MEDCH 527 1/20/2017

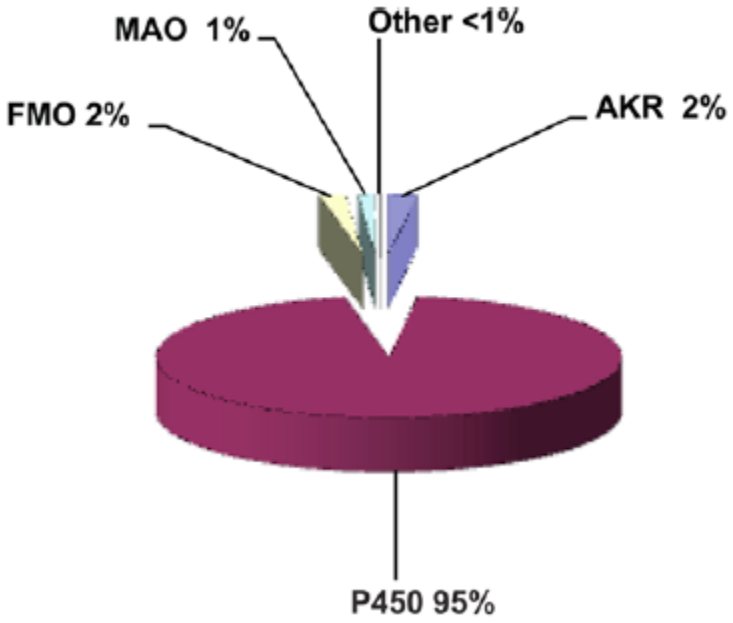
Non-P450 Oxidases and Oxygenases

Flavin-Containing Monooxygenases (microsomal FMOs)

Molybdenum Hydroxylases (cytosolic AO and XO)

Monoamine Oxidases (mitochondrial MAO-A, MAO-B)

Aldehyde Dehydrogenases (cytosolic and mitochondrial ALDH)



Rendic and Guengerich, *Chem. Res. Tox.* (2015)

Useful literature

- Foti, RS and Dalvie DK. Cytochrome P450 and Non–Cytochrome P450 Oxidative Metabolism. *Drug Metab. Dispos.* 44:1229 (2016).
- Krueger SK and Williams DE. Mammalian flavin-containing monooxygenases: structure/ function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther* 2005; 106:357-387.
- Fenema et al. Trimethylamine and Trimethylamine N-Oxide, a Flavin-Containing Monooxygenase 3 (FMO3)-Mediated Host-Microbiome Metabolic Axis Implicated in Health and Disease. *Drug Metab. Dispos.* 44:1839 (2016).
- Hutzler et al., Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin. Drug Metab. Toxicol*. 2013 (2):153-68.
- Youdim MB, Edmondson DE and Tipton KF. The therapeutic potential of monoamine oxidase inhibitors. *Nature Rev. Neurosci.* 7:295 (2006).
- Koppaka V et al. Aldehyde dehydrogenase inhibitors. *Pharmacol Rev.* 2012 Jul;64(3):520-39.

Why should we care about non-P450 pathways?

• P450-mediated metabolism dominates oxidative metabolic clearance of drugs, and so, multiple co-administered drugs can be competing for the same clearance pathways, leading potentially to serious drug-drug interactions.

• Genetic polymorphisms that reduce P450 function are well documented and can also cause serious drug-gene interactions, at least for low therapeutic index drugs.

• For both reasons, designing **away** from P450-dependent clearance mechanisms could be considered a useful strategy during early drug discovery.

• Moreover, as pharmaceutical companies have tried to design ligands for increasingly more complicated biological targets, there has been an inevitable increase in the lipophilicity and molecular volumes of new chemical entities, which have been associated with safety failures in the clinic.

• Therefore, replacement of easily incorporated carbocyclic rings (benzene, naphthyl) rings with heteroaromatic rings (pyrimidine, pthalazine, etc) has become an increasingly employed strategy to reduce lipophilicity.

• This has had the effect of switching away from P450, and towards aldehyde oxidase, as the main metabolic enzyme, for many newer drugs.

Outline

- History/General Enzyme Characteristics
- Structure/Catalytic Mechanism
- Multiplicity/Regulation
- Substrates and Reaction Pathways
- In Vitro Methodologies for;

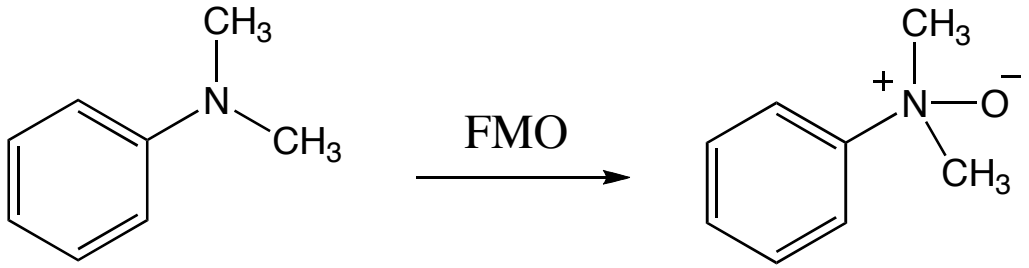
  - differentiating FMO-mediated from P450-mediated Catalysis

  - identifying AO- *versus* XO-dependent catalysis

  - discriminating between MAO-A and MAO-B catalysis

FMO History

1960s A liver microsomal enzyme system (E.C. 1.14.13.8) that utilizes NADPH and molecular oxygen to convert N,N'-dimethylaniline to N,N-dimethylaniline N-oxide first described by Dr. Daniel Ziegler and colleagues.



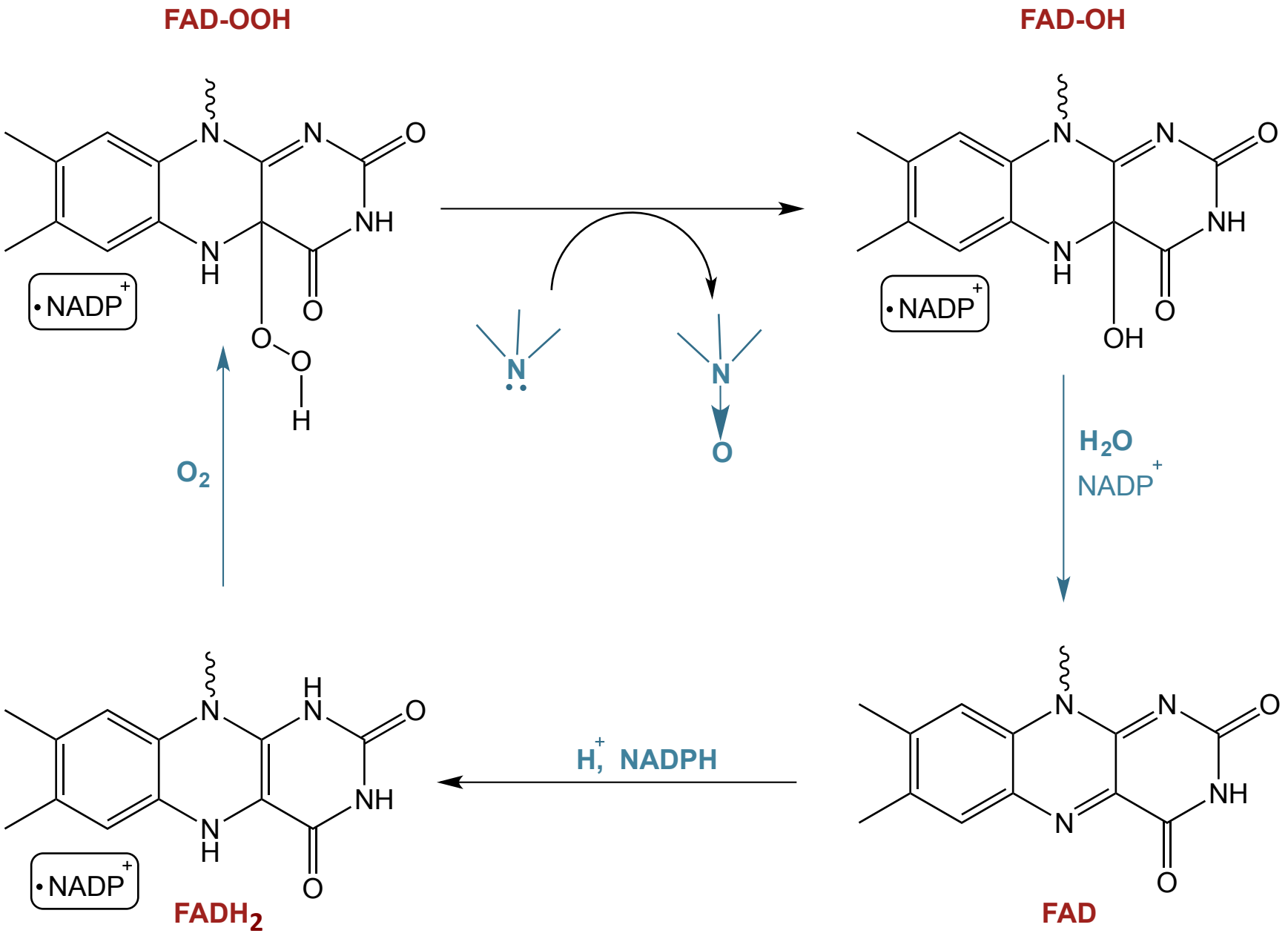
1970s 'Ziegler's enzyme', purified from hog liver, shown to contain flavin, but no heme, thereby distinguishing this flavin-containing monooxygenase (FMO) from the microsomal hemoprotein-containing cytochrome P450s. [1980s – 'lung' FMO]

1990s FMO Nomenclature Committee names hog liver FMO as FMO1 and the 'lung' form as FMO2. Other forms with <60% sequence identity are named with ascending arabic numerals, FMO3, 4, 5 in all mammals.

Comparison of General Properties: FMO *vs* P450

	FMO	**P450**
Function	Monooxygenase	Monooxygenase
	$S + O_2 + NADPH + H^+ \rightarrow SO + H_2O + NADP^+$	
Reducing cofactor	NADPH	NADPH
Cellular Location	Microsomal	Microsomal
Size	60-65 kDa	50-60 kDa
Substrates	Oxidizes at N,S,P	N,S,P and C
Prosthetic group	FAD	Heme

FMO Catalytic Cycle

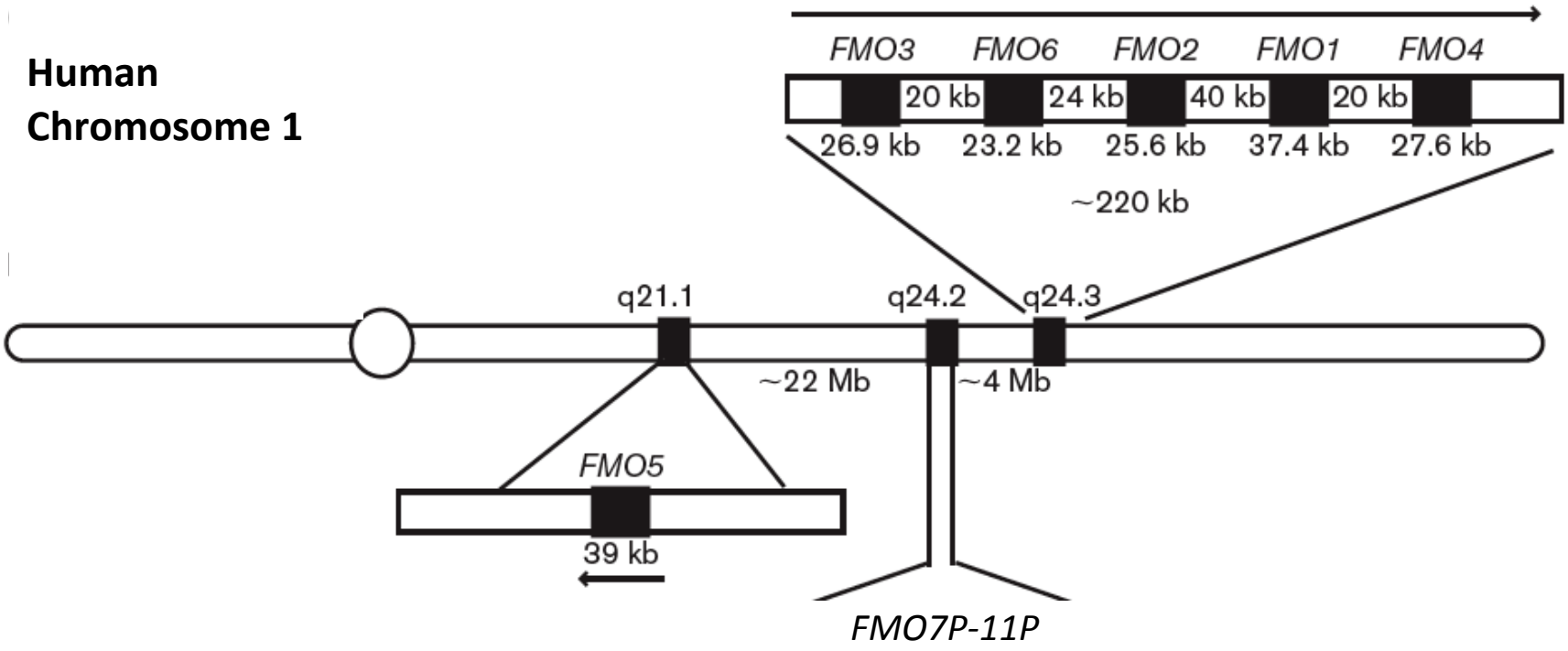


Aspects of FMO Catalysis

- The reaction mechanism is ordered, i.e. NADPH, oxygen and oxidizable substrate add to the enzyme before any of the products leave.
- The enzyme-bound hydroperoxyflavin [FAD-OOH] is a very stable, albeit relatively weak oxidant.
- Release of water and/or $NADP^+$ is believed to be the rate-limiting step.
- FMO can oxidize practically any soft nucleophile that the enzyme's FAD-OOH active center encounters.
- In general, uncharged and single positively charged substrates can gain access, in preference to negatively charged and multiple positively charged compounds.

