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CYTOSOL MEDIATED METABOLISM OF THE EXPERIMENTAL ANTITUMOUR AGENT ACRIDINE CARBOXAMIDE TO THE 9-ACRIDONE DERIVATIVE

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Abstract—The acridine antitumour agent *N*-[2′-(dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316; acridine carboxamide) is oxidized efficiently *in vitro* by rat and mouse hepatic cytosolic fractions. Under these conditions the oxidase activity has an apparent K_m of 11 μM towards AC. A single product is formed which has been identified as the corresponding 9(10*H*)-acridone carboxamide by ^{1}H-NMR and mass spectrometry. Inhibition with menadione and amsacrine, but not allopurinol, indicates that this reaction is most likely to be catalysed by aldehyde oxidase (EC 1.2.3.1). Several AC analogues with modifications to the side chain (the *N*-oxide, *N*-monomethyl-, and amino-derivatives) are also metabolized to the equivalent acridone product but the 7-hydroxylated and 4-carboxylic acid acridine derivatives are not.

Acridine carboxamide {AC§; *N*-[2′-(dimethylamino)ethyl]acridine-4-carboxamide; **1a**; see Fig. 1} is a third generation experimental antitumour agent derived from the antileukaemic agent amsacrine [4′ - (9 - acridinylamino)methanesulphon- *m* - anisidide, NSC 249992] [1, 2]. AC lacks the 9-anilino ring side chain but is substituted at the 4-position. This agent has been selected for further development because of its high activity against subcutaneously implanted Lewis lung tumour cells with little myelosuppression in the mouse at curative doses [1, 2].

By analogy with drugs of similar structure, we would anticipate metabolism of the side chain to yield the *N*-oxide or N-demethylated derivatives or hydrolysis of the amide linkage to yield the carboxylic acid [3]. Similarly, hydroxylation of the acridine ring is possible and 9-acridone formation has been observed with acridine [4, 5]. We wish to report here on the cytosol mediated formation of the 9-acridone carboxamide from AC.

MATERIALS AND METHODS

Materials. [^{3}H]AC (>98% pure, sp. act. 165 μCi/μmol), AC and analogues (mono- or di-hydrochloride salts), amsacrine (isethionate salt) and menadione were synthesized in the Cancer Research Laboratory and were kindly provided by Dr W. A. Denny. [^{3}H]-AC was prepared from [^{3}H]acridine-4-carboxylic acid which had been labeled by catalytic exchange in tritiated aqueous medium by Amersham International (Amersham, U.K.). All AC derivatives were formulated in MilliQ water (to 20 mM) except the 4-carboxylic acid derivative which was first dissolved in DMA and then diluted in water (to 5% DMA). Allopurinol was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and was dissolved in dimethyl sulfoxide. All other reagents and solvents were of analytical or HPLC grade.

Subcellular fractions. Hepatic microsomal and cytosolic fractions from either male Wistar rats (200–300 g) or male BDF$_1$ mice (25–30 g) were prepared in sodium phosphate buffer (100 mM, pH 7.4) by differential centrifugation, essentially as described previously [6]. Protein concentrations were determined by the method of Lowry *et al.* [7] using bovine serum albumin as standard.

Incubations. Incubations were for up to 10 min at 37° in sodium phosphate buffer (either 20 or 50 mM, pH 7.4). The reaction was stopped by addition of an aliquot to 10 volumes of ice-cold methanol. The sample was further extracted with methanol (10 vol.) and methanol/0.1 M ammonium acetate, pH 5 (90:10, v/v; 2 × 10 vol.). The extracts were pooled, reduced to dryness using a Speed-Vac (Savant Instruments Inc., Farmingdale, NY, U.S.A.) and the samples were resuspended in mobile phase for HPLC. In experiments with [^{3}H]AC (100 μM) the percentage recovery of radiolabel after extraction, resuspension and HPLC was 87% ± 7 (SD, N = 14).

Enzyme kinetics. The kinetic parameters K_m and V_{max} were determined by unweighted non-linear least square regression with curve fit by Marquardt analysis on a Hewlett–Packard HP89500 UV/Vis ChemStation.

