[CANCER RESEARCH 50, 252-255, January 15, 1990]

Efficacy, Toxicity, Pharmacokinetics, and *in Vitro* Metabolism of the Enantiomers of Ifosfamide in Mice[1]

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ABSTRACT

The enantiomers of the anticancer drug ifosfamide, (+)-(R)-IFF and (−)-(S)-IFF, were prepared from the racemic compound rac-IFF using enantioselective liquid chromatographic techniques. The efficacy, toxicity, and pharmacokinetics of the individual enantiomers and rac-IFF were studied in mice. The results of the studies indicate that there were no statistically significant differences between the efficacy of (+)-(R)-IFF, (−)-(S)-IFF, and rac-IFF against childhood rhabdomyosarcoma (HxRh28) maintained *in vivo* as a xenograft in immune-deprived female CBA/CaJ mice. Similar results were found in toxicity and pharmacokinetic studies conducted in non-tumor-bearing female CBA/CaJ mice. The production of two major metabolites, aldoifosfamide and isophosphoramide mustard, by mice hepatic microsomes from non-tumor-bearing female CBA/CaJ mice was also investigated. There were no statistically significant differences in the calculated kinetic parameters, V_{max} and K_m, of the production of these two metabolites when the separate enantiomers or the racemic mixture were used as substrate.

INTRODUCTION

In the past few years, it has become clear that the individual stereoisomers, especially the enantiomers, of a biologically active chiral molecule may differ in potency, pharmacological action, metabolism, toxicity, plasma disposition, and urine excretion kinetics (1, 2). This possibility exists for a number of anticancer drugs which are administered as stereoisomeric mixtures.

Ifosfamide is a cyclophosphamide analogue which belongs to a class of anticancer drugs known as oxazaphosphorine nitrogen mustards. IFF[3] is a chiral molecule which contains an asymmetrically substituted phosphorus atom and which exists in two enantiomeric forms, (+)-(R)-IFF and (−)-(S)-IFF. The clinical preparation of this agent is a racemic (50:50) mixture of the enantiomers, rac-IFF.

The pharmacological differences between stereoisomers often arise during the metabolism of the agents (1, 2). This possibility is particularly relevant in the case of IFF since the compound itself is not an active antitumor agent and must be metabolically transformed into the alkylating agent, isophosphoramide mustard (Fig. 1). The first step of this metabolism involves an oxidation of the molecule by hepatic microsomal enzymes. This oxidation can occur either at the 4 position of the oxazaphosphorine ring or at one of the chloroethyl groups. The former pathway leads to the formation of 4-hydroxyifosfamide and eventually to IPM, while the latter pathway leads to the formation of the inactive metabolites 2- and 3-*N*-dechloroethyl-IFF and the central nervous system toxin chloroacetaldehyde. Dechloroethylation can account for up to 48% of the administered IFF (3, 4).

Other metabolic pathways leading to inactive metabolites involve further oxidation of 4-hydroxyifosfamide and its tautomeric form aldoifosfamide to form the inactive metabolites 4-ketoifosfamide and carboxyifosfamide, respectively (5). One or more of these metabolic pathways can be stereoselective leading to a difference in efficacy and toxicity between (+)-(R)-IFF and (−)-(S)-IFF. Some initial reports have, in fact, indicated that there is a substantial stereoselectivity in IFF efficacy, toxicity, disposition, and metabolism (6–9).

Kusnierczyk *et al.* (6) have reported the synthesis of the pure enantiomers of IFF and tested them *in vivo* against four transplanted tumor models, L1210 and P388 lymphoid leukemias, Lewis lung carcinoma, and mouse mammary carcinoma 16C MAC. They observed a higher therapeutic index against all tumors except L1210 when the (−)-(S)-IFF was administered alone. The toxicity of this enantiomer was also found to be higher (6). However, Blaschke *et al.* (10) have chromatographically prepared the enantiomers of IFF from the racemate and observed no difference between (+)-(R)-IFF, (−)-(S)-IFF, and rac-IFF in acute toxicity and activity against L5222 leukemia in rats.

