Biochemical Pharmacology, Vol. 45, No. 10, pp. 2159–2162, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Inhibition by SKF-525A of the aldehyde oxidase-mediated metabolism of the experimental antitumour agent acridine carboxamide

(*Received* 23 *December* 1992; *accepted* 1 *March* 1993)

Abstract—Oxidation of the experimental anti-tumour agent *N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316; acridine carboxamide) to the 9(10*H*)acridone, followed by ring hydroxylation and glucuronidation, appears to be the main pathway of detoxication of AC in the rat and mouse. The acridone formation has been further characterized *in vitro* using an enzyme-enriched fraction where activity per milligram protein is increased approximately 10-fold compared with the cytosolic fraction. Inhibition by amsacrine [4′-(9-acridinylamino)methanesulphon-*m*-anisidide; NSC 249992] and menadione (50% inhibition at 6.4 and 1.8 μM, respectively) but not allopurinol (to 30 μM) indicates that the activity is due to aldehyde oxidase, without the involvement of xanthine oxidase. Interestingly, acridone formation in both the cytosolic and enzyme-enriched fractions is highly sensitive to the classical cytochrome P450 inhibitor SKF-525A [proadifen hydrochloride; 2′-(diethylamino)ethyl 2,2-diphenylpentenoate] (50% inhibition at 9.2 and 1.9 μM, respectively). Further analysis indicates mixed non-competitive type inhibition by SKF-525A (K_{ii}, 0.3 μM; K_{ii}, 4.9 μM). Little or no inhibition was seen with cimetidine, metyrapone or methimazole. No NADPH-dependent acridone formation was observed with the microsomal fraction. These data indicate that acridone formation previously observed in isolated rat hepatocytes and *in vivo* is most likely due to aldehyde oxidase rather than cytochrome P450.

Acridine carboxamide {AC; NSC 601316; *N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide} is an experimental antitumour agent, derived from the antileukaemic agent amsacrine [4′-(9-acridinylamino)methanesulphon-*m*-anisidide; NSC 249992], which 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]. The metabolism of AC has been characterized in the mouse [3] and rat* and in isolated rat hepatocytes [4]. These studies indicate that the main pathways of metabolism are (a) N-oxidation, mediated by flavin-containing monooxygenase, (b) N-demethylation and (c) 9(10*H*)-acridone formation, either from AC or *N*-monomethyl-AC, followed by 7-hydroxylation and glucuronidation. Both N-demethylation and acridone formation are inhibited by SKF-525A [proadifen hydrochloride; 2′-(diethylamino)ethyl 2,2-diphenylpentenoate] the classical inhibitor of cytochrome P450 [5]. This indication of involvement of cytochrome P450 in acridone formation contrasts with previous work in this laboratory on the metabolism of AC using hepatic subcellular fractions [6]. In these experiments AC and *N*-monomethyl-AC were efficiently oxidized by rat and mouse hepatic cytosolic fractions to the corresponding 9(10*H*)acridones. No acridone formation occurred with the hepatic microsomal fractions (in the absence of NADPH) and inhibition with menadione and amsacrine, but not allopurinol, indicated that the cytosol-mediated reaction is most likely catalysed by aldehyde oxidase (EC 1.2.3.1).

Amsacrine, the compound from which AC has been derived, is a potent competitive inhibitor of aldehyde oxidase obtained from rabbit liver [7] and rat liver.† Further, amsacrine has been shown to decrease the formation of 7-hydroxy-methotrexate in both the rabbit and rat [8, 9]. Thus, while AC is at present only in preclinical development, the potential exists for interaction with other drugs if used ultimately in combination chemotherapy with antitumour agents such as methotrexate and amsacrine.

There have been reports of inhibition of aldehyde oxidase-mediated reduction by SKF-525A [10, 11]. We therefore wished to clarify the role of aldehyde oxidase in the metabolism of AC by investigating the effect of SKF-525A on crude and enriched aldehyde oxidase preparations.

Materials and Methods

Materials. AC, *N*-monomethyl-AC and AC-9(10*H*)acridone (as the mono- or dihydrochloride salts), amsacrine (isethionate salt) and menadione were synthesized in the Cancer Research Laboratory and were kindly provided by Dr W. A. Denny. AC and derivatives were formulated in MilliQ water and amsacrine in dimethyl acetamide. Glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, and the inhibitors allopurinol and methimazole were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was from Ciba-Geigy NZ (Auckland, New Zealand); SKF-525A and cimetidine were kindly provided by SmithKline Beecham (Sydney, Australia). Allopurinol was formulated in dimethyl sulphoxide and the remaining inhibitors were dissolved in water. All other reagents and solvents were of analytical or HPLC grade.

