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Plasma pharmacokinetics of the antitumour agents 5,6-dimethylxanthenone-4-acetic acid, xanthenone-4-acetic acid and flavone-8-acetic acid in mice*

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Summary. Although the antitumour agent flavone-8-acetic acid (FAA) exhibits remarkable activity against murine solid tumours, its clinical use has a number of pharmacological drawbacks, including low dose potency and dose-dependent pharmacokinetics. Xanthenone-4-acetic acid (XAA) and its 5,6-dimethyl derivative (5,6-MeXAA) were synthesised during a search for better analogues of FAA. The maximal tolerated doses (MTDs) of 5,6-MeXAA, XAA and FAA in BDF_1 mice were 99, 1,090 and 1,300 μmol/kg, respectively. At the MTD, 5,6-MeXAA displayed the following pharmacokinetic properties: maximal plasma concentration, 600 μM; mean residence time, 4.9 h; AUC, 2,400 μmol $h\ l^{-1}$; and volume of steady-state distribution, 0.2 l/kg. All compounds displayed nonlinear elimination kinetics at the MTD, but when the logarithm of the AUC was plotted against that of the delivered dose, the slope of the regression line for 5,6-MeXAA was found to be 1.2 as opposed to 1.4 for XAA and 1.98 for FAA. 5,6-MeXAA thus showed only a slight deviation from dose-independent kinetics. 5,6-MeXAA bound to plasma proteins in a manner similar to that exhibited by FAA, although the plasma concentration of free drug was lower for the former than for the latter. As a consequence, the calculated maximal free drug concentration for 5,6-MeXAA in plasma was 23 times lower than that for FAA.

Introduction

Flavone-8-acetic acid (FAA) was synthesised in 1984 [1] and has shown an unusual spectrum of antitumour activity, displaying high activity against murine solid tumours [20, 24] and little activity against murine leukaemia [19, 20, 24]. It seems to act as a biological response modifier [6], inducing the synthesis of cytokines [18] and of nitric oxide [25] and reducing tumour blood flow [3, 11, 29]. These unusual properties have caused considerable speculation about its potential as a clinical agent for human solid tumours. FAA entered clinical trials in 1987 [15, 27] and has thus far yielded disappointing results as a single agent [16]. Its synergy with the cytokine interleukin 2 [28] has renewed interest in FAA and prompted clinical trials.

It is apparent that the clinical use of FAA has a number of pharmacological drawbacks. First, its low dose potency means that high doses (5–10 g/m^2) are required for intravenous infusions [15, 16, 27]. Since the free acid form of FAA is poorly soluble in aqueous solution, there is a risk of renal toxicity after such high doses due to the precipitation of FAA free acid within the low pH environment of the kidney tubules. Alkalinization has been used in some studies to prevent such toxicity, but apart from its adverse effect on the pharmacokinetics of the drug and its association with severe hypokalaemia [26], this procedure has been shown to reduce the activity of FAA in experimental systems [12]. Second, FAA displays highly dose-dependent pharmacokinetics [5, 7, 8, 14, 15, 27]. Enhancement of the FAA dosage is associated with prolongation of the plasma elimination half-life and with disproportionately high increases in the AUC. These factors may cause problems during dose escalation and make it difficult to achieve an optimal dose. The third pharmacological problem is that FAA plasma protein binding is saturable, with the free fraction of plasma FAA varying between 3% and 30% of total plasma FAA over the range of plasma concentrations encountered clinically [4, 27].

In a search for compounds exhibiting antitumour properties similar to those of FAA but displaying fewer pharmacological disadvantages, a series of xanthenone-4-

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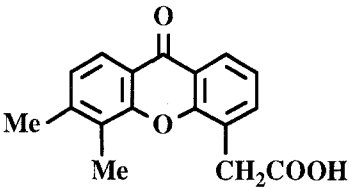

Fig. 1. Structure of 5,6-MeXAA. XAA lacks the two methyl groups

acetic acid (XAA) derivatives have been synthesised, several members of which show antitumour spectra similar to that of FAA [2, 21, 22]. The 5,6-dimethyl derivative (5,6-MeXAA; see Fig. 1 for structure) is curative in 80% of mice bearing advanced colon 38 tumours and is 12 times more dose-potent than FAA [22]. We report the pharmacokinetics of 5,6-MeXAA and XAA in mice and contrast the data with those previously reported for FAA.

