DMD Fast Forward. Published on April 20, 2012 as doi:10.1124/dmd.112.045195

DMD #45195

HYDRALAZINE AS A SELECTIVE PROBE INACTIVATOR OF ALDEHYDE OXIDASE IN HUMAN HEPATOCYTES:

ESTIMATION OF THE CONTRIBUTION OF ALDEHYDE OXIDASE TO METABOLIC CLEARANCE

TIMOTHY J. STRELEVITZ, CHRISTINE C. OROZCO, and R. SCOTT OBACH

PHARMACOKINETICS, DYNAMICS AND METABOLISM

PFIZER GLOBAL RESEARCH AND DEVELOPMENT

GROTON, CT

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Hydralazine as a Selective AO Inhibitor in Hepatocytes

Address Correspondence to:

Tim Strelevitz

Pfizer Inc.

Eastern Pont Rd.

Groton, CT

Timothy.J.Strelevitz@Pfizer.com

Number of Words in:

Abstract: 234

Introduction: 654

Discussion: 1615

Number of Tables: 3

Number of Figures: 5

Number of References: 29

List of non-standard abbreviations:

Aldehyde Oxidase (AO)

$f_{m(AO)}$ (fraction of metabolism by aldehyde oxidase)

N-[(2′-dimethylamino)ethyl]acridine-4-carboxamide (DACA)

1-Aminobenzotriazole (ABT)

cytochrome P450 (CYP)

Williams E. Media (WEM)

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT:

Aldehyde oxidase (AO) metabolism could lead to significant underestimation of clearance when predicting human pharmacokinetics as well as unanticipated exposure to AO-generated metabolites, if not accounted for early in drug research. We report a method utilizing cryopreserved human hepatocytes and the time-dependent AO inhibitor hydralazine (K_I=83±27 μM, k_{inact}=0.063±0.007min^{-1}), which estimates the contribution of AO metabolism relative to total hepatic clearance. Using zaleplon as a probe substrate and simultaneously monitoring the AO catalyzed formation of oxozaleplon and the CYP3A catalyzed formation of desethyzaleplon in the presence of a range of hydralazine concentrations, it was determined that >90% inhibition of the AO activity with minimal effect on the CYP3A activity could be achieved with 25-50 μM hydralazine. This method was employed to estimate the fraction metabolized due to AO ($f_{m(AO)}$) for six compounds with clearance attributed to AO along with four other drugs not metabolized by AO. The $f_{m(AO)}$ values for the AO substrates ranged between 0.49 and 0.83. Differences in estimated $f_{m(AO)}$ between two batches of pooled human hepatocytes suggest that sensitivity to hydralazine varies slightly with hepatocyte preparations. Substrates with a CYP2D6 contribution to clearance were affected by hydralazine to a minor extent, due to weak inhibition of this enzyme. Overall, these findings demonstrate that hydralazine, at a concentration of 25-50 μM, can be used in human hepatocyte incubations to estimate the contribution of AO to the hepatic clearance of drugs and other compounds.

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Aldehyde oxidase (AO) is a soluble molybdenum cofactor containing enzyme that is capable of oxidizing aldehydes, imines, and aromatic azaheterocyclic compounds (Pryde, et al., 2010; Garattini and Terao, 2011; Beedham, 2002). On aromatic azaheterocyclic compounds, it catalyzes the oxidation of relatively electrophilic carbons adjacent to the nitrogen to generate lactam metabolites, with the molybdopterin cofactor participating in a nucleophilic attack on the electrophilic carbon. Although oxygen is the ultimate electron acceptor, the oxygen inserted into the lactam product derives from water; the reducing equivalents from the substrate are passed along to oxygen via FAD and FeS cofactors (Pryde, et al., 2010; Garattini et al., 2012). A specific endogenous substrate has not been definitively identified but AO potentially participates in the metabolism of neurotransmitters, oxidation of products involved in various metabolic pathways as well as degradation of vitamins (Garattini et al., 2003). Notable substrates used in in vitro work include N-methylnicotinamide and phthalazine. Drugs known to have an important contribution of aldehyde oxidase in human include zaleplon (Lake, et al., 2002; Renwick, et al., 2002) and famciclovir in which AO is involved in metabolism of a prodrug to the active antiviral agent penciclovir (Clarke, et al., 1995; Rashidi, et al., 1997).

While there has been a very high focus on the cytochrome P450 family of drug-metabolizing enzymes in the research and development of new drugs, there has been considerably less attention on AO. However, an increase in the prevalence in the use of aromatic azaheterocyclics as substituents in drug design has caused an increase in the importance of AO in drug metabolism (Pryde, et al., 2010). When left unexamined in drug design, an impact of AO on the clearance of a new chemical entity can result in an unexpected low exposure in humans.

Examples of instances where human pharmacokinetics were unacceptable because it was not known that AO contributed a large extent in metabolic clearance prior to administration to humans include carbazeran (Kaye, et al., 1985), zoniporide (Dalvie, et al.,2010), BIBX1382 (Dittrich, et al., 2002), and a ketolide antibiotic (Magee, et al., 2009). It is also possible that AO generated metabolites could be responsible for toxicity (Diamond, et al., 2010). One of the challenges in drug discovery regarding AO is that enzyme expression in commonly employed laboratory animal species (mouse, rat, and dog) differs from human. In particular, the dog does not express the AOX1 gene that is important in human (Terao, et al., 2006).

Prediction of human in vivo clearance of new drug candidates is an important activity in drug discovery so that the pharmacokinetics in humans will be consistent with a reasonable dosing regimen (i.e. low hepatic first pass metabolism that can result in good oral bioavailability; clearance that will yield a half-life that permits an appropriate dosing frequency). Methods to predict human clearance from in vitro metabolism data have been well-established for the cytochrome P450 enzymes (Emoto, et al., 2010; Obach, 2011), and more recently the glucuronyl transferase enzymes (Kilford, et al., 2009). However, quantitative prediction of human clearance for AO metabolized agents has not been accomplished. This may be in-part due to the distribution of AO in extra-hepatic tissues including lung, gastrointestinal tract and kidney (Pryde et al., 2010). Species dependent tissue distribution also is confounding development of predictive tools for AO (Garattini et al., 2011). Recently, an in vitro-in vivo correlation approach has been described wherein 11 compounds with varying rates of AO mediated clearance in humans were studied (Zientek, et al., 2010). The investigators proposed that new compounds

which are subject to AO catalyzed metabolism could be placed into this correlation to gain an estimate of whether clearance would be unacceptably high, moderate, or low.

However, when attempting to predict in vivo clearance for a new compound from in vitro data it is important not only to measure the rate of metabolism, but also the relative contribution that the enzyme (or enzyme family) makes to overall metabolism. Such information is important for predictions of clearance, drug interactions, and inter-patient pharmacokinetic variability from in vitro data. In this report, we describe the development of a method whereby the relative contribution that AO makes to overall hepatic metabolic clearance in humans is quantified. We show that the AO time-dependent irreversible inhibitor, hydralazine (Johnson, et al., 1985) can be used to selectively and completely inhibit AO in human hepatocytes without inhibiting P450 enzymes.

MATERIALS AND METHODS

Materials. Hydralazine, zaleplon, PF-0945863, carbazeran, zoniporide, oxozoniporide, propranolol, midazolam, DACA, naloxone, PF-05218881 and dextromethorphan, were obtained from the Pfizer sample bank (Pfizer, Groton, CT). 1-Aminobenzotriazole (ABT) and O^6-benzylguanine was purchased from Sigma-Aldrich (St Louis, MO). Desethylzaleplon was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cryopreserved human hepatocytes from five individual donors, male and female, were purchased from In Vitro Technologies (Baltimore, MD) (Batch 1: lots AGR, FKM, EHI, TDH, ZFB) as well as a 10-donor mixed gender pre-pooled lot (Batch 2: lot RTH). Both were stored in liquid nitrogen until use. Williams E Media (WEM, GIBCO-BRL cat#C1984, custom formula #91-5233EC) supplemented with 26 mM $NaHCO_3$ and filtered through a 0.22 μm sterile filtration flask. Pooled human liver cytosol was purchased from Celsis IVT (Chicago, IL). Pooled human liver microsomes were purchased from BD Bioscience (San Jose, California). All other reagents and chemicals were of the highest purity available.

