Aldehyde Oxidase and Donor Variability

Challenges to Estimating Human Clearance

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Emergence of Aldehyde Oxidase in Metabolism

Recent Literature

- Pryde et al. (2010) Aldehyde oxidase: An enzyme of emerging importance in drug discovery. *J Med Chem*. 53, 8441-60.
- Garattini and Terao (2011) Increasing recognition of the importance of aldehyde oxidase in drug development and discovery. *Drug Metab Rev*. 43, 374-86.
- Garattini and Terao (2012) The role of aldehyde oxidase in drug metabolism. *Expert Opin Drug Metab Toxicol*. 8, 487-503.
- Morrison et al. (2012) The Role of Aldehyde Oxidase and Xanthine Oxidase in the Biotransformation of a Novel Negative Allosteric Modulator of Metabotropic Glutamate Receptor Subtype 5. Drug Metab Dispos. 40(9), 1834-45.
- Garattini and Terao (2013) Aldehyde oxidase and its importance in novel drug discovery: present and future challenges. *Expert Opin Drug Discov*. 8, 641-54.
- Hutzler JM, Obach RS, Dalvie D and Zientek M (2013) Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin Drug Metab Toxicol*. 9, 153-68.
- Barr JT et al. (2014) Enzyme kinetics, inhibition, and regioselectivity of aldehyde oxidase. *Method Mol Biol*. 1113, 167-86.
- Hutzler et al. (2014) Cynomolgus monkey as a surrogate for human aldehyde oxidase metabolism of the EGFR inhibitor BIBX1382. *Drug Metab Dispos*. 42(10), 1751-60.

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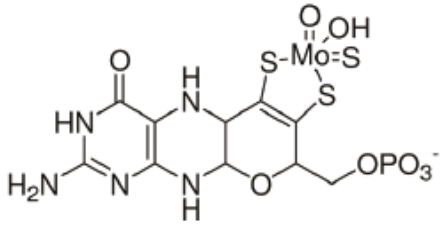
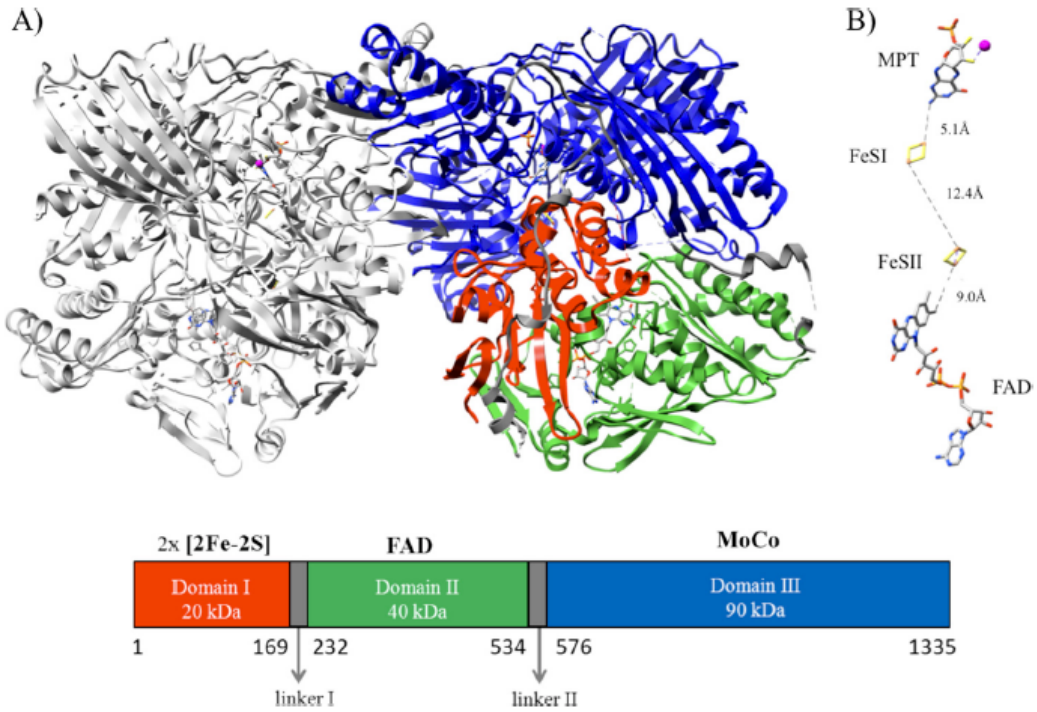
The Enzymology of Aldehyde Oxidase (AO)

- Member of molybdenum cofactor-containing enzyme family that require a flavin adenine dinucleotide (FAD) and molybdenum cofactor (MoCo) for catalytic activity

MoCo



- Same family as xanthine oxidoreductase (XOR)
- Active as a homodimer composed of two identical monomer subunits ~150 kDa
- Each monomer is subdivided into three distinct domains



