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Predicting the maximum-tolerated dose of PNU-159548 (4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyl-daunorubicin) in humans using CFU-GM clonogenic assays and prospective validation

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Abstract

A haematotoxicity model was proposed by Parchment in 1998 to predict the maximum-tolerated dose (MTD) in humans of myelosuppressive antitumour agents by combining data from *in vitro* clonogenic assays on haematopoietic progenitors and *in vivo* systemic exposure data in animals. A prospective validation of this model in humans was performed with PNU-159548, a novel agent showing selective dose-limiting myelosuppression in animals. PNU-159548 and its main metabolite, PNU-169884, were tested *in vitro* on murine, canine and human colony forming units-granulocyte macrophages (CFU-GM) and *in vivo* on mice and dogs. The IC_{90x} ratios (IC_x = concentration inhibiting x% of colony growth) for CFU-GM and drug plasma protein binding were used to adjust the target plasma concentrations versus time curve (AUC) and predict the human MTD. The predicted MTD was compared with values achieved in phase I studies. Canine CFU-GM were 6-fold more sensitive ($P<0.01$) and murine CFU-GM 1.7-fold less sensitive ($P<0.05$) to PNU-159548 treatment than the human progenitors. PNU-169884 behaved similarly to PNU-159548. The predicted MTDs in humans calculated from data in mice and dogs were 15 and 38 mg/m^2, respectively. Overall, 61 patients were treated in two phase I studies, at doses ranging from 1.0 to 16 mg/m^2. Thrombocytopenia was dose-limiting with a MTD of 14 and 16 mg/m^2 in heavily and minimally pretreated/non-pretreated patients, respectively. Adjusting animal MTD data by means of the CFU-GM ratio between species can predict the human MTD with a good quantitative accuracy. Inhibition of common haemopoietic progenitors by PNU-159548 induced neutropenia/thrombocytopenia in animals and thrombocytopenia in patients, probably due to the higher sensitivity to the compound observed in human colony forming units-megakaryocyte (CFU-MK).

Keywords: Cytotoxic; *In vitro* myelotoxicity; IC_{90} ratios; PNU-159548

1. Introduction

Selection of the starting dose and the design of dose escalation are crucial steps in the performance of phase I clinical studies of new cytotoxic antitumour agents. Inappropriate decisions might affect patients' safety and clinical development. Presently, decisions are made mainly on the ground of traditional toxicology in rodents and dogs, even though the limits of using such data in humans are very well known.

A pharmacokinetically-guided dose-escalation design and integrating toxicology and exposure data in animals to overcome interspecies differences has been proposed and prospectively evaluated in humans. However, its clinical application is limited for a variety of reasons including interspecies differences in metabolism, protein binding, normal tissue sensitivity and variability of pharmacokinetics (PKs) [1,2]. *In vitro* haematotoxicity

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is the application of specialised cell cultures, using CFU-GM as a surrogate marker of human haematopoietic tissue, in order to study the adverse effects of exposure to xenobiotics, like cytotoxic antineoplastic agents, with exposure levels comparable to those likely to be achieved at toxic doses in phase I studies [3]. The haematotoxicity model proposed by Parchment and colleagues, to predict the maximum tolerated dose (MTD) of myelosuppressive agents in humans, can be considered for those drugs which have shown dose-limiting myelotoxicity in all animal species [4]. In particular, Model 2 is able to predict the plasma concentration versus time curves (AUC) at the MTD in humans starting from the target AUC at the MTD in animals, and duly adjusted for the interspecies differences in IC_{90x} values (IC_x = concentration inhibiting x% of colony growth). This model can be used to anticipate the target exposure that should be reached during the phase I dose escalation, allowing clinicians to accelerate the overall process. The model requires the active compound and main metabolite(s) to have a linear PK within the dose range tested.

PNU-159548 (4-demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyl-daunorubicin, Fig. 1), the lead compound of the class of alkycyclines, new cytotoxic agents with a promising spectrum of antitumour activity in preclinical models [5], was judged to be a good candidate for assessing the clinical value of the haematotoxicity model. PNU-159548 is endowed with a novel mechanism of action, DNA intercalation and alkylation [6], and is cytotoxic against both murine and human tumour cell lines *in vitro*. The molecule shows a wide spectrum of antitumour activity *in vivo* against both rapidly proliferating murine leukaemias and slowly growing transplantable human tumours and displays a remarkable synergistic effect on xenograft tumours when tested in combination with CPT-11, paclitaxel, docetaxel, etoposide, doxorubicin and gemcitabine [7,8]. PNU-159548 is also able to circumvent resistance to all major classes of cytotoxics including multi-drug resistant-related drugs, alkylating agents and topoisomerase I and II inhibitors [9]. Myelosuppression was the main dose-limiting toxicity (DLT) in all of the tested animal models [5].

