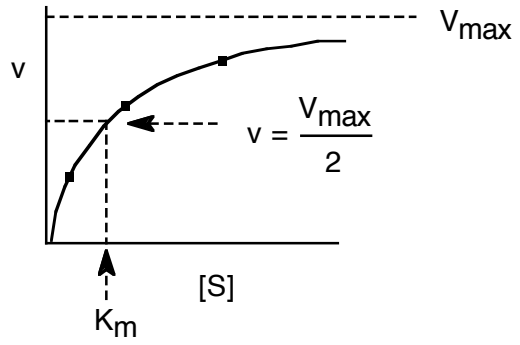
P450 Enzyme Kinetics and Reversible Inhibition

(MedChem/Pceut 527; Winter 2011; Kent Kunze)

The equation took the curse off enzymes. They were brought down from the status of a mysterious name. to a level where at least they were amenable to mathematical treatments

Issac Asimov on the contribution of Leonor Michaelis and Maude Menten to enzyme kinetics

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$



Major areas of interest that we will touch on in the next two lectures are:

1) Characterizing the kinetics of P450 catalyzed oxidation reactions with respect to:
   a) Substrate clearance and metabolite formation rates
   b) Reversible inhibition of enzyme activity
   c) Time dependent inhibition of enzyme activity.

2) Major goals
   a) Introduce the important concepts in enzyme kinetics
   b) Familiarize you with the important terms and assumptions
   c) Improve your "kinetic intuition" (does this make sense?) and pattern recognition (plots).

3) Kinetics is the study of how substrates, enzymes and products in a system change with time. Our goal is to describe the system with a set of parameters and a kinetic model.

   a) We would like our parameters to be constants and to have "real" meaning.

   b) We would like our model to be simple and generally applicable to different systems.

   c) We would like to determine our parameters by systematically varying substrate and inhibitor concentrations and observing effect in the "steady state" if at all possible.

$$E + S \underset{fast}{\overset{K_m}{\rightleftharpoons}} [ES] \xrightarrow[slow]{V_{max}} P$$

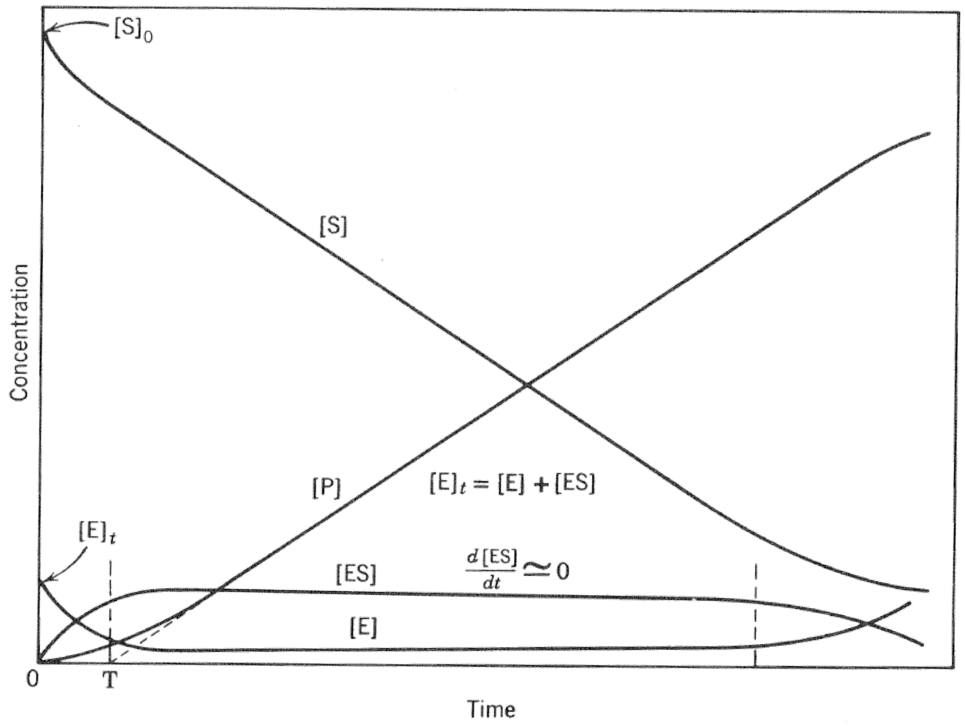


Fig. II-1. Progress curve for a catalyzed reaction where the initial substrate concentration, $[S]_0$, is significantly greater than the initial enzyme concentration, $[E]_t$. As the ratio of $[S]_0/[E]_t$ increases, the steady-state region accounts for an increasing fraction of the total reaction time.

4) Steady State Kinetics of P450 Catalyzed Reactions: Michaelis-Menten Kinetics

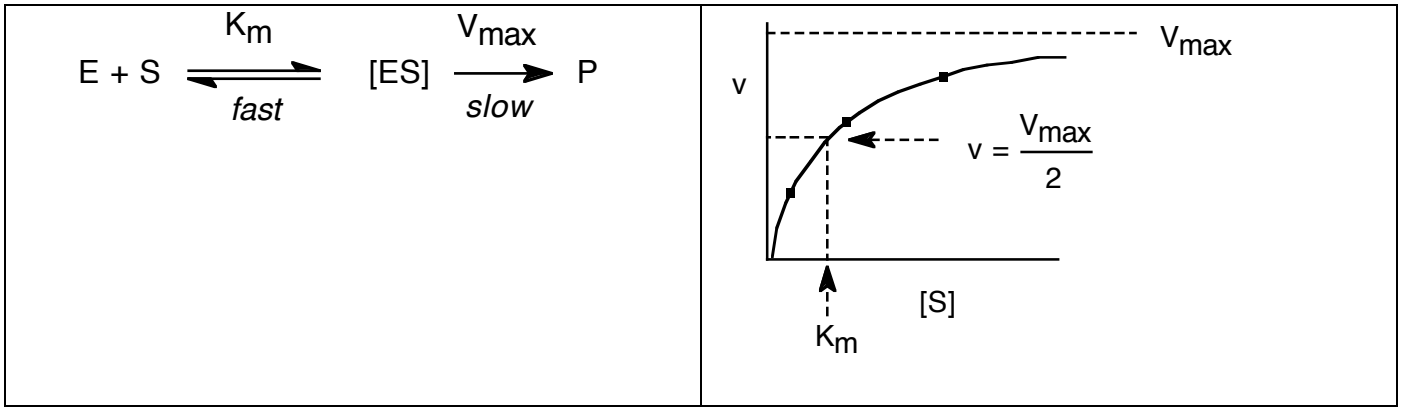
Enzyme-catalyzed reaction kinetics are commonly studied by varying the concentration of substrate S and measuring the amount of product P formed by the enzyme per unit time.

a) The goals of this type of experiment are to (1) determine parameters and (2) verify mechanism:

i) The maximum rate that the enzyme can form product (V_{max}) or k_{cat}.

ii) The concentration of substrate that is required to produce a rate of product formation (v) that is half of the maximum rate ($V_{max}/2$). This value is called a K_m which a special type of dissociation constant. At this concentration of substrate, one-half of the enzyme is complexed with substrate (ES; Michaelis Complex) and one-half is free in solution (E).

iii) Whether or not the enzyme-catalyzed reaction follows Michaelis Menten kinetics (is the v vs [S] plot a true rectangular hyperbola.



b) Features of the v vs [S] plot.