Coelho et al. (2012) *J Biol Chem* 287; 40690-40702

A) Ribbon representation of mouse AOX3 crystal structure

B) Arrangement of protein cofactors and electron flow

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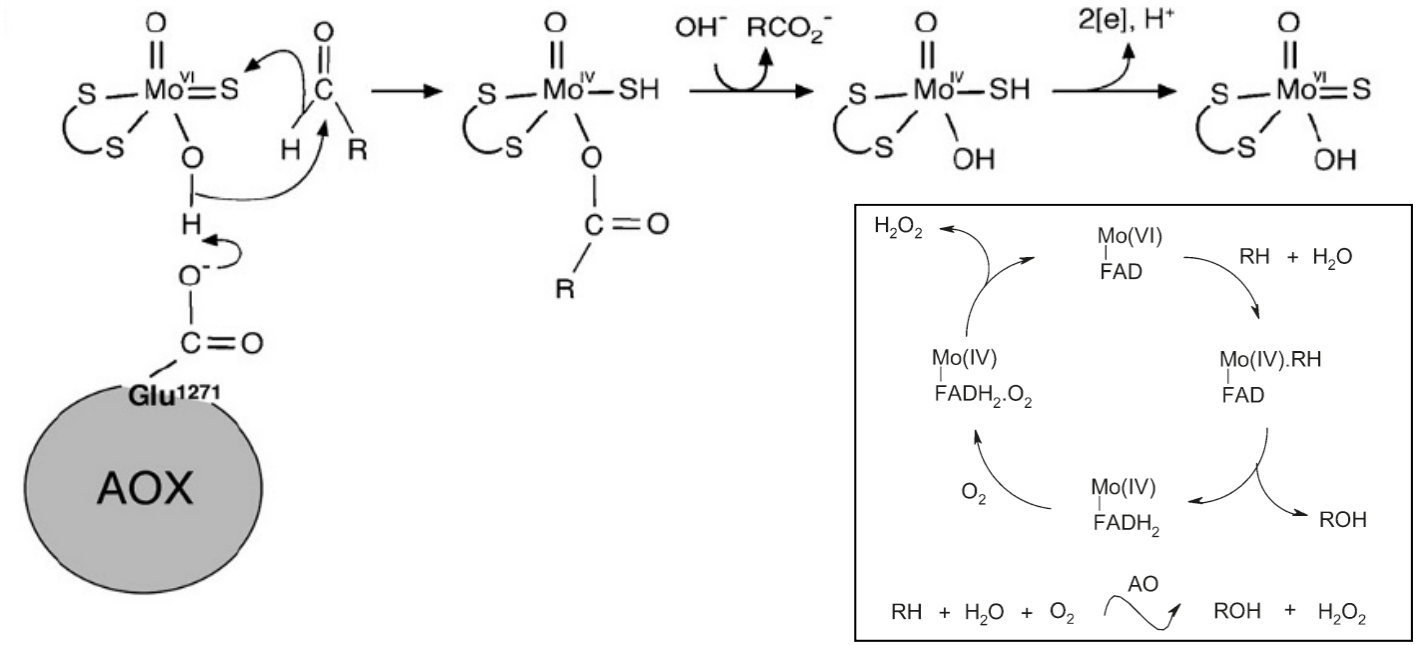
Catalytic Mechanism of AO

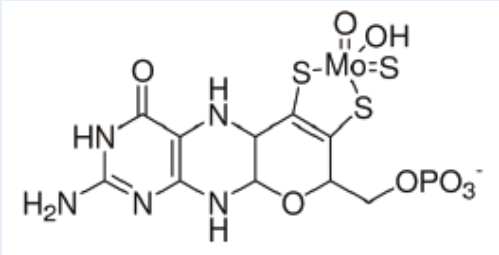
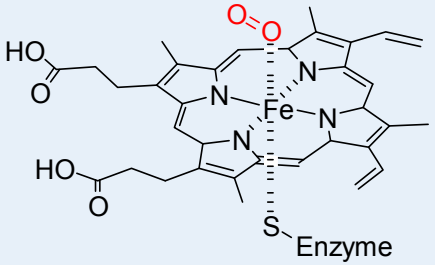


- Conserved Glu residue in substrate pocket important for catalysis
- Nucleophilic attack on electron-deficient carbon
- Hydride transfer to Mo-sulfur (rate-limiting step)
- Oxygen incorporated into substrate comes from water (not molecular O_2)
- Reducing equivalents are passed to the flavin (FAD) via 2Fe/2S centers to form $FADH_2$, which is then reoxidized by molecular oxygen, generating H_2O_2

Alfaro and Jones. (2008) *J Org Chem. 73*; 9469-9472

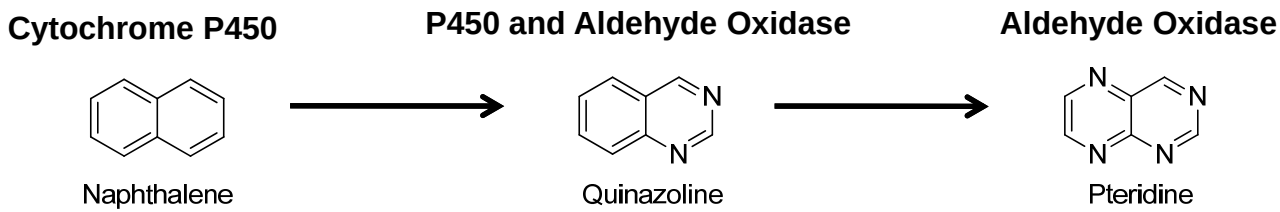
Garattini et al. (2008) *Cell Mol Life Sci. 65*; 1019-1048

