Reduction of Androgen Induced Reactive Oxygen Species (ROS) Production as a Method of Preventing Prostate Cancer

Abstract - Prostate cancer is a leading cause of cancer deaths among men and development of a preventative agent is urgently needed. Reactive oxygen species (ROS) are carcinogens and are linked to prostate tumor development. ROS production in the prostate is linked to the acetyl polyamine oxidase (APAO) enzyme of the polyamine catabolic pathway. Inhibition of APAO should reduce ROS levels in the prostate and consequently reduce the progression of cancer. CPC-200 is an APAO inhibitor. Previously, our lab conducted studies which indicated that CPC-200 could be effective in reducing ROS levels in the prostate. The purpose of this study was to build on the previous data with additional cell culture and animal studies. Here, we confirm that CPC-200 pretreatment reduces androgen induced oxidative stress. In contrast to previous studies, the current animal studies showed no effect of the CPC-200 treatment. Additional studies are being conducted to further determine the effectiveness of CPC-200.

Christopher T. Martin / Biology and Genetics
Author Name/Major

Author Signature

George Wilding / Oncology
Mentor Name/Department

Mentor Signature

05/03/2007
Date

COVER SHEET

TITLE: ___ Reduction of Androgen Induced Reactive Oxygen Species (ROS) production as a Method of Preventing Prostate Cancer _____

AUTHOR'S NAME: __Christopher Thomas Martin _____

MAJOR: _Biology and Genetics _____

DEPARTMENT: _Oncology ____ _____

MENTOR: _George Wilding, Dawn Chruch, and Hirak Basu _____

DEPARTMENT: __Oncology _____

MENTOR(2): _____

DEPARTMENT(2): _____

YEAR: ___2007 _____

(The following statement must be included if you want your paper included in the library's electronic repository.)

The author hereby grants to University of Wisconsin-Madison the permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part in any medium now known or hereafter created.

Reduction of Androgen Induced Reactive Oxygen Species (ROS) Production as a Method of Preventing Prostate Cancer

Christopher Martin, Dawn Church, Hirak Basu, and George Wilding
Comprehensive Cancer Center, University of Wisconsin-Madison

Abstract

Prostate cancer is the second leading cause of cancer deaths among US men and no effective treatment exists to control the advanced stages of the disease. Thus, development of a preventative agent that can reduce the progression of the disease is urgently needed. Reactive oxygen species (ROS) are DNA damaging agents and have previously been directly linked to prostate tumor development. Preliminary studies have linked ROS production in the prostate to the polyamine catabolic pathway. The rate limiting enzyme in this pathway is spermidine/spermine acetyltransferase (SSAT). Androgen upregulates transcription of the SSAT gene and thus increases the rate of polyamine catabolism and ROS production in the prostate. This ROS production is a byproduct of the activity of acetyl polyamine oxidase (APAO). Thus, inhibition of APAO should reduce ROS levels in the prostate and consequently reduce the occurrence and progression of prostate cancer. N,N'-butadienyl-butanediamine MDL 72,527 (MDL, currently developed by Colby Pharmaceutical Company under the drug name CPC-200) is an APAO inhibitor. In previous studies, our laboratory tested the efficacy of CPC-200 as a chemopreventive agent in cell culture and animal models. The preliminary i*n vitro* studies were conducted with LNCaP prostate cancer cells and indicated that CPC-200 reduced androgen induced oxidative stress. In addition, three animal studies indicated that CPC-200 treatment prolonged survival and delayed the progression of prostate cancer in a mouse model. The purpose of this study was to strengthen the previous observations by conducting additional cell culture and animal studies. Here, we confirm that CPC-200 pretreatment reduces androgen induced oxidative stress in LNCaP prostate cancer cells. Animal studies were conducted with the TRAMPF1 mouse model under slightly different conditions from the previous study. These studies did not show a significant effect of the CPC-200 treatment on animal survival. This discrepancy could be explained through the natural variance of the model or through differences in treatment conditions between the studies. Additional studies are being conducted to further determine the effectiveness of CPC-200 as a chemopreventive agent in the treatment of human prostate cancer.

Background

The American Cancer Society data show that in 2006, over 27,000 men in the US died of prostate cancer *(1).* Most prostate cancer patients at the time of initial diagnosis have androgen dependent tumors that regress quickly after surgery or radiation therapy. Unfortunately, in a large number of the patients the cancer recurs as an advanced hormone refractory metastatic disease. There exists no effective therapy to control this disease in its advanced state. Thus, development of a successful preventive agent to reduce the occurrence and the progression of prostate cancer is urgently needed.

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, hydroxyl free radical and nitric oxide have been directly linked to an increase in tumor development in various

tissues, including in the prostate *(2-7)*. The ROS have direct mutagenic effects on DNA and likely play a role in carcinogenesis *(1,4,5,7)*.

In the last decade, multiple laboratories have reported that ROS levels are relatively higher in prostate tumors than in normal prostate tissues (*19*). Previous studies from ours and other laboratories have shown that androgen or androgen analogs at physiological concentrations induce the production of ROS in androgen dependent prostate cancer cells *(8-10,20)*. The exact molecular mechanism of androgen induced ROS production remains unknown. However, our preliminary studies indicate that polyamine catabolism is one of the major contributors of androgen induced ROS production in the prostate cancer cells *(11)*.

Prostate cancer, like other cancers, causes an accumulation of polyamines in the tissue (*12*), but is distinct in that the prostate itself is a source of polyamine production. Specifically, the normal prostate creates the polyamines spermidine and spermine from their precursor diamine putrescine *(12)*. The semen of healthy men contains large amounts of spermine (~ 3 mM) that originate mainly from prostatic secretion. No other human organ has such high polyamine concentrations nor does any other organ secrete polyamines *(12)*.