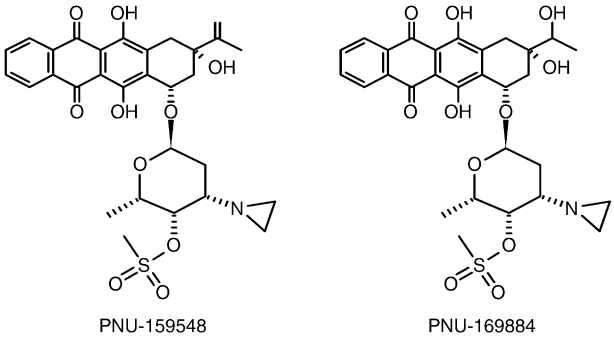

Fig. 1. Chemical structure of PNU-159548 and PNU-169884.

The major route of PNU-159548 metabolism is *via* reduction of the 13-keto group to the 13-dihydro derivative PNU-169884 (Fig. 1). The metabolite of PNU-159548 showed *in vitro* cytotoxicity and myelotoxicity comparable to that of the parent compound. Unlike 13-dihydro metabolites of anthracyclines [10], PNU-169884 maintained *in vivo* antitumour activity against doxorubicin-resistant tumours.

On the basis of these preclinical findings, PNU-159548 was selected for clinical development and the prospective validation of the haematotoxicity model was performed in two concomitant phase I studies using the same design, study population and treatment plan.

The present paper reports the validation of the model and the results of the *in vitro* haematotoxicity studies with plasma samples of patients, while the phase I clinical and PK results are the subject of a separate paper [11].

2. Materials and methods

2.1. Drugs

PNU-159548 and PNU-169884 were synthesised by Pharmacia Corporation, Milan, Italy. The chemical structure of PNU-159548 and PNU-169884 are reported in Fig. 1. The compounds were provided as dry powders. For *in vitro* studies, the compounds were reconstituted with dimethylsulphoxide (drug solubility limits >50 mg/ml) and serial dilutions were performed in the serum-free culture medium containing fixed concentrations of dimethylsulphoxide. For *in vivo* studies, PNU-159548 was reconstituted in sterile water as a pharmaceutical formulation and PNU-169884 was dissolved in cremophor/ethanol. The solutions were freshly prepared before each experiment.

2.2. Animals

Albino Crl:CD-1(ICR)BR mice of both sexes, aged approximately 5–7 weeks (Charles River, Italy) and Beagle dogs of both sexes, aged approximately 1 year (Green Hill, Italy), were used for bone marrow cell cultures, preclinical toxicology and PK studies.

2.3. Haematopoietic progenitor cells and clonogenic assays

Murine and canine bone marrow cells were isolated according to the method previously described by Parchment in Ref. [12] with slight modifications. Murine cells were obtained from animals euthanised by cervical dislocation. The femurs were aseptically removed and the marrow was flushed from the bone and collected in tubes

containing 6 ml of Hank's Balanced Salt Solution (HBSS). Canine cells were obtained by bone marrow aspiration from the iliac crest of anaesthetised dogs. The marrow was drawn into a 30 ml syringe using a Jamshidi needle and then transferred into sterile heparinised tubes. HBSS was then added to each tube to a volume of 3–4 ml to reach a final volume of 6 ml.

Human cord blood (hCB) cells were obtained from placentas after physiological deliveries (with the informed consent of the mothers) according to the method described by Ghielmini in Ref. [13]. In view of the reduced availability of human bone marrow (BM) cells, only hCB cells were used, since the equivalence of both sources of human haematopoietic cells for *in vitro* myelotoxicity tests has already been well demonstrated [14]. Cells from each hCB sample were processed and used within 24 h of the delivery.

Clonogenic assays on murine, canine and human colony forming units-granulocyte macrophages (CFU-GM) were performed according to the method described by Parchment in Ref. [12].

Human CFU-MK were obtained and the clonogenic assay was performed as described by Hogge in Ref. [15].

2.4. *In vitro cytotoxicity*

Drug sensitivity was determined on cells exposed for 1 h to increasing concentrations of PNU-159548 or PNU-169884 in Roswell Park Memorial Institute (RPMI)1640 (canine cells) or Iscove's modified Dulbecco's Medium (IMDM) (murine and human cells), containing 2 mM L-glutamine, penicillin (5000 Units/100 ml) and streptomycin (5 mg/100 ml). The 1-h exposure period was selected for the *in vitro* assessment of myelosuppressive potential of PNU-159548 on the basis of drug stability data and its plasma half-life [5]. At the end of the incubation period, cells were washed twice with HBSS, suspended in 1 ml medium and counted. Then triplicate cultures were prepared by adding a volume corresponding to 250 000 cells to 2.5 ml of methyl cellulose medium for each sample. The components were mixed and plated in 35 mm tissue culture dishes and incubated for 14 days at 37 °C in a fully humidified atmosphere containing 5% CO_2.

