TITLE: New ifosfamide analogues designed for lower associated neurotoxicity and nephrotoxicity with modified alkylating kinetics leading to enhanced in vitro anticancer activity.

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RUNNING TITLE: Metabolism & anticancer activity of new oxazaphosphorines

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LIST OF NON STANDARD ABBREVIATIONS:

ALDH1: aldehyde dehydrogenase
C7,C9-diMe-IFO :dimethyl ifosfamide analogues
CAA : chloroacetaldehyde
CPM : cyclophosphamide
FBS : fetal bovine serum
IFO : ifosfamide
ESI-MS: electrospray ionisation mass spectrometry

$^{31}$P-NMR : Phosphorus Nuclear Magnetic Resonance

mesna : sodium mercaptoethanesulfonate

4-OH-IFO : 4-hydroxy-ifosfamide

IPM : isophosphoramide mustard

$N^2$-DCE-IFO: $N^2$-deschloroethylifosfamide

$N^3$-DCE-IFO: $N^3$-deschloroethylifosfamide

$N^2$,$N^3$-diDCE-IFO: $N^2$-$N^3$-dideschloroethylifosfamide

TS121: $7S,9R$ dimethyl-ifosfamide

TS125 : $7S,9S$ dimethyl-ifosfamide

diMe-IPM : dimethylated analog of the isophosphoramide mustard

$N^2$-DCP-Me-IFO: $N^2$-deschloropropylmethyl-ifosfamide

$N^3$-DCP-Me-IFO: $N^3$-deschloropropylmethyl-ifosfamide

4-OH-diMe-IFO: 4-hydroxy-dimethyl-ifosfamide

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ABSTRACT

Ifosfamide is a well-known prodrug for cancer treatment with CYP450 metabolism. It is associated with both antitumor activity and toxicities. Isophosphoramide mustard is the bisalkylating active metabolite and acrolein a urotoxic side-product. Since acrolein toxicity is limited by co-administration of mesna, the incidence of urotoxicity has been lowered. Current evidence suggests that chloroacetaldehyde, a side-chain oxidation metabolite is responsible for neurotoxicity and nephrotoxicity. The aim of our research is to prevent chloroacetaldehyde formation using new enantioselectively synthetized ifosfamide analogues i.e. C7,C9-dimethyl-ifosfamide. We hypothesise that reduced toxicogenic catabolism may induce less toxicity without changing anticancer activity. Metabolite determinations of the dimethyl-ifosfamide analogues were performed using liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) after in vitro biotransformation by drug-induced rat liver microsomes and human microsomes expressing the main CYP 3A4 and minor CYP2B6 enzymes. Both human and rat microsomes incubations produced the same N-dechloroalkylated and 4-hydroxylated metabolites. A co-culture assay of 9L rat glioblastoma cells and rat microsomes was performed to evaluate their cytotoxicity. Finally, a mechanistic study using Phosphorus Nuclear Magnetic Resonance (31P-NMR) kinetics allowed estimating the alkylating activity of the modified mustards. The results showed that C7,C9-dimethyl-ifosfamide exhibited increased activities although they were still metabolized through the same N-deschloroalkylation pathway. Analogues were 4 to 6 times more cytotoxic than ifosfamide on 9L and the generated dimethylated mustards were 28 times faster alkylating agents than ifosfamide mustards. Among these new ifosfamide analogues, the 7S,9R enantiomer will be assessed for further in vivo investigations for its anticancer activity and its toxicological profile.
INTRODUCTION

The bisalkylating agent ifosfamide (IFO) was introduced into clinical trials in the 1970's, but its early use was limited by severe urotoxicity consisting in haemorrhagic cystitis. This side effect led to the development of sodium mercaptoethanesulfonate (mesna) associated with hydration as a safe and effective regional uroprotection. Further studies have demonstrated IFO activity against a wide range of tumour types, from soft tissue sarcomas to lymphomas both in adult and paediatric patients (Kerbusch et al., 2001). Main adverse effects of IFO include urotoxicity, myelosuppression, nausea and vomiting, neurotoxicity and nephrotoxicity. Le Cesne et al. have shown that high-dose regimen of IFO (HD-IFO) allowed the circumvention of resistance to standard-dose ifosfamide in advanced soft-tissue sarcomas, indicating that there is a dose-effect relationship (Le Cesne et al., 1995). Since the systematic use of adjuvant treatments such as mesna, GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) and setrons, and the increase of IFO dosages, neurotoxicity and nephrotoxicity are the limiting factors for IFO-based chemotherapy (Aleksa et al., 2001, Lee et al., 2001). Indeed, in some studies, up to 40% of the treated patients show neurological disorders depending on the dose quantity and the administration mode and another 5% present a Fanconi syndrome (Patzer et al., 2006). These toxicities seem to be more frequent in children (Dufour et al., 2006).

Ifosfamide (1) is a prodrug with metabolism being required to obtain its active form. The initial activation reaction in IFO metabolism is mainly mediated by the cytochrome P450 enzyme CYP3A4 (Fig. 1). Hydroxylation on the C-4 of the oxazaphosphorine ring leads to 4-hydroxy-Ifosfamide (4-OH-IFO, 2), which is in equilibrium with its tautomeric form, the aldo-Ifosfamide (3). The latter may then either be oxidised by aldehyde dehydrogenase (ALDH1) to carboxy-Ifosfamide (4), an inactive metabolite, or spontaneously to acrolein (5), by a retro-Michael reaction, and the isophosphoramide mustard (IPM, 6) which is the active moiety. IPM is the bisalkylating
moiety and acrolein is believed to be responsible for urotoxicity. Up to 50% of an IFO dose undergoes enzymatic oxidative N-dealkylation, resulting in the loss of chloroethyl side-chains and producing N²-deschboroethylIfosfamide (N²-DCE-IFO, 7), N³-deschboroethylIfosfamide (N³-DCE-IFO, 8) and N²-,N³-dideschboroethylIfosfamide (N²,N³-diDCE-IFO, 9). An equimolar amount of chloroacetaldehyde (CAA, 10) is formed in each of these N-dealkylation reactions. This metabolite is known to be responsible for both nephrotoxicity and neurotoxicity which may be associated with IFO treatment (Aijthkumar et al., 2007, Chatton et al., 2001, Boddy and Yule, 2000) even if it has been recently reported that CAA could contribute to IFO cytotoxicity (Brueggemann et al., 2006). Reducing CAA formation may reduce frequency of IFO related neurotoxicity and nephrotoxicity.

