Characterizing the role of APE1 inhibitor by Mutation of its Covalent Warhead binding site

Noah King, Biochemistry Lab, Organic Chemistry Labs, Lyons Research Group

Simpson College

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Introduction

Cancer is not the leading cause of death in the United States, but it remains one of the most difficult to control. Unlike many chronic diseases, cancer is often not preventable, rarely detected early, and affects individuals across all demographics, regardless of age or lifestyle. Although survival rates have improved, late-stage diagnoses are still common and significantly harder and more expensive to treat. One reason for chemotherapy failure is the ability of cancer cells to survive and recover through DNA repair mechanisms, allowing resistant mutations to emerge. One such repair mechanism is the Base Excision Repair (BER) pathway, which includes the enzyme APE1. APE1 plays a critical role by cleaving the DNA backbone at abasic sites, enabling downstream repair processes. Inhibiting APE1 has been shown to sensitize cells to chemotherapy by preventing repair of toxic DNA lesions, as discussed in Xue and Demple's 2022 study on APE1 knockout and inhibition in DNA repair and gene regulation[1]. To inhibit APE1, a newer strategy of a covalent warhead has been theorized instead of more traditional inhibitors just sitting in the active site. The covalent warhead creates specificity and permanently removes the protein from it's duties. The inhibitor designed requires a Cys residue, which is located at the 57 spot in APE1. This is close enough to interfere with DNA getting into the active site. Previously, inhibition has shown to work. To ensure site specificity, a mutant without the cysteine should be produced to ensure that the inhibition is working as expected. To test this inhibition strategy, a recombinant APE1 protein was expressed in E. coli with a targeted mutation at Cys57. The use of E. coli allows for high-yield, and simple

rapid extraction, making it far more practical than isolating mutated proteins from human cells.

Methods

The original Plasmid of APE1 contained instructions for a Cysteine at position 57, which is the residue for the Covalent Warhead Inhibitor. The primary goal is to mutate APE1 Cysteine57 to Alanine. This mutation serves as a negative control, ensuring that the loss of the reactive sulfur group does not affect activity, thereby confirming that the APE1 inhibitor functions as intended. On Cysteine, the reactive group in Cysteine is Sulfur. Alanine is the most similar amino acid to Cysteine while removing only the Sulfur group. This is important, as larger modifications could've caused larger issues with protein function. Originally, Cys57 had the DNA Codon Sequence TGT. This needs to be mutated with the least number of base changes, so the best way to get to Alanine was GCT. The TG was mutated to a CG, which effectively removes the reactive group Sulfur.

The original unmutated plasmid was provided in bacterial DNA. To isolate it, miniprep extraction was utilized. The bacteria was lysed, exposing the DNA. The DNA was bound and centrifuged utilizing a silica spin column and then washed. 30 µL of water was utilized to elute the DNA, still in Plasmid form. Miniprep averaged 50ng/µL.

Mutagenesis PCR was utilized to make the Cys57 Mutation. A forward and reverse primers hybridize to the plasmid to make copies. The forward primer contains the mutation, which will mutate the copied plasmids. The mutation was located in the center of the forward primer. The primers were designed utilizing NEBaseChanger online tool[1]. The

tool was provided with the mutation and plasmid sequence, and it generated the forward and reverse primers necessary. The primer sequences were GGAGACTAAGGCTTCCGAGAATAAGTTG for the forward, and TGCAGACATAAAATATCTGG for the reverse. For this Mutagenesis PCR, 25 ng of Plasmid template was incorporated. There was 1 µL of both forward and reverse primer. 25 cycles of PCR were run. Initial Denaturation occurred at 98° C for 30 seconds. For each cycle, Denaturation occurred at 98° C for 10 seconds, Annealing occurred at 56° C for 30 seconds, Extension occurred at 72° C for 210 seconds. The PCR has a capability of 1000 base pairs per 30 seconds, and our mutation was 7 thousand base pairs, generating a long extension time. A final extension after all cycles was done at 72° C for another 120 seconds, it was then held at 4° C after completed.