HPLC. The Waters HPLC system consisted of a WISP 710B automatic sample injector, 6000A pump,

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§ Abbreviations: AC, acridine carboxamide, *N*-[2′-(dimethylamino)ethyl]acridine-4-carboxamide; AOC, 9(10*H*)-acridone carboxamide; LSIMS, liquid secondary ion mass spectrometry; DMA, dimethylacetamide; TEAP, triethylammonium phosphate.

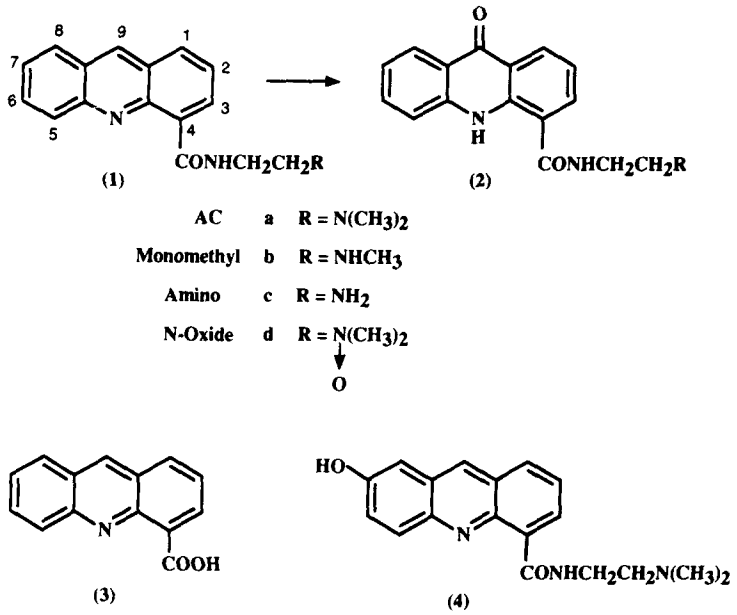

Fig. 1. Structure of AC and derivatives.

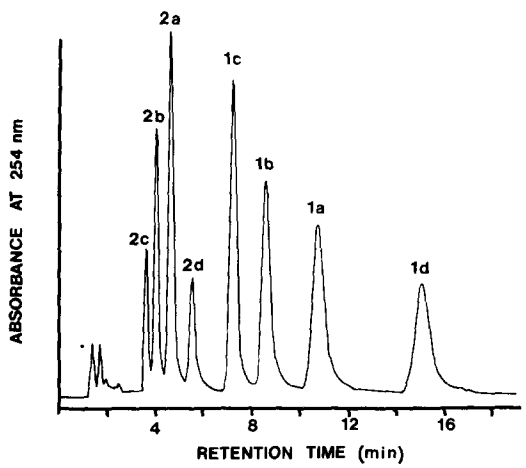

Fig. 2. Representative HPLC profile of AC analogues and respective 9-acridone products. HPLC analysis was performed on a reverse-phase C_{18} Novapak column using a mobile phase of 18% acetonitrile containing 100 mM TEAP, pH 3, at a flow rate of 2.0 mL/min.

RCM-100 compression module fitted with a Waters Novapak reverse-phase C_{18} Radial Pak cartridge (8 mm × 10 cm), a 490E variable wavelength absorbance detector, and data collection and analysis with Waters Baseline software. Separation was achieved with a mobile phase of 18% acetonitrile, 100 mM triethylammonium phosphate (TEAP), pH 3, at 2.0 mL/min and UV detection at 254 nm (Fig. 2). Concentrations were estimated after peak collection and liquid scintillation counting of [^{3}H]-AC of known specific activity and the [^{3}H]AOC product generated by metabolism of [^{3}H]AC. The acridone products **2b–2d** of the AC analogues **1b–1d** were assumed to have the same response as AOC (**2a**). A representative HPLC chromatograph of **1a–1d** and **2a–2d** is shown in Fig. 2.

For product collection, the appropriate peak fractions were pooled and concentrated on C_{18} Bond-Elut cartridges (Analytichem International, Harbor City, CA, U.S.A.). The samples were eluted with 97.5% methanol/2.5% 1 M ammonium acetate, pH 4, and the eluates reduced to dryness using the Speed-Vac.