Subcellular fractions. The hepatic 10,000 *g* supernatant, cytosolic and microsomal fractions from male Wistar rats (200–300 g) were prepared in sodium phosphate (50 mM, pH 7.4) by differential centrifugation essentially as described [6]. Protein concentrations were determined by the method of Lowry *et al.* [12] using bovine serum albumin as standard.

Enrichment of rat liver aldehyde oxidase. Enrichment of aldehyde oxidase from rat liver cytosolic fraction (see Table 1) was based on the modifications described by Bauer and Howard [13] of the methods of Rajagopalan *et al.* [14] and Felsted *et al.* [15]. However, the greatest activity with AC as substrate was found in the fraction precipitating at zero

* Robertson IGC, Bland TJ, Paxton JW and Palmer BD, Characterisation of the polar metabolites of the experimental anti-tumour agent *N*-[2′-(dimethylamino)ethyl]-acridine-4-carboxamide (AC) in the rat, manuscript in preparation.

† Robertson IGC, Bland TJ, Gamage RSKA and Denny WA, Kinetics of some acridine antitumour agents as substrates and inhibitors of rat liver aldehyde oxidase, manuscript in preparation.

Table 1. Preparation of an enriched aldehyde oxidase fraction from rat liver

Step	Protein (mg/mL)	AC-9(10*H*)acridone formation (nmol/min/mg protein)
10,000 *g* homogenate	24.3	0.8
Cytosol	9.8	2.5
Cytosol + 55°/11 min	4.5	3.0
0–35% ammonium sulphate	3.6	32.0

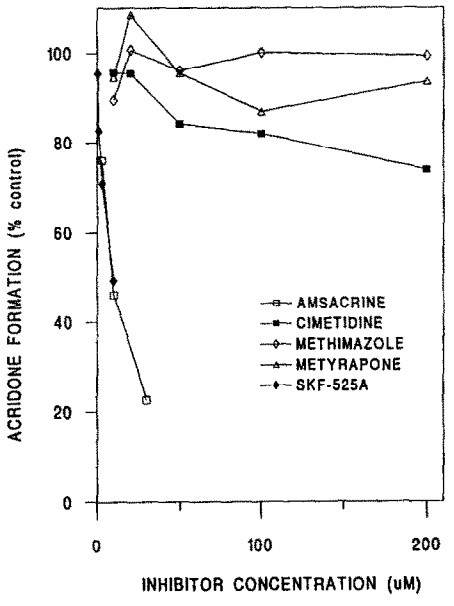

Fig. 1. Effect of amsacrine, cimetidine, methimazole, metyrapone and SKF-525A on AC-9(10*H*)acridone formation from AC (50 μM) by rat hepatic cytosolic fraction. Values are the means from the combined results of two separate experiments.

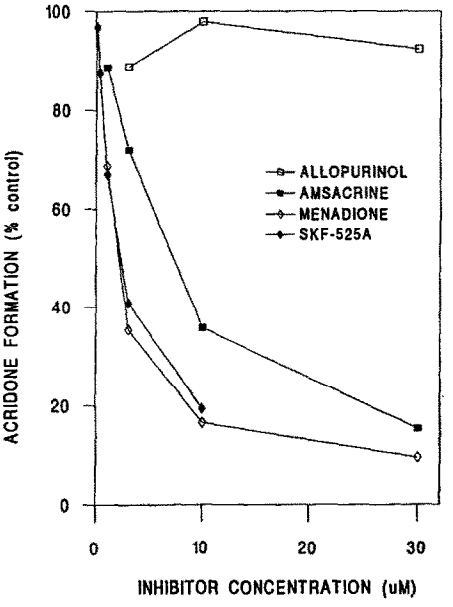

Fig. 2. Effect of allopurinol, amsacrine, menadione and SKF-525A on AC-9(10*H*)acridone formation from AC (50 μM) by an aldehyde oxidase-enriched preparation from rat liver. Values are the means from the combined results of two separate experiments.

to 35% ammonium sulphate (at 25°) and this activity was decreased after further precipitation with acetone.

