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14. ABSTRACT

The goal of these studies is to develop and validate cell-penetrating bi-specific antibodies as an agent that can selectively inhibit the function of intracellular proteins. We have developed 3E10-AR441 bi-specific antibody to inhibit the function both ligand-dependent and independent forms of the androgen receptor (AR), key drivers of prostate carcinogenesis. Key accomplishments in this reporting period are the production of a prototype 3E10-AR441 bi-specific antibody, and demonstration of target engagement under denaturing and non-denaturing conditions, as well as in intact cell systems. The antibody inhibited the growth of prostate cancer cells. We will continue to characterize the biochemical lesions produced by this antibody, while developing higher affinity analogs with improved production characteristics.

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INTRODUCTION

This report covers research activities supported by DOD contract W81XWH-12-1-0534, entitled ***Cell-penetrating bispecific antibodies for targeting oncogenic transcription factors in advanced prostate cancer***. The research is a collaborative effort between Michael Lilly, MD (Principal Investigator) and Richard Weisbart (Co-investigator). Dr. Lilly is based at the Hollings Cancer Center, Medical University of South Carolina, while Dr. Weisbart is at the Sepulveda VA, affiliated with the University of California Los Angeles. This contract was activated at MUSC on October 1, 2012. The reporting period is therefore from 10/1/2014 through 9/30/2015. Work under this contract began in Dr. Lilly's laboratory about November 1, 2012. Dr. Weisbart's group has worked from December, 2012, following the completion of a subcontract agreement between MUSC and Dr. Weisbart's institution.

Three specific aims were proposed. In the first, Dr. Weisbart's group would produce a prototype bispecific antibody targeting the androgen receptor (AR) and optimize its structure and production. The second specific aim, to be carried out by Dr. Lilly's laboratory, focuses on the biochemical and biologic properties of the bispecific antibody, through the use of biochemical and biologic assay systems. The final specific aim was to examine the ability of the bispecific antibody to perturb the growth of prostate cancer cells in murine models. Additional studies would characterize PK/PD parameters of the antibody. Substantial progress has been made in the first two aims. Work on the third specific aim has been delayed because of the tenuous supply of bispecific antibody. The issue was discussed at length with DOD/CDMRP staff and a modified SOW was accepted in early 2015, limiting the animal work to an initial PK/PD experiment. Final DOD/CDMRP animal use approval was obtained in June, 2015. This work will be performed during a NCE, as soon as an adequate amount of antibody is produced.

BODY: CURRENT RESEARCH

1. Weisbart Group

During the grant period we have produced three bispecific antibody constructs composed of 3E10-AR441 in order to identify one with the highest binding affinity and yield. The constructs are shown below:

3E10-AR441 Constructs:

A. 3E10Vk-(GGGGS)3-3E10vH-CH1 Linker-AR441 Vk-(GGGGS)3-AR441 VH-myc-his6
B. B. 3E10Vk-(GGGGS)3-3E10VH-CH1 Linker-AR441 VH-GGSSRSSSSGGGGSGGGGS-AR441 Vk
C. 3E10Vk-(GGGGS)3-3E10vH-myc-his6-AR441 VH-GGSSRSSSSGGGGSGGGGS-AR441 Vk

Although construct "B" had the highest binding affinity, the amount of secreted protein was insufficient for animal studies. Construct "C" was the best overall in both binding affinity and secretion. This construct worked well in killing prostate cancer cells in vitro and was, therefore, selected for use in subsequent animal experiments performed in Dr. Lilly's laboratory. However it required enormous effort and time to produce sufficient amounts of construct "C" for efficacy studies in animals.

There are multiple reasons for the limited secretion of some bispecific antibodies. These include a. structural considerations that interfere with secretion and b. toxicity of the secreted product. 3E10 is an unusual antibody that penetrates cells, and we have shown previously that 3E10 can penetrate and re-enter the cells responsible for its production. We also have recent evidence that blocking reentry may increase the yield of some bispecific antibodies. We are currently exploring re-uptake toxicity as a cause of limited secretion of 3E10-AR441 in Pichia pastoris, and we are exploring methods to inhibit re-uptake.

