Point Mutations Conferring Gleevec Resistance

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Section 3 - 1

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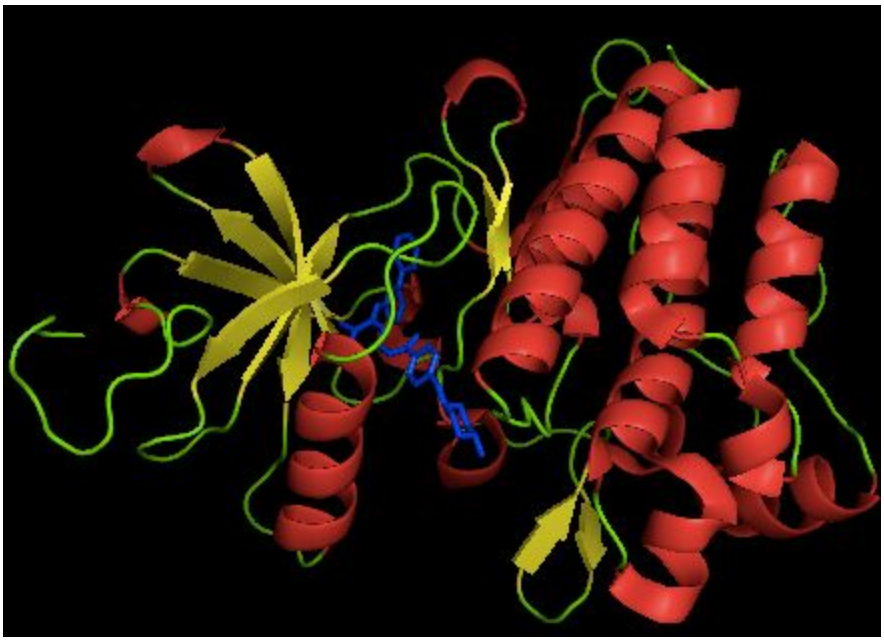


ABSTRACT

Mutations in the Abelson kinase (Abl) domain have been implicated in Gleevec treatment resistant Chronic Myelogenous Leukemia (CML). CML is primarily caused by a chromosomal translocation between chromosomes 9 and 22 that results with a constituently active Abelson kinase domain due to the insertion of the break cluster region gene (BCR) near Abelson kinase gene. The mutated Abelson kinase protein found in CML is Bcr-Abl, however only the Abl domain was used in this experiment as an analogue to mimic the constituently active form of Bcr-Abl. One such mutation in BCR-ABL, H396P, has been linked to increased resistance of the Abelson kinase domain to the Gleevec inhibitor drug. The goal of this experiment is to explore the relative kinase activity between the mutant H396P and native forms of Abelson kinase to confirm the increased resistance of this mutant domain to Gleevec. To accomplish this, wild type vectors and mutant vectors, from site directed mutagenesis at H396P, were created of the Abl domain. Following purification, the mutant vectors were subsequently transformed in vitro to express wild type and mutant Abl protein. Quantitative assays of the kinetic activity of the wild type and H396P mutant Abl in the presence of Gleevec showed a 104.5% inhibition of the wild type protein and a 92.6% inhibition of the mutant H396P. The H396P mutant Abl showed a decreased sensitivity to the Gleevec drug relative to the wild type conformation.

INTRODUCTION

Abelson kinase (Abl) is protein tyrosine kinase that acts as a regulatory element in cell division and differentiation, among other roles within the cell and has been implicated as a potential oncogene.[1] A chromosomal translocation between chromosomes 9 and 22 leads to a BCR-ABL fused gene, and a Bcr-Abl fused protein. The carcinogenic translocation removes the residues responsible for the auto-inhibition quality of the kinase, leading to diseases such as Chronic Myelogenous Leukemia (CML).[1] In 2001, a drug called imitinib, or brand name Gleevac, was approved by the FDA to act as a competitive inhibitor for the ATP-binding site of BCR-Abl. However, patients soon began developing resistance to the drug due to point mutations at a few residues in the Abl protein.[2] One such mutant with a decreased sensitivity to imitinib was identified as H396P Bcr-Abl.[3] This experiment set out to quantify the relative kinase activity of the wild type and mutant H396P Abl protein in the presence and absence of the Gleevac inhibitor. The experimenters hypothesize that the H396P point mutation in Abl kinase will increase Gleevec resistance.

METHODS

Luria broth media was prepared. All bacteria was grown in this media with a final concentration of 50 ug/mL kanamycin and 100 ug/mL streptomycin. Bacteria were incubated at 37°C for growth unless otherwise specified. The bacteria used was *E. coli* Bl21DE3 and *E. coli* DH5a competent cells. Bacterial growth was assayed with a spectrophotometer. Protein expression in *E. coli* BL21DE3 was induced with IPTG. Transformation was done with the *E. coli* DH5a competent cells.

Primers were designed with the use of the OligoAnalyzer found online (URL: www.idtdna.com/analyzer/Applications/Oligoanalyzer/). Primers were ordered from Integrated

DNA technologies and diluted in TE buffer at pH 8.0. Concentrations were determined at 260 nm using UV/vis and further diluted to 25 uM using TE buffer.

DNA size analysis was done with a 1% agarose gel with ethidium bromide to visualize the DNA. The DNA ladder used is a 1 KB plus ladder from Invitrogen. All PCR products were purified with the Q1Aquick PCR purification kit. Isolation of DNA was performed by excising the desired gel band and using the Qiagen QIAquick gel extraction kit. DNA purity and concentration was assayed with the ratio of absorbance between 260 nm and 280 nm.

