Evaluation of Icotinib as a Potent and Selective Inhibitor of Aldehyde Oxidase for Reaction Phenotyping in Human Hepatocytes

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Abbreviations:

AO  Aldehyde oxidase
CL\textsubscript{int,app}  Apparent intrinsic clearance
P450  Cytochrome P450
DMSO  Dimethyl sulfoxide
f\textsubscript{m,AO}  Fraction metabolized by aldehyde oxidase
UGT  Glucuronosyltransferase
K\textsubscript{I}  Inactivator concentration at half maximal inactivation rate
IC\textsubscript{50}  Inhibitor concentration at half maximal activity
IC\textsubscript{90}  Inhibitor concentration required for 90% inhibition
IVIVE  \textit{In vitro} to \textit{in vivo} extrapolation
k\textsubscript{inact}  Maximal inactivation rate
K\textsubscript{m}  Michaelis constant
MoCo  Molybdenum cofactor
k\textsubscript{obs}  Observed first-order inactivation rate
TDI  Time-dependent inhibitor/inhibition
Abstract

Aldehyde oxidase (AO) is a molybdenum cofactor-containing cytosolic enzyme that has gained prominence due to its involvement in the developmental failure of several drug candidates in first-in-human trials. Unlike cytochrome P450s (P450) and glucuronosyltransferase, AO substrates have been plagued by poor \textit{in vitro to in vivo} extrapolation, leading to low systemic exposures and underprediction of human dose. However, apart from measuring a drug’s AO clearance rates, it is also important to determine the relative contribution to metabolism by this enzyme ($f_{m,\text{AO}}$). Although hydralazine is the most well-studied time-dependent inhibitor (TDI) of AO and is frequently employed for AO reaction phenotyping in human hepatocytes to derive $f_{m,\text{AO}}$, multiple studies have expressed concerns pertaining to its utility in providing accurate estimates of $f_{m,\text{AO}}$ values due to its propensity to significantly inhibit P450s at the concentrations typically utilized for reaction phenotyping. In this study, we characterized icotinib, a cyclized analogue of erlotinib, as a potent TDI of AO – inactivating human liver cytosolic zoniporide 2-oxidation equipotently with erlotinib with a $k_{\text{inact}}/K_{\text{I}}$ ratio of 463 and 501 min$^{-1}$mM$^{-1}$, respectively. Moreover, icotinib also exhibits selectivity against P450 and elicits significantly weaker inhibition against human liver microsomal UGT1A1/3 as compared to erlotinib. Finally, we evaluated icotinib as an inhibitor of AO for reaction phenotyping in cryopreserved human hepatocytes and demonstrated that it can yield more accurate prediction of $f_{m,\text{AO}}$ compared to hydralazine and induce sustained suppression of AO activity at higher cell densities – which will be important for reaction phenotyping endeavors of low clearance drugs.
Significance Statement:

In this study, we characterized icotinib as a potent time-dependent inhibitor of AO with ample selectivity margins against the P450s and UGT1A1/3 and demonstrated its utility for reaction phenotyping in human hepatocytes to obtain accurate estimates of $f_{m, AO}$ for victim DDI risk predictions. We envisage the adoption of icotinib in place of hydralazine in AO reaction phenotyping.
Introduction

Recent medicinal chemistry design strategies, which have endeavored to reduce lipophilicity through the introduction of electron-withdrawing azaheterocyclic rings to attenuate lability towards cytochrome P450 (P450)-mediated oxidation, also have the inadvertent effect to shunt metabolism towards non-P450 enzymes (Pryde et al., 2010). One such enzyme is aldehyde oxidase (AO), a molybdenum cofactor (MoCo)-containing cytosolic enzyme that can play an instrumental role in phase I drug metabolism (Dalvie and Di, 2019). AO is a highly promiscuous enzyme that can catalyze the oxidative metabolism of aldehydes, iminium ions, and azaheterocyclic rings as well as the reduction of nitro-containing compounds (Kitamura et al., 2006; Pryde et al., 2010; Garattini and Terao, 2012). Over the last few decades, AO has gained notoriety for being liable in the clinical failures of several drugs – such as carbazeran, BIBX1382, PF-945863, and LuAF09535 – following first-in-human dosing due to inadequate systemic exposures (Kaye et al., 1985; Dittrich et al., 2002; Magee et al., 2009; Jensen et al., 2017). Although significant progress has been made over the last couple of years to enhance our understanding on the in vitro to in vivo extrapolation (IVIVE) of AO clearances for human dose predictions (Izat et al., 2023), there still remains a disparity in our AO predictive capacity as compared to drugs which are substrates of P450s and/or glucuronosyltransferase (UGTs) (Zientek et al., 2010).

Apart from possessing robust methods for IVIVE of AO clearance rates, it is also imperative to develop approaches that can accurately predict in vivo fraction metabolized by AO ($f_{m,\text{AO}}$) from in vitro data as this would help refine IVIVE capabilities and help inform the potential for victim drug-drug interactions (DDI). Hydralazine, an irreversible time-dependent inhibitor (TDI) of AO (Johnson et al., 1985), is frequently harnessed to estimate $f_{m,\text{AO}}$ of prospective clinical drug
candidates in human hepatocytes (Strelevitz et al., 2012). However, the success of this approach is predicated on the core idea that the AO chemical inhibitor employed engenders minimal ‘off-target’ inhibition against other major drug metabolizing enzymes. Strelevitz et al., 2012 previously noted that hydralazine also impacted propranolol and dextromethorphan metabolism in human hepatocytes, suggesting spill over inhibition of other enzymes. Further work has shown that the utility of hydralazine in AO reaction phenotyping is hampered by its non-selectivity towards the P450s. Yang et al., 2019 has demonstrated that hydralazine also non-selectively inhibits CYP1A2, 2B6, 2D6 and 3A activity in human hepatocytes at the concentrations which are frequently employed for AO reaction phenotyping (i.e., 25–50 µM). Additionally, as the IC$_{50}$ of hydralazine against CYP2D6 closely mirrors its IC$_{90}$ against AO, it is recommended that hydralazine should not be utilized to derive f$_{m,AO}$ values for CYP2D6 substrates (Zientek and Youdim, 2015). Moreover, there also appears to be considerable variation in the magnitude of inhibition – ranging from ~5–20% – elicited by a fixed concentration of hydralazine amongst different lots or batches of human hepatocytes (Strelevitz et al., 2012). Taken together, it implies that f$_{m,AO}$ values obtained using hydralazine could be under/overestimated. At this juncture, it also underscores the impetus towards identifying a more potent and/or selective inhibitor of AO for reaction phenotyping in human hepatocytes.

Erlotinib (Fig. 1), a tyrosine kinase inhibitor against epidermal growth factor receptor (EGFR), is a potent competitive inhibitor of human AO wherein it inhibited human liver cytosolic carbazeran 4-oxidation and zaleplon 5-oxidation with a $K_i$ of 0.26 and 0.10 µM, respectively (Tan et al., 2020). Additionally, several groups – including us – have recently shown that erlotinib is a TDI/mechanism-based inactivator of AO (Dick et al., 2023; Liu et al., manuscript in-press). However, erlotinib is also known to inhibit UGT1A1 potently, with $K_i$ values in the
low micromolar range (Liu et al., 2010). Consequently, it is unlikely to fulfill the criteria needed as a chemical inhibitor of AO when used to evaluate its impact on substrate depletion. Interestingly, it has been reported that icotinib (Fig. 1), a cyclized analogue of erlotinib, exhibited a dramatic ~8-fold reduction in its UGT1A1 potencies (Cheng et al., 2017). However, the propensity for icotinib to elicit TDI of AO had not been determined.

