Biochemical Pharmacology, Vol. 59, pp. 161–165, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00323-8

SHORT COMMUNICATION

Inter-species Variation in the Metabolism and Inhibition of *N*-[(2′-Dimethylamino)ethyl]acridine-4-carboxamide (DACA) by Aldehyde Oxidase

*Philip C. Schofield, Iain G. C. Robertson and James W. Paxton**
Department of Pharmacology and Clinical Pharmacology, University of Auckland School of Medicine, Auckland, New Zealand

ABSTRACT. *N*-[(2′-Dimethylamino)ethyl]acridine-4-carboxamide (DACA) is a new anticancer agent currently undergoing clinical trials. The metabolism of DACA to acridone metabolites by aldehyde oxidase (AO) (EC 1.2.3.1) appears to play a major role in its elimination in human patients and rodents. The aim of this study was to compare the ability of human, guinea pig, and rat AO preparations to metabolise DACA, and to determine if either animal model was appropriate for predicting AO-mediated DACA–drug interactions in humans. Both human and rodent liver samples were homogenised in buffer before sequential centrifugation to produce the cytosol fraction. Human supernatant underwent an additional ammonium sulphate precipitation procedure, which produced a 2-fold increase in enzyme activity per milligram of protein. After incubations with DACA (range, 0–200 μM), DACA-9(10*H*)-acridone formation was determined by HPLC analysis. Michaelis–Menten parameters, K_m and V_{max}, were determined from the best fit curves by nonlinear regression. Three of the four human liver preparations had similar DACA intrinsic clearance values (V_{max}/K_m) ranging from 0.27 to 0.35 mL/min/mg protein, whereas both the rat and guinea pig had approximately 7- and 160-fold greater intrinsic clearances, due to lower K_m values in rats (4.5 ± 0.7 μM) and guinea pigs (0.15 ± 0.1 μM) compared with humans (28.3 ± 8.3 μM, N = 4). Amsacrine, menadione, and 7-hydroxy-DACA were potent inhibitors of DACA metabolism in all three species, but 10-fold differences in IC_{50} values were apparent between species. In addition, SKF-525A was a potent inhibitor of the metabolism of DACA in rat cytosol but caused minimal inhibition in the guinea pig or human preparations. These results suggest that neither rat nor guinea pig AO preparations are suitable for predicting AO-mediated DACA–drug interactions in humans. BIOCHEM PHARMACOL **59**;2:161–165, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. aldehyde oxidase; species; acridine carboxamide

The anticancer agent DACA† recently has completed Phase I and now has entered Phase II clinical trials. DACA was chosen for clinical study due to its high activity against various solid murine tumours (including Lewis lung and Colon 38 carcinomas) and various multidrug-resistant human tumour cell lines [1].

Previously, we demonstrated that DACA is metabolised extensively *in vivo* in both mice and rats, producing complex metabolite profiles [2]. The main urinary and biliary metabolites identified were DACA-7-hydroxy-9(10*H*)-acridone and *N*-monomethyl-DACA, excreted as either aglycones or glucuronides. Further studies using isolated rat hepatocytes, subcellular fractions, and enriched rat liver enzyme preparations [2–4] have suggested that AO (EC 1.2.3.1) is an important enzyme in the metabolism of DACA. The rat hepatocyte studies indicated that DACA is oxidised initially to DACA-9(10*H*)-acridone, followed by microsomal oxidation of the side-chain nitrogen [4]. DACA-*N*-oxide also was examined as a substrate for AO in rat cytosol and was found to have a 30-fold lower intrinsic clearance (V_{max}/K_m) compared with DACA [3]. There is also evidence that DACA-*N*-oxide is reduced rapidly in rat hepatocytes to DACA, which then proceeds down the AO metabolism pathway with the formation of the acridone metabolites [4]. A study using trace amounts of [^{11}C]DACA in rats and humans implied that similar metabolites are observed in both species [5]. However, in the Phase I trial, the major urinary metabolite (≈34% of the total dose) was DACA-*N*-oxide-9(10*H*)acridone, with DACA-9(10*H*)-acridone (≈3%) and *N*-monomethyl-DACA-acridone (≈2%) also being observed. Unlike the findings in rodents, no aromatic ring-hydroxylated derivatives or glucuronide conjugates were detected in urine from human patients [6].

