NEW LIMITATION CHANGE

TO
Approved for public release, distribution unlimited

FROM
Distribution authorized to U.S. Gov’t. agencies only; Proprietary Info.; Sep 2000. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702-5012.

AUTHORITY
USAMRMC ltr, 8 Jan 2003
Award Number: DAMD17-99-1-9531

TITLE: Modulation of Androgen-Induced Oxidative Stress Responses as a Chemopreventive Measure in Prostate Cancer

PRINCIPAL INVESTIGATOR: Todd A. Thompson, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin - Madison
Madison, Wisconsin 53706-1490

REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Sep 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-99-1-9531
Organization: University of Wisconsin - Madison
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

[Signature]
05/01/99
### 13. ABSTRACT (Maximum 200 Words)

Changes in cellular redox status are believed to be contributing factors to the development of cancer. We have previously reported that physiologic levels of androgens induce the production of reactive oxygen species in the androgen-responsive LNCaP prostate carcinoma cell line. We hypothesize that androgen-induced changes in prostate redox status may contribute to prostate carcinogenesis. Chemopreventive measures may reduce the development of prostate cancer. This study addresses several molecular mechanisms that have been implicated in cellular redox status changes including cellular calcium homeostasis, redox-sensitive transcription factor activation, nitric oxide generation, and the role of redox-sensitive apoptotic factors. Calcium homeostasis studies are currently in progress. Activity of the redox sensitive transcription factor NF-KB was found to be induced by androgen exposure in LNCaP cells, but not in the androgen-independent DU-145 cell line. Inhibition of nitric oxide synthase activity did not change the production of androgen-induced reactive oxygen species in LNCaP cells. Therefore, nitric oxide is an unlikely mediator of androgen-induced oxidative stress in LNCaP cells. The antiapoptotic Bcl-2 whole cell protein levels were decreased by exposure to $1\text{ nM R1881}$ and $20\mu M\alpha$-tocopherol succinate whereas mitochondrial Bcl-2 protein levels were found to be increased under these conditions. The mitochondrial Bax protein levels were found to be decreased by exposure to $1\text{ nM R1881}$ and $20\mu M\alpha$-tocopherol succinate. These results may provide valuable information for pathways to investigate as targets for chemopreventive measures in prostate carcinogenesis.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover</td>
<td></td>
</tr>
<tr>
<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5 - 9</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>10</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>11</td>
</tr>
<tr>
<td>Conclusions</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>13</td>
</tr>
<tr>
<td>Appendices</td>
<td>14</td>
</tr>
</tbody>
</table>
Prostate cancer is a disease whose incidence markedly increases with age. Many factors may contribute to both the initiation and progression of prostate cancer. Androgens are known to play a role in prostate cancer development. For example, eunuchs, men whose androgen levels have been reduced through castration, do not develop prostate cancer. Yet, the role of androgens in prostate carcinogenesis is poorly understood. We have previously shown that exposure of the LNCaP androgen-responsive prostate carcinoma cell line to physiologic levels of androgen can lead to the production of reactive oxygen species (ROS). ROS have been implicated as etiological factors in aging and may have a role in the development of cancer. We hypothesize that androgen exposure may alter the redox status of the prostate gland in a way which contributes to the development of prostate cancer. Our initial efforts in addressing our hypothesis have focused on understanding the mechanism(s) through which androgens lead to the development of ROS in prostate cells. This study addresses several molecular mechanisms that have been implicated in cellular redox status changes including cellular calcium homeostasis, redox-sensitive transcription factor activation, nitric oxide generation, and the role of redox-sensitive apoptotic factors. Epidemiological studies on dietary antioxidants, including vitamin E, have reported reduced prostate cancer incidences (1). Importantly, these studies suggest that chemopreventive measures which target cellular redox pathways may be effective in reducing the age-related onset of prostate cancer. By determining which pathways contribute to changes in androgen-induced cellular redox in prostate cells we hope to identify pathways that may serve as targets for chemopreventive measures in prostate carcinogenesis.
Body

