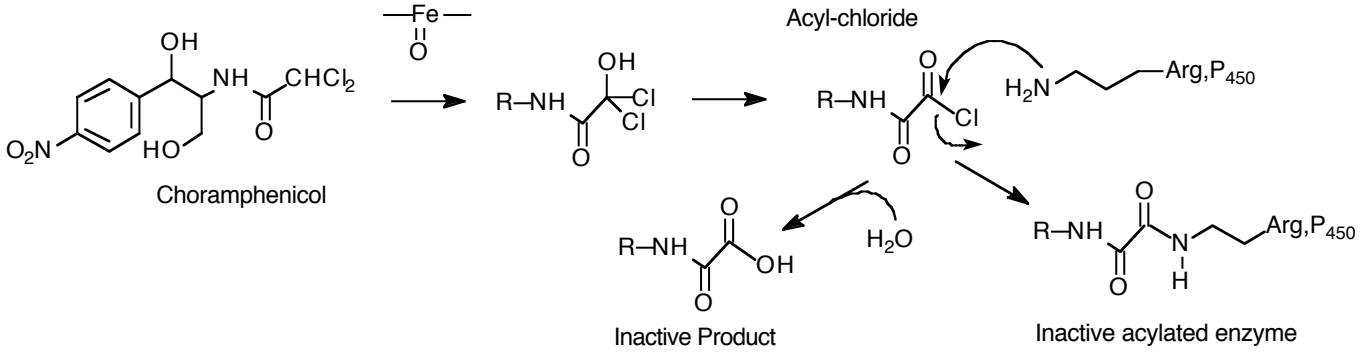
MEDCH 527 Enzyme Kinetics II
Mechanism-Based Inhibition of P450 Enzymes (2019)

Definition: A mechanism-based enzyme inactivator is a relatively unreactive compound that is converted by an enzyme into a species which, without prior release from the active site binds, covalently to the enzyme rendering it inactive.



Characteristic of MBI and P450 enzymes (criteria to establish an MBI)

1. Enzyme activity falls with time in the presence of the MBI.

2. Rate of inactivation (λ or k_{obs}) is first order with time and a hyperbolic function of MBI concentration, or at least saturable.

3. The inclusion of a normal substrate for the enzyme reduces the rate of inactivation by competing with the MBI for the active site of the enzyme.

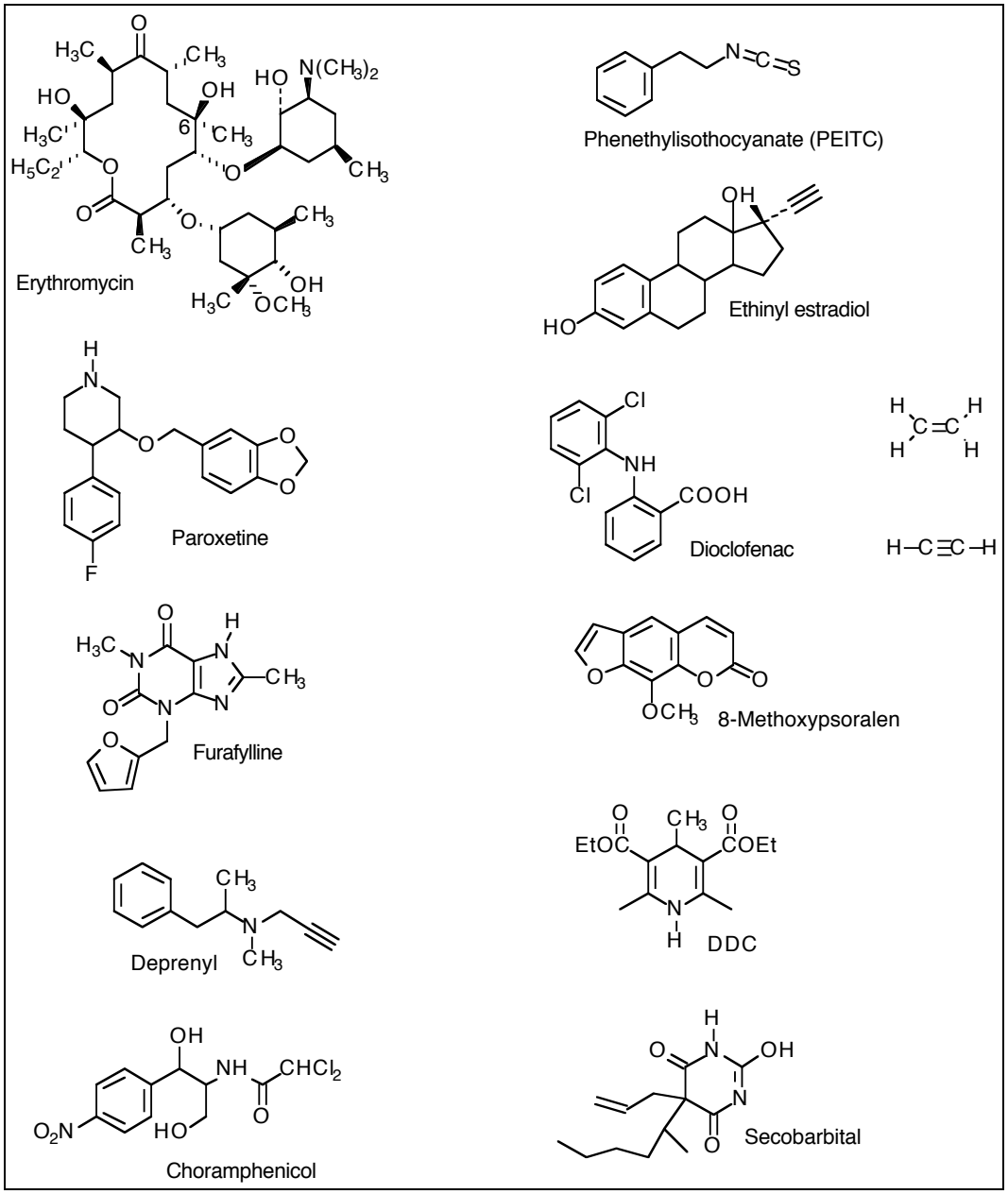
4. Inactivation is irreversible (common test: dialysis does not return enzyme activity).

5. A 1:1 stoichiometry of enzyme and adduct (radiolabeled substrates are useful here).

6. A catalytic step is required (O_2 and NADPH dependent in the case of P450's).

7. Inactivation must occur prior to the release of the activated species from the active site. Addition of trapping agents for electrophilic species, such as glutathione, should not attenuate the rate of inactivation. Note that this does not mean that reactive intermediates are not also released to solution. Often they are released and can be trapped with nucleophiles such as glutathione. Various compounds and enzymes that deactivate reaction oxygen species produced by the cycle or that prevent their propagation are also used (deferroxamine, catalase, superoxide dismutase).

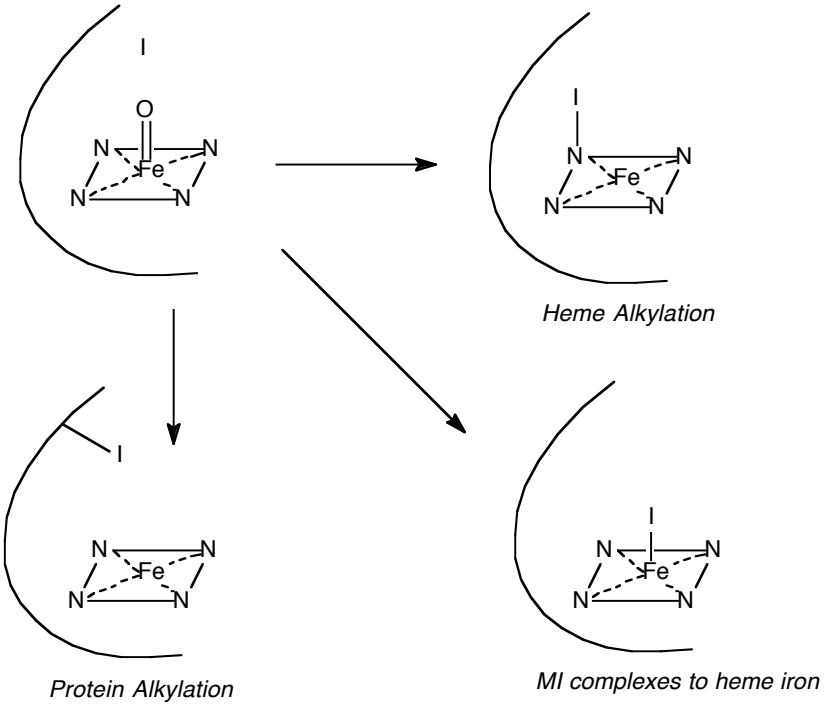
Examples of Mechanism Based Inhibitors of P450 Enzymes



Processing of certain types of structural features (eg: acetylene, olefin, alkylamine) in these substrates by P450 enzymes often causes mechanism based inactivation due to the creation of reactive intermediates.

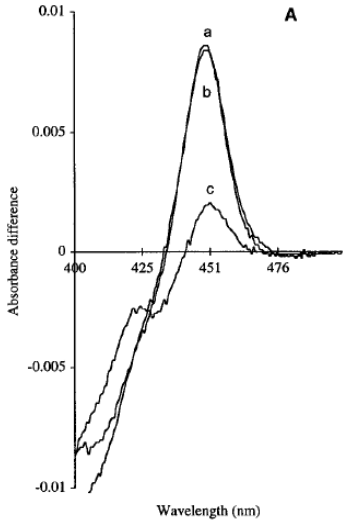
Targets for covalent adduction by reactive intermediates are the heme prosthetic group and/or active site amino acids.

As we will see later some inactivators produce more than one type of adduct with a single enzyme and it appears that bioactivation may produce more than one type of reactive intermediate.



Characterizing enzyme adducts.

1. Heme pyrrole nitrogen alkylation is usually detected by determining if the amount of P450, measured as the ferrous CO complex, is decreased by treatment with the MBI. The alkylated hemes can be removed from the protein and characterized by mass spectrometry and NMR.



2. Protein alkylation can be detected in two ways.

- Using radiolabeled MBI and detecting covalently adducts, preferably after separation on gels.
- HPLC purification of adducted protein and MW analysis to the intact protein or peptide fragments produced by proteolysis using mass spectrometry.