Spectrometry. Absorption spectra of peak fractions were obtained during HPLC with the Waters 490E variable wavelength detector. ^{1}H-NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz) and are referenced to internal standards of tetramethylsilane ($CDCl_3$ solutions) or 3-(trimethylsilyl)propanesulfonic acid sodium salt (D_2O solutions). The aromatic resonances were unambiguously assigned by the use of proton–proton correlation (COSY) techniques. Liquid secondary ion mass spectra (LSIMS) were obtained from a VG70-250S double focusing magnetic sector mass spectrometer (VG Analytical, Manchester, U.K.) equipped with a standard VG ion source and associated caesium ion gun. Samples were dissolved in nitrobenzyl alcohol or acidified glycerol and bombarded with 35 keV caesium ions. Where appropriate, accurate masses were determined using proprietary software designed for multichannel analysis.

RESULTS

Hepatic subcellular fractions. In initial experiments, AC (20 μM) was incubated (10 min at 37°) with either hepatic 9000 *g* supernatant or cytosolic

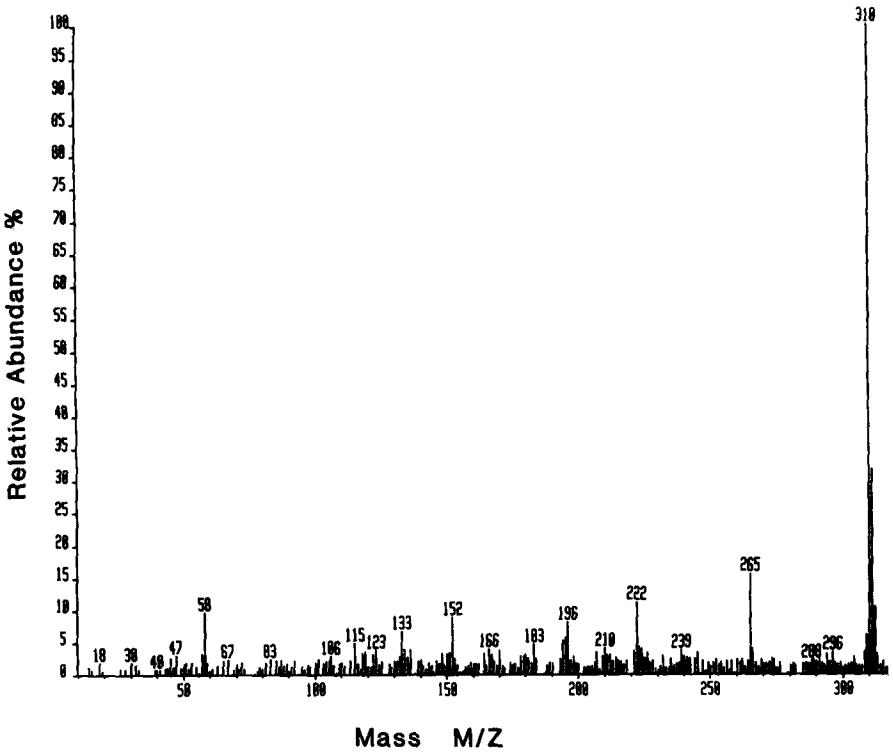

Fig. 3. LSIMS mass spectrum of AOC.

or microsomal fractions (approx. 10, 11 and 2 mg protein/mL, respectively) from male rats or mice. No loss of AC was observed with the microsomal fractions but in the incubations with 9000 *g* supernatant or cytosolic fractions ≥ 90% of AC was metabolized to a single product (results not shown) (see Fig. 2, peak **2a**).

