ml) of the 10^{-3} and 10^{-4} dilutions were plated on complete agar for detection of survival; colonies were counted after incubation for 24 hr at 37°.

Teratogenicity testing. The fetotoxicity and teratogenicity of cyclophosphamide, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide were tested by injection into the amniotic fluid of embryos on day 13 of gestation. Timed gestation pregnant Sprague–Dawley rats (225–250 g) were obtained from Charles River Canada Inc. (St. Constant, Quebec). The day on which spermatozoa were found in the vaginal smear was considered day zero of pregnancy. Pregnant rats were housed on Beta chips in the McIntyre Animal Center (McGill University, Montreal, Quebec) and given Purina rat chow and water *ad lib*. On day 13 of gestation rats were laparotomized under ether anesthesia and the uterus was exposed. Embryos in one uterine horn received an intra-amniotic injection, using a 28-gauge hypodermic needle, of 10 μl of saline (controls) or of drug dissolved in 10 μl of saline. The uterus was repositioned in the abdominal cavity, and the laparotomy was closed with nylon sutures in two layers. Rats were killed by decapitation on day 20 of gestation. Fetuses were removed, examined for external malformations, blotted dry, and weighed.

Statistical analysis. Mutagenicity data were evaluated by linear regression analysis; teratogenicity data

Table 1. Mutagenicity of cyclophosphamide and two of its structural analogs to *S. typhimurium* TA 1535

Drug	Concentration (μg/plate)	Revertant colonies/plate	
		Plus activation*	No activation
Cyclophosphamide	25	37 ± 16†	5 ± 3
	50	85 ± 40	5 ± 3
	100	166 ± 25	15 ± 5
	250	259 ± 61	29 ± 3
	500	585 ± 63	64 ± 6
5,5-Dimethylcyclophosphamide	25	4 ± 2	0
	50	6 ± 1	5 ± 3
	100	11 ± 2	6 ± 1
	250	18 ± 5	1 ± 1
	500	24 ± 5	9 ± 4
Diethylcyclophosphamide	25	4 ± 3	1 ± 1
	50	4 ± 2	1 ± 1
	100	5 ± 3	2 ± 2
	250	3 ± 2	1 ± 1
	500	7 ± 4	1 ± 1

* With the microsomal fraction from the livers of phenobarbital-pretreated rats.

† Each value represents the mean ± S.E.M. for three determinations done in duplicate (N = 3). The spontaneous reversion rate (approximately 20 colonies/plate) has been subtracted for each experiment.

Table 2. Teratogenicity of cyclophosphamide and two of its structural analogs after intra-amniotic administration

Drug	Treatment group: Dose per fetus (mg)	No. of litters	Uninjected fetuses: Dead or resorbed: Total	Uninjected fetuses: Dead or resorbed: Mean per litter*	Uninjected fetuses: Malformed: Total	Uninjected fetuses: Malformed: Mean per litter†	Injected fetuses: Dead or resorbed: Total	Injected fetuses: Dead or resorbed: Mean per litter	Injected fetuses: Malformed: Total	Injected fetuses: Malformed: Mean per litter
Saline		9	5/42	0.10	0/37	0	32/59	0.56	0/27	0
Cyclophosphamide	0.1	7	1/33	0.04	0/32	0	23/52	0.44	0/29	0
	1.0	6	8/34	0.29	26/26‡	1.0‡	35/47	0.71	11/12‡	0.94
5,5-Dimethyl-cyclophosphamide	0.1	6	1/29	0.05	0/28	0	18/36	0.44	0/18	0
	1.0	6	8/30	0.24	0/22	0	27/36	0.76	0/9	0
Diethylcyclo-phosphamide	0.1	7	5/42	0.12	0/37	0	22/48	0.44	1/26‡	0.03
	1.0	6	4/38	0.10	0/34	0	22/36	0.57	6/14‡	0.45

* The mean per litter index for embryolethality is the number of dead or resorbed fetuses/treated implantations per litter.
† The mean per litter for teratogenicity is the number of structurally abnormal fetuses/treated live fetuses per litter.
‡ $P \leq 0.05$ by chi-square analysis for total numbers and Mann–Whitney U test for means per litter, comparing treated groups with the saline controls.

were analyzed by the chi-square test with Yate's correction for discontinuity and by the Mann–Whitney U test and the fetal weight data by the Kruskal–Wallis test. These procedures are described by Snedecor and Cochran [24].

RESULTS

Mutagenicity. The relative mutagenicities of cyclophosphamide, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide were assessed by their abilities to revert *S. typhimurium* TA 1535 to histidine independence (Table 1). As previously reported [22, 23], cyclophosphamide was only weakly mutagenic in the absence of an activating system. In the presence of a microsomal enzyme activating system, cyclophosphamide was mutagenic to *S. typhimurium* TA 1535; there was a linear relationship between the concentration of cyclophosphamide and the number of revertant colonies per plate (333 revertant colonies/μmole per plate).

Neither of the two structural analogs of cyclophosphamide tested, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide, was mutagenic itself and mutagenicity was not enhanced by liver microsomes (Table 1). None of the drugs was significantly bacteriotoxic at the concentrations tested (data not shown).

