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*Original Research Article*

# Individual Variation in Hepatic Aldehyde Oxidase Activity

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*Summary*

**Aldehyde oxidase (AO) is a molybdo-flavo enzyme expressed predominantly in the liver, lung, and kidney. AO plays a major role in oxidation of aldehydes, as well as oxidation of various N-heterocyclic compounds of pharmacological and toxicological importance including antiviral (famciclovir), antimalarial (quinine), antitumour (methotrexate), and nicotine. The aim of this study was to investigate cytosolic aldehyde oxidase activity in human liver. Cytosolic AO was characterised using both the metabolism of N-[(2-dimethylamino)ethyl] acridine-4-carboxamide (DACA) and benzaldehyde to form DACA-9(10H)-acridone (quantified by HPLC with fluorescence detection) and benzoic acid (quantified spectrophotometrically). Thirteen livers (10 female, 3 male) were examined. The intrinsic clearance (Vmax/Km) of DACA varied 18-fold (0.03–0.50 ml/min/mg). Vmax ranged from 0.20–3.10 nmol/min/mg, and Km ranged from 3.5–14.2 $\mu$M. In the same specimens, the intrinsic clearance for benzaldehyde varied 5-fold (0.40–1.8 ml/min/mg). Vmax ranged from 3.60–12.6 nmol/min/mg and Km ranged from 3.6–14.6 $\mu$M. Furthermore, there were no differences in AO activity between male and female human livers, nor was there any relationship to age of donor (range 29–73 years), smoking status, or disease status. In conclusion, our results showed that there are variations in AO activity in human liver. These variations in aldehyde oxidase activity might reflect individual variations or they might be due to AO stability during processing and storage.**

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**Keywords** Aldehyde oxidase; benzaldehyde; DACA; individual variation.

## INTRODUCTION

Aldehyde oxidase (AO) is a dimeric molecule of approximately 300,000 molecular weight having two identical independent subunits, each containing molybdenum, flavin adenine dinucleotide (FAD) and 2Fes/2S iron centres (*1*). AO plays a major role in oxidation of aldehydes, as well as bioactivation and detoxication of compounds of pharmacological and toxicological importance. AO is the main enzyme in the bioactivation of 5-fluoropyrimidine to 5-fluorouracil, the major drug to treat colorectal cancer metastasised to the liver (*2*). In addition to bioactivation, AO plays a major role in detoxification of antimalarials (quinine) (*3*), anticancer drugs, (methotrexate) (*4*), and nicotine (*5*). Recently, AO was found to be the main enzyme that detoxifies N-[(2-dimethylamino)ethyl]acridine-4-carboxamide (DACA), a novel DNA-intercalating agent (*6*) that can overcome multidrug resistance (*7*) and can cross the blood-brain barrier (*8*). Therefore, it may play a future role in the treatment of brain tumours.

Hepatic AO activity varies greatly between animal species (*9*) and strains of the same species (*10*). Substrate-dependent variation of hepatic AO activity has been reported in different strains of rats. Benzaldehyde detected 63.5-fold variation in hepatic AO activity between Sea:SD and WKA/sea rats (*10*). This variation increased to 104-fold with methotrexate (*11*). It has also been suggested that there is a large variation in AO activity in human liver (*12*). Because the pharmacological action of a drug is closely related to its rate of metabolism, individual differences in AO activity is of considerable importance for clinical applications of drugs that are metabolised by this enzyme. The aim of this study was to investigate the variability of AO in human liver cytosolic preparations using both DACA and benzaldehyde.

## EXPERIMENTAL PROCEDURES

### *Chemicals*

Menadione, amsacrine, DACA, DACA-9(10H)-acridone, and N-ethyl DACA (internal standard) were provided by

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Auckland Cancer Society Research Centre, University of Auckland. Ethylenediaminetetraacetic acid (EDTA), benzaldehyde, copper (II) sulphate, bovine serum albumin (BSA), and bicinchoninic acid (BCA) were obtained from Sigma Chemicals Co (Sydney, Australia). Ammonium chloride was provided by Research Organics Inc (Cleveland, Ohio, USA); potassium chloride was provided by Scharlau (Barcelona, Spain). Both acetonitrile and methanol were HPLC grade and were provided by Scientific Supplies, Auckland, New Zealand. Phosphoric acid and triethylamine were from Ridel deHaan, Germany.