Site directed mutagenesis was performed via a forward and a reverse reaction. Reaction 1 contained H_2O, pET28a-Abl plasmid, T7 promoter primer , the mutant reverse primer and GoTaq Green Master Mix. Reaction 2 contained H_2O, pT28a-Abl plasmid, T7 terminator primer, of Mutant forward primer, and GoTaq Green Master Mix. The samples were spun down and added to a thermocycler to perform PCR. The results of this reaction was confirmed and purified. The PCR reaction contained H_2O, T7 Promoter primer, T7 terminator primer, purified PCR reaction 1, purified PCR reaction 2, and GoTaq Green Master Mix. The reaction ran through a thermocycler to generate products. This results were confirmed and purified.

The purified PCR product was treated with Xbal and Xhol restriction enzymes to generate the insert. The reactions conditions included the final PCR product, H_2O, 10x Cutsmart Buffer, Xhol enzyme, and Xbal enzyme. This reaction is incubated in a 37°C water bath for an hour. Loading dye is added and the reaction is stored at 4°C until needed. Similarly, the double digest of the empty vector was also prepared for gel analysis. The reaction was made 10x CutSmart Buffer, Xhol, Xbal, and miniprepped DNA. The reaction was diluted with endonuclease free DI water. It was incubated at 37°C for an hour. Calf intestinal phosphatase was added and incubated at 37°C for 30 minutes. Digested DNA was isolated.

Ligation was performed with the purified insert, pET28a-Abl WT plasmid, and T4 DNA ligase. The ligation reaction was run with a vector to insert ratio of 1:3, 2x Rapid Ligation Buffer, and T4 DNA ligase. A positive control of just the plasmid and a negative control of digested plasmid was also generated.

To calculate amount of plasmid and insert, the following equation was used:

$$\frac{(\text{ng of vector})(\text{kb of insert})}{(\text{kb of vector})} \times \text{molar ratio of (insert/vector)} = \text{ng insert}$$

DNA transformation was performed with positive control, negative control, and mutant transformation conditions. Competent cells were transformed with a heat shock procedure and incubated on S.O.C media. Cells were plated on LB/kan plates. The mutations were confirmed via Sanger Sequencing.

Isolating the WT Abl protein

Ni-NTA binding, washing, and elution buffers were prepared, and Tris-HCl buffers at varying concentrations and pHs were prepared.
A frozen pellet of WT Abl cells were thawed at room temperature, and lysed with the addition of B-PER detergent, protease inhibitor cocktail solution, and a centrifuge. The detergent and protease inhibitor cocktail solution is decanted and chilled Ni-NTA binding buffer added. Ni-NTA resin was prepared and with this resin the WT abl kinase domain was purified by Ni-NTA affinity chromatography. The binding, washing, and elution buffer flow throughs were saved and prepared for used in a SDS-PAGE gel.
The purified WT Abl protein was concentrated and washed with a centrifuge. The concentrated protein was quantified with the aid of a Bradford assay.

Cloning and expressing Mutant Abl

E. coli BL21DE3 cells were transformed with the H396P plasmid and YopH plasmid, and plated on LB/kan/Strep plates. With the grown cells a LB/kan/strep culture was inoculated with the mutant Abl/Yop culture. This cultures optical density at 600 nm was checked periodically until the absorbance was high enough. A sample was taken that was to be our pre-induction sample. IPTG was added to the culture. After some time cells were harvested from the culture and pelleted.
SDS-PAGE was run with loading buffer, MW standard, our pre-induction sample, our post-induction sample, our binding, washing, and elution flow throughs, and the concentrated protein. Once this gel was run it was stained with coomassie blue, and analyzed.

Isolating Mutant Abl protein

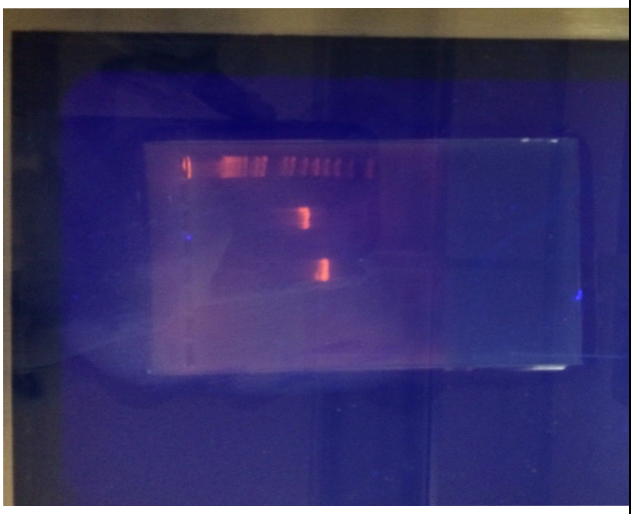
Frozen mutant Abl pellet was thawed and and lysed with B-PER, protease inhibitor cocktail, and a centrifuge. The B-PER and protease inhibitor cocktail was decanted and replaced with chilled Ni-NTA binding buffer. The Mutant Ble kinase domain was purified by Ni-NTA affinity chromatography. The binding, washing, and elution buffer flow through was collected and prepared for SDS-PAGE. The mutant Able purified protein was concentrated with the use of a centrifuge, and quantified with the use of the bradford assay. SDS-PAGE was run with SDS, MW standard, our pre-induction sample, our post-induction sample, our binding, washing, and elution flow through, and our concentrated protein. Once the gel had run it was transferred to coomassie stain, and analyzed.

