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CLINICAL TRIAL REPORT

Pharmacokinetics of 5,6-dimethylxanthenone-4-acetic acid (AS1404), a novel vascular disrupting agent, in phase I clinical trial

M. B. Jameson · B. C. Baguley · P. Kestell · L. Zhao · J. W. Paxton · P. I. Thompson · S. Waller · on behalf of the Cancer Research (UK) Phase I/II Trials Committee

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Abstract
Purpose 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) (AS1404) is a novel antitumour agent that selectively disrupts tumour vasculature and induces cytokines. The purpose of this study was to determine the pharmacokinetics (PK) of DMXAA in cancer patients enrolled in a phase I clinical trial.
Methods DMXAA was administered as a 20-min i.v. infusion every 3 weeks and doses were escalated in cohorts of patients according to a predefined schema. PK samples were taken over the first 24 h of at least the first cycle.
Results DMXAA was administered to 63 patients at 19 dose levels from 6 to 4,900 mg m^{-2}, and 3,700 mg m^{-2} was established as the maximum tolerated dose. The PK observed over the dose range showed a non-linear fall in clearance from 16.1 to 1.42 l h^{-1} m^{-2} and resultant increase in the area under the concentration–time curve (AUC) from 1.29 to 12,400 μM h. In contrast, the increase in peak plasma concentrations from 2.17 to 1,910 μM approximated linearity. DMXAA was highly protein-bound to albumin (>99%) until saturation occurred at higher doses, leading to a rapid increase in the free fraction (up to 20%) and greater concentrations of DMXAA bound to non-albumin proteins. However, the main determinant of the non-linearity of the PK appeared to be sequential saturation of elimination mechanisms, which include hydroxylation, glucuronidation and perhaps hepatic transport proteins. This resulted in an exaggerated non-linear increase in free DMXAA plasma concentrations and AUC compared to total drug.
Conclusions The PK of DMXAA are well-defined, with a consistent degree of non-linearity across a very large dose range.

Keywords DMXAA · Non-linear · Pharmacokinetic · Protein binding · Phase I

M. B. Jameson · P. I. Thompson
Department of Clinical Oncology, Auckland Hospital,
Private Bag 92024, Auckland, New Zealand

M. B. Jameson · B. C. Baguley · P. Kestell · L. Zhao
Auckland Cancer Society Research Centre,
University of Auckland, Private Bag 92019,
Auckland, New Zealand

J. W. Paxton
Department of Pharmacology and Clinical Pharmacology,
University of Auckland, Private Bag 92019,
Auckland, New Zealand

S. Waller · on behalf of the Cancer Research (UK)
Phase I/II Trials Committee
Drug Development Office, Cancer Research (UK),
10 Cambridge Tce, London, NW1 4JL, UK

M. B. Jameson (✉)
Oncology Department, Waikato Hospital,
Private Bag 3200, Hamilton 2001, New Zealand
e-mail: jameson@waikatodhb.govt.nz

Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a novel antitumour agent developed at the Auckland Cancer Society Research Centre that selectively disrupts tumour vasculature and induces cytokines such as tumour necrosis factor and interferons [2]. DMXAA was the most active compound of those synthesised in a programme to develop analogues of flavone acetic acid (FAA), an agent with remarkable antitumour activity in mice, but little efficacy in humans [11]. DMXAA was

found to be both more effective and 12-fold more dose-potent in vivo against colon 38 tumours than FAA [20], and could also induce cytokines in both human and murine cell lines, whereas the activity of FAA was restricted to murine cells alone [5], which prompted the advancement of DMXAA into clinical trial.

The pharmacokinetics (PK) of FAA in both preclinical and clinical studies were complex, showing dose-dependence and saturable protein binding [4, 8, 9, 12, 16, 18, 25]. Preclinical PK data on DMXAA from in vitro human hepatic microsomal preparations, isolated perfused rat liver and in vivo murine studies yielded conflicting predictions regarding possible dose-dependence and saturation of elimination and protein binding in humans [16–18].

The clinical phase I trials of DMXAA, sponsored by Cancer Research (UK), escalated over 800-fold from the starting dose [10, 21]. In this paper we examine the PK of DMXAA over this very large dose range in patients with advanced cancer treated in the phase I trial conducted in Auckland, New Zealand [10].