The polyamine catabolic pathway results in a net production of ROS (Fig. 1). Acetyl polyamine oxidase (APAO) contains FAD that is reduced to $FADH_2$ during this process. In order to regenerate active APAO, $FADH_2$ must be oxidized back to FAD. The process that regenerates active APAO produces the ROS hydrogen peroxide. Spermidine/spermine-N-acetyltransferase (SSAT) is the rate-limiting enzyme in the polyamine catabolic pathway and is found at low levels in a normal prostate *(13)*. Recent studies conducted by our laboratory demonstrate that androgen induces an overexpression of the SSAT gene *(11)*. This results in an increased rate of polyamine catabolism and an increased production of ROS in cells that overexpress SSAT (*14*).

Because the prostate normally has high levels of polyamines, SSAT induction should result in an increase of polyamine catabolism, and therefore ROS production, *specifically* in the prostate. Thus, it is likely that androgen-induced overexpression of SSAT results in an increased production of ROS in the prostate, which may contribute to carcinogenesis. Therefore, an inhibition of polyamine catabolism should reduce ROS levels in the prostate and consequently prevent the occurrence and progression of prostate cancer.

Commercial laboratories have synthesized inhibitors that can selectively inhibit each enzyme in the polyamine catabolic pathway. Previous studies by our laboratory focused on the APAO inhibitor CPC-200 (MDL). Results of the *in vitro* studies showed that CPC-200 selectively reduced androgen induced oxidative stress in prostate cancer cells *(14)*. In addition, the CPC-200 caused an accumulation of the acetylated end product of polyamine synthesis and a reduction in the starting products. This supported the hypothesis that CPC-200 acted to reduce ROS levels by acting on the polyamine catabolic pathway as an APAO inhibitor. Furthermore, preliminary efficacy studies in TRAMP and TRAMPF1 mouse models of prostate cancer indicated that CPC-200 was well-tolerated and prolonged survival by delaying tumor occurrence and tumor growth (*14*). The objective of this study was to firmly establish CPC-200 as an effective chemopreventive agent against prostate cancer in cell culture and animal models. The drug could then further be developed for clinical trials in high-risk human males.

Materials and Methods

Animals – For our *in vivo* studies with CPC-200, [C57BL/6 TRAMP x FVB]F1 (TRAMP F1) mice from our colony were used. These mice are the F1 generation of a cross between transgenic adenocarcinoma in mouse prostate (TRAMP) mice from our colony and standard laboratory white mice (FVB) purchased from Harlan Sprague Dawley (Madison, WI). The TRAMPF1 mice were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH number 85-23, 1985). The mice were housed under laminar air flow in an isolated animal unit in rooms maintained at 25-28°C and 60-70% humidity with a 12-hour light/dark cycle. A maximum of four mice were housed per sterilized cage with free access to sterilized food and water.

The TRAMP F1 mice have a transgenic sequence that is hormonally regulated by androgens *(15).* Androgens are produced in large quantities as the mouse approaches sexual maturity. This results in expression of the transgene and development of invasive prostate cancer as the mouse reaches 12-16 weeks of age. The progression of prostate cancer in the TRAMP F1 model closely mimics the observed development of human prostate cancer *(15).* The survival studies with TRAMP F1 mice were initiated at age 5-6 weeks, prior to development of the tumor. The timed sacrifice studies were initiated at age 19-20 weeks, during which time palpable prostate tumors developed in this model.

Cell lines –LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). The LNCaP cell line is androgen dependent and was established from a metastatic lesion of human prostatic adenocarcinoma *(17).* This cancer cell line grows readily *in vitro*.

Cell Culture - Prostate cancer cells were seeded into 10 cm diameter tissue culture plates with 10 ml of Dulbecco's minimal essential medium (DMEM) (Invitrogen Corportation, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Invitrogen Corportation, Grand Island, NY), nonessential amino acids and 1% equimolar mixture of streptomycin-penicllin (SIGMA, St. Louis, MO). The plates were then incubated in a humidified 95% air/5% CO_2 atmosphere. The cells were grown for at least 24 h to ensure that they were in the log phase of growth. Cells were harvested for experiments by treatment for 3 min with STV (saline A, 0.05% trypsin, 0.02% EDTA, Invitrogen Corporation, Grand Island, NY) at 37 ^{o}C. After harvest, the cells were counted on a standard hemacytometer and seeded in medium containing 4% charcoal-stripped serum plus 1% non-stripped serum. This combination of stripped and non-stripped serum depletes androgen content while limiting adverse growth effects. In all experiments, the cells were then seeded in 96-well tissue culture plates at a density of 4,000 cells per well in 200 µL of medium. 48 hours after plating the cells were treated with CPC-200 at a dose of 10µM, 17.7µM, 20µM, 26.6µM, or 30µM. Twenty-four hours after CPC-200 treatment, the cells were treated with the androgen analog R1881 (PerkinElmer, Waltham, Massachusetts). The plates were incubated for 72-96 hours and then assayed for estimation of ROS levels in intact cells and for DNA content as an estimate of the number of cells present in each well using the fluorescent dyes 2',7' -dichlorofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR) and Hoechst No. 33258 Trihydrochloride (bisBenzimide) (Sigma, St. Louis, MO), respectively.

Analysis of Oxidative Stress in vitro – Analysis of oxidative stress *in vitro* was completed through the use of a DCF assay followed by a DNA assay. The DCF assay was used as a measurement of reactive oxygen species and as an indicator of the redox status of the cells according to a standardized protocol *(9).* In brief, cell cultures were washed with 200 µL Kreb's Ringer buffer prewarmed to 37°C, incubated in dark at 37°C in 100 µL Kreb's Ringer buffer containing 10 µg/mL (final concentration) DCF dye for 45 minutes. Each 96-well culture plate was then scanned on a CytoFluor 2350™ plate scanner (Applied Biosystems, Foster City, CA) using the 485/530 nm filter excitation and emission set, then frozen at -70°C for subsequent assay of DNA content.

