Evaluation of Rhesus Monkey and Guinea Pig Hepatic Cytosol Fractions as Models for Human Aldehyde Oxidase.

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Running Title: Page-Species differences in AOX activity and inhibition

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Abbreviations: guinea pig liver cytosol (GPLC), rhesus monkey liver cytosol (RMLC), human liver cytosol (HLC) electrospray ionization (ESI), aldehyde oxidase isoform 1 (AOX1), aldehyde oxidase (AOX), (N-[2-(dimethylamino)ethyl]acridone-4-caboxamide) (DACA)
DMD #52985

ABSTRACT

Aldehyde oxidase (AOX) is a cytosolic enzyme expressed across a wide range of species including guinea pig and rhesus monkey. These species are believed to be the best preclinical models for studying human AO mediated metabolism. We compared AO activity in rhesus monkey, guinea pig and humans using phthalazine and DACA as substrates and raloxifene as an inhibitor. Michaelis-Menten kinetics was observed for phthalazine oxidation in rhesus monkey, guinea pig and human liver cytosol whereas substrate inhibition was seen with DACA oxidase activity in all the 3 livers. Raloxifene inhibited phthalazine and DACA oxidase activity uncompetitively in guinea pig whereas mixed mode inhibition was seen in rhesus monkey. Our analysis of the primary sequence alignment of rhesus monkey, guinea pig and human AOX1 along with homology modelling has led to the identification of several amino acid residue differences within the active site and substrate entrance channel of AOX1. We speculate that some of these residues might be responsible for the differences observed in activity. Overall, our data indicate that rhesus monkey and guinea pig would overestimate intrinsic clearance in human and would be unsuitable to use as animal models. Our study also showed that AO metabolism in species is substrate dependent and no single animal model can be reliably used to predict every drug response in humans.
INTRODUCTION

Aldehyde oxidase (AOX) catalyses the redox reaction of a broad range of substrates with varying functional groups and sizes, thus making it significant to drug and xenobiotic metabolism. Zaleplon (hypnotic), famciclovir (antiviral), methotrexate (anticancer), zonisamide (antiepileptic) and ziprasidone (antipsychotic) are some of the drugs metabolised by AOX (Rashidi et al., 1997; Beedham et al., 2003; Obach et al., 2004). Recent reviews have indicated an increasing role for AOX in drug discovery (Pryde et al., 2010; Hutzler et al., 2013). As more and more drugs become AOX substrates, the number of drug-drug interactions associated with this enzyme may also rise. An in vitro study carried out on 239 prescription drugs and related compounds showed that 14% of the compounds had IC\textsubscript{50} values less than 10 \textmu M (Obach et al., 2004). This indicates that AOX might have a significant role to play in clinical drug-drug interactions and related toxicity in the future.

Evaluation of potential drug metabolism and drug-drug interactions in humans mediated by AOX is hindered by the lack of an appropriate animal model. Although AOX is widely expressed in many species, including insects and fish, the level of AOX hepatic activity is markedly different between species (Kitamura et al., 2006; Garattini and Terao, 2012). This has been attributed to the variable complement of AOX isoforms expressed in the liver. For example, humans are predicted to synthesise AOX1 as the only isoform in liver whereas AOX1 and AOX3 have been identified and characterized as the major isoforms expressed in rabbit, mouse and rat liver. AOX3 and not AOX1 is believed to make a significant contribution to drug metabolism in the liver of rodents (Garattini and Terao, 2013). Other popular experimental models like the beagle dog routinely used in drug metabolism studies are devoid of AOX activity (Austin et al., 2001). For quite some time now guinea pig and rhesus monkey have been thought to recapitulate the drug metabolism brought about by AOX in human liver. This is because in the liver both these animals are thought to synthesize a sole active AOX1 which is an ortholog of human and mouse AOX1.
(Garattini and Terao, 2013). Whilst there are a few studies that support the monkey (rhesus and cynomologous) as a potential surrogate for human AOX metabolism in vivo (Dittrich et al., 2002; Morrison et al., 2012), there is no evidence in support of the guinea pig.