Identification of AC product (**2a**). Sufficient product was obtained by incubation of AC (100 μM) in rat hepatic cytosol (approx. 10 mg protein/mL) followed by purification as described in Materials and Methods for ^{1}H-NMR and mass spectral analysis. By LSIMS mass spectrometry (resolution, 5000) an exact $[M + H]^+$ ion was found at 310.1555 daltons (±15.1 ppm) for $C_{18}H_{20}O_2N_3$ (Fig. 3). ^{1}H-NMR spectra (see below and Fig. 4) were obtained for the AOC isolate (in D_2O), authentic AOC (free base; in D_2O and $CDCl_3$) and AC (free base; in $CDCl_3$). The spectrum of authentic AOC in deuteriated water (not shown) was identical to that of the AOC isolate. For additional comparison, the spectrum of the AC dihydrochloride salt (the clinical formulation of AC) is also reported. Comparison of the NMR spectrum of AOC with that of the parent compound AC indicated that the characteristic singlet at δ 8.73 ppm in the NMR spectrum of AC (in $CDCl_3$) resulting from the C-9 proton was absent in the spectrum of the metabolite, while the remaining aromatic resonances were all present with similar multiplicities (Fig. 4). Two-proton triplets at δ 3.52 and 2.52 ppm, together with a six-proton singlet at δ 2.31 ppm, in the ^{1}H-NMR spectrum of the metabolite confirmed the presence of an intact side chain. The structure of the metabolite was verified subsequently by direct comparison of UV spectra and retention times on HPLC with the authentic sample of *N*-[2'-(dimethylamino)ethyl]-9(10*H*)-acridone-4-carboxamide (Figs 2 and 5).

^{1}H-*NMR*. AC (**1a**) δ ($CDCl_3$): 8.99 (br, 1H, NH), 8.92 (dd, 1H, J = 7.1, 1.5 Hz, H-3), 8.73 (s, 1H, H-9), 8.16 (dd, 1H, J = 8.8, 0.8 Hz, H-5), 8.02 (dd, 1H, J = 8.4, 1.5 Hz, H-1), 7.94 (dd, 1H, J =8.5, 0.7 Hz, H-8), 7.80 (m, 1H, H-6), 7.58 (dd, 1H, J = 8.4, 7.1 Hz, H-2), 7.54 (m, 1H, H-7), 3.81 (dd, 2H, J = 6.2, 5.0 Hz, $CONHC\underline{H}_2$), 2.72 (t, 2H, J =6.2 Hz, $C\underline{H}_2NMe_2$), 2.45 (s, 6H, NMe_2).

AOC (**2a**) δ ($CDCl_3$): 8.64 (dd, 1H, J = 8.0, 1.4 Hz, H-3), 8.62 (br, 1H, acridone-NH), 8.44 (dd, 1H, J = 8.1, 1.2 Hz, H-5), 7.94 (dd, 1H, J = 7.5, 1.4 Hz, H-1), 7.67 (m, 1H, H-7), 7.40 (dd, 1H, J = 8.2, 0.4 Hz, H-8), 7.33 (br t, 1H, CONH), 7.26 (m, 1H, H-6), 7.22 (dd, 1H, J = 8.4, 7.1 Hz, H-2), 3.52 (dd, 2H, J = 6.1, 5.1 Hz, $CONHC\underline{H}_2$), 2.52 (t, 2H, J = 6.1 Hz, $C\underline{H}_2NMe_2$), 2.31 (s, 6H, NMe_2).

AOC (**2a**; isolate) δ (D_2O): 7.74 (d, 1H, J = 7.9 Hz, H-5), 7.69 (d, 1H, J = 8.0 Hz, H-3), 7.48 (m, 2H, H-1,7), 7.09 (t, 1H, J = 7.4 Hz, H-6), 6.82 (m, 2H, H-2,8), 3.64 (t, 2H, J = 6.2 Hz, $CONHC\underline{H}_2$) 3.42 (t, 2H, J = 6.2 Hz, $C\underline{H}_2NMe_2$), 3.05 (s, 6H, NMe_2).

AC.2HCl (**1a**) δ (D_2O): 9.61 (s, 1H, H-9), 8.63 (dd, 1H, J = 7.2, 0.8 Hz, H-5), 8.50 (d, 1H, J = 8.3 Hz, H-3), 8.30 (d, 1H, J = 8.5 Hz, H-1), 8.26 (m, 2H, H-2,8), 7.91 (m, 2H, H-6,7), 4.04 (t, 2H, J = 6.1 Hz, $C\underline{H}_2NMe_2$), 3.62 (t, 2H, J = 6.1 Hz, $CONHC\underline{H}_2$), 3.13 (s, 6H, NMe_2).

