Goal: Real world application of inhibitors → retroviral inhibitors
(i) HIV life cycle (iii) HIV protease mechanism (v) rational drug design in response to mutations.
(ii) Discussion of drug targets (iv) HIV protease inhibitors.

Lecture 20: Retroviruses & Inhibitors - HIV Protease.

- Identify potential drug targets, based on viral life cycle.
- Compare and contrast serine to aspartyl proteases
- Measure inhibitor binding to characterize drug efficiency.
- Rational drug design in response to mutations.

Human Immunodeficiency Virus (HIV)

- Infects specialized cells in the immune system – T-helper cells (T_H) cells, killing them.
- Retroviruses, the genetic information is stored in RNA (viral RNA, vRNA) which must be first be copied into DNA: vRNA → DNA → mRNA → viral protein
  ↳ vRNA

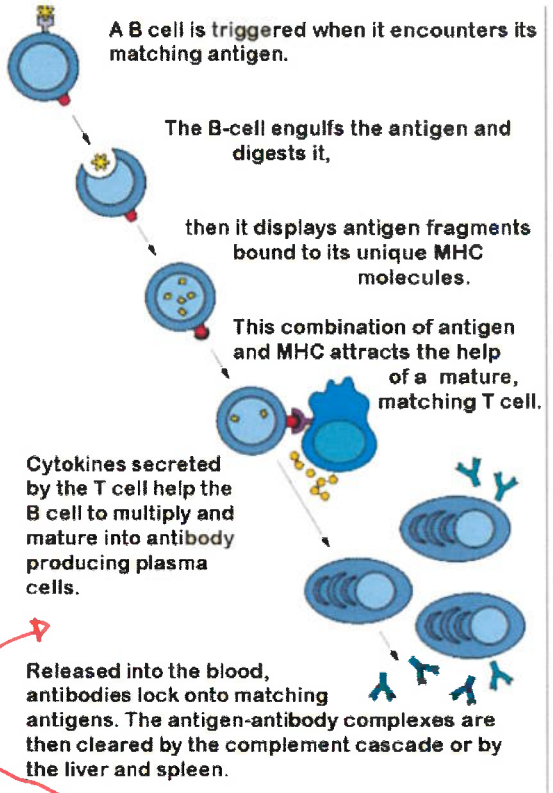
retro step.

Central Role of T-helper (T_H) Cells in Immunity:

Key Cell Types:

B-cells: Have antibody on their cell surface.
T_H-cells: Responsible for activating B-cells
Plasma cells:

- secrete soluble antibody to fight pathogens.
- Develop from B-cells that have been activated by T_H cells.



process of Antibody generation

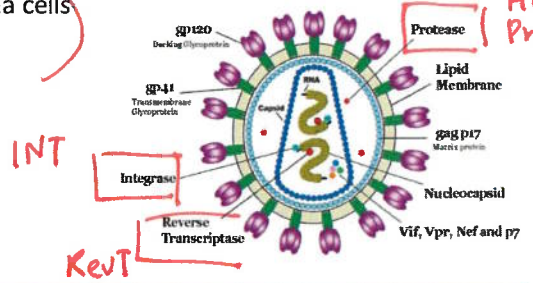
Steps in the production of Antibodies:

1. Antigen (pathogen) binds to antibody on the surface of B-cells.
2. Pathogen is internalized by B-cell and digested into peptides.
3. Peptides from the pathogen are exported to the external surface bound to MHC II molecules.
4. T_{Helper} (T_H) cells recognize peptides on MHC as foreign, starting immune response.
5. T_H cells activate B-cells so that they can differentiate into plasma cells → secrete antibodies that inactivate pathogens

required for steps in replication.

