Plasma Protein Binding of the Experimental Antitumour Agent Acridine-4-carboxamide in Man, Dog, Rat and Rabbit

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Abstract—The plasma binding of N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (AC) was investigated in-vitro by equilibrium dialysis for 3 h at 37°C against isotonic phosphate buffer (pH 7·35) using [^{3}H]AC. There were significant species differences with the smallest % free fraction (mean ± s.d.) occurring in human plasma (3·4 ± 0·2), followed by dog (8·1 ± 0·4), mouse (14·8 ± 0·8), rat (16·3 ± 0·9) and rabbit (20·2 ± 0·7). In plasma from healthy individuals (n = 5), the % free fraction ranged from 2·7 to 3·8. In physiological solutions of human proteins, the greatest binding was observed for α_1-acid glycoprotein (AAG) (0·75 g L^{-1}) with a mean free fraction of 24·1 ± 2·2%, followed by albumin (40 g L^{-1}) with 31·6 ± 0·7 and 39·8 ± 2·5% for fatty-acid-free and globulin-free, respectively. There was also some binding to globulins (5 g L^{-1}) with a mean % free fraction of 70·3 ± 1·6 and 84·8 ± 2·2 for Cohn's fraction I and IV, respectively. Binding data from the displacement of [^{3}H]AC by increasing concentrations of AC in human AAG (0·75 g L^{-1}) or albumin solution (40 g L^{-1}) indicated that AAG had 10-fold greater binding affinity for AC (K_a, 7·8 × 10^4 M^{-1}) compared with albumin (K_a, 6·8 × 10^3 M^{-1}). In human plasma enriched with AAG there was a significant negative linear correlation (r = 0·932; $P < 0·001$) between % AC free fraction and increasing AAG concentration over the range 0·6–4·5 g L^{-1}. Small but significant ($P < 0·05$) increases in AC free fraction occurred in the presence of various metabolites (50 and 100 μM) but, of those tested, only N-monomethyl-acridine carboxamide increased the free fraction to the same extent as parent AC.

N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316) is a third generation acridine anticancer agent developed by the Cancer Research Laboratories, University of Auckland School of Medicine (Atwell et al 1987). Its predecessors were amsacrine which is currently being used for the treatment of leukaemia (Arlin 1983), and CI-921 which is undergoing Phase 2 clinical trials for evaluation against solid tumours. AC was more active against solid tumours in-vitro than amsacrine or CI-921 and was curative towards Lewis lung carcinoma in mice (Finlay & Baguley 1989). The high activity of AC against solid tumours in-vivo is postulated to occur partly due to better distributive properties as it is more lipophilic than amsacrine and CI-921, and at physiological pH, ionization of the acridine nitrogen is suppressed (Atwell et al 1987). Like its predecessors, AC is a DNA-intercalating agent and also a topoisomerase II inhibitor (Atwell et al 1987; Finlay & Baguley 1989). However, AC differs from the other acridine agents in that it exhibits unusual pharmacodynamics. Longer exposure times at lower concentrations were more cytotoxic than higher concentrations for a shorter time, although the area-under-the-curve was the same (Haldane et al 1992). These results indicate the importance of pharmacokinetics to achieve the optimal antitumour effect for AC. Plasma and tissue binding are well recognized factors which may influence the pharmacokinetics and pharmacodynamics of drugs (Aungst et al 1990). In addition, the extent of binding to particular plasma proteins has been reported to affect the brain uptake of several compounds (Machard et al 1989). Both amsacrine and CI-921 were highly bound to plasma proteins with significant species differences (Paxton et al 1990). In human plasma, the free drug fraction was 0·3 and 0·1% for

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amsacrine and CI-921, respectively. The AC free fraction in normal mouse plasma was 15–16%. This was concentration-dependent and increased significantly at AC concentrations ≥ 56 μM (Paxton et al 1992). The aim of this study was to investigate the protein binding of AC, in particular to human plasma proteins.

