NAMPT as a Potential Biomarker for Daporinad Treatment: Analyzing Protein Expression in Gene-Modified Prostate Cancer Cell Lines

Lauren Lee,[1] Adam Lee,[2] Weijie Zhang,[2] Yingbo Huang,[2] R. Stephanie Huang[2]

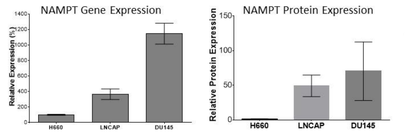
1 = University of Minnesota Undergraduate Research Opportunities Program, 2 = University of Minnesota Department of Experimental and Clinical Pharmacology, Minneapolis, MN 55455

UROP

I. Background

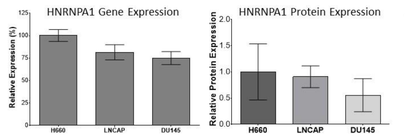
- Neuroendocrine prostate cancer (**NEPC**) is an aggressive form of late stage prostate cancer that typically arises from androgen deprivation therapy (ADT) resistance.[1]
- **Daporinad** (FK866) is an investigational drug that inhibits nicotinamide phosphoribosyltransferase (**NAMPT**), decreasing the biosynthesis of nicotinamide adenine dinucleotide (NAD^+). This alters energy reserves in metabolically active tumor cells and induces tumor cell apoptosis.[2]
- **hnRNP A1** is a heterogeneous nuclear ribonucleoprotein that was nominated along with NAMPT as a potential marker for daporinad sensitivity.[3,4]
- Daporinad was selected in our laboratory as a potential treatment for NEPC using computational dose-response modeling.[3]
- **NCI-H660** is an androgen receptor (AR) negative NEPC strain that expresses lower NAMPT levels compared to **LNCAP**, which is an androgen sensitive non-NEPC strain that expresses higher NAMPT levels. NCI-H660 displays increased sensitivity to daporinad treatment compared to LNCAP.
- **DU-145** is an androgen-independent castration-resistant PC line with high NAMPT expression and displays sensitivity to daporinad treatment similar to LNCAP.
- NAMPT and hnRNP A1 knockdown models were created for the cell lines using shRNA. NAMPT overexpression models were created using mammalian gene expression vectors obtained from Vector Builder.
- RNA was extracted from each cell line, and validation of overexpression and knockdown was quantified by RT-PCR. While this indicates knockdown/overexpression on the RNA level, it is not certain whether or not the same changes are influencing NAMPT protein levels (FIG. 1a, 1c).
- The models underwent a preliminary daporinad drug screening to test for changes in sensitivity (FIG. 2).

1a.



1b.

1c.



1d.

2.

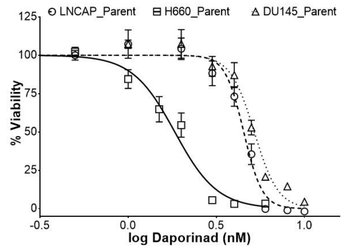


FIG. 1: Gene expression for each line was measured via RT-PCR following an RNA extraction (1a, 1c). Protein expression was measured in this experiment with western blot protein quantification (1b, 1d). The gene and protein expression levels match each other, indicating that an alteration to one (such as a gene knockdown) would strongly impact the other. **FIG. 2:** This figure depicts the results of a WST reagent viability assay following exposure to daporinad treatment. NCI-H660 is the most sensitive to treatment, and DU-145 is the most resistant to treatment.

Objective: To confirm NAMPT and hnRNP A1 knockdown/overexpression on the protein level of the created models.

Hypothesis: Western blot analysis of NAMPT-overexpression models will result in corresponding higher levels of protein synthesis, whereas NAMPT-knockdown models will result in corresponding lower levels of NAMPT protein synthesis compared to the respective control models.

II. Methodology

Cell Culture

- LNCAP/DU-145: Adherent cell model grown in RPMI 1640 media with 10% fetal bovine serum (FBS).
- NCI-H660: Suspension cell model grown in HITES media (5% FBS). All lines were cultured at 37℃ in a 5% CO_2 incubator, and treated for mycoplasma following passage 9.

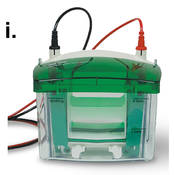
Continuing Daporinad Drug Assay

- Cell lines were seeded at approximate density of 5,000 cells per well. Daporinad was added in serial dilutions between 0.08 nM and 20 nM. Longitudinal cell growth was measured during >72 hour drug exposure time on a Cytation Live Cell Imaging System.

Western Blot

- Quantification of total protein was measured in protein lysates taken from harvested cells by a Bradford assay.
- 10-12 µg of total protein mixed with Tris-Glycine SDS Running Buffer and NUPAGE sample reducing agent in a total volume of 40 µl prior to loading on a 4-20% Mini-PROTEAN TGX Precast protein gel (i.). SeeBlue Plus2 Pre-stained Protein Standard was loaded into the edge wells. Gels ran at 100V for 1 hour.
- An Invitrogen iBlot2™ Gel Transfer Device (ii.) was used to dry-transfer gel results onto a PVDF membrane.
- Membranes were incubated with TBST buffer containing 5% Bovine Serum Albumin (BSA), then blocked with first primary antibody (Recombinant Anti-Visfatin antibody Rabbit monoclonal to Visfatin). Secondary antibody used was HRP-conjugated Goat anti-Rabbit IgG (H+L), and second primary antibody was Recombinant Anti-hnRNP A1 antibody Rabbit monoclonal to hnRNP A1.
- GAPDH Rabbit mAb was used as the loading control.
- A LiCOR™ Western blot imaging system (iii.) was used to read the membrane results.

i.



ii.

iii.

