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TITLE: Overcoming Drug-Resistant Prostate Cancer with APE1/Ref-1 Blockade

PRINCIPAL INVESTIGATOR: Travis Jerde

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14. ABSTRACT

We have identified a new target that might explain how advanced prostate cancer cells avoid being killed by chemotherapy: Apurinic/apyrimidinic endonuclease/redox-factor 1, or simply, Ref-1, for short. In this report, we provide evidence that APE1/Ref-1 is induced in prostate cancer cells and in human prostate cancer. This expression correlates to inflammation and to survivin signaling in human prostate cancer specimens. Genetic knockdown of APE1/Ref-1 disrupts prostate cancer cell growth and survival in cell culture. In addition, inhibition of the redox function selectively of Ref-1 results in cell growth inhibition, with this therapy preferentially inhibiting prostate cancer cell growth above that in non-cancerous cells. Specific blockade of Ref-1 redox activity in tumors is a novel concept in tumor therapy. If we are successful, we will have defined a critical therapeutic target for drug-resistant prostate cancers, and logically clinical trials would follow targeting this pathway.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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11. Appendices	N/A

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

   We hypothesize that Human apurinic/apyrimidinic endonuclease/redox-factor 1 (APE1/Ref-1, or Ref-1) enhances transcriptional activator activity thereby inducing expression of the survival protein survivin during drug resistance. In Aim 1 of this work, we have proposed to determine Ref-1's role in prostate cancer cells and evaluate the mechanisms by which it exerts its effect. In Aim 2 of the funded proposal, we have proposed to determine if STAT3 inhibition results in activation of compensatory pathways of survival protein induction, and if Ref-1 activates AP-1 in response to STAT3 inhibition, specifically in taxane-resistant cells. In Aim 3 of the proposal, we he proposed determine if Ref-1 mediates taxane-resistance in prostate cancer cells and if pharmacological inhibition of Ref-1 with novel but tested inhibitors renders drug-resistant cells sensitive to taxanes, both in vitro and in vivo. The proposal integrates human prostate tissue specimens including drug-resistant metastases with cell culture and *in vivo* animal models. This work may therefore provide the mechanistic basis for a novel combination therapy trial in chemo-resistant prostate cancer, and may therefore be the genesis of a therapeutic for prostate cancer patients.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

   Prostate cancer, taxane resistance, Human apurinic/apyrimidinic endonuclease/redox-factor 1 (APE1/Ref-1), survivin

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

The following is a timeline for tasks, year by year, in the approved statement of work:
The tasks to be completed in year one are highlighted in yellow, and bolded.

