Aldehyde Oxidase Activity in Donor Matched Fresh and Cryopreserved Human Hepatocytes and Assessment of Variability in 75 Donors

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Abstract

Studies were conducted to evaluate the impact of time and cryopreservation on aldehyde oxidase (AO) activity in human hepatocytes isolated from ten donor livers, using \( O^6 \)-benzylguanine as a probe substrate. In addition, variability in activity was assessed using cryopreserved hepatocytes from 75 donors. Substantial donor-dependent loss in AO activity within 24 hours following isolation of hepatocytes was observed (average loss of 42%, range 15-81%). Meanwhile, AO activity in cryopreserved hepatocytes more closely represented the activity observed in fresh hepatocytes that were incubated immediately following isolation for the same donors (within 81% of fresh, range 48-100%). Activity of AO in cryopreserved hepatocytes from 75 donors varied by at least 17-fold (\( \leq 5.4 \) to 90 mL/min/kg), with 63% of the donors having higher activity than a pooled 19-donor lot (34.2 mL/min/kg). When comparing demographics such as gender, body mass index, age, and ethnicity, no statistically significant correlations with activity were observed. Evaluation of medical histories uncovered that three out of the five donors with no measurable activity had immediate histories of extensive alcohol abuse. Meanwhile, two SNPs for \( AOX1 \) (rs3731772 and rs55754655) were detected in our donor pool and showed allelic frequencies similar to those reported from other cohort studies. However, these SNPs did not correlate with a statistically significant difference in intrinsic clearance compared to wild-type donors. With a general lack of clarity around what causes highly variable AO activity, pre-screening donors for AO activity, and creating a custom high-activity pooled lot of cryopreserved hepatocytes is advised to minimize under-predictions of clearance.
Introduction

Aldehyde oxidase (AO) is a cytosolic molybdenum-containing hydroxylase that has rapidly ascended in recent years to be a drug-metabolizing enzyme of high relevance within drug discovery and development (Pryde et al., 2010; Garattini and Terao, 2011, 2012; Hutzler et al., 2013). This enzyme is comprised of two identical ~150 kDa subunits, is active as a homo-dimer, and demonstrates broad substrate selectivity, oxidizing a variety of aldehydes and azaheterocyclic-containing drug molecules (Beedham, 1987; Kitamura et al., 2006; Garattini et al., 2008). Due to the subcellular location of this enzyme, metabolic stability investigations utilizing human liver microsomes fail to identify the contribution of AO-mediated metabolism to total metabolic clearance, which has led to early clinical failures for otherwise promising drug candidates. In particular, poor pharmacokinetic properties were observed following dosing of humans in phase I studies for drugs such as carbazeran, BIBX1382, and FK3453 (Kaye et al., 1984; Dittrich et al., 2002; Akabane et al., 2011). In addition, an AO-mediated metabolite of SGX523 not identified pre-clinically was reported to cause acute renal toxicity due to extremely poor solubility and crystallization in the kidney (Diamond et al., 2010). Unfortunately, all of these aforementioned examples of early clinical failures can be attributed to a lack of complete understanding of metabolic clearance mechanisms, due to in vitro metabolism studies only being conducted in microsomal liver fractions.

In the drug discovery setting, use of human hepatocytes has become more commonplace for predicting total metabolic clearance, as this in vitro system theoretically contains the full complement of drug-metabolizing enzymes and co-factors in the liver. In addition, advancement of cryopreservation techniques has enabled easier access to hepatocytes. However, cytochrome P450 activities are the primary focus of enzyme characterization using selective probe substrates.
Activities for non-P450 enzymes such as aldehyde oxidase have typically not been sufficiently characterized. Utilizing a number of probe substrates of AO, including $O^6$-benzylguanine ($O^6$-BG), our lab has recently demonstrated that pooled cryopreserved human hepatocytes contain reasonably high AO activity, and may be suitable in a drug discovery setting to screen out compounds that undergo rapid metabolism by AO (Hutzler et al., 2012). Despite these findings, some reports in the literature suggest the AO enzyme may be unstable (Duley, 1985), which may contribute to observed under-predictions of clearance using hepatocytes (Akabane et al., 2012), as well as other in vitro systems (e.g. cytosol and S-9 fractions) where AO is present (Zientek et al., 2010). An additional complication impeding progress towards scaling AO-mediated clearance is reported high donor-to-donor variability in AO activity. For example, AO activity as measured by DACA (N-[2-(dimethylamino)ethyl]acridone-4-carboxamide) hydroxylation varied 18-fold ($V_{\text{max}}/K_m$ method) across 13 donors when using liver cytosol as the source of enzyme (Al-Salmy, 2001). Similarly, methotrexate 7-hydroxylase activity assayed in liver cytosol from six human donors varied 48-fold (Kitamura et al., 1999). Meanwhile, variability of AO activity has only been reported in cryopreserved human hepatocytes using vanillin as the probe substrate across five donors, with a 5-fold range of activity observed (Sahi et al., 2008). While these and other reports of variable activity (summarized in Table 1) highlight a question around potential clinical variability in pharmacokinetics for substrates of AO, analysis of a larger donor set is necessary to attempt to draw conclusions as to what donor characteristics and factors contribute to observed variability in activity.

