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Contribution of extra-hepatic aldehyde oxidase activity to human clearance

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Abbreviations: amu, atomic mass unit; AOX, aldehyde oxidase; BW, body weight, CL_{AOX}, total aldehyde oxidase clearance; CL_{int}, intrinsic clearance; CYP, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; FAD, flavin adenine dinucleotide; f_u, fraction unbound; LC/MS/MS, liquid chromatography-tandem mass spectrometry;

Moco, molybdenum pterin cofactor; Q_b , blood flow rate; R_{BP} , blood to plasma distribution.

Abstract:

Aldehyde oxidase (AOX) is a soluble, cytosolic enzyme that metabolizes various N-heterocyclic compounds and organic aldehydes. It has wide tissue distribution with highest levels found in liver, kidney, and lung. Human clearance projections of AOX substrates by in vitro assessments in isolated liver fractions (cytosol, S9) and even hepatocytes have been largely under-predictive of clinical outcomes. Various hypotheses have been suggested as to why this is the case. One explanation is that extra-hepatic AOX expression contributes measurably to AOX clearance and is at least partially responsible for the often observed under-predictions. Although AOX expression has been confirmed in several extra-hepatic tissues, activities therein and potential contribution to overall human clearance have not been thoroughly studied. In this work, the AOX enzyme activity using the S9 fractions of select extra-hepatic human tissues (kidney, lung, vasculature, and intestine) were measured using carbazeran as a probe substrate. Measured activities were scaled to a whole-body clearance using best-available parameters and compared to liver S9 fractions. Here, the combined scaled AOX clearance obtained from the kidney, lung, vasculature and intestine is very low and amounted to <1% of liver. This work suggests that AOX metabolism from extra-hepatic sources plays little role in the underprediction of activity in human. One of the notable outcomes of this work has been the first direct demonstration of AOX activity in human vasculature.

SIGNIFICANCE STATEMENT

This work demonstrates AOX activity is measurable in a variety of extra-hepatic human tissues, including vasculature, yet activities and potential contributions to human clearance are relatively low and insignificant when compared to the liver. Additionally, the modeling of the tissue specific in vitro kinetic data suggests that AOX may be influenced by the tissue it resides in and thus show different affinity, activity, and modified activity over time.

Introduction

Aldehyde oxidase is a molybdenum cofactor-containing cytosolic enzyme recognized for its potential to oxidize various nitrogen-containing heterocyclic compounds and organic aldehydes (Beedham, 2001; Kitamura et al., 2006; Garattini et al., 2008; Pryde et al., 2010; Terao et al., 2016; Rashidi and Soltani, 2017). Catalytically, the mechanism involves nucleophilic attack at an electron-deficient carbon and insertion of oxygen from water. Apart from oxidation reactions, research shows that AOX could also act as a reductase toward N-oxides, sulfoxides and heterocycles under hypoxic conditions (Kitamura and Tatsumi, 1984a; Kitamura and Tatsumi, 1984b). It has also been demonstrated more recently that AOX can catalyze the hydrolysis of amide bonds (Sodhi et al., 2015).

Interest in AOX has increased considerably in recent years, and DMPK laboratories have implemented assays to screen for its activity. This interest primarily stems from changing chemical-derivation or synthesis trends that, while improving drug properties, may result in clearance by non-CYP pathways. Thus, while introduction of groups such as azaheterocycles may improve log P, solubility and overall CYP liabilities, it may also increase potential for AOX-mediated metabolism. Carbazeran (Kaye et al., 1984), zoniporide (Dalvie et al., 2010), SGX523 (Diamond et al., 2010), and Lu AF09535 (Jensen et al., 2017) are examples of drug candidates that failed clinically as a result of unexpected poor pharmacokinetics or toxic metabolite formation due in part to aldehyde oxidase.

AOX-mediated metabolism can be readily captured in vitro through the use of human cytosolic and S9 liver fractions. Where AOX activity is suspected, confidence can be increased through co-incubation of test substrates with an AOX-specific inhibitor, such as hydralazine (Strelevitz et al., 2012). Though AOX-mediated metabolism can be correctly identified pre-clinically, the clearance in human has generally been under-predicted by in vitro assays, and sometimes quite substantially. A number of estimates suggest the in vitro to in vivo correlation (IVIVC) under-

prediction of $CL_{int,in vivo}$ could be as much as 10-fold on average for AOX substrates, when plasma protein binding is taken into account (Zientek et al., 2010; Akabane et al., 2012; Hutzler et al., 2013; De Sousa Mendes et al., 2020). Various explanations have been offered in the literature as to why this may be the case, and these include possible loss of enzyme activity during tissue collection and preservation and/or a loss of enzyme activity over the time course of an in vitro assay. Regarding the latter, it has recently been proposed that regeneration of the AOX enzyme in vitro may be rate-limited by flow of electrons from the molybdenum pterin cofactor (MoCo) to the flavin adenine dinucleotide (FAD) for the reduction of oxygen (Abbasi et al., 2019). In that work, the authors proposed a modulated activity model to capture the early fast-rate of the enzyme to improve clearance predictions. However, even with this model, some residual under-prediction persists. Another possibility, often speculated in the literature, is that contributions to AOX metabolism from extra-hepatic tissue could be a key factor in the underpredictions. Immunohistochemical studies have shown that AOX is broadly distributed throughout the human body (Moriwaki et al., 2001). The kidneys, adrenal glands, lungs and reproductive tissue are rich sources, amongst other tissues. A more recent study has confirmed the presence and activity of AOX in human skin (Manevski et al., 2014). While there is no current evidence suggesting that AOX metabolism at any one organ or tissue bed could rival that occurring at the liver, it cannot be ruled out that the collective contributions of many compartments could be significant.