Specific Aim 1 :To determine the impact of Ref-1 mediated STAT3 activity on survival pathway activation in prostate cancer cell lines and human cancer tissues.	**Timeline (Months)**	**Site 1 PI: Jerde**
Major Task 1: ***Ref-1 co-localization with STAT3 and survival signaling in human prostate cancer:***	1-12	
Subtask1: IRB in hand; acquire specimens	**1-3**	Drs. Jerde/ Chang
Subtask 2: Stain and correlation of human specimens	**1-6**	Dr. Jerde
Subtask 3: Correlation of staining and quantification	**6-7**	Dr. Jerde
Milestone(s) Achieved: determining co-localization; writing and publishing manuscript	***7-12***	
Local IRB/IACUC Approval	**1**	
Milestone Achieved: HRPO/ACURO Approval	**1**	
Major Task 2: ***Effect of Ref-1 on STAT3 activity and survival signaling***		
Subtask 1: Ref-1 siRNA	**1-3**	Drs. Jerde/ Fishel
Subtask 2: Overexpression of wt-Ref-1	**3-6**	Drs. Jerde/ Fishel
Subtask 3: Mutant Ref-1 constructs (C65A)	**4-8**	Drs. Jerde/ Fishel
Subtask 4: selective Ref-1 redox inhibitor, E3330	**6-10**	Drs. Jerde/ Fishel
Milestone(s) Achieved: Determining that Ref-1 regulates STAT3-initiated transcription of target genes, that STAT3 activity and survival will be dependent upon intact Ref-1, functioning through its redox function. Manuscript	***7-12***	
Specific Aim 2 : To determine if Ref-1 inhibition circumvents transcriptional functional compensation triggered by STAT3 inhibitors.	**Timeline (Months)**	**Site 1 PI: Jerde**
Major Task 3: ***Compensatory response in drug resistant and sensitive cell lines-establishing the compensatory response***	6-15	
Subtask1: Constructs of STAT3 and AP-1 promoters driving GFP-Luc gene reporters	**6-8**	Drs. Jerde/ Fishel
Subtask 2: Assess cell lines with STATTIC	8-12	Drs. Jerde/ Fishel
Subtask 3: Assess pathway activation for each transcription activator	12-15	Drs. Jerde/ Fishel
Milestone(s) Achieved: Characterization of the STAT3 inhibition compensatory response	*15*	
Major Task 4***: Assessing Ref-1's role in compensatory signaling***	6-24	
Subtask 1: Constructs of STAT3 and AP-1 promoters driving GFP-Luc gene reporters	**6-8**	Drs. Jerde/ Fishel
Subtask 2: Treatment with E3330, STATTIC,	15-21	Drs. Jerde/ Fishel/ Kelley
Subtask 3: Mutant Ref-1 constructs (C65A)	21-24	Drs. Jerde/ Fishel
Milestone(s) Achieved: Determining role of STAT3 compensatory response on Ref-1 function in docetaxel-resistant lines-quantified data	24	
Major Task 5: ***Assessing Ref-1 driven compensatory signaling in graft tumors***	12-30	
Subtask 1: Grow graft tumors and treat animals with STAT3 inhibitors-assess outcome by luminescence and gene expression.	12-24	Drs. Jerde/ Ratliff/ Kelley

2 cell lines x 3 constructs x 2 treatments x8 per group x 3 sacrifice times=288 mice. 21 day tumor growth, plus 3 day analysis, plus 7 day acclimation time=31 days house per mouse		
Milestone(s) Achieved: Determining in vivo STAT3 compensatory response. Publish manuscript	24-30	
Specific Aim 3: To determine if Ref-1 inhibition sensitizes drug resistance in prostate cancers to chemotherapy.		**Site 1 PI: Jerde**
Major Task 6: ***Evaluating Ref-1 expression in drug-resistant human prostate cancer metastases***	1-30	
Subtask1: Acquire specimens	**1-24**	Drs. Jerde/ Chang
Subtask 2: Stain and correlation of human specimens	**1-25**	Dr. Jerde
Subtask 3: Correlation of staining and quantification	25-26	Dr. Jerde
Milestone(s) Achieved: determining co-localization; writing and publishing manuscript	*26-30*	
Major Task 7*: Determining the effect of Ref-1 on docetaxel-resistant prostate cancer*	12-21	
Subtask 1: Transfect docetaxel-resistant C4-2 (C4-2^{doc}) and PC3 cells (PC3^{doc}) with siRNA	12-15	Drs. Jerde/ Fishel
Subtask 2: CRC in transfected cells, with or without inhibitors	15-18	Drs. Jerde/ Fishel
Subtask 3: Mutant Ref-1 constructs (C65A)	19-21	Drs. Jerde/ Fishel
Milestone(s) Achieved: Determining Ref-1 function in docetaxel-resistant lines-quantified data	12-21	
Major Task 8: ***Effect of Ref-1 inhibition on in vivo models of prostate cancer***	18-36	
Subtask 1: Docetaxel-resistant and parental C4-2 and PC3 cells in vivo by subcutaneous implant 4 cell lines x 4 treatments x 8 per group =128 mice. 21 day tumor growth, plus 12 day analysis, plus 7 day acclimation time=40 days house per mouse	18-30	Drs. Jerde/ Ratliff
Subtask 2: Molecular analysis of tumor graft.	28-36	Drs. Jerde/ Ratliff
Milestone(s) Achieved: demonstrate Ref-1 inhibition in vivo blocks tumor growth and metastasis	24-30	
Milestone(s) Achieved: Publish manuscript demonstrating this effect. Begin to acquire clinical trial documents for future study in humans	30-36	Drs. Jerde/ Kelley

