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TITLE: Targeting Ligand-Dependent and Ligand-Independent Androgen Receptor Signaling in Prostate Cancer

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14. ABSTRACT

Last year, we made significant progress in testing over a hundred analogues of our lead compound targeting ligand-dependent androgen receptor signaling in Prostate Cancer. We had overcome problems with solubility, off-target effects, toxicity and formulation. However, on rigorous evaluation, we found that we could not be sure that these agents were targeting specifically the interaction between the androgen receptor and coregulators. Towards this end, we developed a novel in vivo FRET assay, that validated the activity of our compounds in blocking the Androgen receptor interaction with specific coregulators.

15. SUBJECT TERMS

Prostate cancer, androgen receptor

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Introduction

The androgen receptor (AR) is critical in the normal development and function of the prostate, as well as in prostate carcinogenesis[1]. Androgen deprivation therapy is the mainstay in treatment of advanced PCa (PCa); however, after an initial response, the disease inevitably progresses to castration-resistant PCa (CRPC)[2]. Recent evidence suggests that continued AR activation, either in a ligand-dependent (LD) or in a ligand-independent (LI) manner, is commonly associated with CRPC[1]. There is an unmet need for novel agents to target both LI and LD AR signaling in CRPC. Our overarching hypothesis is that the disruption of interactions between AR and critical cofactors by targeting structural motifs involved in protein-protein interactions (PPIs) may block both LD and LI activation of AR and represent a novel therapeutic approach for patients with CRPC.

In this grant, we had proposed to design and synthesize peptidomimetics that can more specifically disrupt LD and LI activation of AR. We then wanted to evaluate the mechanism of specific peptidomimetics in blocking AR signaling. Finally, we wanted to evaluate the utility of specific peptidomimetics in animal models and on primary PCa tissue.

In our first year, we had made significant strides in these endeavors. We have created and tested more than 23 variants of the peptidomimetics and have learned to build a better more potent peptidomimetic.

In our second year, we continued on our work from the first year and evaluated pharmacologic properties of our best compounds from year 1. We further improved the solubility of our compounds in year 2 and are finalizing a manuscript from that effort.

In the third year, we developed a novel assay to ascertain that the peptidomimetics were targeting direct interactions between the coregulators and the androgen receptor in vivo.

Body

In this grant, our overall goals were to target the androgen receptor in prostate cancer using peptidomimetics for the LxxLL and WxxLF motif using oligo-benzamide scaffolds that are highly specific for and can disrupt the AR-PELP-1 interaction.

We had had a highly productive first two years and hadmade significant strides in our work with peptidomimetics. Towards this end, we had published a critical manuscript in Nature Communications (Ravindranathan et al, Nature Communications 2013) that outlines our work with our leading D2 compound and its remarkable activities on prostate cancer cell lines. This manuscript had been well-received and garnered significant collaborations to further explore these agents. We have further worked on our peptidomimetics and refined their activities.

We then designed tris-benzamide-based molecules to introduce additional functional group from the flanking residues around the LXXLL motif to our lead D2 compound. We created over a hundred compounds to improve the activity of D2. We did find several that were more potent than D2 (year 2 report).

Our primary assay to evaluate the interaction between the AR and coregulators such as PELP1 was a co-immunoprecipitation assay, as shown below:

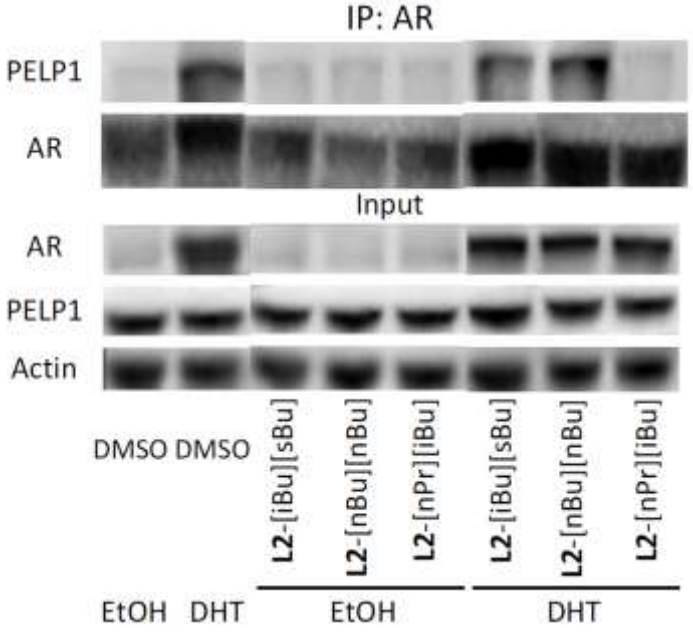

Fig. 1 Effect of bis-benzamides on the AR-PELP1 interactions. LNCaP cells were treated with bis-benzamides in the presence of 10 nM DHT. Cell lysates were then immunoprecipitated with specific antibody for AR and immunocomplexes were subjected to Western blotting with AR and PELP1 antibodies.

