Biochemical Characterization of DNA Repair Enzyme Inhibitors – Molecules with Possible Applications to Improving Cancer Treatment

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Abstract

Polynucleotide kinase-phosphatase (PNKP) is a critical DNA repair enzyme responsible for processing DNA damage caused by radiation. A loss of function in this enzyme results in increased cell susceptibility to radiation-induced DNA damage and subsequent cell death. As radiation therapy is commonly used in cancer treatment, targeted inhibition of PNKP has been proposed to increase the effectiveness of radiation therapy at lower doses. We characterized two previously identified PNKP inhibitors, Candesartan and S4, by their effects on the kinase activity, kinase substrate binding, and phosphatase substrate binding of *Caenorhabditis elegans* and mouse PNKP. The binding assays were conducted using electromobility shift assays (EMSA), while *in vitro* kinase assays were performed to assess kinase activity. Both inhibitors had an effect on both domains of PNKP, but were more effective at displacing the phosphatase substrate than the kinase substrate. Comparisons of kinase activity inhibition by new and older samples of inhibitors showed that both Candesartan and S4 degrade over a span of 3-4 months and lose their effectiveness. These inhibitors show promise for applications in cancer treatment, but further research is needed.

Introduction

Polynucleotide kinase phosphatase (PNKP) is a DNA repair enzyme responsible for processing DNA damaged by radiation.[1] The mammalian enzyme has three domains: a kinase domain that processes 5’ hydroxyl ends, a phosphatase domain that processes 3’ phosphate ends, and an FHA domain that targets the protein to damaged DNA.[1] These ends, which are commonly seen in radiation-induced DNA damage, cannot be ligated by DNA ligase, and must be fixed for DNA repair to proceed.[1]

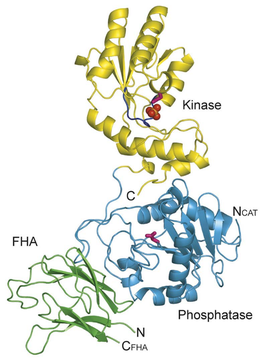

Figure 1: Structure of mouse PNKP. Image taken from Bernstein et al. (2005)

While PNKP has both kinase and phosphatase functionality, the phosphatase domain is generally more active than the kinase.[2] Previous studies have identified two inhibitors of the phosphatase domain, Candesartan and S4. Both compounds were identified to interfere with human PNKP phosphatase substrate binding during a screen of a chemical library (M. Glover, unpublished). Candesartan-Cilexetil is currently in use as an anti-hypertensive drug.

Targeted inhibition of PNKP has been considered as a potential method to improve radiation therapy for cancer.[3] Previous studies have shown that inhibition of PNKP sensitizes cancer cells to radiation therapy, allowing lower doses of radiation to be used.[3]

The aim of this study was to compare the effect of the two inhibitors on *C. elegans* and mouse PNKP to characterize the similarities and differences in their interactions. Mouse PNKP is a close homolog of human PNKP (over 90% sequence similarity), while *C. elegans* PNKP shares approximately 40% sequence identity. The effect of the inhibitors on the phosphatase activity of both enzymes has been assessed in previous studies and found to be effective (N. Steed, unpublished). This study analyzed the effect of these inhibitors on kinase activity, as well as kinase and phosphatase substrate binding. The biochemical information obtained from these studies will be used to guide modelling of inhibitor binding to human PNKP.

Methods

Protein Quantification:

Purified *C. elegans* PNKP (CePNKP) and mouse PNKP (mPNKP) in were quantified by measuring absorbance at 280 nm. Wild-type proteins were used for the kinase assay and kinase EMSA experiments. Phosphatase-inactivated mutant PNKP was used for the phosphatase EMSAs (D57A PNKP and D170A mPNKP).

Kinase Assays:

All kinase assays were conducted using a denaturing polyacrylamide gel. The optimal protein concentration to use with the inhibitors was first determined by testing a range of protein concentrations in serial dilution. The enzyme was reacted with the kinase DNA substrate KSN1, ATP, kinase assay buffer (KAB), water and DMSO on ice for 30 minutes. Samples were boiled at 95°C for 5 minutes, then were loaded twice into the gel, 90 minutes apart. Gels were imaged by detecting the fluorescein probe on KSN1 using the UVP gel imager. The optimal protein concentration was determined to be 5.5 uM, where all reactant had been converted to product. This concentration was used for subsequent experiments with either Candesartan (Cs) or S4. To assess the effects of the inhibitors on CePNKP and mPNKP, the enzyme was preincubated with a range of inhibitor concentrations or DMSO for 10 minutes, then the samples were prepared and run as described above.