The **HIV virus particle** contains three essential enzymes for viral replication:

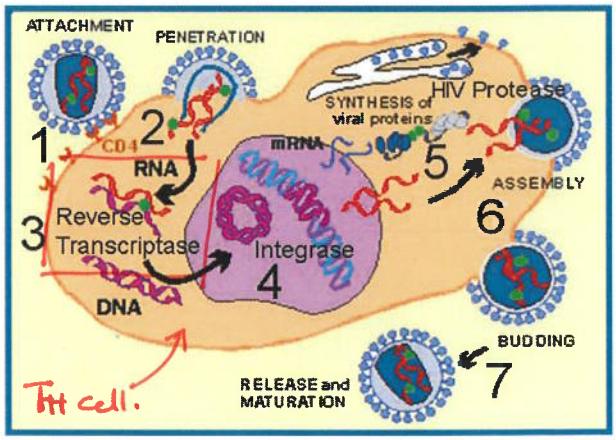
i) reverse transcriptase,
Copies vRNA to DNA, error prone → drug resistant HIV.
ii) integrase,
Inserts DNA copy into host cell chromosome.
iii) HIV protease
Cleaves immature viral proteins to make mature proteins, that assemble into the virus.



HIV Protease.
INT
RevT

HIV Viral Infection of T-Helper Cells:

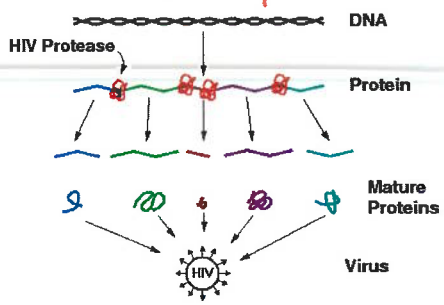
1. Viruses bind to molecules displayed on the T_H cell surface.
2. The virus then fuses with the cell membrane and releases its RNA genome from its lipid envelope.
3. The enzyme **reverse transcriptase** first makes a double-stranded DNA copy of the viral RNA molecule. This process is error prone, leading to



TH cell.

mutation in HIV proteins that affect drug binding

Role of HIV protease.



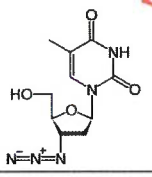
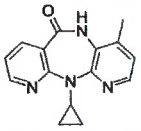
mutations in the virus. ***These mutations cause drug resistant strains of the virus to arise.***

4. The DNA is integrated into the host cell's DNA by an enzyme called **integrase**.
5. Integrated DNA produces vRNA, the genetic material for new virus particles. mRNA is also made from this DNA, to produce proteins for new particles.
6. **HIV protease** required for maturation of viral proteins, by cleaving them into smaller proteins that form the mature virus.
7. Mature virus buds out of cell.

Drug Targets to Combat the HIV Virus - *Why are these good targets for inhibitors that can act as anti-virals?*

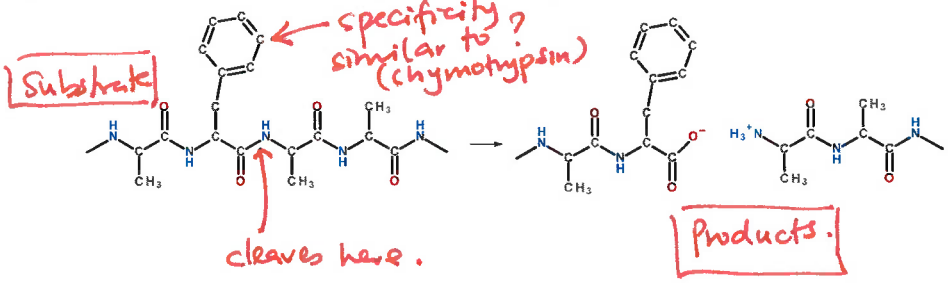
1. Viral fusion
2. Reverse transcriptase
3. Integrase
4. HIV Protease

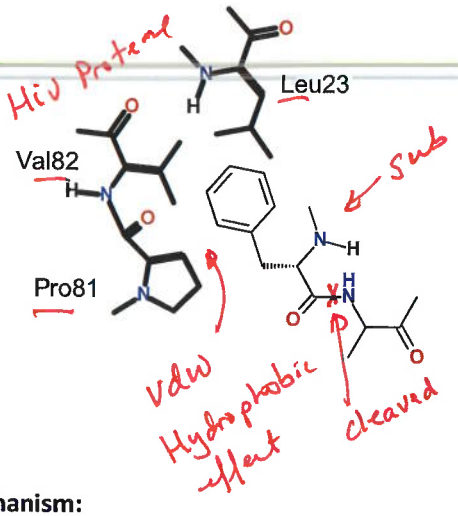
all are essential for replication of the virus. No human analog ∴ no off-target effects