To date, only one study that evaluates the metabolism of a clinical drug, zoniporide, has been conducted in human and compared to both guinea pig and rhesus monkey. The intrinsic clearance of this compound observed in liver of guinea pig and rhesus monkey was lower than human. The authors concluded that rhesus monkey and guinea pig could potentially under predict the clearance of zoniporide in humans if used as surrogates (Dalvie et al., 2013).

In order to better understand xenobiotic and drug metabolism in these species and their correlation to humans we studied the in vitro metabolism of phthalazine and the anticancer agent (N-[2-(dimethylamino) ethyl] acridone-4-carboxamide); DACA by using liver cytosol from human, guinea pig and rhesus monkey. The objectives of this study were to establish the relative clearance values for the three species and investigate inhibition of metabolism by raloxifene, a selective estrogen receptor modulator used in the long term treatment of osteoporosis. Raloxifene is a very potent inhibitor of phthalazine (Obach, 2004) and DACA oxidase activity (Barr and Jones, 2013) in human liver with the IC₅₀ and Kᵢ values in the low nanomolar range. The mode of inhibition with phthalazine was determined to be entirely uncompetitive (Obach, 2004) whereas Barr and Jones, 2013 have shown that inhibition of DACA oxidase with raloxifene in humans was entirely competitive. Another purpose of this study was to determine if raloxifene would inhibit phthalazine and DACA oxidase activities in guinea pig and rhesus monkey in a manner that is similar to the human counterpart and whether the data obtained in this study can be used to predict drug-drug interactions by AOX inhibition in humans.
MATERIALS AND METHODS

Materials

DACA was synthesised in our laboratory (Barr and Jones, 2013) and DACA acridone was kindly provided by Dr. William A. Denny from University of Auckland (Auckland, New Zealand). Phthalazine, 1-phthalazinone, raloxifene and the internal standard 2-methyl-4(3H)-quinazolinone were purchased from Sigma-Aldrich (St. Louis, MO). Guinea pig liver cytosol (GPLC) was pooled from 50 male donors and rhesus monkey liver cytosol (RMLC), pooled from 6 male donors was purchased from Xenotech LLC (Lenexa, KS). Human liver cytosol (HLC), pooled from 150 individual donors (male and female) was purchased from BD Biosciences (Woburn, MA).

In vitro incubations- Phthalazine and DACA oxidase activity

The DACA oxidase activity assay was performed in a similar manner as phthalazine oxidase activity assay with minor differences. DACA oxidase activity in GPLC and RMLC was linear up to 30 min. All incubations were carried out for 5 min using 0.05 mg/ml liver cytosol and the same incubation buffer. For determination of saturation kinetics constants, $V_{\text{max}}$ and $K_m$, stock solutions of DACA were made in DMSO (the affect of DMSO on activity is shown in Supplemental Figure 1) and added to the incubation mixture such that the final DMSO concentration was 0.5% (v/v). For determination of inhibition constant, $K_i$, inhibitor raloxifene too was made up in DMSO and added to the reaction mixture at a final DMSO concentration of 0.5% (v/v). Raloxifene concentration in the range of 0.1-1 μM and 1-10 μM was used to inhibit DACA oxidase activity in guinea pig and rhesus monkey liver cytosol respectively. The final concentration of DMSO in the reaction mixture for DACA oxidase assay with inhibitor was 1% (v/v). The reaction was started, terminated and samples collected for analysis in the same way as with phthalazine.
Assays were performed using modified techniques previously described (Obach et al., 2004; Barr and Jones, 2011; Barr and Jones, 2013). An initial determination of the linearity of reaction velocity with time and protein concentration had revealed that phthalazine oxidase activity in GPLC and RMLC was nonlinear after 2.5 min and hence all incubations with phthalazine were carried out for 2.5 min with 0.075 mg/ml liver cytosol in 25mM potassium phosphate buffer, pH 7.4, containing 0.1mM ethylenediaminetetraacetic acid (EDTA) in a final incubation volume of 0.8ml. Stock solutions of phthalazine were made up in potassium phosphate buffer. For determination of the inhibition constant, Kᵢ, raloxifene was made up in DMSO (the affect of DMSO on activity is shown in Supplemental Figure 2 and 3) and added to the reaction mixture at a final DMSO concentration of 0.5% (v/v). Raloxifene concentration in the range of 10-100nM and 0.5-6μM was used to inhibit phthalazine oxidase activity in guinea pig and rhesus monkey liver cytosol respectively. For determination of saturation kinetics constants, Vₘₐₓ and Kₘ, no inhibitor was added to the reaction mixture. Incubation mixture with or without raloxifene was pre-warmed at 37°C for 5 min and reaction was initiated by the addition of 0.2ml liver cytosol. After 2.5 min, the reaction was terminated by the addition of 0.2 ml of 1M formic acid containing a known concentration of 2-methyl-4(3H)-quinazolinone as internal standard (IS). Quenched samples were centrifuged in a 5415D eppendorf centrifuge at 5000rpm for 10 min and the supernatant was collected for analysis.