Kinase Assay to test WT and Mutant Abl

Kinase assay with the WT and mutant able was performed, with and without the gleevec inhibitor. With the aid of Malachite Green reagent A and B the optical density of each sample was taken, and the results compared and analyzed.

RESULTS

Figure 1: Electrophoretic gel



Top Lane: DNA Ladder
Middle Lane: Reverse Reaction
Bottom Lane: Forward Reaction

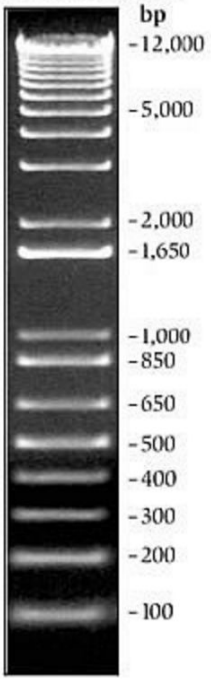
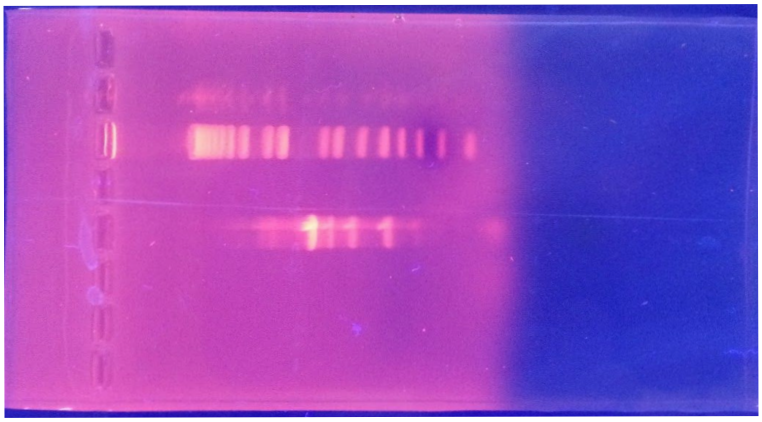


Figure 1 shows the size of the forward primer and the negative primer by comparison to a 1 Kb Plus ladder.The reverse primer was found to be about 400bp in size and the forward primer was found to be about 600bp in size.

Figure 2: Electrophoresis of PCR Product



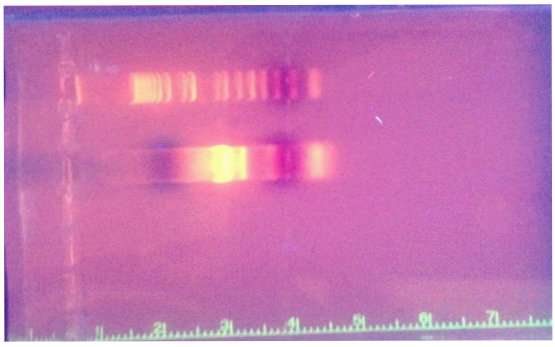
Counting from the top:

Lane 3: 1 KB DNA Ladder

Lane 5: PCR product

Figure 2 shows the final PCR product is what was expected. Fragments were found to be at 1000 base pairs, 900 base pairs, 700 base pairs, and 500 base pairs in length. The expected length of the DNA band is 1114 bp.

Figure 3: Electrophoresis of H396P Mutant Insert

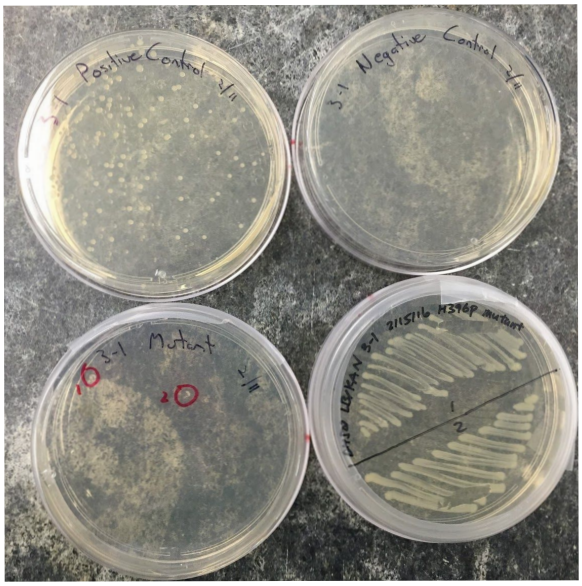


Top lane: 1 kb Plus DNA ladder

Bottom lane: entire digest reaction

Figure 3 shows the the predicted product size based on the vector used is 974 BP. A band appeared at around 1000 bp.