Materials and Methods

Materials

AC as the di-hydrochloride salt and the metabolites; N-[2-(dimethylamino)ethyl]-9(10H)-acridone-4-carboxamide (acridone), N-[2-(dimethylamino-N-oxide)ethyl]-acridine-4-carboxamide (N-oxide), N-[2-(methylamino)ethyl]-acridine-4-carboxamide, (N-monomethyl-AC), N-[2-(methylamino)ethyl]-9(10H)-acridone-4-carboxamide (N-mono methylacridone), and the radiochemical starting material, acridine-4-carboxylic acid sodium salt were synthesized by Professor W. Denny at the Cancer Research Laboratory. The radiochemical starting material was labelled by catalytic exchange in tritiated aqueous media by Amersham International (Amersham, UK), and then conjugated with 2-dimethyl-amino-ethylamine to form the labelled di-hydrochloride salt of AC ([^{3}H]AC) by Professor Denny. The chemical purity of AC, [^{3}H]AC and metabolites was confirmed by HPLC (Young et al 1990). The radiochemical purity (> 98%) was confirmed by thin-layer chromatography and HPLC as previously described (Paxton et al 1992). The specific activity was 164·6 μCi μmol^{-1} and the [^{3}H]AC was used without further purification.

Seamless cellulose tubing (UC #27, Union Carbide Corp., New York) was used for all equilibrium dialysis experiments. Before use, the membrane was soaked in Milli-Q water and heated to 80°C with constant stirring. When cool, the membrane was rinsed twice with Milli-Q water and then

soaked in isotonic phosphate buffer (pH 7·35) for a minimum of 30 min before use. Isotonic phosphate buffer was used for the dialysis experiments and all dilutions. All reagents were of analytical grade. Human serum albumin, one essentially fatty acid-free and the other essentially globulin-free, human α_1-acid glycoprotein (AAG) and human globulin fractions (Cohn fractions I and IV) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Some human plasma was obtained from the local blood bank and contained up to 10% citrate, phosphate and dextrose as anticoagulant.

Methods

Blood samples from healthy volunteers (who had not taken any medication for two weeks) and a male beagle dog were collected by venipuncture. Blood from male New Zealand rabbits and male Wistar rats was obtained by cardiac puncture under general anaesthesia. All samples were collected into heparinized Venoject glass tubes (Terumo Corp., Japan). Blood samples from mice were collected into heparinized microcentrifuge tubes by ocular extraction under anaesthesia with ether or halothane. The plasma was separated at 10°C by centrifugation at 1500 g for 10 min and was subsequently stored in glass vials at −20°C until used. All animal procedures were approved by the Animal Ethics Committee of the University of Auckland.

Dialysis experiments were performed using a Dianorm dialysis apparatus (Diachema, Zurich, Switzerland) which allows for the simultaneous dialysis of 20 samples. Each dialysis cell consisted of two 1-mL Teflon half-cells separated by the dialysis membrane. The appropriate volume of stock AC solution was added to a glass test-tube and the methanol was evaporated under a stream of oxygen-free nitrogen. The required volume of plasma (or protein solution) was then added, followed by [^{3}H]AC to give approximately 20000 d min^{-1} mL^{-1}. Plasma (or protein solution) was adjusted to pH 7·35 using CO_2 or 0·2 M NaOH before loading into one side of the dialysis chamber with isotonic phosphate buffer in the other. Dialysis was performed at 20 rev min^{-1} in a water bath at 37°C for 3 h. Previous experiments had indicated that an equilibrium had been achieved by 3 h (Evans 1992). In addition, dialysis at room temperature (20°C) resulted in a significantly ($P < 0.05$) higher free AC fraction compared with 37°C (Evans 1992). Thus, all experiments were carried out at 37°C to simulate in-vivo conditions. After dialysis, plasma (100 μL) and buffer (500 μL) were obtained from either side, added to scintillation vials with 10 mL Beckman Ready Safe scintillation fluid and counted for 10 min. The plasma solution before dialysis was also counted. All counting was carried out on a Canberra Packard 2200 liquid scintillation counter with quench correction using the transformed spectral index of the external standard spectrum method. The unbound AC fraction (f_u') was calculated as d min^{-1} mL^{-1} in buffer (D_b) divided by d min^{-1} mL^{-1} in plasma (D_p) after dialysis. Adsorption or loss of label does not affect this calculation as a sample from both sides of the membrane was counted. It has been reported that this equation can give an over-estimation of the free fraction due to fluid shifts from the buffer to the plasma side of the membrane (Tozer et al 1983). This is due to the osmotic pressure caused by the protein gradient across the membrane and can result in dilution of the bound drug concentration

Table 1. Free AC fraction in plasma from different species.

