5.36 Biochemistry Laboratory
Spring 2009

5.36 Lecture Summary #1 February 3, 2009

Reading assignment for Thursday: Weisberg, E. et al. Second Generation Inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukamia. *Nature Rev. Cancer* **7**, 345-356 (2007). Posted on MIT server :

Next Laboratory Session: #1

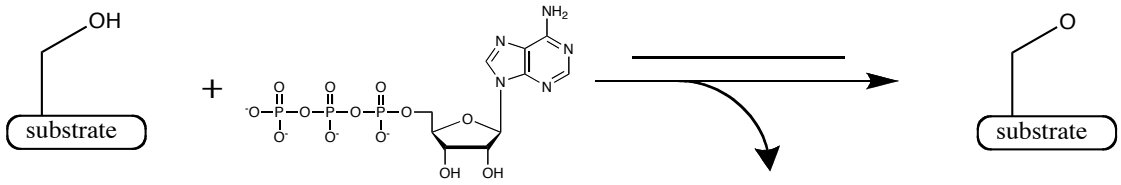
Topics: **I.** Background chemistry for Modules 4 and 5 (kinases!)
- **A.** Review of kinases and phosphatases
- **B.** Abelson kinase (Abl) and Bcr-Abl
- **C.** Kinase inhibitors as cancer drug targets

II. Overview of Modules 4 and 5

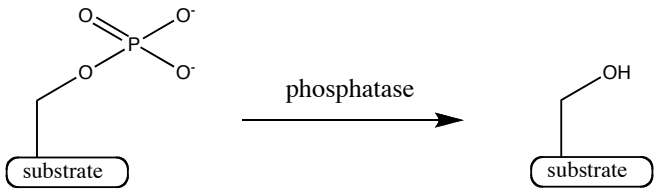
I. BACKGROUND CHEMISTRY FOR MODULES 4 AND 5 (KINASES!)

A) REVIEW OF KINASES AND PHOSPHATASES

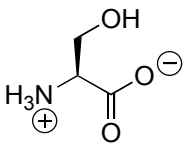
A **kinase** is an enzyme that catalyzes the transfer of a _____ group from ATP onto a _____ (target molecule).



A **phosphatase** is an enzyme that catalyzes the _____ of a phosphate group from a target molecule: the reverse reaction.



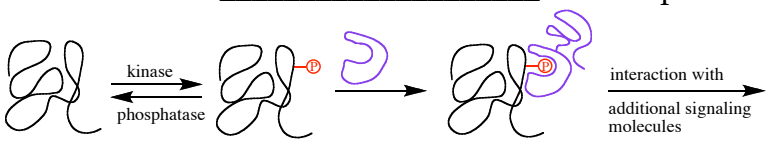
Serine/threonine-kinases phosphorylate target proteins on a Ser or Thr residue. Tyrosine-kinases phosphorylate target proteins on a Tyr residue.



serine (Ser, S) threonine (_____ , T) tyrosine (Tyr, ___)

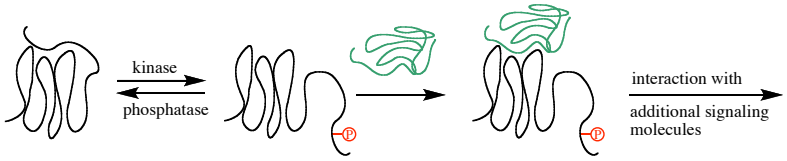
Biological Relevance of Protein Kinases. Kinases (and phosphatases) can regulate the activity of a target protein in various ways. For example, phosphorylation of a target protein can:

- Create new _____ for that protein.



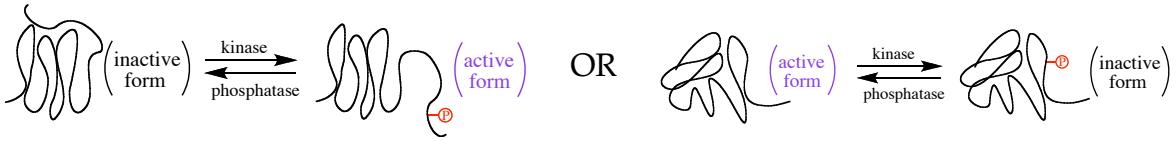
Signaling pathways are _____ or inhibited.