In this study, we report for the first time that icotinib inactivates human liver cytosolic AO in a time- and concentration-dependent manner and is equipotent to that of erlotinib. Additionally, we also examined its interactions with UGT1A1/3 and P450s and determined that it possesses adequate selectivity margins against AO. Finally, we evaluated icotinib as a chemical inhibitor of AO for reaction phenotyping in human hepatocytes and demonstrated that it can yield more accurate prediction of $f_{m, AO}$ compared to hydralazine.
Materials and Methods

Chemicals and Reagents. Carbazeran, capmatinib, N-(2-dimethylamino)ethyl)acridine-4-carboxamide (DACA), erlotinib, hydralazine, idelalisib, JNJ38877605, 3-descladinosyl-11,12-dideoxy-6-Omethyl-3-oxo-12,11-(oxycarbonyl-(1-(1R-(1,8-naphthyridin-4-yl)-ethyl)azetidin-3-yl)-imino)-erythromycin A (PF-0945863), and zoniporide were obtained from Pfizer Global Material Management (Groton, CT). Alamethicin, β-estradiol, lithocholic acid, O\textsuperscript{6}-benzylguanine, and uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). Icotinib hydrochloride was procured from MedChem Express (Monmouth Junction, NJ). Zaleplon was acquired from USP (Rockville, MD). Human liver microsomes (Lot HLM103, pooled from 36 male and 14 female donors) were obtained under contract from Sekisui Xenotech (Kansas City, KS). Human liver cytosol (Lot FDD, pool of 16 donors comprising 8 males and 8 females) was purchased from Celsis IVT (Chicago, IL). Cryopreserved human hepatocytes (Lot SPB, custom donor mixed consisting of 6 male and 7 female) were obtained under contract from BioIVT (Westbury, NY). Williams’ E medium (WEM) was purchased from Gibco (Dublin, Ireland). All other chemicals and reagents utilized in this work were of the highest purity available.

IC\textsubscript{50} Shift of AO in Human Liver Cytosol. IC\textsubscript{50} shift experiments were performed as described previously with some minor modifications (Obach et al., 2007). Briefly, incubations were performed in duplicates (n=2) in a reaction mixture consisting of 100 mM potassium phosphate buffer (pH 7.4), human liver cytosol (0.06 mg/mL), the AO probe substrate zoniporide (10 µM – ∼\textit{K}_m), and either vehicle (0.5% v/v dimethyl sulfoxide – DMSO), erlotinib (0.003 – 30 µM) or icotinib (0.001 – 10 µM). The final organic content was 0.5% v/v DMSO – which was previously demonstrated to not significantly affect cytosolic AO activity (Behera et al., 2014).
Following a 5 min prewarming step at 37°C on a dry heater block, the reaction was initiated with the sequential addition of probe substrate (i.e., zoniporide) and inhibitor (i.e., erlotinib or icotinib). Parallel experiments which incorporated a 30 min preincubation step with the inhibitor were also conducted to determine if the inhibition elicited by either test inhibitors were time-dependent in nature. In these incubations, the probe substrate was introduced after 30 min. In both cases, the reaction was allowed to proceed for 15 min – under which formation of 2-oxozoniporide by human liver cytosolic AO was previously determined to be linear (data not shown) – after which the reaction was quenched via the addition of three volumes of ice-cold acetonitrile containing internal standard (50 ng/mL tolbutamide). The samples were then vortexed and centrifuged at 2100 g for 5 min. Subsequently, aliquots of supernatant were withdrawn, dried down under a gentle stream of N₂ gas and reconstituted in a lower volume of 5:95 water/acetonitrile mixture for tandem liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

**Time-Dependent Inhibition of AO in Human Liver Cytosol.** Evaluation of TDI was performed as described previously with minor modifications (Tang et al., 2022). Briefly, reaction mixtures comprising 100 mM potassium phosphate buffer (pH 7.4), human liver cytosol (0.6 mg/mL), along with 100× working stock solutions of erlotinib or icotinib (DMSO <0.5% v/v) were prepared. Following a 5 min prewarming at 37°C on a dry heater block, the reaction mixtures (n=2) were preincubated with varying concentrations of erlotinib (0.02 – 50 µM), icotinib (0.02 – 50 µM) or vehicle (0.5% v/v DMSO). At various predefined timepoints (1, 3, 6, 10, 15, and 23 min), an aliquot of this primary preincubation mixture was sampled and diluted 20-fold into a secondary activity incubation mixture containing the AO probe substrate zoniporide (100 µM - ~10-fold $K_m$) in 100 mM potassium phosphate buffer (pH 7.4). The
secondary incubation was terminated after 15 min via the addition of three volumes of ice-cold acetonitrile containing internal standard (50 ng/mL tolbutamide). The samples were then vortexed, centrifuged, concentrated, and reconstituted as described above for LC-MS/MS analysis.

**Inhibition of UGT1A1 and UGT1A3 in Human Liver Microsomes.** IC₅₀ experiments against UGT1A1 was conducted as previously described (Walsky et al., 2012), except the substrate concentrations were reoptimized for the specific lot of human liver microsomes employed in these studies. Briefly, incubations mixtures consisting of 100 mM Tris HCl buffer (pH 7.5), human liver microsomes (0.025 mg/mL), 5 mM MgCl₂, and an isoform-selective probe substrate at concentrations approximating the Kᵣ or S₅₀ (100 µM β-estradiol for UGT1A1 and 120 nM lithocholic acid for UGT1A3) were prepared prior to the start of the assay. The incubation mixture was pre-incubated with alamethicin (10 µg/mL) on ice for 15 min to allow for pore formation. The incubation mixture was warmed to 37°C on a dry heater block prior to the addition of either erlotinib (0.006 – 60 µM), icotinib (0.006 – 60 µM) or vehicle (1% v/v DMSO). Reactions were initiated by the addition of 5 mM UDPGA and stopped after 60 min for UGT1A1 and 5 min for UGT1A3 via the addition of ice-cold acetonitrile containing internal standard (30 nM d₃-β-Estradiol-3-glucuronide for UGT1A1 and 50 ng/mL tolbutamide for UGT1A3). The samples were then vortexed, centrifuged, concentrated, and reconstituted as described above for LC-MS/MS analysis.

**Evaluation of Time-Dependent Inhibition of Human Liver Microsomal P450 by Icotinib.**

The propensity for icotinib to evoke TDI of several relevant P450 isoforms (i.e., CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A) was evaluated using a cocktail TDI assay at 3 µM as described previously (Yates et al., 2012). Briefly, reaction mixtures comprising 100
mM potassium phosphate buffer (pH 7.4), human liver microsomes (0.3 mg/mL), NADPH (1.3 mM), and MgCl$_2$ (3.3 mM) were prepared and prewarmed to 37°C on a dry heat block for 5 min. Erlotinib or icotinib (3 µM) was then added and the reaction mixture was preincubated. At various timepoints (1, 5, 10, 20, 30, and 40 min), an aliquot of the primary preincubation mixture was transferred to a secondary activity mixture containing the P450 cocktail substrates (at concentrations ~10× $K_m$) (Supplementary Table 1), NADPH (1.3 mM), and MgCl$_2$ (3.3 mM) in 100 mM potassium phosphate buffer (pH 7.4). The secondary incubation was allowed to proceed for 6 min after which it was terminated via the addition of an equal volume of ice-cold acetonitrile containing a cocktail of internal standards (Supplementary Table 1). The samples were then vortexed, centrifuged, concentrated, and reconstituted as described above for LC-MS/MS analysis.