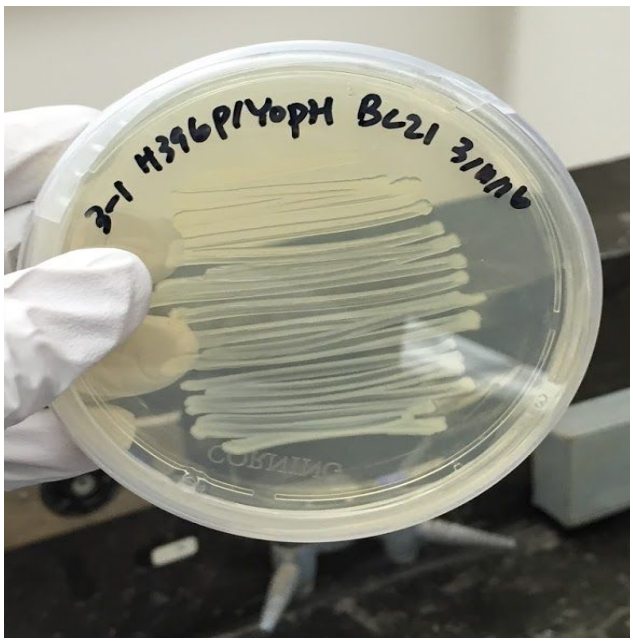
Figure 4: Transformed Bacterial Colonies



Legend:
Top left: Positive control
Top right: Negative control
Bottom Left: Transformation
Bottom right: Streaked Transformation Colonies (1&2)

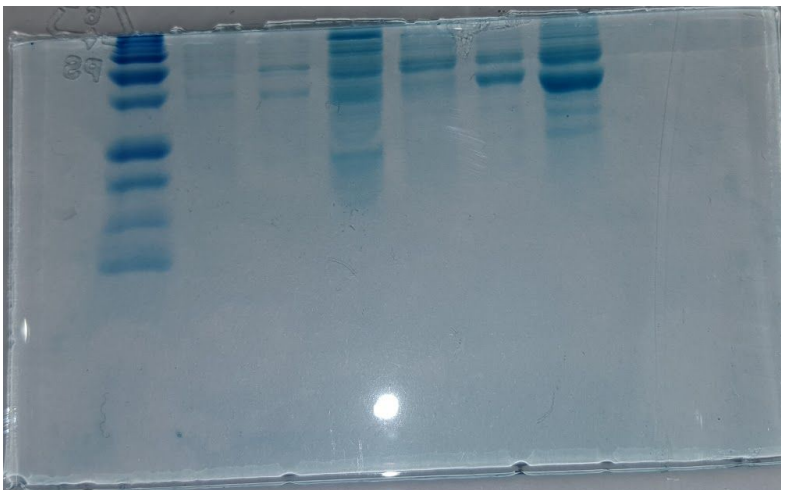
Positive control: vector(full) added
Negative control: digested vector added
Transformation: vector and insert

Figure 5: Successful transformation of mutant vector and YOPH



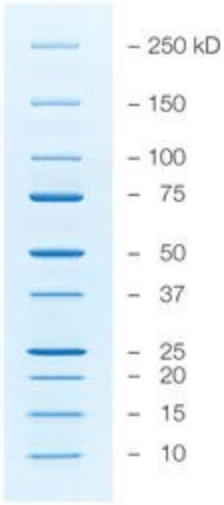
I don't have a picture of the controls but they were the same as figure 4. SHould we combine the figures?

Figure 6:



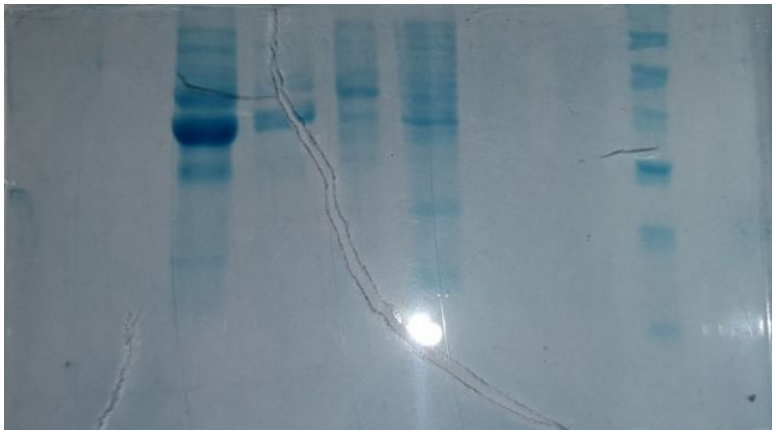
Lanes (Left to Right)

1
2. Ladder
3. Pre-induction sample
4. Post-induction sample
5. Binding sample
6. Wash sample
7. Elution sample
8. Concentrated protein
9
10



A band appeared at less than 37 kD and a band appeared at above 50 kD but less than 75 Kd.

Figure 7:

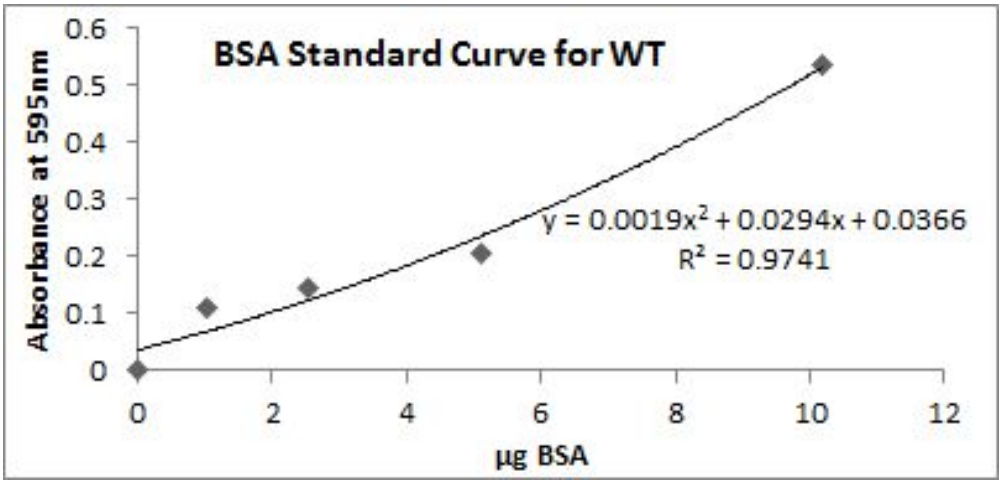


Lanes (Right to left)

