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

Kanika V. Choughule, John T. Barr, and Jeffrey P. Jones

Department of Chemistry, Washington State University, Pullman, Washington

Received May 16, 2013; accepted August 5, 2013

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 AOX-mediated metabolism. We compared AOX activity in rhesus monkeys, guinea pigs, and humans using phthalazine and *N*-[2-(dimethylamino)ethyl]acridone-4-carboxamide (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 three 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 aldehyde oxidase isoform 1 (AOX1) along with homology modeling 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 monkeys and guinea pigs would overestimate intrinsic clearance in humans and would be unsuitable to use as animal models. Our study also showed that AOX 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) catalyzes 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 metabolized 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 IC₅₀ values less than 10 μ 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 synthesize aldehyde oxidase isoform 1 (AOX1) as the only isoform in the 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, such as the beagle dog, routinely used in drug metabolism studies are devoid of AOX activity (Austin et al., 2001). For quite some time now, guinea pigs and rhesus monkeys have been thought to recapitulate the drug metabolism brought about by AOX in human liver. This is because, in the liver, both of these animals are thought to synthesize a sole active AOX1 which is an ortholog of human and mouse AOX1 (Garattini and Terao, 2013). Although there are a few studies that support the monkey (rhesus and cynomolgous) 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 evaluating the metabolism of a clinical drug, zonisamide, has been conducted in humans and compared with both guinea pigs and rhesus monkeys. The intrinsic clearance of this compound observed in the livers of guinea pigs and rhesus monkeys was lower than in humans. The authors concluded that rhesus monkeys and guinea pigs could potentially underpredict the clearance of zonisamide in humans if used as surrogates (Dalvie et al., 2013).

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 humans, guinea pigs, and rhesus monkeys. 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

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM100874].

dx.doi.org/10.1124/dmd.113.052985.

[§]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: AOX, aldehyde oxidase; AOX1, aldehyde oxidase isoform 1; DACA, (*N*-[2-(dimethylamino)ethyl]acridone-4-carboxamide); DMSO, dimethylsulfoxide; GPLC, guinea pig liver cytosol; IS, internal standard; RMLC, rhesus monkey liver cytosol.

oxidase activity (Barr and Jones, 2013) in human liver, with IC_{50} and K_i 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 pigs and rhesus monkeys 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

DACA was synthesized in our laboratory (Barr and Jones, 2013), and DACA acridone was provided by Dr. William A. Denny from the 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) pooled from 50 male donors, and rhesus monkey liver cytosol (RMLC), pooled from 6 male donors, were purchased from XenoTech, LLC (Lenexa, KS). Human liver cytosol, 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 the phthalazine oxidase activity assay, with minor differences. DACA oxidase activity in GPLC and RMLC was linear up to 30 minutes. All incubations were carried out for 5 minutes using 0.05 mg/ml liver cytosol and the same incubation buffer. For determination of saturation kinetics constants V_{max} and K_m , stock solutions of DACA were made in dimethylsulfoxide (DMSO; the effect of DMSO on activity is shown in Supplemental Fig. 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 was also 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 and 1–10 μ M 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 the 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, 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 minutes, and hence all incubations with phthalazine were carried out for 2.5 minutes with 0.075 mg/ml liver cytosol in 25 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA in a final incubation volume of 0.8 ml. Stock solutions of phthalazine were made up in potassium phosphate buffer. For determination of the inhibition constant K_i , raloxifene was made up in DMSO (the effect of DMSO on activity is shown in Supplemental Figs. 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 10–100 nM and 0.5–6 μ M was used to inhibit phthalazine oxidase activity in guinea pig and rhesus monkey liver cytosol, respectively. For determination of saturation kinetics constants V_{max} and K_m , no inhibitor was added to the reaction mixture. Incubation mixture with or without raloxifene was prewarmed at 37°C for 5 minutes and reaction was initiated by the addition of 0.2 ml of liver cytosol. After 2.5 minutes, the reaction was terminated by the addition of 0.2 ml of 1 M 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 (Eppendorf, Mississauga, ON, Canada) at 5000g for 10 minutes, and the supernatant was collected for analysis.