In order to prevent CAA toxicities, different strategies have been proposed. First, Kupfer and colleagues relieved patients from neurotoxicity using methylene blue to decrease CAA toxicity (Kupfer et al., 1996). Visarius and colleagues demonstrate the pharmacological mechanism of methylene blue usage (Visarius et al., 1998). Unfortunately, methylene blue is not conveniently used in prophylaxis, but is mainly limited to relieve symptoms.

Second, as some CYP450 isoforms are more implicated in IFO N-deschboroethylation, specific isoform inhibition in the toxicogenic pathway should prevent CAA formation (Scripture et al., 2005). Nevertheless, this approach is not compatible with clinical use. Indeed, cancer patients receive several drugs that are mainly metabolised by CYP450 enzymes in the liver. The benefit associated with blockage of CAA formation from IFO may be largely overshadowed by increased toxicity of associated treatments.

The third option we developed involved the synthesis of new IFO analogues, C7,C9-dimethyl-ifosfamide (C7,C9-diMe-IFO, 11) (Fig. 1), designed to lower the susceptibility of side-chain oxidation through methylation of the C7 and C9 positions. Firstly, the steric hindrance created should decrease enzymatic access. Secondly, the methyl group electron-donor effect reduces
oxidability. Finally, even if the extra-cyclic oxidation occurs, the side-product will be chloroacetone and not CAA. From a chemical point of view, α-halogeno methylketones are less reactive than their aldehyde homologues.

Two C7,C9-diMe-IFO analogues [7S,9R dimethyl-ifosfamide (TS121) and 7S,9S dimethyl-ifosfamide (TS125)] were chosen for the pharmacological study on the basis of their easy access from the natural isomer of alanine. Their evaluation results are presented. As side chains were modified from IFO, it was mandatory to investigate the N-deschloroalkylation metabolism, the cytotoxic activity and the alkylation potential of these newly synthesized analogues.
METHODS

Chemicals and reagents

Ifosfamide (HOLOXAN®) (2-(2-chloroethylamino)-3-(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide) and cyclophosphamide (ENDOXAN®) (2-[bis-(2-chloroethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide) were obtained from Baxter SA (Maurepas, France). N2-DCE-IFO ([3-(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphonan-2-yl] amine), N2-DCE-IFO (N-(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphinan 2-oxide), C7,C9-diMe-IFO analogues (TS121 and TS125) and their desalkylated metabolites were enantioselectively synthesized according to previously developed techniques (Paci et al., 2001). Their purity and chemical structures were assessed by 31P-, 13C-, 1H-NMR study, and electrospray mass spectrometry (ESI-MS). HPLC grade solvents (methanol, ethyl acetate) and ammonium formate were obtained from Carlo Erba (Rodano, Italy). Sodium thiosulphate pentahydrate (Na2S2O3, 5 H2O >99.5% purity) and magnesium chloride were obtained from Acros Organics, Geel Belgium. Deionised water was prepared using a UHQ II system (USFELGA SA, Trappes, France).

Phenobarbital, aroclor 1254, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT stain) were provided by Sigma-Aldrich (St Louis, USA).

Culture reagents (DMEM, ref. 12800-116; concentrated penicillin/streptomycin antibiotic mixture; fetal bovine serum (FBS); trypsin-EDTA) were purchased from Gibco (Paisley, UK).
Metabolic investigations

Preparation of rat liver microsomes

Procedures involving animals were carried out in compliance with the conditions established by the European Union (Directives N°86/609/CE and N°2003/65/CE). Livers were obtained from non-induced Sprague-Dawley male rats or from male rats treated with 500 mg/kg Aroclor 1254 once daily for 3 days, with 80 mg/kg phenobarbital once daily for 3 days. Microsomes were prepared as previously described (Abernathy et al., 1971).

Human microsomes expressing P450 enzymes.

Three batches HG42 (ref. Nr. 452042), HG112 (ref. Nr. 452112), and HH74 (ref. Nr. 452174) human microsomes expressing mainly CYP3A4 and CYP2B6, provided by BD Gentest, (Woburn, MA) were used for IFO and diMe-IFO analogues incubations.

Microsomal incubations

A typical incubation sample contained 1 mg of rat or human liver microsomal proteins, 0.1M potassium phosphate buffer (pH 7.4), 5 mM magnesium chloride, 40 mM glucose-6-phosphate, 2 mM NADP, and 20 IU of glucose-6-phosphate dehydrogenase in a final volume of 0.5 mL (Storme et al., 2005). Samples were pre-incubated for 10 min at 37°C. Afterward, 1 µmol of the compound (IFO or C7-C9-diMe-IFO) was added to give a final concentration of 2 mM. Incubations were carried for 0.5 or 1 h for rat and human microsomes and stopped by adding 5.0 mL of ice cold ethyl acetate. After centrifugation at 6,000 x g, the organic layer was decanted and evaporated to dryness under nitrogen. The residue was stored at -20°C until analysis. It was then dissolved in methanol and diluted five fold in the 5 mM ammonium formate solution and a 20 µL aliquot was analyzed by HPLC-MS/MS.