Biosynthesis of Oxozaleplon. Zaleplon (20 μM) was incubated with human liver cytosol (10 mg/mL) in a total volume of 10 mL potassium phosphate buffer (0.1 M, pH 7.4). The incubation was carried out at 37^{o}C open to air for 3 h. The reaction was terminated by addition of 8 mL CH_3CN containing 0.32 mL formic acid. The mixture was spun in a centrifuge at 1700 x g for 5 min. To the supernatant was added 0.1% formic acid to a final volume of 100 mL, followed by centrifugation at 40000 x g for 30 min. The supernatant was applied through a Jasco HPLC

pump at 0.5 mL/min onto a Varian Polaris C18 column equilibrated in 0.1% formic acid containing 10% CH_3CN. After the entire supernatant was loaded, the column was moved to an HPLC-MS system (Thermo-Finnigan LTQ with Surveyor HPLC system) and the oxozaleplon product was eluted using a mobile phase gradient that commenced with 0.1% formic acid containing 10% CH_3CN, held for 5 min, followed by a linear gradient to 70% CH_3CN at 50 min. The eluent was collected into 20 second fractions; fractions containing oxozaleplon (which eluted at ~27 min) were pooled, evaporated by vacuum centrifugation, and the residue was reconstituted in 0.075 mL [$^2H_6$]DMSO for analysis by quantitative proton NMR (Walker, et al., 2010). The resulting stock solution was 1.86 mM and was diluted as appropriate to make standard curves for bioanalysis.

Substrate and Inhibitor Preparations. All substrate stock solutions were prepared at 3 mM in DMSO. Further dilutions were made with WEM for a final substrate concentration of 1 µM (propranolol had a final concentration of 0.1 µM). For studies in which the zaleplon metabolites were quantified, zaleplon final concentration was increased to 20 µM. The CYP inhibitor, ABT was prepared at 400 mM in DMSO for a final concentration of 1 mM. Hydralazine was prepared in water prior to each study at various concentrations.

Hepatocyte Preparations. Immediately prior to each experiment, the individual donor hepatocytes were thawed by gently shaking in a 37^{o}C water bath for 90 seconds then pooled and diluted 25x the hepatocyte volume into pre-warmed and O_2/CO_2 (95/5) bubbled WEM. The pooled mixture was centrifuged at 100 x g for five min at room temperature. Following centrifugation, the supernatant was discarded and the hepatocyte pellet was resuspended in

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

WEM to either 0.75 x 10^6 cells/mL or 2.25 x 10^6 cells/mL. The hepatocyte number and viability were determined using trypan blue exclusion staining in a hemocytometer. Cell preparations with viability greater than 80% were diluted with WEM and using a Thermo Labsystems Multidrop DW instrument 30 µL of cell suspension was added to individual wells of 96-well tissue culture treated polystyrene plates (final cell density was 0.5 x 10^6 cells/mL or 1.5 x 10^6 cells/mL). For the donor variability study individual donors were kept separate and prepared by the same method as the pooled hepatocytes.

Hepatocyte Incubations: Cells were placed in a 37°C incubator under an atmosphere of O_2/CO_2 (95/5) with 95% relative humidity for 30 min. Following the 30 minute incubation, 15 µl of 3 µM substrate or substrate/inhibitor mix was added to individual wells using an Apricot Designs Personal Pipettor 550. Incubations were performed in triplicate and were initiated by the addition of substrate or substrate/inhibitor solution to the hepatocytes. Reactions were terminated at 0, 5, 15, 30, 60, 120 and 240 min by adding 135 µL of cold CH_3CN containing internal standard (100 ng/mL PF-05218881). Following the termination of the reaction, plates were centrifuged at 3000 x g at 4°C for 5 min. The supernatants were transferred to 96-deepwell plates for LC-MS/MS analysis.

LC-MS/MS Analysis. Samples were analyzed by LC-MS/MS using a Shimadzu quaternary HPLC pump with an Agilent 1100 series membrane degasser (Agilent Technologies, Palo Alto, CA) and Leap autosampler (CTC Analytics, LEAP Technologies Inc. , Carrboro, NC) coupled to a PE Sciex API 4000 QTrap mass spectrometer (Applied Biosystems/MDS-SCIEX). Electrospray ionization in positive mode (ESI+) with multiple reaction monitoring (MRM) was

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

used. Mass spectrometer parameters were individually optimized for each compound and internal standard (Table 1). Chromatographic separation was achieved using a Phenomenex Synergy Polar-RP 4µ 50×2.0mm column. The mobile phases, both containing 0.1% formic acid, were water/CH_3CN (95/5) (solvent A) and water/CH_3CN (5/95) (solvent B). A linear gradient of solvent B from 5 to 95% was applied over 3.5 min on the column at a flow rate of 0.5 mL/min. The column was then re-equilibrated to initial conditions. The total sample analysis time was approximately four min. All analytes eluted between 1.5 and 2.5 min. A standard curve was used to quantify zaleplon, oxozaleplon and desethylzaleplon. Linearity was observed between 0.5µM to 25µM, 0.001µM to 5µM, and 0.0025µM to 25µM, respectively. Acceptable assay performance was based on linearity throughout the dynamic range of the standard curve. Also, standards were included only if within 20% of the nominal value. AB Sciex Analyst 1.4.2 software was used to analyze all data.

Calculations for $Cl_{int,app}$ and f_{mAO}. The area under the concentration-time curve ($AUC_{(0-\infty)}$) was calculated from the substrate depletion time course using the linear trapezoidal approximation and extrapolation from the last quantifiable time to infinity from the estimated half-life ($t_{1/2}$). All $CL_{int,app}$ were calculated as:

$$CL_{int,app} = \frac{AUC_{(0-\infty)} \bullet V}{[S] \bullet \text{cell number}}$$

The $t_{1/2}$ was estimated as ln2/slope, where the slope is that of the plot of the terminal elimination phase on a logarithmic scale. Fraction metabolized by AO ($f_{m(AO)}$) was calculated by:

$$f_{m(AO)} = \frac{CL_{int,app} - CL_{int,app,hyd}}{CL_{int,app}}$$

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

in which $CL_{int,app,hyd}$ is the apparent intrinsic clearance in the presence of hydralazine, as calculated as above.

Cytochrome P450 Inhibition Study. The cytochrome P450 (CYP) inhibition assay utilized a cocktail of six probe substrates metabolized by major CYP isoforms and human liver microsomes (HLM) to assess the inhibition potential of a test compound for each CYP isoform (Zientek et.al., 2008).

Time-Dependent Inhibition of Aldehyde Oxidase by Hydralazine. A progress-curve approach was utilized to determine the time-dependent inhibition of human AO activity by hydralazine. Incubation mixtures consisted of pooled human liver cytosol (5 mg/mL), zoniporide (20 µM), hydralazine (0-500 µM), in 0.1 M potassium phosphate, pH 7.4. Reactions were commenced with the addition of cytosol and incubated at 37^{o}C. At times of 0, 2.5, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min an aliquot (0.075 mL) of the incubation mixture was removed and added to 0.025 mL CH_3CN containing 5% formic acid and 0.02 mM metoprolol as an internal standard. The mixtures were centrifuged (Eppendorf; 14000 rpm, 5 min), and supernatants were analyzed by HPLC-MS. The injection volume was 10 µL. The HPLC consisted of an Inertsil C8 column (100 x 4.6 mm; 3µ) equilibrated in 0.1% formic acid at a flow rate of 0.8 mL/min. This mobile phase composition was maintained for 1.5 min followed by a linear increase in CH_3CN composition to 80% at 6 min, held at this condition for 1 minute, followed by a 3 minute re-equilibration period at initial conditions. The eluent was introduced into the source of a Finnigan LTQ mass spectrometer operated in the positive ion mode. The mass transitions of m/z 337→278 and 268→191 were monitored for oxozoniporide and internal

standard, respectively. Oxozoniporide was quantitated from a standard curve ranging from 0.05-10 µM. Data analysis was done for progress curve analysis as described in Morrison and Walsh (1988).

