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Aldehyde Oxidase: Why In Vitro *Absolute Activity Doesn't Matter*

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Friday, September 27th, 2013

Presentation Overview

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- **Importance of aldehyde oxidase (AO) in drug metabolism**
- AO activity in human liver cytosol or S9 is variable between sources: Possible reasons
- The impact of low vs. high AO activity lots on *in vitro* to *in vivo* extrapolation of clearance of drugs metabolized by AO
- Calculation of $f_{m,\mathrm{CYP}}$ vs. $f_{m,\mathrm{AO}}$
- Concluding remarks

Summary of aldehyde oxidase characteristics

- A molybdenum-containing enzyme that does NOT require NADPH
- Relatively wide tissue distribution (high in liver, kidney and lung)
- Generally high but variable activity in humans
- Cytosolic localization
- Catalyzes oxidations (wide substrate selectivity):
  - Aromatic aldehydes
  - Aromatic azaheterocycles
  - Iminium ions
- Reductions (e.g., ziprasidone, some isoxazoles, nitroaromatics, *N*-oxides and sulfoxides)

Major species differences may lead to erroneous prediction of human clearance
Dog: Absent; rat: moderate, but variable; mouse, guinea pig and rhesus monkey: high
Led to withdrawal of CP-945863, carbazeran and RO1 from development

Importance of aldehyde oxidase (AO) in drug metabolism

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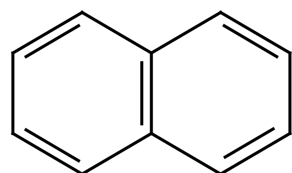
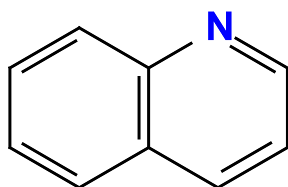
A simplistic overview of drug design for low CYP "liability"

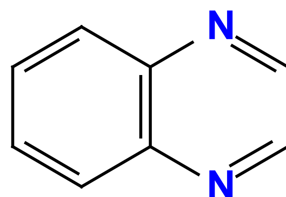
- Reduce overall logP (correlation between high logP and CYP metabolism, but NOT AO metabolism)
- Addition of fluorine (etc.) to block oxidation by CYPs
- Reduce electron density of aromatic carbons (e.g., introduce nitrogen)
  - CYPs tend to oxidize carbon atoms with high electron density
  - **BUT**: AO tends to oxidize carbon atoms with low electron density

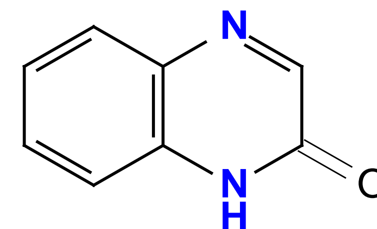


Naphthalene
CYP only



Quinoline
CYP and AO





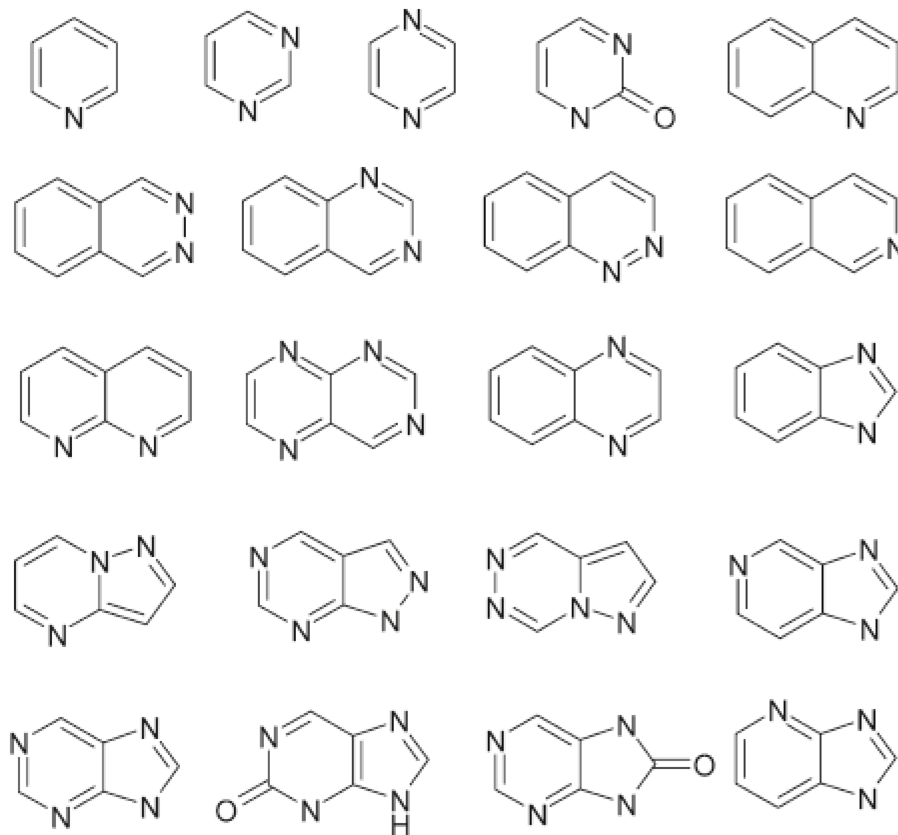
Quinoxaline
AO only

Quinoxaline-2-one

Deepak Dalvie. Gordon Research Conference, 2011

Spatial orientation in the binding pocket of AO is also important!

Model azaheterocycles that may be metabolized by AO



Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, Tran T (2010) *J Med Chem* **53**:8441

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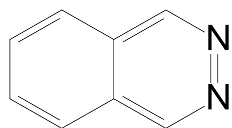
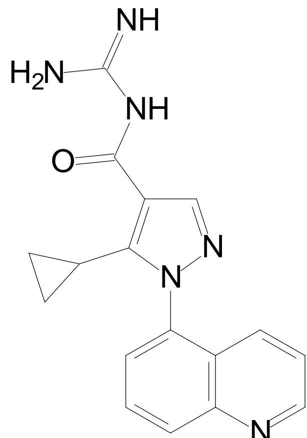
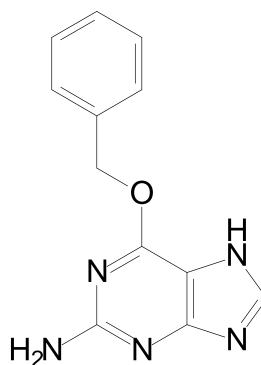
Model AO substrates used at XenoTech