Our objective in this current work was to measure the rates of AOX activity in the S9 fractions from various extra-hepatic tissues known or suspected to express the AOX enzyme and to use best-available physiological parameters to estimate potential contributions to total human AOX clearance. Studies were conducted using carbazeran as a probe substrate, a classic example of a compound with human clearance that is significantly underpredicted by liver tissue fractions. In this work, carbazeran is an ideal tool due to its high intrinsic activity, and although it

has been observed to undergo glucuronidation in hepatocytes (Sharma et al., 2012), it is metabolized exclusively by AOX to a single product, 4-oxo-carbazeran in human cytosol, or S9 without cofactors (Kaye et al., 1985; Xie et al., 2019) (**Figure 1**).

While we cannot capture AOX activity in every extra-hepatic tissue compartment, the exercise does reveal the potential significance of extra-hepatic contribution to the overall human AOX clearance of a drug and the collective role AOX containing extra-hepatic tissue may play in the often-observed underprediction phenomenon.

Materials and Methods

Materials

The human vasculature S9 fractions, obtained as a gift from BioIVT (Baltimore, MD), were prepared from the interior linings of iliac arteries collected during standard liver harvesting. The yield of vascular S9 per weight of tissue was calculated from the total protein yielded from the preparation as measured by the BCA protein assay (Thermo Fisher Scientific, Rockford, IL) divided by the weight of tissue used in the preparation, and this is represented as the average of three donors (one male and two females). The human kidney (whole kidney, mixed sex, pool of 8), lung (non-smoker, mixed sex, pool of 4) and intestinal (duodenal and jejunal, prepared by elution, mixed sex, pool of 15) S9 fractions were procured from XenoTech (Kansas City, KS). Human liver S9 fractions were procured from BioIVT (mixed sex, pool of 150). Carbazeran and 4-oxo-carbazeran were procured from Toronto Research Chemicals (ON, Canada), and hydralazine was procured from Sigma Aldrich (St. Louis, MO). Dialysis membranes (6-8 kDa molecular weight cut-off) for protein binding were procured from HTDialysis (Gales Ferry, CT). Water and acetonitrile were of HPLC grade and were procured from Fisher Scientific (Fair Lawn, NJ). All other reagents used in the study were of extra-pure grade.

Methods

Tissue S9 Incubations. Each tissue-specific extra-hepatic S9 fraction (kidney, lung, vasculature, intestinal) was incubated at a final protein concentration of 1 mg/mL in phosphate buffer (100 mM, pH 7.4) with 1, 3, 10 and 30 μ M carbazeran. Liver S9 fraction was incubated at a final protein concentration of 0.1 mg/mL in an identical manner. The lower end of the carbazeran concentration range (1 μ M) was chosen specifically to represent concentrations

lower than the Michaelis-Menten constant (K_M) values reported in the literature (3-6 μM), and assumed to provide the highest rate of metabolite formation (Chen et al., 2019; Tan et al., 2020; Uehara et al., 2020). Thus, the lower end of the concentrations range is assumed to provide an accurate account of maximum CL_{int} ($\sim V_{\text{max}}/K_M$). Pooled S9 products were incubated in duplicate (lung, kidney, intestinal) or triplicate (liver) at each substrate concentration. For vasculature S9, each individual donor was tested separately at each test concentration. No cofactors were added to any incubations. Incubations (300 μL starting volume) were conducted in 96 deep well plates (Thomson Instruments, Oceanside, CA) using a Thermomixer (Eppendorf, Hamburg, Germany) at 37 °C with a shaking speed of 600 rpm. Aliquots (30 μL) were removed at 0, 3, 7, 15, 30, 60 and 90 minutes post incubation and quenched with 200 μL of chilled acetonitrile containing a proprietary internal standard. Parallel incubations were conducted in the presence of hydralazine (100 μM final concentration), a known AOX inhibitor. All collected samples were centrifuged at 4000 x g for 10 minutes, and supernatants were diluted 3-fold in water prior to LC/MS/MS injection. Authentic standards of 4-oxo-carbazeran were prepared in the non-fortified S9 tissue matrices and processed in an identical manner.

