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Development and Characterization of Recombinant Human Aldehyde Oxidase Expressed in HEK293 Cells

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PURPOSE

Aldehyde Oxidase (AO) is a cytosolic enzyme that plays an important role in xenobiotic biotransformation. With broad substrate specificity, AO catalyzes the oxidation of aldehydes, aromatic azaheterocycles and nitrogen-carbon double bond containing compounds. In recent years, AO has gained considerable attention in the pharmaceutical industry due to 1) multiple drug failures in clinical trials due to issues with the AO pathway; and 2) recent growth in the number of aromatic aza-heterocycle moieties found in many drug leads that are AO substrates. Currently, most recombinant AO is expressed in bacteria and this prokaryotic expression system has disadvantages such as different protein post-translation modification leading to different function compared to mammalian systems. In this study, we developed a recombinant human AO enzyme using a mammalian cell based expression system to enable the early assessment of the liability of AO for drug metabolism and clearance.

METHOD

Materials: Recombinant AO and cytosol control are Corning® Supersomes™ Ultra Human Aldehyde Oxidase (AOX1) and control cytosol (coming soon), human liver cytosol (HLC) is Corning UltraPool™ 150 cytosol (Cat. No. 452115), Corning human hepatocytes (Cat. No. 454503, 454504, 454551, or 454427), Corning Gentest™ high-viability cryohepatocyte recovery medium (Cat. No. 454560). All chemicals were purchased from Sigma-Aldrich.

AO enzymatic assay for liver cytosol and recombinant AO cytosol: For each reaction, 175 µL pre-warmed assay buffer (25 mM Potassium Phosphate, pH 7.4) was mixed with 20 µL pre-warmed substrate working stock, and maintained at 37°C on a heat block. The reaction was initiated with 5 µL 20 mg/mL human liver cytosol pool (HLC), or recombinant human rAO. The vial caps were left open for oxygen permeation. At the end of the desired incubation period, the reaction was stopped with AAA stop solution (phthalazine as substrate) or 100% ACN containing internal standard (zaleplon as substrate) . For the "Blank" incubation, the stop solution was added before addition of enzyme. Next, the sample was centrifuged at 13,000 rpm for 3 min. The supernatant was subjected for HPLC analysis (phthalazine as substrate) or LC-MS analysis (zaleplon as substrate).

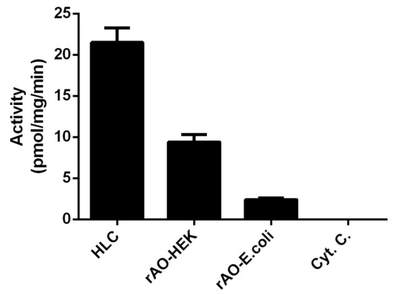
AO enzymatic assay for hepatocytes: individual lot of cryopreserved human hepatocytes or pooled hepatocytes from three individual donors were thawed rapidly at 37°C and processed using the Hepatocyte High Viability Recovery Kit per the instruction. The cells were then resuspended in William's Medium E (WME) to 1 x 10^6 viable cell/mL. Cell suspension was added to each well (250 µL for 48-well plate, and 100 µL for 96-well microplate) and the plate was incubated in a 37°C incubator with 5% CO_2 for 15 min. The reaction was initiated with pre-warmed substrate solution prepared in WME media and incubated in a 37°C incubator with 5% CO_2 for desired amount of time. For assay with phthalazine, the reaction was stopped by taking 200 µL reaction, mixing with 100 µL AAA stop solution, then centrifuging for 3 min. at 14,000 rpm, and the supernatant was subjected for HPLC analysis. For assay with zaleplon, the reaction was stopped by adding 50 µL of 100% ACN containing internal standard directly to the incubation plate, the metabolite formation is analyzed by LC-MS/MS.

Freeze/Thaw study. rAO sample were thawed on ice, small sample was aliquoted out and frozen at -80ºC immediately, this is the 1X sample. The rest of the sample was left on ice for 6 hours followed by being frozen at -80ºC freezer overnight. The process was repeated 5 times. After all samples were collected, samples were assayed together using zaleplon as substrate.