Summary, year one tasks:

Specific Aim 1 : To determine the impact of Ref-1-mediated STAT3 activity on survival pathway activation in prostate cancer cell lines and human cancer tissues.
Major Tasks:
1: Ref-1 co-localization with STAT3 and survival signaling in human prostate cancer:

We have published a manuscript (referenced in the publication section, below) that identifies, among other things, that inflammation-generated survivin is the dominant survival factor induced by prostate cancer in humans. It localizes to inflammation, a major cause of STAT3 induction in human prostate cancer. (Figure 1; Task 1, subtask 2) This is the basis of our selection of survivin as the critical survival

factor induced by APE1/Ref-1 – driven STAT3 signaling in prostate cancer. This figure, figure 6 from our manuscript, demonstrates the induction of survival signaling in prostate cancer:

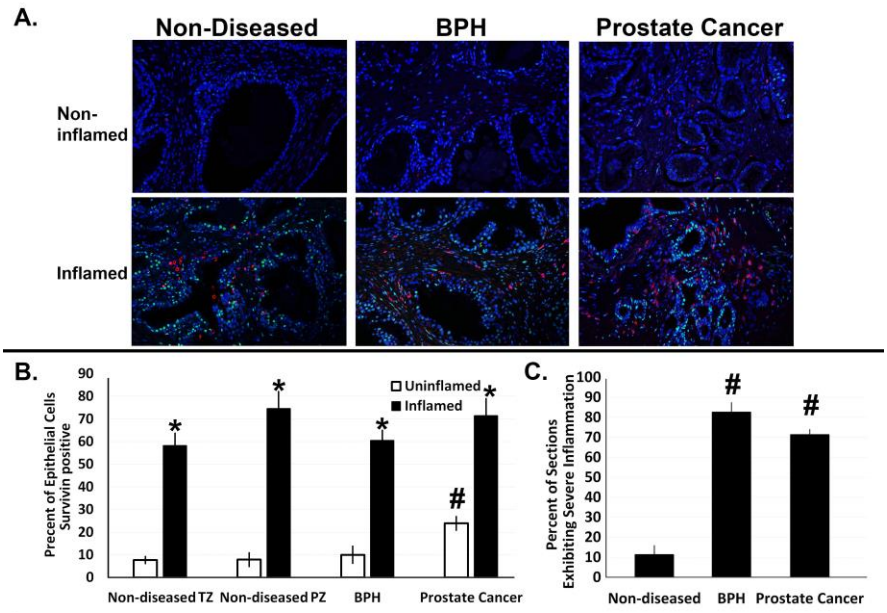


Figure 1. Human prostate specimens demonstrate intense survivin staining juxtaposed to regions of inflammation. **A,** immunofluorescent images of non-inflamed or non-inflamed human prostates representing non-diseased controls, BPH specimens, or prostate cancer specimens as indicated. Sections were stained for survivin (green) and CD45, a pan leukocyte marker (red) to identify regions of inflammation. Sections were deemed non-inflamed if they exhibited less than 10 leukocytes per 20x field, and inflamed if they exhibited greater than 30 leukocytes per field. **B,** quantified cell counting of epithelial cells positive for survivin expression in human prostates; data expressed are the percentage of epithelial cells expressing survivin in non-inflamed and inflamed prostates at each time point of inflammation—three 20x fields per prostate section were averaged for each data point, and all data are expressed as mean ± s.e.m. *p<0.05 inflamed versus non-inflamed prostate; #p<0.05 disease condition versus non-diseased control. Analysis of variance (ANOVA), n=12 human prostates. **C,** percent of sections from each human prostate group (non-diseased, BPH, and cancer) that exhibited 30 leukocytes per 20x section. Three 20x fields per prostate section were averaged for each data point, and all data are expressed as mean ± s.e.m. #p<0.05, disease condition versus non-diseased control. Analysis of variance (ANOVA), n=12 human prostates.