* Corresponding author: Dr. James W. Paxton, Department of Pharmacology and Clinical Pharmacology, University of Auckland School of Medicine, Private Bag 92019, Auckland, New Zealand. Tel. (64) 9-373-7599, Ext. 6413; FAX (64) 9-373-7556; E-mail: j.paxton@auckland.ac.nz

† *Abbreviations*: DACA, *N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide; AO, aldehyde oxidase; 2,3-diOMeNQ, 2,3-dimethoxy-1,4-naphthoquinone; 2-OMeNQ, 2-methoxy-1,4-naphthoquinone; $9NH_2$-DACA, 9-amino-*N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide; 7OH-DACA, *N*-[(2′-dimethylamino)ethyl]-7-hydroxyacridine-4-carboxamide; and IC_{50}, concentration causing 50% inhibition of enzymic reaction.

Received 29 December 1998; accepted 13 July 1999.

TABLE 1. Human liver preparations

Donor	Age (years)	Sex	Time since death	Cause of death
A	62	Male	4 hr 32 min	Heart disease
B	26	Male	10 hr 15 min	Hit by falling tree
C	28	Female	7 hr	Viral myocarditis
D	58	Female	8 hr	Ischaemic heart disease

AO is a ubiquitous enzyme with distinct iso-enzymic inter-species variations, whose various isoforms are located primarily in the liver and kidney [7–9]. Previously, we have shown that menadione (2-methyl-1,4-naphthoquinone), SKF- 525A, and methadone are potent inhibitors of rat AO [10, 11]. The aim of this study was to determine: (a) whether acridone formation could be detected in an AO preparation from human liver; and (b) whether rat or guinea pig hepatic preparations are appropriate models for predicting AO-mediated DACA–drug interactions in humans.

MATERIALS AND METHODS

Chemicals

SKF-525A was supplied by SmithKline Beecham Pharmaceuticals, and morphine sulphate was obtained from Douglas Pharmaceuticals. Menadione, 2,3-diOMeNQ, and 2-OMeNQ were provided by Dr. R. Munday (NZ Pastoral Agriculture Research Institute, Ltd.). 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (amsacrine), DACA, $9NH_2$-DACA, 7OH-DACA, and methyl-*N*-[4-(9-acridinylamino)phenyl]carbamate (methyl-carbamate) were provided by Professors B. C. Baguley and W. Denny of the Auckland Cancer Society Research Centre (The University of Auckland). All compounds were formulated in the appropriate buffer immediately before use.

Subcellular Fractions

Permission was received from the appropriate local Ethics Committees to use human and animal tissues. The livers from freshly killed male Wistar rats (200–300 g) and male guinea pigs (340–360 g) were weighed, perfused, and homogenised with 20 mM sodium phosphate (pH 7.4) at 3 vol./g. All processing and centrifugation steps were performed at 2–4°, with final preparations being stored at −80°. The homogenate was centrifuged for 2 min at 450 *g*. Then the supernatant was removed and subjected to the following sequential centrifugation steps: 20 min at 8000 *g*; then 60 min at 130,000 *g*; and finally 30 min at 130,000 *g* to give the cytosol preparation used in the animal experiments. Human post-mortem liver (150–200 g) (Table 1) was weighed, washed, and homogenised with 3 vol./g of 50 mM sodium phosphate/1 mM EDTA (pH 7.4) before being subjected to the above centrifugation steps. Then ammonium sulphate [$(NH_4)_2SO_4 \cdot H_2O$, enzyme grade] was added with slow stirring to the final supernatant to achieve 29% saturation. After 10 min of periodic stirring, the solution was centrifuged for 20 min at 5500 *g*, and the supernatant was removed for further addition of ammonium sulphate to give 37% saturation. After 10 min of periodic stirring, the latter was centrifuged for 20 min at 5500 *g* to obtain the active AO 29–37% precipitate, which contained 95% of the AO activity of the homogenate. The precipitate was redissolved in 5 mL of 50 mM sodium phosphate/1 mM EDTA (pH 7.4) buffer and used immediately or else stored frozen at −80. Subjecting the human cytosol to this precipitation procedure increased the activity per milligram of protein by 2-fold. Total protein content was determined in the incubations by the method of Lowry *et al.* [12].