Species	Mean f_u (%)	s.d.	n
Man	3·4	0·2	5
Dog	8·1	0·4	4
Rabbit	20·2	0·7	4
Rat	16·3	0·9	4
Mouse	14·8	0·8	12

n = number of replicates from a single sample, except for mice where a pooled sample was used.

Table 2. Free AC fraction in various human protein solutions.

Protein	Concn (g L^{-1})	f_u (%)	s.d.	n
AAG	0·75	24·1	2·2	4
HSA-EFAF	40	31·6	0·7	4
HSA-EGF	40	39·8	2·5	5
Globulins (fraction I)	5	70·3	1·6	7
Globulins (fraction IV)	5	84·8	2·2	4

HSA, human serum albumin; EFAF, essentially fatty acid-free; EGF, essentially globulin-free.

that causes the over-estimation of the free fraction. All free fractions were corrected for this effect by measurement of the plasma protein concentrations before and after dialysis, and the f_u' corrected for volume changes by the equation proposed by Huang (1983):

$$f_u = f_u'R/(f_u'R + 1 - f_u')$$

where R is the ratio of plasma protein concentration after and before dialysis.

The suitability of using [^{3}H]AC to determine the AC free fraction after dialysis was confirmed by measuring absolute concentrations of AC in plasma and buffer by HPLC after extraction with acetonitrile (Young et al 1990). This method is specific for AC without interference by known AC metabolites or breakdown products. The intra- and inter-assay coefficients of variation were 4·1 and 7·7%, respectively, and the lowest concentration that could be measured with acceptable accuracy (± 10%) was 20 nM. The % free AC fraction in human blood bank plasma determined after dialysis by HPLC (5·93 ± 0·86; n = 5) was not significantly different from that determined in the same set of samples by [^{3}H]AC (6·08 ± 0·31; n = 5). For this reason all experiments were carried out using [^{3}H]AC.

Where appropriate, mean values were statistically evaluated using Student's t-test (double sided) or, where multiple comparisons were undertaken, by analysis of variance using MKMODEL. Differences were considered to be significant at $P < 0.05$. The binding data were modelled as a displacement experiment with MKMODEL (Elsevier-Biosoft, Cambridge, UK) (Holford 1985).
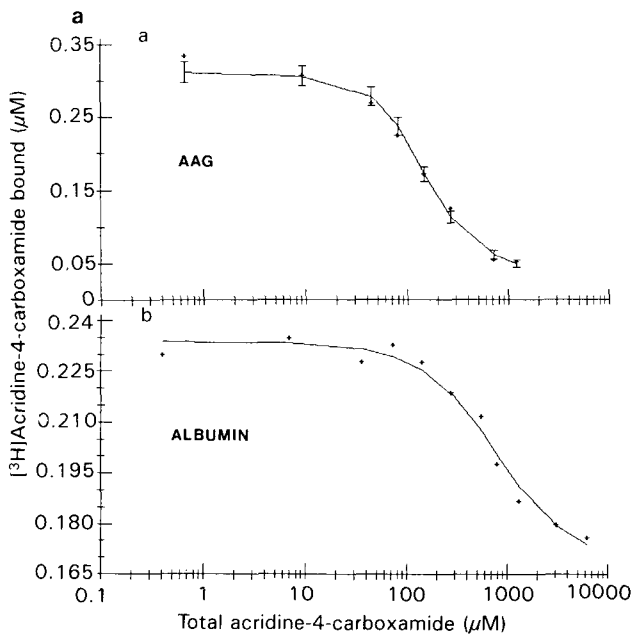
FIG. 1. Displacement curves for the binding of AC to (a) human AAG (0·75 g L⁻¹), and to (b) human albumin (40 g L⁻¹). The vertical axis represents the concentration (μM) of radioactive AC bound by the protein. The curve represents the MKMODEL fit and the error bars the standard errors of the model fit.
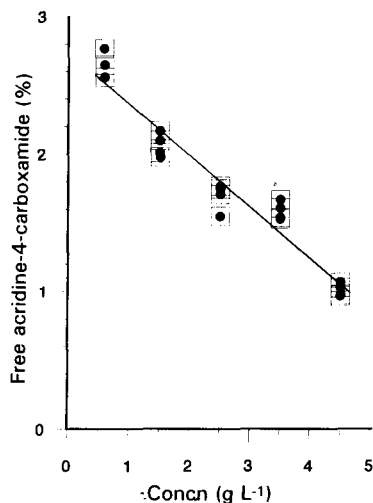