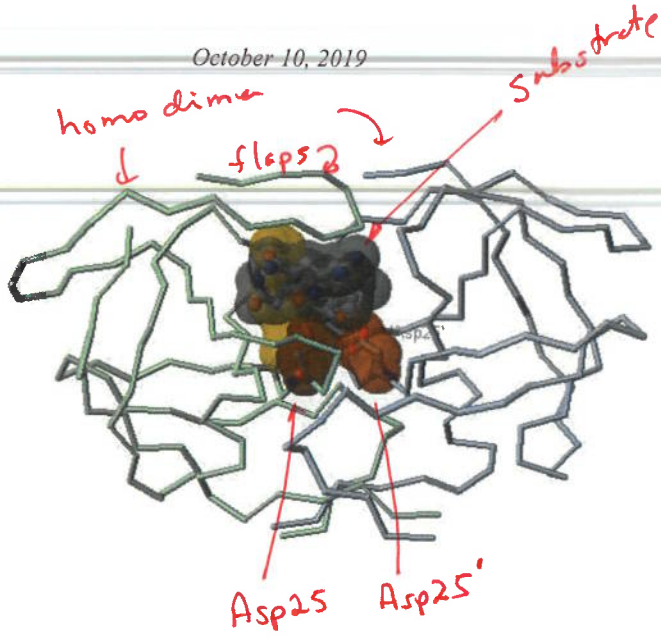
1980-84 HIV virus Characterized	**Fusion Inhibitors**	**RevTrans Inhibitors – NRTI (competitive)**	**RevTrans Inhibitors – NNRTI (allosteric)**	**Integrase Inhibitors**	**Protease Inhibitors (competitive)**
1985-89		AZT 	still used in limited cases.		
1990-94		Didanoisine, Zalcitabine, Stavudine			
1995-99		Lavidudine	Nevirapine,  Delavirdine, Efavirenz		Saquinavir, Ritonovair, Indinavir, Nelfinavir
2000-04	Enfuvirtide	Didanosine Emtricitabine			Atazanavir, Fosamprenavir
2005-09	Maraviroc		Etravirine	Raltegravir	Darunavir, Tipranavir
2010-14	still active development for therapy.		Nevirapine XR, Rilpivirine	Dolutegravir, Evitegravir	

HIV Protease (Aspartyl protease)

1. An essential enzyme in the maturation of the HIV virus. If inhibited, the virus cannot replicate.
2. The HIV protease is a homo-dimeric protein, containing two catalytic Asp residues, Asp25 and Asp25', the same residue on each chain. However, the pK_a values of these two differ widely, one is about 4.0 and the other about 6.0. It is a member of the general class of ***Aspartate proteases.***
3. One of the Asp residues must be protonated the other must be deprotated for full activity.
4. Water is the activated nucleophile, no acyl-intermediate.
5. Prefers hydrophobic substrates due to Val82 plus other non-polar residues in its active site.

