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ORIGINAL ARTICLE

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The *N*-dechloroethylation of ifosfamide: using stereochemistry to obtain an accurate picture of a clinically relevant metabolic pathway

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Abstract The cumulative urinary excretions of the enantiomers of ifosfamide [(*R*)-IFF, (*S*)-IFF)] and their 2-*N*-dechloroethylated (2-DCE-IFF) and 3-*N*-dechloroethylated (3-DCE-IFF) metabolites were determined in 11 adult cancer patients who received a single 3-h infusion of IFF (3 g/m^2) with mesna uroprotection. The urine samples were analyzed for the compounds of interest using an enantioselective gas chromatographic-mass spectrometric assay. The results indicated an enantioselective excretion of the parent and *N*-dechloroethylated metabolites: the urinary recovery of (*R*)-IFF was significantly greater than that of (*S*)-IFF (1.73 ± 0.45 vs 1.43 ± 0.41 mmol, $P < 0.0001$); the excretion of (*S*)-2-DCE-IFF (0.75 ± 0.53 mmol) was greater than that of (*R*)-2-DCE-IFF (0.42 ± 0.22 mmol, $P = 0.071$) while the excretion of (*R*)-3-DCE-IFF (1.64 ± 0.76 mmol) was greater than that of (*S*)-3-DCE-IFF (0.77 ± 0.59 mmol, $P = 0.012$). The study also revealed two distinct metabolic patterns in which the urinary recoveries of (*R*)-2-DCE-IFF and (*R*)-3-DCE-IFF were linked as were those of (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF. The results suggest that at least two enzymes are involved in the *N*-dechloroethylation of IFF. The data also demonstrate the importance of following the metabolic fate of (*R*)-IFF and (*S*)-IFF and of determining the relative urinary excretion of all dechloroethylated metabolites.

Key words Microsomal metabolism · N-dechloroethylation · Ifosfamide enantiomers

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Introduction

Ifosfamide (IFF; Fig. 1) is an alkylating agent which has demonstrated activity against a wide range of tumors [3, 5]. In its original form, IFF is not an active antitumor agent and must be metabolically transformed into the cytotoxic agent, isophosphoramide mustard. The initial metabolic step in this transformation is the oxidation of the 4-carbon of the oxazaphosphorine ring by cytochrome P450 (CYP) enzymes to form 4-hydroxyIFF [4]. CYP-dependent oxidations also occur (up to 48% of the dose) [4] at one of the two β-chloroethyl side-chains producing *N*-dechloroethylated metabolites, 2-dechloroethyl-IFF (2-DCE-IFF) and 3-dechloroethyl-IFF (3-DCE-IFF; Fig. 1), and chloroacetaldehyde [1, 2, 5, 6, 17].

2-DCE-IFF and 3-DCE-IFF have no antitumor activity, but may play a role in clinically important adverse effects. Neurotoxic side effects such as cerebellar dysfunction, seizures and changes in mental status have been reported in up to 30% of patients on high-dose parenteral IFF and are dose-limiting toxicities in up to 50% of patients on oral IFF [12, 14]. Oral administration and dose fractionation result in higher urinary excretion of metabolites suggesting an important first-pass effect and autoinduction of metabolism [12, 13, 14]. These observations explain the higher incidence of neurotoxic effects after multiple oral dosing [14].

The neurotoxicity has been associated with *N*-dechloroethylation [8, 11, 14, 16] and an understanding of this pathway is critical to a safer and more effective clinical use of IFF. Fundamental to the delineation of the *N*-dechloroethylation pathway is the recognition that IFF is a chiral molecule. The compound contains an asymmetrically substituted phosphorus atom and exists in two enantiomeric forms, (*R*)-IFF and (*S*)-IFF; the 2- and 3-DCE-IFF metabolites are also chiral and exist in (*R*) and (*S*) forms. In clinical practice, IFF is administered