FMO Multiplicity

- Five active genes, *FMO1-FMO5,* are present in most species.
- *FMO6* is inactive due to alternative splicing.



- FMO1, FMO2 and FMO3 are the best characterized enzymes

FMO1: General Characteristics

- The majority of our knowledge of the FMO enzymes is derived from detailed biochemical studies conducted in the 1970s and '80s on hog liver FMO1 - the first FMO to be purified.

- FMO1 is characterized by its **promiscuous substrate specificity**, which is the widest of all of the FMO isoforms.

- FMO1 is the dominant form of the enzyme in the liver of many experimental animal species, but is not expressed in adult human liver.

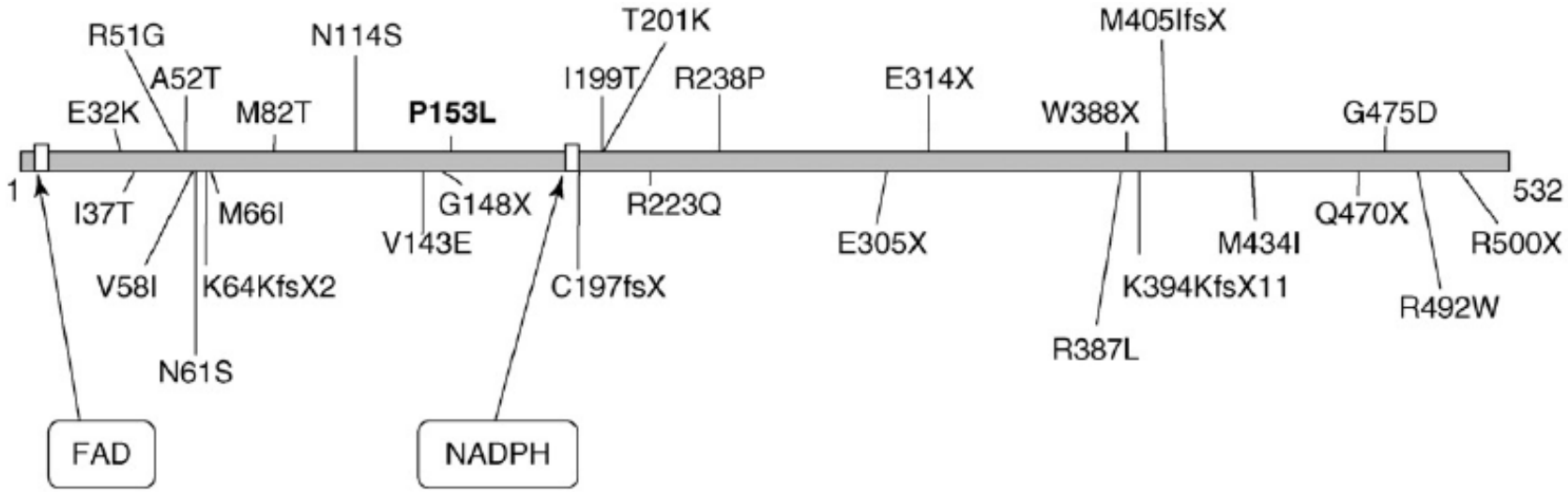
- **In humans, liver FMO1 is a fetal enzyme**, but is also expressed at relatively high levels in **adult kidney**.

FMO2: General Characteristics

- Generally found at highest levels in lung tissue of experimental species.
- FMO2 is also characterized by its **unusual thermostability.**
- Structure-function data obtained with phenothiazines and rabbit FMO2 suggest the C4a-hydroperoxyflavin lies about 6-8 angstroms below the enzyme surface in a channel no more than 8 angstroms in diameter.
- Genetic polymorphisms in humans, commonly Q472X, renders human FMO2 inactive in all but a small proportion of the population.

FMO3: General Characteristics

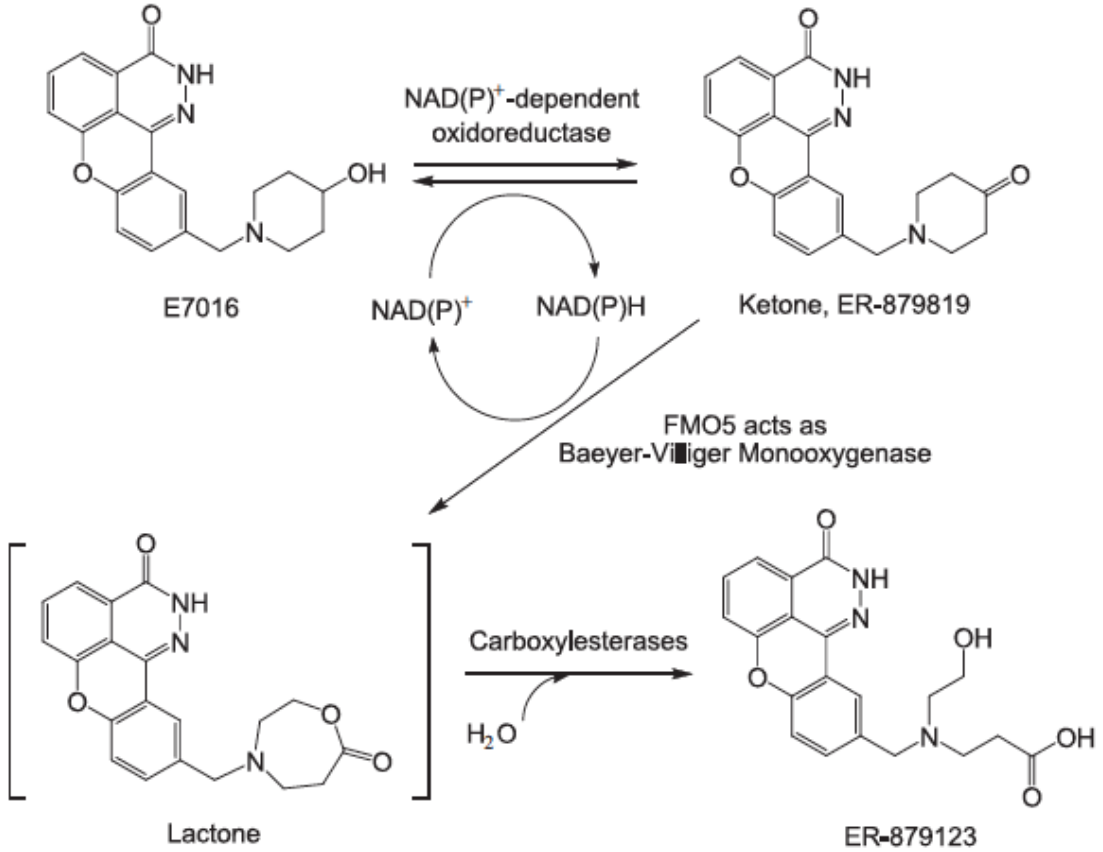
- The major FMO enzyme present in adult human liver ~50 pmol/mg.
- Possesses reasonably broad substrate specificity, but is more selective than FMO1; i.e. FMO1 selectivity < **FMO3** < FMO2.
- Trimethylamine (TMA) is the classical endogenous substrate.
- In **1º trimethylaminuria** (TMAU), affected individuals cannot metabolize TMA and so excrete the odoriferous compound in their urine, sweat and breath due to TMAU **mutations in the *FMO3* gene**.



FMO4 and FMO5: General Characteristics

- FMO4 appears to be ubiquitously expressed at low levels in a variety of tissues, but little is known of its substrate specificity, due largely to difficulties in expression of the recombinant enzyme.

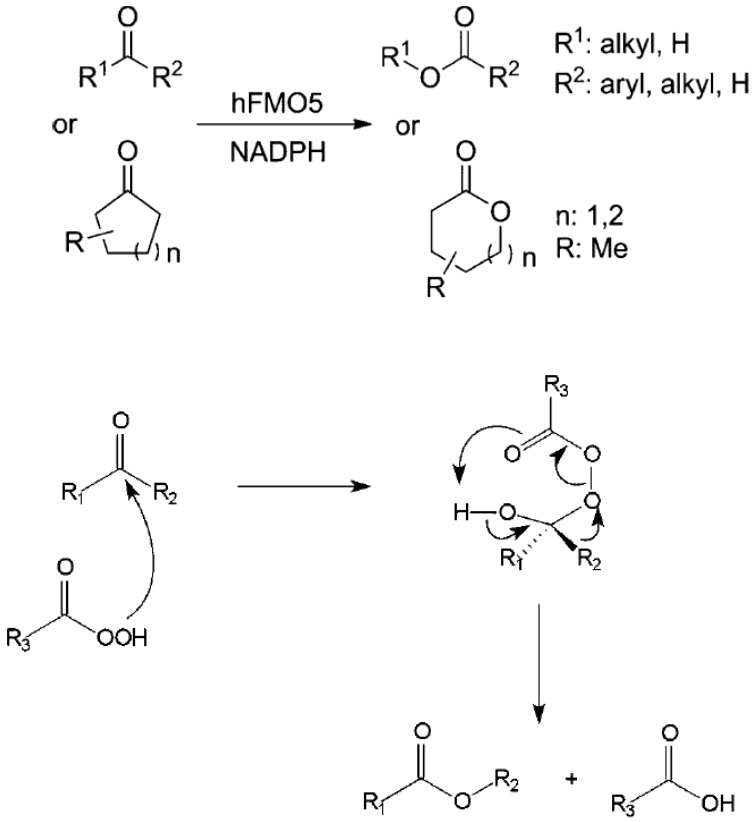
- FMO5 is a major FMO form present in human liver (~30 pmol/mg), but exhibits little or no catalytic activity towards common FMO substrates. Acts as a Bayer-Villigerase!



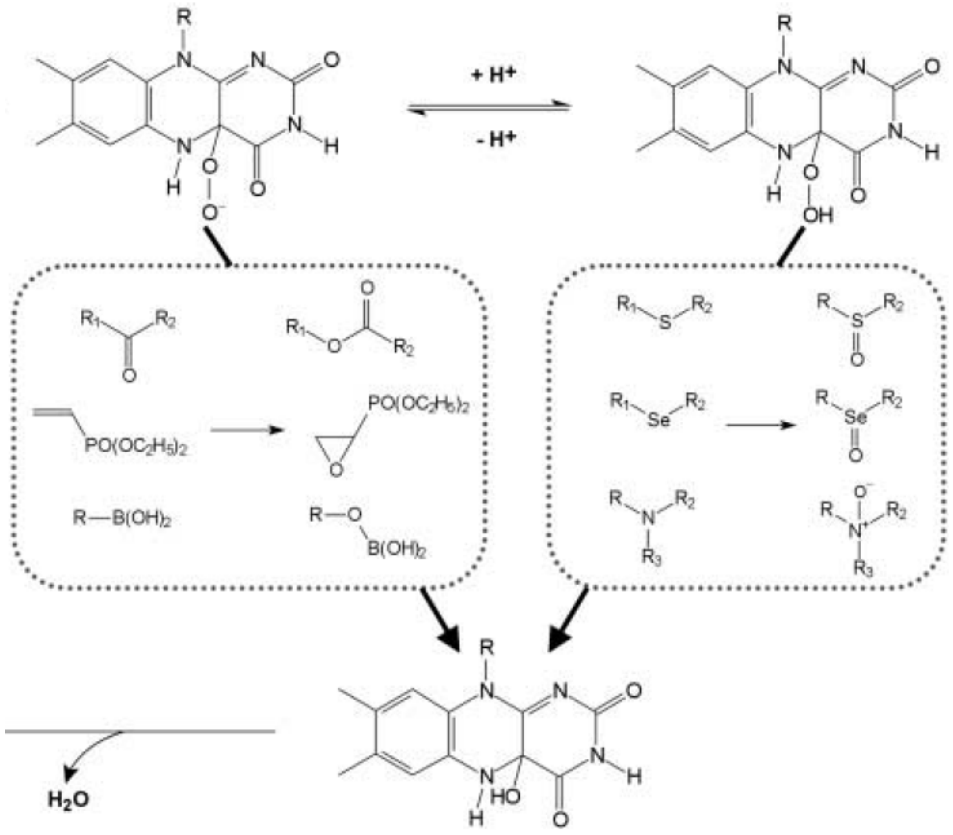
Lai et al., DMD, 2011
Fiorentini et al., ACS Chem Biol, 2016

FMO5 – Bayer Villiger Reactions

- Rationalized by the active enzyme species being the hydroperoxy anion – FAD-O-O$^-$.



Scheme 1. Mechanism of the Baeyer–Villiger oxidation by peracids.



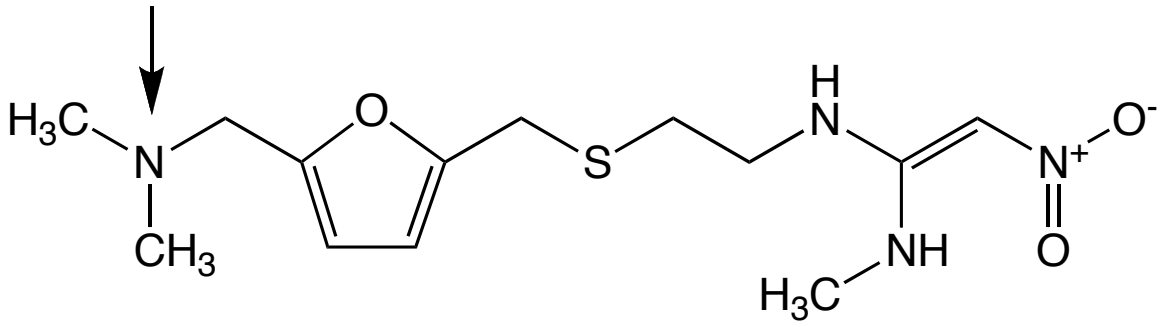
Common FMO Metabolites

- Microsomal FMOs oxidize substrates containing nucleophilic nitrogen, sulfur, phosphorous and selenium atoms.
- Common metabolites are sulfoxides (S-oxides) generated by FMO from alkyl aryl sulfides.
- The prototypical FMO reaction is N-oxygenation of a tertiary amine, e.g. trimethylamine, N,N-dimethylaniline, to the respective N-oxide metabolite.
- N-oxides and S-oxides are highly polar metabolites contain coordinate covalent bonds between the oxygen and heteroatom.
- N.B. Both FMO and P450 can catalyze formation of N-oxides and S-oxides and so tools have been developed to discriminate between these two enzymatic processes in biological tissues.