Intrinsic Clearance Determination of Prototypical AO Substrates in Human Hepatocytes.

Human hepatocytes were freshly thawed prior to the start of each experiment and resuspended in prewarmed and oxygenated WEM supplemented with 26 mM NaHCO$_3$ and 50 mM HEPES. Cells were counted and viability was assessed using the trypan blue exclusion method. All vials of thawed hepatocytes utilized in this study were determined to have ≥80% viability. The hepatocytes were preincubated with either vehicle (0.2% v/v methanol) or icotinib (3 µM) on an orbital shaker (VWR, Radnor, NJ) set to 150 rpm for 30 min in a humidified incubator (75% relative humidity, 5% CO$_2$) maintained at 37°C. Thereafter, 100× working stock solutions of each individual AO substrate (i.e., carbazeran, zoniporide, zaleplon, DACA, O$_6$-benzylguanine, idelalisib, capmatinib, JNJ38877605, and PF-0945863) were added to initiate the substrate depletion experiment. The final concentration of each AO substrate and hepatocyte cell density was 1 µM and 0.5 million cells/mL, respectively. At various predetermined timepoints ranging
from 0 – 4 h, aliquots were sampled and transferred into four volumes of an ice-cold acetonitrile quenching solution containing internal standard (50 ng/mL tolbutamide). The samples were then vortexed and centrifuged at 2100g for 5 min to pellet the cells. Subsequently, the supernatants were withdrawn, dried down under a gentle stream of N₂ gas, and reconstituted in a lower volume of 5:95 water/acetonitrile mixture for LC-MS/MS analysis.

**Effect of Icotinib Concentration and Hepatocyte Cell Density on the Inhibition of Zaleplon Oxidase and Deethylase Activity.** Incubations were performed as described in the previous section with some minor alterations. Briefly, freshly thawed human hepatocytes were resuspended into two different densities representative of those typically employed in hepatocyte stability assays. Hepatocytes were preincubated with either vehicle (0.2% v/v methanol), icotinib (3 µM) or hydralazine (25 µM) as described in the previous section. Thereafter, 100× working stock solutions of zaleplon were added to initiate the substrate depletion experiment. The final concentration of zaleplon was 1 µM whereas the final hepatocyte cell densities were either 0.5 million cells/mL or 1.5 million cells/mL. After 30 min, the reaction was quenched, and the resulting samples were then vortexed, centrifuged, concentrated, and reconstituted as described in the previous section for LC-MS/MS analysis. Residual zaleplon oxidase activity – which acts as a surrogate of AO enzymatic activity – in human hepatocytes at the two different densities after a 30 min preincubation with either icotinib or hydralazine was expressed as a percentage of its corresponding vehicle control incubations. Desethylzaleplon, a minor metabolite of zaleplon which is predominantly catalyzed by CYP3A (Renwick et al., 1998), was also measured. Additionally, parallel experiments which sought to interrogate the impact of different icotinib concentrations (0.001 – 10 µM) on zaleplon oxidase and deethylase activities in hepatocytes (0.5 million cells/mL) were also conducted.
LC-MS/MS Methodology. All samples were analyzed using a LC-MS/MS system consisting of an Agilent 1290 binary pump (Santa Clara, CA), a CT PAL autosampler (Carrboro, NC), and a Valco 2-position switching valve interfaced with an AB Sciex 6500 triple quadrupole mass spectrometer (Framingham, MA) equipped with an electrospray ionization source operating in mixed polarity mode. Chromatographic separation of all analytes described in this work was achieved using an ACQUITY ultra-performance liquid chromatography ethylene bridged hybrid (BEH) C18, 1.7 µm, 2.2 x 50 mm column (Waters, Milford, MA). Unless or otherwise stated, the aqueous mobile phase (A) was 0.1% formic acid in water and the organic mobile phase (B) was 0.1% formic acid in acetonitrile. The gradient elution conditions were as follows: linear gradient from 5% to 95%B (0 – 1.20 min), isocratic at 95%B (1.20 – 1.50 min), and re-equilibration at 5%B (1.50 – 2.00 min). Mobile phases were delivered at a flow rate of 0.6 mL/min. The autosampler temperature was maintained at 4°C and the injection volume ranged from 2 – 10 µL, depending on the analyte being quantified. The multiple reaction monitoring ion transitions and compound-dependent MS parameters (i.e., declustering potential and collision energy) are summarized in Supplementary Table 2. Chromatographic peak integration was performed using the Analyst software (Sciex, Framingham, MA). For all analytes the peak area of the analyte was expressed as a ratio to the peak area of the corresponding internal standard. Specific information pertaining to the LC-MS/MS conditions for the UGT1A1 and UGT1A3 assay are detailed in Supplementary Table 3.

Data Analysis.

Estimation of IC\textsubscript{50}. IC\textsubscript{50} values for the direct inhibition of zoniporide 2-oxidation in human liver cytosol, β-estradiol 3-O-glucuronidation and lithocholic acid glucuronidation in human liver
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Microsomes by erlotinib and icotinib were determined by nonlinear regression fitting to a four-parameter model with variable slope (Equation 1) on GraphPad Prism 9 (La Jolla, CA).

\[
Y = Min + \frac{Max - Min}{1 + \left(\frac{IC_{50}}{X}\right)^h}
\]

where \(Y\) is percent activity remaining, \(Min\) is the minimum activity asymptote, \(Max\) is the maximal activity asymptote, \(IC_{50}\) is the inhibitor concentration at the inflection point between maximum and minimum activities, \(X\) is the unbound inhibitor concentration in the incubation, and \(h\) is the Hill coefficient.

**Estimation of \(K_I\) and \(k_{inact}\).** Inactivation kinetic parameters for the TDI of erlotinib and icotinib against zoniporide 2-oxidation in human liver cytosol were computed according to methods previously described by Yates *et al.*, 2012. Briefly, the observed first-order inactivation rate \((k_{obs})\) was determined by normalizing the peak area ratios of 2-oxozoniporide in each sample to that of the corresponding mean solvent control peak area ratios in the initial time point after which a natural logarithmic transformation was applied, and the resulting data points were fitted to a straight line. The \(k_{obs}\) at each inhibitor concentration was obtained by calculating the slope of line in the initial linear portion of the curve. Finally, a plot of \(k_{obs}\) against the nominal concentrations of erlotinib or icotinib employed in the assay allowed for the estimation of \(K_I\) and \(k_{inact}\) by nonlinear regression of the three-parameter Michaelis-Menten model (Equation 2).

\[
k_{obs} = k_{obs\ (solvent\ control)} + \frac{k_{inact} \times [I]}{K_I + [I]}
\]
where \( k_{\text{obs (solvent control)}} \) is the observed first-order inactivation with solvent control, \( k_{\text{inact}} \) is the maximal inactivation rate, \( K_I \) is the inactivator concentration at half \( k_{\text{inact}} \) and \([I]\) is the nominal concentrations of the test inhibitor in the primary preincubation mixture.