Protein Binding. The protein binding assay for extra-hepatic S9 fractions was conducted using equilibrium dialysis. Tissue-specific S9 fractions (1 mg/mL protein) fortified with 1 μM compound and blank 100 mM phosphate buffer, pH 7.4 were placed on opposite sides of a divided 96-well dialysis membrane device. Samples (triplicates) were dialyzed against buffer for a total of 6 hr at 37 °C and 5% CO_2 on an oscillating platform inside a standard cell culture incubator. At the end of the incubation period, donor and receiver samples were matrix matched (10 μL of S9 study sample and 40 μL of blank assay buffer, or 40 μL of buffer study sample and 10 μL of non-fortified S9 matrix) and were quenched with 200 μL of chilled acetonitrile containing a proprietary internal standard. Quenched samples were centrifuged at

4000 x g for 10 minutes, and supernatants were diluted 3-fold in water prior to LC/MS/MS injection.

LC/MS/MS Analysis. Analysis was conducted with an API4500 triple quadrupole mass spectrometer (AB Sciex, Ontario, Canada). Samples were analyzed with electrospray ionization in the positive mode with a source temperature of 500 °C. The parent > daughter transition monitored for carbazeran was 361.2 > 218.1 amu, and the collision energy was set at 38 volts. The parent > daughter transition monitored for 4-oxo-carbazeran was 377.1 > 288.1 amu, and the collision energy was set at 23 volts. The HPLC column (Kinetex C18-XB, 50 x 2.1 mm, 5 µm particles) was procured from Phenomenex (Torrance, CA). The mobile phase consisted of HPLC-grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid for components A and B, respectively. The mobile phase was held at 5% B and ramped to 95% B over 1.3 minutes at a flow rate of 0.6 mL/min. The total run time was 1.5 minutes. LC/MS/MS chromatograms were collected and integrated with Analyst software (version 1.6). A standard curve (1-500 nM) was used to quantify 4-oxo-carbazeran formation in all extra-hepatic S9 study samples (kidney, lung, vasculature, intestinal). For liver S9 study samples, the standard curve range was shifted up to 8-fold higher (8-4000 nM), depending on substrate incubation concentration, to capture the higher amounts of metabolite formed in these incubations. Assay performance was accepted based on linearity throughout the dynamic range of the curve ($R > 0.99$ with $1/x^2$ weighting) and back-calculation of standards to within 20% of the nominal value.

Data Analysis. Consistent with supporting data acquired during the studies presented within this manuscript, the AOX pathway was presumed to be the only pathway contributing to carbazeran clearance utilizing S9 tissue fractions, with 4-oxo-carbazeran being the sole metabolite formed. As such, carbazeran intrinsic clearance (CL_{int}) in each tissue was estimated based on 4-oxo-carbazeran time-course data obtained in the various S9 incubations, and calculated according to

$$CL_{int} = \frac{V_{max,tissue}}{K_{M,tissue}} \quad (\text{Equation 1})$$

In Equation 1, AOX maximum 4-oxo-carbazeran formation rate ($V_{max,tissue} = E_{tissue} \times k_{cat,fast}$) and Michaelis-Menten constant ($K_{M,tissue} = \frac{k_{on} + k_{cat,fast}}{k_{off,tissue}}$) were calculated using parameters of AOX modulated activity model (Abbasi et al., 2019), which were estimated by fitting the model (**Figure 2**) to the 4-oxo-carbazeran time-course data. In the model, $k_{cat,fast}$ is the initial fast AOX catalytic rate constant, E_{tissue} is the initial active AOX concentration in each of the tissues, k_{on} is the binding rate constant, and $k_{off,tissue}$ is the tissue-specific unbinding rate constant. The model was implemented in MATLAB R2019 (MathWorks, Natick, MA) and simultaneously fit to 4-oxo-carbazeran mean concentration time-course data from all tissue S9 incubations using default fitnlm algorithm settings with a proportional error model and all model parameters assumed to be log-normally distributed. Description of the full model characterization can be found in the Supplementary Materials.

The CL_{int} values were scaled to in vivo ($CL_{int,scaled}$) for each tissue according to

$$CL_{int,scaled} = CL_{int} * \frac{mg \text{ S9 protein}}{g \text{ tissue}} * \frac{g \text{ tissue}}{kg \text{ body wt}} \quad (\text{Equation 2})$$

Total AOX clearance (CL_{AOX}) contributions from liver, kidney, lung and intestinal compartments were estimated by the well-stirred model as follows:

$$CL_{AOX} = \frac{Q_b * \left(\frac{f_{u,plasma}}{R_{BP}} \right) * \left(\frac{CL_{int,scaled}}{f_{u,S9}} \right)}{Q_b + \left(\frac{f_{u,plasma}}{R_{BP}} \right) * \left(\frac{CL_{int,scaled}}{f_{u,S9}} \right)} \quad (\text{Equation 3})$$

where Q_b is blood flow to the tissue compartment, $f_{u,plasma}$ is unbound free fraction in plasma, R_{BP} is the blood-to-plasma concentration distribution, and $f_{u,S9}$ is the free fraction in the S9

incubations. For vasculature, it was considered that there is continuous exposure to the blood throughout the body, and the blood flow restriction was removed. Furthermore, it was assumed that the iliac arterial tissue was representative of the entirety of the human vasculature. CL_{AOX} from the vasculature was estimated as follows:

$$CL_{AOX} = \left(f_{u,plasma} / R_{BP} \right) * \left(CL_{int,scaled} / f_{u,S9} \right) \quad (\text{Equation 4})$$

