Lecture 3:

Cytochromes P450 enzymes (III):
polymorphism and QSAR

Flavin containing monoxygenases (FMO):
structure-function and catalytic cycle

Cytochromes P450: polymorphism

Drug Metabolising Enzymes: polymorphism

- Phase 1 enzymes (activation):
  - Cytochrome P450s
  - Monoamino oxidase
  - Microsomal flavin monooxigenase
  - *Alcohol dehydrogenase
  - *Aldehyde dehydrogenase
  - Esterase
  - Epoxid hydrolases
- Phase 2 enzymes (conjugation):
  - UDP-Glucuronosyltransferases UGTs
  - Glutathione-S-transferase GSTs
  - *Sulphotransferases SLTs
  - *N-acetyltransferases NATs

Inter-individual differences in drug metabolism

- Many factors: Genetic factors, Drug-drug interactions, Enzyme induction, Dietary factors, Inhibition, Disease

- Genetic polymorphism:

Enzyme	Mutation (major)	Consequence	Frequency in Caucasians
CYP2A6	Leu160His	Defect enzyme	5%
CYP2C9	Arg144Cys Ile359Leu	Impaired interaction with CPR Higher K_m	20% 6%
CYP2C19	Cryptic splice site in exon 5	No enzyme	10%
CYP2D6	Splice defect in 4/ex5 junction	No enzyme	23%
CYP2E1	Arg76His	Much less enzyme	1%

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Toxicology Letters

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Genetic variability in susceptibility and response to toxicants

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Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future

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Table 1. Relative importance of polymorphisms in human cytochrome P450 enzymes involved in drug metabolism

Enzyme	Fraction of drug metabolism (%)[a]	Substrates	Major allelic variants[b]	Clinical effects of the polymorphism	Significance of the polymorphism[c]
CYP1A2	5	Drugs, carcinogens	*CYP1A2*1K*	Less enzyme expression and inducibility	+
CYP2A6	2	Nicotine, drugs, carcinogens	*CYP2A6*4, CYP2A6*9*	Altered nicotine metabolism	+
CYP2B6	2–4	Drugs	–	Significant for the metabolism of cancer drugs	+
CYP2C8	1	Drugs	*CYP2C8*3*	Altered taxol metabolism	+
CYP2C9	10	Drugs	*CYP2C9*2, CYP2C9*3*	Drug dosage[d]	+++
CYP2C19	5	Drugs	*CYP2C19*2, CYP2C19*3*	Drug dosage[d], drug efficacy	+++
CYP2D6	20–30	Drugs	*CYP2D6*2x*n	No response	+++
			*CYP2D6*4*	Drug dosage[d]	+++
			*CYP2D6*10*	Drug dosage[d]	+
			*CYP2D6*17*	Drug dosage[d]?	+
			*CYP2D6*41*	Drug dosage[d]?	+
CYP2E1	2–4	Carcinogens, solvents, drugs	–	No conclusive studies	–
CYP3A4	40–45	Drugs, carcinogens	Rare	No conclusive studies	–
CYP3A5	<1	Drugs	*CYP3A5*3*	No conclusive studies	–

[a]The estimated fraction of responsibility of the respective enzyme for drug metabolism in phase 1 reactions as a percentage of drugs metabolized by all cytochrome P450 enzymes. Data are from Bertz and Granneman [1], Relling and Evans [2] and M. Ingelman-Sundberg, unpublished.

[b]A description of the alleles can be found on the human cytochrome P450 allele nomenclature committee home page (http://www.imm.ki.se/CYPalleles/).

[c]The significance of the polymorphism is based on the number of reports showing impact of the P450 polymorphism on the pharmacokinetics of drugs that are substrates for the enzyme in question. Increasing numbers of '+' illustrate the increasing importance of the polymorphism relative to the other forms of P450.

[d]The dose of the drug is advantageously adjusted depending on the genotype with respect to the individual enzyme.

- 4 phenotypes can be identified:
  - PM = Poor Metabolizers:
    - Lack the functional enzyme
  - IM = Intermediate Metabolizers
    - Heteozygous for one deficient allele or carry two alleles that cause decreased activity
  - EM = Extensive Metabolizers
    - Have 2 normal alleles
  - UM = Ultra-rapid Metabolizers
    - Have multiple gene copies, trait that is dominantly inherited

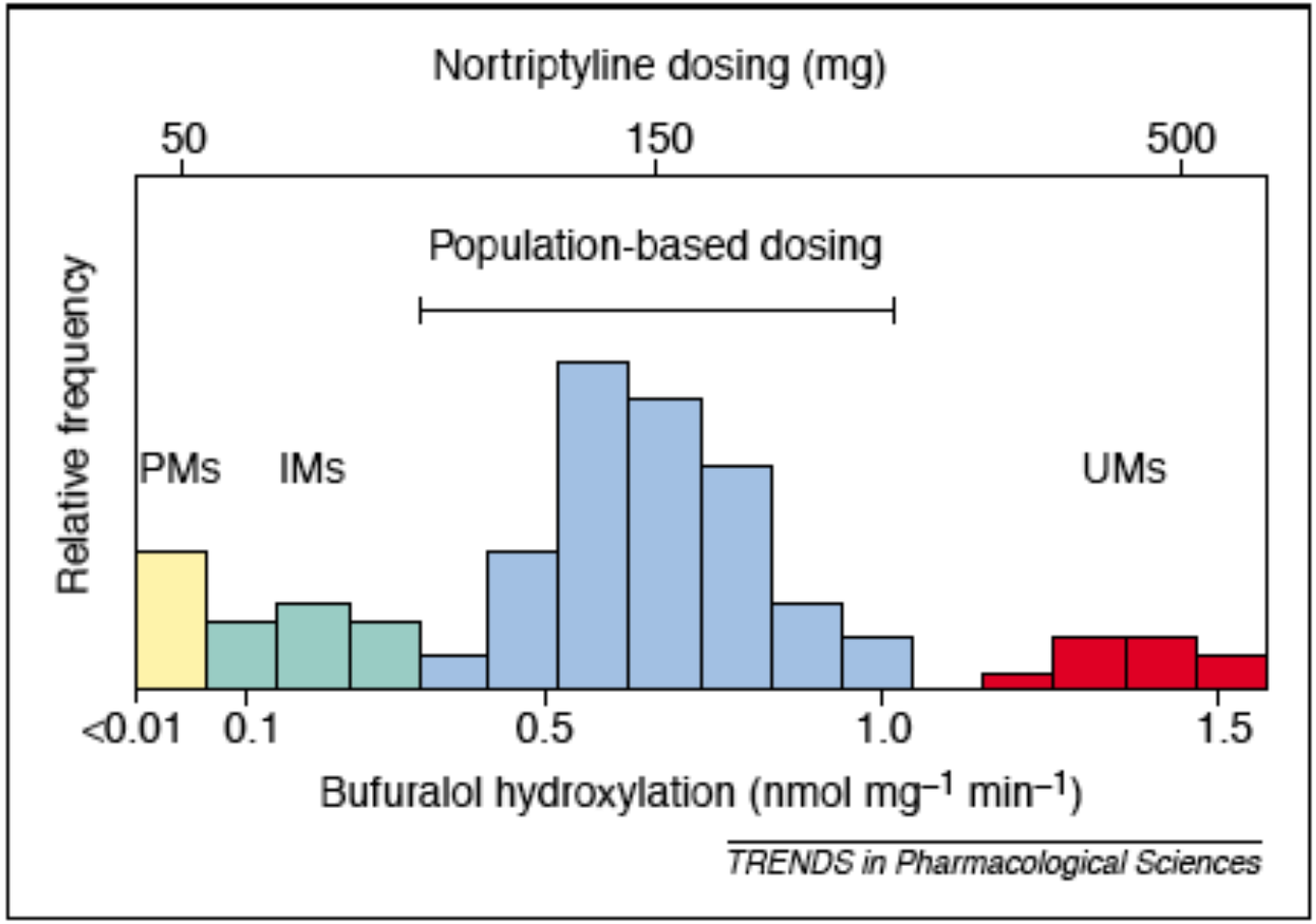


Figure 1. Variation in drug metabolism and nortriptyline dosing in the European population, based on cytochrome P450 CYP2D6 activity (hydroxylation of bufuralol). Within the population four phenotypes can be identified: poor metabolizers (PMs), who lack the functional enzyme; intermediary metabolizers (IMs), who are heterozygous for one functional allele or have two partially defective alleles encoding the enzyme; extensive metabolizers (EMs), who have two normal alleles; and ultrarapid metabolizers (UMs), who carry duplicated or multiduplicated functional *CYP2D6* genes. The relative frequency of these phenotypes refers to the European population as a whole. The doses of nortriptyline that are required to achieve therapeutic levels in all phenotypes are given. Despite this variation in metabolizing capability, population-based dosing is used today, and is based on the average plasma levels obtained in a given population for a given dose. Figure is adapted from Zanger *et al.* [84] and extrapolated to the whole European population.

