Drug metabolism (3)

Sheila Sadeghi
Metabolic Biochemistry

Role of human cytochromes P450 in drug development

P450s responsible for 95% of drug metabolism
25-35% of Drug failures are due to P450s
50% of ADR's due to P450s
Role of P450 testing in Drug Discovery

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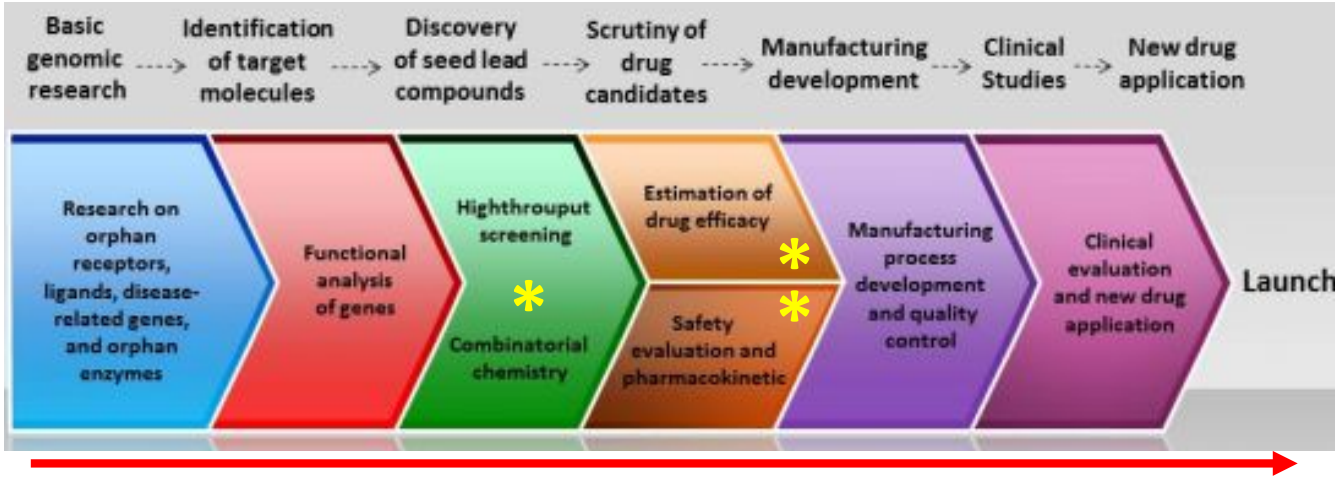
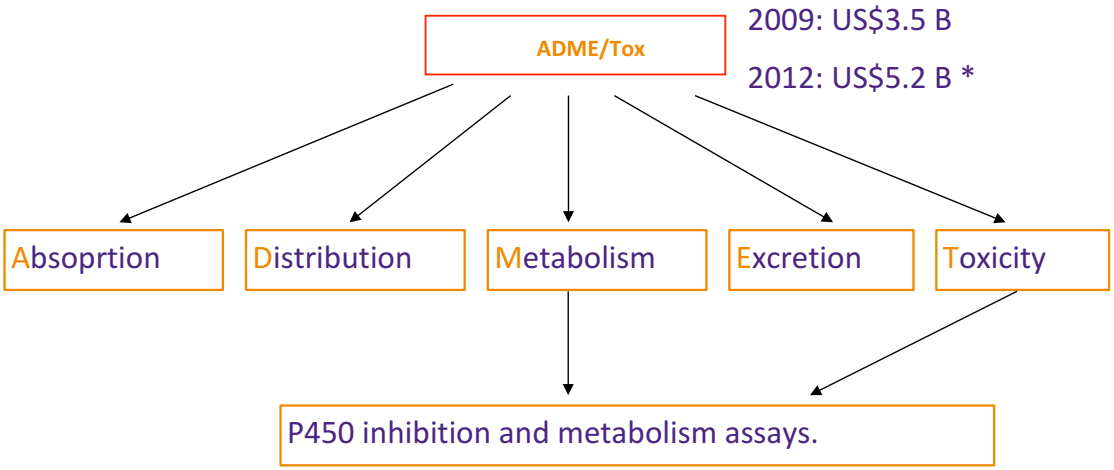


The ADME/Tox and *in vitro* P450 Markets



* Predictive ADME/Tox Strategies.

Dr N. Patel, Business Insights, 2006.

In vitro methods for metabolic studies on NCE

- Human liver microsomes:
  - Source rare
  - Contains mixtures of DMEs (P450s, FMOs, UGTs) - requires the use of mixtures of isoform specific substrates/inhibitors
- Human hepatocytes and liver slices
  - Closely mimics *in vivo* situation
  - Must be fresh; long term storage is a problem
- Recombinant-engineered DMEs:
  - *E.coli:*
    - N-ter modifications. 1991 Henry Barnes, bovine CYP17A, changed first 7 codons - silent mutations - increased AT richness (codons 4 and 5) and minimised potential mRNA IIary structure (codons 6 and 7).
    - Best vector pCWori with tandem lac promoter plus unusual spacing between the ribosome binding site and the initial codon (3 bases instead of 8-12).
    - Some different K_m have been observed