A stereospecific difference in the metabolism and clearance of the enantiomers of IFF has also been reported. Misiura *et al.* (7) observed that the urines of two patients who were administered rac-IFF were enriched with (+)-(R)-IFF and (−)-(S)-2-dechloroethyl-IFF. In this instance, the production of the dechloroethylated metabolite from (−)-(S)-IFF was 2.7- to 6.7-fold higher than from the corresponding (+)-(R)-IFF. Blaschke *et al.* have studied 50 patients receiving 3000 mg/m^2 of rac-IFF as a 48-h continuous infusion and found a stereoselective formation of the 2- and 3-dechloroethyl-IFF.[4] They have found (S):(R) ratios for the 3-dechloroethylated metabolite of as high as 85:15. In addition, initial studies in pediatric patients receiving rac-IFF have demonstrated a significantly higher plasma clearance for (−)-(S)-IFF relative to the (+)-(R)-enantiomer (8).

Blaschke and Widey (9) have also investigated the metabolism of the enantiomers of IFF in mice by using the individual radiolabeled enantiomers. The results indicated that (*a*) The ketoifosfamide was almost entirely produced from (−)-(S)-IFF, refuting the previously suggested rapid equilibrium between 4-hydroxyifosfamide and aldoifosfamide; (*b*) the 2-*N*-dechloroethyl-IFF was produced to a greater extent from (+)-(R)-IFF [(R):(S) of about 2:1]; and (*c*) the 3-*N*-dechloroethyl-IFF was produced to a greater extent from (−)-(S)-IFF [(R):(S) of 1:3].

In order to further investigate the efficacy, toxicity, and disposition of the enantiomers of IFF, we have prepared enantiomerically pure (+)-(R)-IFF and (−)-(S)-IFF by using enantioselective liquid chromatography (11). The enantiomers have been studied alone and in combination with each other in both *in vivo* and *in vitro* systems. The *in vivo* investigations involved efficacy studies with a childhood rhabdomyosarcoma (HxRh28) maintained as a xenograft in immune-deprived female CBA/CaJ mice and toxicity and pharmacokinetic studies conducted in non-tumor-bearing female CBA/CaJ mice. The *in vitro* stud-

Received 5/8/89; revised 9/27/89; accepted 10/13/89.

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[1] This work was supported by Grant CA23099 from the National Cancer Institute, by the Cancer Center Support (CORE) Grant CA21765, and by the American Lebanese Syrian Associated Charities.

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[3] The abbreviations used are: IFF, ifosfamide; IPM, isophosphoramide mustard.

[4] G. Blaschke, personal communication.

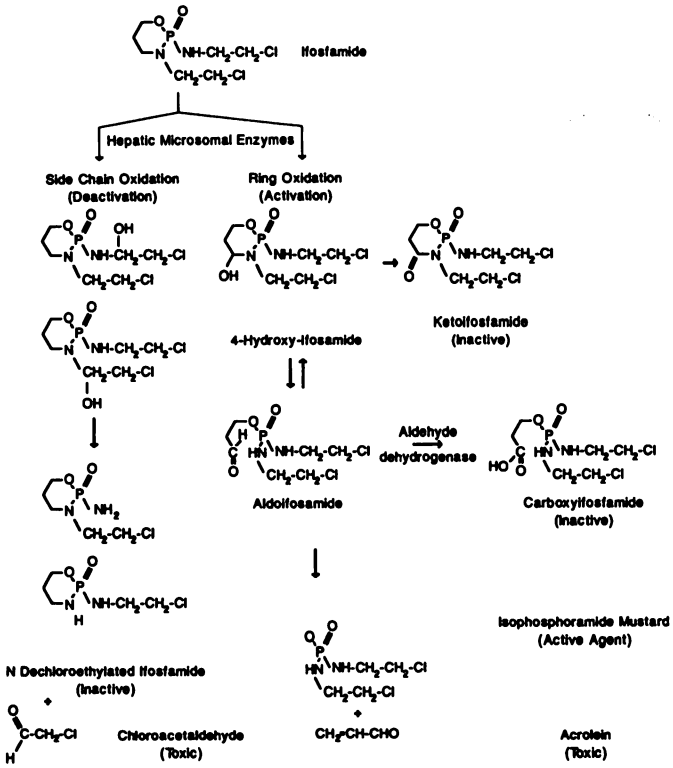

Fig. 1. Metabolism of ifosfamide.

ies investigated only one of the two possible metabolic pathways arising from the oxidation of IFF at the 4 position of the oxazaphosphorine ring. These studies determined the production of two major metabolites, aldoifosfamide and IPM, by mice hepatic microsomes from non-tumor-bearing female CBA/CaJ mice.