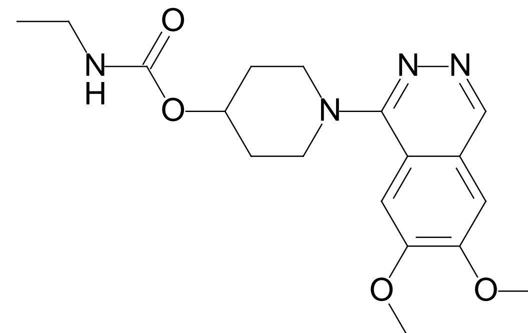
Phthalazine



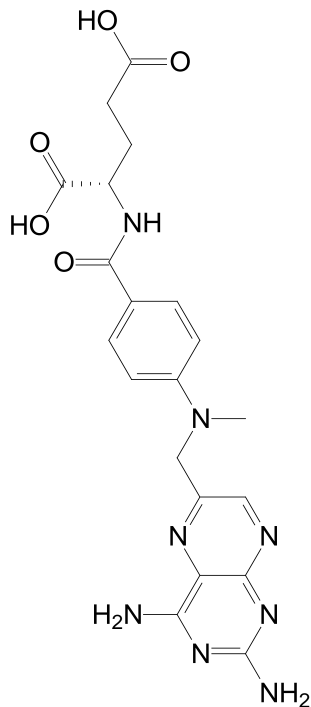
Zoniporide
Medium CL_{int}



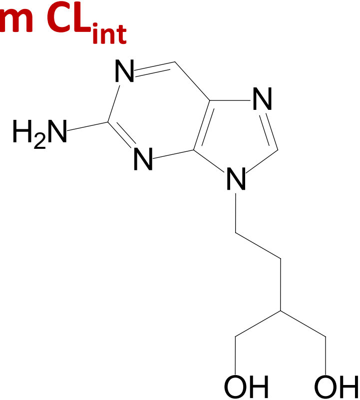
O(6)-Benzylguanine
Medium CL_{int}



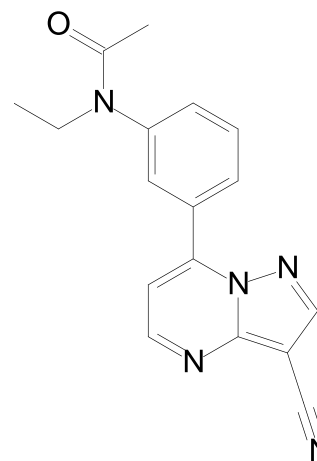
Carbazeran
High CL_{int}



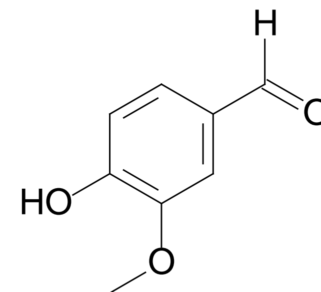
Methotrexate
Low CL_{int}



6-Deoxypenciclovir (prodrug)
Medium CL_{int}



Zaleplon
Low CL_{int}



Vanillin

Importance of aldehyde oxidase (AO) in drug metabolism: Summary

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- AO can be very important enzyme for high $f_{m,AO}$ drugs:
  - A move away from P450 metabolism can lead to more AO "liability"
  - Higher clearance in human hepatocytes vs. HLM can be a clue
  - Look for aromatic azaheterocyles and other AO structural motifs
- Dramatic species differences for some AO substrates
  - Clearance: reasonable in preclinical species; very high in humans
  - Dogs lack AO
  - No single species (even rhesus monkey or guinea pig) can predict human clearance for every AO substrate
- Highly variable activity of AO in different lots of human liver cytosol or S9
  - Scale-up from human liver cytosol generally underpredicts *in vivo* clearance by AO (average 11-fold, Zientek et al., 2010)
  - This is in contrast to the generally correct IVIVE for CYP-mediated clearance from microsomes or hepatocytes from the same lots

Zientek et al., (2010) *Drug Metab Dispos* **38**:1322

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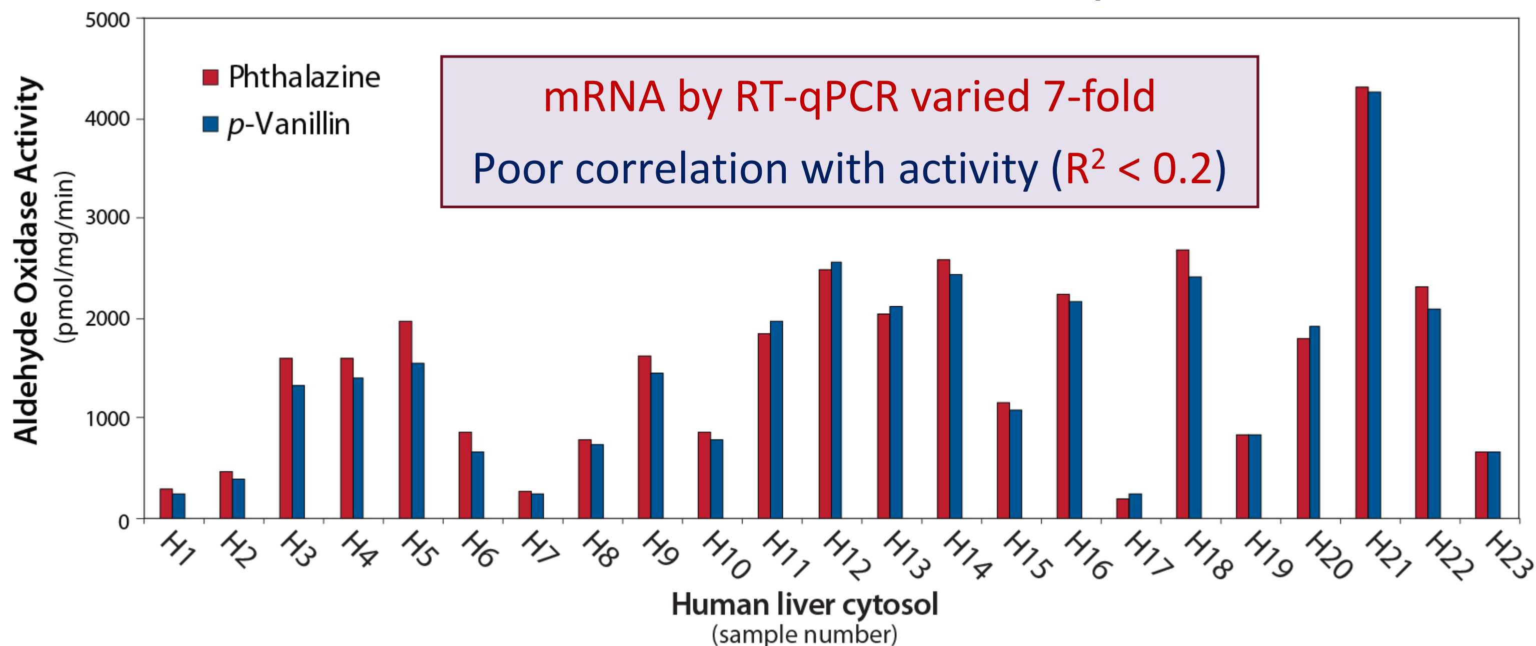
AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

- Variability in human liver cytosolic AO **activity**:
  - XenoTech ~20-fold across 24 individuals with phthalazine and vanillin