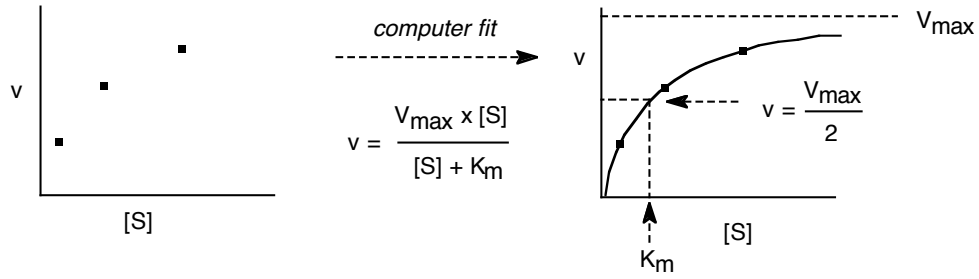
i) At low substrate concentrations ($[S]<<K_m$) the observed product formation rates v are directly proportional to substrate concentrations (v is 1st order with respect to substrate; double the substrate concentration doubles the rate of product formation). In this region, a constant percentage of substrate is cleared from solution per unit time.

ii) At high, saturating concentrations of substrate ($[S]>>K_m$) the observed product formation rates are independent of substrate concentrations (v is zero order with respect to substrate; double the substrate concentration, no change in rate). In this region a constant amount of substrate is cleared from solution per unit time.

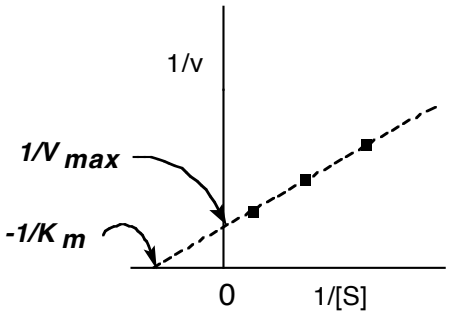
iii) At substrate concentrations in the region of the K_m ($[S]=K_m$) the reaction order is approximately 0.5 (double the substrate concentration increase v by 50%.

c) Methods for determining kinetic constants

i) We can fit the data obtained to the Michaelis-Menten equation using non-linear regression packages. This generates a hyperbolic curved line of best fit through the data points and provides us with estimates of the two parameters.

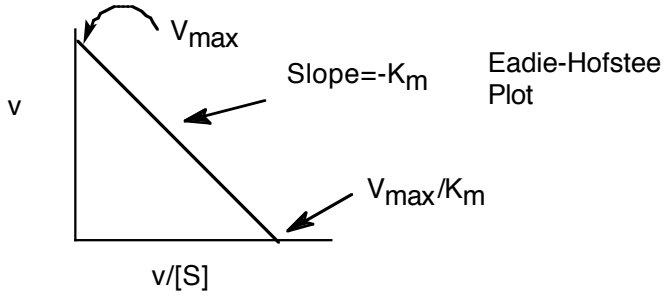


ii) Lineweaver-Burke Plot (a double reciprocal plot of the data (1/v vs 1/[S])). We can calculate the reciprocal values of the velocities and substrate concentrations and plot each pair of reciprocal values. If the points lie on a straight line we can draw or calculate that line and calculate V_{max} and K_m from the intercepts.



Lineweaver Burke Plot
of a hyperbolic function
is a straight line.
A double reciprocal plot

iii) Eadie Hofstee Plot (a reciprocal plot (v vs v/[S]). Again data points should lie on a line. A very sensitive and discriminating plot.