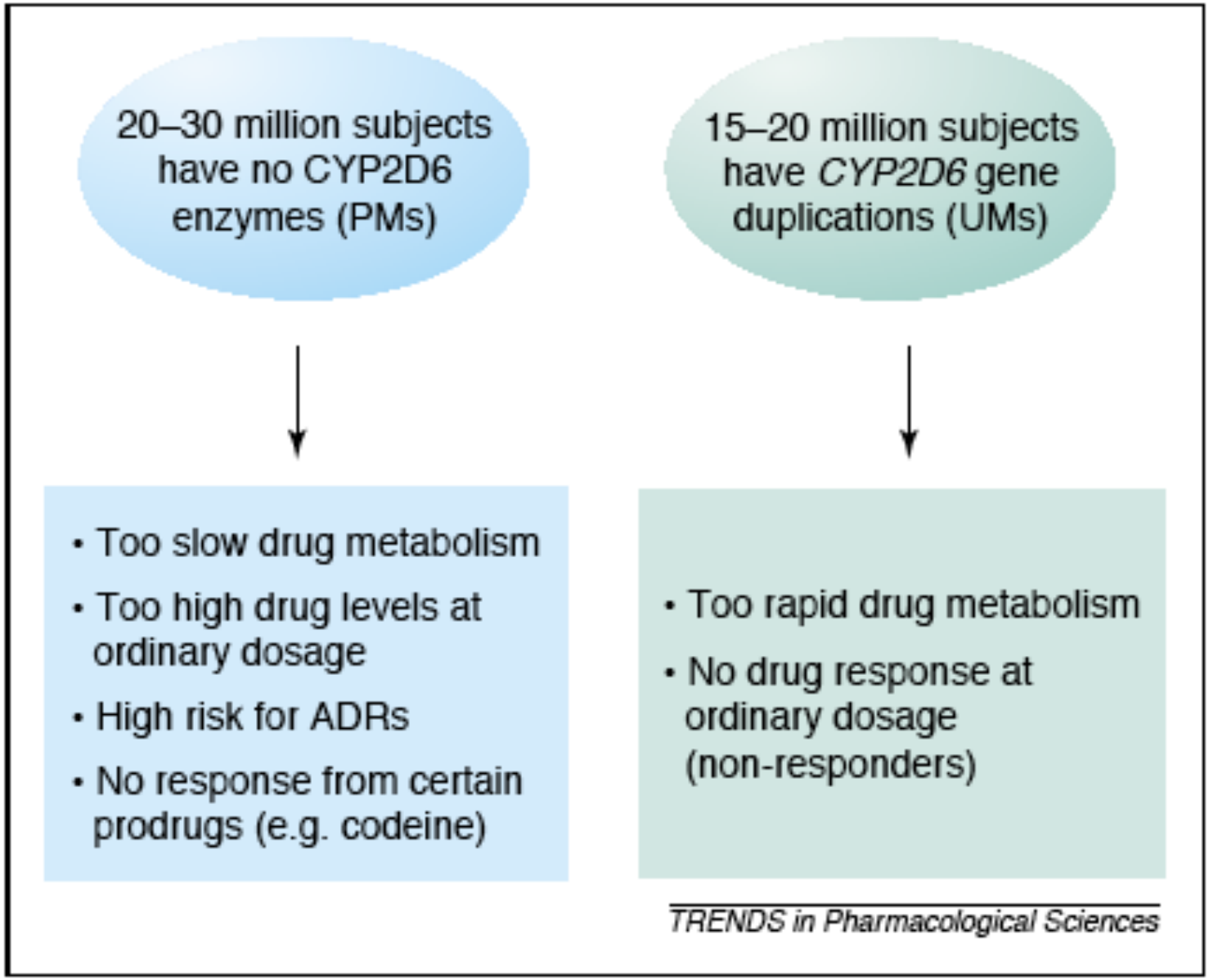


Figure 2. The consequences of outlier cytochrome P450 CYP2D6-dependent drug metabolism. 35–50 million Europeans are either poor metabolizers (PMs) (i.e. lack the functional enzyme) or ultrarapid metabolizers (UMs) (i.e. have multiple gene copies of *CYP2D6*, resulting in elevated enzyme levels), with respect to CYP2D6. As a result of the use of population-based dosing, drug treatment can result in many different effects in these subjects. Abbreviation: ADRs, adverse drug reactions.

Table 2. Examples of the clinical impact of cytochrome P450 pharmacogenetics[a]

Disease	Enzyme	% of dose[b]		Examples
		UMs	PMs	
Depression	CYP2C9	–	–	Bipolar disorders and valproate
	CYP2C19	–	40	PMs and SSRIs
	CYP2D6	200	30	Non-responders (UMs) and side-effects of tricyclic antidepressants (PMs)
Psychosis	CYP2D6	160	30	Haloperidol and parkinsonian side-effects; oversedation and perphenazine, thioridazine
Ulcer	CYP2C19	–	20	Dosing of PPIs; pH and gastrin changes
Cancer	CYP2B6	–	–	Cyclophosphamide metabolism
	CYP2D6	250	60	Non-response to anti-emetic drugs (UMs)
Cardiovascular	CYP2C9	–	30	Warfarin dosing (acenocoumarol); irbesartan and blood pressure response
	CYP2D6	160	30	Perhexiline neuropathy and hepatotoxicity
Pain	CYP2D6	–	–	Codeine, no response (PMs)
Epilepsy	CYP2C9	–	–	Phenytoin pharmacokinetics and side-effects

[a]Abbreviations: CYP, cytochrome P450; PMs, poor metabolizers; PPIs, protein pump inhibitors; SSRIs, selective serotonin reuptake inhibitors; UMs, ultrarapid metabolizers.
[b]The doses shown for depression and psychosis are weighted as related to the size of samples in all studies published, as reviewed by Kirchheiner *et al.* [55]. The other doses are based on data presented in the main text. All doses are percentages of the normal dose.

Cytochromes P450: QSAR

Structure-activity relationships of NCE

- Quantitative Structure-Activity Relationship (QSAR):
  - identifies the physico-chemical properties responsible for a biological activity.
  - relationship = equation that quantifies the effects
  - allows predictions on the effects of new substitutions over the biological activity.
- 3 main structural-physical-chemical charact. of a molecule:
  - hydrophobicity;
    - Crucial for membrane crossing = bioavailability
    - Measured by the partition coefficient P:

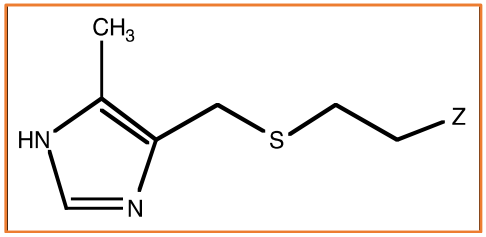
      P = [conc. mol. in octanol] / [conc. mol. in water]
  - electronic effects;
  - steric factors.

QSAR: hydrophobicity

- Relationship between hydrophobicity (P) and biol. activity (C, conc. of drug required for a given effect):