The exact same method was adopted for the calculation of \( k_{\text{obs}} \) of erlotinib and icotinib in the single concentration cocktail TDI assay. A parallel line test (Equation 3) was conducted for both compounds against each of the six P450 isoforms evaluated to determine if the \( k_{\text{obs}} \) obtained with erlotinib or icotinib at 3 \( \mu \text{M} \) was significantly different (i.e., \( p < 0.05 \)) from the respective solvent control.

\[
z = \frac{|k_{\text{obs}} - k_{\text{obs (solvent control)}}|}{S.E. k_{\text{obs}} - S.E. k_{\text{obs (solvent control)}}}
\]  

(3)

where \( k_{\text{obs}} \) and \( k_{\text{obs (solvent control)}} \) is the observed first-order inactivation with erlotinib/icotinib at 3 \( \mu \text{M} \) and solvent control, respectively.

**Estimation of \( f_{m,AO} \).** Estimation of fraction metabolized by AO (\( f_{m,AO} \)) was adapted from Strelevitz et al., 2012 with some minor modifications. Briefly, the \( \text{CL}_{\text{int,app}} \) of each AO substrate in human hepatocytes was derived using Equation 4 – 6.

\[
t_{1/2} \text{(min)} = \frac{\ln 2}{k}
\]

(4)

\[
V \text{ (\( \mu \text{L/million cells} \))} = \frac{\text{incubation volume}}{\text{number of cells in incubation}}
\]

(5)

\[
\text{CL}_{\text{int,app}} \text{ (\( \mu \text{L/min/million cells} \))} = \frac{\ln 2 \times V}{t_{1/2}}
\]

(6)
where 

\[ t_{1/2} \]

is the estimated half-life of substrate in the hepatocyte stability assay, \( k \) is the elimination rate determined from the plot of the terminal elimination phase on a natural logarithmic scale, and \( \text{CL}_{\text{int,app}} \) is the apparent intrinsic clearance.

Thereafter, the \( f_{m,AO} \) value was then estimated using **Equation 7**.

\[
f_{m,AO} = \frac{\text{CL}_{\text{int,app}} (\text{vehicle}) - \text{CL}_{\text{int,app}} (\text{icotinib})}{\text{CL}_{\text{int,app}} (\text{vehicle})}
\]

(7)

where \( \text{CL}_{\text{int,app}} (\text{vehicle}) \) and \( \text{CL}_{\text{int,app}} (\text{icotinib}) \) is the apparent intrinsic clearance calculated in the absence and presence of icotinib, respectively.
Results

**Equipotent Time-Dependent Inhibition of Human Liver Cytosolic AO by Erlotinib and its Structural Analogue Icotinib.** Both erlotinib and icotinib were found to potently inhibit human liver cytosolic AO-mediated zoniporide 2-oxidation in a concentration-dependent manner with IC\textsubscript{50} values in the sub-micromolar range (**Fig. 2A and B**) (**Table 1**). When we incorporated a 30 min inhibitor preincubation step into the assay, a characteristic leftward shift in its IC\textsubscript{50} curve was observed for both test inhibitors. This was accompanied by an 8.0- and 6.2-fold shift in the apparent IC\textsubscript{50} values of erlotinib and icotinib, respectively, to 0.280 ± 0.018 µM and 0.717 ± 0.181 µM (**Fig. 2A and B**) (**Table 1**). Taken together, these results suggested that the inhibition of AO elicited by both erlotinib and icotinib are time-dependent in nature. We then followed-up these preliminary studies with a more exhaustive TDI assay at multiple inhibitor concentrations and preincubation times to elucidate inactivation kinetics parameters (K\textsubscript{I} and k\textsubscript{inact}). These experiments confirmed that erlotinib and icotinib inactivated human liver cytosolic AO-mediated zoniporide 2-oxidation in a time- and concentration-dependent manner (**Fig. 3A and B**). Plotting the k\textsubscript{obs} against the nominal concentrations of erlotinib and icotinib employed in the primary preincubation mixture yielded a characteristic hyperbolic Michaelis-Menten profile with K\textsubscript{I} values of 0.719 ± 0.087 µM and 1.18 ± 0.108 µM, and k\textsubscript{inact} values of 0.360 ± 0.014 min\textsuperscript{-1} and 0.546 ± 0.018 min\textsuperscript{-1} for erlotinib and icotinib, respectively (**Fig. 3C and D**) (**Table 1**). Importantly, our results also revealed that icotinib evoked equipotent TDI of AO as erlotinib as substantiated by its similar k\textsubscript{inact}/K\textsubscript{I} ratio (i.e., 463 min\textsuperscript{-1}mM\textsuperscript{-1} in icotinib vs. 501 min\textsuperscript{-1}mM\textsuperscript{-1} in erlotinib) (**Table 1**).

**Icotinib Engenders Less Potent Direct Inhibition of Human Liver Microsomal UGT1A1 and UGT1A3 Compared to Erlotinib.** Erlotinib is known to be potent direct inhibitor of
UGT1A1, with $K_i$ values in the low micromolar range (Liu et al., 2010). These previous findings were confirmed in this study where we demonstrated that erlotinib inhibited human liver microsomal UGT1A1-catalyzed $\beta$-estradiol 3-glucuronidation with an IC$_{50}$ value of 1.34 ± 0.110 µM (Supplementary Fig. 1A). Furthermore, we demonstrated that erlotinib could also potently inhibit human liver microsomal UGT1A3-mediated lithocholic acid 24-glucuronidation with an IC$_{50}$ value of 3.02 ± 0.733 µM (Supplementary Fig. 1B) (Table 2). Conversely, we determined that while icotinib could also engender inhibition of both UGT1A1 and UGT1A3 in a concentration-dependent manner, the corresponding IC$_{50}$ were 12.2 ± 2.20 µM and 25.6 ± 2.52 µM which were 9.1- and 8.5-fold higher, respectively, as compared to erlotinib (Supplementary Fig. 1A and B) (Table 2).

### Icotinib Does Not Yield Meaningful Time-Dependent Inhibition of P450 Enzymes.

Our TDI screen of erlotinib and icotinib against the six clinically-relevant P450 isoforms revealed that, at the single studied concentration of 3 µM, both compounds were devoid of TDI against CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 (Supplementary Fig. 2). Rather, TDI was only discernable with CYP3A wherein the $k_{obs}$ values were 0.012 min$^{-1}$ and 0.009 min$^{-1}$ for erlotinib and icotinib, respectively, which differed significantly ($p < 0.05$) from the solvent control ($k_{obs} = 0.0051$ min$^{-1}$) as determined by the parallel line test (Supplementary Fig. 2). However, it is known that TDI assays for CYP3A in human liver microsomes yield markedly higher estimates of $k_{obs}$ as compared to hepatocytes (Eng et al., 2021), hence a question arises as to whether the apparent TDI of CYP3A by icotinib identified in our single concentration TDI screen would be recapitulated in hepatocytes. Measurement of CYP3A-catalyzed zaleplon deethylase activity in 0.5 million cells/mL of hepatocyte revealed that CYP3A activity did not decline after a 30 min
preincubation with icotinib (0.001 – 10 µM) (Fig. 4). Rather, we observed a dose-dependent decrease in AO-mediated zaleplon oxidase activity with an IC₅₀ of 0.175 µM (Fig. 4).