This study addresses several molecular mechanisms that have been implicated in cellular redox status changes including cellular calcium homeostasis, redox-sensitive transcription factor activation, nitric oxide generation, and redox-sensitive apoptotic factors. For the purposes of this report, the data acquired thus far is presented as Tasks as listed in the original Statement Of Work. The times listed in the original Statement of Work for each Task represent the approximate time it was estimated to perform the Task and not the chronological order in which the Tasks were intended to be performed. Therefore, the progress made for each Task varies. For example, the measurement of calcium homeostasis as specified in Task 1 has only just started whereas the initial studies for Task 3 have provided information which may require a change in the studies as specified.

Vitamin E (i.e. \( \alpha \)-tocopherol) is poorly water soluble. Also, different forms of \( \alpha \)-tocopherol have different water solubilities. In these studies \( \alpha \)-tocopherol succinate (ATS), which is converted to \( \alpha \)-tocopherol by cellular esterases and is moderately water soluble, was used. The importance of controlling the \( \alpha \)-tocopherol levels in preparations was realized due to the heterogeneity of responses observed by different investigators. For example, our original studies reported that 500\( \mu \)M concentrations of ATS were needed to abrogate androgen-induced ROS in LNCaP cells (2). In contrast, using a different method of reagent preparation, we found that 10 to 20 \( \mu \)M concentrations of ATS were sufficient to reduce androgen-induced ROS in LNCaP cells (Figure 1). A high performance liquid chromatography method was developed to standardize the ATS/media preparations used in these studies. Using this method to determine ATS levels has resulted in consistent observations between studies in our laboratory.

In all studies performed, the LNCaP androgen-responsive prostate carcinoma cell line and the androgen-independent DU-145 prostate carcinoma cell line were used. The purpose of Task 1 is to determine whether or not changes in calcium homeostasis occur after androgen exposure and ATS treatment. Determination of calcium homeostasis in these studies has only just begun. To date, it has been determined that LNCaP cells will efficiently load with the calcium sensitive probe Indo-1/AM (data not shown). This is important since not all cell lines load equally well with the Indo-1 dye. The methods used to study calcium homeostasis in prostate carcinoma cells are as originally developed for the Jurkat cell line (3), which is the cell line being used as a control for these studies. The Jurkat cell line is non-adherent whereas the LNCaP and DU-145 cell lines are adherent and require removal from the cell culture plate prior to analysis by flow cytometry (using a Becton-Dickinson FACStar, as originally proposed). The removal of adherent cells from the tissue culture plate may introduce artifact into the measurement of calcium levels using Indo-1, therefore a 96-well plate method is also
being developed that will allow measurements of Indo-1 fluorescence without removing cells from the plate. If this method is not effective, calcium levels will be determined using the calcium sensitive probe Fura-2/AM, which has been used effectively for the measurement of cellular calcium levels in cell culture plates. Thus, Task 1 is in progress and is not anticipated to require any alterations to the original proposal other than the technical consideration of supporting the flow cytometry data with analysis using a Perkin Elmer LS50B luminescence spectrometer.