The results of the *in vivo* studies indicate that there are no statistically significant differences in the efficacy, toxicity, or pharmacokinetic disposition of (+)-(R)-IFF, (−)-(S)-IFF, and rac-IFF. The results of the *in vitro* studies were consistent with these findings. There were no statistically significant differences in the calculated kinetics parameters, V_{max} and K_m, of the production of aldoifosfamide or IPM when the separate enantiomers or the racemic mixture were used as substrate.

MATERIALS AND METHODS

Chemicals

Rac-IFF was a gift from Bristol-Myers Co. (Evansville, IN). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO).

Chromatographic Preparation of Enantiomers of IFF

The preparation of the pure enantiomers of IFF was accomplished on a cellulose-based high-pressure liquid chromatography chiral stationary phase (Chiralcel OD; J. T. Baker, Phillipsburg, NJ). The chromatographic conditions have been reported elsewhere (11). The assignment of the steric configurations was accomplished by the comparison of chromatographic and absolute rotation with samples of the (+)-(R)- and (−)-(S)-enantiomers provided by G. Blaschke (University of Munster, Munster, Federal Republic of Germany). The enantiomeric purity of the collected compounds was 99.6% for both enantiomers as determined by enantioselective gas chromatography (8).

In Vivo Studies

Efficacy Studies. The enantiomers of IFF were evaluated against a childhood rhabdomyosarcoma (HxRh28) maintained *in vivo* as a xenograft in immune-deprived female CBA/CaJ mice (12). Groups of 7 mice were treated with a single i.p. administration of rac-IFF, (+)-(R)-IFF, or (−)-(S)-IFF in doses of 525 mg/kg and 600 mg/kg. The growth of tumors was assessed from the measurement of two perpendicular diameters at 7-day intervals by using vernier calipers over a 42-day period.

Toxicity Studies. The comparative toxicity of the enantiomers of IFF was investigated by treating groups of 7 female CBA/CaJ mice weighing 22 ± 2 g with a single i.p. administration of rac-IFF, (+)-(R)-IFF, or (−)-(S)-IFF in doses ranging from 475 to 650 mg/kg. The experiments were run in duplicate and the data (weight loss and mortality) were collected for 21 days.

Pharmacokinetic Studies. The pharmacokinetic parameters of the enantiomers of IFF were determined after the administration of 450 mg/kg rac-IFF i.p. to female CBA/CaJ mice weighing 22 ± 2 g. The mice were sacrificed at 15 (5 mice), 30 (5 mice), 60 (13 mice), 120 (8 mice), 240 (7 mice), and 480 (8 mice) min after dosing. The concentrations of IFF enantiomers in the plasma were measured by an enantioselective gas chromatographic assay, using a chiral stationary phase (Chirasil-Val III, Alltech Associates, Deerfield, IL) and a Hewlett-Packard Model 5890 gas chromatograph equipped with a Model 5790 mass selective detector (Hewlett-Packard Co., Avondale, PA) (8). A sum of exponentials was fit to the data to determine a plasma disappearance rate constant by using maximum likelihood with the assumption that the measurement error was proportional with a coefficient of variation of 10% for weighting each observation.

In Vitro Studies

The preparation of liver microsomes and the incubation mixture were based on the method of Sladek (13) and were accomplished in the following manner.

Preparation of Microsomes. Normal female CBA/CaJ mice were sacrificed and their livers were excised. The livers were homogenized in 1.15% KCl in 0.1 M phosphate buffer, pH 7.4 (1 ml/g of tissue), by 20 strokes in a Potter-Elvehjem homogenizer. Homogenates were then centrifuged in a Beckman L8-M centrifuge at 9,000 × *g* for 25 min (Ti 50 rotor, Beckman Instruments Inc., Houston, TX). Supernatants were transferred, avoiding the lipid layer, to a fresh tube and centrifuged at 105,000 × *g* for 60 min. The supernatant was discarded and the pellet was resuspended and homogenized (20 strokes) with 1 ml 1.15% KCl in 0.1 M phosphate buffer, pH 7.4, per g of original liver. The whole procedure was conducted at temperatures between 0 and 4°C. The microsomes were used the same day.

Incubation Mixture. The microsomal incubation mixture was composed of 1 ml of the liver microsomal fraction, 0.4 mM nicotinamide adenine dinucleotide phosphate, 4 mM glucose 6-phosphate, 0.4 unit/ml of glucose-6-phosphate dehydrogenase, 3 mM magnesium chloride, 0.68% potassium chloride in 0.06 M phosphate buffer, pH 7.4, in a final volume of 5 ml. The mixture was kept on ice.