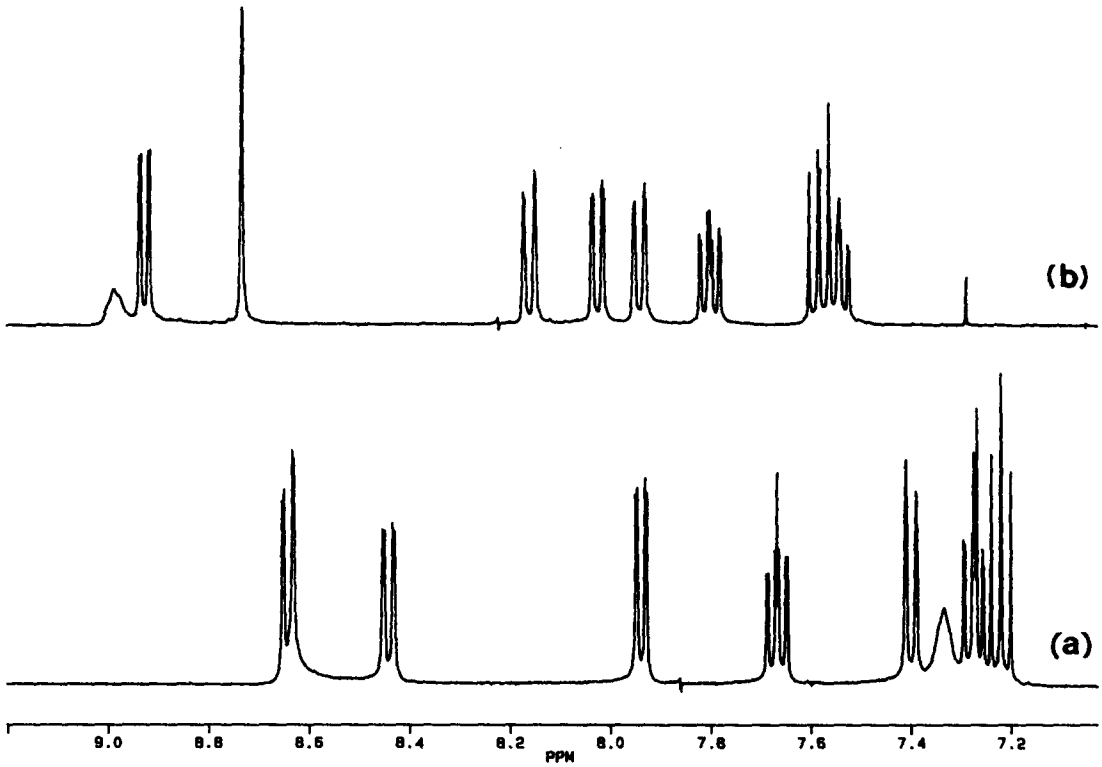

Fig. 4. 400 MHz ^{1}H-NMR spectra of (a) AOC and (b) AC, for $CDCl_3$ solutions, in the region of δ 9.2–7.2.

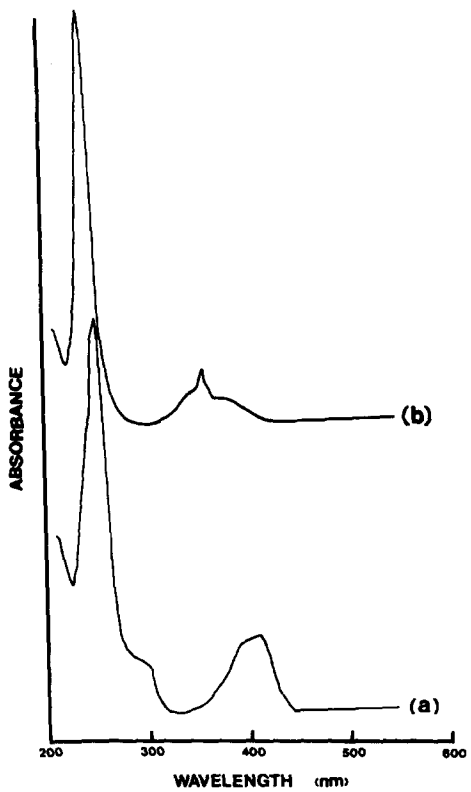

Fig. 5. UV/Vis absorption spectra of (a) AOC and (b) AC obtained during HPLC in 18% acetonitrile, 100 mM TEAP, pH 3. Maxima are 250 and 356 nm for AC, and 254 and 410 nm for AOC.

