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PRECLINICAL STUDIES

Toxicokinetics of the tumour cell mitochondrial toxin, PENAO, in rodents

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Summary

PENAO (4-(N-(S-penicillaminylacetyl)amino)phenylarsonous acid) is a second-generation peptide arsenical that inactivates mitochondria in proliferating tumour cells by covalently reacting with mitochondrial inner-membrane adenine nucleotide transferase. The toxicokinetics of PENAO has been investigated in Sprague-Dawley rats to inform route of administration and dosing for human clinical trials. PENAO was well tolerated at 3.3 mg/kg daily intravenous injections but associated with significant toxicity at 10 mg/kg, primarily in the males. The major target organ for toxic effects was the kidney, with changes observed in tubular dilation, presence of casts, basophilic tubules, lymphoid aggregates and interstitial fibrosis. Kidney function was impaired in males with dose-dependent increase in serum creatinine concentration. The severity of the microscopic lesions was reduced in the females, but not the males, at the completion of the four-week recovery period. The elimination phase half-life of PENAO varied between 0.4 and 1.7 h and volume of distribution ranged from 0.25 to 0.88 L/kg for the different dose groups and treatment days, suggesting that PENAO distributes in the extracellular fluids at the doses tested. The area under the curve and clearance values indicate that male rats had reduced elimination of PENAO compared to females, which may account for the increased toxicity in males. PENAO is significantly better tolerated in rodents than its predecessor, GSAO. As GSAO was generally well tolerated with few side effects in a phase I trial in patients with solid tumours, these findings bode well for the tolerability of intravenous dosing of PENAO in patients.

Keywords cancer · PENAO · GSAO · organoarsenical · toxicokinetics · mitochondria

Introduction

Mitochondria are an attractive cancer drug target because their proper functioning is required for tumourigenesis [1]. Peptide trivalent arsenicals have been developed as mitochondrial toxins for human cancer cells. The first-generation molecule, GSAO (4-(N-(S-glutathionylacetyl)amino)phenylarsonous acid) [2–4], has been tested in a Phase I trial in patients with solid tumours [5]. GSAO was generally well tolerated with a maximum tolerated dose of 22 mg/m^2/day.

GSAO is processed at the cell surface and in the cytosol before the As(III) atom of the compound reacts with mitochondria. The final metabolite is CAO (4-(N-(S-cysteinylacetyl)amino)phenylarsonous acid) [4], which reacts with two cysteine thiols located on the matrix facing loops of mitochondrial inner-membrane adenine nucleotide transferase (ANT). A stable cyclic dithioarsinite complex is formed where both ANT sulfur atoms are bound to the As(III) atom of CAO [6]. ANT is a 30 kDa trans-membrane protein that functions in oxidative phosphorylation by exchanging newly formed ATP in the matrix with spent ADP across the inner-membrane [7]. Reaction of CAO with ANT blocks ADP/ATP exchange that leads to partial uncoupling of oxidative phosphorylation, an increase in superoxide production and arrest of proliferation of the cell [2, 8]. CAO reacts more efficiently with ANT when cells are proliferating due to the higher mitochondrial matrix calcium levels in proliferating cells.

PENAO (4-(N-(S-penicillaminylacetyl)amino)phenylarsonous acid) is a cysteine mimetic analogue of CAO that bypasses the extracellular and cytosolic processing of GSAO [8]. PENAO accumulates in cells much more rapidly than GSAO, which translates to more potent effects on proliferating tumor cells in culture

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and markedly better anti-tumor efficacy in mice [8]. In pre-clinical studies, PENAO combines very effectively with mammalian target of rapamycin (mTOR) inhibitors of the rapalog class [9, 10]. A Phase I/IIa trial of intravenous PENAO alone or in combination with rapamycin in patients with solid tumours with an activated mTOR pathway is in the planning stages.

Here, the pharmacokinetics of intravenous PENAO has been investigated in adult male and female Sprague-Dawley rats to inform route of administration and dosing for clinical trials. I report that PENAO is significantly better tolerated than GSAO in rats. The finding that GSAO is generally well tolerated in cancer patients [5] is a positive sign that PENAO will also be well tolerated.