Detection and Analysis of the Formation of 1-Phthalazinone and DACA-9(10H) Acridone Using High-Performance Liquid Chromatography–Tandem Mass Spectrometry Assay. The high-performance liquid chromatography tandem mass spectrometry 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 the DACA metabolite DACA-9(10H) acridone was carried out as per Barr and Jones (2013). Samples were analyzed on an 1100 series high-performance liquid chromatography system (Agilent Technologies, Santa Clara,

CA) coupled to an API 4000 tandem mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster City, CA). A Synergi Polar reverse-phase column (30 × 3.0 mm, 4 μ M; 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 comprised 90% acetonitrile, 9.9% water, and 0.1% formic acid. Total chromatographic separation time was 5 minutes per sample. Retention times for IS and 1-phthalazinone were 2.5 and 2.8 minutes, respectively, and for IS and DACA-9(10H) acridone were 1.4 and 1.8 minutes, respectively. All samples were analyzed using positive ion mode on an electrospray ionization interface. 1-Phthalazinone and 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 μ M. DACA-9(10H) acridone and 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 the mean $\pm$ S.E.M. for three experiments performed on different days. Enzyme kinetics parameters were obtained by fitting to the appropriate nonlinear regression model. The Michaelis-Menten equation was used to fit phthalazine substrate saturation data, whereas DACA substrate inhibition data were fitted to the substrate inhibition model.

Michaelis-Menten model:

$$V = V_{max}[S]/(K_m + [S])$$

Substrate inhibition model:

$$V = V_{max}[S]/(K_m + [S](1 + [S]/K_i))$$

[S] is substrate concentration and V_{max} is the maximum reaction velocity. K_m is the Michaelis-Menten constant or substrate concentration at half V_{max} and K_i is the inhibition constant at a high concentration of the substrate (in this case, it is DACA).

Data obtained from linear regression of the Lineweaver-Burk plot were used to generate a 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 analyzed using F-test, a feature built into the GraphPad Prism software (version 4.04; GraphPad Software, Inc., San Diego, CA). The 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 $P \leq 0.05$ were considered significantly nonzero. Graphs with significant nonzero 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 nonzero slope in the regression line of the y intercept replot, but not for slope replot, were determined to be uncompetitive, and graphs with significant nonzero slope in the regression line of both the slope and y intercept replot were determined to be a mixed-mode type of inhibition (Barr and Jones, 2011). K_i values were calculated by fitting data to a global fit nonlinear regression analysis using the appropriate kinetic model. All statistical analysis and data fitting was done using GraphPad Prism.

Uncompetitive inhibition model:

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

Mixed mode inhibition model:

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

V is the reaction velocity; V_{app} is the apparent V_{max} ; [S] is the substrate concentration; K_{app} is the apparent Michaelis-Menten constant; [I] is the inhibitor concentration; K_{is} is the dissociation constant for the enzyme inhibitor complex; and K_{ii} is the dissociation constant for the enzyme-substrate-inhibitor complex.

Homology Modeling. Homology modeling uses a template structure (a protein of known structure) to construct a hypothetical tertiary structure model of a target protein from a primary sequence. In general, the higher the homology is 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). Modeling was done with Schrödinger’s Prime module (Schrodinger, Portland, Oregon). to generate a protein structure followed by induced fit docking workflow using DACA as a ligand to refine residues within 5 Å of the DACA ligand. The template structure was from PDB ID 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 modeled using the energy-based method in Prime. However, residues 168–200 could not be replaced and are not included in the model. Both the rhesus monkey and the guinea pig were modeled from the final human structure.

Results

Enzyme kinetic parameters for phthalazine and DACA oxidase in humans, guinea pigs, and rhesus monkeys are listed in Table 1. Oxidation of phthalazine in all three species followed Michaelis-Menten kinetics (Fig. 1). In contrast, substrate inhibition was seen for DACA oxidase activity in guinea pig, rhesus monkey, and human liver cytosols (Fig. 2). Phthalazine oxidase from rhesus monkeys was found to be most efficient, with a high V_{max} and low K_m . The intrinsic clearance (V_{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 pigs and humans, respectively. The order of AOX activity with phthalazine was rhesus monkey > guinea pig > human. In contrast to the phthalazine oxidase activity, DACA oxidase activity

TABLE 1
Saturation kinetic parameters for AOX-catalyzed oxidation of phthalazine and DACA in GPLC, RMLC, and human liver cytosol

Each value represents an average ± S.E.M. for triplicate determinations except DACA and human liver cytosol (HLC) which was done in duplicate.