Data Analysis: Each data point represents the mean value of two replicates (phthalazine as substrate, HPLC detection) or three replicates (zaleplon as substrate, LC-MS detection). Kinetic parameters were determined by non-linear regression using SigmaPlot. For inhibition assay, IC_{50} values were determined by using a Sigmoidal Hill three parameter equation using GraphPad Prism™.

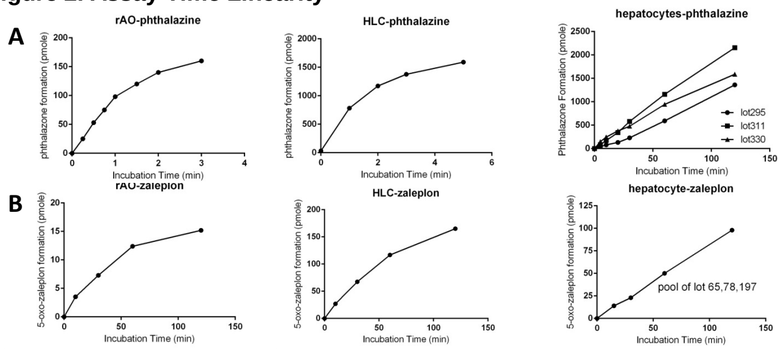
RESULTS

Figure 1. Corning Ultra Human Aldehyde Oxidase Performance



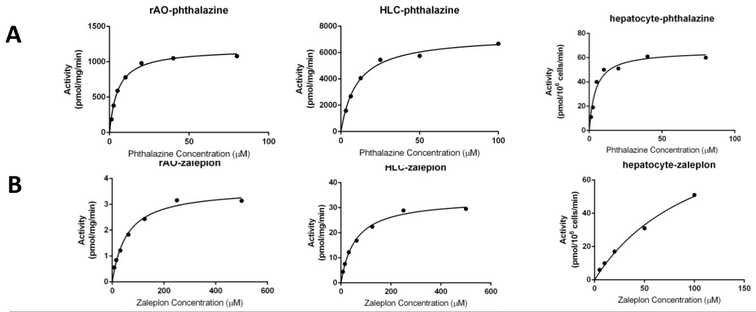
Activity comparison of native AO in human liver cytosol (HLC), recombinant AO expressed in HEK293 cells (rAO-HEK), recombinant AO expressed in *E. coli* (rAO-E.coli), and cytosol control from HEK293 cells (Cyt. C.) using probe substrate zaleplon. AO expressed in HEK293 cells exhibited ~40% activity comparing to native AO in HLC, and it is 4-fold higher activity than AO expressed in *E. coli*. There is no activity found in the cytosol control from HEK293 cells. The substrate concentrations used in the assay is 250 µM.

Figure 2. Assay Time Linearity



The timecourse of metabolite formation by recombinant AO (rAO), human liver cytosol (HLC) and human hepatocytes. (A) 100 µM phthalazine or (B) 500 µM zaleplon was incubated with rAO, Corning UltraPool HLC, or human hepatocytes at 37°C, the reaction was stopped at various time points . The metabolite phthalazone was analyzed with HPLC, the metabolite 5-oxo-zeleplon was analyzed using LC-MS. The phthalazine metabolism is linear for up to 2 min. for rAO and HLC, but 120 min. for human hepatocytes. The zaleplon metabolism is linear for up to 60 min. for rAO and HLC, and 120 min. for human hepatocytes.

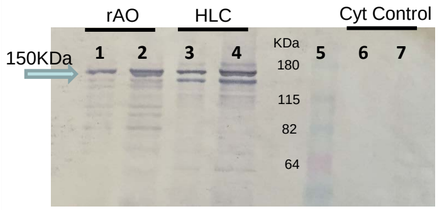
Figure 3. Kinetic Analysis of Substrates in rAO, HLC, and Hepatocytes



Substrate	Ultra AO Supersomes	UltraPool 150 Donor HLC	Human Hepatocytes	Literature	
Phthalazine	5.7	10.2	4.5	8.0 (HLC)	Barr JT, 2011, DMD,2381-6
Zaleplon	56.7	56.8	105	124 (HLC)	Renwick AB, 2002, Xenobiotica, 849-62

