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ORIGINAL ARTICLE

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Comparative preclinical toxicology and pharmacology of isophosphoramide mustard, the active metabolite of ifosfamide

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Abstract *Background*: Isophosphoramide mustard (IPM) is the cytotoxic alkylating metabolite of Ifosfamide (IFOS). IPM is being readied for a phase I clinical trial. In the present preclinical study, IPM was evaluated for usage in multidose intravenous (IV) infusion protocols. *Methods*: Mice and dogs received IV IPM daily for 3 days. Single-day dosing—oral and IV—to mice, rats, and monkeys is also reviewed for comparison. Complete toxicology studies were completed in the mice and dogs. For mice, dogs and monkeys, IV pharmacokinetic studies were conducted and compared. *Results*: For mice, the LD_{10} for the 3-day IV schedule for IPM was calculated to be 119 mg/kg (with 95% confidence limits of 87–134 mg/kg) (combined sexes), and for adult male dogs the maximum tolerated dose (MTD) was 5 mg/kg. Pharmacokinetic studies in mice, dogs and monkeys were compared and projected to human dosing. For dogs that received 10 mg/kg of IPM, $T_{1/2\beta}$ was 0.99 h, and clearance was constant (1.01 l/h/kg). IPM was detected from 0 h to 1.5 h after the 5 mg/kg dose and from 0 h to 2 h after the 10 mg/kg dose; none was detected after 2 h. The IV MTD in dogs was 5 mg/kg per day for 3 days. Renal tubular necrosis and bone marrow failure were the causes of death. Transient liver, renal and bone marrow toxicity and gastrointestinal dysfunction were seen at low doses (< 5 mg/kg) in dogs. In mice (receiving 100 mg/kg IV) plasma concentrations disappeared in less than 1 h ($T_{1/2\alpha}$ 2 min), with a clearance of 8.44 l/h/kg. For monkeys, the mean $T_{1/2}$ was 4.2 h. Median clearance was 1.65 l/h/kg and no IPM was detected 4 h after dosing. No potential IPM metabolites could be detected in any of the studies. In vitro, plasma protein bound 90% of IPM within 5 min of incubation. *Conclusions*: Predictions for human pharmacokinetic parameters and dosing are made from allometric analysis using the above three species. Data predicted an acceptable starting dose of 30 mg/m^2 with a clearance of 39.5 l/h, and a $T_{1/2}$ of 1 h 45 min for a 70-kg patient.

Keywords Isophosphoramide mustard · Pharmacology/toxicology · Mice · Rat · Dogs · Monkey

Abbreviations 5-FU: 5-Fluorouracil · BW: Body weight · DMA: Dimethylacetamide · IFOS: Ifosfamide · IPM: Isophosphoramide mustard · LD_n: Lethal dose n% · MTD: Maximum tolerated dose · TBDMF: *N*-(*t*-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide

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Introduction

Isophosphoramide mustard (IPM) is the cytotoxic metabolite of the anticancer drug ifosfamide (IFOS;

Fig. 1) [7]. IFOS is included in combination chemotherapy as treatment for sarcoma, breast cancer, germ cell tumors and non-Hodgkin's lymphomas [1, 2, 8, 5]. IFOS is a prodrug that requires metabolic activation to the active alkylating agent (Fig. 1). First, IFOS is hydroxylated by cytochrome P450, resulting in 4-hydroxyifosfamide. The latter metabolite may also serve as a transport proform of IPM and has the potential to be transformed intracellularly into IPM (Fig. 1) [6]; however, IPM is generally considered to be the major form transported by blood in patients [3, 7].

Antitumor activity of IPM in vivo has been well documented with significant activities in L1210 and P388 leukemias, Lewis lung carcinoma, B16 melanoma, CD8F1 mammary carcinoma and colon 38 tumor. At doses lower or equivalent to the LD_{10} in mice, IPM retains high activity against CP-resistant L1210 and P388 leukemias [10]. Thus, the rationale for the development of IPM is well established and timely. The use of IPM in place of IFOS can eliminate both the main renal toxicity due to acrolein formation and neurological toxicity due to chloroacetaldehyde [9].

In the present study, the pharmacokinetics and toxicology of IPM in four animal species (mice, rats, dogs and monkeys), the total protein binding and the stability of IPM were investigated. A computational analysis is described to predict pharmacokinetic parameters in humans.

Materials and methods

Drug formulation and chemicals

IPM (DEKK-TEC, New Orleans, La.) was prepared as a lyophilized preparation by the University of Iowa, Pharmaceutical Services from saline solution. It was stored in a liquid nitrogen freezer until use. A method of synthesis has been described [10]. In all of the acute toxicity studies, IPM was dissolved in saline immediately prior to use. When a control group was included in the study, saline served as the control article. IPM was prepared as a free salt. IPM is stable in dimethylacetamide (DMA) at 4°C for 72 h and this was used as a stock solution for the pharmacokinetic calibration and reference curves [3].