How drug metabolising P450s are studied

Enzyme	Chrom. loc.	Poly mor.	Inducible	Marker activity	Inhibitor
CYP1A2	15q22	YES	YES	Ethoxyresorufin-O-deethylation Phenacetin-O-deethylation	Fluvoxamine Furafylline
CYP2A6	19q13	YES	YES	Coumarin-7-hydroxylation	
CYP2C9	10q24	YES	YES	Diclofenac-4'- hydroxylation S-Warfarin-7- hydroxylation	Sulfaphenazole
CYP2C19	10q24	YES	YES	S-Mephenytoin-4'- hydroxylation R-Omeprazole-5- hydroxylation	
CYP2D6	22q13	YES	NO	Debrisoquine-4- hydroxylation Bufuralol-1'- hydroxylation Dextromethorphan-N-demethylation	Quinidine
CYP2E1	10q24	(YES)	YES	Chlorzoxazone-6- hydroxylation	4-Methylpyrazole Diethyldithiocarbamate
CYP3A4	7q22	YES	YES	Testosterone-6ß- hydroxylation Nifedipine oxidation	Ketoconazole

Recombinant-engineered DMEs:

» *E.coli:*
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» N-ter modifications of P450s for expression in *E.coli* :

CYP450 isozyme	Native N-terminus	Modified N-terminus
1A2	MALSQSVPFSATELLLASAIFCLV	MALLLAVFLFCLV
2C9	MDSLVVLVLCLSCLLLLSLWRQSS	MARQSS
2C19	MDPFVVLVLCLS	MALLAVFLVLCL
2D6	MGLEALVPLAVVAIPL	MALEALVPLAVIVAIFL
3A4	MALIPDLAMETWILLAVSLVLLYL	MALLLAVFLVLLYL
2E1	MSALGVTVALLVWAAFLLLVSMWRQV	MARQVH

» Baculovirus-infected insect cells:
- Allows co-expression of DME systems in microsomes that resemble the human ones;
- Co-infection with multiple viruses and/or dual promoter viruses: CPR, cyt b_5 ,CYP450 isozymes;
- K_m slightly lower, v_{max} slightly higher - due to ratio CPR:P450 = 1:10 in human liver microsomes, 8:1 in Baculosomes.

Use of recombinant DMEs

- Isozyme identification
  - » To estimate *in vivo* clearance
  - » To predict genetic differences in metabolism
- Determination of K_m and v_{max}
  - » Use isolated enzymes for full charact. of parameters related to clearance
- Inhibitor screening
  - » Very important, see example Terfenadine-Ketoconazole
- Synthesis of metabolites
  - » Large scale production of metabolites (>100 mg) for study structure, toxicity and further metabolism
- High throughput screening

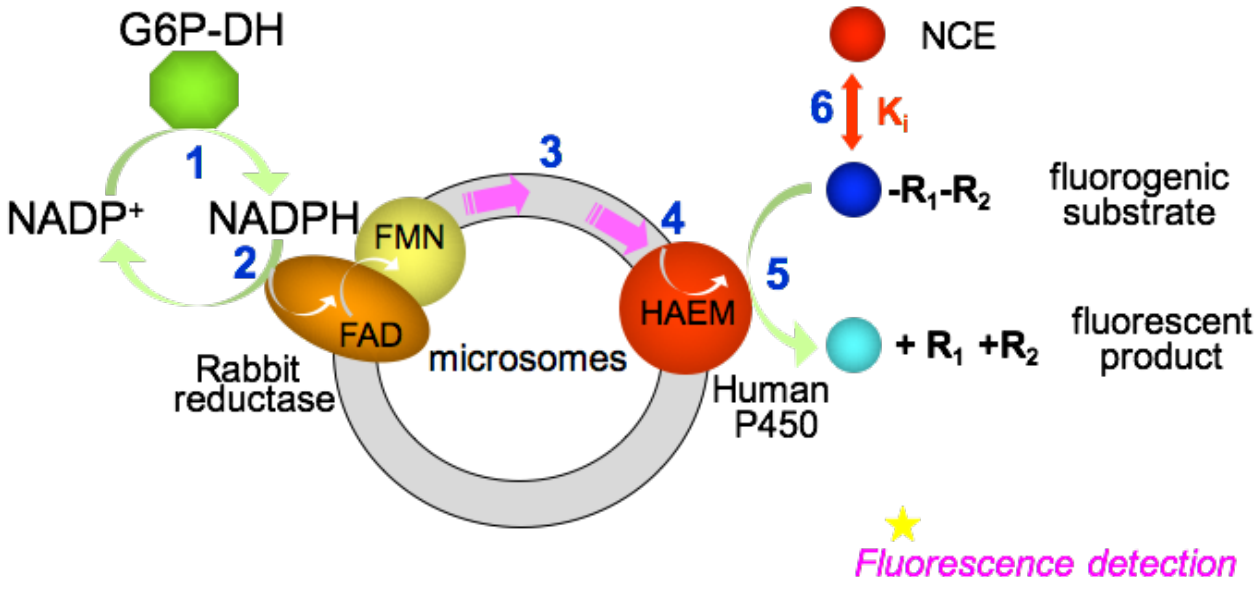
Cytochromes P450: HTS

High Throughput Screening (HTS)