Eadie-Hofstee
Plot

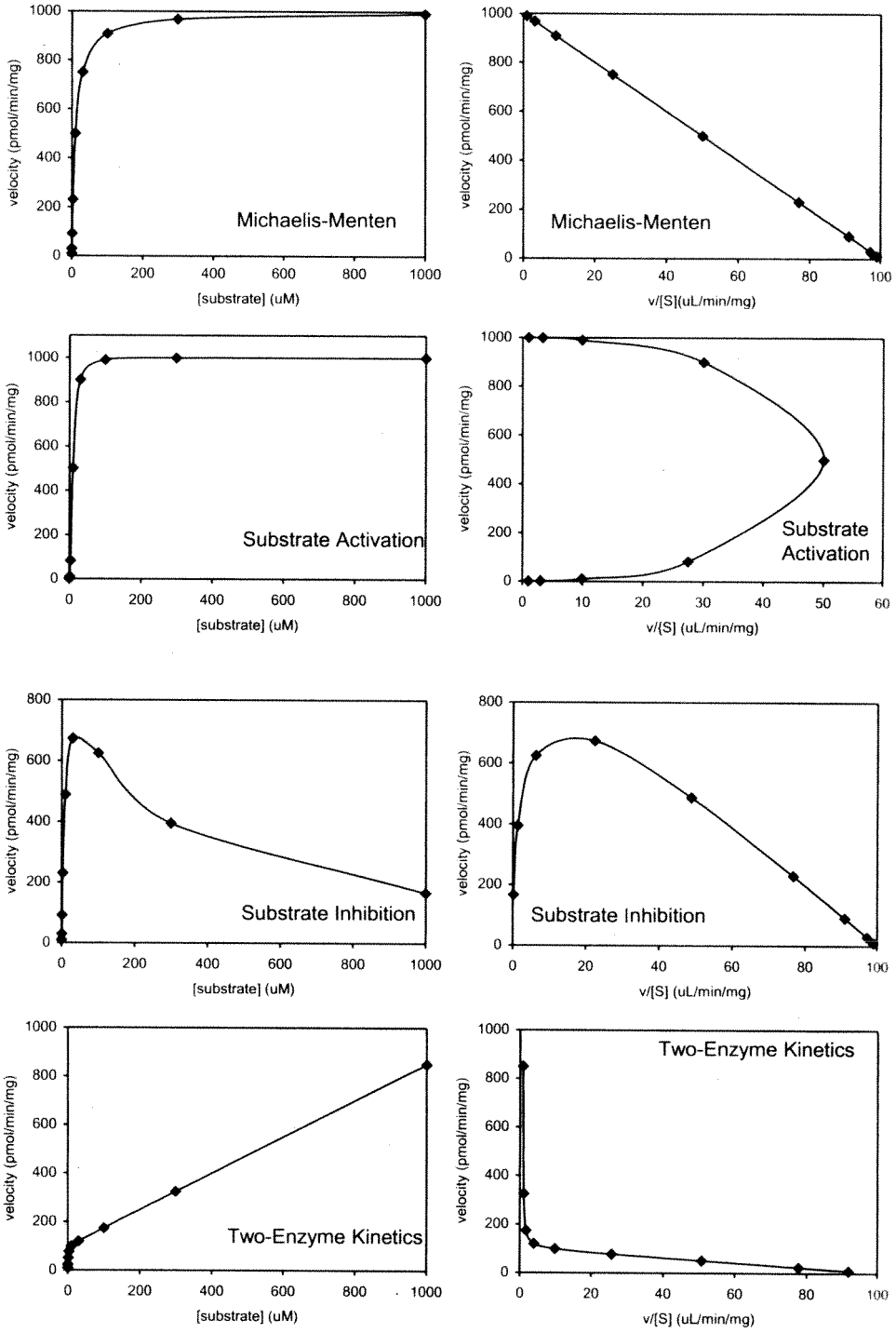


Figure 3 Four common enzyme kinetic models for cytochrome P450–catalyzed reactions in human liver microsomes. Substrate saturation curves (*left*). Eadie-Hofstee transformed plots of the data on the left (*right*).

d) The reciprocal plots are very useful for detecting non-Michaelis-Menten behavior. Three major types of non-classical behavior are:

i) When multiple enzymes in liver microsomes catalyze the formation of the same product.

ii) Allosteric behavior when more than one substrate binding site exists on a single enzyme.

iii) Non-specific binding of substrates to protein or lipid. When free substrate concentrations available to the enzyme vary significantly from the nominal concentrations (or you have messed up on your serial dilutions of substrate).

e) Important aspects of K_m

i) K_m values are reported in units of substrate concentration (molar (M), millimolar (mM), micromolar (μM), etc).

ii) The K_m is the same value as the concentration of substrate that produces a rate of product formation that is half of V_{max}.

iii) The K_m is a measure of the affinity of a particular substrate for a particular enzyme. The lower the K_m the higher the affinity of the substrate for the enzyme.

iv) K_m is not dependent on enzyme concentration and is a constant for a given substrate enzyme pair under standard conditions.

v) The K_m approximately equal to the dissociation constant K_d.

f) Important aspects of V_{max}

i) V_{max} is equal to the rate v (<u>this can be product formation or substrate consumption</u>) that would be observed in an incubation if the enzyme was saturated with substrate (not always possible due to solubility limitations of many substrates). It is expressed as a rate (nmol product/min) and must be sourced to some reference value characteristic of that particular incubation such as mg microsomal protein, nmol of total P450, nmol of CYP2C9, mg wet weight liver used, 10^6 hepatocytes etc.

$$V_{max} = \frac{17 \text{ nmol P formed}}{\text{min} \cdot \text{mg mic. protein}} = 17 \text{ pmol P} \cdot \text{min}^{-1} \cdot \text{mg mic. protein}^{-1}$$

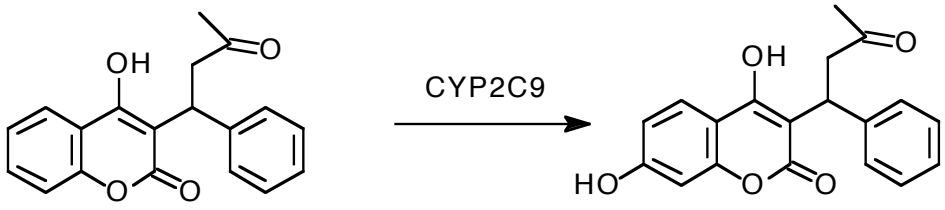
$$k_{cat} = \frac{8.5 \text{ nmol P formed}}{\text{min} \cdot \text{nmol CYP3A4}} = 8.5/\text{min} = 8.5 \text{ min}^{-1}$$

ii) When we know the amount/concentration of the enzyme itself we can calculate a k_{cat} or turnover number. The turnover number is the number of times a single enzyme molecule can

makes a product molecule per unit time (seconds or minutes usually) under saturating conditions.

iii) The k_{cat} is a constant for a given substrate enzyme pair under standard conditions however it may vary depending on the preparation (microsomes, supersomes etc) Why?.

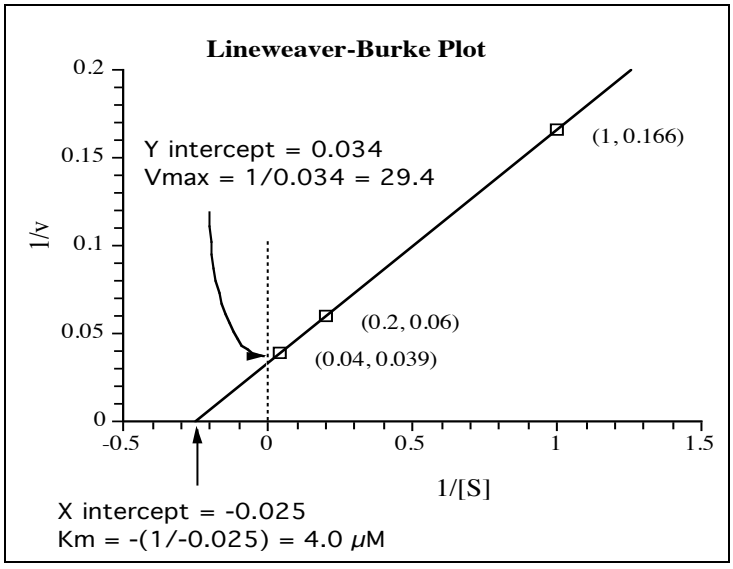
g) So let's do an example using a drug being metabolized by a cytochrome P450 enzyme in samples of a human liver (1mg tissue) where we measure the amount of product formed in 5 min:



warfarin
Coumadin

7-hydroxywarfarin
(major metabolite in man)

Concentration of substrate in tube containing 1 mg samples of the liver	Amount of the product we measure after a 5 minute incubation	The rate or velocity that we calculate (v)	Reciprocal of the substrate concentration	Reciprocal of the velocity we calculated
Warfarin	7-OH warfarin	7-OH warfarin	1/[S]	1/V
[S] (μM)	P amount produced (pmole mg liver^{-1})	v (nmole · mg liver^{-1} ·minute^{-1})	μM^{-1}	(nmole^{-1} mg liver minute)
1	30	6.0	1.00	.166
5	83.3	16.7	0.20	.060
25	129	25.9	0.04	.039



$$V_{max} = 29.4 \text{ nmole product} \cdot \text{ mg liver}^{-1} \cdot \text{ minute}^{-1}$$