FIG. 2. Relationship between the AC free fraction and AAG concentration in human plasma enriched with increasing AAG concentrations. The AAG concentration in the unenriched plasma was 0·6 g L⁻¹. The relationship is satisfactorily represented by the linear equation $y = 2·74 - 0·38x$ ($r^2 = 0·932$).

Table 3. Free AC fraction in normal human plasma from five subjects.

No.	Sex/age	f_u (%)	s.d.	n
1	M/43	3·40	0·20	5
2	M/25	2·60	0·11	4
3	F/40	2·80	0·47	4
4	F/40	3·60	0·10	4
5	M/23	3·22	0·04	4
	Mean	3·34	0·34	5

Table 4. Free AC fraction in human plasma in the presence of various metabolites.

Plasma samples	Concn (μM)	f_u (%)	s.d.	n
Controls (1 μM AC)	—	3·60	0·04	4
	—	3·37	0·09	4
AC	50	7·66*	0·13	4
	100	8·73*	0·03	4
N-Monomethyl-AC	50	7·22*	0·10	4
	100	9·00*	0·23	4
N-Oxide	50	6·34*	0·26	4
	100	5·79*	0·14	4
Acridone	50	4·73*	0·21	4
	100	4·23	0·29	4
N-Monomethyl-acridone	50	3·79	0·17	4
	100	4·95*	0·15	4

* $P < 0·05$ compared with control.

Results

Species differences

The free AC fraction observed in the plasma (containing 1 μM AC) from various animal species is reported in Table 1. AC was highly bound in plasma from all species studied but showed a 6-fold variation in free fraction across species, ranging from 3·4±0·2% in fresh human plasma to 20·2±0·7% in the rabbit.

Specific human plasma proteins

The free AC fractions in buffer solutions containing 1 μM AC and individual human plasma proteins at physiological concentrations are shown in Table 2. There was significant binding to all proteins studied. Although AAG was present in the lowest concentration (0·75 g L⁻¹), the greatest binding of AC was observed for this protein. Further ligand saturation/displacement experiments were undertaken with a small fixed amount of [³H]AC (0·37 μM) with increasing amounts of unlabelled AC in an AAG solution (0·75 g L⁻¹; 19 μM) or in human serum albumin (40 g L⁻¹; 700 μM). The binding data were modelled as a displacement experiment, using a model which assumes the presence of two ligands and one binding site. The resultant fit for AAG (Fig. 1a) gave a binding constant (K_a) of $7·8 \times 10^4$ M⁻¹ and a binding site concentration (B_{max}) of 73 μM. The fit for human albumin (Fig. 1b) gave a K_a of $6·8 \times 10^3$ M⁻¹ and a B_{max} of 272 μM.

The effect of changes in plasma AAG concentrations was examined by adding increasing amounts of AAG to human plasma containing a known concentration of AAG (0·6 g L⁻¹ as determined by laser nephelometry in the Department of Immunology, Auckland Hospital). Fig. 2 shows the relationship between AC free fraction in plasma as a function of AAG concentration. Over the range studied (0·6–4·5 g L⁻¹) there was a significant negative linear correlation (r = 0·932; $P < 0·001$) between free fraction and increasing AAG concentrations in human plasma.

The free AC fraction in plasma from five healthy volunteers of varying age (23–43 years) and of both sexes is reported in Table 3. At an AC concentration of 1 μM, the free fraction varied from 2·7±0·1 to 3·9±0·5 with a mean value of 3·3±0·3% for these healthy individuals.

Effects of metabolites on AC free fraction

The metabolites, acridone, N-oxide, N-monomethyl-AC and N-monomethyl-acridone were individually added to plasma (containing 1 μM AC) at concentrations of 50 and 100 μM, and the free AC fraction measured. The results are reported in Table 4. Small but significant increases in the AC free fraction were observed with all the metabolites studied. However, only the N-monomethyl-AC derivative increased the free fraction to a similar extent as the same concentration of AC.