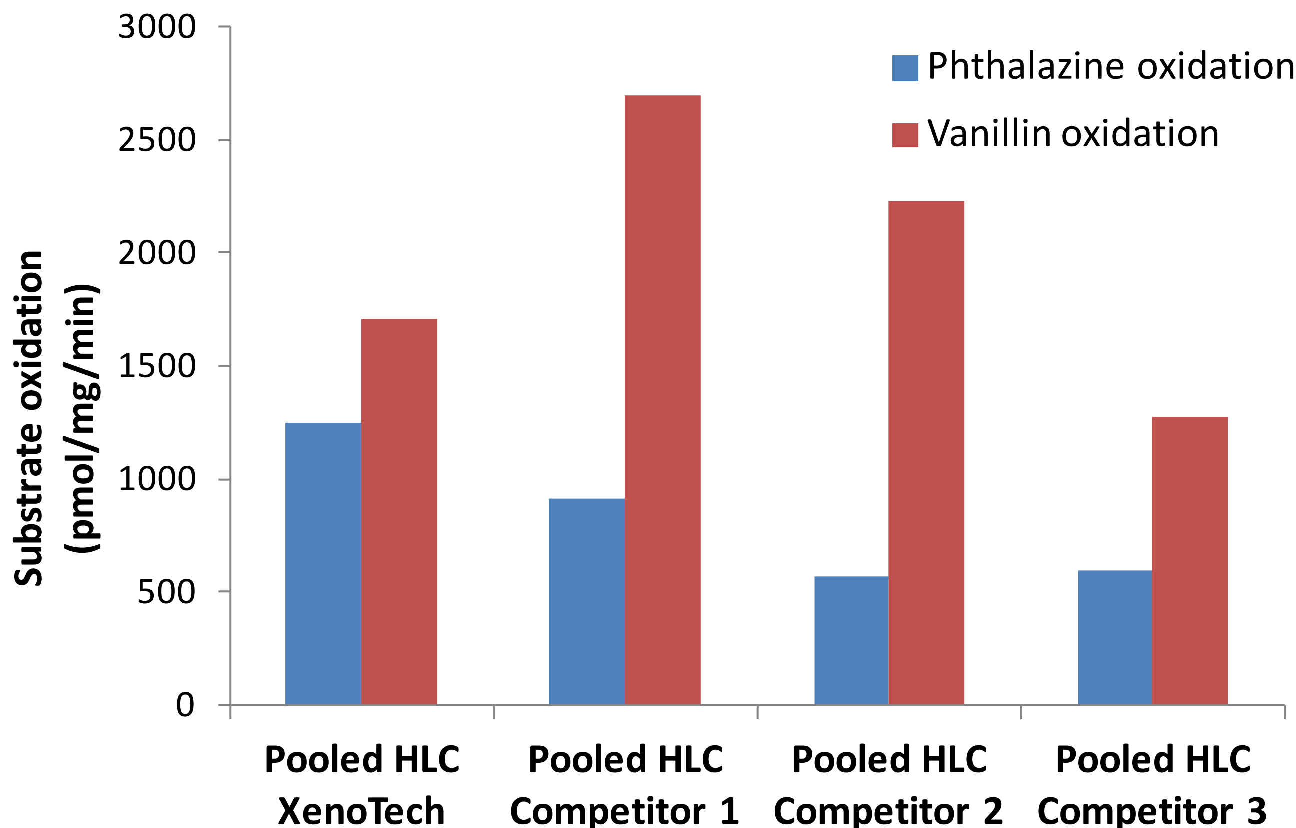


  - 19-, 43- and 90-fold variability in CL_{int} for phthalazine, zoniporide and carbazeran across 20 individuals (50:50 ♂:♀) (Fu et al., 2013)
  - 1.5-fold range in V_{max} for DACA oxidation across three lots of pooled human liver cytosol from the same vendor (Barr et al., 2013)

Yerino, Toren and Parkinson (2007) *Drug Metab Rev* **39**:
Barr JT et al. (Sept. 5, 2013) *Mol Pharmaceutics:* dx.doi.org/10.1021/mp4003046
Fu C et al. (2013) *Drug Metab Dispos* **41**:1797

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

- Variability in human liver cytosolic AO **activity**: Vendor comparison



Within the same experiment, vendor comparisons can be substrate-dependent, but all provide rates within approximately 2-fold of one another

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

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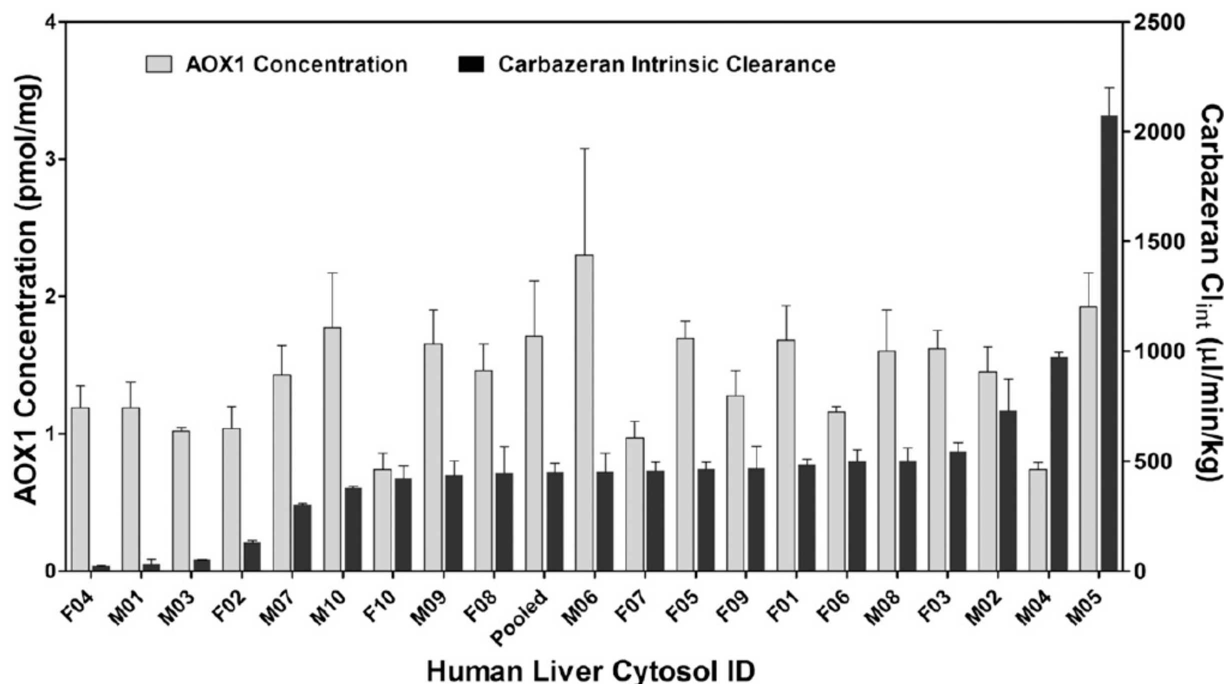
- Variability in human liver cytosolic AO **content**:
  - 3.4–fold by LC-MS/MS across 20 individuals (0.74 – 2.3 pmol/mg protein) (Fu et al., 2013)
  - 1.9-fold by LC-MS/MS across three lots of *pooled* human liver cytosol from the same vendor (21 – 40 pmol/mg protein) (Barr et al., 2013)
  - 17 to 28-fold difference in reported [AO] (/mg protein basis) between publications (Note: two different vendors compared)
  - Correlation between activity and [AO] not reported by Fu et al., but for 80% of samples, [AO] fell between ~ 1 and 1.8 pmol/mg protein (median = 1.45 pmol/mg protein)