If there was 10 nmole of enzyme in 1 mg liver then

$$k_{cat} = \frac{29.4 \text{ nmole product} \cdot \text{ mg liver}^{-1} \cdot \text{minute}^{-1}}{10 \text{ nmole enzyme} \cdot \text{ mg liver}^{-1}} = 2.94 \text{ minute}^{-1}$$

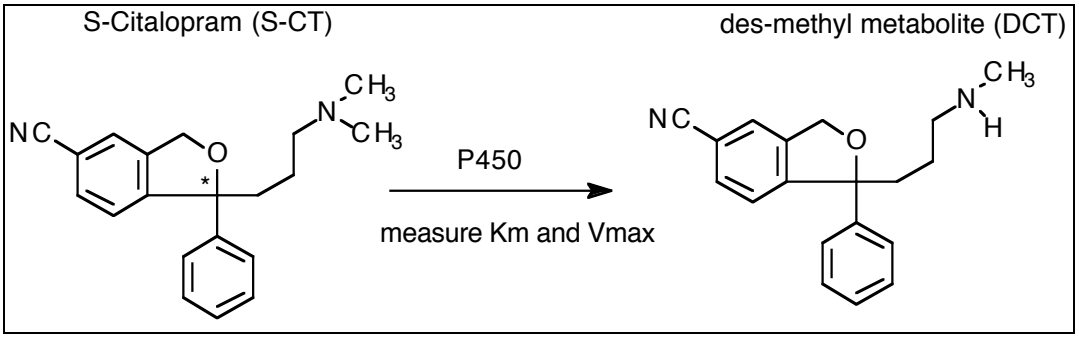
h) The ratio of V_{max}/K_m or (enzyme catalytic efficiency when we know the amount of enzyme) is a useful parameter.

i) Prediction of whole body hepatic or tissue clearances from in vitro incubation data using pharmacokinetic models and scaling factors.

ii) Comparing the effects of amino acid changes on enzyme function.

iii) Predicting the relative contribution of different enzymes to the clearance of a drug.

5) Using the numbers (K_m, k_{cat} and V_{max}) for a purpose estimating the contribution of 3 enzymes to the clearance of citalopram (Focus on S enantiomer; escitalopram). Here we will pretend that this transformation is the primary route of metabolism and clearance. DMD **29** 1102 (2001)



a) First we look at formation of DCT from S-CT in HLM and note that the v vs [S] curve appears to be hyperbolic and that velocity of product formation is given as pmol DCT min^{-1} mg microsomal $protein^{-1}$. The apparent K_m ($K_{m,app}$)is 165 μM. V_{max} is approximately 1200 pmol DCT min^{-1} mg microsomal $protein^{-1}$ (Note we had to get this info from the fit, not directly from the plot).

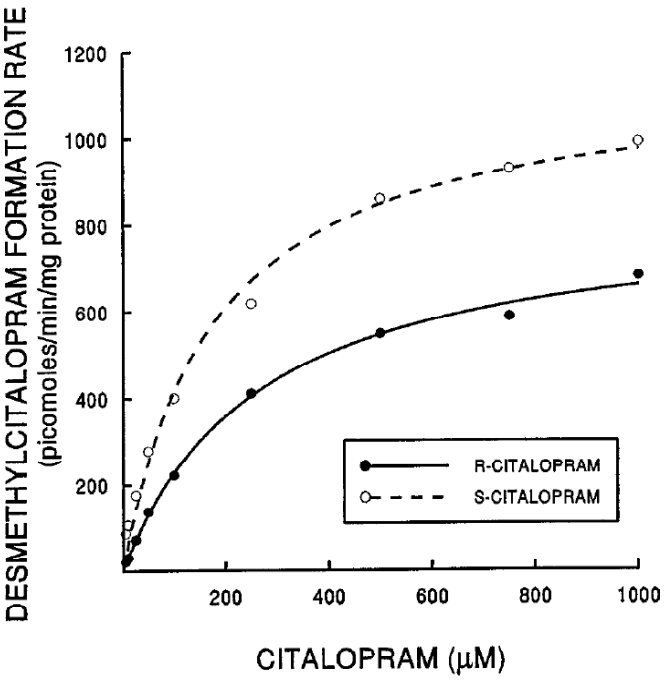
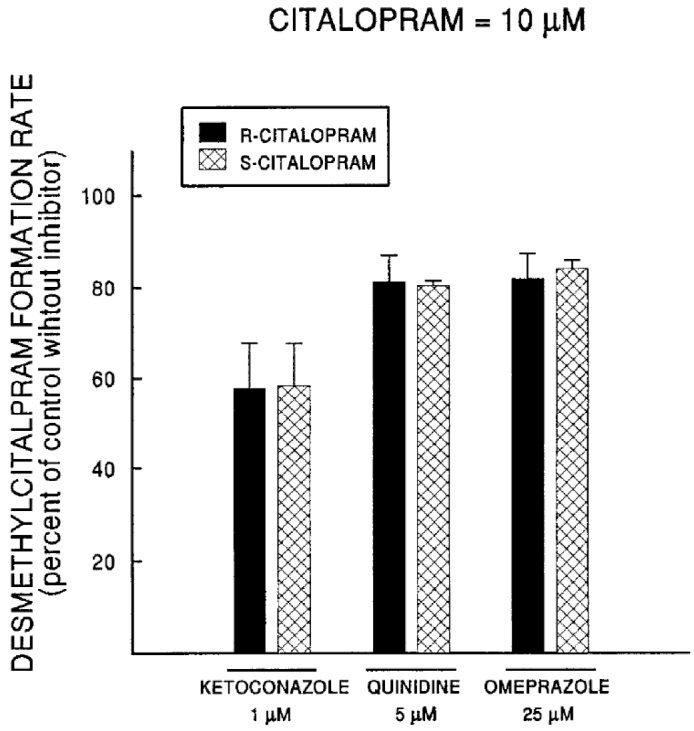


FIG. 1. *Formation of DCT from R- and S-CT by liver microsomes from a representative human liver sample.*

b) Next we look at the effects of isoform selective inhibitors on product formation rates to see if more than one enzyme is involved. Looks like 3A4, 2D6 and 2C19 may be important; that is if our probe inhibitors are selective.



c) The authors then looked at the kinetics of product formation by each of these enzyme alone in supersomes

