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Induction of specific-locus and dominant lethal mutations in male mice by ifosfamide (Holoxan)†

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Summary

Ifosfamide induced dominant lethal mutations in spermatozoa of mice at doses of 200 and 300 mg/kg and in spermatids and spermatocytes at 600 mg/kg. The highest dose also induced specific-locus mutations in post-spermatogonial germ-cell stages of mice but not in spermatogonial stem cells. The nature of the induced mutations suggests they are intergenic. The spermatogenic specificity of ifosfamide in mouse germ cells is similar to that of the structurally related cytostatic drugs cyclophosphamide and trofosfamide. Due to the post-spermatogonial germ cell specificity of ifosfamide, the genetic risk is limited to a few weeks after exposure.

1. Introduction

Ifosfamide is a phosphamide ester of nitrogen mustard. It is structurally closely related to cyclophosphamide and trofosfamide and differs only in the side chains of the N-atoms (Brock, 1983). It undergoes metabolic transformation to cytostatic and immunosuppressive metabolites (Brock *et al*., 1973; Struck *et al*., 1994; Wagner, 1994; Fleming, 1997). The most important therapy-limiting factor is its urotoxicity, which manifests itself mainly in haemorrhagic cystitis due to renal elimination of aggressive metabolites (Brock, 1983; Zalupski & Baker, 1988; Springate & van Liew, 1995).

Ifosfamide is mutagenic in *Escherichia coli* and in *Salmonella typhimurium* after biotransformation through extracts of rodent liver (Ellenberger & Mohn, 1975; Benedict *et al*., 1977; Della Morte *et al*., 1986). It also induces gene conversion in yeast in the rat host mediated assay or when yeast was treated with urine of ifosfamide-treated rats (Siebert, 1974). Genetically engineered V79 cells that express cytochrome P450IIBI and P450IA1 are able to transform ifosfamide to mutagenic metabolites (Doehmer *et al*., 1990). In *Drosophila melanogaster*, ifosfamide induces sex-linked recessive lethal mutations mainly in mature sperm (Vogel, 1975). Sister-chromatid exchanges, as well as gene mutations, are found in V79 cells implanted in diffusion chambers into mice treated with ifosfamide (Sirianni & Huang, 1980). Chromosomal aberrations were induced by ifosfamide in bone marrow cells of mice, rats and Chinese hamsters (Röhrborn & Basler, 1977). Given the relevant human exposure by chemotherapy it is important to determine whether ifosfamide poses a genetic risk to humans, i.e. whether it is mutagenic in germ cells. In the present experiments ifosfamide was tested for induction of dominant lethal and specific-locus mutations in germ cells of male mice.

2. Materials and methods

(i) *Animals and treatment*

Ifosfamide (CAS no. 3778-73-2, Holoxan, Asta Pharma, Frankfurt, Germany) was dissolved in bi-distilled water. The solution was injected intraperitoneally into male mice within 20 min of preparation. The injected volume was 0·5 ml per animal and the weight of the mice ranged from 24 to 29 g. Males were selected such that the range of body weights did not deviate more than 5% from the group

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† This paper is dedicated to our friend and colleague Bruce Cattanach on the occasion of his retirement.

Table 1. *Induction of dominant lethal mutations in (102/E1 × C3H/E1)F1 male mice by ifosfamide (Holoxan)*

