

TITLE PAGE

Why Do Most Human Liver Cytosol Preparations Lack Xanthine Oxidase Activity?

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Document Summary

Text Pages 14

Tables 1

Figures 1

References 23

Word Count:

Abstract 248

Introduction 611

Results and Discussion 1201

Abbreviations- 6-MP, 6-mercaptopurine; 6-NO₂Q, 6-nitroquinazolinone; AO, aldehyde oxidase; AP, allopurinol; DACA, N-[(2-dimethylamino)ethyl]acridine-4-carboxamide; HLC, human liver cytosol; IS, internal standard; MDL, method detection limit; MRM, multiple reaction monitoring; LC-MS/MS, liquid chromatography tandem mass spectrometry; UW, university of Wisconsin; XO, xanthine oxidase

ABSTRACT

When investigating the potential for xanthine oxidase (XO) mediated metabolism of a new chemical entity *in vitro*, selective chemical inhibition experiments are typically used. Most commonly, these inhibition experiments are performed using the inhibitor allopurinol (AP) and commercially prepared human liver cytosol (HLC) as the enzyme source. For reasons detailed herein, it is also a common practice to perfuse livers with solutions containing AP prior to liver harvest. The exposure to AP in HLC preparations could obviously pose a problem for measuring *in vitro* XO activity. To investigate this potential problem, an HPLC-MS/MS assay was developed to determine whether AP and its primary metabolite, oxypurinol, are retained within the cytosol for livers that were treated with AP during liver harvest. Differences in enzymatic activity for XO and aldehyde oxidase (AO) in human cytosol that can be ascribed to AP exposure were also evaluated. The results confirmed the presence of residual AP (some) and oxypurinol (all) human liver cytosol preparations that had been perfused with an AP containing solution. In every case where oxypurinol was detected, XO activity was not observed. In contrast, the presence of AP and oxypurinol did not appear to have an impact on AO activity. Pooled HLC that was purchased from a commercial source also contained residual oxypurinol and did not show any XO activity. In the future, it is recommended that each HLC batch is screened for oxypurinol and/or XO activity prior to testing for XO mediated metabolism of a new chemical entity.

INTRODUCTION

Human xanthine oxidase (XO) and aldehyde oxidase (AO) are two closely related molybdoflavoenzymes that share a remarkable degree of amino acid sequence identity, require the same cofactors (Garattini et al., 2003) and appear to share a similar mechanism (Alfaro and Jones, 2008). Despite their similarities, each enzyme is thought to have distinct substrate specificities with AO being much more promiscuous (Garattini and Terao, 2012). In the field of drug metabolism in particular, AO has burgeoned as an important enzyme primarily for its ability to oxidize a number of azaheterocycle containing drugs (Pryde et al., 2010). A number of drugs candidates were also recently discontinued in early clinical studies either because of low bioavailability or toxicity attributed to rapid AO metabolism (Hutzler et al., 2013). In contrast, XO drug substrates have rarely been noted in recent literature. For many years, allopurinol (Elion et al., 1966), and 6-mercaptopurine (Rashidi et al., 2007) have remained the major XO substrates of clinical significance.

To distinguish between XO and AO metabolic activity for a new chemical entity *in vitro*, selective chemical inhibition experiments are typically used (Hutzler et al., 2013). Most commonly, allopurinol is used to selectively inhibit XO (Panoutsopoulos et al., 2004), and menadione is used to target AO activity (Pryde et al., 2010). Recently, hydralazine has also been suggested as a potent and highly selective AO inhibitor (Strelevitz et al., 2012). These inhibition experiments are most often performed with human liver cytosol (HLC) which appears to be the most common source of this enzyme.

HLC is prepared from donor livers that have been harvested using either of two methods. Most commonly, livers are procured from deceased donors via a full hepatectomy in which the entire liver is removed and subsequently processed and separated into microsomal and cytosolic fractions. The second procurement method involves live donors in which a partial hepatectomy, or liver resection, is performed. When livers are obtained via full hepatectomy, the livers are typically flushed, or perfused, with a standardized solution prior to being removed. Perfusion is important prior to liver transplantation, in which the solution acts to preserve the liver tissue and protect it from reperfusion injury. Reperfusion

injury is a phenomenon that results from oxidative damage that occurs to the tissue when the blood supply returns to the liver after a period of ischemia (Mendes-Braz et al., 2012). Additives in the perfusion solution can mitigate the damage caused by these deleterious oxidative processes. Since the late 1980s, the gold standard perfusion solution has been University of Wisconsin (UW) solution (Mühlbacher et al., 1999; Feng et al., 2007). Despite other alternative solutions, UW solution has continued to be the most commonly used preservation solution in liver transplantation surgery (Watson and Dark, 2012). UW solution is principally composed of potassium, lactobionate, and hydroxyethyl starch, but also contains 1 mM of allopurinol (Janssen et al., 2004) which is of primary concern to the present study.