1
2. Ladder
3. Pre-induction sample
4. Post-induction sample
5. Binding sample
6. Wash sample
7. Elution sample
8. Concentrated protein
9
10

A band appeared at 33kD and at around 60 kD.

Figure 8: BSA Standard Curve for WT



Standard Curve Data Entry:

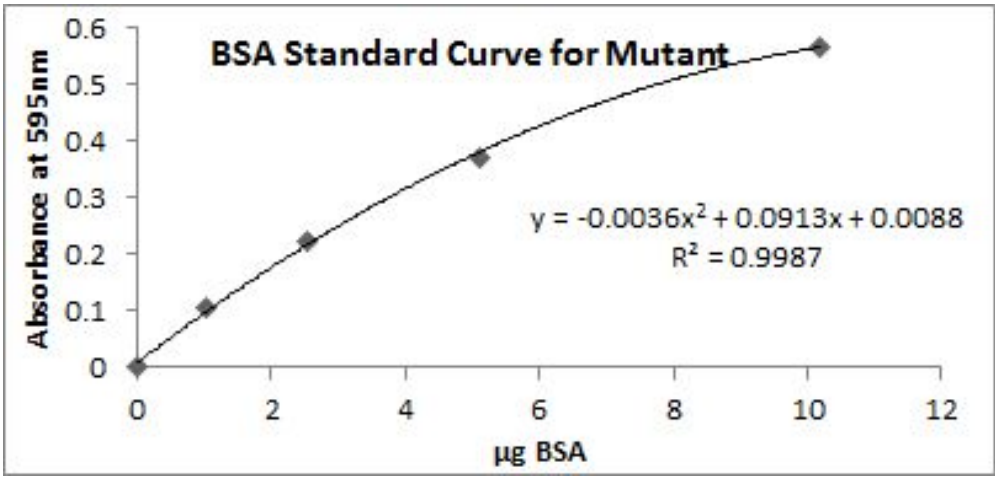
µL of BSA added	µg BSA	Absorbance values at 595nm replicate 1	Absorbance values at 595nm replicate 2	Average of A595 replicates
0	0	0	0	0
2	1.02	0.149	0.069	0.109
5	2.55	0.175	0.113	0.144
10	5.1	0.156	0.256	0.206
20	10.2	0.593	0.483	0.538

Unknown Data Entry: C-value from quadratic equation of standard curve: 0.0366

Purified and Concentrated Wild-type Abl kinase

	Abs at 595nm Rep 1	Abs at 595nm Rep 2	Average of Reps 1 and 2	[C-value from graph]-[y value or Avg of abs reading for sample]	Solve for X-intercept with Quadratic equation (link above)	Concentration	Average to yield Final Concentration
5µL of sample	0.451	0.378	0.4145	-0.378	8.35	8.35	**7.11**
10µL of sample	0.687	0.603	0.645	-0.608	11.75	5.88	**mg/mL**

Figure 9: BSA Standard Curve for Mutant



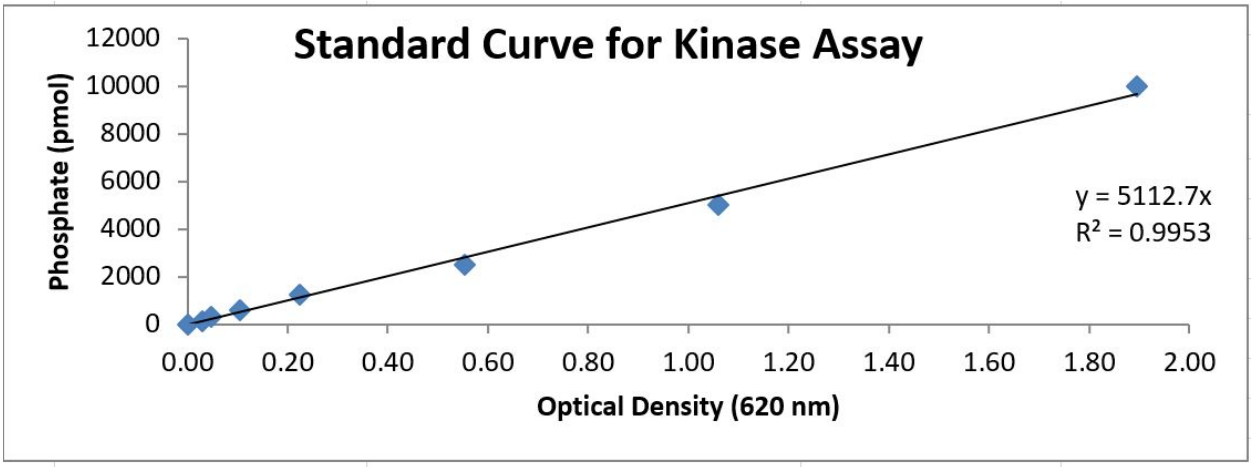
µL of BSA added	µg BSA	Absorbance values at 595nm replicate 1	Absorbance values at 595nm replicate 2	Average of A595 replicates
0	0	0	0	0
2	1.02	0.098	0.116	0.107
5	2.55	0.227	0.223	0.225
10	5.1	0.364	0.382	0.373
20	10.2	0.57	0.566	0.568