Incubations. Incubations were typically for 2 min at 37° in sodium phosphate (either 20 or 50 mM, pH 7.4) in glass tubes. The reaction was initiated, after preincubation at 37° for 1 min, by addition of substrate and stopped by addition of 10 vol. of ice-cold methanol. In experiments with the NADPH generating system, incubations also contained glucose-6-phosphate (5 mM), $NADP^+$ (4 mM) and glucose-6-phosphate dehydrogenase (1 U/mL). The samples were reduced to dryness in the same tube using a Speed-Vac (Savant Instruments Inc., Farmingdale, NY, U.S.A.) and resuspended in mobile phase (100 mM triethylammonium phosphate, pH 3 containing 19% acetonitrile). Product recoveries were equivalent to that obtained after more exhaustive extraction and transfer [6]. The concentration of inhibitor needed to reduce acridone formation by 50% was determined at a substrate concentration of 50 μM. In all cases the inhibitors were added to the enzyme fraction on ice 1 min before preincubation at 37°. The final percentage of dimethyl acetamide (amsacrine) or dimethyl sulphoxide (allopurinol) was 0.05% or 0.2%, respectively. These solvents alone had no significant effect.

HPLC. AC-9(10*H*)acridone formation was monitored by HPLC as described [6].

Enzyme kinetics. The kinetic parameters, K_m, V_{max}, and the inhibition constants were determined by unweighted non-linear least squares regression with curve fit by Marquardt analysis on a Hewlett Packard HP89500 UV/Vis ChemStation or alternately by using MKMODEL [16]. The 50% inhibition values were calculated using MKMODEL and a simple Emax model.

Results

The effects of amsacrine, cimetidine, methimazole, metyrapone and SKF-525A on the rat hepatic cytosol-mediated formation of AC-9(10*H*)acridone are shown in Fig. 1. The reaction was inhibited by amsacrine and SKF-525A with 50% inhibition at 8.8 ± 3.0 (SE) and 9.2 ± 2.8 μM, respectively. Approximately 20% inhibition was obtained with cimetidine (at 200 μM) and no inhibition was seen with methimazole or metyrapone (up to 200 μM). Formation of the equivalent acridone of the *N*-monomethyl-AC was also inhibited by SKF-525A with 50% inhibition at 8.0 ± 2.9 μM (data not shown).

A 10-fold enriched preparation of aldehyde oxidase was

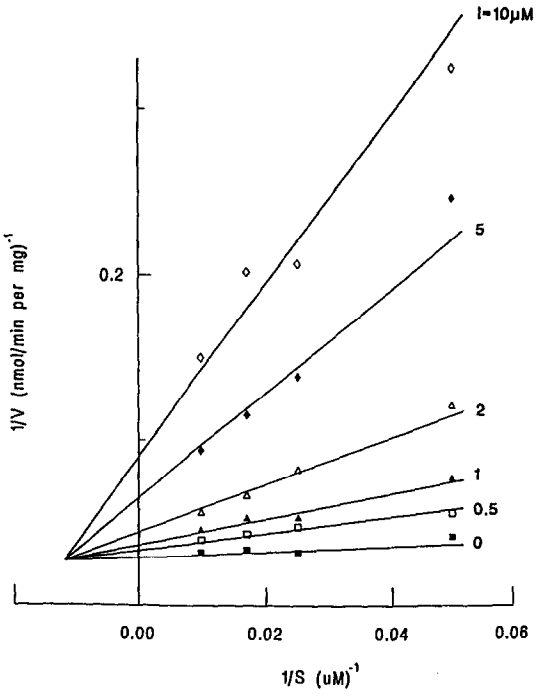

Fig. 3. A double reciprocal plot of inhibition of 9(10*H*)acridone formation from AC by SKF-525A. Values are the means from the combined results of two separate experiments.

obtained from rat hepatic cytosolic fraction as outlined in Table 1. This preparation was used to examine further the inhibition of AC-acridone formation. The effects of allopurinol, amsacrine, menadione and SKF-525A are shown in Fig. 2. No inhibition was observed with allopurinol but amsacrine, menadione and SKF-525A were inhibitory with 50% inhibition at 6.4 ± 1.4 (SE), 1.8 ± 0.3 and 1.9 ± 0.4 μM, respectively.

A more detailed kinetic analysis of the inhibition of acridone formation by SKF-525A was carried out. The best fit using Marquardt analysis indicated mixed non-competitive inhibition with inhibition constants (K_{is} and K_{ii}, respectively) of 0.3 ± 0.1 (SE) and 4.9 ± 1.2 μM. A double reciprocal plot of the data is shown in Fig. 3.