Methods

Patients and dosing

Full eligibility criteria are described elsewhere [10]. In brief, adult patients with proven malignancy but no effective therapy available were eligible if they had adequate performance status, > 3 months life expectancy, adequate haematopoietic, renal and hepatic function (including normal bilirubin) and gave written informed consent prior to study entry. The regional ethics committee approved the study, which was conducted in accordance with the principles of the Declaration of Helsinki.

5,6-Dimethylxanthenone-4-acetic acid (MW 304) was administered intravenously in a minimum volume of 100 ml of normal saline over 20 min every 3 weeks. Subdued lighting was used during drug preparation and administration to prevent decarboxylation and precipitation [19]. Most patients received a 20 mg ml^{-1} formulation of the sodium salt in 0.1 M phosphate buffer, pH 7.7, but a more concentrated formulation (100 mg ml^{-1} in 0.02 M phosphate buffer, pH 7.9) was used late in the trial to reduce infusion volumes at higher dose levels. The starting dose of 6 mg m^{-2} was one-tenth of the LD_{10} in mice and a modified Fibonacci scheme was constructed to guide dose escalation, subject to toxicity, PK and data from the parallel UK phase I study.

Cohorts of three patients were to be treated at each dose level (up to six patients if significant toxicity occurred). When dose-limiting toxicity (DLT) was observed in two of three patients at a dose level, an additional three patients were to be treated at the previous dose level, designated the maximum tolerated dose (MTD), to determine the recommended dose for phase II trials.

Pharmacokinetic sampling

On course 1 (and course 2 in one patient at each dose level) blood samples were collected into heparinised tubes, taking 20 ml prior to the infusion then 10 ml samples at the following times from infusion completion: −10, 0, 15, 30 and 45 min then 1, 1.5, 2, 4, 6, 8, 12 and 24 h. The blood was centrifuged at 1,000*g* for 10 min and aliquots of plasma (and packed red cells in the last five patients) were stored at −20°C until analysed. Urine was collected prior to the infusion then over 24 h post-infusion. Aliquots of each urine sample passed within 6 h of the infusion were immediately diluted (1:3, v/v) with 10 mM ammonium acetate/methanol (60:40, v/v) pH 5.5 and frozen at −20°C. Urine was collected for the remaining 18 h into a bottle containing 200 ml of 100 mM ammonium acetate/methanol (60:40, v/v) pH 5.5 and an aliquot was stored at −20°C. Tumour biopsies to assess the PK of DMXAA were performed immediately before drug infusion and repeated at 3–4.5 h and 24 h after infusion where feasible, and frozen at −80°C until analysed.

Drug determination and sample processing

Plasma and urine concentrations of DMXAA were determined in triplicate using automated solid-phase extraction (Gilson Medical, Middleton, WI, USA) and high-performance liquid chromatography (HPLC) with fluorescence detection, as previously described [14]. Samples were diluted up to 20-fold with 1 μM ammonium acetate (depending on the expected drug concentration) and 20 μM 2,5-DMXAA was used as the internal standard. Plasma free drug concentrations were determined after centrifugation of 500 μl plasma samples in Centrisart (Sartorius AG, Goettingen, Germany) ultrafiltration devices (MW cut-off 20,000 Da) at 2,000*g* for 45 min at room temperature. The ultrafiltrate was injected directly onto the HPLC column without the addition of internal standard. Packed red cell aliquots were processed in the same manner as described for plasma. Samples of diluted urine were mixed with the internal standard and processed as above. Urine samples were also hydrolysed with 1 M NaOH and then treated as above to determine alkali-labile glucuronide metabolites. Frozen tumour samples

were homogenised in 10 mM ammonium acetate at 4°C then centrifuged at 14,000*g* for 10 min at 4°C. The supernatant was treated in the same manner as for plasma detailed above.