Discussion

These studies have shown that AC is approximately 97% bound in normal human plasma and that a 6-fold variation in the free fraction exists across the species examined. These differences may be of importance when extrapolating AC's animal pharmacokinetic and toxicity data to man. Improved allometric relationships across species for plasma clearance and volume of distribution were observed for CI-921 and amsacrine when species differences in plasma protein binding were taken into account (Paxton et al 1990). Some variation in the free AC fraction was also apparent in plasma from healthy individuals, although not to the same extent as across species. This variation is not unexpected as our results indicate that the acute phase reactant protein AAG is of major importance in the plasma binding of AC. In normal subjects a 4-fold range (0·36–1·46 g L^{-1}) in AAG plasma concentrations has been reported to occur (Routledge et al 1980) and a 2-fold variation has been observed in healthy individuals over a six month period (Paxton 1983). This variation would be expected to be magnified in various pathological conditions associated with physiological stress, such as active metastatic cancer when AAG concentrations may be significantly increased (Paxton & Briant 1984). Our results suggest that a maximum 3-fold reduction in the free fraction to 1% could be achieved in plasma with the highest AAG concentrations (e.g. 4·5 g L^{-1}). However, human albumin also binds AC, although with lesser affinity but greater capacity than AAG. Thus, it would appear difficult to predict the free fraction in cancer patients as, although they typically have elevated AAG levels, they usually also have decreased albumin concentrations. In addition it is worthwhile noting that the binding of AC to physiological solutions of human AAG (76%) and albumin (68%) is not perhaps as high as expected compared with 97% in human plasma. Possible explanations for this include specific ligands (or other proteins) in plasma inducing conformational changes in the binding protein to enhance its overall binding, or other proteins such as lipoproteins contributing to the total binding in human plasma.

Another factor which may contribute to a variable free AC fraction in patients' plasma is the concentration-dependent binding to both AAG and albumin. In mouse plasma, concentrations $\geq$56 μM were required to cause a significant increase in AC free fraction (Paxton et al 1992). In human plasma 50 μM caused a 2-fold increase in AC free fraction. However, it is unlikely that such high plasma concentrations would be achieved in patients. In mice receiving a single curative dose intraperitoneally (150 mg kg^{-1}) for advanced Lewis lung carcinoma, the maximum concentration achieved was 20·9 $\pm$ 3·6 μM which rapidly fell to below 10 μM within 1 h (Evans et al 1992). In-vitro studies have suggested that a sustained concentration (e.g. 2 μM for 6 h) was optimal to achieve the greatest cell kill (Haldane et al 1992). Thus concentrations sufficiently high enough to cause a significant increase in AC free fraction may not be achieved or may only be short-lived in patients. Of potentially greater importance with regard to the alteration of the plasma free AC fraction in-vivo may be the effect of metabolites. AC is extensively and rapidly metabolized in the mouse and high concentrations of metabolites with slow elimination accumulate in the plasma (Evans et al 1992; Paxton et al 1992). The major metabolism pathways for AC in the mouse appear to be demethylation and N-oxidation of the aliphatic tertiary amine side chain, oxidation at the C9 position of the acridine ring to form the acridone, and 7-hydroxylation of acridone metabolites followed by conjugation with glucuronic acid (Robertson et al 1993). The metabolites used in our binding studies have been identified in both in-vivo and in-vitro rodent studies, but as yet the slow-eliminating metabolites have not been identified (Robertson et al 1993; Schlemper et al 1993). Examination of plasma after an intravenous dose (90 μmol kg^{-1}) in mice indicated a significant rise in the % free AC from 14·6$\pm$0·5 in controls to 20·8$\pm$0·4 and 18·4$\pm$0·9 in samples collected at 1 and 3 h, respectively, after drug administration (Paxton et al 1992). An increased AC free fraction in human plasma was observed with all the metabolites studied, but only the N-monomethyl-AC metabolite was as effective as the parent AC in competing with [^{3}H]AC for plasma binding sites. Whether similar metabolites or metabolite concentration-time curves will occur in patients is not known.

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