Metabolite Profiles of Drugs in Human Hepatocytes. O6-benzylguanine, PF-0945863, zaleplon, zoniporide, DACA, carbazeran, propranolol, were incubated at 10 µM with pooled human hepatocytes (~750000 cells/mL) in 2 mL. Incubations were carried out at 37^{o}C under an atmosphere of O_2/CO_2 (95/5). Aliquots were removed at time zero, 30 min, 1 h, or 3 h (depending on the expected turnover of the individual drug) and terminated with five volumes of CH_3CN. The mixture was centrifuged at 1700 g for 5 min and the supernatant removed under nitrogen. The residue was reconstituted in 0.2 mL of 1% formic acid and injected onto a Thermo-Finnigan Surveyor HPLC in line with a diode array detector (200-400 nm) and ion trap mass spectrometer (LTQ). The HPLC system consisted of a Varian Polaris C18 column (4.6 x 250 mm; 5 µ) equilibrated in 0.1% formic acid containing 5% CH_3CN at a flow rate of 0.8 mL/min. This mobile phase condition was held for 5 min followed by a linear gradient to 80% CH_3CN at 30 min, which was held for 5 min more before returning to initial conditions to re-equilibrate the column. The LTQ was operated in the positive ion mode with data-dependent scanning; tune file parameters and collision energies were optimized for each compound based on the response for the protonated molecular ion and fragment ions, respectively.

RESULTS

Metabolism of Zaleplon in Human Hepatocytes. The metabolism of zaleplon in cyropreserved pooled human hepatocytes was examined to determine the enzyme kinetic parameters for the formation of the AO metabolite oxozaleplon and the CYP metabolite desethylzaleplon. These are the two major metabolic pathways reported for this drug (Kawashima et. al. 1999). Preliminary experiments had determined 30 min to be an optimal incubation time for formation of both metabolites. Clinical concentrations for zaleplon are in the low µM range (Greenblatt et al. 1998), however, it was determined from this study that a 20 µM zaleplon incubation concentration was necessary to produce both metabolites in a readily measurable amount. Zaleplon concentrations above 50 µM did not result in a corresponding increase in either metabolite. Since 20 µM zaleplon produced both metabolites in sufficient quantity for quantification throughout the incubation time course, this concentration was selected for subsequent experiments. Although zaleplon kinetics have been reported (Lake et.al. 2002) in human liver cytosol and liver slices, to date, the enzyme kinetics of zaleplon metabolism in cryopreserved human hepatocytes have not been reported. However, the data shown in Figure 1 preclude making reliable estimates of K_M and V_{max} due to the apparent complexity of the v vs. [S] relationship.

Effect of Hydralazine and ABT on Zaleplon Metabolism in Human Hepatocytes. Zaleplon (20µM) was incubated with hydralazine between 0 and 200 µM in a five donor pool of human hepatocytes for 30 min to determine the inhibitory effect on the formation of the AO mediated oxozaleplon metabolite and the CYP mediated desethylzaleplon metabolite (Figure 2).

Oxozaleplon decreased as a percent of control with increasing hydralazine concentration. Greater than 90% inhibition of AO mediated metabolite formation resulted from 25 μM hydralazine while not inhibiting the desethylzaleplon CYP mediated metabolite formation. At concentrations greater than 100 μM readily measurable inhibition of desethylzaleplon formation was observed. At 50 μM hydralazine, there was a slight effect on the CYP3A catalyzed deethylation reaction, thus it is concluded that concentrations should not exceed this value to selectively inhibit aldehyde oxidase.

The pan-CYP inhibitor ABT was co-incubated with zaleplon to better characterize the reliability of assessing a specific AO inhibitor in hepatocytes (Figure 3). Zaleplon (20 μM) was co-incubated with between 0 and 1.5 mM ABT. Desethylzaleplon decreased with increasing ABT concentration. CYP metabolite formation was inhibited >90% in the presence of 1 mM ABT. The AO derived oxozaleplon metabolite formation was unaffected by ABT at the concentrations tested. This result confirms that oxozaleplon is generated by AO and desethylzaleplon is generated by CYP and that these pathways can be useful for probing the selectivity of inhibitors of these two enzymes.

Effect of Hydralazine on Individual Human Cytochrome P450 Enzymes. While the experiment described above shows that hydralazine does not affect CYP3A catalyzed zaleplon N-deethylation, it is important to determine the potential potency at inhibiting other P450 enzymes. Across the major drug metabolizing P450 enzymes, hydralazine at 25 μM showed little to no inhibition (Table 2). When hydralazine was tested at 50 μM the percent of control for CYP2D6 and CYP3A was reduced to 77% and 76%, respectively.

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Inactivation of Human Aldehyde Oxidase by Hydralazine. In a previous report, it was proposed that hydralazine was a time-dependent inhibitor of aldehyde oxidase, using guinea pig enzyme (Critchley et al. 1994). However, this was not known for human aldehyde oxidase, thus measurement of the time-dependence and determination of inactivation kinetic parameters was undertaken for the human enzyme. Using the oxidation of zoniporide to oxozoniporide as a probe reaction (Dalvie et al., 2010) and pooled human cytosol as the source of enzyme, the inactivation kinetics of aldehyde oxidase by hydralazine were determined. The maximum inactivation rate constant (k_{inact}) was 0.063±0.007 min^{-1} and the concentration yielding 50% of the maximum inactivation rate (K_I) was 83 ± 27 μM. This was determined using a progress-curve approach, in which substrate and inactivator are simultaneously incubated (Figure 4).

Determination of Fraction Metabolized by Aldehyde Oxidase in Pooled Human Hepatocytes. The use of hydralazine to determine fraction metabolized for compounds that are metabolized by AO was tested using ten compounds with diverse enzymatic pathways (Table 3). O^6-benzylguanine, PF-0945863, zaleplon, zoniporide, DACA and carbazeran were selected because these drugs have been shown to possess an AO contribution to their total clearance (Zientek, 2010). The results showed that hydralazine can have a substantial effect on drugs possessing an aldehyde oxidase component to their metabolic clearance (Table 3). Two different batches of pooled hepatocyte lots as well as four individual lots of hepatocytes were examined to assess inter-lot variability (Figure 5). Some minor differences were observed in sensitivity to hydralazine, with batch 1 demonstrating an apparent greater effect of 25 μM hydralazine, while batch 2 required the use of 50 μM hydralazine.

Naloxone, propranolol, midazolam and dextromethorphan were selected because they have no AO mediated clearance. These compounds were considered negative controls that could expose an effect by hydralazine on other metabolic enzymes. Dextromethorphan is primarily metabolized by CYP2D6 (Gorski et.al., 1994), which is one of the enzymes that can be inhibited by hydralazine (Table 2), and propranolol has a component of its metabolism catalyzed by CYP2D6 (Yoshimoto et. al., 1995). Hydralazine has an effect on intrinsic clearance of propranolol and dextromethorphan, whereas minimal effect was notable for midazolam or naloxone.

To confirm that these AO substrates have other metabolic pathways besides the aldehyde oxidase mediated reactions, the profile of metabolites was qualitatively determined in human hepatocytes (Table 3). DACA, zaleplon, and PF-0945863 all demonstrated other types of oxidative pathways commonly associated with P450 enzymes, zoniporide demonstrated a hydrolysis reaction (as previously described; Dalvie, et al., 2010), and carbazeran demonstrated a considerable extent of direct glucuronidation (presumably on the phthalazine nitrogen). Only O^6-benzylguanine appeared to demonstrate a single metabolite that is presumably generated by aldehyde oxidase, but this oxidation could also possibly be catalyzed by other enzymes (e.g. xanthine oxidase, P450s). The large effect of hydralazine on O^6-benzylguanine intrinsic clearance would support that AO is the dominant enzyme involved in its clearance.

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

While aldehyde oxidase has been an enzyme known to be involved in the metabolism of some drugs for several years, it has been gaining importance in drug metabolism over recent years (Pryde, et al., 2010). This has been posing new challenges in drug design, since methods for predicting various human pharmacokinetic attributes (e.g. clearance, drug-drug interactions, interpatient variability) which have been reasonably well-established for compounds metabolized by cytochrome P450 enzymes (Houston, 1994, McGinnity et. al., 2004), are not well-known for drugs metabolized by aldehyde oxidase. Recently, Zientek et al. proposed a correlative method for categorizing new compounds shown to be metabolized by aldehyde oxidase as potentially high, moderate, or low clearance drugs (Zientek, et al., 2010). That method utilized human liver cytosol or S-9 fraction as a source of enzyme, measurement of in vitro CL_{int}, and comparison to a set of eleven drugs known to be metabolized by aldehyde oxidase and for which human pharmacokinetic data were available. By comparison of the CL_{int} value for a new compound to the eleven known drugs, the in vivo CL can be predicted, albeit with low precision. More recently Hutzler, et al., extended this type of approach to human hepatocytes as an in vitro system and showed quantitative prediction of clearance by aldehyde oxidase for substrates of high clearance (Hutzler, et al., 2011).