In an effort to address the knowledge gaps described above, AO activity was measured in fresh human hepatocytes from ten individual donors over 24 hours immediately following isolation of hepatocytes from intact livers, as well as in cryopreserved hepatocytes prepared from
the same donors (75 total donors). In addition, characteristics of tested donors were examined to understand the impact of demographic variables such as age, gender, ethnicity, medical history, and common single nucleotide polymorphisms (SNPs) on AO activity. AO enzymatic activity was estimated utilizing O₆-benzylguanine (O₆-BG) as the probe substrate (Figure 1). O₆-BG has been reported to be exclusively metabolized by AO-mediated oxidation to the 8-oxoguanine metabolite in cancer patients, where the area under the concentration vs. time curve (AUC) of the metabolite was 12 to 29-fold greater than parent O₆-BG (Dolan et al., 1998). In addition, we have demonstrated in our lab that when hydralazine, a selective inhibitor of AO (Strelevitz et al., 2012), is co-incubated with O₆-BG in cryopreserved hepatocytes, metabolism is effectively inhibited (Hutzler et al., 2012). Thus, O₆-BG appears to be a suitable probe substrate for estimating AO activity in hepatocytes.
Materials and Methods

Chemical and Biological Reagents. O\textsuperscript{6}-Benzylgua\textntilde{n}ine and toltubamide were purchased from Sigma Chemicals (St. Louis, MO). Human hepatocytes (fresh, cryopreserved individual donor, and pooled cryopreserved mixed gender, 19-donor, Lot OOO), hepatocyte media (\textit{InVitroGro\textsuperscript{TM} HT} thawing and \textit{InVitroGro\textsuperscript{TM} KHB} for incubation) and additional cell culture reagents were obtained from BioreclamationIVT (Baltimore, MD). All other reagents and chemicals were of the highest purity available.

Hepatocyte Isolation Procedure. Human livers from ten donors that were not suitable for transplantation for various reasons (demographic constraints, lifestyle history, anatomical concerns such as size, vascularization, fat content, etc.) were obtained through organ procurement organizations (OPOs) from donors with proper consent. The livers were flushed at the time of harvest with Viaspan\textsuperscript{®} or similar preservation solution and transported on wet ice to BioreclamationIVT between 12 and 23 hours following harvest. A two-step collagenase isolation with differential centrifugation procedure was employed to isolate the hepatocytes (Li et al., 1999). Three separate 10 million hepatocyte suspensions from each donor stored in DMEM-based medium (25% Viaspan\textsuperscript{®}) were prepared for incubation and assessment of AO activity immediately following isolation (within 2 hours), ~24 hours post-isolation (incubations conducted at BioreclamationIVT), and ~24 hours post-isolation following shipment of hepatocytes to Boehringer-Ingelheim (common practice when ordering “fresh” hepatocytes from a vendor). Hepatocyte suspensions for ~24 hour post-isolation incubations conducted at BioreclamationIVT were stored at 4°C without mixing to mimic shipping conditions. The remainder of the suspension from each donor was cryopreserved according to protocol used by BioreclamationIVT. Donor demographics are shown in Table 2.
**Hepatocyte Incubations.** Freshly isolated human hepatocytes (as described above) were gently mixed prior to centrifugation at 50 x g for 5 min at 4°C. The supernatant was discarded and the hepatocyte pellet was re-suspended in *InVitroGro™* KHB. Pooled mixed gender (lot number OOO, 19 mixed gender donors) and individual donor cryopreserved human hepatocytes were stored in liquid nitrogen until use. Immediately prior to experiments, sufficient aliquots of hepatocytes were thawed rapidly (~2 min) in a shaking water bath at 37°C. The contents of each vial were diluted 1/50 in pre-warmed (37°C) *InVitroGro HT* thawing medium and gently mixed prior to centrifugation at 50 x g for 5 min at room temperature. The hepatocyte pellet was re-suspended in *InVitroGro* KHB. The cell number and viability were determined using a hemocytometer after staining with trypan blue. Viabilities for each hepatocyte experiment were ≥70%. Cell suspension was diluted to provide a 2x stock and pre-warmed at 37°C for 15 min. Incubations were initiated by the addition of O₆-BG substrate solution (250 μl) to the hepatocyte suspension (250 μl, 250,000 viable cells) in a 48-well polypropylene incubation plate (1 μM final substrate concentration, 0.02% DMSO, 0.5% acetonitrile), followed by gentle swirling in an atmosphere of 5% CO₂ and 95% relative humidity (n=4 replicates). Reactions were terminated at 0, 5, 15, 30, 60 and 120 min by aliquoting 50 μl of incubate into 150 μl of cold acetonitrile (0.1% acetic acid) containing internal standard (0.25 μM tolbutamide). Quench plates were then centrifuged at 3000 x g for 5 min, and supernatants were transferred to 96 well plates for bioanalysis by tandem LC/MS/MS (for incubations conducted at Boehringer-Ingelheim), or stored at -80°C and then shipped on dry ice to Boehringer-Ingelheim for tandem LC/MS/MS bioanalysis (for incubations conducted at BioreclamationIVT).