The DNA assay provides an estimate of the number of cells in each well and allows for standardization of the results from different plates. Prior to the DNA assay, each culture plate was equilibrated to room temperature under protection from light. Hoechst dye was then added to each well in 200 µL of high salt TNE buffer (10 mM Tris, 1mM EDTA, 2 M NaCl [pH 7.4]) at a final concentration of 6.7 µg/mL. After further incubation in dark at room temperature for 2 hours, culture plates were scanned on the CytoFluor 2350™ scanner using the 360/460 nm filter excitation and emission set. The DCF fluorescence units were normalized to the Hoechst-DNA fluorescence units for each well and used as a measure of the level of reactive oxygen species being generated. The DNA fluorescence units were also used as an indirect measure of cell growth. DCF/DNA ratings were deemed indicative of oxidative stress when the rating exceeded 100% of the control by one standard deviation.

In Vivo Survival Study of TRAMP F1 Mice – TRAMP F1 mice were treated beginning at 6 weeks of age with either a saline control or a 25mg/kg CPC-200-saline mixture. There were two groups of CPC-200 treated mice. One group (CPC-200Q2) was given an intraperitoneal (i.p.) injection once every 2 weeks over 12 weeks for a total of 7 doses, while the other group (CPC-200Q4) was given an i.p. injection once every four weeks over 12 weeks for a total of 4 doses. The vehicle group was on the same treatment schedule as CPC-200Q4. After the treatment period expired the mice were followed for overall survival. The mice were sacrificed when gross palpations indicated that the tumor had reached a diameter greater than 15mm. After sacrifice, all necropsy data was recorded and the prostate tissue was formalin fixed, paraffin blocked, microtome sectioned, H&E stained and observed for tumor formation under the microscope. The necropsy data was compared across the groups to determine whether the CPC-200 treatment had a significant effect on the survival of mice or on the progression of their prostate tumors. Mice were removed from the study when the mouse lost 15% or more of its body weight, or when other significant health issues, such as disease, warranted removal. Mice removed from the study for one of these reasons were not included in the data analysis.

In Vivo Timed Sacrifice study of TRAMP F1 mice - TRAMP F1 mice were treated beginning at 5-6 weeks of age with either a saline control or a 25mg/kg CPC-200-saline mixture. There were two groups of CPC-200 treated mice. One group (CPC-200Q2) was given an i.p. injection once every 2 weeks over 12 weeks for a total of 7 doses, while the other group (CPC-200Q4) was given an i.p. injection once every four weeks over 12 weeks for a total of 4 doses. The vehicle group was on the same treatment schedule as CPC-200Q4. After 12 weeks, the treatment was stopped and the mice were observed for an additional two weeks. In week 14 of the study all mice were sacrificed. Prior to sacrifice, a subset of the mice were injected with hydroethidine dye as described below. After sacrifice, all necropsy data was recorded and the prostate tissue,

seminal vesicles, kidney, and tumor were formalin fixed, paraffin blocked, microtome sectioned, H&E stained and observed for tumor formation under the microscope. The histology and necropsy data was compared across the groups to determine whether the CPC-200 treatment had a significant effect on the incidence or progression of prostate cancer in this model. Mice were removed from the study when the mouse lost 15% or more of its body weight, or when other significant health issues, such as disease, warranted removal. Mice removed from the study for one of these reasons were not included in the data analysis.

Hydroethidine Treatment - The dye hydroethidine (HEt) (Polysciences Inc., Warren, PA) is oxidized to ethidium by superoxide *(18).* This oxidation reaction causes fluorescence when HEt is exposed to ROS and can therefore be used as an indication of oxidative stress. HEt was dissolved in DMSO (80 mg/ml) and diluted in isotonic saline to 0.8 mg/ml. The dye was injected into TRAMP F1 animals through the tail vein at 8 mg/kg. At this concentration the dye is non-toxic. One hour after injection with HEt the animals were sacrificed and their tissues were collected as described in the timed sacrifice study. Fluorescence microscopy was performed using 488 excitation, 595 emission in a Olympus VX-51 fluorescence microscope (Melville, NY). The relative fluorescence of the various tissues was used as an indicator of ROS formation.

Results

CPC-200 reduces androgen induced oxidative stress in LNCaP cells – LNCaP cells were treated with either 10 μM, 20 μM, or 30 μM CPC-200. Twenty four hours later they were treated with a graded concentration of the androgen analog (R1881) ranging from 0 - 2nM. The cells were incubated for 72 hours and then harvested for the DCF assay and subsequent DNA assay. DCF/DNA ratios were deemed significant when the reading exceeded 100% of the control by one standard deviation. These studies show that androgen treatments result in a significant decrease in DCF/DNA units at treatments of 0.0156, 0.0313, and 0.0625 nM R1881 (84%, 79%, and 82% control respectively) (Figure 2). This is indicative of a decrease in oxidative stress at these levels of androgen treatment. These data correlate well with the previous studies conducted in our laboratory (*9*).

In addition, these studies show that androgen treatment significantly increases the DCF/DNA reading at R1881 treatments of 0.5, 1, and 2 nM (149%, 159%, and 149% control respectively) (Figure 2), which also correlates with the previous studies conducted in our laboratory (*9*). Thus, androgen induces oxidative stress at treatments greater than 0.5 nM.

