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14. ABSTRACT The goal of this research is to firmly establish the mechanism of androgen receptor (AR)-JunD heterodimer induction of the SSAT gene leading to oxidative stress that contributes to the development and progression of prostate cancer (PCa), and to identify small molecules that specifically inhibit this AR-JunD interaction and prevent development/progression of PCa in pre-clinical models. Data from this research will identify the most efficacious drug to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. Significant findings during Year 1 of the research include: identification of sequences in the SSAT promoter that are relatively more important in the androgen activated AR-JunD induction of SSAT; identification of 13 small molecules that specifically inhibit AR-JunD; and classification of 12 of the 13 small molecules as non-antiandrogens to be further tested for efficacy against PCa models as proposed.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8
Appendices.....	8

INTRODUCTION

We hypothesize that activated androgen receptor (AR) forms a heterodimer with JunD and this AR-JunD heterodimer induces SSAT gene expression leading to polyamine oxidation and consequent production of excess reactive oxygen species (ROS) in prostate cells. Excess ROS, in turn, contribute to the development and progression of prostate cancer (PCa). The purpose of this research is to firmly establish the mechanism of the induction of SSAT by the AR-JunD complex that leads to an excess ROS production in PCa cells, identify small molecules that specifically inhibit the AR-JunD interaction downstream of androgen activation of AR in this pathway, validate the activity of the inhibitors against ROS production and growth in PCa cells, and determine the chemotherapeutic/chemopreventive efficacy of the lead inhibitors against pre-clinical mouse models of PCa. Data from this research will identify the most efficacious drug to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. This report summarizes the progress that has been achieved over the past year toward completing the proposed aims.

BODY

The following are the data collected with regards to tasks listed in our statement of work (SOW):

Task 1. Establish the mechanistic pathway for androgen-induced SSAT expression in CaP cells (months 1-18):

1.1. Create mutant SSAT promoter-luciferase reporter constructs (months 1-4).

Four different constructs with various degrees of truncation of the SSAT promoter that is connected to a luciferase reporter were constructed in a pGL4 vector following a standardized protocol. The constructs are shown schematically in Fig. 1 below.

Table 1

Effect of 1 nM R1881 on the induction of Luciferase activity in LNCaP cells transfected with SSAT-luc plasmid

pGL4		
197-SSAT-luc (0.28kb)		
358-SSAT-luc (0.44kb)		
659-SSAT-luc (0.74kb)		
Full-SSAT-luc (3.2kb)		

Constructs	Fold increase +R/-R
197-SSAT-luc 160	4576
358-SSAT luc	1.297968
659-SSAT-luc 086	4553
Full SSAT-luc	1.656394
pGL4 0.30	5446

Figure 1. Various SSAT promoter reporter constructs. Each different length of SSAT promoter sequence was amplified and inserted into a basic pGL4 vector. Each vector separately was transfected into LNCaP cells. After transfection the cells were treated with 1nM R1881 or left untreated in F1C4 for 72h. The cells were then lysed and the luciferase activity were measured. Each construct has important binding sequences for transcription factors. (figure adapted from Babbar, N *et al*, 2006 [1]).

1.2 Transiently transfect mutant constructs into cells and compare 1) SSAT promoter activity by luciferase reporter assay and 2) reduction in androgen-induced ROS production by DCFH dye oxidation assay (months 4-12).

All constructs shown in Fig. 1 were transiently transfected in LNCaP cells. The cells were incubated in androgen-depleted media and then treated with 1 nM androgen analog R1881 for

72 h. The effects of R1881 treatment on the transfected cells are listed in Table 1. While all but one construct showed an enhancement of luciferase activity upon R1881 treatment, the luciferase activity was maximum for the full length (FL) promoter and its truncated section with 197 bp lead sequence. A slight decrease in SSAT activation was observed for the truncated section with 659 bp lead sequence. These data suggest a three dimensional structure of the promoter sequence that may block certain sections in the SSAT promoter that are important for androgen induction.

Our published chromatin immunoprecipitation (ChIP) data showed that a 77 bp section between 550-650 bp lead sequence is the JunD binding domain of the SSAT promoter in the presence of androgen [1].

1.3 Perform ChIP assay to identify the AR-JunD binding sequence in most and least effective mutants (months 12-18).

These studies have been planned.

Task 2. Screen a small molecule library to identify inhibitors of AR-JunD interaction (months 1-15):

2.1 Optimize the GL-reconstitution screening assay conditions using the “hit” obtained from the partial screening assay performed in preliminary studies (months 1-4).