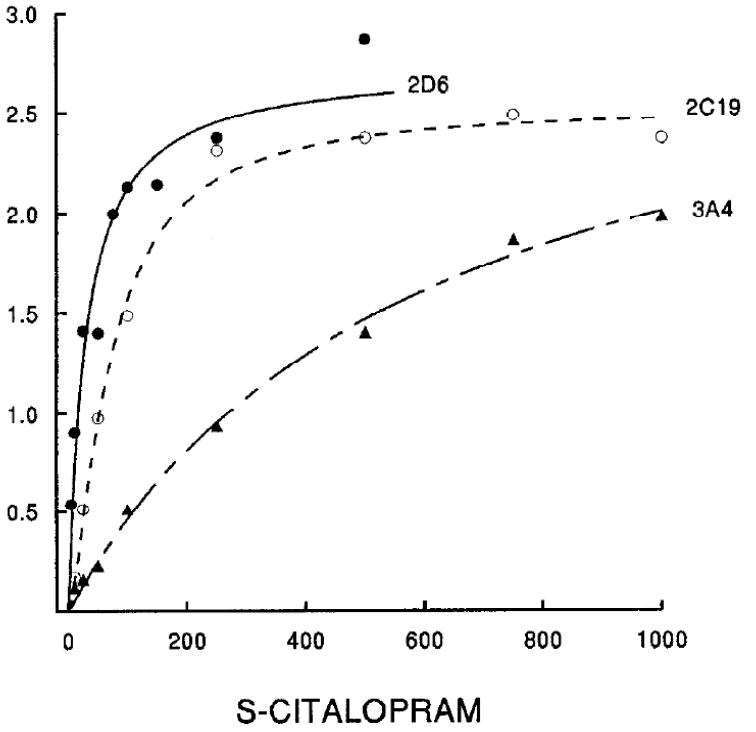


TABLE 3

Kinetic analysis of biotransformation of citalopram and desmethycitalopram enantiomers by heterologously expressed human cytochromes

Units are: V_{max}, picomoles per minute per picomole of P450; K_m, micromolar concentration; V_{max}/K_m, nanoliters per minute per picomole of P450; Relative V_{max}/K_m, percentage of total. Table entries are parameter estimates with asymtotic standard errors in parenthesis.

Transformation Reaction	Cytochromes		
	CYP2C19	CYP2D6	CYP3A4
R-CT to DCT			
V_{max}	1.65 (±0.09)	2.18 (±0.11)	2.15 (±0.21)
K_m	77 (±15)	47 (±8)	454 (±103)
V_{max}/K_m	2.13	4.63	0.47
Abundance-adjusted relative V_{max}/K_m	32.9%	20.7%	46.3%
S-CT to DCT			
V_{max}	2.52 (±0.08)	2.73 (±0.17)	3.19 (±0.22)
K_m	69 (±6)	29 (±7)	588 (±86)
V_{max}/K_m	3.63	9.54	0.54
Abundance-adjusted relative V_{max}/K_m	36.9%	28.1%	34.9%

d) We note that the Y axis of the plots and the V_{max} values in the table are in units of product formation rates given as pmol DCT min^{-1} pmole $P450^{-1}$ so the V_{max} values are actually $k_{cat's.}$

e) Levels of (S)-citalopram in plasma are 200 nM which is far below the K_m values for these enzymes. Thus the metabolic clearance of substrate to metabolite by each enzyme is given as the V_{max}/K_m ratio.

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m} \approx \frac{V_{max} \cdot [S]}{K_m} \quad so \quad Cl_{f,m} = \frac{v}{[S]} = \frac{V_{max}}{K_m}$$

f) Finally these V_{max}/K_m values (catalytic efficiency and intrinsic clearance for each enzyme) are weighted by the amounts of enzyme nomally present in an average human liver to provide the expected percent contribution of each P450 to the overall reaction.

g) Thus the authors scaled this data to reflect the relative amounts of the P450 enzymes present in the average liver to provide estimates of the relative importance of the 3 enzymes in the clearance of (S)-citalopram. The FDA requires this information in an NDA. Drug companies like to develop drugs that are cleared from the body by multiple enzymes and or other clearance pathways. (Why?)

h) Since citalopram is a known drug with PK studies in the literacture we could take the microsomal data and see how well it predicts our best guess, after scaling, of the hepatic clearance. What would we be missing? Other metabolites, other clearance mechanisms (renal clearance), other types of enzymes.

6) Current practice in development focuses on obtaining data about substrate clearance first. This is called metabolic stability. Here we determine how rapidly a substrate disappears in an incubation with single enzymes, microsomes, 10,000 x G supernatents and hepatocytes. A key feature of this approach is to use low concentrations of substrates (e.g. 1 μM) since the approach works best when $S<K_m$ for all relevant enzymes.

a) The rates of substrate loss can be scaled to a human liver.

b) Hepatocytes are particularly useful since they contain all of the enzymes and many of the transporters. There are some problems however. What might they be?

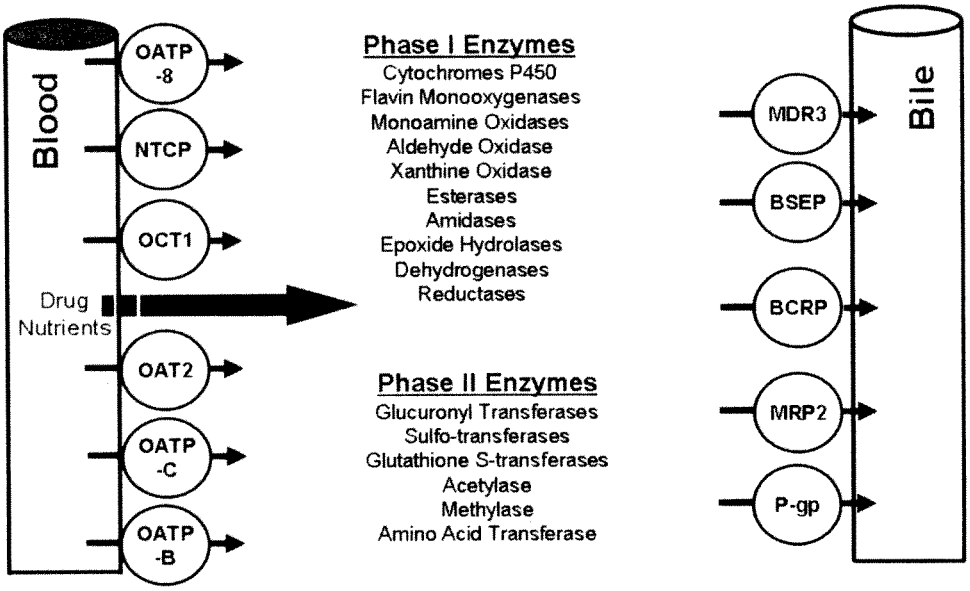


Figure 1 Drug transporters (uptake and efflux) and drug-metabolizing enzymes (phases I and II) of the human hepatocyte.