	V_{max} $nmol\ min^{-1}\ mg^{-1}$	K_m μM	K_i μM	V_{max}/K_m $ml\ min^{-1}\ mg^{-1}$
Phthalazine				
GPLC	26.3 ± 0.9	4.5 ± 0.7	NA	5.8
RMLC	55.8 ± 2.7	3.4 ± 0.8	NA	16.4
HLC	9.2 ± 0.4	12.1 ± 1.7	NA	0.8
DACA				
GPLC	2.2 ± 0.2	0.3 ± 0.07	3.2 ± 0.8	7.3
RMLC	14.8 ± 3.7	8.7 ± 3.3	22.32 ± 9.4	1.7
HLC	3.7 ± 0.08	8.8 ± 0.4	652.2 ± 78.35	0.4

NA, not applicable.

observed with rhesus monkeys was between guinea pig and human clearances. Guinea pigs had the highest V_{max}/K_m value mainly due to a much lower K_m relative to monkeys and humans. A general caveat to be aware of is that, although human and guinea pig liver cytosol was obtained from 150 and 50 donors, respectively, rhesus monkey liver cytosol was pooled from only six donors. Given the small pool size, activity observed in this pool might not be representative of the general population.

Fig. 1. Saturation kinetics plots for AOX-catalyzed oxidation of phthalazine in guinea pig (A), rhesus monkey (B), and human liver (C). Each value represents an average ± S.E.M. for triplicate determinations.

Fig. 2. Saturation kinetics plots for AOX-catalyzed oxidation of DACA in guinea pig (A), rhesus monkey (B), and human liver (C). Each value represents an average $\pm$ S.E.M. for triplicate determinations except the DACA human liver measurement, which was performed in duplicate. Shown as an inset are Eadie-Hofstee plots for each species.

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. Similar to Obach's human data (Obach, 2004), our findings for guinea pigs also indicate that raloxifene is an uncompetitive inhibitor of phthalazine oxidase activity (Fig. 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 42 nM for guinea pig compared with the K_i value of 0.8 nM that is observed with human liver (Table 2). Raloxifene also uncompetitively inhibits DACA oxidase activity in guinea pigs, with a K_i value of 230 nM, whereas a previous investigation carried out by Barr and Jones (2013) showed raloxifene is a competitive inhibitor of DACA oxidase activity in human liver cytosol. Differences in inhibition patterns between monkeys and humans were also seen for both substrates. Mixed-mode inhibition was seen in rhesus monkey liver with phthalazine and DACA as substrates (Fig. 3), with K_{is} values of 3.6 and 2.1 μ M and K_{ii} values of 0.5 and 1.3 μ M, respectively.

Analysis of primary sequence alignment of guinea pig, rhesus monkey, and human AOX in combination with homology modeling (see *Materials and Methods*) led to the identification of several residue differences within the active-site and substrate entrance channel of the enzyme. It should be noted that, although 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 humans and monkeys in active-site and entrance channel residues are as follows: K718E, V811A, L812F, F885L, F1014Y, I1085V, and M1149I.

Discussion

Several studies have reported high intrinsic clearance for different AOX substrates in monkeys and humans followed by rabbits, guinea pigs, and rodents (Takasaki et al., 2005; Kitamura et al., 2006; Fukiya et al., 2010). Sahi et al. (2008) reported to have seen the highest AOX activity with vanillin in monkeys followed by mice, humans, and rats. Beedham et al. (1987) showed monkey hepatic cytosol to have the highest AOX activity with *N*-heterocyclic compounds. However, recent results by Dalvie et al. (2013) indicate that zonisamide intrinsic clearance by rhesus monkey S9 is low when compared with humans and guinea pigs. 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 pigs, and rhesus monkeys, and attempt to develop structural hypotheses consistent with differences in inhibition and clearance for each species.