CFU-GM colonies containing at least 50 cells were considered and were scored on an inverted microscope [16]. Each experiment was considered to be valid for the analysis only if a miminal cloning efficiency of 60–70 (hCB and murine BM) and 20–30 (canine BM) GM-CFC/10^5 mononuclear cells (MNC) was obtained. CFU-MK colonies in which cells showed a pink membrane and blue nuclei staining, were scored on an inverted microscope according to the established criteria in Ref. [15].

The effect of the compounds was evaluated as IC_{70} and IC_{90} and calculated by log-linear regression analysis. The unpaired *t*-test of Student was applied to compare mouse and dog to human values.

The plasma of 9 patients treated at doses of 12–14 mg/m^2 of PNU-159548, was collected before the drug administration, at the end of infusion (time 0) and at 15, 30, 60 min and 24 h post-dosing. Samples were immediately frozen at −80 °C. Plasma samples were incubated for 1 h with hCB cells to determine their capacity to inhibit CFU-GM growth. The concentrations inhibiting 50–70–90% of the colony growth were read from the plotted dose–response curves.

2.5. In vivo *animal toxicology*

Single-dose toxicity studies after intravenous (i.v.) bolus administration were performed in mice and dogs of both genders with PNU-159548 (2.5–5.8 and 0.1–0.6 mg/kg in mice and dogs, respectively) and in mice with PNU-169884 (2.5–7.5 mg/kg).

Blood sampling for the evaluation of haematological parameters (erythrocytes, platelets, leucocytes, neutrophils, eosinophils, basophils, lymphocytes and monocytes) were performed at doses of 2.5 and 4.5 mg/kg in mice and at 0.1, 0.3 and 0.6 mg/kg in dogs up to 28 days after treatment. The animals were checked daily for mortality, behaviour and general condition, body weight was recorded twice a week. Clinical observations and weekly laboratory examinations were performed during the study and a post-mortem examination was performed in all animals. Target organs and MTDs were identified in both species. The MTDs was defined as the dose level inducing a moderate (50–60%) decrease in leucocyte and/or platelet counts without any other major toxicities.

2.6. *Clinical studies*

The safety profile and PKs of PNU-159548 administered as a single i.v. dose every 21 days were evaluated in two separate phase I studies carried out in a total of 61 adult patients with a variety of solid tumours. The clinical results of these studies are the object of a separate publication [11]. PNU-159548 was administered as a 10-min infusion from a starting dose of 1 mg/m^2, corresponding to one-tenth of the LD_{10} (LD = lethal dose to *x*% of animals) in mice. Toxicity was graded by National Cancer Institute- Common Toxicity Criteria (NCI-CTC) version 2.0. The MTD was defined as the dose at which $\geqslant 2/3$ or $\geqslant 2/6$ patients experienced DLT after the first cycle. DLTs were grade 4 neutropenia lasting $\geqslant 5$ days or complicated by grade 3 or 4 infection febrile neutropenia, grade 3 or grade 4 thrombocytopenia, grade 4 anaemia, grade 3 or grade 4 non-haematological toxicity.

2.7. *Pharmacokinetics*

The systemic exposure of PNU-159548 and PNU-169884 were evaluated after single i.v. administration in

mice, dogs and patients during the first cycle. Samples were collected in pre-cooled heparinised tubes, immediately placed in an ice/water bath, centrifuged at 1200*g* for 10 min at 4 °C and frozen at −80 °C until analysis. Compounds were assayed by using a fully automated dual-column liquid chromatography-mass spectrometry assay. The limit of quantitation was 0.05 ng/ml for PNU-159548 and 0.1 ng/ml for PNU-169884.

PK data analysis was carried out using a non-compartmental analysis approach with the aid of the WinNonlin package (Scientific Consulting, Inc).

2.8. Plasma protein binding

Pooled fresh plasma obtained from mice, dogs and one human healthy volunteer were stored at −20 °C and used according to the stability tests (98% unchanged drug). PNU-159548 or PNU-169884 were added to plasma to yield concentrations between 300 and 3000 ng/ml. After equilibration at 37 °C for 15 min, plasma samples were immediately transferred to Centrifree® tube micropartition system and centrifuged at 1500*g* for 10 min. The unbound fraction of the drug was estimated as the ratio between the drug concentration in the plasma filtrate and the total drug concentration ×100.

3. Results

3.1. In vitro *myelotoxicity*

The myelotoxic effect of PNU-159548 and of its 13-dihydro metabolite PNU-169884 was assessed in mouse, dog and human CFU-GM cells. After a 1-h exposure, PNU-159548 induced a concentration-dependent inhibition of CFU-GM colony formation (Fig. 2) with IC_{70} and IC_{90} values in mice of 77.12 and 125.44 ng/ml, respectively (Table 1). Dog progenitors were the most sensitive (IC_{70}: 7.79 ng/ml; IC_{90}: 12.88 ng/ml) while an intermediate effect was shown on human CFU-GM (IC_{70}: 44.15 ng/ml; IC_{90}: 70.92 ng/ml). It appears that canine progenitor cells were approximately 6-fold more sensitive to the myelotoxic effect of PNU-159548 than human progenitors ($P<0.01$) whereas murine CFU-GM were approximately 1.7-fold less responsive ($P<0.05$). The *in vitro* myelotoxicity of PNU-169884 was similar to that of the parent compound, the dog CFU-GM cells being 8.6 times more sensitive than human progenitors.

