Comparison of the Mutagenicity and Teratogenicity of Cyclophosphamide and Its Active Metabolites, 4-Hydroxycyclophosphamide, Phosphoramide Mustard, and Acrolein

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ABSTRACT

Cyclophosphamide must be metabolically activated to have maximal mutagenic or teratogenic activity. The first step in this activation is hydroxylation to 4-hydroxycyclophosphamide; this metabolite breaks down to form two cytotoxic metabolites, phosphoramide mustard and acrolein. In this report, the mutagenicity and teratogenicity of cyclophosphamide, 4-hydroperoxycyclophosphamide (which forms 4-hydroxycyclophosphamide spontaneously in solution), phosphoramide mustard, and acrolein were compared. Mutagenicity was assessed using a Salmonella typhimurium TA 1535 test system; teratogenicity was studied in rats on Day 20 of gestation after intra-amniotic injection of drug on Day 13. The activation of cyclophosphamide to mutagenic metabolites was dependent on the presence of liver microsomes and reduced nicotinamide adenine dinucleotide phosphate while both phosphoramide mustard and 4-hydroperoxycyclophosphamide were mutagenic without activation, with the latter being the most potent. The third metabolite, acrolein, was bacteriotoxic at low concentrations; it was not mutagenic in the absence and only very weakly mutagenic in the presence of liver microsomes. All four compounds tested were teratogenic. The malformations produced by cyclophosphamide, 4-hydroperoxycyclophosphamide, and acrolein included edema, hydrocephaly, open eyes, cleft palate, micrognathia, omphalocele, bent tail, and forelimb and hindlimb defects, whereas phosphoramide mustard produced only hydrocephaly and tail, forelimb, and hindlimb defects. 4-Hydroperoxycyclophosphamide and its breakdown product, acrolein, were more potent as teratogens than either cyclophosphamide or phosphoramide mustard. Cyclophosphamide produced malformations in both the injected and contralateral uninjected fetuses while the other three compounds all produced malformations only in the injected fetuses. Thus, the site of activation of cyclophosphamide to a teragen is probably maternal. Because acrolein plays a major role in the teratogenicity of cyclophosphamide but is only weakly, if at all, mutagenic, the teratogenicity and mutagenicity of metabolites of this drug are dissociable.

INTRODUCTION

Cyclophosphamide is widely used as a cancer chemotherapeutic agent and as an immunosuppressant. Its use is associated, however, with certain undesirable or toxic effects including fetal malformations, secondary cancer, and cystitis (32, 34, 35). Metabolic activation of cyclophosphamide is required to produce both the therapeutic and the toxic effects (3, 5, 13, 14, 21, 24, 26, 31, 36, 37, 39). Several attempts have been made to modify this drug or its metabolism to improve the relative ratio of desirable to undesirable effects (11, 15, 18, 19, 22, 33, 36).

Activation of cyclophosphamide is thought to occur predominantly in the liver and is catalyzed by the microsomal mixed-function oxidase system. The first step in the activation of cyclophosphamide is C-4 hydroxylation to form the unstable intermediate, 4-hydroxycyclophosphamide (Chart 1). This metabolite, or its aldehyde tautomer, aldophosphamide, decomposes spontaneously to yield phosphoramide mustard and acrolein. Several investigators (6, 7, 23, 25, 37) have presented evidence that the selective therapeutic specificity of cyclophosphamide is due to the intermediate, 4-hydroxycyclophosphamide-aldophosphamide. With respect to one of the undesirable toxic effects, the bladder toxicity or sterile hemorrhagic cystitis, it has been possible to demonstrate that the responsible metabolite is acrolein (8, 10). Administration of a thiol compound provides protection from the bladder toxicity of a high dose of cyclophosphamide without affecting the cytotoxic specificity (11, 33). With respect to mutagenicity, Ellenberger and Mohn (12) found that, with the exception of acrolein, all the metabolites of cyclophosphamide tested were mutagenic. The identity of the metabolite(s) of cyclophosphamide that is responsible for the teratogenic effects of this drug remains to be resolved. Evidence has, however, been obtained in different laboratories that either phosphoramide mustard or acrolein is the "ultimate" teratogenic metabolite of cyclophosphamide (9, 27, 39).