- Result in _____ changes that affect binding



Signaling pathways are actived or inhibited.

or modulate enzyme _____.



Depending on the enzyme, the phosphorylated form may be active OR inactive.

Phosphorylation by kinases can "switch on" an enzyme substrate to produce significant quantities of active enzyme on timescale of _____ to _____.

Compare this to protein expression, which can take _____ to _____ to produce significant quantities of protein.

Phosphorylation (and other post-translational modifications) thereby allows cells to respond to their environment on a much faster timescale than if they relied solely on expression to modulate quantities of active protein.

B) ABELSON KINASE (Abl)

Abl is a 120 kDa (_____ amino acid) protein tyrosine kinase (PTK).

The Abl "kinase domain" is _____ kDa (amino acids 229-551*). See Appendix B for the Abl kinase domain DNA and amino acid sequences.* Sub domains bind signaling proteins, DNA, and actin.

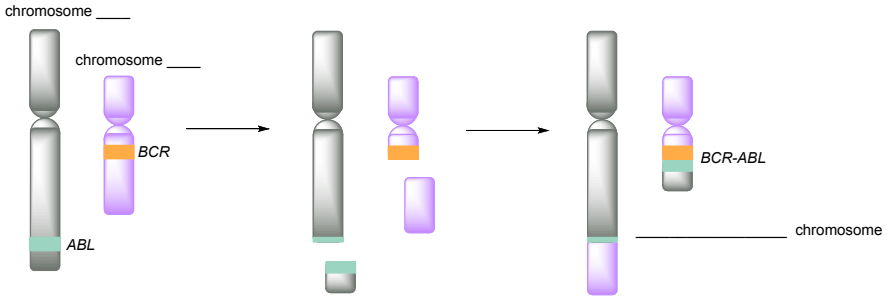
Abl is _____ by the binding of the "cap" domain and other N-terminal domains to the kinase active site. **The Abl default setting is _____.**

Abl in healthy cells. The precise biological roles of Abl are still unknown. However Abl appears to be involved in

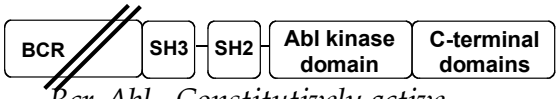
- cell division.
- stress response.
- cell adhesion and migration.

Abl in disease.
Mutations and / or overexpression of kinases lead to a wide range of diseases.

A reciprocal _____ between the Abl-enconding chromosome 9 and the Bcr-encoding chromosome 22 results in a fused _____ gene.



The fused BCR-ABL gene product is the mutant protein Bcr-Abl. The Bcr-Abl protein lacks the residues responsible for Abl inhibition and is _____ (always ON).



Bcr-Abl. Constitutively active.

Abl. Auto-inhibited. Activity is _____.

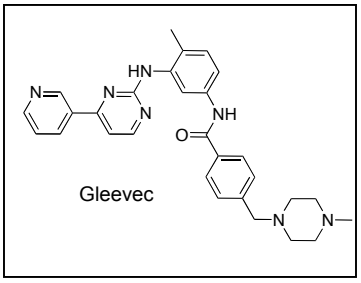
Aberrant kinase activity of Bcr-Abl is the underlying cause **chronic myloid leukemia** (_____), a cancer of the bone marrow.

- CML white blood cells do not function correctly and take up room, resulting in _____ normal white blood cells and red blood cells.
- Incidence: ~5000 new cases each year in US. This represents _____% of all cases of adult leukemia in Western populations
- The only well-described risk factor is exposure to ionizing radiation.

C) ABL INHIBITORS AS CANCER DRUG TARGETS

Chemists at Novartis used rational drug design combined with high throughput screening technologies to find drug targets that inhibit Abl activity.