Gas chromatographic–mass spectrometry analysis (GC–MS) of IPM

TBDMF was used as a derivatizing agent in the GC–MS analysis of IPM (Fig. 2). To prevent the occurrence of

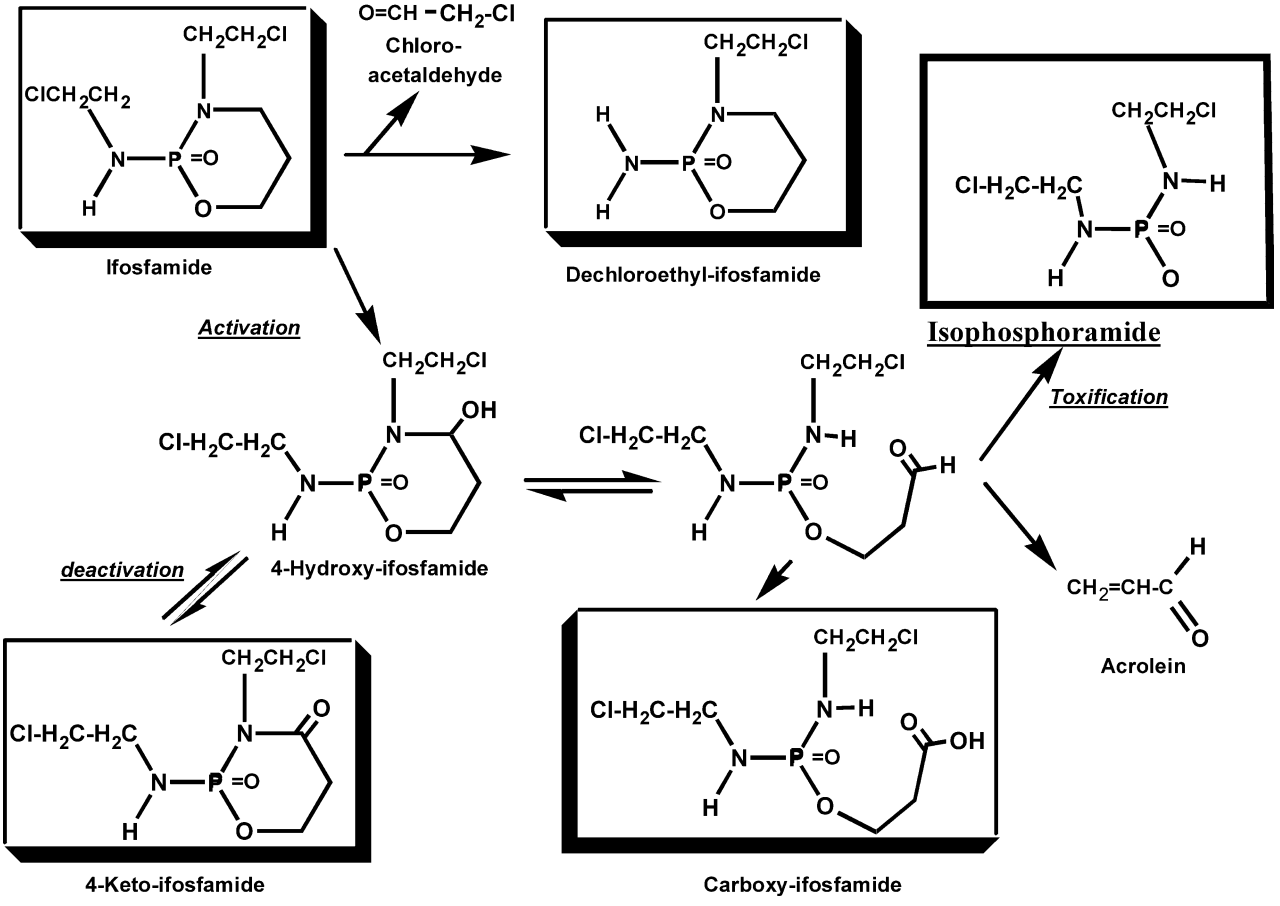

Fig. 1 Ifosfamide metabolism pathway leading to isophosphoramide mustard

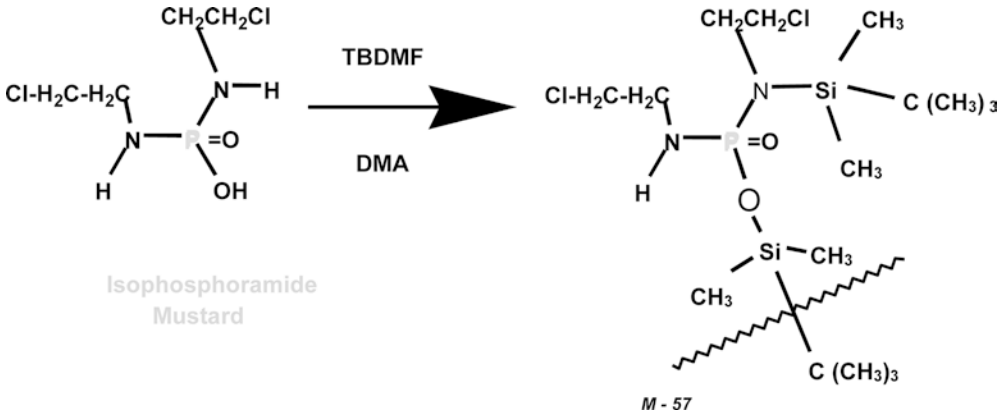

Fig. 2 IPM chemical derivatization and characteristic fragment $m/z = 355$. (m 412-57)