Dose (mg/kg)	Mating interval (days)	Females with implants		Corpora lutea per female	Preimplantation loss per female	Total implants		Lie implants		Dead implants			Dominant lethal mutations[a]
		n	%			*n*	Per female	*n*	Per female	*n*	%	Per female	
First experiment													
0	1–4	39	97·5	12·3	1·0	441	11·3	402	10·3	39	8·8	1·0	
	5–8	40	100	12·5	1·2	452	11·3	405	10·1	47	10·4	1·2	
	9–12	37	92·5	12·3	1·2	410	11·1	360	9·7	50	12·2	1·4	
	13–16	40	100	12·3	1·7	422	10·5	378	9·4	44	10·4	1·1	
	17–20	38	95·0	12·8	1·2	438	11·5	408	10·7	30	6·8	0·8	
	21–24	39	97·5	12·6	1·5	430	11·0	393	10·1	37	8·6	0·9	
300	1–4	37	92·5	11·6	2·5	337	9·1	244	6·6	93	27·6*	2·5	36·0
	5–8	38	95·0	12·4	1·9	401	10·6	335	6·8	66	16·5*	1·7	12·9
	8–12	40	100	12·6	1·4	450	11·3	384	9·6	66	14·7	1·6	1·3
	13–16	40	100	12·6	1·7	436	10·9	384	9·6	52	11·9	1·3	−1·6
	17–20	40	100	12·4	1·6	430	10·8	380	9·5	50	11·6	1·3	11·5
	21–24	39	97·5	12·6	1·0	451	11·6	402	10·3	49	10·9	1·3	−2·3
600	1–4	35	89·7	11·4	4·5	243	6·9	125	3·6	118	48·6*	3·4	65·4
	5–8	35	89·7	11·9	3·3	302	8·6	182	5·2	120	39·7*	3·4	48·6
	9–12	37	94·9	12·8	2·2	391	10·6	315	8·5	76	19·4*	2·1	12·5
	13–16	35	89·7	12·4	1·6	378	10·8	294	8·4	84	22·2*	2·4	11·1
	17–20	37	94·9	12·3	2·2	375	10·1	309	8·4	66	17·6*	1·8	22·2
	21–24	33	84·6	12·7	3·3	311	9·4	247	7·5	64	20·6*	1·9	25·7
Second experiment													
0	1–4	34	100	12·7	1·4	385	11·3	349	10·3	36	9·4	1·1	
	5–8	35	100	12·9	1·3	406	11·6	372	10·6	34	8·4	1·0	
	9–12	35	100	12·7	1·1	404	11·5	372	10·6	32	7·9	0·9	
	13–16	34	97·1	12·6	1·0	393	11·6	360	10·6	33	8·4	1·0	
	17–20	34	97·1	12·5	1·5	374	11·0	349	10·3	25	6·7	0·7	
	21–24	35	100	12·5	1·1	400	11·4	361	10·3	39	9·7	1·1	
	25–28	35	100	12·5	1·1	399	11·4	364	10·4	35	8·8	1·0	
	29–32	34	97·1	13·0	1·4	396	11·6	362	10·6	34	8·6	1·0	
	33–36	34	97·1	12·6	1·4	382	11·2	346	10·2	36	9·4	1·1	

200	1–4	35	100	12·4	1·8	373	10·7	285	8·1	88	23·6*	2·5	20·7
	5–8	35	100	12·6	1·6	385	11·0	329	9·4	56	14·5*	1·6	11·6
	9–12	35	100	12·8	1·5	395	11·3	350	10·0	45	11·4	1·3	5·9
	13–16	35	100	12·4	1·3	387	11·1	353	10·1	34	8·8	1·0	4·7
	17–20	32	91·4	12·4	1·1	364	11·4	327	10·2	37	10·2	1·2	0·4
	21–24	34	97·1	12·8	1·4	386	11·4	351	10·3	35	9·1	1·0	−0·1
	25–28	35	100	12·9	1·9	384	11·0	340	9·7	44	11·5	1·3	6·6
	29–32	35	100	13·1	1·2	417	11·9	387	11·1	30	7·2	0·9	−3·9
	33–36	34	97·1	13·1	2·1	374	11·0	337	9·9	37	9·9	1·1	2·6
600	1–4	18	66·7	11·4	5·7	103	5·7	49	2·7	54	52·4*	3·0	73·5
	5–8	23	85·2	12·1	3·8	192	8·3	104	4·5	88	45·8*	3·8	57·5
	9–12	25	92·6	12·1	1·9	256	10·2	205	8·2	51	19·9*	2·0	22·8
	13–16	25	92·6	12·7	2·0	268	10·7	203	8·1	65	24·3*	2·6	23·3
	17–20	25	92·6	12·8	1·9	273	10·9	214	8·6	59	21·6*	2·4	16·6
	21–24	24	88·9	12·3	3·0	223	9·3	169	7·0	54	24·2*	2·3	31·7
	25–28	25	92·6	13·3	2·3	276	11·0	244	9·8	32	11·6	1·3	6·2
	29–32	25	92·6	12·8	1·3	288	11·5	260	10·4	28	9·7	1·1	2·3
	33–36	27	100	12·7	2·0	288	10·7	260	9·6	28	9·7	1·0	5·4

* $p < 0{\cdot}05$ for the comparison with the corresponding control group.
[a] Calculation is based on absolute figures.

mean. Control males in the dominant lethal experiments were injected with an equal volume of the solvent. The ifosfamide exposure doses were 200, 300 and 600 mg/kg body weight (b.w.) in the dominant lethal experiments and 600 mg/kg b.w. in the specific-locus experiment. These doses were sublethal.