N-ethyl DACA and DACA were dissolved in 10% (v/v) methanol. Benzaldehyde was dissolved in 20% (v/v) methanol. Menadione was dissolved in 10% (v/v) methanol and then dissolved in 0.5 mM sodium phosphate buffer.

### *Tissue Preparation*

Fresh human liver samples were obtained from 13 Caucasians patients who underwent liver resections for colorectal metastasis carried out in the Auckland Hospital. Table 1 shows the demographic and donor data. Ethical approval was granted for the use of these liver samples in our study.

Liver samples were collected soon after removal. Liver sections were then divided into approximately 15–20-g portions and snap frozen in liquid nitrogen. Thereafter the liver portions were stored at −80°C until use. Portions of the same liver were pooled and homogenized in four volumes of 67 mM phosphate buffer containing 1.15% KCl at pH 7.4. The homogenates were then subjected to successive centrifugation at 9,000 *g* for 20 min and at 100,000 *g* for 65 min (*13*). The cytosolic fractions formed were collected, and protein concentrations were determined spectrophotometrically (Spectra Max Plus) using the bicinchoninic acid method with bovine serum albumin as a protein standard.

### *Benzaldehyde Assay for Hepatic Aldehyde Oxidase Activity*

Hepatic AO activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 247 nm consequent upon the oxidation of 50 $\mu$M benzaldehyde (extinction coefficient = 17.54 $cm^{-1}$ $mM^{-1}$) to benzoic acid. The decrease in absorbance was monitored in 0.5 mM sodium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 1 M ammonium chloride at 25°C (*14*). To obtain kinetic parameters five different concentrations of benzaldehyde were used ranging from 5 $\mu$M–50 $\mu$M together with 1.3 mg/ml cytosolic protein concentration.

### *DACA Assay for Hepatic Aldehyde Oxidase Activity*

The rate of DACA-9(10H)-acridone formation was examined over the concentration range of DACA (0–300 $\mu$M) and cytosolic protein concentration of 1.3 mg/ml. For the inhibition studies, 50 $\mu$M DACA was used with various concentrations of the AO inhibitor, menadione. Incubations were typically carried out in glass tubes for 2 min at 37°C in a 0.5 mM sodium phosphate buffer solution pH 7.8. Concentrations of menadione (0, 1, 3, 10, 30 $\mu$M) were added to the cold (0–4°C) enzyme mixture and stored on ice for 1 min before preincubation for 1 min at 37°C. The reaction was initiated by the addition of DACA, with gentle vortexing, followed by a 2-min incubation at 37°C. The reaction was stopped by the addition of 10 vol of ice-cold methanol with 1 $\mu$M internal standard (N-ethyl DACA). The mixtures were vortexed for 1 min and then centrifuged (3000 × *g*) for 3 min to precipitate proteins. Aliquots of the supernatant (30 $\mu$l) were injected on to the HPLC column in duplicate to measure the rate of DACA-9(10H)-acridone formation.

**Table 1**
Demographic and liver donors details

| Human liver | Age | Gender | Smoker | Viral hepatitis | HIV status | Recent medication | Alcohol intake |
|---|---|---|---|---|---|---|---|
| HL1 | 48 | F | Yes | Negative | Unknown | None | None |
| HL2 | 73 | F | No | Unknown | Unknown | None | None |
| HL3 | 54 | F | No | Unknown | Unknown | None | None |
| HL4 | 63 | F | No | Unknown | Unknown | None | None |
| HL5 | 59 | F | No | Surface AB | Unknown | Warfarin dopamine | None |
| HL6 | 53 | F | No | Unknown | Unknown | Albendazole | None |
| HL7 | 41 | M | No | Negative | Unknown | Augmentin | Occasional |
| HL8 | 55 | F | No | Unknown | Unknown | None | None |
| HL9 | 46 | M | No | Unknown | Unknown | Norstat | None |
| HL10 | 29 | F | No | Unknown | Unknown | None | None |
| HL11 | 49 | M | No | Unknown | Unknown | None | None |
| HL12 | 63 | F | No | Unknown | Unknown | None | None |
| HL13 | 70 | F | No | Unknown | Unknown | None | None |

## HPLC

The HPLC system consisted of a Waters 501 pump, WISP 710B automatic sample injector, and Waters Maxima software to collect and analyse data. A guard column was positioned ahead of the phenomenex C18 Bondaclone stainless steel column (300 × 3.9 mm). Detection was carried out by 474 Waters scanning fluorescence detector at 257 nm excitation and 445 nm emission. Separation and fractionation of metabolites were achieved using a mobile phase that consisted of 25% acetonitrile, 100 mM triethylammonium phosphate (TEAP) at pH 3. The mobile phase flow rate was 1.6 ml/min.