The 30 μM CPC-200 pretreatment kept the DCF/DNA reading within one standard deviation of the control for all tested levels of androgen treatment. This suggests that 30 μM CPC-200 completely blocked both the ROS reducing as well as the ROS inducing effects of androgen at all androgen concentrations tested (Figure 2). The 10 and 20 μM CPC-200 treatments did not have a significant effect on the androgen induced oxidative stress.

Furthermore, the CPC-200 pretreatments had no significant effect on oxidative stress in the absence of R1881 (Figure 3). This suggests that the CPC-200 effect is specific to an androgen induced pathway. Overall, these data correlate with the previous cell culture studies conducted in our laboratory (*11*) and support our hypothesis that CPC-200 reduces androgen induced ROS levels by inhibiting the androgen induced polyamine catabolic pathway.

CPC-200 is stable in solution for a minimum of 10 months – The CPC-200 used by our laboratory in previous animal and cell culture studies was prepared from different stocks and at different times than the CPC-200 used in the current studies. In order to demonstrate that the efficacy of the drug had not been affected by differences in handling or methods of preparation, we ran a series of comparison studies. We used three different batches of CPC-200. The first and second batches had been previously used and had shown effectiveness in reducing androgen induced oxidative stress (*11*,*14*). The third batch was used in this study and showed similar effectiveness in reducing androgen induced oxidative stress in cell culture (Figure 2).

For the current cell culture comparison study, the first batch had been kept in solution for 25 months, the second batch 10 months, and the third batch less than one month at -20 ^{o}C. Both the second and third batches of CPC-200 showed a similar efficacy in blocking androgen induced oxidative stress (batch 3 data in Figure 2, batch 2 data not shown). This indicates that the CPC-200 from the two stocks is of similar efficacy to that seen in the earlier studies (*14*), and that CPC-200 is stable in solution for a minimum of 10 months. However, the CPC-200 from batch 1 showed no efficacy in reducing androgen induced oxidative stress (Figure 4), whereas it had indeed shown efficacy when it was originally used in the preliminary studies (*11*). This indicates that CPC-200 may not be stable for 25 months in solution. Future studies should make use of CPC-200 that has been in solution for 10 months or less in order to minimize the possibility of losing drug efficacy.

CPC-200 treatment has no overt toxic effects on animals - The body weight of the mice was measured once weekly. The CPC-200 treated mice showed no significant difference in body weight as compared to the vehicle control (Figure 5). Thus, the CPC-200 treatments were not overtly toxic to the animals at a 25 mg/kg dose given once monthly or once biweekly. These data held true for both the timed sacrifice study as well as the survival study.

CPC-200 treatments did not show any effect on time to tumor incidence or overall survival - The time to tumor incidence was determined by palpating mice from the survival study once weekly. Palpable tumors appeared in 40%-60% of mice by 16 weeks of age in all treatment groups (Figure 6a). Thus, treatment with CPC-200 had no observed effect on the time to tumor incidence for mice.

Survival study mice were sacrificed when the diameter of the tumor exceeded 15mm. Mice that had to be sacrificed for a reason other than tumor size were not included in the final data set. One hundred percent of mice in all groups survived until 16 weeks of age. Subsequently, there was a steady loss of mice each week. By 24 weeks of age, survival had dropped to 50% in each CPC-200 treatment group and to 57% in the vehicle group. By 33 weeks of age, survival was below 25% in all groups, and by 51 weeks of age survival had dropped to 0% in all groups. Overall, there was no significant difference in the survival of the mice amongst the three treatment groups (CPC-200Q4 $p=0.32$, CPC-200Q2 $p=0.45$, student's t-test) (Figure 6b).

CPC-200 treatments had no detectable effect on the pathology of prostate tissues in vivo – Mice in the timed sacrifice study were all sacrificed at a similar age (19-20 weeks). At this age, only five mice had macroscopic tumor development (two out of ten vehicle treated and three out of eight CPC-200Q2 mice). However, it remained a possibility that microscopic changes had occurred in the prostate. In order to determine whether CPC-200 affected the pathology of the

prostate, the TRAMPF1 tissues from both CPC-200 treated and untreated animals were observed by a pathologist at the University of Wisconsin – Madison Comprehensive Cancer Center, Dr. Weixiong Zhong. Dr. Zhong examined the tissues and reported on the type and extent of development of tumors that had occurred on the microscopic level. The grading system used was based on the publication from Kaplan-Lefco *et. al*., 2003 (*15*). In some cases, the pathologist found several stages of cancer within one mouse. Here, we report only the worst stage of cancer observed for each mouse.

Dr. Zhong found that the vehicle treated mice had the greatest percentage of well-differentiated carcinoma and the lowest percentage of poorly differentiated carcinoma (60% and 20% respectively). The CPC-200Q4 treatment group had high percentages of poorly differentiated carcinoma and the lowest percentage of well differentiated carcinoma (42.9% and 14.3% respectively). The CPC-200Q2 treatment group had similar percentages of both the poorly and the well differentiated carcinoma (50% and 37.5% respectively) (Figure 7). These data seem to indicate that the vehicle group suffered fewer of the advanced stage tumors than did either of the CPC-200 treated groups. However, the subjectivity of the observer and the relatively few tissue samples used in these studies preclude a definitive conclusion.

CPC-200 treatments did not show any effect on oxidative stress in vivo - In order to determine the effect of CPC-200 on oxidative stress *in vivo,* three CPC-200 treated and three vehicle treated mice from the timed sacrifice study were treated with HEt prior to sacrifice, which was two weeks after the last administration of CPC-200 or the vehicle control. All tissues were observed under a fluorescence microscope for fluorescence intensity. The tissues were scored based on a relative scoring system developed in the laboratory (Figure 8a). Both the CPC-200 and the vehicle treated mice had an average "+" rating of 2 for the anterior prostate (AP) and of 0.4 for the tumor tissues. This suggests that on average, normal prostate is under relatively greater oxidative stress than is the tumor tissue. In addition, we observed no significant difference in fluorescence between the tissues of the CPC-200 treated animals and the vehicle control (Figure 8b). Because the fluorescence is indicative of ROS formation, these data suggest that CPC-200 had no effect on oxidative stress *in vivo* two weeks after the last treatment.