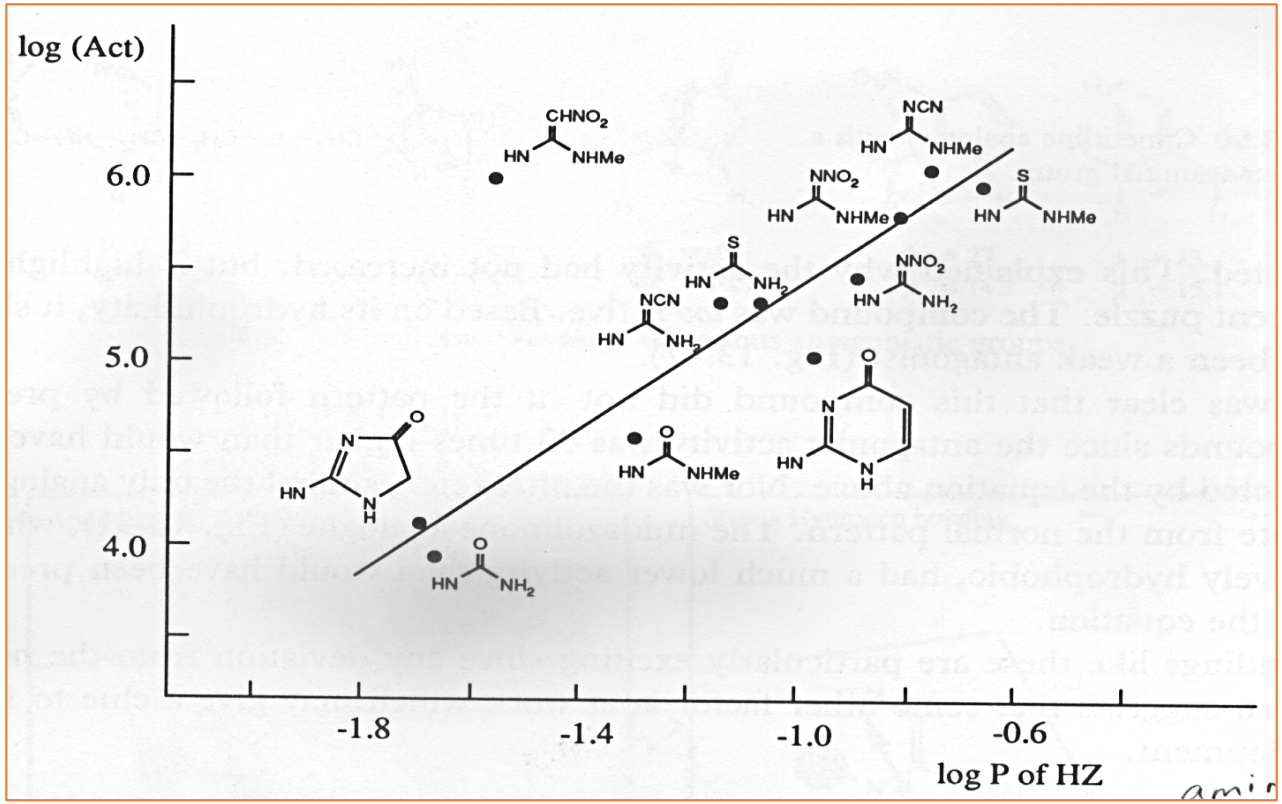
$$Log(1/C) = k_1 LogP + k_2$$

- Cimetidine: different planar amines (Z) were investigated:



The relationship was found to be:

Log(activity) = 2.0LogP + 7.4



Parabolic Log(1/C) *vs* Log P

- An optimum was found for butyl or pentyl substituent;
  - a benzyl substituent was found particularly active, but toxic in its side effects.
- The straight line is observed because of the narrow range of LogP values usually investigated.
  - In reality, the biological activity increases as LogP until a maximum is reached; beyond this point ($LogP^o$), an increase in LogP causes a decrease in activity:

$$Log(1/C) = -k_1(LogP)^2 + k_2 LogP + k_3$$

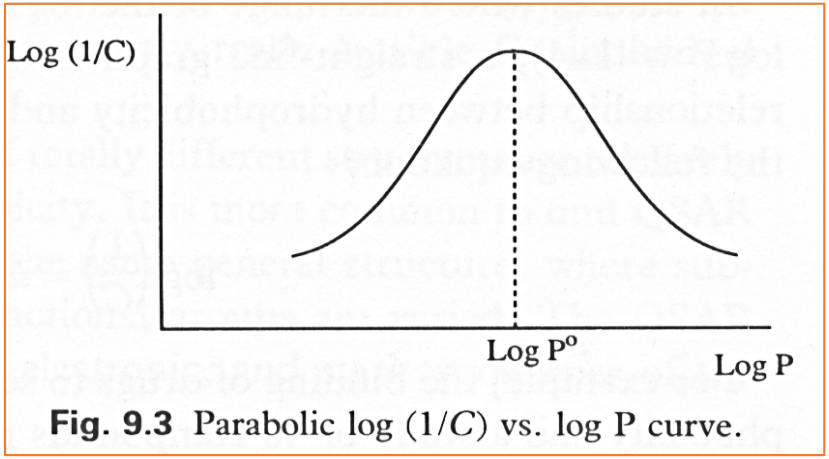


Fig. 9.3 Parabolic log (1/*C*) vs. log P curve.

P450 and QSAR

- The site of metabolism by P450s is determined by the disposition of the H-bonds donors/acceptors in the S
- LogP or $LogD_{7.4}$ takes into account molecular size, volume, surface and polarity.
  - $LogD_{7.4}$ is important for substrates containing ionizable groups; it contains LogP and pK_A carrying information on ionisation:

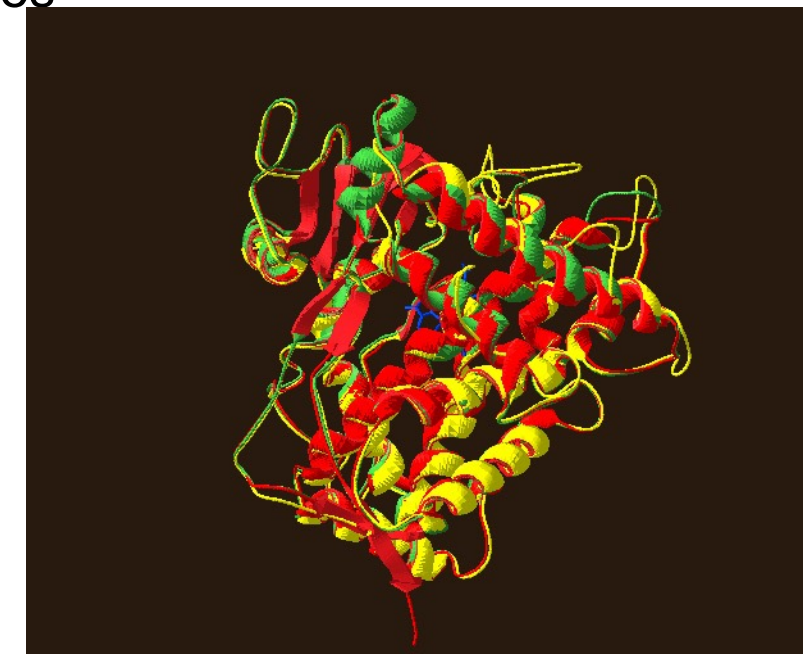
monoprotic acids: $LogD_{7.4} = LogP - Log(1 - 10^{pH-pK_A})$

monoprotic bases: $LogD_{7.4} = LogP - Log(1 - 10^{pK_A-pH})$

CYP	Average LogP	LogP range	Substrates close to average LogP
1A1	4.51	2.25-6.75	6-aminochrysene (4.98)
1A2	1.57	0.01-3.15	Phenacetin (1.57)
2A6	1.66	0.07-2.79	Losigamone (1.46)
2B1/6	2.53	0.23-5.14	Phenytoin (2.47)
2C9	3.15	1.56-5.18	Tienilic acid (3.15)
2C19	2.12	1.49-2.53	Moclobemide (2.13)
2D6	3.18	0.75-5.40	Dextromethorphan (3.36)
2E1	0.63	-1.35-3.15	Pyridine (0.65)
3A4	2.94	0.97-5.71	Cyclosporin A (2.92)

P450 active site: *in silico* predictions.

- QSAR would ideally provide a full correlation between:
  - 3D structure or model of the human enzymes
  - experimental K_m and $v_{max;}$
  - LogP and $LogD_{7.4}$ of the S;
  - map of the electronic properties of S



- This should allow to predict the products derived from metabolism of lead compounds.

- 3D structures of the human P450s is of paramount importance.

http://www.astex-technology.co.uk/servlet/astex

Flavin containing monoxygenases (FMO):

structure-function and catalytic cycle

Introduction hFMO

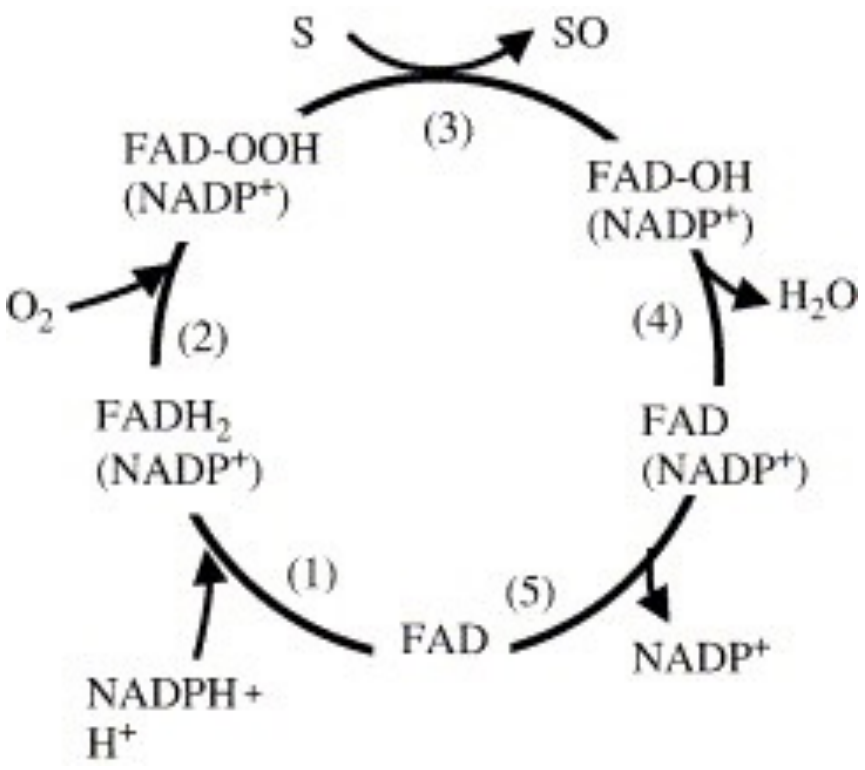
- Second most important family of monooxygenases in terms of drug metabolism
  - family of flavin (FAD) monoxygenases
  - Involved in metabolism of xenobiotics (drugs)
  - Catalyse the NADPH-dependent oxygenation of soft nucleophiles
  - No crystal structures available
  - 5 different isoforms, most important one is FMO3
    - Present in adult liver
    - Membrane-bound