Results

Toxicity of PENAO in rats

The toxicity study included three groups of 16 male and 16 female Sprague Dawley rats treated by intravenous injection with either saline vehicle or PENAO at 3.3 and 10 mg/kg dose levels for two five-day cycles separated by a two-day period without dosing. This is a commonly used and acceptable rodent species for toxicology studies of experimental cancer drugs. Terminal examinations were conducted on eight male and eight female rats from each dose group on days 15/16 after completion of the second cycle of dosing, and on days 38/39 following a four-week treatment free period. The vehicle and PENAO formulations were administered at approximately the same time each day (plus or minus 90 min) by a bolus intravenous injection into a lateral tail vein at a dose rate of 5 mL/kg. The left and right veins were used on alternate days where possible to allow recovery between injections. The dose volume per rat was based on body weight measurements obtained on the same day.

The interim examinations included daily clinical observations for signs of toxicity throughout the study. Body weight and food consumption were also measured. Interim blood samples were collected for haematology and clinical biochemistry analyses on day 8. Urine samples were collected overnight (approximately 18 hours) with animals singly housed in metabolic cages in the last week of the study. Terminal examinations included haematology and clinical biochemistry analyses. A detailed necropsy with external and internal examinations was also completed. Full histopathology was conducted on control and high dose animals from the main and recovery study animals.

PENAO was well tolerated at 3.3 mg/kg but was associated with significant toxicity at 10 mg/kg, primarily in the males. One high dose male was found dead on day four. Treatment at 10 mg/kg was associated with a progressive decrease in body weight in several individuals (Fig. 1), which resulted in the discontinuation of dose administration in one male from the toxicity study. Treatment at 10 mg/kg was also associated with an initial reduction in the mean rate of body weight gain on days two through four. Clinical signs associated with PENAO treatment included the induction of hunched body posture and gait.

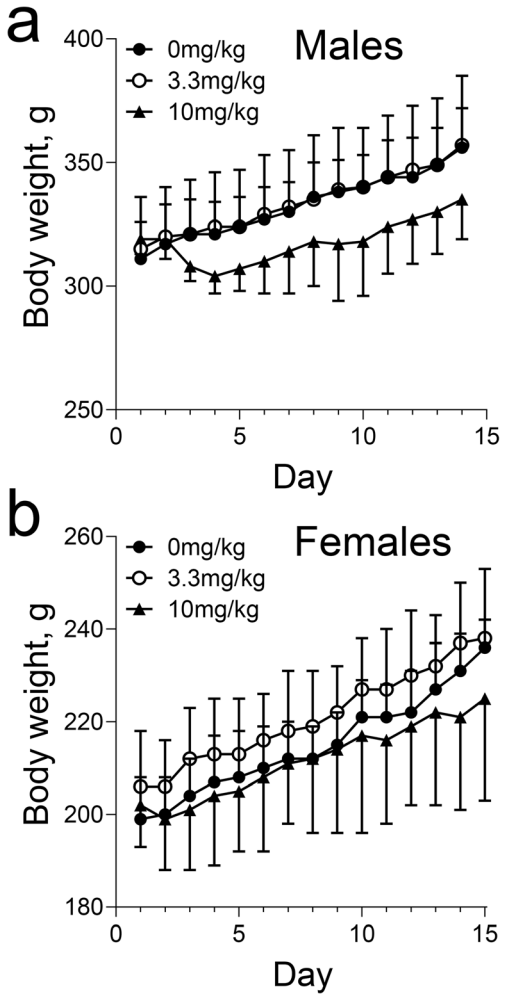

Fig. 1 Toxicity of PENAO in male and female rats. Adult male (part a) or female (part b) Sprague-Dawley rats were administered 0, 3.3 or 10 mg/kg PENAO daily by intravenous injection for 15 days. Data points and errors are mean and SD for n = 8 per group. There is no significant differences between groups

The major target organ for toxic effects of PENAO treatment was the kidney. Macroscopic findings at necropsy of pale kidneys, blotchy kidneys, granular/pitted kidneys and enlarged kidneys were made on days 15 and 38 in males treated with 10 mg/kg PENAO. These findings were also associated with an increase in kidney weight. There were microscopic findings of injury to the kidney, which included tubular dilation, the presence of casts, basophilic tubules, lymphoid aggregates and interstitial fibrosis (Table 1). The lesions were detected primarily in the high dose rats following treatment with 10 mg/kg PENAO and were more severe in the males than the females.