However, this assay showed that AR and PELP1 were in a complex and not necessarily directly bound to each other. Reviewers of our manuscript suggested that we show that the peptidomimetics were directly targeting the interaction between AR and PELP1, especially within a cellular context. Towards this end, we spent most of

year 3 developing and optimizing an in vivo FRET assay to validate that the peptidomimetics were able to block the AR-PELP1 interaction. Because of the complexity of this assay, we had to coptimize conditions for the assay and develop a novel platform for in vivo FRET. This assay is technically challenging and took us a greater part of a year to optimize.

We were the beta-users of a novel NanoBiT assay from Promega that utilizes a structural complementation-based approach to monitor protein–protein interactions within living cells. Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 1 kDa) subunits of NanoLuc Luciferase were optimized for the analysis of protein interaction dynamics. When LgBiT and SmBiT subunits are separated, the Large BiT part loses the majority of luciferase activity. However, when the direct interaction between fusion proteins on LgBiT and SmBiT occurred, the interaction promotes structural complementation between LgBiT and SmBiT and results in full luciferase activity (Fig. 2). Protein–protein interaction are then monitored in living cells following addition of the Nano-Glo Live Cell Reagent, a non-lytic detection reagent containing the cell-permeable furimazine substrate and observed luminescent signals. The primary advantage of this assay was that we could test the activity of the peptidomimetics on the interaction between AR and PELP1 within the cell and within the native cellular context.

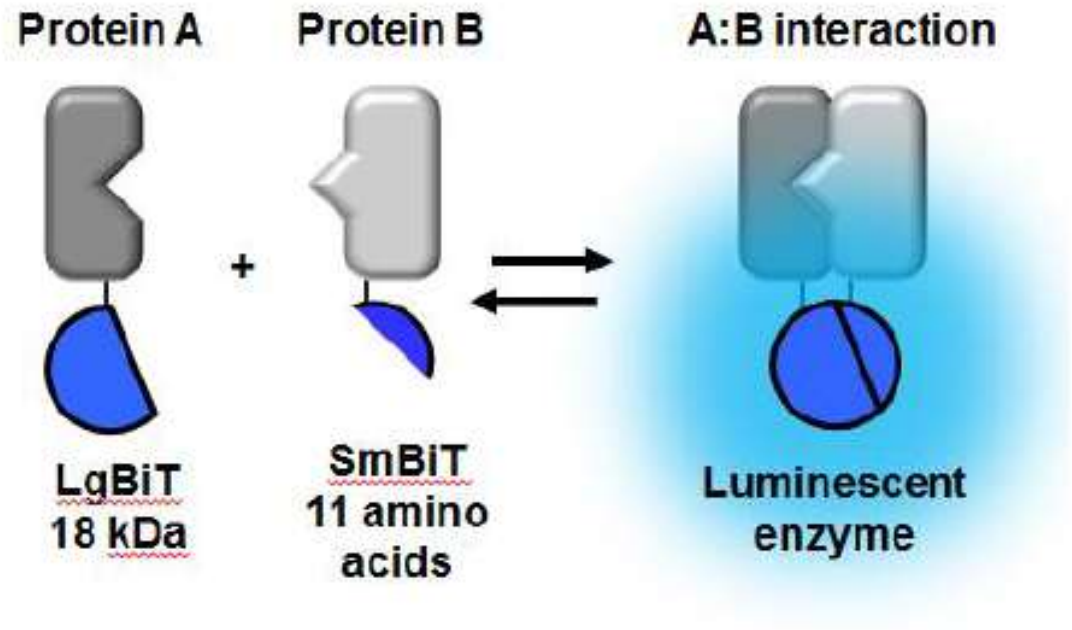

Fig 2: schematic of the nanobit assay showing that the interaction between protein A and protein B results in luminescence.

