# 5.36 Biochemistry Laboratory
Spring 2009

# URIECA Biochemistry

I. Scientific background

A. Review of kinases and phosphatases

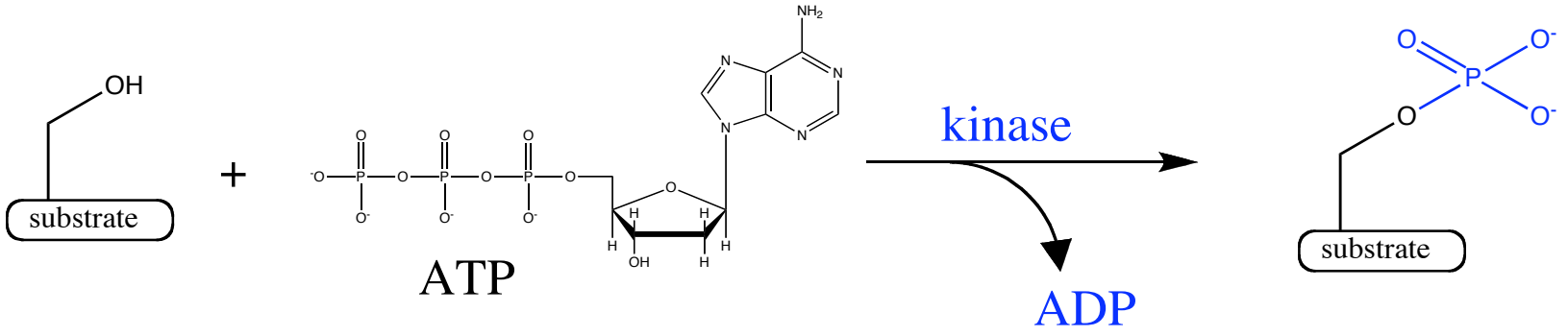
B. Abl kinase and Bcr-Abl

C. Kinase inhibitors as cancer drug targets

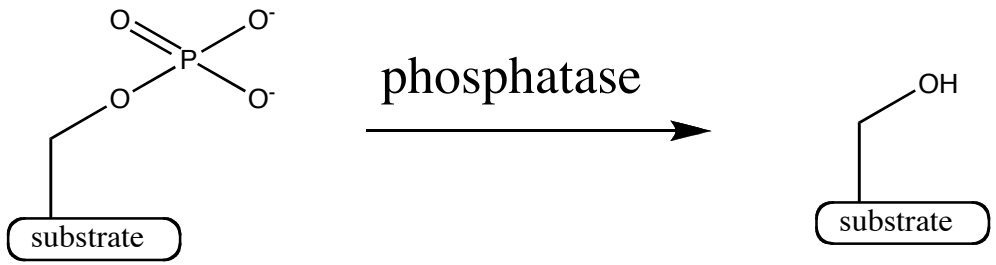
II. Overview of labs

# A Review of Kinases

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

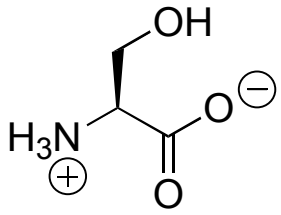



A **phosphatase** is an enzyme that catalyzes the removal of a phosphate group from a target molecule: the reverse reaction.

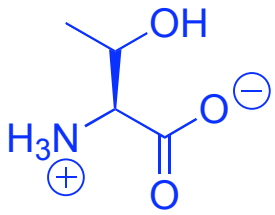


# Protein kinases modify other proteins

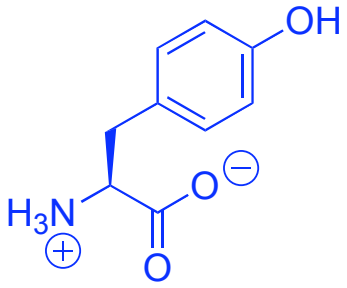
Serine/threonine-kinases phosphorylate target proteins on a Ser or Thr residue.




Ser, S




Thr, T




Tyr, Y

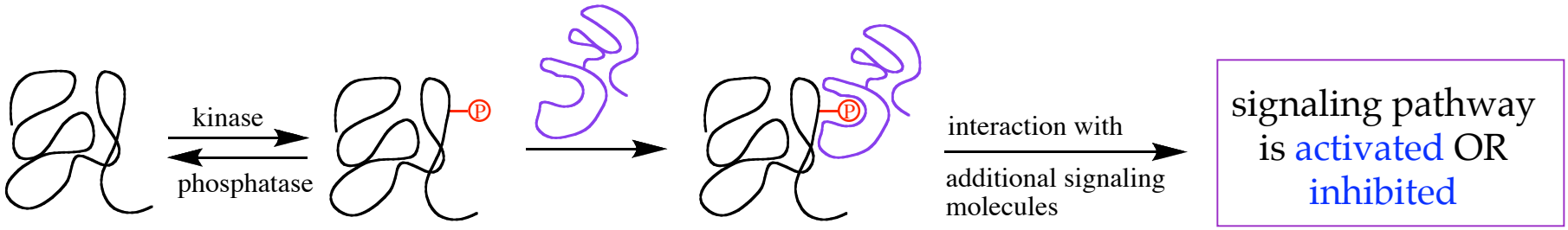
Tyrosine-kinases phosphorylate target proteins on a Tyr residue.