**Detection and analysis of the formation of 1-phthalazinone and DACA -9(10H) acridone using HPLC-MS/MS assay**

The HPLC-MS/MS assay for the detection of 1-phthalazinone in GPLC and RMLC was carried out as described previously by (Barr and Jones 2011) and the detection of DACA metabolite, DACA -9(10H) acridone was carried out as per (Barr and Jones 2013). Samples were analysed on an 1100 series high performance liquid chromatography system (Agilent technologies, Santa Clara, CA) coupled to API 4000 tandem mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster city, CA). Synergi Polar reverse-phase column (30 x 3.0 mm,
4 μM; Phenomenex, Torrance, CA) was used for chromatographic separation. Mobile phase A contained 0.05% formic acid and 0.2% acetic acid in water whereas mobile phase B was comprised of 90% acetonitrile, 9.9% water and 0.1% formic acid. Total chromatographic separation time was 5 min per sample. Retention time for IS and 1-phthalazinone were 2.5 and 2.8 min respectively and for IS and DACA-9(10H) acridone was 1.4 and 1.8 min respectively. All samples were analysed using positive ion mode on an electrospray ionisation (ESI) interface. 1-phthalazinone and the IS were detected in liver cytosol using multiple reaction monitoring for the m/z transition of 147 -> 118 and 161 -> 120 respectively. Quantitation was achieved by extrapolation of a 1-phthalazinone standard curve ranging from 0.01 to 10 μM. DACA-9(10H) acridone and the IS were detected in liver cytosol using multiple reaction monitoring for the m/z transition of 310->265 and 161 -> 120 respectively and quantitation was achieved by extrapolation of DACA-9(10H) acridone standard curve ranging from 2 to 1000 nm.

Data analysis

All V_{max} and K_{m} determinations were measured in triplicate. Data obtained were expressed as mean ± standard error of the mean (SEM) for 3 experiments done on different days. Enzyme kinetics parameters were obtained by fitting to appropriate nonlinear regression model. Michaelis-Menten equation was used to fit phthalazine substrate saturation data whereas DACA substrate inhibition data was fitted to substrate inhibition model.

*Micahelis-Menten model*

\[
V = \frac{V_{\text{max}} [S]}{(K_{m} + [S])}
\]

*Substrate inhibition model*

\[
V = \frac{V_{\text{max}} [S]}{(K_{m}+[S] (1+[S]/K_{i}))}
\]
[S] is substrate concentration and \( V_{\text{max}} \) is the maximum reaction velocity. \( K_m \) is the Michaelis-Menten constant or substrate concentration at half \( V_{\text{max}} \) and \( K_i \) is the inhibition constant at high concentration of the substrate (in this case it is DACA).

Data obtained from linear regression of Lineweaver-Burke plot was used to generate replot of slope and y intercept data. Statistical analysis performed on the replot data was used to determine the mode of inhibition. The slope and y intercept values obtained from the replot graph were analysed using ‘F-test’, a feature built into the GraphPad Prism software (version 4.04; GraphPad Software Inc. San Diego, CA). \( P \) value generated by this test was used to determine if the slope and y-intercept were statistically significant. Slope and y-intercept replots with a \( p \leq 0.05 \) were considered significantly non zero. Graphs with significant non zero slope in the regression line for slope replot but not for the y-intercept replot were considered to be strictly competitive. Likewise graphs with significant non zero slope in the regression line of y-intercept replot but not for slope replot were determined to be uncompetitive and graphs with significant non zero slope in the regression line of both slope and y-intercept replot were determined to be mixed mode type of inhibition. (Barr and Jones, 2011) \( K_i \) values were calculated by fitting data to a global fit non linear regression analysis using the appropriate kinetic model. All statistical analysis and data fitting was done using GraphPad Prism.