Discussion

The summary of the *in vitro* data suggests that CPC-200 treatment is able to completely block both the ROS reducing as well as the ROS inducing effects of androgen *in vitro* in LNCaP prostate cancer cells. Furthermore, these *in vitro* data suggest that the CPC-200 effect is specific to an androgen induced pathway. These data correlate with the preliminary cell culture studies published from our laboratory (*11*) and support our hypothesis that CPC-200 reduces ROS levels by inhibiting the androgen induced polyamine catabolic pathway.

However, the current *in vivo* survival data do not support the data from the previous survival studies. In the current studies, CPC-200 did not delay the progression of prostate cancer in TRAMPF1 mice and did not increase the likelihood of survival for treated TRAMPF1 mice. This is in contrast to the previous studies performed in our laboratory in which the CPC-200 treatment both delayed the time to tumor incidence and prolonged survival of the treated mice (*14*).

One major difference between the previous survival studies and the current survival studies was the age at which the mice received treatment. TRAMPF1 mice in the previous study

received treatment from 8-20 weeks of age. TRAMPF1 mice in the current study received treatment from 6-18 weeks of age. Thus, mice in the current study started and stopped treatment at earlier ages than the mice that were used in the previous studies. Androgens in the mouse reach their peak levels as the mouse approaches puberty at eight weeks of age (*22*). Thus, mice in the current study received their initial CPC-200 treatments at a time when androgen concentrations were possibly lower than that of adult mice, whereas the mice in the previous studies (*11*) received the entirety of their treatments after androgen was at or near its adult levels. The *in vitro* data show that CPC-200 has no effect in the absence of androgens. Therefore, the fact that mice in the previous study received all of their treatments after they had attained normal serum androgen levels may account for the variation between the previous data and the current study.

Alternatively, it is possible that the difference between these studies is attributable to the natural variance of the TRAMPF1 model. In both studies, the CPC-200 treated mice showed similar longevity and the median survival in both studies was 21 weeks of age (see Figure 6B in the current study and ref. *11*). The median survival of vehicle treated mice from both studies was 20 weeks of age. However, in the previous study all vehicle treated mice had died by 26 weeks of age. This is in contrast to the current study in which three mice lived until 32 weeks of age. The longevity of those three mice may account for the lack of significant separation between the CPC-200 treated and the vehicle control groups in the current study. It is possible that those three mice were simply aberrations that can be accounted for in the natural variance of the model.

In order to analyze this variance, we collaborated with a statistician in the University of Wisconsin Comprehensive Cancer Center Biostatistics Department, Dr. Mary Lindstrom. Dr. Lindstrom conducted an analysis across both the first and second TRAMPF1 studies in an attempt to determine the natural variance of the model. She found that if these studies do indeed represent the natural variability of the model, greater than 90 mice per treatment group would be necessary in order to observe a statistically significant effect of the CPC-200 treatment. Currently, our laboratory is conducting additional animal studies with the TRAMPF1 mouse model in an attempt to test the statistical significance of the observation.

The variability seen between these studies may be attributable to the molecular mechanism through which the mice develop cancer. The expression of androgen activates a transgene (*15).* This transgene interferes with and leads to the inactivation of p53 (*15).* p53 affects many cellular processes and plays an important role in regulating the cell cycle and DNA repair (*23*). Thus, disruption of p53 in the prostate leaves the tissue susceptible to DNA damage from many types of mutagens.

The expression of the transgene is known to be detectable by as early as eight weeks of age (*15*). However, it is possible that the transgene is expressed at an earlier age at a lower serum androgen level, possibly in the weeks that lead up to puberty. Therefore, the mice may become susceptible to damage from a variety of mutagens other than ROS at an early age. Androgen induced ROS production does not occur until the androgens reach normal levels at the onset of puberty (*9*). Thus, the mice may develop tumors as a result of p53 inactivation prior to and separate from the onset of ROS production. In addition, it is possible that the CPC-200 shielded the treated mice from the effects of ROS, but that some other carcinogenic effect ultimately lead to the tumor formation in this set of animals. Therefore, the susceptibility of this model to many types of mutagens may account for the variability seen between the studies.

Furthermore, it is possible that a model more specific to the effects of ROS will be needed in order to conclusively demonstrate an effect of CPC-200 treatment. Along those lines, our laboratory is in the process of developing a more suitable mouse model that specifically induces a transgene responsible for ROS production. Studies with these animals may be necessary to establish the role of ROS in prostate tumor development.

Also in our current study, there was an apparent trend in the pathology data in that the worst stages of cancer were more prevalent in the CPC-200 treated groups than in the vehicle control (Figure 7). However, the pathology grading system used in this study is very subjective. Different pathologists often give significantly different ratings to the same tissue samples and a single pathologist may give a different rating to the same sample upon subsequent viewings. Our pathologist had only a single viewing of the tissues before tabulating the readings shown here. Thus, we do not feel that the apparent trends are significant. In the future, we will be moving to a more quantitative measurement of tissue pathology that uses specific molecular markers to differentiate between the different stages of cancer (*21*). This new method, now being standardized in the University of Wisconsin – Comprehensive Cancer Center, should decrease the subjectivity in measurements and increase the significance of the data.