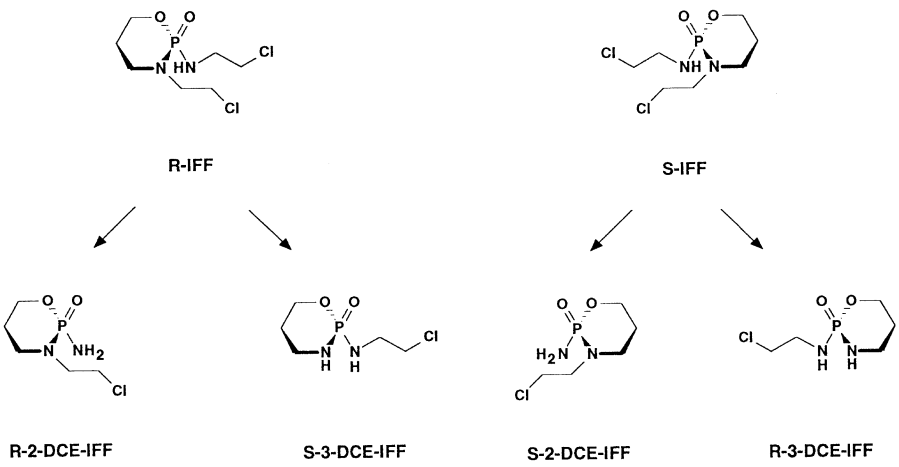

Fig. 1 The structure of the enantiomers of ifosfamide, (*R*)-IFF and (*S*)-IFF, and their respective 2-*N*-dechloroethylated (2-DCE-IFF) and 3-*N*-dechloroethylated (3-DCE-IFF) metabolites

as a racemate, i.e. a 50 : 50 mixture of the two enantiomers.

Studies have shown that the metabolism and pharmacokinetics of the IFF isomers are different, with (*S*)-IFF more extensively cleared by the *N*-dechloroethylation pathway [2, 7, 10, 16]. Boos et al. [2] have demonstrated interindividual differences in the stereoselectivity of 3-DCE-IFF excretion. In a study of multiple IFF dosing in 11 children, 5 patients excreted more (*S*)-3-DCE-IFF than (*R*)-3-DCE-IFF (*S* : *R* ratio 57 : 43), while the other 6 displayed an S : R ratio of 39 : 61.

The clinical relevance of interindividual differences in IFF *N*-dechloroethylation have been indicated by a study which identified a relationship between the occurrence of CNS toxicity and the urinary excretion of (*R*)-3-DCE-IFF [16]. The one patient who experienced severe neurotoxicity excreted elevated levels of (*R*)-3-DCE-IFF (28% of the administered dose vs an average of 13%) and (*R*)-2-DCE-IFF (8% vs 4%), while no significant changes were detected in the urinary excretion of (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF.

The data suggest that the identification of the CYP(s) responsible for the *N*-dechloroethylation of IFF is an important first step in the prediction and management of IFF-related CNS toxicity. The in vitro 4-hydroxylation and *N*-dechloroethylation of IFF have been studied using characterized human microsomes, and the results indicate that CYP3A is the major isoform responsible for the 4-hydroxylation of IFF [4, 17] as well as the *N*-dechloroethylation of IFF [17]. However, despite the recognized stereochemical differences in the in vivo fate of IFF stereoisomers, these studies were carried out without consideration of the enantioselectivity of the process. In addition, the relative production of the two positional isomers, 2-DCE-IFF and 3-DCE-IFF, was also ignored as only the total production of chloroacetaldehyde was measured [17].

The study reported here demonstrates the importance of following the metabolic fate of (*R*)-IFF and (*S*)-IFF and of determining the relative urinary excretion of all of the DCE-IFF metabolites. This conclusion is based upon the observation of two distinct urinary metabolite patterns arising from a single intravenous (i.v.) administration of racemic IFF. While the results do not identify the individual microsomal isoforms responsible for the patterns, they suggest that at least two distinct enzymes must be involved in the process.

Patients and Methods

Clinical protocol

Included in this study were 11 female patients suffering from histologically proven recurrent pelvic carcinoma. The patients were part of a clinical protocol which has been previously described [10]. The study protocol was approved by the McGill University Ethics Committee and written informed consent was obtained from each patient before treatment.

Drug Administration

(*R*, *S*)-IFF (Ifex, Bristol-Myers Squibb Canada, Montréal, Quebec, Canada) was administered by a 3-h i.v. infusion at 3 g/m^2 with 2 g/m^2 mesna (Uromitexan, Bristol-Myers Squibb Canada) and 3 h after the end of the (*R*, *S*)-IFF infusion, 1 g/m^2 mesna was administered in a 60-min period.

Urine Collection

Total urinary excretion was collected from the start of the infusion until 24 h following the end of the infusion during the following time periods: 0–2 h, 2–4 h, 4–8 h, 8–12 h, 12–24 h. The collected volumes were measured and 50-ml aliquots were stored at -20° C until analysis.