Aldoifosfamide Assay. The incubation mixture was preheated at 37°C for 1.5 min. At time 0 the ifosfamide was added to the reaction mixture so that the final IFF concentration ranged from 0.153 to 2.299 μmol/ml. Samples (0.75 ml) were taken at time 1.3 and 6 min. The reaction was linear over this time period.

The samples were consecutively treated with 0.3 ml of 5.5% zinc sulfate, 0.3 ml barium hydroxide (saturated), and 150 μl of 0.01 M HCl. The resulting mixture was centrifuged (1000 × *g*, 5 min). The supernatant was filtered on a syringe-adaptable 0.45-μm nylon filter. A sample of the filtrate (0.75 ml) was derivatized with 0.4 ml of a solution containing 6 mg/ml of aminophenol, 6 mg/ml of hydroxylamine·HCl (3 M in HCl). The solution was heated at 100°C for 20 min, cooled to room temperature, and the concentration of the resulting derivative was determined in a spectrofluorimeter (340 nm excitation wavelength, 500 nm emission wavelength) (14). The concentration of aldoifosfamide was determined from standard curves constructed by using acrolein (14).

The kinetics of the production of aldoifosfamide were analyzed with a Michaelis-Menten model on the Enzfitt program (Elsevier-Biosoft, Cambridge, United Kingdom).

Isophosphoramide Mustard Assay. IFF was incubated at 37°C with

one-fourth of the microsomal incubation mixture and the remaining three-fourths were added in three equal portions every 15 min. The final IFF concentration was 7.66 μmol/ml. A 1-ml sample was taken 15 min after the addition of the last batch. It was consecutively treated with 0.4 ml zinc sulfate (5.5%) and barium hydroxide (saturated). The resulting mixture was centrifuged at 1000 × *g* for 5 min; 0.5 ml sodium acetate buffer (0.5 M, pH 4.0) and 0.2 ml *p*-nitrobenzylpyridine (5% in acetone) were added to 1 ml of the supernatant. The solution was heated at 100°C for 2.5 min, cooled to room temperature, and filtered. The filtrate was extracted twice with 3 ml of ethyl acetate. Sodium hydroxide (0.5 ml of a 6.5 N solution) was added to 1 ml of the extract and the absorption at 628 nm was measured 15 s after alkalinization (15). The reaction was followed at 628 nm rather than the previously reported 530 nm because the maximum absorbance was observed at this wavelength. The concentration of IPM was determined from a standard curve which was constructed with nitrogen mustard (Mustargen) as the alkylating agent.

RESULTS AND DISCUSSION

In Vivo Studies

Efficacy. The rhabdomyosarcoma xenograft was selected for this study on the basis of preliminary studies where it was shown to be moderately sensitive to IFF (data not shown). The growth of tumors in mice receiving (+)-(R)-IFF and (−)-(S)-IFF was examined at 525 and 600 mg/kg, and compared to that in mice receiving saline injection. Both enantiomers caused regressions at these dosage levels. The effect of the 600-mg/kg dose on cumulative relative tumor volume is presented in Fig. 2. At the lower dose, (−)-(S)-IFF caused a slightly but not significantly greater mean volume regression, although at 600 mg/kg, responses of tumors treated with either enantiomer were similar. In neither study did one enantiomer demonstrate significantly greater antitumor activity in terms of tumor regression or regrowth delay.

Toxicity. Toxicity was studied in non-tumor-bearing immune-deprived mice at dose levels up to 650 mg/kg. Mice received a single i.p. administration of rac-IFF or purified enantiomers. Toxicity was manifested by weight loss, which reached a nadir on day 9, and death. Survival data with percentage of weight loss are presented in Table 1. At all dose levels, (+)-(R)-, (−)-(S)-IFF, and rac-IFF demonstrated similar toxicity. At 650 mg/kg, the mean weight loss at the nadir was 30, 20, and 17% for (+)-(R)-IFF, (−)-(S)-IFF, and rac-IFF, respectively. Only in mice treated with (−)-(S)-IFF enantiomer were there obvious signs of bladder toxicity, as blood was observed in the urine.