Fig. 3. Lineweaver-Burk 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.

Phthalazine is oxidized by rhesus monkeys with the highest intrinsic clearance, followed by guinea pigs and then humans. Using guinea pigs to estimate intrinsic clearance by humans would lead to a significant 11-fold overestimation of clearance based on specific activity (k_{cat}/K_m). These differences in clearance result from about a 3-fold higher affinity for AOX as judged by the lower K_m values in both monkeys and guinea pigs, and a higher V_{max} . 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 k_{cat} reflect the ability of the different AOX 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 K_m as a measure of affinity since debinding is fast relative to the forward oxidation step. Both rhesus monkey and guinea pig AOX 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 pigs to estimate human clearance would lead to a about a 20-fold overestimation of clearance, not correcting for specific activity, which is unknown, whereas rhesus monkeys would only lead to a modest 2-fold overestimation when the specific activity of human and monkey AOX is accounted for. Interestingly, monkeys and humans bind the cationic DACA with almost identical affinities, whereas guinea pigs bind DACA almost 2 kcal/mol tighter. Overall, results from measuring the clearance of both DACA and phthalazine indicate that both guinea pigs and rhesus monkeys would

TABLE 2
Inhibition constants for the inhibition of phthalazine and DACA oxidase activity in guinea pig, rhesus monkey, and HLC by raloxifene

	Phthalazine			DACA		
	K_{is}	K_{ii}	K_{ii}/K_{is}	K_{is}	K_{ii}	K_{ii}/K_{is}
	μM			μM		
GPLC	NA	0.042 ± 0.002	NA	NA	0.23 ± 0.017	NA
RMLC	3.6 ± 2.4	0.5 ± 0.027	0.14	2.14 ± 0.246	1.34 ± 0.1	0.6
HLC	NA	0.00087 ± 0.00005 ^a	NA	0.0023 ± 0.0006 ^b	NA	NA

HLC, human liver cytosol; NA, not applicable.
^aObach, 2004.
^bBarr and Jones, 2012.

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 later. One caveat is that the V/K (V_{Max}/K_M) 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 (Enzyme-substrate complex), inhibiting turnover. Substrate inhibition was observed with DACA in guinea pigs and rhesus monkeys, with K_i values of 3.2 and 22 μM , respectively, which indicate that the second substrate binds with significantly higher affinity for these species than for human AOX, which has a K_i value of 652 μM . Although inhibition could occur by binding to the allosteric site removed from the active catalytic site for oxidation, the broad substrate selectivity associated with AOX makes this enzyme potentially similar to the cytochrome 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 pigs, 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 (2013) found raloxifene inhibition of DACA oxidase activity in human liver to be competitive. Although raloxifene inhibition of DACA and phthalazine activity in guinea pigs was entirely uncompetitive, mixed-mode inhibition was observed for both substrates in rhesus monkeys. 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; Garattini et al., 2003; Schumann et al., 2009). To understand the

structural determinants underlying these differences in activity, active-site and substrate channel residues were identified based on homology models built for each species based on the crystal structure of mAOX3 (mouse AOX3) (Coelho et al., 2012). Two-dimensional interaction diagrams for DACA and the active site are shown in Fig. 4. Figure 4 shows identical interactions around the acridine ring for all three species with mainly lipophilic interactions and a π - π 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. Although all three species have an electrostatic interaction via Asp881, guinea pigs have two methionines (Met885 and Met1023) interacting with the methyl groups and the cationic nitrogen. These methionine residues could enhance DACA binding in guinea pig AOX 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 nor in monkey AOX does DACA interact with amino acid 1023, which is an alanine for both species, and amino acid 885 is a phenylalanine in humans 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 pigs and monkeys. It can be hypothesized that the anionic glutamate can function as a hydrogen bond acceptor from the protonated nitrogen of DACA and would explain why substrate inhibition is so much stronger in guinea pigs and monkeys than in humans.