Barr JT et al. (Sept. 5, 2013) *Mol Pharmaceutics:* dx.doi.org/10.1021/mp4003046
Fu C et al. (2013) *Drug Metab Dispos* **41**:1797

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

- Correlation between AO activity and expression is weak
  - XenoTech 2007: lack of correlation with mRNA ($r^2 < 0.2$)
  - Fu et al., 2013: little correlation between [AO] and carbazeran CL_{int}



  - Barr et al., 2013: Weak correlation between DACA oxidation V_{max} and [AO]: $r^2 = 0.48$ (for three lots of pooled human liver cytosol)

Yerino, Toren and Parkinson (2007) *Drug Metab Rev* **39**:
Barr JT et al. (Sept. 5, 2013) *Mol Pharmaceutics:* dx.doi.org/10.1021/mp4003046
Fu C et al. (2013) *Drug Metab Dispos* **41**:1797

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

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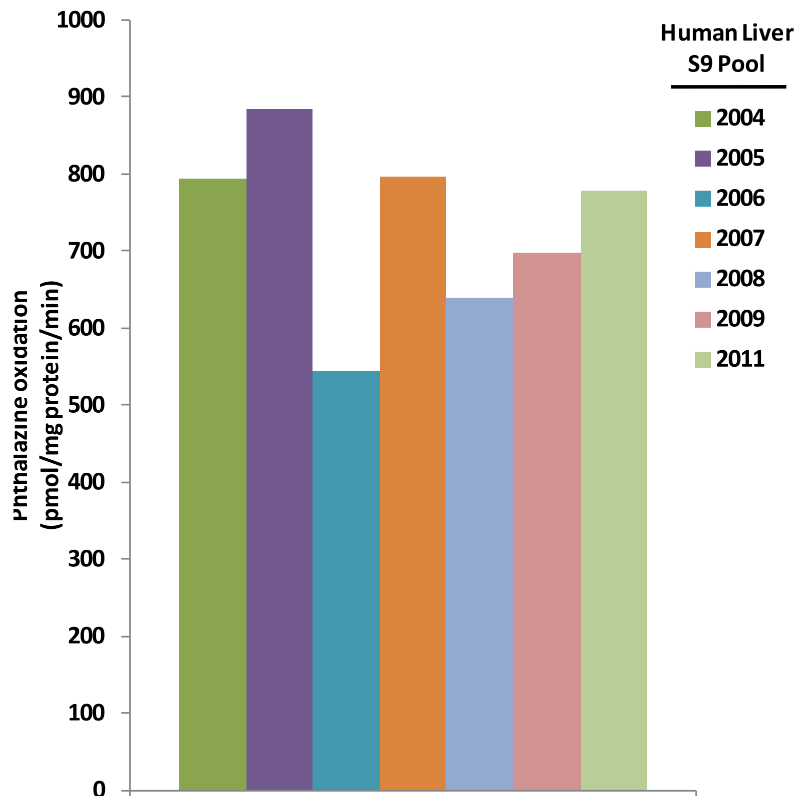
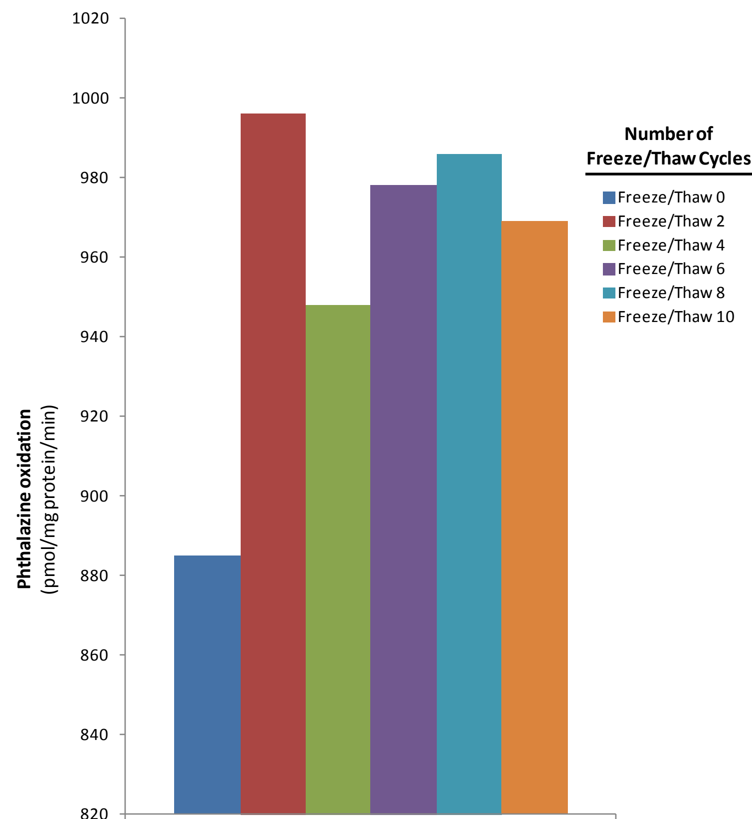
- Possible reasons for poor correlation between activity and expression
  - Individual factors: Age, disease state, smoking, alcohol use. *Not* gender (Fu et al., 2013)
  - Stability: Warm ischemia time, sample preparation, handling and storage, buffer components
  - AO properties: homodimer dissociation, protein misfolding, chemical or enzymatic modification, variable incorporation or de-sulfurization of the MoCo (note: various inactive or "less active" forms would not be differentiated by LC-MS/MS)
  - Genetic varibility: Poor or fast metabolizers (Hartmann et al., 2012)
  - Translational regulation, adiponectin levels? (Barr et al., 2013)
  - Chemical inhibition?
- Alcohol use? Fu et al.: two individual donors with lowest activity (but "normal" [AO]) were heavy, chronic alcohol users
  - Analysis of XenoTech data from 2007 revealed that 5 of the lowest activity donors (i.e., <33% of average) were alcohol users

Hartmann T, Terao M, Garattini E, Teutloff C, Alfaro JF, Jones JP, Leimkühler S (2012) *Drug Metab Dispos* **39**:856

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

- Freeze-thaw and long-term storage stability of XenoTech human liver cytosolic AO





No impact of multiple freeze-thaw cycles or long term storage

To be presented at ISSX, 2013, Toronto: Otwell et al.: poster #75

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AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