In addition to the prediction of human CL for aldehyde oxidase substrates, it is also important to understand the relative contribution of this enzyme to overall clearance. This is essential to understand the potential for interindividual variability in pharmacokinetics that can arise by interindividual differences in enzyme expression or drug-drug interactions. Furthermore, it has been recently reported that human aldehyde oxidase is subject to genetic polymorphisms that can impact activity (Hartmann, et al., 2012), thus potentially serving as a

source of inter individual variability. The greater percentage to which a specific enzyme contributes to clearance, the greater the potential impact that variability in the activity of that enzyme will have on interindividual variability in clearance. For example, it has been previously estimated that zaleplon is metabolically cleared by both aldehyde oxidase and cytochrome P450 3A4 by approximately a two-thirds/one-third ratio (Renwick et al., 2002). Thus, even if CYP3A4 were completely inhibited in vivo, the exposure to zaleplon would only increase by about 30%, which is what is observed with co-administration of erythromycin, a potent CYP3A4 inhibitor (Sonata Product Label, http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/020859s011lbl.pdf; accessed December 12, 2011). Thus, the aldehyde oxidase route of clearance serves to blunt the effect of a potent CYP3A4 inhibitor. It is therefore important to be able to predict the relative contribution of aldehyde oxidase to the overall clearance of individual drugs. The development of an in vitro method to make this prediction was the objective of the studies described in this paper. To develop an in vitro method to predict the impact of aldehyde oxidase to overall clearance, two elements are important: (a) an in vitro system that possesses aldehyde oxidase activity within as complete a complement of drug metabolizing enzymes as is possible, and (b) a selective tool that will knock-out aldehyde oxidase activity with acceptable selectivity. Pooled cryopreserved human hepatocytes used in suspension were selected for the in vitro system, based on an assumption that aldehyde oxidase and other drug metabolizing enzyme activities are representative of what is present in the human liver in vivo. The potential for extrahepatic clearance and the possibility that relative enzyme activities significantly change throughout the tissue acquisition, cell preparation, storage, and in vitro incubation processes must be accepted as possible limitations (as they are for any drug metabolism study conducted in hepatocytes)

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

(Akabane et al., 2011). As for a tool compound that will selectively inhibit aldehyde oxidase as completely as possible, several were considered. Menadione has been used extensively as a selective inhibitor of aldehyde oxidase relative to the related enzyme xanthine oxidase. However, menadione is subject to rapid metabolism and in preliminary experiments it also showed substantial inhibition of several cytochrome P450 activities and was thus not pursued further (data not shown). Raloxifene is a very potent uncompetitive inhibitor of human aldehyde oxidase in cytosol preparations (Obach, 2004), however raloxifene has also been shown to be an inactivator of CYP3A4 (Chen et al., 2002) and was therefore deemed not selective enough to be used for this purpose. Hydralazine had been previously shown to be an inactivator of guinea pig aldehyde oxidase (Critchley et al. 1994), and was selected for further exploration as a selective inhibitor of human aldehyde oxidase that could be used in hepatocytes. It should be noted that during our investigations, another group showed that hydralazine could inhibit aldehyde oxidase in human hepatocytes (Hutzler, et al., 2011), albeit it in that report it was not used for the estimation of $f_{m(AO)}$.

Our investigations showed that hydralazine possessed suitable properties as a selective inhibitor of aldehyde oxidase in human hepatocytes. It possessed little activity at the major drug metabolizing P450 enzymes (Table 2). Using zaleplon oxidase and deethylase activities as simultaneous probes for aldehyde oxidase and cytochrome P450 activities, respectively, hydralazine at 25 μM demonstrated the necessary selectivity for the former enzyme (Figures 2 and 3). Higher concentrations (i.e. ≥100 μM) started showing some effect on P450 activity. Hydralazine was shown to be a time-dependent inhibitor of human aldehyde oxidase (Figure 4), as it had been previously shown to be for the guinea pig enzyme (Critchley et al. 1994). Overall,

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

hydralazine demonstrated acceptable properties as an aldehyde oxidase selective probe inhibitor in human hepatocytes.

Once the experimental conditions were established (i.e. pooled cryopreserved human hepatocytes as the in vitro system, hydralazine as the selective inhibitor at 25 µM, and monitoring the decline in test compounds at 1 µM over 4 h as the end point measurement) we tested these conditions with a wide array of drugs known to be metabolized, at least in part, by aldehyde oxidase. Several drugs were shown to have half or more of their metabolic clearance catalyzed by aldehyde oxidase including O6-benzylguanine, PF-0945863, zaleplon, zoniporide, DACA and carbazeran (i.e. $f_{m(AO)} \geq 0.50$; Table 3). Four negative controls were also tested. Naloxone and midazolam which are primarily metabolized by UGT and P450 enzymes respectively, were minimally affected by hydralazine. However, a more significant effect was observed on the consumption of propranolol and dextromethorphan (Table 3). Both of these compounds are metabolized by CYP2D6 and it was noted that among the P450 enzymes tested, hydralazine had the greatest effect on CYP2D6 (Table 2). Thus it will be important that when using this method to estimate $f_{m(AO)}$, a known CYP2D6 probe substrate also be included as a control, and that if an effect of hydralazine is also observed on that drug, it is possible that the new compound(s) being tested may be a substrate of CYP2D6 and not aldehyde oxidase. This could be easily accessed by using a CYP2D6 inhibitor in a parallel incubation. A second potential limitation of the approach is that there must be measurable turnover of the test compound in order to determine the impact of hydralazine. To this end, we used a concentration of 1.5×10^6 cells/mL to reduce the observed half-life of substrates, leading to a measurable difference between the CL_{int} for a substrate exposed to hydralazine and not, thus enabling estimation of $f_{m(AO)}$. . Compounds with very low CL_{int} will not be readily addressed using a

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

substrate depletion approach; an alternate experimental design will need to be employed to estimate $f_{m(AO)}$, such as quantitative monitoring of the formation of metabolites in the presence and absence of hydralazine. Nevertheless, these findings support the use of hydralazine (at 25-50 µM) in human hepatocytes as an acceptable probe inhibitor of aldehyde oxidase.

Examination in a second batch of human hepatocytes showed that a greater concentration of hydralazine was needed (50 µM), and demonstrates the potential for inter-lot variability in the sensitivity to hydralazine and/or differences in the content of AO and various CYP isoforms in the hepatocytes. Based on our observations of the subtle differences in the effect of hydralazine among two hepatocyte batches, it is recommended that investigators employing this method establish a concentration of the inhibitor between 25 and 50 µM that is optimal for their own hepatocyte preparations. This can be done using one or more of the AO substrates described in this work, along with a CYP2D6 substrate to ensure that too high a concentration is not used that would sacrifice selectivity.

The best way to determine whether the $f_{m(AO)}$ values estimated using this in vitro method match the contribution of AO to drug clearance in humans would be to use data from a human metabolism and excretion study using radiolabeled substrate, and to sum up the excretory metabolites that can be attributed to AO catalysis. However, among the six AO substrates that we tested, zoniporide is the only one that also has such clinical metabolism data reported (Dalvie et al., 2010). In that study $f_{m(AO)}$ can be estimated to be between 0.52 and 0.69. This range correlates well with our in vitro estimates of 0.64 and 0.55. Despite this agreement, there is insufficient clinical metabolism and excretion data for drugs with known aldehyde oxidase mediated clearance to assess the quantitative correlation of $f_{m(AO)}$ between in vitro and in vivo

measurements. The reported $f_{m(AO)}$ are relative estimations based on the described in vitro studies.

