Biotransformation of Drug Candidates by Non-CYP Metabolic Pathways

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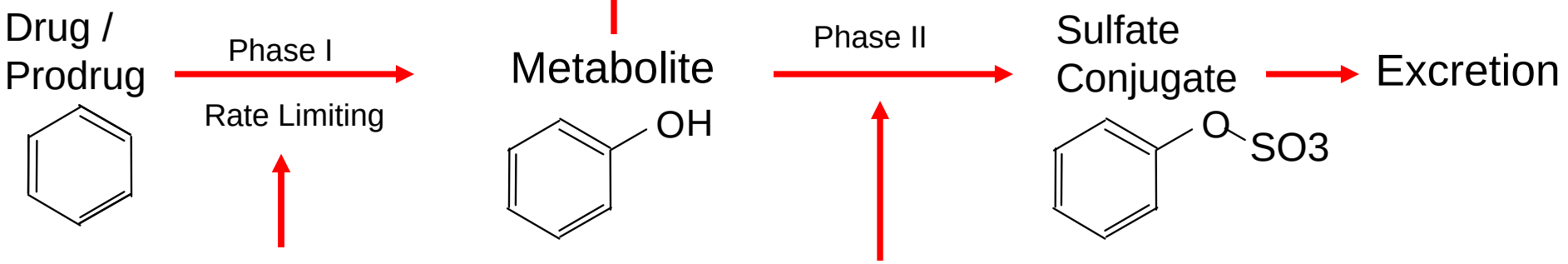
Biotransformation in Drug Metabolism

- Drug metabolism
  - Main function is to facilitate removal of compounds from the organism, thereby preventing unwanted accumulation of foreign compounds orendogenous compounds to reach potentially toxic levels
- Functional group biotransformations:
  - Phase I metabolism reaction: functionalization
    - Oxidations: Introduce a new polar functional group to the parent drug
    - Reductions: modify an existing functional group to be more polar
    - Hydrolyses: Unmask existing polar functional group
    - Most common functional groups: -OH, -NH-, -COOH.
  - Phase II metabolism reaction: conjugation
    - Glucuronidation, sulfonation

Phase I and Phase II Enzymes

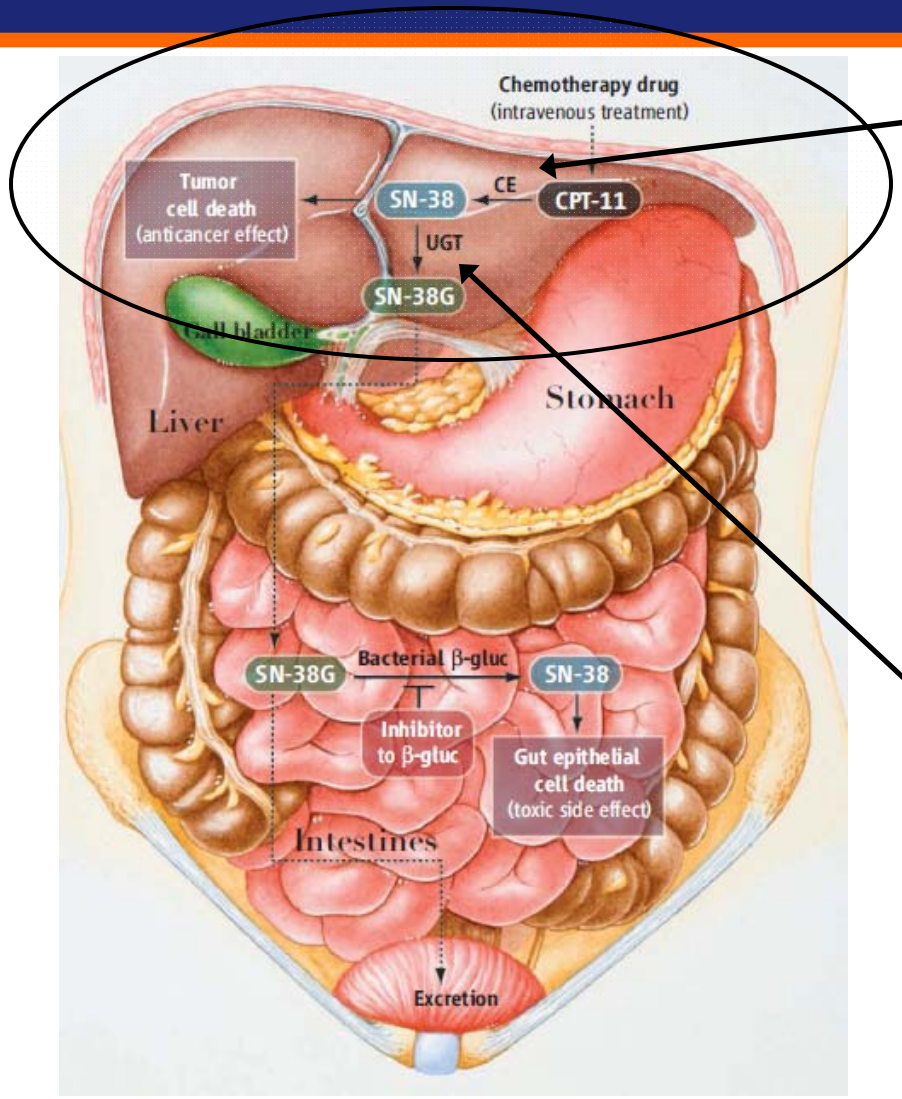
- Can be active or inactive at target site
- Toxic
- Mutagen or Carcinogen



- Expose functional group that can be conjugated
- Small increase in hydrophilicity
- Include: CYPs, AO, CESs,

- Large increase in hydrophilicity
- Conjugates are generally inactive
- Include: UGTs, SULTs

Case Study (CPT-11): Non-CYP pathways



- CES converts prodrug (CPT-11) to active drug (SN-38)
- SN-38 is active against many cancers but has a dose-limiting toxicity effect
- UGTs convert SN-38 to SN-38 Glucuronide, an inactive compound that is excreted into the GI tract

Patel et al., Science **330**, 766 (2010)