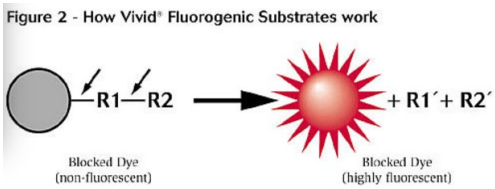
- Problem: large numbers of NCE need to be screened.
  - Classic method:
    - » 1) incubation with DME; 2) extraction; 3) HPLC separation; 4) char. of products ➔ time consuming, not suitable for HTS.
- One solution: competitive inhibition assay. Evaluation of the conversion rate of a substrate in the presence/absence of a potential inhibitor or substrate. Commercially available kit:
  - » Aurora VIVID™ fluorogenic substrates:
    - Series of substrates for all major human P450s. They are turned over at high rates yielding a fluroscent product with high ε (>40,000 $cm^{-1}M^{-1}$ and high fluorescence quantum yield.
  - » Panvera Baculosomes® reagents:
    - Include major human P450s from Baculosomes plus rabbit reductase and NADPH regenerating system.

VIVID™ kit

The VIVID™ kit

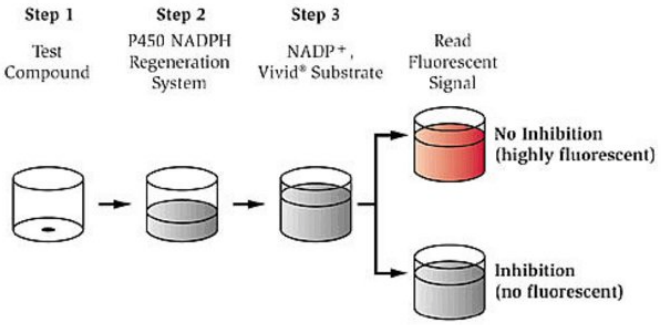


‘Typical’ experiment:



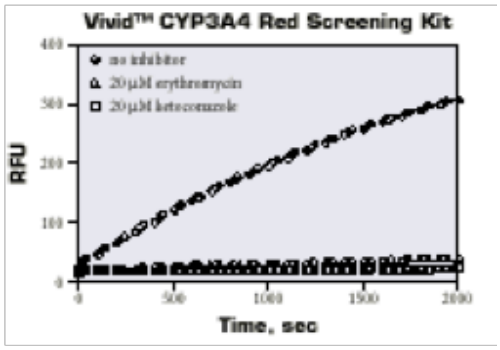
■ Procedure:

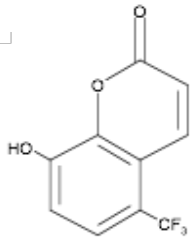
Figure 1 - Three-step Vivid® screening protocol



■ NB: All P450s must be checked; for each different fluorogenic compounds must be tested

Results:





Vivid™ CYP3A4 Red Screening Kit
Vivid™ CYP3A4 Green Screening Kit
Vivid™ CYP3A4 Blue Screening Kit
Vivid™ CYP2C9 Red Screening Kit
Vivid™ CYP2C9 Green Screening Kit
Vivid™ CYP2C9 Blue Screening Kit
Vivid™ CYP2D6 Cyan Screening Kit
Vivid™ CYP1A2 Blue Screening Kit
Vivid™ CYP2C19 Blue Screening Kit

11

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.

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.

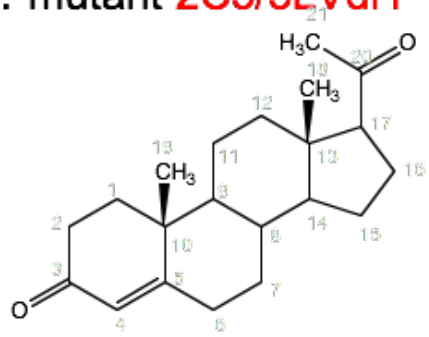
- 3D structures of the human P450s is of paramount importance.

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

How Protein Engineering has helped solving the structure of the mammalian P450s

Rational design of rabbit CYP2C5

- Extensive protein engineering has led to the first (Jan 2000) mammalian P450 structure, a template for modelling other P450s and for making predictions for drug discovery.
- Homology modelling was used to engineer CYP2C3 and CYP2C5 to make a soluble and active mammalian P450 suitable for crystallisation: mutant 2C5/3LVdH
- Both 2C3 and 2C5 hydroxylate progesterone:
  - 2C3: 16-α hydroxylation
  - 2C5: C-21 methyl hydroxylation



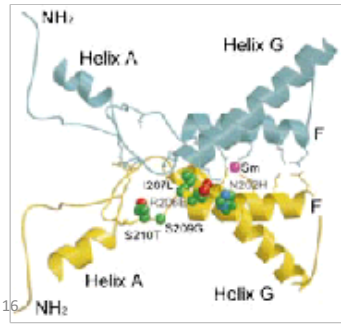
Mutations to achieve 2C5/3LVdH

- Membrane association: leader sequence for co-translational insertion in SER, N-ter helix followed by non-contiguous hydrophobic portions that generate hydrophobic patches that cause association to the membrane.
- Mutants 2C3dH and 2C5dH:
  - removed N-ter, res. 3-21, plus addition of 4 His at the C-ter
    - » fully active, released from membranes at high salt conc.
    - » purified without addition of detergents
    - » but tend to aggregate in dimers/tetramers