Unknown Data Entry: C-value from quadratic equation of standard curve: 0.0088

Purified and Concentrated Wild-type Abl kinase

	Abs at 595nm Rep 1	Abs at 595nm Rep 2	Average of Reps 1 and 2	[C-value from graph]-[y value or Avg of abs reading for sample]	Solve for X-intercept with Quadratic equation (link above)	Concentration	Average to yield Final Concentration
5µL of sample	0.501	0.516	0.5085	-0.500	1.6	1.60	**1.56**
10µL of sample	0.222	0.285	0.2535	-0.245	3.05	1.53	**mg/mL**

Figure 10: Standard Curve for Kinase Activity



				Conversion Factor from Standard Curve or Slope (pmol/OD 620nm) =				5112.7
Sample Results:		**From spect:**			**Use Conversion Factor from slope of standard curve**	**Divide by Incubation time (min) and µg protein added (see below)**	**Coupling rate from product information/conditions of reaction**	**Compare with or without inhibitor**
Tube #	**Samples**	**620 nm absorbance**	**Average 620 nm absorbance**	**Subtract background reading at 620 nm**	**pmol of phosphate transferred**	**Specific Activity* (pmol/min/µg)**	**Corrected Specific Activity for coupling rate (pmol/min/ug)**	**% Inhibition with Gleevec drug**
a	Negative Control	0.239						
b	(no enzyme)	0.247	0.243	0	0.0			
c	Positive Control	0.757						
d	(ADP)	1.593	1.175	0.932	4765.0			
e	WT Abl - Gleevec	0.517						104.5%
f		0.592	0.5545	0.3115	1592.6	17.2	25.5	
g	WT Abl +	0.221						
h	Gleevec	0.237	0.229	-0.014	-71.6	-0.8	-1.1	
i	Abl-H396P -	0.591						92.6%
j	Gleevec	0.561	0.576	0.333	1702.5	18.4	27.2	
k	Abl-H396P +	0.295						
l	Gleevec	0.24	0.2675	0.0245	125.3	1.4	2.0	
*units of specific activity defined: pmol of phosphate transferred to substrate per min of reaction per µg of protein								

Reaction Time (min)	Protein (µg/µL)	Volume added (µL)	µg added
10	0.33	28	9.24

DISCUSSION

The PCR product produced matches the expected length of the reaction (453 bp) because the gel band appeared between 400 BP and 500 Bp, indicating that the reaction was successful. Also, the forward reaction generated a band at around 600 bp, indicating that the reaction successful too. The expected lengths of the PCR product was determined by examining the base pair locations of the points of interest.

Forward Reaction
Abl-H396P Reverse: 631
Flanking region = 45 bp
Expected size = 631 bp + 45 bp = 676 bp

Reverse Reaction
Abl-H396P Forward: 616 bp
T7 Terminator: 1069 bp
Expected size = 1069 bp - 616 bp = 453 bp

Because the initial PCR reaction generated its expected results, the purified DNA could be used to generate the full length H396P mutant. The expected size of the final reaction product is 1114 bp.

Final Reaction
Flanking region = 45 bp
T7 Terminator = 1069 bp
Expected size = 45 bp + 1069 bp = 1114 bp

The agarose gel made generated a band at around 1000 bp, confirming that the PCR reaction was successful in generating the desired product. The band at 700 bp and 500 bp is believed to be contaminants from the previous PCR reactions as their products were predicted to be 676 bp and 474 bp in size. Because the PCR reaction was successful, the purified DNA could be ligated to the expression vector.

A DNA gel for the final digested PCR product was used to confirm a successful ligation (Figure 3). Based on the restriction sites for Xbal and Xhol on the ABL plasmid, the expected size of the mutated gene is interest is 974 bp. Thus, from the gel, the correct product was produced.

To confirm a successful transformation of the E. Coli CH5a cells, a screening procedure was used (figure 4). The transformation was successful because both the single mutant transformation and the YOPH/mutant transformation were able to grow on the Kan/Strep plate.

To confirm that the mutation did occur, sequencing was done on the miniprepped plasmid DNA from the transformation culture. Sequencing showed that for the forward reaction, a A to C mutation was generated at 624 BP on the WT plasmid (650 BP on the mutation reaction), indicating a successful site-directed mutagenesis experiment. The DNA around the mutation had high fidelity to the original plasmid, showing that only the site of interest was mutated. Similarly, the reverse reaction was also successful. At site 624 on the original plasmid (site 883 on the reverse reaction), there was a A to C mutation, indicating that the mutation was successful for the reverse reaction as well. The sites around the mutation were conserved, showing that only the site of interest was mutated.

To determine the concentration of the purified wild type and mutant protein, a Bradford assay was used. From the standard curve, the concentration was found and used to standardize concentrations used for the SDS-PAGE analysis and the enzyme activity assay.