Table 1 Incidence of microscopic findings in the kidney from PENAO administration

	Non-Recovery (day 15/16)						Recovery (day 38/39)					
Sex	M	M	M	F	F	F	M	M	M	F	F	F
Dose (mg/kg)	0.0	3.3	10.0	0	3.3	10.0	0.0	3.3	10.0	0	3.3	10.0
Number examined	8	8	8	8	8	8	8	8	8	8	8	8
Tubular dilation	1	6*	8***	0	3	1	0	5*	7***	0	6**	0
Basophilic tubules	3	8*	8*	1	2	8	1	8***	7**	0	5*	2
Casts	1	4	7***	0	0	1	0	0	6**	0	0	0
Lymphoid aggregates	0	5*	6**	0	0	2	0	1	7***	2	0	0
Interstitial fibrosis	0	0	0	0	0	0	0	2	5*	0	1	0

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

The lesions identified in the kidney correlated with measures of functional impairment. There was a dose-related increase in serum creatinine concentrations in the male rats, which reached significance on day 15 ($p < 0.01$). Baseline creatinine levels were 24.71 ± 3.54 μM that increased to 27.44 ± 5.02 μM at 3.3 mg/kg PENAO and 32.11 ± 1.87 μM at 10 mg/kg PENAO. Serum urea concentrations were also elevated in the males and females. Urinalysis findings related to treatment included an increase in the incidence of blood in the urine in the second week of treatment at 10 mg/kg (Table 2). The severity of the microscopic kidney lesions was reduced in the females, but not the males, at the completion of the four-week recovery period. The presence of interstitial fibrosis in the recovery animals indicates that these lesions are chronic and persistent in the high dose males. Furthermore, the increases in serum creatinine and urea were also not fully reversed in the males and high dose females.

PENAO treatment at 10 mg/kg was associated with an increase in total white blood cell counts on day 15/16, which was attributed to an increase in lymphocyte counts. These increases are likely to reflect the observed immune cell infiltration of the kidney in these animals and were partially reversed at the completion of the four-week treatment free period. There was a mild reduction in haemoglobin concentration and haematocrit values on day 15 in the 10 mg/kg males, which was fully recovered on day 38 following the four-week treatment free recovery period. There was also evidence for minor toxic effects of PENAO on haemopoiesis which were reversed following a four-week treatment free period. A no-observed-effect level was not detected in this study.

Table 2 Urinalysis findings from PENAO administration

	Non-Recovery (day 15/16)						Recovery (day 38/39)					
Dose	0 mg/kg		3.3 mg/kg		10 mg/kg		0 mg/kg		3.3 mg/kg		10 mg/kg	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Glucose (+)	0	0	0	0	1	1	0	0	0	0	0	0
Bilirubin	5	5	4	4	3	3	0	0	0	0	0	0
Ketones (trace/positive)	5	0	4	0	2	1	5	0	3	0	4	0
Specific gravity>1.025	0	0	0	0	0	0	0	0	0	2	0	0
Specific gravity<1.010	3	0	1	2	0	1	3	1	3	0	1	0
Blood (trace/positive)	1	0	0	1	6*	5*	1	0	0	1	0	0
pH>7.0	0	5	8***	6	7**	7	6	5	7	3	6	5
pH<6.0	0	0	0	0	0	0	0	1	0	1	0	0
Protein (trace/positive)	8	8	8	7	8	8	8	8	8	8	8	8
Nitrite (trace/positive)	0	0	0	0	0	0	0	1	0	1	1	0
Leukocytes (trace/positive)	0	0	1	0	5*	2	1	0	0	0	1	0
Squamous cells	3	7	5	7	5	7	7	6	6	5	7	4
Erythrocytes	1	2	1	1	6*	5	0	0	0	1	1	0
Leukocytes	2	1	1	1	5	2	1	0	1	0	1	0
Crystals	1	0	1	0	1	2	1	3	1	1	0	1

*, ** Significant difference versus 0.0 mg/kg group; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Pharmacokinetics of PENAO in rats

The pharmacokinetic study included 54 adult Sprague Dawley rats with 12 male and 12 females treated with 0, 3.3 and 10 mg/kg of PENAO for 12 days, which corresponds to the maximum tolerated dose (MTD) and one third of the MTD, respectively. The low and high dose groups were further subdivided into four sub-groups for collection of blood samples for analyses at two time points each, for a total of 8 time points from 0 to 240 min, after dosing on days one and 12. Plasma PENAO concentration was measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Figs. 2 and 3).