High performance liquid chromatography and mass spectrometry HPLC-MS/MS

Microbore HPLC
The HPLC-MS/MS method was adapted from the previously described technique (Storme et al., 2005). Sample extracts and IFO standards were analyzed with a 1100 series HPLC system (Agilent Technologies, Massy, France), including a binary pump, a by-pass loop autosampler and a Nucleosil® C18HD 5 µm column (1 mm i.d. x 150 mm long; Interchim, Montluçon, France). Gradient elution was performed at a flow-rate of 60 µL/min with the eluent A (5 mM ammonium formate pH 5.5/methanol) (80/20, v/v) and eluent B (5 mM ammonium formate pH 5.5/methanol) (20/80, v/v). IFO and its metabolites were analyzed with the following gradient method: starting with 100% eluent A, the gradient mixture was increased up to A/B (50/50, v/v) from 5.0 to 6.0 min and was kept until 15.0 min, then back again to initial conditions for 10.0 min. C7,C9-diMe-IFO (TS) and their metabolites were analyzed with the following gradient method: starting with 100% eluent A, the gradient mixture was increased up to A/B (20/80, v/v) from 5.0 to 6.0 min and was kept for 9.0 min, then back again to initial conditions until 30.0 min.

**Electrospray mass spectrometry**

Detection was performed with a Quattro-LCZ triple-quadrupole mass spectrometer equipped with an orthogonal electrospray source (Micromass, Manchester, UK). The ESI source and nitrogen gas temperatures were set at 90 and 200°C, respectively. The ESI voltage and the cone voltage were set at 3500 V and 30 V, respectively. Product ion scan mode was used with collision-induced dissociation. The collision energy was set at 20 eV, with argon in the collision cell. Continuous positive ion electrospray spectra were obtained over the scan range m/z 50 to 300 in 0.5 s using the Masslynx™ software (Micromass, Manchester, UK).
Cytotoxic activity on 9L cells.

9L cells

The rat gliosarcoma cell line 9L is a brain-tumor-derived adherent cell line and was cultured in DMEM supplemented with sodium hydrogenocarbonate (3.7 g/L), with 1% dilution of concentrated penicillin/streptomycin antibiotic mixture and with 2% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified incubator containing 5% CO2 atmosphere. Cells growing in 25 cm² tissue cultured flask (Cellstar®, Greiner Bio-One, Frickenhausen, Ge) filled with 5 mL of culture medium.

Microsome-prodrug co-culture assay

As previously described by Baldwin and colleagues (Baldwin et al., 2003), the 9L cells were seeded in 96-well tissue culture microplates (Cellstar®, Greiner Bio-One, Frickenhausen, Ge) at a density of 1000 cells/100µL/well and allowed to attach for 24 h. The top row of each plate remained empty as a cell-free staining negative control. The same culture media was used as described above except for FBS concentration, increased to 10%. For cytotoxicity investigations of the prodrug at different concentrations, NADPH (1.5 mM final concentration) and 1 µg of rat liver microsomes were added to each well (final volume of 100 µL). Increasing concentrations of prodrug from 0 to 0.3 µM diluted in culture media were then added to each well. For cytotoxic investigations according microsomes concentration, NADPH (1.5 mM final concentration) and prodrugs at IC 50 were added to each well (final volume of 100 µL). Increasing amounts of microsomal proteins from 0 to 4 µg diluted in culture media were then added to each well. The top two rows were used as blank row and negative control (no prodrug/microsome). Each prodrug/microsomes concentration combination was assayed in triplicate. After six hours, 100 µL of culture media was added for routine culture over 4 days after the addition of prodrug. Cells were stained according to the MTT
assay adapted from Mosmann (Mosmann 1983). After incubation for 2 hours at 37°C with 100 µL of a 10 % w/V MTT solution in culture media, the plate was then gently shaken and 100 µL of a lysis solution (200 g of SDS in 1 L DMF/H2O 1:1 v/v, pH 4.7) was added into each well. After one hour of shaking at room temperature, the plates were read on a Multiscan 355 (Thermo-Electron, Courtaboeuf, Fr) using a test wavelength of 570 nm and a reference wavelength of 630 nm.

As described by Baldwin et al., data are expressed in individual figures as relative growth rate at each drug concentration (with error bars representing standard deviation), determined by dividing the absorbance value for a given prodrug concentration by the absorbance of the drug-free control. A value of 1 corresponds to 100% cell growth rate, relative to the drug-free control. Trends presented in each figure are representative of two or three independent experiments; graphs shown and IC50 values mentioned in the text are based on single experiments deemed most representative. Pictures of cell culture wells were achieved using an Axiovert 135 (Zeiss, Ge) fluorescence microscope.

31P NMR kinetic study of mustard alkylating activity

NMR experiments were performed on a BRUKER AVANCE-400 NMR spectrometer (Bruker, Wissembourg, France). The 31P NMR kinetic technique and mustard syntheses were adapted from Boal (Boal et al., 1989) and Springer (Springer et al., 2004).

31P NMR kinetic studies. General Procedure.

NMR sample solutions of IPM and its dimethylated analogue (diMe-IPM) were prepared immediately prior to their use by adding buffer containing the trapping agent to a glass flask which contained the compound of interest (usually around 30 mg in 1 mL of buffer). This latter consisted in 0.1 M sodium phosphate buffer (pH 7.4), where a trapping agent in excess (5 M equiv. at least)
e.g. sodium thiosulphate, pentahydrate was added before dissolution of IPM or diMe-IPM. The buffer contained 10% v/v of deuterium oxide to facilitate NMR experiments. The pH of the resultant solution was adjusted to 7.4 as necessary (1 M HCl, or NaOH) and the solution was transferred to a 5-mm NMR tube. Only kinetic runs presenting variations of less than 0.5 pH units were included for the analysis. When experiments were not performed at room temperature, the sample was allowed to thermally equilibrate for several minutes prior to data collection.