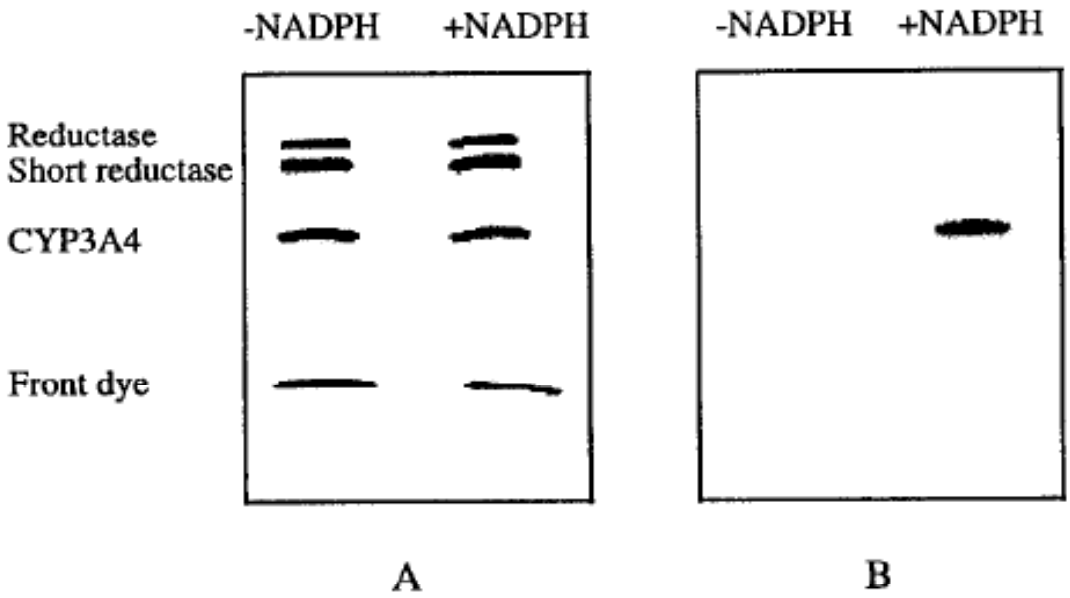
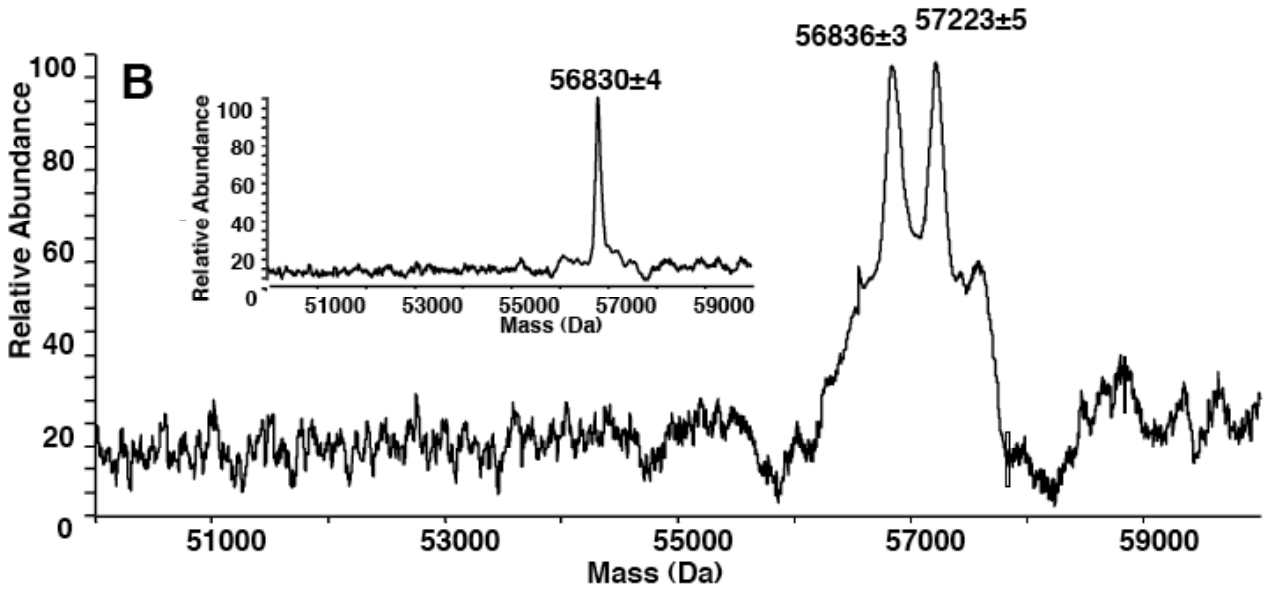
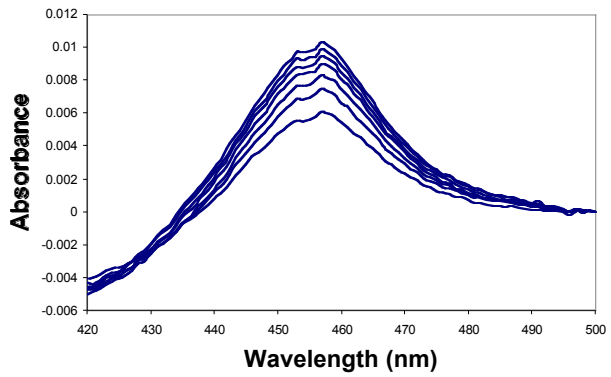


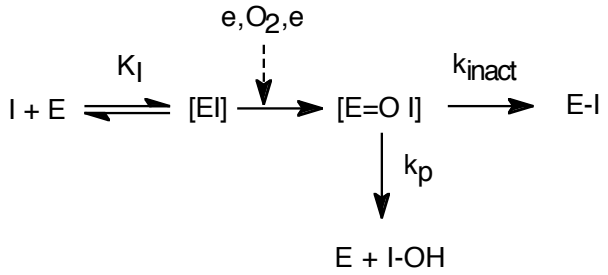
Fig. 5. SDS-PAGE analysis of the CYP-3A4 reconstituted reaction mixture after incubation with [^{3}H]mifepristone in the presence or absence of NADPH. A, Coomassie blue-stained gel; B, autoradiography of the gel. The experimental details were as described in *Materials and Methods*.



3. MI complexes have distinct spectral properties and are conveniently measured directly as they are formed in time using a UV-VIS spectrophotometer by difference spectroscopy (λmax at 456 nm).



- MBI's must be activated, usually by reaction with perferryl oxygen. Thus MBI's are substrates for the enzyme (hence the old name suicide substrate) and side products of the reaction are often observed. These may or may not be related to the reactive intermediate



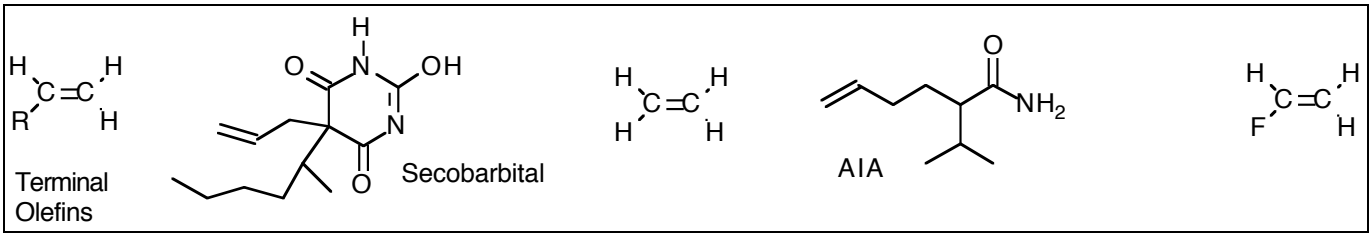
A portion of the catalytic events produce product(s) (I-OH above) that are released to the medium. The products observed often provide clues as to the nature of the reactive intermediate.

Partition Ratios The ratio of k_p/k_{inact} (products other than dead enzyme I-OH above) /(dead enzyme) is called the partition ratio. Partition ratios can vary from less than 1 to 5000 and more

Enzyme adducts exhibit a range of stabilities. Most protein and heme adducts are reasonably stable however some of the adducts may be cleaved under more stringent chemical environments. As we will see later on heme iron adducts such as MI complexes (nitroso-heme iron complexes) are quite stable yet they can be oxidized. This treatment restores enzyme activity. Thus we have to consider that some adduct types are pseudo-irreversible.

Some Examples of What We Know about Mechanism

Activation of olefins such as secobarbital, ethylene, octene usually leads to adducts with P450 heme pyrrole nitrogens. Occasionally covalent binding with active site amino acids is observed:



- Transfer of oxygen from the heme iron of P450s to terminal olefin pi bonds produces intermediates that react with the pyrrole nitrogens of heme (N-alkylated heme) at the terminal carbon.

- Epoxides are not involved in the inactivation process with respect to heme alkylation. Partition ratios tend to be high (>100) so heme alkylation is usually a low probability event.