# Biological Relevance of Protein Kinases

Kinases (and phosphatases) can regulate the activity of a protein in several ways:

For example, phosphorylation of a protein target can

- create new binding site(s) for that protein.



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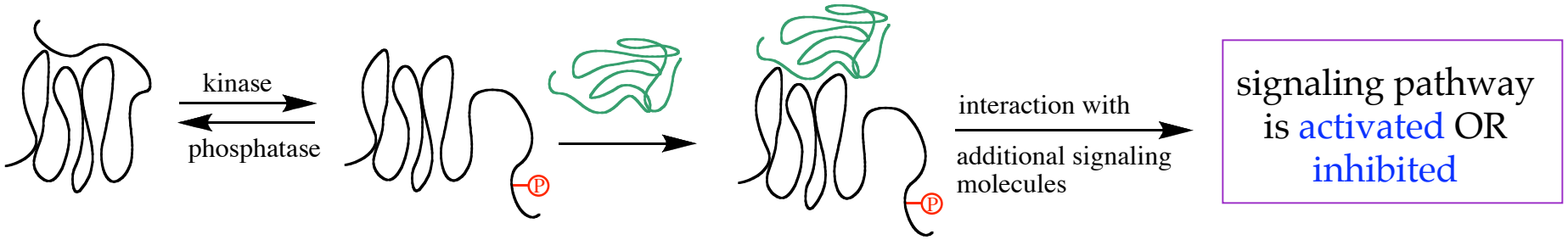
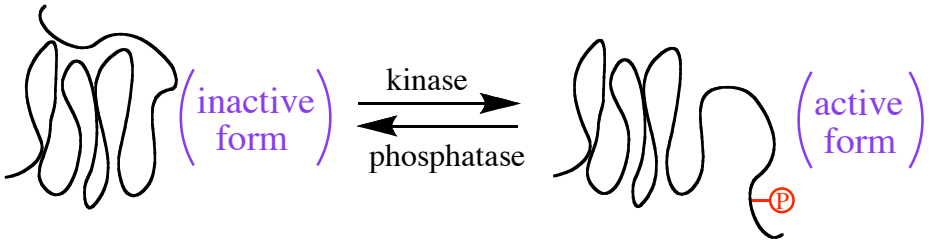
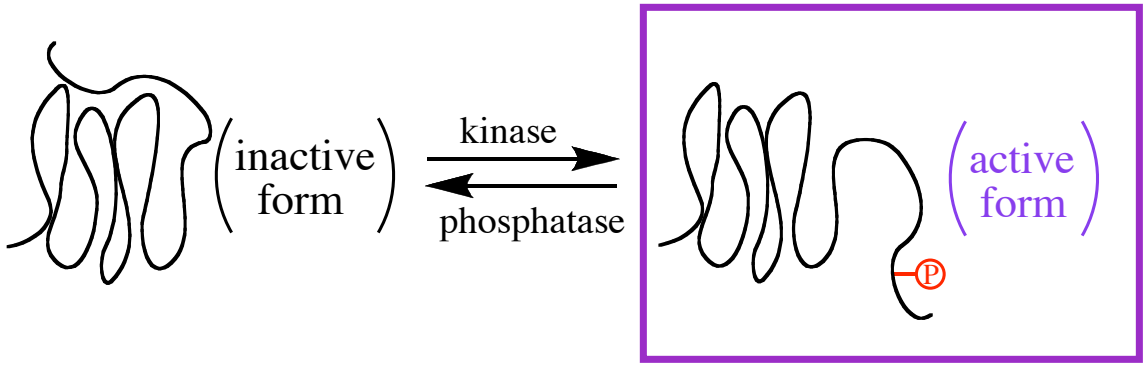
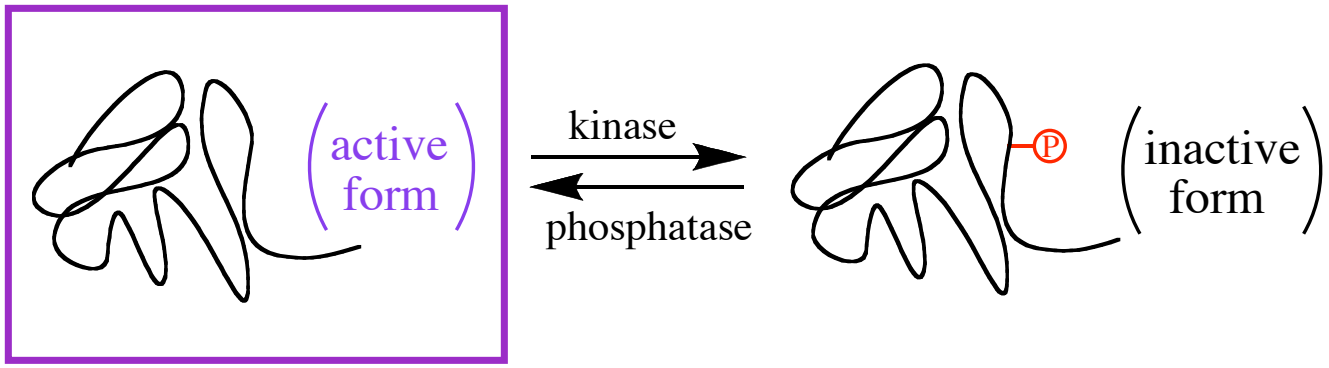



or modulate enzyme activity.





