Teratogenicity and Neonatal Toxicity of Ifosfamide in Mice[1] (37450)

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Ifosfamide {3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-2H-1,2,3-oxazaphosphorine-2-oxide; NSC 109724} is a structural analog of cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide; Cytoxan; Endoxan; NSC 26271} and was developed in an attempt to improve upon the antineoplastic activity of cyclophosphamide. The antineoplastic activity of ifosfamide occurs with bioactivation which proceeds maximally in the microsomal fraction of rat liver homogenate (1) as does cyclophosphamide (2–6). Cyclophosphamide is teratogenic in chicks (7), rabbits (8), rats (9–11) and mice (12–14). Nordlinder (15), Short and Gibson (16, 17) and Bus, Short and Gibson (18) found that cyclophosphamide administration to newborn mice produces abnormal development such as shortened tails and decreased body weight at maturity.

The purpose of this investigation was to determine the teratogenic effects of ifosfamide in mice and to examine postnatal toxicity resulting from a single administration of ifosfamide to newborn mice.

Methods. Virgin Swiss Webster mice (Spartan Research Animals, Inc., Haslett, MI) were housed in groups of five in stainless steel cages with wire mesh bottoms and allowed food and water *ad libitum*. A 12 hr light–dark cycle (lights on at 8 AM) was maintained to synchronize estrous cycles. Mating was accomplished by placing 1 male in a cage of 5 females for 1 hr starting at 8 AM. The day vaginal plugs were found was designated Day 1 of gestation.

Ifosfamide (Cancer Chemotherapy, National Cancer Institute, NIH, Bethesda, MD) was administered intraperitoneally (ip) at doses of 5, 10, and 20 mg/kg on Day 11 of gestation. Ifosfamide was prepared in normal saline and injected in a volume of 0.1 ml/10 g body weight. Control animals received normal saline on Day 11. On Day 19 of gestation, gravid females were sacrificed by ether anesthesia and the uterine horns were externalized. The number and position of live, dead and resorbed fetuses was recorded. Fetuses were removed by cautery of the umbilical cord, dried, weighed and examined for gross anomalies. Half of the litter was fixed in Bouin's solution and half in 95% ethanol. Fetuses fixed in Bouin's solution were hand sectioned and examined under a dissecting microscope for soft tissue anomalies (19) and those fixed in 95% ethanol were cleared, stained with alizarin red S and examined for skeletal anomalies.

Postnatal toxicity of ifosfamide was determined by administration of a single dose, 45 mg/kg subcutaneously (sc), to members of litters of 1 day old mice. Each litter was normalized to 10 mice before injection. Control litters received normal saline. Litters were housed individually in clear plastic shoe box cages with access to food and water *ad libitum*. At various times after treatment total litter weights and number of surviving animals were recorded. All litters were weaned on Day 28 and segregated by sex. Litters were sacrificed on Day 49 and the tail length and body length (nose to base of tail) were determined for each animal.

The *in vivo* appearance of alkylating

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TABLE I. Resorption Rate and Fetal Size of Offspring of Pregnant Mice Given Ifosfamide on Day 11 of Gestation.

Ifosfamide[a] dosage (mg/kg)	No. pregnant mice treated	Mean response per litter ± SE				
		No. of implantations	No. of fetuses	% Resorption	Body wt (g)	Crown–rump length (cm)
0	6	13.5 ± 1.7	13.5 ± 1.7	0.0 ± 0.0	1.263 ± 0.055	2.3 ± 0
5	6	15.0 ± 1.4	14.7 ± 1.4	2.5 ± 1.7	1.199 ± 0.047	2.3 ± 0
10	6	11.3 ± 1.2	11.2 ± 1.4	2.1 ± 2.1	1.038 ± 0.054[b]	2.1 ± 0.1[b]
20	7	11.8 ± 1.7	2.5 ± 0.8[b]	81.2 ± 5.6[b]	0.587 ± 0.065[b]	1.7 ± 0.1[b]

[a] Ifosfamide, ip, given on Day 11 of gestation.
[b] Significantly different from controls, $p < 0.05$.

metabolites of ifosfamide and cyclophosphamide in plasma of adult female mice was determined at various times after ip administration of each drug (100 mg/kg). Three animals were used for each time point. Alkylating activity was measured colorimetrically (20) in plasma by alkylation of 4-(*p*-nitrobenzyl)-pyridine (NBP) to the highly colored quaternary pyridinium state. Blood samples were obtained by cardiac puncture. One half milliliter aliquots of plasma were reacted with NBP (5% in acetone) and the colored product was extracted into ethylacetate and the optical density was measured at 540 nm. Alkylating activity was expressed as equivalent to micrograms nor-nitrogen mustard per 1.0 ml plasma.