The objective of this study was to compare the mutagenicity and teratogenicity of cyclophosphamide with that of some of its active metabolites: 4-hydroxycyclophosphamide; phosphoramide mustard; and acrolein. Since 4-hydroxycyclophosphamide is unstable, we have used 4-hydroperoxycyclophosphamide; this compound is rapidly hydrolyzed to 4-hydroxycyclophosphamide in aqueous solution (Chart 1). In this report, it is demonstrated that metabolically activated cyclophosphamide, 4-hydroperoxycyclophosphamide, and phosphoramide mustard are all mutagenic to Salmonella typhimurium TA 1535. The most potent mutagen is 4-hydroperoxycyclophosphamide. Cyclophosphamide, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein are all teratogenic after intra-amniotic injection. Only 4-hydroperoxycyclophosphamide and acrolein produce the same spectrum of malformations as cyclophosphamide; these compounds are at least 100 times more potent as teratogens than cyclophosphamide. Therefore, it is possible to dissociate the mutagenic and teratogenic...
Cyclophosphamide: Mutagenicity and Teratogenicity

**Chart 1. Activation of cyclophosphamide.**

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**MATERIALS AND METHODS**

**Chemicals.** Cyclophosphamide was purchased from Koch-Light Laboratories Ltd., Colnbrook, England. 4-Hydroperoxycyclophosphamide and phosphoramide mustard (ASTA-5317) were gifts from Dr. N. Brock, Asta-Werke, Bielefeld, Germany. Acrolein was purchased from Eastman Chemical Co., Rochester, N. Y. Solutions of these chemicals were prepared immediately prior to use. Phenobarbital was purchased from Allen & Hanburys, Toronto, Ontario, Canada. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were purchased from Sigma Chemical Co., St. Louis, Mo.

**Mutagenicity Testing.** *S. typhimurium* strain TA 1535 (kindly provided by Dr. Bruce Ames) was used to assay for mutagenic 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. (2) as described previously (21, 22). This mutagenicity assay was linear with both the concentration of cyclophosphamide and the microsomal fraction (21). 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 (0.1 ml), microsomal activation system (0.5 ml), and bacteria (0.1 ml) were incubated for 60 min at 37°C. Aliquots (0.1 ml) of the 10^{-8} and 10^{-4} dilutions were plated on complete agar for detection of survival; colonies were counted after incubation for 24 hr at 37°C.

**Teratogenicity Testing.** The tetotoxicity and teratogenicity of cyclophosphamide, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein were tested by injection into the amniotic fluid of embryos on Day 13 of gestation. Timed-gestation pregnant Sprague-Dawley rats (225 to 250 g) were obtained from Charles River Canada, Inc. (St. Constant, Québec, Canada). The day on which spermatozoa were found in the vaginal smear was considered Day 0 of pregnancy. Pregnant rats were housed on Beta chips in the McIntyre Animal Centre (McGill University, Montreal, Québec, Canada) and given Purina rat chow and water ad libitum. On Day 13 of gestation, the pregnant rats were laparatomized under ether anesthesia and the uterus was exposed. Embryos in one uterine horn received an intraamniotic injection, using a 28-gauge hypodermic needle, of 0.9% NaCl solution (controls) or drug dissolved in 0.9% NaCl solution (38). The uterus was repositioned in the abdominal cavity and the laparotomy was closed with nylon sutures in 2 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 and 2-way analysis of variance; teratogenicity data were analyzed by the Mann-Whitney U test and the fetal weight data by paired and unpaired t tests as appropriate. These procedures are described by Snedecor and Cochran (38).

**RESULTS**

**Mutagenicity.** The relative mutagenicity of cyclophosphamide, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein was assessed by their ability to revert *S. typhimurium* TA 1535 to histidine independence (Chart 2). In the absence of a microsomal enzyme-activating system, cyclophosphamide was only weakly mutagenic. After activation mediated by liver microsomes and a NADPH-generating system,
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Chart 2. Mutagenicity of cyclophosphamide, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein. Mutagenicity was measured using S. typhimurium TA 1535 in the presence and absence of an activating system, as described in the text. Columns, means for 3 experiments (n = 3); bars, S.E.; *, bacteriotoxicity.

Cyclophosphamide was mutagenic to S. typhimurium TA 1535. There was a linear relationship (correlation coefficient, 0.943) between the concentration of cyclophosphamide per plate and the number of revertant colonies per plate.