Steady State Kinetics of the Reversible Inhibition of P450 Catalyzed Reactions:

1) Why do we care?

   a) Prediction of and prevention of drug drug interactions.

   b) Develop safer drugs.

   c) Study P450 enzymology.

2) The primary goal is to determine inhibitor affinity and mechanism of inhibition. Reversible inhibitors reduce enzyme activity by binding to the enzyme and preventing catalysis.

   a) Standard equilibrium binding concepts such as mass balance and affinity constants apply to reversible binding of inhibitors to enzymes.

$$[IE] \underset{}{\overset{K_I}{\rightleftharpoons}} I + E \qquad K_I = \frac{[I]\,[E]}{[IE]}$$

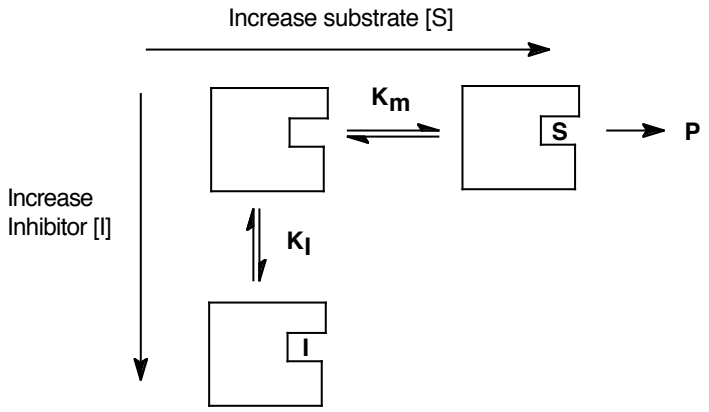
   b) We call the binding constant a K_I. Like the binding constant K_m for substrates, the K_I is a dissociation constant of an enzyme inhibitor pair.

3) Competitive Inhibition:

   a) The binding site for the inhibitor is the active site of the enzyme. The inhibitor competes with the normal substrate for the active site of the enzyme.

   b) By mass balance we see that increasing the inhibitor concentration at a given a fixed concentration of substrate S decreases the amount of free enzyme and the amount of enzyme that is present as the [ES] complex. The equilibrium shifts to the left as the inhibitor concentration is increased and the rate is reduced.

   c) By mass balance we also see that increasing the substrate concentration in the presence of a fixed amount of inhibitor increases the amount of enzyme in the [ES] complex and decreases the amount of enzyme in the [EI] complex as well as free enzyme. Equilibrium shifts to the right and the rate is increased.



d) <u>The equation for competitive inhibition:</u>

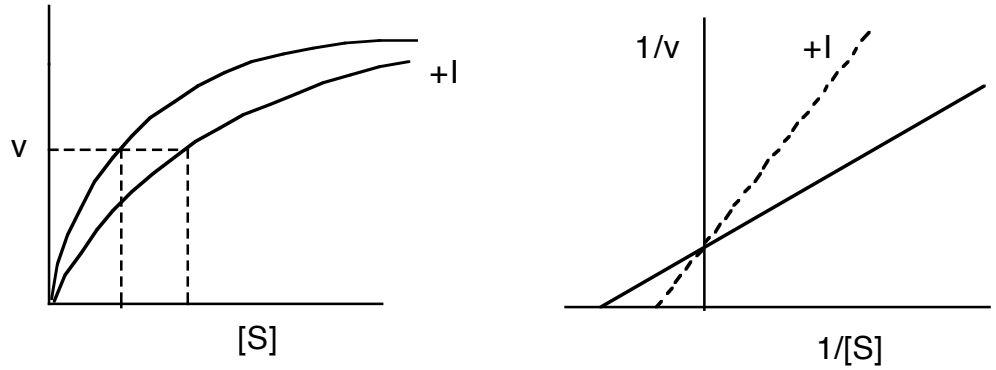
$$v = \frac{V_{\max} \cdot [S]}{[S] + K_m \cdot \left(1 + \frac{[I]}{K_I}\right)} = \frac{V_{\max}}{1 + \frac{K_m}{[S]} \cdot \left(1 + \frac{[I]}{K_I}\right)}$$

i) When we carry out a kinetic experiment (vary [S] and measure P) in the presence and absence of a competitive inhibitor we find that the K_m in the presence of the inhibitor is increased relative to the control but that the V_{max} is not affected.

ii) In the presence of a fixed concentration of an inhibitor we measure an apparent K_m. <u>The true affinity of the substrate for the enzyme has not changed, only the apparent affinity.</u>

iii) <u>A hallmark of competitive inhibition is that increasing the substrate concentration will overcome the effects of a fixed concentration of inhibitor</u>.

<u>Competitive Inhibitor increases K_m but doesn't affect V_{max}</u>



4) Non-competitive inhibition:

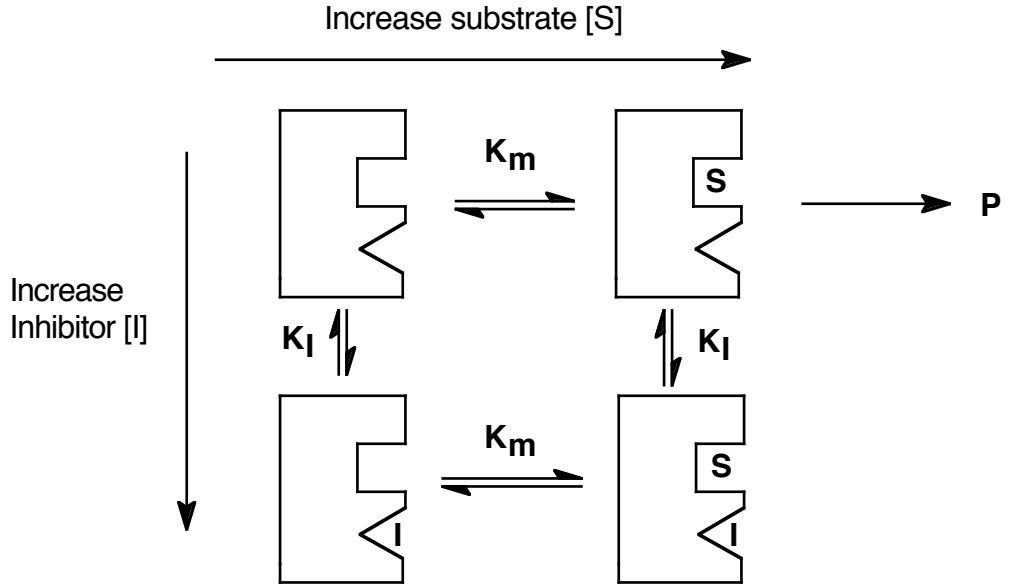
a) The inhibitor binds to the enzyme either in the substrate binding site or elsewhere on the enzyme.

b) The key to understanding non-competitive inhibition is that the inhibitor and the substrate can both be bound to the enzyme at the same time. Therefore the inhibitor and the substrate do not compete for a site on the enzyme. In strictly non-competitive inhibition inhibitor and substrate binding is random and independent.

c) High concentrations of inhibitor and substrate drive the equilibrium in favor of the inactive [ESI] complex.

d) The net effect of an experiment where substrate concentration is increased in the presence of a fixed concentration of inhibitor is that the Km is not affected but Vmax is decreased.