**Bioanalytical Procedures.** Quantitation of all analytes was performed using an AB Sciex (Foster City, CA) API-5000 triple quadrupole mass spectrometer equipped with electrospray
ionization (ESI) interface in positive ion mode and connected in-line to a Waters Acquity UPLC® system (Waters, Milford, MA). Separation of analytes was performed using a Waters Acquity BEH high-pressure C18 1.7μm (2.1 x 50 mm) column held at 50°C. Mobile phase was flowed at 0.5 mL/min with a rapid gradient starting at 95% A (0.1% formic acid in water) and 5% B (0.1% formic acid in acetonitrile), held for 0.5 min, then ramped linearly to 5:95 A:B, and held for 1.4 min, followed by returning to initial conditions (2 min total run time). The multiple reaction monitoring (MRM) transitions for each analyte were as follows: O6-benzylguanine (m/z 242.0>199.1), and tolbutamide (m/z 271.3>91.1). Standard curves for O6-benzylguanine were prepared and found to be linear within concentration range of 0.025-2.0 μM. The limit of quantitation (LOQ) for O6-benzylguanine was determined to be 0.07 μM. All data were analyzed using AB Sciex Analyst 1.4.2 software.

**Intrinsic Clearance Estimates.** In vitro intrinsic clearance (Cl\textsubscript{int}) was estimated (mL/min/kg) following incubations in human hepatocytes for 2 hours using substrate depletion methodologies and Equation 1:

\[
Cl_{\text{int}} = 0.693 \times \frac{1}{t_{1/2}} \times \frac{ml \text{ incubation}}{hepatocytes} \times \frac{gm \text{ liver wt}}{kg \text{ body wt}} \times \frac{hepatocytes}{gm \text{ liver wt}}
\]

where \(t_{1/2}\) is the observed in vitro substrate depletion half-life (min) as determined by linear regression. Scaling factors for intrinsic clearance calculations were as follows: 25.7 gm liver weight per kg body weight, and 120 \(\times\) \(10^6\) hepatocytes per gram liver.

**Single Nucleotide Polymorphism (SNP) Analysis.** Genomic DNA was prepared from cryopreserved hepatocytes (n=71 donors) using the Qiagen QIAamp DNA Mini kit (Gaithersburg, MD). TaqMan® Genotyping SNP assays and TaqMan® Genotyping Master Mix were obtained from Life Technologies (Carlsbad, CA) and were used in reactions according to manufacturer instructions. Reactions were run on an ABI7500 Fast Real-Time PCR System and
data was captured and analyzed using SDS v1.3 software. All of the SNP markers detected were in Hardy-Weinberg equilibrium as tested by Fisher’s Exact Test. Statistical analysis was performed using GraphPad Prism (v5.02) (La Jolla, CA).
Results