2C5dH: Δ 3-21 to eliminate N-ter helix
D2A, Q22K, N23T, G25S, R26K to facilitate expression
plus 4 His tag at C-ter to facilitate purification

- Further engineering on 2C5:

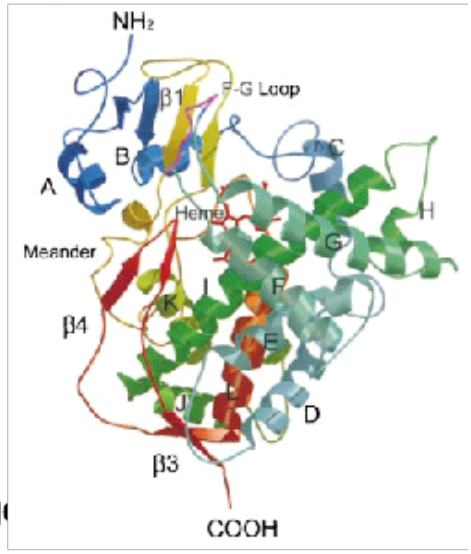
2C5/3LVdH: 2C5dH plus parts of 2C3:
N202H, R206E, I207L, S209G, S210T to decrease aggregation, tetramer ➔ monomer

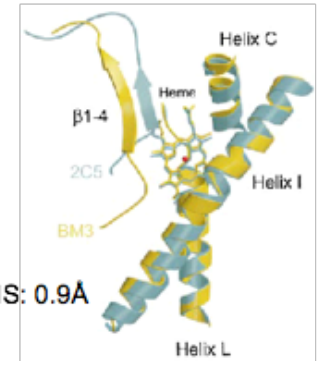


2C5/3LVdH structure.

- The cys60, that is in two symmetry related molecules, was found to form dimers.
- Overall folding similar to bacterial P450s, especially P450BM3.

Haem sandwiched between helic L and I.
Axial ligand cys432, 'expected'
water not modelled.



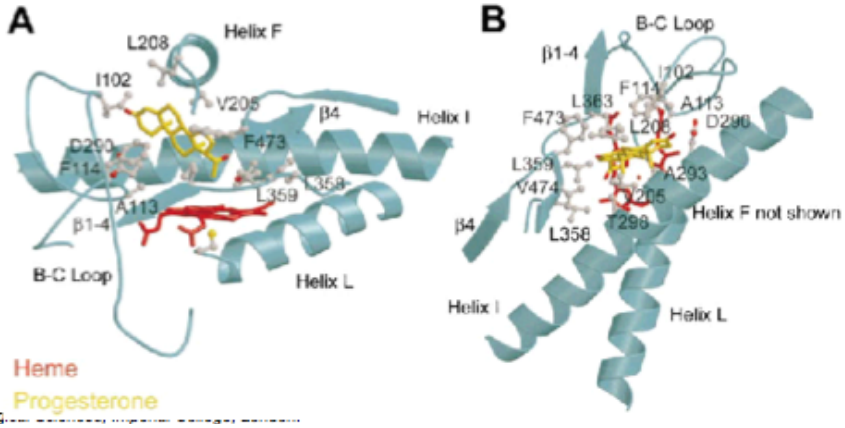


RMS: 0.9Å

Thr298 on helix I is
the proton relay
from exterior surface
to protonate reduced oxyg

Substrate binding site

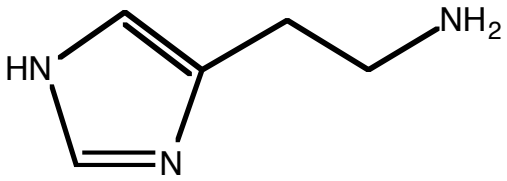
- Progesterone was docked in the active site using AUTODOCK.
- The 6 topologically important elements, SRS, identified by modelling and mutagenesis are confirmed in the structure.



The first rationally designed drug: Cimetidine

- In the 1970s, a team at Smith Kline & French (now GSK) opened the new era of rational drug design with the discovery of the histamine H_2-receptor antagonists.
- The problem:
  - Gastric ulcer was a serious life-threatening problem 50 years ago;
  - The cause is the release of excess gastric acid by the parietal cells in the stomach;
    - The *parietal cells* of the stomach are innervated by the autonomous nervous system;
    - Under certain stimuli the termini of these nerves release *acetylcoline*;
    - Acetylcholine reaches the parietal cells, activates their *cholinergic receptors*, and ultimately causes the release of gastric acid in the stomach;
    - This also stimulates the *G cells* of the stomach, which produce the hormone *gastrin*, a peptide that moves in the blood supply and further stimulates the parietal cells.

Strategy

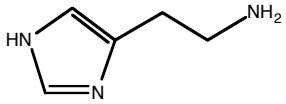


- Anticholinergic drugs that block the acetylcholine receptors are extremely aspecific;
- Histamine could also stimulate gastric acid release:
  - hence the SKF team proposed to start a research programme looking at histamine receptors and antagonists

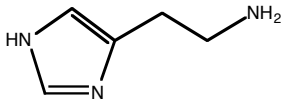
- Histamine:
  - Released during:
    - Cell damage: dilation of blood vessels to prevent infection;
    - Allergic reactions (hay fever, rashes, insect bites, asthma)
    - Drugs developed towards these targets failed to inhibit gastric acid release!