Quantitation of DMXAA

Standard curves for each assay were consistently linear ($r^2 > 0.99$) over the concentration range 0.125–100 μM and inter-assay accuracy (87–107%) and precision [coefficients of variation (CV) of 2–5%] for the calibration standards were acceptable. The assay was validated using quality control (QC) human plasma spiked with known DMXAA concentrations and frozen at −20°C. In the early stages of the trial the QC plasma concentrations were 0.125, 1 and 10 μM but these were increased progressively to a maximum of 800 μM because of the increasing sample concentrations of DMXAA. The intra-assay relative recoveries and CV for QC plasma samples with DMXAA concentrations of 5, 40 and 400 μM were 88–113 and 0–5%, respectively ($n = 5$, 3 assays), and inter-assay accuracy and precision were also acceptable (relative recoveries 93–104% and CV 7–8%, 25 assays). The respective values for QC samples of lower and higher concentrations were comparable.

Pharmacokinetic calculations

Model-independent pharmacokinetic parameters were estimated using MKMODEL (Elsevier-Biosoft, Cambridge, UK), an extended least-squares modelling system. The area under the concentration–time curve (AUC) was calculated using the log trapezoid rule for total drug concentrations to 24 h and was extrapolated to infinity by addition of the value of C_t/k_{el} (where C_t is the concentration at the last timepoint and k_{el} is the elimination rate constant, calculated as the terminal slope of the log concentration–time profile determined by linear regression). The trapezoid rule was used to calculate the free drug AUC over the time range sampled (Prism 2.01, Graphpad Software Inc., San Diego, CA, USA). Other parameters included steady-state volume of distribution (V_{ss}) and plasma clearance (Cl), calculated as dose/AUC. Results are presented as mean ± SD.

Curve fitting was undertaken using non-linear least-squares fitting with a weighting function of $1/y^2$ (Sigmaplot, SPSS Inc., Chicago, IL, USA). The choice of mono-, bi- or tri-exponential equations was made by applying Akaike's information criterion [1, 24], comparing the standard errors of the parameter estimates, the correlation coefficient for the estimate and a visual inspection of the curves.

Results

5,6-Dimethylxanthenone-4-acetic acid was administered to 63 patients at 19 dose levels ranging from 6 to 4,900 mg m^{-2} (Table 1). Non-haematological DLT at 4,900 mg m^{-2} established 3,700 mg m^{-2} as the MTD. Dose escalation deviated from the planned Fibonacci series after the first escalation because of comparative PK data in mice, and further escalation was guided by PK, pharmacodynamic data and toxicity from both the NZ and UK trials (discussed further in Jameson et al. [10]).

The pharmacokinetic parameters determined for each patient are averaged by dose cohort (six patients treated at 500 and 3,700 mg m^{-2} and three patients in all other cohorts) and summarised in Table 1. The plasma total DMXAA concentration–time profile at lower doses best fitted a tri-exponential equation, suggesting a three-compartment pharmacokinetic model. However, these characteristics were progressively obscured with increasing dose, exemplified in Fig. 1, most likely due to saturation of elimination pathways. The model-independent mean terminal elimination half-lives (7.6 ± 10.5 h for total drug and 2.3 ± 4.1 h for free drug) increased at higher dose levels (Table 1).

The plasma PK of DMXAA were dose-dependent as demonstrated by clearance falling with increasing dose, from 16.1 ± 4.07 to 1.42 ± 0.56 l h^{-1} m^{-2} over the dose range 6–4,900 mg m^{-2} (clearance = dose$^{-0.36}$; Fig. 2a). As a result, the AUC increased non-linearly (AUC = dose$^{1.36}$) from 1.29 ± 0.36 to 12,400 ± 401 μM h over the same dose range. In contrast, the increase in C_{max} (from 2.17 ± 0.59 to 1,910 ± 603 μM) remained linear with a consistent slope on a log–log plot (slope = 1.05 ± 0.14, $r = 0.99$) but it did not increase at all at the two highest dose levels (Fig. 3).

5,6-Dimethylxanthenone-4-acetic acid was highly protein-bound (>99%) at doses up to 650 mg m^{-2} but saturation of protein binding at higher doses occurred in both this trial and in vitro studies, with free plasma DMXAA reliably exceeding 1% at total drug concentrations in excess of 500 μM and the plasma percent free drug fraction rose as high as 20% (Fig. 4). At the two highest dose levels, peak red cell concentrations were approximately double the total concentrations in plasma (Fig. 5).