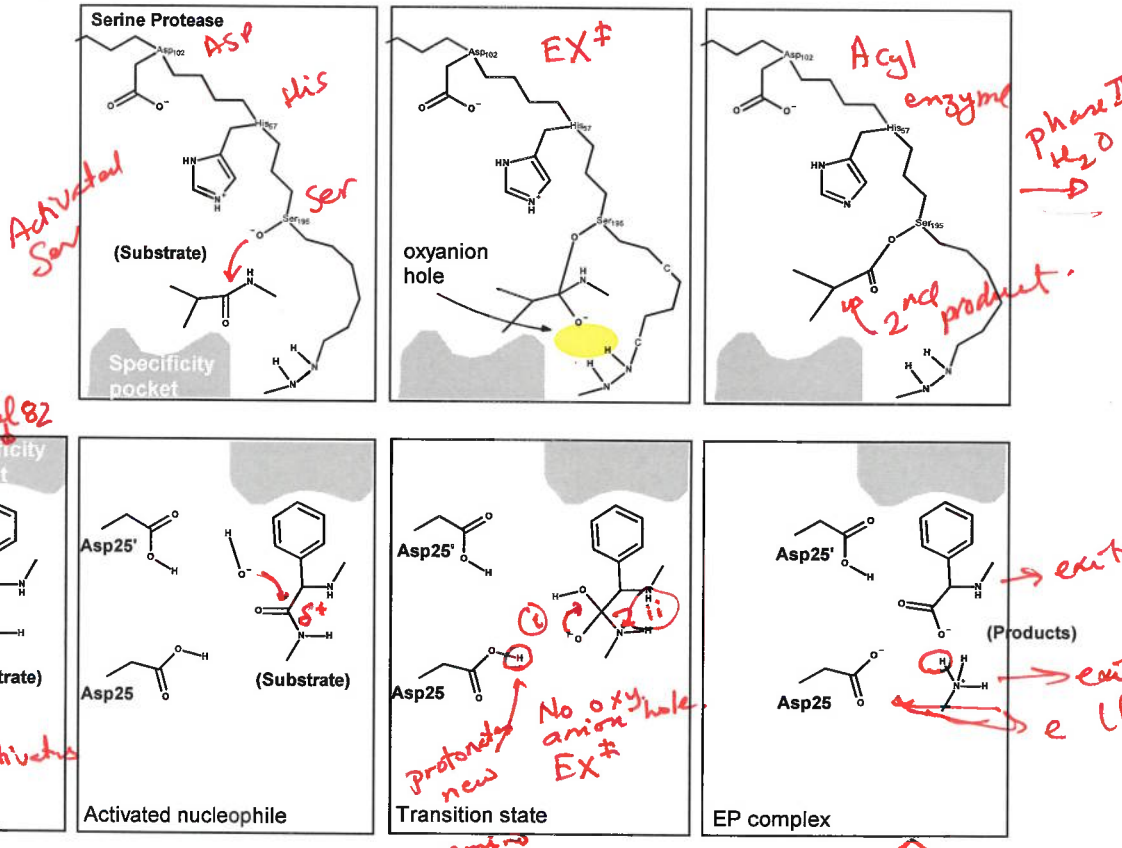






Mechanism:

1. Activation of H_2O by Asp25'
2. Nucleophilic attack on C=O of substrate
3. Tetrahedral transition state
4. Peptide bond cleavage
5. Protonation of new NH_2 by Asp25



Reflection: Compare and contrast the difference between Serine and Aspartyl Proteases.

Ser
i) Ser, His, Asp — Asp, Asp

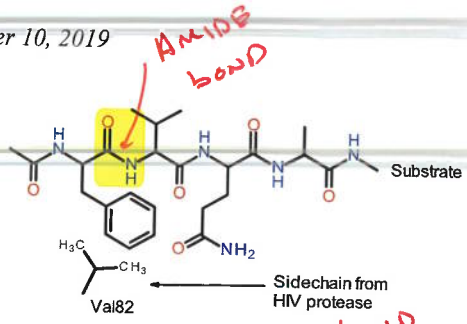
ii) Activation of Ser by His — Activation of H_2O by Asp25' (deprotonates)

iii) oxyanion hole — No oxyanion hole

iv) Acyl intermediate — No covalent intermediate ∴ both products are released

Inhibition of HIV Protease (HIV Drugs):

- Most drugs are small peptide-like analogs with non-cleavable bonds that resemble peptide bonds.

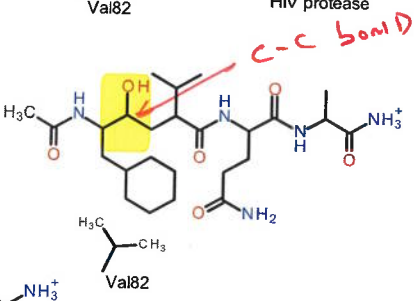


Where will they bind on the enzyme?