Differentiation from Cytochrome P450

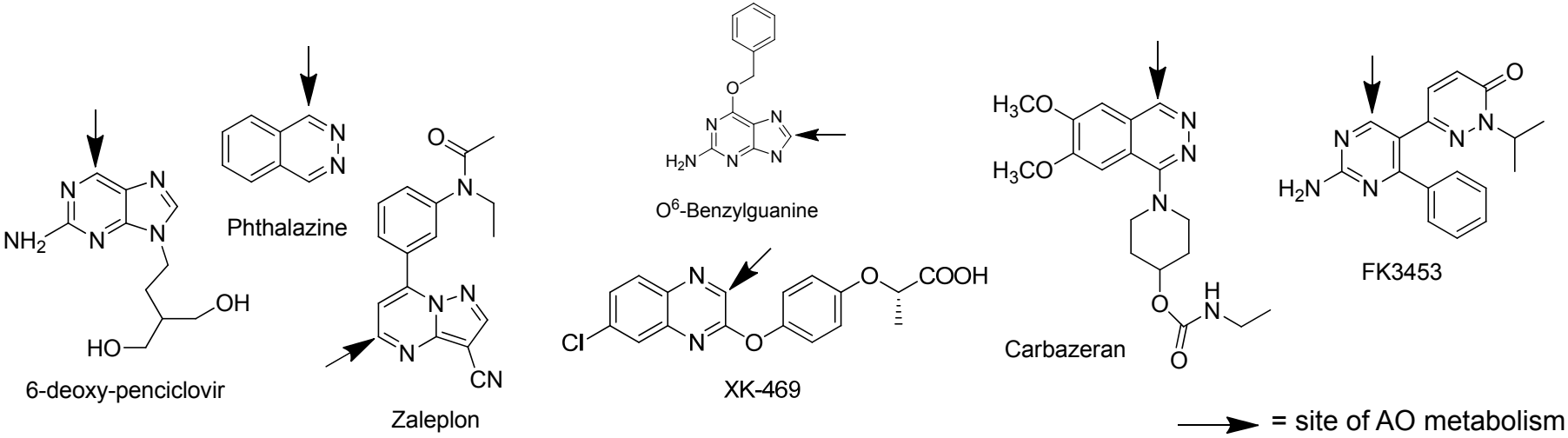
	Aldehyde Oxidase	Cytochrome P450
Sub-Cellular Location	Cytosol	Microsome (membrane-bound)
Prosthetic Group	Molybdopterin (Mo) 	Heme (Fe) 
Isoforms	One enzyme in human (AOX1) Two pseudogenes	Large super-family
Substrates	Aldehydes, Azaheterocycles (some reductive pathways)	Wide range
Mechanism	Nucleophilic	Electrophilic
Products	Oxidized product and H_2O_2	Oxidized product and water (may uncouple to form ROS)
Reducing equivalents	Produces	Consumes
Oxygen source in product	Water	Molecular O_2

Oxidation of Heterocyclic Rings

➢ As medicinal chemists introduce nitrogens into aromatic ring systems to circumvent P450-mediated metabolism or optimize pharmacological target potency (e.g. kinases), the potential for AO-mediated oxidation increases



➢ Oxidation involves cleavage of a C-H bond in azaheterocyclic compounds, typically adjacent to the nitrogen atom (electron deficient carbon)



AOX1 and Species Differences

Table 1. The table lists the complement of functionally active aldehyde oxidases expressed in the liver and other tissues of humans, chimpanzees and popular animal experimental models.

Animal species	Liver isoenzyme(s)	Other isoenzyme(s)	Pseudogenes
Human	AOX1	-	*AOX3, AOX3L1*
Chimpanzee	AOX1	-	*AOX3, AOX3L1*
Rhesus monkey	AOX1	AOX3l1	*AOX3, AOX4*
Guinea pig	AOX1	AOX4, AOX3l1	-
Dog	-	AOX4, AOX3l1	*AOX1, AOX3*
Cat	-	AOX3l1	AOX1, AOX4
Pig	-	AOX3L1	*AOX1, AOX3, AOX4*
Mouse	AOX3, AOX1	AOX4, AOX3l1	-
Rat	AOX3, AOX1	AOX4, AOX3l1	-
Rabbit	AOX3, AOX1	AOX4, AOX3l1	-

The number and type of inactive aldehyde oxidase pseudogenes are also indicated. Please note that the data in mice and rats refer to the C57BL/6J and Wistar strains, since variability of AOX isoenzyme expression has been observed in other strains, as discussed in the article.

- AO expression in animal species varies considerably, from no active gene (dog) to expression of one (human and some primates) up to 4 isoenzymes (rodents)
- Results of drug disposition studies in rat/dog species should not necessarily be extrapolated to humans
- Rhesus monkey and guinea pig considered potentially good models for human

Species	Sequence Similarity
Human	100%
Chimpanzee	99%
Rhesus Monkey	96%
Cyno Monkey	95%

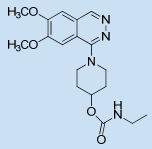
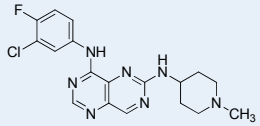
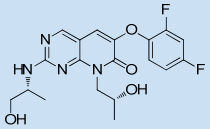
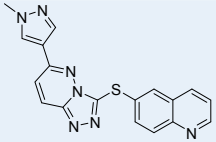
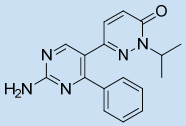
UniProtKB Website