**Evaluation of Icotinib as a Chemical Inhibitor of AO for Reaction Phenotyping in Human Hepatocytes.** Nine prototypical AO substrates (carbazeran, zoniporide, zaleplon, DACA, O⁶-benzylguanine, idelalisib, capmatinib, JNJ38877605, and PF-0945863) were employed to assess the utility of icotinib for AO reaction phenotyping in human hepatocytes. The representative substrate depletion plots for carbazeran (high AO clearance), zoniporide (moderate AO clearance), and zaleplon (low AO clearance) generated from the hepatocyte stability incubations in the presence and absence of icotinib (3 µM) preincubation are depicted in Fig. 5. The CL_{int,app} (vehicle) of carbazeran, zoniporide and zaleplon are 24.1, 11.3, and 7.48 µL/min/million cells, respectively, whereas the corresponding CL_{int,app} (icotinib) are 3.99, 2.24, and 2.53 µL/min/million cells. This in turn translated to an estimated f_{m,AO} value of 0.83 for carbazeran, 0.80 for zoniporide and 0.66 for zaleplon (Table 3). The f_{m,AO} for the rest of the AO substrates determined in this work by utilizing the icotinib approach as well as those using the conventional hydralazine methodology are summarized in Table 3. Plotting the f_{m,AO} values revealed a good correlation between both orthogonal approaches (Fig. 6A), with the f_{m,AO} values of 6 out of 9 compounds being within 1.5-fold of each other. There was only one compound (capmatinib) in which the f_{m,AO} value deviated by more than 2-fold. Comparing the in vitro f_{m,AO} values gleaned from hepatocyte inhibition studies with those obtained from in vivo human mass balance studies (Lake et al., 2002; Dalvie et al., 2013; Jin et al., 2013; Glaenzel et al., 2020) revealed that the icotinib approach yielded more accurate predictions of f_{m,AO} values in 3 out of 4 compounds (Fig. 6B). Furthermore, there was a substantial improvement to the R² value obtained when comparing f_{m,AO} values determined using the newer paradigm involving icotinib (0.899) as
compared to hydralazine (0.802) (Supplementary Fig. 3). Notably, the $f_{m, AO}$ of the aforementioned AO substrate, capmatinib, was grossly underpredicted with hydralazine (~5-fold) but was successfully predicted well within 1.5-fold of its corresponding in vivo $f_{m, AO}$ value (0.40 vs. 0.33).

**Inhibition of AO-mediated Zaleplon Oxidase Activity by Icotinib is Preserved at Higher Hepatocyte Cell Densities.** It is also imperative to consider if inhibition of AO can be preserved at higher hepatocyte cell densities – which may be employed for CL$_{int, app}$ determination of compounds with lower metabolic clearance – so that $f_{m, AO}$ values are not underpredicted due to insufficient inactivation of AO enzymatic activity. Here, we showed that residual AO-catalyzed zaleplon oxidase activity (normalized to vehicle controls) after a 30 min preincubation with icotinib (3 µM) was relatively unchanged – 3.55 ± 0.07 % at 0.5 million cells/mL and 5.33 ± 0.43 % at 1.5 million cells/mL (Fig. 7) (Table 4). On the contrary, we observed that at the higher hepatocyte cell density, there was a marked ~5-fold increase in residual zaleplon oxidase activity from 2.87 ± 0.23 % at 0.5 million cells/mL to 14.2 ± 0.33 % at 1.5 million cells/mL with hydralazine (Fig. 7) (Table 4).
**Discussion**

AO is a drug metabolizing enzyme that has gained prominence in pharmaceutical research due to its involvement in the developmental failure of several drug candidates. Unlike P450s and UGTs, AO substrates have been plagued by poor IVIVE, leading to low systemic exposures and underprediction of human dose (Hutzler *et al.*, 2013). In addition to measuring a drug’s AO clearance rates, it is also important to determine the relative contribution to metabolism by this enzyme. This is known as $f_m$ and is a key factor in determining susceptibilities towards victim DDI (Rowland and Matin, 1973) and inter-patient pharmacogenetically-based pharmacokinetic differences. While hydralazine is the most well-studied TDI of AO and is frequently employed for AO reaction phenotyping, several studies have highlighted concerns pertaining to hydralazine derived $f_{m,AO}$ values due to its propensity to significantly inhibit some P450s at the concentrations typically utilized for reaction phenotyping (Zientek and Youdim, 2015; Yang *et al.*, 2019; Toselli *et al.*, 2022). In this study, we characterized icotinib as a potent and selective inhibitor of AO and further evaluated its utility as an alternative to hydralazine for AO reaction phenotyping in human hepatocytes. In broad strokes, we presented nascent evidence that icotinib may yield more accurate estimations of $f_{m,AO}$ compared to hydralazine.

Icotinib is a cyclized analogue of erlotinib that is approved for the therapy of non-small-cell lung cancer with EGFR-positive mutations (Li *et al.*, 2022). Here, our findings revealed for the first time that the cyclization of the two 2-methoxyethoxy groups of the quinazoline nucleus in icotinib did not negate its TDI of AO. Juxtaposing the $k_{inact}/K_i$ ratio – which is a surrogate measure of inactivation potency (Tseng *et al.*, 2021), obtained for both erlotinib and icotinib against the inactivation of human liver cytosol zoniporide 2-oxidation revealed modest differences and indicated equipotent TDI against AO. This is perhaps unsurprising as the alkyne
motif, which is preserved in both erlotinib and icotinib, is likely the structural alert that precipitates inactivation of AO. This is corroborated by findings from Dick et al., 2023 whom recently reported the importance of the alkyne moiety in erlotinib to AO inactivation. Those investigators elegantly demonstrated this by showing that the potency of AO inactivation was unaffected in OSI-420, a metabolite of erlotinib which was demethylated on the 2-methoxyethoxy side chain, but drastically blunted with 4-methyl erlotinib which has a methyl group added to the 4-position of the phenylacetylene moiety. Moreover, replacing the alkyne with an alkene group (3-vinyl erlotinib) significantly attenuated its AO inactivation whereas reverting to an ethyl group (tetrahydro erlotinib) completely abolished the inactivation of AO. Consequently, we also propose that mechanism of AO inactivation by erlotinib and icotinib could arise from nucleophilic attack of electron-deficient alkyne carbon by the thiol group in the reduced form of MoCo in AO resulting in the formation of an irreversible thioester bond via a thiol-alkyne addition (Fig. 8) which may be chemically reduced upon resulfization of the enzyme with dithionite (Dick et al., 2023). The potential for alkynes to function as latent electrophiles and induce irreversible covalent inactivation of thiol groups in proteins has been expounded upon (Mons et al., 2019) and a similar reactivity of molybdenum liganded sulfide with alkynes has been reported previously (Casewit and DuBois, 1986). Importantly, as such thiol-alkyne addition reactions are driven by proximity rather electrophilicity (Sommer et al., 2013), it further concurs with Dick et al., 2023’s findings as to why 4-methyl erlotinib – which possesses a sterically hindered methyl group on the phenylacetylene ring – exhibited marked decreases in its AO inactivation potency. Future work involving native mass spectrometry with intact AO protein will be conducted to validate the postulated mechanism of AO inactivation as these novel
findings would aid future rational design of potent and selective inactivators against AO and other molybdenum-containing hydrolases (i.e., xanthine oxidase).