Statistical analyses were by analysis of variance, completely randomized design. The level of significance was chosen at $p < 0.05$.

Results. Ifosfamide, 20 mg/kg on Day 11, but not 5 and 10 mg/kg significantly increased resorption rate as compared to controls (Table I). Fetal body weight and crown–rump length were decreased by 10 and 20 mg/kg ifosfamide (Table I). A wide variety of externally visible anomalies were apparent in Day 19 fetuses whose dams received 20 mg/kg of ifosfamide on Day 11 (Table II). Open eyes, external hydrocephalus, micromelia, adactyly, syndactyly, microcaudate, and kinky tails occurred more frequently ($p < 0.05$) than in controls. Soft tissue examination revealed statistically

TABLE II. Gross Anomalies in Offspring of Pregnant Mice Given Ifosfamide on Day 11 of Gestation.

	Mean % response per litter ± SE			
Ifosfamide[a] (mg/kg):	0	5	10	20
No. litters examined:	6	6	6	5
Gross anomalies				
Open eyes	0.0 ± 0.0	0.0 ± 0.0	7.8 ± 5.0	90.0 ± 10.0[b]
External hydrocephalus	0.0 ± 0.0	1.3 ± 1.3	1.3 ± 1.3	90.0 ± 10.0[b]
Micromelia	0.0 ± 0.0	3.9 ± 3.9	0.0 ± 0.0	63.3 ± 18.6[b]
Adactyly	0.0 ± 0.0	0.0 ± 0.0	15.8 ± 13.9	83.3 ± 16.7[b]
Syndactyly	0.0 ± 0.0	1.0 ± 1.0	2.4 ± 2.4	65.0 ± 15.9[b]
Polydactyly	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 6.7
Microcaudate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	78.3 ± 14.8[b]
Kinky tail	0.0 ± 0.0	1.0 ± 1.0	1.3 ± 1.3	26.7 ± 19.4[b]
Gastroschisis	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0

[a] Ifosfamide, ip, on Day 11 of gestation.
[b] Significantly different from controls, $p < 0.05$.

TABLE III. Soft Tissue Anomalies in Offspring of Pregnant Mice Given Ifosfamide on Day 11 of Gestation.

	Mean % response per litter ± SE			
Ifosfamide[a] (mg/kg):	0	5	10	20
No. litters examined:	6	6	6	5
Soft tissue anomalies				
Cleft palate	0.0 ± 0.0	0.0 ± 0.0	9.3 ± 4.4	20.0 ± 20.0
Internal hydrocephalus	0.0 ± 0.0	0.0 ± 0.0	9.9 ± 4.9	90.0 ± 10.0[b]
Microphakia	0.0 ± 0.0	0.0 ± 0.0	7.1 ± 7.1	46.7 ± 22.6[b]
Kidney ectopia	0.0 ± 0.0	0.0 ± 0.0	2.4 ± 2.4	66.7 ± 21.1[b]
Hydronephrosis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	76.7 ± 14.5[b]
Exencephaly	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 10.0
Cryptorchidism	0.0 ± 0.0	0.0 ± 0.0	20.8 ± 16.3	40.0 ± 24.5

[a] Ifosfamide, ip, on Day 11 of gestation.
[b] Significantly different from control, $p < 0.05$.

significant increases in internal hydrocephalus, microphakia, kidney ectopia, and hydronephrosis at 20 but not 5 and 10 mg/kg ifosfamide (Table III). Ifosfamide, 10 and 20 mg/kg, significantly increased the number of skeletal defects (Table IV). The incidence of supernumerary ribs was significantly increased by 5 mg/kg ifosfamide.

Ifosfamide, 45 mg/kg sc, given to 1 day old mice significantly altered growth and

TABLE IV. Skeletal Anomalies in Offspring of Pregnant Mice Given Ifosfamide on Day 11 of Gestation.