At the same time, magnetic field homogeneity was made on a blank NMR tube filled with buffer plus trapping agent. A glass insert containing 25% aqueous H₃PO₄ was added to the blank NMR tube to be used as a chemical shift standard. The sample was then placed in the NMR probe and a final adjustment of the magnetic field homogeneity was performed before the initiation of spectral acquisition. For the first kinetic analysis of a compound, the spectra were obtained every 10-20 min. In repeated kinetic analyses, the spectra were obtained at time intervals appropriate to the reactivity of the compound as indicated by the initial experiment. ³¹P NMR acquisitions at 162.0 MHz used a 3200 Hz spectral window, 4k data points, a 1D sequence with ¹H broadband decoupling using WALTZ16 decoupling program (Shaka et al., 1983). Each experiment consisted of 200 scans lasting 2 min 33 sec, unless otherwise indicated.

The free induction decay (FID) signal was automatically stored and the next spectral acquisition was initiated at time t, relative to the “zero” time (initiation of first acquisition). The stored FID signals were exponentially multiplied so as to result in an additional 1 Hz of line broadening in the frequency-domain spectra.

For the diMe-IPM alkylation kinetics, the accumulation time (2 min 33 sec) was too long compared to the speed of transformation to obtain meaningful results. Thus, acquisition parameters were modified in order to shorten each NMR experiment duration and maintain adequate resolution: i.e. a spectral window of 2260 Hz, 2k data points and 64 scans. Moreover, the line broadening in the frequency-domain spectra was modified to 0.3 Hz. These modifications allowed good sensitivity in
for an acquisition period of 40 seconds. This was compatible with the faster transformation kinetics reported for diMe-IPM.

NMR data were analysed as the sum of integrals normalised to 100%. Peak areas were used to measure the relative proportions of the corresponding compounds. Kinetic progress curves (proportions versus time) were analysed using the non-linear squares fitting program DynaFit, with the following kinetic diagram (Kuzmic, 1996): $\text{A} \rightleftharpoons \text{B} \rightleftharpoons \text{C}$. 
RESULTS

Biotransformation of IFO and its analogues

Dealkylated IFO metabolites are the best markers of IFO neurotoxicity. According to Yu and Waxman, rat liver cytochrome P450 2B leads to IFO N-deschloroethylation (Yu et al., 1996). As phenobarbital is a strong inducer of these P450 2B, metabolism experiments were performed using phenobarbital or Aroclor-induced rat microsomes and compared with non-induced rat microsomes, both in the presence of NADPH.

The HPLC-MS/MS analyses of IFO incubations (Fig.2a) were performed to identify IFO metabolites by means of their mass spectra and to evaluate the metabolism yield of the prodrug into N-deschloroethylated metabolites (toxic pathway) and into 4-hydroxylated metabolite (active pathway). Specific MS/MS detections were used for IFO (m/z 261→m/z 154), for $N^2$-DCE-IFO (m/z 199→m/z 92), for $N^3$-DCE-IFO (m/z 199→m/z 78), for 4-OH-IFO (m/z 277→m/z 118). The parent ion of $N^2$-DCE-IFO (m/z 199) produced the ion CH$_2$=NH+-CH$_2$-CH$_2$-Cl (m/z 92), whereas $N^3$-DCE-IFO generated the ion NH$_3$+-CH=CH-Cl (m/z 78).

The HPLC-MS/MS analyses of C7,C9-diMe-IFO incubations (Fig. 2b) are shown to identify the corresponding metabolites and to evaluate the metabolism yield of the prodrug into N-deschloropropylated metabolites (inactivating pathway) and into 4-hydroxylated metabolite, probably responsible of the diMe-IPM liberation (active pathway). Specific MS/MS detections are used for C7,C9-diMe-IFO (m/z 289→m/z 168), for $N^2$-DCP-Me-IFO (m/z 213→m/z 168), for $N^3$-DCP-Me-IFO (m/z 213→m/z 92), for 4-hydroxy-diMe-IFO (m/z 305→m/z 132). The difference between the fragment ion (m/z 168) of C7,C9-diMe-IFO and the fragment ion (m/z 154) of IFO corresponds to an additional methyl on the side-chain. By the same token, the parent ion of $N^3$-DCP-Me-IFO (m/z 213) produced the ion CH$_3$-NH$_2$+-CH=CH-Cl (m/z 92) with a difference of 14
mass units, corresponding to a methyl group, confirming fragments ions previously described for IFO, whose interpretation was facilitated by various deuterated IFO analogues (Liu et al., 2005).

Induced and non-induced rat microsomes were incubated over 1h, instead of 0.5h, to detect significant amounts of active metabolites of IFO and IFO analogues (4-OH-IFO and 4-OH-diMe-IFO) (data not shown).

Phenobarbital-induced rat microsomal incubations (n=3) of 2 mM IFO and of C7,C9-diMe-IFO (TS121 and TS125) induced the following three metabolites after 1h incubation time (summarized in Table1). For IFO metabolism, yields were 1.2% for \(N^2\)-DCE-IFO, 5.2% for \(N^3\)-DCE-IFO and 10.0% for 4-OH-IFO. For the metabolism of the 7S,9R analogue (TS121), yields were 12.5% for \(N^2\)-DCP-Me-IFO, 22.3% for \(N^3\)-DCP-Me-IFO and 6.0% for 4-OH-diMe-IFO. For the 7S,9S analogue (TS125), they were 29.5% for \(N^2\)-DCP-Me-IFO, 22.8% for \(N^3\)-DCP-Me-IFO and 3.0% for 4-OH-diMe-IFO. As 83.6% IFO remained unchanged, 59.1% C7,C9-diMe-IFO (TS121) and 44.7% C7,C9-diMe-IFO (TS125) were not metabolised using phenobarbital-induced rat microsomes.