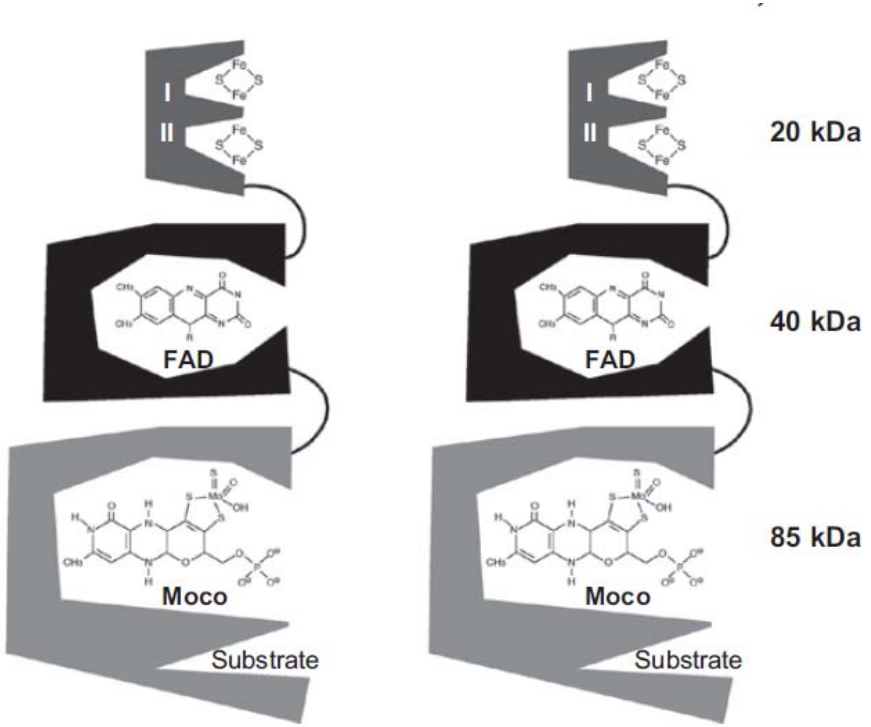
Webinar Agenda

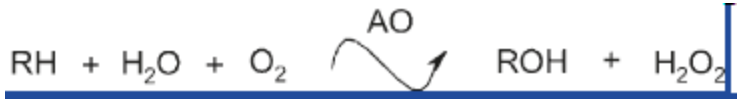
- Aldehyde Oxidase
  - Background
  - Native AO in Human Liver Cytosol, and Human Hepatocytes
- Carboxylesterase
- UDP-Glucuronosyltransferase

Aldehyde Oxidase Background

- Aldehyde Oxidase (AO) is a molybdo-flavoenzyme present in the cytosolic compartment of many tissues in various animal species, including humans.

- AO Oxidize a wide range of aldehydes and heterocyclic compounds.
  - Increasing importance in drug metabolism, primarily due to the recent growth in the # of aromatic aza-heterocycle moieties that are found in many drug leads.





BD

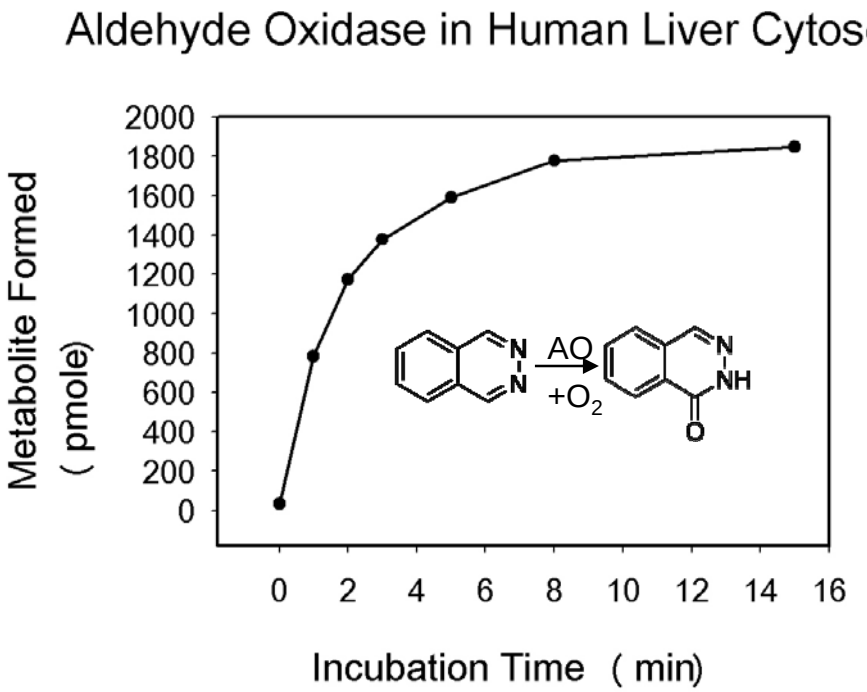
E. Garattini and M. Terao, *Drug Metab. Rev*., 2011, **43**, 374-86; D. C. Pryde, *et al*, *J. Med. Chem*, 2010, **53**, 8441-60.

The Importance of AO Metabolism

- AO: present in the cytosolic fractions, thus, standard metabolic stability studies using Human Liver Microsomes do not capture AO-mediated metabolism.
  - That the AO pathway has been overlooked leads to clinical failures, either due to toxicological outcomes (Diamond et al., 2010), or to higher-than-predicted clearance in human, yielding unacceptable pharmacokinetic properties (e.g. FK3453 from Astellas Pharma).
  - David Pryde et al (2010) in Pfizer has proposed Decision Tree to guide decision-making during the screening of potential AO substrates.
- There is limited information about the ability to scale *in vitro* clearance for compounds that are substrate of AO for accurate human predictions.
  - First paper to correlate in vitro metabolism data with *in vivo* pharmacokinetic data using liver S9/cytosol (Zientek et al, 2010), no explanation for the underestimation of the *in vitro*-scaled intrinsic clearance comparing to the *in vivo* values.
  - The liability of AO causes concern. A stable and reproducible source of AO is needed.

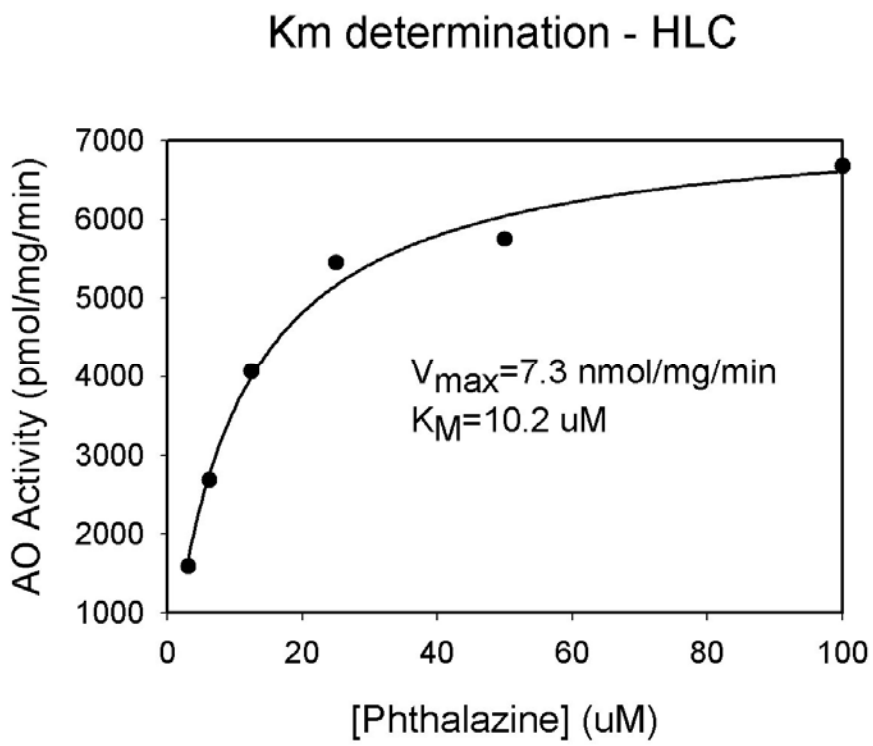
N. Gerst et al, Astellas, **P269**, 2011 ISSX poster; J. M. Hutzler et al, Boehringer Ingelheim, **P264**, 2011 ISSX poster; D. C. Pryde, *et al*, *J. Med. Chem*, 2010, **53**, 8441-60; Diamond et al, *DMD*, 2010, **38**, 1277-85; Zientek et al, *DMD*, 2010, **38**, 1322-27.