Currently, there are large amounts of a mutated strand of DNA, along with small amounts of the original unmutated strand. A KLD mix is utilized to prepare PCR DNA for transformation. PCR outputs unphosphorylated DNA, which will not form a plasmid. The KL in KLD, Kinase and Ligase, combine work to phosphorylate the DNA and immediately ligate it to form a plasmid. The D in KLD, Dpn1 is utilized next. PCR creates methylated DNA, the Dpn1 enzyme digests non methylated DNA, in this case, the original unmutated strands. Next, the Plasmids are ready to be transformed back into E. coli. The DH5Alpha strain of E. coli was utilized. The Plasmid included an Ampicillin resistance gene; thus Ampicillin was utilized as the antibiotic. It was placed in SOC media for nutrient growth and then incubated at 37° C for 60 minutes while shaking. 100 µL of plasmid was plated on Ampicillin plates and then incubated overnight at 37° C.

The miniprep from E. coli to DNA was done with the same methodology as originally stated above.

The Sanger Sequencing was sent off to a lab to be done. The lab was provided with 100ng of plasmid and 5μL of primer. Sanger Sequencing has a resolution of a few hundred base pairs. Sanger Sequencing uses fluorescent dideoxynucleotides(ddNTPs) to obtain the sequence of the Plasmid. PCR is utilized, with a very small amount of ddNTPs in there, with plenty of other nucleotides. There is a small chance a ddNTP will be incorporated instead of a regular nucleotide. This molecule will fit, but it will not continue the reaction. The net output is several hundred different lengths of DNA. These are sorted based on length, and knowing which color is fluoresced by each base pair, the sequence can be obtained. The primer sequence GGTATCACGTTAGGGATGGATG was utilized for this PCR reaction.

The successfully mutated plasmid was transformed again into BL21 E. Coli. This strain contains a T7 promoter in order to control transcription of the recombinant protein. The transcription was performed with the same methodology as reported above, with a shorter heat shock time of 20 seconds.

To express the protein in E. Coli, IPTG was utilized. IPTG is a lactose analog, which controls the lactose promoter. The transcription of T7 RNA Polymerase is controlled by the lactose promoter, so addition of IPTG produces T7 RNA Polymerase, allowing the protein to be expressed.

A colony was selected from the plate and combined with 3mL LB media with Ampicillin. This was incubated overnight at 20C while shaking. 1 mL of this culture was combined with

30mL LB and incubated for 2.5 hours at 37C while shaking. At OD600 0.4-.08, the protein expression was induced by addition of 1mM IPTG. This was incubated for 90 minutes at 37C.

1 mL of culture was taken from the plate and spun at 16,000 RPM for one minute to pellet the cells, with the supernatant discarded.

An SDS-Page was run to confirm proper protein expression. To do this, the concentrations were determined through a Bradford assay. The Coomassie stain is a dye that changes color from red to blue when binding to a protein. 200 uL of sample was added to 2.0 mL of the Coomassie stain. This was left for 5 minutes to allow the dye to bind to the protein. Next, the absorbance at 595nm was measured. A standard curve with known protein concentrations was utilized. With a linear regression, a relationship between protein concentration and absorbance can be obtained for the spectrometer. Utilizing the previously obtained absorbance, a proper protein concentration can be obtained, which is necessary to properly dose the amount of protein for the SDS-Page.

Utilizing this protein concentration data, 100µg was combined with the SDS Loading Dye up to a total volume of 50µL. This solution was placed in boiling water for five minutes to denature the proteins. 10µL of the sample was placed in the SDS-Page well, with electrophoresis being ran at 200V for 30 minutes. The gel was washed with water, then stained with the Coomassie stain while shaking for one hour. The gel was destained overnight with 0.1M acetic acid and then analyzed to determine if protein is present at the expected size of 10kDa.

With confirmation of recombinant protein presence, the next goal was to remove all other proteins present to fully purify. The BL21 strain E. Coli cells were lysed with 1.5mL of BugBuster Lysis Cocktail. This was then incubated for 20 minutes at room temperature on a shaker, and then centrifuged at 16,000xg for 20 minutes at 4c.

Our APE1 recombinant protein contains a Maltose Binding Protein(MBP) affinity tag. Utilizing small beads coated with amylose sugar binding to the MBP-APE1 complex , other proteins can be removed. 15mL of wash buffer was added along with 1.5mL of the amylose beads. After incubating on a shaker for 60 minutes at 4C, it was centrifuged at 3500 RPM for 3 minutes at 4C.