Histamine receptors

- SKF team hypothesis:
  - There are 2 histamine receptors, that histamine cannot distinguish, but good antagonists would**:**
    - **H_1-receptors:** inflammation processes.
    - **H_2-receptors** proposed for gastric acid secretion.
- Searching for a lead compound:
  - First clue: modified imidazole ring gave no antagonists, but produced an agonist, 4-methylhistamine, capable of stimulating acid secretion, but not producing the other typical histamine responses. This proved the existence of the two separate classes of receptors
  - The researchers relied on the physical and chemical characteristics of histamine:
    - conformationally flexible,
    - highly polar and hydrophilic,
    - strong capacity of hydrogen bonding.



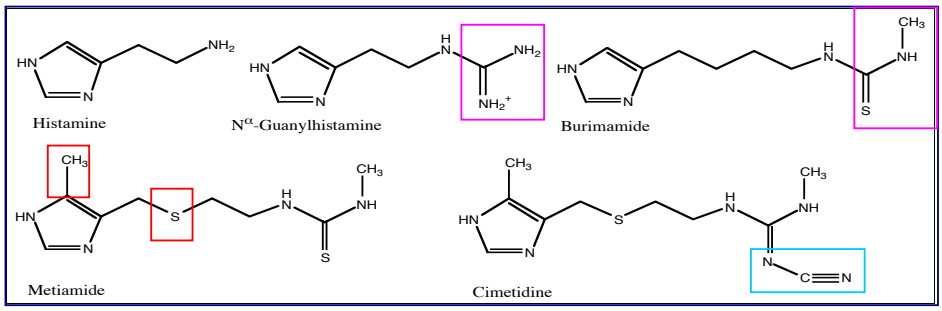
Searching for a lead

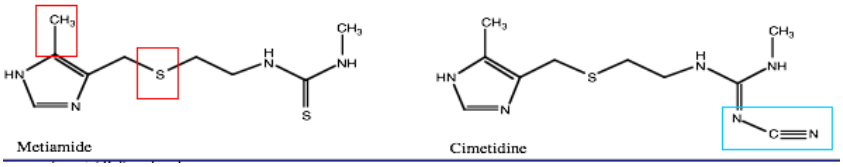


- End of 1968:
  - more than 200 derivatives synthesised,
    - different ionisation properties,
    - different substituents on the imidazole ring,
    - added lipophilic substituents to increase interactions with purported non-polar parts of the receptor,
    - altered side chain substitutions.
  - none was active.
- Assay:
  - 1. Infusion of histamine in an anaesthetised rat's stomach to stimulate acid production
  - 2. Addition of the test compound
  - 3. Measurement of the possible change in pH.
- Revision of assay procedure:
  - Histamine levels were increased,
  - N^{α}-guanylhistamine became the lead compound.

Synthesis of new H_2-antagonists

- The guanidine group was substituted with a neutral thiourea:
  - Burimamide: more active antagonist
    - published in Nature in 1972.
- Analysis of the electronic properties:
  - Metiamide: new molecule with a thioether linkage on the side chain and a methyl group on the C5 of the imidazole ring
    - 10 times more potent than burimamide. active by oral ingestion and clinical trials started in 1973 showed ulcers clearing up within three weeks.





- The thiourea group on the side chain presented potential toxicity problems:
  - Cimetidine : a new compound containing a cyanoguanidine group instead of the thiourea was synthesised.
- The methodical approach must be emphasised.
  - No structure of the receptor was known, continuous updates of sketches of the possible interaction sites were made.

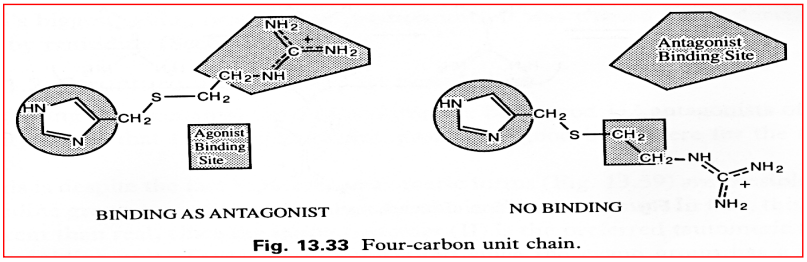


Fig. 13.33 Four-carbon unit chain.

- Cimetidine was launched in 1976 as Tagamet (anTAGonist and ciMETidine)
  - 10 years after its introduction had achieved sales worth 1000million dollars.

- **Cytochrome P450 inhibition by cimetidine**

- Cimetidine is a potent inhibitor of certain cytochrome P450 enzymes, including CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP 2E1, and CYP3A4.

- The drug appears to primarily inhibit CYP1A2, CYP2D6, and CYP3A4, of which it is described as a moderate inhibitor.

- This is notable since these three CYP450 isoenzymes are involved in CYP450-mediated drug biotransformations;

- As a result, cimetidine has the potential for a large number of pharmacokinetic interactions:
- include warfarin, theophylline, phenytoin, carbamazepine, pethidine and other opioid analgesics, tricyclic antidepressants, lidocaine, terfenadine, amiodarone, flecainide, quinidine, fluorouracil, and benzodiazepines.