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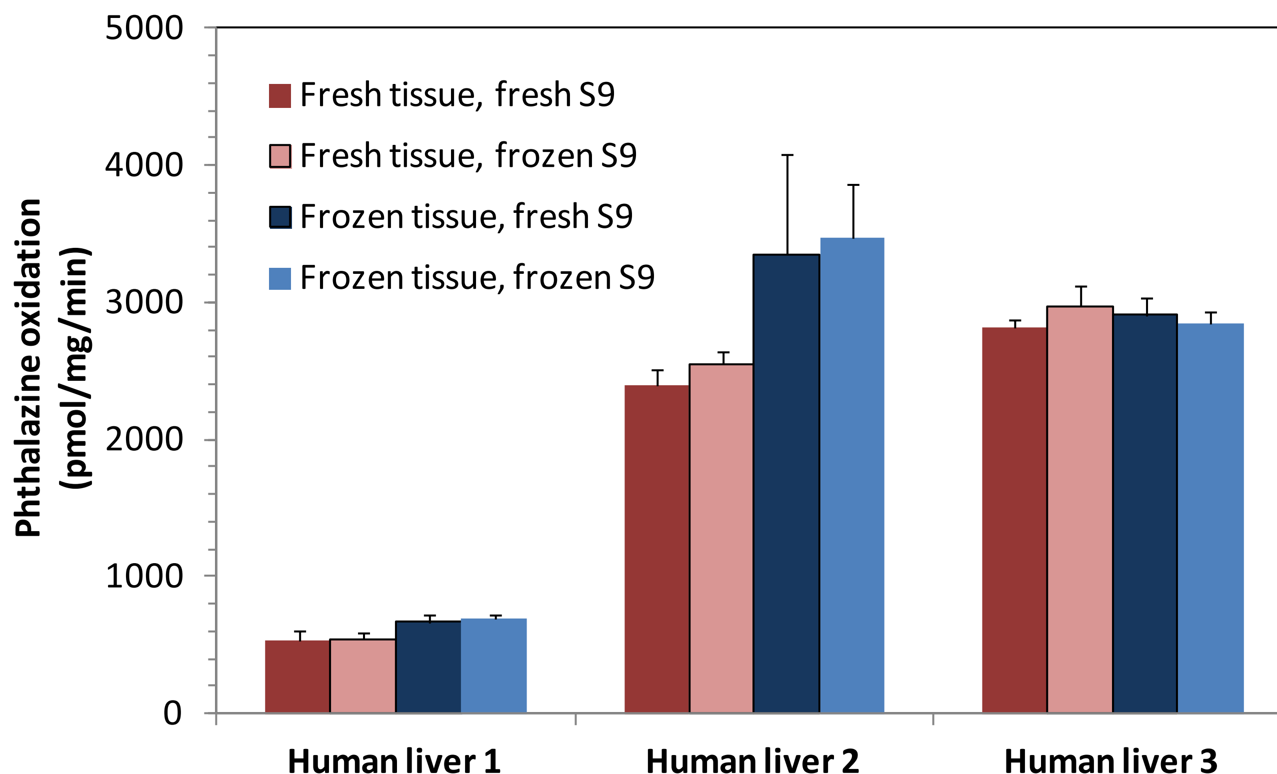
- Impact of fresh vs. frozen tissue on AO activity



Activities are within 50% of one another regardless of the use of fresh or frozen tissue, and fresh or frozen and thawed S9

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons

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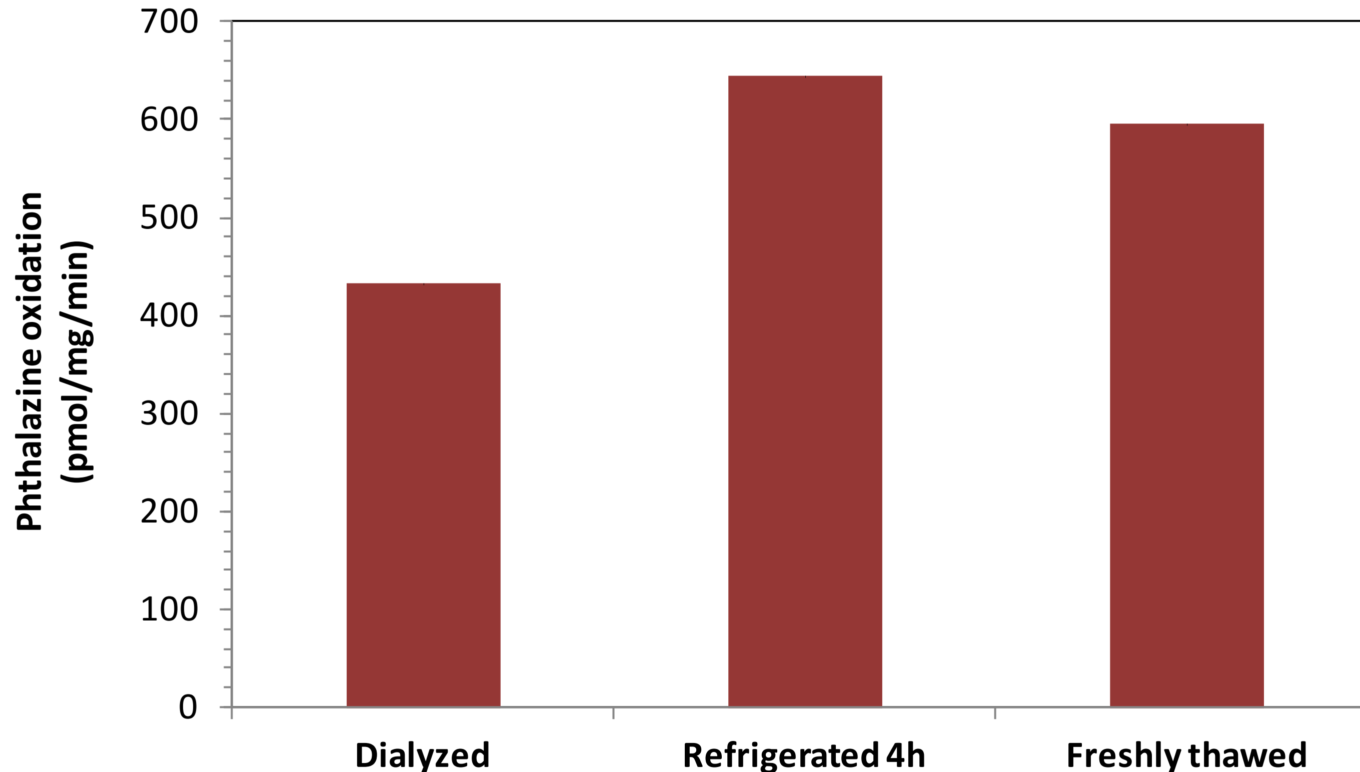
- Impact of dialysis on AO activity in human liver S9



Dialysis of S9 does not increase activity:
no apparent endogenous or contaminating inhibitor present

AO activity in human liver cytosol or S9 is variable between sources: Possible reasons: Summary

- Individual/environmental factors
- Stability
- AO properties
- Genetic variability
- Translational or other regulatory mechansims?