	Mean % response per litter ± SE			
Ifosfamide[a] (mg/kg):	0	5	10	20
No. litters examined:	6	6	6	3
Skeletal anomalies				
Skull bones absent or not ossified	0 ± 0	0 ± 0	0 ± 0	66.7 ± 33.3[b]
Sternebrae absent or not ossified	0 ± 0	4.8 ± 4.8	6.5 ± 4.4	55.6 ± 29.4[b]
Sternebrae fused	0 ± 0	3.3 ± 3.3	22.0 ± 10.8	44.4 ± 29.4[b]
Supernumerary ribs	0 ± 0	55.2 ± 14.1[b]	10.4 ± 8.2	0 ± 0
Fused ribs	0 ± 0	0 ± 0	0 ± 0	100 ± 0[b]
Ribs absent or not ossified	0 ± 0	0 ± 0	2.1 ± 2.1	16.7 ± 16.7
Vertebrae fused	0 ± 0	0 ± 0	49.8 ± 16.1[b]	100 ± 0[b]
Fibula absent or not ossified	0 ± 0	0 ± 0	0 ± 0	100 ± 0[b]
Ulna absent or not ossified	0 ± 0	0 ± 0	0 ± 0	61.1 ± 20.0[b]
Radius absent or not ossified	0 ± 0	0 ± 0	0 ± 0	61.1 ± 20.0[b]
Metatarsals absent or not ossified	0 ± 0	2.4 ± 2.4	25.3 ± 13.0	61.1 ± 20.0[b]
Metacarpals absent or not ossified	0 ± 0	0 ± 0	25.3 ± 13.0	77.8 ± 22.2[b]
Phalanges absent or not ossified	0 ± 0	15.2 ± 10.5	32.1 ± 15.1	61.1 ± 20.0[b]
Exostosis	0 ± 0	0 ± 0	4.2 ± 4.2	16.7 ± 16.7

[a] Ifosfamide, ip, on Day 11 of gestation.
[b] Significantly different from control, $p < 0.05$.

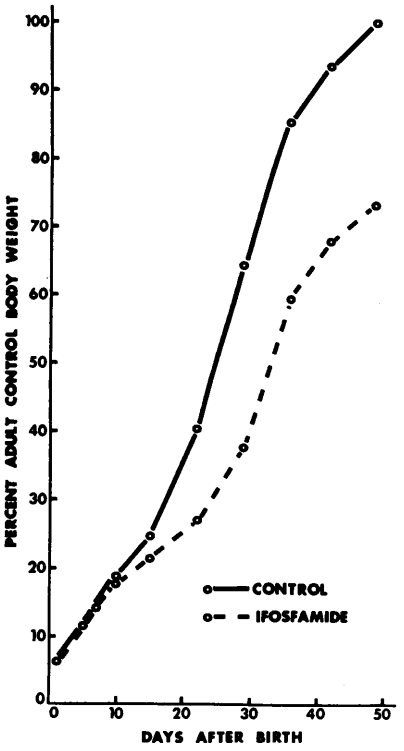

FIG. 1. Effect of ifosfamide, 45 mg/kg sc on Day 1, on growth and development of newborn mice. Each point is the mean mouse body weight within litters for 4–7 litters. There were 10 animals/litter on Day 1.

development. Mean body weight (per litter basis) was significantly reduced compared to controls from 15 days after treatment until termination of the experiment. Treated animals weighed 73.2% of controls at 49 days (Fig. 1). No mortalities were observed among control or ifosfamide-treated animals throughout the experiment. Ifosfamide, 45 mg/kg sc on Day 1, significantly decreased the ratio of tail length to body length at 49 days (0.86 ± 0.01 vs 0.97 ± 0.01 for controls), which indicated a disproportionate shortening of the tail.

Peak plasma levels of NBP alkylating products in adult mice after ifosfamide and cyclophosphamide (Fig. 2) were higher for cyclophosphamide (13.8 μg/ml) than for ifosfamide (4.5 μg/ml). Peak levels were reached 16 min after administration and declined to negligible levels 256 min after administration. Ifosfamide alkylating activity was significantly less than cyclophosphamide alkylating activity 16 and 32 min after injection.

Discussion. Ifosfamide administration to mice on Day 11 of gestation produced teratogenic and embryotoxic effects qualitatively similar to those of cyclophosphamide (14). Doses used in the two studies were equimolar. Several effects of prenatal administration of ifosfamide, however, were different from those observed with cyclophosphamide. Cyclophosphamide, but not ifosfamide, showed dose-related embryotoxicity (fetal resorption) at 5, 10 and 20 mg/kg. Ifosfamide caused resorptions only at the higher dose. Cyclophosphamide produced no significant skeletal anomalies at 5 and 10 mg/kg, whereas ifosfamide significantly increased the incidence of fused vertebrae and supernumerary ribs at those doses. Thus, induction of skeletal anomalies appears more sensitive to administration of ifosfamide than to cyclophosphamide. Ifosfamide, 20 mg/kg, produced a higher incidence of hydronephrosis (77.7%) than cyclophosphamide. A recent clinical study (21) reported that single high doses of ifosfamide produced marked nephrotoxicity.

Ifosfamide, 45 mg/kg, given to 1 day old mice produced neonatal toxicity similar to cyclophosphamide, 45 mg/kg (18). The two drugs, however, induced different incidences of mortality resulting from administration of equivalent doses. Ifosfamide produced no mortalities during the 49 day experimental period while cyclophosphamide mortality reached 35% after 49 days.