Due to the possibility that livers may be exposed to high amounts of allopurinol (AP) during the harvesting process, and the fact that AP is a potent XO inhibitor, the question was raised: does the method of liver harvesting have an impact on *in vitro* XO enzymatic activity? The goal of this study was to answer the aforementioned question, thereby documenting the presence of residual AP within the cytosol as well any effect of using a solution containing AP in the liver harvesting process on both XO and AO activity. Herein, the activity differences were evaluated in human cytosol prepared from liver tissue harvested in three different ways: full hepatectomy with UW perfusion solution present, full hepatectomy with a perfusion solution not containing AP, and partial hepatectomy (resection).

MATERIALS AND METHODS

Materials. Intact liver tissue from individual donors was obtained from the University of Washington (n = 10) and St Jude Children's Research Hospital (n = 10) human liver banks. Both were maintained with complete anonymity of the tissue donor and thus exempt from human subjects research regulations, as determined by the respective Institutional Review Boards and federal regulation. Liver tissue obtained from St. Jude Children's Research Hospital was processed through the St. Jude Liver Resource by the Liver Tissue Procurement and Distribution System (NIH Contract #N01-DK-9-2310) and by the Cooperative Human Tissue Network. Commercial human liver cytosol (pooled from 150 individual donors of mixed gender) was purchased from BD Biosciences (Franklin Lakes, NJ). Allopurinol, oxypurinol, 6-nitroquinazolinedione, 6-mercaptopurine, and 2-methyl-4(3H)-quinazolinone were obtained from Sigma-Aldrich (St. Louis, MO). 6-thiouric acid and heavy isotope labeled oxypurinol were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). N-[(2-dimethylamino)ethyl]acridine-4-carboxamide (DACA) (Barr and Jones, 2013) and 6-nitroquinazolinone (Alfaro et al., 2009) were synthesized according to previously published methods. DACA-9(10H)-acridone was kindly provided by Dr. William A. Denny from University of Auckland (Auckland, New Zealand). All other reagents used were analytical grade or better.

Preparation of Cytosol from Liver Tissue.

For each liver, approximately 0.25-1.6 g tissue was combined with 3.5 mL homogenization buffer (50 mM KPi, 0.25 M sucrose, 1 mM EDTA, pH 7.4) and 18 ceramic beads (2.8 mm, Omni International, Kennesaw, GA) in a 7 ml polypropylene tube. A homogenate was generated using the Omni Bead Ruptor 24 Homogenizer with attached BR-Cryo cooling unit, operated under the following settings: 5.8 m/sec, 45 sec/cycle, 30 sec dwell time and 2 cycles between -10 to -15° C. The homogenate was transferred to a clean 10 ml polycarbonate centrifuge tube and subjected to 15,000 x g for 30 min. at 4°C. The resulting supernatant was transferred to a clean ultracentrifuge tube, diluted with homogenization buffer to balance

and centrifuged at 120,000 x g for 70 min at 4 °C. The cytosol (supernatant) was removed, transferred into 1.5 mL storage tubes and stored at -80°C.

Enzyme Activity Assays. All assays were performed in 25mM potassium phosphate buffer, pH 7.4 in a shaking water bath incubator at 37 °C. All substrate stocks were made in DMSO, and diluted such that the final concentration of DMSO did not exceed 1% v/v. All reactions were stopped by the addition of a solution composed of 1 M formic acid and a known concentration of IS. Incubations were performed as described below and immediately analyzed by LC-MS/MS.