III. Results

3.

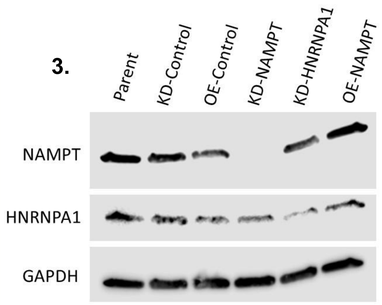
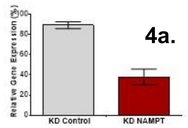
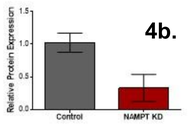


FIG. 3. Representative western blot image for DU-145 cell line. NAMPT protein appears at approximately 55 kDa, and hnRNP A1 at approximately 35 kDa. Significant NAMPT knockdown was observed compared to the knockdown control, and some NAMPT overexpression was observed. hnRNP A1 showed some knockdown compared to the control, but to a lesser extent compared to NAMPT.

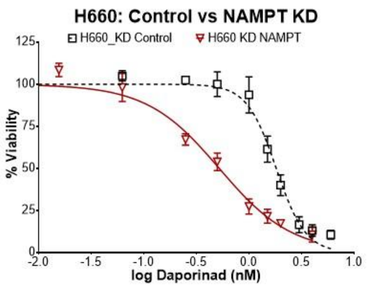
4a.



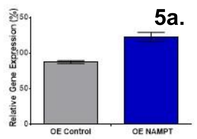
4b.



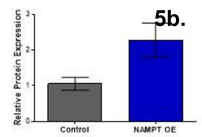
4c.



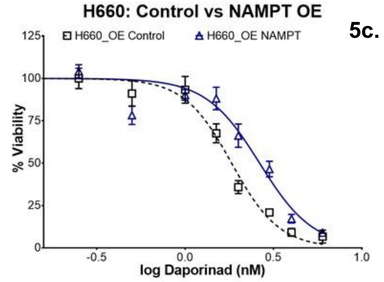
5a.



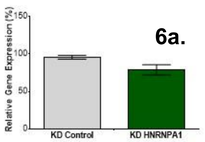
5b.



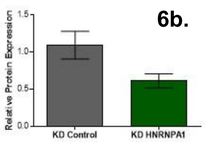
5c.



6a.



6b.



6c.

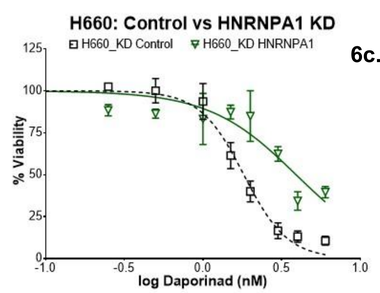


FIG. 4-6: Parts a) and b) of each figure depict the comparison between relative gene/protein expression and their respective controls in NCI-H660. To confirm actual gene knockdown and overexpression, the protein expression of that line should be changed to the same extent. Since parts a) and b) of each figure match and the protein expression roughly equals the gene expression, these models are validated. Part c) of each figure depicts the results of a WST reagent viability assay following a daporinad drug screen. For the NAMPT knockdown (4c), viability is lower over the increasing daporinad concentrations, indicating that increased sensitivity to treatment. The NAMPT overexpression (5c) and hnRNP A1 knockdown (6c) both resulted in decreased drug sensitivity.

7.

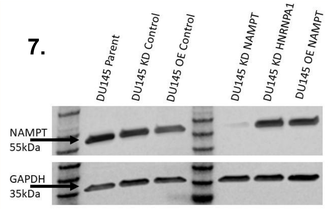


FIG. 7: Western blot results for DU-145 line after NAMPT primary antibody. GAPDH loading control presented as a consistent single line, indicating no major protein loading variation. Compared to the control, significant NAMPT protein knockdown and some protein overexpression was achieved.

8.

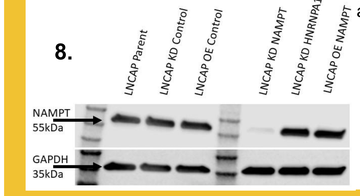


FIG. 8: Western blot results for LNCAP line after NAMPT primary antibody. Significant NAMPT protein expression knockdown was observed compared to the control. Minimal NAMPT overexpression was achieved.

IV. Conclusions

- Hypothesis was supported in the NCI-H660 line. Significant NAMPT gene knockdown corresponded with a significant NAMPT protein expression knockdown (FIG. 4a, 4b). NAMPT gene overexpression resulted in similar increased levels of expression on the protein level (FIG. 5a, 5b).
- Hypothesis was also partially supported in the DU-145 and LNCAP lines. Significant NAMPT protein knockdown was achieved (FIG. 7, 8), but overexpression appeared to a lesser extent.
- Knockdown of hnRNP A1 was also confirmed for relative protein and gene expression in the NCI-H660 line, but to a lesser extent than NAMPT.

V. Future Directions

- Additional replicates of the cell line western blots will need to be completed, with emphasis on quality overexpression images.
- Potential mechanism of daporinad could be explored.
- Sensitivity to other drugs could be tested to see effect of hnRNP A1 knockdown.

VI. Acknowledgements

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VII. References

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