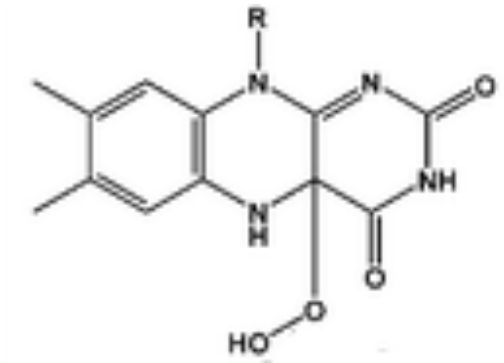
Properties

- Phase I drug metabolising enzyme
- Microsomal like CYPs
- NADPH dependent enzyme
  - FAD co-factor
- Oxidation of nucleophillic heteroatom contaning small molecules
  - soft centres such as nitrogen and sulfur i.e. N-oxidation and S-oxidation
- Cannot oxidise carbon – not as powerful as CYPs
- 5 genes (FMO 1-5) and 6 pseudogenes in humans

Model of human FMO1 showing FAD (pink) and NADPH (green) bound

Reaction mechanism



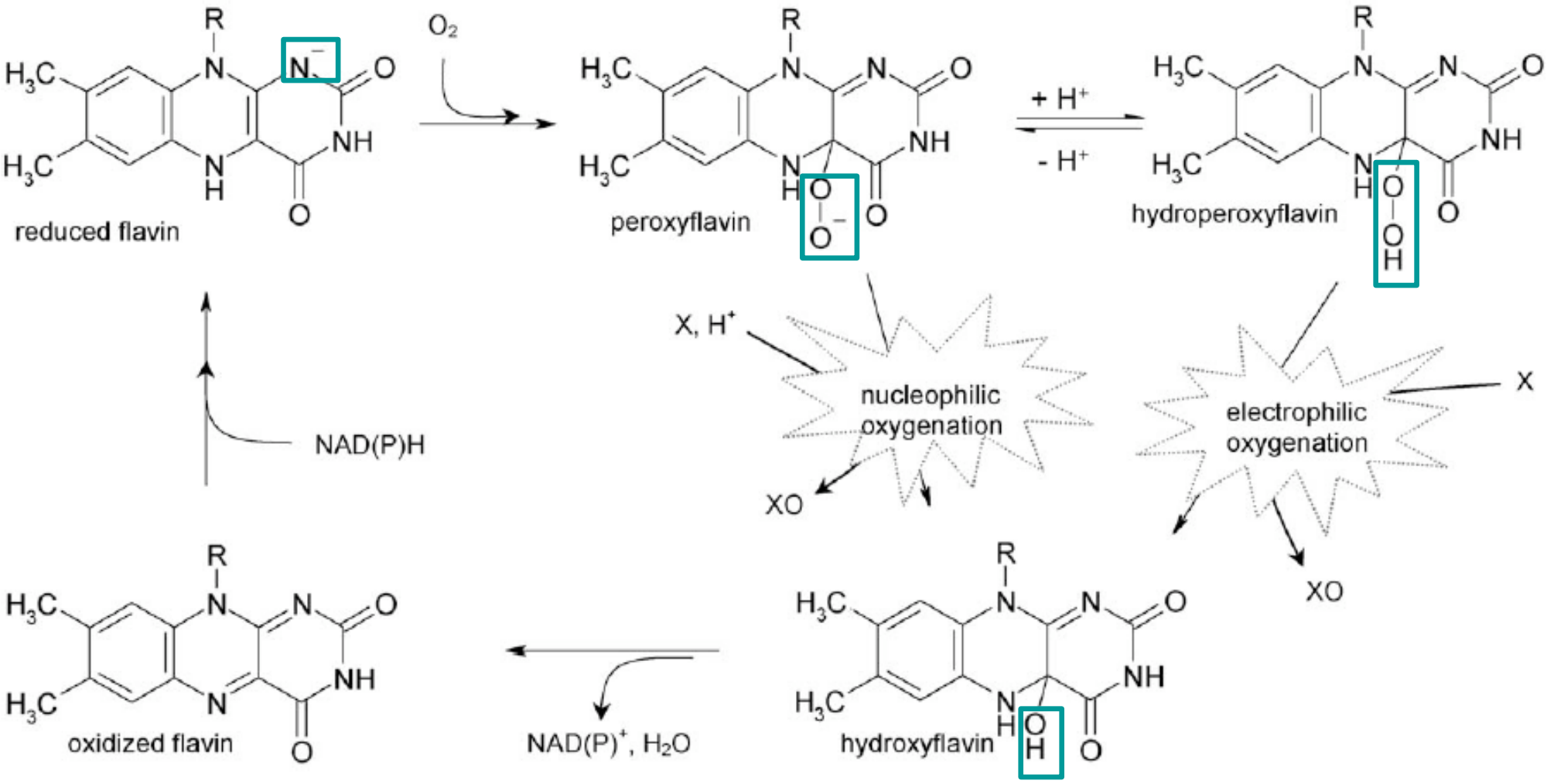


C4a-hydroperoxyflavin

"Loaded gun"

- Enzyme is reduced by NADPH and binds oxygen to form a stable C4a-hydroperoxyflavin prior to substrate binding
- Substrate spends very little time in active site
  - Higher turn-over number than human CYPs
- C4a-hydroperoxyflavin stable unlike compound I of P450s
  - Protein environment prevents decomposition of hydroperoxyflavin ?
  - Minimises uncoupling and formation of reactive oxygen species
  - Conservation of NAPDH but unproductive cycles can occur

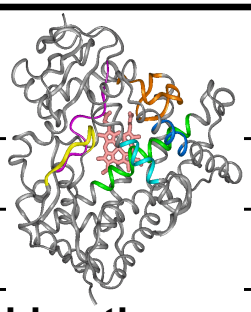
Catalytic cycle

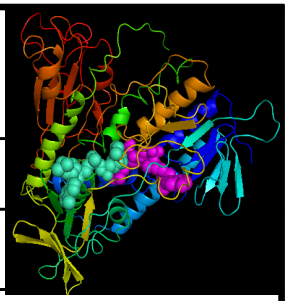


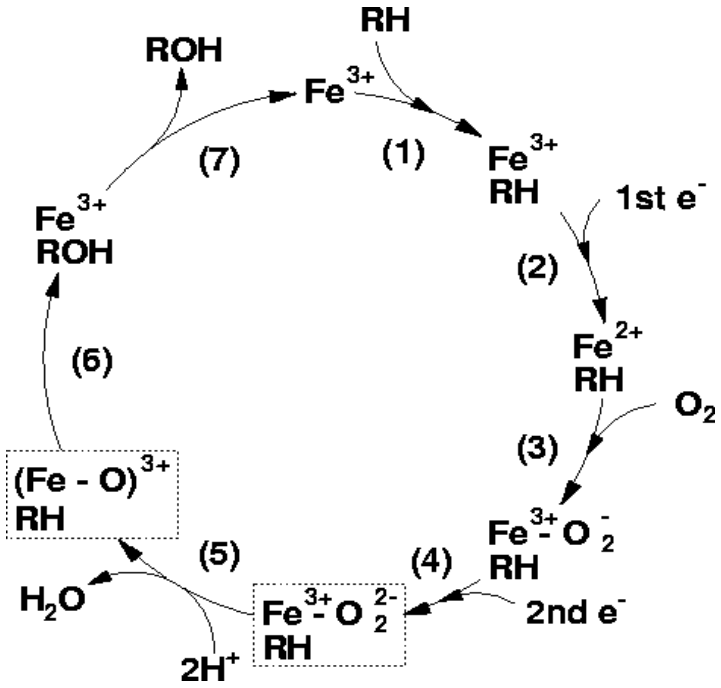
Ref: W.J.H. van Berkel et al. Journal of Biotechnology 124 (2006) 670–689