Lastly, we observed no significant effect of CPC-200 on oxidative stress *in vivo* two weeks after the last CPC-200 treatment had been completed (Figure 8). It is possible that the CPC-200 treatment did indeed have an effect on oxidative stress *in vivo*, but that this effect did not last over the two week lapse between the last CPC-200 treatment and the date of sacrifice. Thus, we cannot exclude the possibility that CPC-200 had a short term effect on the oxidative stress *in vivo*. Currently, our laboratory is conducting studies in which a CPC-200 injection is given 24 hours prior to the injection of HEt. These studies should help to clarify whether or not CPC-200 is able to have a short term effect on oxidative stress *in vivo*.

Overall, cell culture data strongly indicates that MDL acts to reduce ROS levels by inhibiting the androgen induced polyamine pathway. The observed discrepancies between the *in vivo* data from the current study and the previous studies could be explained through the natural variation of the model or through differences in treatment conditions. We will conduct additional animal studies with the TRAMPF1 model and will continue with the development of a new animal model that more specifically examines the role of ROS production in tumor development.

Literature Cited

1. Cancer Reference Information. http://www.cancer.org/docroot/STT/content/STT_1x_Cancer Facts Figures_ 2007.asp (accessed April 2007).
2. Cerutti, P.A. Prooxidant states and tumor promotion. *Science* 1985, 227: 375-381.
3. Eeles, R.A., and Powles, T.J. Chemoprevention options for BRCA1 and BRCA2 mutation carriers. *J Clin Oncol*. 2000, 18(21 Suppl):93S-9S.
4. Lee, W.H., Morton, R.A., Epstein, J.D., Brooks, J.D., Campbell, P.A., Bova, G.S., Hsieh, W.S., Isaacs, W.B., and Nelson, W.G. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl Acad Sci* 1994, 91:11733-37.
5. Oberley, T.D., and Oberley, L.W. Oxygen radicals and cancer. In: Yu, BP (ed), F*ree Radicals In Aging* 1993, 247-267.

6. Rautalahti, M., and Huttunen, J. Antioxidants and carcinogenesis. *Ann Med* 1994, 26:435-441.
7. Wilding, G. Endocrine control of prostate cancer. *Cancer Surveys* 1995, 23:43-62.
8. Ripple, M.O., Hagopian, K., Oberley, T.D., Schatten, H., and Weindruch, R. Androgen-induced oxidative stress in human LNCaP prostate cancer cells is associated with multiple mitochondrial modifications. *Antiox. Redox Signal* 1999, 1:71-81.
9. Ripple, M.O., Henry, W.F., Rago, R.P., and Wilding, G. Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. *J Natl Cancer Inst* 1997, 89:40-48.
10. Ripple, M.O., Henry, W.F., Schwarze, S.R., Wilding, G., and Weindruch, R. Effect of antioxidants on androgen-induced AP-1 and NF-κB DNA-binding activity in prostate carcinoma cells. *J Natl Cancer Inst* 1999, 91:1227-1232.
11. Clower, C., Thompson, T.A., Church, D., Lopez, C., Woster, P., Basu, H.S., and Wilding, G. Androgen Induced Polyamine Catabolic Enzyme Expression is a Major Cause of Oxidative Stress in Prostate Cancer Cells. AACR annual meeting, 2005, Anaheim, CA.
12. Cohen, S.S. A guide to the polyamines. *Oxford University Press* 1998, 296-319.
13. Schipper R.G., Deli G., Deloyer P., Lange W.P., Schalken J.A., and Verhofstad A.A. Antitumor activity of the polyamine analog N(1), N(11)-diethylnorspermine against human prostate carcinoma cells. *The Prostate* 2000, 44(4):313-21.
14. Basu HS, Thompson TA, Clower C, Church D, Mehraein F, Amlong C, Madapathage T, Woster P and Wilding G. An inhibitor of acetyl polyamine oxidase specifically blocks androgen induced oxidative stress and prevents occurrence of prostate cancer in TRangenic Adenocarcinoma of Mouse Prostate (TRAMP). The 18th Annual EORTC-AACR-NCI Conference on Molecular Targets and Cancer Therapeutics, Nov. 2006, Prague, Czech Republic.
15. Kaplan-Lefko, P.J., Chen, T., Ittmann, M.M., Barrios, R.J., Ayala, G.E., Huss, W.J., Maddison, L.A., Foster, B.A., and Greenberg, N.M. Pathobiology of autochthonous prostate cancer in pre-clinical transgenic mouse model. *The Prostate* 2003, 55:219-237.
16. Klein, K.A., Reiter, R.E., Redula, J., Moradi, H., Zhu, X.L., Brothman, A.R., Lamb, D.J., Marcelli, M., Belldegrun, A., Witte, O.N., and Sawyers, C.L. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nature Medicine* 1997, 3(4):402-408.
17. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Ming Chu, T., Mirand, E.A., and Murphy, G.P. LNCaP model of human prostatic carcinoma. *Cancer Research* 1983, 43:1809-1818.
18. Zhao H., Kalivendi S., Zhang H., Joseph J., Nithipatikom K., Vasquez-Vivar J., and Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med.* 2003, 34(11):1359-68.
19. Oberley, T.D., Zhong, W., Szweda, L.I., and Oberley, L.W. Localization of antioxidant enzymes and oxidative damage products in normal and malignant prostate epithelium. *The Prostate* 2000, 44: 144-155.
20. Pathak, S.K., Sharma, R.A., Steward, W.P., Mellon, J.K., Griffiths, T.R., and Gescher, A.J. Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies. *Eur J Cancer* 2005, 41(1): 61-70.