PNU-159548 was tested on human colony forming units-megakaryocyte (CFU-MK) and its effect was compared with that on CFU-GM cells (Table 2). PNU-159548 treatment resulted in approximately 2-fold more toxicity ($P<0.05$) on CFU-MK than on CFU-GM cells (IC_{70}: 20.53 and 44.15 ng/ml, respectively).

3.2. PNU-159548 and PNU-169884 toxicology in animal models

PNU-159548 toxicity studies were performed in mice and dogs after single i.v. administrations and PNU-169884 was tested i.v. in mice.

In mice, the LD_{10} and LD_{100} were 2.5 and 5.8 mg/kg in males and 3.5 and 4.5 mg/kg in females, respectively, with 2.5 mg/kg as the MTD. The main cause of death was severe myelotoxicity. In surviving animals, a dose-related decrease in leucocytes, from approximately 64% at 2.5 mg/kg ($P<0.01$) to 82% at 4.5 mg/kg ($P<0.01$) was observed on day 5 with recovery between days 22 and 27 (Fig. 3a). The decrease in circulating leucocytes was mainly due to a decrease of both neutrophils and lymphocytes. Neutrophils recovered rapidly by day 14 while lymphocytes showed a delayed, and sometimes incomplete, recovery (data not shown). A dose-related decrease in platelets from approximately 20% at 2.5 mg/kg ($P<0.01$) to 56% at 4.5 mg/kg ($P<0.01$) on day 5 with recovery by day 14 was also observed (Fig. 3b). The toxicological profile of PNU-169884 in mice was comparable to that of the parent compound (data not shown).

The toxicity profile of PNU-159548 in dogs is reported in Fig. 4. No mortality was observed in the dog study and the MTD was 0.3 mg/kg. A marked statistically significant dose-related decrease occurred in peripheral leucocytes from approximately 45% at 0.1 mg/kg to 83% at 0.6 mg/kg and the neutrophil nadir was observed on day 11 (Fig. 4a). Complete recovery from myelosuppression was achieved by day 15 at the lowest dose and by day 28 at the intermediate and highest doses. Platelet counts were also reduced at all dose levels, with some dose relationships being observed, the decrease ranging from approximately 68% at 0.1 mg/kg to 98% at 0.6 mg/kg (Fig. 4b).

3.3. Systemic exposure evaluation

In mice, the systemic exposure to PNU-159548 increased with the dose (Table 3) with mean AUC values between genders of 778, 2284 and 5207 ng h/ml at 2.5, 4.5 and 7.5 mg/kg, respectively. The metabolite was formed within 0.5–1 h after dosing and showed a systemic exposure comparable to that of the parent drug at all of the tested doses (Table 4). On the basis of these data, the AUCs at the MTD were 778 and 897 ng h/ml, for PNU-159548 and PNU-169884, respectively.

In dogs, the mean systemic exposure to PNU-159548 was 47.9, 115.3 and 258.6 ng h/ml after the administration of 0.1, 0.3 and 0.6 mg/kg, respectively (Table 3), with comparable values for PNU-169884 (Table 4). On the basis of these data, the AUCs at the MTD were 116 and 92 ng h/ml, for the parent compound and the metabolite, respectively. From the point of view of systemic exposure