Determination of XO activity. A final concentration of 1 mg/ml protein was used in all incubations. In the 6-mercaptapurine assay, the incubation was performed for 60 minutes with 100 µM substrate. Activity was quantified by monitoring the formation of the metabolite, 6-thiouric acid. For 6-nitroquinazolinone, the incubation was performed for 10 minutes with 20 µM substrate. Activity was quantified by monitoring the formation of the metabolite, 6-nitroquinazolinone.

Determination of AO activity. A final protein concentration ranging between 0.15-0.66 mg/ml was used. Preliminary experiments showed that product formation was linear with respect to protein concentration within the range used in these activity experiments. The incubation was performed for 10 minutes with 100 µM substrate (DACA). DACA activity was quantified by monitoring the formation of the metabolite, DACA acridone.

AO IC₅₀ assays. DACA was used as the probe substrate for AO activity at approximately K_m concentrations (5 µM). Reactions were carried out for 5 minutes using commercially purchased pooled HLC with a final protein concentration of 0.05 mg/ml.

Liver cytosol treatment for allopurinol and oxypurinol detection. Human liver cytosol samples were treated with a formic acid solution containing a known amount of 2-methyl-4(3H)-quinazolinone as internal standard, such that the final concentration of formic acid was 200 mM. Samples were vortexed and subsequently frozen at -80 °C to induce protein precipitation. Upon thawing, the samples were

centrifuged for 10 min at 5000 rpm using an Eppendorf 5415D centrifuge. The supernatant was collected and analyzed immediately by HPLC-MS/MS.

HPLC-MS/MS. All samples were detected using an 1100 series high performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) coupled to an API 4000 tandem mass spectrometry system (Applied Biosystems/MDS Sciex, Foster City, CA) with a turbospray electrospray ionization source. Mobile phases comprised 0.05% formic acid and 0.2% acetic acid in water (A), and 90% acetonitrile, 9.9% water, and 0.1% formic acid (B).

Detection of Allopurinol and Oxypurinol. Chromatography was performed on a Zorbax Eclipse XDB-C8 column (4.6 X 150 mm, 5 μ M; Agilent, Santa Clara, CA). Using a flow rate of 800 μ L/min, the column was equilibrated at initial conditions of 99% mobile phase A for 2.5 min. Chromatographic separation was performed using a linear gradient over the next 17.5 min to 5% mobile phase A, and was held at 5% A for 1 minute. Over the next minute, mobile phase A was ramped back to 99 % and then held constant for a re-equilibration time of 3 min. The total chromatographic assay time was 25 min per sample, and the retention times for allopurinol, oxypurinol, and 2-methyl-4(3H)-quinazolinone (IS) were 4.2, 4.9, and 8.5 minutes, respectively. The MRM m/z transition monitored for allopurinol, oxypurinol, and 2-methyl-4(3H)-quinazolinone (IS) were 137.1 \rightarrow 110.1, 153.1 \rightarrow 136.1, and 161.2 \rightarrow 120.1, respectively. Quantitation was achieved by extrapolating from a standard curve of authentic allopurinol and oxypurinol using a concentration range of 0.06 to 60 μ M. The method detection limit for allopurinol and oxypurinol was determined to be 0.027 and 0.025 μ M, respectively.

To validate the assay, heavy isotope labeled oxypurinol (oxypurinol-¹³C¹⁵N₂) was spiked into each sample and monitored using a MRM transition of 156.1 \rightarrow 139.1. It was ensured that the retention time for all peaks for oxypurinol matched exactly with the heavy isotope labeled standard. Further validation of analyte recovery and linearity in a complex matrix was also performed. A sample of HLC that contained no detectable amount of AP or oxypurinol was used as a matrix blank for this experiment. Known concentrations of AP and oxypurinol were added to both buffer and the matrix blank in separate vials, and

the measured values were compared (Supplemental Figure 2). A total of 7 points was used for allopurinol and 8 points for oxypurinol. The concentration range for each analyte covered the minimum and maximum concentration values for any tested HLC in this study.