5 mL of wash buffer was added, and mixed gently then centrifuged at 3500 RPM for 3 minutes at 4C, removing the supernatant afterwards. This was repeated three times to ensure all other proteins were washed away. Remaining, is just the amylose beads bound to the MBP bound to the APE1. To remove the MBP and beads, an Xa Protease factor was utilized. The beads were suspended in 10mL of wash buffer, combined with 2µL of Factor Xa Protease, and then incubated overnight while mixing at 4C. This cleaves the protein from the MBP.

At this point, four different samples were taken for the final SDS-Page analysis, totally bound beads after incubation, the supernatant flow through unbound proteins, the beads after washing before cleavage, and the beads after cleavage.

P-aminobenzamidine coated agarose beads have affinity for Xa Protease. These can be bound to the Xa Protease, and then pelleted out to remove. 0.5mL of these beads was

added to 15mL Factor Xa Protease and mutant APE1 solution. This was incubated at room temperature for 30 minutes, and then spun at 4000xg for 5 minutes. The flow through supernatant, now containing only the mutant APE1, was saved. The solution was spun through a 3kDa membrane to capture protein and expel water, concentrating the enzyme. This utilized a Amicon Ultra-15 Spin Filter, with 15mL sample volume, centrifuged at 4000xg for 30 minutes.

To measure protein concentration of the purified, a simpler, nanodrop assay was utilized. This measures absorbance at 295nm, a small sample of the protein was placed on the nanodrop spectrometer, to obtain protein concentration. The SDS-Page was ran again with only the purified protein as described in methods previously.

A kinetics assay was utilized to determine repair activity in vitro. A specifically made strand of partially cut DNA was utilized to perform this. A quencher and fluorescent dye were placed on opposite sides of the DNA strand. While the DNA remains attached, the quencher quenches the dye, however if APE1 were to cut the DNA strand the dye and quencher would no longer be close, and we'd see fluorescence. The ends of the DNA were hair pinned in order to specify only one possible site for APE1 activity, as it has been shown to have some ability to cut at the 3' and 5' ends. The strand can be seen as followed, with FAM indicating the DYE, Ab indicating the Abasic site the APE1 is active in, and IABlk the quencher utilized. /FAM/ TT CCTA AC TCCT GT TAGG AA GC CTA GTA CAT /Ab/ TGT CAA CC GGAT AC TCCT GT ATCC GG TTG ACA C ATG TAC TAG GC /IABlk/.

An Eppendorf MasterCycler Realplex was utilized to run this assay with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The reaction recipe required 100 µL total volume, with 10 µM of DNA, 0.1 nM of APE1, 1% DMSO, and 10 mM of Inhibitor. Three different inhibitor types, Positive, Negative, None, were combined with the Wild Type and Mutant proteins. In each well, the DNA was placed as a drop on the side of the plate, then centrifuged to start all reactions at the same time. The plate was placed in the Eppendorf MasterCycler Realplex to read the assay results. Final calculations utilized the relative reaction rates, where the no inhibitor is used as the benchmark. This formula is (Inhibitor/No Inhibitor) and theoretically should be between 0 and 1. The instrument took excitation values along the way and read until the 10 minute mark.

A chemotherapy assay was ran in combination with MTT and HeLa cervical cancer cells. MMS is a methylating agent attaching a carbon wherever it can, destroying cells. It's commonly used as a chemotherapy drug. The MTT assay utilizes a yellow dye that's metabolized by living cells and turns into purple. An Assay can be read measuring the presence of the purple at 570, and more purple meaning more cells alive. This allows us the ability to measure what percentage of the cells died, again using the relative survival similar to the Kinetics Assay. A 96 well plate was used, with every sample in triplicate to reduce error. Each well included 100 µL of cell growth media, approximately 5000 cells, and a variable amount of inhibitor and MMS. Three concentrations of inhibitor, 0 , 10 µM, and 100 µM, along with three dosage levels of MMS predicided based on literature suggesting what can kill around half. A good range is necessary, if all are too high, they all die, and if all are too low, none of them die. Neither of these would allow the ability to

measure how many of them die over inhibitor concentrations based on chemotherapy concentration. 100 µm, 400 µM, 1200 µM were the three variable levels of MMS used.

Results

The Sanger Sequencing showed the mutation expected. The Sanger Sequence can be shown below in Figure 1. In Blue, the highlighted bases are where the mutation occurred. This GCT describes an Alanine, which is what was expected as a mutation. This shows the pairs T and G were successfully changed to C and G to change the Cysteine to Alanine.