**Uncompetitive inhibition model**

\[
V = \frac{(V_{\text{app}} [S])}{(K_{\text{app}} + [S] (1 + [I]/ K_{ii}))}
\]

**Mixed mode inhibition model**

\[
V = \frac{(V_{\text{app}} [S])}{((K_{\text{app}} (1 + [I]/K_{is}) + [S] (1 + [I]/ K_{ii})))}
\]

\( V \) is the reaction velocity

\( V_{\text{app}} \) is the apparent \( V_{\text{max}} \).
[S] is the substrate concentration

\( K_{\text{app}} \) is the apparent Michaelis-Menten constant.

[I] is the inhibitor concentration.

\( K_{i_s} \) is the dissociation constant for the enzyme inhibitor complex

\( K_{i_{ii}} \) is the dissociation constant for the enzyme-substrate-inhibitor complex

**Homology Modelling**

Homology modelling uses a template structure (a protein with of known structure) to construct a hypothetical tertiary structure model of a target protein from a primary sequence. In general the higher the homology between the template and the target primary sequences, the better the model. Proteins with higher than 20% homology are normally considered to have similar tertiary structure (Shimoji et al., 1998). Modelling was done with Schrödinger’s Prime module to generate a protein structure followed by induced fit docking workflow using DACA as a ligand to refine residues within 5 angstroms of the DACA ligand. The template structure was from pdb file 3zyv of mouse AOX3 (Coelho et al., 2012). Sequence alignment used ClustalW and the alignment did not need to be adjusted due to the high homology (79% homology) of the two primary sequences. For the portions of the mouse structure that did have good electron density, the human enzyme was modelled using the energy-based method in Prime. However residues 168-200 could not be replaced are not included on the model. Both the rhesus monkey and the guinea pig were modelled from the final human structure.

**RESULTS**

Enzyme kinetic parameters for phthalazine and DACA oxidase in human, guinea pig and rhesus monkey have been listed in Table 1. Oxidation of phthalazine in all three species followed Michaelis-Menten kinetics (Figure 1). In contrast, substrate inhibition was seen for DACA
oxidase activity in guinea pig, rhesus monkey and human liver cytosols (Figure 2). Phthalazine oxidase from rhesus monkey was found to be most efficient with a high \( V_{\text{max}} \) and low \( K_m \). The intrinsic clearance \( (V_{\text{max}}/K_m) \) observed with rhesus monkey phthalazine oxidase was 16.4 ml min\(^{-1}\)mg\(^{-1}\) which was 3 and 20 fold greater than the intrinsic clearance for guinea pig and human respectively. The order of AOX activity with phthalazine was rhesus monkey > guinea pig > human. In contrast to the phthalazine oxidase activity, DACA oxidase activity observed with rhesus monkey was between guinea pig and human clearances. Guinea pig had the highest \( V_{\text{max}}/K_m \) value mainly due to a much lower \( K_m \) relative to monkey and humans. A general caveat to be aware of is that while human and guinea pig liver cytosol was obtained from 150 and 50 donors respectively, rhesus monkey liver cytosol on the other hand was pooled from only 6 donors. Given the small pool size, activity observed in this pool might not be representative of the general population.