We next sought to examine if icotinib possesses increased AO selectivity against the UGTs and P450s, relative to erlotinib. A major issue hindering the adoption of erlotinib as a chemical inhibitor of AO is its potent inhibition of UGT1A1 (Liu et al., 2010), hence it does not have the necessary selectivity margins needed for reaction phenotyping in human hepatocytes possessing the full complement of hepatic drug metabolizing enzymes (Soars et al., 2007). However, icotinib appealed to us because of a previous report indicating that it induced weaker inhibition of UGT1A1 compared to erlotinib (Cheng et al., 2017). Indeed, we managed to recapitulate the less potent inhibition of UGT1A1 by icotinib (~9-fold). Additionally, we also evaluated the inhibition of both erlotinib and icotinib against UGT1A3, as there are frequent overlaps in the substrate specificities between these two UGT isoforms (Lépine et al., 2004; Lapham et al., 2018), and the same fold-decrease (~9-fold) in UGT1A3 inhibition potency as with UGT1A1 was observed. This is also somewhat unsurprising because most tyrosine kinase inhibitors have been shown to inhibit UGT1A1 most potently with the IC$_{50}$ against other UGT isoforms (i.e., 1A3, 1A4, 1A6, 1A9 and 2B7) being considerably lower (Miners et al., 2021). On the other hand, our TDI screen in human liver microsomes revealed that icotinib presumably engendered TDI of CYP3A. Indeed, while icotinib has been reported to inactivate CYP3A (Sun et al., 2021), the $K_I$ value obtained in human liver microsomes is 20.8 µM – which ~18-fold higher than the corresponding value obtained against AO and is therefore unlikely to be an issue in our reaction phenotyping studies in hepatocytes which employs 3 µM icotinib. This is further substantiated by direct evidence garnered from simultaneously measuring both AO-catalyzed zaleplon oxidase and CYP3A-mediated zaleplon deethylase activity in hepatocytes under initial rate conditions.
following a 30 min preincubation of icotinib in which only AO activity was abrogated in a dose-dependent manner. Conversely, CYP3A activity remained unperturbed at the concentration ranges evaluated (0.001 – 10 µM) and instead found to be amplified relative to vehicle controls. Such a phenomenon is not unexpected and is emblematic of metabolic shunting away from AO towards its uninhibited CYP3A pathway (Crouch et al., 2016). Taken together, our data strongly suggests that icotinib is a potent inhibitor of AO and has ample selectivity margins over P450s and UGT1A1/3 at the proposed concentration for reaction phenotyping in human hepatocytes.

A major finding emanating from this work is the improvement in $f_{m, AO}$ estimates yielded by icotinib over hydralazine. This is based on the greater concordance of $f_{m, AO}$ values obtained from hepatocyte inhibition studies using icotinib with those derived from human metabolism and excretion studies involving radiolabeled substrate. However, there is a paucity of such in vivo $f_{m, AO}$ data and we only managed to identify four compounds in the literature where such data was available. Nevertheless, we observe a greater improvement in $f_{m, AO}$ estimates in all but one of the four compounds evaluated. However, it should be noted that the $f_{m, AO}$ for idelalisib derived using the icotinib approach was still within 1.25-fold of the reported in vivo value and the discrepancy is likely due to experimental variability. Another striking finding in this work is the impact of different hepatocyte cell densities on the inhibition of AO activity by icotinib and hydralazine. With hydralazine there was a profound ~5-fold increase in residual zaleplon oxidase activity when the hepatocyte cell density was increased from 0.5 million cells/mL to 1.5 million cells/mL. As the current drug development paradigm favors clinical candidates with comparatively lower metabolic clearances, a higher cell density (~2 million cells/mL) in hepatocyte stability assays would likely be needed for $f_{m, AO}$ phenotyping. Therefore, if hydralazine is utilized there could be insufficient suppression of AO activity which leads to
underpredictions in $f_{m,AO}$ values and may necessitate elevations in hydralazine concentrations from 25 to 50 µM which would also invariably engender a greater degree of non-selective inhibition of P450 isoforms and further confound $f_{m,AO}$ estimates. This issue could potentially be circumvented with icotinib as tripling the cell density only had a modest increase on residual zaleplon oxidase activity with AO activity still largely suppressed to well below the critical 10% threshold (IC$_{90}$) (Zientek and Youdim, 2015). Thus, this represents another advantage icotinib has over hydralazine in AO reaction phenotyping. Finally, it should be noted that at the concentration of 3 µM, icotinib elicits about ~23% inhibition of UGT1A1 – further reiterating the challenges associated with identifying chemical inhibitors that are ‘clean’ of any off-target inhibition liabilities. As the inhibition of UGT1A1 is reversible, study teams may opt to incorporate a washout step to remove the inhibitor before the addition of the substrate to further improve inhibitor selectivity. Notwithstanding this limitation, we believe that icotinib is superior to hydralazine as a chemical inhibitor of AO for reaction phenotyping in human hepatocytes.

In conclusion, this study illuminated the utility of the AO inactivator icotinib for reaction phenotyping in human hepatocytes to obtain accurate estimates of $f_{m,AO}$. We envisage the adoption of icotinib in place of hydralazine in AO reaction phenotyping. The proposed biochemical and structural mechanism of AO inactivation by icotinib and the reasons underscoring the loss of inactivation potency with hydralazine at higher cell densities will be interrogated in future studies.
Acknowledgements:

The authors would like to thank and acknowledge Samantha Jordan for her help in the LC-MS/MS tuning of AO substrates; Nicholas Ferguson for discussions concerning the lithocholic acid 24-glucuronidation assay; Elaine Tseng, Yuanyuan Shi, and Xiaofeng Wu for generously donating their time to contribute valuable scientific discussions towards this project.
Footnotes

- LWTT, ED, KL and RSO are employees of Pfizer Inc., New York, NY, USA and may own shares/stock options in Pfizer Inc., New York, NY, USA.

- This study was sponsored by Pfizer Inc., New York, NY, USA.

- The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data

- This article has supplemental material available at dmd.aspetjournals.org.
Authorship Contributions:

Participated in research design: Tang, Lapham, Obach

Conducted experiments: Tang, DaSilva

Performed data analysis: Tang, DaSilva

Wrote or contributed to the writing of the manuscript: Tang, Lapham, Obach
References


Figure Legends

**Fig. 1.** Chemical structure of erlotinib and its structural analogue icotinib. The phenylacetylene motif (highlighted in red) is preserved in icotinib.

**Fig. 2.** IC$_{50}$ curves representing the inhibition of human liver cytosolic AO-catalyzed zoniporide 2-oxidation by (A) erlotinib and (B) icotinib. An 8.0- and 6.2-fold apparent shift in IC$_{50}$ values can be observed which suggests that the inhibition elicits by both test compounds are time-dependent in nature.

**Fig. 3.** Time- and concentration-dependent inhibition of human liver cytosolic AO-mediated zoniporide 2-oxidation by (A) erlotinib and (B) icotinib. Plot of $k_{\text{obs}}$ against nominal concentrations of (C) erlotinib and (D) icotinib in the primary preincubation mixture yields a characteristic hyperbolic profile with $K_I$ values of 0.719 ± 0.087 µM and 1.18 ± 0.108 µM, respectively, and $k_{\text{inact}}$ values of 0.360 ± 0.014 min$^{-1}$ and 0.546 ± 0.018 min$^{-1}$, respectively. The corresponding transformed Kitz-Wilson plot (inset in Fig. 3C and D) is also illustrated.

**Fig. 4.** Inhibition of AO-catalyzed zaleplon 5-oxidation but not CYP3A-mediated zaleplon N-desethylation by icotinib in human hepatocytes.