- Cimetidine may decrease the effects of CYP2D6 substrates that are prodrugs, such as codeine, tramadol, and tamoxifen

Structure-activity relationships

- 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.
- Three main structural-physical-chemical charact. of a molecule:
  - hydrophobicity;
    - Crucial for membrane crossing = bioavailability
    - Measured by the partition coefficient P:

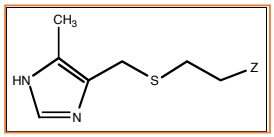
      P = [conc. mol. in octanol] / [conc. mol. in water]
  - electronic effects;
  - steric factors.

QSAR: hydrophobicity

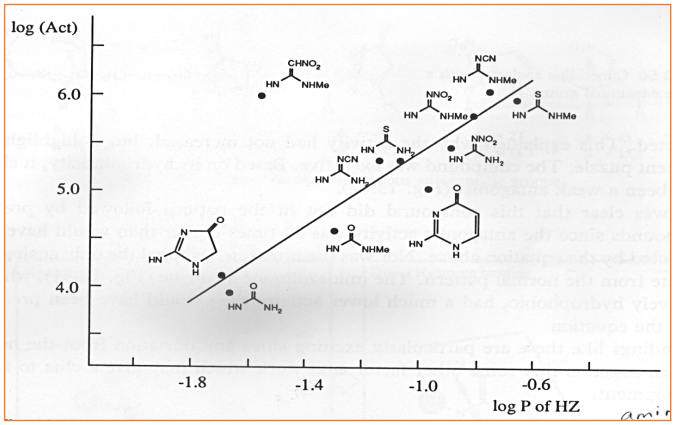
- Relationship between hydrophobicity (P) and biol. activity (C) - conc. of drug required for a given effect:

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

- 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.

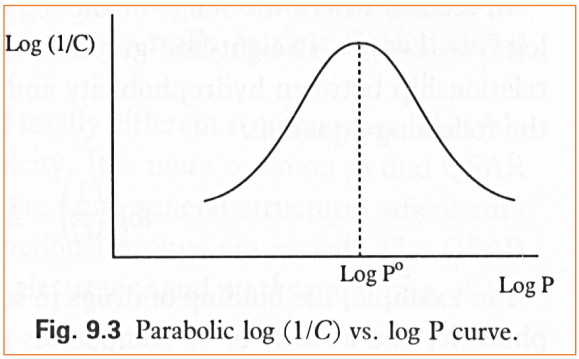


Fig. 9.3 Parabolic log (1/*C*) vs. log P curve.

- 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°), an increase in LogP causes a decrease in activity:

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

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

Monoprotic acids:
acids that are able to donate only one proton.

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)

Cytochromes P450: polymorphism

Drug Metabolising Enzymes: polymorphism

- Phase 1 enzymes (activation):
  - Cytochrome P450s
  - Monoamino oxidase
  - Microsomal flavin monooxygenase
  - *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 120 (2001) 259–268

Toxicology Letters

www.elsevier.com/locate/toxlet

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

Magnus Ingelman-Sundberg

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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*2*xn	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
    - Heterozygous 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

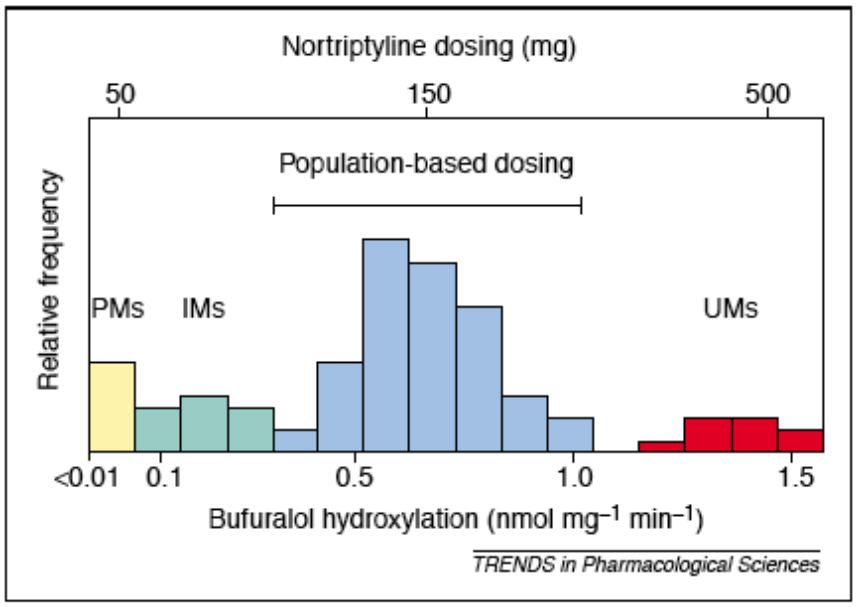


Figure 1. Variation in drug metabolism and nortriptyline dosing in the European population, based on cytochrome P450 CYP2D6 activity (hydroxylation of bufuralol). Within the population four phenotypes can be identified: poor metabolizers (PMs), who lack the functional enzyme; intermediary metabolizers (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.