Raloxifene is a potent inhibitor of AOX with IC\(_{50}\) and \( K_i \) values in the low nanomolar range. Previous reports have shown that raloxifene is an uncompetitive inhibitor of phthalazine oxidation and a competitive inhibitor of DACA oxidation in humans. Like Obach’s human data, our findings for guinea pig also indicate that raloxifene is an uncompetitive inhibitor of phthalazine oxidase activity (Figure 3). However, the inhibitory potency observed in guinea pig liver was much lower than what was reported by Obach in human liver. We observe a \( K_i \) value of 42nM for guinea pig compared to the \( K_i \) value of 0.8nM that is observed with human liver (Table 2). Raloxifene also uncompetitively inhibits DACA oxidase activity in guinea pig with a \( K_i \) value of 230nM, while a previous investigation carried out by Barr and Jones showed raloxifene is a competitive inhibitor of DACA oxidase activity in human liver cytosol (Barr and Jones, 2013). Differences in inhibition patterns between monkeys and humans were also seen for both substrates. Mixed mode inhibition was seen in rhesus monkey liver for phthalalazine and DACA
as substrates (Figure 3) with $K_{\text{in}}$ values of 3.6 and 2.1μM and $K_{\text{i}}$ values of 0.5 and 1.3μM respectively.

Analysis of primary sequence alignment of guinea pig, rhesus monkey and human AOX in combination with homology modelling (see Methods section) led to the identification of several residue differences within the active site and substrate entrance channel of the enzyme. It should be noted that while the three proteins have very high homology with the template mouse AOX3, which increases confidence in the structure, this is still a model and the active-site structures could be different than those predicted by the homology models. Since rhesus monkey shows 96% identity, difference in rates and inhibition patterns can be hypothesized about using the monkey sequence in comparison with the human sequence. In primary sequence order the differences between human and monkey in active-site and entrance channel residues are: K718E, V811A, L812F, F885L, F1014Y, I1085V, and M1149I.
DISCUSSION

Several studies have reported high intrinsic clearance for different AOX substrates in monkey and human followed by rabbit, guinea pig and rodents (Takasaki et al., 2005; Kitamura et al., 2006; Fukiya et al., 2010). Sahi et al. reported to have seen the highest AOX activity with vanillin in monkey followed by mouse, human and rat (Sahi et al., 2008). Beedham et al. showed monkey hepatic cytosol to have highest AOX activity with N-heterocyclic compounds (Beedham et al., 1987). However, the recent results by Dalvie indicate that zoniporide intrinsic clearance by rhesus monkey S9 is low when compared to human and guinea pig (Dalvie et al., 2013). The molecular basis for these species and substrate differences are not known. Herein we investigate AOX mediated metabolism of a physiological cation, DACA, and a neutral small molecule AOX substrate, phthalazine, in humans, guinea pig and rhesus monkey and attempt to develop structural hypotheses consistent with differences in inhibition and clearance for each species.

Phthalazine is oxidized by rhesus monkey with the highest intrinsic clearance, followed by guinea pig and then human. Using guinea pig to estimate intrinsic clearance by humans would lead to a significant 11-fold overestimation of clearance based on specific activity (kcat/Km). These differences in clearance result from about a 3-fold higher affinity for AOX as judged by the lower Km values in both monkey and guinea pig, and a higher Vmax. Based on the isotope effects that have been measured for oxidation by AOX of numerous substrates (Alfaro et al., 2009; Sharma et al., 2012), oxidation appears to be rate limiting and the substrate is not committed to catalysis. This means that differences in kcat reflect the ability of the different AO active-sites in the different species to accommodate the transition state associated with oxidation. Thus, both monkey and guinea pig AOX stabilize the transition state by around 0.5 kcal/mol relative to the human enzyme. Furthermore, the low commitment to catalysis supports the use of Km as a measure of affinity since debinding is fast relative to the forward oxidation step. Both rhesus monkey and guinea pig AO bind phthalazine about 0.5 kcal/mol tighter than human AOX.
DACA, in contrast to phthalazine, is cleared faster by guinea pig AOX than monkey AOX. Using guinea pig to estimate human clearance would lead to about a 20-fold overestimation of clearance not correcting for specific activity, which is unknown, while rhesus monkey would only lead to a modest 2-fold overestimation when the specific activity of human and monkey AO is accounted for. Interestingly, monkeys and humans bind the cationic DACA with almost identical affinities, while guinea pig binds DACA almost 2 kcal/mol tighter.