Incubations

The incubation conditions and estimations of IC_{50}, K_m, and V_{max} were performed as previously reported [10]. The kinetic parameters of K_m, V_{max}, and inhibition constants were determined by unweighted nonlinear least squares regression analysis using MKMODEL [13]. The rate of DACA-9(10*H*)-acridone formation was examined over the concentration range of 0–200 μM DACA. For the inhibition studies, 5, 50, and 100 μM DACA were used for the guinea pig, rat, and human studies, respectively, with various concentrations of the potential inhibitors (amsacrine, $9NH_2$-DACA, 7OH-DACA, methyl-carbamate, menadione, 2,3-diOMeNQ, 2-OMeNQ, SKF-525A, and morphine). The IC_{50} value for DACA-9(10*H*)-acridone formation was calculated for each test compound.

Incubations were typically carried out in glass tubes for 2 min at 37° in a sodium phosphate buffer solution. In all cases, the test inhibitory compounds were added to the cold (0–4°) enzyme mixture and stored on ice for approximately 1 min before being preincubated for 1 min at 37°. The reaction was initiated by the addition of DACA, with gentle vortexing, followed by a 2-min incubation at 37°. The reaction was stopped by the addition of 10 vol. of ice-cold methanol, and the samples were reduced to dryness using a Speed-Vac (Savant Instruments Inc.). HPLC mobile phase was added to each sample, vigorously vortexed, and then centrifuged. Duplicate HPLC injections were performed on each sample to determine the concentration of DACA remaining and the amount of DACA-9(10*H*)-acridone formed.

HPLC

Analysis of DACA and DACA-9(10*H*)-acridone was performed using a Waters HPLC system, consisting of a WISP

TABLE 2. Kinetic parameters for DACA-9(10)-acridone formation by cytosolic AO in different species

Species	K_m (μM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m (mL/min/mg protein)
Human			
A	30 ± 5.7	0.7 ± 0.5*	0.023 ± 0.004
B	37 ± 3.6	10 ± 0.4*	0.27 ± 0.03
C	29 ± 5.2	6.5 ± 0.4*	0.33 ± 0.06
D	17 ± 3.6	6.0 ± 0.4*	0.35 ± 0.08
Rat	4.5 ± 0.7	9.8 ± 0.4	2.2 ± 0.4
Guinea pig	0.15 ± 0.1	8.0 ± 0.8	53.3 ± 6.3

Values are means ± SD, N = 4 experiments performed in duplicate, i.e. 8 data points. The DACA concentration range = 0–200 μM.
* Human V_{max} values have been corrected (×0.5) for the 2-fold AO purification step to allow comparison with the rodent cytosol values.

710B automatic sample injector, a 6000A pump, an RCM-100 compression module fitted with a Waters C18 μBondapak Radial Pak cartridge (8 mm × 10 cm), and a 490E variable wavelength absorbance detector. HPLC data collection and analysis were performed using Waters software. Separation and fractionation of the metabolites were achieved using a linear gradient of ammonium formate at pH 4.5 (0.24 to 0.17 M) and acetonitrile (37–50%) over 7.5 min with UV detection (250, 254, and 261 nm).