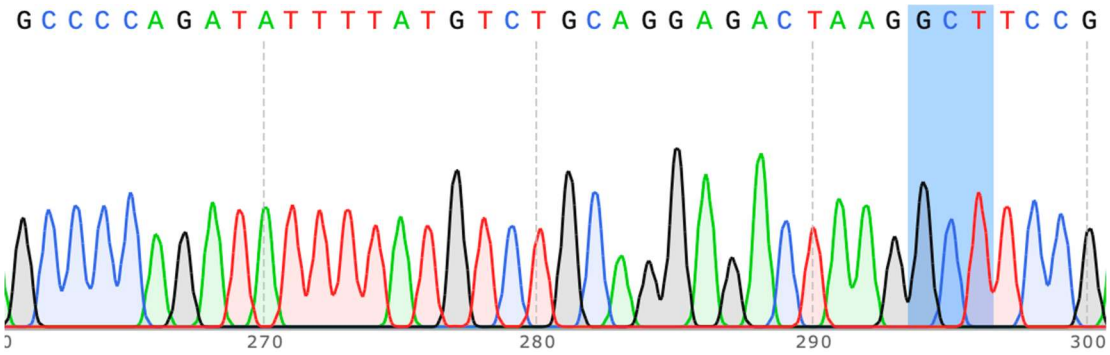

Figure 1: The Sanger Sequence of the Mutation Plot

The x axis is sorted by length of base pair. For each x axis integer, the color of the peak represents the base pair in that location. The three bases highlighted in the blue section are the mutated Codon. The sequence showed GCT, which was previously TGT. This shows a successful mutation to GCT thus changing Cysteine57 change to Alanine

The results from the SDS-Page 1, analyzing the unpurified but expressed APE1-mGold-MBP complex can be seen in Figure 2. The expected size for this fusion protein is 107.1 kDa, a faint line can be seen below this, at a relatively close size, albeit smaller than expected. The main expectation is that this promoter should've expressed this protein at a much higher level than other natural E. Coli proteins, which is shown with a clear dark line. It can be noted that this faint line

occurred at around ~65kDa. This is the size of the mGold-APE1 fusion protein without MBP. It's possible that an unknown Protease natural in E. Coli was able to cleave the MBP off.

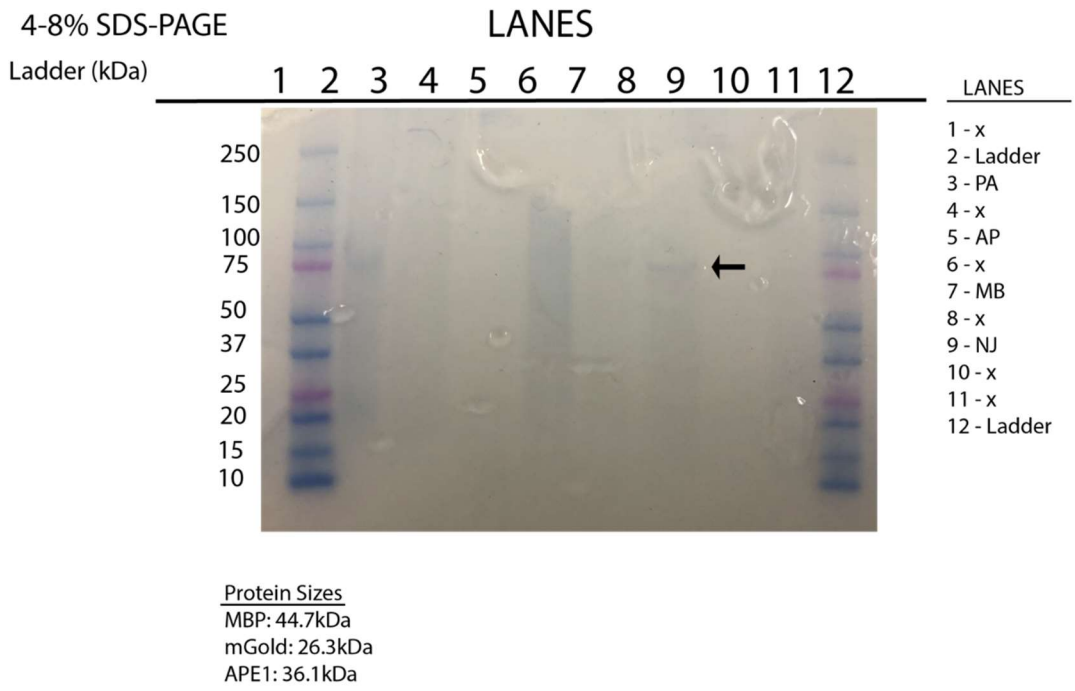

Figure 2: SDS-PAGE Analysis of Fusion Protein Expression.

