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

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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. Michaels-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), famiclovir (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 μM (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 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 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.
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-phenazinomethanol, raloxifene, and the internal standard 2-methyl-l-(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 activity in GPLC and RMLC was linear up to 30 minutes. All incubations were 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:
\[ V = \frac{V_{max}}{1 + \frac{S}{K_m}} \]

Substrate inhibition model:
\[ V = \frac{V_{max}}{1 + \frac{S}{K_m} + \frac{[S]}{K_i}} \]

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

Mixed mode inhibition model:
\[ V = \frac{V_{app}[S]}{([K_{app}(1 + [I]/K_a)] + [S](1 + [I]/K_a))} \]

\( V \) 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; \( K_i \) is the dissociation constant for the enzyme inhibitor complex; and \( K_{di} \) 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 (Schrödinger, 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 $V_{\text{max}}$ 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 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 observed with rhesus monkeys was between guinea pig and human clearances. Guinea pigs had the highest $V_{\text{max}}/K_m$ 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.

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

NA, not applicable.

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.
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 $K_i$ values of 3.6 and 2.1 $\mu$M and $K_{ii}$ values of 0.5 and 1.3 $\mu$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 zoniporide 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.
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 an 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>
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<tr>
<th>TABLE 2</th>
<th>Inhibition constants for the inhibition of phthalazine and DACA oxidase activity in guinea pig, rhesus monkey, and HLC by raloxifene</th>
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</thead>
<tbody>
<tr>
<td>Phthalazine</td>
<td>$K_i$</td>
</tr>
<tr>
<td>GPLC</td>
<td>NA</td>
</tr>
<tr>
<td>RMLC</td>
<td>3.6 ± 2.4</td>
</tr>
<tr>
<td>HLC</td>
<td>0.00087 ± 0.00005</td>
</tr>
<tr>
<td>DACA</td>
<td>$K_i$</td>
</tr>
<tr>
<td>GPLC</td>
<td>NA</td>
</tr>
</tbody>
</table>

HLC, human liver cytosol; NA, not applicable.

*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, in human liver the allosteric site is more 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 indicate that 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.
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 developed to be predictive for regioselectivity and intrinsic clearance (Torres et al., 2007; Jones and Korzekwa, 2013).

Authorship Contributions

**Conducted experiments:** Choughule, Barr.

**Performed data analysis:** Choughule, Jones.

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

**References**


Address correspondence to: Jeffrey P. Jones, Department of Chemistry, Washington State University, P.O. Box 646430, Pullman, WA 99164-4630. E-mail: jpj@wsu.edu
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