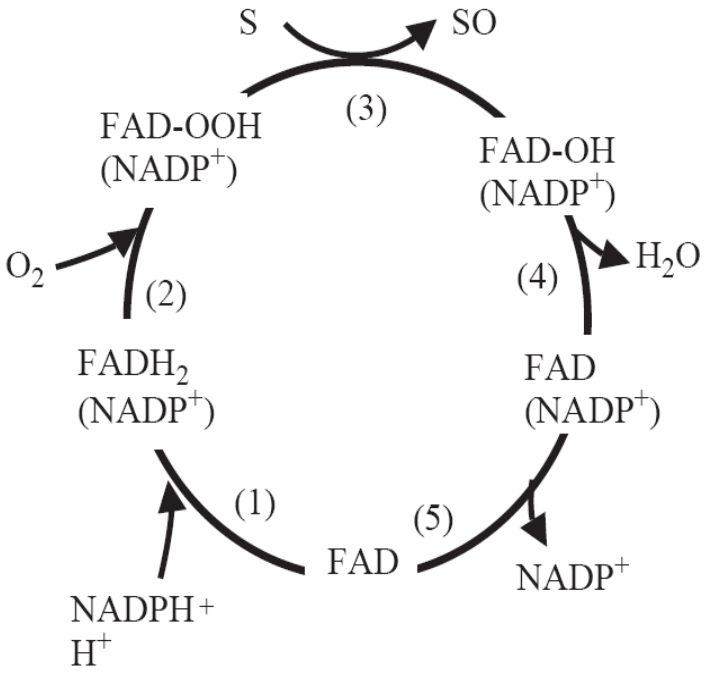
P450 versus FMO

P450	FMO
Huge family	**Small family**
Active site haem	Active site FAD
Binds the substrate before reaching the active form	**Ready to oxidise before substrate binds**
Induced by substrate	**Not induced by substrate**









Reaction mechanism

- Very few true competitive inhibitors of FMOs
  - Dietary indoles
  - (dimethylamino)stilbene carboxylic acids
  - Less potential for drug-drug interactions
- Enzyme not inactivated by reactive metabolites
- Enzyme not inducible
- FMOs could be used as detoxification route instead of P450s but very limited substrate specificity and reactions carried out.

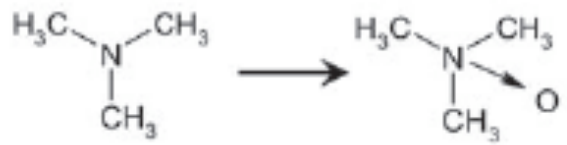
Tissue specific expression of hFMO

	FMO1	FMO2	FMO3	FMO4	FMO5
Fetal brain	56.4	17.6	5.6	14.6	21.0
Adult brain	3.1	140.9	10.7	19.6	56.5
Fetal liver	945.7	93.1	445.6	488.3	4406.8
Adult liver	96.0	988.7	**23088.6**	4881.7	**26539.5**
Adult kidney	**6198.2**	4682.7	530.9	**2509.9**	1628.3
Adult lung	595.7	**115895.5**	2223.9	738.1	2274.9
Adult small intestine	522.9	928.7	74.2	403.3	2586.3

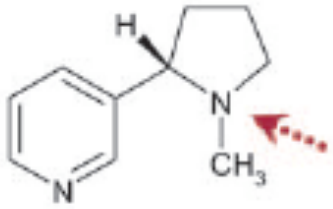
	Tissue
FMO1	kidney
FMO2	lung
FMO3	liver
FMO4	kidney
FMO5	liver

Tissue specific expression of the FMO isoforms in humans expressed as copies per ng RNA

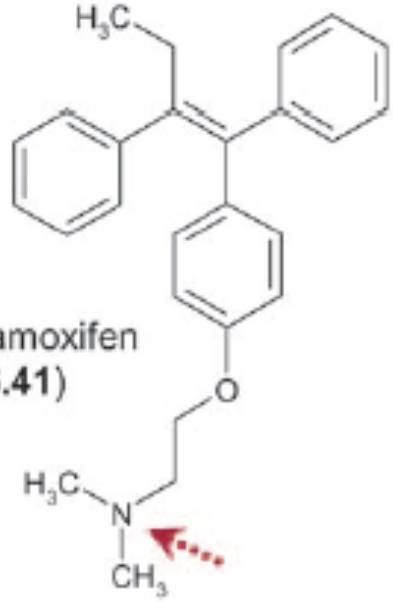
FMO3: Substrates



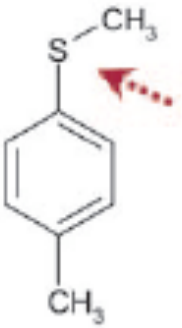
Trimethylamine (**6.49**) → **6.50**



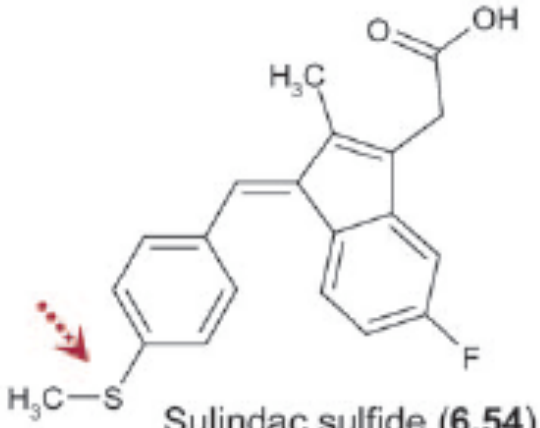
(*S*)-Nicotine (**6.24**)



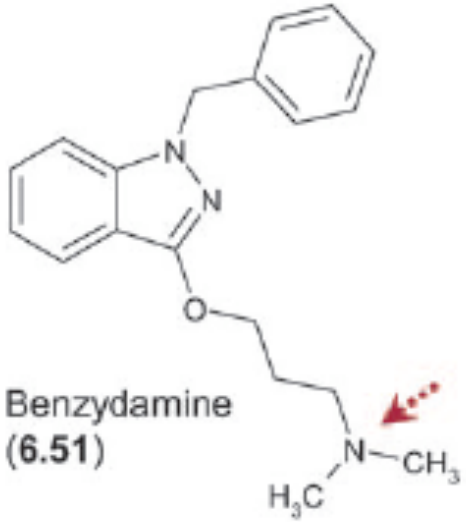
Tamoxifen (**6.41**)



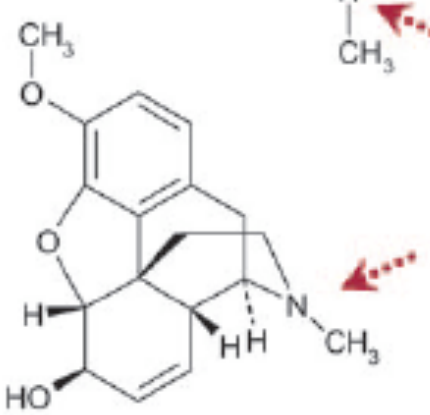
Methyl *p*-tolyl sulfide (**6.53**)



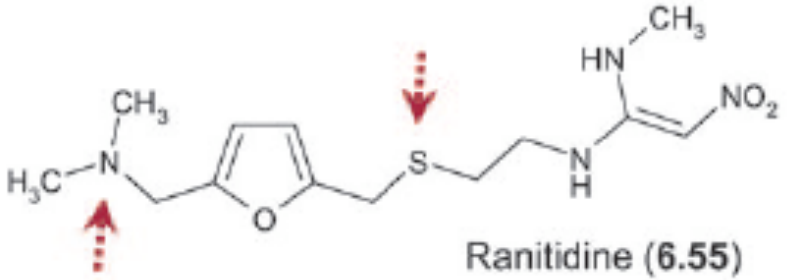
Sulindac sulfide (**6.54**)



Benzydamine (**6.51**)



Codeine (**6.52**)



Ranitidine (**6.55**)

Human FMO1

- Primarily expressed in adult kidneys and fetal liver
  - Expression in liver drops immediately after birth
- Polymorphic with 20 allelic variants
  - Most result in increased K_m and or altered V_{max}
  - FMO1*6 variant – low expression of enzyme
- Does not oxygenate primary amines
- Broadest specificity of all human FMOs
- Substrates include
  - Imipramine and chlorpromazine (anti-depressants)
  - Disulfiram (used to treat alcohol dependance)
- Purified human enzyme thermolabile and inhibited by low concentration of anionic detergents

Human FMO2

- Primarily expressed in the lung
- Polymorphic with 5 allelic variants
  - Most result in no activity at all
- Very active towards bioactivation of small MW thioureas and detoxification of thioethers
  - Increased risk of toxicity following thiourea exposure in individuals with wild-type alelle
  - Decreased risk of toxicity following thioether containing organophosphate exposure in individuals with wild-type alelle
- Restricted active site and therefore very substrate specific enzyme
  - Substrate access channel estimated to be 8 Å long by 8 Å wide cylinder.
- Tertiary amines are excellent substrates
- Purified enzyme is thermostable compared to FMO1 and FMO3 and not inhibited by anionic detergents like FMO1 and FMO3

Human FMO4 and FMO5

- Primarily found in adult liver and kidney
- Polymorphic but few variants reported to date
- Very limited substrate specificity and little contribution to drug metabolism identified to date
  - Difficult to express
  - Emerging role in drug metabolism metabolism

Most relevant to drug metabolism: Human FMO3

- Primarily expressed in the liver
  - Expression levels 60% of human CYP3A sub-family
- Polymorphic with 26 allelic variants
  - Most result in reduced activity
- Most relevant to both drug metabolism and metabolism of endogenous compounds
- Intermediate substrate specificity compared to FMO1
- Substrates include
  - Tamoxifen (breast cancer treatment)
  - Clozapine (antipsychotic)
  - Nicotine
  - Trimethylamine (dietary compound)
  - Ranitidine (anti-ulcer)

Genetic variants and Polymorphisms

Variant	Substrate	K_m (μM)
FMO1		
wild type	Methimazole	7
p.H97Q		14
p.I303V		7
p.I303T		16
p.R502X		-
FMO2		
wild type	Ethylenethiourea	16
p.N413K		40
p.71D		-
p.V113X		-
p.S1195L		-
p.X472		-

Enzymatic activity of human FMO1, FMO2 and FMO3 allelic variants.