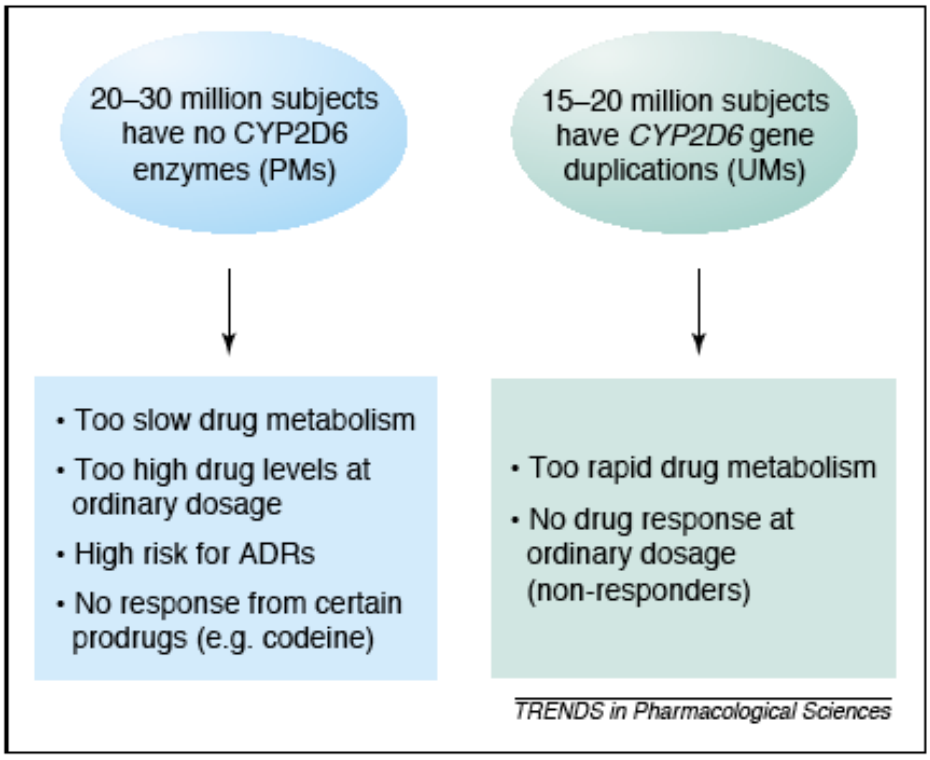


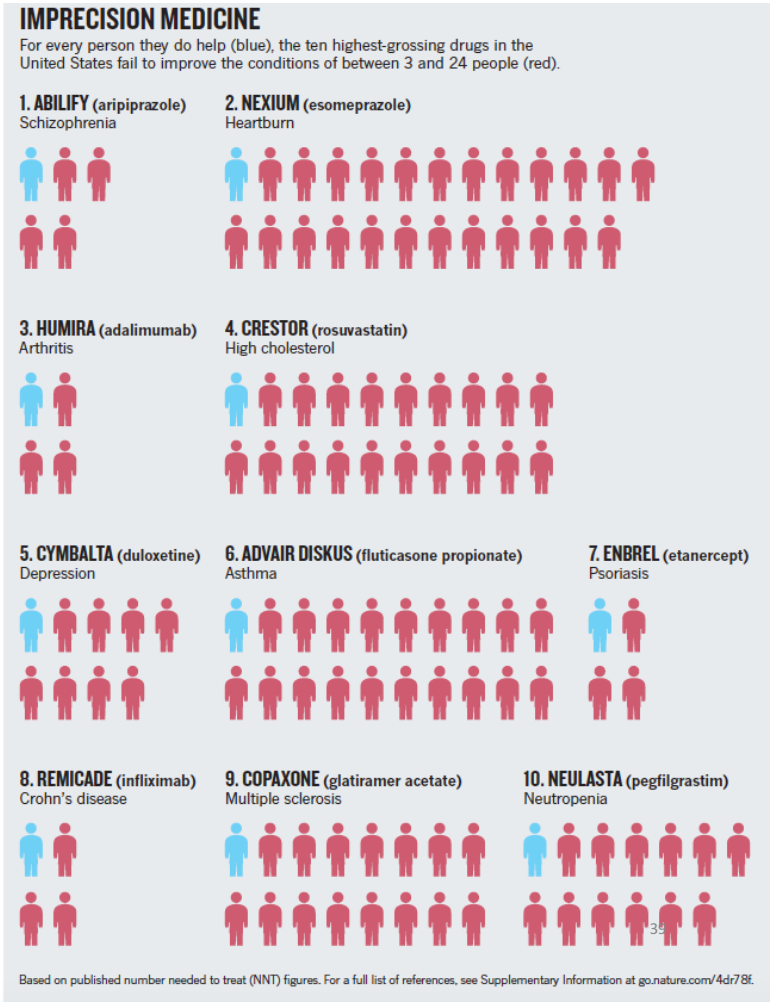
Figure 2. The consequences of outlier cytochrome P450 CYP2D6-dependent drug metabolism. 35–50 million Europeans are either poor metabolizers (PMs) (i.e. lack the functional enzyme) or ultrarapid metabolizers (UMs) (i.e. have multiple gene copies of *CYP2D6*, resulting in elevated enzyme levels), with 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.