AO Activity in Human Liver Cytosol (HLC)



- Phthalazine is probe substrate of AO
- Conclusion:
  - AO activity in HLC is approximately linear to 2 min
  - AO activity in HLC is 5.9 nmol/mg/min (2 min incubation).
- Assay Conditions:
  - 0.5 mg/ml BD Gentest™ Human Liver Cytosol 150 donor pool (452115) --- in linear range
  - Substrate: 100 µM Phthalazine
  - Metabolite: Phthalazone

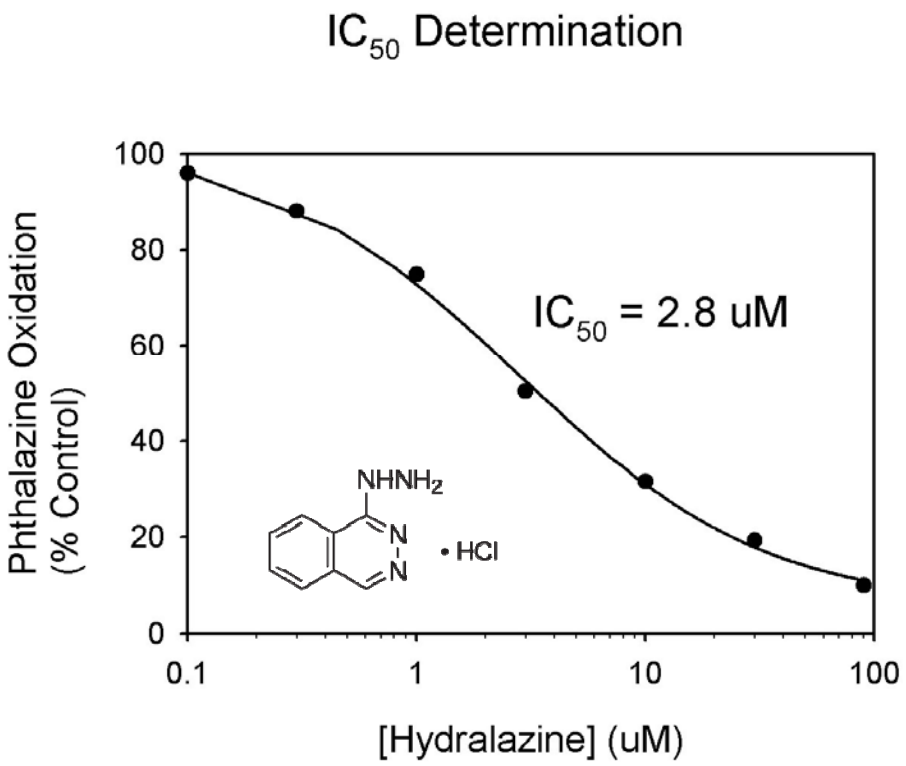
Km – Phthalazine - HLC



- Conclusion: The Km for probe substrate Phthalazine is 10.2 µM, matches publication: Km=8.0 µM by Jeff Jones, 2011 DMD
- Assay Conditions:
  - 0.5 mg/ml BD Gentest™ Human Liver Cytosol 150 donor pool (452115)
  - Incubation Time: 2 min

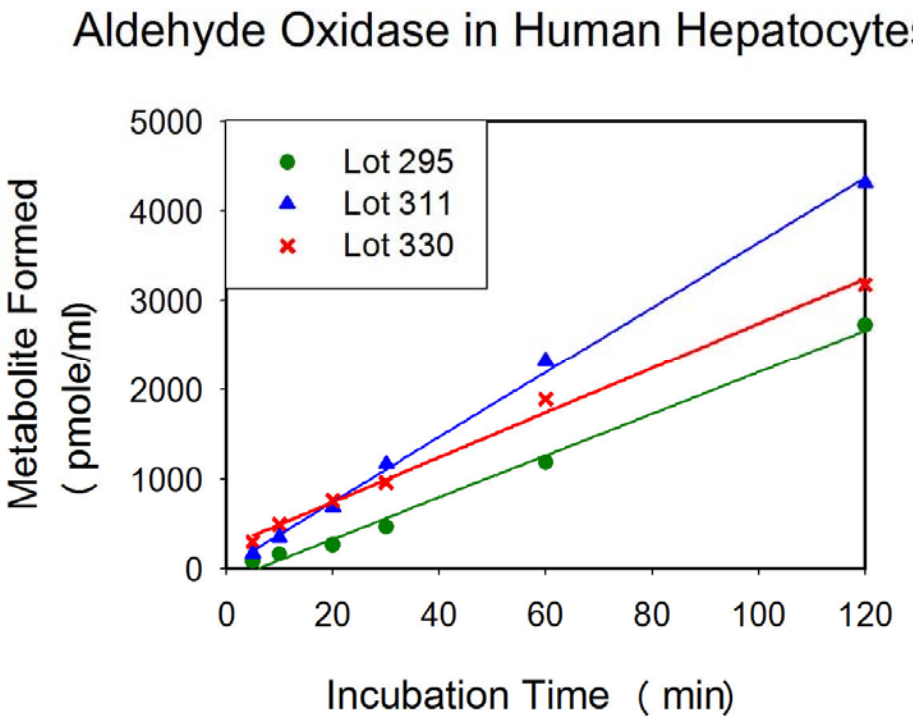
John T. Barr and Jeff P. Jones, *DMD*, **39**, 2381-6

IC_{50} - Hydralazine - HLC



- Hydralazine is a probe inhibitor for AO
- Conclusion:
  - IC_{50} for probe inhibitor Hydralazine is 2.8 µM
  - At [Hydralazine]=100 µM, 90% of AO activity was inhibited.
- Assay Conditions:
  - [Phthalazine] = 10 µM
  - 0.1 mg/ml BD Gentest™ Human Liver Cytosol 150 donor pool (452115)
  - Incubation Time: 2 min

AO Stability in hepatocytes



- AO Activity is linear up to 2 hours in cryopreserved hepatocytes (much more stable than HLC)
- Assay condition:
  - 250K cells per well in 48 well plate
  - [Phthalazine]=100 uM