Kinetics of formation of AOC in rat hepatic cytosolic fraction. The rate of formation of AOC (at 100 μM AC) was linear up to at least 2.5 mg protein/mL and to 5 min incubation, and the reaction was inhibited 100% by prior heat treatment of cytosol at 90° for 10 min (results not shown). For determination of kinetic parameters, an incubation time of 2 min and a cytosol dilution equivalent to 1.75 mg protein/mL were chosen. Apparent K_m and V_{max} values are given in Table 1 (11 μM and 3.1 nmol/min/mg protein, respectively).

The effects of menadione, amsacrine and allopurinol on the formation of the AC-acridone are shown in Table 2. The reaction was inhibited by menadione and amsacrine with 50% inhibition occurring at 12 and 16 μM, respectively. No inhibition was seen with allopurinol over the same concentration range (0–30 μM).

Acridone formation from AC derivatives. In incubations with rat hepatic cytosolic fraction no product was observed with the 4-carboxylic acid (**3**) and 7-hydroxy- (**4**) analogues of AC (results not shown). The percentage recovery of 7-hydroxy-AC after extraction and HPLC was 93% ± 5 (SD, N = 6) but recovery of the 4-carboxylic acid analogue was 69% ± 12 (SD, N = 4). However, with the *N*-monomethyl-, amino- and *N*-oxide analogues (**1b, c** and **d**, respectively) significant metabolism occurred. A single product was formed for each of the *N*-monomethyl- and amino-analogues. These and the major product observed with the *N*-oxide had a similar relative retention time to the parent as that observed with AOC (Fig. 2). In addition, all three

Table 1. Kinetics of 9-acridone formation from AC and AC analogues by rat hepatic cytosolic fraction

Substrate	Concentration range (μM)	K_m (μM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m
AC (**1a**)	0–200	11 ± 3	3.10 ± 0.14	0.280
N-Monomethyl (**1b**)	0–100	7 ± 1	2.85 ± 0.09	0.406
Amine (**1c**)	0–100	9 ± 3	2.46 ± 0.19	0.280
N-Oxide (**1d**)	0–500	134 ± 16	1.23 ± 0.06	0.009

Values are means ± SD calculated from the combined results of two separate experiments.

Table 2. Effect of menadione, amsacrine and allopurinol on AOC formation

Concentration (μM)	AOC formation (nmol/mL)		
	Amsacrine	Allopurinol	Menadione
0	8.39 ± 0.16 (0)*	8.06 ± 1.26 (0)	9.58 ± 1.24 (0)
3	7.01 ± 0.44 (16)	8.12 ± 0.78 (0)	7.72 ± 0.14 (19)
10	5.57 ± 0.14 (34)	8.96 ± 0.45 (0)	5.62 ± 0.78 (41)
30	2.40 ± 0.26 (71)	9.71 ± 0.16 (0)	1.13 ± 0.52 (88)

Values are means ± SD of duplicate incubations with 50 μM AC, 2 min, 37°, 1.63 mg protein/mL. Amsacrine and menadione were dissolved in DMA (final concentration 0.125 and 0.25%, respectively) and allopurinol in dimethyl sulfoxide (final concentration 0.5%). The inhibitors were added immediately before AC. Product formation in the absence of solvent was: 8.81 ± 0.54 nmol/mL (experiment 1, amsacrine and allopurinol) and 9.45 ± 0.72 nmol/mL (experiment 2, menadione).

* % inhibition.

products had UV/visible spectra identical to that of AOC (Fig. 5). Finally, by LSIMS mass spectrometry, ions at 296, 282 and 326 daltons were obtained for these products of reaction with the *N*-monomethyl-, amino- and *N*-oxide analogues, respectively, (results not shown). Apparent K_m and V_{max} values for formation of these AOC derivatives are also given in Table 1. Similar values to those for AC were obtained for the *N*-monomethyl- and amino-analogues but the *N*-oxide was a poor substrate. For the *N*-oxide, the incubation time and protein concentration were increased to 5 min and 3.5 mg/mL, respectively. Formation of the *N*-oxide acridone was linear to 10 min and 4 mg/mL. In addition, products corresponding to AC, *N*-monomethyl-AC and their respective acridone products were observed; the sum of these additional products was equivalent to 27–77% of the *N*-oxide acridone formed over a dose range of 50 to 500 μM (results not shown).