To initially calibrate the system, we injected ^{3}H-labelled DACA standards repetitively into the HPLC apparatus. Each fractionated HPLC peak was collected, and its radioactivity was determined using a Packard Tri-carb 2200CA liquid scintillation counter and Amersham ACS-11 scintillation fluid. For general compound identification, we compared retention times and UV spectral analyses of parent drug and metabolites against their synthetic standards using a diode array detector. Identification was confirmed further using NMR and mass spectrometry analysis on some samples, as previously reported [2, 3].

RESULTS

Intra- and Inter-species Comparison of DACA-9(10H)-acridone Formation

Typical DACA-9(10*H*)-acridone formation rates varied with DACA concentration for human, rat, and guinea pig AO preparations, according to Michaelis–Menten kinetics (Table 2). Three of the four human liver preparations had similar intrinsic clearance values (V_{max}/K_m) for acridone formation, ranging from 0.27 to 0.35 mL/min/mg protein (Table 2). One human liver (A) displayed approximately 10-fold lower intrinsic clearance for DACA, due to a 10-fold reduction in its V_{max}. Except for the latter, the V_{max} values were similar across species. However, the rodent AO preparations appeared to have a greater affinity for DACA, indicated by their lower K_m values (4.5 ± 0.7 and 0.15 ± 0.1 μM for rat and guinea pig, respectively) when compared with the human liver K_m values (range, 17–37 μM), resulting in greater intrinsic clearances for DACA (approximately 7-fold for rat and 160-fold for guinea pig) when compared with human livers (B, C, D).

Intra- and Inter-species Comparison of the Inhibition of DACA-9(10H)-acridone Formation

The most potent inhibitor of DACA-9(10*H*)-acridone formation by human liver AO was 7OH-DACA, indicated by the IC_{50} values (range, 0.02 to 0.05 μM) (Table 3). Similar IC_{50} values were observed for 7OH-DACA in the guinea pig (0.03 ± 0.01 μM), but the IC_{50} increased 10-fold in the rat (0.32 ± 0.03 μM). Both $9NH_2$-DACA and methyl-carbamate were less potent inhibitors of AO in all

TABLE 3. IC_{50} Values for DACA-9(10)-acridone formation by AO in different species

Inhibitor	Concentration range tested (μM)	IC_{50} (μM)					
		Human				Rat (N = 4)	Guinea pig (N = 5)
		A	B	C	D		
Amsacrine	0–30	2.5 ± 0.5	4.8 ± 0.6	1.9 ± 0.1	3.7 ± 0.4	7.7 ± 0.06	7.7 ± 3.0
7OH-DACA	0–20	0.02 ± 0.03	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.32 ± 0.03	0.03 ± 0.01
$9NH_2$–DACA	0–10	NT	8.1 ± 1.5	NT	NT	9.5 ± 0.6	0.1 ± 0.01
Methyl-carbamate	0–100	NT	1.0 ± 0.1	NT	NT	9.6 ± 0.6	2.0 ± 0.4
Menadione	0–50	0.8 ± 0.02	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.5	0.73 ± 0.04	3.1 ± 0.04
2,3-diOMeNQ	0–500	332 ± 23	411 ± 37	NT	NT	167 ± 20	Potentiation
2-OMeNQ	0–400	25.6 ± 2.3	23.5 ± 1.7	23.3 ± 2.0	NT	53.5 ± 11.0	24.7 ± 3.4
SKF-525A	0–500	NT	> 200	NT	NT	1.1 ± 0.1	>500
Morphine	0–1000	NT	438 ± 10	NT	NT	273 ± 18	>600

Values are means ± SD, N = 4–5 experiments performed in duplicate. NT = not tested in this species sample. DACA concentration: human, 100 μM; rat, 50 μM; and guinea pig, 5 μM.