All physiological parameter values used in the above equations are recorded in Table 1. The $f_{u,plasma}$ and R_{BP} values for carbazeren, 0.08 and 0.7, respectively, were taken from a previous publication (Zientek et al., 2010). Carbazeren $f_{u,S9}$ was measured by equilibrium dialysis and determined to be 0.72, 0.82, 0.72 and 0.74 for kidney, lung, vasculature and intestinal S9 fractions (1 mg/mL), respectively. For the liver S9 fraction (0.1 mg/mL), carbazeren consumption by AOX is too extensive to measure binding by equilibrium dialysis. Here, liver $f_{u,S9}$ was calculated as 0.97 per the non-specific binding model (Austin et al., 2002) as follows:

$$f_{u,liverS9} = \frac{1}{\left(\frac{[P]_{extra-hepaticS9}}{[P]_{liverS9}} * \left(\frac{1 - f_{u,extra-hepaticS9}}{f_{u,extra-hepaticS9}} \right) + 1 \right)} \quad (\text{Equation 5})$$

where $[P]_{extra-hepaticS9}$ is 1 mg/mL, $[P]_{liverS9}$ is 0.1 mg/mL, and $f_{u,extra-hepaticS9}$ is 0.75, which is the average f_u measured across all extra-hepatic tissues (kidney, lung, vasculature, intestinal) studied.

Results

Following the in vitro incubations with carbazeran, non-linear formation of 4-oxo-carbazeran metabolite was observed and quantified against authentic standards in the liver, kidney, lung and vasculature S9 fractions at all incubation concentrations (**Figure 3a, b, c, d, e, respectively**). Across all incubations, initial faster rate of 4-oxo-carbazeran formation was quickly followed by a slower rate of formation. Only negligible amounts of metabolite were detected in the intestinal S9 fractions, and specifically only observed at the higher incubation concentrations (**Figure 3f**) and associated with the slower rate of formation. While the extent of carbazeran elimination was too low to be accurately measured in extra-hepatic S9 fractions, it was easily observed in liver S9 fractions, and there was an approximate mass balance between substrate consumption and metabolite formation. A representative plot of carbazeran consumption versus 4-oxo-carbazeran formation for 1 μ M incubations in liver S9 fraction is shown in **Figure 3a**. Co-incubation of carbazeran with 100 μ M hydralazine, a selective mechanism-based inhibitor of AOX, significantly blocked metabolite formation in all incubations. In liver S9, there was a 93% decrease in metabolite formation at the highest test concentration (30 μ M) of carbazeran. Similarly, all extra-hepatic tissues showed at least 81% decrease in metabolite formation at the same conditions. Relative concentrations of 4-oxo-carbazeran quantified at the end of the incubation period (90 minutes) for all carbazeran test concentrations, with and without presence of hydralazine, are presented in **Figure 4** for each tissue S9 fraction tested. Taken together, observed data in human S9 fractions without co-factors suggests that carbazeran is metabolized to a single product, 4-oxo-carbazeran, and this is mediated by the AOX pathway.

Despite minimal consumption of carbazeren by the various extra-hepatic tissue S9 samples, 4-oxo-carbazeren metabolite formation quickly followed non-linear kinetics that can be described by the AOX modulated activity model (Abbasi et al., 2019). This model was able to accurately capture ($\text{adj. } R^2 = 0.99$) 4-oxo-carbazeren formation time-course data in all tissue S9 incubations (**Figure 3**), with estimated parameter values captured in Table 2. In the final model, liver and kidney were found to have a lower Michaelis-Menten constant value ($5.2 \mu\text{M}$) relative to the other tissues ($21 \mu\text{M}$), as well as different enzyme inactivation and slow catalytic rate constant values (Table 3). In order to estimate intestinal S9 CL_{int} ($0.038 \mu\text{L}/\text{min}/\text{mg}$ S9) despite minimal 4-oxo-carbazeren formation, it was necessary to assume that the intestinal AOX enzyme rate constants were the same as in other tissue's S9 fractions (lung and vasculature). Similarly, to estimate active AOX enzyme level in each tissue (E_{tissue}), it was assume that the AOX fast catalytic rate constant was the same across all tissues. Thus, we determined that the liver has the highest estimated AOX enzyme concentration level at $21 \text{ pmol}/\text{mg}$ S9 protein (Table 2). AOX intrinsic clearance estimates, calculated according to Equation 1, were 670, 1.2, 0.35, 0.58, $0.038 \mu\text{L}/\text{min}/\text{mg}$, respectively, for liver, kidney, lung, vasculature, and intestine S9 fractions incubated with carbazeren (Table 3). The scaled intrinsic clearance ($\text{CL}_{\text{int,scaled}}$) and total AOX clearance (CL_{AOX}) the values estimated in various extra-hepatic tissues were very low relative to the liver (Table 3). Collectively, CL_{AOX} estimates in the extra-hepatic tissues sampled in this current work represent $<1\%$ compared to that of the liver.