We have recently reported that AP-1 and NF-κB response element binding is increased after androgen exposure in LNCaP cells and this effect is decreased by ATS treatment (2). The ability of this binding to affect transcriptional activity has not yet been evaluated. In Task 2, I proposed to investigate the modulation of AP-1 and NF-κB response elements on transcriptional regulation in LNCaP cells after androgen exposure using AP-1 and NF-κB luciferase reporter constructs. These studies were initiated by performing transient transfection analysis of the NF-κB luciferase reporter construct in LNCaP and DU-145 cells with and without androgen exposure. The NF-κB expression vector used in these studies, pNFκBTKluc, contains 3 NF-κB response elements that regulate the expression of the luciferase gene from a minimal thymidine kinase promoter. A control vector, pmutNFκBTKluc, in which the NF-κB response elements have been mutated, was also used in these studies. Exposure of LNCaP cells to 1 nM R1881 (the dose of the synthetic androgen R1881 used to produce ROS in LNCaP cells) for 2 days resulted in a 2.4-fold increase in luciferase activity compared to unexposed (F1C4) cells (Figure 2A). The increase in luciferase activity due to NF-κB response element activation has been repeated twice and found to be statistically significant. This result was specific to the NF-κB response elements as no statistically significant increases were observed under these conditions with the pmutNFκBTKluc construct (Figure 2B). Furthermore, the increase in NF-κB activity was found to be androgen-dependent since no changes in luciferase expression were observed in the androgen-independent DU-145 cell line after androgen exposure (Figure 3). Currently, these studies are being performed to determine the effects of ATS treatment on androgen-induced NF-κB activation in LNCaP cells. Also, the specificity of this response to ATS will be evaluated in the DU-145 cell line.

In addition to increases in NF-κB response element binding, we have also reported that AP-1...
response element binding is significantly increased in LNCaP cells after androgen exposure (2).

I originally used a collagenase promoter construct, which is AP-1 responsive, to assess androgen-induced modulation of AP-1 activity. Studies using this construct were criticized since response elements other than AP-1 are present in the collagenase promoter and a control construct in which the AP-1 site is mutated is not available. Therefore, an AP-1 reporter vector and the corresponding mutant control are being constructed. The techniques used to produce these constructs are routinely performed in our laboratory and should not present any problem in the continued efforts for completing Task 2. Once these constructs are available, studies comparable to those done with the NF-κB sites will be performed. Thus, Task 2 is progressing as anticipated and no changes to this protocol are requested.

Studies to assess androgen-induced ROS generation in our laboratory were performed using the peroxide/hydroxyl radical sensitive probe dichlorodihydrofluorescein diacetate (DCF). Nitric oxide (NO) has been reported to produce the same fluorescent changes in DCF as peroxide and hydroxyl radical (4). In addition, it was reported that androgen exposure significantly increases NO synthase expression in LNCaP cells (5). Since NO production would allow a very specific pathway to target redox changes in prostate cells it was proposed in Task 3 to investigate the possible role of NO in redox changes in androgen-exposed LNCaP cells. To initiate these studies, a dose-response to the non-specific NO synthase inhibitor N⁵-methyl-L-arginine (NMA) was performed in LNCaP cells after 1 nM R1881 exposure. No decrease in the production of ROS was observed after a 4 day treatment with up to 100μM NMA (Figure 4). In addition, the presence of ROS was determined following short-term exposure (i.e. 1 hour) of NMA and no changes were observed in the androgen-induced production of ROS in LNCaP cells (data not shown). Thus, it is

![Figure 3](image-url)

![Figure 4](image-url)
unlikely that NO is the source of ROS that produces DCF fluorescence after androgen exposure in LNCaP cells. We have continued to pursue a collaboration with the electron paramagnetic resonance facility at the National Biomedical EPR Center as proposed in Task 3. In addition to using spin traps that are sensitive to NO production, which will help confirm our NMA studies, I will use other spin traps (e.g. α-phenyl-tert-butylnitrone and 5,5-dimethylpyrroline-N-oxide) that will help identify the specific ROS species that are generated from androgen-exposure in LNCaP cells. Furthermore, following a suggestion from our collaborators at the National Biomedical EPR Center, I will perform an analysis of ROS production on mitochondrial preparations as well as whole cell preparations. In addition to helping determine which ROS species are responsible for the changes in redox status, this will help determine the subcellular source of ROS that develop after androgen exposure in LNCaP cells. If these studies are successful in measuring androgen-induced ROS, then further studies will be performed to determine the effect of ATS exposure on ROS production in androgen exposed LNCaP cells using EPR. Thus, I request that Task 3 be expanded to determine the specific ROS species responsible for the changes observed in redox changes in LNCaP cells after androgen exposure since our initial studies suggest that NO is an unlikely candidate as the ROS responsible for these changes. This will continue our collaboration with the National Biomedical EPR Center as originally proposed in Task 3.