Detection of 6-thiouric acid. Chromatography was performed on a Zorbax Eclipse XDB-C8 column (4.6 X 150 mm, 5 μ M; Agilent, Santa Clara, CA). Using a flow rate of 800 μ L/min, the column was equilibrated at initial conditions of 99% mobile phase A for 3 min. Chromatographic separation was performed using a linear gradient over the next 1.5 min to 25% mobile phase A, and was held at 25% A for 1.5 minutes. Over the next minute, mobile phase A was ramped back to 99% and then held constant for a re-equilibration time of 1 min. The total chromatographic assay time was 8 min per sample, and the retention times for 6-thiouric acid and 3,5-dibromo-4-hydroxybenzoic acid (IS) were 4.3 and 6.8 minutes, respectively. The MRM m/z transitions monitored for 6-thiouric acid and 3,5-dibromo-4-hydroxybenzoic acid (IS) were 183.0 \rightarrow 140.2 and 294.8 \rightarrow 251.0, respectively. Quantitation was achieved by extrapolating from a standard curve of authentic 6-thiouric acid using a concentration range of 0.2 to 10 μ M. The method detection limit for 6-thiouric acid was determined to be 0.140 μ M.

Detection of 6-nitroquinazolinedione. Chromatography was performed on a Synergi Polar reverse-phase column (30 X 3.0 mm, 4 μ m; Phenomenex, Torrance, CA). Using a flow rate of 800 μ L/min, the column was equilibrated at initial conditions of 95% mobile phase A for 0.3 min. Chromatographic separation was performed using a linear gradient over the next 2.2 min to 25% mobile phase A, and was held at 25% A for 0.5 minutes. Over the next 1.5 minutes, mobile phase A was ramped back to 95 % and then held constant for a re-equilibration time of 1 min. The total chromatographic assay time was 4.5 min per sample, and the retention times for 6-nitroquinazolinedione and 2-methyl-4(3H)-quinazolinone (IS) were 1.8 and 1.3 minutes, respectively. The MRM m/z transitions monitored for 6-nitroquinazolinedione and 2-methyl-4(3H)-quinazolinone (IS) were 208.0 \rightarrow 162.0 and 161.2 \rightarrow 120.1, respectively. Quantitation was achieved by extrapolating from a standard curve of authentic 6-nitroquinazolinedione using a

concentration range of 1 to 25 μM . The method detection limit for 6-nitroquinazolinedione was determined to be 0.85 μM .

Detection of DACA acridone metabolite. LC-MS/MS conditions were performed as described previously (Barr and Jones, 2013).

Determination of Method Detection Limit. For calculating MDL, a repeat injection approach was used as described previously (Wells et al., 2011; Barr et al., 2013). In short, a concentration of each authentic analyte that produced a signal approximately 5-10 times the noise was chosen. Each standard was injected onto the column a total of 5 times and the peak areas were determined. The MDL was calculated using the following formula:

$$\text{MDL} = S_{\bar{x}} T_{\alpha}$$

Where $S_{\bar{x}}$ is equal to the standard deviation of the mean for peak area and T_{α} is the value chosen from a t-table for $n=5$ at a 99% confidence interval.

RESULTS AND DISCUSSION

To ascertain any differences between liver harvesting conditions, each individual cytosol sample was first screened for residual AP, and its primary metabolite, oxypurinol. Due to the complexity of the sample matrix, contaminate peaks were observed. This was particularly an issue for oxypurinol because contaminate peaks with a similar retention time to the analyte were seen for some samples. To overcome this and ensure the validity of the results, each sample was spiked with a known concentration of heavy isotope labeled oxypurinol (oxypurinol- $^{13}\text{C}^{15}\text{N}_2$). Supplemental Figure 1 shows the peaks observed for oxypurinol and oxypurinol- $^{13}\text{C}^{15}\text{N}_2$ as well as typical contaminate peaks. For the determination of oxypurinol in each sample, it was ensured that the peak retention time matched exactly with the heavy isotope labeled standard. The method was further validated by generating a quality control curve for both analytes in which a known amount of AP and oxypurinol were added into a blank cytosol matrix (Supplemental Figure 2).

Table 1 shows the amount of AP and oxypurinol found in each sample of HLC. As indicated on the table, the leftmost group originated from donor livers that were harvested via a full hepatectomy and perfused with a solution that did not contain AP (Group 1). The center group of livers were harvested via full hepatectomy and perfused with UW solution containing 1mM AP (Group 2). The group of liver samples on the right was obtained from partial hepatectomies (resections) (Group 3). For the livers in group 1, no AP or oxypurinol was detected. Two of the five livers from Group 2 contained a varied amount of residual AP, whereas all 5 had detectable amounts of oxypurinol. Of the livers in Group 3, eight out of ten showed no trace of residual AP or oxypurinol. Interestingly, two livers from the resected group (344 and 947) did show a residual amount of oxypurinol. When the origins of these two liver samples were thoroughly investigated, it was found that they were flushed with UW solution and shipped on ice prior to being frozen. This exposure to UW solution clearly explains why oxypurinol was detected in these two samples.