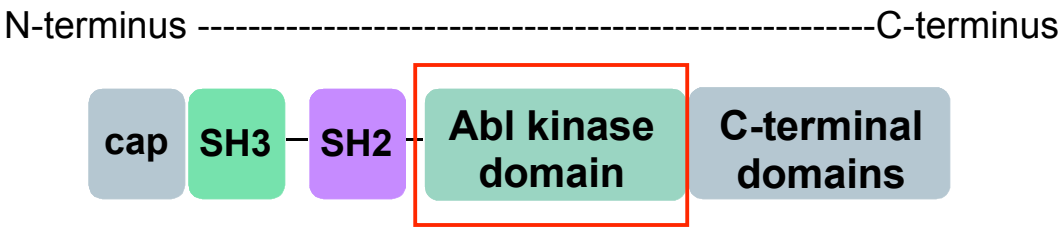

The phosphorylated form of an enzyme may be the active or the inactive form.



Phosphorylation by kinases can “switch on” an enzyme substrate to produce significant quantities of active enzyme on timescale of seconds to minutes.

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

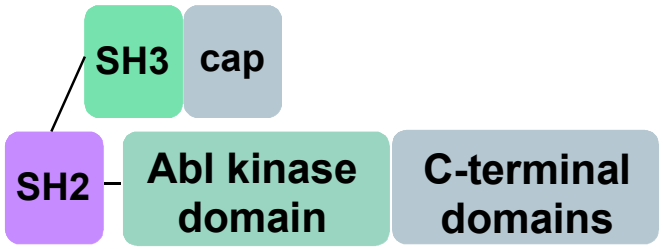
# Abelson kinase (Abl)




- Abl is a 120-kDa ( 1,130 amino-acid) protein tyrosine kinase.
- The Abl "kinase domain" is 33 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.



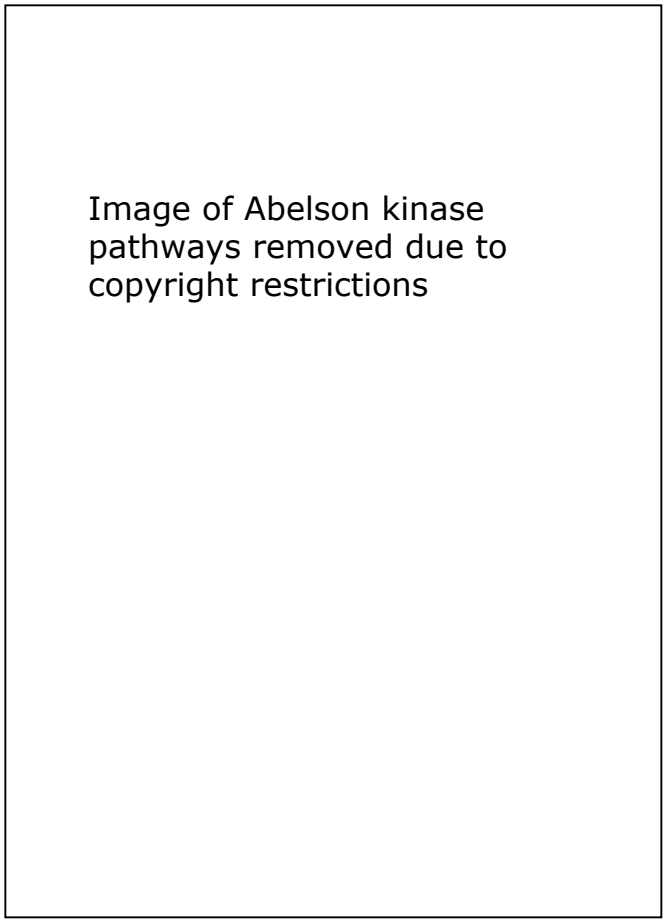

- Abl is autoinhibited by the binding of the "cap" domain to the kinase active site. The Abl default setting is OFF .

# Abelson kinase (Abl) in healthy cells

Precise biological functions are unknown.

Abl appears to be involved in

- **cell division** (via cell cycle regulation) and differentiation.
- **stress response** (response to DNA damage /oxidative stress).
- **cell adhesion** (via integrin signaling).