- Epoxides are likely be involved in protein alkylation reactions

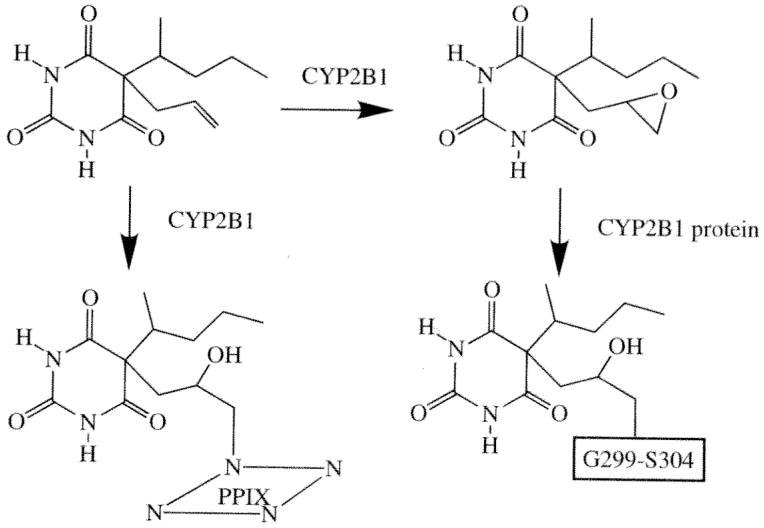
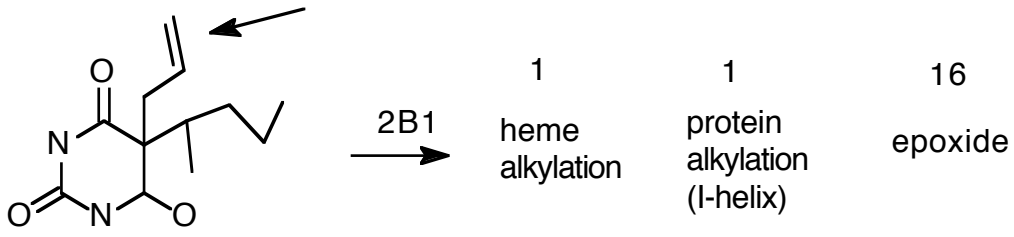
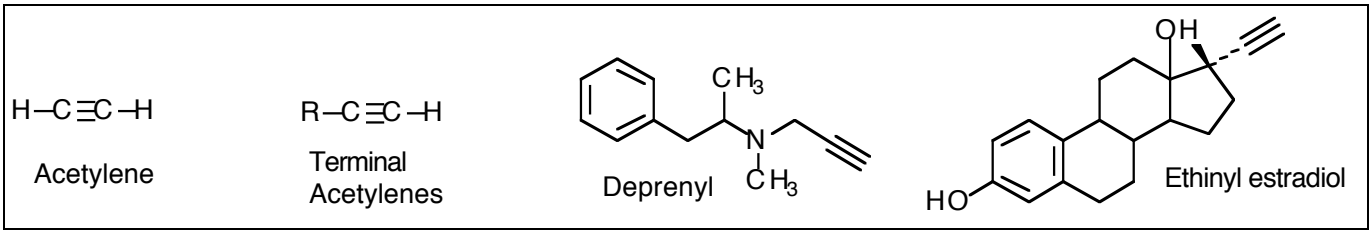


Figure 7.7. The oxidation of secobarbital by CYP2B1 results in both alkylation of one of the residue Gly299 and Ser304 and *N*-alkylation of the heme group as well as the generation of an epoxide.

In the case of secobarbital protein alkylation also occurs presumably due reaction of the epoxide with an active site amino acid. Generally heme alkylation appears to be the major event. Partition ratio? 8.

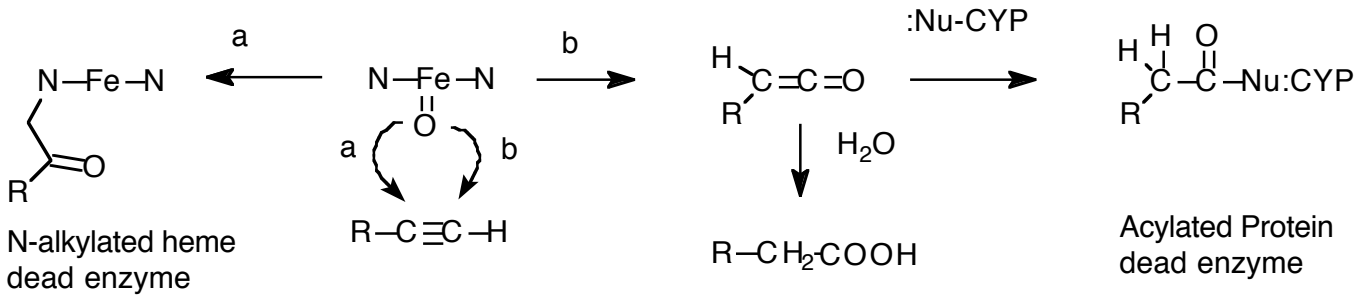


Acetylenes:

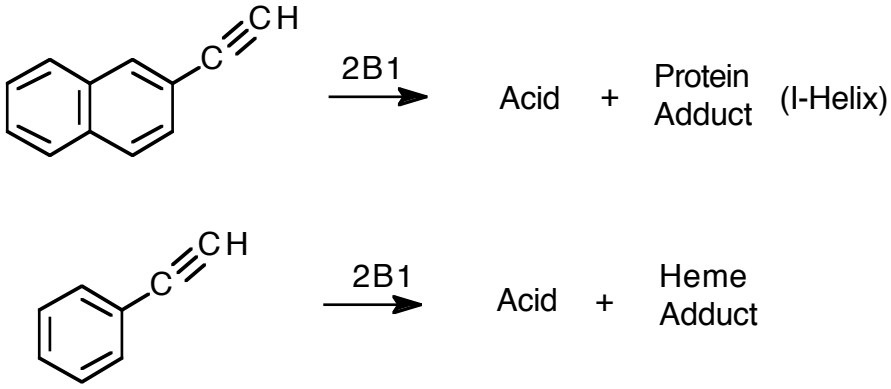


Transfer of oxygen from the iron of P450 to acetylenic triple bond is asymmetric leading to reactive intermediates that can form covalent bonds with heme pyrrole nitrogens or nucleophilic active site amino acids.

- Oxygen attack at the terminal carbon produces ketene intermediates that:
  - Alkylate amino acid of the P450 protein.
  - React with water in the active site or medium to form the carboxylic acid via hydrolysis.
- Heme N-alkylation seems to be the result of oxygen attack on the internal acetylenic carbon.

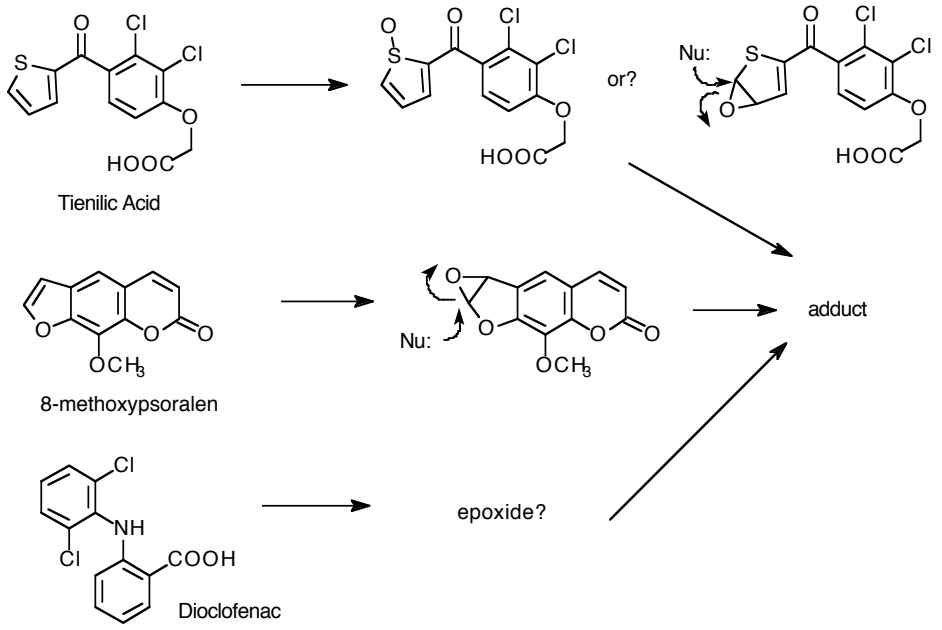


Here we see that slight changes in actetylene structure can affect the dominant alkylation event.