(ii) *Dominant lethal test*

Dominant lethal mutation tests were conducted using 13- to 14-week-old (102/E1 × C3H/E1)F1 male mice, following the standard protocol (Ehling, 1977; Ehling *et al*., 1978). Groups of 40 males each were dosed with the test compounds at 0, 300 and 600 m/kg b.w. in the first experiment and groups of 35 males were dosed with the test compound at 0, 200 and 600 mg/kg b.w. in the second experiment. After injection, each male was caged with one virgin (102/E1 × C3H/E1)F1 female mouse, 13–14 weeks old. Females were replaced every 4 days for six sequential matings in the first experiment and nine sequential matings in the second experiment. Successful matings were detected by daily examination of females for vaginal plugs. The females were killed 14–17 days after observation of a vaginal plug. The uterine contents were inspected for the number of total, live and dead implantations. The corpora lutea were visualized with the aid of a low-power dissecting microscope. The frequency of induced dominant lethal mutations (DL) was calculated as follows:

$$\mathrm{DL}\ (\%) = \left[1 - \frac{\left[\dfrac{\text{live embryos}}{\text{female in experimental group}}\right]}{\left[\dfrac{\text{live embryos}}{\text{female in control group}}\right]}\right] \times 100.$$

(iii) *Specific-locus test*

For the specific-locus experiments, (102/E1 × C3H/E1)F1 male mice, 9–16 weeks old, received an intraperitoneal injection of 600 mg ifosfamide per kilogram b.w. After treatment, the males were mated with one untreated test-stock female, 10–13 weeks old. The sequential mating procedure used in this experiment extended over five intervals of 4 days. For each new mating interval untreated virgin test-stock females were used. A permanent monogamic mating schedule started 43 days after treatment to recover offspring derived from treated stem cell spermatogonia. The day of conception was determined as the day of birth minus the gestation time of 19 days. The experiment comprised two replicates of 175 treated males.

The test-stock females were homozygous for the

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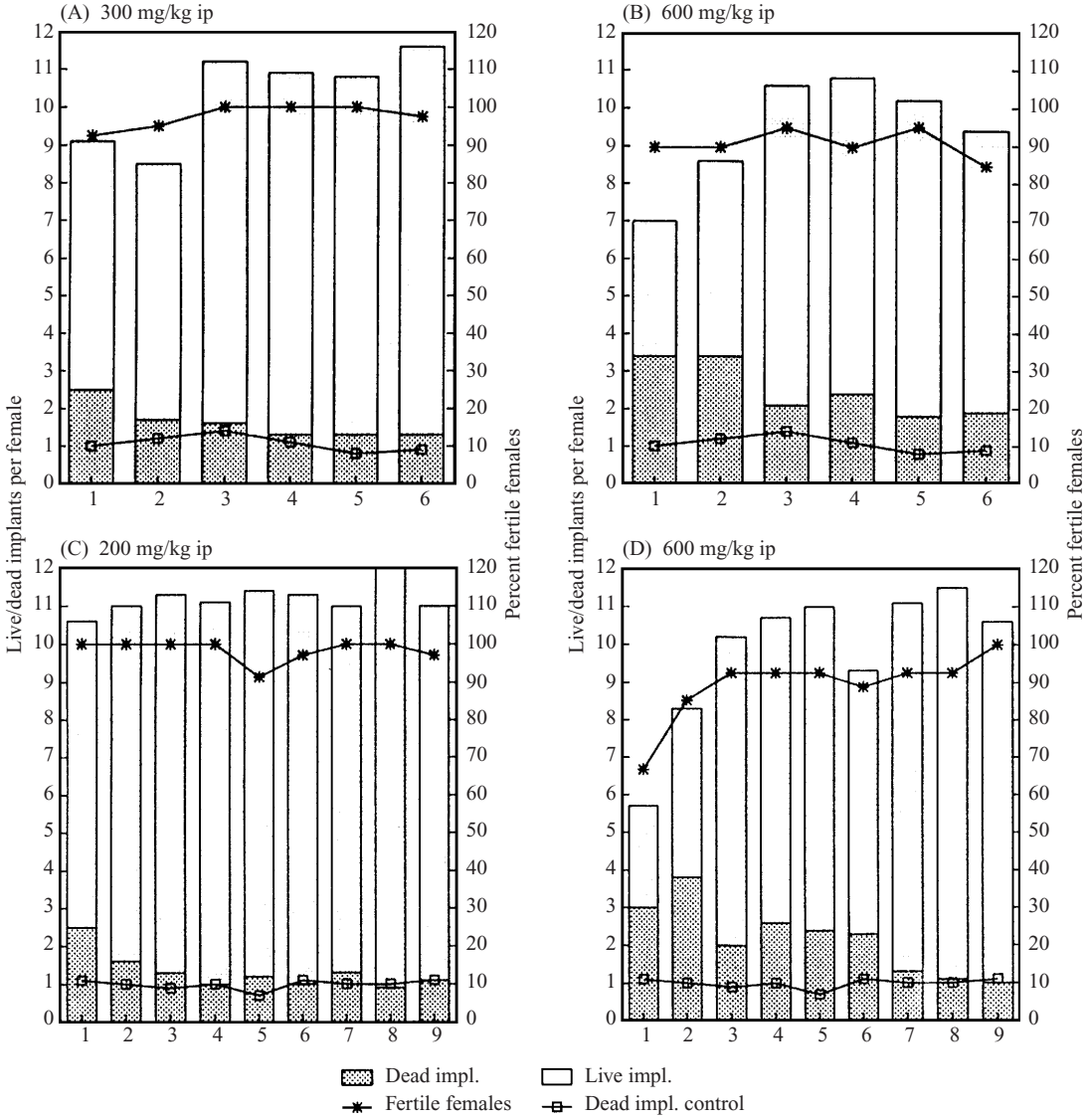