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The impact of low vs. high AO activity lots on in vitro *to* in vivo *extrapolation of clearance of drugs metabolized by AO*

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- Allometric scaling of in vitro CL_{int} to in vivo CL is well established for CYPs but not AO
- IVIVE is a challenge due to in vitro under prediction
  - This may be why “high” activity AO subcellular fractions are generally preferred

In Vitro-In Vivo Correlation for Intrinsic Clearance for Drugs Metabolized by Human Aldehyde Oxidase

Michael Zientek, Ying Jiang, Kuresh Youdim, and R. Scott Obach

Pharmacokinetics, Pharmacodynamics, and Drug Metabolism Pfizer Inc., La Jolla, California (M.Z., Y.J.); Pharmacokinetics, Pharmacodynamics, and Drug Metabolism Pfizer, Inc., Sandwich, United Kingdom (K.Y.); and Pharmacokinetics, Pharmacodynamics, and Drug Metabolism Pfizer, Inc., Groton, Connecticut (R.S.O.)

Received March 26, 2010; accepted May 5, 2010

First report to correlate in vitro metabolism data with in vivo PK data for AO substrates

Describe a **yard-stick or rank order** approach to characterize AO clearance

Zientek et al., (2010) *Drug Metab Dispos* **38**:1322

The impact of low vs. high AO activity lots on in vitro *to* in vivo *extrapolation of clearance of drugs metabolized by AO*

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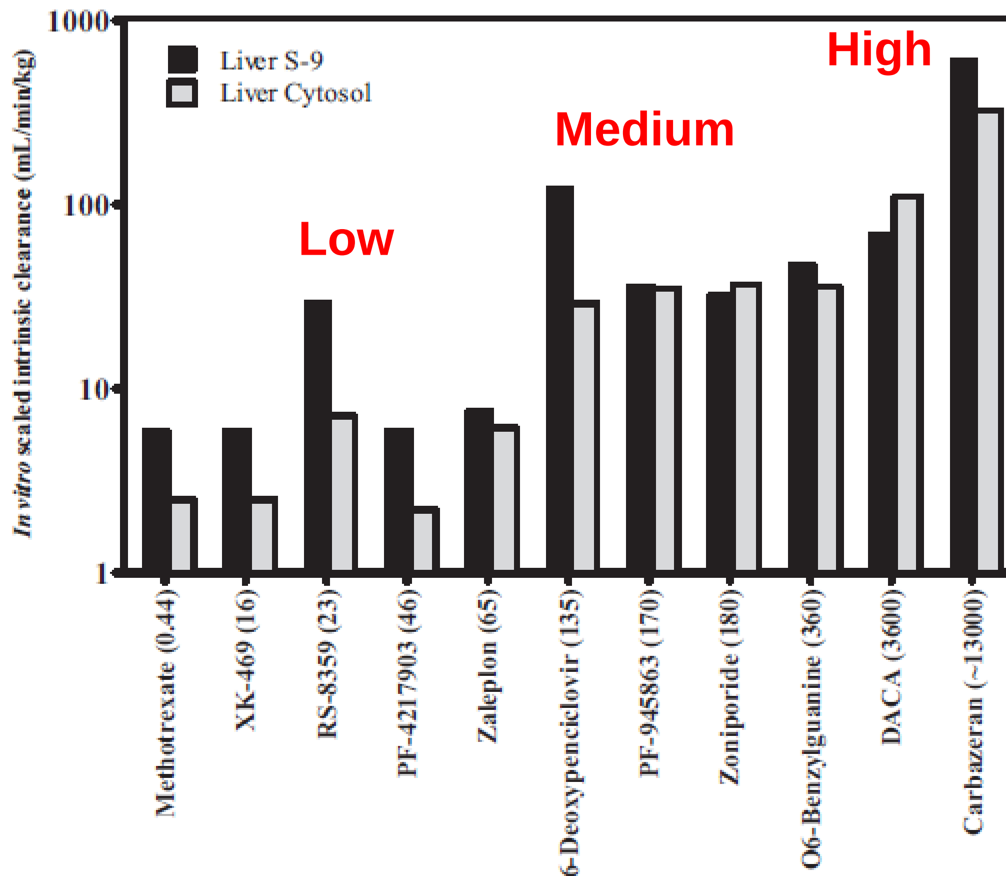
- Zientek and colleagues examined 11 drugs metabolized by AO and with in vivo pharmacokinetic data
- Determined in vitro CL_{int} with pooled human cytosol and S9 and compared to the free in vivo CL_{int}



Correlation allows for the qualitative scaling of a new drug (low, medium or high in vivo CL_{int})

Select compounds from this test set can be run as calibrators to determine the rank order with a new drug

Zientek et al., (2010) *Drug Metab Dispos* **38**:1322

Question: Is the yard-stick or rank order approach dependent on the absolute AO activity in S9 or cytosol?

XenoTech will be presenting a poster at **ISSX 2013**:

Yerino et al., Selection of human Liver S9 and cytosol fractions for evaluating clearance by aldehyde oxidase (AO): The impact of low versus high AO activity lots. **Poster 331**

- XT examined the Zientek et al. (2010) test set compounds with diverse in vitro $CL_{int,AO}$ (high, medium, low)
  - Used the rank order approach
  - Used S9 and cytosol isolated from 5 human individuals and pooled products (cytosol, n = 50; S9, n = 200) spanning a **10-fold difference** in AO activity
  - Determined half-lives of AO substrates and compared the rank order to the AO activity of each lot (determined by phthalazine)

The impact of low vs. high AO activity lots on in vitro *to* in vivo *extrapolation of clearance of drugs metabolized by AO*

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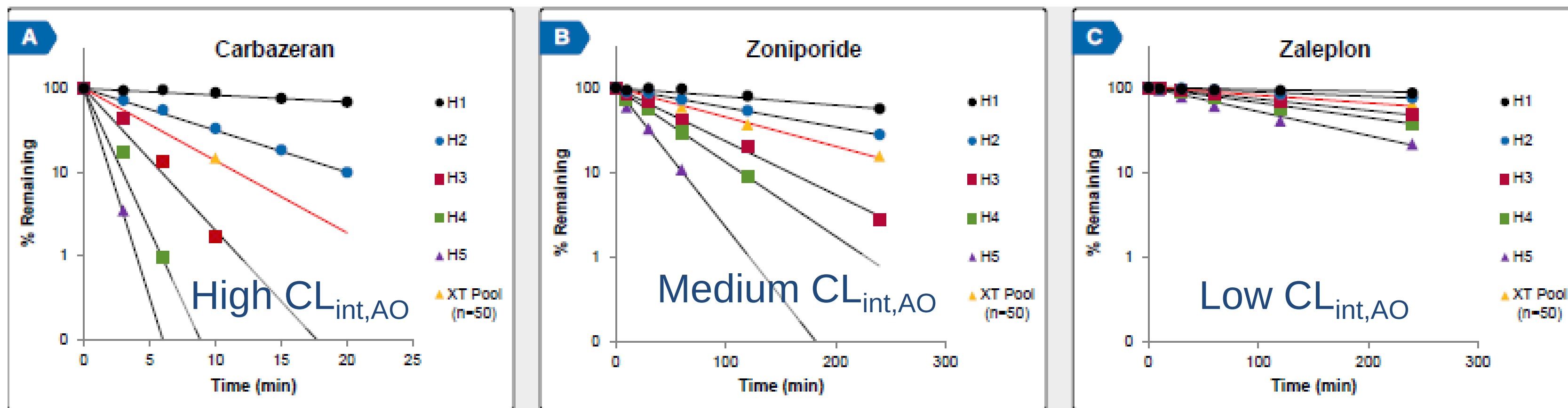
Metabolic stability (clearance) of AO substrates in human cytosol