significant drug degradation during workup analysis, aliquots of the different sample specimens were individually frozen at −80°C. 5-FU was used as internal standard. Plasma samples were stored at −5°C until analyzed. Random plasma samples were spiked with IPM at 0.1–10 μg/ml in DMA. Extractions were made using 3 ml cold acetonitrile, vortexed for 3 min and centrifuged. The acetonitrile was recovered and evaporated to dryness under nitrogen. The dry residue was chemically derivatized by treatment with a mixture of 20 μl DMA and 75 μl TBDMF at 60°C for 15 min and 3 μl of the mixture injected into the GC–MS. The IPM GC–MS assay was conducted on a Hewlett–Packard 5890 series II gas chromatograph coupled with a mass spectrometer (MS Engine Hewlett–Packard). The chromatographic column used was CPSIL-19CB (Chrompak), diameter 0.3 mm, and length 60 cm, with a helium column flow at 0.5 ml/min. Injection was initiated at 200°C, increasing to 280°C at 10°C/min. Under these conditions, IPM quantification was performed by selected ion monitoring (SIM) at m/z 355 and 301, fragments corresponding, respectively, to IPM and 5-FU. m/z 355 represents the main fragment of the molecule corresponding to the loss of the well-known *t*-butyl group (m-57) (Fig. 2). Retention times for IPM and 5-FU were 14.8 min and 9 min, respectively. The quantification limit of IPM was 100 ng/ml. The extraction efficiency was 85%.

Verification of the assay included a calibration curve assaying five plasma standards prepared with IPM concentrations ranging from 0.1 to 20 μg/ml. Plasma was obtained from healthy volunteers and spiked with IPM. Standard curves were constructed by plotting the ratio of the peak area of IPM to that of internal standard against known drug concentrations. Drug concentrations in all samples were calculated using the results of linear regression analysis. Reproducibility was higher than 85%.

Protein binding studies

In order to assess protein binding of IPM, 188 μg IPM was dissolved in 5 ml fresh human plasma (obtained from the blood bank). (Maximum binding of IPM after IV infusion of 5 mg/ml was determined by extrapolation to be 188 μg.) In duplicate, the exposure time of IPM was evaluated at 0, 5, 15, 30 and 60 min intervals at 37°C. At each time point, 800 μl plasma was placed into centrifugal filter devices MPS1 (Amicon) and 400 μl plasma retained for IPM plasma binding determinations. The protein-free filtrates were centrifuged for 30 min at 3000 *g* at 2°C, and the IPM concentration in the resulting filtrates determined by GC–MS analysis as described above. The ratio of IPM in the protein-free filtrate to that in the plasma was the free plasma protein fraction.

Stability studies

IPM stability was investigated under sterile conditions in RPMI 1640 medium at 37°C under an atmosphere containing 5% CO_2. Concentrations of IPM were in the 150–220 μl range. This was the steady-state concentration range for IPM after extrapolation from infusion studies. Aliquots were withdrawn at 0, 10 and 30 min, and 1, 2, 4, 7 and 24 h and analyzed by GC–MS as described above.

Animals

Adult Sprague-Dawley rats (males 259–308 g and females 163–209 g) and C_3H mice (males 21–29 g and females 18–28 g) were obtained from Harlan Industries (Indianapolis, Ind.), and housed in groups of three to five per cage in light-controlled (12 h/day) and temperature-controlled (24°C) animal isolators with filtered vents and exhausts. They were fed a diet of Purina Laboratory Chow (Purina Feed) and received tap water ad libitum.

Adult male and female beagle dogs (6.8–8.7 kg) were raised and maintained at MPI (Mattawan, Mich.). They were fed a diet of Purina Dog Chow (Purina Feed) and received tap water ad libitum.

Adult male Rhesus macaque (*Macaca mulata*) monkeys (one 10.2 kg and the other 12.2 kg) were raised and

maintained at the Tulane Primate Center (Covington, La.), and housed singly in controlled cages. The animals were fed Purina Monkey Chow (Purina Feed) and fresh fruit daily and received tap water ad libitum.

Mice, rats and dogs were killed with phenobarbital/ketamine anesthesia and/or carbon dioxide inhalation. The monkeys were not killed. An institutional animal care and use committee (IACUC) reviewed and approved all the studies.