The current DoD grant has enabled us to establish the feasibility of treating prostate cancer with the exquisite specificity of antibodies that can be delivered inside of cancer cells as bispecific sc Fv fragments. Preliminary studies suggest that we will be able to establish methods to increase production for clinical studie

2. Lilly Group

A. Inhibition of AR genomic signaling by bispecific antibody. A key goal of specific aim #2 was to demonstrate that the 3E10-AR441 antibody can engage its target (AR) and interrupt AR-dependent signaling and effects. During the 01 year we developed an AR-dependent luciferase reporter gene system in LNCaP cells (LNCaP/ARELuc cells), and used it to show that 3E10-AR441 can disrupt genomic AR signaling. During the 02 year we expanded these studies to show that the bispecific antibody can also disrupt AR signaling to an endogenous promoter. During the 03 year with have expanded these studies further by examining the effects of the 3E10-AR441 antibody on genomic signaling by the AR/v7 splice variant of AR (Fig. 1). This protein is constitutively active, and induces signaling from an AR-dependent luciferase reporter gene without the presence of testosterone. To study this variant receptor we performed transient transfection of LNCaP/ARELuc cells, then cultured them for 48hrs in the absence of androgen. Test antibodies or other inhibitors were added for the final 24hrs of culture. The 3E10-AR441 again showed dose-dependent inhibition (>60%) of luciferase activity, while the 3E10 scFv alone had only a minimal effect at very high concentrations. Unlike the previous observations with DHT-stimulated luciferase activity assays, enzalutamide had no effect on the constitutive induction of luciferase activity by the AR/v7 splice variant receptor (not shown). The 3E10-AR441 bispecific antibody is now only the second known agent that will inhibit the effects of this variant receptor on genomic signaling. A previously-described small molecule (EPI-001) is now in preclinical development, but has recently been found to have off-target effects.

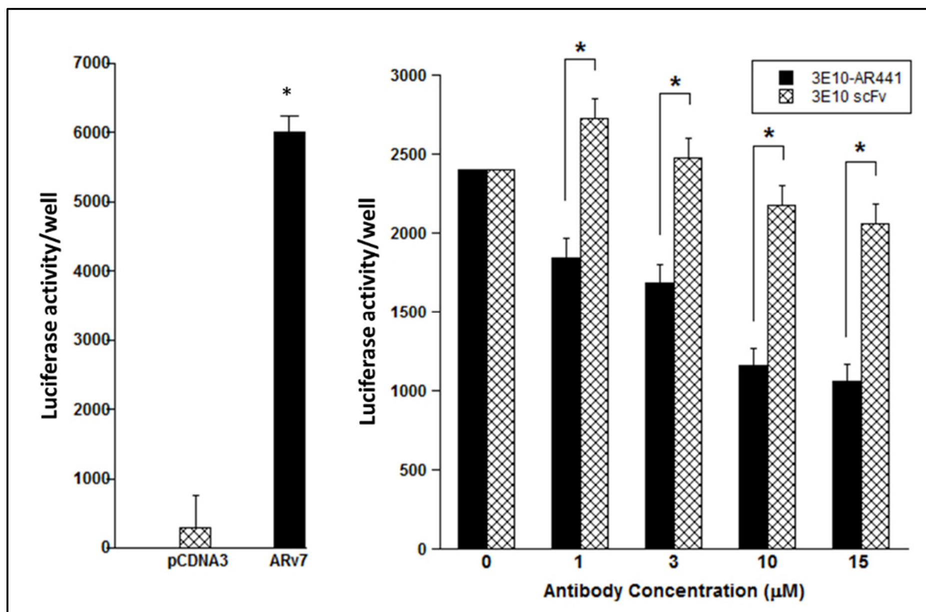

Fig 1. *Left panel*: Activation of genomic AR signaling following transfection of LNCaP cells with ARv7. *Right panel*: Inhibition of ARv7-directed genomic signaling by 3E10-AR441 biAb.

B. Binding affinity of bispecific antibody 3E10-AR441 and 3E10-Myc-His-AR441. During the 03 we have continued to use our sandwich ELISA-based assay to measure the binding affinity of newer versions of 3E10-AR441 to WT and variant AR proteins. We have now produced a comprehensive catalog of binding affinities for the original AR441 mAb, our initial 3E10-AR441 bispecific Ab, the AR441 scFv alone, and a new version of the bispecific, 3E10-Myc-His-AR441. This latter protein was produced by Dr. Weisbart to see if the antigen binding affinity would be enhanced. The changes included moving the Myc, His tags from the C-termiinus to the linker region between the 2 scFvs. In addition the ordering of the VH and VK sequences was reversed in the AR441scFv motif. We demonstrated that the binding

affinity increased from about 271nM to 49nM, more than a 5-fold improvement. Unfortunately the 3E10-Myc-His-AR441 protein is still not produced well in a *Pichia* system.

C. Development of a sandwich ELISA to detect 3E10-containing antibodies in biologic samples. In order to proceed to PK/PD studies in rodents, we proposed to develop an assay capable of detecting very small amounts of 3E10-AR441 in biologic specimens. During the 02 year we prepared a polyclonal rabbit antibody agains the 3E10 scFv antibody, to allow us to measure not just 3E10-AR441, but any monovalent or bivalent antibody containing the 3E10 scFv. However initial attempts to formulate a useful ELISA met with repeated technical challenges.