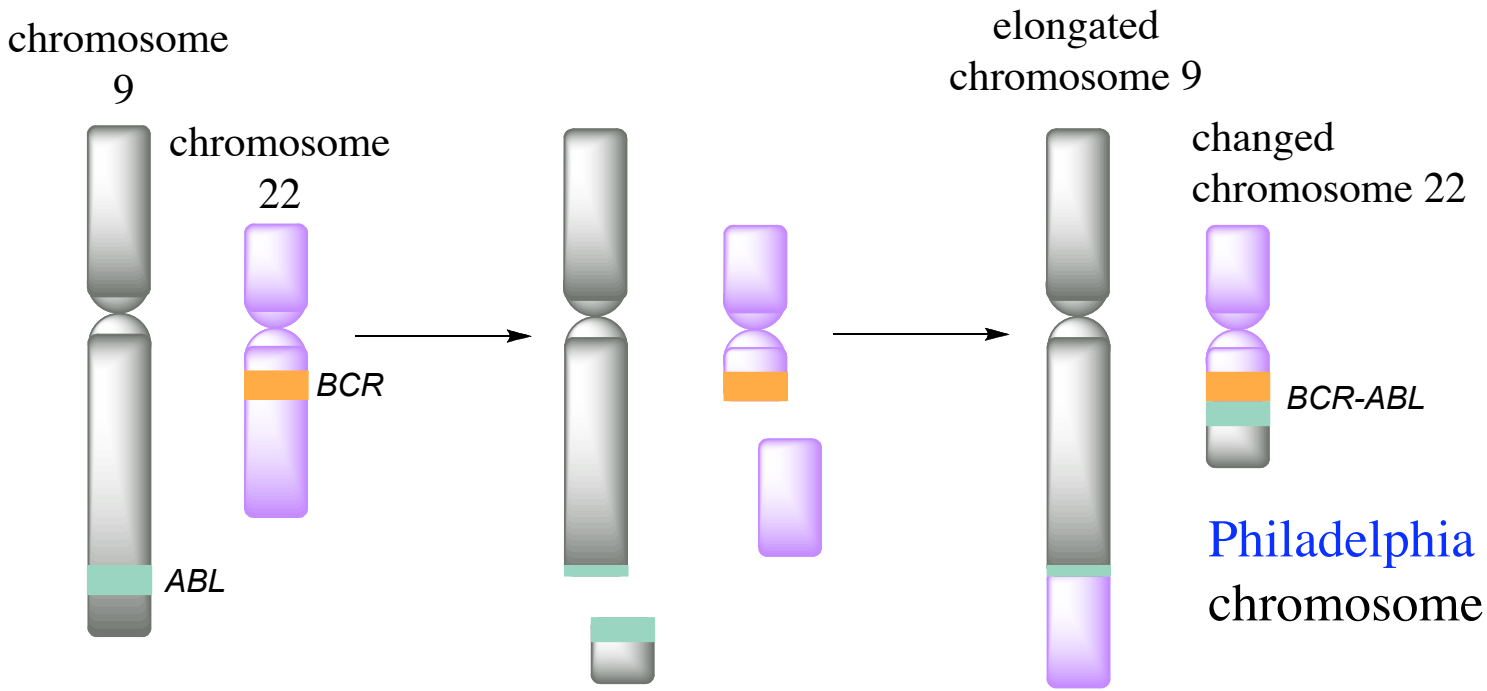



*Recall that Abl activity is **controlled**.*

# Abl kinase (Abl) in disease

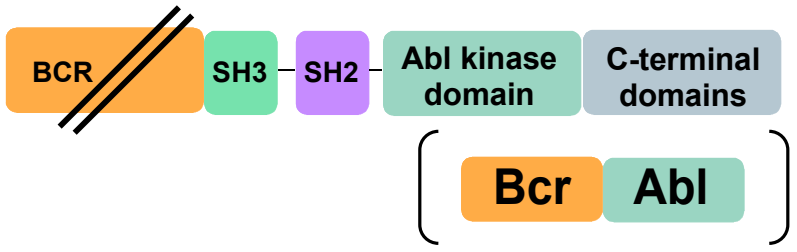
Mutations and / or overexpression of kinases lead to a wide range of diseases.

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



# Abl kinase (Abl) in disease

The fused BCR-ABL gene product is the mutant protein Bcr-Abl.




**Bcr-Abl**

*Constitutively active*


**Abl**

*Auto-inhibited. Activity is controlled.*

The Bcr-Abl protein lacks the residues responsible for Abl inhibition and is constitutively active (always ON).

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

# Chromic Mylogenous Leukemia
# CML

Slow growing cancer of the white blood cells in the bone marrow.

Diagram showing which cells CML can start in removed due to copyright restrictions. See image from Cancer Research UK http://www.cancerhelp.org.uk/help/default.asp?page=4835

CML white blood cells do not function correctly and take up room, resulting in fewer normal white blood cells and red blood cells.