Mouse/rat/dog toxicity studies were conducted at MPI (Mattawan, Mich.) under GLP regulations as described in the Guide for the Care and Use of Laboratory Animals, Office for Laboratory Animal Welfare, NIH. Monkey studies were conducted at Tulane University Primate Center (Covington, La.) using good research practice techniques. Mouse/rat IV and oral toxicity studies included doses of IPM in saline (5–10 mg/ml) administered by IV push via a tail vein or by oral gavage.

Pharmacokinetic studies

Two adult male Rhesus macaque monkeys were given IPM (5 mg/kg) as a slow IV bolus, and samples were collected at various time points. Two predosing samples were taken. An 18-gauge venous catheter was inserted into the right saphenous vein for bleeding, and a 20-gauge catheter was placed in the left saphenous vein for drug administration. The dose of IPM was 5 mg/kg administered in 10 ml sterile saline as a bolus injection. Blood samples were collected at the following time points: 5, 15, 30, 60, 90 and 120 min, and 1, 1.5, 2, 4, 6 and 24 h. Animals were anesthetized with Telazol (tiletamine/zolazepam) and ketamine, lightly restrained through the bleed, and returned to their cages. The catheter for collecting samples was flushed after each bleed with 5% dextrose/lactated Ringer's solution and 2 ml blood was discarded prior to each bleed.

IPM was administered as a slow bolus injection daily to eight male and eight female adult beagle dogs. Dose escalations were: 1, 1.5, 3, 5, and 10 mg/kg. A 20-gauge venous catheter was inserted into a saphenous vein for bleeding. The drug was administered though a separate femoral vein. Blood samples were withdrawn at 0, 5, 15 and 30 min, and 1, 1.5, 2, 4, 8, 12 and 24 h. Animals were anesthetized with ketamine. The plasma was separated and stored at −70°C.

IPM (100 mg/kg) was administered IV via the tail vein to 30 adult female C_3H mice. Blood samples were collected from three to five mice at 0, 5, 15 and 30 min, and 1, 2, 4 and 6 h and combined to obtain a sufficient quantity of plasma. The plasma was separated and stored at −70°C.

Animal pharmacokinetic data analysis

Model parameters were estimated using Micropharm software, and nonlinear least squares regression was performed using Simplex and Gauss Newton algorithms [11]. An open two-compartment model provided the best fit. Clearance, volume of distribution and half-lives were derived from estimates of the model parameters.

Allometry

An allometric pharmacokinetic modeling was performed using a population, nonlinear mixed-effect modeling [4]. The data from the three animal species were analyzed in a single step according to a two-compartment open model. The parameters of the model were V_1 and V_2 (central and peripheral volumes of distribution) and CL and Q (elimination and intercompartmental clearances). The body weight (BW) in kilograms for each species was used as a covariate in the modeling of pharmacokinetic parameters, according to the typical relationship, $P = a \times BW^b$, where P represents a pharmacokinetic parameter, and a and b are the parameters to be estimated. Interspecies and residual variabilities were modeled as proportional and mixed (proportional plus additive) errors, respectively.

Results

Toxicity

Acute oral and IV toxicity study results for mice and rats are presented in Table 1 which shows the median 50% lethal dose (LD_{50}) values. These values were calculated by combining the data from the acute single-dose studies according to route and species and are available only for mice and rats. Specific lethal values are discussed below for dogs, as well as multidosing in the mouse.

Clinical signs generally reflecting the deteriorating state of both mice and rats prior to death were observed in both sexes in a dose-dependent manner and included body surface staining, decreased activity, lethargy, loss

Table 1 IPM lethal single doses

Species and strain	Number/sex	Route	LD_{50} (g/kg)	Time until last death (days)	Length of study (days)
Rat (Sprague-Dawley) [Crl: CD^R(BR)]	31 M 25 F	Oral	3.56	2	14
Mouse (Sprague-Dawley) [Crl: CD^R(BR)]	31 M 25 F	Oral	1.43	2	14
Rat (Sprague-Dawley) [Crl: CD^R(BR)]	28 M 19 F	IV	0.43	2	14
Mouse (Sprague-Dawley)[Crl: CD^R(BR)]	23 M 20 F	IV	0.69	2	14

Table 2 Subacute toxicity in the dog following treatment with IPM i.v daily for 3 days

Dose (mg/kg)	Number and sex	Observations
1.0	2 M 2 F	No deaths
1.5	2 M 2 F	No deaths
3.0	2 M 2 F	No deaths
5.0	2 M 2 F	No deaths
10.0	2 M 2 F	All died
100.0	2 M 2 F	All died

of appetite, decreased defecation, tremors, and/or whole-body edema. The lethal experience was sedation followed by respiratory arrest. No seizures or gross pathologies were observed for the survivors. As discussed in the section on multiday dosing for mice, splenic depletion of lymphocytes, aplastic bone marrow and renal tubular necrosis in the kidney were observed and considered the underlying cause of death. Although death at 2 days is considered to be early from renal tubular necrosis, the pathology supports the diagnosis (D.G.S.).