Effect of Time and Cryopreservation on Intrinsic Clearance in Hepatocytes from Ten Donors. In vitro intrinsic clearance was determined utilizing $O^6$-BG as a marker for AO activity immediately following isolation of fresh hepatocytes from intact livers (process for isolation and preparing for incubation takes $\leq$ 2 hours from receipt of liver), as well as ~24 hours post-isolation, and following cryopreservation. Donors included seven males and three females, with ages ranging from 4-74 years (Table 2). Intrinsic clearance data for the ten donors is presented in Figure 2. Overall, the data suggests that substantial loss in AO activity within the first 24 hours following isolation of hepatocytes is common (average loss of 42%, range 15-81% for 7/10 donors). Interestingly, this downward trend in activity was not observed for donors D3, D4, and D7, which suggests that loss of activity is likely donor-dependent. When comparing AO activity 24 hours post-isolation (stored at 4°C) to 24 hours in hepatocytes that were shipped overnight (mimicking process for how “fresh” hepatocytes are typically acquired), intrinsic clearance values were comparable for 5/10 donors, with measurably higher activity in donors D1, D2, D8, and D10 (Figure 2). Lastly, the effect of cryopreservation was evaluated using the same ten donors. Overall, activity in cryopreserved hepatocytes fairly well represented the activity observed in fresh hepatocytes that were isolated and incubated immediately for the same donors (within 81%, range 48-100%), which suggests that cryopreserved hepatocytes may be preferable from an activity standpoint compared to fresh hepatocytes that typically are at least 24 hours old prior to conduct of in vitro metabolism studies. Lastly, when comparing activity across the ten donors, high donor-to-donor variability was observed (5 to 8-fold) regardless of condition.
Donor Variability in AO Activity. Given that cryopreserved hepatocytes appear to maintain reasonable activity for AO when compared to fresh human hepatocytes that are incubated in suspension immediately following isolation, intrinsic clearance for 75 individual donors was subsequently assessed in cryopreserved human hepatocytes using $O_6$-BG. Donor characteristics for the 75 donors were collected and can be found in the supplemental information (Supplemental Table 1). The data for $O_6$-BG intrinsic clearance across 75 donors is presented graphically in Figure 3. First, it is worth noting that the activity observed in a lot of 19 pooled mixed-gender donors ($Cl_{int} = 34.2\pm1.4 \text{ mL/min/kg (n=4 replicates)}$, dotted line in Figure 3) was less than ~63% the donors in the current 75 donor pool population. Also, the first 5 donors (LNE, JGM, GTD, GTV, and TYS) had unmeasurable AO activity as determined by substrate depletion. The cut-off used for extrapolation of half-life was ~790 minutes (e.g. half-life $\geq$ 790 min), which is derived from estimating that at a minimum, $\geq$10% of substrate must be consumed by end of the 120 min incubation time to conclude measurable enzyme activity. This scales to an estimated intrinsic clearance of $\leq 5.4 \text{ mL/min/kg (equation 1)}$. Overall, activity for the 75 tested donors varied by at least 17-fold, with the lowest activity being $\leq 5.4 \text{ mL/min/kg}$, and highest being 90 mL/min/kg. The average activity across 70 donors (not including the five donors that had unmeasurable activity) was 39.9 mL/min/kg, with a high standard deviation of 19 mL/min/kg (solid line in Figure 3), indicating the donor variability. When evaluating medical history information of the donors, 3 out of the 5 low-activity donors (LNE, GTD, and TYS) had histories of extensive alcohol abuse up until time of death (Table 1 in Supplemental Information). Meanwhile, donor GTV was a “social drinker”, without quantitative information. Two donors (ORM and CNM) with higher AO activity than what was observed in the pooled cryopreserved lot had past medical histories of alcohol abuse, but quit years prior to death.
Overall, when comparing demographics such as gender, body mass index (BMI), ethnicity (African American, Hispanic, and Caucasian) and age (range 1-88), no statistically significant differences were observed with respect to activity when utilizing two-tailed t-test and 1-way ANOVA analysis (Figure 4).

With respect to disease factors, a history of diabetes was noted for 15 out of 75 donors (20%), but was a disease characteristic in donors spanning the entire range of activities (Table 1 in Supplemental Information). No additional trends were identified.

**Single Nucleotide Polymorphism (SNP) Analysis.** Single nucleotide polymorphism (SNP) data for 4 donors (JVA, UFN, BLC, and CNM) was unable to be collected due to lack of sufficient cells for DNA isolation following initial metabolism studies. Thus, known SNPs for *AOX1* were analyzed in the donor population (n=71) using Taqman PCR assays (Table 3). Four of the six SNPs tested were not detected in our donor population. Two SNPs for *AOX1* (rs3731772 and rs55754655) were detected and showed allelic frequencies similar to those reported from other cohort studies such as the NHLBI Exome Sequencing Project (Table 4). However, the presence of these mutant alleles did not correlate with a statistically significant difference in intrinsic clearance values for rs55754655 (p=0.2502) or rs3731722 (p=0.0514) when compared to the wild-type group using a two-tailed t-test (Figure 5).
Discussion

Aldehyde oxidase (AO) is a cytosolic drug-metabolizing enzyme that has emerged in recent years in the metabolism of azaheterocyclic drug molecules (Pryde et al., 2010; Garattini and Terao, 2013; Hutzler et al., 2013). The ideal in vitro system for comprehensively evaluating metabolic clearance is hepatocytes, which theoretically contain the full complement of drug-metabolizing enzymes. However, the activity of non-P450 metabolic enzymes, such as aldehyde oxidase, has generally not been well characterized. Recently, we reported AO activity in two separate lots of pooled human cryopreserved hepatocytes (Hutzler et al., 2012). However, an appraisal of the activity of AO within the first 24 hours following isolation of hepatocytes from a human liver has not been reported. Acute loss of AO enzymatic activity over this time remains an open question, as under-predictions of clearance have been reported (Zientek et al., 2010; Akabane et al., 2011). To this end, in vitro studies were designed and conducted utilizing fresh and donor matched cryopreserved hepatocytes to evaluate the impact of time and cryopreservation on enzymatic activity. In addition, donor variability in the largest set of subjects reported to date (75 donors) was evaluated using cryopreserved human hepatocytes.