Varient	Substrate	Km (μM)
FMO3		
wild type	Trimethylamine	32
p.K158L		206
p.V257M		1151
p.F510X		1034
p.E132H		26
p.E132H/K158L		11
p.K308G		42
p.K158L/K308G		23
p.L360P		11
wild type	Methimazole	12
p.K158L		10
p.E132H		15
p.E132H/K158L		10
p.K308G		17
p.K158L/K308G		12
p.L360P		8

Human FMO3 and Trimethylaminuria

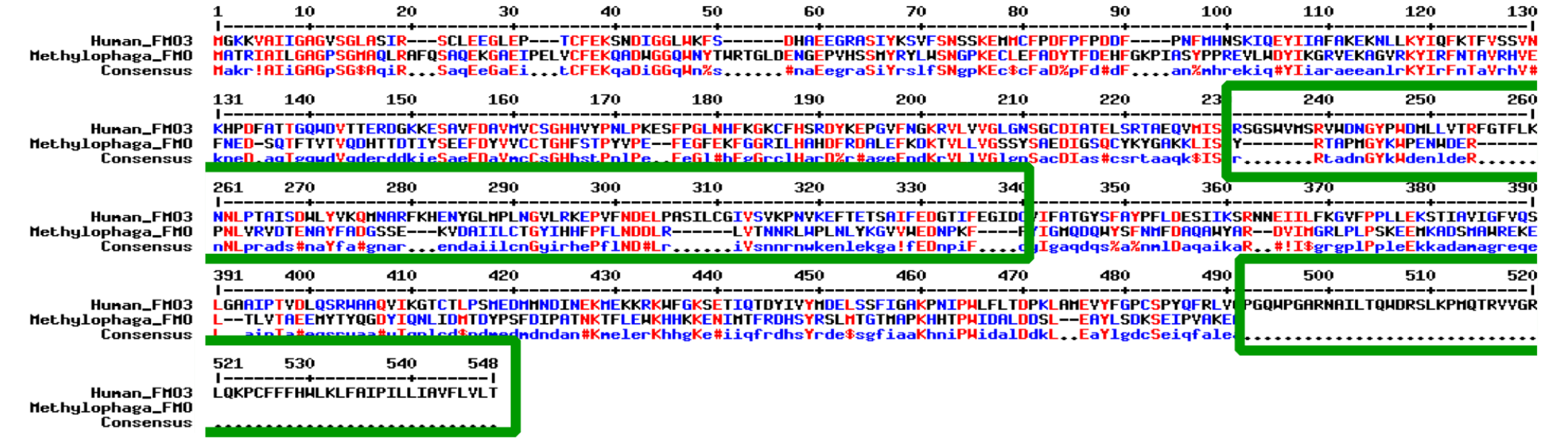
- Trimethylamine –smelly compound found in diet (eggs, legumes, certain meats, fish)
- Excreted from body *via* urine after oxidation to trimethylamine *N*-oxide by FMO3
- Genetic polymorphisms leading to low FMO3 activity result in an inability to secrete trimethylamine *via* urine (trimethylaminuria)
  - Secreted in sweat and urine as parent compound (trimethylaminuria)
  - Leads to odour – “Fish-odour” syndrome
  - First reported as early as 1400 BC.

FMO and Disease

- Polymorphisms in FMO3 have been shown to cause disease
- Fish Odour Syndrome or Trimethylaminuria (TMAU)
  - caused by a rare genetic defect :
    - TMAU is a metabolic disorder whereby abnormal amounts of TMA are present in the urine, sweat, expired air, and other bodily secretions
    - TMA has a powerful smell of rotting fish which causes patients suffering from TMAU to have highly objectionable body odour
  - 2 relatively common polymorphisms, P153L and E305X, result in a large decrease in turnover of Trimethylamine (TMA) to Trimethylamine N-oxide (TMANO)
  - TMAU patients excrete up to 80% of their TMA (from diet) as free amine
    - healthy individuals convert 96% of the TMA into TMANO before excreting them
- single M82T mutation in FMO3
  - completely abolished enzyme function leading to TMAU.

Molecular Modeling

Target-template alignment

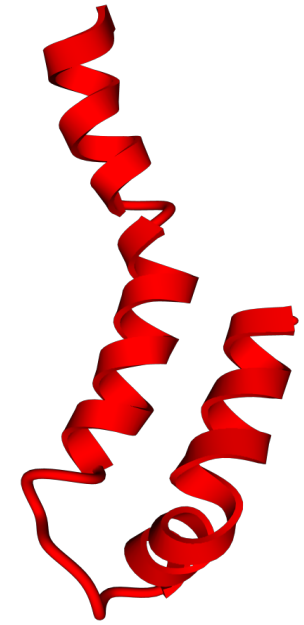


Known crystal structures (≈28% homology)

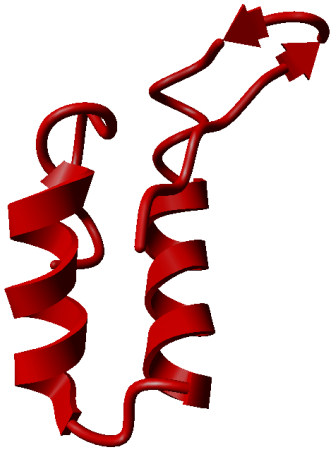
- Yeast FMO (Eswaramoorthy et al., PNAS, 2006)
- Bacterial FMO (Alfieri et al., PNAS, **2008**)