Subacute multidose toxicity in mouse and dog

A subacute IV study of IPM administered daily for 3 days to mice was performed to obtain LD_{10}/LD_{10} values and toxicity that could be projected to a human study. The doses ranged from 75 to 562 mg/kg and included five male and five female animals per dose. The LD_{10}/LD_{10} values were 119 mg/kg and 149 mg/kg, respectively. The mortality observed reflected a typical dose-response effect, with IPM being slightly more toxic in females than in males.

The IV LD_{10} of IPM for multidosing was calculated as 119 mg/kg (95% CI 87–134 mg/kg) in mice (combined sexes), while the IV LD_{50} was calculated as 149 mg/kg (95% CI 132–169 mg/kg). The LD_{10} values for males and females separately were 168 and 125 mg/kg, respectively, while the LD_{50} values for males and females were 176 and 132 mg/kg, respectively; in neither of the latter cases could the 95% confidence limits be calculated.

In mice, severe bone marrow depletion was present in males at 178 mg/kg and higher and in females at 133 mg/kg and higher. There were essentially no hematopoietic cells present in the bone marrow of any of the animals graded as severe. Secondary to the absence of hematopoietic cells, congestion (increased blood) was present in the marrow of many of the animals. Necrosis of kidney tubules occurred in males with IPM doses of 237 mg/kg and higher and in females with doses of 133 mg/kg and higher. In the males, the incidence of animals with more severe lesions increased with increasing dose. This was also true for females, except that there was essentially no difference between the two highest doses. Basophilic tubules, indicative of regeneration and secondary to the necrosis, were present in many of the kidneys. Lymphoid depletion occurred in most of the males and all of the females that died. While females were more severely affected than males, the most severe depletion occurred at 316 mg/kg and higher in both sexes.

The 3-day dosing of dogs included 12 adult beagle dogs (6 male and 6 female) which were divided into three groups and received daily IV injections of IPM for 3 days (Table 2). Treatment-related toxicity of IPM in dogs treated IV for up to 3 days was seen at all doses tested, with the findings demonstrating dose relationships. Doses of 10 and 100 mg/kg resulted in 100% treatment-related mortality.

Mortality at I00 mg/kg was the result of severe, diffuse necrosis of renal cortical tubule cells, while mortality at 10 mg/kg was the result of bone marrow depletion, leading to almost a complete absence of hematopoietic cells. Microscopically, kidney and bone marrow effects were not observed at doses up to 5 mg/kg, but minimal effects were noted in the liver (trace neutrophilic infiltration in the centrilobular region and/or trace increase in the number of leukocytes within the sinusoids). Clinically, dose-related depressions of both leukocyte and platelet counts were noted at doses from 1 to 10 mg/kg. Somewhat lower hemogram values were seen at doses from 1.5 to I0 mg/kg. For the 5 mg/kg group, pancytopenia with reductions in platelet counts on day 11 after treatment were noted, which reversed. Serum electrolytes and albumin were reduced at doses as low as 3 mg/kg and were generally further reduced at doses up to I0 mg/kg. Alkaline phosphatase and cholesterol were increased at 5 and I0 mg/kg, while aspartate aminotransferase, alanine aminotransferase, sorbital dehydrogenase, total bilirubin, blood urea nitrogen, and creatinine were increased, and glucose was decreased at 10 mg/kg. Many of these findings at I0 mg/kg may be secondary to the general debilitated state that occurred in the dogs, although the increases in blood urea nitrogen and creatinine values correlated with the observed renal toxicity at I00 mg/kg. A finding of enlarged pupils seen at doses from 1.5 to 5 mg/kg may reflect a specific pharmacological effect of IPM. At the 1–5 mg/kg doses, diarrhea and soft stools were noted; all animals recovered. Based on the conditions and findings of this study, the maximum tolerated dose (MTD) of IPM following daily IV administration for 3 days was considered to be 5 mg/kg. However, some toxicity was noted at doses as low as 1 mg/kg.

Plasma protein binding and stability in dogs

Protein binding values were determined in plasma samples obtained from dogs. The proportions of IPM bound in ultrafiltered plasma were 98.9% at T_0, 98.5% at $T_{5\ min}$, 98% at $T_{15\ min}$ and 98% at $T_{30\ min}$. Therefore, IPM protein binding was instantaneous and represented $>98\%$ of IPM available. After a 7-h incubation in phosphate buffer, pH 7.4, at 37°C, 40% of the IPM was

recovered and after a 24-h incubation, 10% was recovered. This supports the conclusion that in clinical trials the duration of IV infusion should not exceed 1 h.

Pharmacokinetic studies in monkeys, dogs and mice

Plasma concentration-time profiles of IPM in the two adult monkeys after single injections of 5 mg/kg are presented in Fig. 3. Mean pharmacokinetic parameters (Table 3) were $T_{1/2\beta}$ 4.2 h and clearance 1.65 l/h/kg. The IPM level 4 h after IPM administration was below the limit of quantification.