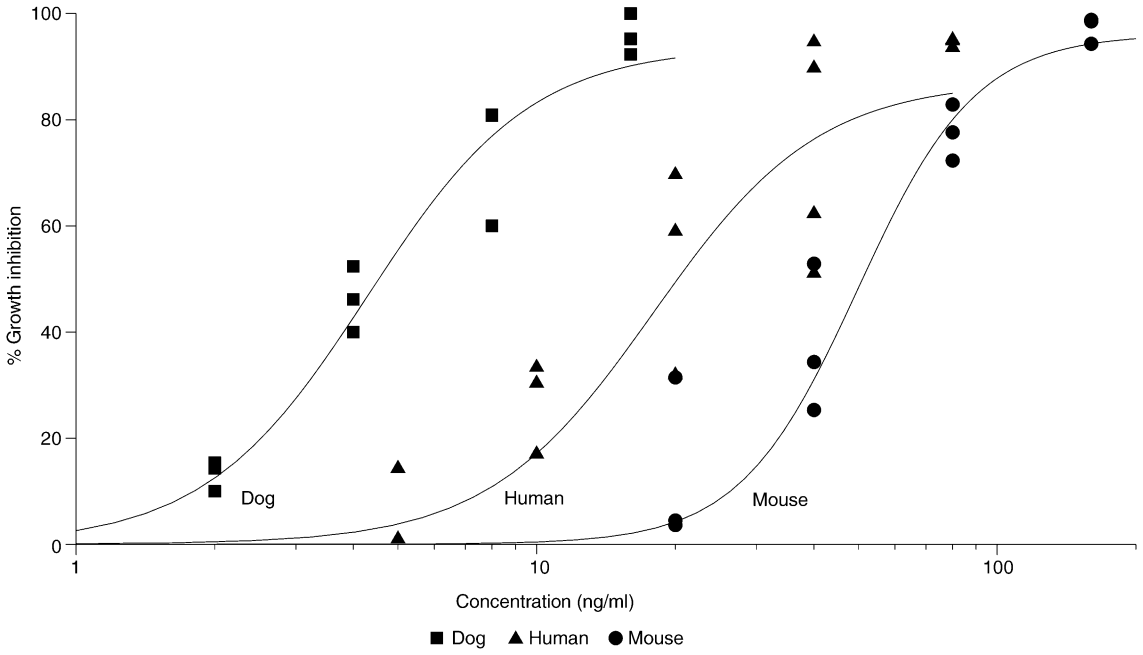

Fig. 2. *In vitro* myelotoxicity of PNU-159548 on murine (●—●), canine (■—■) and human (▲–▲) haematopoietic progenitor cells (1 h treatment).

Table 1
Myelotoxic effect of PNU-159548 and PNU-169884 on CFU-GM colony formation: cross-species comparison[a]

Species	PNU-159548		PNU-169884	
	IC_{70} (ng/ml0)	IC_{90} (ng/ml)	IC_{70} (ng/ml)	IC_{90} (ng/ml)
Mouse	77.12±10.45*	125.44±4.18**	91.87±18.83*	154.32±25.01*
Dog	7.79±0.73**	12.88±0.96**	4.44±0.28**	7.26±0.13***
Human	44.15±12.11	70.92±18.56	38.17±4.1	62.48±6.25

S.D., standard deviation; CFU-GM, colony forming units-granulocyte macrophages. * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$ (Student unpaired *t*-test).

[a] Cells incubated with the compound for 1 h. Results are the mean±S.D. of three individual experiments.

Table 2
Comparative effect of PNU-159548 on human CFU-GM and CFU-MK colony formation[a]

Cell lineage	IC_{70} (ng/ml)	IC_{90} (ng/ml)
CFU-GM	44.15±12.11	70.92±18.56
CFU-MK	20.53±13.96*	38.33±20.02

[a] CFU-MK, colony forming units-megakaryocyte. * = $P<0.05$ (Student S.D. unpaired *t*-test).

at MTD, dogs appeared to be 6.7-fold more sensitive to the myelotoxic effects of PNU-159548 than mice.

3.4. *Prediction of the human MTD from* in vitro *and* in vivo *preclinical toxicity and pharmacokinetic data*

The *in vitro* data with human, murine and canine haematopoietic progenitor cells and the *in vivo* toxicity and pharmacokinetic data in both animal species were used to predict the human MTD for PNU-159548. The relative toxicity coefficient (IC_{90} differential) of PNU-159548, calculated as the ratio between the IC_{90} on human and IC_{90} on murine or canine CFU-GM cells, was 0.57 and 5.5 for mouse and dog, respectively (Table 5). PNU-169884 showed similar results.

A target exposure level to PNU-159548 in humans (AUC at MTD) of 443 and 639 ng h/ml was calculated by multiplying the AUC at the MTD in mice and dogs respectively, by the relative toxicity coefficient between the two species according to the following equation:

$$\text{Target Human AUC} = \text{Animal AUC at MTD} \times IC_{90}\ \text{differential}$$

In order to obtain the corresponding MTD in patients, an allometric model, based on the regression of the logarithm of plasma clearance (CL) in mice and dogs and the corresponding body weight, was used to estimate a predicted human CL of 1614 mL/h/kg [17].

Multiplying the target human AUC by the human CL, the corresponding MTD (mg/kg) was obtained. The MTD predicted in humans was 0.72 mg/kg from the mouse data (corresponding to 29.5 mg/m^2) and 1.03 mg/kg from the dog data (corresponding to 38 mg/m^2). These values were corrected by the differences in plasma protein binding observed *in vitro* between mice (99%) or dogs (98%) and humans (98%). The MTD predicted from murine data was cautiously reduced, taking into account that in humans the free-drug concentrations could increase from 1 to 2% of the total plasma concentrations.