Additionally, we have stained our human primary tumor specimens with APE1/Ref-1 and survivin, and found 71% correlation between survivin and APE1/Ref-1 in human prostate cancer specimens (Figure 2: Task 1, subtask 3). These data were all performed with our newly acquired human specimens, Task 1, subtask 1.

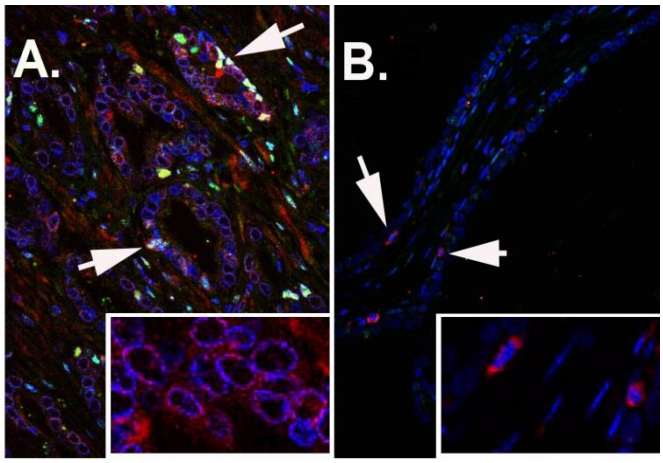


Figure 2. Immunofluorescence of Ref-1 (red) and survivin (green) in human prostate cancer **[A]** and normal human prostate **[B]**. Ref-1 is highly expressed in human prostate cancer, and is localized to survivin-expressing cells (71% co-localization). Expression is localized to select basal cells in normal prostate. While Ref-1 co-localizes with nuclear chromatin (blue) in prostate cancer,**(A,inset)** it is expressed in the cytosol of normal tissues.

Specific Aim1, Major Task 2: Effect of Ref-1 on activity and survival signaling

We have found that siRNA knockdown of APE1/Ref-1 inhibits cell growth in PC3, LNCaP, and C4-2 prostate cancer cell lines. **Task 2, subtask 1. Figures 3-5.** These data indicate that APE1/Ref-1 is essential for prostate cancer cell survival and growth. We used two distinct siRNA transfections, as indicated to demonstrate these effects over a 6 day experiments of prostate cancer cell growth. Cells shown are in the pictures are dyed with methylene blue, and quantified as shown in the graphs. These data will be submitted later this year for publication.

PC-3 6 Days Post Transfection

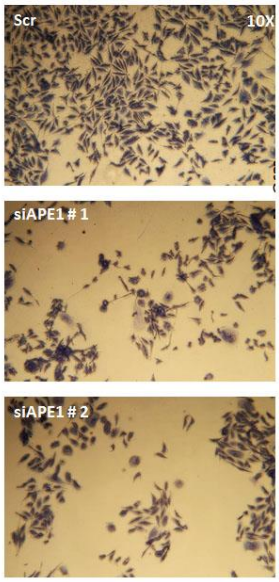
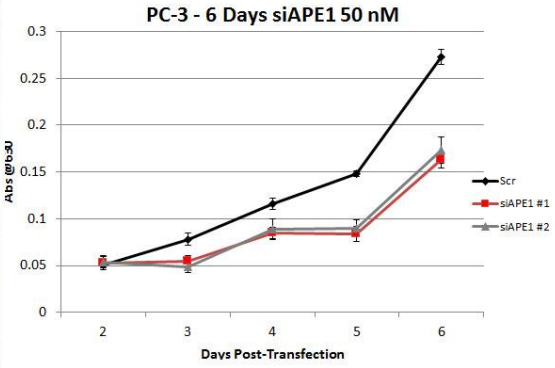