In conclusion, a method whereby $f_{m(AO)}$ can be estimated for the metabolism of drugs in humans using hepatocytes with hydralazine as a selective inhibitor has been demonstrated. This method should prove useful in the design of new drugs when the prediction of human pharmacokinetic attributes such as clearance and potential for drug-drug interactions is important. It should also prove useful when designing a drug-drug interaction study strategy, in that observation of a substantial contribution to total CL by aldehyde oxidase will have a bearing on the types of drug-drug interaction clinical studies that should be considered.

Acknowledgements

The authors would like to thank Mike West for support with the DDI assay and Greg Walker for NMR quantification of oxozaleplon. We would also like to thank Larry Tremaine and Louis Leung for their review of this work and helpful suggestions.

Authorship Contributions

Participate in research design: Obach, Orozco, Strelevitz

Conducted experiments: Obach, Orozco, Strelevitz

Contributed new reagents or analytical tools: n/a

Performed data analysis: Obach, Orozco, Strelevitz

Wrote or contributed to the writing of the manuscript: Obach, Orozco, Strelevitz

References

Akabane T, Gerst N, Naritomi Y, Masters J, Tamura K. (2011) A practical and direct comparison of intrinsic metabolic clearance of several non-CYP enzyme substrates in freshly isolated and cryopreserved hepatocytes. Drug Metab Pharmacokinet doi: 10.2133/dmpk.DMPK-11-RG-097

Beedham C (2002) Molybdenum hydroxylases, in: Enzyme Systems that Metabolise Drugs and Other Xenobiotics (Loannides C ed). pp. 147-188, John Wiley & Sons, Ltd., New York, NY

Chen Q, Nqui JS, Doss GA, Wang RW, Cai X, DiNinno FP, Blizzard TA, Hammond ML, Stearns RA, Evans DC, Ballie TA, Tang W. (2002) Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem Res Toxicol* **7**:907-14.

Clarke SE, Harrell AW, and Chenery RJ. (1995) Role of aldehyde oxidase in the in vitro conversion of famciclovir to penciclovir in human liver. *Drug Metab. Dispos* **23**: 251-254.

Critchley DJ, Rance DJ, Beedam C. (1994) Biotransformation of carbazeran in guinea pig: effect of hydralazine pretreatment. *Xenobiotica* **1**: 37-47.

Dalvie D, Zhang C, Chen W, Smolarek T, Obach RS, and Loi CM. (2010) Cross-species comparison of the metabolism and excretion of zoniporide: contribution of aldehyde oxidase to interspecies differences. *Drug Metab. Dispos* **38**: 641-654.

Diamond S, Boer J, Maduskuie TP, Falahatpisheh N, Li Y, and Yeleswaram S. (2010) Species-specific metabolism of SGX523 by aldehyde oxidase and the toxicological implications. *Drug Metab. Dispos* **38**: 1277-1285.

Dittrich C, Greim G, Borner M, et al (2002) Phase 1 and pharmacokinetic study of BIBX1382, an epidermal growth factor receptor (EGFR) inhibitor, given in a continuous daily oral administration. *Eur. J. Cancer* **38**: 1072-1080.

Emoto C, Murayama N, Rostami-Hodjegan A, Yamazaki H. (2010) Methodologies for investigating drug metabolism at the early drug discovery stage: prediction of hepatic drug clearance and P450 contribution. *Curr Drug Metab* **8**: 678-85.

Garattini E, Mendel R, Romao M, Wright R, Terao M. (2003) Mammalian molybdo-flavenzyme, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. *Biochem. J.* **372**: 15-32

Garattini E, Terao M (2011) Increasing recognition of the importantce of aldehyde oxidase in drug development and discovery. *Drug Metab Rev.* **43**: 374-386

Garattini E, Terao M. (2012) The role of aldehyde oxidase in drug metabolism. Expert Opin *Drug Metab Toxicol*. **8**: 487-503

Greenblatt D, Harmatz J, von Moltke L, Ehrenberg B, Harrel L, Corbett K, Counihan M, Graf J, Darwish M, Mertzanis P, Martin P, Cevallos W, Shader R. (1998) Comparative kinetics and dynamics of zaleplon, zolpidem, and placebo. *Clin. Pharmacol Ther* **64**: 553-561

Gorski CJ, Jones DR, Wrighton SA, Hall SD (1994) Characterization of dextromethorphan N-demethylation by human liver microsomes. *Biochem Pharm* **48**: 173-182.

Hartmann T, Terao M, Garattini E, Teutloff C, Alfaro JF, Jones JP, and Leimkuhler S (2012) The impact of SNPs on human aldehyde oxidase. *Drug Metab. Dispos.* doi: 10.1124/dmd.11.043828Houston BJ. (1994) Utility of invitro drug metabolism data in predicting in vivo metabolic clearance. *Biochem Pharm* **9**:1469-1479.

Hutzler, MJ, Yang YS, Albaugh D, Fullenwider, CL, Schmenk J, Fisher MB. (2012) Characterization of aldehyde oxidase enzyme activity in cryopreserved human hepatocytes. *Drug Metab Dispos* **40**:267-275

Johnson C, Stubley-Beedham C, Stell JG. (1985) Hydralazine: a potent inhibitor of aldehyde oxidase activity in vitro and in vivo. *Biochem Pharmacol* **34**: 4251-4256.

Kawashima K, Hosoi K, Naruke T, Shiba T, Kitamura M, Watabe T. (1999) Aldehyde oxidase-dependent marked species differences in hepatic metabolism of the sedative-hypnotic, zaleplon, between monkeys and rats. *Drug Metab Dispos* **27**: 422-428.

Kaye B, Rance DJ, Waring L. (1985) Oxidative metabolism of carbazeran in vitro by liver cytosol of baboon and man. *Xenobiotica* **3**: 237-242.

Kilford PJ, Stringer R, Sohal B, Houston BJ, Galestin A. (2009) Prediction of drug clearance by glucuronidation from in vitro data: use of combined cytochrome P450 and UDP-Glucuronosyltransferase cofactors in alamethicin-activated human liver microsomes. *Drug Metab Dispos* **37**: 82-89.

Lake BG, Ball SE, Kao J, Renwick AB, Price RJ, Scatina JA. (2002) Metabolism of zaleplon by human liver: evidence for involvement of aldehyde oxidase. *Xenobiotica* **10**:835-847.

Magee T, Ripp SL, Li R, Buzon RA, Chupak L, Dougherty TJ, Finegan SM, Girard D, Hagen A, Falcone MJ, Farley KA, Granskog K, Hardink JR, Huband MD, Kamicker BJ, Kaneko T, Knickerbocker MJ, Liras JL, Marra A, Medina I, NguyenTT, Noe MC, Obach RS, O'Donnel JP, Penzien JP, Reilly UD, Schafer JR, Shen Y, Stone GG, Strelevitz TJ, Sun J, Tait-Kamradt A, Vaz AD, Whipple DA, Widlika DW, Wishka DG, Wolkowki JP, Flanagan ME. (2009) Discovery of azetidinyl ketolide for the treatment of susceptible and multidrug resistant community-acquired respiratory tract infection. *J Med Chem* **52**: 7446-7457.

McGinnity DF, Soars MG, Urbanowicz RA, Riley RJ. (2004) Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. *Drug Metab Dispos* **32**: 1247-1253.

Morrison J, Walsh, C. (1988) The behavior and significance of slow-binding enzyme inhibitors. *Advances in Enzymology and Related Areas of Molecular Biology* **61**: 201-301.

Obach RS. (2011) Predicting clearance in humans from in vitro data. *Curr Topics in Med Chem* **11**: 334-339.

Obach RS. (2004) Potent inhibition of human liver aldehyde oxidase by raloxifene. *Drug Metab Dispos* **32**:89-97.

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, 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.

Renwick AB, Ball SE, Tredger RJ, Price RJ, Walters DG, Kao J, Scatina JA, Lake BG (2002) Inhibition of zaleplon metabolism by cimetidine in the human liver: in vitro studies with subcellular fractions and precision cut liver slices. *Xenobiotica* **32**: 849-862.

Terao M, Kurosaki M, Barzago MM, Varasano E, Boldetti A, Bastone A, Fratelli M, Garattini E (2006) Avian and canine aldehyde oxidases: novel insights into the biology and evolution of molydbo-flavoenzymes. *J. Bio. Chem* **28**: 19748-19761.