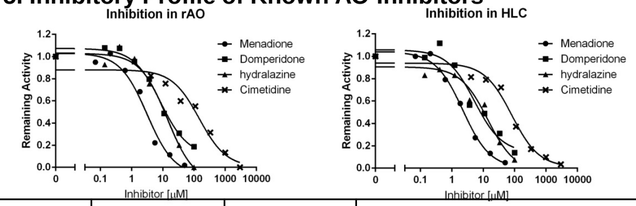
Concentration-dependent oxidation of phthalazine (A) and zaleplon (B) by recombinant AO, human liver cytosol (HLC) and human hepatocytes. The recombinant AO expressed in HEK293 cells showed comparable kinetic (K_m) properties as native AO in human liver cytosol (HLC) or human hepatocytes, as well as literature report. The incubation time is 2 min. for phthalazine assays with rAO and HLC, 20 min. for phthalazine assay with hepatocytes, and 60 min. for zaleplon assays. Six or eight substrate concentrations were used in each kinetic determination. There is no activity detected in the cytosol control made from HEK cells for both substrates.

Figure 4. Recombinant AO Expression



Western blot analysis of rAO expressed from HEK293 cells (lane 1 and 2: 1.5 µg and 5 µg), HLC (lane 3 and 4: 0.6 µg and 2 µg) and cytosol control of HEK293 cells (lane 6 and 7: 1.5 µg and 5 µg). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with a primary antibody (rabbit polyclonal anti-AO, Abcam, ab92519) and a secondary antibody (AP-anti-rabbit). rAO expressed in HEK293 cells shows a correct molecular weight of 150kDa, consistent with native AO in HLC.

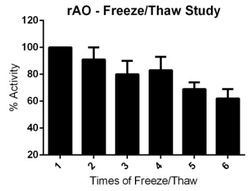
Figure 5. Inhibitory Profile of Known AO Inhibitors



Inhibitor	Ultra AO Supersomes	UltraPool 150 Donor HLC	Literature	
Menadione	3.1	2.2	0.2-7.0	Pryde DC, 2010, J. Med. Chem., 8441-60
Domperidone	8.4	5.3	5.3 (Ki)	Barr JT, 2011, DMD, 2381-6
Hydralazine	13.9	13.9	83 (Ki)	Strelevitz TJ, 2012, DMD, 1441-8
Cimetidine	146	81.5	231	Renwick AB, 2002, Xenobiotica, 849-62

The inhibitory effect of menadione, domperidone, hydralazine, and cimetidine on enzymatic activity of rAO expressed in HEK293 cells, and Corning UltraPool 150 donor HLC. IC_{50} was determined by co-incubating 1 mg/mL rAO or 1 mg/mL HLC with 50 µM zaleplon in the presence of a range of concentration of menadione (0 - 50 µM), domperidone (0 - 100 µM), hydralazine (0 - 100 µM), and cimetidine (0 - 3,000 µM). Each point represents the mean value of three replicates and the solid lines represented the non-linear regression fitting. The curve represents one of 2 independent experiments. The IC_{50} values generated using recombinant AO expressed in HEK293 cells are comparable to the native AO in HLC, and consistent with those published in the literature.

Figure 6. Freeze/Thaw Study



Recombinant AO activity is sensitive to freeze/thaw cycles. On average the activity decreases 8% after each freeze/thaw cycle. The activity remains 80% or above if the sample is thawed less than 4 times. It is recommended to aliquot rAO sample to minimize freeze/thaw cycles and maintain the high activity.

CONCLUSION

- Human aldehyde oxidase was successfully overexpressed in HEK293 cells and the recombinant enzyme is more active than rAO expressed in *E. coli* and has consistent molecular weight with native AO enzyme in human liver cytosol.
- Time-dependent catalytic enzyme activity of rAO was evaluated for two probe substrates. The kinetics of probe substrates and inhibition profiles of known AO inhibitors determined by rAO are aligned well with native AO enzyme in HLC or primary human hepatocytes.
- The rAO enzymatic activity is sensitive to freeze/thaw cycles. The activity remains 80% or above if the sample is thawed less than 4 times.
- The rAO expressed in HEK293 cells is demonstrated to be a stable and reproducible in vitro tool for studying AO mediated drug metabolism.