It is worth noting that oxypurinol concentrations were found at much higher levels than AP itself. Also, AP was not observed in all livers that were perfused with UW solution whereas in all cases oxypurinol was observed. This is likely a result of metabolism that is occurring throughout the processing time of the cytosol prior to being frozen. Because oxypurinol is a product of AP metabolism, the formation of oxypurinol is dependent on the time in which the liver was exposed to AP. Therefore, variable handling time may explain the large variation in oxypurinol concentration observed between AP dosed livers.

Once the presence or absence of AP and oxypurinol in certain liver cytosol batches was established, the possible effect this had on XO enzymatic activity was evaluated. Two compounds were chosen as selective XO substrates (Scheme 1). The first, 6-mercaptopurine (6-MP), is metabolized in two steps to 6-thiouric acid (Kalra et al., 2007; Rashidi et al., 2007). The second probe, 6-nitroquinazolinone (6-NO₂Q), is a known substrate for XO (Skibo et al., 1987) but not for AO (Alfaro et al., 2009). Figure 1 shows the results of activity screening for human livers that were procured in three different ways, again with groups 1-3. From these results, it is clear that the presence of AP in the administered perfusion solution has a marked impact on XO activity. In every case when livers were perfused with an AP-containing solution prior to harvest, XO activity was not observed. In contrast, XO activity was observed in every case where the liver was perfused with a solution that did not contain AP. Not surprisingly, XO activity was also observed in all of the resected livers except the two that had residual amounts of AP and oxypurinol present. Without exception, the presence of oxypurinol in the cytosol was an indicator of an absence of XO activity. Interestingly, the lack of XO activity was not dependent at all on the concentration of AP or oxypurinol. This is likely because AP and oxypurinol are also known time dependent inactivators of XO (Elion, 1966; Massey et al., 1970), and thus exposure time during liver processing may eradicate all XO activity even at relatively low concentrations (Scheme 2).

The impact of liver harvesting conditions on AO activity was also examined. Using the probe substrate DACA (Schofield et al., 2000; Barr and Jones, 2013), each liver was screened for enzymatic activity. AO activity was observed for livers in all three groups, and the presence of AP and oxypurinol appeared to have no observable effect (Figure 1). To confirm this, an IC_{50} inhibition assay was performed with AP and oxypurinol (Supplemental Figure 3). For both compounds, the IC_{50} exceeded 1 mM, an amount that is far greater than any residual amount of AP or oxypurinol in the cytosol. From these results, it is clear that AO activity is not affected by AP or oxypurinol.

Similar to the cases of individual donors that were perfused with AP, commercial pooled HLC did contain residual oxypurinol (Table 1), and, not surprisingly, XO activity was not observed (data not shown). As discussed in the introductory section, accurate knowledge of XO activity towards new chemical entities is largely dependent on selective chemical inhibition experiments using commercial HLC as the enzyme source. Adding AP to cytosol that is already devoid of any XO activity would obviously have no effect, and thus the experimenter may erroneously draw the conclusion that XO has no role in metabolism of the tested compound. Therefore, it is certainly possible that some compounds may have been overlooked as potential XO substrates using the standard *in vitro* selective chemical inhibition approaches. In the future, it is recommended that batches of cytosol be screened for AP and oxypurinol prior to testing for XO activity. Also, testing each batch for XO activity using a known active substrate such as 6-MP would be advisable. Due to variability in liver sources, it is possible that variation in AP and oxypurinol concentrations as well as XO activity would be observed between different cytosol vendors and possibly even within different batches from the same vendor. In addition, it seems likely that the presence of AP during liver harvest would also impact XO activity in other *in vitro* systems, including liver S9 and hepatocytes.