Of the 3 metabolites of cyclophosphamide tested, 2 [4-hydroxycyclophosphamide (4-hydroperoxycyclophosphamide) and phosphoramide mustard] were mutagenic themselves; mutagenicity was not enhanced in the presence of liver microsomes. There was a linear relationship between the concentration of the compound per plate and the number of revertant colonies for both 4-hydroperoxycyclophosphamide (range, 5 to 50 μg/plate; correlation coefficient, 0.935) and phosphoramide mustard (range, 25 to 500 μg/plate; correlation coefficient, 0.957). At a concentration of 100 μg/plate, 4-hydroperoxycyclophosphamide was highly bacteriotoxic (Chart 3, <20% survival); phosphoramide mustard was less bacteriotoxic than 4-hydroperoxycyclophosphamide while activated cyclophosphamide was not toxic to the bacteria.

The mutantic potencies of bioactivated cyclophosphamide, 4-hydroperoxycyclophosphamide, and phosphoramide mustard are compared in Chart 4. The relative mutagenicity of these 3 compounds, expressed in number of revertant colonies per nmol, is calculated from linear regression analysis of the data in Chart 2. 4-Hydroperoxycyclophosphamide is 6.2 times more mutagenic than cyclophosphamide following activation. Phosphoramide mustard is 3.2 times more mutagenic than activated cyclophosphamide. Thus, 4-hydroperoxycyclophosphamide is a more potent mutagen than its breakdown product, phosphoramide mustard.

The third metabolite of cyclophosphamide tested, acrolein, was bacteriotoxic even at concentrations as low as 10 μg/plate (Chart 3); at concentrations of 0.01, 0.1, and 1.0 μg, survival rates were adequate (97, 91, and 77%, respectively) to permit mutagenicity testing. Acrolein was not mutagenic itself and was only weakly mutagenic in the presence of liver microsomes (Chart 2). There was no linear relationship between acrolein concentration and number of revertant colonies per plate by regression analysis.

Chart 3. Bacteriotoxicity of cyclophosphamide, 4-hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein. Bacteriotoxicity was measured with S. typhimurium TA 1535 in the presence of an activating system, as described in the text. Survival is expressed as the log of the surviving cell fraction (N/N0). Point, mean of 2 experiments.

Chart 4. Relative mutagenicity of bioactivated cyclophosphamide, 4-hydroperoxycyclophosphamide, and phosphoramide mustard. The relative mutagenicity (revertant colonies/nmol) is calculated from linear regression analysis of the data in Chart 2, as described in the text.
Teratogenicity. The injection of 0.9% NaCl solution into embryos in one uterine horn significantly (p < 0.05) increased the number of dead or resorbed fetuses in that horn (Chart 5). However, all the injected fetuses alive on Day 20 of gestation were normal. Intraamniotic injection of 100 µg (0.36 µmol) of cyclophosphamide per fetus had no effect on pregnancy outcome measured by the number of fetuses dead, resorbed, or malformed. Injection of 1000 µg (3.6 µmol) of cyclophosphamide per fetus did not significantly increase the number of injected fetuses that were dead or resorbed but did increase the number of fetuses that were malformed. In addition to finding 91.7% of the injected fetuses malformed, 100% of the live uninjected fetuses on the contralateral side were also malformed. The malformations observed included edema (29%), hydrocephaly (97%), open eyes (55%), micrognathia (18%), cleft palate (16%), omphalocele (18%), bent tail (5%), and forelimb (5%) and hindlimb (74%) defects (adactyly, syndactyly, polydactyly).

A dose of 1000 µg (3.4 µmol/fetus) of 4-hydroperoxycyclophosphamide, in the same range as the dose of cyclophosphamide required to produce malformations, was toxic to all the injected fetuses. In contrast to the observation with cyclophosphamide, 4-hydroperoxycyclophosphamide had no effect on the contralateral uninjected fetuses. Lower doses of 4-hydroperoxycyclophosphamide (10 µg, 0.034 µmol/fetus; 100 µg, 0.34 µmol/fetus) were still fetotoxic but now, to the surviving fetuses, were also teratogenic. The 2 lower doses of this compound (1 µg, 0.003 µmol/fetus; 10 µg, 0.03 µmol/fetus) caused malformations in 41.2 and 66.7%, respectively, of the injected fetuses. The spectrum of malformations induced by 4-hydroperoxycyclophosphamide was identical to that induced by cyclophosphamide. These malformations were: edema (21%), hydrocephaly (36%), open eyes (29%), micrognathia (14%), cleft palate (14%), omphalocele (7%), bent tail (14%), and forelimb (18%) and hindlimb (39%) defects.