Alterations in the Bcl-2 family of proteins have been observed following changes in cellular redox status. Bcl-2 proteins affect cellular apoptotic responses with some Bcl-2 family members being proapoptotic (e.g. Bax, Bak, Bad) and others acting to prevent apoptosis (e.g. Bcl-2, Bcl-xL). Androgens have been reported to modulate Bcl-2 protein levels in LNCaP cells (6). In Task 4, we have proposed to investigate the modulation of Bcl-2 family members after androgen exposure in LNCaP cells and how these changes may be affected by ATS treatment. Interestingly, we have found that androgen exposure potentiates ATS-induced apoptotic responses in LNCaP cells (see appended AACR abstract). Exposure of LNCaP cells to 0.1 nM R1881 (a concentration of R1881 that produces increased cellular proliferation in LNCaP cells) did not significantly change protein levels of Bax or Bcl-2 as measured by immunoblot analysis (Figure 5). Exposure of LNCaP cells to 1 nM R1881 for 4 days produced a decrease in whole cell Bcl-2 protein levels, which was also observed in cells treated with 1 nM R1881 and 20 μM ATS. Interestingly, the levels of Bax and Bcl-2 proteins in mitochondrial preparations were significantly altered. For example, mitochondrial Bax protein levels were decreased and Bcl-2 protein levels were increased in LNCaP cells exposed to 1 nM R1881 and treated with 20 μM ATS (Figure 5), which are changes typically associated with antiapoptotic conditions in cells. Yet, we consistently observe an increase in apoptotic cell death under these conditions. This paradoxical observation requires analysis of the remaining Bcl-2 family members in addition to a systematic evaluation of their role in ATS- and androgen-induced apoptosis before any conclusions can be drawn. Thus, the determination of Bcl-xL,
Unpublished data

Bak, and Bad protein levels in androgen exposed LNCaP cells are currently being performed as specified in Task 4 of this proposal. Bax and Bcl-2 protein levels were not altered in DU-145 cells exposed to androgen or treated with 20 μM ATS (data not shown).

In summary, I believe significant progress has been achieved in all Tasks as presented in the original Statement of Work. No problems have been encountered that should prevent these studies from being completed in the following year. Therefore, it is anticipated that all Tasks will be completed as originally proposed and Task 3 will be expanded to further investigate the specific ROS species produced by androgen exposure in LNCaP cells. In addition to the studies presented in this report, I have also made significant progress in collaborations with other investigators on the University of Wisconsin campus interested in prostate cancer. I continue to coordinate our monthly Prostate Group meeting. Also, I am currently teaching 3 lectures on prostate cancer for the U.W. Department of Pathology and 2 lectures on carcinogenesis for the U.W. Environmental Toxicology Center. Dr. George Wilding continues to be an outstanding mentor for my studies in prostate cancer. In addition to the other ongoing studies in Dr. Wilding’s laboratory, the studies in this proposal have provided valuable information for pathways to investigate as targets for chemopreventive measures in prostate carcinogenesis.
Key Research Accomplishments

- **Task 1** - Determination of intracellular calcium levels
  - Indo-1/AM calcium probe loading accomplished in LNCaP cells

- **Task 2** - Determination of AP-1 and NF-κB transcription factor activation in either AP-1 or NF-κB luciferase reporter constructs
  - NF-κB luciferase reporter construct activity stimulated by androgen in LNCaP cells
  - NF-κB luciferase reporter construct activity not stimulated by androgen in DU-145 cells

- **Task 3** - Measure reactive nitric oxide levels using spin trapping and electron paramagnetic resonance
  - Studies performed with NMA suggest that androgens do not induce NO in LNCaP cells