A black arrow can be seen pointing to a faint darker line, showing there was a high expression for a protein, at a size reasonably close to the expected. All two lettered lanes are the same sample from different groups.

When SDS-Page 2 was ran, the protein complex was purified to APE1-mGold, with an expected size of 62.4kDa. In Figure 3 below, the SDS-Page can be seen, while there are several different samples doing multiple things, it's important to note that many of them have a distinct line appearing to be very close to that 62.4kDa value. This shows that APE-mGold fusion protein is clearly present at multiple stages as expected. It can be seen in lane 6, from a sample taken before the beads were cut off. It can be seen in high concentration on the supernatant, where we lost

some, along with all other native E. Coli proteins.

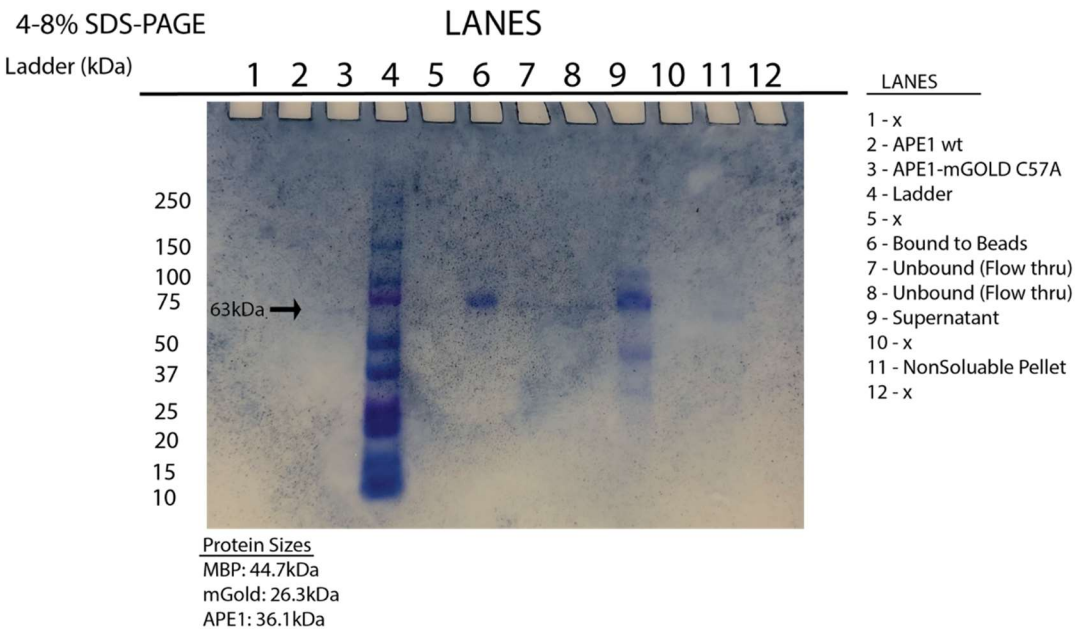

Figure 3: SDS-PAGE Analysis of SDS-PAGE Analysis of Fusion Protein Purification

Post purification, the mGold-APE1 fusion protein can be seen expressed at 62.4kDa. It can primarily be seen in the Bound to bead format, where the final purified product was. Additionally, it can be seen in the Supernatant lane. This suggests that some amount of protein was still lost during purification. ‘

The inhibitors previously characterized are believed to bind covalently to the Sulfur group on Cys57, the mutated fusion protein doesn’t include Cys57, which suggests that the mutant protein should show no loss in activity due to inhibition. The assay can be seen in Figure 4 below. The kinetics assay showed a moderate success. It showed that at lower inhibition concentrations, the APE1 rate was not affected. However, at 1000mM, the mutant protein showed large decreases in activity, likely due to pure saturation of the entire protein. Future work would include data points at 100mM and possibly elsewhere, to see if this is a larger problem starting at 30-40 mM, or only exists near 1000mM.