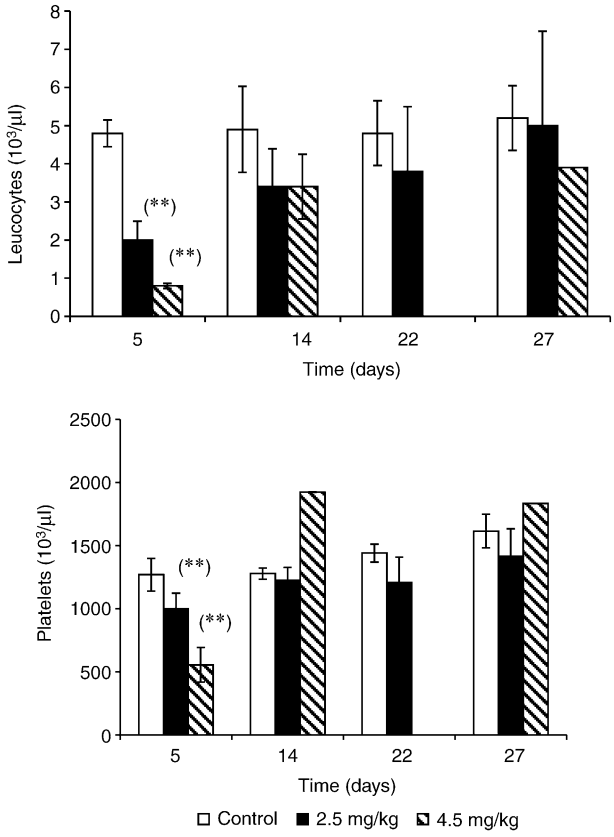

Fig. 3. Haematological toxicity of PNU-159548 in mice: (a) leucocytes and (b) platelets. Drug toxicity was evaluated after a single injection. Control groups were given the vehicle. Results are the mean ±S.D. (** = $P<0.01$) of 20 animals (10 males/10 females). S.D., standard deviation.

Therefore, the final predicted human MTD was between 15 (mice) and 38 (dogs) mg/m^2.

3.5. *Clinical results*

A total of 61 patients were treated at eight different dose levels, ranging from 1 to 16 mg/m^2. The DLT was thrombocytopenia with the MTD fixed at 14 and 16 mg/m^2 in heavily pretreated ($\geqslant$2 prior chemotherapies) and minimally pretreated/non-pretreated patients ($\leqslant$1 prior chemotherapy) respectively. The recommended doses for phase II studies in the same patient categories were 12 and 14 mg/m^2, respectively.

The plasma levels of PNU-159548 and PNU-169884 were determined in 49 patients treated at all of the dose levels. Overall, the systemic exposure to PNU-159548 increased with the dose in the range of doses investigated, with mean AUC values (±S.D.) of 324 (±116) ng h/ml at 14 mg/m^2 and 441 (±85) at 16 mg/m^2. Maximum plasma levels of the reduced metabolite, PNU-169884, were reached approximately 1 h after treatment with a $T_{1/2}$ similar to that of the parent drug. The systemic exposure to PNU-169884 was similar or slightly higher than that to the parent drug and increased with the dose;

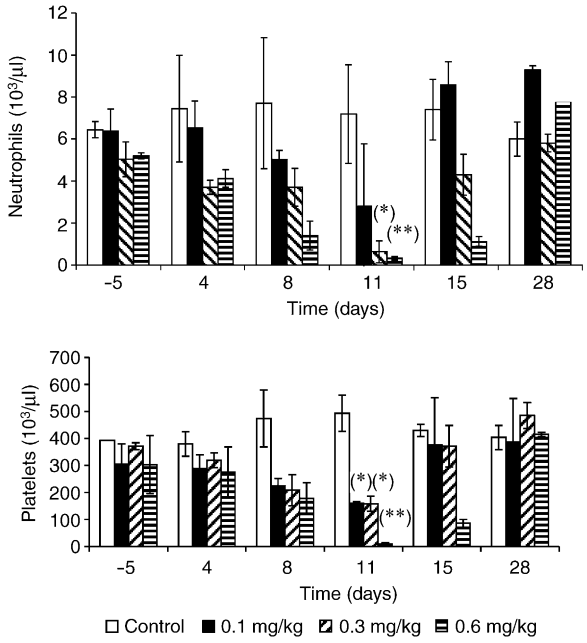

Fig. 4. Haematological toxicity of PNU-159548 in dogs: (a) neutrophils and (b) platelets. Drug toxicity was evaluated after a single injection. Control groups were given the vehicle. Results are the mean ±S.D. of two animals (one male/one female). S.D., standard deviation.

mean AUC values (±S.D.) were 327 (±115) ng h/ml and 435 (±87) at 14 and 16 mg/m^2 respectively. The AUC values found in patients are superimposable to the target human AUC obtained from the murine model (Table 5).

3.6. 3.6.In vitro *myelotoxicity of patients' plasma*

The plasma of 9 patients treated at doses of 12–14 mg/m^2 of PNU-159548 was tested *in vitro* on human CFU-GM cells. The mean% CFU-GM inhibition (±S.D.) induced by the patients' plasma taken at 15, 30 and 60 min after dosing was 55±12, 37±18 and 19±11, respectively (data not shown). Fig. 5 shows the correlation between CFU-GM inhibition by patients' plasma and the corresponding PNU-159548 concentrations. The IC_{70} and IC_{90} were 338 and 1305 ng/ml, respectively. These cytotoxicity values appeared to be 8 and 18 times higher than the IC_{70} and IC_{90} obtained when the compound was tested in medium (Table 1).