Discussion

While the human liver is the richest source of AOX, the current study has demonstrated that AOX activity is clearly present and measurable in a variety of extra-hepatic human tissues. Of significance, we demonstrate for the first time that AOX activity is measurable in the human vasculature, as represented by S9 fractions prepared from iliac arteries. Given that AOX has been found to be expressed in a variety of extra-hepatic human tissues beyond the scope of this current work (adrenal glands, reproductive tissue, adipose), it can be fully expected that measurable extra-hepatic AOX activity can be found throughout the human body, which would further contribute to the metabolism of AOX substrates.

Given this backdrop, it seems plausible that the collective contributions to AOX metabolism by extra-hepatic sources could, at a minimum, partially explain the underprediction of human clearance scaled from in vitro assays conducted with pooled hepatic S9 or cytosolic fractions. Admittedly, various assumptions were made in the current study when estimating a potential CL_{AOX} contribution from a particular tissue compartment, and physiological parameters may not be as accurate as those commonly used for liver tissue scaling. Regardless, these assumptions likely have little impact on the overall findings presented in this study, where the extra-hepatic tissues only provide a minor contribution to overall systemic clearance. Here, the collective estimated CL_{AOX} of kidney, lung, vasculature and intestinal compartments together contribute <1% of that predicted by the liver.

The tissue fractions tested were chosen due to the identified expression levels of AOX in the tissue (Basit et al., 2020), the volume of tissue of the organ (Davies and Morris, 1993), and/or

the lack of blood flow restriction pertaining to the tissue. While it would have been desirable to study additional human tissues beyond the scope of this work, it is unlikely, based on these criteria that any would contribute significantly to whole-body AOX clearance. For example, the adrenal glands are often cited as having rich AOX expression (Moriwaki et al., 2001; Gomez-Sanchez, 2007). However, adrenal glands are very small, and the combined weight of adult human adrenal glands is only roughly 10 grams (Kreiner, 1982). While adrenal glands may have a rich blood supply on a per gram basis, total blood flow has been estimated at roughly 0.14% of cardiac output (Gomez-Sanchez, 2007), or what would amount to approximately 0.1 mL/min/kg. Similarly, contribution of human skin, which was demonstrated to have limited AOX activity (Manevski et al., 2014), to whole-body AOX clearance would also be insignificant when compared to liver.

Carbazeran, a PDE-1 inhibitor that produces concentration-dependent positive inotropic responses, known for its greater than or equal to hepatic blood flow systemic clearance (≥ 21 mL/min/kg), and being extensively metabolized to the 4-oxo-metabolite in vivo, was chosen as an ideal probe substrate to identify AOX activity in tissue S9 fractions. Carbazeran is also known for its specificity for AOX versus other enzymes in S9 fractions, especially since enzymes that require cofactors in vitro (i.e. UGTs and CYPs) are of little consequence without addition of cofactors, which were excluded from the protocol. Additionally, and perhaps most importantly to these experiments, carbazeran exhibits a rapid turnover rate providing its sensitivity as a marker even with very low AOX expression levels in tissue. Due to this fact, the number of potential substrates available to assess extra-hepatic AOX activity was limited. In addition to carbazeran, zoniporide was tested during the course of these experiments using all of the aforementioned tissues. With protein concentrations up to 1 mg/ml, zoniporide did not result in adequate substrate turnover, even at enzyme saturating substrate concentrations (30 μ M), and failed to provide measurable metabolite formation rates, thus disqualifying it as an applicable substrate (**Supplemental Figure 3**).

The analysis of the data and scaling of organ specific AOX clearance utilized the well-stirred model. This particular model, as opposed to the dispersion model or parallel tube model, was used because it presents the highest possible in vivo clearance estimate for each tissue's contribution to the overall systemic clearance in humans. For vasculature, the blood flow restriction was removed entirely. Therefore, the possibility of an under-prediction based on the scaling method has been minimized. An interesting observation of this work is that, in all extra-hepatic tissues studied, the initial rate of 4-oxo-carbazeran production was followed by a rapid deceleration in product formation despite minimal consumption of carbazeran. It has been hypothesized by Abbasi and co-workers that AOX is likely slow to reduce oxygen in vitro, resulting in enzyme reduction that leads to the decelerated substrate turnover. We employed an AOX modulated activity model (Abbasi et al., 2019) in order to accurately capture the early rapid-rate of AOX activity, characterized by the fast catalytic rate constant. Since the early rapid rate of AOX activity in vitro is believed to most accurately represent in vivo AOX activity, the application of the AOX modulated activity model provided us with a more accurate estimate of intrinsic clearance. Despite the more accurate estimate of the initial faster rate of substrate turnover, extra-hepatic AOX contribution estimates were found to be very low.