Figure 3



C4-2 6 Days Post Transfection

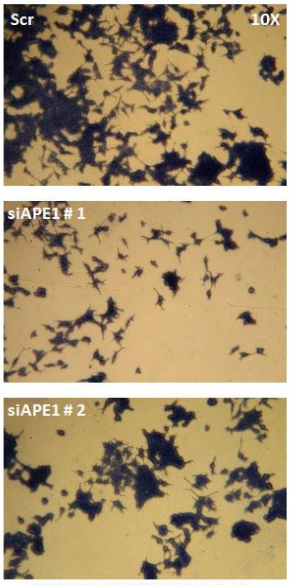
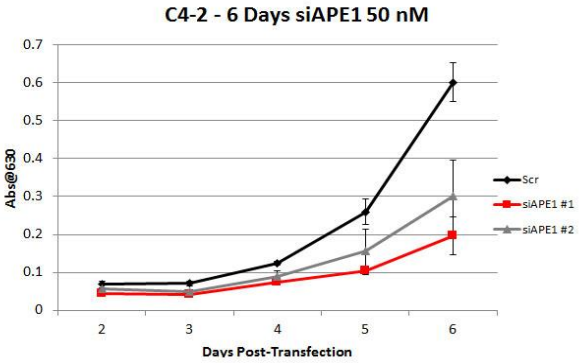


Figure 4



LNCaP 6 Days Post Transfection

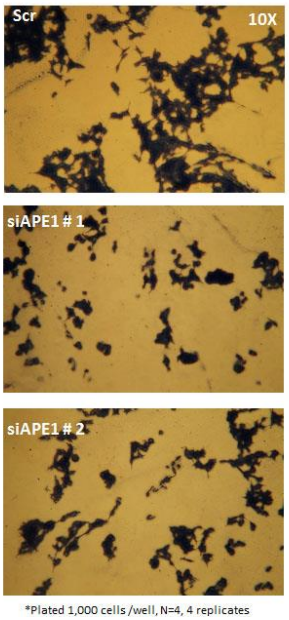
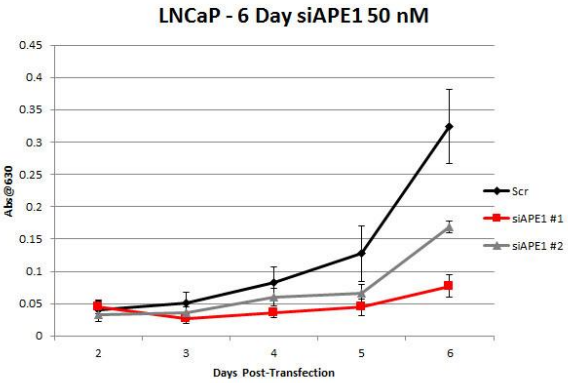