Overall, results from measuring the clearance of both DACA and phthalazine indicate that both guinea pig and rhesus monkey would overestimate intrinsic clearance by significant amounts and that the differences are substrate dependent. These differences can be attributed to structural differences in the AOX active site as discussed below. One caveat is that the V/K of both DACA and phthalazine is high, so for these compounds scaling to in vivo would lead to the conclusion that they are both blood flow limited for all species.

Another very noticeable, and problematic species difference, is the level of homotropic negative allosterism (substrate inhibition) associated with DACA. Substrate inhibition results from a second substrate binding to the ES complex inhibiting turnover. Substrate inhibition was observed with DACA in guinea pig and rhesus monkey with Kᵢ values of 3.2 and 22μM respectively which indicate that the second substrate binds with significantly higher affinity for these species than for human AOX which has a Kᵢ value of 652μM. While inhibition could occur by binding to allosteric site removed from the active catalytic site for oxidation, the broad substrate selectivity associated with AOX makes this enzyme potentially similar to the P450 enzymes that can bind multiple substrates at or near the active-site (Roberts et al., 2011).

Raloxifene is a potent AOX inhibitor in vitro in humans and has a potential to produce clinical drug interactions (Obach, 2004; Barr and Jones, 2013). In guinea pig, raloxifene inhibited phthalazine and DACA oxidase activity uncompetitively. This mechanism of inhibition is in agreement with data obtained by Obach 2004 who also observed uncompetitive inhibition of...
phthalazine, vanillin and nicotine iminium oxidase activity in human liver cytosol. In contrast, Barr and Jones found raloxifene inhibition of DACA oxidase activity in human liver to be competitive. Whilst raloxifene inhibition of DACA and phthalazine activity in guinea pig was entirely uncompetitive, mixed mode inhibition was observed for both substrates in rhesus monkey. Mixed mode inhibition occurs when the inhibitor can bind to the free as well as the enzyme substrate complex. Thus, the mechanism of inhibition is both species and substrate dependent. Furthermore, as observed for mouse AOX3, raloxifene inhibits both monkey and guinea pig with much less potent $K_i$ values.

Previous reports have shown that differences in size, shape and amino acid residues in the active site and substrate access channel of AOX can perturb interactions and binding affinity (Beedham et al., 1995; Schumann et al., 2009; Garattini et al., 2003). In order to understand the structural determinants underlying these differences in activity, active site and substrate channel residues were identified based on a homology models built for each species based on the crystal structure of mAOX3 (Coelho et al., 2012). Two dimensional interaction diagrams for DACA and the active-site are shown in Figure 4. Figure 4 shows identical interactions around the acridine ring for all three species with mainly lipophilic interactions, and a $\pi-\pi$ stacking interaction associated with PHE923. In addition GLU882 is conserved and interacts by accepting a hydrogen bond from the amide proton of DACA. The major difference is in the binding environment around the positively charged aliphatic nitrogen. While all three species have an electrostatic interaction via ASP881, guinea pig has two methionines (MET885 and MET1023) interacting with the methyl groups and the cationic nitrogen. These methionine residues could enhance DACA binding in guinea pig AO by lipophilic interactions with methyl groups, electrostatic interactions with the nitrogen cation, and/or hydrogen bonding interactions (Kollman et al., 1975). In contrast, neither in human or monkey AOX does DACA have interactions with amino acid 1023, which is an alanine for both species, and amino acid 885 is a phenylalanine in human and a leucine in
monkeys. It can be hypothesized that these two methionines may play an important role in the lower $K_M$ observed for DACA binding in guinea pig AOX.

Figure 5 shows the substrate access channel and binding site for each species color coded by the amino acid properties. The red regions around the positively charged nitrogen are from interactions with negatively charged ASP881 and GLU882. The green lipophilic patches show strong lipophilic binding interactions around DACA’s acridine ring and are prevalent in the entrance channel to the binding pocket. Substrate inhibition can be explained by the binding of a second substrate in the entrance channel to the active-site. The large lipophilic patches just outside the binding site provide a location for a second DACA acridine ring to bind. Interestingly, directly adjacent to this lipophilic site in humans is a lysine (LYS718), which would be a cation at physiological pH. In contrast, amino acid 718 is a glutamate, an anion at physiological pH, in both guinea pig and monkey. It can be hypothesized that the anionic glutamate can function as a hydrogen bond acceptor from the protonated nitrogen of DACA and explain why substrate inhibition is so much stronger in guinea pig and monkey than in humans.