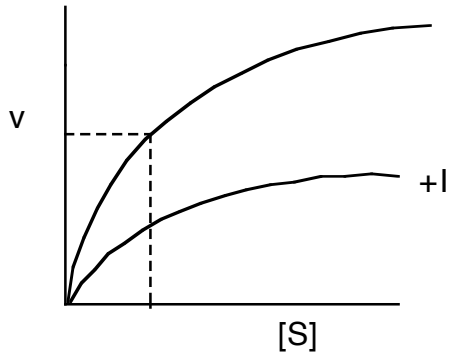
e) A hallmark of this type of inhibition is that increasing the substrate concentration cannot overcome the effect of the inhibitor.

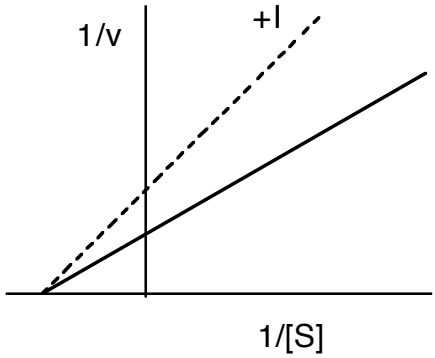


The equation for non-competitive inhibition

$$v = \frac{V_{\max} \cdot [S]}{[S] \cdot \left(1 + \frac{[I]}{K_I}\right) + K_m \cdot \left(1 + \frac{[I]}{K_I}\right)} = \frac{V_{\max} \cdot [S]}{\left(1 + \frac{[I]}{K_I}\right) \cdot \left([S] + K_m\right)}$$

Non-competitive inhibitor decreases V_{max} but doesn't affect K_m





5) Summary of the differences between competitive and non-competitive inhibitors. These inhibitors, when present, convert kinetic constants to apparent kinetic constants.

$$v = \frac{V_{max}\ [S]}{[S] + K_m}$$

Type of Inhibitor	[I] = K_I		Any [I]	
	K_m	V_{max}	K_m	V_{max}
Competitive	↑ 2-fold	N.E.	↑ $(1+[I]/K_I)$	N.E.
Non-competitive	N.E.	↓ 2-fold	N.E.	↓ $(1+[I]/K_I)$

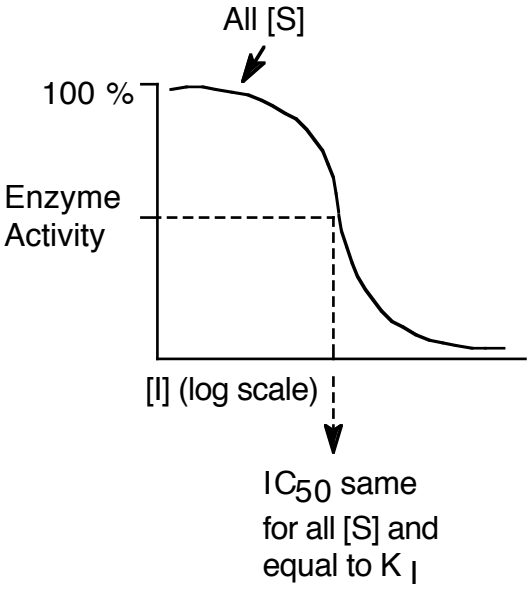
6) Often the potency of an inhibitor is given as IC_{50} rather than K_I.

a) IC_{50} is defined as the concentration of inhibitor that reduces enzyme activity by 50%.

b) IC_{50} is commonly measured in enzyme assays for inhibitor effect because the experiments are less resource intensive.

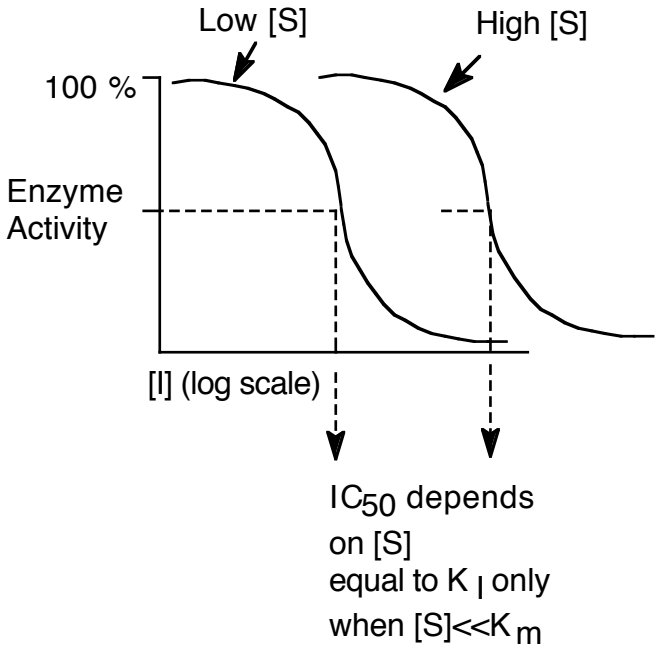
c) It is also used as measure the potency of antagonists for receptor activity in the presence of the natural ligand.

Non-competitive



$$IC_{50} = K_I$$

Competitive



$$IC_{50} = K_I \cdot \left(1 + \frac{[S]}{K_m}\right)$$

- Thus we see that IC_{50} values depend on substrate concentration for competitive inhibitors but not for non-competitive inhibitors.

- Typically industry will use concentrations of test isoform selective substrates at or below their respective Km's in order to evaluate inhibitor potency as IC_{50}. This is because they want to be sure that any errors in the estimates will overpredict inhibitory potency (K_I).