QUINTILES

Garattini and Terao (2012) *Expert Opin Drug Metab Toxicol* 8(4); 487-503

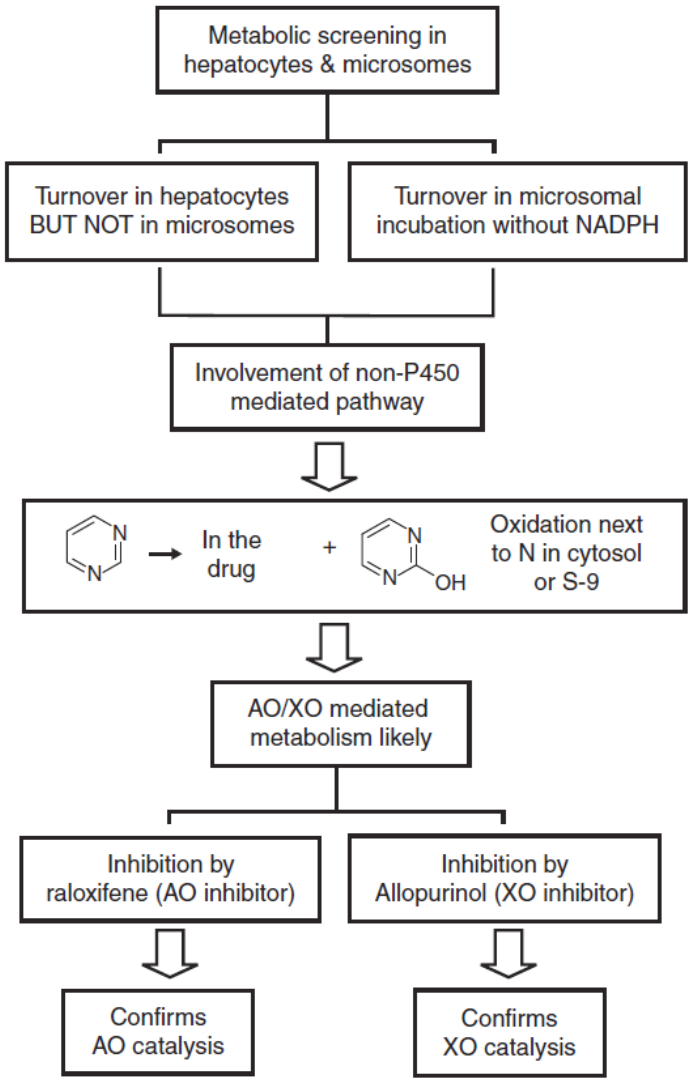
Clinical Implications of AO Metabolism

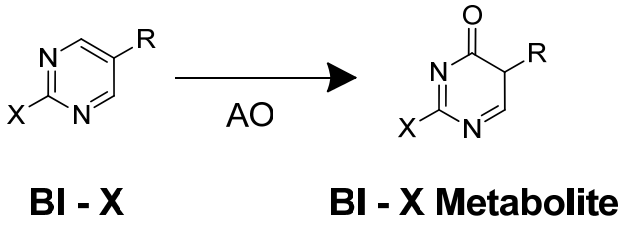
Poor Human Pharmacokinetics

Drug	Structure	Preclinical DMPK Data	Clinical Outcome	Year
Carbazeran		Dog PK (62-74% F)	<5% F	1984
BIBX1382		HLM (stable) mouse, rat and dog PK (50-100% F)	5% F	2002
RO1		Rat PK (6.2 hr half-life) Dog and Monkey PK (4 hr half-life)	0.7 hr half-life *(6 hrs projected by allometric scaling)	2010
SGX523		Tox species (rat and dog) selected based on metabolite profile in microsomes	Acute Renal Toxicity (low soluble metabolite)	2010
FK3453		HLM (stable) Rat PK (31-55% F) Dog PK (71-93% F)	Low oral exposure	2011

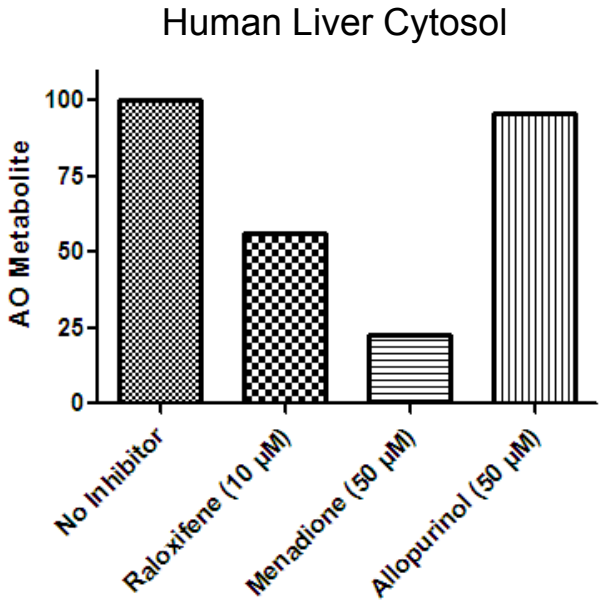
- Numerous clinical candidates have demonstrated poor pharmacokinetic properties in humans due to extensive metabolism mediated by aldehyde oxidase (AO)
- AO metabolic pathway missed…studies conducted in wrong subcellular fraction and species!
- Need to understand clearance mechanisms in human, as well as species differences