Both maximum plasma concentration (C_{max}) and area under the curve (AUC_{0-last}) increased with dose (Table 3). The elimination phase half-life varied between 0.4 and 1.7 h for the different dose groups and treatment days. The observed volume of distribution for the different dose groups and treatment days ranged from 0.25 to 0.88 L/kg, suggesting that PENAO distributes in the extracellular fluids at the doses tested.

The AUC and clearance (CL_{Obs}) values obtained indicate that there may be differences in the effect of 12 days of PENAO treatment between male and female rats – in both dose groups the male rats had reduced clearance and increased AUC after 12 days treatment, suggesting that elimination may be impaired. In the female rats given 3.3 mg/kg/day the AUC and clearance increased, and at 10 mg/kg/day the AUC dropped while the clearance increased, suggesting increases in elimination.

Discussion

PENAO was designed to bypass the pro-drug mechanism of action of GSAO [8]. GSAO is processed at the cell surface and in the cytosol before it reacts with mitochondrial ANT. Cancer cell surface γ-glutamyl transpeptidase cleaves the γ-glutamyl residue of GSAO to produce GCAO (4-(N-(S-cysteinylglycylacetyl)amino)phenylarsonous acid) that enters the cell via a plasma membrane organic ion transporter. GCAO is processed further to CAO by removal of the glycine residue, which then enters the mitochondrial matrix and reacts with ANT [4]. Cytosolic concentrations of CAO are limited due to export by the plasma membrane multidrug resistance associated protein isoforms 1 and 2 (MRP1/2) [3]. PENAO is a cysteine mimetic analogue of CAO and so is not subject to the extracellular and cytosolic processing of GSAO.

PENAO accumulates in cells 85-times faster than GSAO due to increased rate of entry and decreased rate of export via MRP1/2, which translates to 44-fold increase in anti-proliferative activity *in vitro* and ~20-fold better anti-tumor efficacy *in vivo* [8]. This faster rate of accumulation of PENAO in cells was expected to result in increased toxicity of PENAO *in vivo*. The contrary is the case, however.

The GSAO MTD in Sprague-Dawley rats is ~5 mg/kg using the same dosing schedule used here for PENAO. GSAO treatment resulted in mild effects on blood chemistry and haematology and some microscopic changes in the kidney. The effects were dose-related and more marked in male animals than in females [5]. GSAO and PENAO have molecular weights of 570.04 Da and 390.02 Da, respectively, so on