As Aroclor is a strong inducer of a wide range of CYP 450 isoforms (1A, 2B and 3A) of the total oxidase activity (Ryan and Levin, 1990), metabolism experiments were first performed using Aroclor-induced rat microsomes (n=3). As shown in Table 1, whereas 92.1% IFO remained unchanged, 70.0% TS125 and 75.5% TS121 were metabolised using Aroclor-induced rat microsomes with 25% of its of activated metabolite, 4-hydroxy-diMe-IFO versus 5.2% for 4-hydroxy-IFO. The latter results with activated metabolites may be interesting for cytotoxic activity, however the values should be interpreted with caution as 4-hydroxy-compounds are strongly labile.

The IFO incubation time was set at 1h rather than 0.5h with human microsomes expressing CYP3A4 and CYP2B6 in order to produce the 4-hydroxy-IFO active metabolite in sufficient yields.
and the two $N$-DCE-IFO metabolites. For both IFO analogues, the $N$-DCP-methyl-IFO and the 4-hydroxy-diMe-IFO metabolites were detected by LC-MS/MS either after 0.5 or 1h of incubation. Relative estimation of biotransformation is shown for 1h in table 2. The biotransformation rates were however very low. Human microsomal incubations of TS121 (7S,9R) and TS125 (7S,9S) dimethylated IFO analogues produced the same hydroxylated metabolite and the two $N$-DCP-methyl-IFO metabolites observed after rat microsomes incubations, thus confirming the same metabolism pathway. Moreover, the metabolism rates appeared to be faster for analogues compared to IFO as observed with the rat microsomes. The hydroxylated metabolites were produced more readily for TS121 and TS125, but side-chain oxidations also generated metabolites.

**Cytotoxic activity on 9L cells**

**9L cells growth inhibition assay**

Evaluation of oxazaphosphorine prodrugs cytotoxicity, thanks to microsomal CYP using 9L rat gliosarcoma cells, was first described by Baldwin et al. in 2003. In fact, their experiments demonstrated that an extra-cellular source of CYP activity, such as liver microsomes extract, can enhance CYP prodrug cytotoxicity in a manner comparable to that obtained using CYP-expressing tumor cell lines (Baldwin et al., 2003).

After a preliminary study, the number of cells per well (450 vs 1000 cells/well), the culture media (DMEM supplemented with 2% vs 10% FBS) and the growth time (3, 4, 5, 6 or 7 days) were slightly adapted. The retained conditions (1000 cells/well in DMEM supplemented with 10% FBS and pyruvate, after 5 days of growth) allowed the production of sufficient active metabolites according to the number of cells per plate for the MTT assay.

Microsome-prodrug co-culture assays were designed such that each well contained NADPH, prodrug and liver microsomes isolated from either non-induced or drug-induced rats. Microsomes
isolated from drug-induced rat liver were first tested for their ability to activate the CPM and IFO prodrugs. After a 4-day period of cell incubation with the prodrug and liver microsomes, the cells were stained according to the MTT method and the relative cell growths were determined following subtraction of background staining (microsomal protein in the absence of cells).

The IC₅₀ obtained for IFO and CPM (0.500 mM and 0.150 mM) were consistent with previously described results (Baldwin et al., 2003). For IFO analogues (TS125, TS121), IC₅₀ were first calculated at 0.130 mM and 0.080 mM, respectively and was consistent with a higher cytotoxicity which can be due to a higher activation rate or to a higher intrinsic cytotoxicity of the diMe-IPM, or a combination of both.

As shown in Fig. 3A, no significant cytotoxicity was observed for IFO and for IFO analogues in the absence of microsomes. As the drug concentration increased, cell viability decreased to 75% after 4 days with a prodrug concentration of 0.3 mM and a 1 µg-microsomes protein for IFO but decreased to 25%, for TS 121 and TS 125.

In accordance with increased drug concentrations, the IC₅₀ results and cell viability prompted comparison of IFO and CPM analogues with non-induced and phenobarbital-induced microsomes. Fig 3b indicates cell cultures with rat phenobarbital-induced microsomes at the concentration used with the determined concentration of prodrugs (0.5 mM for IFO and CPM, and 0.15 mM for dimethyl analogues) showed significant differences between analogues and oxazaphosphorine drugs used in clinical practice (IFO or CPM). TS121 and TS125 seemed here to have same cytotoxic efficiency (21% and 28% of cell viability at 2 µg microsomes protein for TS 121 for TS 125, respectively). The two tested analogues were more cytotoxic than CPM (32%) and moreover IFO (66%). Fig 3c indicates that the non-induced microsome concentration used with the same prodrug concentration (0.5 mM for IFO and CPM, and 0.15 mM for dimethyl analogues), gave significant differences between analogues and IFO. These experiments demonstrated that they were 4 to 6-fold more cytotoxic than ifosfamide on 9L gliosarcoma cells. TS 121 and TS 125 were much more
cytotoxic than clinically available oxazaphosphorine drugs; 48% and 52% of cell viability at 2 µg microsomes protein for TS 121 and TS 125, respectively, and 68% and 71% for IFO and CPM. The results can be readily observed as microscopic views depicted on Fig. 4. All compounds were activated giving cytotoxic moieties in the presence of microsomes, while no significant cytotoxicity was confirmed in the absence of microsomes for IFO, CPM and C7,C9-diMe-IFO compounds, permitting their description as prodrugs.

31P MNR kinetic study of mustard alkylating activity

The alkylating activity of diMe-IPM was compared to IPM to check that IFO analogues produce an effective mustard that could help explain the observed cytotoxicity.

To obtain a kinetic reference, the previously described 31P NMR kinetic measurements with IPM in the presence of excess sodium thiosulphate (used as trapping nucleophilic agent) was reproduced in the NMR instrument.