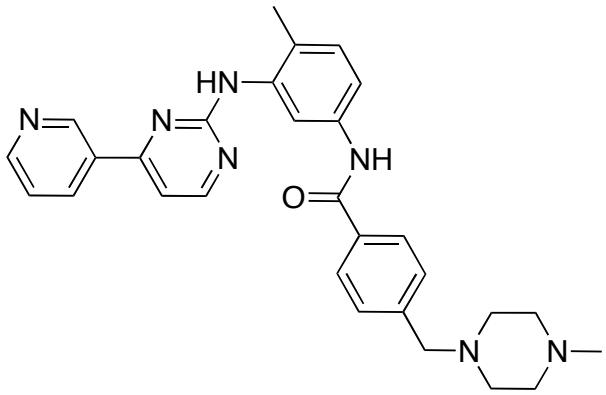
Incidence: ~5000 new cases each year in US. represents 15–20% of all cases of adult leukemia in Western populations

Only well-described risk factor is exposure to ionizing radiation

# Abl as a drug target

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

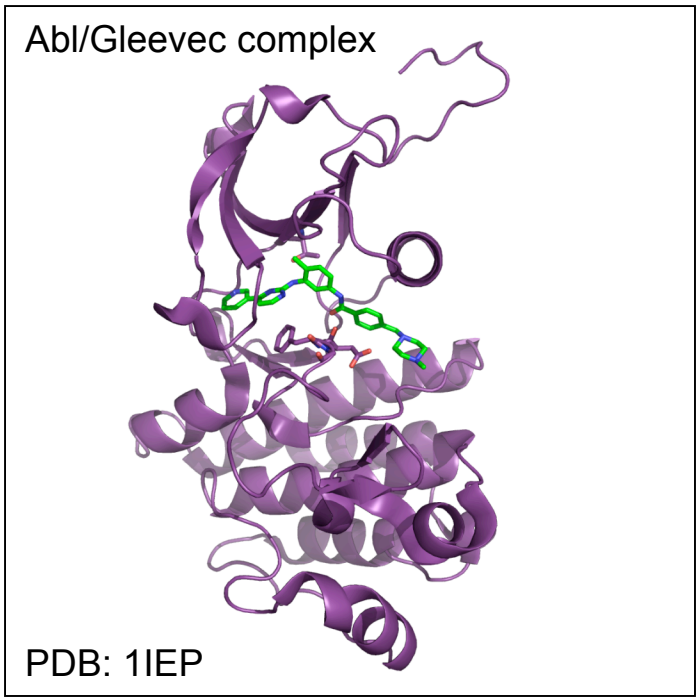
These efforts culminated in the development of the drug Gleevec, which was approved by the FDA in 2001 for CML treatment.

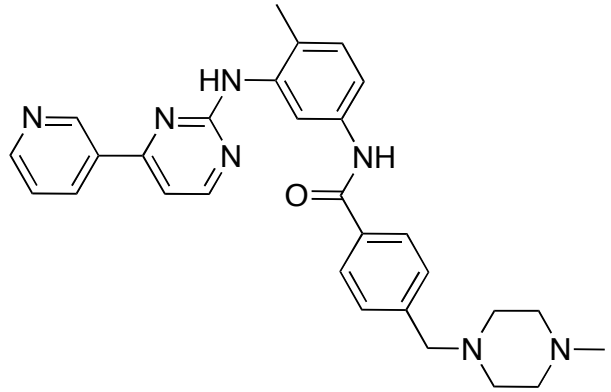




**Gleevec** ($IC_{50}$ = 250 nM)

Gleevec binds to the active site of the Abl kinase domain and stabilizes the inactive conformation of the protein.

The Gleevec-Abl interaction is highly specific. Gleevec inhibits only two other kinases at physiological levels, neither of which result in major side effects.




**Gleevec** ($IC_{50}$ = 250 nM)

Note: Other names for Gleevec are imatinib and STI-576.

**The New York Times**
May 29, 2001, Tuesday
**Scientists View New Wave of Cancer Drugs**
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~ 80% of CML patients diagnosed in the chronic stage experience remission.

Gleevec is the first drug that selectively inhibits a tyrosine kinase. This is very exciting from the standpoint of drug discovery!