Molecular Modeling

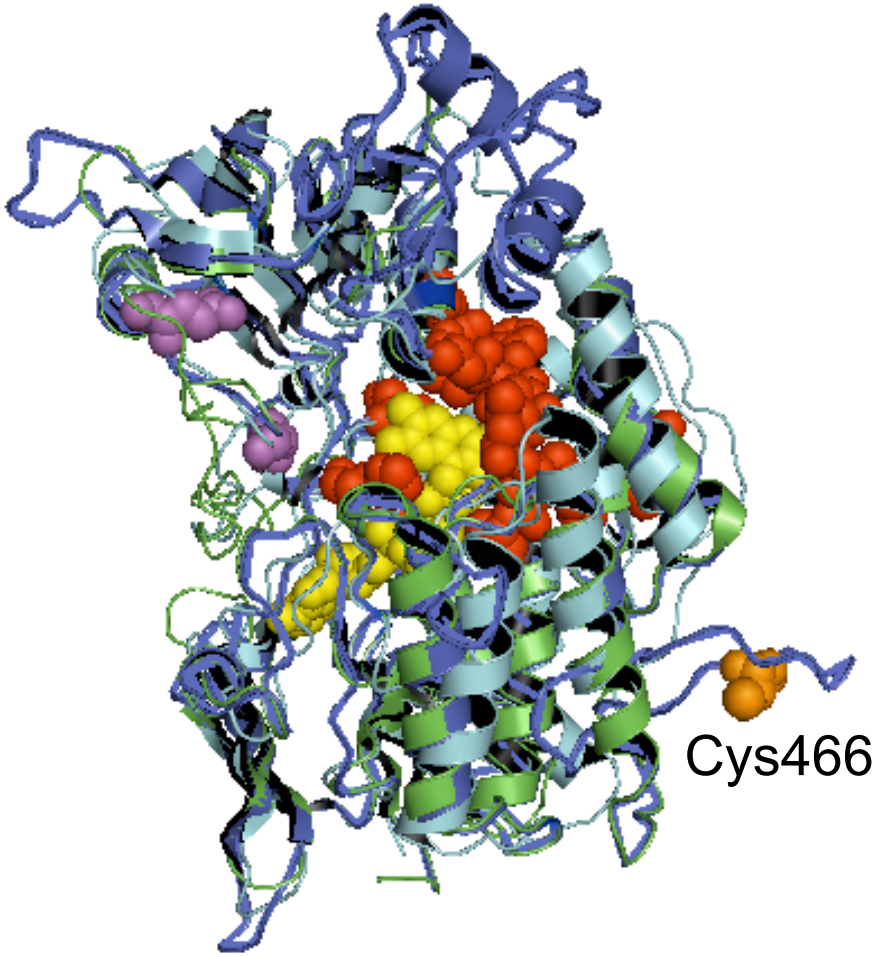
Ab initio and homology modeling



C-terminus

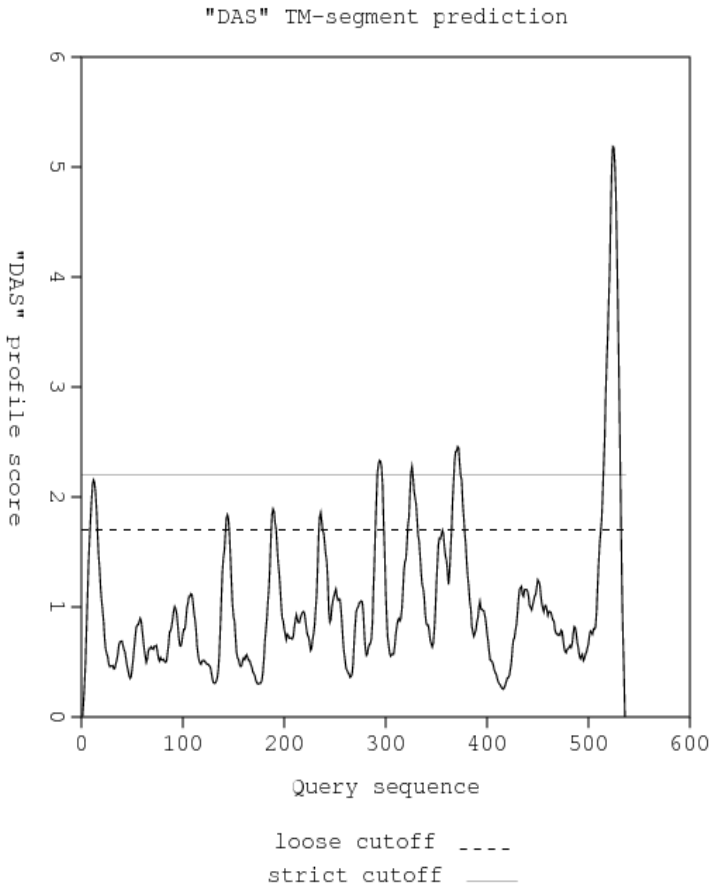


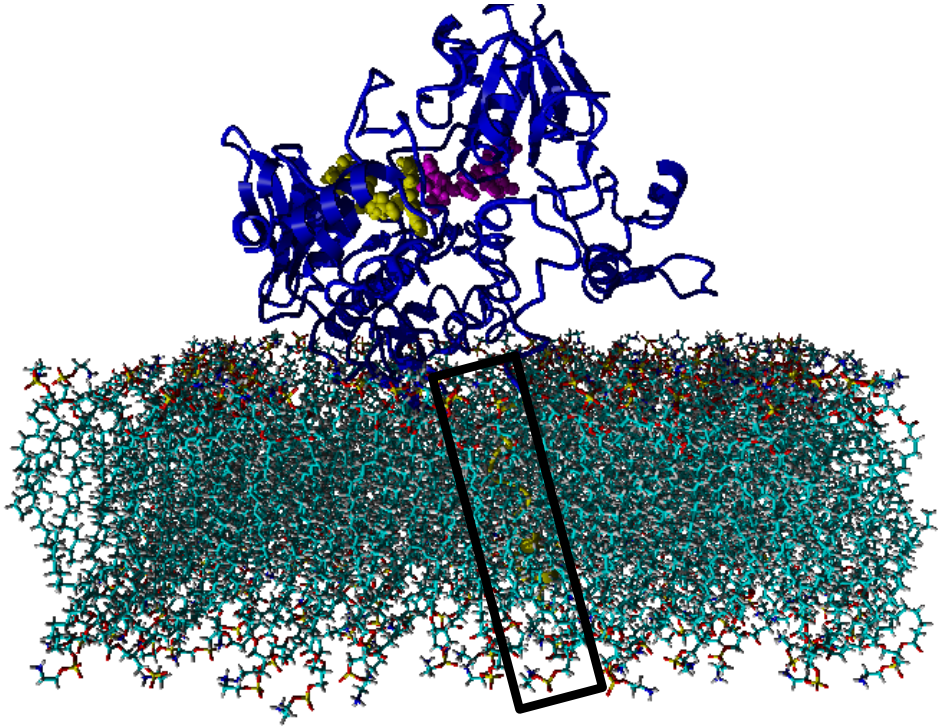
Insert

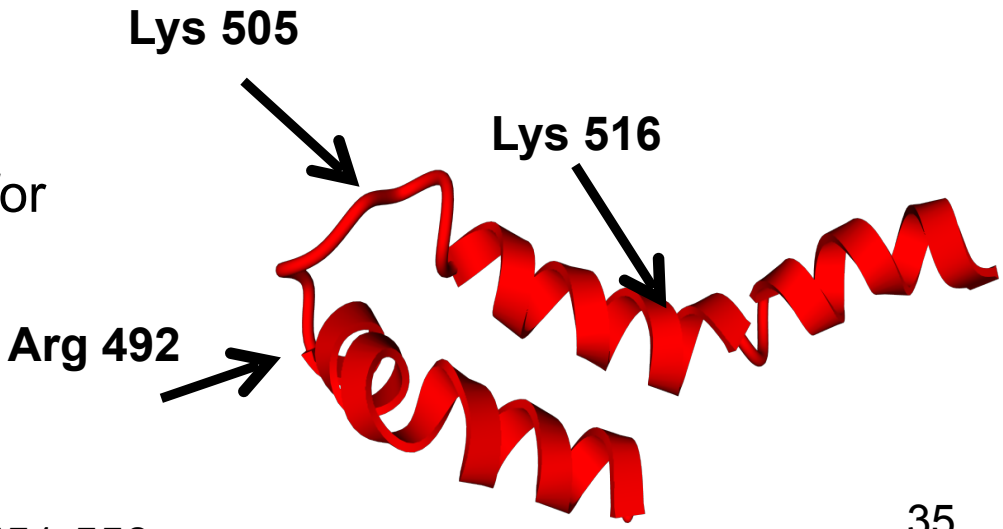


Superimposition of yeast (green, PDB:2GV8), bacterial (cyan, PDB:2VQ7) and human FMO3 (model; blue); RED = active site, YELLOW = FAD, PURPLE = access channel

Deletion of the membrane anchor







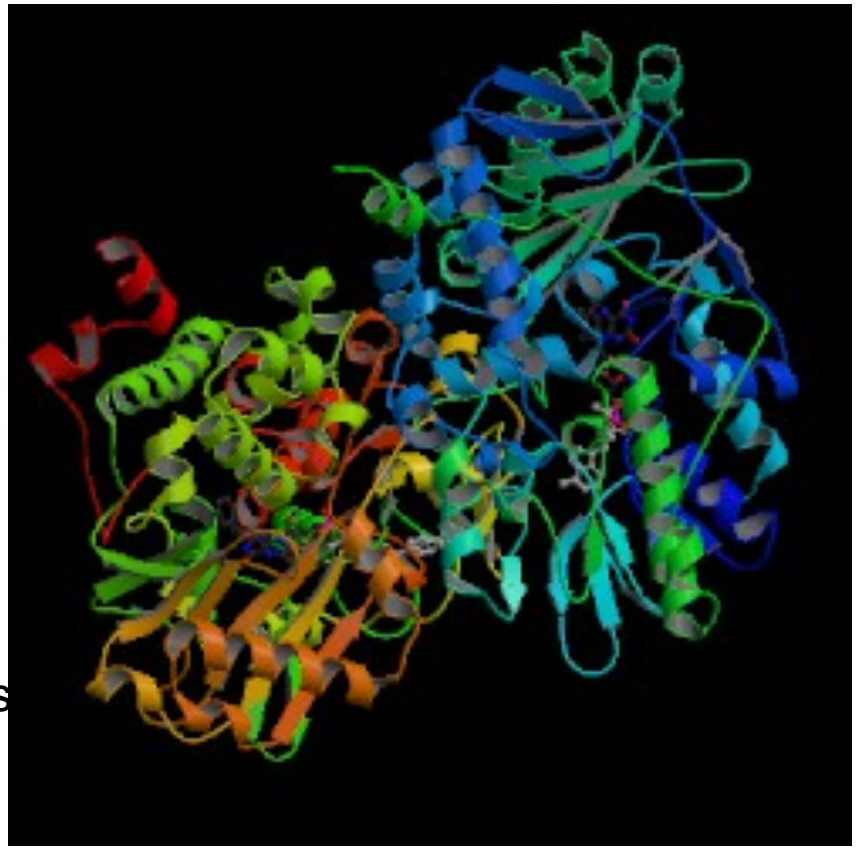
C-terminal region of hFMO3 responsible for the insertion of the enzyme in membrane. Three different clones were generated carrying a stop codon at different residues

Ref: Catucci et al., Biochem Pharm (2012) 83:551-558.