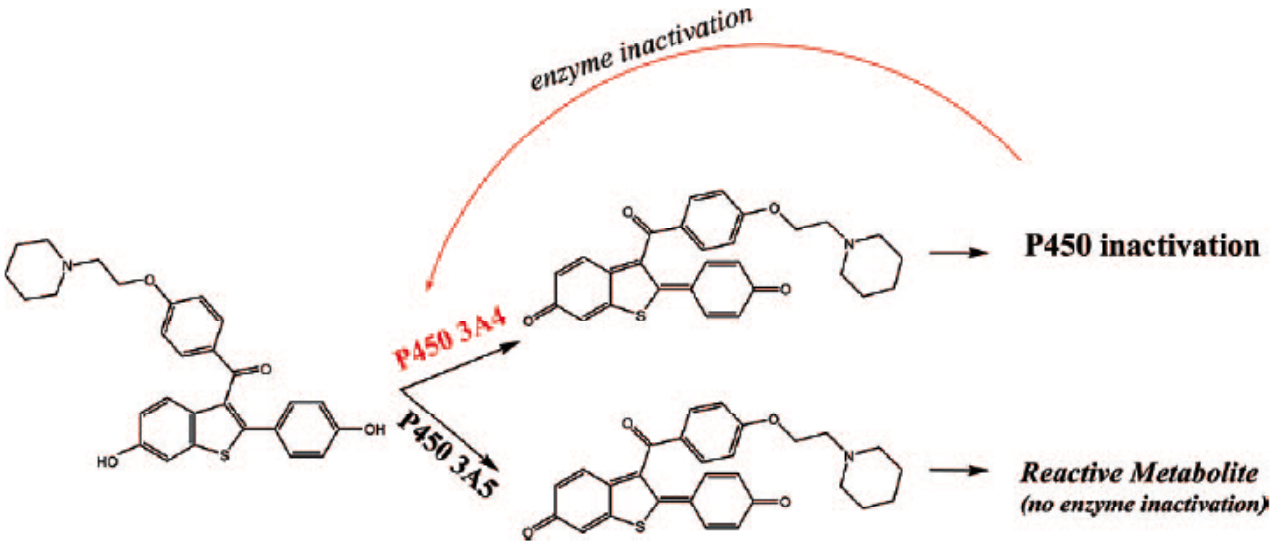


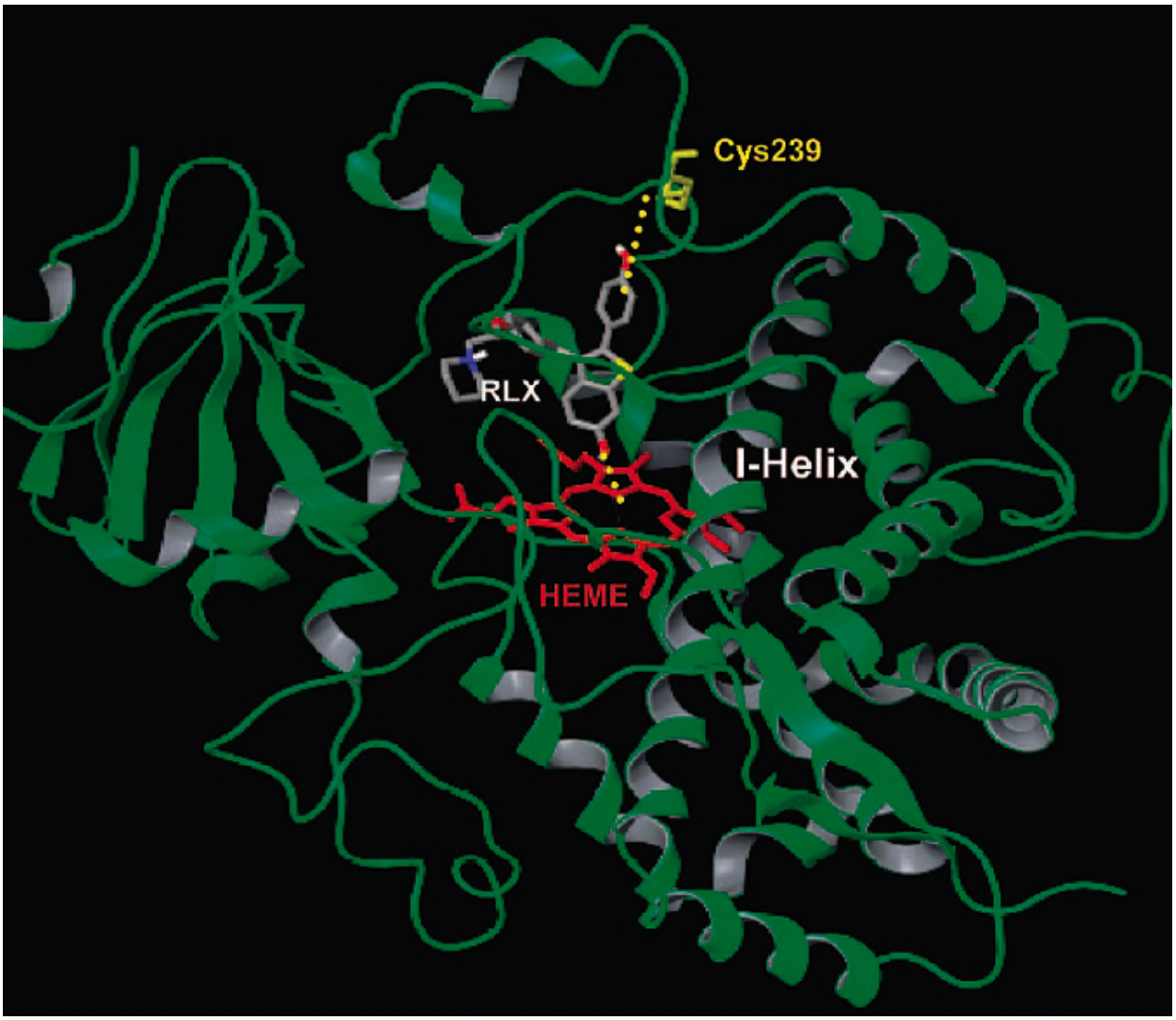
Heteroaromatics and Aromatics

Five membered heteroaromatic compounds thiophenes and furans can be activated to reactive intermediates that form adducts with protein. Very often adducts to other proteins in solution are also observed indicating that reactive intermediates are released. The non-specific binding can be suppressed by the addition of nucleophiles such as glutathione. Enzyme inactivation rates are generally not significantly reduced. This turns out to a critical distinction when one is trying to make the argument that inactivation is occurring in the active site of the enzyme.

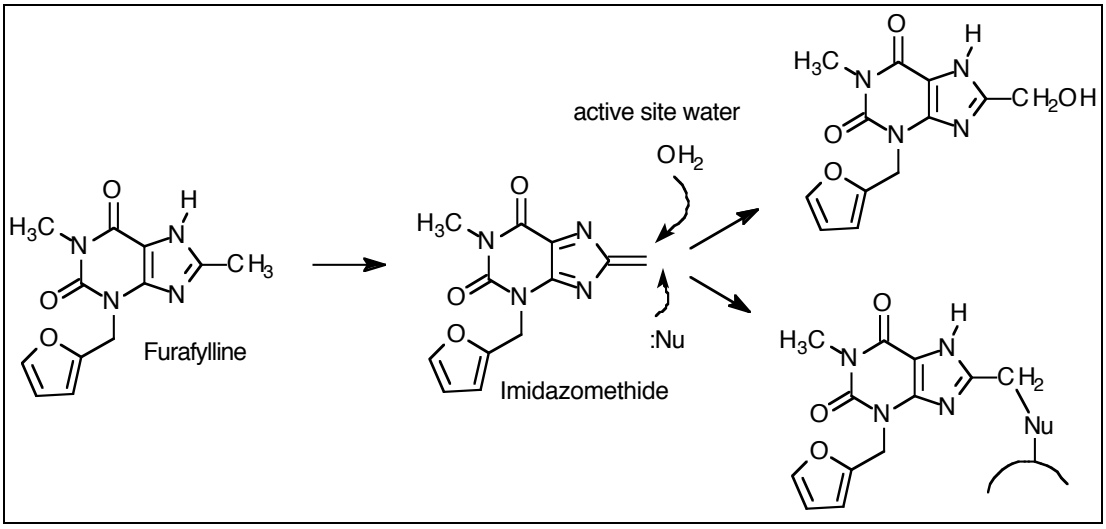


Quinone Methides (Raloxifene: Chem Res Tox 20 1778-86 (2007).



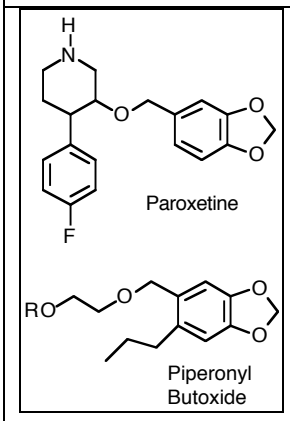
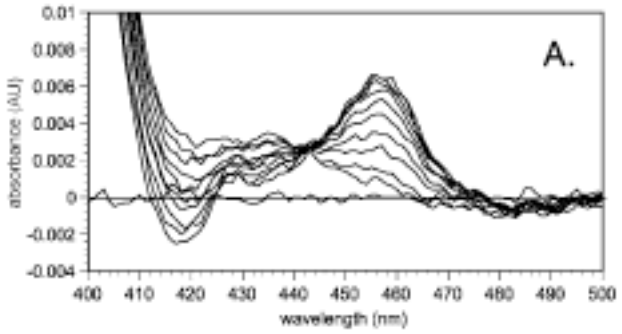

Cys239
RLX
I-Helix
HEME

Furafylline is a selective inhibitor of CYP1A enzymes that acts by protein alkylation:



Quasi-irreversible inhibitors bind to the heme iron to produce spectroscopically unique species called MI complexes.

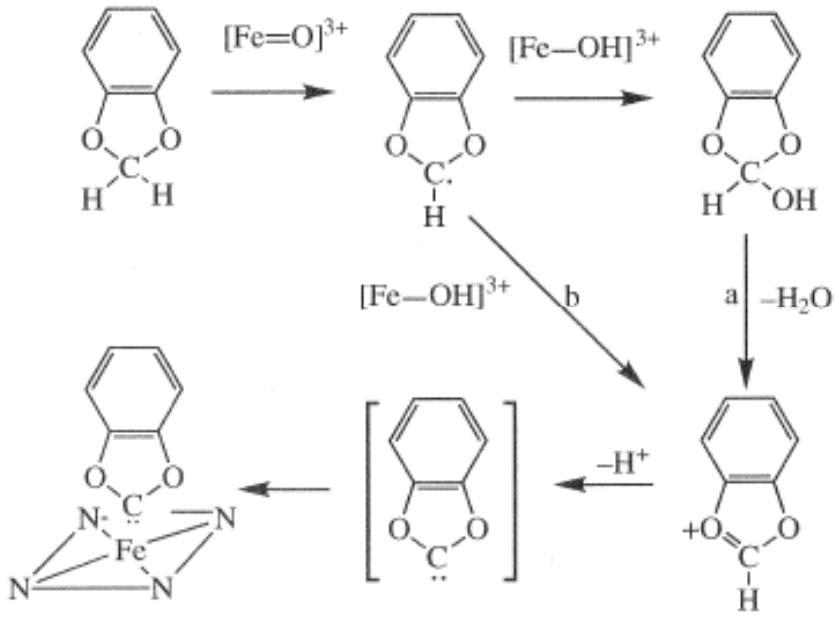
Metheylene dioxyphenyl compounds such as the insecticide synergist piperonyl butoxide and the drug paroxetine form carbene complexes with ferrous heme iron to form carbon ligated MI complexes (Metabolite Intermediate Complexes): These MI complexes have a Soret band at 450-458 nm.

	MI complex formation with CYP2D6 and paroxetine
	

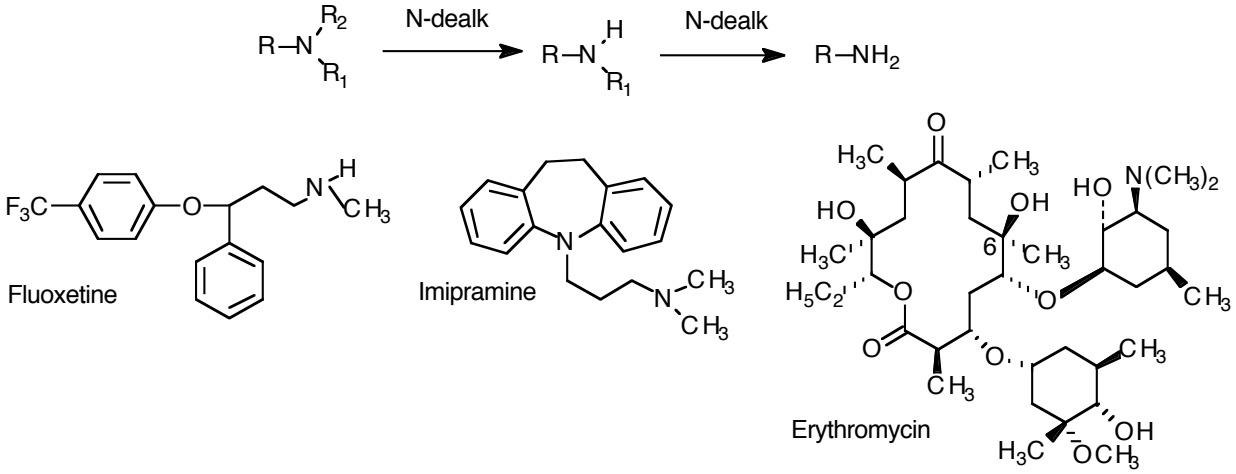
Piperonyl butoxide is used to inhibit the biotransformation and deactivation of insecticides.