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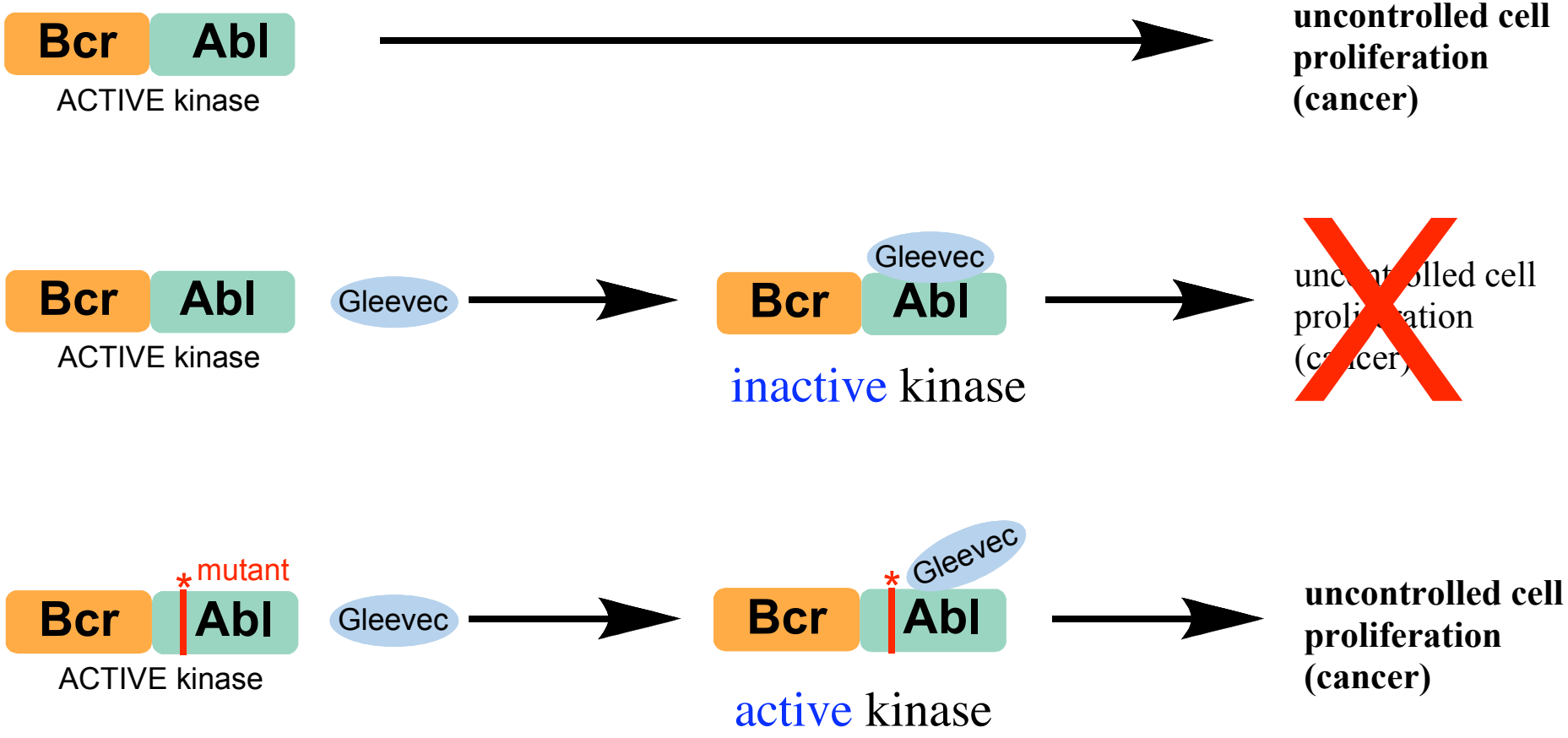
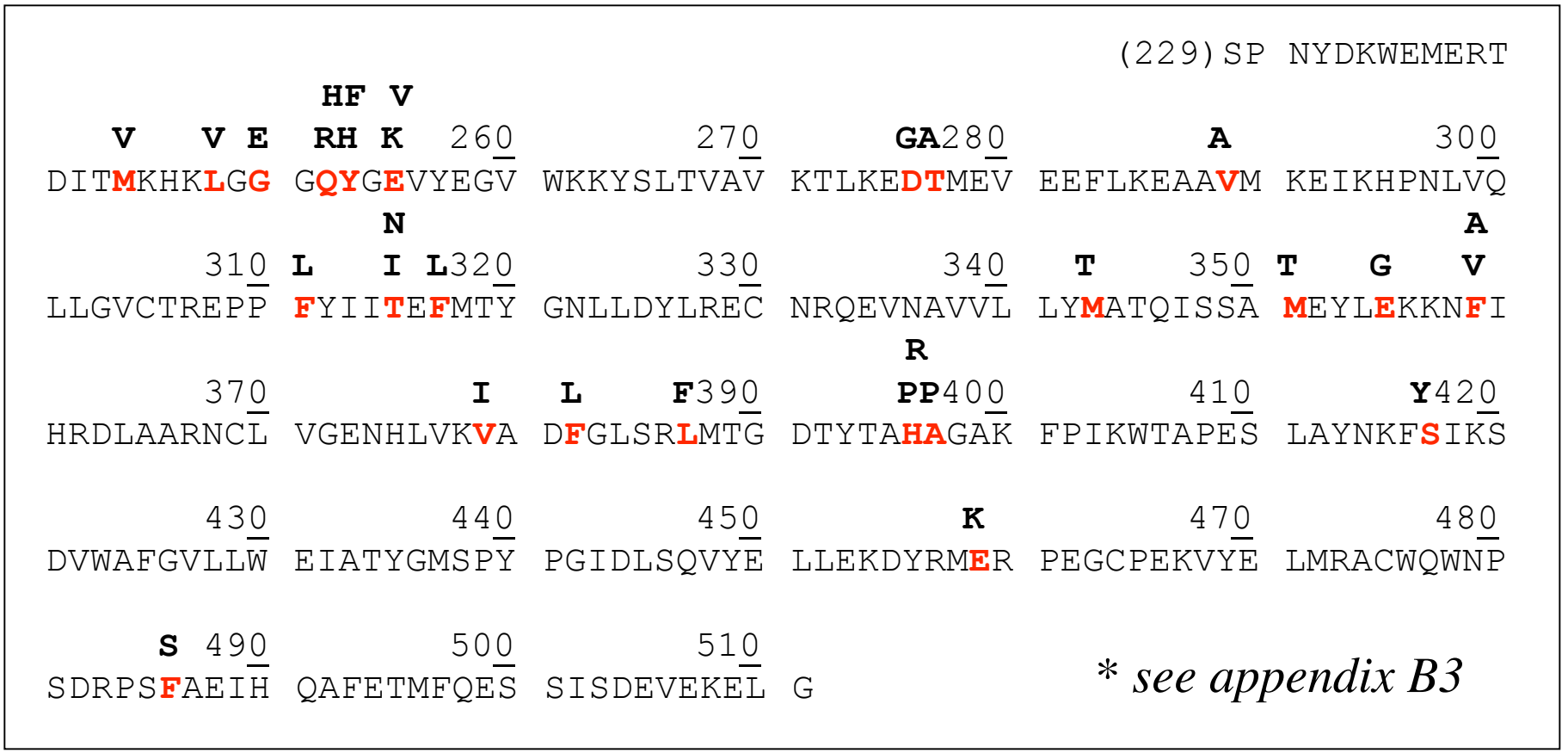
**However….**