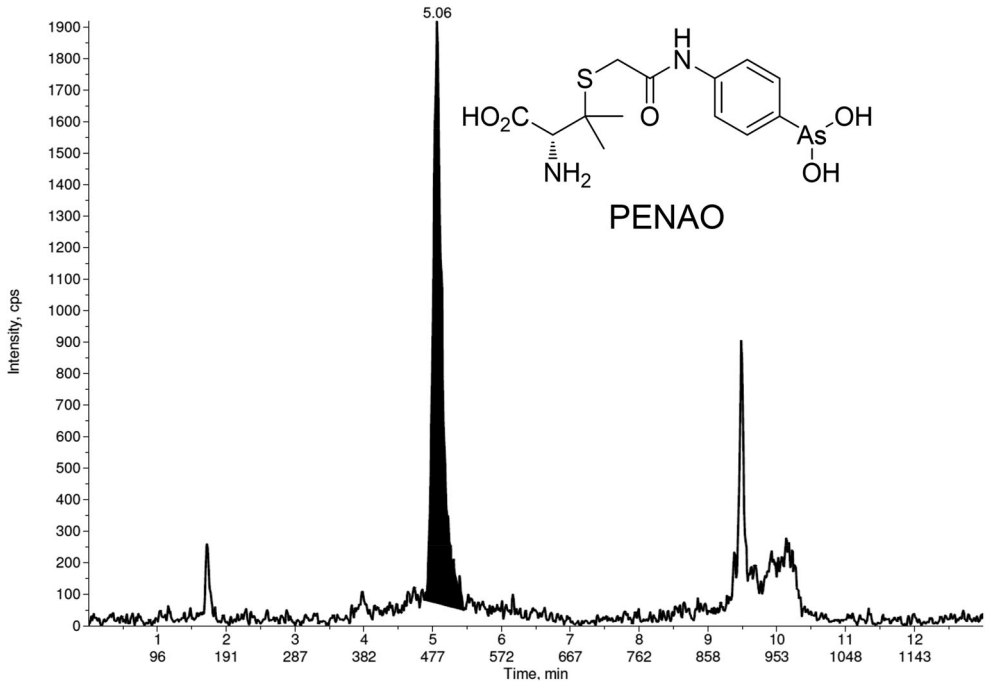

Fig. 2 HPLC chromatogram of PENAO from rat plasma. A representative HPLC chromatogram of a rat plasma extract containing 5 ng/mL PENAO. PENAO (structure shown) elutes at 5.06 min