		Half-life (minutes)					
Substrate (1 µM)	$CL_{int, AO}$ (in vitro)	H1	H2	H3	H4	H5	XT Pool (n=50)
Carbazeran	High	36	6	1.7	0.9	0.6	4
Deoxypenciclovir	Med	>240	96	25	22	12	71
Zoniporide	Med	>240	133	47	35	19	89
Benzylguanine	Med	>240	144	56	38	21	113
Zaleplon	Low	>240	>240	218	163	108	>240
Methotrexate	Low	>240	>240	>240	>240	>240	>240
Phthalazine		382	711	1524	2417	3148	1286

Threshold value

Pooled cytosol reflected median activity

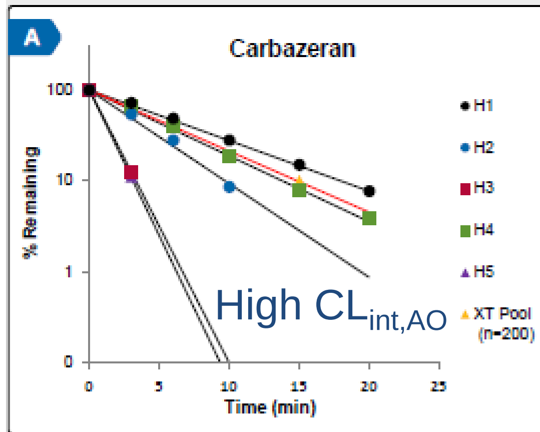
Rank order approach worked in every case except lowest activity individual (H1)

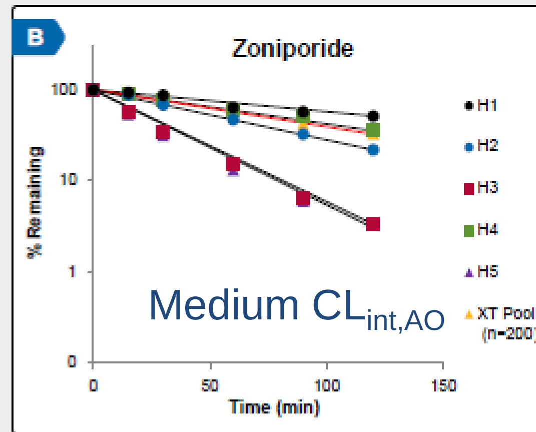
Suggests that for cytosol a threshold value of AO activity may need to be established to utilize the rank order approach

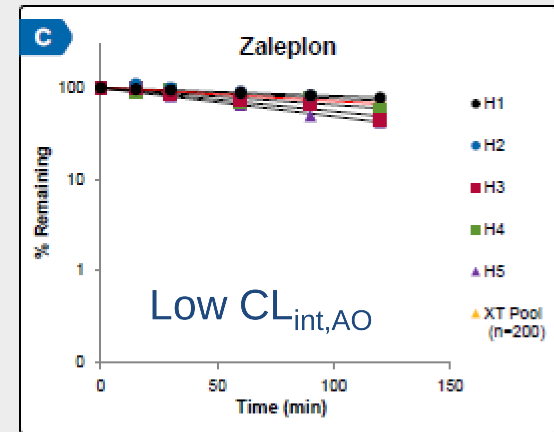
To be presented at ISSX, 2013, Toronto: Yerino et al.: poster #331

The impact of low vs. high AO activity lots on in vitro *to* in vivo *extraplation of clearance of drugs metabolized by AO*

Metabolic stability (clearance) of AO substrates in human S9







		Half-life (minutes)					
Substrate (1 µM)	**$CL_{int, AO}$ (in vitro)**	**H1**	**H2**	**H3**	**H4**	**H5**	**XT Pool (n=200)**
Carbazeran	High	6	3	1	4	1	5
Deoxypenciclovir	Med	72	38	15	63	12	57
Zoniporide	Med	104	54	25	84	21	74
Benzylguanine	Med	101	58	25	132	22	86
Zaleplon	Low	>120	>120	105	>120	86	>120
Methotrexate	Low	>240	>240	>240	>240	>240	>240
Phthalazine		256	554	1080	1266	1946	609

Rank order approach worked in every case (including lowest activity individual)

Suggests that AO activity in H1 is above the threshold necessary for rank order

Above threshold value

Pooled S9 reflected median activity

To be presented at ISSX, 2013, Toronto: Yerino et al.: poster #331

Question: Is the yard-stick or rank order approach dependent on the absolute AO activity in S9 or cytosol?

Answer: The rank order (yard-stick) approach is **not dependent** on the absolute AO activity, with the exception of values below a defined threshold of AO activity

- To determine threshold activity, we recommend **characterization** of S9 and cytosol with the AO test set of compounds to determine if a particular S9 or cytosol product is appropriate for the rank order approach
- XT has done this for our **pooled S9 (n = 200)** and **pooled cytosol (n = 50)**
- Pooled products by their nature will have AO activity **above the threshold** values (if n is high enough)

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Calculation of fm_{CYP} *vs.* fm_{AO}

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How do I know if my drug is metabolized by AO?

- Traditionally for CYPs, the use of chemical inhibitors is used with microsomes, S9 or hepatocytes to determine fm_{CYP}
  - In many cases, specific isoform inhibitors are used to determine specific CYP contribution (e.g. fm_{CYP3A4})
  - A general CYP inhibitor may also be used to determine total fm_{CYP} (e.g. 1-ABT)
- A similar approach may be used to determine fm_{AO} by using a specific AO inhibitor

Hydralazine As a Selective Probe Inactivator of Aldehyde Oxidase in Human Hepatocytes: Estimation of the Contribution of Aldehyde Oxidase to Metabolic Clearance

Timothy J. Strelevitz, Christine C. Orozco, and R. Scott Obach

Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Groton, Connecticut

Received February 22, 2012; accepted April 20, 2012

Hydralazine may be a good candidate to determine fm_{AO}

Strelevitz et al., (2012) *Drug Metab Dispos* **40:**1441-1448

Calculation of fm_{CYP} *vs.* fm_{AO}

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TABLE 3

Metabolic pathway, apparent intrinsic clearance, and $f_{m(AO)}$ *identified for 10 selected compounds and compared in two human hepatocyte batches*

Metabolic pathways were confirmed using biotransformation. Human hepatocytes were suspended at 1.5 million cells/ml. Termination time points = 0, 5, 15, 30, 60, 120, and 240 min; *n* = 3/time point. $Cl_{int, app}$ values were calculated from averaged $AUC_{0-\infty}$ extrapolated data. Batch 1: pooled lots AGR, FKM, EHI, TDH, and ZFB; batch 2: lot RTH.