Paroxetine is an antidepressant that causes drug drug interactions with CYP2D6 substrates

Proposed mechanism of carbene complex formation is shown below. Free active enzyme can be regenerated from these complexes by gentle oxidation with ferricyanide.



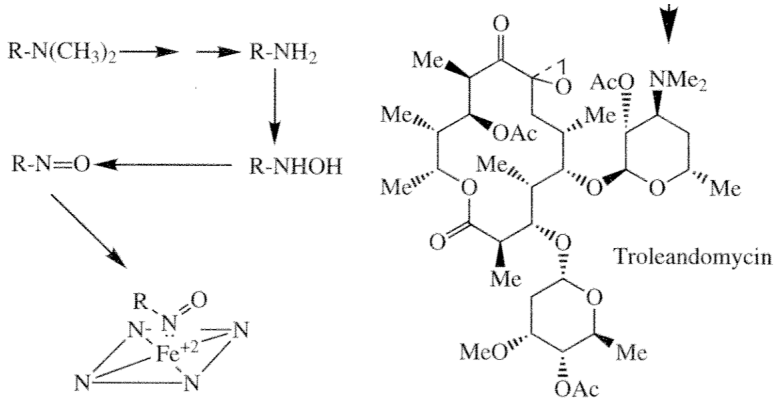
Alkylamines are also processed to MI complexes. The Soret band for these nitroso-ligated hemes also occurs at 455 nm. In man, the CYP3A enzymes are particularly susceptible to formation of MI complexes.



- A number of drugs in use and NCE's in combinatorial libraries contain this functionality. As we know alkyl amines are metabolized to simpler amines via N-dealkylation reactions. Often the half-lives of the metabolites are longer than the parent drugs in vivo.

- Many alkyl amines are processed to stable, catalytically inactive MI complexes.

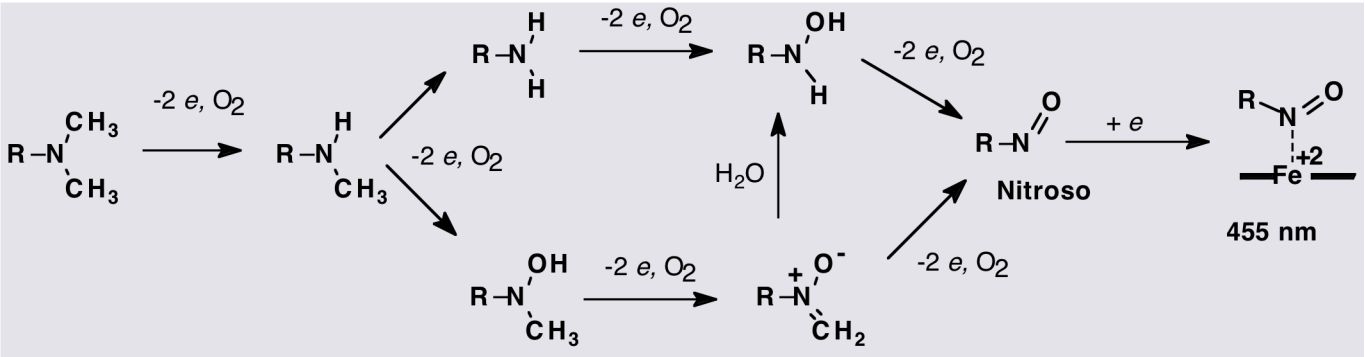
- A classic example is troleandomycin (TAO) that forms an MI complex with CYP3A enzymes in humans and animals.



- Here we note that four oxidations are required to produce the nitroso species that forms the stable MI complex with the ferrous heme of P450. Somewhat surprisingly these complexes can form rather rapidly in microsomes despite the requirement for multiple reactions.

- NADPH and oxygen dependent formation of MI complexes can be observed spectroscopically as a 450-458 nm species in microsomes. MI complexes are reasonably stable. They have been observed in microsomes prepared from animals and liver biopsy specimens from humans treated with TAO and other alkyl amines such as erythromycin, diltiazem, fluoxetine, desipramine, nortryptilline, amphetamine.

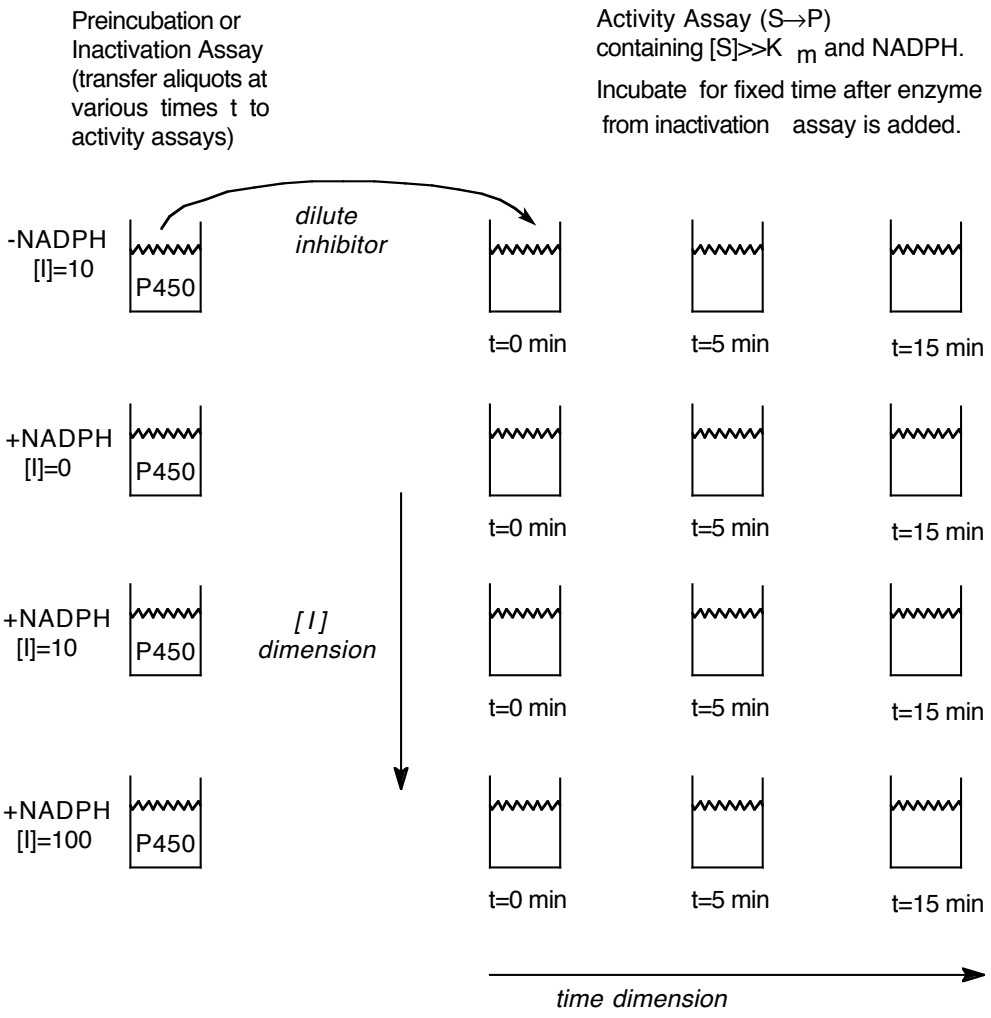
Multiple pathways and intermediates have been suggested to be involved in MI complex formation.



Characterizing Mechanism-Based Inhibition by the Numbers (Kinetics)

Method I Measuring the rate of loss of enzyme activity by determining the amount of active enzyme remaining as a function of time and MBI concentration.

- Experimental designs focus on determining the rate of loss of enzyme activity caused by the MBI. Because MBI must be catalytically activated by the enzyme itself we must use two types of incubations.
- Inactivation incubations that exposes the enzyme to different concentrations of the MBI for various periods of time.
- Activity assays in separate tubes with normal substrates for the enzyme to determine remaining activity.

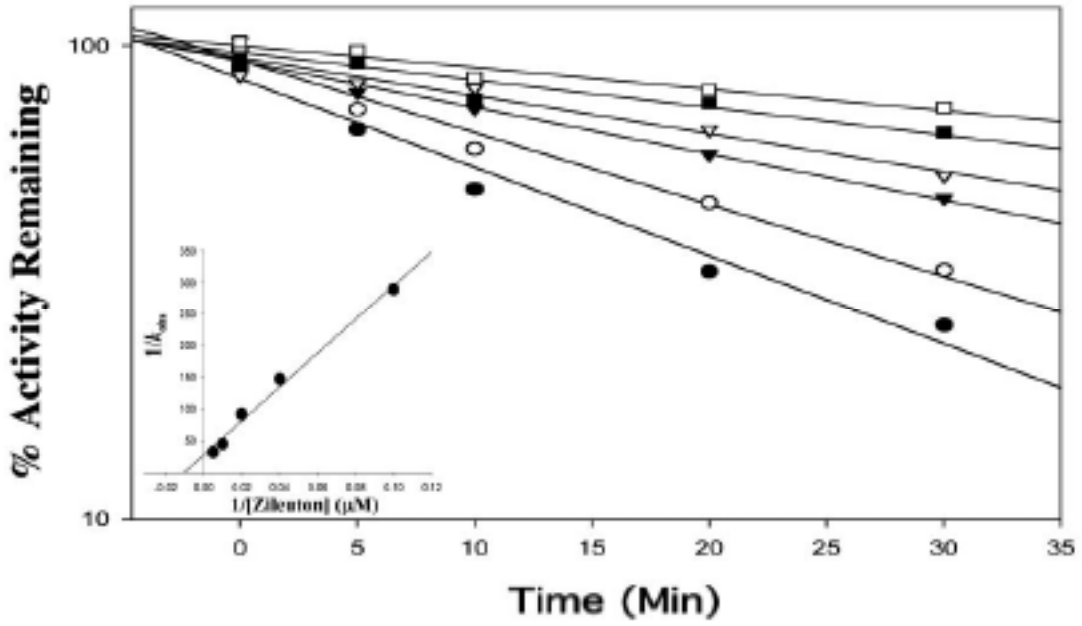


Features of the experimental design:

Dilute inhibitor into activity assays to prevent any further time dependent inhibition
Control for NADPH dependent loss of enzyme activity in the absence of inhibitor
Further evidence such as the demonstration of covalent binding to the enzyme is required bu not always obtained.