A rapid increase in free drug concentrations was also observed at doses above 650 mg m^{-2}: an almost tenfold dose escalation from 500 to 4,900 mg m^{-2} increased the total plasma C_{max} fivefold, whereas the free plasma C_{max} escalated by a factor of 66. Over the same dose range, the total drug AUC increased 20-fold whereas free drug AUC increased 200-fold, with a corresponding non-linear fall in clearance (Fig. 2b). The total drug V_{ss} of 0.36 l kg^{-1} did not change significantly

Table 1 Model-independent pharmacokinetic parameters at each dose level on the first cycle; mean (SD)

Dose (mg m^{-2})	Dose (μmol kg^{-1})	C_{max} (μM)	AUC (μM h)	Cl (l h^{-1} m^{-2})	Free C_{max} (μM)	Free AUC (μM h)	Free Cl (l h^{-1} m^{-2})	V_{ss} (l kg^{-1})	$t_{1/2}$ (h)
6	0.46 (0.04)	2.17 (0.59)	1.29 (0.36)	16.1 (4.07)	0.01 (0.01)	NM	NM	0.41 (0.11)	1.24 (0.16)
10.2	0.87 (0.13)	6.07 (2.31)	5.24 (1.55)	6.88 (2.44)	0.04 (0.01)	NM	NM	0.28 (0.14)	1.76 (1.26)
20.4	1.82 (0.11)	10.1 (4.22)	9.09 (6.27)	11.30 (9.26)	0.07 (0.04)	NM	NM	0.62 (0.18)	4.48 (4.00)
40.8	3.32 (0.90)	29.7 (12.3)	31.5 (13.1)	4.84 (2.14)	0.17 (0.06)	NM	NM	0.38 (0.28)	4.51 (0.30)
81.6	7.60 (1.02)	40.0 (5.01)	35.9 (11.9)	8.13 (2.99)	0.24 (0.06)	NM	NM	0.71 (0.18)	5.30 (0.71)
160	12.4 (1.93)	101 (16.9)	103 (11.1)	5.20 (0.54)	0.64 (0.15)	NM	NM	0.30 (0.06)	4.11 (0.89)
240	21.9 (4.10)	254 (99.9)	248 (73.7)	3.40 (1.22)	2.38 (1.16)	NM	NM	0.19 (0.03)	4.68 (1.99)
360	31.0 (5.60)	264 (37.6)	456 (214)	3.00 (1.35)	2.36 (0.34)	NM	NM	0.22 (0.05)	4.50 (0.82)
500	42.1 (5.00)	367 (58.4)	638 (372)	3.04 (0.98)	3.69 (1.15)	2.74 (0.69)	620 (156)	0.30 (0.15)	5.86 (1.67)
650	56.1 (1.60)	479 (110)	1,130 (903)	2.68 (1.48)	5.29 (2.75)	5.28 (2.69)	466 (238)	0.28 (0.09)	6.41 (0.35)
850	61.9 (12.9)	564 (77.3)	1,180 (63.5)	2.37 (0.13)	10.9 (5.68)	15.0 (3.83)	196 (54.4)	0.26 (0.08)	7.50 (4.29)
1,100	97.5 (9.5)	762 (94.8)	1,430 (251)	2.58 (0.47)	15.4 (6.12)	16.8 (5.31)	231 (74.0)	0.35 (0.16)	7.25 (1.93)
1,375	127 (13.6)	905 (103)	4,660 (2,200)	1.19 (0.73)	32.1 (25.2)	49.7 (51.4)	204 (196)	0.28 (0.10)	7.94 (1.77)
1,650	131 (12.0)	1,160 (236)	2,890 (806)	1.99 (0.65)	29.6 (3.21)	38.3 (13.8)	155 (56.6)	0.31 (0.09)	10.2 (4.38)
2,000	200 (11.6)	1,320 (70.2)	5,030 (2,540)	1.55 (0.75)	50.4 (11.0)	66.8 (20.8)	104 (28.1)	0.40 (032)	9.97 (8.52)
2,600	218 (27.9)	1,450 (66.6)	5,920 (437)	1.45 (0.11)	107 (3.46)	216 (19.1)	39.8 (3.65)	0.24 (0.09)	6.05 (2.64)
3,100	267 (31.5)	1,430 (55.1)	6,910 (1,080)	1.50 (0.22)	186 (104)	271 (98.5)	40.6 (12.4)	0.30 (0.09)	7.10 (1.64)
3,700	287 (28.5)	1,910 (523)	13,400 (6,370)	1.08 (0.47)	125 (20.4)	275 (57.6)	46.2 (10.8)	0.25 (0.08)	9.19 (3.05)
4,900	461 (73.3)	1,910 (604)	12,400 (4,010)	1.42 (0.56)	242 (54.6)	554 (175)	30.9 (8.95)	0.41 (0.18)	8.73 (2.29)