DISCUSSION

AC is metabolized efficiently to a single product *in vitro* in rat and mouse hepatic cytosolic fraction. This metabolite was identified as the 9-acridone carboxamide from a comparison of its ^{1}H-NMR spectrum with that of the parent AC together with its mass spectrum. Thus, the mass spectrum contained a molecular ion at 310 daltons, corresponding to the addition of an oxygen atom to the AC molecule. The characteristic singlet in the NMR spectrum of AC resulting from the C-9 proton was absent in the spectrum of the metabolite, while the remaining aromatic resonances were all present with similar multiplicities, thereby establishing the C-9 position as the site of hydroxylation. The structure of the metabolite was verified subsequently by direct comparison with an authentic sample of *N*-[2′-(dimethylamino)ethyl]-9-(10*H*)-acridone-4-carboxamide [8].

Formation of AOC from AC is inhibited by menadione and amsacrine. Thus the most likely enzyme catalysing this reaction is aldehyde oxidase (aldehyde: O_2 oxidoreductase, EC 1.2.3.1) [9, 10]. Thus, oxidation to the 9-hydroxyl derivative is followed by tautomerization to the 9(10*H*)acridone. No inhibition was observed with allopurinol, indicating that xanthine oxidase is not contributing to this reaction in the cytosolic fraction [9].

Aldehyde oxidase has been shown to catalyse the formation of the 7-hydroxy derivative from methotrexate [11]. Inhibition of the formation of this metabolite (which is thought to contribute to renal toxicity) has been shown to occur in the rabbit on treatment with amsacrine [12]. While AC is at present only in preclinical development, a similar potential exists for interaction *in vivo* if used ultimately in combination therapy with antitumour agents such as methotrexate and amsacrine.

The *N*-oxide, *N*-monomethyl-, amino-, 7-hydroxy-, and 4-carboxylic acid derivatives of AC are potential metabolites of AC. To date, the *N*-oxide and *N*-monomethyl-derivatives have been detected in rat and mouse *in vivo* and in samples *in vitro* (Robertson *et al.*, unpublished observations). It was, thus, of interest to determine the efficiency of formation of the respective acridone products of these derivatives. The N-demethylated analogues were as efficiently metabolized as the parent compound. A higher K_m and lower V_{max} were observed for the side chain *N*-oxide. However, the side chain hydrolysis and the ring hydroxylated (**3** and **4**) derivatives were not metabolized. It is also noteworthy that significant aldehyde oxidase catalysed reduction of the *N*-oxide to the parent AC did occur to a limited extent under these conditions. The reduction of tertiary amine *N*-oxides by aldehyde oxidase has been reported [13] and it appears that the *N*-oxide itself is able to serve as the electron donor for its reduction to AC. Further, incubation of AC-*N*-oxide, but not AC, also resulted in the production of *N*-monomethyl-AC and the *N*-monomethyl-AC-acridone. Thus, demethylation may be occurring via the *N*-oxide rather than from AC.

The significance of this reaction *in vivo* is not known. In rat hepatocyte incubations three minor products have been observed which appear to correspond to the acridones of the parent, *N*-oxide and demethylated analogues; however, while these acridone products are not readily detected in rat and mouse bile or urine, preliminary evidence indicates that two secondary acridone derivatives are major metabolites in rat and mouse bile (Robertson *et al.*, unpublished observations). AOC has much reduced antitumour activity *in vitro* compared with AC and is inactive *in vivo* against intraperitoneally implanted P388 leukaemia tumour cells in the mouse [8]. Furthermore, with AOC a maximal tolerated dose 2.3 times the optimal dose for AC antitumour activity was achieved. Thus, AOC formation, at least, appears to result in inactivation and detoxication of AC.

Several studies have examined the structure–activity relationship in the metabolism of various agents by aldehyde oxidase [14–17]. AC analogues as well as other acridone and anilinoacridine analogues, including the known inhibitor amsacrine, are available from the Cancer Research Laboratory and further characterization of the kinetics of this reaction, using these derivatives, is in progress.

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