- active site similar to substrate.

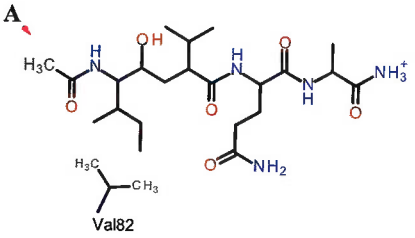
What will happen to them after they bind?

- No cleavage

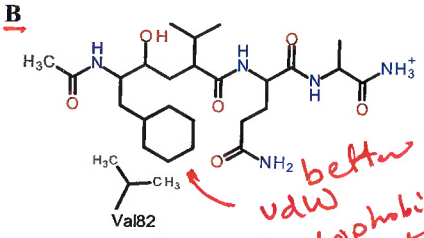


Drug Design: Compounds A (Isobutyl) and B (cyclohexane) are candidates for HIV protease inhibitors. Which of the two drugs will be more effective at inhibiting the wild-type protease?

A



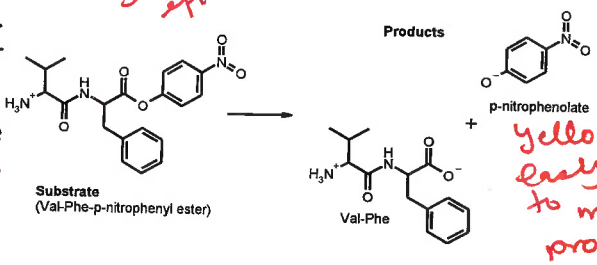
B



better vdW hydrophobic effect.

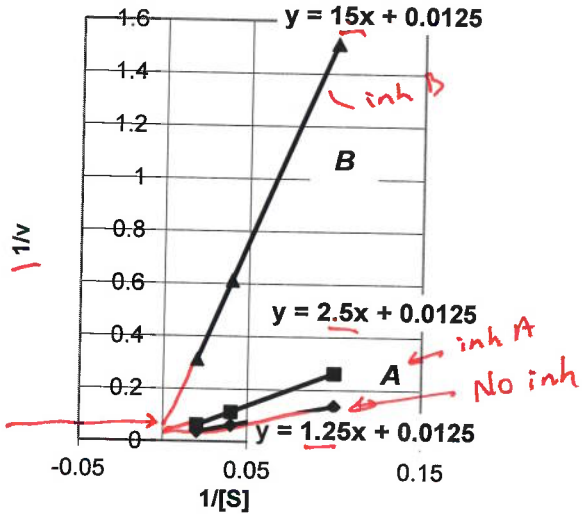
$K_I = K_D$

Answer: We will assume that these are competitive inhibitors. Therefore, we need to compare the K_I values for each inhibitor binding to the protease, using the following steps:



yellow easy to measure product.

1. Select a suitable substrate for steady-state assays. The kinetics of HIV protease can be measured using this substrate, producing a bright yellow nitrophenolate ion.
2. Measuring K_I for both Drugs:
   a) Acquire velocity versus substrate, no inhibitor.
   b) Acquire velocity versus substrate, fixed inhibitor.
   Analysis - Graphical
   i) Plot double reciprocal plots.
   ii) Obtain α from ratio of slopes
   iii) $K_I = [I]/(\alpha-1)$
   Analysis – Model Fitting (see next page)
   i) Fit the data directly to the appropriate kinetic equation: $v = k_{cat}E_{TOT} \times [S]/(\alpha K_M + [S])$



[S] µM	v (I=0)	v ([A]=10 nM)	v ([B]=10 nM)
10	7.3	3.8	0.7
25	16	8.9	1.6
50	26.7	16.0	3.2
1/[S]	**1/V (I=0)**	**1/V ([A]=10nM)**	**1/V ([B]=10nM)**
0.10	0.138	0.263	1.513
0.04	0.063	0.113	0.613
0.02	0.038	0.063	0.313

The units of velocity are µmoles product/sec.