Drug	Metabolic Pathways	$Cl_{int, app}$						$f_{m(AO)}$	
		Batch 1			Batch 2				
		No Hydralazine	25 μM Hydralazine	50 μM Hydralazine	No Hydralazine	25 μM Hydralazine	50 μM Hydralazine	Batch 1[a]	Batch 2[b]
		μl · min⁻¹ · 10⁶ cells⁻¹							
O^6-Benzylguanine	AO	23.8	N.C.	N.C.	21.0	7.50	3.65	N.C.	0.83
PF-0945863	AO, N-demethylation	23.9	N.C.	N.C.	24.8	11.5	9.61	N.C.	0.61
Zaleplon	AO, N-deethylation	11.5	3.47	3.08	10.9	6.95	4.82	0.70	0.56
Zoniporide	AO, hydrolysis	22.0	7.97	5.08	18.3	12.9	8.16	0.64	0.55
DACA	AO, N-demethylation	55.5	25.9	21.2	51.7	22.6	12.6	0.53	0.76
Carbazeran	AO, glucuronidation	73.9	37.8	27.2	67.2	37.1	30.1	0.49	0.55
Propranolol	Hydroxylation, glucuronidation	305	198	179	294	245	236	0.35	0.20
Midazolam	Hydroxylation	34.3	31.7	32.5	43.5	45.9	41.3	0.08	0.05
Naloxone	Hydroxylation, glucuronidation	56.9	52.9	54.5	48.3	57.4	56.9	0.07	N.D.
Dextromethorphan	N-Demethylation[c]	—	—	—	30.9	29.0	18.7	—	0.39

N.C., $Cl_{int, app}$ in the presence of hydralazine could not be calculated because the slope of the ln[*C*] versus time curve was not statistically different from zero, which precluded a reliable measurement; N.D., not determined; —, compound not run in assay.

[a] $f_{m(AO)}$ was determined using 25 μM hydralazine data.

[b] $f_{m(AO)}$ was determined using 50 μM hydralazine data.

[c] Data from Gorski et al. (1994).

The contribution of CYP vs. AO metabolism can be identified with chemical inhibitors (1-ABT and hydralazine)

Strelevitz et al., (2012) *Drug Metab Dispos* **40:**1441-1448

Calculation of fm_{CYP} vs. fm_{AO}

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Fractional metabolism CYP vs. AO characterized with 1-ABT (1 mM) and hydralazine (25 µM) in pooled hepatocytes

Substrate	Metabolic pathway	CL_{int} (µL/10^6 cells/min) Control	25 µM Hydralazine	1 mM ABT	fm AO (XT hepatosure)
Midazolam, 1 µM	CYP, UGT	15.38	15.35	2.67	0.00
Dextromethorphan, 1 µM	CYP	16.18	0.92	0.08	0.94
Naloxone, 1 µM	UGT, CYP	39.35	39.18	31.34	0.00
Propranolol, 0.1 µM	CYP	15.53	6.32	2.95	0.59
Carbazeran, 1 µM	AO > UGT	36.49	14.35	34.68	0.61
Benzylguanine, 1 µM	AO	7.53	2.79	10.29	0.63
Zaleplon, 1 µM	AO > CYP	2.20	0.23	1.78	0.89
Zoniporide, 1 µM	AO > hydrolysis	6.65	0.66	6.76	0.90

Confirmed that the fm_{AO} can be determined by chemical inhibition with hydralazine

Apparent contribution of AO for CYP2D6 substrates – **Why?**

Calculation of fm_{CYP} *vs.* fm_{AO}

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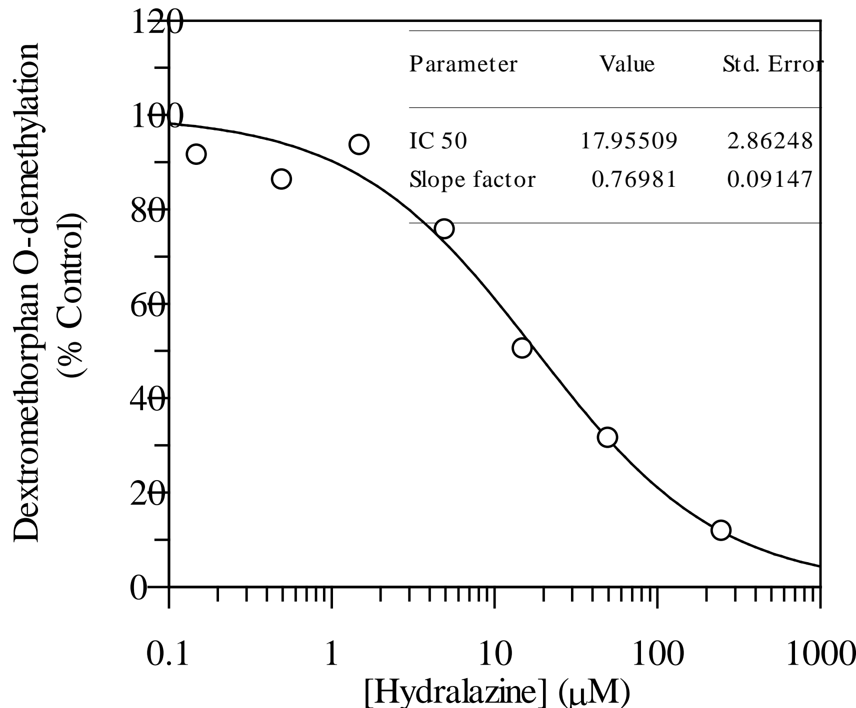
Fractional metabolism CYP vs. AO: Hydralazine is a CYP2D6 inhibitor in hepatocytes

	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
IC50 (µM)	**>250**	**215**	**>250**	**>250**	**>250**	**18**	**235**



Hydralazine may be useful to identify drugs as AO substrates that ***are not substrates of CYP2D6***

For substrates of CYP2D6, additional controls may be necessary (such as a specific CYP2D6 inhibitor or substrate)

Chemical inhibition approach with hydralazine can also be used with **S9**

Presentation Overview

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- Importance of aldehyde oxidase (AO) in drug metabolism
- AO activity in human liver cytosol or S9 is variable between sources: Possible reasons
- The impact of low vs. high AO activity lots on *in vitro* to *in vivo* extrapolation of clearance of drugs metabolized by AO
- Calculation of fm_{CYP} vs. fm_{AO}
- **Concluding remarks**

Concluding remarks

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- A move away from P450 can lead to more AO “liability”
- Variability in AO activity and content in human liver cytosols or S9 is high. Content does not correlate with activity
- Sample preparation and handling do not appear to explain variability
- The rank order approach to qualitatively benchmark in vivo $CL_{int,AO}$ is not dependent on the absolute AO activity of cytosol or S9 once threshold values have been established
- Characterization of S9 or cytosol using the rank order AO test set to determine if product meets threshold criteria
  - XT cytosol and S9 pooled products exceed threshold criteria
- Inhibition of AO by hydralazine may be used to determine fm_{AO} but only for drugs that are not substrates for CYP2D6

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