As of 2006, 16% of patients diagnosed in the chronic stage show 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 1 amino acid in the kinase domain.

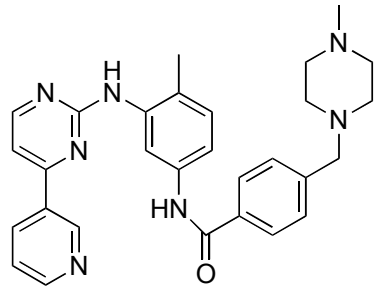


Over 30 *different* point mutations in the Abl kinase domain of Bcr-Abl have been identified in Gleevec-resistant CML patients to date.

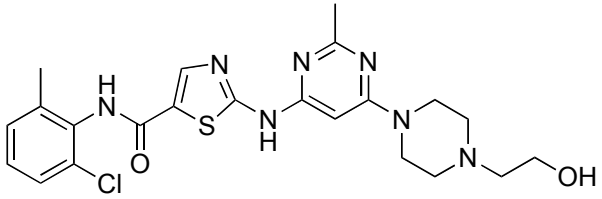



A point mutation is a single base substitution. For the mutations shown above, each point mutation results in an amino acid change.

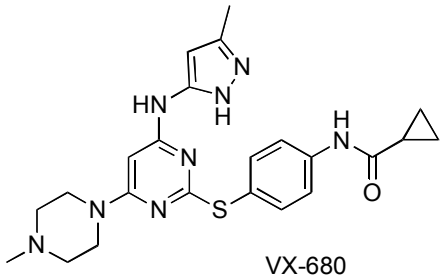
# New inhibitors are currently being explored to target the Gleevec-resistant Bcr-Abl mutants



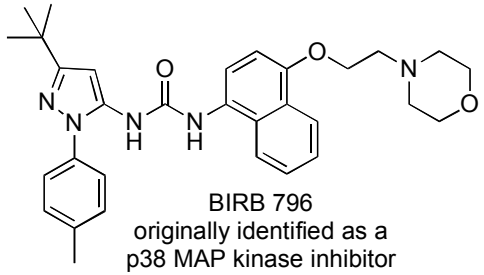
Imatinib (Gleevec, STI571)



Dasatinib (Sprycel, BMS-354825)



VX-680
originally identified as an Aurora inhibitor



BIRB 796
originally identified as a
p38 MAP kinase inhibitor

Scientists are using computer modeling, x-ray crystallography, high throughput screens and rational design to find more leads.

# Our goals in Modules 4 and 5

1) Express and purify the kinase domain of a Gleevec-resistant Bcr-Abl mutant (the H396P mutant).

2) Use an in-vitro kinase activity assay to investigate inhibition of the H396P mutant by Gleevec and other Abl inhibitors.

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1) Express and purify the kinase domain of a Gleevec-resistant Bcr-Abl mutant (the H396P mutant).

2) Use an in-vitro kinase activity assay to investigate inhibition of the H396P mutant by Gleevec and other Abl inhibitors.

3) Evaluate crystal structures of the wt and the H396P Abl kinase domains bound to kinase inhibitors to investigate various modes of inhibition.