In conclusion, three groups of individual donor livers, procured in different ways, were examined for residual oxypurinol and AP concentration and presence of XO and AO enzymatic activity. The presence of residual AP (some) and oxypurinol (all) was observed in human liver cytosol preparations that had been perfused with UW solution. In every case where oxypurinol was detected, XO activity was not observed. In contrast, the presence of AP and oxypurinol did not appear to impact AO activity. This was supported by the fact that AP and oxypurinol inhibited AO activity with IC_{50} values that exceeded 1mM. Commercial pooled HLC also contained residual oxypurinol and did not show any XO activity. In the future, it is recommended that each HLC batch is screened for oxypurinol and/or XO activity prior to testing the metabolism of a new chemical entity.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Barr, Choughule, Zientek, Strom, Schuetz, Thummel, and Jones.

Conducted experiments: Barr, Choughule, Nepal, Wong, Chaudhry, and Joswig-Jones.

Contributed new reagents or analytic tools: N/A

Performed data analysis: Barr, Choughule, Jones, and Nepal.

Wrote or contributed to the writing of the manuscript: Barr, Zientek, Schuetz, Thummel, and Jones.

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FOOTNOTES

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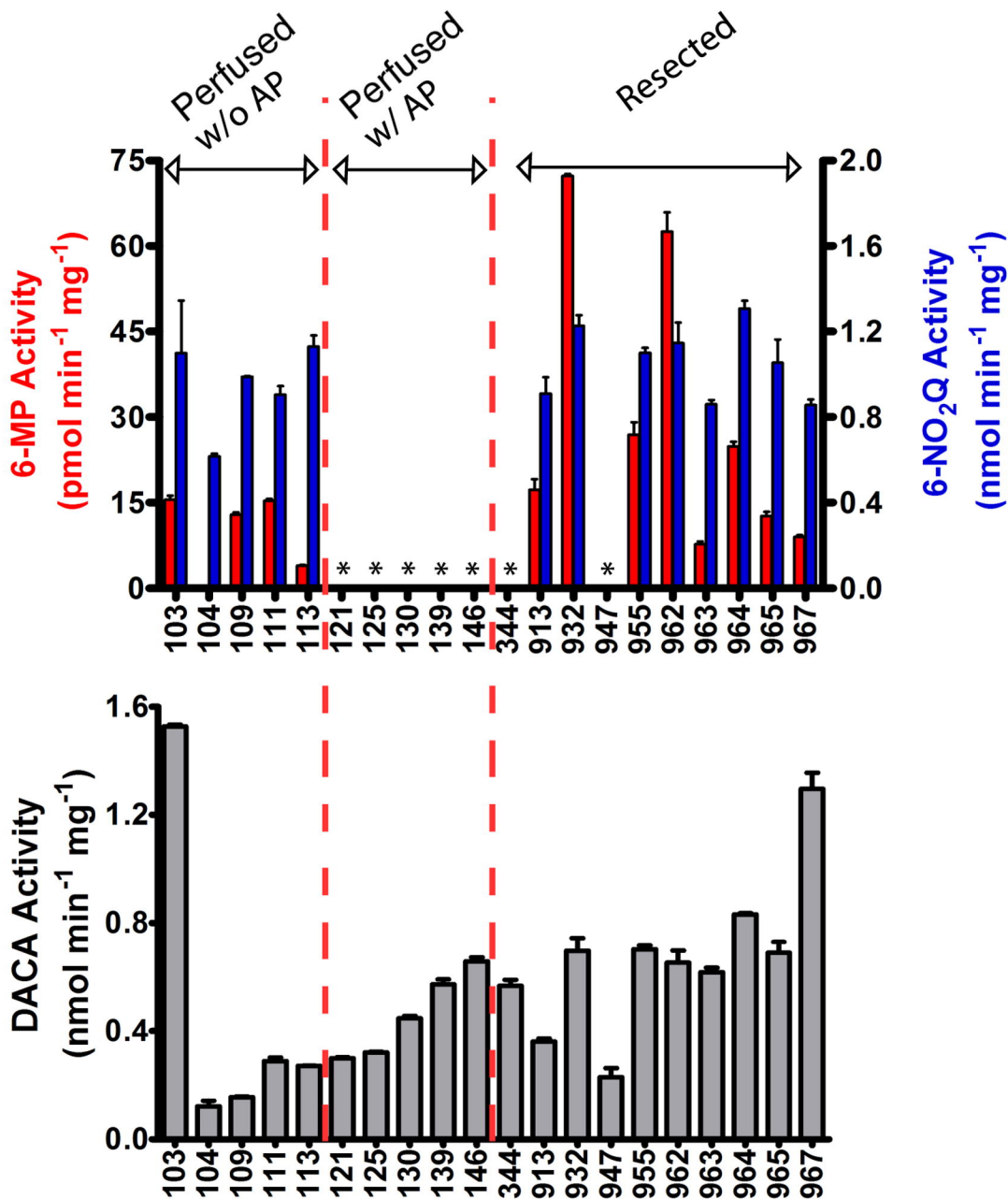
FIGURE LEGENDS