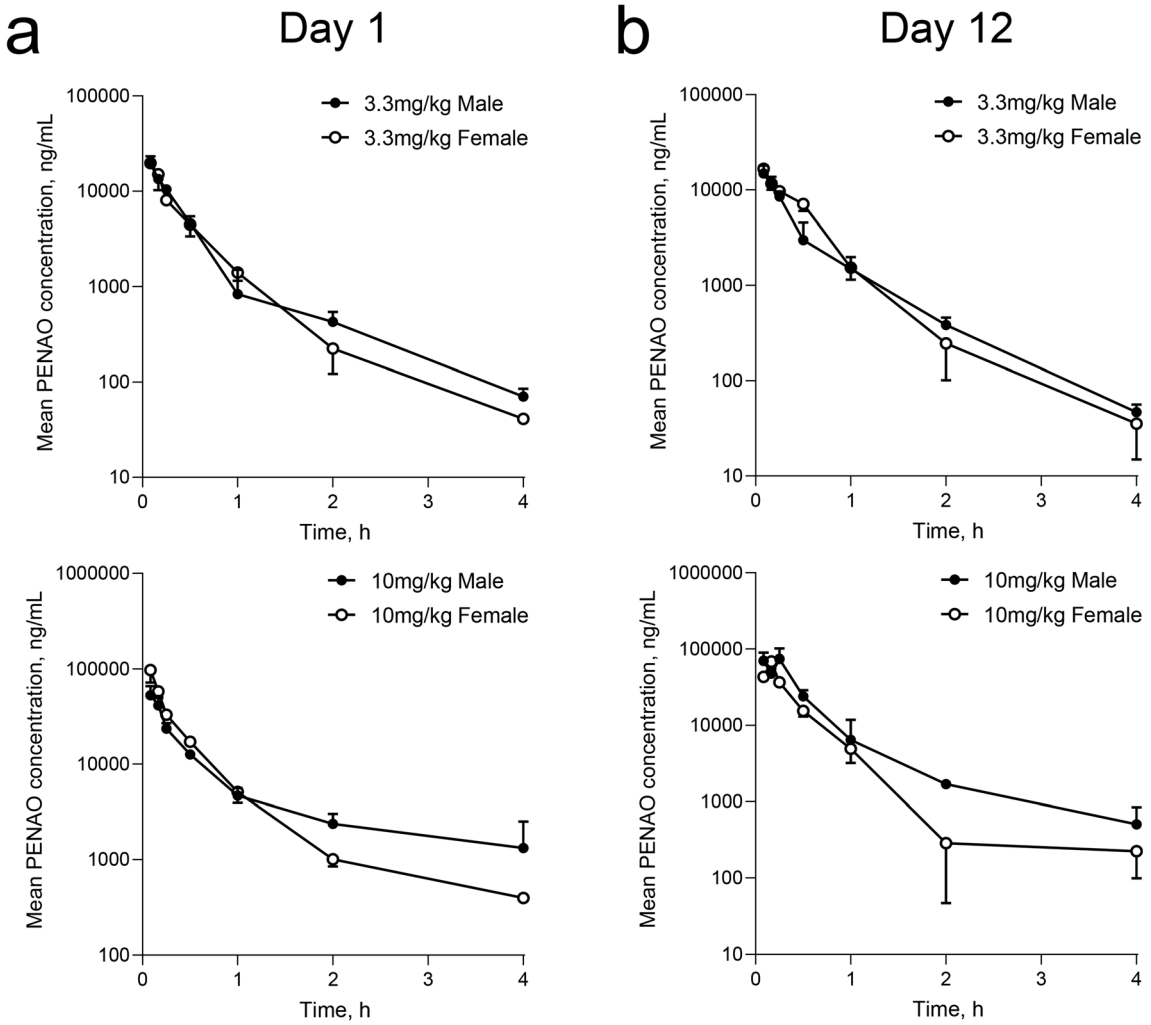

Fig. 3 Pharmacokinetics of PENAO in male and female rats. Adult male or female Sprague-Dawley rats were administered 3.3 or 10 mg/kg PENAO daily by intravenous injection for 12 days. Blood samples were taken on day 1 (part a) and 12 (part b) at 8 time points from 0 to 240 min post-dose and plasma PENAO concentration measured by LC-MS/MS. Data points and errors are mean and SD for n = 3 per group

a molar basis PENAO with a 10 mg/kg MTD is better tolerated than GSAO in rats. This is also unexpected based on the pharmacokinetics of the two compounds.

The clearance and elimination phase half-life for GSAO [5] and PENAO (Table 3), when administered at MTD to male rats on day one, is 0.76 and 0.35 L/h/kg and 0.05 and 1.72 h,

Table 3 Pharmacokinetic parameters of PENAO in rats

Dose (mg/kg)	Sex	Day	AUC_{0-last} (h.μg/mL)	AUC_{0-last}/Dose (h.kg.μg/mL/mg)	C_{max} (μg/mL)	C_{max}/Dose (kg.μg/mL/mg)[1]	T_{max} (h)	λz (1/h)	$T_{1/2}$ el (h)	Vd_{Obs} (L/kg)	CL_{Obs} (L/h/kg)	Regression coefficient
3.3	M	1	6.4	2.0	14.8	4.5	0.0833	1.18	0.59	0.43	0.51	1.00
3.3	M	12	7.5	2.3	19.3	5.8	0.0833	0.83	0.83	0.52	0.43	1.00
3.3	F	1	8.3	2.5	18.3	5.6	0.0833	1.60	0.43	0.25	0.40	0.96
3.3	F	12	7.3	2.2	19.7	6.0	0.0833	1.39	0.50	0.32	0.45	0.95
10	M	1	24.9	2.5	53.0	5.3	0.0833	0.40	1.72	0.88	0.35	0.95
10	M	12	39.3	3.9	74.4	7.4	0.25	0.82	0.85	0.31	0.25	0.95
10	F	1	29.2	2.9	82.3	8.2	0.0833	1.35	0.51	0.25	0.34	0.87
10	F	12	25.7	2.6	68.5	6.9	0.1667	1.50	0.46	0.26	0.39	0.82

1 Determined at 5 min, except 10/M at 15 min and 10/F at 10 min

respectively. The same differences are observed for female rats. GSAO, therefore, is cleared significantly faster than PENAO from rats despite that fact that GSAO is more toxic in this species. The greater toxicity may relate to high spiking concentrations of GSAO in the kidney, the primary organ for toxicity for both compounds.

GSAO has a predominately intravascular biodistribution in rats [5]. In contrast, the observed volume of distribution for PENAO suggests that it distributes in the extravascular space as well. This difference in biodistribution of the compounds is predicted to limit the peak concentrations of PENAO in the kidney. Perhaps the mechanism(s) of clearance of the compounds in the kidney tubules is overwhelmed by very high transient levels of GSAO, whereas clearance of PENAO is more manageable because blood levels do not reach the same peak. The reason for the sex specific difference in the toxic effects of PENAO (and GSAO) is unknown.

GSAO was generally well tolerated with few side effects in a phase I trial in patients with solid tumours [5]. Three dose-limiting toxicities occurred during the study: derangement of liver function tests at dose level 12.4 mg/m^2/day, and paroxysmal atrial fibrillation and encephalopathy at dose level 44.0 mg/m^2/day. The GSAO MTD was calculated as 22.0 mg/m^2/day. Notably, there was no impairment of kidney function in the patients, in contrast to what was observed for GSAO in rats. This bodes well for the tolerability of PENAO in humans.