Other N-containing Drug Substrates

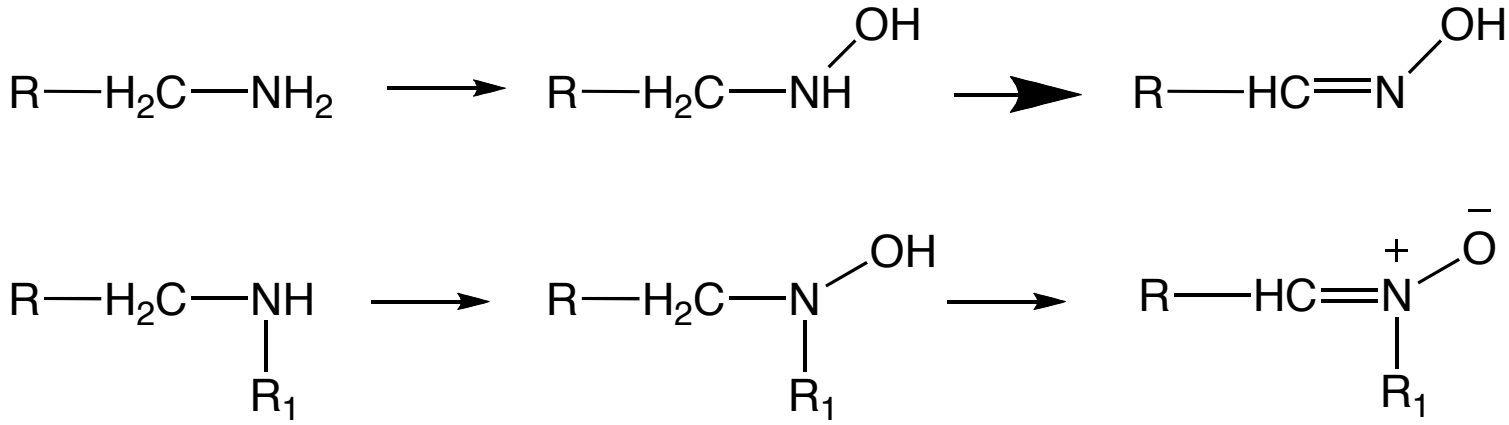
- While FMO has the capacity to metabolize a vast range of amine-containing compounds, a significant contribution to the metabolic clearance of drugs approved in the US is much more limited.

- Examples include; *ranitidine*, benzydamine, itopride, olanzapine, pargyline, xanomeline and chlorpheniramine.



FMO-catalyzed N-oxygenation

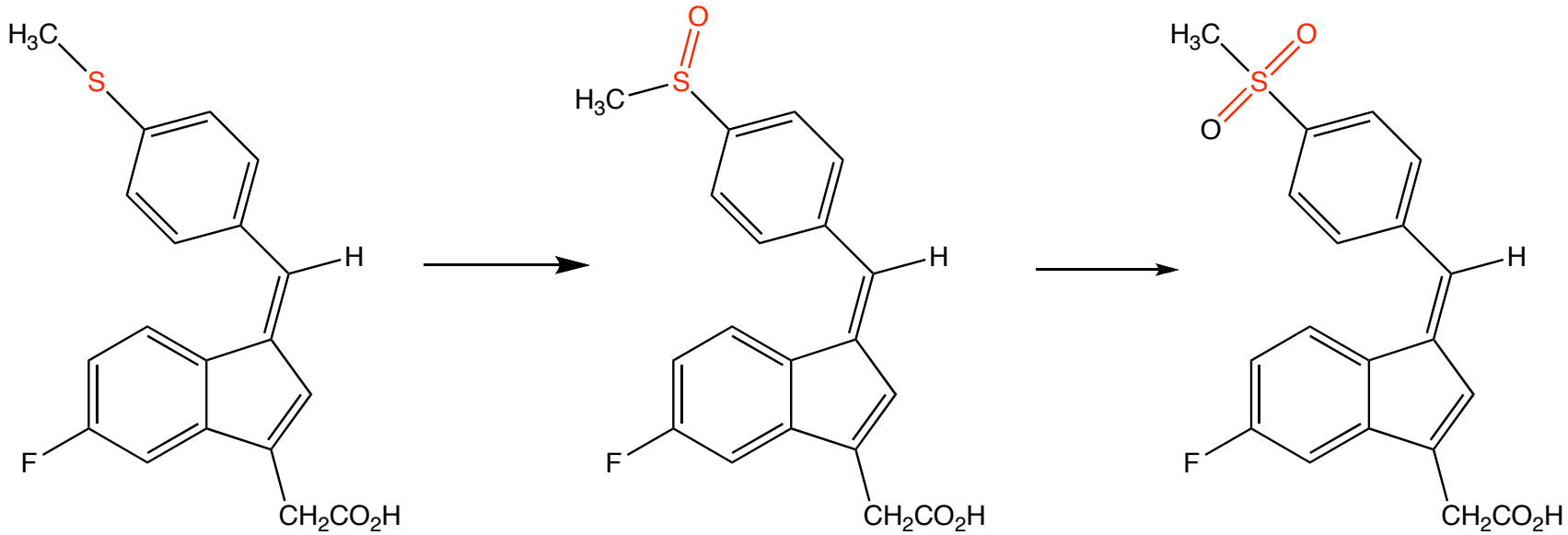
- FMOs can also convert 1° and 2° amines to hydroxylamine, nitrone and oxime metabolites.



- In general, these FMO reactions are considered detoxification pathways.
- However, some secondary hydroxylamines, e.g. those derived from 3,3-iminodipropionitrile and N-deacetyl ketoconazole, have been associated with neurotoxicity and hepatotoxicity, respectively.
- This may occur *via* reactive species derived from the nitrone metabolite (see Fig 2 in Foti and Dalvie, 2016).

FMO-Catalyzed S-Oxygenation

- FMOs catalyze sulfoxide formation from sulfides and (less efficiently) sulfone formation from sulfoxides,

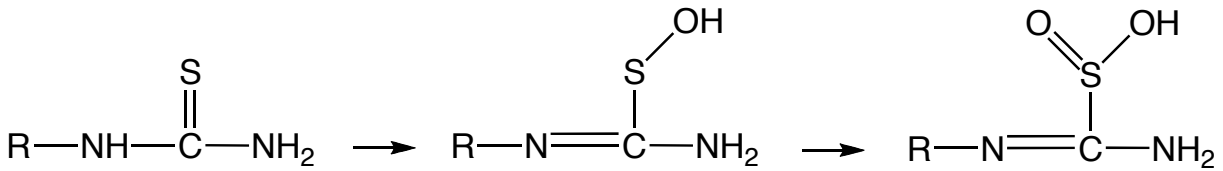


Sulindac sulfide **Sulindac** **Sulindac sulfone**

- Other S-containing drug substrates include; cimetidine, methimazole, ethionamide and SM-12502.

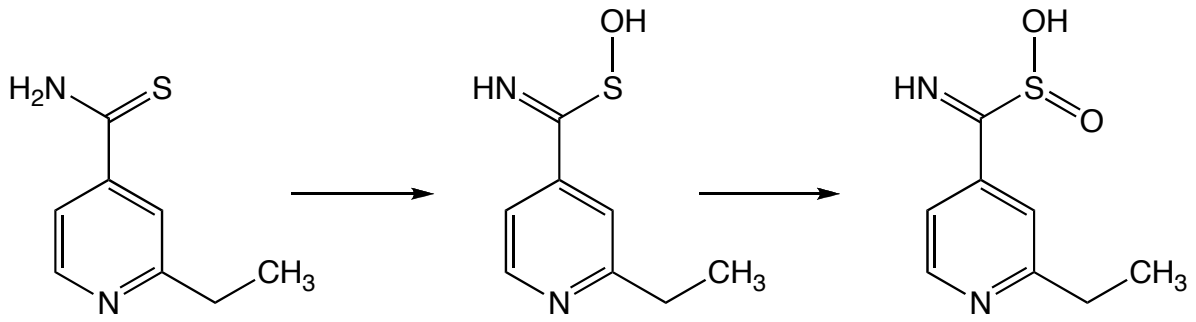
FMO-catalyzed Bioactivation

- Most commonly associated with metabolism of S-containing substrates, e.g. thioureas, that can be converted sequentially to reactive sulfenic and sulfinic acids.



- Classical substrates activated in this fashion are the hepatotoxins, thioacetamide and thiobenzamide.

- A drug example is ethionamide, which *requires* bioactivation by a *M. tuberculosis* FMO for its antitubercular activity, whereas organ toxicities may be due to human FMO-dependent (off-target) bioactivation.



Tools for Identifying FMO Catalysis in Microsomes

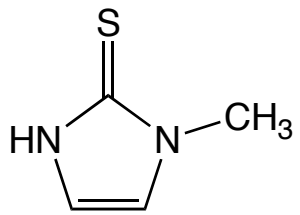
- P450s can catalyze essentially all the same reactions as FMO.
- Main goal is to differentiate FMO activity from that of P450 in microsomes.
- Approach is to

  (A) selectively inhibit P450 or,

  (B) selectively activate or inactivate FMO activity.

Diagnostic inhibitors for FMO?

- **No inhibitory antibodies** have generated against any FMO isoform.
- **No mechanism-based inhibitors** of FMO have been described.
- Reversible inhibitors include alternate substrates, such as methimazole.



- However, methimazole is **not a specific inhibitor** for FMO.
- Other potentially isoform-selective alternate substrates are imipramine (FMO1) and trimethylamine (FMO3).

(A) Selective inhibition of P450

1. Use a P450 reductase antibody.
   - All P450s use the same reductase to transfer electrons from NADPH.

2. Use a mechanism-based inhibitor of the P450s, e.g. 1-aminobenzotriazole (ABT) is a pan inhibitor of microsomal P450, but not FMO.
   - Relies on P450-mediated conversion of ABT to benzyne, which then reacts with P450 heme.

(B) Selective inhibition of FMO

1. Exploit thermolability of FMOs (except FMO2)

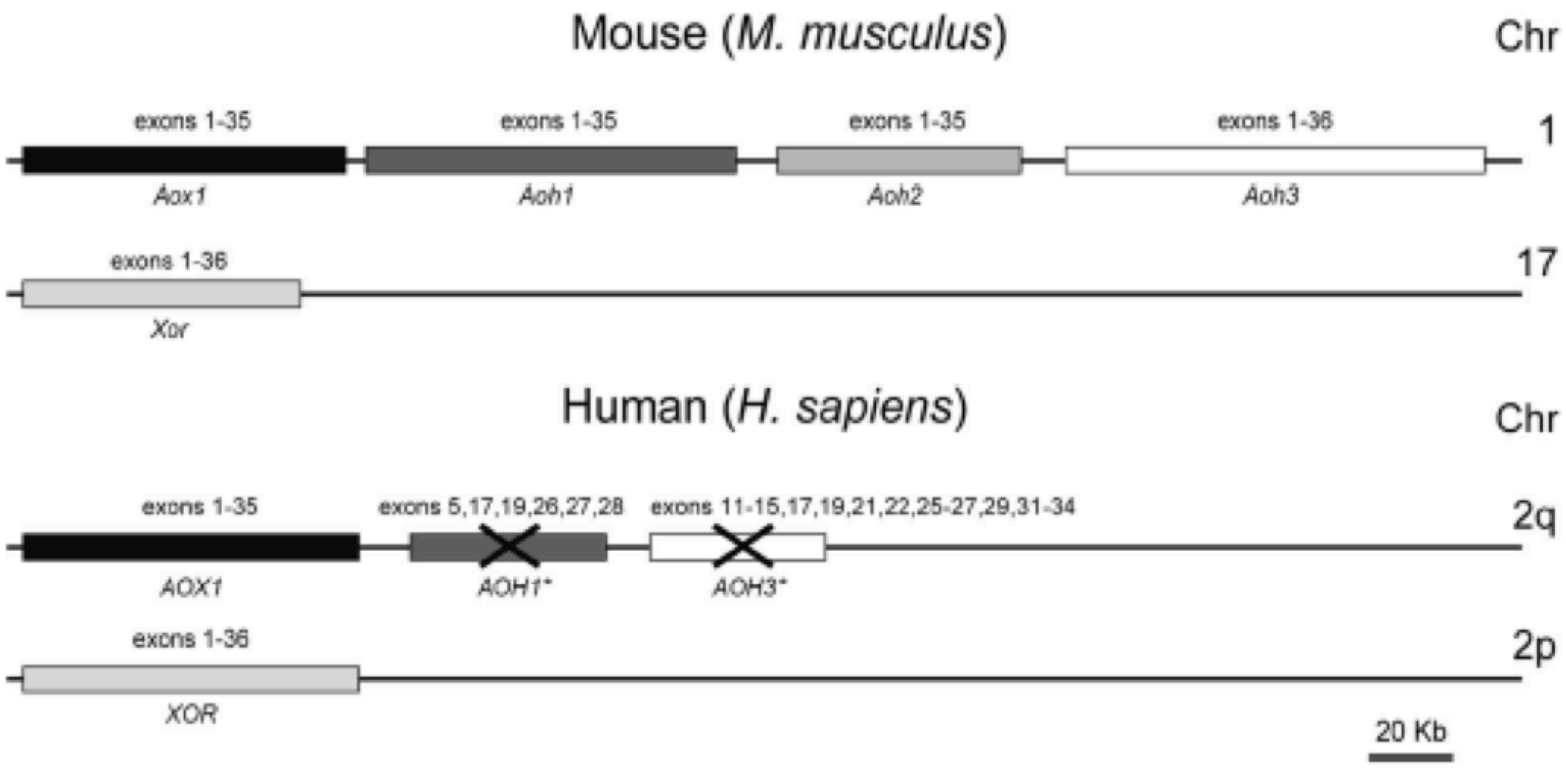
   - Heat microsomes at 45^{o}C in the absence of NADPH for ~2 min selectively inactivates FMO1 and FMO3.