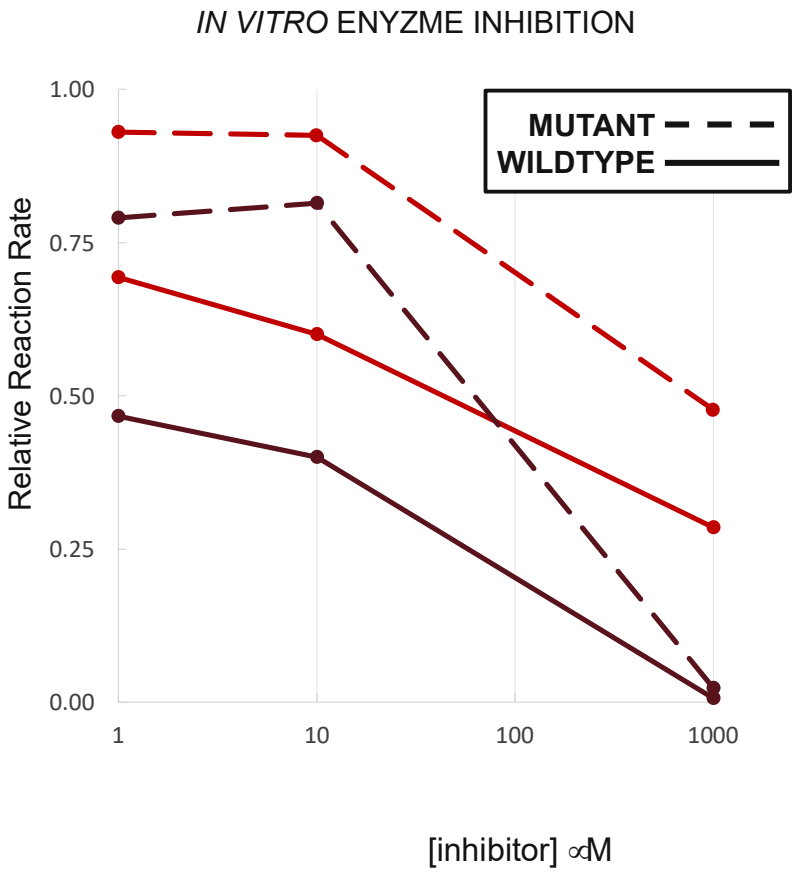

Figure 4: In Virto APE1 Kinetics Inhibition assay using a fluorescent DNA to determine APE1 base repair activity. The positive inhibitor is in red, with carboxylic acid. The negative inhibitor in brown, which is only one carbocyclic short of the positive inhibitor. On the X axis, we show the concentration of inhibitor, either 1,10, or 1000 mM. The Y axis shows the relative readout of fluorescence after 30 minutes. The mutant APE1 can be seen to not be affected by the inhibitor between the 1 and 10 mM, while the wildtype shows clearer inhibition. At 1000mM, everything shows inhibition, possibly due to the fact there is so much inhibitor that it will interfere with the mutant without a binding site.

In the HeLa Cell MTT Assay, it was hypothesized that the inhibitor combined with MMS would increase cell death by preventing any DNA repair. The results can be seen below in Figure 5. Part a shows that as expected, more chemotherapy kills more cells. Part b shows that the inhibitor is non toxic at reasonable concentrations, an important future step for medicinal usage. Part c shows the dual therapy results. There are 9 different data

points, three dosages of inhibitor each with three dosages of MMS. The low and medium strength dosages of MMS show little movement across large movement of inhibitor. The large dosage of MMS shows a dose dependence where cell survival significantly drops as inhibition went from 1 to 10 mM. This matches what was expected by the assay.