When a drug metabolism research lab orders fresh hepatocytes from a vendor, by the time they receive cells for incubations, they are at least 24 hours old (next day following isolation). Understanding the potential loss in AO activity over this time period is important, especially if using metabolism data for scaling and prediction of human metabolic clearance. When in vitro intrinsic clearance was determined immediately following isolation of fresh hepatocytes from ten donor livers (within 2 hours post-isolation), and then measured again in the same cells roughly 24 hours following isolation, substantial loss in AO activity was observed (average loss of 42%, range 15-81%). Interestingly, this trend was dependent on the donor, as no
activity loss was observed over this time period for donors D3, D4, and D7 (Figure 2). When evaluating the available donor characteristics, it was not clear why some donors displayed a loss in activity within the initial 24 hours, whereas others did not. Since all donor hepatocytes were treated the same, it is unlikely to be due to the process of cell isolation or storage over the initial 24 hours. One could plausibly suggest a link between the general health of the donor livers or cells and AO activity over time, but this is entirely speculative. Additional research utilizing more donors would need to be conducted to provide convincing evidence. When comparing activity of AO 24 hours post-isolation (e.g. stored) to hepatocytes that were shipped overnight (mimicking process for how “fresh” hepatocytes are typically acquired), intrinsic clearance values were overall comparable, but with a currently unexplained increase of activity for donors D1, D2, D8 and D10 following shipment and incubation (Figure 2). The impact of cryopreservation was also evaluated using the same ten donors. Overall, activity in cryopreserved hepatocytes fairly well represented the activity observed in hepatocytes that were isolated and incubated immediately for the same donors (within 81%, range 48-100%), which suggests that cryopreserved hepatocytes would be preferable to fresh hepatocytes that commonly are at least 24 hours old prior to conduct of in vitro metabolism studies. This is further rationalized when one considers the variability in activity loss observed over the initial 24 hours, and the uncertainty around availability of fresh donor hepatocytes. A similar evaluation was undertaken by Akabane and colleagues (Akabane et al., 2012), where they compared activity for various non-P450 enzymes in fresh and cryopreserved hepatocytes. Aldehyde oxidase was among the non-P450 enzymes tested, and they used multiple probe substrates to estimate activity, including O⁶-BG. Their results were dependent on AO substrate, but with O⁶-BG as the marker, cryopreserved hepatocytes from the same donors (n=4 donors) appeared to have higher
activity than fresh hepatocytes (Akabane et al., 2012). It is worth noting that the “fresh” hepatocytes used in their studies were acquired from multiple vendors in the traditional manner (e.g. ordered and shipped next day). Although speculative, it may be possible based on our observations that substantial AO activity was lost during the isolation and shipment time, which could explain the higher activity observed in cryopreserved hepatocytes from the same donor.

When comparing activity across the ten donors, regardless of whether comparing the intrinsic clearance values in fresh or cryopreserved hepatocytes, donor variability was observed (5 to 8-fold). Several reports in the literature demonstrate high donor-to-donor variability for AO activity, although the magnitude appears substrate- and methodology- (e.g. metabolite formation or substrate depletion) dependent (Table 1). Given that cryopreserved hepatocytes from the ten donors tested appear to possess comparable activity to hepatocytes that were immediately incubated following isolation, use of cryopreserved hepatocytes was determined to be suitable for evaluating variation in AO activity across a larger donor pool (n=75 donors), where characteristics (e.g. gender, age, ethnicity, and medical history) could be evaluated in effort to elucidate potential causes for this variation. To our knowledge, this represents the largest donor pool from which variability in activity has been assessed, in particular, using human hepatocytes. Overall, activity for the 75 tested donors varied by at least 17-fold, with the lowest activity being ≤5.4 mL/min/kg (5 donors), and highest being 90 mL/min/kg (average intrinsic clearance, 39.9 ± 19 mL/min/kg). No significant trends emerged when comparing activities across ethnic backgrounds (African American, Hispanic, and Caucasian). In addition, when comparing gender, age and body mass index (BMI), no significant differences were observed with respect to activity (Figure 4). Interestingly, when evaluating medical history data of the donors, we discovered that 3 out of the 5 low-activity donors (LNE, GTD, and TYS) had histories of
extensive alcohol abuse up until time of death. This acute effect of alcohol abuse was also recently reported by scientists at Pfizer (Fu et al., 2013). The exact mechanism of this alcohol effect on AO activity was not elucidated, although it was found that \( AOX1 \) protein expression level appeared unaffected, despite significant decreases in enzyme activity (Fu et al., 2013). Thus, the presence of AO protein apparently does not correlate with activity, which complicates the assessment of variability factors. Fu and colleagues suggest that several additional factors may contribute to variable or low AO activity, including disruption of active homo-dimer formation, and molybdenum (Mo) cofactor deficiency (Fu et al., 2013). Meanwhile, in our studies, other donors with a history of alcohol abuse that abstained from alcohol years prior to death (donors ORM and CNM), demonstrated moderate (45.3 ± 4.9 mL/min/kg) to high (77 ± 13 mL/min/kg) AO activity, suggesting that the alcohol effects may be more acute in nature. This finding is worthy of additional research, as there could be pharmacokinetic/pharmacodynamic drug interaction implications for alcoholics taking any drug that has a predominant clearance mechanism through AO.