During the 03 year we have succeeded in formulating a useful ELISA. This was accomplished by using the rabbit polyclonal anti-scFv 3E10 for both the capture and detection antibodies. Since this polyclonal antibody reacts with multiple epitopes on the 35Kd antigen, it is likely that multiple "pairs" of antibody are present, allowing the same antibody to serve both roles. The detection antibody was biotinylated, and a streptavidin-HRP reagent was used as the secondary detection agent. This ELISA proved to be sensitive and facile to use. An unexpected problem however was that the anti-3E10 antibody cross-reacts with normal mouse serum proteins, presumably *kappa* light chains. This will require that we do all PK/PD studies in rats.

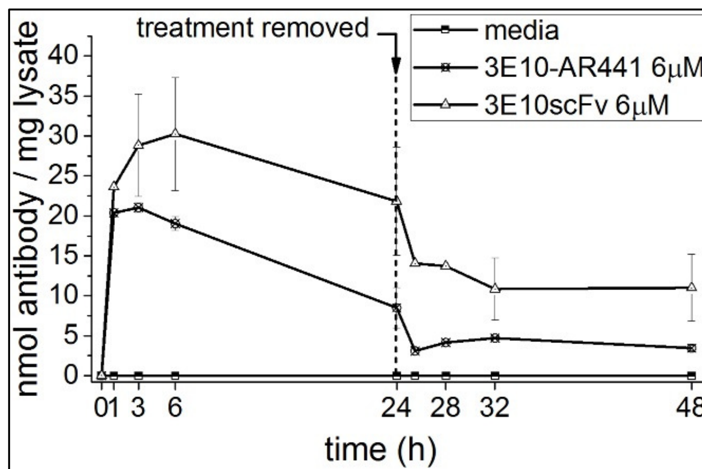

Fig. 2. Measurement of intracellular 3E10scFv or 3E10-AR441 biAb In LNCaP cells,using 3E10scFv ELISA

We have used our ELISA assay to quantitatively describe the ingress and egress of 3E10-AR441 and the 3E10scFv alone, in and out of LNCaP cells in culture (Fig. 2). Uptake of either antibody occurred rapidly, and was maximum between 1 and 3hrs after antibody addition. When antibody was removed from the culture medium a substantial quantity of the cell associated antibody was lost, appearing into the fresh medium rapidly. However between 25-50% of the initial cell-associated antibody remained, and there was little or no change over the subsequent 24hrs. It is likely that this stable, cell-associated antibody is bound to DNA through the 3E10scFv motif. There may be continuing interaction with nuclear AR by these antibody molecules "trapped" in the cell nucleus. The 3E10 scFv protein was taken up more rapidly, and remained in the cells at higher levels, than did the 3E10-AR441 bispecific antibody.

D. Demonstration that scFv AR441 sequences prevent secretion of fusion proteins in yeast. We have used a yeast display platform to directly demonstrate that sequences present in the scFv AR441 motif of the 3E10-AR441 bispecific antibody limit secretion of the protein from yeast (Fig. 3). The plasmid pTCON2 encodes the Saccharomyces *aga2* gene, with Myc tag. When transfected into yeast, the *aga2* protein is secreted and then binds to *aga1* already present on the yeast surface. This can be detected as a Myc-positive protein of about 30kd on the surface of the yeast. When the

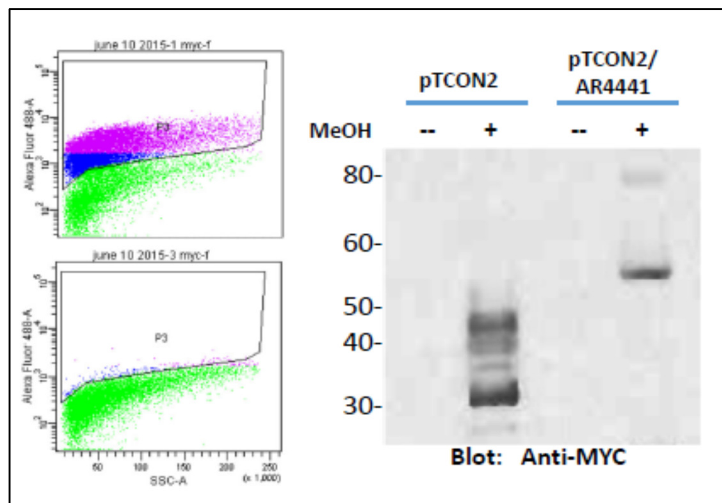

Fig. 3. Expression of aga2-AR441 fusion protein in yeast

AR441 scFv sequence is cloned in-frame with *aga2*, the fusion protein should be expressed also as a Myc-tagged protein on the yeast surface. Yeast transfected with pTCON2 or pTCON2/AR441 express their respective 30kd and 55kd proteins following methanol stimulation, as shown by the immunoblot (R panel) probed with anti-Myc. However the flow cytometry analysis shows that only yeast transfected with pTCON2 show a Myc-tagged protein on their surface (blue and purple). The yeast transfected with pTCON2/AR441 cannot secrete the fusion protein and so it does not appear on the surface. These data show that a key problem with producing the 3E10-AR441 biAb in yeast is the prevention of secretion by AR441scFv sequences. We may be able to use yeast surface display to identify mutants of AR441 scFv with enhanced binding or secretion properties to improve production.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 03

1. **Weisbart Group**

   Construction of variant 3E10-AR441 antibodies "B" and "C" as outlined above, with production of several milligrams of the corresponding antibody.