2. Exploit FMOs relative insensitivity to non-ionic detergents.

   - Treatment of microsomes with 0.2% Lubrol or Emulgen will inactivate P450s , but not FMOs.

Molybdenum Hydroxylases

Two forms: Xanthine oxidase (XO) and aldehyde oxidase (AO) are separate gene products exhibiting ~50% amino acid homology. There is only one gene for each enzyme in humans - *XOR* and *AOX1*, respectively.

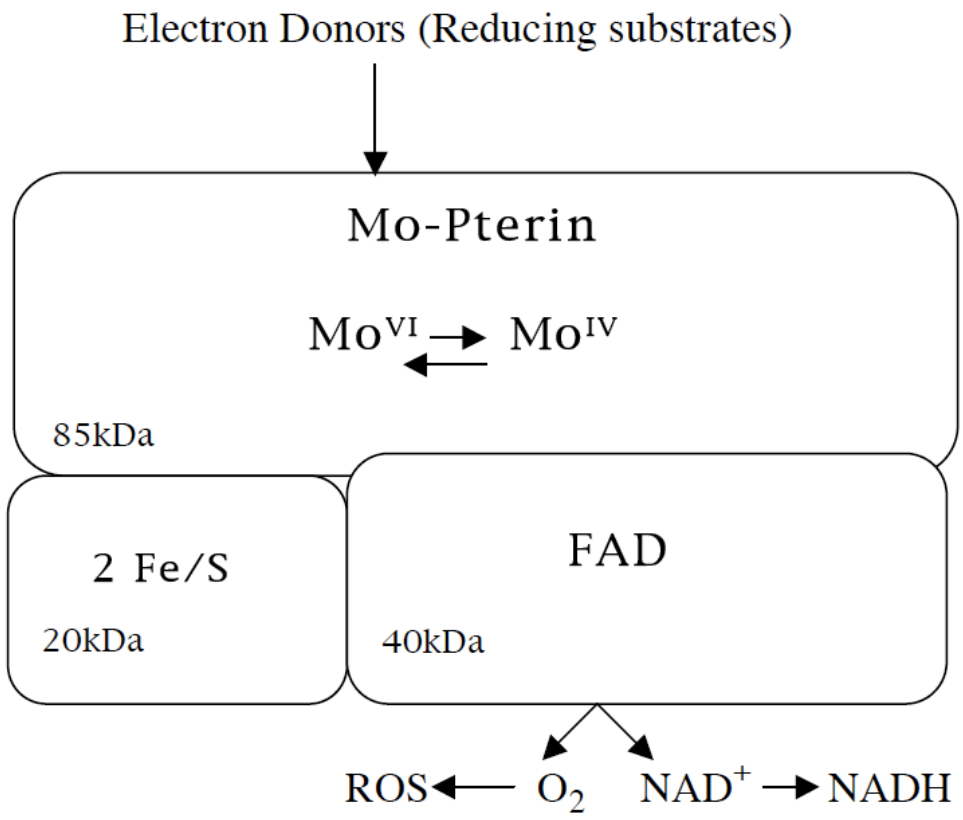


Molybdenum Hydroxylase Complex

$$RH + H_2O \longrightarrow ROH + 2e^- + 2H^+$$

The enzymes have a complex tri-partite (flavoprotein) structure, comprising two identical subunits of ~145 KDa each.

The enzyme complex typically shuttles electrons from substrates (i.e, the **substrate gets oxidized** in the process) to an electron acceptor, usually oxygen, generating ROS. N-oxides, sulfoxides, aromatic nitro compounds can also be **reduced**.



e.g. Superoxide anion radical
Hydroxyl radical

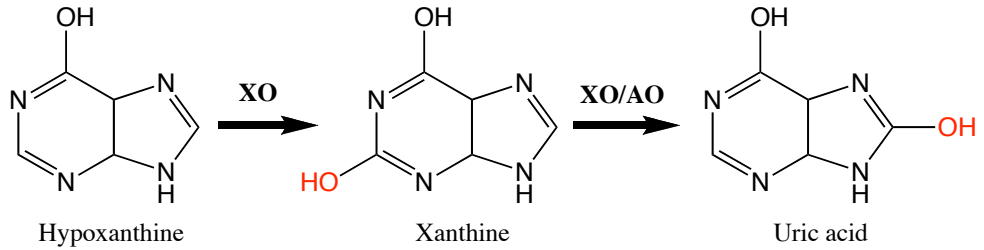
Substrate Specificity – Guanine derivatives

In the main, XO and AO target **sp^2-hybridized carbon atoms rendered electron-deficient** by a nitrogen atom to which they are linked by a double bond, -CH=N.

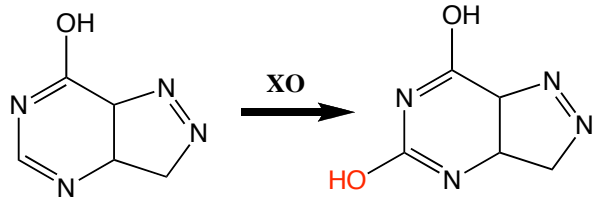
The major substrates for XO and AO are **nitrogen heterocycles**. Xanthine is the prototypic substrate for XO and allopurinol is a selective XO inhibitor.

XO and AO bioactivate 6-deoxy guanine prodrugs for the anti-viral, penciclovir. This strategy was helpful because the active agents were <u>poorly bioavailable.</u>

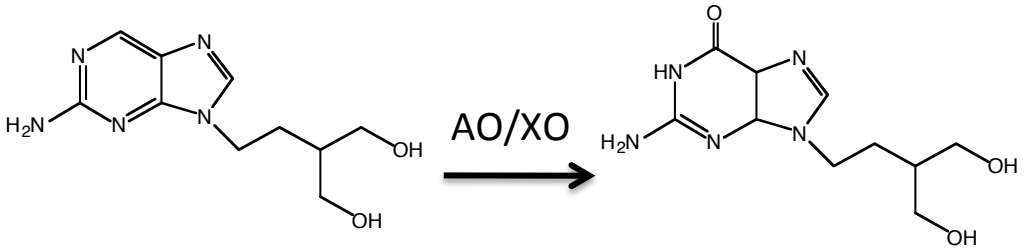
XO also metabolizes the anticancer drug, 6-MP (minor pathway)



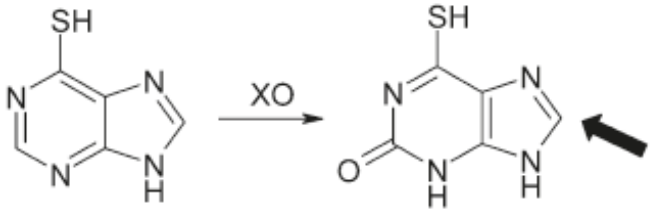
Hypoxanthine Xanthine Uric acid



Allopurinol Oxypurinol

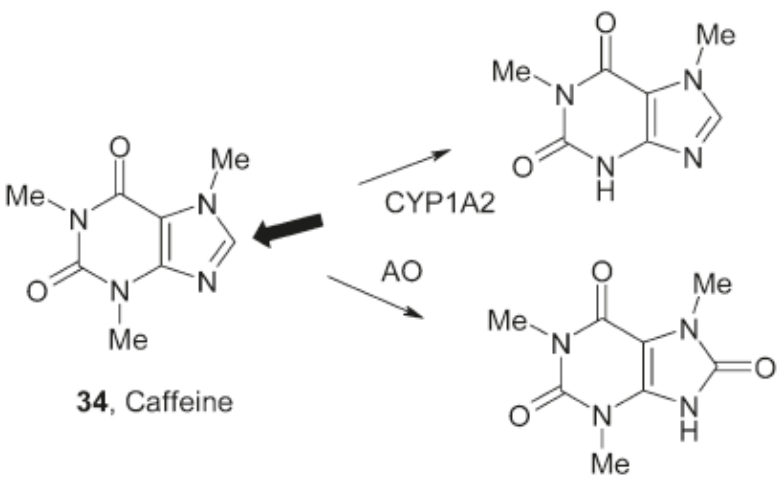


6-Deoxypenciclovir Penciclovir



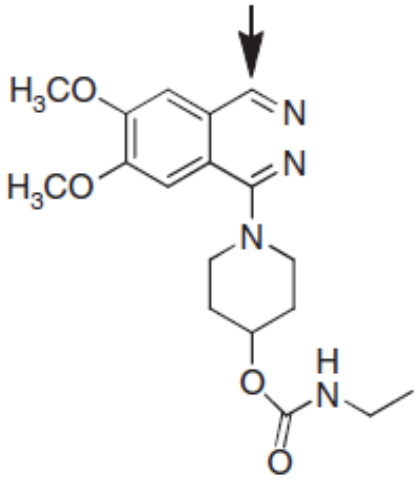
32, 6-mercaptopurine **33**, 6-thioxanthine

Additional heterocyclic substrates for AO

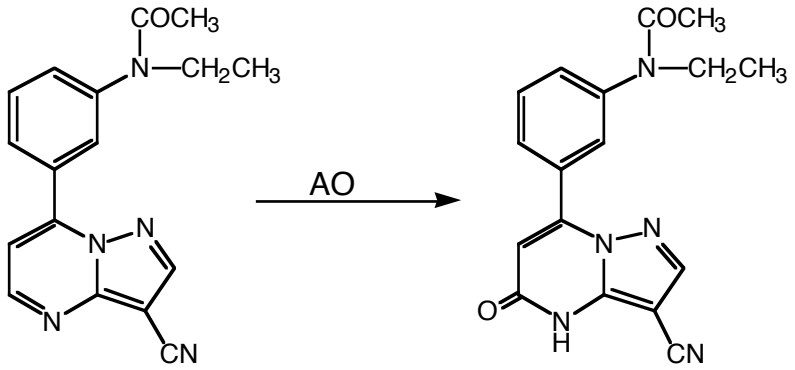


34, Caffeine

In vivo probe for XO?



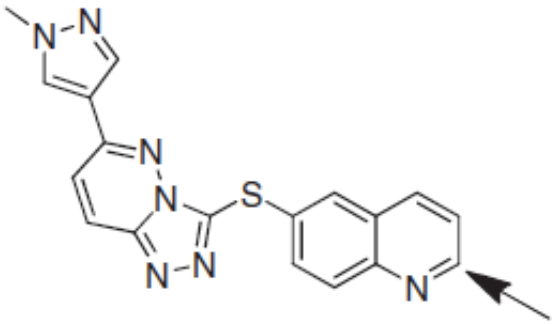
Carbazeran – <5% oral bioavailability, rapid metabolism



Zaleplon

5-Oxo-Zaleplon

Short-acting hypnotic

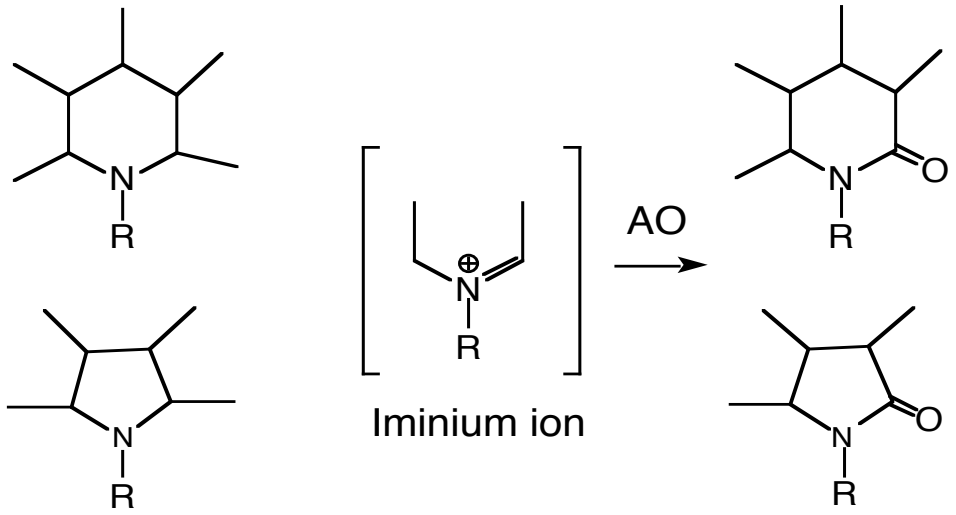


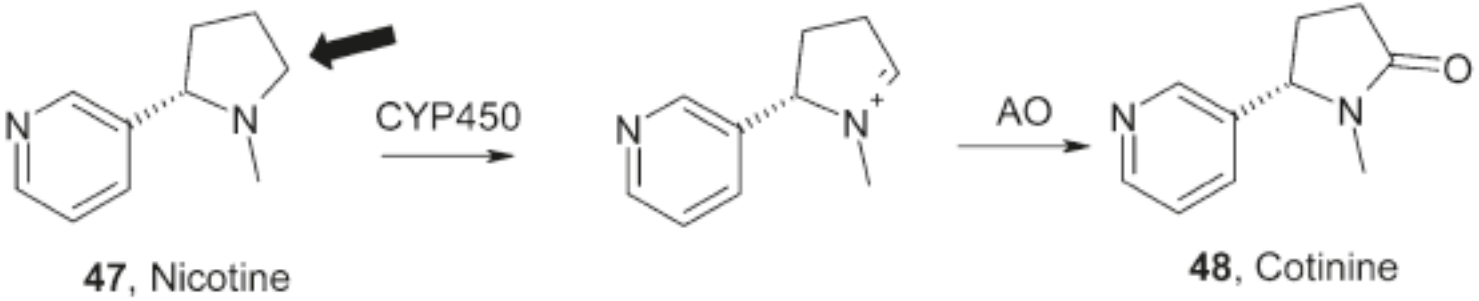
SGX 523 – renal toxicity due to insoluble (!) lactam metabolite

Metabolism of Iminium ions by AO

AO also plays an important role in the detoxification of potentially reactive iminium ions that can be generated by P450 or MAO often from cyclic tertiary amines.