- **Task 4** - Measure Bcl-2 family members using Western blotting
  - Whole cell Bcl-2 protein decreased by 1 nM R1881 and 20 μM ATS
  - Mitochondrial Bcl-2 protein decreased by 1 nM R1881
  - Mitochondrial Bcl-2 protein increased by 1 nM R1881 and 20 μM ATS
  - Mitochondrial Bax protein decreased by 1 nM R1881 and 20 μM ATS
Reportable Outcomes

- 1 poster was presented at the 2000 AACR Meeting from this work - Appendix 1 Abstract
- No manuscripts have yet resulted from these studies
- No patents or licenses have been applied for or issued as a result of these studies
- No degrees have been supported by this award
- No unique cell lines, tissues, or serum has been generated from these studies
- No informatic data bases or animal models have resulted from these studies
- No funding has been applied for based on this award
- No employment opportunities or research opportunities have yet occurred as a result of this award
All of the studies proposed in this award are in progress. No problems have been encountered that should prevent these studies from being completed in the following year. The current findings suggest that transcriptional changes in NF-κB activity may play a role in androgen-induced oxidative stress. However, nitric oxide production does not appear to be a factor in androgen-induced oxidative stress in LNCaP cells. Modulation of Bcl-2 family members may also contribute to the changes observed following androgen exposure in LNCaP cells and these changes can be modified by vitamin E exposure. Significant opportunities in my study of prostate cancer have been afforded by the efforts supported by this award. For example, I have made significant progress in collaborations with other investigators interested in prostate cancer research on the University of Wisconsin campus, in the organization of prostate cancer study groups, and in prostate cancer education. Dr. George Wilding continues to be an outstanding mentor for my studies in prostate cancer. The results from this award should provide valuable data for developing strategies in chemopreventive efforts of prostate cancer.
References


Appendix 1

Vitamin E Potentiates Apoptosis in the Presence of Androgens
in the LNCaP Human Prostate Carcinoma Cell Line

Michelle M Lucido, Todd A Thompson, George Wilding, Univ of Wisconsin-Madison, Madison, WI.

Epidemiological studies suggest that a diet supplemented with vitamin E can reduce the incidence of prostate cancer, yet the role of vitamin E remains unclear. Our previous data has shown that the androgen responsive LNCaP human prostate carcinoma cell line when treated with the synthetic androgen R1881 exhibited a dose dependent increase of reactive oxygen species (ROS) and takes on more differentiated characteristics. Our subsequent data has shown that vitamin E in the form of a-tocopherol succinate (ATS) can decrease the DNA binding activity of redox-sensitive transcription factors such as AP-1 and NF-κB. The potentiation of apoptosis was investigated in LNCaP cells co-treated with vitamin E and androgens. All analysis were performed 4 days after treatment. The generation of ROS was affected with increasing treatment of 5, 10, 15, and 20 μM ATS in the presence of 1nM R1881 by a decrease of 0, 9.6, 25.8, and 46.5%, respectively. LNCaP cells treated with either 1nM R1881 or 20 μM ATS had a decrease in viability of 5.4% and 12%, respectively, whereas co-administration of 1nM R1881 and 20 μM ATS resulted in a 43.4% decrease in cell viability. Apoptotic populations increased in a dose dependent manner of R1881 in the presence of 20 μM ATS; 1nM R1881 with 20 μM ATS apoptotic populations were at least 5-fold higher than single agent treatments. Levels of the anti-apototic Bcl-2 protein were increased in the presence of R1881, whereas the addition of 20 μM ATS counteracted these effects. This data suggests that androgens act synergistically with vitamin E to alter cell signaling towards a pathway of apoptosis, which may contribute to vitamin E’s action in the prevention of prostate cancer.

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

[Signature]

PHILIS M. RINEHART
Deputy Chief of Staff for Information Management