A high dose (1000 µg, 4.5 µmol/fetus) of phosphoramide mustard killed both injected and uninjected fetuses. A lower dose (100 µg, 0.45 µmol/fetus) of phosphoramide mustard was fetotoxic only to injected fetuses; this treatment induced open eyes in one fetus of the 5 surviving injected fetuses. Treatment with a 10-fold lower dose (10 µg, 0.05 µmol/fetus) did not significantly increase fetal deaths compared to the 0.9% NaCl solution controls but did induce malformations on the injected side in 16% of the live fetuses; these included hydrocephaly (4%) and forelimb (8%), hindlimb (16%), and tail (4%) defects. No malformed fetuses were observed at lower doses of phosphoramide mustard (1 or 0.1 µg/fetus).

High doses (100 µg, 1.8 µmol/fetus; 10 µg, 0.18 µmol/fetus) of acrolein induced malformations in 85.7% of the injected live fetuses. The types of malformations induced by acrolein were very similar to those induced by cyclophosphamide and 4-hydroperoxycyclophosphamide. These included: edema (7%), hydrocephaly (43%), open eyes (7%), cleft palate (7%), omphalocele (29%), and forelimb (50%), hindlimb (50%), and tail (4%) defects.
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(21%) defects. A 10-fold lower dose (0.1 µg, 0.002 µmol/fetus) of acrolein had no effect on the resorption or malformation rate relative to 0.9% NaCl solution-treated controls. Thus, the dose range of acrolein capable of producing malformations is narrower than that for either 4-hydroperoxycyclophosphamide or phosphoramide mustard.

4-Hydroperoxycyclophosphamide, phosphoramide mustard, and acrolein were all embryolethal at the doses equivalent to the dose of cyclophosphamide required to produce teratogenic effects. Both 4-hydroperoxycyclophosphamide and acrolein induced the same spectrum of malformations as cyclophosphamide while phosphoramide mustard induced only hydrocephaly and limb and tail defects. The doses of 4-hydroperoxycyclophosphamide and acrolein required to produce the teratogenic effects were at least 100 times less than the dose of cyclophosphamide necessary to produce the same defects. Moreover, cyclophosphamide was the only compound tested that induced malformations in the contralateral un injected fetuses as well as in the injected fetuses.

Administration of a low dose (100 µg/fetus) of cyclophosphamide had no significant effect on fetal weight [3.55 ± 0.61 (S.D.) g, as compared to 3.67 ± 0.21 g for 0.9% NaCl solution-injected controls]. However, treatment with a teratogenic dose of cyclophosphamide (1000 µg/fetus) caused a marked decrease (60%) not only in the weight of injected fetuses (1.59 ± 0.68 g) but also in the weight of the contralateral uninjected fetuses (1.82 ± 0.59 g). Administration of 4-hydroperoxycyclophosphamide (doses from 1 to 100 µg/fetus) caused decreases varying from 19 to 48% in the weight of injected fetuses relative to the 0.9% NaCl solution-injected controls. In contrast to the results with cyclophosphamide, weights of the contralateral uninjected fetuses were less affected (decreases from 3 to 12% relative to the 0.9% NaCl solution-injected controls). Treatment with doses (10 to 100 µg/fetus) of phosphoramide mustard that did induce malformations caused small decreases (9 to 18%) in the weight of injected fetuses. The weights of the contralateral uninjected fetuses were not significantly affected. Treatment with acrolein (0.1 or 1 µg/fetus) also decreased (24%) the weight of injected fetuses without significantly affecting the weight of the un injected contralateral controls.

DISCUSSION

The objective of this study was to investigate the relative mutagenicity and teratogenicity of cyclophosphamide, its synthetic preactivated analog, 4-hydroperoxycyclophosphamide, and its cytotoxic metabolites, phosphoramide mustard and acrolein. With the exception of acrolein which was extremely bacteriotoxic, all the compounds tested were mutagenic to S. typhimurium TA 1535. Unlike cyclophosphamide, neither 4-hydroperoxycyclophosphamide nor phosphoramide mustard required metabolic activation to be mutagenic.