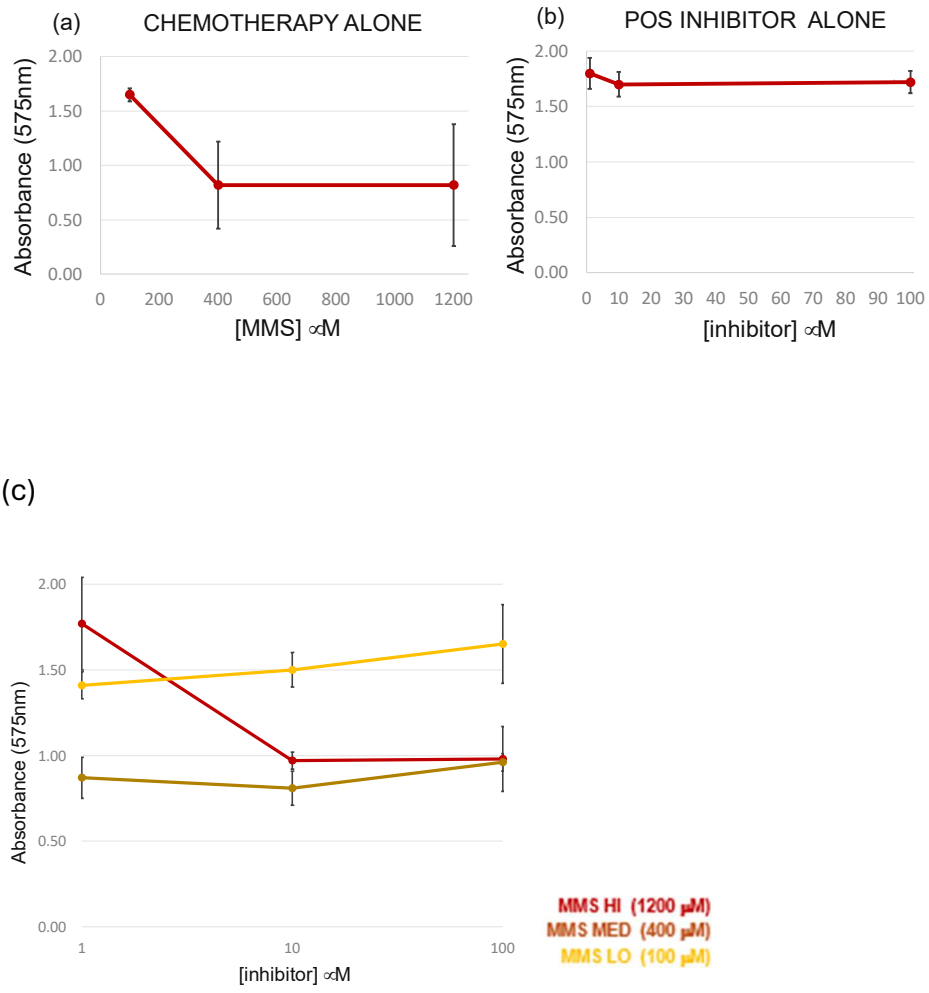

Figure 5: Chemotherapy assay using HeLa cervical cancer cells. Cells were dosed with varrying amounts of MMS chemotherapy, and given various amounts of positive inhibitor. On the Y axis, values are correlated with absorbance at 575 nm, which is the Purple that has been metabolized by cells, suggesting that higher values mean cell survival.

A- No APE1 or Inhibitor. Shows more cells die with more chemotherapy.

B- Inhibitor only. Shows inhibitor doesn't have affect on cell death, not toxic.

C- Dual therapy. Combination of MMS and Inhibitor at 3 doses each. No significant difference can be seen in the low or medium dosages of MMS. The high dosage can be seen to have some dose dependance.

Discussion

The mutant protein was shown to have successfully been expressed. Sanger sequencing showed the correct mutation of the plasmid, with the SDS-PAGE showing a blob at the expected size. The in vitro kinetics assay showed that the mutated protein was functional and worked as expected.

The assays ran to determine site specificity of the warhead inhibitor were a mixed bag. The kinetics assay did show inhibition at very high concentrations of inhibitor, but this was hypothesized to be non-covalent due to sheer amounts. This can likely be seen in future works to confirm the hypothesis.

Though not fully conclusive, the MTT Assay showed that at high MMS concentrations, there was a level of dose dependance. While it only worked here, this should still be considered a success. The entire goal medicinally is to inhibit APE1 to let chemotherapy kill more cancer cells, and that's exactly what was suggested by the high dosage data.

Future work would address the effect of this covalent inhibitor on something traditionally done non covalently. Covalent inhibitors don't float around just filling up active sites, they bind. In real physiological conditions, unlike our *E. coli* expression, proteins being made constantly. This means that proteins made after the original dosage of MMS are

still functional. A study to consider what impact a second—or possibly even more dosages would have on the inhibition success.

References

1. Xue Z, Demple B. Knockout and Inhibition of Ape1: Roles of Ape1 in Base Excision DNA Repair and Modulation of Gene Expression. Antioxidants (Basel). 2022 Sep 15;11(9):1817.