Our experiments were adapted from Boal et al. (Boal et al., 1989) and from Springer et al. (Springer et al., 1998). As seen in Fig. 5A, the gradual disappearance of the 31P NMR signal for IPM-Cl₂ occurred alongside the steady growth of a new signal (labelled as IPM-Alk₂), assigned to the anticipated and previously described bisalkylation product. A signal was also observed for a transient species (labelled as the monoalkylated intermediate IPM-Alk/Cl), kinetically associated with the two other species. This signal has been previously assigned to IPM-Alk/Cl (Springer et al. 1998). It has been shown that in the reaction sequence, IPM-Cl₂ → IPM-Cl/Alk → IPM-Alk₂, the formation of both IPM-Alk/Cl and IPM-Alk₂ was preceded by an intramolecular cyclization of the chloroethylamido functionality, resulting in the formation of an aziridine. In the presence of an excess of nucleophiles, such as thiosulphate, the electrophilic aziridine was shortlived and this sequence (IPM-Cl₂ → IPM-CIAlk → IPM-Alk₂) can be treated kinetically as two
simple, consecutive "first-order" reactions. As there was an excess of nucleophile, its concentration can be considered as a constant and included in the $k_{\text{exp}}$.

$$\text{IPMCl}_2 \rightarrow \text{IPMCI}/\text{Alk} : k_{\text{exp1}}$$

$$\text{IPMCl}/\text{Alk} \rightarrow \text{IPMAIK}_2 : k_{\text{exp2}}$$

$k_{\text{exp1}}$ represents either the disappearance rate of IPM-Cl$_2$ (independent of the presence of intermediates between IPM-Cl$_2$ and IPM-Alk/Cl), or the composite production rate of IPM-Cl/Alk from IPM-Cl$_2$; similarly, for $k_{\text{exp2}}$ and IPM-Alk/Cl and IPM-Alk$_2$. Since intermediate species were not observed in the presence of trapping agents, their conversion rates to IPM-Alk/Cl and IPM-Alk$_2$ must be fast, and thus the composite rate closely approximates to the conversion rate of the observed species. The proportions of the different forms as a function of time is depicted (Fig. 6). The $k_{\text{exp1}}$ and $k_{\text{exp2}}$ rates were fitted to the observed proportions of the different forms applying classical first order kinetic equations.

Least-squares fitting of the $^{31}\text{P}$ NMR data (signal relative intensities of IPM-Cl$_2$, IPM-Cl/Alk, IPM-Alk$_2$) resulted in values of $k_{\text{exp1}}$ and $k_{\text{exp2}}$ of $11.636 \times 10^{-3}$ min$^{-1}$ +/- 0.79% (eq.1a) and $6.119 \times 10^{-3}$ min$^{-1}$ +/- 0.71% (eq.2a), respectively at 37°C, consistent with previously obtained results (Boal et al. 1989). Rate constants for diMeIPM were determined under similar reaction conditions. At 37°C, alkylation is so fast that there is no associated result (data not shown). Thus diMe-IPM kinetic has been performed at room temperature (20°C). It was found that $k_{\text{exp1}} = 34.184 \times 10^{-3}$ min$^{-1}$ +/- 1% (eq.1b) and $k_{\text{exp2}} = 16.170 \times 10^{-3}$ min$^{-1}$ +/- 1.4% (eq.2b) for the diMe-IPM at room temperature. IPM transformation kinetics experiments were performed at room temperature for comparison. As expected, the kinetics were much slower, borne out by the rate constants: $k_{\text{exp1}} = 1.129 \times 10^{-3}$ min$^{-1}$ +/- 0.64% (eq.1a') and $k_{\text{exp2}} = 0.602 \times 10^{-3}$ min$^{-1}$ +/- 0.69% (eq.2a') (Fig. 6 B & C).

Easier signal integration was allowed by the addition of 10% v/v D$_2$O in the buffer splitting the overlapping Na$_2$S$_2$O$_3$ signals. It was thus possible to obtain good estimations of IPM-Cl$_2$, IPM-
Cl/Alk, IPM-Alk₂ abundances as a function of time and hence estimations of $k_{\text{exp}1}$ and $k_{\text{exp}2}$. These rate constants values are in close agreement with those previously described (Springer et al., 1998).

**DISCUSSION**

From metabolic investigations, the major finding was that dimethylated analogues were metabolised in the same way as IFO. However, three modifications in this metabolic pathway are to be noted. Firstly, the metabolism rates of C7,C9-diMe-IFO were systematically higher than IFO’s irrespective of the human or rat drug-induced microsomes used (about 50% with phenobarbital and 30% with Aroclor). Secondly, with rat microsomes, TS 125 metabolism mainly generated $N^2$-DCP-Me-IFO, while TS 121 produced higher rate of $N^3$-DCP-Me-IFO. The opposite case was found when human microsomes were used. These results confirm the hypothesis that steric hindrance can modify metabolic regioselectivity. Finally, 4-hydroxylation rates for TS 125 and IFO appeared very similar while TS 121 exhibited a higher rate of 4-hydroxylation using aroclor-induced rat microsomes. This may explain the higher cytotoxic activity and the higher alkylating reactivity found with this enantiomer (see below). A 4-hydroxylated metabolite was detected with both IFO analogues, tracing the formation of the nitrogen mustard but the non-productive side-chain oxidation products were also observed in important amounts for both analogues. However, CAA is not produced with these compounds as $N$-dechloroalkylation should lead to chloroacetone. This ketone, known to be toxic for external exposures at high concentrations. It is chemically less reactive than an aldehyde, as is CAA. Unfortunately, this labile ketone could not have been observed using ESI-MS.