These efforts culminated in the development of the drug _____, which was approved by the FDA in _____ for CML treatment.



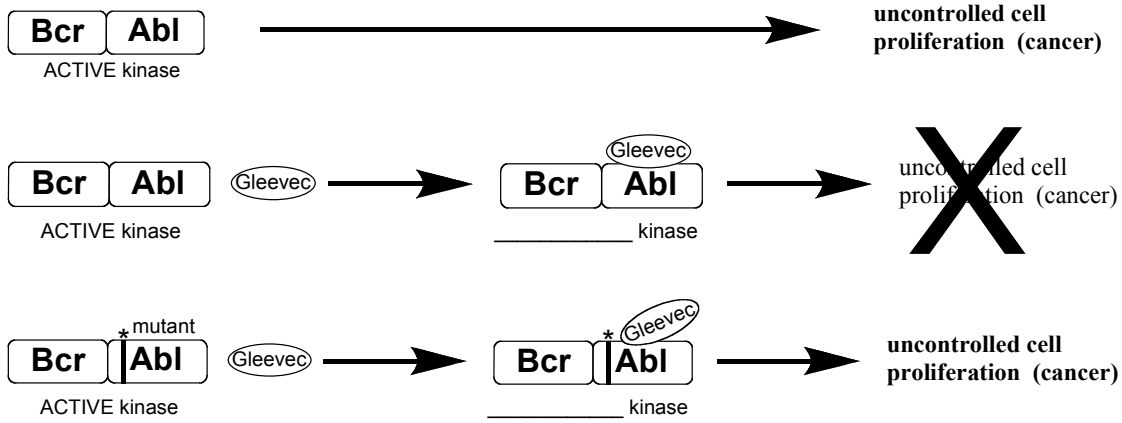
- Gleevec binds to the active site of the Abl kinase domain and stabilizes the _____ conformation of the protein.
- The Gleevec-Abl interaction is highly _____. Gleevec inhibits only ____ other kinases at physiological levels, neither of which result in problematic side effects.
- Other names for Gleevec are _____ and _____.
- Approximately ____% of CML patients diagnosed in the chronic stage experience remission.

Gleevec is the first drug that selectively inhibits a _____. This is incredibly exciting from the standpoint of drug discovery!

Resistance to Gleevec:

As of 2006, _____% of patients diagnosed in the _____ stage showed Gleevec resistance. More than half of patients in advanced stages of CML show Gleevec resistance.

In patients with Gleevec resistant CML, mutations are found in the Bcr-Abl gene, usually at just __ _____ in the kinase domain.

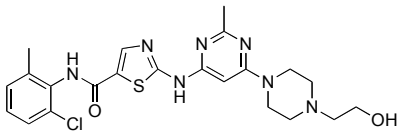


Over 30 *different* _____ mutations in the Abl kinase domain of Bcr-Abl have been identified in Gleevec-resistant CML patients to date.

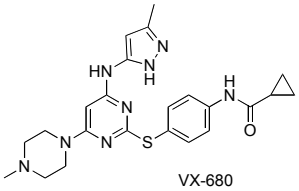
```
                                                    (229)SP NYDKWEMERT
                HF V
   V   V E   RH K  260        270       GA280          A          300
DITMKHKLGG GQYGEVYEGV WKKYSLTVAV KTLKEDTMEV EEFLKEAAVM KEIKHPNLVQ
                 N                                                A
        310 L    I L320        330        340    T    350 T   G   V
LLGVCTREPP FYIITEFMTY GNLLDYLREC NRQEVNAVVL LYMATQISSA MEYLEKKNFI
                                      R
        370          I   L    F390       PP400        410        Y420
HRDLAARNCL VGENHLVKVA DFGLSRLMTG DTYTAHAGAK FPIKWTAPES LAYNKFSIKS

        430        440        450         K          470        480
DVWAFGVLLW EIATYGMSPY PGIDLSQVYE LLEKDYRMER PEGCPEKVYE LMRACWQWNP

     S 490        500        510
SDRPSFAEIH QAFETMFQES SISDEVEKEL G
```

A point mutation is a single base _____. For the mutations shown above, each point mutation results in an amino acid change.