21. Witkiewicz, A.K., Varambally, S., Shen, R., Mehra, R., Sabel, M.S., Ghosh, D., Chinnaiya,n A.M., Rubin, M.A., and Kleer, C.G. Alpha-methylacyl-CoA racemase protein expression is associated with the degree of differentiation in breast cancer using quantitative image analysis. *Cancer Epidemiol* 2005, 14(6):1418-23.
22. Wilks-Martinello, R., Dane, A., Mortensen, E., Jeyakumar, G., Wang, X.Y., and Russel, P.J. Application of the transgenic adenocarcinoma mouse prostate (TRAMP) model for pre-clinical therapeutic studies. *Anticancer Research* 2003, 23:2633-2642.
23. Levine, J.A. p53, the cellular gatekeeper for growth and division. *Cell* 1997, 88: 323–331.

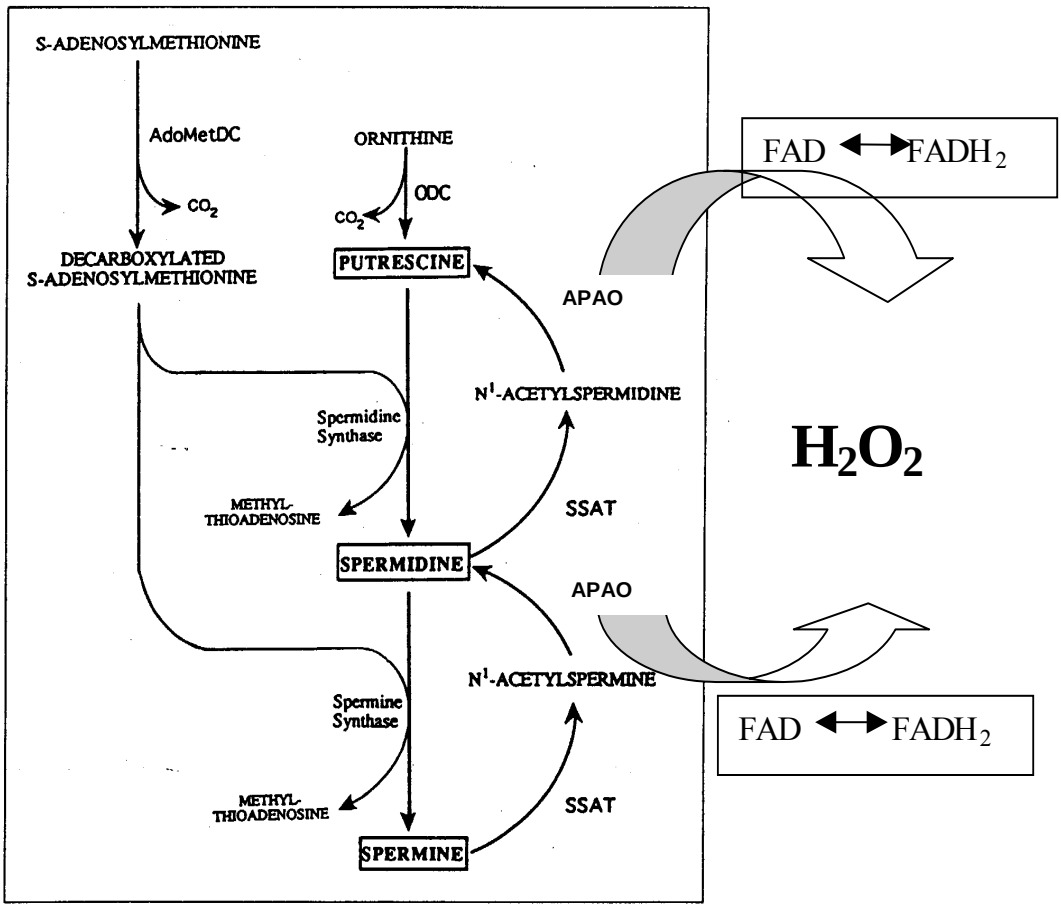


Fig. 1: The polyamine biosynthetic/catabolic cycle results in a net production of ROS. Acetyl polyamine oxidase (APAO) uses FAD that is reduced to $FADH_2$ during this process. In order to regenerate active APAO, $FADH_2$ must be oxidized back to FAD. The process that regenerates active APAO creates the ROS hydrogen peroxide