three species, but once again marked differences occurred between the species, especially for $9NH_2$-DACA, with IC_{50} values of 0.1 ± 0.01, 0.95 ± 0.07, and 8.1 ± 1.5 μM in the guinea pig, rat, and human (only B was tested) livers, respectively. Similar IC_{50} species values were also observed for amsacrine (humans, 1.9 to 4.8 μM; rat, 7.7 ± 0.06 μM; guinea pig, 7.7 ± 3.0 μM) and menadione (humans, 0.8 to 1.7 μM; rat, 0.73 ± 0.04 μM; guinea pig, 3.1 ± 0.04 μM). The other menadione analogues were less potent. SKF-525A, which inhibited rat preparations (IC_{50} 1.1 ± 0.1 μM), caused minimal inhibition in the guinea pig or the single human preparation tested. Similarly, morphine required high concentrations (>273 μM) to inhibit DACA-9(10*H*)-acridone formation in all species tested.

DISCUSSION

Based on intrinsic clearance, our results suggest that the guinea pig cytosol has the greatest ability to metabolise DACA, followed by the rat, and then the human preparation. In S-9 fractions, Rodrigues [14] reported the rank order of AO activity (using N^1-methyl-nicotinamide as substrate) as monkey > rat > dog > humans, with an approximately 10-fold difference between rat and humans. However, caution is advisable when comparing across species, as a 64-fold difference in liver AO activity has been reported across different strains of rats [15]. Also in six human livers, a 40-fold variation in AO activity was observed, which was dependent on the substrate used [14]. Similarly, a 50-fold variation also was reported in seven fresh human livers using benzaldehyde as substrate [16]. In our study, one human liver (preparation A) had 10-fold less capacity to metabolise DACA compared with the other three, which were very similar. Preparation A was the freshest human liver sample (received 4 hr 32 min after patient death), suggesting that this reduction in activity was not caused by the stability or degradation of the enzyme with time after death. Age and cause of death (male, 62 years, died of heart disease) may be factors, but this appears unlikely when compared with donor D, who also died of heart disease at age 58. However, the latter comparison may be complicated by sex differences, which have been reported to occur in mice [17]. Other factors such as cigarette smoking or concurrent drug therapy also are known to affect drug metabolism capacity, but unfortunately these details were not available for our donors.

Our results also emphasise the difficulty of using animal tissues to model and predict the outcomes of human AO-mediated DACA–drug interactions. Although 7OH-DACA, menadione, and amsacrine were potent inhibitors in the three species examined, 10-fold differences in IC_{50} values were apparent between species. Indeed, SKF-525A, while potently inhibiting DACA-9(10*H*)-acridone formation in the rat, had minimal effect on guinea pig and human AO preparations. Similar marked differences in substrate specificity for human liver AO and hepatic enzyme from rabbit, guinea pig, and baboon have been demonstrated by Beedham *et al.* [18], who concluded that different AO isozymes were present in the different species, with guinea pig and human showing the closest spectrum of activity.

It is of interest that 7OH-DACA was the most potent inhibitor of AO activity in all three species, as this compound may be formed *in vivo*. However, in a previous study, no metabolism was observed when 7OH-DACA was incubated with rat liver cytosol, suggesting that the latter is an inhibitor but not a substrate for AO [3]. Metabolism studies in rat and mouse have identified the 7-hydroxylated DACA-9(10*H*)-acridone and its corresponding glucuronide [3, 4, 10], but neither metabolite was detected in measurable amounts in urine from patients in the Phase I trial [6]. However, feedback inhibition of acridone formation by metabolites *in vivo* remains a possibility.

In conclusion, these results suggest caution in extrapolating the data obtained from rodent AO studies to humans. The most appropriate method/model for predicting possible drug–drug interactions involving AO in patients would appear to be human isolates, or else cDNA-cloned, expressed, and purified human AO.

This work was supported by the Health Research Council, the Cancer Society of New Zealand, and the Auckland Medical Research Foundation. We also wish to thank Grant Knight and Saskia Geurts for their technical assistance.