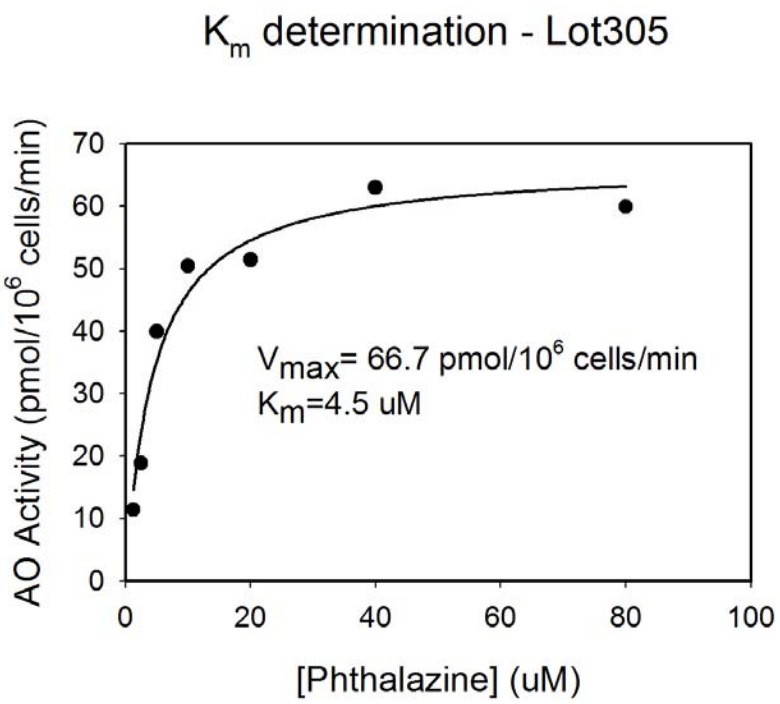
AO Activity in hepatocytes

Cryo Hepatocytes	Catalog #	AO Activity pmol/(min* 10^6 cells)
Lot 251	454504 (Susp)	7.5
Lot 295	454551 (Ind)	45.4
Lot 301	454427 (Tra)	16.2
Lot 305	454427 (Tra)	61.2
Lot 311	454551 (Ind)	71.8
Lot 330	454551 (Ind)	52.9

- AO activity ranges from 7.5 to 71.8 pmol/(min*10^6 cells), with an average of 43 pmol/min/10^6 cells.
- This could be due to polymorphisms of hAO in population
  - Recent publication from Leimkuhler lab showed single NT polymorphisms changed the wild-type AO activity with Phthalazine to ~35% (R921H) or 1.4-fold (N1135S)

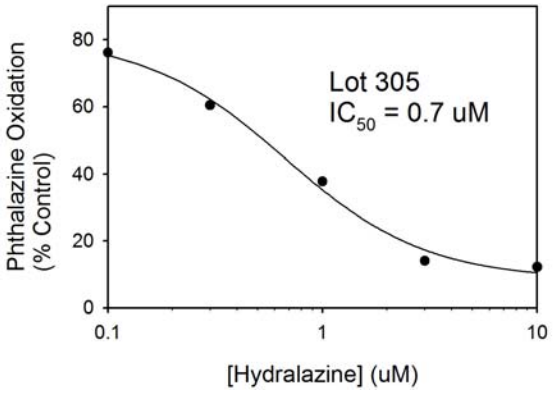
T. Hartmann, et al and S. Leimkuhler, "The Impact of Single Nucleotide Polymorphisms on Human Aldehyde Oxidase", 2012, *DMD*, **40**, 856-64.

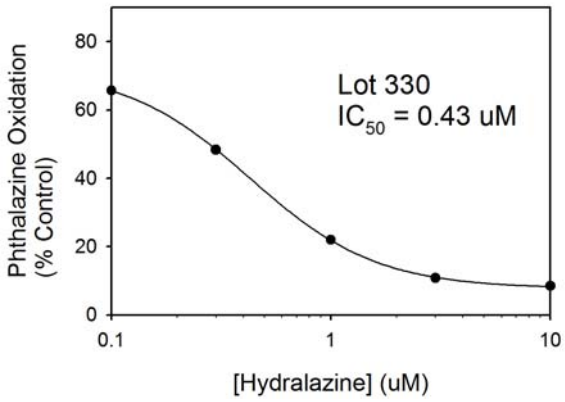
Km --- Phthalazine

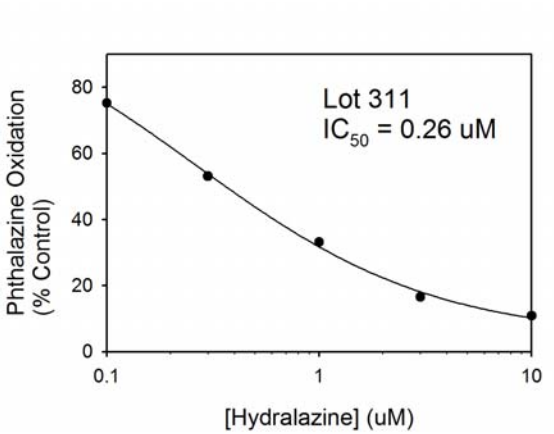


- K_m=4.5 uM

IC_{50} in Hepatocytes







- >90% activity is inhibited by hydralazine
- IC_{50} ranges from 0.3 to 0.7 uM

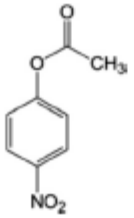
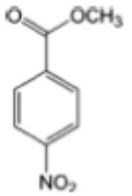
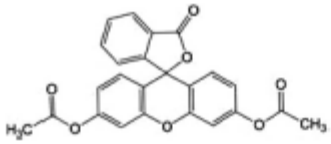
Summary

	Assay Linearity	Activity	Km (uM) – Phthalazine	IC50 (uM) - Hydralazine
Human Liver Cytosol	2 min	5900 pmol/mg/min	10.2 uM	2.8 uM
Hepatocytes	120 min	1.4 – 71.8 pmol/min/10^6 cells	4.5 uM	0.3-0.7

CES Introduction

- Carboxylesterases (CESs) are Phase I metabolizing enzymes.
- CESs are important for metabolism of ester containing drugs, and for rational drug design to increase bioavailability
  - Prodrug activation: CPT-11, Capecitabine (both are anti cancer drug)
  - Drug metabolism: Cocaine (Narcotics), Temocapril (Angiotensin-converting enzyme inhibitors)
- Human CESs mainly belong to the CES1 and CES2 family.
  - CES1 mainly expressed in liver, also in intestine
  - CES2 mainly expressed in intestine.