By assuming that the AOX fast catalytic rate constant is the same across all tissues, the difference in AOX activity between various tissues, as determined by the early rapid-rate of 4-oxo-carbazeran formation, can be attributed to the abundance of active AOX enzyme in S9 fractions. Thus, using the AOX modulated activity model, estimates of active AOX enzyme abundance in each tissue's S9 fraction were obtained. It was found that the active AOX abundance in the liver (21 pmol/mg S9 protein) is in general agreement with measured liver AOX abundance (11.96 \pm 4.46 pmol/mg S9 protein) described by Basit and colleagues (Basit et al., 2020). In addition, the published extra-hepatic AOX abundance levels for the heart and intestine were below the limit of quantification, which agrees with much lower estimated active AOX levels that we obtained using AOX activity (Table 2). In contrast, Basit et al., 2020 showed

only an 8-fold difference in AOX abundance between kidney (1.53 ± 0.79 pmol/mg S9 protein) and liver, while our analysis of AOX activity data showed greater than 500-fold reduction in active kidney AOX abundance (0.038 pmol/mg S9 protein) relative to liver. Since enzyme abundance does not always correlate with activity, it is possible that our assumption of tissue-independent enzyme fast catalytic rate is incorrect, which could explain the observed disconnect. Tissue sample preparation differences among suppliers and laboratories, and perhaps between tissues, could also impact the resulting AOX activity and thus the model-derived active AOX enzyme tissue abundance estimates.

In support of potential differences in enzyme activity between tissues, it was necessary to introduce tissue-specific differences in several of the model's enzyme rate constants in order to accurately describe the full 4-oxo-carbazeran time-course data in kidney and liver S9 fractions (Supplementary Material). This observation suggests that it is possible that AOX enzyme activity could be different in various tissues and thus complicates the total metabolic clearance extrapolation based on a recombinant protein enzyme activity and tissue enzyme abundance levels as proposed by Basit et al., 2020. It is possible that sample differences between liver, kidney, and other tissues could have affected observed 4-oxo-carbazeran formation, which would impact interpretation of model-derived active AOX enzyme tissue abundance estimates.

This work suggests that future efforts may be best directed toward understanding aspects of tissue isolation and fraction preparation which may negatively impact AOX activity. Sanoh and co-workers, who isolated human hepatocytes from humanized PXB mice, and then used them to predict human $CL_{int, in vivo}$, showed a three-fold or greater under-prediction from the line of unity (Sanoh et al., 2012). This direct comparison of in vivo clearance in humanized mice and the scaled hepatic clearance from hepatocytes, isolated from the same animal, suggests that the loss in AOX activity during preparation of liver cells and fractions may impact our ability to predict human in vivo AOX clearance. Furthermore, despite accounting for the lack of the active AOX enzyme regeneration during the experiment through the modulated activity model,

unaccounted factors may affect the level of active AOX enzyme prior to the incubation. Perhaps additional systematic work pertaining to the complexity of enzyme activation may shed more light on the limited enzymatic activity of tissue isolates and ways to correct for it.

In conclusion, this work advances the narrative that AOX is active and measurable in a variety of extra-hepatic human tissues, including the vasculature. However, the collective contribution of the tissues tested in these experiments to whole-body clearance of AOX substrates is very low compared to the liver and are unlikely to contribute significantly toward the often-observed underprediction of human AOX clearance.

Authorship Contributions

Participated in research design: Kozminski and Zientek

Conducted experiments: Kozminski

Performed data analysis: Kozminski and Selimkhanov

Wrote or contributed to the writing of the manuscript: Kozminski, Selimkhanov, Heyward, and Zientek

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Footnotes

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Legend for Figures

Figure 1. Metabolism of carbazeran to 4-oxo-carbazeran by AOX.

Figure 2. The AOX modulated activity model describes product P formation from substrate S by enzyme E through the formation of the enzyme-substrate complex (ES), which also leads to the formation (k_{inact}) of a less catalytically active version of the enzyme E^* ($k_{\text{cat,slow}} < k_{\text{cat,fast}}$). The substrate and enzyme binding (k_{on}) and dissociation (k_{off}) rate constants are unaffected by the change in the enzyme's catalytic activity.

Figure 3. Time course of carbazeran depletion and 4-oxo-carbazeran formation in liver S9 fraction after incubation with 1 μM carbazeran (A). Time course of 4-oxo-carbazeran formation after incubation with 1 μM (blue), 3 μM (red), 10 μM (yellow), 30 μM (purple) carbazeran in liver (B), kidney (C), lung (D), vasculature (E), and intestinal (F) S9 fractions. Points represent average data with error bars representing standard errors associated with technical and biological (vasculature only) replicates. The curves show AOX modulated activity model simulations based on simultaneous fit to 4-oxo-carbazeran time course data from all tissue S9 fractions.

Figure 4. Formation of 4-oxo-carbazeran in liver (A), kidney (B), lung (C), vasculature (D), and intestinal (E) S9 fractions after 90 minutes of incubation with 1, 3, 10, and 30 μM carbazeran in absence (■) and presence (□) of 100 μM hydralazine.