NM not measured

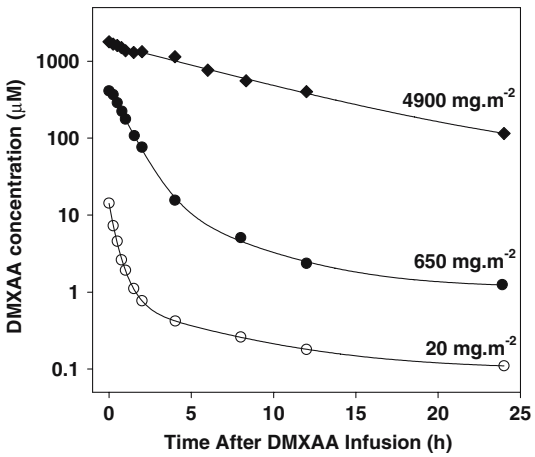

Fig. 1 Effect of increasing dose on the plasma DMXAA concentration time-course on a log–linear plot. Each time-course represents data from single patients treated at 20, 650 and 4,900 mg m^{-2}

with dose ($r^2 = 0.02$) and free drug V_{ss} was nearly 50-fold greater at 16.6 l kg^{-1}. Toxicokinetics appeared to correlate with free drug PK, as the frequency of clinical toxicities recorded per patient increased substantially as free drug concentrations rose ($r^2 = 0.91$).

Repeat plasma concentration time-courses were obtained on a second cycle in 15 patients at doses from 6 to 2,600 mg m^{-2} and there were no significant changes in plasma AUC on paired *t*-test ($P = 0.32$), suggesting no induction of metabolising enzymes. While C_{max} was significantly lower on second cycles ($P = 0.046$), this is thought to reflect altered distribution kinetics due to falling plasma albumin concentrations in these patients with advancing disease ($P = 0.0001$ for paired *t*-test on albumin concentrations). Subclinical ureteric obstruction on the first cycle of DMXAA in one patient led to renal impairment and reduced Cl of DMXAA, which increased threefold on the second cycle when ureteric patency had been restored. There was no correlation between the creatinine clearance (range 49–149 ml min^{-1}) and DMXAA clearance for individual patients with essentially normal renal function who were treated at higher dose levels ($r = -0.18$).

Examination of urine from patients revealed that up to 6% of the dose was present as unchanged DMXAA, while up to 70% was excreted as metabolic products of glucuronidation and 6-methylhydroxylation. Tumour drug concentrations following DMXAA administration were measured in biopsies taken from three patients, one at each of the three highest dose levels, and levels ranged from 62 to 163 μmol kg^{-1} in tissue taken 3–4.5 h after dosing to 3.8–14.3 μmol kg^{-1} in tissue taken at 24 h. These greatly exceeded drug concentrations found in murine tumours at comparable timepoints (37.2 μmol kg^{-1} at 3 h and 0.24 μmol kg^{-1} at 24 h), following administration of DMXAA at the murine MTD (approximately 90 mg m^{-2}) (personal communication, Dr P. Kestell).

Discussion

This trial provided an excellent opportunity to examine the PK of DMXAA across an unusually large dose