The mean plasma curves for IPM after administration of 5 and 10 mg/kg as single doses to dogs are presented in Fig. 4. Pharmacokinetic data for the study are summarized in Table 3. IPM was non-detectable 1.5 h after administration of 5 mg/kg and at 2 h after administration of 10 mg/kg. No plasma IPM was detected at doses of 1 and 1.5 mg/kg. Laboratory errors prevented interpretation of the data for the 3 mg/kg dose. The $T_{1/2}$ was calculated as 48 min. Plasma clearance was constant between 5 and 10 mg/kg, with good linearity for the pharmacokinetics of IPM at the 5 and 10 mg/kg dose levels.

The pharmacokinetics of IPM in mice are shown in Fig. 5. IPM plasma concentrations decreased rapidly in less than 1 h ($T_{1/2\alpha}$ 2 min), with a clearance of 8.44 l/h/kg and a terminal $T_{1/2\beta}$ of 451 min. A total of 30 female mice were dosed with 100 mg/kg IV, and three to five animals were killed at each of the times indicated and the plasma pooled. Figure 5 shows data from one experiment.

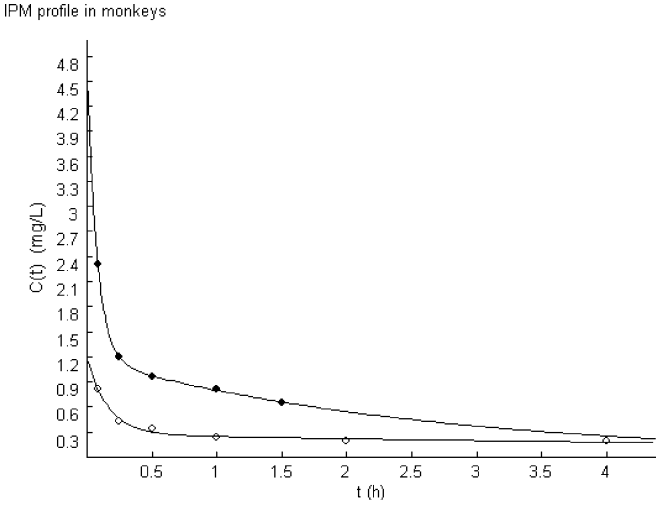

Fig. 3 Plasma concentration-time profiles of IPM in two monkeys after a 5 mg/kg IV injection

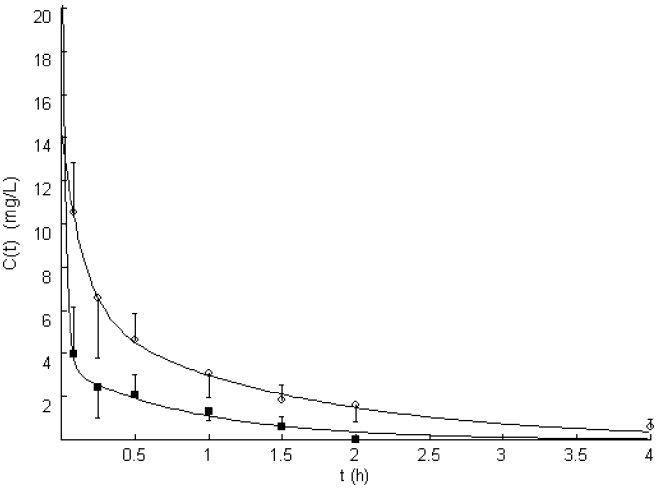

Fig. 4 IPM plasma concentration-time profiles in dogs after IV administration of 5 mg/kg (*filled squares*) and 10 mg/kg (*open circles*)

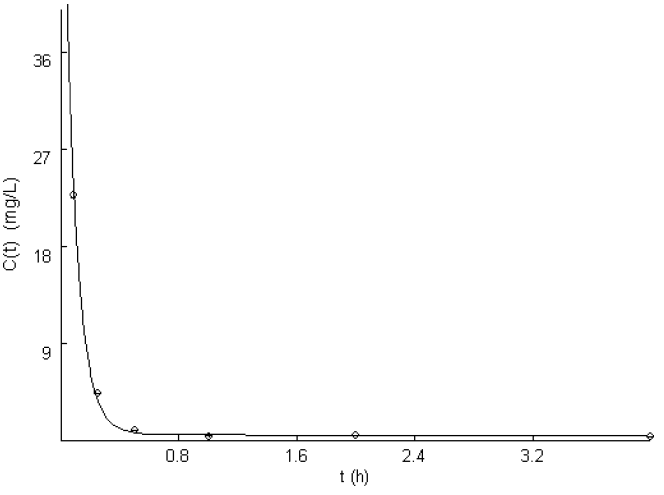

Fig. 5 IPM plasma concentration-time profile after 100 mg/kg IV injection in mice

Allometry

The data for all species were simultaneously fitted to a two-compartment model. The population analysis allowed estimation of the parameters a and b relating BW

Table 3 Pharmacokinetic parameters for IPM after a single IV administration to monkeys, dogs and mice. The data are presented as mean values