Once the α values are found, we can calculate the K_I for each inhibitor using the formula: $K_I=[I]/(\alpha-1)$.

	Slope	α = slope(I>0)/slope(I=0)	K_I
No Inh.	1.25		
Inhibitor A	2.50	α = 2.50/1.25 = 2	10nM/(2-1) = 10nM
Inhibitor B	15.00	α = 15.00/1.25 = 12	10nM/(12-1) = 0.9 nM.

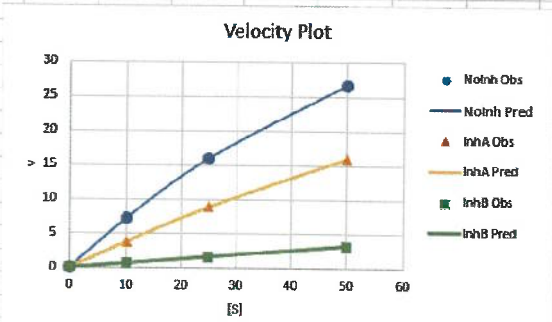
$K_I = K_D$

Inhibitor B binds better.

$v = V_{max} \cdot \frac{[S]}{\alpha K_m + [S]}$ — α 1 for 1st column; αA; αB

SUM =$M$1*A2/(A2+$M$2*$M$3)

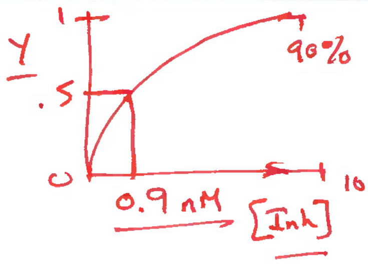
	A	B	C	D	E	F	G	H	I	J	K	L	M
1	[S]	v (no inh)	Pred	Diff	v (+inh A)	Pred	Diff	v(+inh B)	Pred	Diff			
2	0	0	0	0	0	$M$3)	0	0	0	0		Vmax	80.5668
3	10	7.3	7.266508	0.033492	3.8	3.804444	0.004444	0.7	0.660991	0.039009		Km	100.8742
4	25	16	16.00146	0.001457	8.9	8.881986	0.018014	1.6	1.632388	0.032388		alpha (A)	2.000217
5	50	26.7	26.7	1.05E-06	16	16.00006	6.43E-05	3.2	3.19994	5.98E-05		alpha (B)	11.98404
6												Sum Diff	0.128929



Inh B

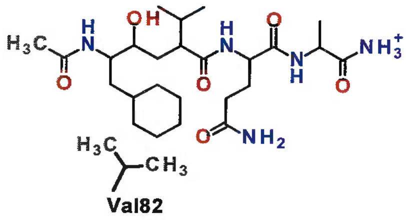
$K_I = K_D = 0.9$ nM

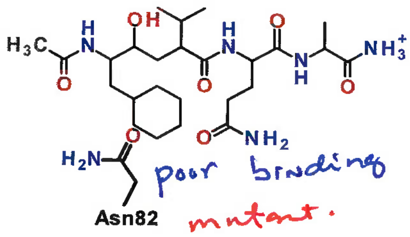
HIV Protease

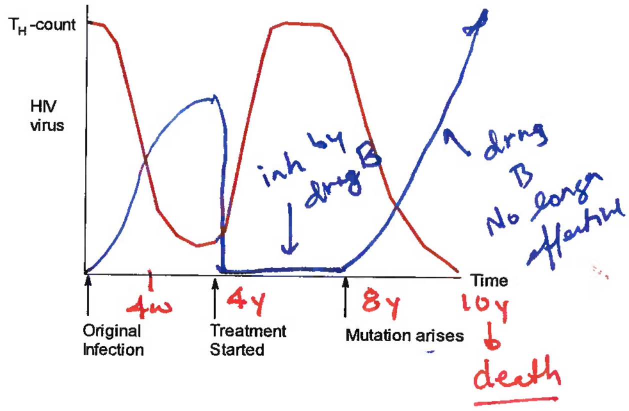


Drug resistance & rational drug design:

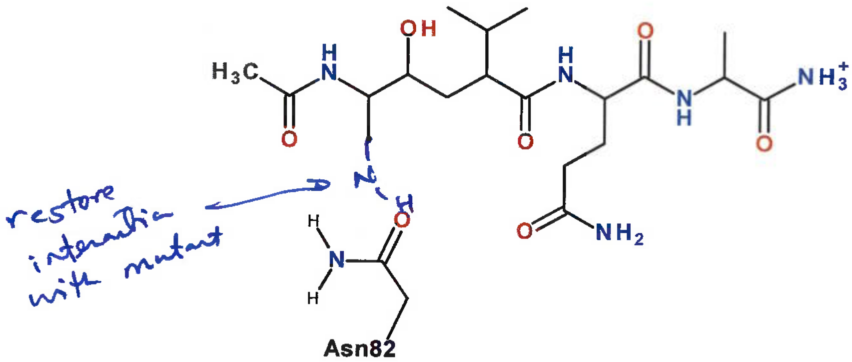
- Error prone copying of vRNA to DNA introduces changes in the sequence of the viral RNA (mutations), leading to altered amino acids in the viral proteins.
- changes in the residues that are involved in drug binding may reduce binding.
- The mutant virus is no longer inhibited from growing and will quickly overgrow the wild-type virus.
- A common mutation that arises in many HIV patients is changing Val82→Asn82 in HIV protease.
- The altered HIV protease can be inhibited with modified protease inhibitors.







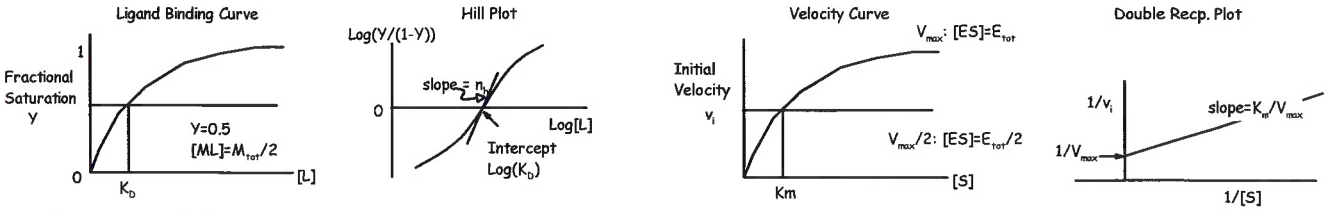
i) How might you alter the existing inhibitor to be effective at binding to HIV protease with the Asn82 mutation?



Equations and plots:

Ligand binding

$$Y = \frac{[L]}{K_D + [L]} \qquad Y = \frac{[L]^{n_h}}{K_D^{n_h} + [L]^{n_h}} \qquad v = V_{MAX}\frac{[S]}{K_M + [S]} = k_{CAT}E_{TOT}\frac{[S]}{K_M + [S]} \qquad \frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$



Summary of Parameters:

Parameter	Related Formula	Importance	From Velocity Curve: $v=V_{MAX}[S]/(K_M+[S])$	From Double Reciprocal Plot
V_{MAX}	= $k_{CAT}[E_{TOTAL}]$	Maximum rate of product produced at a given enzyme concentration (Enzyme saturated, [ES]=E_{TOT}.	Reaction velocity at high substrate	V_{MAX}=1/*y*-int
k_{CAT}	=$V_{MAX}/[E_{TOTAL}]$	Turnover number: Number of product molecules produced/sec by a single enzyme molecule. ***Higher k_{CAT}, more efficient the chemistry*** **(ES→EP)**	see V_{MAX}	see V_{MAX}
K_M	=(k_{OFF} +k_{CAT})/k_{ON} ≈ K_D	Related to dissociation constant for substrate binding. ***Lower K_M, better binding of*** **(S)** **(E + S → ES)**	Substrate concentration to give a velocity that is ½ V_{MAX}	K_M = slope*V_{MAX} = slope/*y*-int
k_{CAT}/K_M		Specificity constant, velocity at low [S]		
K_I (Competitive *and* Mixed inhibition)	α=1+([I]/K_I) K_I=[I]/(α-1) K_I =[E][I]/[EI]	Dissociation constant for inhibitor binding to free enzyme: (EI) → (E) + (I). ***Lower K_I, better binding of*** **(I) to (E).** *(Identical to K_D. $K_I = K_D$)*	Best to use double reciprocal plot, or direct fitting with Solver.	$\alpha = \frac{\text{slope}([I]>0)}{\text{slope}([I]=0)}$ K_I=[I]/(α-1)
K_I' (Only mixed inhibition)	α'=1+([I]/K_I') K_I'=[I]/(α'-1) K_I'=[ES][I]/[ESI]	Dissociation constant for inhibitor to bind to ES complex: (ESI) → (ES) + (I) ***Lower K_I', better binding of*** **(I) to (ES).** *(Identical to K_D. $K_I' = K_D$)*	Best to use double reciprocal plot, or direct fitting with solver.	$\alpha' = \frac{\text{Y-int}([I]>0)}{\text{Y-int}([I]=0)}$ K_I'=[I]/(α'-1)