Analytical Procedures

The enantiomers of IFF and of its *N*-dechloroethylated metabolites were quantitated in urine using a previously reported enantioselective gas chromatography-mass spectrometry (GC-MS) method [9].

Results and discussion

Over 27 h, the mean cumulative urinary excretion of unchanged IFF was 3.16 mmol (27.6% of the administered dose). The cumulative excretion of (*R*)-IFF was significantly greater than that of (*S*)-IFF (1.73 $\pm$ 0.45 vs 1.43 $\pm$ 0.41 mmol, $P < 0.0001$). The total cumulative excretion of the *N*-dechloroethylated metabolites was 3.56 $\pm$ 0.45 mmol, and when this total was separated into 2-DCE-IFF and 3-DCE-IFF, the respective cumulative excretions were 1.17 $\pm$ 0.19 mmol (10.3% of the administered dose) and 2.38 $\pm$ 0.20 mmol (20.9% of the administered dose; Fig. 2A). When the stereochemical distributions of the two DCE-IFF metabolites were considered, the excretion of (*S*)-2-DCE-IFF (0.75 $\pm$ 0.53 mmol) was greater than that of (*R*)-2-DCE-IFF (0.42 $\pm$ 0.22 mmol, $P = 0.071$) while the excretion of (*R*)-3-DCE-IFF (1.64 $\pm$ 0.76 mmol) was greater than that of (*S*)-3-DCE-IFF (0.77 $\pm$ 0.59 mmol, $P = 0.012$). These results indicate an enantioselective excretion of the parent and *N*-dechloroethylated metabolites which is consistent with previously reported observations [2, 7, 10, 15, 16].

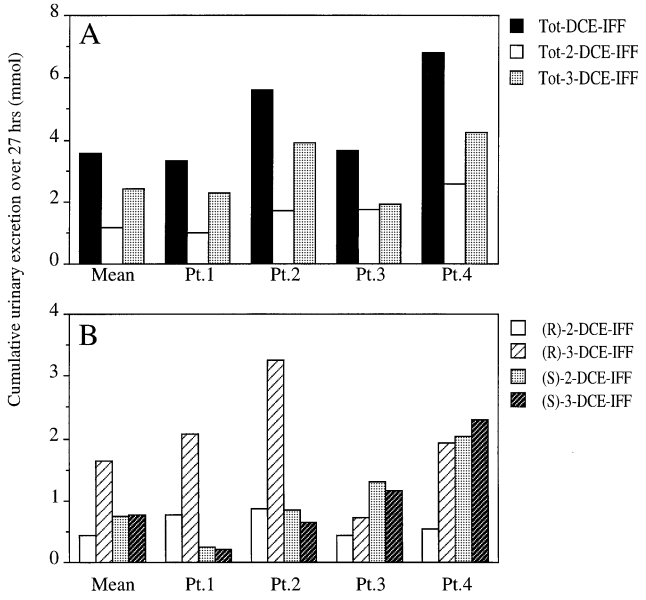

Fig. 2A, B The cumulative 27-h urinary excretions (in mmols) of the *N*-dechloroethylated metabolites of ifosfamide presented as the mean of 11 patients (*Mean*) and the individual totals from four patients (*Pt.* 1, *Pt.*2, *Pt.*3, *Pt.*4). **A** Combined excretions of: all the *N*-dechloroethylated metabolites (Tot-DCE-IFF), the 2-*N*-dechloroethylated metabolites (Tot-2-DCE-IFF) and the 3-*N*-dechloroethylated metabolites (Tot-3-DCE-IFF). **B** cumulative excretions of each of the stereoisomers of the 2- and 3-*N*-DCE metabolites of IFF

An examination of the stereochemical distribution of 2-DCE-IFF and 3-DCE-IFF in each patient profiled in this report revealed two distinct metabolic patterns. To illustrate each pattern, the cumulative excretions of the (*R*) and (*S*) enantiomers of 2-DCE-IFF and 3-DCE-IFF from 4 of the 11 patients are presented in Fig. 2B. In patients 1 and 2, the cumulative urinary excretions of (*R*)-2-DCE-IFF and (*R*)-3-DCE-IFF were elevated while (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF levels were either decreased (patient 1) or relatively unchanged (patient 2). The opposite pattern was observed in patients 3 and 4, where the cumulative urinary excretions of (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF levels were elevated while that of (*R*)-3-DCE-IFF was reduced (patient 3) or slightly elevated (patient 4) and (*R*)-2-DCE-IFF levels were relatively unchanged in both patients.