Pharmacokinetic Studies. The plasma samples were collected over an 8-h period after a single i.p. injection of rac-IFF (450 mg/kg equivalent to 225 mg/kg of each isomer administered simultaneously). The plasma concentrations of the IFF enantiomers were quantifiable over the first 2 h of the study. The 4- and 6-h concentrations were too low to be included in the pharmacokinetic calculations. The data were well described by a single exponential ($r^2 = 0.97$ for both enantiomers). Over this time course, the estimated half-lives were 6.9 min for both enantiomers.

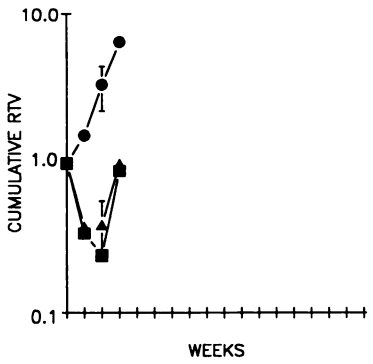

Fig. 2. Response of Rh28 xenografts to treatment with enantiomers of ifosfamide. Mice received a single i.p. administration of saline (●), 600 mg/kg (+)-(R)-IFF (▲), or 600 mg/kg (−)-(S)-IFF (■). Curves show the cumulative relative tumor volume for each set of experimental mice. See text for experimental details. *Bars*, relative SD where they exceeded symbol size.

Table 1 *Survival of non-tumor-bearing mice treated with a single i.p. dose of IFF*

Dose (mg/kg)	Deaths/total		
	(+)-(R)-IFF	(−)-(S)-IFF	Rac-IFF
550	0/7 (7)[a]	0/7 (13)	0/7 (12)
600	1/7 (14)	1/7 (13)	0/7 (17)
650	4/7 (30)	3/7 (20)	3/7 (17)

[a] Numbers in parentheses, percentage of maximal loss of weight for surviving mice in each treatment group.

These results are consistent with the findings of Blaschke and Widey (9). In this study, the enantiomers of IFF were administered separately to NMRI mice as a single 100-mg/kg i.p. injection of the ^{3}H-labeled compound. There were no statistically significant differences in plasma concentration-time curves of the enantiomers.

In Vitro Studies

Aldoifosfamide. The assay used to quantitate the aldoifosfamide produced by the microsomes involves the initial conversion of the aldoifosfamide into acrolein, followed by a further conversion into a fluorescent compound. It had been previously demonstrated that the naturally produced acrolein (Fig. 1) trapped in macromolecules does not interfere with the assay (14). The limit of sensitivity of the assay is 0.1 nmol/ml. Representative kinetic curves obtained by this method are presented in Fig. 3 and the kinetic parameters V_{max} and K_m are summarized in Table 2.

The results of this study indicate that there is no statistically significant difference in the microsomal production of aldoifosfamide when either rac-IFF, (+)-(R)-IFF, or (−)-(S)-IFF is used as substrate. These findings are consistent with the results obtained *in vivo* by Blaschke *et al.* (9), who did not observe a significant difference in plasma levels of aldoifosfamide or 4-hydroxyifosfamide in mice treated with the separate isomers of IFF.

Isophosphoramide Mustard. It has been demonstrated that *p*-nitrobenzylpyridine will react with the chloroethyl groups of the IFF and IPM molecules to produce a derivative which can be quantitated at 628 nm. IPM is the more reactive of the two, but IFF and other metabolites of IFF also react with *p*-nitrobenzylpyridine. We found that after 2.5 min at 100°C the alkylation of IPM was almost complete, whereas there was no significant alkylation by IFF or IFF metabolites. Therefore, limiting the reaction time to 2.5 min prevented any background due to IFF or other inactive metabolites produced by the microsomes.

The detection limit of the assay was 7 nmol/ml. After the incubation of rac-IFF, (+)-(R)-IFF, and (−)-(S)-IFF with the liver microsomes, the observed levels of IPM were 35, 40, and 38 nmol/ml, respectively, and were not significantly different ($P = 0.619$). These results also correlate with the findings of Blaschke *et al.* (9), who found no significant difference in the IPM levels found in the urine of mice treated with either (+)-(R)-IFF or (−)-(S)-IFF.