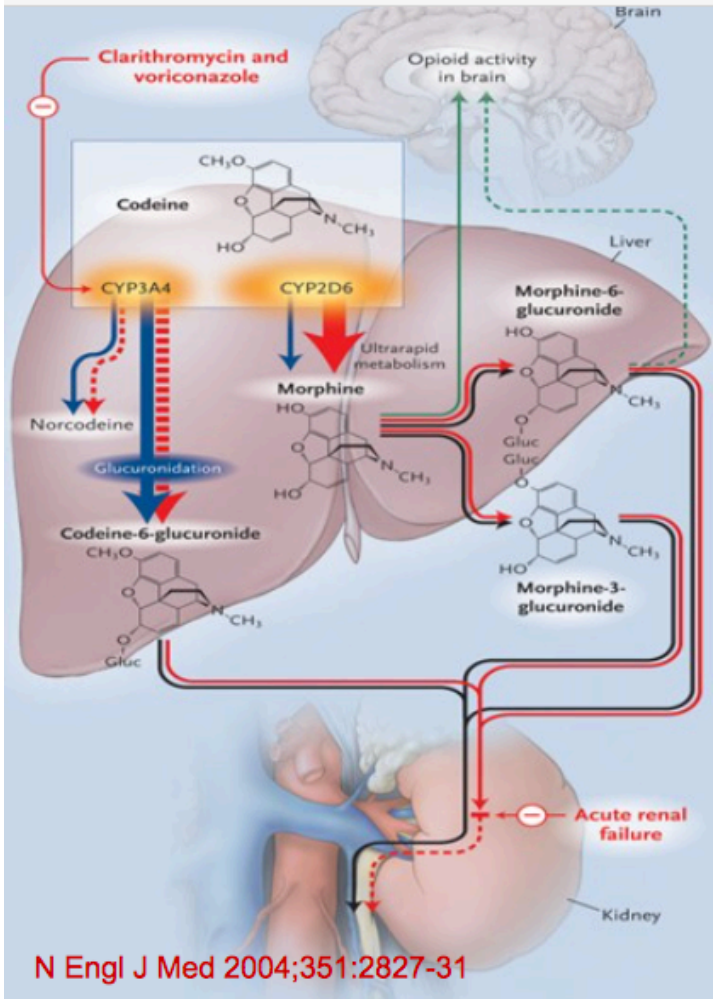
Table 2. Examples of the clinical impact of cytochrome P450 pharmacogenetics[a]

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.

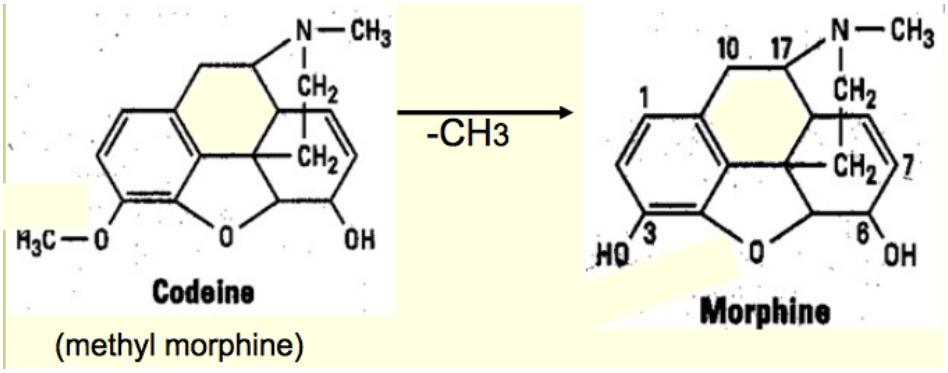
Ref: Nature, vol. 520 2015





FDA website

- Codeine and tramadol are a type of narcotic medicine called an opioid.
- Codeine is used to treat mild to moderate pain and also to reduce coughing. It is usually combined with other medicines, such as acetaminophen, in prescription pain medicines. It is frequently combined with other drugs in prescription and over-the-counter (OTC) cough and cold medicines.
- Tramadol is a prescription medicine approved only for use in adults to treat moderate to moderately severe pain. However, data show it is being used in children and adolescents despite the fact that it is not approved for use in these patients.



41

Safety Announcement

[4-20-2017] The Food and Drug Administration (FDA) is restricting the use of codeine and tramadol medicines in children. Codeine is approved to treat pain and cough, and tramadol is approved to treat pain. These medicines carry serious risks, including slowed or difficult breathing and death, which appear to be a greater risk in children younger than 12 years, and should not be used in these children. These medicines should also be limited in some older children. Single-ingredient codeine and all tramadol-containing products are FDA-approved only for use in adults.

https://www.fda.gov/downloads/drugs/drugsafety/UCM553814.pdf

FDA's strongest warning, called a *Contraindication*, to the drug labels of codeine and tramadol alerting that codeine should not be used to treat pain or cough and tramadol should not be used to treat pain in children younger than 12 years. to treat pain after surgery to remove the tonsils and/or adenoids

A new *Warning* -against their use in adolescents between 12 and 18 years who are obese or have conditions such as obstructive sleep apnea or severe lung disease, which may increase the risk of serious breathing problems.

https://www.fda.gov/downloads/drugs/drugsafety/UCM553814.pdf

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