**Fig. 5.** Representative substrate depletion plots of (A) carbazeran (high AO clearance), (B) zoniporide (moderate AO clearance), and (C) zaleplon (low AO clearance) in human hepatocytes in the presence and absence of icotinib (3 µM) 30 min preincubation. The CL$_{\text{int,app}}$ (vehicle) of
carbazelan, zoniporide and zaleplon are 24.1, 11.3, and 7.48 µL/min/million cells, respectively. Whereas the corresponding CL_{int,app} (icotinib) are 3.99, 2.24, and 2.53 µL/min/million cells.

**Fig. 6.** (A) Correlation between f_{m,AO} values determined using the icotinib approach outlined in this work and literature f_{m,AO} values obtained from the conventional hydralazine methodology. (B) Correlation between f_{m,AO} values estimated from *in vitro* hepatocyte inhibition studies and those determined from *in vivo* human mass balance studies (Lake *et al.*, 2002; Dalvie *et al.*, 2013; Jin *et al.*, 2013; Glaenzel *et al.*, 2020). The closed circles refers to f_{m,AO} values calculated using hydralazine (25 µM) whereas the open circles represent f_{m,AO} values computed using icotinib (3 µM). In both correlation plots, the solid line, dotted lines, and dashed lines represent the line of unity, 1.5-fold, and 2-fold deviation from unity, respectively.

**Fig. 7.** Residual AO-catalyzed zaleplon oxidase activity (normalized to vehicle controls) in hepatocytes after a 30 min preincubation with either icotinib (3 µM) or hydralazine (25 µM) at two different cell densities.

**Fig. 8.** Proposed mechanism of AO inactivation involving nucleophilic attack of electron-deficient alkyne carbon in erlotinib and icotinib by the thiol group in the reduced form of MoCo in AO resulting in the formation of an irreversible thioester bond via a thiol-alkyne addition.
**Table 1.** Summary of IC$_{50}$ values and inactivation kinetic parameters obtained from the direct and time-dependent inhibition of human liver cytosolic AO-mediated zoniporide 2-oxidation by erlotinib and icotinib.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$</th>
<th>Shifted IC$_{50}$</th>
<th>Fold-shift</th>
<th>$K_I$</th>
<th>$k_{\text{inact}}$</th>
<th>$k_{\text{inact}}/K_I$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td></td>
<td>$\mu M$</td>
<td>min$^{-1}$</td>
<td>min$^{-1}$mM$^{-1}$</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>0.280 ± 0.018</td>
<td>0.035 ± 0.002</td>
<td>8.0-fold</td>
<td>0.719 ± 0.087</td>
<td>0.360 ± 0.014</td>
<td>501</td>
</tr>
<tr>
<td>Icotinib</td>
<td>0.717 ± 0.181</td>
<td>0.116 ± 0.009</td>
<td>6.2-fold</td>
<td>1.18 ± 0.108</td>
<td>0.546 ± 0.018</td>
<td>463</td>
</tr>
</tbody>
</table>

Data (n=2) are presented as means ± S.E
Table 2. Summary of IC$_{50}$ values obtained from the direct inhibition of human liver microsomal UGT1A1-mediated β-estradiol 3-glucuronidation and UGT1A3-mediated lithocholic acid 24-glucuronidation by erlotinib and icotinib.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>UGT1A1 IC$_{50}$</th>
<th>UGT1A3 IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.34 ± 0.110</td>
<td>3.02 ± 0.733</td>
</tr>
<tr>
<td>Icotinib</td>
<td>12.2 ± 2.20</td>
<td>25.6 ± 2.52</td>
</tr>
<tr>
<td>Fold-difference</td>
<td>9.1-fold</td>
<td>8.5-fold</td>
</tr>
</tbody>
</table>

Data (n=2) are presented as means ± S.E.
Table 3. Comparison of $f_{m, AO}$ values for 9 prototypical AO substrates obtained using either the icotinib methodology outlined in this study or the conventional hydralazine approach (Strelevitz et al., 2012).

<table>
<thead>
<tr>
<th>AO Substrate</th>
<th>$f_{m, AO}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Icotinib (3 µM)</td>
<td>Hydralazine (25 µM)$^a$</td>
</tr>
<tr>
<td>Capmatinib</td>
<td>0.33</td>
<td>0.08</td>
</tr>
<tr>
<td>Carbazeran</td>
<td>0.83</td>
<td>0.65</td>
</tr>
<tr>
<td>DACA</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td>Idelalisib</td>
<td>0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>$O^6$-</td>
<td>0.76</td>
<td>0.79</td>
</tr>
<tr>
<td>Benzylguanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-0945863</td>
<td>0.63</td>
<td>0.87</td>
</tr>
<tr>
<td>JNJ38877605</td>
<td>0.84</td>
<td>0.70</td>
</tr>
<tr>
<td>Zaleplon</td>
<td>0.66</td>
<td>0.80</td>
</tr>
<tr>
<td>Zoniporide</td>
<td>0.80</td>
<td>0.84</td>
</tr>
</tbody>
</table>

$f_{m, AO}$ values derived using the hydralazine methodology were obtained from the literature. When multiple $f_{m, AO}$ values were available, the arithmetic mean value was reported. As hydralazine is known to elicit nonselective inhibition of P450 enzymes at higher concentration (Yang et al., 2019), we opted for a more conservative estimate by only including $f_{m, AO}$ estimates determined at 25 µM hydralazine.
Table 4. Residual zaleplon oxidase activity normalized to vehicle controls after a 30 min preincubation with either icotinib (3 µM) or hydralazine (25 µM) at two different hepatocyte cell densities.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hepatocyte Cell Density</th>
<th>% zaleplon oxidase activity</th>
<th>% zaleplon oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 million cells/mL</td>
<td>1.5 million cells/mL</td>
<td></td>
</tr>
<tr>
<td>Icotinib (3 µM)</td>
<td>3.55 ± 0.07</td>
<td>5.33 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>Hydralazine (25 µM)</td>
<td>2.87 ± 0.23</td>
<td>14.2 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

Data (n=2) are presented as means ± S.E.
Fig. 1

Erlotinib

Icotinib
Fig. 2

Panel A: Zoniporide 2-Oxidation (% Control) in response to Erlotinib (μM) with No Preincubation and 30 min Preincubation.

Panel B: Zoniporide 2-Oxidation (% Control) in response to Icotinib (μM) with No Preincubation and 30 min Preincubation.
Fig. 3

A

B

C

D

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Fig. 4

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Fig. 6

A

\[ f_{m, AO} \text{(Hydralazine)} \]

\[ f_{m, AO} \text{(Icotinib)} \]

B

\[ f_{m, AO} \text{(in vitro; hepatocyte inhibition)} \]

\[ f_{m, AO} \text{(in vivo; human mass balance)} \]

- Capmatinib
- Idelalisib
- Zaleplon
- Zoniporide
Fig. 7

Icotinib Hydralazine

0 5 10 15 20
% Zaleplon Oxidase Activity

0.5 million cells/mL
1.5 million cells/mL

Icotinib  Hydralazine

% Zaleplon Oxidase Activity

0 5 10 15 20
Fig. 8
Evaluation of Icotinib as a Potent and Selective Inhibitor of Aldehyde Oxidase for Reaction Phenotyping in Human Hepatocytes

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Pharmacokinetics, Dynamics, and Metabolism, Pfizer Inc., Groton, Connecticut (LWTT, ED, KL, RSO)
**Supplementary Table 1.** Probe substrates and internal standards for cocktail single concentration TDI assay