Fig. 1. Dominant lethal test with ifosfamide. Male mice were mated consecutively to individual females at 4 day intervals. Mating interval 1 corresponds to 1–4 days post-treatment, 2 to 5–8 days, 3 to 9–12 days, 4 to 13–16 days, 5 to 17–20 days, 6 to 21–24 days, 7 to 25–28 days, 8 to 29–32 days, and 9 to 33–36 days post-treatment. Detailed data are given in Table 1. (*A*) and (*B*) First experiment; (*C*) and (*D*) second experiment.

following seven recessive markers: *a* (non-agouti), *b* (brown), c^{ch} (chinchilla), *d* (dilute), *p* (pink-eyed dilution), *s* (piebald) and *se* (short-ear). These seven loci are distributed among five autosomes. The *c* and *p* loci are linked on chromosome 7, 9–15 cM apart, and the *d* and *se* loci are closely linked on chromosome 9, only 0·12 cM apart (Roderick *et al.*, 1996).

The offspring were counted, sexed and carefully examined externally at birth for variant phenotypes. The litters were re-examined when cages were changed, with a final examination at weaning age. The presumed mutations were genetically tested and confirmed by an allelism test. A detailed description of the specific-locus test was published by Cattanach (1971) and Ehling (1978).

(iv) *Statistics*

The allocation of the animals to the various treatment groups was based on a statistically randomized procedure (Ehling, 1977). For the dominant lethal experiments, statistical comparisons were made with concurrent controls and were confined to post-implantation losses and per cent fertile matings as described by Vollmar (1977). For the specific-locus experiments statistical comparisons were based on the data from the historical control of the laboratory. For the analysis of the specific-locus mutation data the calculations of the probabilities were based on Fisher's exact treatment of a 2×2 table employing computer routines available from SAS PROC FREQ (release

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6.10, SAS System for Windows, 1994, SAS Institute, Cary, NC).

3. Results

If the dynamics of spermatogenesis is not affected by the treatment, the treated gametogenic stage from which a conception arose may be ascertained by the time interval between treatment and conception as follows: spermatozoa (1–7 days), spermatids (8–21 days), spermatocytes (22–35 days) followed by differentiating spermatogonia (36–42 days) and stem-cell spermatogonia ($\geqslant$ 43 days) (Oakberg, 1968).

(i) *Dominant lethal mutations*

The frequency of induced dominant lethal mutations as calculated reflects any reduction in the number of live implants due to treatment when compared with the concurrent control group. This may include an increase in the frequency of dead implants as well as an increase in the frequency of preimplantation losses. An increase in the frequency of preimplantation loss may be due to a dominant lethal effect (death of fertilized eggs at an early embryonic stage) or an excess of unfertilized ova, a cytotoxic effect. The difference between total implantations per female and live embryos per female represents post-implantation death. The relationship between sample size and our ability to detect dominant lethal mutations was analysed by Vollmar (1977).

The results of the dominant lethal experiments are summarized in Table 1. Single doses of 200 or 300 mg ifosfamide per kilogram b.w. induced dominant lethal mutations primarily in spermatozoa, corresponding to mating intervals 1 and 2 (1–8 days post-treatment). A single dose of 600 mg ifosfamide per kilogram b.w. also affected spermatids and spermatocytes, corresponding to mating intervals 1–6 (1–24 days post-treatment). With the higher dose the proportion of dominant lethal mutations due to preimplantation loss increased as indicated by a reduction in the total number of implants per female. Effects on fertility were observed only in the highest dose group. Fig. 1 demonstrates graphically the induction of dominant lethal effects following treatment of male mice with ifosfamide expressed as dead implants per female.