Lactam metabolites are the result, e.g. AO-catalyzed formation of the nicotine metabolite, cotinine, that is formed in by sequential P450/AO metabolism.

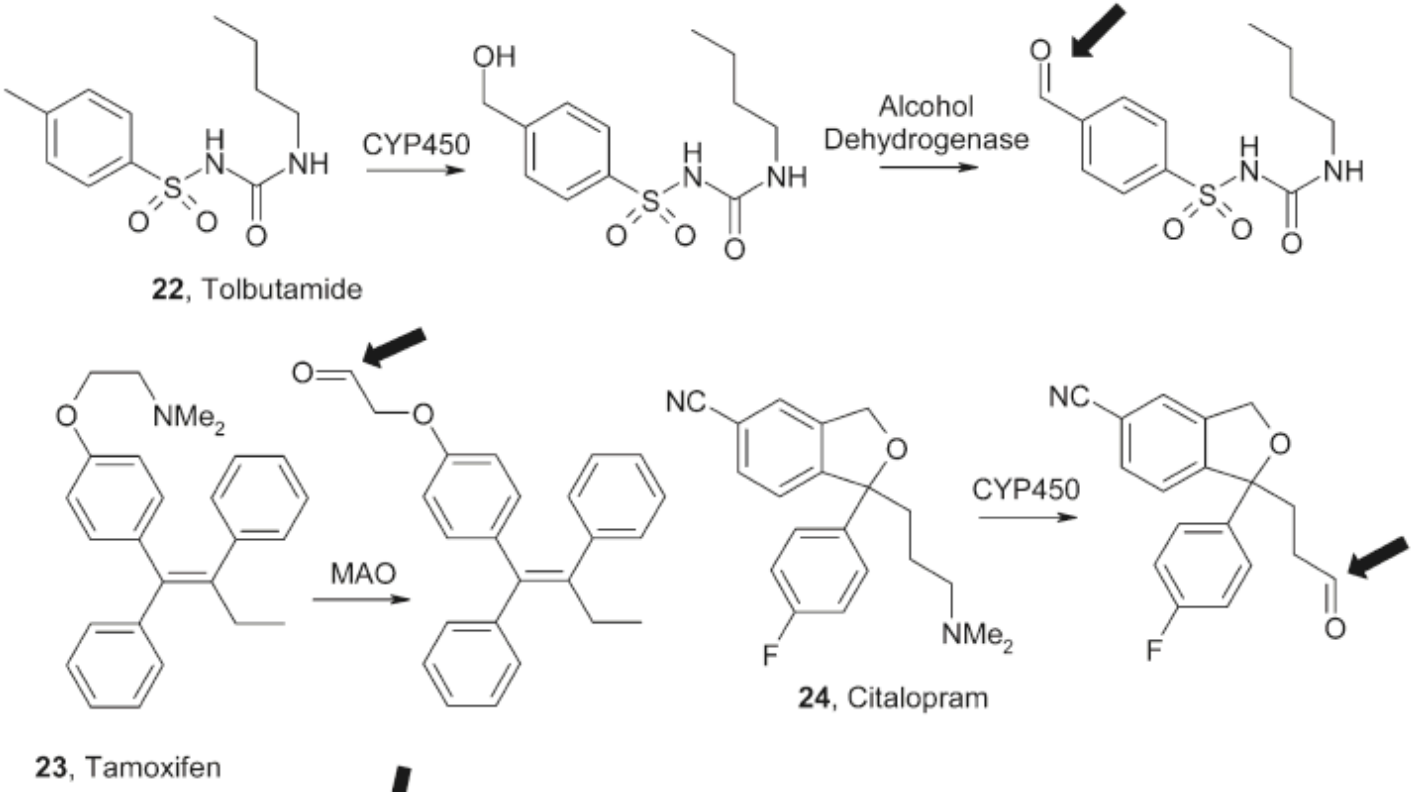




Metabolism of Aldehydes by AO

AO can catalyze the oxidation of aldehydes to carboxylic acids, and both endogenous (e.g. retinaldehyde) and xenobiotic aldehydes (below) may be substrates.
As with iminium ions, these processes can be sequential.

Note: AO-catalyzed oxidation of aldehydes *in vivo* may not be a highly significant process. Other enzymes like ALDHs and P450s may have a higher affinity for certain aldehydes.



Reductive Metabolism by AO (may not happen in vivo!)

AO and XO can catalyze reductive metabolism (in vitro) in the presence of a good electron donor such as xanthine (XO) and 2-hydroxypyrimidine or N-methylnicotinamide (both AO), and in the absence of air.

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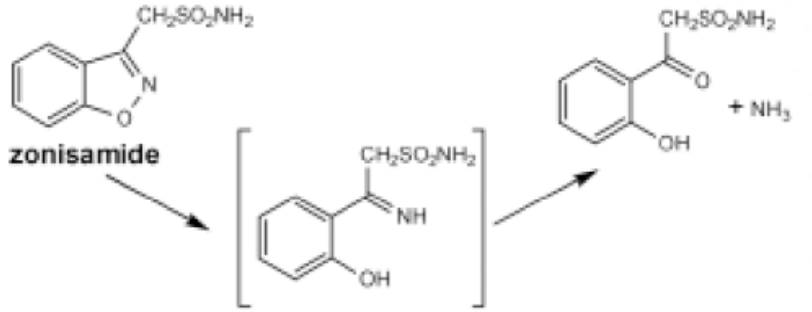
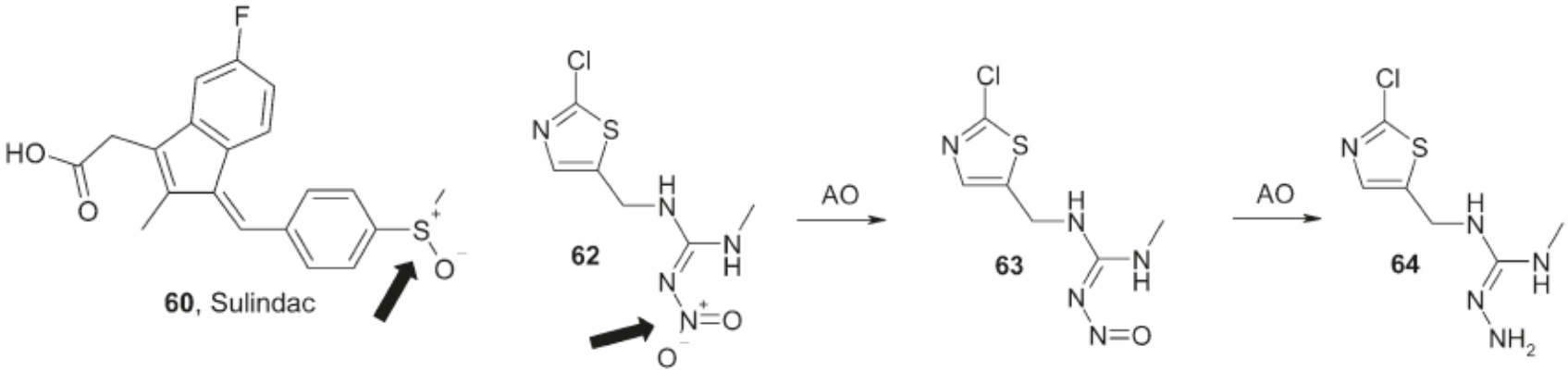


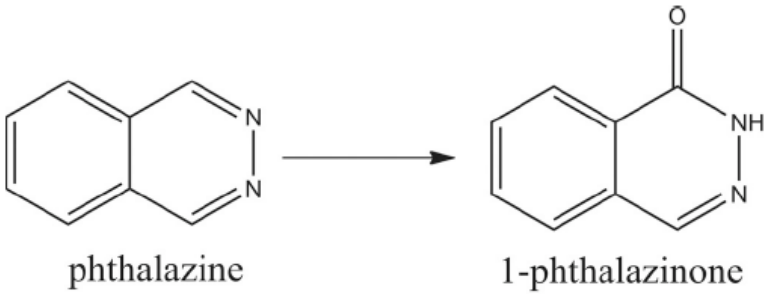


Prodrug activation

Two and 6-electron reductions!

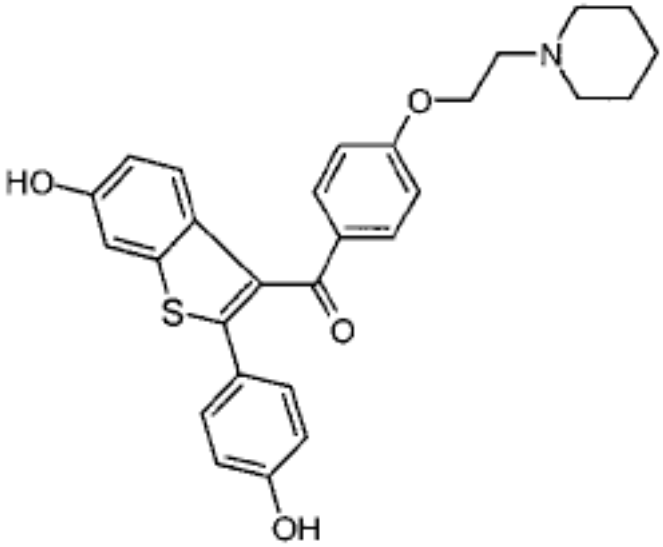
Substrate and inhibitor probes for AO

Pthalazine – substrate probe

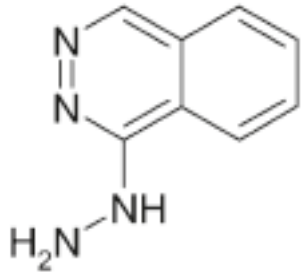


phthalazine 1-phthalazinone

Raloxifene – very potent(low nM), uncompetitive AO inhibitor, MBI for CYP3A4, so avoid in hepatocytes



Hydralazine – selective time-dependent inhibitor of AO. Low potency (Ki ~80 μM), but can use at 25 μM in hepatocytes to estimate fraction metabolized by AO with no effect on P450s (Strelevitz et al., DMD 2012).



Vanillin and Menadione are other 'classical' AO inhibitors

Inter-species, inter-organ and inter-individual variability

General dogma: AO activity is high in humans and monkeys, low in rodents, and absent in dogs.

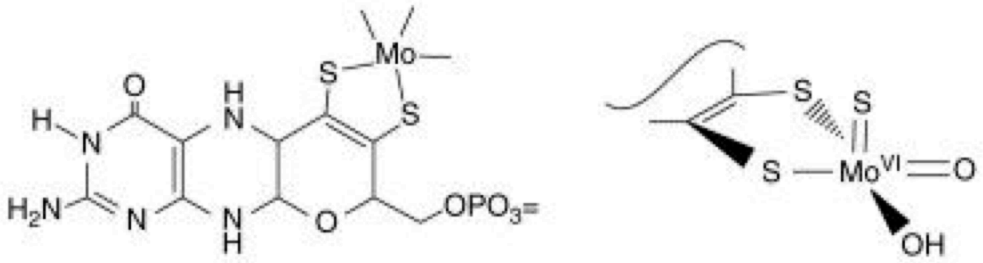
Caveats: Large strain difference in rats and mice. Gender differences in mice, with males exhibiting 3-4x activity of females.

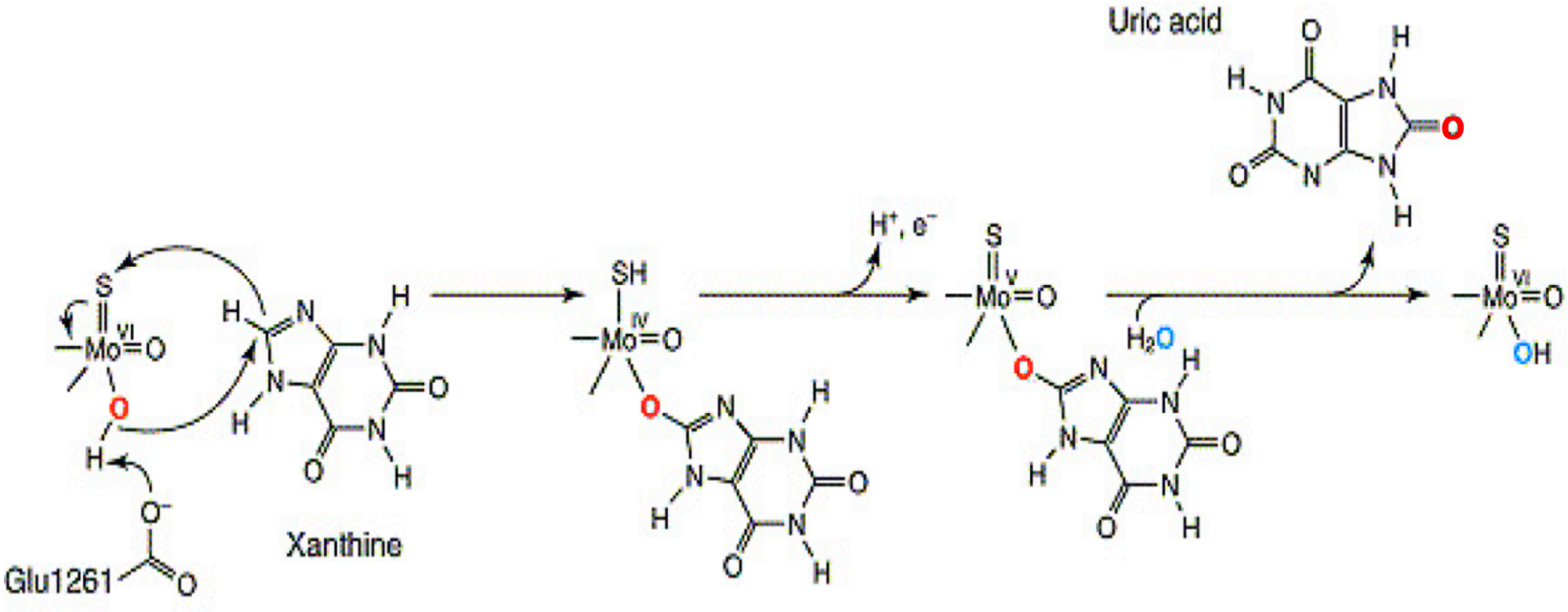
Human liver cytosolic activities: Efforts to scale in vitro activity to in vivo typically under-predict AO-mediated clearance.