Materials and methods

Chemicals and mice. FAA was supplied by the Pharmaceutical Resources Branch, National Cancer Institute (Bethesda, Md., USA). XAA, 5,6-MeXAA, 3-methyl-XAA and 5-propoxy-XAA were synthesised according to published methods [2, 21, 22] and were formulated as the sodium salts. Male B6D2F$_1$ mice that had been bred in the laboratory animal facility were housed under constant temperature and humidity using sterile bedding, water and food according to institutional ethical guidelines. Animals aged 8–12 weeks (body weight, 20–30 g) were used in all experiments.

Drug administration and blood collection. Immediately before their use, drug solutions were made up in sterile 5% sodium bicarbonate under subdued lighting to prevent photolytic degradation [23]. Drug solutions were given to mice by intravenous bolus injection (100–150 µl) via the tail vein. Blood was collected in heparinised tubes from each of three mice at the appropriate time points (up to 72 h) using ether anaesthesia. Plasma was separated by centrifugation, removed and stored at –80° C until analysis.

Determination of the MTD. The MTD was determined by treating groups of mice at a nontoxic dose and escalating the dose in 50% increments until mortality was first encountered. At this point, an intermediate dose was given. Any animals appearing to be distressed during these determinations were killed by cervical dislocation.

Drug analysis. Plasma samples were processed under subdued lighting to minimise photolytic degradation [23]. Previously reported methods [5, 17] were applied for the analysis of XAA and FAA using 3-methyl-XAA as an internal standard. For 5,6-MeXAA analysis, the previously described method [17] was modified in that 5-propoxy-XAA was used as the internal standard and water: acetonitrile: acetic acid (60:40:2, by vol.) was used as the mobile phase at a flow rate of 1.8 ml/min. The intra-assay variation (%CV) was 3.3%, the inter-assay variation (%CV) was 4.5% and the theoretical recoveries ranged from 98.7% to 102.2%. The standard curve was linear up to a concentration of 20 µM ($r = 0.999$) and the limit of detection was 0.1 µM. Plasma samples were diluted with 10 mM ammonium acetate buffer (pH 5.5) to the appropriate concentrations for assay.

Plasma protein binding. The method used to evaluate plasma protein binding was similar to that described by Brodfuehrer et al. [4] and utilised the Amicon Centrifree micropartition system (Amicon Corp., Danvers, Mass.). Drugs were dissolved in mouse plasma (obtained from BDF$_1$ mice) at appropriate concentrations (50–2,000 µM) and were incubated at 37° C for 15 min. Aliquots (100 µl) of plasma were analyzed for drug content by HPLC using the methods described above. Next, 300 µl aliquots of plasma were placed in the upper compartment of the micropartition system and centrifuged at 2,000 *g* for 20 min. The ultrafiltrate was then analyzed by HPLC and the percentage of free drug was determined as the ratio of ultrafiltrate/unfiltered drug concentrations. Control experiments established that the drugs did not bind to the membranes.

Data analysis. Pharmacokinetic analysis was performed using the non-compartmental method of Gibaldi and Perrier [13] and MKMODEL version 3.34, and extended least-squares modelling system developed by Dr. N. Holford, Department of Pharmacology, University of Auckland School of Medicine. The AUC and area under the first moment curve (AUMC) were calculated by the trapezoid rule when successive values were increasing and by the log trapezoid rule when successive values were decreasing and were then extrapolated to infinity. The apparent volume of distribution at steady state (V_{ss}) was determined by dividing the product of the dose and the AUMC by the square of the AUC. The mean residence time (MRT), representing the time required for 63.2% of the delivered dose to be eliminated, was estimated by dividing the AUMC by the AUC. The slope of the concentration-time profile was obtained by linear regression analysis of all time points and was used to calculate the plasma elimination half-life ($t_{1/2}$). Plasma clearance (C) was calculated by dividing the delivered dose by the AUC.