Teratogenicity. The relative teratogenicities of cyclophosphamide, 5,5-dimethylcyclophosphamide and diethylcyclophosphamide were evaluated following intra-amniotic injection into embryos in one uterine horn (Table 2). The injection of saline alone significantly ($P \leq 0.05$) increased the number of dead or resorbed fetuses among those injected but did not produce any malformations. Intra-amniotic injection of a low dose (0.1 mg/fetus) of cyclophosphamide had no effect on pregnancy outcome as evaluated by the number of fetuses that were dead, resorbed or malformed (Table 2) or by fetal weight (Table 3). A 10-fold higher dose (1.0 mg/fetus) did not increase significantly the number of fetuses that were dead or resorbed but did increase significantly the number of malformed fetuses on both the uninjected (100%) and the injected (91.7%) side. The malformations observed included hydrocephaly (37/38), open eyes (21/38), edema (11/38), micrognathia (7/38), omphalocele (7/38), cleft palate (6/38), bent tail (2/38) and fore (2/38) and hindlimb (28/38) defects (adactyly, syndactyly, polydactyly). At this dose, a marked (60%) decrease in fetal weight was also observed in both the injected and contralateral uninjected fetuses ($P \leq 0.05$).

Administration of 5,5-dimethylcyclophosphamide had no effect on fetal weight (Table 3) or on the number of fetuses dead, resorbed or malformed (Table 2) on either the injected or uninjected side of the uterus. [The apparent increase in the number of dead or resorbed fetuses (Table 2) was not statistically significant because it was due to an increase in only one or two litters.]

Unlike cyclophosphamide, administration of diethylcyclophosphamide had no effect on the contralateral uninjected fetuses. However, this compound did increase significantly ($P \leq 0.05$) the number of malformed fetuses on the injected side. The

Table 3. Effects of cyclophosphamide and two of its structural analogs on fetal weight

Treatment group		Fetuses	
Drug	Dose per fetus (mg)	Uninjected	Injected
Saline		3.78 ± 0.06* (9)	3.63 ± 0.69 (9)
Cyclophosphamide	0.1	4.15 ± 0.39 (7)	3.57 ± 0.24 (6)
	1.0	1.89 ± 0.33† (4)	1.67 ± 0.41† (4)
5,5-Dimethyl-cyclophosphamide	0.1	3.77 ± 0.15 (6)	3.37 ± 0.11 (6)
	1.0	3.48 ± 0.15 (5)	3.43 ± 0.23 (4)
Diethylcyclo-phosphamide	0.1	3.52 ± 0.16 (7)	3.21 ± 0.13 (7)
	1.0	3.68 ± 0.12 (6)	2.86 ± 0.27 (6)

* Mean of the treated fetuses alive on day 20 of gestation per litter ± S.E.M. (number of litters).

† $P < 0.05$ by the Kruskal–Wallis test, comparing the treated group with the saline controls.

higher dose of diethylcyclophosphamide (1.0 mg/fetus) produced the same spectrum of malformations as cyclophosphamide (above); the highest incidence of malformations observed was that for limb defects (42.9%). The decrease (21%) in fetal weight that was observed was not statistically significant (Kruskal–Wallis test, $P \geq 0.05$). The one fetus that was malformed after treatment with the lower dose of diethylcyclophosphamide (0.1 mg/fetus) had a bent tail.

DISCUSSION

In this study the mutagenicity and teratogenicity of cyclophosphamide were compared to those of two of its structural analogs. One of these compounds, 5,5-dimethylcyclophosphamide, was inactive as either a mutagen or as a teratogen. Because this compound can be metabolized to form an hydroxylated intermediate, it is unlikely that it is such an intermediate (i.e. 4-hydroxycyclophosphamide-aldophosphamide) that is responsible for the mutagenicity and teratogenicity of cyclophosphamide. The second analog, diethylcyclophosphamide, was teratogenic but not mutagenic. After metabolic activation diethylcyclophosphamide can form acrolein but not phosphoramide mustard [18]. Thus, acrolein, which is teratogenic [13, 14] but not mutagenic [14, 15], may be responsible for the observed effects of diethylcyclophosphamide.

Diethylcyclophosphamide was less potent as a teratogen than cyclophosphamide [14]. The difference in teratogenic potency between cyclophosphamide and diethylcyclophosphamide may be due to a difference in the rate of formation of the hydroxylated intermediate and of acrolein *in vivo*. A previous study [18] demonstrated that the rate of formation of acrolein from diethylcyclophosphamide in the presence of liver microsomes *in vitro* is 60% that from cyclophosphamide. Alternatively, this difference may represent the greater contribution of phosphoramide mustard than of phosphoric acid diamide to embryotoxicity.

In addition, diethylcyclophosphamide induced malformations only in injected fetuses, whereas cyclophosphamide was equitoxic to both the injected and contralateral uninjected fetuses. At present there is too little information available with respect to the site(s) of metabolism and pharmacokinetics of the metabolites of these drugs to explain these results in terms of an ultimate teratogen. However, these findings do support the conclusion from previous studies that cyclophosphamide itself requires maternal metabolic activation to be teratogenic [6–12].

Neither of the structural analogs of cyclophosphamide tested was mutagenic toward *S. typhimurium* TA 1535. These results suggest that the metabolite responsible for the mutagenicity of activated cyclophosphamide is phosphoramide mustard.

This study demonstrates that the hydroxylated intermediates formed from cyclophosphamide and its analogs (e.g. 4-hydroxy-5,5-dimethylcyclophosphamide) probably do not account for either the teratogenicity or the mutagenicity of these compounds. The cytotoxic metabolite, acrolein, is probably responsible for the teratogenic effects of diethylcyclophosphamide and some of the teratogenic effects of cyclophosphamide after *in vivo* administration, but it is not mutagenic. Thus, the teratogenicity and mutagenicity of metabolites of cyclophosphamide do not appear to be correlated. Further investigations are required to explain the apparent differences in acrolein teratogenicity in *in vitro* and *in vivo* studies and even *in vivo* after intra-amniotic versus intravenous administration.

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