Plots

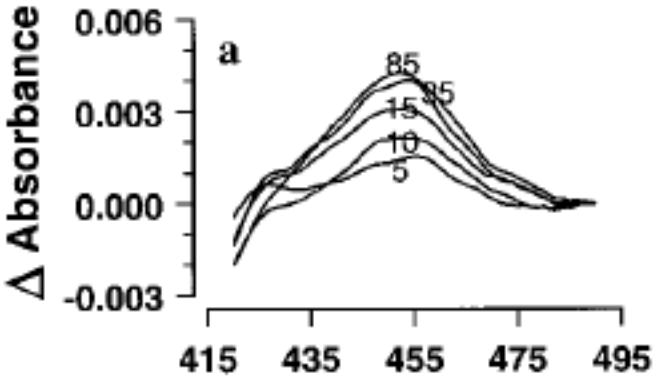
Typically, particularly at the early time points loss of enzyme activity is first order with time. Thus we plot log or ln percent remaining activity as a function of time and calculate the slopes of the lines to obtain values of λ or k_{obs} at each inhibitor concentration.



(S)-Zileuton Parameters for CYP1A2 inactivation using phenacetin in the activity assay: K_I= 98 μM; k_{inact} .037 min^{-1}. Enzyme $t_{1/2}$ is 20 min at saturating concentrations of (S)-Zileuton.

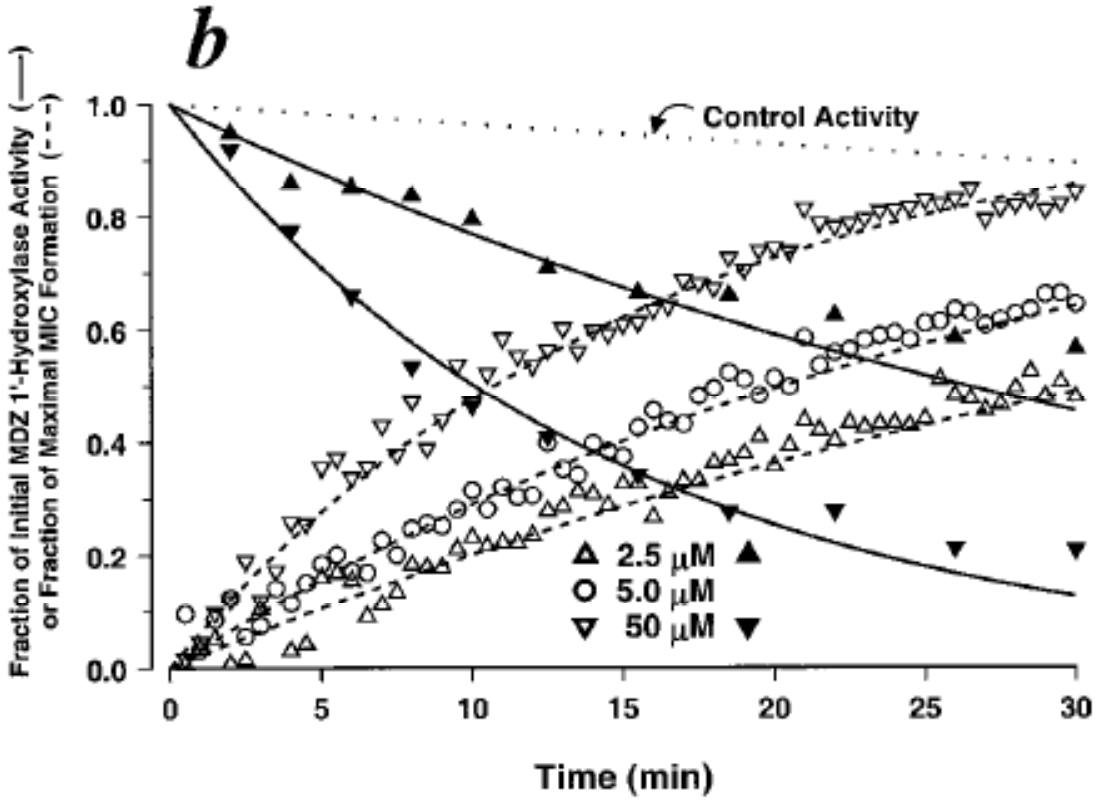
Reciprocal plots of λ or k_{obs} vs 1/I produce linear plots from which K_I and k_{inact} can be determined from X and Y intercepts (above inset).

Method 2: For alkylamine and methylenedioxyphenyl MBI's we can measure the appearance of inactivated enzyme directly in a spectrophotometer. For instance we can look at the formation of an MI complex formed upon incubation of the macrolide antiobiotic clarithormycin with CYP3A4.

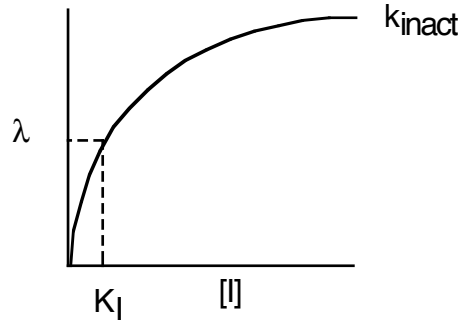


Below we see plots for the appearance of MI complex and loss of enzyme activity of with time of exposure of active enzyme to clarithromycin.

By either method we can calculate rates of inactivation λ at a given concentration of inhibitor.



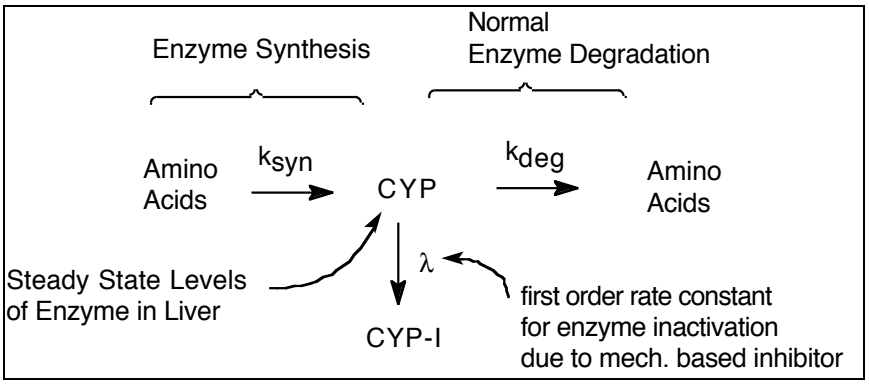
- A direct plot of λ as a function of inhibitor concentration is hyperbolic. The maximum rate for enzyme inactivation at saturating concentrations of the MBI is given as k_{inact}
- The concentration of inhibitor required to produce an inactivation rate (λ) that is one-half that of k_{inact} is the K_I. The K_I is approximates the reversible affinity of the MBI for the enzyme.
- Units of k_{inact} and λ are reciprocal time min^{-1}, hr^{-1} etc and enzyme half-life is given as 0.693/λ.
- Values of k_{inact} and K_I are constants for a given enzyme with a MBI.



$$\lambda_{[I]} = k_{\deg.[I]} = \frac{k_{inact} \cdot [I]}{[I] + K_I}$$

A little bit about the kinetics of inactivation helps us get a handle on *in vivo* relevance and potential problems detecting the phenomenon in vitro:

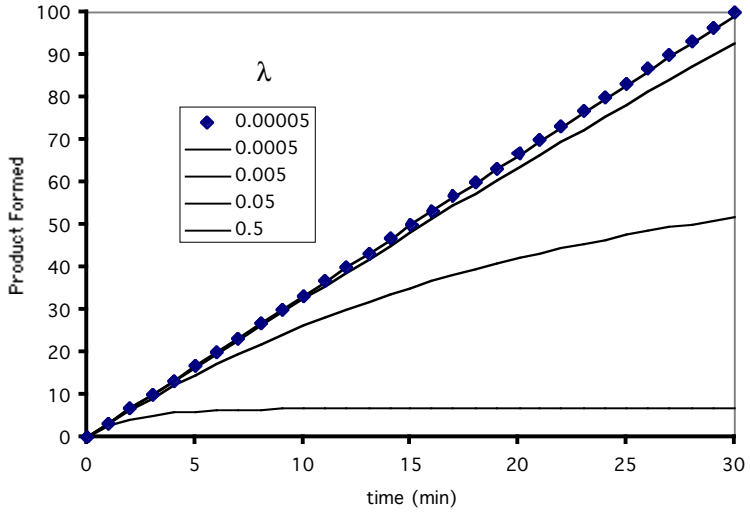
How do we know when inactivation that we might observe is significant? To do this we need to know the concentration of inhibitor available to the enzyme in the liver or other target organ and the endogenous rate of turnover of the P450 enzyme pool of interest (k_{deg} below).



- Our benchmark is the normal rate of enzyme degradation (k_{deg}) which is normally equal to k_{syn}.to provide some steady state level of enzyme $[E]_{ss}$.

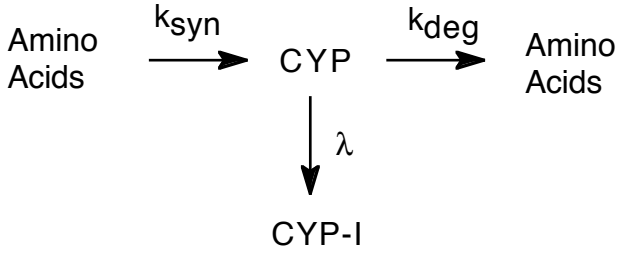
$$[E]_{ss} \propto \frac{k_{syn}}{k_{\deg}} \text{ normal} \qquad [E]_{ss} \propto \frac{k_{syn}}{k_{\deg} + \lambda} \text{ in the presence of MBI}$$

- Using a benchmark half-life of 24 hrs for a P450 enzyme we calculate that normal k_{deg}.is approximately 0.0005 min^{-1}. Thus a value of λ equal to this rate would reduce active enzyme concentrations by 50%.



We see that we could never see this value of λ in *in vitro* incubations so we may miss some slow inactivators.

- Heightened likelihood of drug-drug interactions where pool of active CYP in the liver is irreversibly depleted.