Table 1: Physiological parameters used for scaling CL_{int} and estimating CL_{Aox} contributions

Tissue	mg S9 protein / g tissue	g tissue / kg BW	Q_b (mL/min/kg)	References
Liver	120	21.4	20.7	(Zientek et al., 2010; Cubitt et al., 2011)
kidney	39 ^a	4.6 ^a	16.4	(Obach et al., 2006; Scotcher et al., 2017)
lung	28 ^a	10.4 ^a	77.9	(Myers et al., 2011; Levitzky, 2013; Scotcher et al., 2017)
Vasculature	8.5 ^b	14.3	n/a	(Aird, 2005; Myers et al., 2011)
intestine	27 ^a	1.1 ^{a,c}	3.54	(Obach et al., 2006)

n/a = not applicable

^apersonal communication from XenoTech, LLC

^bpersonal communication from BioIVT

^cbased on total wet weight of eluted enterocytes

Table 2: Modulated Activity Model (MAM) parameter estimates

Parameter	Liver	Kidney	Lung	Vasculature	Intestine	Units
k _{on}	1.2e8 [3.1e7 - 4.5e8]					μM-1min-1
k _{off}	6.2e8 [1.7e8 - 2.3e9]		2.4e9 [5.0e8 - 1.2e10]			min-1
k _{cat,fast}	167 [125 - 224]					min-1
k _{inact}	0.35 [0.26 - 0.46]	0.16 [0.11 - 0.23]	1.1 [0.77 - 1.5]			min-1
k _{cat,slow}	4.5 [2.6 - 7.6]	25 [17 - 38]	6.9 [5.2 - 9.2]			min-1
E _{tissue}	21 [17 - 26]	0.038 [0.028 - 0.050]	0.043 [0.032 - 0.059]	0.072 [0.053 - 0.098]	4.7e-3 [3.4e-3 - 6.5e-3]	pmol/mg S9

Values in brackets represent 95% confidence intervals.

Table 3: Calculated parameters and scaled AOX clearance contributions

Tissue	K_M (μM)	V_{max} (pmol/min/mg S9)	CL_{int} (μL/min/mg S9)	CL_{int,scaled} (mL/min/kg)	Total CL_{AOX} (mL/min/kg)
liver	5.2	3507	670	1721	19
kidney	5.2	6.3	1.2	0.22	0.034
lung	21	7.2	0.35	0.10	0.014
vasculature	21	12	0.58	0.071	0.011
intestine	21	0.78	0.038	0.0011	0.00017