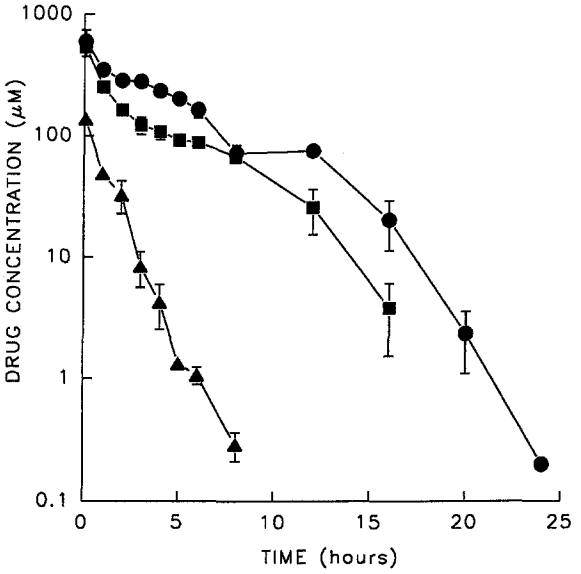

Fig. 2. Plasma drug concentrations in mice following the administration of 5,6-MeXAA at doses of 99 (●), 66 (■) and 9.9 (▲) µmol/kg

Table 1. The MTD and the dose at which mortality was first encountered in BDF$_1$ mice for 5,6-MeXAA, XAA and FAA

Drug	5,6-MeXAA		XAA		FAA	
	MTD	D	MTD	D	MTD	D
Dose (µmol/kg)	99	115	1,090	1,180	1,300	1,440
Deaths/number treated	0/5	1/6	0/5	1/5	0/3	2/8

D, Dose at which mortality first occurred

Results

Determination of the MTD

Table 1 shows the MTD and the doses at which mortality was first encountered for 5,6-MeXAA, XAA and FAA. The MTD for XAA was 16% lower than that for FAA, whereas that for 5,6-MeXAA was 13 times lower than that

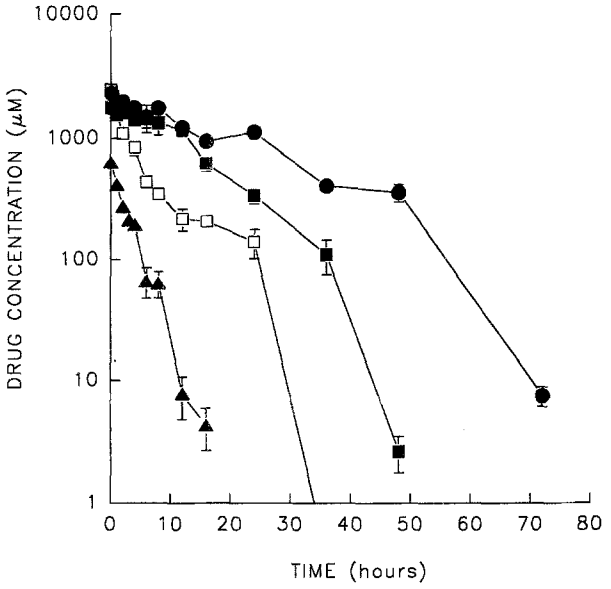

Fig. 3. Plasma drug concentrations in mice following the administration of XAA at doses of 1,090 (●), 730 (■) and 109 (▲) μmol/kg and of FAA at a dose of 1,300 μmol/kg (□)

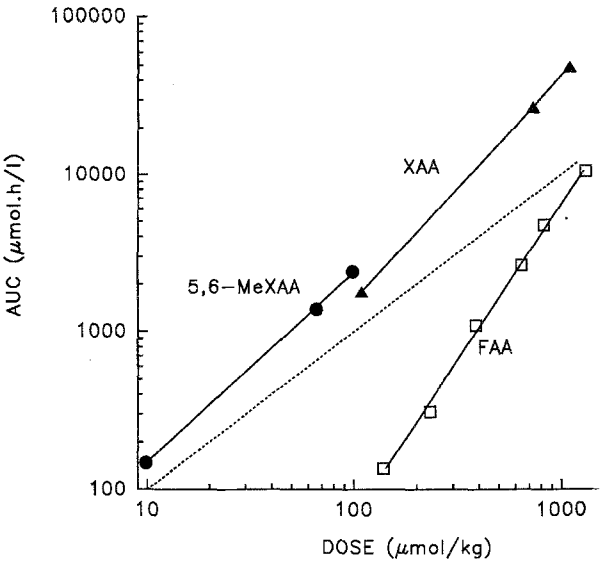

Fig. 4. Relationship of AUC to dose for 5,6-MeXAA (●), XAA (▲) and FAA (□). FAA data derives from Table 2 (*right-hand points*) and from the work of Chabot et al. [5] (*remaining points*). The *dotted line* indicates the slope required for dose-independent pharmacokinetics

for the latter. The main side effect at the MTD was sedation, the onset of which occurred within 1 h for FAA and XAA but between 4 and 6 h after treatment for 5,6-MeXAA.