To generate different NanoBiT fusion constructs, human AR and PELP1 coding sequences were amplified by PCR and separately subcloned into NB-MCS vectors (Promega). To test the protein–protein interaction between AR and PELP1 by using the NanoBiT assay, C-LgBit-AR paired with C-SmBit-PELP1 HEK or C-LgBit-PELP1 with C-SmBit-AR constructs were transiently transfected into HEK293T cells by using Fugene HD transfection reagent (Promega). After a 24-hour incubation, the cells were treated with DMSO or peptidomimetics (at different doses) for 2 hours and then treated cells

with EtOH or DHT (10 nM) for 30 mins. After treatment, Nano-Glo live cell reagents were added into cells and luminscence was measured after 10 mins.

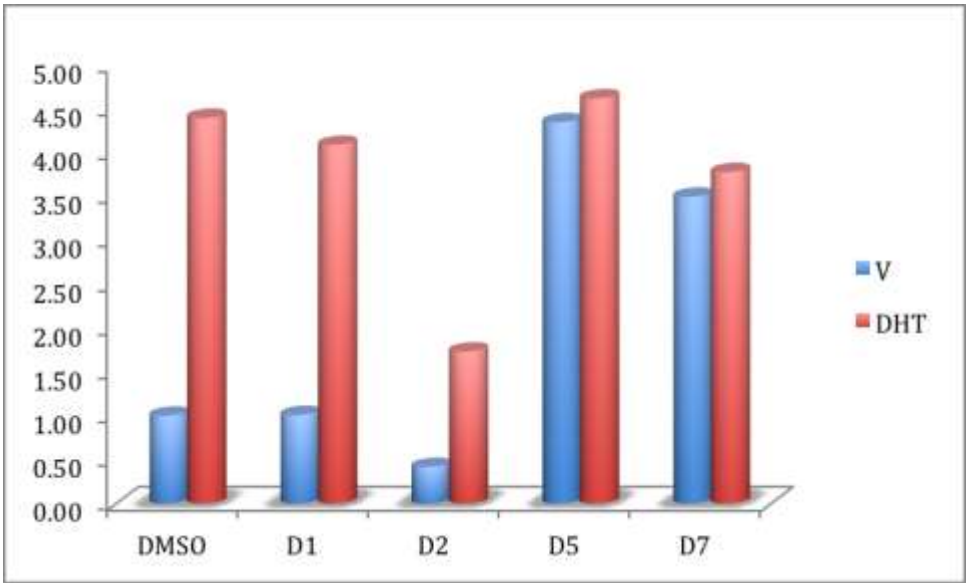

Fig. 3 Effect of bis-benzamides on the AR-PELP1 interactions. 293 cells were transiently transfected with large and small bit AR and PELP1 constructs and then treated with bis-benzamides in the absence and presence of 10 nM DHT. Evaluation of the fluorescent signal reveals the degree of interaction between AR and PELP1 within the cell.

We show that the D2 peptidomimetic was able to block the direct interaction between AR and PELP1 in 293 cells and PC3 cells (Fig. 3). These data strongly support the activity of the peptidomimetics on direct protein-protein interactions. This critical component of our story enables completion of the D2 variant manuscript and it is being readied for resubmission.

Because of the technical challenges in getting this assay done, we have applied for a no-cost extension to complete the proposed experiments for year 3, including validation of the effects of the peptidomimetics.

Key Research Accomplishments

1. We have systematically designed, synthesized and evaluated over a 100 compounds to improve pharmacologic properties of D2
2. We have created a lead compound that appears to be active at 10nM
3. We have developed a novel in vivo FRET assay to evaluate the interaction between AR and specific coregulators. This is a critical step forward in further understanding the activity of our platform.
4. I am the coPI of a DOD transformative grant designed to bring another of our drugs forward into clinic.
5. We have published our approach to peptidomimetic design

   Tailoring peptidomimetics for targeting protein-protein interactions. Akram ON[1], DeGraff DJ[2], Sheehan JH[3], Tilley WD[4], Matusik RJ[5], Ahn JM[6], Raj GV[7]. Mol Cancer Res. 2014 Jul;12(7):967-78. doi: 10.1158/1541-7786.MCR-13-0611. Epub 2014 Mar 18.

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6. We have published extensively using the explant model

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Reportable Outcomes for last year

1. We have developed a novel in vivo FRET assay to ascertain direct protein-protein interactions between AR and coregulators
2. We have shown that the peptidomimetics block AR and coregulator interactions
3. We are now further testing our panel of peptidomimetics using our novel assay

Conclusion

We continue to make progress in our work with peptidomimetics targeting ligand-dependent and ligand-independent androgen receptor signaling in prostate Cancer. We have now developed a novel in vivo FRET assay for evaluation of our peptidomimetic platform.

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