Species	No. of animals	Dose (mg/kg)	AUC (mg/kg/l/h)	Clearance (mg/kg/h)	$T_{1/2\beta}$ (h)	Vd (l)	Cmax (mg/l)
Mouse	5	100	11.85	8.44	7.53	50.62	66.00
Dog	2	5	3.67	1.36	0.61	1.00	3.81
	2	10	9.84	1.01	0.99	1.29	10.52
Monkey	2	5	3.00	1.65	4.2	10.1	1.5

Table 4 Coefficient and exponent power functions relating pharmacokinetic parameters of IPM to BW (*NE* not evaluated)

Parameter	V_1	CL	Q	V_2
a	1.29 l	3.82 l/h	2.84 l/h	2.38 l
b	0.90 kg^{-1}	0.55 kg^{-1}	0.82 kg^{-1}	0.65 kg^{-1}
Interspecies variability (%)	NE	47	NE	NE
Extrapolation to 70-kg human	59 l	40 l/h	92 l/h	38 l

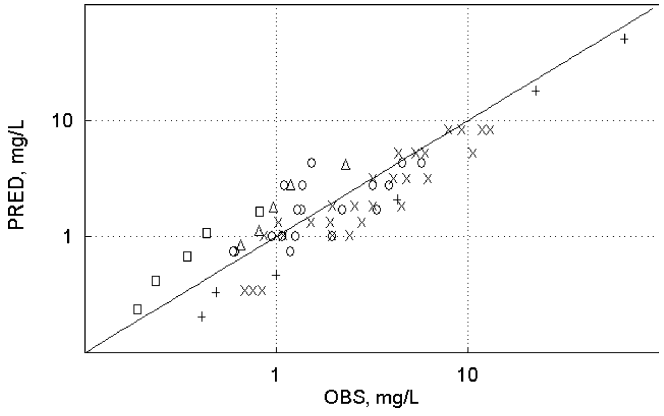

Fig. 6 Predicted plasma concentrations (*PRED*) using the population-allometric modeling versus observed concentrations (*OBS*). Data are presented on a log scale. The straight line is the line of identity (PRED = OBS) (*plus symbols* mice 100 mg/kg, *open circles* dog 5 mg/kg, *crosses* dog 10 mg/kg, *open squares* monkey 1 5 mg/kg, *open triangles* monkey 2 5 mg/kg)

Table 5 Actual versus allometric-predicted clearance values for IPM (*NA* not available)

Species	Clearance (mg/l)	
	Actual	Predicted
Mouse (100 mg/kg)	8.44	22
Dog (5 mg/kg)	1.36	1.40
Dog (10 mg/kg)	1.01	1.01
Monkey (5 mg/kg)	1.65	1.34
Humans	NA	0.56

Table 6 Estimated comparable human IV dosages

Species	Subacute IV MTD (mg/m^2 per day)	Comparable human IV dose (mg/m^2 per day)
Mouse	357	36
Dog	100	100

to the pharmacokinetic parameters (Table 4). The predicted-observed concentration plots had an acceptable distribution of the points along the line of unity, except for one monkey (Fig. 6). The interspecies variability on CL and the residual variability were in the mean superior ranges, 47% and 48%, respectively. Table 5 shows allometric-predicted and actual clearance values.

Table 6 shows the MTD for mice and dogs and the estimated values for humans. The latter values were used to design the clinical trial in the IND studies [13].

Discussion

IFOS is a prodrug alkylating anticancer drug with a wide spectrum of activity. Prior to being an effective alkylator, IFOS must be metabolized to IPM, the active anticancer agent [7]. Limitation of the therapeutic effectiveness of IFOS results from the toxicity of two metabolites, acrolein and chloroacetaldehyde, which are also formed along with IPM (Fig. 1) [2]. The former two chemicals have been related to the neurological and renal toxicity noted with IFOS therapy [7, 9]. Thus, the use of IPM, alone or in combination therapy should eliminate the exposure to acrolein and chloroacetaldehyde [2]. The rationale for the clinical development of IPM is based on its significant antitumor activity with anticipated reduced toxicity (e.g., hemorrhagic cystitis and encephaloneuropathies) in comparison with cyclophosphamide or IFOS [10].

We report here the results of acute and subacute toxicity studies of IPM in rodents, dogs, and primates, and of experimental therapy studies in mice. The endpoint of all the studies was identification of an MTD to develop acceptable starting doses for the phase I trial. The animal toxicity studies included data from mice, rats and dogs. Single oral LD_{50} values in rats were calculated as 3560 mg/kg for both sexes combined. In mice, oral (single dose) LD_{50} values were calculated as 1432 mg/kg for both sexes combined.

The IV single-dose LD_{50} values of IPM in rats and mice (sexes combined) were calculated as 428 and 688 mg/kg, respectively, and the LD_{10} value in mice (sexes combined) was calculated as 93 mg/kg.

After administration of IPM to mice IV daily for 3 days, the LD_{10} and LD_{50} values were calculated as 119 and 149 mg/kg, respectively, a steep curve.