### *Data Analysis*

The formation rate of benzoic acid and DACA-9(10H)-acridone were calculated as nmol/min per mg cytosol protein and were used with their corresponding substrate concentrations to calculate the kinetic parameters $K_m$ (Michaelis-Menten constant) and $V_{max}$ (maximum velocity). In the inhibition studies, the concentration which caused 50% inhibition of AO activity ($IC_{50}$) was calculated. The Prism 2.01 program (Graphpad software Co., CA, USA) was used to estimate these parameters by non-linear regression with a single binding site model. The single binding site model (v = Vmax *S/(Km + S)) gave the best fit, with acceptable standard errors for the parameters Vmax and Km (v = the rate of oxidation; S, the substrate concentration).

## RESULTS

Cytosolic AO from 13 human livers was characterised by the formation of DACA-9(10H)-acridone from DACA and benzoic acid from benzaldehyde. There was individual variation in all kinetic parameters for DACA-9(10H)-acridone formation (Table 2). Km values averaged 8.3 $\mu$M (range 3.5 to 14.2), Vmax averaged 1.2 nmol/min/mg (range 0.2 to 3.1), intrinsic clearance averaged 0.20 mL/min/mg (range 0.03 to 0.50). Similarly, Km values for benzoic acid formation averaged 8.6 $\mu$M (range 3.6 to 14.6), Vmax averaged 6.1 nmol/min/mg (range 3.6–12.6), intrinsic clearance averaged 0.74 mL/min/mg (range 0.4 to 1.1). The kinetic parameters for benzoic acid formation are shown in Table 3. There was no correlation between benzaldehyde Vmax (DACA vs benzaldehyde), nor Km (DACA vs benzaldehyde) (data not shown). There was no relationship between age and gender and both DACA and benzaldehyde clearance. Table 4 shows $IC_{50}$ values for DACA-9 (10H)-acridone formation in human liver. There was 28.7-fold variation in $IC_{50}$ values with average of 3.8 $\mu$M (range 0.3 to 8.6).

## DISCUSSION

The aim of our investigation was to study cytosolic aldehyde oxidase in human liver. In our study, we found variation in kinetic parameters for both DACA-9(10H) formation and benzoic acid formation. The difference in intrinsic clearance (Vmax/Km) was 16.6-fold with DACA and 2.75-fold with benzaldehyde. Although this variation might indicate individual variation in aldehyde oxidase activity, caution is advised as human aldehyde oxidase activity has been reported to be labile. AO activity has been shown to decrease upon homogenisation and storage (*15*). Therefore, whether variation in human AO reflects differences in activity or stability of AO is still to be determined. In this study, 3.5-fold variation in human AO activity was found with

**Table 2**
Kinetic parameters for DACA-9(10H)-acridone formation in human livers

| Human livers | Km ($\mu$M) | Vmax (nmol/min/mg) | Intrinsic clearance (mL/min/mg) |
|---|---|---|---|
| HL1 | 4.9 | 0.5 | 0.1 |
| HL2 | 6.8 | 1.6 | 0.2 |
| HL3 | 11.4 | 0.7 | 0.1 |
| HL4 | 7.0 | 0.4 | 0.1 |
| HL5 | 8.3 | 0.2 | 0.03 |
| HL6 | 12.7 | 1.4 | 0.1 |
| HL7 | 14.2 | 0.8 | 0.1 |
| HL8 | 11.3 | 2.0 | 0.2 |
| HL9 | 5.0 | 0.7 | 0.1 |
| HL10 | 9.4 | 3.1 | 0.3 |
| HL11 | 8.1 | 1.2 | 0.2 |
| HL12 | 4.6 | 0.8 | 0.2 |
| HL13 | 3.5 | 1.8 | 0.5 |
| Mean | 8.3 | 1.2 | 0.2 |
| ±SE ranges | (0.5–6.8) | (0.1–0.8) | (0.1–0.3) |

Results are expressed as mean of two experiments.