Plasma pharmacokinetics

Semilogarithmic plasma concentration-time curves obtained for 5,6-MeXAA are shown in Fig. 2. The curves were slightly sigmoid for the two highest doses (99 and 66 μmol/kg) and included an intermediate phase involving a lower elimination rate and a more rapid terminal elimination phase. At the lowest dose (9.9 μmol/kg; 10% of the MTD), elimination conformed to a one-compartment (monoexponential) model. Corresponding curves for XAA are shown in Fig. 3. Again, the curves were sigmoid at the two highest doses (1,090 and 730 μmol/kg) and linear at the lowest dose (109 μmol/kg). At the MTD, there was a slow decrease in XAA concentration with time for up to 48 h after drug administration, followed by a more rapid elimination rate that occurred at between 48 and 72 h. At an intermediate dose (730 μmol/kg), XAA was undetectable in plasma at 72 h, whereas at the lowest dose it was undetectable at 24 h after drug administration. For comparison, FAA was also tested at its MTD (Fig. 3). The initial clearance phase was more rapid than that for XAA and slightly slower than that for 5,6-MeXAA; it was followed by a slower intermediate phase and, finally, by a rapid terminal clearance phase. The concentration after 36 h was at the limit of detection. The pharmacokinetic parameters estimated for the data in Figs. 2 and 3 are shown in Table 2, and the relationship between the AUC and the dose is plotted in Fig. 4.

Plasma protein-binding properties

5,6-MeXAA, XAA an FAA were added to mouse plasma at a range of drug concentrations similar to those encountered in vivo, and the percentage of free and bound drug was analyzed (Fig. 5). Scatchard plots provided evidence of saturation of a protein-binding site at bound drug concentrations of around 1,000 μM. All three compounds

Table 2. Pharmacokinetic parameters for 5,6-MeXAA, XAA and FAA

Drug	Dose (μmol/kg)	c_{max} (μM)	$t_{1/2}$ (h)	MRT (h)	AUC (μmol h l^{-1})	C ($l\ h^{-1}\ kg^{-1}$)	V_{ss} (l/kg)
5,6-MeXAA	9.9	137	0.89	1.3	148	0.067	0.086
	66	540	2.7	4.2	1,400	0.047	0.2
	99	600	4.2	4.9	2,400	0.041	0.2
XAA	109	630	3.1	3.2	1,800	0.063	0.2
	730	1,760	8.3	11.3	27,000	0.027	0.31
	1,090	2,300	15.1	18.9	48,000	0.023	0.43
FAA	1,300	2,500	6.1	6.7	10,400	0.13	0.84

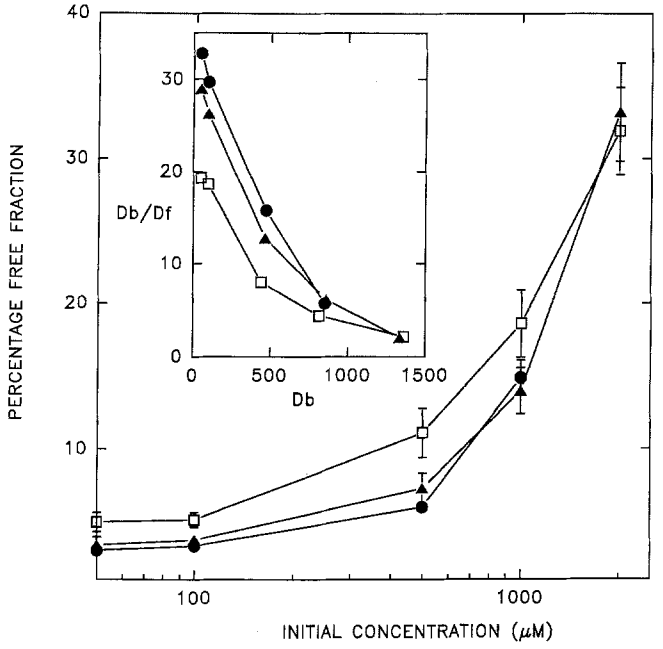

Fig. 5. Relationship between the free drug fraction and added drug concentration for 5,6-MeXAA (●), XAA (▲) and FAA (□) in the presence of mouse plasma. *Inset:* Plot of the same data using Scatchard plots of the ratios of bound to free drug (*Db/Df*) versus the corresponding micromolar bound drug concentrations (*Db*)

showed similar plasma protein-binding characteristics, although 5,6-MeXAA exhibited a free fraction that was 1.6 times lower than that of FAA at nonsaturating drug concentrations, with XAA displaying intermediate binding properties.