To confirm that the protein was successfully made and purified, a SDS-Page gel was used and stained with Coomassie blue. As expected, a band at about 33 kDa appeared in the post-induction, elution, and concentrated protein lanes, verifying by size that the correct protein was made and was purified (Figure 6). Likewise, the same 33 kDa band appeared the mutant protein as well, indicating that the H396P mutation in the Abl kinase was also successfully induced and purified (Figure 7). In both gels, there is a band at above 50 kDa, which could possibly be purified protein dimerizing.

To quantify Gleevec resistance in the H396P mutation, the specific activity of mutant and wild type enzymes were compared with and without Gleevec (Figure 8). From the raw data, the percent inhibition of the wild type was 104.5% and the percent inhibition of the mutant was 92.6%, indicating that the H396P mutation conferred some amount of Gleevec resistance.

A possible mechanism behind the inhibition of Gleevec is that the H396P mutation occurred on the A-loop region of the kinase. Mutations in the activation could bias the kinase towards the active conformation, preventing Gleevec from binding to the kinase.[4]

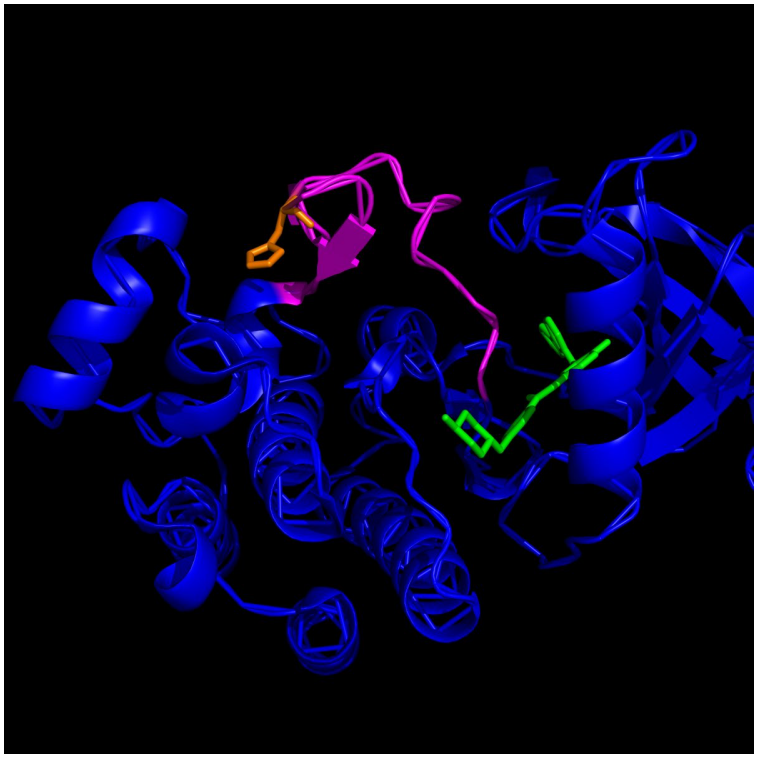
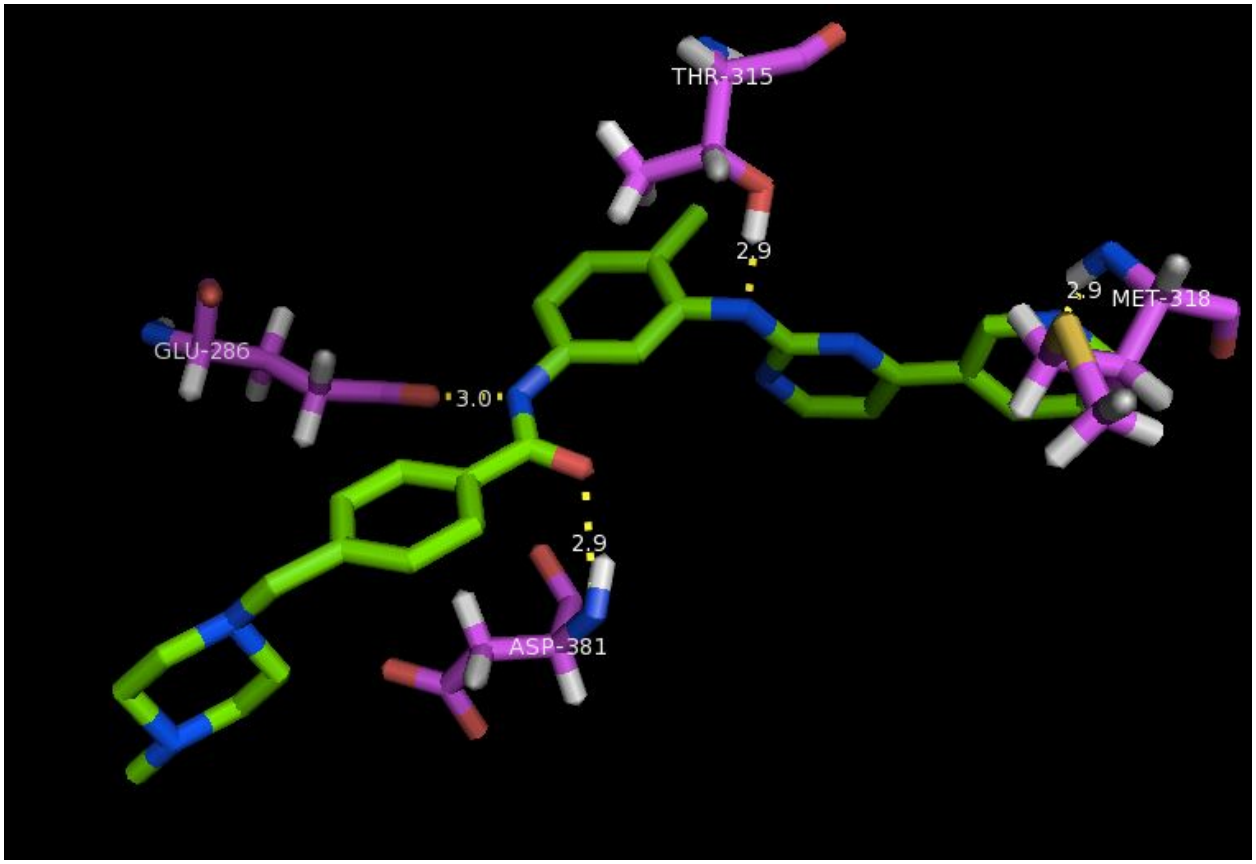