Oxazaphosphorines are activated by a 4-hydroxylation reaction catalyzed essentially by human P450 CYP2B6 and rat P450 CYP2B1 enzymes (Chang et al., 1993, Clarke and Waxman, 1989). As
phenobarbital is a strong inducer of these later isoforms (Waxman et al., 1990), and as the 9L-microsome co-culture system has been previously described with phenobarbital-induced microsomes (Baldwin et al., 2003), we choose this system to evaluate the cytotoxic activity of these IFO analogues with non-induced and phenobarbital-induced microsomes. As with IFO and CPM, phenobarbital-induced liver microsomes metabolize these two dimethylated IFO analogues to a greater extent than non-induced rat liver microsomes, leading to higher cytotoxic values. This difference is low for IFO, while the differences for dimethylated analogues and for CPM are comparable. These results are consistent with the higher catalytic activity of phenobarbital inducible CYP 2B1 described by Baldwin et al. with CPM, which is more efficiently activated than IFO.

Two differences have been noticed when comparing alkylation kinetics of IPM and diMe-IPM. Firstly, in the presence of trapping agents, both values of $k_{\text{exp}1}$ and $k_{\text{exp}2}$ are considerably higher for diMe-IPM than for IPM. Secondly, for the dimethyl mustard, $k_{\text{exp}1}$ is approximately twice $k_{\text{exp}2}$, as for the alkylation reactions of isophosphoramide mustard. The diMe-IPM alkylates around 28 times faster than IPM and follows the same mechanism. Of course, the alkylation activity may be different with nucleotides or DNA, but the large differences between the two mustard alkylation rates clearly demonstrates that the diMe-IPM mustards are much more reactive through nucleophiles. This faster alkylation may be explained by the donor effect of the methyl group or by a modification of the 3D conformation of the chloroethyl chains due to its steric hindrance. Thus, methyl aziridines should be stabilized and their formation facilitated.

The very rapid alkylation rate might jeopardize the ability of diMe-IPM to reach DNA tumor cells to cross-link them. DiMe-IPM may also be deactivated by nucleophilic trapping agents. In order to answer this question, the activities oxazaphosphorine analogues is planned to be evaluated on mice xenografted with a panel of solid human tumours.
In the present work, new ifosfamide analogues with methylated side-chains have been evaluated. The metabolism experiments with rat liver microsomes showed that metabolism rates were much higher for C7,C9-diMe-IFO analogues than for IFO. These investigations demonstrated that ring oxidation was enhanced especially with TS121, in the case of the Aroclor microsomes. But the increase in non-productive side-chain oxidation is many times greater. As the alkylating moiety was modified, the bisalkylating potential of these active compounds was studied with $^{31}$P-NMR experiments showing that the diMe-IPM was 28 times a faster alkylating agent than IPM. These results have to be correlated with six-fold higher cytotoxicity found during the 9L-microsomes cytotoxicity tests. The tested analogues are metabolised into a more reactive mustards than IPM produced following IFO metabolism. Considering the cytotoxic activity, the co-cultured microsome cell assays demonstrated that the cytotoxic activities of C7,C9-diMe-IFO analogues were greater than IFO and CPM, in agreement with the alkylating activity assay and the metabolic investigations. One of these analogues will be evaluated on mice xenografted with a panel of human solid tumours in the near future.

As side-chain oxidation still occurred, leading most probably to chloroacetone after N-dechloropropylation, knowing that chloroacetone should be less toxic than chloroacetaldehyde and as very little is known about its effects on kidneys and nervous system (Sargent et al., 1986), chloroacetone and analogues’ toxicities will be investigated on rat renal model.
References


Footnotes:

T. Storme and A. Paci contributed equally to this work.

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LEGENDS FOR FIGURES:

**Figure 1**: Ifosfamide metabolism pathways and structure of C7,C9-diMe-IFO analogues.

**Figure 2**: HPLC-MS/MS chromatograms of incubations of (A) ifosfamide and of (B) TS121 (7S,9S-diMe-IFO) with phenobarbital induced rat microsomes. (*similar chromatograms for TS 125 (7S,9R-diMe-IFO)*).

**Figure 3**: Cytotoxicity of oxazaphosphorine drugs (CPM and IFO) and dimethylated ifosfamide analogues (TS 121 and TS 125) after microsomal activation by liver microsomes from non-induced or phenobarbital-induced rats. (A) Prodrug concentrations of IFO, TS 121, and TS 125 used were from 0 to 0.3 mM. Cells were cultured with 1 µg microsomal protein for IFO (-■-), for TS 121 (-△-) and for TS 125 (-×-). Cells cultured with IFO and without microsomes constituted the reference. (B) Prodrug concentrations were set at 0.5 mM for IFO and CPM, and at 0.15 mM for TS 121 and TS 125. Cells were cultured with microsomes prepared from phenobarbital-induced rat livers. Concentration of microsomal protein increased between 0 to 4 µg. (C) Prodrug concentration were set at 0.5 mM for IFO and CPM, and at 0.15 mM for TS 121 and TS 125. Cells were cultured with microsomes prepared from non-induced rat livers. The concentration of microsomal protein increased between 0 to 4 µg. Mean values and standard deviations are shown for 4 replicates.

**Figure 4**: Contrast microscopic pictures of 9L cells culture without prodrug (A) with IFO 0.5 mM (B), with TS 121 0.15 mM (C) and with TS 125 0.15 mM (D).

**Figure 5**: Stack plotting of $^{31}$P NMR kinetic of mustard alkylation in the presence of an excess of sodium thiosulphate is faster at 37°C (on the left, A) than at 20°C (on the middle, B) for IPM. At
20°C, $^{31}$P NMR kinetic alkylation is faster for diMe-IPM (on the right, C) than for IPM (on the middle).