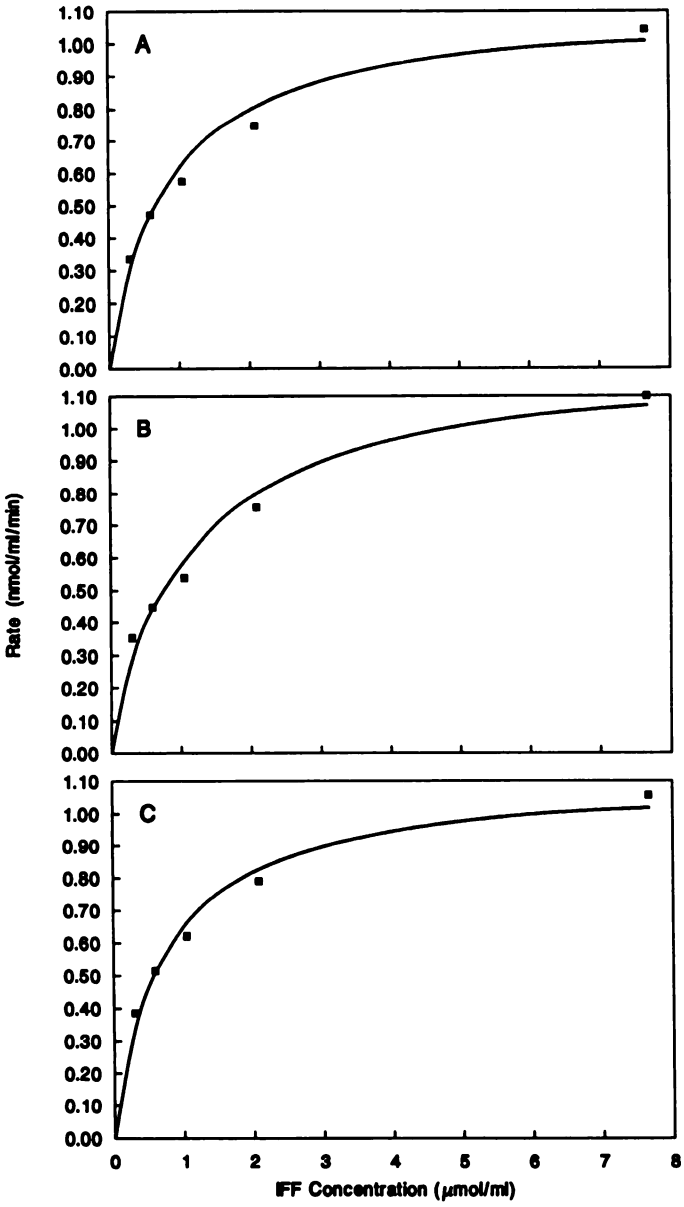

Fig. 3. Rate of aldoifosfamide production (nmol/ml/min) from IFF by hepatic microsomes, where (A) rac-IFF; (B) (+)-(R)-IFF; (C) (−)-(S)-IFF.

Table 2 *Kinetic parameters of the microsomal production of aldoifosfamide*

	K_m (μmol/ml)	V_{max} (nmol/ml/min)
Rac-IFF	0.89 ± 0.15[a]	1.13 ± 0.06
(+)-(R)-IFF	1.10 ± 0.26	1.22 ± 0.10
(−)-(S)-IFF	0.72 ± 0.12	1.12 ± 0.06

[a] Mean ± SD.

Conclusion

The results from the *in vivo* efficacy studies and the *in vitro* microsomal metabolism studies of the enantiomers of IFF have demonstrated no statistically significant difference between (+)-(R)-IFF and (−)-(S)-IFF and between the individual enantiomers and rac-IFF. These findings are internally consistent since they both reflect the microsomal conversion of IFF to aldoifosfamide, followed by the metabolic production of IPM.

However, this study has only addressed one of the two possible metabolic pathways of IFF. The pathway leading to the production of the *N*-dechloroethylated metabolites and chloroacetaldehyde has not been investigated. Since stereochemical differences in the excretion of the *N*-dechloroethylated metabolites have been reported in humans (7) and mice (9), the microsomal conversion of IFF to these metabolites needs to be investigated. In addition, a stereochemical difference in the plasma clearance of IFF has been observed in humans (8) but not in mice (this work and Ref. 9). These results raise the question of the validity of mice as a model for human efficacy, toxicity, and pharmacokinetic studies of IFF. Further work along both of these lines is currently in progress.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to Pamela Cheshire for her excellent technical assistance, and to Drs. John Rodman and Marc Sunderland for assistance in the analysis of the pharmacokinetic data.

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