In addition to donor demographics and medical histories, another potential contributor to variable AO activity is the presence of single nucleotide polymorphisms (SNPs), which are known and have been reported (Smith et al., 2009; Hartmann et al., 2012; Kurzawski et al., 2012). While the two variants observed in our donor population (rs55754655 and rs3731722) have been reported to have clinical implications in use of azathioprine (Smith et al., 2009), the intrinsic clearance differences in our cohort did not reach statistical significance despite a trend towards higher activity compared to wild-type individuals (Figure 5). The limited number of individuals and the high variability of the activity may require a larger cohort to establish this connection. Hartmann and colleagues reported that both \( AOX1 \) SNPs rs55754655 (N1135S) and
rs3731722 (H1297R) led to 2- to 4-fold higher catalytic activities, but this conclusion was derived using purified expressed AO enzyme, and thus was independent of additional donor variable factors. When considering all of the aforementioned factors involved in impacting AO activity (Mo deficiency, etc.), it is unlikely that genotype alone dictates the phenotypic activity of AO. While this SNP analysis was limited in scope, this premise is supported by a recent report where variability in XK469 clearance was found to be independent of AOX1 polymorphisms (Ramirez et al., 2013).

In summary, it has been demonstrated that “fresh” hepatocytes obtained from a vendor, which are in reality at least 24 hours old, may have lost substantial AO activity depending on donor, whereas cryopreserved hepatocytes appear to better represent the activity in a donor liver when incubations are conducted immediately following isolation of hepatocytes. Thus, cryopreserved human hepatocytes appear to be a suitable in vitro model for determining intrinsic clearance activity for substrates of AO as part of an integrated approach to understanding total metabolic clearance. In addition, there is substantial donor-to-donor variability for AO activity, which requires careful characterization of donors or donor pools when selecting hepatocyte lots that contain adequate activity for the multitude of drug-metabolizing enzymes. Since it appears that multiple factors may contribute to variable or low AO activity, it is not recommended to use hepatocytes from a single donor to estimate AO activity, especially if alcohol abuse is noted in the donor medical history. Instead, pre-screening donors for AO activity, and creating a custom high-activity pooled lot of cryopreserved hepatocytes is advised to minimize under-prediction of metabolic clearance in human.
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Authorship Contributions

Participated in research design: Hutzler, Yang, Moeller, Brown, Heyward

Conducted experiments: Yang, Moeller, Brown, Heyward

Contributed new reagent or analytic tools: n/a

Performed data analysis: Yang, Hutzler, Moeller, Brown, Heyward

Wrote or contributed to the writing of the manuscript: Hutzler, Yang, Moeller, Brown, Heyward
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Footnotes

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Legend for Figures

Figure 1. Structure of aldehyde oxidase probe substrate O6-benzylguanine. Arrow indicates the site of metabolism by aldehyde oxidase.

Figure 2. Bar chart demonstrating aldehyde oxidase activity (O6-benzylguanine intrinsic clearance) for n=10 human donors over initial 24 hours following hepatocyte isolation from intact liver, as well as activity following cryopreservation of same donor hepatocytes. Clearance values are mean of n=4 replicates, with error bars representing standard deviation (SD). See Materials and Methods for incubation procedures.

Figure 3. Variability in intrinsic clearance (mL/min/kg) for O6-benzylguanine oxidation in cryopreserved hepatocytes from 75 individual donors. Dotted line represents observed intrinsic clearance for O6-benzylguanine in a pooled cryopreserved hepatocyte lot consisting of 19 mixed gender donors. Clearance values for all are mean of n=4 replicates, with error bars representing standard deviation (SD). See Supplemental Information for donor characteristics, including medical histories.