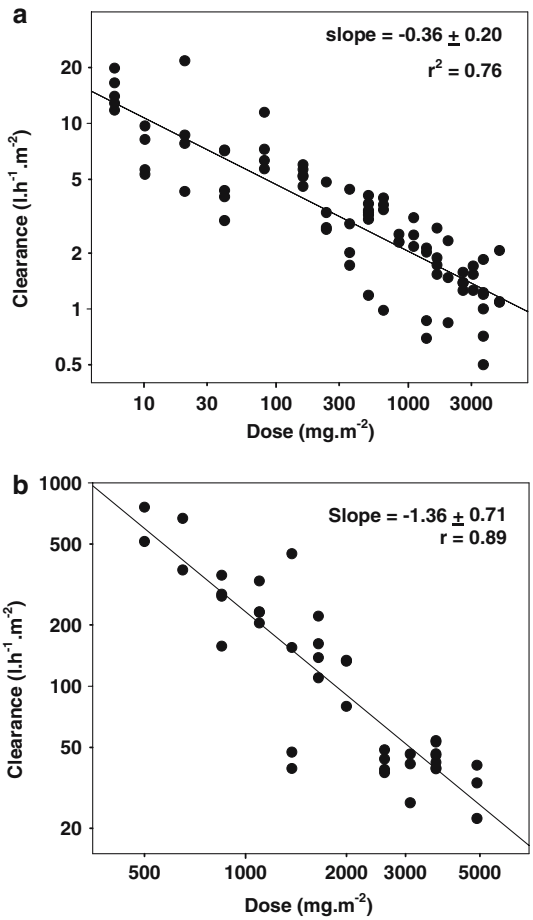

Fig. 2 Non-linear relationship between Cl and DMXAA dose, shown on log–log plots **a** for total drug and **b** for free drug

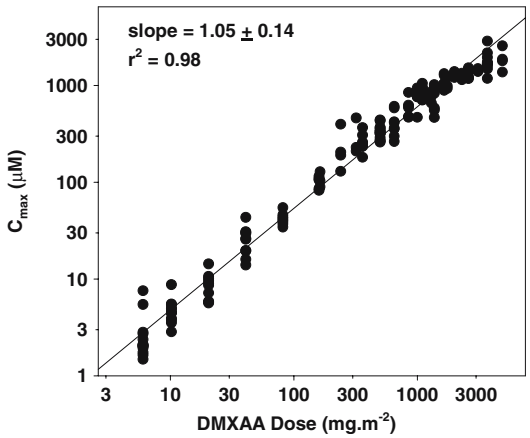

Fig. 3 Linear relationship between peak plasma concentration and DMXAA dose except at the highest dose levels; log–log plot

range. The non-linearity (dose-dependence) of the PK (also observed in the other phase I trial [21]) is remarkably consistent over the large dose range, best demonstrated on the log–log plots that examine the > 10-fold

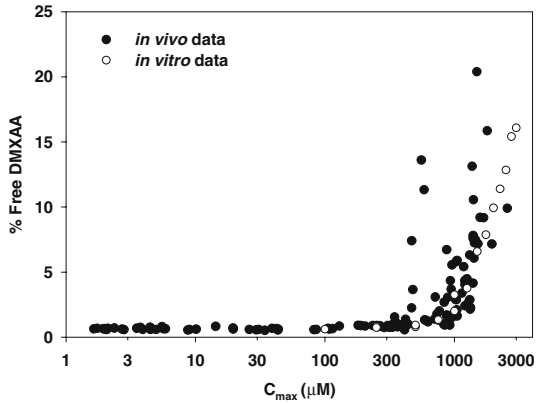

Fig. 4 Relationship between percentage plasma free drug and DMXAA concentration; linear–log plot, showing both in vivo trial data and in vitro data using human plasma

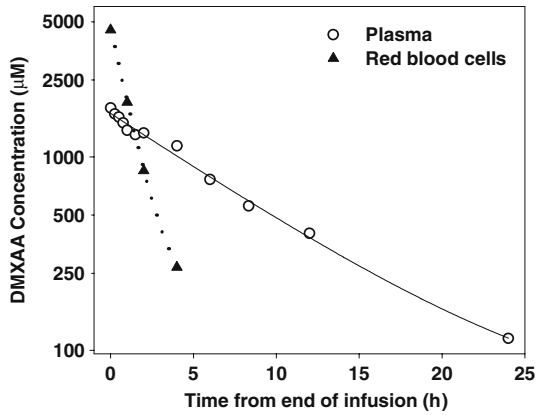

Fig. 5 5,6-Dimethylxanthenone-4-acetic acid concentration–time profile in red blood cells and plasma in a patient treated at 4,900 mg m^{-2}; log–linear plot

fall in total and free drug clearance with increasing dose (Fig. 2 a, b). Non-linear PK of DMXAA were also observed in other species [13, 16].