Walker GS, Ryder TF, Sharma R, Smith E, Freund A. (2011) Validation of isolated metabolites from drug metabolism studies as analytical standards by quantitative NMR. *Drug Metab Dispos* **39**:433-440.

Yoshimoto K, Echizen H, Chiba K, Tani M, Ishizaki T. (1995) Identification of human CYP isoforms involved in the metabolism of propanolol enatiomers—N-desisopropylation is mediated maily by CYP1A2. *Br J Clin Pharmac* **39**: 421-431.

Zientek M, Miller H, Smith D, Dunklee MB, Heinle L, Thurston A, Lee C, Hyland R, Fahmi O, Burdette D. (2008). Development of an in vitro drug-drug interaction assay to simultaneously monitor five cytochrome P450 isoforms and performance assessment using drug library compounds. *J Pharmacol Toxicol Methods* **58**: 206-214.

Zientek M, Jiang Y, Youdim K, Obach RS. (2010) In vitro- in vivo correlation for intrinsic clearance for drugs metabolized by human aldehyde oxidase. *Drug Metab Dispo.* **38**:1322-1327

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Reprint requests to:

Timothy Strelevitz

Pfizer Global Research and Development

Pharmacokinetics, Dynamics and Metabolism

MS: 8220-8559

Eastern Point Rd., Groton, CT 06340

Email: Timothy.J.Strelevitz@Pfizer.com

Figure Legends

Figure 1. Enzyme kinetics of zaleplon metabolism in five lot pool of cryopreserved human hepatocytes (0.5 M cell/mL) monitored for the formation of oxozaleplon (AO mediated) and desethylzaleplon (CYP mediated)

Figure 2. Inhibition of zaleplon (20 µM) metabolism by hydralazine (0 200 µM) in a five lot pool of cryopreserved human hepatocytes (0.5 M cell/mL), error bars represent the standard deviation of n=3 data points

Figure 3. Inhibition of zaleplon (20 µM) metabolism by ABT (0 – 1.5 µM) in a five lot pool of cryopreserved human hepatocytes (0.5 M cell/mL), error bars represent the standard deviation of n=3 data points

Figure 4. Inactivation of AO in human cytosol by hydralazine. Upper panel: time course of formation of oxozoniporide; Lower panel: relationship between inactivation rate constants and hydralazine concentration to determine K_I and k_{inact} that was derived from the data in the upper panel according to the method described by Morrison and Walsh (1988).

Figure 5. Effect of hydralazine (25 µM) on individual lot and pooled hepatocytes (1.5 M cell/mL) relative to Control (0 µM hydralazine), error bars represent the standard deviation of n=3 data points

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Compound Mass Spectrometer Parameters

Compound	Mass Transitions	Declustering Potential	Collision Energy	Collision Cell Exit Potential
Zaleplon	306→236	80	40	26
Oxozaleplon	322→252	80	40	26
Desethylzaleplon	278→208	80	40	26
O6-Benzylguanine	242→91	80	25	12
Carbazeran	361→272	80	30	12
DACA	294→249	90	25	12
PF-945863	825→668	90	40	12
Zoniporide	321→262	90	20	12
Midazolam	326→291	100	35	12
Naloxone	328→212	90	45	12
Propranolol	259→116	60	25	12
Dextromethorphan	272→215	75	35	12

DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. **Cytochrome P450 inhibition by hydralazine in human liver microsomes**

	% Control Activity					
Hydralazine (μM)	**CYP1A2**	**CYP2C8**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4**
25	100	108	105	103	92	98
50	89	95	95	90	77	76

The following probe substrates were used: 10 μM phenacetin for CYP1A2, 5 μM paclitaxel for CYP2C8, 5 μM diclofienac for CYP2C9, 40 μM s-mephenytoin for CYP2C19, 5 μM dextromethorphan for CYP2D6, and 2 μM midazolam for CYP3A4.

Table 3. Metabolic pathway, apparent intrinsic clearance and $f_{m(AO)}$ identified for ten selected compounds and compared in two human hepatocyte batches

Drug	Metabolic Pathways	Batch 1 $Cl_{int,app}$ (µL/min/10^6 cells)			Batch 2 $Cl_{int,app}$ (µL/min/10^6 cells)			$f_{m(AO)}$	
		No hydralazine	25 µM hydralazine	50 µM hydralazine	No hydralazine	25 µM hydralazine	50 µM hydralazine	Batch 1[a]	Batch 2[b]
O6-Benzylguanine	AO	23.8	NC	NC	21.0	7.50	3.65	NC	0.83
PF-0945863	AO, N-demethylation	23.9	NC	NC	24.8	11.5	9.61	NC	0.61
Zaleplon	AO, N-deethylation	11.5	3.47	3.08	10.9	6.95	4.82	0.70	0.56
Zoniporide	AO, hydrolysis	22.0	7.97	5.08	18.3	12.9	8.16	0.64	0.55
DACA	AO, N-demethylation	55.5	25.9	21.2	51.7	22.6	12.6	0.53	0.76
Carbazeran	AO, glucuronidation	73.9	37.8	27.2	67.2	37.1	30.1	0.49	0.55
Propranolol	Hydroxylation, glucuronidation	305	198	179	294	245	236	0.35	0.20
Midazolam	Hydroxylation	34.3	31.7	32.5	43.5	45.9	41.3	0.08	0.05
Naloxone	Hydroxylation, glucuronidation	56.9	52.9	54.5	48.3	57.4	56.9	0.07	ND
Dextromethorphan	N-demethylation*	-	-	-	30.9	29.0	18.7	-	0.39

Metabolic pathways were confirmed using biotransformation. Human hepatocytes were plated at 1.5 million cells/mL. Termination time points = 0, 5, 15, 30, 60, 120 and 240 min; N=3/time point. $Cl_{int,app}$ (µl/min/10^6 cells) values were calculated from averaged $AUC_{0\text{-}inf}$ extrapolated data.

Batch 1: pooled lots AGR, FKM, EHI, TDH, ZFB

Batch 2: lot RTH

[a] $f_{m(AO)}$ was determined using 25 µM hydralazine data

[b] $f_{m(AO)}$ was determined using 50 µM hydralazine data

- compound not run in assay

NC: $Cl_{int,app}$ in the presence of hydralazine could not be calculated because the slope of the ln[C] vs. time curve was not statistically different from zero, which precluded a reliable measurement.