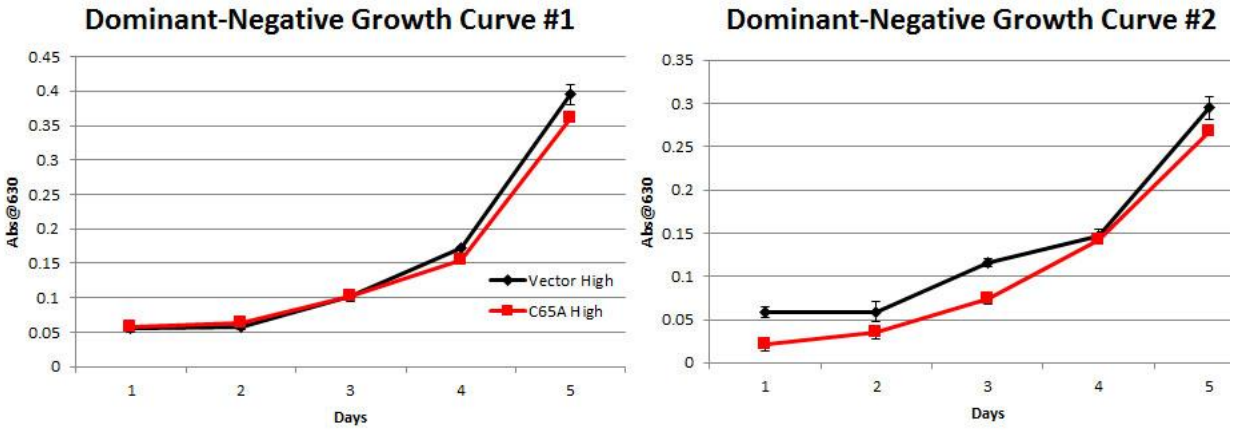
Figure 5



*Plated 1,000 cells /well, N=4, 4 replicates

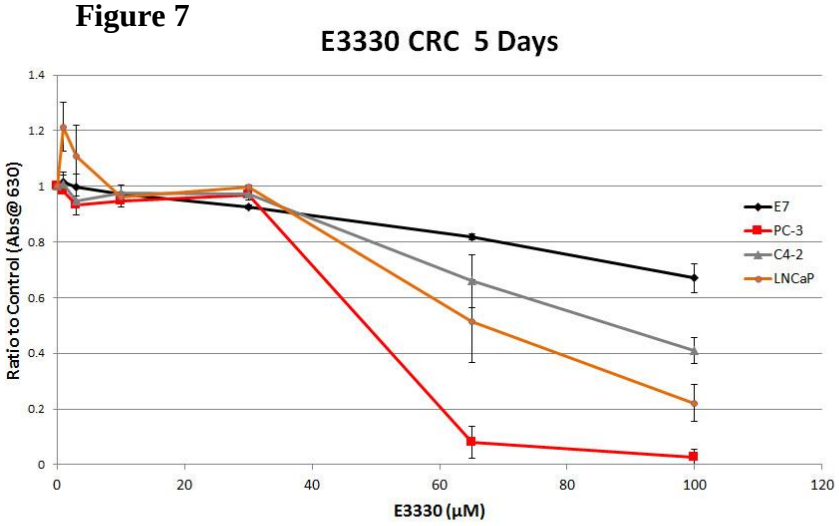
We then transfected cells with an overexpression of wildtype APE1/Ref-1, or a redox-dead C65 mutant form of Ref-1 (see proposal). This experiment did not include knockdown of the endogenous form of the protein in these cells, but we had anticipated that overexpression of the wild-type form would induce cell growth and survival, while the C65 redox dead form would not. In this case, the endogenous Ref-1 activity appears to have been enough to maintain cell numbers in prostate cancer cells. We will repeat this experiment while knocking down endogenous APE1/Ref-1. Figure shown in **Figure 6** are growth curves, with wild-type and C65 mutant protein, as indicated, in PC3 and C4-2 cells, Curve 1, and Curve 2, respectively. **(Task 2, sub tasks 2,3)**

Figure 6



Next, we treated prostate cells with the redox-selective inhibitor E3330 and determined the effect on cell growth. We compared the non-cancer cell line E7 to that of 3 cancer cell lines, PC3, C4-3, and LNCaP. Selective redox function inhibition of Ref-1 activity preferably inhibited prostate CANCER cell growth, but did little to affect that of the noncancerous E7 line. The EC25 and EC50 concentrations for each cell line are shown in the table. **(Figure 7) (Task 2, subtask 4)**

Figure 7



	E7	PC-3	C4-2	LNCaP
IC_{25}	82.7+/- 8.7	36.0 +/- 1.0	57.4 +/- 3.8	43.8+/- 4.2
IC_{50}	>100	54.7 +/- 1.6	89.5 +/- 7.8	71.9+/- 7.2

Specific Aim 2 : To determine if Ref-1 inhibition circumvents transcriptional functional compensation triggered by STAT3 inhibitors.