Phosphatase EMSAs:

The phosphatase EMSAs were conducted as described for the kinase EMSAs, except using the mutant PNKP and the phosphatase DNA substrate F1. The optimal protein concentration was determined to be 84.25 uM for both enzymes. The DNA was detected using the fluorescein probe on F1, and protein was detected by subsequent staining with Coomassie Blue (BioRad).

Kinase EMSAs:

EMSA experiments were conducted on native polyacrylamide gels. The optimal protein concentration where all free DNA was bound was first determined by testing a range of protein concentrations with 0.2 uM KHP2 DNA substrate. The samples were incubated for 10 minutes at room temperature and 10 minutes on ice, then run on a native gel for one hour at 100V. The DNA was detected by SYBR-Safe staining. Protein was detected by staining with Coomassie Blue. The optimal protein concentration was determined to be 142 uM for CePNKP and 22 uM for mPNKP. The inhibitors were tested as described but replacing DMSO with a range of inhibitor concentrations.

Results

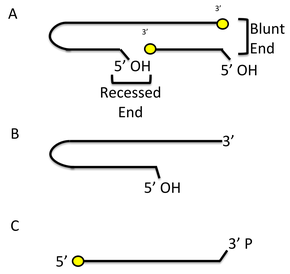

Figure 2: DNA substrates used in all experiments. (A) Kinase assay substrate KSN1. (B) Kinase EMSA substrate KHP2. (C) Phosphatase EMSA substrate F1. Fluorescein probes are shown by yellow circles

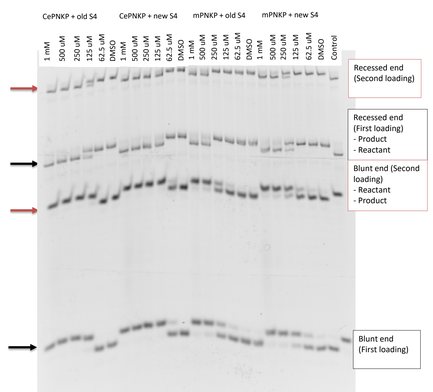

Figure 3: Kinase activity assay comparing the effectiveness of 5-month-old S4 and new S4 against CePNKP and mPNKP. The control contains no protein. Samples were loaded twice on the gel, 90 minutes apart, to achieve optimal separation of the bands and to obtain duplicate data. The disappearance of the product band and the reappearance of the reactant band indicates inhibition of kinase activity. Inhibition of CePNKP kinase activity occurred starting at 125 uM of both old and new S4 for the recessed end of KSN1, and 125uM of old S4 and 62.5 uM of new S4 for the blunt end. Inhibition of mPNKP kinase activity occurred starting at 500 uM of old S4 and at 250 uM of new S4 for the recessed end, and at 250 uM of both old and new S4 for the blunt end.

Results

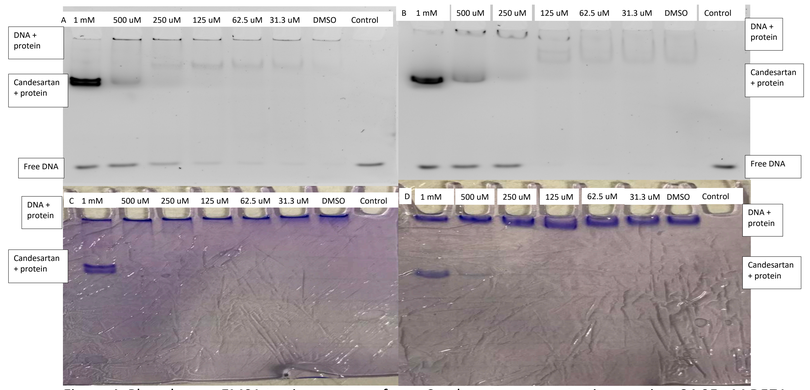

Figure 4: Phosphatase EMSA testing a range of new Candesartan concentrations against 84.25 uM D57A CePNKP (A,C) and 84.25 uM D170A mPNKP (B,D). The control contains no protein. (A) Substrate F1 with CePNKP. (B) Substrate F1 with mPNKP. (C) D57A CePNKP stained with Coomassie. (D) D170A mPNKP stained with Coomassie. The band corresponding to free DNA reappears due to dissociation of the PNKP-DNA complex, which begins at 250 uM of Candesartan for both enzymes. The change is more abrupt for mPNKP. For both CePNKP and mPNKP, an additional smaller shift is seen with higher Candesartan concentrations, potentially corresponding to the fluorescent signal of Candesartan bound to the protein. Candesartan has a characteristic UV absorbance maximum at 254 nm.[4]