Substrate Specificity for CESs

Substrate	Structure	Specificity	Activity				
			HLM	CES1-b	CES1-c	HIM	CES2
			$\mu mol \cdot mg^{-1} \cdot min^{-1}$				
4-Nitrophenyl acetate		CES1≈ CES2	2.86	1.43	1.40	1.18	0.682
Methyl 4-nitrobenzoate		CES1	2.39	0.957	0.680	0.223	0.0990
Fluorescein diacetate		CES2	16.9	0.357	0.281	37.2	13.8

- CES1 prefers substrate with small alcohol group and large acyl group.
- CES prefers substrates with large alcohol group and small acyl group.
- BD Supersomes™ CES1 and CES2 show consistent substrate specificity

Wang J., Williams ET, Bourgea J, Wong YN, and Patten CJ (2011). *DMD* **39**:1329-1333

Three isoforms of CES1

A

```
CES1-a.pro   MWLRAFILATLSASAAWAGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPPLGPLRFTPPQPAEPWSFVKN  80
CES1-b.pro   MWLRAFILATLSASAAWSGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPPLGPLRFTPPQPAEPWSFVKN  79
CES1-c.pro   MWLPALVLATLSASAAWSGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPPLGPLRFTPPQPAEPWSFVKN  79

CES1-a.pro   ATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYLNIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAA  160
CES1-b.pro   ATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYLNIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAA  159
CES1-c.pro   ATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYLNIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAA  159

CES1-a.pro   HENVVVVTIQYRLGIWGFFSTGDEHSRGNWGHLDQVAALRWVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPLAKNL  240
CES1-b.pro   HENVVVVTIQYRLGIWGFFSTGDEHSRGNWGHLDQVAALRWVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPLAKNL  239
CES1-c.pro   HENVVVVTIQYRLGIWGFFSTGDEHSRGNWGHLDQVAALRWVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPLAKNL  239

CES1-a.pro   FHRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELLETTLKMKFLSLDLQGDPRESQPLL  320
CES1-b.pro   FHRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELLETTLKMKFLSLDLQGDPRESQPLL  319
CES1-c.pro   FHRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELLETTLKMKFLSLDLQGDPRESQPLL  319

CES1-a.pro   GTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLIPMQLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEA  400
CES1-b.pro   GTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLIPMQLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEA  399
CES1-c.pro   GTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLIPMLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEA  398
                                                                  362

CES1-a.pro   TEKYLGGTDDTVKKKDLFLDLIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAP  480