2. **Lilly Group**
   A. Demonstration that 3E10-AR441 inhibits androgen-dependent genomic and genomic AR-dependent signaling. Genomic signaling studies used LNCaP cells engineered to expess firefly luciferase under the control of a synthetic ARE. Similar studies were also performed with intact LNCaP cells stimulated with DHT, to show inhibition of PSA mRNA expression as an example of genomic signaling through an endogenous receptor. Controls were 3E10, 3E10-3G5, enzalutamide.
   B. Demonstration that 3E10-AR441 can inhibit non-genomic, AR-dependent signaling. These studies used LNCaP cells stimulated with DHT, with a readout of calcium release detected fluorometrically over time. Controls included ionomycin (positive control), enzalutamide, 3E10
   C. Demonstration that 3E10-AR441 bispecific antibody can bind to wild-type and LBD-deficient splice variants of the AR under "native" conditions, using an immunoprecipitation assay with a protein L-coated solid phase
   D. Development of a competitive EIA assay to measure binding affinity of antibodies to the AR441 epitope. We have used the assay to measure of binding affinities of parental MoAb AR441 to WT, truncated, and splice variant ARs. Binding affinities of 3E10-AR441 bispecific antibody to WT and truncated ARs have also been accomplished.
   E. Development and characterization of test cell lines for study of 3E10-AR441:

REPORTABLE OUTCOMES

1. The 3E10-AR441 bispecific antibody will enter prostate cancer cells and localize predominately in the nucleus, where it remains for more than 24hrs after removal of antibody from the cell environment.
2. The 3E10-AR441 bispecific antibody can bind to a variety of AR forms, including receptors lacking the ligand-binding domain, under denaturing and non-denaturing conditions.
3. The 3E10-AR441 bispecific antibody inhibits genomic and non-genomic signaling by the wild type and AR/v7 variant AR forms in LNCaP cells.
4. The 3E10-AR441 bispecific antibody has a lower binding affinity for both WT and truncated ARs than does the parental MoAb AR441.
5. Binding affinity of biAb is improved by changing the order of the AR441 VH and VK domains in the cDNA construct, and by moving the epitope tags.

6. Dr. Goicochea-Papaffava is preparing a manuscript describing these findings, for submission to a chemistry-oriented journal.
7. Protein sequences withing the AR441 scFv impair secretion of fusion proteins containing scFv AR441 from yeast.

DELIVERABLES

1. Rabbit polyclonal antibody to scFv 3E10, purified by ammonium sulfate precipitation and Protein A affinity chromatography, approximately 8mg. Also a biotinylated variant, and a functional, formulated ELISA assay kit.
2. Poster describing studies up to April, 2015, presented at the American Association of Cancer Research, Philadelphia, PA, April, 2015 (abstract 642)
3. Manuscript describing studies through September, 2015 (now submitted to *Molecular Therapy*)

CONCLUSIONS

1. The bispecific 3E10-AR441 antibody has performed as expected in tissue culture experiments, by entering cells, translocating to the nucleus, and blocking androgen-dependent genomic and non-genomic signaling through the wild-typeAR. In addition it inhibits ligand-independent signaling through the AR/v7 receptor. The antibody binds to both wild-type and LBD-mutant ARs, demonstrating robust target engagement.
2. The binding affinity of the 3E10-AR441 is probably several-fold lower than that of the parental monoclonal AR441. This may require much larger amounts of antibody than expected for a significant *in vivo* biologic effect.
3. Further protein engineering of the 3E10-AR441 antibody is probably needed to 1) enhance the binding affinity to the target protein, and 2) to improve yields from the current yeast expression system
4. The studies accomplished to date by both the Lilly and Weisbart laboratories represent a substantial completion of the propose studies for months 1-24 under specific aims #1, 2, as outlined in the approved Statement of Work. Studies under specific aim #3 as outlined in the modified SOW are beginning, as indicated by our development of an ELISA to measure 3E10-AR441 in biologic samples.
5. A no-cost extension will be requested for 10/1/2015-9/30/2016 to allow completion of the proposed PK/PD measurements in rats. The final DOD approval of our IACUC application was obtained in June, 2015 and we are now preparing and stockpiling enough antibody to complete the work.

REFERENCES

none

APPENDICES

none