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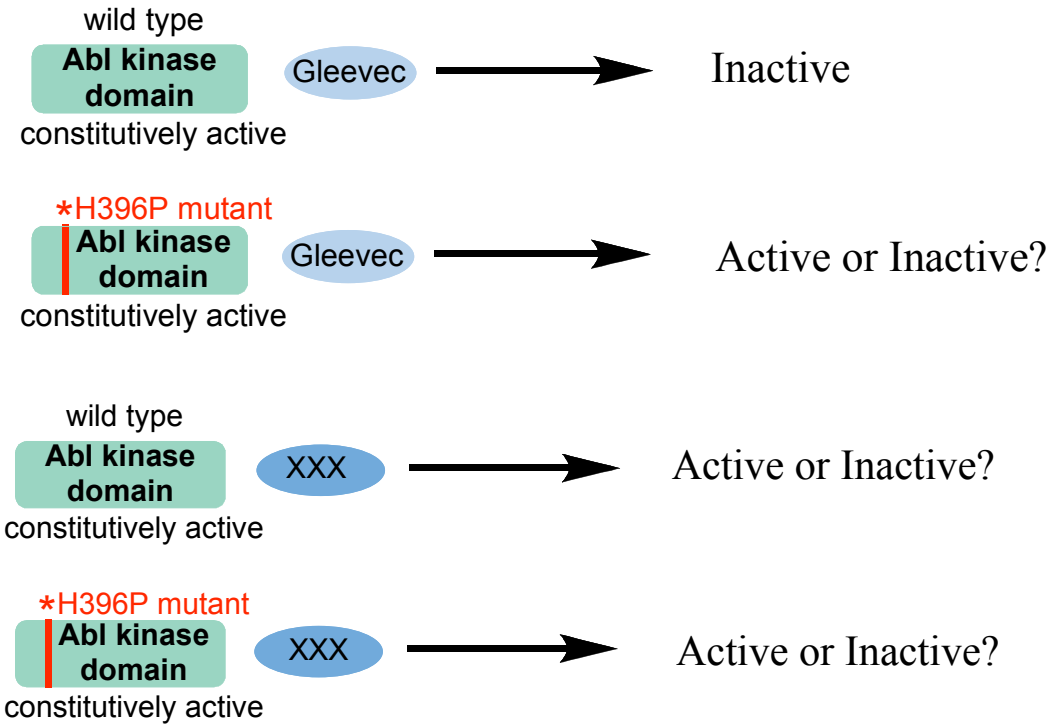
4) Use DNA site directed mutagenesis to create an expression vector with another Bcr-Abl mutant.

*Note that the DNA mutagesis is a related, but different project. We will end up with mutant DNA that can be used in another term. Biochemists often pursue several related projects concurrently.*




## Abl kinase domain as a model for full length Bcr-Abl.

The kinase domain is identical 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.

# Laboratory Sessions: Multitasking in Biochemistry

| | H396P Abl Expression and Purification / Kinase assays | DNA isolation and mutagenesis for a new Abl kinase domain mutant |
|---|---|---|
| **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. |
| **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. |
| **3** | | Digest isolated DNA to check for the wt Abl insert. Run DNA agarose gel. Design primers for an Abl kinase domain mutant. |
| **4** | Prepare protein purification buffers. Create a BSA standard curve for future protein quantification. | |
| **5** | Lyse cells and isolate the H396P Abl kinase domain. Dialyze protein into TBS. | |
| **6** | Prepare an SDS-PAGE protein gel. | |
| **7/ 8** | Run SDS protein gel. Concentrate protein and quantify final protein concentration. | |
| **9** | | Set up PCR for DNA mutagenesis. |
| **10** | | Complete the DPN digest and transform storage cells with mutant DNA. Pour LB/agar plates. |
| **11** | | Isolate (by miniprep) and quantify DNA. Prepare mutant DNA samples for sequencing. |
| **12** | Prepare buffers and reagents for the coupled kinase activity assay. | |
| **13/14** | Complete kinase assays: wt Abl kinase domain and the H396P mutant domain in the absence and presence of inhibitors. | |
| **15** | Complete crystal structure viewing exercises. | Analyze DNA sequencing results. |

# General Course Information

**PRE-LABS and LAB PARTICIPATION.** Prior to each laboratory session (except for session 1), you should outline the procedures you will be carrying out and complete any relevant calculations. Prelabs should include a clear outline, but need not repeat detailed procedures that you will refer to in your lab manual (for example, the step by step procedure for a miniprep). Your TA will assign you a grade of 0, 1 or 2 points for each lab session based on your preparation and participation in lab for that day.

| | | |
|---|---|---|
| **GRADING** | Lab preparation and participation | 40 points |
| | Lecture attendance | 10 points |
| | Laboratory work / lab report | 50 points |
| | **Total** | **100 points** |

# Scientific Lessons

- Kinases and phosphatases in signal transduction
- Enzyme mechanisms
- Links between aberrant enzyme activity and human disease (ie. cancer)
- Drug design and development
- Mechanisms of drug resistance
- Connections between biochemistry and other fields of chemistry.

# Laboratory Techniques

**DNA biochemistry**: how to detect, purify, amplify, and mutate DNA

- Technique lecture: site-directed mutagenesis
- Technique lecture : plasmids and DNA amplification using bacteria

**Protein biochemistry**: how to express, purify, and detect protein. how to assay enzyme activity and inhibition.

- Technique lecture: affinity tags for protein purification
- Technique lecture: Assays for kinase activity
- Activity on structure viewing

# Safety Training (required for lab work)