Fig. 4. Two-dimensional interaction diagrams for the homology models of each AOX1 enzyme. Green indicates lipophilic interactions, and red is negative electrostatic interactions. Phe923 has π -stacking interactions with the substrate in all the structures.

Fig. 5. Binding site of AOX1 from different species. Red represents negative charge regions, green represents lipophilic regions, cyan represents glycine-rich regions, and purple represents positive charge regions. MoCo is the molybdenum cofactor and DACA is the substrate.

In summary, there is a considerable amount of substrate-dependent variation in AOX-mediated drug metabolism and inhibition between humans, guinea pigs, and rhesus monkeys. However, if we can understand the molecular basis of binding and inhibition, we may be able to rationally choose animal 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.

Performed data analysis: Choughule, Jones.

Wrote or contributed to the writing of the manuscript: Choughule, Barr, Jones.

References

- Alfaro JF, Joswig-Jones CA, Ouyang W, Nichols J, Crouch GJ, and Jones JP (2009) Purification and mechanism of human aldehyde oxidase expressed in *Escherichia coli*. *Drug Metab Dispos* **37**:2393–2398.
- Austin NE, Baldwin SJ, Cutler L, Deeks N, Kelly PJ, Nash M, Shardlow CE, Stemp G, Thewlis K, and Ayrton A, et al. (2001) Pharmacokinetics of the novel, high-affinity and selective dopamine D3 receptor antagonist SB-277011 in rat, dog and monkey: in vitro/in vivo correlation and the role of aldehyde oxidase. *Xenobiotica* **31**:677–686.
- Barr JT and Jones JP (2011) Inhibition of human liver aldehyde oxidase: implications for potential drug-drug interactions. *Drug Metab Dispos* **39**:2381–2386.
- Barr JT and Jones JP (2013) Evidence for substrate-dependent inhibition profiles for human liver aldehyde oxidase. *Drug Metab Dispos* **41**:24–29.
- Beedham C, Bruce SE, Critchley DJ, al-Tayib Y, and Rance DJ (1987) Species variation in hepatic aldehyde oxidase activity. *Eur J Drug Metab Pharmacokin* **12**:307–310.
- Beedham C, Critchley DJ, and Rance DJ (1995) 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.
- Beedham C, Miceli JJ, and Obach RS (2003) Ziprasidone metabolism, aldehyde oxidase, and clinical implications. *J Clin Psychopharmacol* **23**:229–232.
- Coelho C, Mahro M, Trincão J, Carvalho AT, Ramos MJ, Terao M, Garattini E, Leimkübler S, and Romão MJ (2012) The first mammalian aldehyde oxidase crystal structure: insights into substrate specificity. *J Biol Chem* **287**:40690–40702.
- Dalvie D, Xiang C, Kang P, and Zhou S (2013) Interspecies variation in the metabolism of zonisamide by aldehyde oxidase. *Xenobiotica* **43**:399–408.
- Dittrich Ch, Greim G, Börner M, Weigang-Köhler K, Huisman H, Amelsberg A, Ehret A, Wanders J, Hanauske A, and Fumoleau P (2002) Phase I and pharmacokinetic study of BIBX 1382 BS, an epidermal growth factor receptor (EGFR) inhibitor, given in a continuous daily oral administration. *Eur J Cancer* **38**:1072–1080.
- Fukuiya K, Itoh K, Yamaguchi S, Kishiba A, Adachi M, Watanabe N, and Tanaka Y (2010) A single amino acid substitution confers high cinchonidine oxidation activity comparable with that of rabbit to monkey aldehyde oxidase 1. *Drug Metab Dispos* **38**:302–307.
- Garattini E, Mendel R, Romão MJ, Wright R, and Terao M (2003) Mammalian molybdo-flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. *Biochem J* **372**:15–32.
- Garattini E and Terao M (2012) The role of aldehyde oxidase in drug metabolism. *Expert Opin Drug Metab Toxicol* **8**:487–503.