The optimization of the screening process was undertaken with rigor and has been accomplished in the course of the last one year. Nine compounds identified during the preliminary screening assay reported in the grant proposal have been reconfirmed with the standardized assay. Two of the compounds that do not have anti-androgenic properties are now being further developed for preclinical testing in cell culture and *in vivo* models.

2.2. Perform the optimized screen on the 14,400 compound Maybridge HitFinder library (months 4-12).

In the process of optimization, a more efficient way of screening in a 384-well plate based assay as compared to the originally planned 96-well plate based assay was standardized. This standardization helped in substantial savings that allowed us to screen a 25,000 compound Life Chemical Library instead of the 14,400 compound Maybridge HitFinder library at no extra cost.

2.3. Eliminate false positive “hits” by performing the positive control (SMAD3-PKB) interaction screen (months 6-15).

The initial screening of the 25,000 compound Life Chemical Library yielded 833 “hits”. These compounds were further checked for “false positives” using the positive control, and 13 compounds have been identified and reconfirmed as real “hits” with greater than 30% inhibition of the AR-JunD interaction in the screening assay.

Task 3. Select inhibitors that act downstream to AR activation and validate compounds for activity against CaP cells (months 8-28):

3.1 Test compounds for their ability to bind AR using an AR-LBD binding fluorescence polarization assay (months 8-26).

The 13 compounds identified as “hits” were further checked for their anti-androgenic properties. Twelve compounds were identified as non-antiandrogenic and are currently being prioritized for further testing. These compounds are currently being submitted for patent process. Details of these compounds will be made available under confidentiality upon request.

3.2 Test compounds for their ability to inhibit the translocation of AR-JunD to the cell nucleus (months 8-26).

The nuclear translocation assay for the AR-JunD complex is currently undergoing standardization and validation process.

3.3 Test compounds for their ability to inhibit growth of CaP cells and block androgen-induced ROS production in CaP cells (months 10-28).

The ability of the “hits” in inhibiting growth of LNCaP cells and their androgen-independent LNCaP C4-2 cells in the presence or absence of androgen has been standardized, and data collection will begin this month following completion of prioritization of the compounds.

Task 4. Select the lead drug candidate for future clinical testing by comparing efficacy of 2 to 3 drug candidates in mouse models of CaP (months 6-36).

4.1 Screen potential drug candidates for oral bioavailability, pharmacokinetics and maximum tolerated dose in mice (months 6-28).

The methods of testing and LC-MS protocol for identification of one of the compounds presented in the preliminary studies section in the proposal have thus far been standardized. The oral bioavailability and pharmacokinetic properties of the compound has been determined. The testing of the lead compound(s) identified from the 13 “hits” from the Life Chemical Library will be undertaken during the course of this year.

4.2 Determine efficacy of drug candidates against a CaP cell xenograft model in nude mice (months 12-32).

These studies will be undertaken during the course of this year.

4.3 Determine efficacy of drug candidates against the TRAMP mouse model of CaP (months 15-34).

These studies will be undertaken during the course of this year.

4.4 Complete a statistical comparison of the efficacy of drug candidates against both CaP animal models to select the lead drug for further pre-clinical testing and future clinical trial (months 34-36).

This task will be performed when all data from proposed experiments have been collected.

KEY RESEARCH ACCOMPLISHMENTS

- Established mutant SSAT promoter-luciferase reporter constructs
- Identified more important sequences in SSAT promoter for androgen induction of SSAT
- Optimized the high throughput screen for 384-well plate based assay
- Completed screening on a 25,000 compound Life Chemical Library
- Identified 13 compounds as true “hits”
- Identified 12 of the 13 “hits” as non-antiandrogenic compounds
- Standardized conditions for testing the compounds for efficacy against growth and ROS production in androgen-dependent and androgen-independent human prostate carcinoma cells in the presence or absence of androgen
- Standardized protocol for development of LC-MS analysis and testing of compounds for oral bioavailability and pharmacokinetics

REPORTABLE OUTCOMES

- Kegel S, Mehraein-Ghomi F, Reuter Q, Schmidt J, Church D, Hoffmann FM, Basu H, and Wilding G. Targeting a novel pathway for prostate cancer therapy [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2583 (see Appendix)
- Plasmids developed: Four different mutant SSAT promoter-luciferase reporter constructs with various degrees of truncation of the SSAT promoter connected to a luciferase reporter in a pGL4 vector
- SBIR Phase I grant proposal submitted December 2010: “Androgen Receptor-JunD Complex Inhibitors to Prevent Prostate Cancer Progression” (Colby Pharmaceutical Company PI Hirak Basu, UW-Madison PI George Wilding)

CONCLUSION

The data from the truncation of SSAT promoter assay yielded the intriguing possibility of studying the secondary structure of the promoter sequence in the cellular chromatin. The planned Chromatin Immunoprecipitation (ChIP) assay should lead to a more detailed mechanistic assay for SSAT induction and cellular ROS production.