Comparison of the PK of DMXAA and FAA revealed striking similarities, in that FAA showed non-linear PK over the full dose range and, at higher concentrations, saturation of protein binding and hepatic elimination mechanisms occurred [6, 9, 12, 23]. Its metabolites were also predominantly glucuronides with significant enterohepatic recirculation [6, 18] and it also had much faster clearance in humans than in mice [25]. In addition, tumour concentrations achieved clinically with FAA were comparable to those in mice at an effective antitumour dose [7, 15], as was seen with DMXAA in this trial, and do not account for the absence of significant tumour responses observed in the phase I trials. It would appear that pharmacodynamic factors are more likely to account for the difference in antitumour activity of FAA and DMXAA between species.

Progressive saturation of at least three capacity-limited biotransformation and elimination pathways appears to be the most plausible basis for the non-linear PK of DMXAA. The fall in clearance at lower concentrations is most likely due to saturation of 6-methylhydroxylation (via CYP1A2), a high affinity–low capacity enzyme (K_m=21 ± 5 μM in human liver microsomes, n = 14) [29]. Another saturable elimination pathway is likely to be hepatic transport of DMXAA-glucuronide via multidrug resistance-associated proteins [30]. However, glucuronidation of DMXAA (via UGT1A9 and UGT2B7 isoenzymes) is only likely to be saturated at the highest doses of DMXAA administered in the clinical trial. This biotransformation pathway was low affinity–high capacity (K_m=138 ± 79 μM in human liver microsomes, n = 14) [29], and plasma free drug concentrations greater than this K_m were only achieved at the highest dose level. While these in vitro systems may substantially underestimate clearance of the drug [28], their relative K_m indicate the order in which they are likely to become saturated. Enterohepatic recirculation of glucuronide metabolites, suggested in this clinical trial (data not shown) and evident in murine studies [16], could also contribute to an apparent long terminal half-life component on the PK profile and hence reduced clearance.

In contrast, while saturation of protein binding was very prominent at higher doses of DMXAA, this is unlikely to substantially contribute to the non-linearity of the PK of the free drug. DMXAA is highly protein-bound with albumin being the main binding species, and the plasma free drug fractions conform very closely to those predicted from the binding constant of DMXAA for albumin [3]. However, saturation of protein binding would tend to increase total drug clearance through making more free drug available to elimination pathways, and free plasma DMXAA concentrations would change minimally because of the nearly 50-fold greater volume of distribution for free drug than total drug. Red blood cells (and probably haemoglobin in particular) provided low affinity–high capacity binding sites that became an increasingly significant component of the free drug volume of distribution with higher doses [27]. This drug "pool" was discarded when separating plasma for analysis and most likely accounted for the lack of increment in plasma C_{max} at higher dose levels.

The 23% intra-individual variability of the AUC on repeated doses of DMXAA was much less than inter-individual differences in PK parameters (up to 300%). Factors that could contribute to the inter-individual variation include differences in albumin and haemoglobin concentrations (influencing plasma C_{max} and protein binding), the extent of liver metastases, genetic factors such as isoenzyme expression polymorphisms, smoking (which induces CYP1A2), use of other medications that compete for or affect the same metabolising enzymes, variation in hepatic blood flow and the degree of enterohepatic recirculation of the glucuronide metabolites.

The prediction of the PK and toxicity of DMXAA in humans from the preclinical data available prior to initiating the trial was poor. While protein binding kinetics and major metabolic pathways were identified, saturation of glucuronidation and protein binding was not expected within the anticipated clinical dose range [17, 22]. In contrast, later studies found both hepatic metabolism and protein binding of DMXAA were saturable [27, 26], and in vivo preclinical studies showed dose-dependent PK in mice [16], rats and rabbits [13], fitting a two-compartment model with capacity-limited elimination. However, the inter-species differences observed in protein binding, routes of elimination and their capacity did not account for the differences in toxicity, with the MTD varying tenfold between the species based on body weight, and over 60-fold when based on BSA [13, 27].

The non-linear plasma PK and major routes of elimination of DMXAA in humans have been elaborated in this trial. This may assist with dosing in future clinical trials, which could further explore the relationship of DMXAA dose, administration schedule and PK with selected pharmacodynamic endpoints and toxicities in order to enable an optimal dose schedule to be determined for future development.

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