ND: not detected

*Gorski et al., 1994

Figure 1

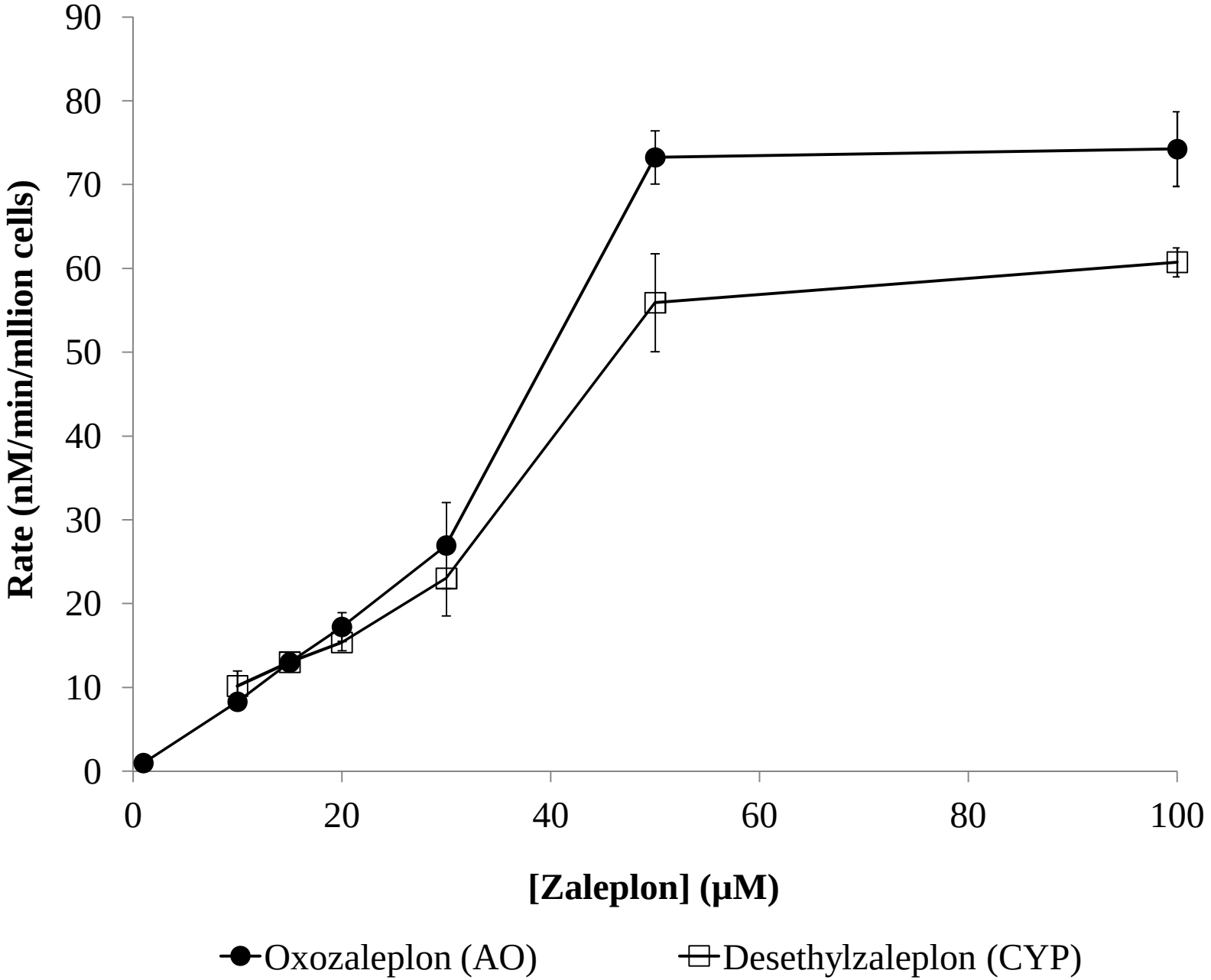


Figure 2

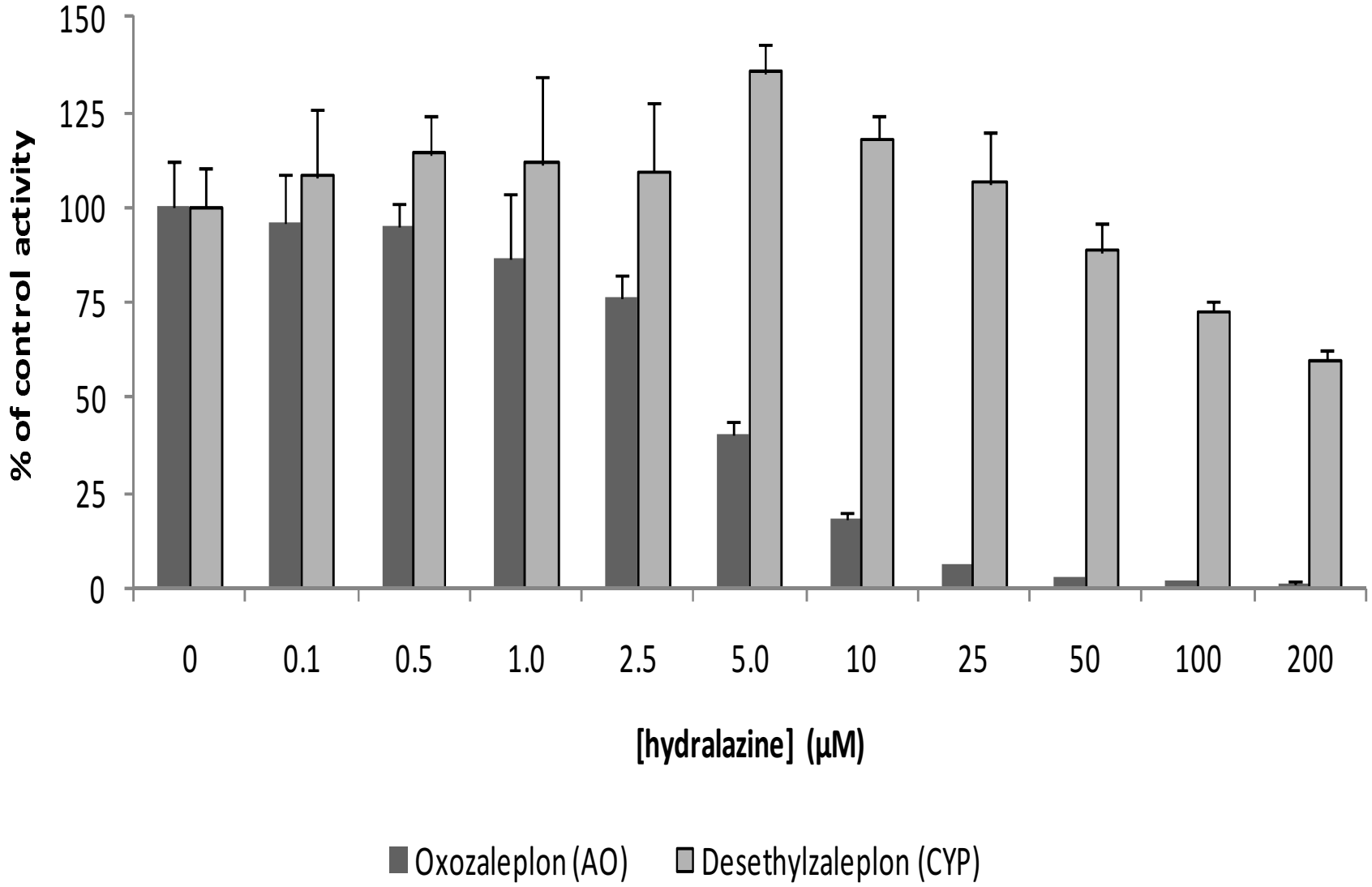
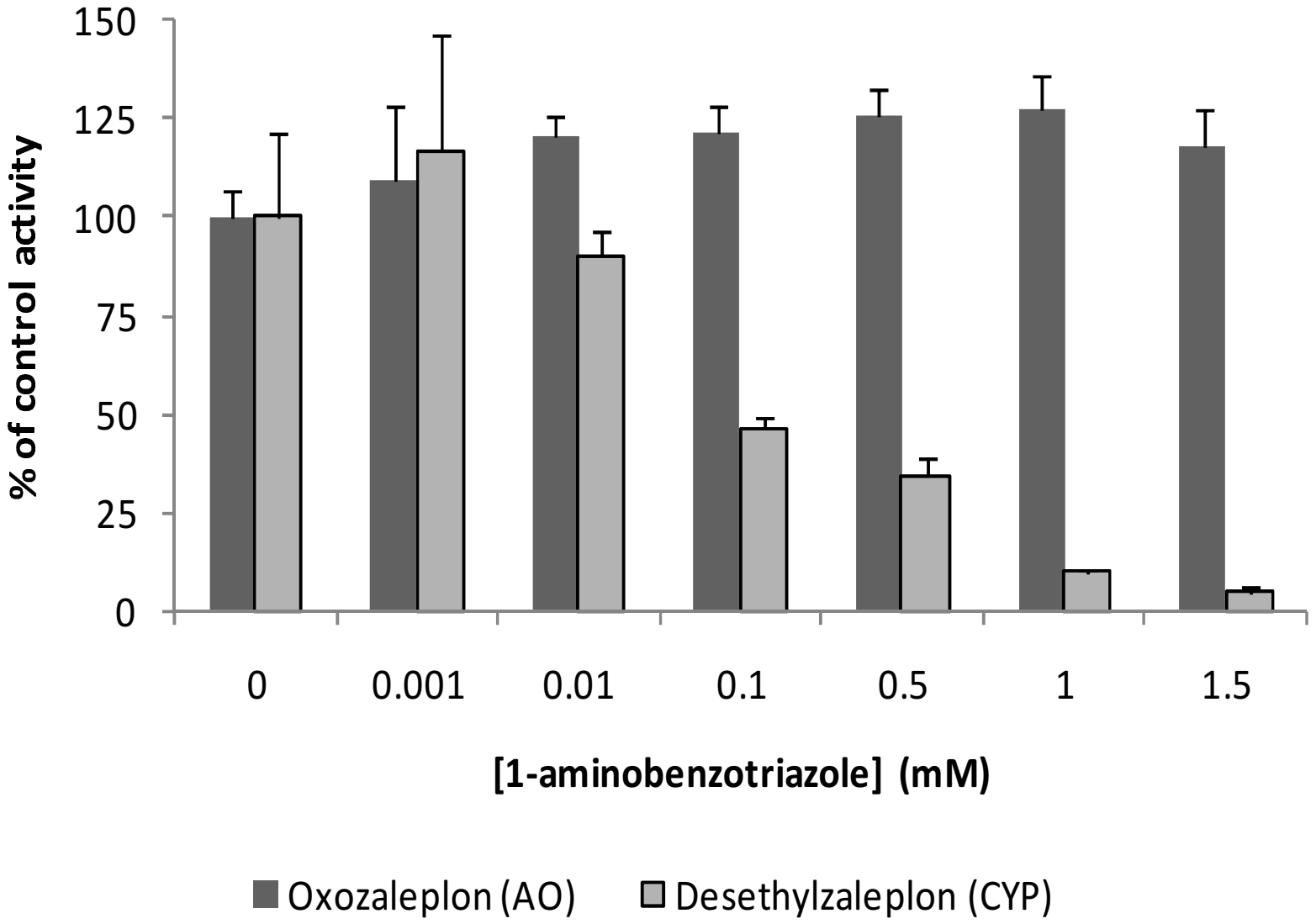
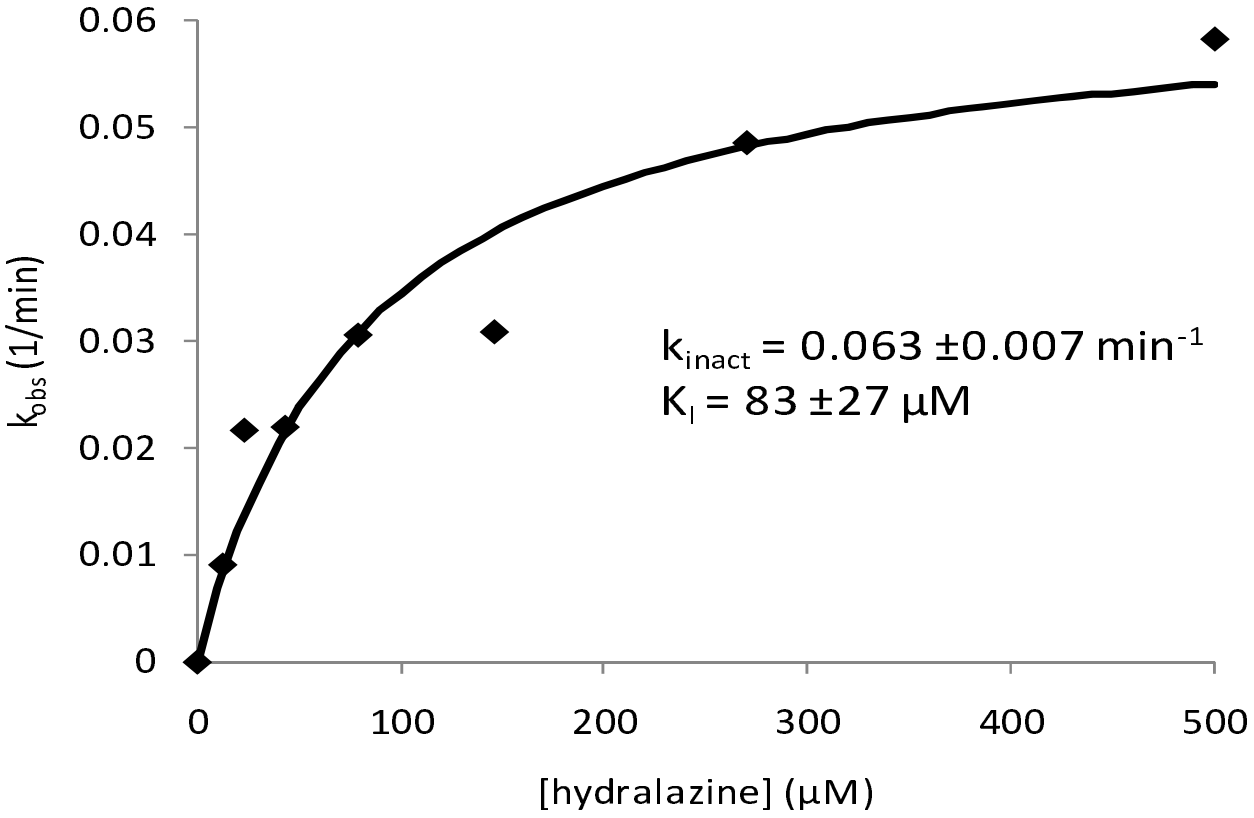