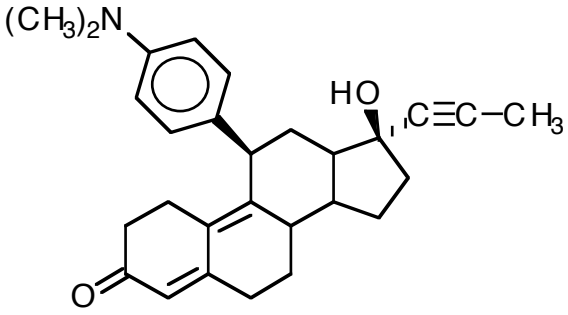
What concentration of the CYP 1A2 inactivator furafylline (K_I= 25 μM; k_{inact} = 0.9 min^{-1}) would produce a value of λ equal to .0005 min^{-1}?

$$I = K_I \div \left(\frac{k_{inact}}{\lambda} - 1 \right) = 25\mu M \div \left(\frac{0.9}{0.0005} - 1 \right) = 0.014\mu M$$

- Given a peak concentration of furafylline in plasma of 6 μM following a single 100 mg dose and its measured half-life of 50 hr in vivo we can calculate that as much as 15 days would be required before CYP1A2 content would return to 50% of control values!

- Since λ varies with inhibitor concentration, inhibitors with values of k_{inact} greater than 0.0005 min^{-1} are all suspect. Many inhibitors will not be a problem since λwill be far lower than k_{inact} at physiologically relevant concentrations of the inhibitor.

- A second factor that is important is mass balance. The amount of inhibitor dosed may simply be far too low to reduce the amount of enzyme contained in the body significantly. The acetylenic contraceptive steroids such as ethinylestradiol falls into this category. An exception to this may be RU486 although no drug interactions have been reported as yet.



Mefipristone (RU-486) and CYP3A4

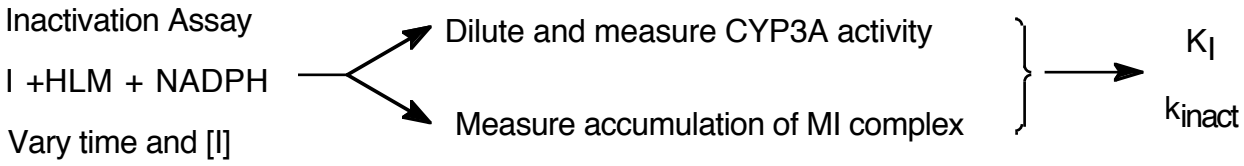
K_I = 5 μM k_{inact} = 0.089 min^{-1}

He *et al* JPET **288** 791-797 (1999)

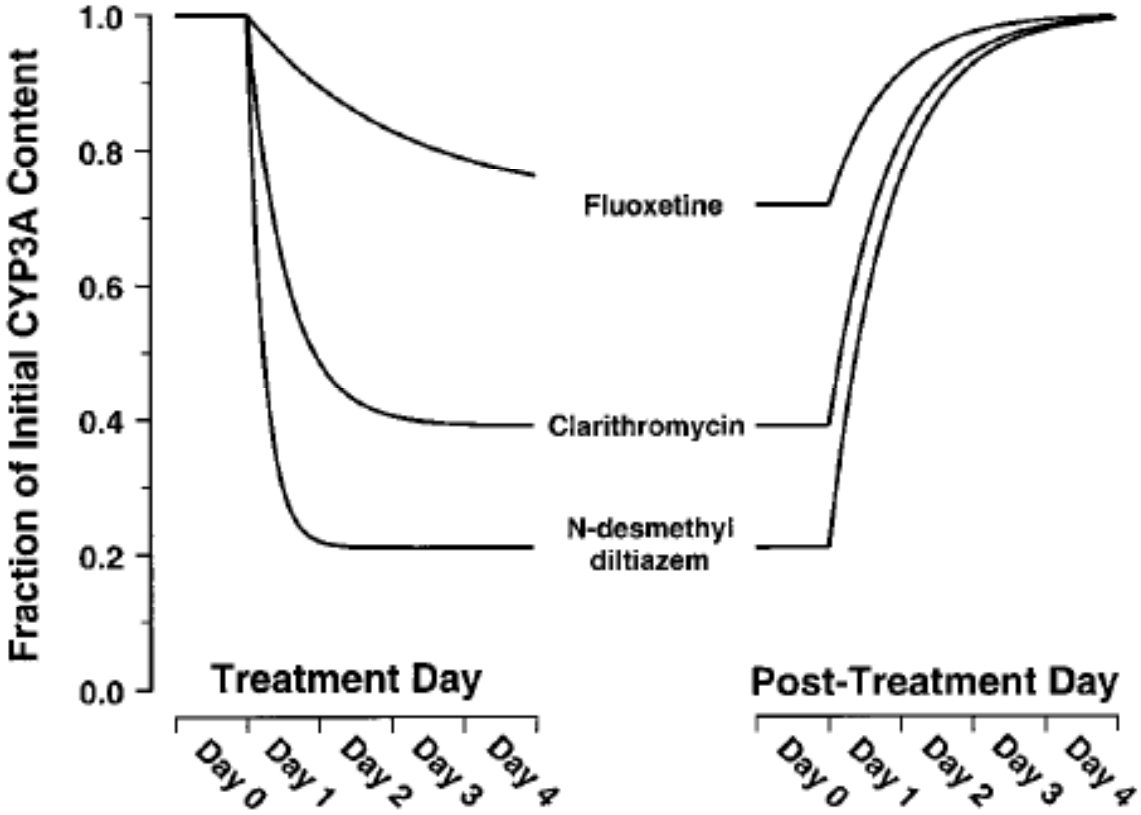
<u>Prediction of In Vivo Effect for Compounds that form MI complexes</u>.

Mayhew *et al* DMD **28** 1031-1037 (2000)

Demonstrate formation of MI complexes with expressed CYP3A4+(B5) for diltiazem, N-desmethyl diltiazem (MA), fluoxetine and clarythromycin and measure K_I and k_{inact}.



Predict the fall in active CYP3A enzyme concentrations when these inhibitors are present at their normal in vivo concentrations. In the case of clarithomycin active enzyme concentrations are predicted to fall to 40% of control.



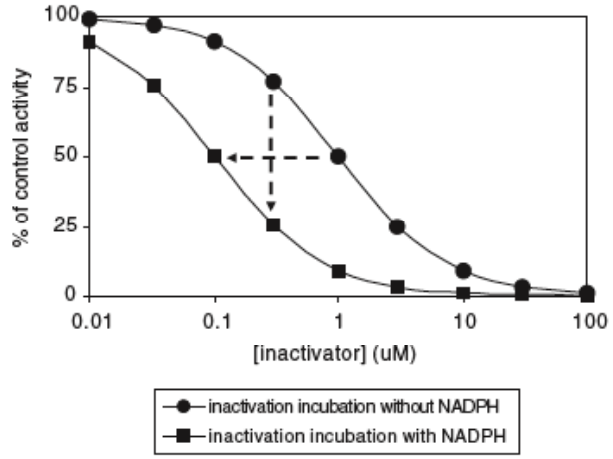
Clarithromycin: KI= 5.5 μM; k_{inact} = .072 min^{-1}: $[I]_p$ in plasma 0.1 μM.

$$\frac{AUC}{AUC'} = \frac{Cl'_{int}}{Cl_{int}} = \frac{[E]'_{ss}}{[E]_{ss}} = \frac{k_{deg}}{k_{deg} + \frac{[I]_p \cdot k_{inact}}{[I]_p + K_I}} = \frac{k_{deg}}{k_{deg} + \lambda}$$

Major issues for time dependent inactivation (TDI) of P450 enzymes by MBI.

1. Accurate determination of inactivation rate constants (K_I and k_{inact}). Not trivial to do well particularly for slow inactivators. How accurate do we have to be?

2. Rapid screening techniques (establishing criteria for acceptance



or rejection of an N. C. E.).

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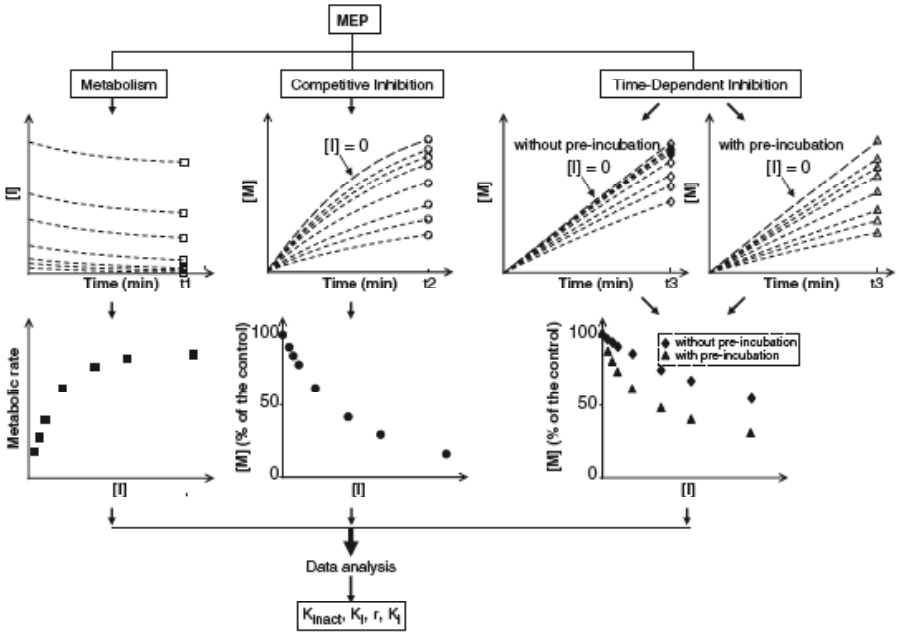

Figure 5. Schematic representation of the three elements of the mechanistically based experimental paradigm (MEP): (1) metabolism of the inactivator; (2) co-incubation paradigm to assess competitive inhibition; and (3) pre-incubation to assess time-dependent inhibition (see the text for details). The plots represent simulations of experiments with the representative inactivator (*R*)-(+)-menthofuran. Reproduced from Yang et al. (2007a), with permission.