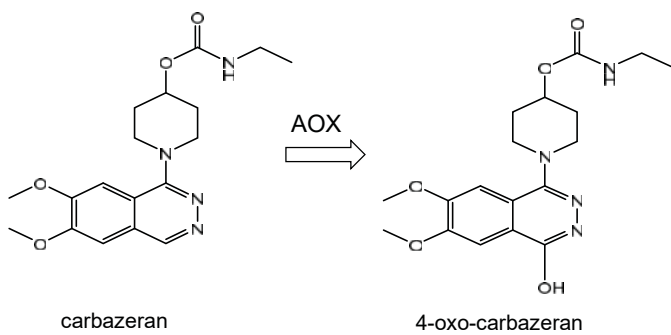


Figure 1

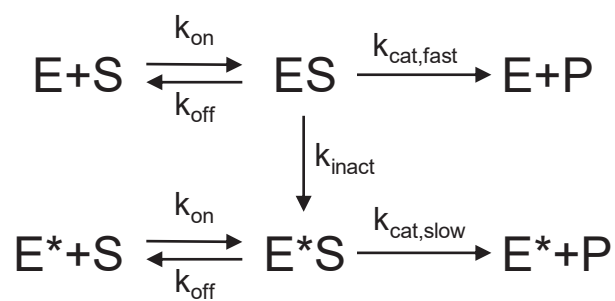


Figure 2

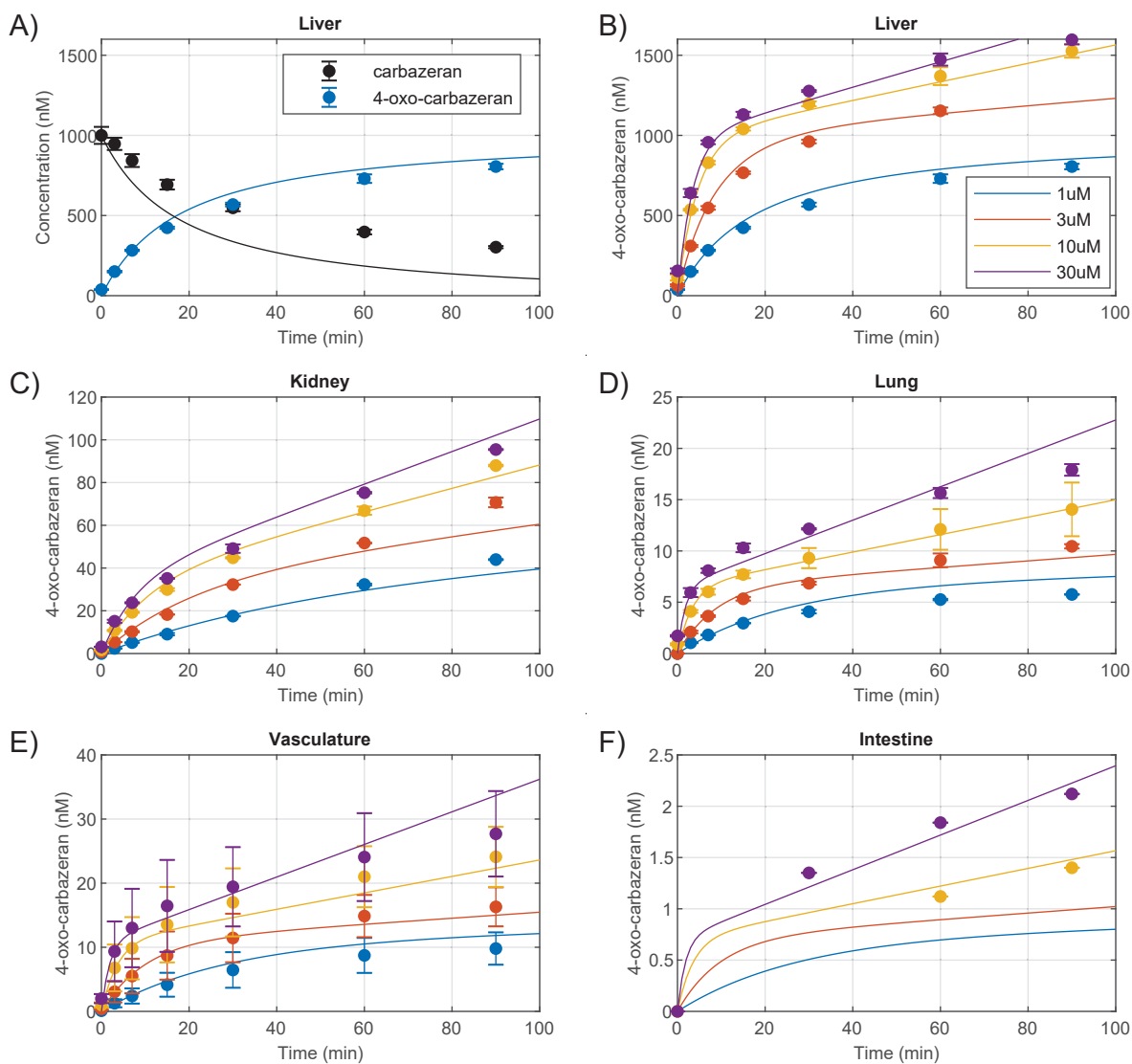


Figure 3

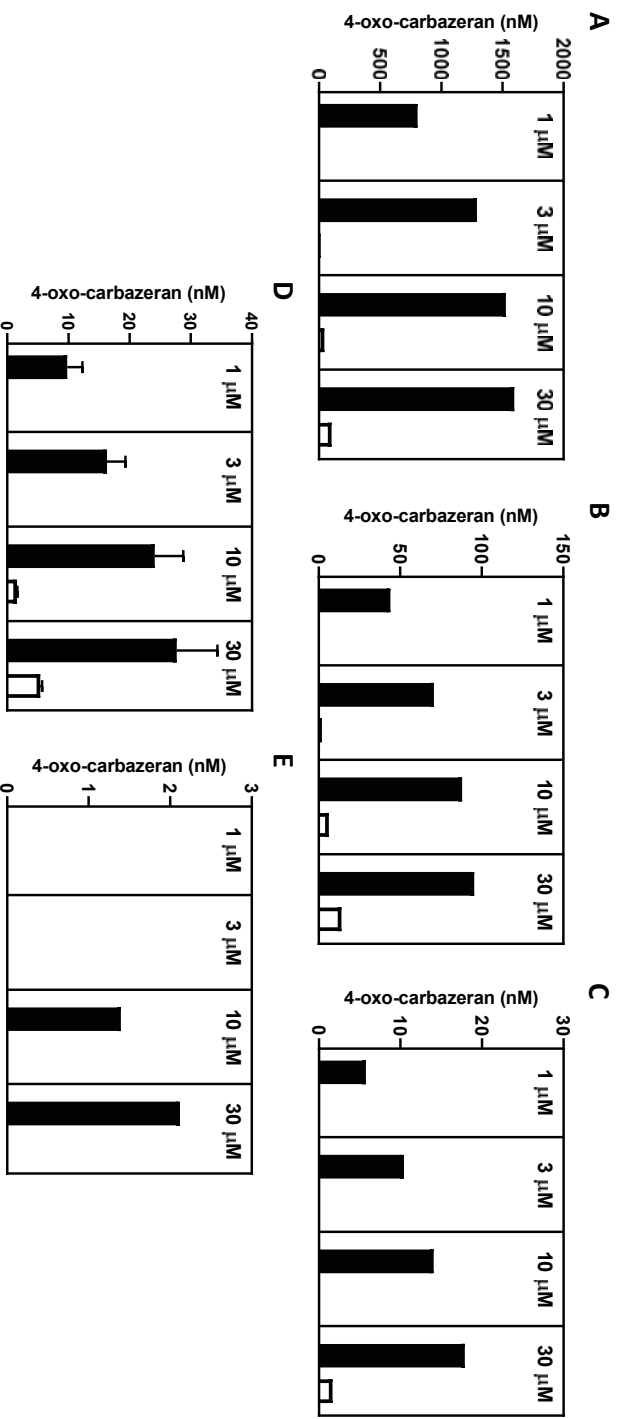


Figure 4