Confirming AO-Mediated Metabolism In Vitro





BI - X **BI - X Metabolite**

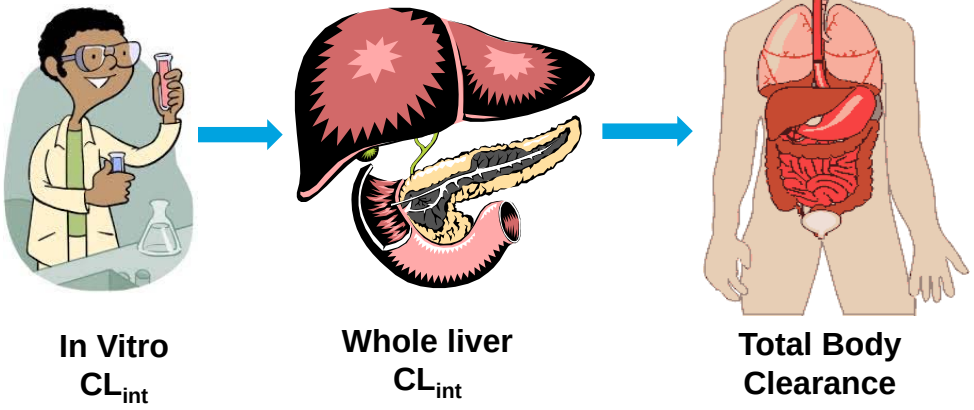


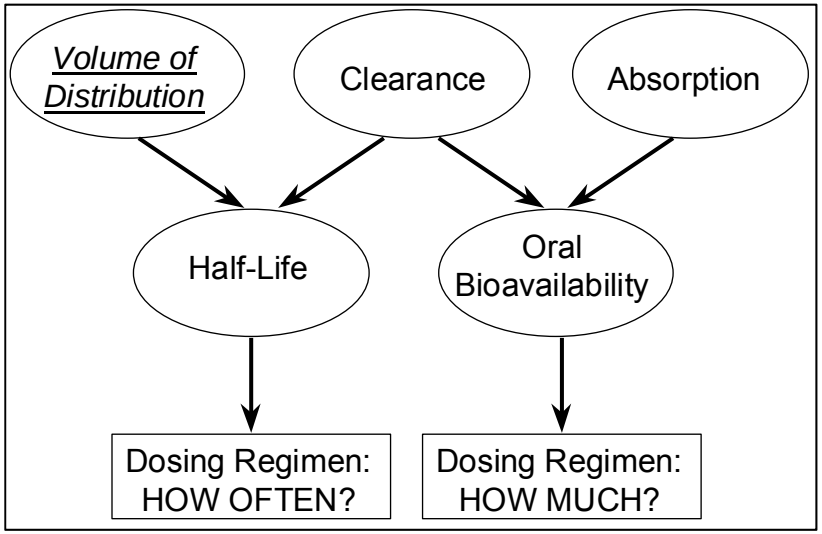
➢ Confirmation of AO-mediated metabolism by simple inhibition study in liver cytosol

QUINTILES

Hutzler et al.(2013) *Expert Opin Drug Metab Toxicol*. 9; 153-68

DMPK Strategies for Human Clearance Projection





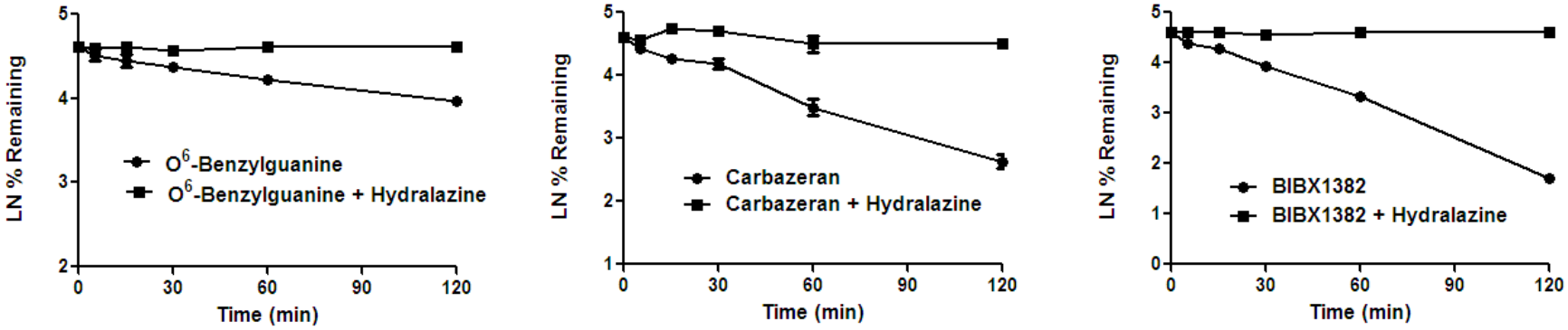
Obach RS (2001) *Curr Opin Drug Discov Devel.* 4; 36-44

- For estimating total metabolic clearance, cryopreserved hepatocytes have become a standard in vitro system, with full complement of drug-metabolizing enzymes
- Necessary for predicting clearance, and human PK/dose
- Until recently, AO activity in human hepatocytes was not tested
- Concerns exist about under-prediction of metabolic clearance

AO Activity in Pooled Cryopreserved Human Hepatocytes

Substrate	Predicted Hepatic Clearance* (mL/min/kg)	Co-Incubation w/Hydralazine (50 µM)	Human $Clearance_{total}$ (mL/min/kg)
O^6-Benzyl Guanine	12.4	<4.3	15.6
Carbazeran	17.2	<4.3	38
BIBX1382	18.2	7.4	25-55

*well-stirred model, fu=1



➢ Hydralazine (50 µM) inhibited the metabolism of O^6-BG, carbazeran, and BIBX1382 in a 20-donor pool of cryopreserved human hepatocytes

➢ AO activity confirmed in cryopreserved hepatocytes (under-predictions noted)

QUINTILES

Hutzler et al. (2012) *Drug Metab Dispos*. 32; 89-97

Expression of AOX1 in Human Tissues

Tissue	AOX1
Adrenal Gland	**1.17**
Bone Marrow	0.00366
Brain	0.00199
Colon	0.00930
Heart	0.0178
Kidney	**0.485**
Liver	**1.45**
Lung	0.0374
Pancreas	**0.269**
Peripheral Leukocytes	<0.0001
Placenta	0.0179
Prostate	0.121

Tissue	AOX1
Salivary Gland	0.00697
Skeletal Muscle	0.0349
Small Intestine	0.00486
Spinal Cord	0.00744
Spleen	0.00632
Stomach	0.0107
Testis	0.0899
Thymus	0.00455
Thyroid Gland	0.0450
Trachea	0.0342
Uterus	0.0153

Data are expressed as the ratio of the target mRNA to PPIA mRNA.