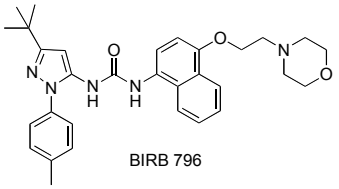
Second generation inhibitors are currently being explored to target the Gleevec-resistant Bcr-Abl mutants. *(See appendix D of the lab manual for information on these and other Abl inhibitors.)*



Dasatinib (Sprycel, BMS-354825)



VX-680



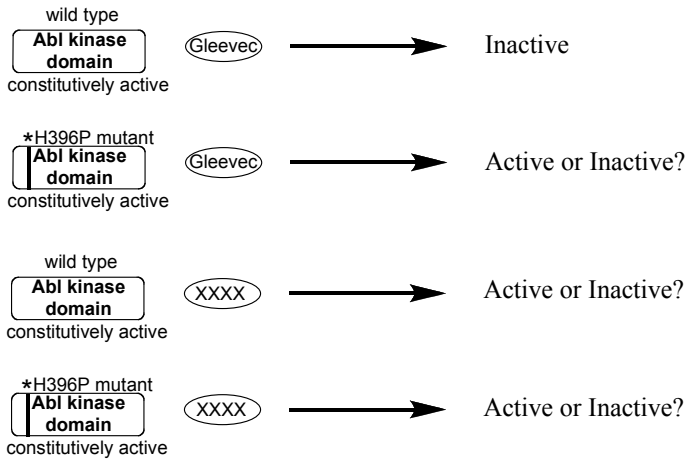
BIRB 796

II. OVERVIEW OF MODULES 4 AND 5

RESEARCH GOALS

1. Express and purify the kinase domain of a Gleevec-resistant Bcr-Abl mutant (this semester we'll focus on the _____ mutant).
2. Use an in-vitro _____ activity assay to investigate inhibition of the H396P mutant by Gleevec and other Abl inhibitors.***
3. Evaluate crystal structures of the wt and H396P Bcr-Abl kinase domains bound to kinase inhibitors to investigate various modes of inhibition.
4. Use DNA site directed mutagenesis to create an expression vector with another Bcr-Abl mutant. *Note that the DNA mutagesis is a related, but separate project. You will create mutant DNA that can be used in another term.*

*** You will use an in-vitro kinase assay to experimentally determine whether the Abl kinase domain is inhibited by Gleevec or by one or more other potential Abl inhibitors. You will run the assay with commercially available wild type Abl kinase domain and with the H396P Abl kinase domain mutant that you express and purify in Module 4.



Notice that you will use the Abl kinase domain as a model for full length Bcr-Abl.

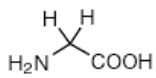
- The kinase domain is _____ between Abl and Bcr-Abl.
- Both the Abl kinase domain and Bcr-Abl are constitutively active.
- The smaller size of the Abl kinase domain aids expression, folding and handling in experiments.

Refer to the laboratory sessions outline (below and on page 4 of your lab manual) and a more detailed session-by-session outline (on page 5 of your lab manual) to keep track of what you will be working on during a given lab day.