Figure 1- Measured enzymatic activity for individual liver samples for two XO probe substrates (top) and one AO substrate (bottom). Livers are grouped into three different harvesting conditions: full hepatectomy using a perfusion solution without allopurinol (left group), full hepatectomy using a perfusion solution containing 1 mM allopurinol (center group), and partial hepatectomy/resection (right group). The activity units denote the amount of product formed (nmol or pmol) per minute per mg of cytosolic protein. Asterisks indicate samples that showed no XO activity for either probe substrate. Asterisks also indicate samples in which residual oxypurinol was detected. The method detection limit for XO metabolites are stated in the methods section of this text.

	Perfused w/o AP					Perfused w/ AP					Resected					Commercial							
Liver Number	103	104	109	111	113	121	125	130	139	146	344	913	932	947	955	962	963	964	965	967			
Residual [AP] (μ M)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	3.5 \pm	3.2 \pm	N.D.	1.1 \pm	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
Residual [OP] (μ M)	N.D.	1.5	0.56	35 \pm	32 \pm	39 \pm	19 \pm	1.1	N/D	N/D	0.66 \pm	N/D	51 \pm 0.61

Table 1- Concentration measurements for residual AP and oxypurinol in individual liver cytosol preparations. Individual livers were harvested in three different ways: full hepatectomy without an AP-containing perfusion solution (Perfused w/o AP), full hepatectomy with an AP-containing perfusion solution (perfused w/ AP), and partial hepatectomy (resected). Pooled HLC purchased from a commercial source (commercial) was also tested. N.D. indicates that no analyte was detected. The method detection limit for AP and oxypurinol are stated in the methods section of this text. Values reflect the mean \pm standard deviation for duplicate determinations.

Figure 1



* no activity observed; trace oxypurinol found in the sample

Supplemental Data

Drug Metabolism and Disposition

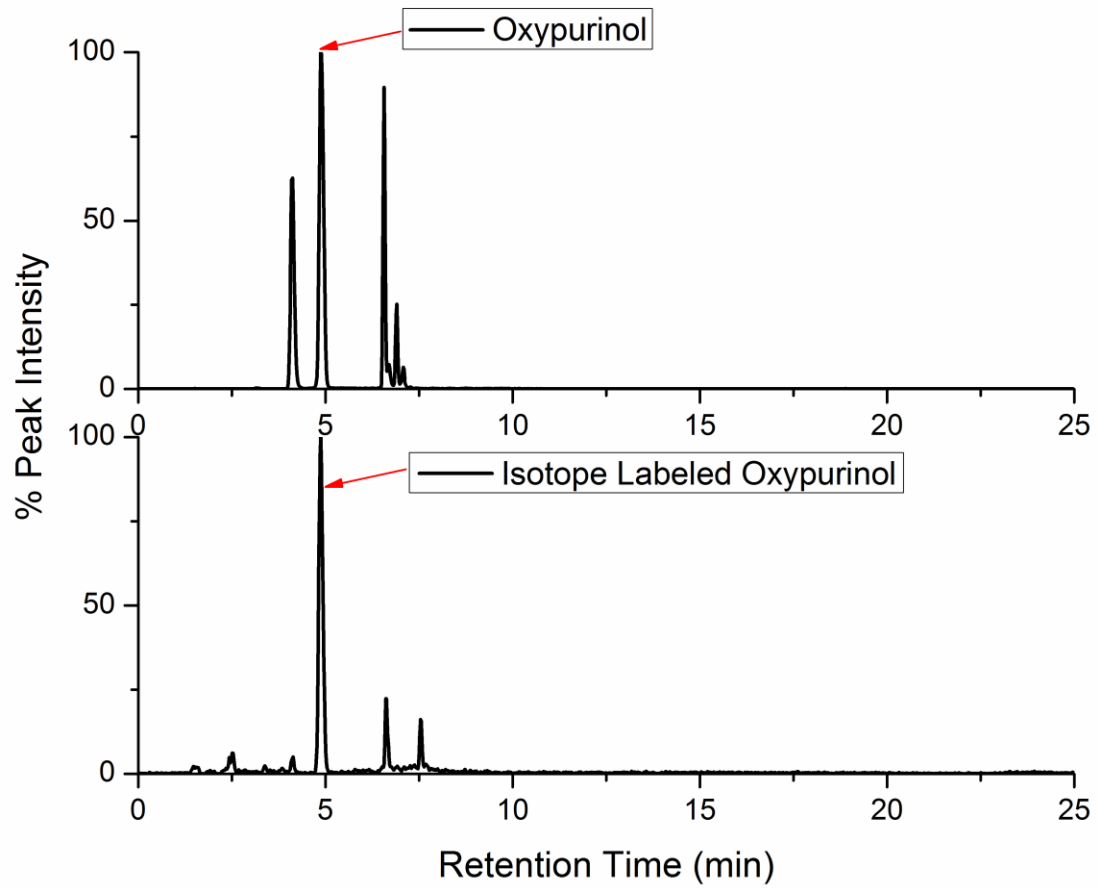
Why Do Most Human Liver Cytosol Preparations Lack Xanthine Oxidase Activity?