The results suggest that the urinary excretions of (*R*)-2-DCE-IFF and (*R*)-3-DCE-IFF are linked, as are the urinary excretions of (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF. These relationships were explored by multiple regression analyses (Pearson Product Moment Correlation). The results, presented in Fig. 3, indicate a good correlation between the excretion of (*R*)-2-DCE-IFF and (*R*)-3-DCE-IFF ($r = 0.619$, $P = 0.042$) and an excellent correlation between (*S*)-2-DCE-IFF and (*S*)-3-DCE-IFF ($r = 0.980$, $P = 0.001$). No other significant relationships were found. It is important to note, that the (*R*)-2/(*R*)-3 and (*S*)-2/(*S*)-3 relationships do not imply a separate metabolic route for each enantiomer. Indeed, according to the Cahn–Ingold–Prelog Nomenclature System, (*S*)-IFF produces (*S*)-2-DCE-IFF and (*R*)-3-DCE-IFF while (*R*)-IFF produces (*R*)-2-DCE-IFF and (*S*)-3-DCE-IFF (Fig. 1).

The cumulative 27-h urinary excretions of the DCE-IFF metabolites from the 11 patients were correlated with their previously reported plasma AUC_{0-27} values [10]. A significant relationship was determined for three of the four metabolites: (*R*)-3-DCE-IFF ($r = 0.761$, $P = 0.007$); (*S*)-2-DCE-IFF ($r = 0.993$, $P < 0.001$); (*S*)-3-DCE-IFF ($r = 0.826$, $P = 0.002$). No relationship was found between the cumulative 27-h urinary excretion of (*R*)-2-DCE-IFF and the corresponding plasma AUC_{0-27} which is probably because of the low cumulative excretion (0.42 $\pm$ 0.22 mmol and low circulating plasma levels ($AUC_{0-27} = 140 \pm 20$ μM h) of this metabolite.

The relationships between cumulative urinary excretions and plasma AUCs demonstrate the suitability of urine measurements as an index of circulating plasma levels and, by extension, of IFF metabolism. Thus, patients excreting lower than average amounts of DCE-IFF could be classified as "poor" *N*-dechloroethylators while increased excretion of DCE-IFF could classify a person as an "extensive" *N*-dechloroethylator.

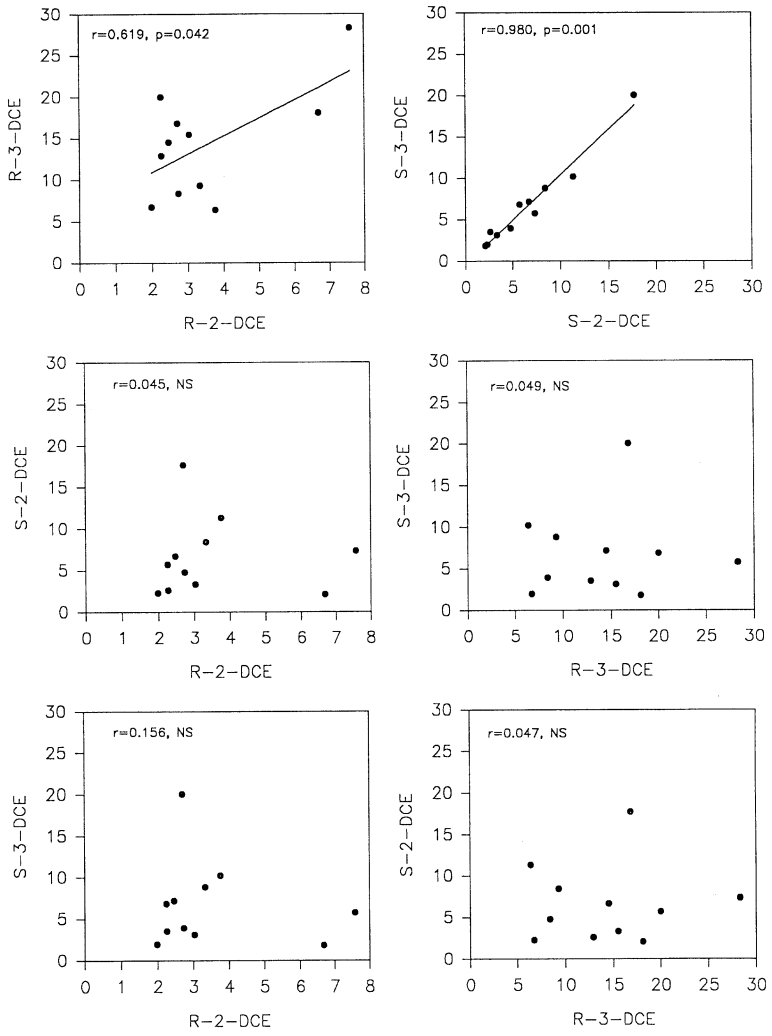

Fig. 3 The correlations between the cumulative 27-h urinary excretions (expressed as percentages of total administered dose) of the stereoisomers of the 2- and 3-N-DCE metabolites of IFF as determined by multiple regression analyses (Pearson Product Moment Correlation)