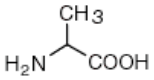
	H396P Abl protein expression/ kinase inhibition assays	***DNA site-directed mutagenesis***
Session 1	Grow a starter culture of cells with the H396P Abl and Yop-encoding vectors.	Grow a starter culture of cells with the wild type Abl vector.
Session 2	Express the H396P Abl protein. (Spin down cells on the following day.)	Isolate wt-Abl vector DNA through a miniprep. Quantify DNA concentration by UV-Vis.
Session 3		Digest isolated DNA to check for the wt Abl insert. Run DNA agarose gel. Design primers for an Abl kinase domain mutant.
Session 4	Prepare protein purification buffers. Create a BSA standard curve for future protein quantification.	
Session 5	Lyse cells and isolate the H396P Abl kinase domain. Dialyze protein into TBS.	
Session 6	Prepare an SDS-PAGE protein gel.	
Session 7/ Session 8	Run SDS protein gel. Concentrate protein and quantify final protein concentration.	
Session 9		Set up PCR for DNA mutagenesis.
Session 10		Complete the DPN digest and transform storage cells with mutant DNA. Pour LB/agar plates.
Session 11		Isolate (by miniprep) and quantify DNA. Prepare mutant DNA samples for sequencing.
Session 12	Prepare buffers and reagents for the coupled kinase activity assay.	
Session 13 and Session 14	Complete kinase assays: wt Abl kinase domain and the H396P mutant domain in the absence and presence of inhibitors.	
Session 15	Complete crystal structure viewing exercises.	Analyze DNA sequencing results.

For your reference, amino acid structures with three-letter and one-letter codes. (http://www.snell-pym.org.uk/sarah/soup.html)

Small

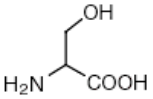


Glycine (Gly, G)
MW: 57.05

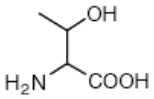


Alanine (Ala, A)
MW: 71.09

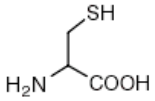
Nucleophilic



Serine (Ser, S)
MW: 87.08, pK_a ~ 16

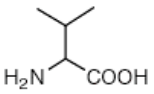


Threonine (Thr, T)
MW: 101.11, pK_a ~ 16

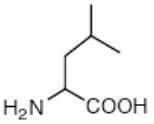


Cysteine (Cys, C)
MW: 103.15, pK_a = 8.35

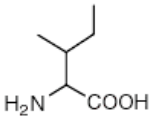
Hydrophobic



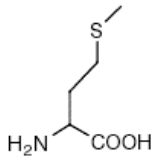
Valine (Val, V)
MW: 99.14



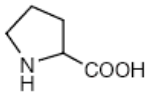
Leucine (Leu, L)
MW: 113.16



Isoleucine (Ile, I)
MW: 113.16

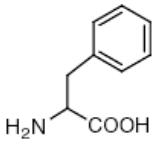


Methionine (Met, M)
MW: 131.19

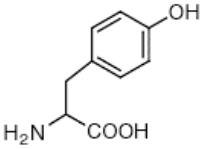


Proline (Pro, P)
MW: 97.12

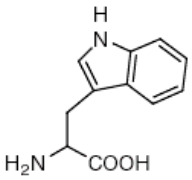
Aromatic



Phenylalanine (Phe, F)
MW: 147.18

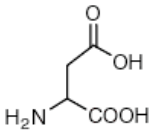


Tyrosine (Tyr, Y)
MW: 163.18

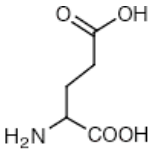


Tryptophan (Trp, W)
MW: 186.21

Acidic

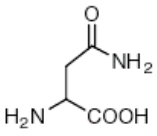


Aspartic Acid (Asp, D)
MW: 115.09, pK_a = 3.9

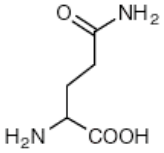


Glutamic Acid (Glu, E)
MW: 129.12, pK_a = 4.07

Amide

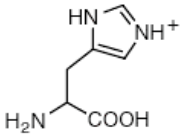


Asparagine (Asn, N)
MW: 114.11

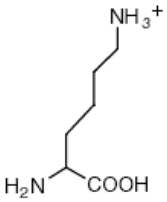


Glutamine (Gln, Q)
MW: 128.14

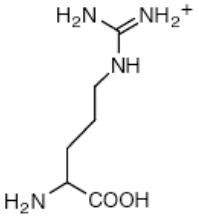
Basic



Histidine (His, H)
MW: 137.14, pK_a = 6.04



Lysine (Lys, K)
MW: 128.17, pK_a = 10.79



Arginine (Arg, R)
MW: 156.19, pK_a = 12.48