**Table 3**
Kinetic parameters for benzoic acid formation in human liver

| Human livers | Km $\mu$(M) | Vmax (nmol/min/mg) | Intrinsic clearance (mL/min/mg) |
|---|---|---|---|
| HL1 | 7.8 | 4.4 | 0.6 |
| HL2 | 12.5 | 7.2 | 0.6 |
| HL3 | 10.5 | 6.0 | 0.6 |
| HL4 | 14.6 | 6.0 | 0.4 |
| HL5 | 7.5 | 5.2 | 0.7 |
| HL6 | 4.8 | 5.4 | 0.4 |
| HL7 | 10.6 | 4.7 | 0.4 |
| HL8 | 3.6 | 6.5 | 1.8 |
| HL9 | 5.1 | 3.6 | 0.7 |
| HL10 | 8.6 | 7.7 | 0.9 |
| HL11 | 7.6 | 4.4 | 0.6 |
| HL12 | 6.8 | 5.5 | 0.8 |
| HL13 | 11.2 | 12.6 | 1.1 |
| Mean | 8.6 | 6.1 | 0.74 |
| ±SE ranges | (0.3–3) | (0.1–1.3) | (0.1–0.2) |

Results are expressed as mean of two experiments.

**Table 4**
The effect of menadione on DACA-9-(10H)-acridone formation in human liver

| Human livers | $IC_{50}(\mu M)$ |
|---|---|
| HL1 | 2.0 |
| HL2 | 7.4 |
| HL3 | 4.9 |
| HL4 | 0.6 |
| HL5 | 2.4 |
| HL6 | 0.3 |
| HL7 | 0.8 |
| HL8 | 5.2 |
| HL9 | 3.1 |
| HL10 | 7.5 |
| HL11 | 3.8 |
| HL12 | 8.6 |
| HL13 | 2.7 |
| Mean | 3.8 |
| ±SE ranges | (0.1–1.5) |

DACA concentration used (50 $\mu$M), menadione concentration (0, 1, 3, 10, 30 $\mu$M).
Results shown are mean of two experiments.

benzaldehyde. This finding is in agreement with Rodrigues et al. who detected 3.6-fold variation with the same substrate (*12*). In contrast, Sugihara et al. found a 50-fold variation in human AO activity with benzaldehyde (*16*). The large discrepancy in AO activity between our results and that of Sugihara et al. might be due the involvement of genetic polymorphism. We found a lack of correlation in either kinetic parameter between DACA and benzaldehyde. This might suggest the involvement of different isozymes in the metabolism of the two substrates. The mechanism of regulation of these isozymes is not known at the present, however, a single gene has been found to encode human AO (*17*). Therefore, it might be possible that post-transcriptional mechanisms are responsible for the production of the isozymes.

Factors such as gender may influence AO activity in the human. Gender has been reported to influence mouse AO activity (*18*). Male mice exhibit higher AO activity than female mice. Although we detected no relationship between gender and AO activity, we cannot exclude such a relationship because of the low number of livers used. Similarly, for other factors such as age, cigarette smoking, and drug usage. Our numbers are too small to exclude a relationship to AO activity.

Further studies are required to determine whether variation of hepatic AO activity in man reflects individual variation or is due to the instability of AO during the processes of storage and homogenization. Such a study might involve the use of a marker enzyme such as glucose-6-phosphate dehydrogenase or lactate dehydrogenase during preparation and storage. Furthermore, additional studies are required to assay for other factors such as cytokines that affect human AO activity. Hepatic drug-metabolising capacity is known to be altered by these mediators (*19–23*). Cytokines are known to be increased in certain diseases (*24*) and can account for individual variation in AO activity. Unfortunately information with respect to cytokine levels was not available in our study. Determining these factors is of great importance, as they should be taken in to account when prescribing drugs that are metabolised by AO, including methotrexate, quinine, and carbazeran. A standard dosage of these drugs may lead to therapeutic failure, which may have fatal consequences or toxicological implications. Therefore, there may be a need for monitoring the plasma drug concentration and possibly for adjusting the dosage of a drug for individual patients. This is of the greatest importance for drugs with a narrow therapeutic index. In conclusion, we found variation in hepatic aldehyde oxidase activity. The basis for such variation might reflect individual variation or might be due to the lability of human AO. Further studies are required to resolve this issue.

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