CES1-b.pro   TEKYLGGTDDTVKKKDLFLDLIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAP  479
CES1-c.pro   TEKYLGGTDDTVKKKDLFLDLIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAP  478

CES1-a.pro   FLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKLKDKEVAFWTNLFAKKAVEKP  560
CES1-b.pro   FLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKLKDKEVAFWTNLFAKKAVEKP  559
CES1-c.pro   FLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKLKDKEVAFWTNLFAKKAVEKP  558

CES1-a.pro   PQTEHIEL                                                                          568
CES1-b.pro   PQTEHIEL                                                                          567
CES1-c.pro   PQTEHIEL                                                                          566
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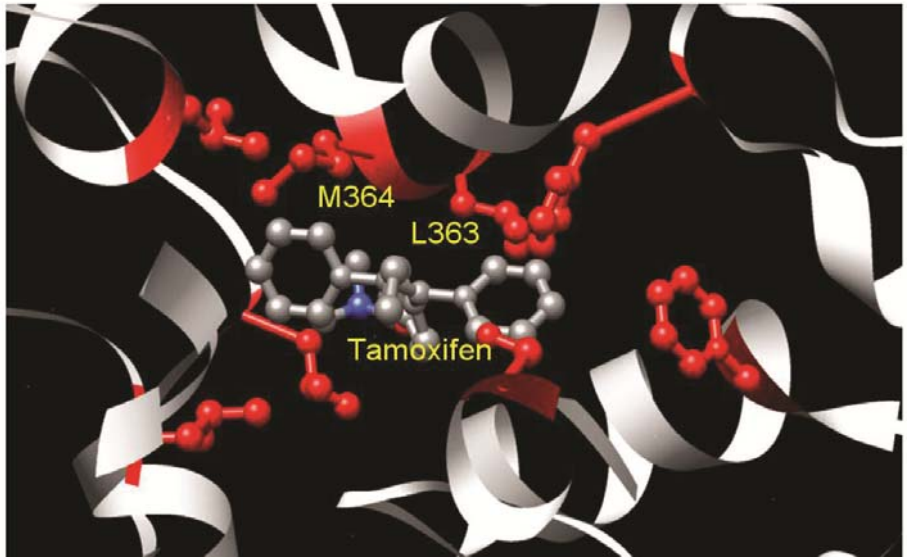
B

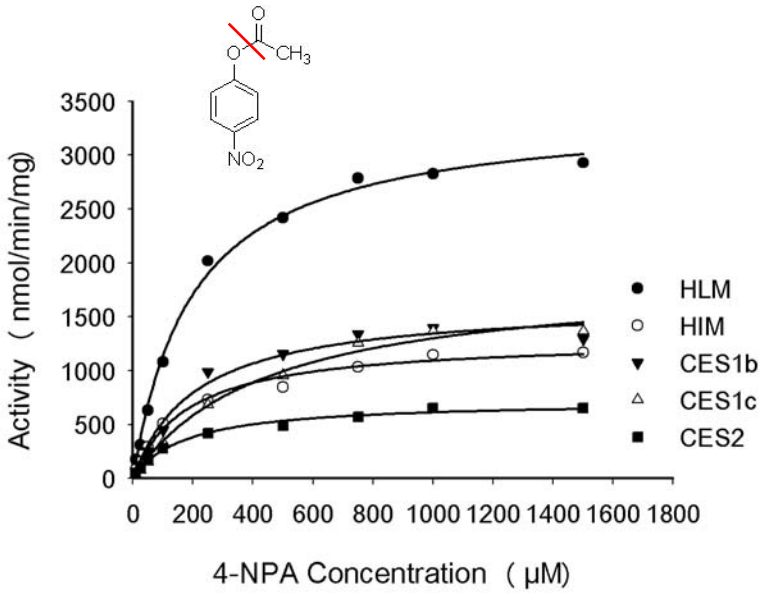


- CES1-a has an extra Ala18 near the N terminus comparing to CES1-b
- CES1-c is lacking Gln362 in the proposed active site comparing to CES1-b

BD

Recombinant CESs show consistent characteristics as human tissues

	4-NPA as substrate	
Enzyme	K_m(μM)	V_{max}(μmol/mg/min)
HLM	198 ± 17	3.41 ± 0.08
CES1b	208 ± 41	1.62 ± 0.09
CES1c	441 ± 67	1.87 ± 0.11
HIM	182 ± 25	1.29 ± 0.05
CES2	173 ± 22	0.718 ± 0.024

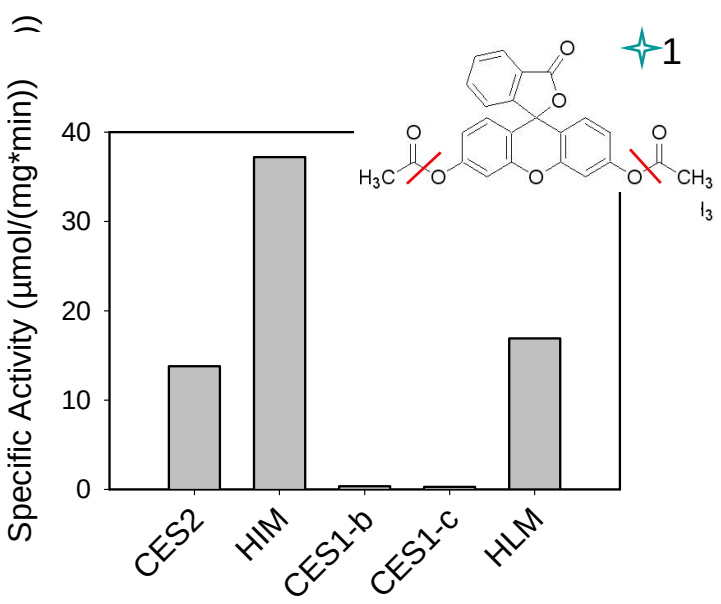


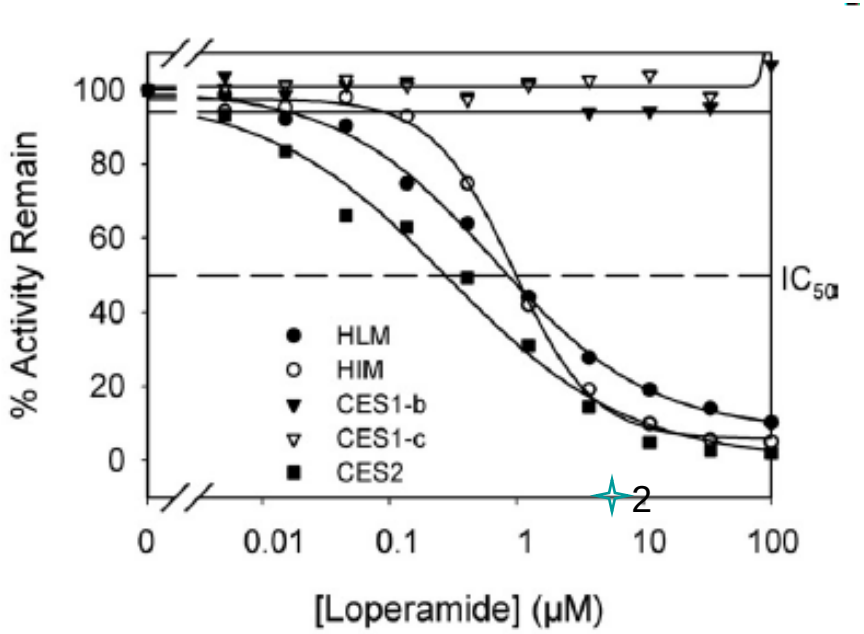
BD Supersomes™ CESs:

- CES1b predominant form in liver for the hydrolysis 4-NPA (K_m matches HLM)
- CES2 is the predominant form in the intestine (K_m matches HIM)
- CES1c found in liver, higher K_m value due to one mutation in the active site

- CES1 and CES2 activity was found to be similar to human liver microsomes and intestinal microsomes respectively

Fluorescein Diacetate is a Probe Substrate for CES2





- Activity for BD Supersomes CES2 is about 50-fold higher vs CES1b or 1c (k_{cat} values of CES2 is roughly 100-fold higher than CES1b or 1c based on estimated expression level)
- CES2 is present in both liver and intestine– FD hydrolysis in HLM is due to CES2
- Loperamide, a known CES2-specific inhibitor, inhibits HLM, HIM, and CES2 with similar IC_{50}, while showing no inhibition towards CES1b/c

Wang J., Williams ET, Bourgea J, Wong YN, and Patten CJ (2011). *DMD* **39**:1329-1333

1 50 µM FD was used as substrate

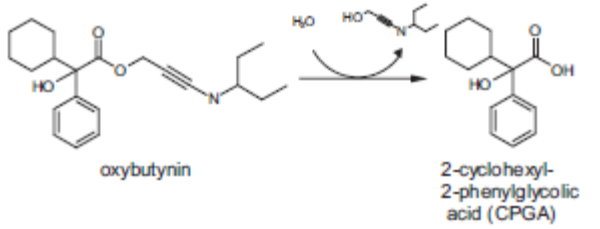
2 5 µM FD was used as substrate

BD CESs application in Pharma

TABLE 1

Kinetics parameters of oxybutynin hydrolysis in HLMs, HLC, and recombinant human CESs

Enzyme Source	K_m	V_{max}	CL_{int}