Figure 3



DMD Fast Forward. Published on April 20, 2012 as DOI: 10.1124/dmd.112.045195
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



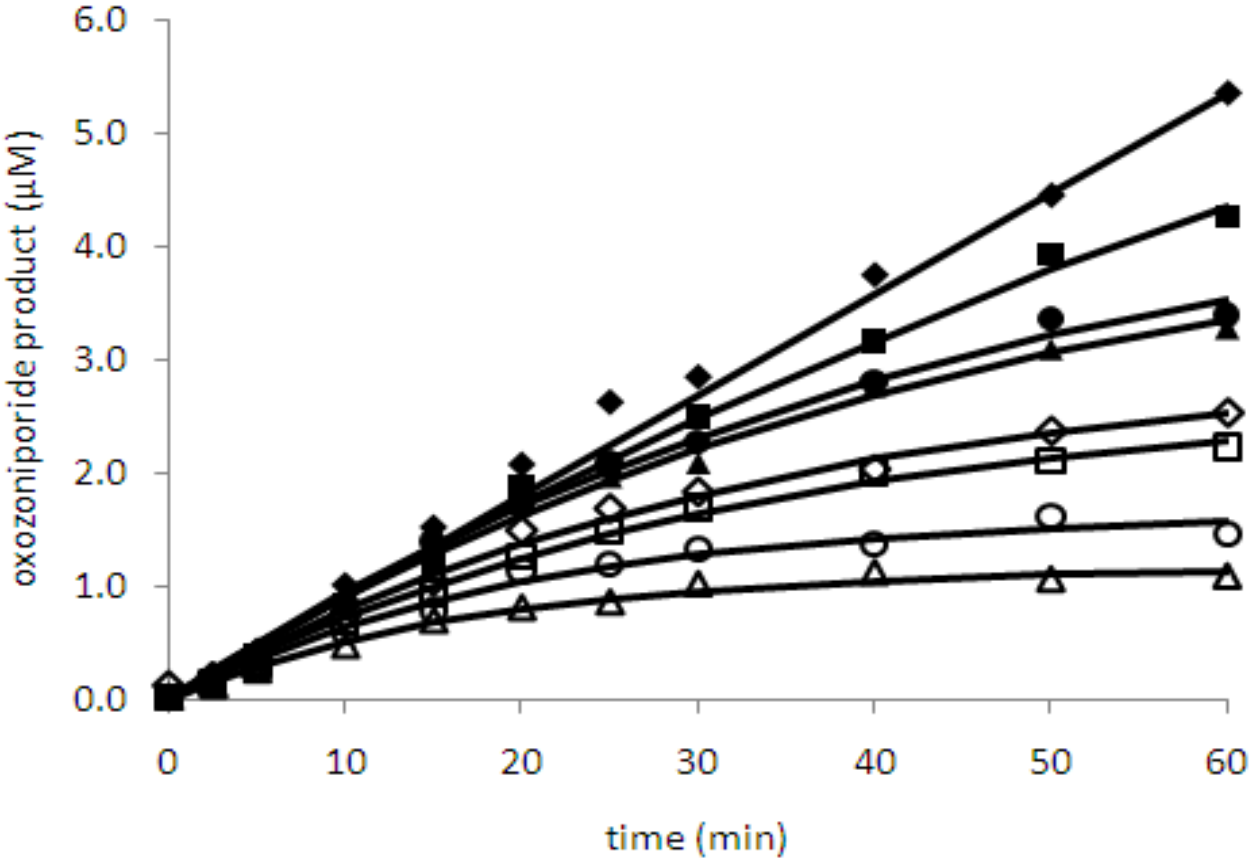


Figure 5