- Contribution of extra-hepatic AO to metabolic clearance needs more research
- Human adrenal homogenate and human skin has considerable AO activity

Nishimura et al. (2006) *Drug Metab Pharmacokinet* . 21(5); 357-74
Manevski et al. (2014) *Drug Metab Dispos.* Sept, epub ahead of print

Variability of AO Activity from Literature

TABLE 1

Summary of literature reports demonstrating high variability of aldehyde oxidase activity in various in vitro systems

In Vitro System	Donors	Methodology	Probe Substrate	Fold Range Activity	Reference
S-9 fraction	6	Metabolite formation	NMN	>40	Rodrigues et al., 1994
			Benzaldehyde	3.6	
			6-Methylpurine	3.6	
Cytosol	7	Depletion	Benzaldehyde	50	Sugihara et al., 1997
		Metabolite formation	NMN	4.3	
Cytosol	6	Metabolite formation	Methotrexate	48	Kitamura et al., 1999
Cytosol	13	V_{max}/K_m	DACA	18	Al-Salmy, 2001
		Depletion	Benzaldehyde	5	
Cryopreserved hepatocytes	5	Metabolite formation	Vanillin	5	Sahi et al., 2008
Cytosol	16	Metabolite formation	NMN	9.4	Tayama et al., 2012
Cytosol	20	Depletion	Carbazeran	90	Fu et al., 2013
			Zoniporide	42	
			Phthalazine	17	

DACA, *N*-[2-(dimethylamino)ethyl]acridone-4-carboxamide; NMN, N^1-methylnicotinamide.

- Wide range of reported variability for AO activity from literature that is highly dependent on substrate used
- Larger pool of donors using same probe(s) necessary to assess any links to donor characteristics

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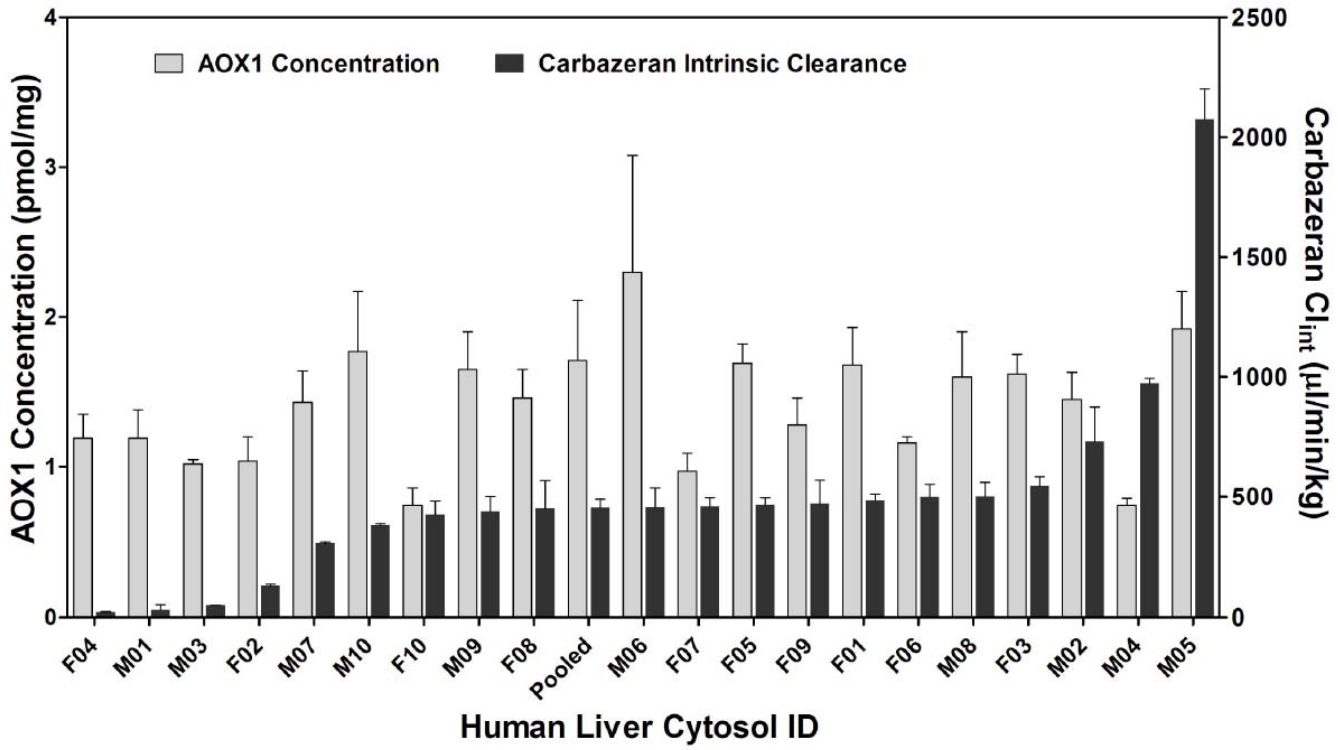
Hutzler et al. (2014) *Drug Metab Dispos. 42; 1090-1097*

Donor Variability in AO Activity

20 Donors in Liver Cytosol



- AOX1 protein similar across donors, but activity highly variable (90-fold)
- F04 and M01 donors had history of chronic alcohol consumption
- Low AOX1 enzyme activity may be due to cofactor deficiency, single nucleotide polymorphism (SNP), or homodimer dissociation