In summary, there is considerable amount of substrate dependent variation in AOX mediated drug metabolism and inhibition between human, guinea pig and rhesus monkey. However, if we can understand the molecular basis of binding and inhibition we may be able to rationally choose animals models for specific classes (e.g. based on charge and size) of compounds. Whole cell systems which have been engineered to epigenetically suppress AOX in human cells might also be better alternatives to using animal models (Garattini and Terao, 2012). Another approach is to use computational models which have been shown to be predictive for regioselectivity and intrinsic clearance (Torres et al., 2007; Jones and Korzekwa, 2013).
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Choughule, Jones

Conducted experiments: Choughule, Barr

Contributed new reagents or analytic tools: N/A

Performed data analysis: Choughule, Jones

Wrote or contributed to the writing of the manuscript: Choughule, Barr and Jones
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I. System-dependent effects of specific deuteration with aldehyde oxidase cleared drugs.

*Drug Metab Dispos* **40**:625-634.


FOOTNOTES

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FIGURE LEGENDS

Figure 1: Saturation kinetics plots for AOX catalysed oxidation of phthalazine in guinea pig (A), rhesus monkey (B) and human liver (C). Each value represents an average ± SEM for triplicate determinations.

Figure 2: Saturation kinetics plots for AOX catalysed oxidation of DACA in guinea pig (A), rhesus monkey (B) and human liver (C). Each value represents an average ± SEM for triplicate determinations except DACA human liver measurement which was done in duplicate. Shown as an inset are Eadie-Hofstee plots for each species.

Figure 3: Lineweaver-Burke plots and slope intercept replots (inset) for raloxifene inhibition of phthalazine (A and B) and DACA (C and D) activity in guinea pig (A and C) and rhesus monkey (B and D) liver cytosol. Each point reflects an average of duplicate determinations.

Figure 4: 2-dimensional interaction diagrams for the homology models of each AOX1 enzyme. Green indicates lipophilic interactions, red is negative electrostatic interactions, PHE923 has pi-stacking interactions with the substrate in all the structures.

Figure 5: Binding site of AOX1 from different species. Red represent negative charge regions, green lipophilic regions, cyan glycine rich regions, and purple represent positive charge regions.
Table 1: Saturation kinetic parameters for AOX catalysed oxidation of phthalazine and DACA in guinea pig (GPLC), rhesus monkey (RMLC) and human (HLC) liver. Each value represents an average ± SEM for triplicate determinations except DACA HLC which was done in duplicate.

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<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>nmol min$^{-1}$ mg$^{-1}$</td>
<td>$\mu$M</td>
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<tr>
<td>GPLC</td>
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Table 2: Inhibition constants for the inhibition of phthalazine and DACA oxidase activity in guinea pig, rhesus monkey and human liver by raloxifene. * Obach, 2004; ¥ Barr and Jones 2012

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Figure 1

A) Reaction velocity (nmol product formed min⁻¹ mg⁻¹) vs. [phthalazine]; µM

B) Reaction velocity (nmol product formed min⁻¹ mg⁻¹) vs. [phthalazine]; µM

C) Reaction velocity (nmol product formed min⁻¹ mg⁻¹) vs. [phthalazine]; µM
Figure 4

Rhesus monkey

Guinea pig

Human
Figure 5

Rhesus monkey

Guinea pig

Human
Evaluation of Rhesus Monkey and Guinea Pig Hepatic Cytosol Fractions as Models for Human Aldehyde Oxidase.

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Supplemental figure 1

Effect of increasing DMSO concentration on DACA oxidase activity in human (HLC), guinea pig (GPLC) and rhesus monkey (RMLC) liver cytosol.
**Supplemental figure 2**

Effect of 0.5% DMSO on phthalazine oxidase activity in guinea pig liver cytosol

**Supplemental figure 3**

Effect of 0.5% DMSO on phthalazine oxidase activity in rhesus monkey liver cytosol