Ellenberger and Mohn (12) previously investigated the mutagenicity of metabolites of cyclophosphamide in the Escherichia coli strain 343/113 test system. They found that acrolein (doses not reported) was not mutagenic whereas both 4-hydroperoxycyclophosphamide and phosphoramide mustard were base substitution mutagens. Our results confirm this observation in a different test system. In addition, in this report, it is demonstrated that 4-hydroperoxycyclophosphamide is a more potent mutagen than either of its breakdown products, phosphoramide mustard or acrolein. Other investigators have also used a S. typhimurium test system to measure mutagenicity of bioactivated cyclophosphamide found in urine or blood (4, 41). In some test systems, acrolein, as well as phosphoramide mustard, causes chromosomal damage (3, 5). However, the mechanism involved in chromosomal breaks or tangling may be different from that in base substitution mutagenicity.

The spectrum of malformations produced by cyclophosphamide can be duplicated by treatment with 4-hydroperoxycyclophosphamide or acrolein at much lower doses. Both of these compounds have a selective local effect on injected fetuses. In these studies, phosphoramide mustard was less potent as a teratogen than either acrolein or 4-hydroperoxycyclophosphamide; this metabolite produced both a lower incidence and fewer types of malformations. Using a variety of model systems, different laboratories have come to different conclusions with respect to the "ultimate" teratogenic metabolite of cyclophosphamide. Studying preimplantation mouse embryos in vitro, Spielmann and Jacob-Müller (39) concluded that, since phosphoramide mustard and 4-hydroperoxycyclophosphamide exhibited identical embryotoxicity, phosphoramide mustard was the ultimate embryotoxic metabolite of cyclophosphamide. Mirkes et al. (27), using rat whole-embryo cultures undergoing organogenesis, also demonstrated that phosphoramide mustard was the teratogenic metabolite of cyclophosphamide. Acrolein, at doses equimolar to those of bioactivated cyclophosphamide, had no effect on any of the parameters measured. However, following in vivo i.p. injection in mice, Gibson and Becker (16) reported that phosphoramide mustard was less potent as a teratogen than cyclophosphamide. Claussen et al. (9) tested embryotoxicity in vivo with injections into the yolk sac in rabbit embryos. These investigators did not study phosphoramide mustard, but they did demonstrate that acrolein was both embryotoxic and teratogenic. They concluded that this metabolite is important in the embryotoxicity of cyclophosphamide. Previously, Müller (29) demonstrated that nitrogen mustard was teratogenic after in utero injection in rats.

Our results suggest that acrolein and phosphoramide must ard both play a role in the embryotoxicity and teratogenicity of cyclophosphamide. The failure to detect the teratogenicity of acrolein in the in vitro embryo culture system is interesting. Acrolein itself may require metabolic activation to be embryotoxic; in vivo, this compound is metabolized to glycaldialdehyde and glyceraldehyde or to acrylic acid (30). This conversion may be possible in vivo near the fetus following an intraamniotic injection but not in vitro in the culture system. Alternatively, because acrolein is extremely volatile, it may not persist in the culture flasks. The narrow dose-response relationship observed in the present study for the teratogenicity of acrolein suggests that this effect could be missed. It is likely that there is a dose threshold for the teratogenicity of this compound, similar to that reported for acetaminophen-induced hepatic necrosis (28). Indeed, acrolein (like acetaminophen) can deplete glutathione (17), and the formation of the glutathione conjugate of acrolein (3-hydroxypropylmercapturic acid) has been suggested as an index of the activation of cyclophosphamide (1).

In this study, the most teratogenic metabolite of cyclophosphamide is acrolein, whereas the most mutagenic metabolite is 4-hydroxycyclophosphamide. Acrolein, although extremely...
bacteriotoxic, does not appear to be mutagenic. Thus, the mutagenicity and teratogenicity of metabolites of cyclophosphamide are dissociable. This comparison of the relative mutagenicity and teratogenicity of "proximal" metabolites of cyclophosphamide should help delineate the mechanism of action of this drug as a teratogen.

The metabolites of cyclophosphamide responsible for its therapeutic effects are probably 4-hydroxyxycyclophosphamide, aldophosphamide or phosphoramide mustard (6, 7, 23, 25, 37, 40). If this is so, it may be possible to modify either cyclophosphamide itself or its disposition to prevent the teratogenic effects mediated by acrolein, as has been suggested for the bladder toxicity produced by this metabolite.

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