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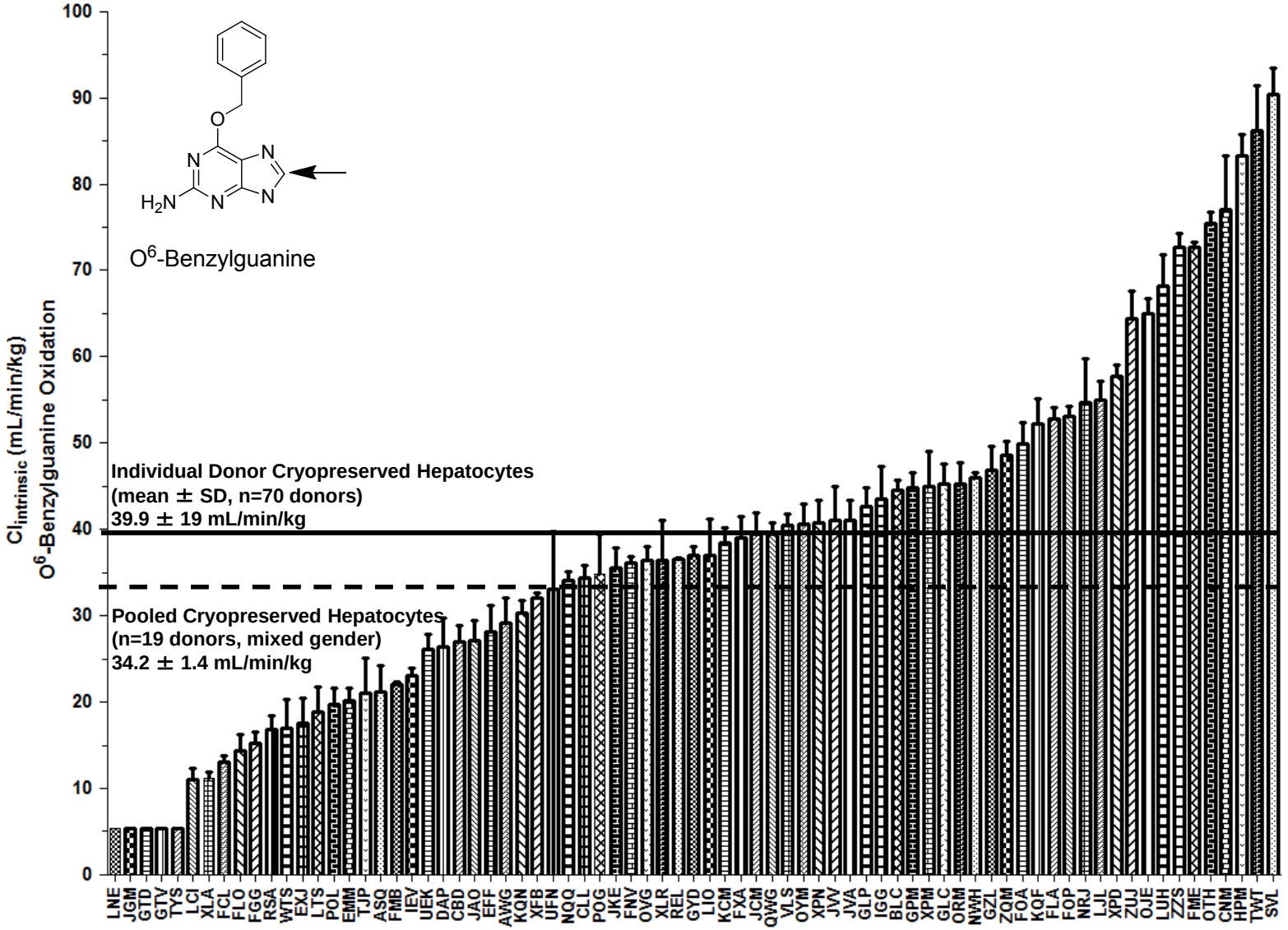
Fu et al. (2013) *Drug Metab Dispos. 41; 1797-1804*

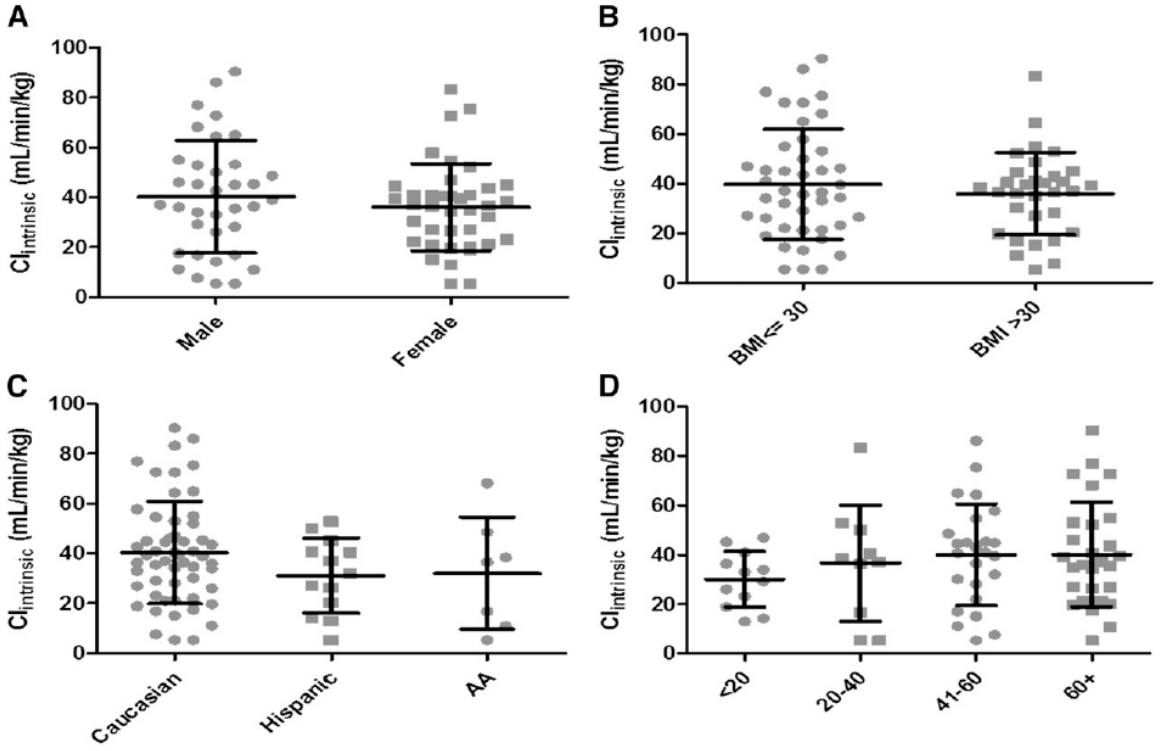
Variability in AO Activity Across 75 donors