4. Discussion

The haematotoxicity Model 2 proposed by Parchment in Ref. [4], predicting the human MTD of myelosuppressive cytotoxic antitumour drugs, was prospectively validated in two concomitant phase I studies of PNU-159548. The model predicts human MTD, rather than the plasma exposure level, by adjusting animal MTD for intrinsic differences in drug tolerance

Table 3
Plasma pharmacokinetic parameters of PNU-159548 after single i.v. administration to mice and dogs[a]

Species (No.)	Dose (mg/kg)	C_{5min}±SE (ng/ml) (M–F)	AUC(0–t_z)±SE (ng h/ml) (M–F)	$t_{1/2}$ (h)	V_{ss} (ml/kg)	CL (ml/h/kg)
Mouse	2.5	1454.5±359–1974.6±35	696.2±101–860.2±3	0.8	1355	3244
[10]	4.5	4884.8±548–5903.7±741	2126.2±160–2442.6±231	5.7	1366	2005
	7.5	10772.4±1672–8295.5±835	5792.4–4621.4	10.1	2574	1440
Dog	0.1	64.6–59.4	41.1–52.6	1.15	2734	2115
[1]	0.3	183.3–160.7	116.8–113.9	1.9	3033	2483
	0.6	344.1–500.7	231–286.1	1.52	2211	2290

C_{5min}, plasma concentration at the first sampling time (5 min); M, male; F, female; AUC(0–t_z), AUC from 0 to the last sampling time; $t_{1/2}$, terminal half-life; V_{ss}, volume of distribution at steady state; CL, total plasma clearance; i.v., intravenous; S.E., standard error.
[a] At 7.5 mg/kg dose level in mice, the evaluation of S.E. was not possible due to death of some animals after treatment.

Table 4
Plasma pharmacokinetic parameters of PNU-169884 after single i.v. administration of PNU-159548 to mice and dogs[a]

Species (No.)	Dose (mg/kg)	C_{5min}±S.E. (ng/ml) (M–F)	AUC (0–t_z)±SE (ngh/mL) (M–F)	$t_{1/2}$ (h)	Ratio AUC (0–t_z) PNU-169884/PNU-159548
Mouse [10]	2.5	251.3±15.0–251.6±11.50	839.8±59–953.7±64	1.8	1.1
	4.5	466.6±3.9–525.0±71.4	2549.2±222–3175±288	2.7	1.2
	7.5	759.2±73.8–620.1±82.4	5981–5629	5.2	1.1
Dog [1]	0.1	12.1–19.9	37.0–70.9	1.34	1.2
	0.3	34–34.6	105.4–79.3	2.29	0.9
	0.6	72.8–89.8	257.1–230	1.77	1.0

C_{5min}, plasma concentration at the first sampling time (5 min); M, male; F, female; AUC(0–t_z), AUC from 0 to the last sampling time; $t_{1/2}$, terminal half-life; V_{ss}, volume of distribution at steady state; CL, total plasma clearance; i.v., intravenous; S.E., standard error.
[a] 4At 7.5 mg/kg dose level in mice, the evaluation of S.E. was not possible due to death of some animals after treatment.

Table 5
Prediction of human MTD of PNU-159548 from *in vitro* and *in vivo* toxicology and pharmacokinetic data

Parameter	Mouse		Dog	
	PNU-159548	PNU-169884	PNU-159548	PNU-169884
Relative toxicity coefficient (IC_{90} differential)	0.57	0.4	5.51	8.61
AUC at MTD (ng·h/ml)	778	897	116	92
Target human AUC_{MTD} (ng h/ml)	443	359	639	792
Estimated human CL (ml/h/kg)	1614		1614	
Predicted human MTD corrected for estimated CL	0.72 mg/kg (29.5 mg/m^2)		1.03 mg/kg (38 mg/m^2)	
Predicted human MTD corrected for protein binding	0.36 mg/kg (15 mg/m^2)		1.03 mg/kg (38 mg/m^2)	

IC_{90} differential, the ratio of IC_{90} values from human and animal CFU-GM assays.

between experimental species and humans. The predicted MTD for PNU-159548 in humans was calculated by taking into account the AUC and clearance in mice and dogs on the premise that myelosuppression was the main toxicity in the preclinical studies. Differences in plasma protein binding between mice, dogs and humans were also included in the model giving a final predicted MTD of 15 and 38 mg/m^2 according to the results in the mice or dogs, respectively.