Methods

Animals and PENAO formulation

All procedures undertaken were approved by the RMIT Animal Experimentation and Ethics Committee (AEEC Project #0801; sub-part 0801F). Males and nulliparous and non-pregnant female Sprague-Dawley rats were supplied by Animal Resources Centre, Canning Vale, Western Australia, originally from Charles River Breeding Laboratories. Animals were approximately 8 weeks of age at the time of the first dose. The starting mean body weight values were within a 20 % range for each sex and corresponded to 315 ± 13.6 g and 200 ± 12.0 g for the males and females, respectively. The study animals were housed in a single room maintained at 22 ± 2 °C and 30–70 % relative humidity with a 12 h light/dark cycle. Animals were fed meat-free rat and mouse pellets (Specialty Feeds, Glen Forrest, Australia) and mains tap water ad libitum. Periodic feed, bedding and water quality testing was undertaken to ensure there were no contaminants which could affect the study. Males and females were housed in groups of two or three rats per cage in standard polypropylene rat boxes with a stainless steel lid and Aspen bedding material. The animals were quarantined on arrival for a period of at least four days. Only healthy animals were used in the study.

PENAO was produced as described [8], dissolved in sterile water for injection at a concentration of 20 mg/mL (weight/volume) and sterilized by passage through a 0.22 μm filter. This solution was retained at ambient temperature and protected from light for up to seven days of use. Dose formulations were prepared on the day of use by dilution of stock solution into sterile saline to final concentrations of 0.66 and 2.00 mg/mL. These formulations were used for dose administration at a rate of 5 mL/kg to achieve final dose rates of 3.3 and 10 mg/kg. The formulations were prepared under aseptic conditions in a laminar flow hood and retained at ambient temperature prior to use on the same day.

Terminal examinations

Terminal examinations were conducted on days 15 (non-recovery) and 39 (recovery). On the evening prior to termination, food was withdrawn but animals were allowed access to water. On the day of termination, the rats were subject to a final measurement of body weight and a blood sample was collected by puncture of the lateral tail vein and transferred into a plain tube. The animals were then exposed to a rising concentration of carbon dioxide. Immediately following the cessation of breathing the rats were killed by exsanguination with puncture of the abdominal aorta. Two blood samples were collected and transferred to two tubes containing EDTA or sodium citrate as anticoagulant. A full haematology profile for each sample was determined on the same day using an Advia 2120 Hematology Analyzer. The haematological parameters measured were erythrocyte count, haemoglobin concentration, haematocrit, total leukocyte count, differential leukocyte count, platelet count and reticulocyte count, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration. Plasma was prepared for measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT) using a semi-automated Start 4 coagulation analyzer. Serum was prepared and analysed for alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, calcium, creatinine, glucose (fasting), potassium, sodium, total bilirubin, total cholesterol, total serum protein, urea, globulin and albumin/globulin ratios using a Konelab 20XTi Biochemistry Analyzer. Urine samples were analysed for specific gravity, pH, protein, glucose, ketones, bilirubin, occult blood, and urobilinogen using dip-stick tests. Microscopy of spun deposits, including an evaluation for epithelial cells, casts, crystals, sperm and other abnormalities, was also performed. Gross necropsy included an external examination, and internal examination of the thymus, heart, vasculature, respiratory and skeletal systems, spleen, urinary and genital systems, adrenals, thyroid, gastrointestinal tract, brain and pituitary. Weights of adrenal gland, brain, heart, kidneys (both), liver, spleen, thymus, ovaries (both) and uterus with uterine cervix and

oviducts, epididymides and testes were recorded, with paired organs weighed together. Bone marrow smears were prepared from all study animals at the time of necropsy.