However, there is an inherent danger in developing a poor/extensive classification for IFF metabolism. This approach usually assumes the involvement of a single metabolizing enzyme. In the case of IFF, the microsomal isoform CYP3A has been identified as the major enzyme associated with IFF *N*-dechloroethylation [17]. If CYP3A is the major or only CYP involved, then an induction or overexpression of this enzyme should increase the urinary excretion of all four metabolites and produce a consistent poor/extensive pattern. This is clearly not the case. The observation of two distinct metabolic patterns is inconsistent with the view that a single microsomal enzyme is responsible for all or most of the *N*-dechloroethylation of IFF. The results suggest the involvement of at least two isoenzymes, each of which is able to *N*-dechloroethylate both (*R*)-IFF and (*S*)-IFF.

It is not difficult to see how ignoring the existence of four distinct DCE-IFF stereoisomers could result in the failure to recognize the involvement of more than one microsomal isoform in this metabolic transformation. The cumulative excretion of DCE-IFF in the 11 patients was indeed consistent with the supposition of the involvement of a single CYP (Fig. 2A). Given a mean cumulative excretion of 3.56 mmol, patients 1 and 3 could be considered "normal" *N*-dechloroethylators (3.24 and 3.61 mmol, respectively) while patients 2 and 4 could be categorized as extensive *N*-dechloroethylators (5.57 and 6.75 mmol, respectively). Even if the cumulative excretion is divided into its 2-DCE-IFF and 3-DCE-IFF components, the underlying pattern is still undetectable; patients 1 and 3 are still "normal" while patients 2 and 4 are "extensive". Only the determination of the stereochemical patterns of the metabolites revealed the actual metabolic situation. These results illustrate that to accurately study the metabolic fate of a chiral compound it is necessary to follow the transformation of each stereoisomer.

The clinical implications of ignoring the stereochemistry of IFF *N*-dechloroethylation are evident in the statement, "the involvement of the same cytochrome P450 enzyme in both reactions (i.e. 4-hydroxylation

and *N*-dechloroethylation) prohibits selective inhibition of the *N*-dechloroethylation pathway, as might be desirable to reduce toxic side effects" (quoted from reference 17). In this regard, one would expect patients 2 and 4, the "extensive *N*-dechloroethylators" to experience toxic side effects. However, patients 2 and 4 had different metabolic profiles and had different reactions to IFF therapy. Patient 4 tolerated the drug while patient 2 had severe CNS toxicity [16]. Since the stereochemistry of the process reveals that at least two CYPs are involved and suggests a metabolic pattern which is associated with CNS toxicity, there is the possibility that once these CYPs are identified, selective inhibition may indeed increase the therapeutic index of IFF.

In fact, in vitro, CYP3As are the major isoforms catalyzing IFF activation, but expressed CYP2A6, -2B6, -2C8 and -2C9 are also catalytically competent in hydroxylating IFF [4]. The same might be true for the dechloroethylation pathway although the authors reported that the contribution of other CYPs would be minor [17]. In vitro incubations with 1 m*M* of IFF may be very different from the in vivo situation where the maximum concentrations of (*R*)- and (*S*)-IFF are 200 μ*M* and fall below 100 μ*M* 3 h after the end of the infusion [10]. At higher concentrations, high-capacity enzyme systems such as CYP3As are likely to predominate at the expense of other enzymes. In vivo, IFF metabolism is, therefore, likely to show the interplay of various enzyme systems.

Thus, the observed interpatient variations in the urinary excretions of the (*R*) and (*S*) enantiomers of 2-DCE-IFF and 3-DCE-IFF may stem from differential expression of one or more of the enzyme systems. Therefore, IFF therapy might be inappropriate for a particular subgroup of patients. Once the key CYPs are identified, the possibility exists that IFF-sensitive patients could be identified before initiation of treatment through the use of appropriate probe drugs or genetic screening.

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