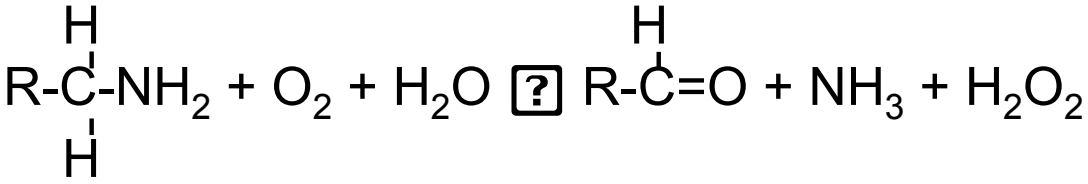
Other Phase-1
Drug metabolising enzymes

Monoamine Oxidase (MAO)

- Catalyses oxidation of monoamines.
- Covalently bound FAD co-factor
- Mitochondrial
- Two types in humans: MAO-A and MAO-B.
- Vital to inactivation of neurotransmitters e.g. seretonin, adrenaline, noradrenaline.
- Inhibitors used in treatment of depression
- Important in dietary tyramine metabolism
  - Drug - food interaction between MAO inhibitors and tyramine containing foods e.g. Chocolate ,cheese, yeast extracts



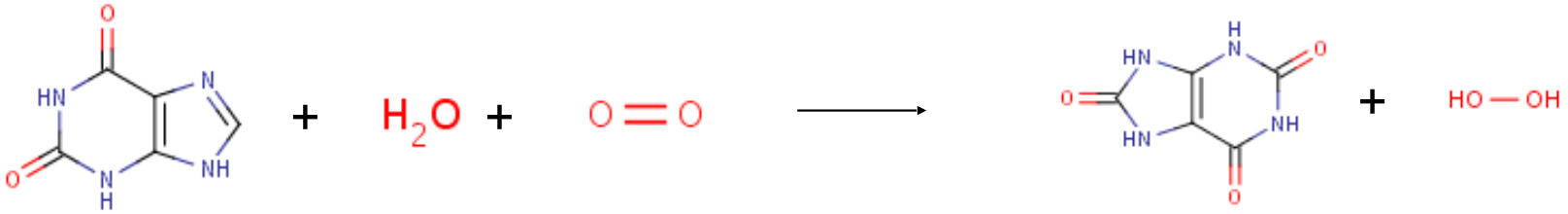
Human MAO-B (pdb: 1GOS)



Xanthine Oxidase (XO)

- Catalyses oxidation of hypoxanthines to xanthines and then to uric acid.
- Large (270 kDa) protein with 2 FAD, 4 2Fe-2S clusters and 2 molybdenum atoms.
- NADH dependent enzyme
- Uses water as source of oxygen atom
- Drugs metabolised include theophylline and 6-mercaptopurine.

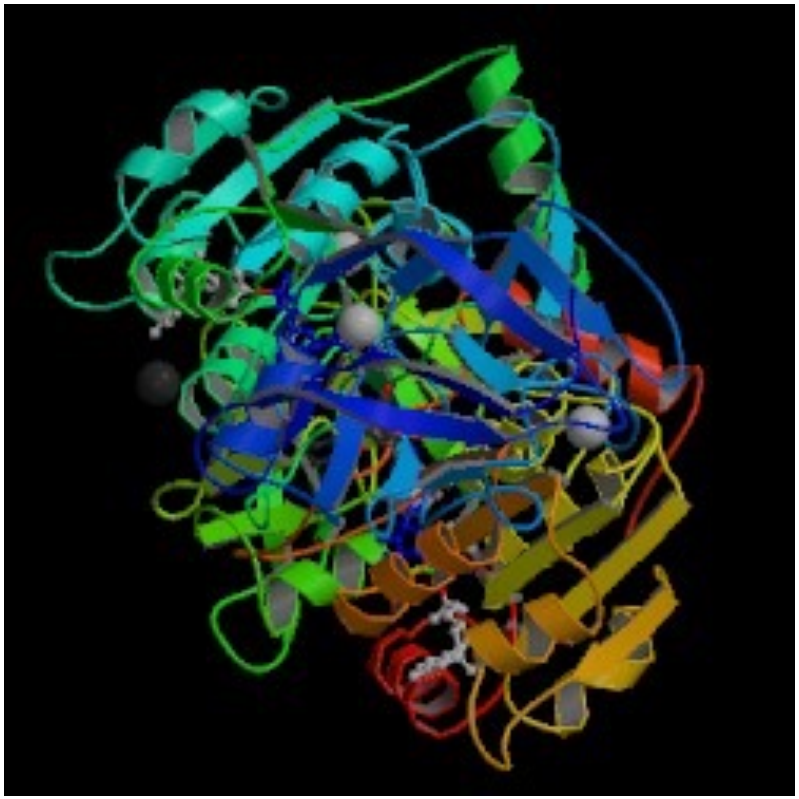
Bovine XO (pdb: 1FIQ)



XANTHINE URIC ACID

Alcohol and aldehyde dehydrogenases

- Multiple forms in humans
  - Smooth ER
  - Mitochondrial
  - Cytosolic
- Alcohol dehydrogenase in humans
  - 2 Zn^{2+} containing dimer
  - Polymorphic
- Applications in fuel cells



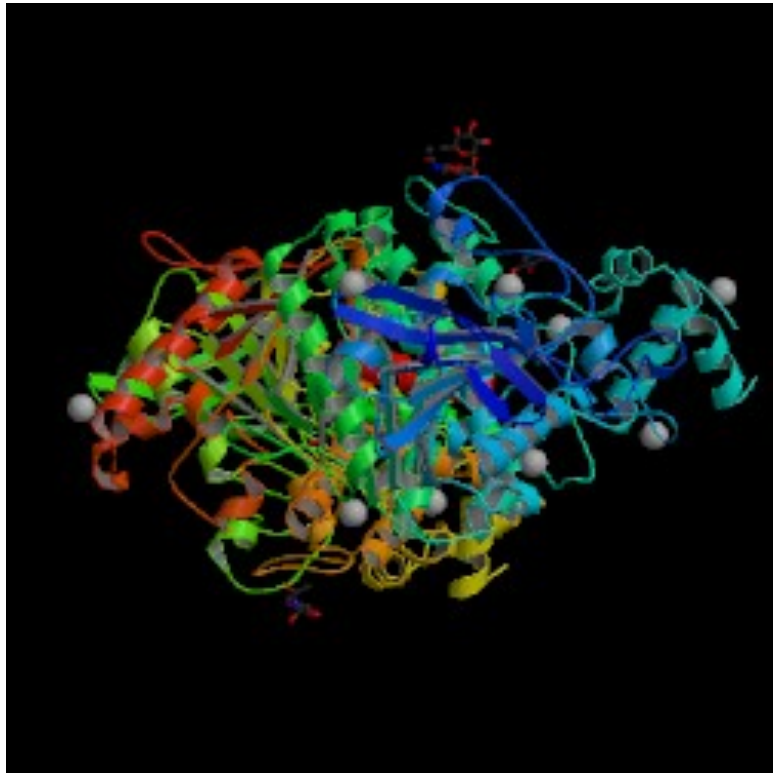
Human alcohol dehydrogenase (pdb: 1HDX)

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+$$

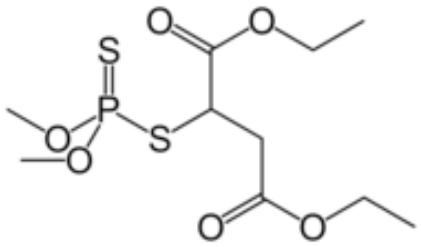
$$CH_3CHO + NAD^+ \longrightarrow CH_3COOH + NADH + H^+$$

Esterases

- Multiple forms in humans
  - Lipases
  - Acetylesterases
  - Thioesterases
  - Amidases
- Responsible for hydrolysis of ester and amide drugs e.g. Aspirin, procaine, lidocaine, peptide drugs
- ß-lactamase in bacteria responsible for penicillin resistance
- Inhibitors of acetylcholinesterase are potent neurotoxins (Chemical warfare) but also used clinically for anaesthesia and two treat glaucoma and Alzheimer's disease and also as pesticides
- Inhibitors e.g Malathion a pesticide
  - Phosphorus atom with two lipophillic groups, a leaving group (halide or thiocyanate) and terminal oxygen.



Mouse acetylcholinesterase (pdb: 1N5M)



Malathion