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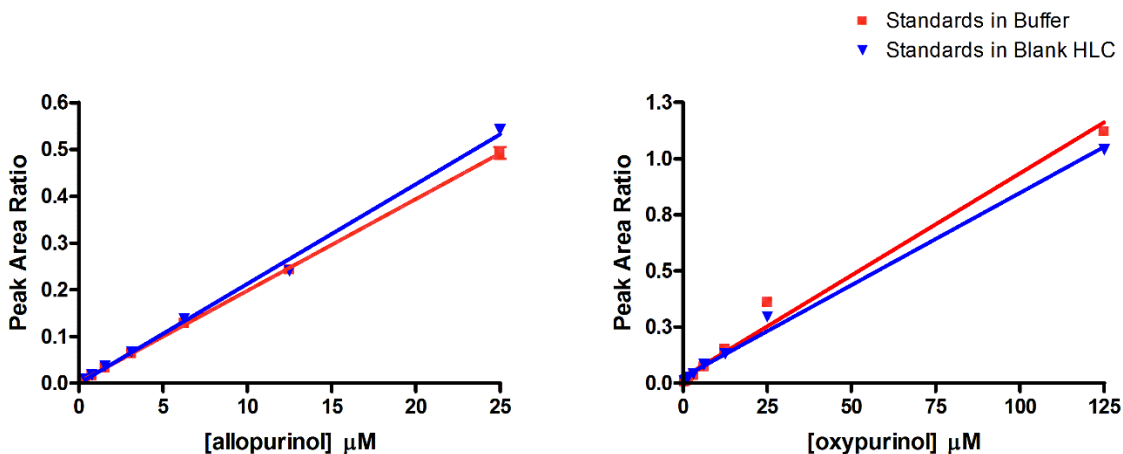
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Supplemental Figure 1- Detection of oxypurinol in individual HLC preparations. Chromatogram traces for a typical sample of HLC analyzed by LC-MS/MS in MRM mode are shown. Heavy isotope labeled oxypurinol was spiked into each sample to validate the exact retention time of oxypurinol.



Supplemental Figure 2- Validation of the detection method for allopurinol (AP) and oxypurinol (OP) in human liver cytosol (HLC). A sample of HLC that contained no detectable amount of AP or OP was used as a matrix blank for this experiment. Known concentrations of AP and OP were added to both buffer and the matrix blank in separate vials. Peak area ratios measured by HPLC-MS/MS for AP (left) and OP (right) are shown. All measured values in HLC are within 10% of the values of standards in buffer. These results show a minimal effect of the matrix on analyte recovery and linearity.



Supplemental Figure 3- Inhibition of AO-mediated metabolism of DACA by allopurinol (red) and oxypurinol (blue). Commercial human liver cytosol was used as the enzyme source. IC_{50} values for both inhibitors exceeded 1 mM, the highest concentration that was tested.