- Is this due to enzyme lability after processing?
- To significant extra-hepatic AO catalysis?
- Or perhaps to the presence of dietary inhibitors in processed human liver cytosols?
- NB: XO activity low in processed human liver due to inclusion of allopurinol in perfusion solutions.

Catalytic Mechanism

- Active site contains the **exotic cofactor - molybdopterin.**
- Substrates (RH) react at the Mo (VI) centre.





- Catalysis is initiated by **base-assisted, nucleophilic** attack of the Mo^{VI}–**O**H group on the electron deficient carbon of the N-C bond, with **concerted hydride transfer** to the Mo=S group.
- Hydrolysis **of the Mo-O-C bond by water** releases oxidized substrate (R**O**H).

MAO: General Characteristics

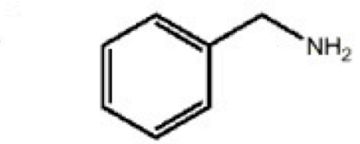
	FMO	AO	**MAO**
Location	Microsomal	Cytosolic	**Mitochondrial (outer membrane)**
Monomer Size	55-65 kDa	145 kDa	**50 kDa**
Catalytic center	FAD N-terminal, GxGxxG motif	Mo-pterin (plus an FAD-subunit)	**FAD C-terminal covalent cysteinyl**
Reducing 'Cofactor'	NADPH	Substrate	**Substrate**
Typical Amine Substrates	Tertiaryamines	N-Heterocycles	**Primary amines**
Xenobiotic induction	No	No	**No**
Tissue distribution	Liver Intestine	Liver Intestine	**Nerve terminals (presynaptic sympathetic) Liver, intestine**
Multiplicity (humans)	FMO 1-5	**AO (and XO)**	**MAO-A, MAO-B**

Examples of commonly used MAO substrates

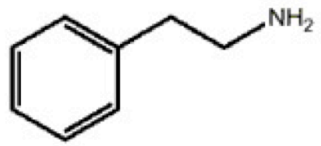
Primary amine neurotransmitters are typically MAO-A substrates

Xenobiotics, including environmental toxins, e.g. MPTP are often MAO-B substrates

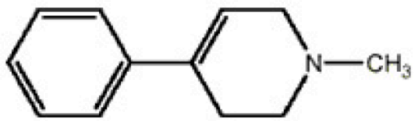
However, molecular size is a determinant, with MAO-A typically preferring larger molecules



BENZYLAMINE

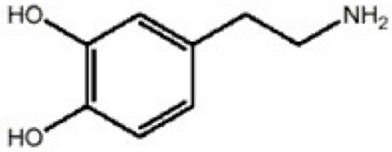


PHENETHYLAMINE

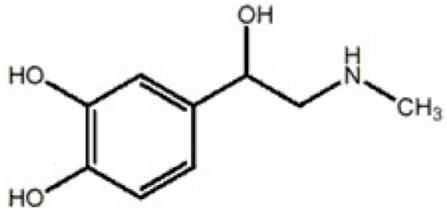


MPTP

SEROTONIN



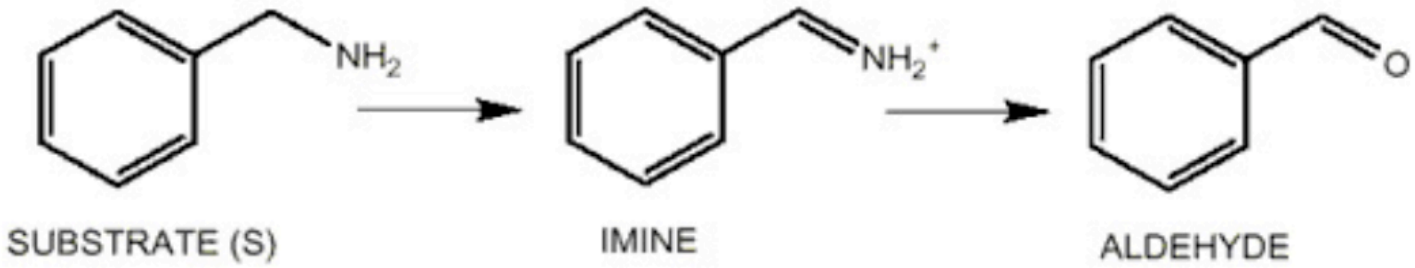
DOPAMINE

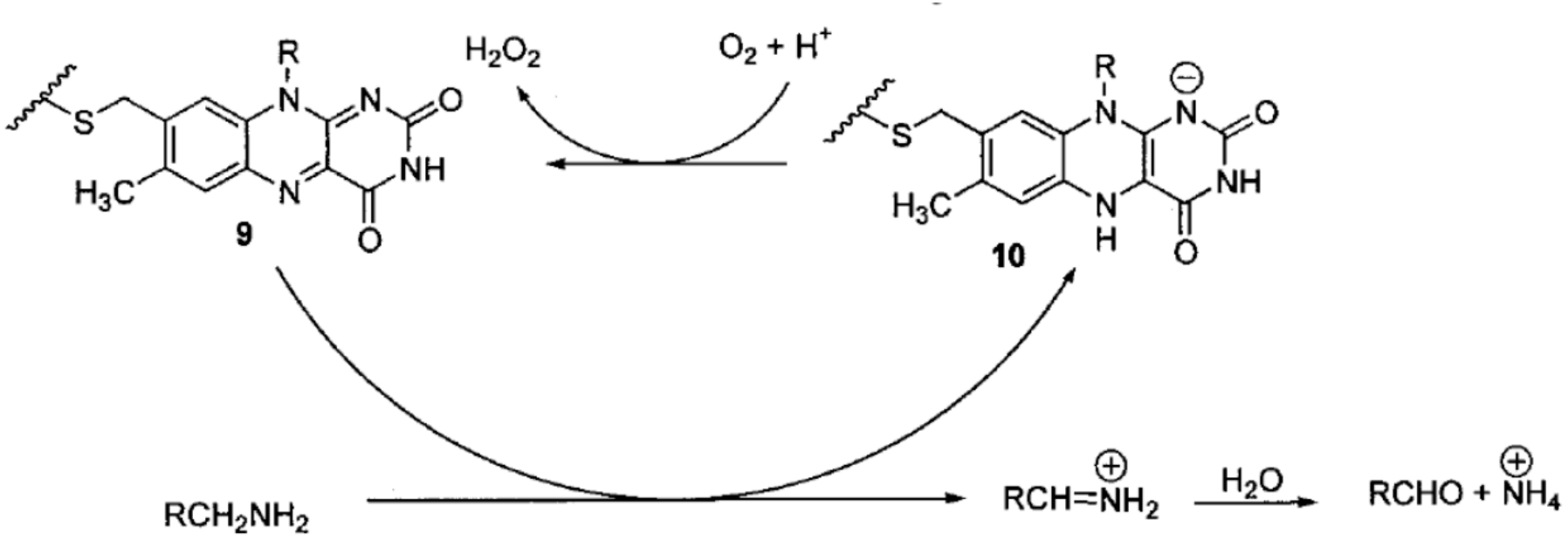


EPINEPHRINE (adrenaline)

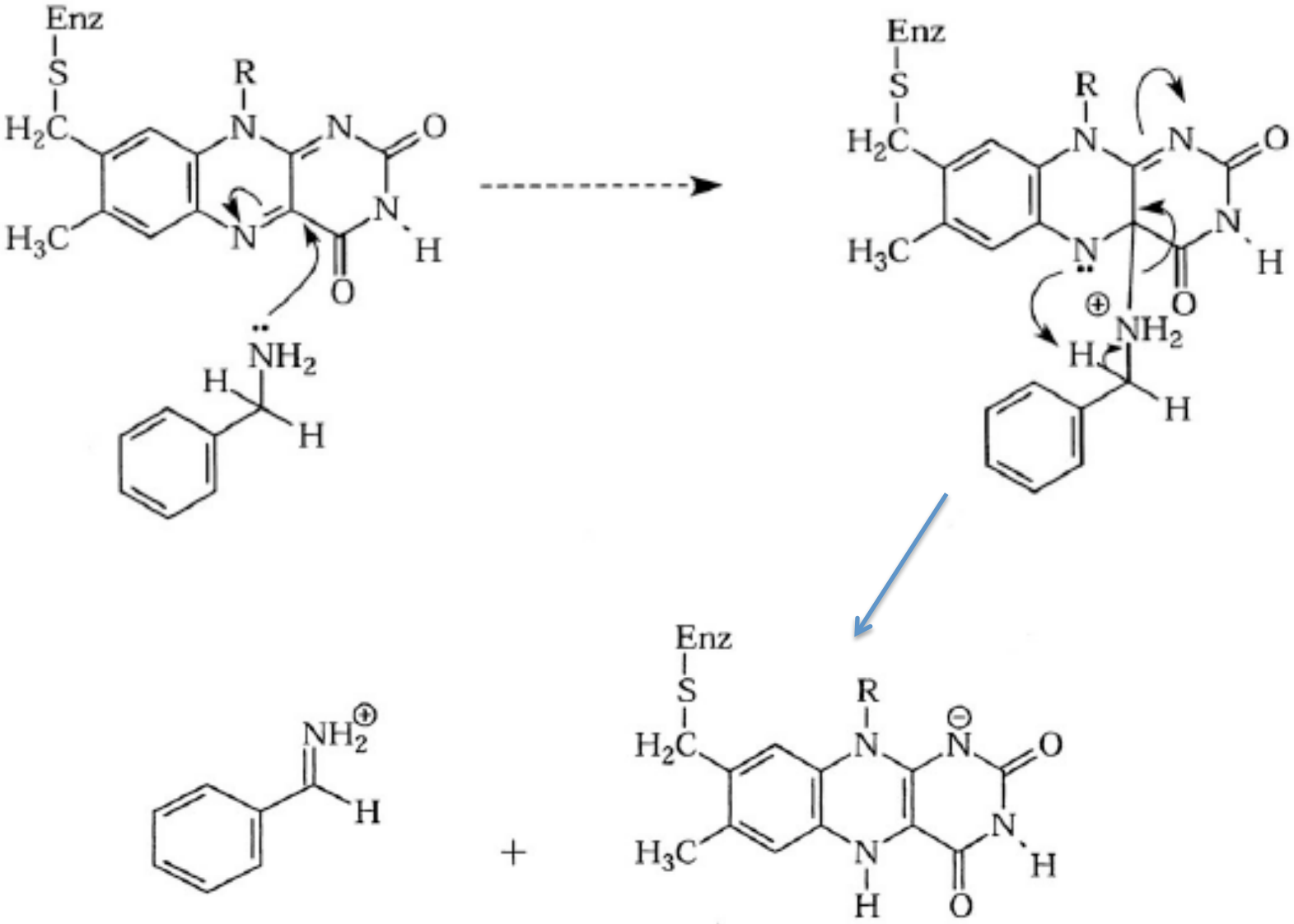
MAO catalyzes oxidative deamination (cleavage of C-N bond) to form aldehydes