Figure 4. Whisker plots comparing AO activity (O6-benzylguanine Clintrinsic) across 75 donors stratified by A. gender, B. body mass index (BMI), C. ethnic background and D. age groups. Black bar indicates mean with error bars indicating standard deviation (SD). No statistically significant differences were observed between gender (A, p=0.3653) and BMI (B, p=0.4189) groups utilizing two-tailed t-test. In addition, no significant differences in activity were observed between race (C) and various age groups (D) using 1-Way ANOVA with Bonferroni’s Multiple Comparison test (p>0.05).

Figure 5. Whisker plots comparing AO activity (O6-benzylguanine Clintrinsic) for individuals genotyped and grouped into wild-type “WT” or with either one or two G alleles “G”. Black bar
indicates mean with error bars indicating standard deviation (SD). No statistically significant differences were observed between genotypes using a two-tailed t-test: A. rs55754655 (n=58, 34 WT/24 G) p=0.2502, B. rs3731722 (n=44, 34 WT/10 G) p=0.0514.
Table 1. Summary of literature reports demonstrating high variability in aldehyde oxidase activity in various in vitro systems.

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<td>Cytosol</td>
<td>13</td>
<td>$V_{\text{max}}/K_m$</td>
<td>DACA 18</td>
<td>Al-Salmy, 2001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Depletion</td>
<td>Benzaldehyde 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryopreserved Hepatocytes</td>
<td>5</td>
<td>Metabolite formation</td>
<td>Vanillin 5</td>
<td>Sahi et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>16</td>
<td>Metabolite formation</td>
<td>NMN 9.4</td>
<td>Tayama et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>20</td>
<td>Depletion</td>
<td>Carbazeman 90</td>
<td>Fu et al., 2013</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zoniporide 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phthalazine 17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NMN=$N^1$-methylnicotinamide
Table 2. Characteristics for ten human donors used to assess impact of time and cryopreservation on aldehyde oxidase activity in suspended hepatocyte incubations.

<table>
<thead>
<tr>
<th>Donor (Abbreviation)</th>
<th>Age (yrs)</th>
<th>Race</th>
<th>Gender</th>
<th>COD</th>
<th>Alcohol</th>
<th>Tobacco</th>
<th>Substance Abuse</th>
<th>Medical History</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 (WOX)</td>
<td>25</td>
<td>CA</td>
<td>Male</td>
<td>Anoxia-drug intox</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>NR</td>
</tr>
<tr>
<td>D2 (POG)</td>
<td>35</td>
<td>CA</td>
<td>Female</td>
<td>CVA</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>D3 (XLA)</td>
<td>42</td>
<td>CA</td>
<td>Male</td>
<td>Head Trauma-Fall</td>
<td>Yes</td>
<td>Yes</td>
<td>NR</td>
<td>Parkinson’s</td>
</tr>
<tr>
<td>D4 (LUH)</td>
<td>64</td>
<td>AA</td>
<td>Male</td>
<td>Stroke/CVA</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Noonan’s syndrome</td>
</tr>
<tr>
<td>D5 (NQQ)</td>
<td>6</td>
<td>CA</td>
<td>Male</td>
<td>Anoxia</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Evan’s Syndrome, Autoimmune Neutropenia</td>
</tr>
<tr>
<td>D6 (IEV)</td>
<td>4</td>
<td>CA</td>
<td>Female</td>
<td>Anoxia</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>NR</td>
</tr>
<tr>
<td>D7 (CNM)</td>
<td>67</td>
<td>CA</td>
<td>Male</td>
<td>ICH</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>HTN with meds</td>
</tr>
<tr>
<td>D8 (ZUJ)</td>
<td>53</td>
<td>CA</td>
<td>Male</td>
<td>CVA</td>
<td>NR</td>
<td>Yes</td>
<td>NR</td>
<td>HTN</td>
</tr>
<tr>
<td>D9 (SVL)</td>
<td>69</td>
<td>CA</td>
<td>Male</td>
<td>ICH</td>
<td>Social</td>
<td>No</td>
<td>No</td>
<td>NR</td>
</tr>
<tr>
<td>D10 (CLL)</td>
<td>74</td>
<td>CA</td>
<td>Female</td>
<td>Head Trauma-Fall</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>NR</td>
</tr>
</tbody>
</table>

CA, Caucasian; AA, African-American; COD, cause of death; CVA, cerebrovascular accident; ICH, intracranial hemorrhage; HTN, hypertension; NR, not reported
Table 3. Summary of AOX1 single nucleotide polymorphisms (SNP) and allelic frequencies observed in 71 donors.