Microscopic examination at autopsy documented treatment-related bone marrow depletion and/or kidney tubular necrosis as the cause(s) of death in the study. Severe bone marrow depletion was present in males following treatment at doses of 178 mg/kg and higher, and in females following treatment at doses of 133 mg/kg and higher. Kidney tubular necrosis occurred in males following treatment at doses of 237 mg/kg and higher, and in females following treatment at doses of 133 mg/kg and higher. The latter finding was confirmed by microscopic examination as early as 2 days after treatment (Table 1) (by one of the authors, D.G.S.). In addition, splenic lymphoid depletion was noted in most

males and in all females that died during the study (all doses). No obvious treatment-related microscopic findings were noted in either sex following treatment at 75 mg/kg. Clinical signs generally secondary to the deteriorating state of the mouse prior to death were observed, but no clear evidence of BW effects were seen in mice surviving to study termination.

A variety of clinical findings were observed in mice at all dose levels except 100 mg/kg IV in both sexes throughout the study. These findings included low carriage, hunched posture, decreased activity, tremors, decreased defecation/soft stool, respiratory difficulty, increased/discolored (red) salivation, discoloration/staining of body areas and partial closure of the eyes in some or all animals. At 100 mg/kg IV, mice appeared normal throughout the study with the exception of a single male, which exhibited low carriage and body surface staining late in the study. Clinical findings observed at the higher dose levels primarily included ptosis, low carriage, decreased activity, tremors, decreased defecation, labored respiration, staining of body areas and scabbed, discolored, and/or ulcerated areas of the tail generally noted towards the end of the study. No seizures or paralysis were noted. No bleeding was noted (vaginal, rectal or urethral orifice).

Adult beagle dogs were treated with an IV dose (1–100 mg/kg) of IPM daily for 3 days. LD_{100} effects were noted at doses of 100 mg/kg and 10 mg/kg. At the 1, 1.5, 3 and 5 mg/kg doses, all animals recovered and survived. Bone marrow toxicity with thrombocytopenia and mild renal tubular changes were noted. Soft stools and loss of appetite were also noted at doses in the range 1–5 mg/kg. The lower doses of IPM were tolerated reasonably well.

Comparison of the MTD in dogs following 3-day IV dosing (100 mg/m^2) with the LD_{10} in mice following 3-day IV dosing (357 mg/m^2) supports IPM as a minimally toxic alkylating agent with a narrow therapeutic range. On a milligram per meter squared basis, the starting dose in humans should be between one-tenth the LD_{10} in mice (36 mg/m^2 per day) and 100 mg/m^2 per day from the dog data (Table 6).

The described GC–MS assay was developed to document IPM's pharmacokinetic parameters. The assay was reproducible (92%), with two steps: organic extraction and chemical derivatization. No interfering IPM metabolites were detected (e.g., 2-phosphoroethanolamine or 2-chloroethylphosphoramidic acid).

Pharmacokinetic studies were conducted in three animal species, monkeys, dogs, and mice. The maximum tolerated 3-day IV dose of IPM in dogs was 5 mg/kg daily; 10 mg/kg per day for 3 days is the lethal dose. In dogs, death following treatment with IPM was due to renal failure and bone marrow depletion. No conclusions can be drawn in monkeys because of the absence of a toxic dose. However, at a single IV dose of 5 mg/kg, no major toxicity was observed in monkeys. Minimal reversible effects in beagle dogs were observed at doses up to and including 5 mg/kg per day for 3 days that consisted of hematological and renal toxicities.

Pharmacokinetic parameters were obtained from Gauss Newton algorithm modeling [11]. The $T_{1/2}$ was calculated as 7.53 h in mice and monkeys and 0.61 h in dogs. These results suggest that in the clinical trials IPM should be administered over a short period of time, e.g., 0.5–1.5 h. This agrees with the stability data for IPM in saline [12] and the observations in the IND application [13]. Mean plasma clearance values for monkeys and dogs that received 5 mg/kg were 1.65 and 1.36 l/h/kg, respectively, with good linearity. No differences in pharmacokinetics between male and female dogs were noticed. In mice, IPM clearance increased with a value of 8.44 l/h/kg.

The allometric analysis of interspecies pharmacokinetic data provided clearance predictions reasonably close to those actually estimated, except in the mouse. Human pharmacokinetic parameters were predicted from allometric analysis using all animal species. Data predicted an acceptable starting dose of 30 mg/m^2 with a clearance of 39.5 l/h, a $T_{1/2\alpha}$ of about 10 min and a $T_{1/2\beta}$ of about 1 h 45 min for a patient of 70 kg. The extrapolated clearance for humans should be considered cautiously, for the interspecies variability for this parameter was high (about 47%). The stability data are surprising in view of the published half-life of about 75 min for IPM in buffer at 37°C [12]. However, the former value agrees with the infusion stability observed and reported in the FDA IND for IPM [13].

The human clinical phase I trial of IPM should start soon on the basis of this preclinical study in order to confirm the antitumor activity of this drug.

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