(ii) *Specific-locus mutations*

The sensitivity of the different germ-cell stages to the induction of specific-locus mutations by ifosfamide is summarized in Table 2. After treatment with 600 mg ifosfamide per kilogram b.w., a total of eight specific-locus mutations were observed in mating intervals 2–5 (5–20 days post-treatment) among 7246 offspring raised. The derived mutation rate for post-spermatogonial stages of $15{\cdot}8 \times 10^{-5}$ mutations per gamete per locus is significantly different from the historical control in our laboratory ($p < 0{\cdot}01$).

With ifosfamide there was no increase in the frequency of specific-locus mutations in stem-cell spermatogonia of mice. One observed mutation among 20071 offspring ($\geqslant$ 43 days after treatment) rules out at the 5% significance level an observed rate greater than 2·2 times the historical control rate (Ehling *et al.*, 1988).

The litter size reduction in the specific-locus experiment corresponds well with the induced frequency of dominant lethal mutations. The simultaneous induction of dominant lethal and specific-locus mutations in post-spermatogonial germ-cell stages by ifosfamide suggests that both types of mutations are due to intergenic changes. In spermatogonia the tested mutation was homozygous-viable.

Table 2. *Induction of specific-locus mutations by isofosfamide* (*Holoxan*) *in germ cells of male mice*

Dose (mg/kg)	Mating intervals (days)	No. of offspring	Average litter size	No. of mutations at 7 loci	Mutations per locus per 10^5 gametes
Historical control		271625	–	22[a]	1·2
600	1–4	304	2·3	–	–
	5–8	1243	4·5	1 (*p*)	11·5
	9–12	2068	6·8	2(2*p*)[b]	13·8
	13–16	1791	6·2	3 (c^a, *p*, *s*)	23·9
	17–20	1840	6·4	2 (*p*, *d*)	15·5
	Total	7246		8	15·8*
	$\geqslant$ 43	20071	8·1	1 (*s*)	0·7

* $p < 0{\cdot}01$.
[a] Includes one cluster of 2 *se* and one cluster of 6 *s* mutants.
[b] Additionally, one sterile possible specific-locus mosaic was observed.

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The great majority of mutations induced by chemical mutagens in spermatogonia are due to intragenic changes (Ehling, 1991).

4. Discussion

Ifosfamide induced mutations only in the post-spermatogonial germ-cell stages of mice. A similar induction pattern for mutations in germ cells of mice was also observed for the structurally related cytostatic drugs cyclophosphamide and trofosfamide (Ehling & Neuhäuser-Klaus, 1988, 1994). The only difference is that with ifosfamide at the highest dose late spermatocyte stages also were affected in the dominant lethal test. The induction of specific-locus mutations in spermatozoa and spermatids of mice allows the characterization of the genetic risk due to ifosfamide treatment of patients.

Specific-locus experiments in mice had been suggested by the EPA (1986) as the basis for the quantification of a potential genetic hazard to humans. Using data from a specific-locus experiment in mice for generating quantitative information concerning heritability of genetic effects, an assessment of the heritable risk of acrylamide was published by Dearfield *et al*. (1995). A similar approach can be used for the quantification of the genetic risk of ifosfamide in humans.

Knowledge of the differential spermatogenic induction of germ-cell mutations in mice is very important for the genetic risk assessment: beyond the stem-cell spermatogonia, cells that go through meiotic divisions and through successive differentiation stages have only a transitory existence. This time interval is 5 weeks in mice (Oakberg, 1968) and almost twice as long in man (Heller & Clermont, 1964). In contrast to post-spermatogonial germ-cell stages, A_s (stem-cell) spermatogonia continue to divide throughout reproductive life. Since ifosfamide affects only post-spermatogonial germ cell stages, the entailed risk is limited to a few weeks after exposure. The doubling doses for induced specific-locus mutations can be compared between the three related drugs. The point estimates for the doubling doses of cyclophosphamide and trofosfamide are 6·0 and 7·5 mg/kg, respectively (Ehling & Neuhäuser-Klaus, 1994). The comparable doubling dose for ifosfamide based on the present experiment is one order of magnitude higher, namely 49·3 mg/kg. The difference may be due to the speed of renal elimination of the reactive metabolites. Thus, the ranking of the three chemicals for their effectiveness in inducing specific-locus mutations in post-spermatogonial germ-cell stages of mice is cyclophosphamide = trofosfamide > ifosfamide. This is in contrast to most other mutagenicity assay systems where the ranking was cyclophosphamide < ifosfamide < trofosfamide (Mohn & Ellenberger, 1976). Our results emphasize the importance of mammalian genetic tests for the genetic risk evaluation of drugs.

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