Major Tasks (to be completed year 1):

Major Task 3: Compensatory response in drug resistant and sensitive cell lines-establishing the compensatory response: Constructs of STAT3 and AP-1 promoters driving GFP-Luc gene reporters.

We have successfully transfected constructs of STAT3 and AP-1, (and HIF1a, and p65) promoter-driven luciferase constructs into C4-2 and PC3 prostate cancer cells, and made stable transfectants. These transfectants will be used for the mechanistic experiments described in the remaining tasks approved for AIM2, major tasks 3 and 4, over the next 12 months.

Specific Aim 3: To determine if Ref-1 inhibition sensitizes drug resistance in prostate cancers to chemotherapy. Evaluating Ref-1 expression in drug-resistant human prostate cancer metastases

We have acquired the first 12 specimens of human prostate cancer metastases from our human pathology core, as described in the proposal. Staining for APE1/Ref-1 in these specimens, and the correlation to surviving has begun. To date, statistical data is lacking as the specimen number is too low. We expect this analysis to be completed in the next year, as described on the statement of work.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

    1. Identified that APE1/Ref-1 is highly expressed in human prostate cancers.
    2. Discovered that APE1/Ref-1 is correlated to survivin expression in human prostate cancers.
    3. Published manuscript showing that inflammation induces survivin expression in experimental models and that it correlates to sites of inflammation in human prostate cancer.
    4. Found that redox-specific Ref-1 inhibition inhibits selectively prostate cancer cell growth above that of non-cancerous prostate cancer cells.

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

    We have found that the multi-functional protein APE1/Ref-1 is induced in prostate cancer cells and in human prostate cancer. This expression correlates to inflammation and to survivin signaling in human prostate cancer specimens. Genetic knockdown of APE1/Ref-1 disrupts prostate cancer cell growth and survival in cell culture. In addition, inhibition of the redox function selectively of Ref-1 results in cell growth inhibition, with this therapy preferentially inhibiting prostate cancer cell growth above that in non-cancerous cells.

    In the coming year, We will complete the experiments outlined in major task 3 and 4 of our statement of work, including the repeat of overexpression of C65 redox-dead mutant Ref-1 concurrent with endogenous protein knockdown as described above. We will also determine if Ref-1 inhibition circumvents transcriptional functional compensation triggered by STAT3 inhibitors. We will begin our experiments on our C4-2 and PC3 docetaxel-resistant cell lines to determine if redox inhibition of Ref-1 sensitizes these cells to taxane-chemotherapy. Finally, we will grow our graft tumors in animal models to assess if redox-specific inhibition of Ref-1 is effective and inhibiting prostate tumor growth, in vitro.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

      (1) Lay Press: Nothing to report
      (2) Peer-Reviewed Scientific Journals:

      McIlwain DM, Snider BM, Zoetemelk M, Myers JD, Edwards ME, Jerde TJ. Coordinated induction of cell survival signaling in the inflamed microenvironment of the prostate. The Prostate, 2015

      (3) Invited Articles: Nothing to report

      (4) Abstracts: Nothing to report

   b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

   Plenary Invitation Society of Basic Urological Research
   To be presented at the Annual Fall Meeting of the Society of Basic Urological Research, Ft. Lauderdale, FL November 11, 2015

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

   Nothing to report

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

   As shown in key research findings, we have identified a mechanism by which prostate cancer cells survive the noxious conditions of the inflamed prostatic microenvironment.

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

   Nothing to report

   For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science, Military Medicine,* etc.).

11. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

Not applicable

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not applicable

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Not applicable

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