Discussion

In the present study, pharmacokinetic data for 5,6-MeXAA were obtained over a 10-fold range of doses (up to the MTD) in mice and were compared with corresponding data for XAA and FAA. The AUC for this drug at the MTD, which is often taken as an indicator of the effective applied dose for anticancer agents [9], was <25% of that for FAA (Table 2). When the AUC was plotted versus the delivered dose for the three drugs (Fig. 4), some interesting differences were revealed. For FAA, the slope of the double logarithmic plot was 1.98, meaning that a 2-fold increase in the dose would result in an almost 4-fold increase in AUC, whereas for 5,6-MeXAA a 2-fold increase in the dose would lead to only a 2.3-fold increase in AUC. XAA displayed intermediate values, with a 2-fold change in dose resulting in a 2.7-fold change in AUC. Thus, although there was evidence of dose-dependent kinetics for 5,6-MeXAA, the effect was slight. Nonlinear pharmacokinetics is commonly encountered in drugs that frequently cause clinical toxicity, and a recent survey of therapeutic drug-monitoring laboratories led to the recommendation that screening of new chemotherapeutic agents should favor drugs whose pharmacokinetics indicates minimal dose dependency [10]. This is probably particularly true for anticancer agents, which are dosed at the limit of acceptable toxicity and exhibit a narrow therapeutic index.

All three compounds showed rather complex concentration-time profiles that varied with dose, the elimination curves for 5,6-MeXAA and XAA at their respective MTDs being slightly sigmoid (Figs. 2, 3). As is evident in Fig. 4, XAA and 5,6-MeXAA were eliminated more slowly from plasma than was FAA, since the former exhibited a higher AUC at the same delivered dose. All compounds showed reduced rates of clearance at intermediate times after drug administration, followed by accelerated terminal elimination phases. In the case of FAA, it is known that glucuronidation of the acid group is followed by biliary excretion [5]. Consequent hydrolysis of the glucuronide in the gut releases free drug, which is then reabsorbed, thus accounting for the altered clearance rates at intermediate times. Studies are under way to investigate whether XAA and 5,6-MeXAA are metabolised in an analogous manner.

The three drugs bound plasma proteins in a similar way, with 5,6-MeXAA displaying the lowest free drug fraction (Fig. 5). The binding of FAA to human and mouse plasma has been hypothesized to occur predominantly at the two indolebenzodiazepine binding sites of the albumin molecule [4]. Since the concentration of albumin in mice is 560 μM [4], the saturation ratios determined for XAA and 5,6-MeXAA from the Scatchard plots in Fig. 5 correspond to approximately two drug molecules being bound per albumin molecule. These data are in good agreement with other results [4]. It is noteworthy that the free drug fraction of 5,6-MeXAA varied only slightly (0.02–0.05) over the physiological range of plasma drug concentrations, whereas that of FAA varied markedly (0.04–>0.3).

The c_{max} value for 5,6-MeXAA was 3.9 times lower than that for FAA when both drugs were given at the MTD (Table 1). However, when protein binding is taken into account (Fig. 5), the maximal free drug concentration for 5,6-MeXAA is 37 μM, 23 times lower than that for FAA (840 μM). This should minimise any risk of renal toxicity at therapeutic doses as a consequence of precipitation of the free acid in renal tubules.

In conclusion, if our understanding of the poor clinical efficacy of FAA could be improved and approaches could be developed to overcome its drawbacks, then FAA analogues exhibiting better pharmacokinetic properties might be considered for further trials. It is not clear whether the pharmacokinetic properties of 5,6-MeXAA in mice described in this report would be mirrored in human studies. However, the 13-fold molar dose potency of 5,6-MeXAA as compared with FAA, together with its high experimental antitumour activity [22], make it an attractive candidate for human studies.

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