The metabolism of AC by rat hepatic 10,000 *g* supernatant, cytosolic and microsomal fractions was examined in the presence and absence of an NADPH generating system. No 9(10*H*)acridone formation was observed with the microsomal fraction and no NADPH-dependent stimulation of acridone formation was observed with the other fractions (data not shown).

Discussion

AC-acridone formation in isolated rat hepatocytes is inhibited by the classical cytochrome P450 inhibitor SKF-525A [4], whereas the acridone is efficiently formed in rat hepatic cytosolic fraction, most likely mediated by aldehyde oxidase [6]. We have now shown SKF-525A to be a potent inhibitor of this reaction. In a preliminary experiment (Fig. 1) with rat hepatic cytosolic fraction 50% inhibition was obtained at a similar concentration of SKF-525A to that of amsacrine (a characteristic inhibitor of aldehyde oxidase [7]). Addition of cimetidine and metyrapone, two other cytochrome P450 inhibitors, and the flavin-containing monooxygenase inhibitor methimazole, which inhibits AC-*N*-oxide formation in rat hepatocytes [4], resulted in little or no inhibition.

Further confirmation of aldehyde oxidase as the target of SKF-525A has been obtained using an enzyme-enriched preparation from rat hepatic cytosolic fraction. Acridone formation by this preparation was inhibited by amsacrine and menadione but not allopurinol, supporting the involvement of aldehyde oxidase but not xanthine oxidase [17].

The extent of inhibition by SKF-525A of the reaction mediated by the enzyme-enriched fraction (Fig. 2) was comparable to that by menadione. Inhibition by menadione is thought to occur by inhibition of electron transport [see 17]. More detailed kinetic analysis indicates that SKF-525A is a mixed non-competitive inhibitor. However, the inhibition constant for binding to the enzyme–substrate complex (K_{ii}) is approximately 16-fold greater than that for binding to the free enzyme (K_{is}). Thus, there may be overlap between the substrate and inhibitor binding sites. The K_{is} value of 0.3 μM for SKF-525A can be compared with the K_m of 5.0 μM for AC and the K_i value (competitive inhibition) of 1.4 μM for inhibition by amsacrine of AC-9(10*H*)acridone formation obtained with the same enzyme-enriched fraction.*

AC is derived from amsacrine [1, 2]. It lacks the aniline ring and is substituted at the 4-position with an alkylamino side chain terminating in a dimethyl tertiary amine. SKF-525A has some structural similarity to AC in containing an alkylamino side chain with a diethyl tertiary amine group and this group may be involved in the interaction with the enzyme. A partial competitive mode of inhibition would also imply that SKF-525A might be a substrate for aldehyde oxidase. However, no metabolism of SKF-525A was observed in the absence of AC (results not shown).

Finally, the characteristics of metabolism by cytochrome P450 and the molybdenum hydroxylases have been summarized and compared [18]. Mechanistically, the two enzymes can be differentiated. The molybdenum hydroxylases catalyse aromatic oxidation by nucleophilic attack, with the oxygen being derived from water, whereas cytochrome P450 oxidation occurs by electrophilic attack with molecular oxygen being incorporated into the substrate. The predicted position of nucleophilic attack in simple acridine is position 9 [19], as found with AC. Further, no acridone formation is detected on incubation of AC with rat hepatic microsomal fraction.

Thus, the AC-9(10*H*)acridone formation observed in rat hepatocytes and *in vivo* is most likely due to aldehyde oxidase rather than to cytochrome P450. This highlights the potential for significant interactions between AC and established antitumour agents such as amsacrine. These results emphasize the well-recognised caution that must be used in the interpretation of results obtained with SKF-525A and other inhibitors in complex systems. For these reasons it is still difficult to make a definitive estimate of the contribution of aldehyde oxidase *in vivo*, for instance by the use of amsacrine or other aldehyde oxidase inhibitors. A possible approach, however, may be selective competitive inactivation of molybdenum-containing enzymes such as aldehyde and xanthine oxidases with tungsten [see 17].

Acknowledgements—This work was supported by the Cancer Society of New Zealand. I.R. is a recipient of a Health Research Council of New Zealand Senior Research Fellowship.

* Robertson IGC, Bland TJ, Gamage RSKA and Denny WA, Kinetics of some acridine antitumour agents as substrates and inhibitors of rat liver aldehyde oxidase, manuscript in preparation.

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