References

1. Finlay GJ and Baguley B, Selectivity of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide towards Lewis lung carcinoma and human tumour cell lines *in vitro*. *Eur J Cancer Clin Oncol* **25:** 271–277, 1989.
2. Robertson IGC, Palmer BD, Paxton JW and Bland TJ, Metabolism of the experimental antitumor agent acridine carboxamide in the mouse. *Drug Metab Dispos* **21:** 530–536, 1993.
3. Robertson IGC, Palmer BD, Officer M, Siegers DJ, Paxton JW and Shaw GJ, Cytosol mediated metabolism of the experimental antitumour agent acridine carboxamide to the 9-acridone derivative. *Biochem Pharmacol* **42:** 1879–1884, 1991.
4. Schlemper B, Siegers DJ, Paxton JW and Robertson IGC, Rat hepatocyte-mediated metabolism of the experimental antitumour agent *N*-[2′-(dimethylamino)ethyl]acridine-4-carboxamide. *Xenobiotica* **23:** 361–371, 1993.
5. Osman S, Luthra SK, Brady F, Hume SP, Brown G, Harte RJA, Matthews JC, Denny WA, Baguley BC, Jones T and Price PM, Studies on the metabolism of the novel antitumor agent [*N-methyl-*^{11}C]*N*-[2-(dimethylamino)ethyl]-acridine-4-carboxamide in rats and humans prior to phase I clinical trials. *Cancer Res* **57:** 2172–2180, 1997.
6. Schofield PC, Robertson IGC, Paxton JW, McCrystal MR, Evans BD, Kestell P and Baguley BC, Metabolism of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide in cancer patients undergoing a phase I clinical trial. *Cancer Chemother Pharmacol* **44:** 51–58, 1999.
7. Beedham C, Bruce RC, Critchley DJ, Al-Tayib Y and Range DJ, Species variation in hepatic aldehyde oxidase activity. *Eur J Drug Metab Pharmacokinet* **12:** 307–310, 1987.
8. Beedham C, Al-Tayib Y and Smith JA, Role of guinea pig and rabbit hepatic aldehyde oxidase in oxidative *in vitro* metabo-

lism of cinchona antimalarials. *Drug Metab Dispos* **20:** 1–6, 1992.
9. Cashman JR, Structural and catalytic properties of the mammalian flavin-containing monooxygenase. *Chem Res Toxicol* **8:** 165–182, 1995.
10. Robertson IGC and Bland TJ, Inhibition by SKF-525A of the aldehyde oxidase-mediated metabolism of the experimental antitumour agent acridine carboxamide. *Biochem Pharmacol* **45:** 2159–2162, 1993.
11. Robertson IGC and Gamage RSKA, Methadone: A potent inhibitor of rat aldehyde oxidase. *Biochem Pharmacol* **47:** 584–587, 1993.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
13. Holford NHG, MKMODEL: A modelling tool for microcomputers. Pharmacokinetic evaluation and comparison with standard computer programs. *Clin Exp Pharmacol Physiol* **12**(Suppl 9)**:** 95, 1985.
14. Rodrigues AD, Comparison of levels of aldehyde oxidase with cytochrome P450 activities in human liver *in vitro*. *Biochem Pharmacol* **48:** 197–200, 1994.
15. Sugihara K, Kitamura S and Tatsumi K, Strain differences in liver aldehyde oxidase activity in rats. *Biochem Mol Biol Int* **37:** 861–869, 1995.
16. Sugihara K, Kitamura S, Tatsumi K, Asahara T and Dohi K, Differences in aldehyde oxidase activity in cytosolic preparations of human and monkey liver. *Biochem Mol Biol Int* **41:** 1153–1160, 1997.
17. Yoshihara S and Tatsumi K, Purification and characterization of hepatic aldehyde oxidase in male and female mice. *Arch Biochem Biophys* **338:** 29–34, 1997.
18. Beedham C, Critchley DJP and Rance DJ, Substrate specificity of human liver aldehyde oxidase toward substituted quinazolines and phthalazines: A comparison with hepatic enzyme from guinea pig, rabbit and baboon. *Arch Biochem Biophys* **319:** 481–490, 1995.