<table>
<thead>
<tr>
<th>P450 Isoform</th>
<th>Probe Substrate</th>
<th>Concentration</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>200 µM</td>
<td>D₇-Acetaminophen</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>16.6 µM</td>
<td>D₅-N-desethylamodiaquine</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>64.5 µM</td>
<td>¹³C₆-4'-hydroxydiclofenac</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin</td>
<td>393 µM</td>
<td>D₃-4'-hydroxymephenytoin</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>18.1 µM</td>
<td>D₃-dextrorphan</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Midazolam</td>
<td>20.9 µM</td>
<td>D₄-1'-hydroxymidazolam</td>
</tr>
</tbody>
</table>
Supplementary Table 2. Optimized compound-dependent MS parameters of analytes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mass Transitions</th>
<th>Declustering Potential</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capmatinib</td>
<td>413.0 → 382.0</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Carbazeran</td>
<td>361.3 → 271.8</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>DACA</td>
<td>294.2 → 249.2</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Idelalisib</td>
<td>416.3 → 176.2</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>O(^6)-Benzy1guanine</td>
<td>241.9 → 91.2</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td>PF-0945863</td>
<td>824.4 → 667.3</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>JNJ38877605</td>
<td>378.2 → 129.1</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Zaleplon</td>
<td>306.2 → 236.2</td>
<td>82</td>
<td>39</td>
</tr>
<tr>
<td>Zoniporide</td>
<td>321.2 → 262.1</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>2-oxozoniporide</td>
<td>337.1 → 278.2</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>5-oxozaleplon</td>
<td>322.0 → 252.0</td>
<td>82</td>
<td>39</td>
</tr>
<tr>
<td>Desethylzaleplon</td>
<td>278.2 → 236.4</td>
<td>82</td>
<td>39</td>
</tr>
<tr>
<td>Estradiol 3-O-glucuronide</td>
<td>447.2 → 113.2</td>
<td>-55</td>
<td>-30</td>
</tr>
<tr>
<td>Lithocholic acid 24-glucuronide</td>
<td>551.3 → 375.3</td>
<td>-60</td>
<td>-55</td>
</tr>
<tr>
<td>17β-Estradiol-3-β-D-glucuronide-d3</td>
<td>450.2 → 113.2</td>
<td>-55</td>
<td>-30</td>
</tr>
<tr>
<td>Compound</td>
<td>Mass (M)</td>
<td>Mass (M’')</td>
<td>T (min)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>----------</td>
<td>------------</td>
<td>---------</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>268.1</td>
<td>116.1</td>
<td>60</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>152.1</td>
<td>110.0</td>
<td>50</td>
</tr>
<tr>
<td>N-desethylamodiaquine</td>
<td>328.2</td>
<td>283.0</td>
<td>50</td>
</tr>
<tr>
<td>4’-hydroxydiclofenac</td>
<td>312.0</td>
<td>230.0</td>
<td>50</td>
</tr>
<tr>
<td>4’-hydroxymephenytoin</td>
<td>235.2</td>
<td>150.1</td>
<td>50</td>
</tr>
<tr>
<td>Dextrophan</td>
<td>258.1</td>
<td>201.1</td>
<td>50</td>
</tr>
<tr>
<td>1’-hydroxymidazolam</td>
<td>342.2</td>
<td>324.0</td>
<td>50</td>
</tr>
<tr>
<td>D7-Acetaminophen</td>
<td>159.1</td>
<td>115.0</td>
<td>50</td>
</tr>
<tr>
<td>D3-N-desethylamodiaquine</td>
<td>333.2</td>
<td>283.0</td>
<td>50</td>
</tr>
<tr>
<td>13C6-4’-hydroxydiclofenac</td>
<td>318.0</td>
<td>236.0</td>
<td>50</td>
</tr>
<tr>
<td>D3-4’-hydroxymephenytoin</td>
<td>238.2</td>
<td>150.1</td>
<td>50</td>
</tr>
<tr>
<td>D3-dextrorphan</td>
<td>261.1</td>
<td>201.1</td>
<td>50</td>
</tr>
<tr>
<td>D4-1’-hydroxymidazolam</td>
<td>346.2</td>
<td>328.0</td>
<td>50</td>
</tr>
</tbody>
</table>
**Supplementary Table 3.** LC-MS/MS analytical conditions for β-estradiol 3-glucuronidation assay (UGT1A1) and lithocholic acid 24-glucuronidation assay (UGT1A3).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Estradiol 3-glucuronidation (UGT1A1)</th>
<th>Lithocholic acid 24-glucuronidation (UGT1A3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile Phase A</td>
<td>0.1% formic acid in water</td>
<td>10 mM ammonium acetate in water + 1% isopropyl alcohol</td>
</tr>
<tr>
<td>Mobile Phase B</td>
<td>0.1% formic acid in acetonitrile</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Gradient Elution Conditions</td>
<td>16% B (0 – 0.70) → 16 to 95% B (0.70 – 2.00) → 95% B (2.00 – 3.00) → 95 to 16% B (3.00 – 3.10) → 16% B (3.10 – 4.00)</td>
<td>5% B (0 – 0.20) → 5 to 96% B (0.20 – 2.20) → 96% B (2.20 – 3.20) → 96 to 5% B (3.20 – 3.25) → 5% B (3.25 – 3.80)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.750 mL/min</td>
<td>0.500 mL/min</td>
</tr>
<tr>
<td>LC Column</td>
<td>Phenomenex Kinetix XB-C18 2.1 x 30 mm, 2.6 um</td>
<td>Aquity UPLC BEH C18 1.7, 2.1 x 100 mm column (heated to 50°C)</td>
</tr>
<tr>
<td>ESI Mode</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Cone Voltage (kV)</td>
<td>-4500</td>
<td>-4500</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>450</td>
<td>450</td>
</tr>
</tbody>
</table>
Supplementary Fig. 1. IC₅₀ curves representing the inhibition of human liver microsomal (A) UGT1A1-mediated β-estradiol 3-glucuronidation and (B) UGT1A3-catalyzed lithocholic acid 24-glucuronidation by erlotinib and icotinib. Icotinib induces ~9-fold less potent inhibition both of UGT isoforms as compared to erlotinib.
Supplementary Fig. 2. Single concentration TDI screen of erlotinib (3 µM) and icotinib (3 µM) against (A) CYP1A2, (B) CYP2C8, (C) CYP2C9, (D) CYP2C19, (E) CYP2D6, and (F) CYP3A. Both test drugs only appear to elicit TDI of CYP3A with $k_{\text{obs}}$ values of 0.012 min$^{-1}$ and 0.009 min$^{-1}$ and for erlotinib and icotinib, respectively, differing significantly (p < 0.05) from the solvent control ($k_{\text{obs}} = 0.0051$ min$^{-1}$) as determined by the parallel line test. The positive controls utilized are 0.1 µM furafylline (CYP1A2), 6 µM gemfibrozil glucuronide (CYP2C8), 0.2 µM tienilic acid (CYP2C9), 0.9 µM ticlopidine (CYP2C19), 0.1 µM paroxetine (CYP2D6), 1 µM verapamil (CYP3A).
Supplementary Fig. 3. Correlation between $f_{m, AO}$ values estimated from in vitro hepatocyte inhibition studies (25 µM hydralazine in blue and 3 µM icotinib in purple) and those determined from in vivo human mass balance studies. The calculated $R^2$ was 0.899 for $f_{m, AO}$ value derived using icotinib and 0.802 with hydralazine.