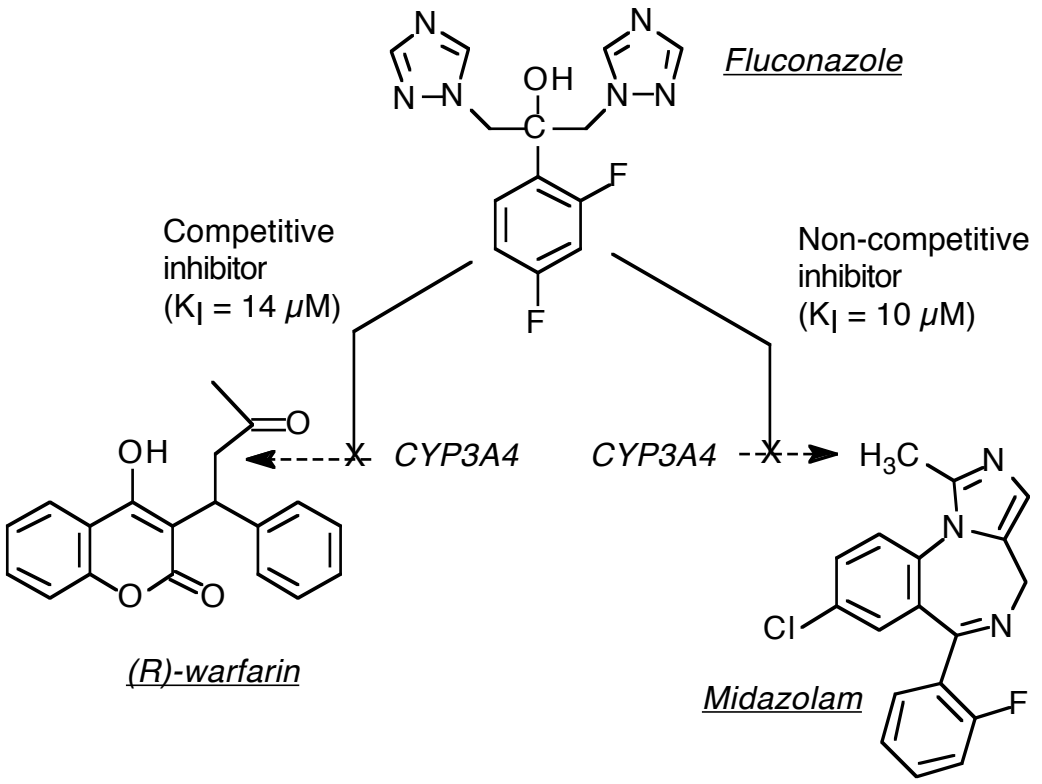
- Mechanism of inhibition is of secondary importance early screens.

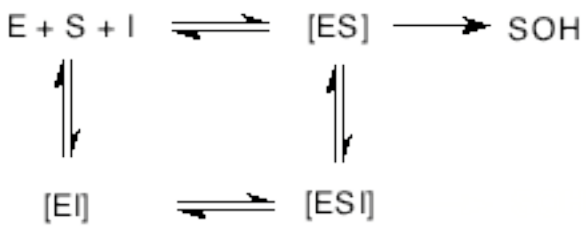
7) The contrast between non-competitive and competitive inhibition is interesting.

a) Fluconazole is a competitive inhibitor of the CYP3A4 catalyzed 10-hydroxylation of (R)-warfarin indicating that fluconazole is capable of binding in the active site of the enzyme.

b) However fluconazole is a non-competitive inhibitor of the CYP3A4 catalyzed 1-hydroxylation of midazolam indicating that fluconazole and midazolam form an [ESI] complex with the enzyme.

c) Does this mean that fluconazole and midazolam co-occupy the active site of the enzyme?





8) Current practice is to screen libraries of compounds in HLM for inhibition of the major CYP's using the IC_{50} type experiments.

a) Isoform specific substrate product pairs are used for each enzyme.
b) The substrate concentrations used are at or less than the Km for the target enzyme.
c) Mixtures or cocktails of non-interacting substrates can be used.
d) Follow-up studies can be performed to determine Ki and mechanism of inhibition.
e) An implicit assumption is that the Ki's observed for an inhibitor for an enzyme with different substrate product pairs will be the same or similar. With CYP3A4 in particular this might not always be the case. Likely culprits are
   i) Allosterism
   ii) Complex inhibition via time dependent inhibition.

TABLE 1

K_i values obtained using pooled HLMs

Global S.E. for data fitting was less than 20% and r^2 = >0.80 for each effector.

Effector	K_i Values with Probe Substrate								
	TST	MDZ	SIL	FLU	BUD	QUI	BUS	SIM	FEL
					μM				
AMG 458	0.55	7.3[a]	1.0	3.3	7.3	5.6	ACT[b]	7.7	8.6[c]
Budesonide	ACT	0.10	0.17	0.14	X	0.18	0.038	1.3	13.1
Buspirone	50	0.54	2.1	9.3	13.2	1.3	X	10.3	50
Clozapine	7.4	0.42	17.6	9.3	2.1	5.4	0.74	17.1	10.4
Cyclosporine	24.2	3.1[c]	3.5	6.9[c]	1.5	0.36	1.1	12.7	4.3
Dextromethorphan	50	8.6	50	50	50	50	26.2	50	24.8[c]
Felodipine	ACT	0.25[c]	0.65	0.44	0.085	0.20[c]	0.87	0.43	X
Fluoxetine	8.7	4.2[c]	3.3	50	47.0	2.7	5.5[c]	ACT	50
Fluticasone	ACT	0.085	0.39	X	0.16	0.93	0.080	17.1	0.79
Fluvoxamine	20.4	2.6	2.7	50	0.68	1.6	5.3[c]	16.1	50
Haloperidol	27.4	2.3[c]	2.4	3.1	0.84	3.6	0.61	9.4	2.9[c]
Itraconazole	0.013	0.013	0.016	0.012	0.044	0.016	0.016[c]	0.052	0.045
Ketoconazole	0.023	0.014[c]	0.017	0.044	0.011	0.009	0.021[c]	0.072	0.079
Midazolam	6.4	X	1.8	0.74	4.2	0.97	0.85	8	30.5
Nifedipine	ACT	1.8[c]	8.7	0.46	0.27	1.9[a]	0.95	1.7	14.9
Sertraline	10.4	3.1[c]	2.1[c]	12.6	0.20	1.2[c]	2.1[c]	1.6	50
Sildenafil	50	0.71	X	3.9	2.1	1.1	2.0	14.6	50
Simvastatin	35.0	0.16	0.37	0.38	0.31	0.81[c]	0.54	X	16.5[c]
Terfenadine	11.2	1.0[c]	0.21	0.31	0.055	0.066	0.13	1.3	1.2
Testosterone	X	2.7[a]	9.5	1.8	5.0	50	3.7	1.2	ACT

TST, testosterone; MDZ, midazolam; SIL, sildenafil; FLU, fluticasone; BUC, budesonide; QUI, quinicine; BUS, buspirone; SIM, simvastatin; FEL, felodipine.
[a] Linear-mixed inhibition.
[b] Activation.
[c] Noncompetitive inhibition.