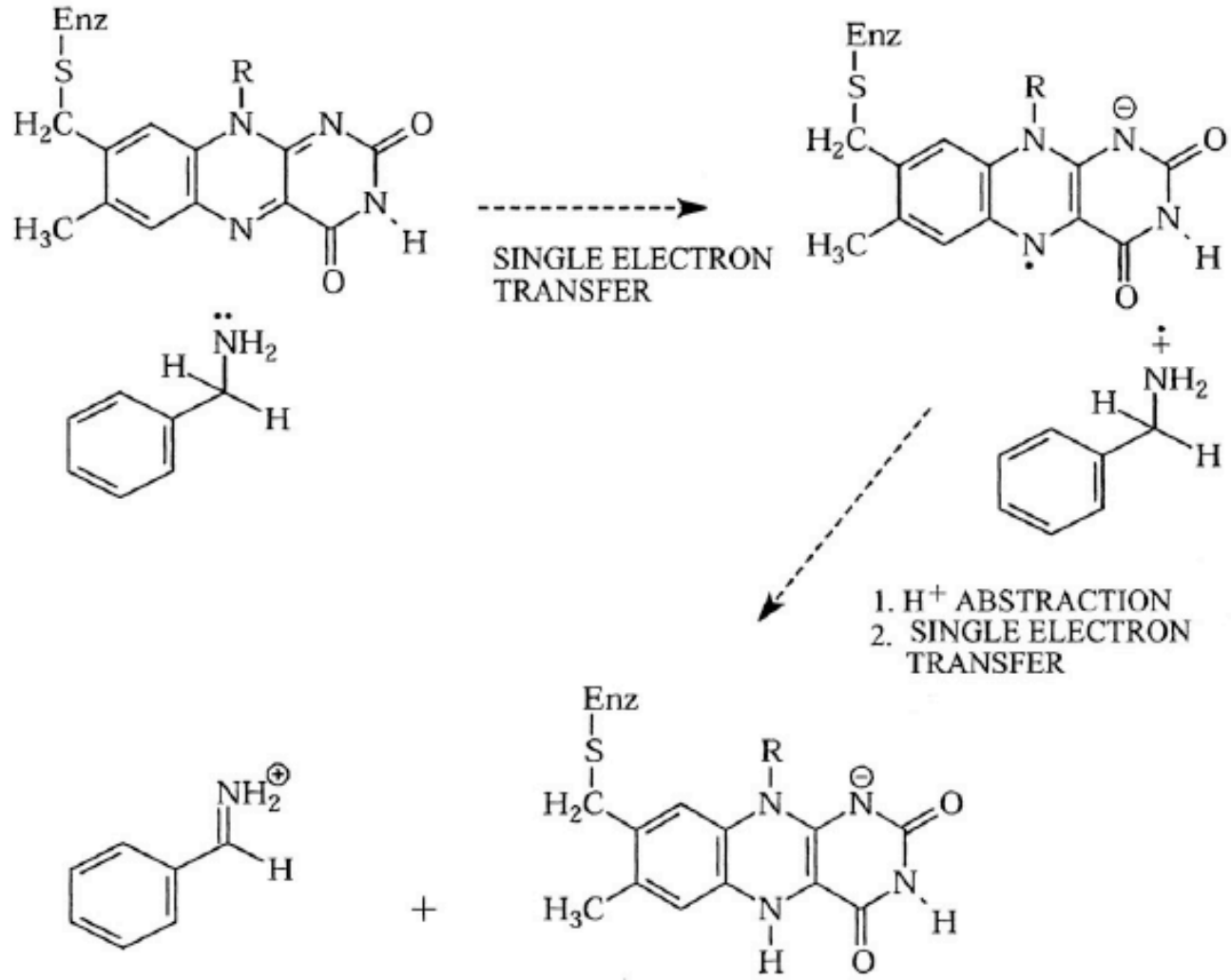
Oxidation of Amines to Imines by MAO-bound FAD





Concerted (polar) mechanism

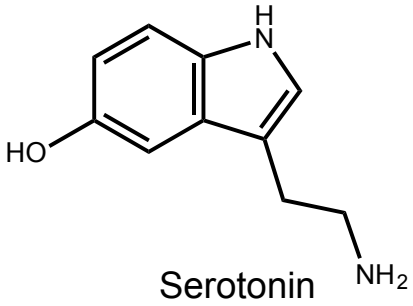




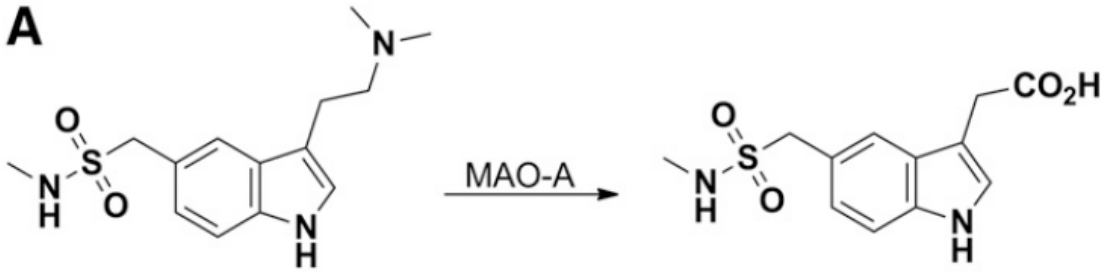
- This mechanism parallels the SET mechanism of P450-mediated N-dealkylation.
- Supported by MAO inactivation by cyclopropyl and cyclobutylamines.
- Hydrogen atom abstraction (HAT) mechanism(s) also proposed for MAO catalysis.

Metabolism of xenobiotics by MAO

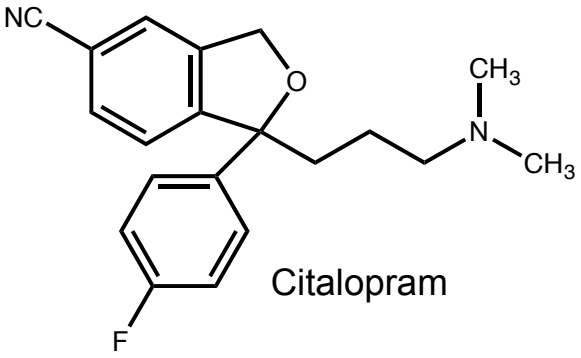
- Not very many examples,
  - drugs with moieties resembling the **indoleamine moiety of serotonin (5HT)**
    e.g. sumitriptan, citalopram, and MPTP - a neurotoxin bioactivated by MAO-B in the brain.



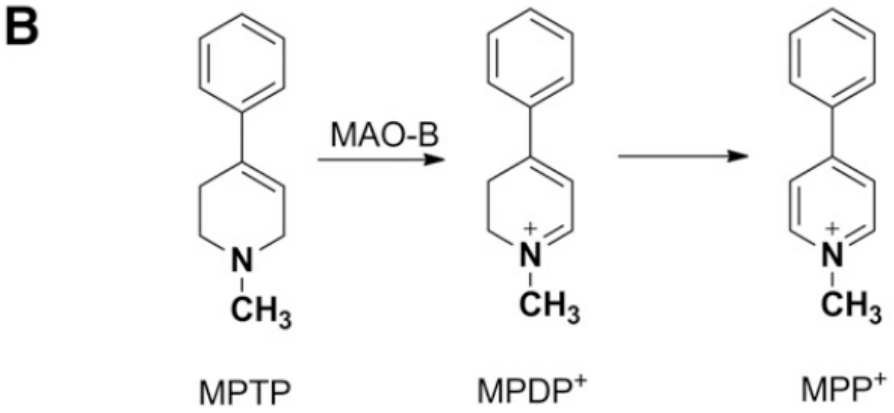
Serotonin



Sumatriptan



Citalopram

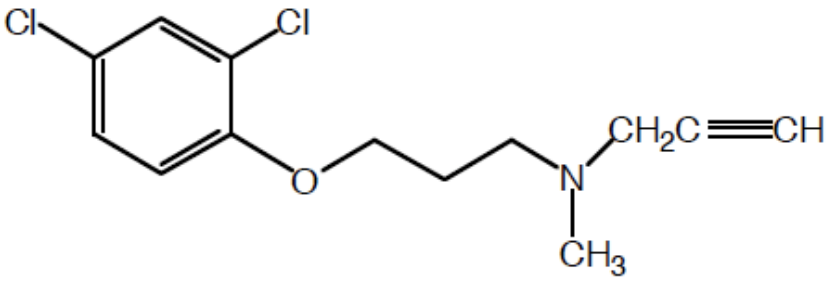


MPTP MPDP+ MPP+

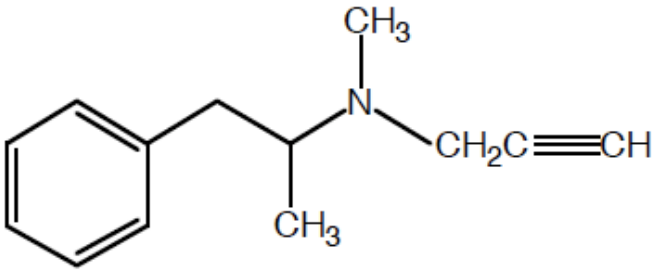
- NOVA – The Case of The Frozen Addict
  http://openvault.wgbh.org/catalog/V_474CF2C8A20B4173988486AC4C605A3C