Prenatal toxicity differences induced by ifosfamide and cyclophosphamide may be explained in several ways. First, the decreased level of plasma alkylating metabolites in adult mice for ifosfamide with respect to cyclophosphamide (Fig. 2) may have resulted in differences in the absolute amount or time period of exposure of drug in contact with embryos. Cyclophosphamide teratogenicity was decreased by phenobarbital pretreatment and was associated with increased maternal plasma alkylating metabolites (22). Furthermore, structural analogs of cyclophosphamide, which were of greater polarity and produced levels of alkylating metabolites higher than

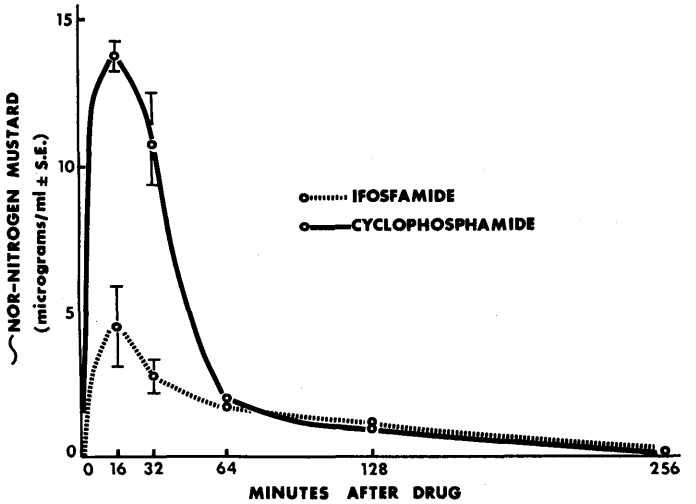

FIG. 2. The levels of alkylating metabolites (measured in the plasma of adult mice after 100 mg/kg ip doses) of ifosfamide and cyclophosphamide. Alkylating activity is expressed as equivalents of nor-nitrogen mustard. Each point is the mean ± SE of 3 determinations.

cyclophosphamide, also induced a lesser degree of teratogenicity (23). Bus, Short and Gibson (18) suggested that cyclophosphamide may cross the placenta more readily than the polar alkylating metabolites and thereupon slowly release toxic alkylating metabolites in the embryo. A similar situation may exist for ifosfamide. Second, reduced absorption of ifosfamide may have decreased alkylating metabolite formation. This appears unlikely, however, considering that peak alkylating activity for both compounds was reached 16 min after injection and that ifosfamide activation is reduced in rat liver homogenates when compared to cyclophosphamide (1). Third, selective pooling of possibly toxic metabolites of either drug due to differential elimination may be discounted in that the disappearance of alkylating metabolites occurred at an equal rate for both agents from 64 to 256 min after injection. The postnatal toxicity of ifosfamide, particularly the mortality rate, may have also been diminished by decreased formation of alkylating metabolites, the level of which have been shown to effect postnatal mortality with cyclophosphamide (18). A final explanation for both pre- and postnatal toxicity differences is that the two drugs may have quantitative differences in their interactions with receptor sites. This has been observed in a comparison of the cardiovascular activities of ifosfamide and cyclophosphamide (24).

Summary. Ifosfamide (IFA) is a structural analog of cyclophosphamide (CP), an alkylating agent which causes perinatal toxicity in mice. The purpose of this study was to examine the teratogenicity and neonatal toxicity of IFA in Swiss Webster mice. IFA, 0, 5, 10 and 20 mg/kg ip, was given to pregnant mice on Day 11 of gestation and teratogenic effects were determined on Day 19. In other experiments, IFA, 45 mg/kg sc, was given to 1 day old mice and growth and development were followed for 49 days. Plasma levels of IFA and CP alkylating metabolites were determined in adult mice given doses of 100 mg/kg ip. IFA, 20 mg/kg, significantly increased resorption rate to 81.2 ± 5.6% and 10 and 20 mg/kg IFA significantly reduced fetal body weight to 82.5 and 48.6% of controls, respectively. IFA, 20 mg/kg, significantly increased the incidence of open eyes, internal and external hydrocephalus, micromelia, adactyly, syndactyly, microcaudate, kinky tail, microphakia, kidney ectopia, hydronephrosis, missing or non-ossified skull bones, sternbrae, vertebrae and long bones. IFA, 5 mg/kg, significantly increased the incidence of supernumerary ribs. IFA admini-

stration to 1 day old mice significantly reduced mean body weight to 73.2% of controls and the ratio of tail length to body length from 0.97 to 0.86 after 49 days. Mortality was not increased. The levels of plasma alkylating metabolites in adult mice after IFA were significantly less than after CP. Neonatal toxicity induced by IFA resembled that of CP, while the teratogenic response to IFA, although qualitatively similar to CP, was quantitatively different.

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