3. Is hepatocyte work necessary? (Transporters, induction vs inhibition, phase II enzymes). Zhao et al DMD 33 853-61 (2005); McGinnity DMD 34 1291-1300 (2006)

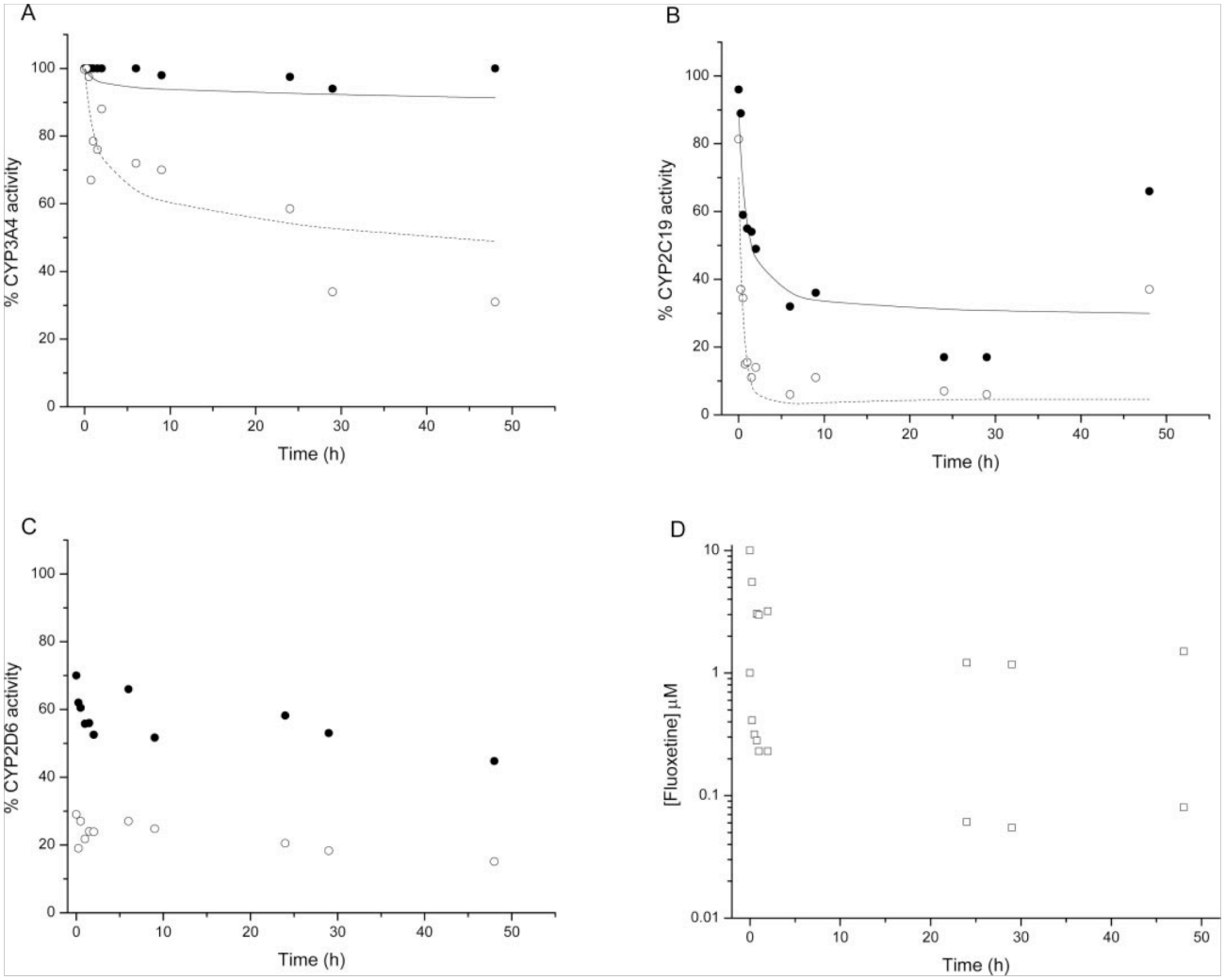

FIG. 3. CYP3A4, 2C19, and 2D6 activity in cultured human hepatocytes after incubation with fluoxetine. Using cultured human hepatocytes, CYP3A4-dependent midazolam 1′-hydroxylation (A), CYP2C19-dependent *S*-mephenytoin 4′-hydroxylation (B), and CYP2D6-dependent bufuralol 1′-hydroxylation (C) were determined after incubation with 1 μM (closed circles) and 10 μM (open circles) fluoxetine, as described under *Materials and Methods*. Data points represent the mean activity relative to a solvent control incubation at the corresponding time point, from donors 1, 3, and 5. Where activities from at least three donors were measured, error bars reflect the standard deviation from the mean. The solid and dashed lines indicate nonlinear regression of the 1 and 10 μM fluoxetine data, respectively, using eq. 4, as described under *Materials and Methods*. Open squares represent the mean concentrations of fluoxetine (D) from the 1 and 10 μM incubations over the time course of the experiment as determined by HPLC-MS/MS.

4. In order to predict the magnitude of the inhibition we have to take into account the fraction of the object drug that is cleared by the inhibited enzyme (f_{mCYP}).

$$\text{predicted}\ \frac{AUC_i}{AUC} = 1 + \frac{\left(\frac{[I] \times k_{inact}}{[I] + K_I}\right)}{k_{deg}} = 1 + [\lambda]/k_{deg}$$

$$\text{predicted}\ \frac{AUC_i}{AUC} = \frac{CL_{int}}{CL_{int,i}} = \frac{1}{\left(\frac{f_{mCYP}}{1 + \left(\frac{[I] \times k_{inact}}{k_{deg} \times ([I] + K_I)}\right)}\right) + (1 - f_{mCYP})}$$

This equation used

TABLE 1

In vivo and in vitro data used for risk assessment

Inactivator	P450	Inactivator Dose	Object Drug	AUC Change of Object Drug (Observed)	K_I	k_{inact}	λ/k_{deg} Using Total C_{max}	λ/k_{deg} Using Unbound C_{max}	References[a]
					μM	min^{-1}			
Amiodarone	3A4	400 mg, q.d., 4 days	Simvastatin acid	1.8	42	0.02	7.8*	0.3*	Becquemont et al., 2007; Shoaf et al., 2005; Mori et al., 2009
Amprenavir	3A4	1200 mg, b.i.d., 10 days	Rifabutin	2.9	0.3	0.73	3268	2876	Polk et al., 2001; Ernest et al., 2005
Cimetidine	2D6	300 mg, q.i.d., 6 days	Imipramine	2.7	77	0.03	15	12	Wells et al., 1986; Kosoglou et al., 2000; Madeira et al., 2004
Clarithromycin	3A4	500 mg, b.i.d., 9 days	Simvastatin	10	5.5	0.07	133	36	Jacobson, 2004; van Haarst et al., 1998; Mayhew et al., 2000
Clopidogrel	2B6	75 mg, b.i.d., 4 days	Bupropion	1.4	1.4	1.9	78	4.7	Turpeinen et al., 2005; Kim et al., 2008; Walsky and Obach, 2007
Dasatinib	3A4	100 mg, single	Simvastatin	1.2	6.3	0.03	5.8	0.4	Product Label[b]; Li et al., 2009
Diltiazem	3A4	60 mg, t.i.d., 2 days	Buspirone	5.3	3.7	0.07	37.6	11	Lamberg et al., 1998; Shum et al., 1996; Zhang et al., 2009
Erythromycin	3A4	500 mg, t.i.d., 2 days	Simvastatin	6.2	11	0.05	57.0	12	Kantola et al., 1998; Olkkola et al., 1993; McConn et al., 2004
Fluoxetine	3A4	20 mg, q.d., 21days	Alprazolam	1.3	5.3	0.02	3.8	0.2	Hall et al., 2003; Harvey and Preskorn, 2001; Mayhew et al., 2000
Isoniazid	3A4	90 mg, b.i.d., 4days	Triazolam	1.5	228	0.08	9.7*	9.7*	Ochs et al., 1983; Dattani et al., 2004; Wen et al., 2002
Mibefradil	3A4	100 mg, single	Midazolam	8.9	2.3	0.40	553	4.0	Veronese et al., 2003; Welker et al., 1998
Nelfinavir	3A4	1250 mg, b.i.d., 14days	Simvastatin	6.1	0.48	0.22	952	77	Hsyu et al., 2001; Fang et al., 2008; Ernest et al., 2005
Paroxetine	2D6	20 mg, q.d., 17days	Atomoxetine	7.1	3.6	0.13	25	1.3	Belle et al., 2002; Perloff et al., 2009
Ritonavir	3A4	600 mg, single	Saquinavir	112	0.038	0.29	1315	913	Hsu et al., 1998; Luo et al., 2003
Rofecoxib	1A2	25 mg, q.d., 4 days	Tizanidine	12	4.8	0.07	44	6.7	Backman et al., 2006; Karjalainen et al., 2006
Saquinavir	3A4	1200 mg, t.i.d., 5 days	Midazolam	5.2	0.2	0.31	1223	102	Palkama et al., 1999; Cook et al., 2004; Ernest et al., 2005
Tadalafil	3A4	20 mg, q.d., 14 days	Lovastatin	1.12	12	0.21	76*	4.9*	Ring et al., 2005; Wrishko et al., 2008
Ticlopidine	2B6	250 mg, b.i.d., 4 days	Bupropion	1.6	0.3	0.43	1067*	174*	Turpeinen et al., 2005; Lu et al., 2006; Walsky and Obach, 2007
Ticlopidine	2C19	200 mg, q.d., 8 days	Omeprazole	6.2	9.2	0.25	108	2.7	Ieiri et al., 2005; Lu et al., 2006; Atkinson et al., 2005
Tienilic acid	2C9	250 mg, q.d., 19 days	(*S*)-Warfarin	2.9	12.5	0.13	846	30	O'Reilly, 1982; Dubb et al., 1979; Hutzler et al., 2009
Verapamil	3A4	80 mg, t.i.d., 2 days	Simvastatin	4.7	4.6	0.43	195	21	Kantola et al., 1998; Johnson et al., 2001; Wang et al., 2004
Zileuton	1A2	800 mg, b.i.d., 5 days	Theophylline	1.9	117	0.04	16	1.2	Granneman et al., 1995; Lu et al., 2003

* The inactivator concentration was calculated with dose normalization.
[a] References for in vivo AUC change, in vivo inactivator concentration (in cases where C_{max} was not measured in the DDI study), and in vitro MBI parameters.
[b] http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/021986s009s010lbl.pdf.

A λ/kdeg value of 1 based on in vitro data predicts a two fold increase in AUC of the parent drug. Note that the fm of the object drug is not taken into account so the predicted value is an overestimate.

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