Cryopreserved Human Hepatocytes



Hutzler et al. (2014) *Drug Metab Dispos*. 42(6), 1090-97.

Observations From 75 Donor Study



- Aldehyde oxidase activity variable, at least 17-fold (≤5.4 – 90 mL/min/kg)
- No significant correlations with donor characteristics
- 3/5 low activity donors had recent medical history of alcohol abuse
- Other possible variables: SNPs, Mo deficiency, dimerization, disease state, diet
- Are custom pools of cryopreserved hepatocytes a practical solution??

Hutzler et al. (2014) *Drug Metab Dispos*. 42(6), 1090-97.

Creation of Custom Pooled Hepatocytes

Low Cl_{int} (<30 mL/min/kg)		Moderate Cl_{int} (30-60 mL/min/kg)		High Cl_{int} (60-90 mL/min/kg)	
Donor	O^6-BG Cl_{int} (mL/min/kg)	Donor	O^6-BG Cl_{int} (mL/min/kg)	Donor	O^6-BG Cl_{int} (mL/min/kg)
EXJ	17.5	IGG	43.5	FME	72.7
GTD	<5.4	NRJ	54.7	OTH	75.5
LCI	11.0	NWH	46.0	SVL	90.4
LNE	<5.4	XPM	45.0	TWT	86.1
RSA	16.8	ZQM	48.6	ZZS	72.6
Mean	**11.2**	**Mean**	**47.6**	**Mean**	**79.5**

- Five donors were selected and pooled to make a low, moderate, and high clearance lot based on O^6-benzylguanine data

- Clearance observed in the pool should be close to the average among the donors included in pool

Study Conditions

Variable	In Vitro Condition
Thawing cells	37deg C water batch <2 min
Thawing medium	InVitroGRO HT
Centrifuge	50 x g for 5 min @ room temp
Re-suspension	InVitroGRO KHB
Cell Viability	Trypan blue (>70%)
Cell suspension stock	Make 2x stock and pre-warm @37deg C for 15 min
Substrate (1 µM final in incubation)	Make 2x stock @ 2 µM and pre-warm @37 deg C
Incubation start	Addition of 250 µL of substrate to 250 µL cell suspension
Cell density	0.5 x 10^6 cells/mL in 500 µL (250,000 cells per well)
Incubation plate	48 well polypropylene plate
% organic in incubation	0.02% DMSO and 0.5% acetonitrile
Shaking speed	200 rpm
Time Points	0, 5, 15, 30, 60, 120 min (n=4 per time point)
Quench	50 µL aliquot into 150 µL cold acetonitrile w/0.1% acetic acid
Internal standard	Tolbutamide (0.25 µM)
LC/MS/MS bioanalysis	API Sciex 4000 ESI^+ (MRM 242>199)

- In vitro incubations conducted at BioreclamationIVT and LC/MS/MS bioanalysis conducted at Quintiles Bioanalytical and ADME Labs, Indianapolis

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Creation of Custom Pools (n=5 donors/pool)

Low Cl_{int} (<30 mL/min/kg)		Moderate Cl_{int} (30-60 mL/min/kg)		High Cl_{int} (60-90 mL/min/kg)	
Donor	O^6-BG Cl_{int} (mL/min/kg)	Donor	O^6-BG Cl_{int} (mL/min/kg)	Donor	O^6-BG Cl_{int} (mL/min/kg)
EXJ	17.5	IGG	43.5	FME	72.7
GTD	<5.4	NRJ	54.7	OTH	75.5
LCI	11.0	NWH	46.0	SVL	90.4
LNE	<5.4	XPM	45.0	TWT	86.1
RSA	16.8	ZQM	48.6	ZZS	72.6
Mean	**11.2**	**Mean**	**47.6**	**Mean**	**79.5**
Pool	**6.7 ± 5.6**	**Pool**	**41.8 ± 12.9**	**Pool**	**80.1 ± 8.0**

- Intrinsic clearance observed in 5-donor pool compared favorably well with average clearance data from individual donors

- Custom pools can be prepared with donors to provide expected rates of AO-mediated metabolism

Conclusions

- Aldehyde oxidase metabolism is easy to diagnose, with in vitro conditions well characterized and reported
- Predicting clearance for AO substrates in human still problematic, likely due to extra-hepatic AOX1 expression
- Extreme donor-to-donor variability also observed, which further complicates prediction of clearance (what kind of donors make up your pool??)
- Literature accounts of under-prediction of clearance may be partly due to widely variable activity
  - Additional factors likely involved (e.g. disease, diet, etc)
  - Relevant SNPs need further characterization
- Characterization of AO activity in donor livers need to be performed routinely
- Custom pools of cryopreserved hepatocytes containing donors with high activity potentially useful for minimizing under-prediction of AO-mediated metabolic clearance

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