- Garattini E and Terao M (2013) Aldehyde oxidase and its importance in novel drug discovery: present and future challenges. *Expert Opin Drug Discov* **8**:641–654.
- Hutzler JM, Obach RS, Dalvie D, and Zientek MA (2013) Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin Drug Metab Toxicol* **9**:153–168.
- Jones JP and Korzekwa KR (2013) Predicting intrinsic clearance for drugs and drug candidates metabolized by aldehyde oxidase. *Mol Pharm* **10**:1262–1268.
- Kitamura S, Sugihara K, and Ohta S (2006) Drug-metabolizing ability of molybdenum hydroxylases. *Drug Metab Pharmacokin* **21**:83–98.
- Kollman P, McKelvey J, Johansson A, and Rothenberg S (1975) Theoretical studies of hydrogen-bonded dimers. Complexes involving HF, H₂O, NH₃, CH₄, H₂S, PH₃, HCN, HNC, HCP, CH₂NH, H₂CN, H₂CO, CH₄, CF₃H, C₂H₂, C₂H₄, C₆H₆, F₂, and H₃O⁺. *J Am Chem Soc* **97**:955–965.
- Morrison RD, Blobaum AL, Byers FW, Santomango TS, Bridges TM, Stec D, Brewer KA, Sanchez-Ponce R, Corlew MM, and Rush R, et al. (2012) The role of aldehyde oxidase and xanthine oxidase in the biotransformation of a novel negative allosteric modulator of metabotropic glutamate receptor subtype 5. *Drug Metab Dispos* **40**:1834–1845.
- Obach RS (2004) Potent inhibition of human liver aldehyde oxidase by raloxifene. *Drug Metab Dispos* **32**:89–97.
- Obach RS, Huynh P, Allen MC, and Beedham C (2004) Human liver aldehyde oxidase: inhibition by 239 drugs. *J Clin Pharmacol* **44**:7–19.
- Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, and Tran TD (2010) Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *J Med Chem* **53**:8441–8460.
- Rashidi MR, Smith JA, Clarke SE, and Beedham C (1997) In vitro oxidation of famciclovir and 6-deoxypenciclovir by aldehyde oxidase from human, guinea pig, rabbit, and rat liver. *Drug Metab Dispos* **25**:805–813.
- Roberts AG, Yang J, Halpert JR, Nelson SD, Thummel KT, and Atkins WM (2011) The structural basis for homotropic and heterotropic cooperativity of midazolam metabolism by human cytochrome P450 3A4. *Biochemistry* **50**:10804–10818.
- Sahi J, Khan KK, and Black CB (2008) Aldehyde oxidase activity and inhibition in hepatocytes and cytosolic fractions from mouse, rat, monkey and human. *Drug Metab Lett* **2**:176–183.
- Schumann S, Terao M, Garattini E, Saggu M, Lenzian F, Hildebrandt P, and Leimkübler S (2009) Site directed mutagenesis of amino acid residues at the active site of mouse aldehyde oxidase AOX1. *PLoS ONE* **4**:e5348.
- Sharma R, Strelevitz TJ, Gao H, Clark AJ, Schildknecht K, Obach RS, Ripp SL, Spracklin DK, Tremaine LM, and Vaz AD (2012) Deuterium isotope effects on drug pharmacokinetics. I. System-dependent effects of specific deuteration with aldehyde oxidase cleared drugs. *Drug Metab Dispos* **40**:625–634.
- Shimoji M, Yin H, Higgins L, and Jones JP (1998) Design of a novel P450: a functional bacterial-human cytochrome P450 chimera. *Biochemistry* **37**:8848–8852.
- Takasaki W, Yamamura M, Nozaki A, Nitani T, Sasahara K, Itoh K, and Tanaka Y (2005) Stereoselective pharmacokinetics of RS-8359, a selective and reversible MAO-A inhibitor, by species-dependent drug-metabolizing enzymes. *Chirality* **17**:135–141.
- Torres RA, Korzekwa KR, McMasters DR, Fandozzi CM, and Jones JP (2007) Use of density functional calculations to predict the regioselectivity of drugs and molecules metabolized by aldehyde oxidase. *J Med Chem* **50**:4642–4647.

Address correspondence to: Jeffrey P. Jones, Department of Chemistry, Washington State University, P.O. Box 644630, Pullman, WA 99164-4630. E-mail: jpj@wsu.edu