During the course of this year, we have also rigorously standardized the small molecule screening protocol to identify inhibitors of AR-JunD interaction that can be further studied for their anti-cancer efficacies. The standardization process yielded a 384 well plate based assay instead of the 96 well plate based assay originally proposed. Therefore, we screened a 25,000 Life Chemical Library, instead of the originally proposed 14,400 Chembridge Hitfinder Library, at no extra cost. Screening the bigger library likely yielded more “hits” (13) for further analysis and lead optimization to be undertaken during the course of this year.

The identification of a significant number of hits during the first year of the project is considered significant as the likelihood of identification of a successful drug increases. Detailed validation and drug development studies to be undertaken during the course of this project should yield a likely drug candidate that can be further advanced to preclinical and clinical development to treat early-stage recurrent prostate cancer patients, who have no approved therapy and represents a long unmet medical need.

REFERENCES

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2. Mehraein-Ghomi F, Basu HS, Church DR, Hoffmann FM, Wilding G. Androgen Receptor Requires JunD as a Coactivator to Switch on an Oxidative Stress Generation Pathway in Prostate Cancer Cells. Cancer Res. 2010 Jun 1;70(11):4560-8. PMID: 20460526. NIHMSID:192513

APPENDICES

Appendix 1: AACR 2011 abstract

APPENDIX 1

Targeting a novel pathway for prostate cancer therapy

Stacy Kegel, Farideh Mehraein-Ghomi, Quentin Reuter, Joseph Schmidt, Dawn Church, F. Michael Hoffmann, Hirak Basu, George Wilding. University of Wisconsin- Madison, Madison, WI

In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2583

Development of an effective therapy to prevent prostate cancer (PCa) progression to castrate-resistant PCa (CRPCa) remains an unmet medical need, mainly due to a poor understanding of the mechanism of PCa progression. Reactive oxygen species (ROS) are produced in high amounts in PCa cells and play a major role in PCa development and progression. We have published that activated androgen receptor (AR)-JunD complex induces spermidine/spermine N1-acetyl transferase (SSAT), the first and regulatory enzyme in a major polyamine catabolism pathway that yields over-production of ROS specifically in the polyamine-rich PCa cells. Our recent data further suggest an intriguing mechanism of PCa progression, where AR-JunD induced SSAT expression and consequent upregulation of the transcription factor NF- κ B may set up an autocrine feed forward loop of SSAT-ROS-NF κ B-SSAT that can sustain ROS production and PCa cell proliferation in the absence of androgen. A focus of our current research is to identify compounds that specifically target and block steps in this novel pathway downstream to AR activation and thereby have potential to be new targeted therapeutic agents to prevent PCa progression to CRPCa in early stage progressing PCa patients with minimal side effects. In the research presented here we utilized a novel high throughput screen (HTS) assay to find compounds that prevent the initiating AR-JunD interaction step in this ROS generating pathway and tested their efficacy in PCa cells.

A high throughput assay based on Gaussia Luciferase enzyme reconstitution via protein-protein interaction was used to screen the NCI diversity set library of drug like molecules to identify inhibitors of the AR-JunD interaction. Selected hits were further screened to determine their ability to bind to the AR using a published fluorescence polarization assay in order to categorize the molecules as non-antiandrogens or antiandrogens. As we intend to target the pathway downstream of AR activation, we focused on non-antiandrogens, further testing them for efficacy against androgen induced ROS generation and effect on cell growth in PCa cells in vitro by our published DCFH dye oxidation and DNA fluorescence assays. Of the 14 hits from the HTS assay of the NCI diversity set library, nine small molecules were chosen based on their drug-like chemical characteristics for further studies along with two synthesized analogs of one of the selected small molecules. We have categorized six agents as non-antiandrogens and five as having some antiandrogenic activity. Our ROS assay identified a lead non-antiandrogen compound that blocks androgen induced ROS production in PCA cells at less than 1 μ M concentration. This agent also significantly inhibited androgen-independent growth of PCa cells at 4 μ M. Data for all compounds will be presented.