Diagnostic MAO Inhibitors

MAO-A Clorgyline



MAO-B Deprenyl

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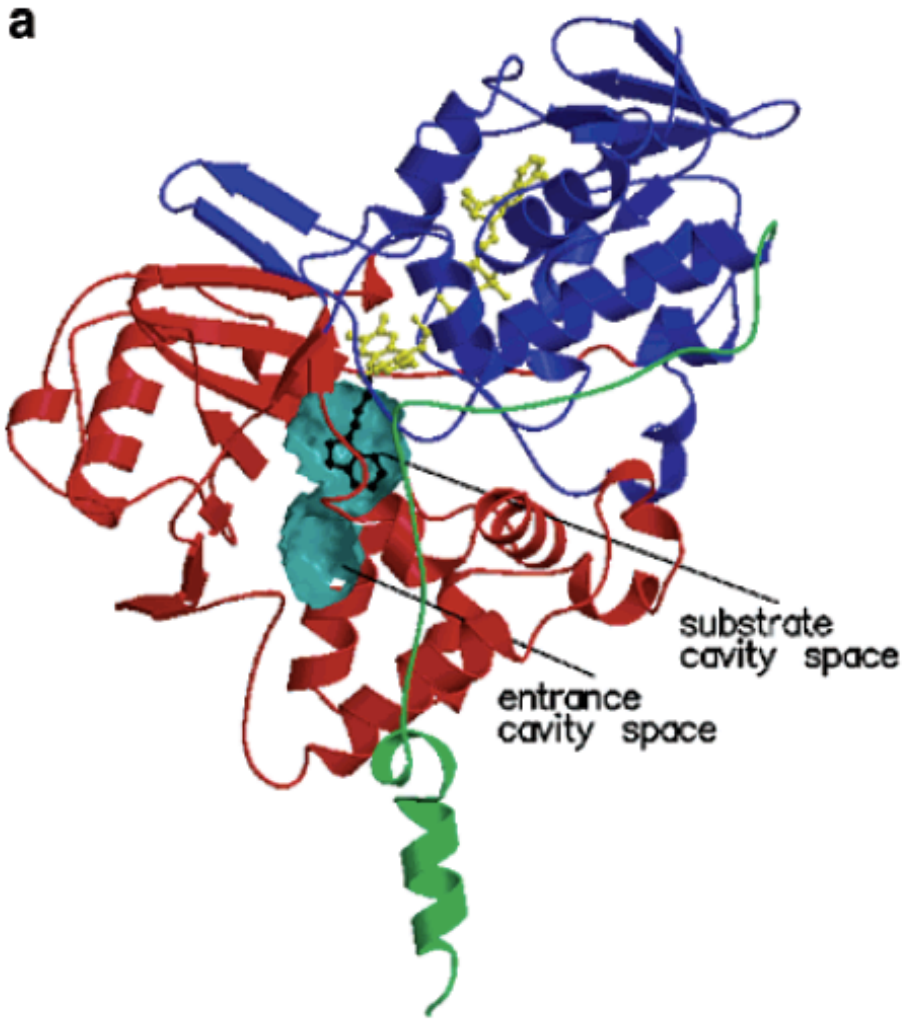
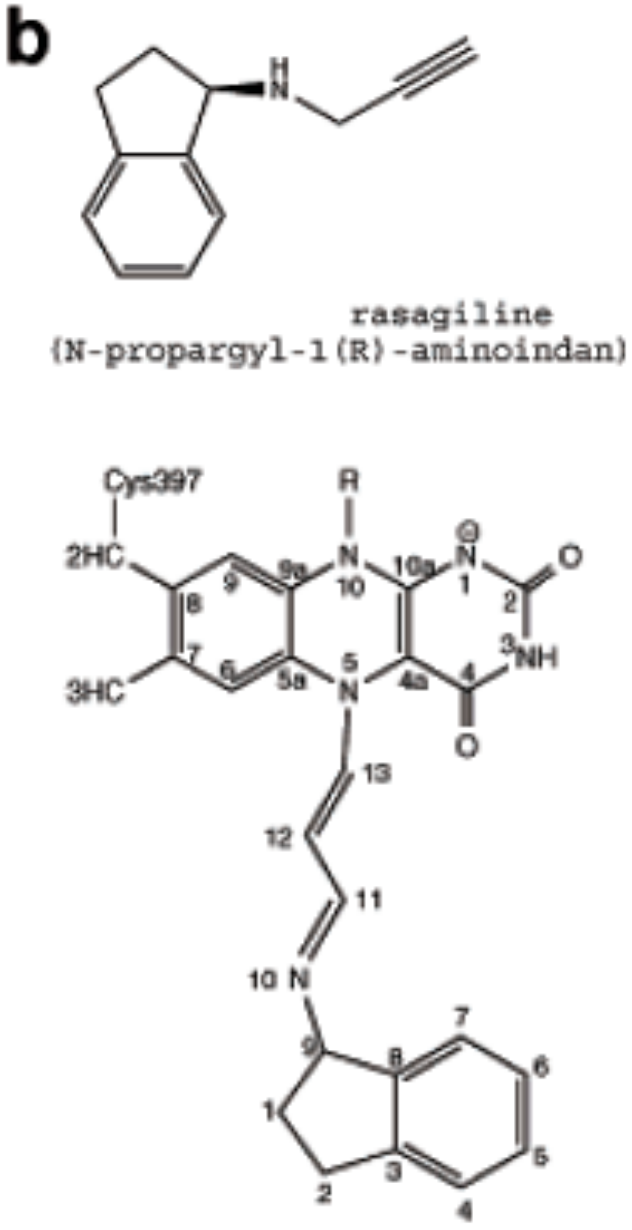
                          MAO A-I335
                          MAO B-Y326
Mouse MAO A   331 APISITLDDTKPDGSMPAIMGFILARKAERLAKLHKDIRKRKICE 375
  Rat MAO A   331 :::A:::::::::::L:::::::::::D:::::::::::::::: 375
Bovine MAO A  331 ::::::::::::::L:::::::::::D::::V:::::::::::: 375
Human MAO A   331 ::::::::::::::L:::::::::::D:::::::E::::K:::: 375
Trout MAO     324 :::GL:::::::::TV:::::::::::CRK:CG:T:EE::KR::: 368
Mouse MAO B   322 :::AY:::::::::TYA::::::::H::RK:VR:T:EE:L::L:: 366
  Rat MAO B   322 :::AY::::::::AGCA::::::::H::RK:VR:T:EE:L::L:: 366
Human MAO B   322 ::VAY:::::::E:NYA::::::::H::RK::R:T:EE:LK:L:: 366
                        *                   *     *  * *
```

- Structural basis for inhibitor (and substrate) selectivities is the Y→I change shown above (Geha, RM et al., JBC, 2001).
- Crystal structures are available for the two enzymes to help further rationalize ligand selectivities (Milczek et al., *FEBS J* (2011).

Mechanism-based inactivation of MAO by acetylenes





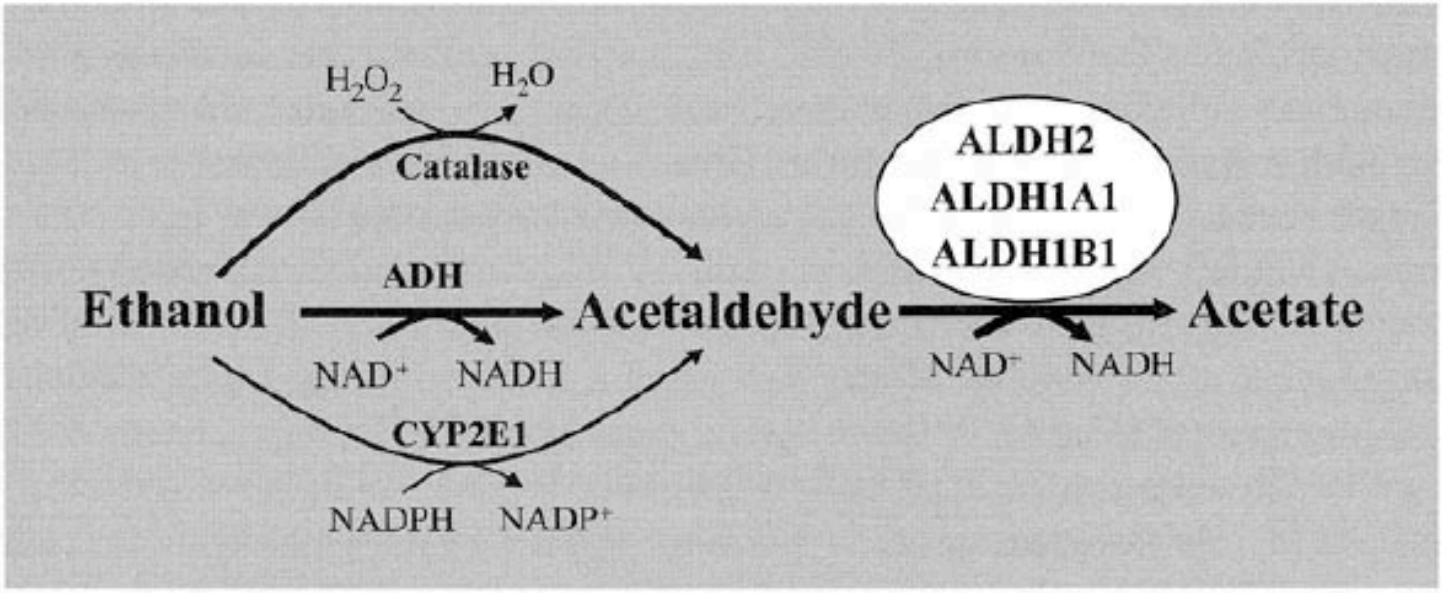
Covalently bound to the N-5 position on FAD

Bindi et al., *J.Med Chem*, 2004

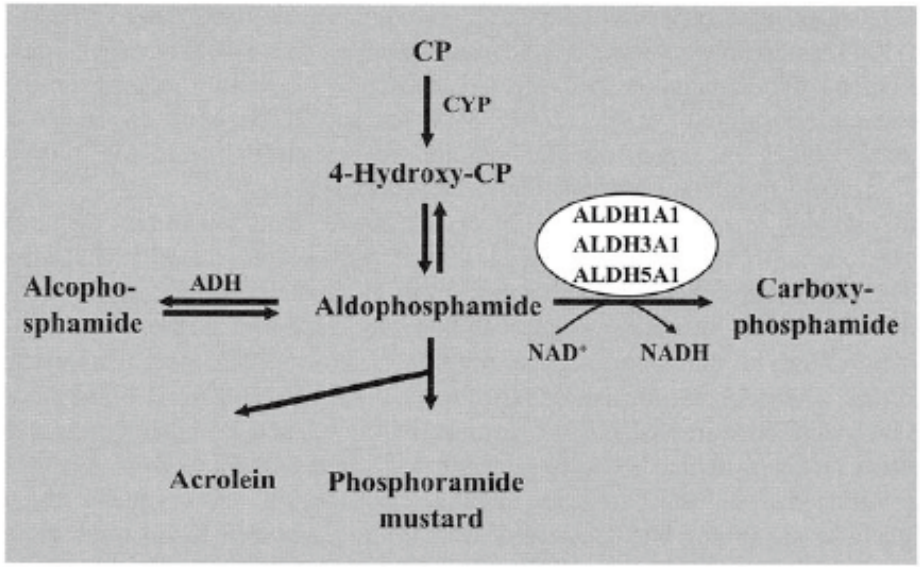
ALDEHYDE DEHYDROGENASE

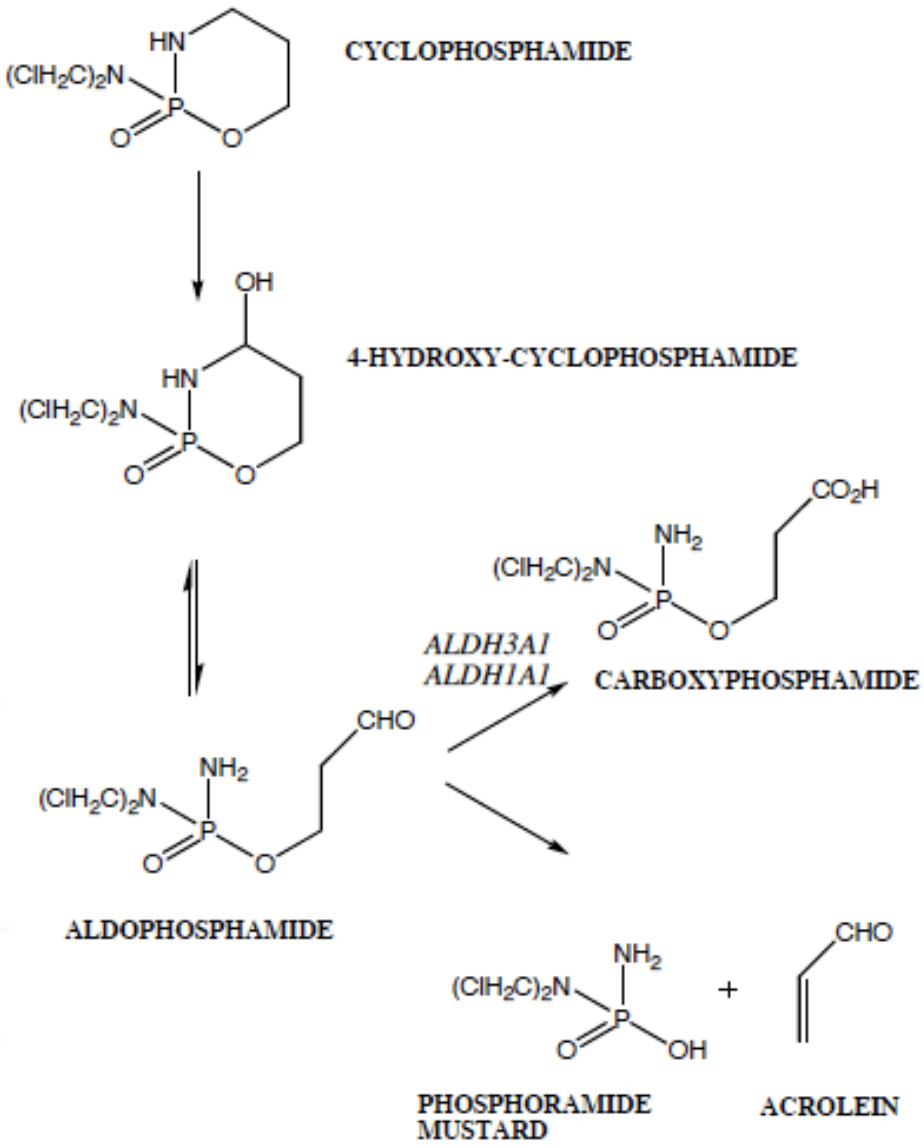
- ALDHs catalyze the NAD^+-dependent irreversible oxidation of aldehydes.
- R-CHO ----> $R\text{-}CO_2H$
- Catalytic cysteine residue, oxygen comes from activated water molecule
- Homotetramer, 4 subunits of 55kDa
- >20 ALDH genes coding for enzymes with with broad substrate specificities exist in the human genome.
- ALDH1-3 are most closely associated with xenobiotic metabolism.

ALDH2 - Mitochondrial form, mainly responsible for the metabolism of ethanol-derived acetaldehyde.



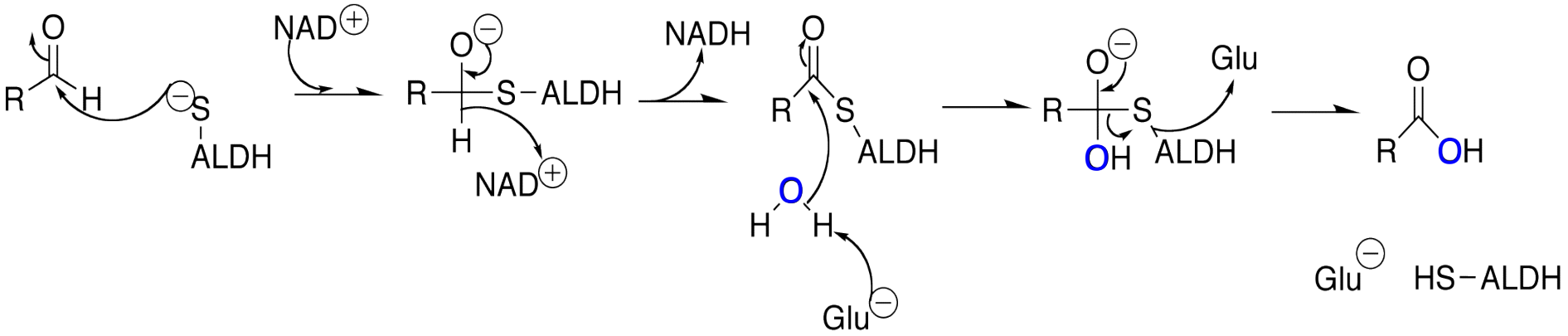
ALDH3A1 is expressed at high levels in tumors and, form that of the enzyme that, alongwith ALDH1A1/5A1, may determine cellular response to the anticancer drug, cyclophosphamide (CP)





ALDH Catalytic Mechanism

- Catalytic cysteine residue
- New oxygen atom in acid metabolite comes from water



CHEAT SHEET FOR NON-P450 ENZYMES

Cofactors, substrates, inhibitors, and primary tissue locations for additional drug-metabolizing enzymes

Enzyme	Cofactor	Examples of Substrates	Examples of Inhibitors	Tissue Location
FMO (FMO1, FMO3, FMO5)	NADPH/FAD	Benzydamine, clozapine, imipramine, tamoxifen, nicotine, voriconazole, sulindac sulfide	Methimazole	Kidney, intestine, fetal liver (FMO1); liver, lung, kidney (FMO3); liver (FMO5)
AO	Molybdenum pyanopterin	Allopurinol, citalopram carbazeran, tamoxifen metabolites	Hydralazine, chlorpromazine, isovanillin	Liver, lung, kidney, small intestine
XO	Molybdenum pyanopterin	1-Methylxanthine, allopurinol	FYX-051, febuxostat	Liver, heart, lung, adipose, mammary gland
MAO (MAO-A, MAO-B)	FAD	5-Hydroxytryptamine and epinephrine (MAO-A); benzylamine and β-phenylethylamine (MAO-B)	Moclobemide, clorgyline (MAO-A)	Liver, placenta, brain
		Sertraline and clomipramine (both)	Deprenyl (MAO-B)	
CES	None	Cocaine, methylphenidate, meperidine	Benzil, trifluoromethyl ketones, organophosphorus compounds	Liver
Epoxide hydrolase (sEH and mEH)	None	Carbamazepine and styrene oxide (mEH); epoxyeicosatrienoic acids (sEH)	1,1,1-Trichloropropylene oxide (mEH)	Liver
			Valproic acid	
Aldo-ketoreductase (multiple isoforms)	NADPH	Haloperidol, ketotifen, oracin	NSAIDs	Liver, kidney, brain, blood
Sulfotransferase (multiple isoforms)	PAPS	Acetaminophen and troglitazone (1A1); salbutamol and dobutamine (1A3); ethynylestradiol (1E1); budenoside (2A1)	Pentachlorophenol	Liver, intestine, platelets, brain, kidney, endometrium, skin, prostate, placenta
glutathione transferase (multiple isoforms)	Glutathione	1-Chloro-2,4-dinitrobenzene, chlorambucil, melphalan	Ethacrynic acid, piriprost, indomethacin	Liver, kidney, lung, brain, skeletal muscle, heart, small intestine, spleen
N-Acetyltransferase (NAT1, NAT2)	Acetyl CoA	*p*-Aminobenzoic acid and *p*-aminophenol (NAT1); dapsone, sulfamethazine, procainamide (NAT2)	Acetaminophen, 5-iodosalicylic acid	Liver, esophagus, small intestine, stomach, colon, bladder, lung
Acyl-CoA synthetase	ATP, CoA	Ibuprofen, flunoxaprofen, clofibrate	Triacsin C, rosiglitazone	Liver, heart, adipose tissue
Methyltransferase (structure-dependent isoforms)	*S*-Adenosyl methionine	6-Mercaptopurine, 6-thioguanine, azathioprine, dopamine, captopril	Entacapone, tolcapone	Liver (adult and fetal), lung, kidney, small intestine