EI ⇌ E + I $K_I = K_D$

ESI ⇌ ES + I $K_I' = K_D$

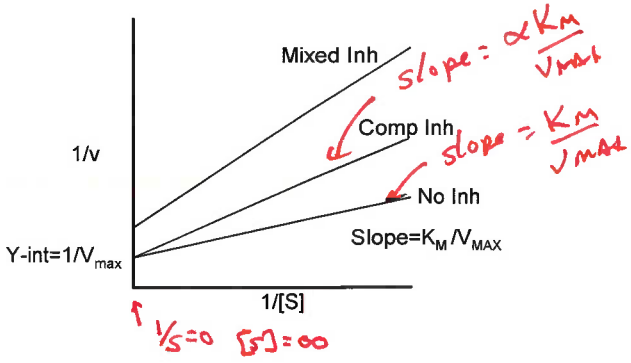
Effect of Inhibition on Kinetic Parameters:

Kinetic Parameter	Competitive Inhibition	Mixed Inhibition
K_M	$K_M^{Observed} = \alpha K_M$	$K_M^{Observed} = \frac{\alpha}{\alpha'}K_M$
V_{MAX}	$V_{MAX}^{Observed} = V_{MAX}$	$V_{MAX}^{Observed} = V_{MAX}/\alpha'$

Double reciprocal Plot: 1/v versus 1/[S]:

$$\frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}} \qquad \frac{1}{v} = \frac{\alpha K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}} \qquad \frac{1}{v} = \frac{\alpha K_M}{V_{MAX}}\frac{1}{[S]} + \frac{\alpha'}{V_{MAX}}$$

	No Inh.	Competitive	Mixed
Slope	K_M/V_{MAX}	$\alpha K_M/V_{MAX}$	$\alpha K_M/V_{MAX}$
***y*-Int**	$1/V_{MAX}$	$1/V_{MAX}$	α'/V_{MAX}
$\alpha = \frac{\text{slope}([I]>0)}{\text{slope}([I]=0)}$		$\frac{\alpha\frac{K_M}{V_{MAX}}}{\frac{K_M}{V_{MAX}}} = \alpha$	$\frac{\alpha\frac{K_M}{V_{MAX}}}{\frac{K_M}{V_{MAX}}} = \alpha$
$\alpha' = \frac{\text{Y}-\text{int}([I]>0)}{\text{Y}-\text{int}([I]=0)}$		$\frac{1/V_{MAX}}{1/V_{MAX}} = 1$	$\frac{\alpha'/V_{MAX}}{1/V_{MAX}} = \alpha'$



Competitive: Bind at active site. *y*-intercept in double reciprocal plot is *unchanged*. Slope *must* change.
Mixed Inhibition: Bind elsewhere. *y*-intercept in double reciprocal plot is higher. Slope *may* change, depending on (α/α').

$\alpha = 1 + \frac{[I]}{K_I}$ if known.

α - from ratio of slopes

$K_I = \frac{[I]}{\alpha}$