In both phase I studies, the MTD was fixed at 14 and 16 mg/m^2 for patients more or less extensively pretreated with chemotherapy, thus validating the haematotoxicity model from a quantitative point of view. Myelosuppression was the principal side-effect observed in patients and DLT was mainly thrombocytopenia, while neutropenia was of minor clinical relevance. Conversely, both neutropenia and thrombocytopenia were reported in *in vivo* preclinical studies in the mice and dogs, with a temporal pattern of appearance comparable to that generally observed with other cytotoxic drugs. When PNU-159548 was investigated *in vitro* in human CFU-MK clonogenic assays, it appeared to be two times more toxic against CFU-MK than CFU-GM. The prediction of human MTD through the haematotoxicity model was based on the results of the CFU-GM assay, in the

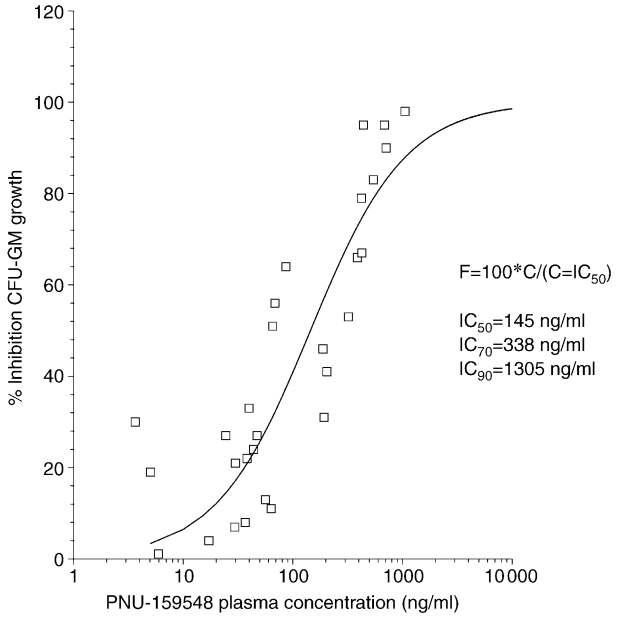

Fig. 5. Myelotoxicity in human CFU-GM induced by plasma samples of patients treated with different doses (12–14 mg/m^2) of PNU-159548. IC_{50}, IC_{70}, IC_{90}: PNU-159548 plasma concentrations inhibiting 50, 70 and 90% of CFU-GM growth. CFU-GM, colony-forming units-granulocyte macrophage.

assumption that neutropenia or myelosuppression, in general, and not thrombocytopenia, was dose-limiting.

The initially predicted human MTD from the mouse model was 29.5 mg/m^2. However, this dose-level might have resulted in dose-limiting neutropenia in humans. This value was corrected because of differences in plasma protein binding between mice and humans (99 and 98%, respectively) and this correction resulted in a significant decrease in the predicted human MTD to 15 mg/m^2, which is in the range of those achieved in the subsequent phase I studies.

The evaluation of the predicted dose in man from the canine data was approximately two-fold higher than that achieved in the clinical setting. This difference as compared with the mouse model should be mainly ascribed to the higher sensitivity of the canine haematopoietic progenitors to PNU-159548, both *in vitro* and *in vivo*.

Nevertheless, exaggerated susceptibility of the dog haematopoietic system with respect to other species is a well known finding which has also been described for other cytotoxic antitumour agents [12,18,19].

The haematotoxicity model therefore proved to be useful in predicting myelosuppression from a quantitative and, to a lesser extent, qualitative point of view; above all, the use of CFU-GM clonogenic assays was useful in providing dose–response curves in the different animal species. The steepness of the curve suggested that dose escalation should follow an accelerated design with limited cohorts of patients only at the first three dose levels, with a relatively early start of the standard phase where more patients are treated per dose level. The implementation of this design was successful, allowing an initial fast evaluation of the non-toxic doses, followed by a safe broader evaluation of the toxic ones.

The success of the prediction model was also due to some key technical issues, such as the evaluation of a time of exposure of clinical relevance and the inclusion of interspecies differences in plasma protein binding. The relevance of the latter aspect is confirmed by the markedly higher IC_{70} and IC_{90} values in the CFU-GM clonogenic assay performed with plasma samples of patients treated at the highest dose levels. Provided that interspecies differences in protein binding are included in the model, the performance of clonogenic assays with patients' plasma samples might be envisaged only in selected situations, as in cases of discrepancies between preclinical toxicology and clinical results. The presence and the effects of some myelotoxic metabolites might also be elucidated in these systems and be instrumental in helping develop future clinical research [16].

The PK studies performed in humans provided information that further justifies the use of the haematotoxicity model, such as the linearity of the PKs and correlation between systemic exposure to both the parent compound and metabolite and thrombocytopenia [11].

In conclusion, retrospective validations based on Model I of Parchment were already described by Pessina and colleagues in Ref. [20,21] but, to our knowledge, this is the first prospective validation of the application of an *in vitro* haematotoxicity model with a purely myelosuppressive agent, PNU-159548. Animal results accurately predicted the human MTD, but not thrombocytopenia as the DLT. Additional information on the effect of a new agent on human megakaryocytopoiesis must be envisaged when thrombocytopenia is expected in humans from preclinical studies.

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