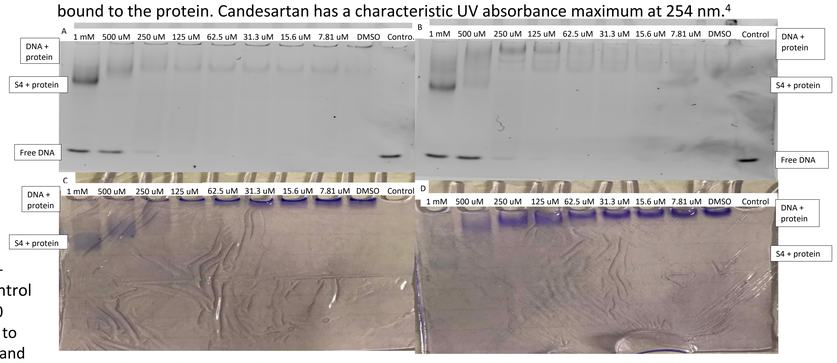

Figure 5: Phosphatase EMSA testing a range of new S4 concentrations against 84.25 uM D57A CePNKP (A,C) and 84.25 uM D170A mPNKP (A,D). The control contains no protein. (A) Substrate F1 with CePNKP. (B) Substrate F1 with mPNKP. (C) D57A CePNKP stained with Coomassie. (D) D170A mPNKP stained with Coomassie. The reappearance of free DNA is seen starting at 500 uM of S4 for both CePNKP and mPNKP. Additional smaller shifts are seen with 1 mM and 500 uM S4 for CePNKP, and from 1 mM to 250 uM S4 for mPNKP, potentially corresponding to S4 bound to the protein.

Results

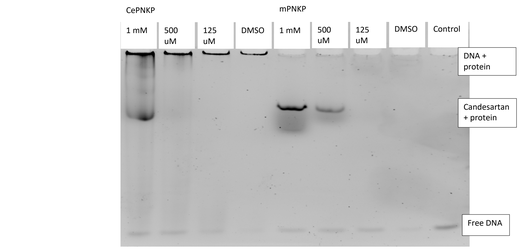

Figure 6: Kinase EMSA testing binding inhibition of new Candesartan solutions on CePNKP and mPNKP. The control contains no protein. Only partial binding inhibition was seen with both CePNKP and mPNKP at higher Candesartan concentrations, but the reappearance of the free DNA does not match the intensity of the control. The inhibitor is unable to completely displace substrate from the kinase active site.

Conclusions

- Candesartan and S4 inhibit both the kinase and phosphatase activities of CePNKP and mPNKP.
- Both inhibitors were more effective against CePNKP.
- Candesartan was more effective at inhibiting phosphatase substrate binding than S4.
- Candesartan was more effective at displacing the phosphatase substrate than the kinase substrate.
- Both Candesartan and S4 degrade over a span of 3 to 4 months when stored at -20°C and lose their effectiveness.

Future Directions:

- Identify the large, intense band seen in the EMSA experiments. It is suspected to be fluorescence of the inhibitor bound to protein, which can be confirmed by doing a control with protein and inhibitor, but no DNA.
- The band potentially corresponding to inhibitor bound to the protein also runs faster than unbound protein. Techniques such as Small Angle X-Ray Scattering (SAXS) and Circular Dichroism (CD) could be used to look for large scale conformation changes upon inhibitor binding, which could explain this finding.
- Quantitative analysis of inhibitor kinetics and interactions with PNKP to help determine the mechanism of inhibition.

References

1. Bernstein N. K., Williams R. S., Rakovszky M. L., Cui D., Green R., Karimi-Busheri F., Mani R. S., Galicia S., Koch C. A., Cass C. E., Durocher D., Weinfeld M. and Glover J. N. M. (2005) The Molecular Architecture of the Mammalian DNA Repair Enzyme, Polynucleotide Kinase. *Molecular Cell*. 17: 657-670.
2. Dobson C. J. and Allinson S. L. (2006). The phosphatase activity of mammalian polynucleotide kinase takes precedence over its kinase activity in repair of single strand breaks. *Nucleic Acids Research*. 34: 2230-2237.
3. Srivastava, P., Sarma, A., and Chaturvedi, C. M. (2018). Targeting DNA repair with PNKP inhibition sensitizes radioresistant prostate cancer cells to high LET radiation. *PLoS ONE 13*: 1-14. https://doi.org/10.1371/journal.pone.0190516
4. Pradhan, K. K., Mishra, U. S., Pattnaik, S., Panda, C. K., & Sahu, K. C. (2011). Development and validation of a stability-indicating UV spectroscopic method for candesartan in bulk and formulations. *Indian journal of pharmaceutical sciences*, *73*(6), 693.

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