**Figure 6:** $^{31}$P NMR kinetics comparing IPM and diMe-IPM at 20 °C over a 120 hours-period (A). For IPM, the IPM Cl$_2$ (●) was transformed into IPM Alk/Cl (■) and then IPM Alk$_2$ (▲) in 120 hours. In the same conditions, but in 5 hours only (B, zoomed), diMe-IPM Cl$_2$ (○) gave IPM Cl/Alk (□), and then IPM Alk$_2$ (△). Mechanism of alkylation is shown on the reaction scheme (C).
### Table 1: Metabolism yields (in percents) of IFO and C7,C9-diMe-IFO (TS121 and TS125) after rat phenobarbital- or aroclor-induced microsomes incubation for 1h (n=3).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IFO</th>
<th>Phenobarbital</th>
<th>Aroclor</th>
<th>Compounds</th>
<th>TS121</th>
<th>Phenobarbital</th>
<th>Aroclor</th>
<th>TS125</th>
<th>Phenobarbital</th>
<th>Aroclor</th>
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<td></td>
<td></td>
<td>Phenobarbital</td>
<td>Aroclor</td>
<td></td>
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<td>Aroclor</td>
<td></td>
<td>Phenobarbital</td>
<td>Aroclor</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;-DCE-IFO</td>
<td>1.2</td>
<td>0.4</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;-DCP-Me-IFO</td>
<td>12.6</td>
<td>5.0</td>
<td>29.5</td>
<td>44.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N&lt;sub&gt;3&lt;/sub&gt;-DCE-IFO</td>
<td>5.2</td>
<td>2.2</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;-DCP-Me-IFO</td>
<td>22.3</td>
<td>45.5</td>
<td>22.8</td>
<td>18.8</td>
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<td></td>
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<tr>
<td>4-OH-IFO</td>
<td>10.0</td>
<td>5.2</td>
<td>4-OH-diMe-IFO</td>
<td>6.0</td>
<td>25.0</td>
<td>3.0</td>
<td>6.6</td>
<td></td>
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<tr>
<td>IFO (unchanged)</td>
<td>83.6</td>
<td>92.1</td>
<td>C7,C9-diMe-IFO</td>
<td>59.1</td>
<td>24.5</td>
<td>44.7</td>
<td>30.0</td>
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</table>

*As experiments do not reflect the initial reaction rates, data do not provide a quantitative measure of the relative formation rates of metabolites.*
Table 2: Metabolism yields (in percents) of IFO and C7,C9-diMe-IFO (TS121 and TS125) after human microsomes incubation for 1h (n=3).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IFO</th>
<th>Compounds</th>
<th>Human microsomes</th>
<th>Metabolite</th>
<th>Human microsomes</th>
<th>Human microsomes</th>
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<tbody>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;-DCE-IFO</td>
<td>0.5</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;-DCP-Me-IFO</td>
<td>0.8</td>
<td></td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>N&lt;sub&gt;3&lt;/sub&gt;-DCE-IFO</td>
<td>1.3</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;-DCP-Me-IFO</td>
<td>0.3</td>
<td></td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>4-OH-IFO</td>
<td>0.1</td>
<td>4-OH-diMe-IFO</td>
<td>0.6</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>IFO (unchanged)</td>
<td>98.1</td>
<td>C7,C9-diMe-IFO</td>
<td>98.3</td>
<td></td>
<td>96.2</td>
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</tr>
</tbody>
</table>

As experiments do not reflect the initial reaction rates, data do not provide a quantitative measure of the relative formation rates of metabolites.
Figure 1

Ifosfamide (IFO) 1

C7,C9 dimethylIFO analogs:
- TS121: 7S,9R
- TS125: 7S,9S

ANABOLISM
Ring oxidation CYP450

CATABOLISM
side chains oxidation CYP450

Chloroacetaldehyde (CAA) 10

4-OH-IFO 2

N²-DCE-IFO 7
N²,N³-diDCE-IFO 9
N³-DCE-IFO 8

carboxy-IFO 4

4-aldo-IFO 3

IPM 6
Acrolein 5

ALDH

retro Mikeal reaction

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Figure 2

(A) 4-OH-IFO

N²-DCE-IFO

N³-DCE-IFO

IFO

(B) 4-OH-diMe-IFO

N²-DCP-Me-IFO

N³-DCP-Me-IFO

diMe-IFO
Figure 3

- **Figure 3A**: Graph showing the effect of drug concentration (mM) on relative cell growth. The x-axis represents drug concentration (mM) ranging from 0 to 0.3, and the y-axis represents relative cell growth ranging from 0 to 1.25. Lines represent different treatments: IFO + no microsome, IFO + 1 µg PB induced microsomes, TS 121 + 1 µg PB induced microsomes, and TS 125 + 1 µg PB induced microsomes.

- **Figure 3B**: Graph showing the effect of microsomal protein (µg) on relative cell growth. The x-axis represents microsomal protein (µg) ranging from 0 to 4, and the y-axis represents relative cell growth ranging from 0 to 1. Lines represent different treatments: IFO (0.5 mM), CPM (0.5 mM), TS 121 (0.15 mM), and TS 125 (0.15 mM).

- **Figure 3C**: Graph showing the effect of microsomal protein (µg) on relative cell growth. The x-axis represents microsomal protein (µg) ranging from 0 to 4, and the y-axis represents relative cell growth ranging from 0 to 1. Lines represent different treatments: IFO (0.5 mM), CPM (0.5 mM), TS 121 (0.15 mM), and TS 125 (0.15 mM).
Figure 4
Figure 5

A: IPM 37°C  B: IPM 20°C  C: DiMeIPM 20°C

t=14h  t=14h

t=4h  t=4h

t=1.5h  t=1.5h

t=0min  t=0min

IFMCl2  IFMAlk2  IFMCl  IFMAlkCl  DiMeIFMCl2  DiMeIFMAlkCl2
Figure 6

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