Figure 9: Abl-Kinase. The activation loop is highlighted in purple, the site of mutation is orange, and Gleevec is green. PBD: 1IEP

An alternative mechanism for Gleevec resistance in Abl-kinase is that the mutations in the activation loop extend the activation loop out, which prevents gleevec from binding.[5]

CONCLUSIONS

This experiment set out to quantify the relative kinetic activity of the wild type and H396P mutant forms of the Abelson kinase protein. While both carcinogenic, the mutated form was theorized to have increased resistance to the ATP binding site inhibitor imitinib. Through site directed mutagenesis and kinetic assays, the H396P mutant was found to have a lower percent of inhibition, 92.6%, compared to the wild type Abl protein at 104.5% inhibition. A possible explanation of this is that the H396P mutation is in the A-loop, which determines if Abl-kinase is in its active conformation or its inactive conformation.[6] The mutation may prevent the Abl-kinase from assuming its inactive conformation, preventing Gleevec from binding to the protein.



REFERENCES

1. Bartram, C., de Klein, A., Hagemeijer, A., van Agthoven, T., van Kessel, A., Bootsma, D., Grosveld, G., Ferguson-Smith, M., Davies, T., Stone, M., Heisterkamp, N., Stephenson, J., and Groffen, J. (1983) Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 306, 277-280

2. Shah, Neil P., et al. "Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia." *Cancer cell* 2.2 (2002): 117-125.

3. Young, Matthew A., et al. "Structure of the kinase domain of an imatinib-resistant Abl mutant in complex with the Aurora kinase inhibitor VX-680."*Cancer research* 66.2 (2006): 1007-1014.

4.Hantschel, Oliver, and Giulio Superti-Furga. "Mechanisms of Activation of Abl Family Kinases." *Abl Family Kinases in Development and Disease*. Springer New York, 2006. 1-10.

5.Von Bubnoff, N., C. Peschel, and J. Duyster. "Resistance of Philadelphia-chromosome positive leukemia towards the kinase inhibitor imatinib (STI571, Glivec): a targeted oncoprotein strikes back." *Leukemia* 17.5 (2003): 829-838.

6. Von Bubnoff, Nikolas, et al. "BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study." *The Lancet* 359.9305 (2002): 487-491.

Not used

3. Greggio, E., Zambrano, I., Kaganovich, A., Beilina, A., Taymans, J., Daniels, V., Lewis, P., Jain, S., Ding, J., Syed, A., Thomas, K., Baekelandt, V., and Cookson, M. (2008) The Parkinson Disease-associated Leucine-rich Repeat Kinase 2 (LRRK2) Is a Dimer That Undergoes Intramolecular Autophosphorylation. *Journal of Biological Chemistry* 283, 16906-16914

4. Brasher, B., and Van Etten, R. (2000) c-Abl Has High Intrinsic Tyrosine Kinase Activity That Is Stimulated by Mutation of the Src Homology 3 Domain and by Autophosphorylation at Two Distinct Regulatory Tyrosines. *Journal of Biological Chemistry* 275, 35631-35637

5. Wu, Z. (2011) Phosphatase-Coupled Universal Kinase Assay and Kinetics for First-Order-Rate Coupling Reaction. *PLoS ONE* 6, e23172

6. Taylor, E., Fortune, J., and Drennan, C. (2010) A research-inspired laboratory sequence investigating acquired drug resistance. *Biochemistry and Molecular Biology Education* 38, 247-252

7. Prof. C. Drennan & Dr. E. Taylor, 5.36 - *Biochemistry Laboratory*, Spring 2009. (Massachusetts Institure of Technology: MIT OpenCourseWare), http://ocw.mit.edu License: Creative Commons BY-NC-SA

8. Bartram, C., de Klein, A., Hagemeijer, A., van Agthoven, T., van Kessel, A., Bootsma, D., Grosveld, G., Ferguson-Smith, M., Davies, T., Stone, M., Heisterkamp, N., Stephenson, J., and Groffen, J. (1983) Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 306, 277-280

9. Schindler, T. (2000) Structural Mechanism for STI-571 Inhibition of Abelson Tyrosine Kinase. *Science* 289, 1938-1942

10. Apperley, J. (2007) Part I: Mechanisms of resistance to imatinib in chronic myeloid leukaemia. *The Lancet Oncology* 8, 1018-1029