<table>
<thead>
<tr>
<th>Reference SNP</th>
<th>Amino Acid Change</th>
<th>Cohort mutant frequency n=142</th>
<th>dbSNP mutant frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs55754655</td>
<td>N1135S</td>
<td>0.218</td>
<td>0.189 $^1$</td>
</tr>
<tr>
<td>rs3731722</td>
<td>H1297R</td>
<td>0.092</td>
<td>0.056 $^1$</td>
</tr>
<tr>
<td>rs199984835</td>
<td>Y126Stop</td>
<td>0</td>
<td>0.026 $^2$</td>
</tr>
<tr>
<td>rs41309768</td>
<td>R802C</td>
<td>0</td>
<td>0.006 $^2$</td>
</tr>
<tr>
<td>rs56199635</td>
<td>R921H</td>
<td>0</td>
<td>0.003 $^2$</td>
</tr>
<tr>
<td>rs141786030</td>
<td>S1271L</td>
<td>0</td>
<td>0 $^1$</td>
</tr>
</tbody>
</table>

$^1$ ESP_Cohort_Population n=4552, $^2$ COLL2006 n=360, n=chromosomes samples analyzed
Table 4. Genotype prevalence by ethnic background for two most common AOX1 SNPs. The prevalence of each genotype is given in total and by race for 71 donors. A large cohort population from the NCBI dbSNP database is included for comparison.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Caucasian (n=50)</th>
<th>Hispanic (n=14)</th>
<th>African American (n=7)</th>
<th>Total (n=71)</th>
<th>dbSNP (^1)</th>
</tr>
</thead>
</table>
| rs55754655 | \(\begin{array}{l}
\text{wt/wt} \\
\text{wt/G} \\
\text{G/G}
\end{array}\) | \(\begin{array}{l}
60.0\% \\
36.0\% \\
4.0\%
\end{array}\) | \(\begin{array}{l}
71.4\% \\
21.4\% \\
7.1\%
\end{array}\) | \(\begin{array}{l}
57.1\% \\
28.6\% \\
14.3\%
\end{array}\) | \(\begin{array}{l}
62.0\% \\
32.4\% \\
5.6\%
\end{array}\) | \(\begin{array}{l}
66.5\% \\
29.4\% \\
4.2\%
\end{array}\) |

| rs3731722 | \(\begin{array}{l}
\text{wt/wt} \\
\text{wt/G} \\
\text{G/G}
\end{array}\) | \(\begin{array}{l}
88.0\% \\
12.0\% \\
0.0\%
\end{array}\) | \(\begin{array}{l}
64.3\% \\
35.7\% \\
0.0\%
\end{array}\) | \(\begin{array}{l}
71.4\% \\
28.6\% \\
0.0\%
\end{array}\) | \(\begin{array}{l}
81.7\% \\
18.3\% \\
0.0\%
\end{array}\) | \(\begin{array}{l}
89.2\% \\
10.3\% \\
0.5\%
\end{array}\) |

\(^1\) ESP_Cohort_Populations: Data is derived from population cohorts participating in the NHLBI Exome Sequencing Project (n=2275). For a list of participants please see the program website at: https://esp.gs.washington.edu/drupal/

n=diploid individuals
Figure 1

O^6^-Benzylguanine  8-oxo Benzylguanine
Figure 2

The figure shows a bar graph comparing intrinsic Cl (mL/min/kg) across different samples labeled as follows:

- D1 (WOX)
- D2 (POG)
- D3 (XLG)
- D4 (LUH)
- D5 (NOO)
- D6 (IEV)
- D7 (CMN)
- D8 (ZUJ)
- D9 (SVL)
- D10 (CLL)

The graph indicates the following conditions:

- Black bars: Immediately following isolation
- Dotted black bars: 24 hrs post isolation
- White bars: 24 hrs post isolation (shipped)
- Gray bars: Cryopreserved

The y-axis represents the intrinsic Cl values ranging from 0 to 120 mL/min/kg.
Figure 3

Individual Donor Cryopreserved Hepatocytes
(mean ± S.D., n=70 donors)
39.9 ± 19 mL/min/kg

Pooled Cryopreserved Hepatocytes
(n=10 donor, mixed-gender)
34.2 ± 1.4 mL/min/kg
Figure 4

A

B

C

D

Cl_intrinsic (mL/min/kg)

Cl_intrinsic (mL/min/kg)

Cl_intrinsic (mL/min/kg)

Cl_intrinsic (mL/min/kg)

Male

Female

BMI < 30

BMI > 30

Caucasian

Hispanic

AA

<60

60+

<60

60+
Figure 5

A

rs55754655 (N1135S)

B

rs3731722 (H1297R)

Cl_{intrinsic} (mL/min/kg)

WT  G  WT  G