PENAO extraction from plasma and analysis

Blood collection was by lateral tail vein puncture on conscious animals into lithium heparin tubes and plasma prepared by centrifugation at 3000 g for 10 minutes at 2 to 8 °C. The resulting plasma samples were divided into two equal aliquots and stored at -80 °C. Frozen plasma samples were thawed at room temperature and a 100 μL aliquot mixed with 0.9 mL of 0.2 % formic acid in H_2O and 10 μL of a 5000 ng/mL solution of the internal standard, GSAO (a closely related molecule). The sample was resolved on Phenomenex C18-E SPE columns preconditioned with MeCN, MeOH, H_2O then 0.2 % formic acid in H_2O. The column was washed with ~ 1 mL of 0.2 % formic acid in H_2O and sample eluted with 1 mL of 80 % MeOH. Eluates (10 μL) were analysed by LC-MS/MS (Applied BioSystems API 4000 QTRAP LC/MS/MS mass spectrometer system including a Prominence Shimadzu HPLC and controlled by Applied BioSystems "Analyst version 1.4.2" software workstation) with quantification based on the ratio of peak area of PENAO with the peak area of internal standard, GSAO. PENAO concentrations were calculated from a standard curve of PENAO in rat plasma. The method was specific for PENAO and the internal standard (GSAO) in six different lots of rat plasma. The method was linear over the range of 5 to 5000 ng/mL with a lower limit of quantitation of 5 ng/mL and upper limit of quantification of 5000 ng/mL. No significant carry-over was observed. Samples of rat plasma prepared at 50,000 and 25,000 ng/mL were diluted 50-fold without loss of neither accuracy nor precision. Recovery of both PENAO and internal standard was acceptable. No stability issues were observed after either three freeze-thaw cycles or long-term storage (up to 23 days) at -80 °C. Stability at room temperature in plasma was observed for 2 h, while stability of processed samples containing both PENAO and internal standard was demonstrated for 48 h in the autosampler. Stock solutions of PENAO and GSAO were prepared in 10 % methanol v/v in H_2O at a concentration of 1 mg/mL and stored at 2 to 8 °C for up to 21 days and was found to be stable over this period. Stock and working solutions of both PENAO and the internal standard were stable for at least 6 h at room temperature and beyond 19 days when stored at 4 °C.

Determination of pharmacokinetic parameters

Toxicokinetic parameters were determined using WinNonLin Pro Node 5.2TM software package (Pharsight Corporation, Mountain View, CA, USA). Values reported as below the lower limit of quantification were replaced with zero for the calculation of mean concentrations and pharmacokinetic parameters. Sample times with two or more individual values below the lower limit of quantification were treated as absent samples and not used in the calculations. A non-compartmental analysis method was used for the calculations. Areas under the plasma PENAO concentration versus time curves were calculated by the linear trapezoidal rule up to last measurable sampling time point (AUC_{0-last}). The last area segment was calculated by the addition of the area increment $Ct/\lambda z$, where λz is the terminal clearance rate constant and Ct is the plasma concentration at the last measurable sampling time point. Parameters relying on the determination of the terminal elimination phase rate constant (K_{el}) were not reported if the coefficient of determination (Rsq) was less than 0.08.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, USA). For comparisons of differences between control and treatment groups, where possible, Bartlett's test was used to assess homogeneity of group variances for each specified endpoint. If Bartlett's test was not significant ($p > 0.05$), a pooled estimate of the variance (Mean Square Error) was computed from a one-way analysis of variance (ANOVA) and utilized for a Dunnett's comparison of each treatment group with the control group. If Bartlett's test was significant ($p < 0.05$), comparisons with the control group were made using Welch's t-test. Body weight and food consumption data were analysed by repeated measures two-way ANOVA with Bonferroni post-test. Results of all pair-wise comparisons were reported at the 0.05, 0.01 and 0.001 statistical significance levels. All endpoints were analysed using two-tailed tests. Historical data indicate that leukocyte counts (total and differential) are not normally distributed; therefore, a log transformation was performed on these data. The transformed data was then analysed as described above. Frequency data such as the incidence of necropsy and histopathology findings were compared using Fisher's exact test.

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Author contributions PJH conceived and funded the study and wrote the manuscript.

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Data availability Raw data is available upon request.

Compliance with ethical standards

Conflicts of interest/Competing interests PJH is the Chief Scientific Officer of PENAO Pty Ltd, a company formed to manage the clinical and commercial development of PENAO.

Ethics approval All procedures undertaken were approved by the RMIT Animal Experimentation and Ethics Committee (AEEC Project #0801; sub-part 0801F).

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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