	μM	$pmol \cdot min^{-1} \cdot mg\ protein^{-1}$	$\mu l \cdot min^{-1} \cdot mg\ protein^{-1}$
HLM	22	130	5.9
HLC	13	110	8.2
CES1	17	310	18
CES2	62	32	0.51



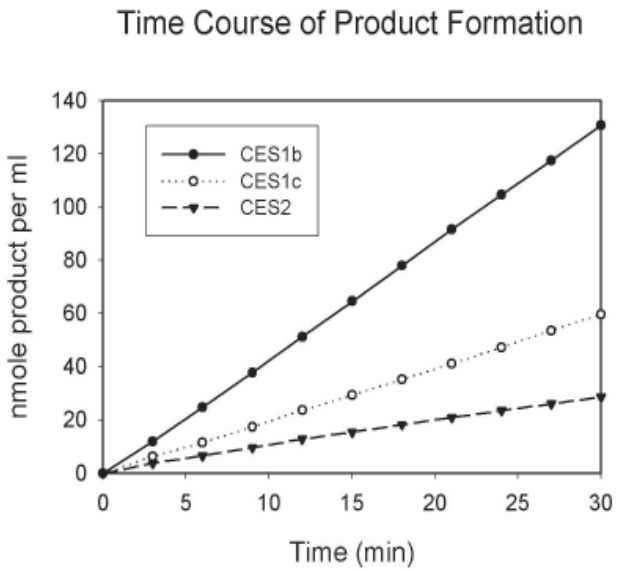
- Application in Pharmas --- Astellas Pharma
  - CES1 and CES2 from BD was tested with CES1 marker reaction (clopidogrel hydrolysis) and CES2 marker reaction (irrinotecan hydrolysis)
  - CES1 and CES2 were tested with oxybutynin, K_m matches human tissue.
  - CES1 and CES2 were tested in inhibition studies with bis-(p-nitrophenyl) phosphate, clopidogrel, nordihydroguaiaretic acid, procainamide, physostigmine, and loperamide.

Y. Sato, et al (2012). *DMD* **40**:902-6, “Conclusive identification of the Oxybutynin-Hydrolyzing Enzyme in Human Liver”

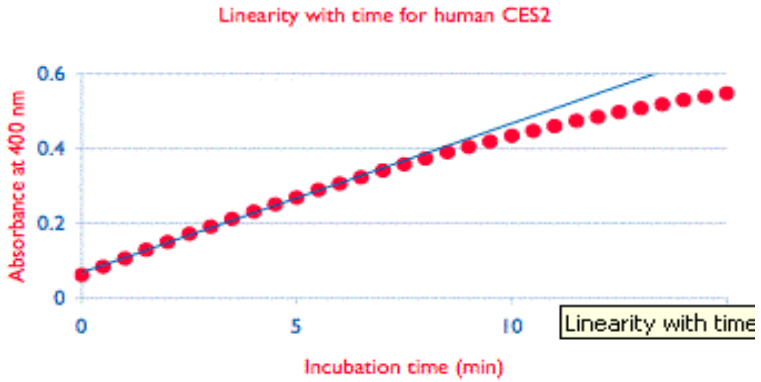
BD

Long Linearity

– Enzyme remains active for more than 30 minutes when incubated with the drug compound, allowing time for slowly metabolizing drug compounds to form metabolites and be identified



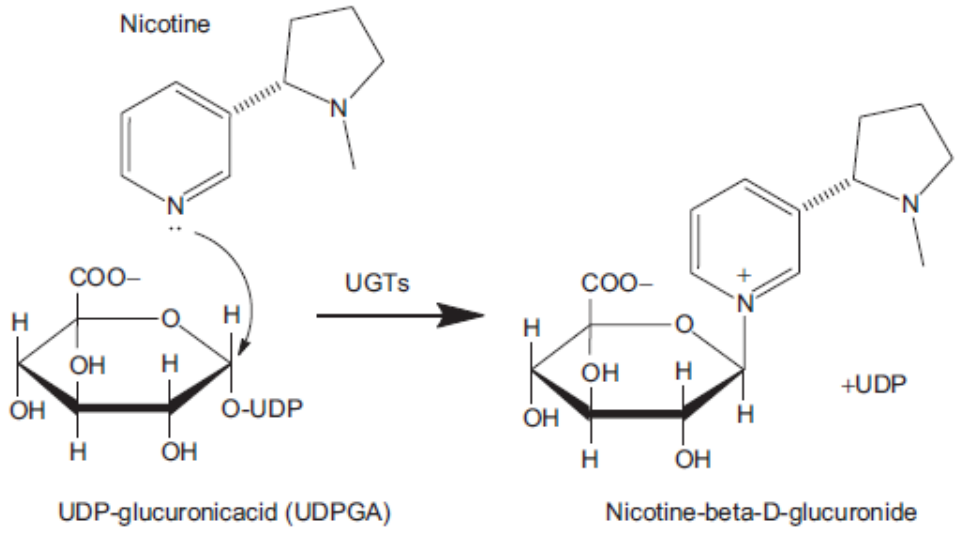
BD - Linearity up to > 30 mins for CES1 and CES2.



Company X - Linearity up to 5 mins for CES1 and CES2.

UDP-Glucuronosyltransferases (UGTs)

- Most important Phase II drug metabolizing enzyme
- UGTs conjugate glucuronic acid to lipophilic substrates to more water-soluble metabolites, glucuronides, to facilitate excretion
- Two most important glucuronidation rxns are:
  - O-glucuronidation
  - N-glucuronidation



UGT Subfamily

UGT Subfamily:

1A Sub-Family (9)	2B Sub-Family (7)	2A Sub-Family (3)
1A1, 1A3, 1A4, 1A5, 1A6	2B4, 2B7, 2B10, 2B11	2A1, 2A2, 2A3
1A7, 1A8, 1A9, 1A10	2B15, 2B17, 2B28	

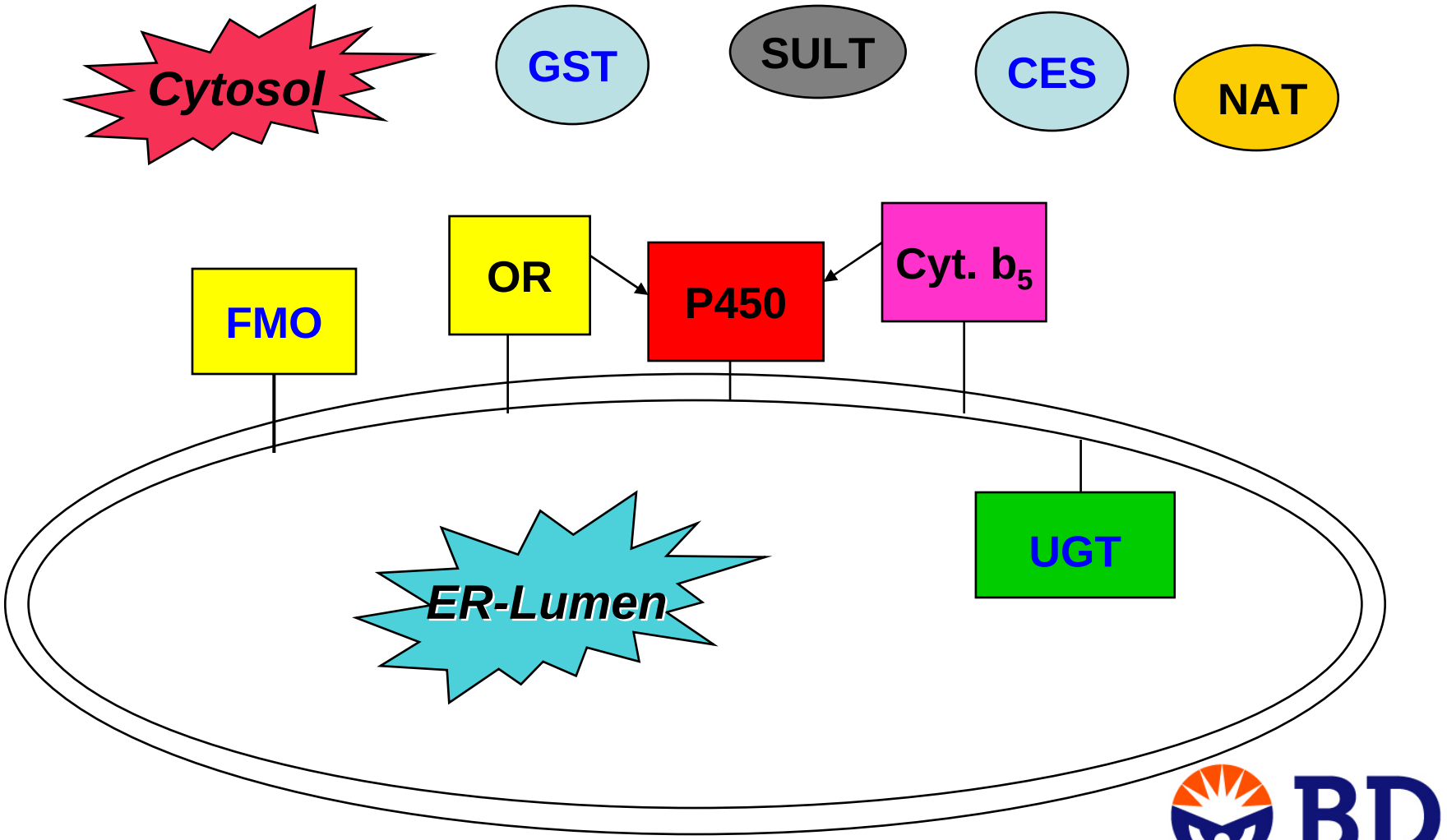
Tissue Expression:

11 UGTs are abundantly expressed in the liver (shown in red), UGTs 1A7 in liver, 1A8 and 1A10 are found in GI tract (shown in blue).

BD UGT Supersomes:

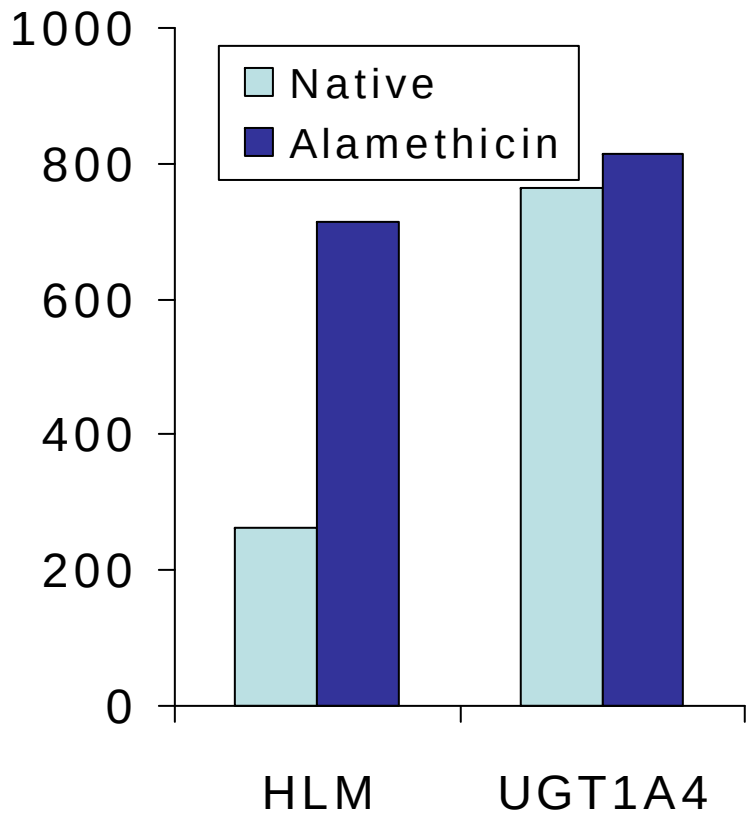
most extensive portfolio in the market with 13 UGTs (shown underlined)

Phase I and Phase II Drug Metabolism Enzymes in ER and Cytosol



UGT Latency in HLM

- UGTs are located on the lumenal face of the microsome.
- This limits access of substrates and UDPGA and reduces activity (latency).
- Treatment with detergents or pore forming agents reduces latency.
- Detergent treatments can be “tricky” and kill CYPs.
- Recommendation is to use pore forming agent “alamethicin”



BD

List of Some UGT Probe Substrates

- UGT1A1: Estradiol (3-glucuronide), Bilirubin
- UGT1A3: 25-Trihydroxy Vitamin D3
- UGT1A4: Trifluoperazine, Amitriptyline/Imipramine (high Km, 100 uM)
- UGT1A6: Serotonin, 5-hydroxytryptophol (5HTOL)
- UGT1A9: Propofol
- UGT2B7: AZT, Morphine
- UGT2B10: Amitriptyline (low Km, 10 uM)
- UGT2B15: S-Oxazepam

List of Some UGT Selective Inhibitors

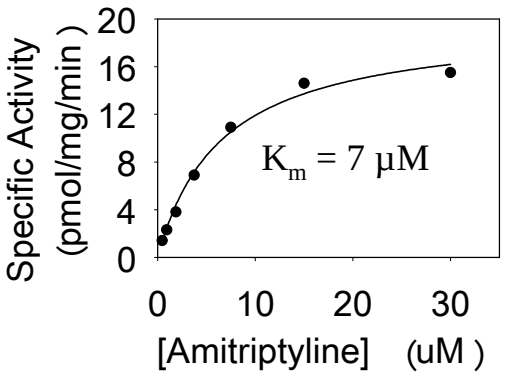
- UGT1A1: Bilirubin, Atazanavir (*in vivo*)
- UGT1A3: 25-Trihydroxy Vitamin D3, 2-Hydroxyestradiol
- UGT1A4: Hecogenin (10uM)
- UGT1A6: Naphthol
- UGT1A9: Niflumic acid (2.5uM), Propofol
- UGT2B7: Fluconazole (2.5mM), Morphine

BD Supersomes™ UGT portforlio

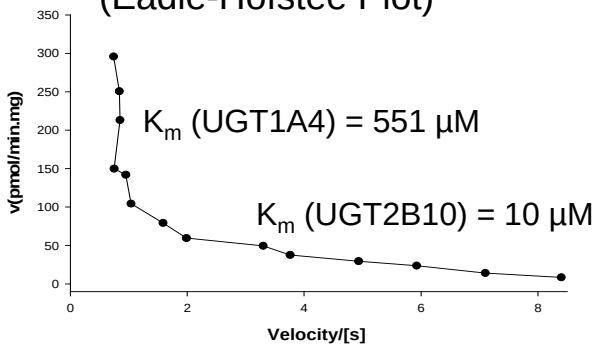
	Substrate	K_m (µM)	
		Supersomes	HLM
UGT1A1	Bilirubin	1.1	0.8
UGT1A4	Trifluoperazine	61	85
UGT1A9	Propofol	10	26
UGT2B7	Morphine (6-Glucuronidation)	766	815
UGT2B10	Amitriptyline	7	10

- K_m of BD Supersomes™ UGTs matches K_m of HLM – native conformation in the insect cell membrane

BD Supersomes UGT2B10



HLM with Amitriptyline (Eadie-Hofstee Plot)



- UGT2B10 is the high affinity enzyme for Amitriptyline N-glucuronidation

Summary

- Non-CYP pathways are becoming more important due to reduced risk of DDI relative to CYP pathways
- Non-CYP pathways are numerous and add complexity to reaction phenotyping studies (each having a unique tissue distribution and co-factor requirement)
- In general, tools for studying non-CYP pathways are lacking compared to CYPs
- Recombinant enzymes (e.g. Supersomes) are currently available for most non-CYP drug metabolizing enzymes; most commonly used tool for studying non-CYP pathways

BD Supersomes™ - Validated for Reaction Phenotyping Studies

- Ensures activity of recombinant enzymes is functionally similar to native enzymes expressed in human tissues.
  - cDNA of human recombinant metabolizing enzymes match the published sequence from the U.S. National Library of Medicine native DNA sequence
  - K_m with probe substrate matches human tissue microsomes or hepatocytes
  - IC_{50} with probe inhibitor matches human tissue microsomes or hepatocytes
- High catalytic activity
  - Activity typically higher or comparable to human tissue microsomes or hepatocytes
  - Provides robust data suitable for high throughput reaction phenotyping and drug inhibition screening assays.
- Linearity
  - Enzymes typically remain active for more than 30 minutes when incubated with the drug compound, allowing time for slowly metabolizing drug compounds to form metabolites and be identified (for some Supersomes activity can be linear for > one hour, e.g. UGTs)
- Availability
  - Extensive portfolio of human CYP and non-CYP drug metabolizing enzymes (phase 1 and phase 2), as well as major rat P450s
  - Highly characterized and ready for use

Questions?

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