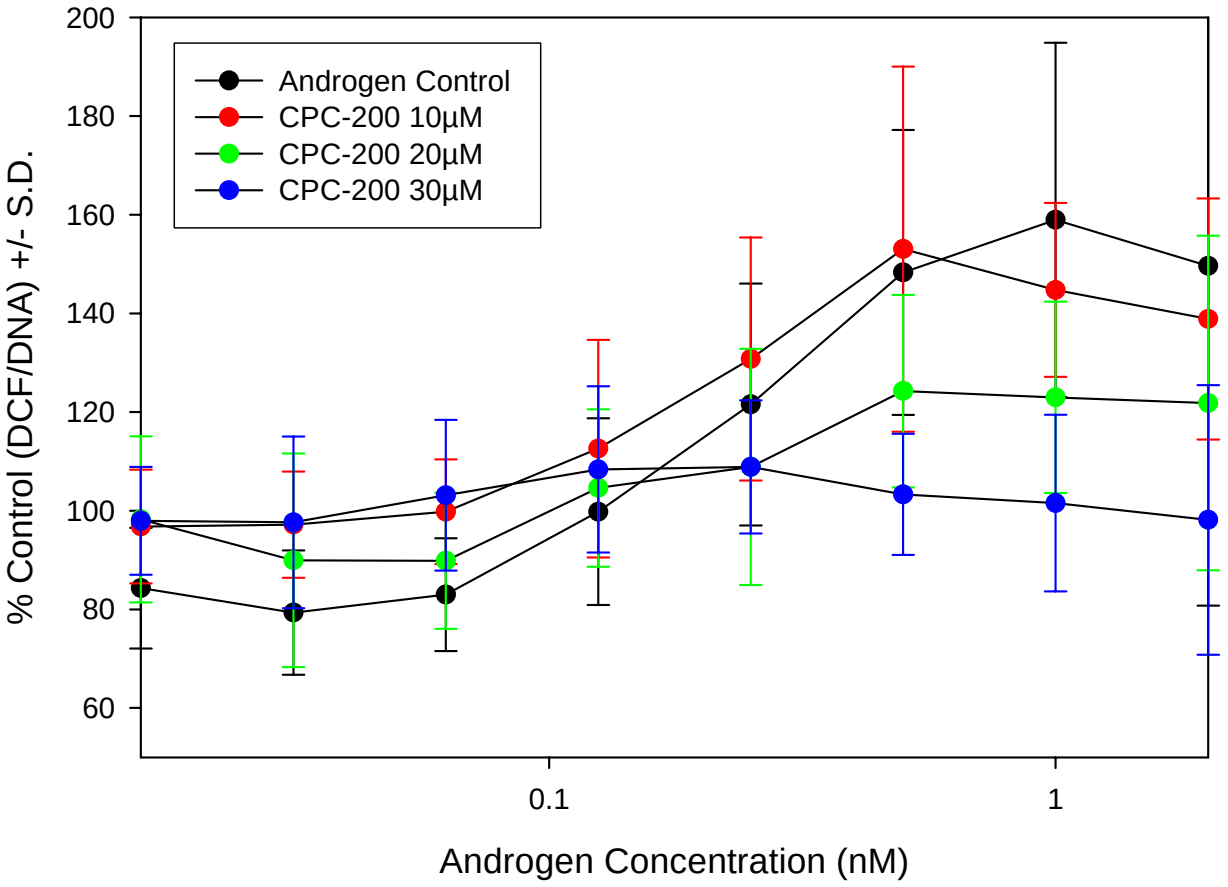


Fig. 2: Effect of CPC-200 Treatment on Androgen Induced Oxidative Stress. LNCaP cells were treated with a serial dilution of androgen from 2nM to 0nM in androgen depleted medium. Twenty four hours later, the cells were treated with CPC-200 to a final concentration of 10, 20, or 30µM. The androgen control received only androgen. The level of oxidative stress was determined through a DCF assay and was normalized to the number of cells in culture using a DNA assay. All CPC-200 data were normalized to the effect of CPC-200 alone. The data are reported as the mean ± S.D. of 12 replicate wells expressed as a percentage of the mean of the control wells that received no androgen or CPC-200 treatments.

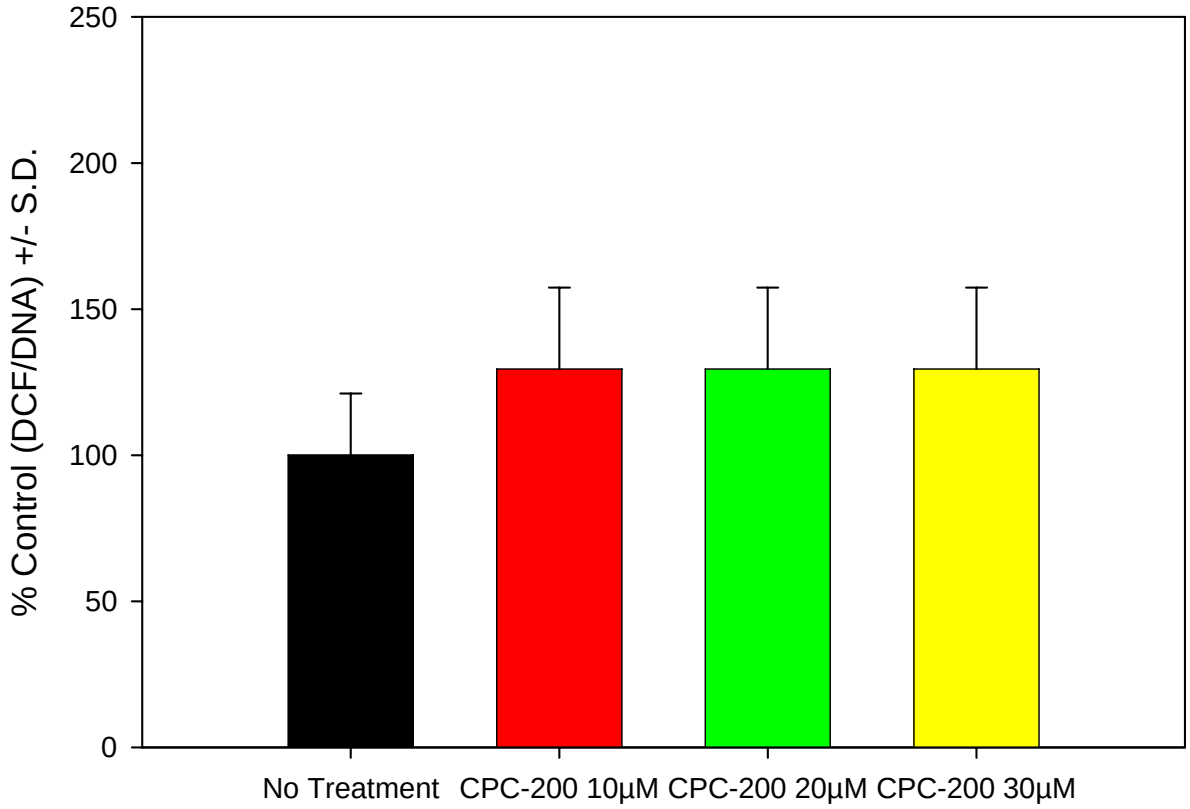


Fig. 3: Effect of CPC-200 on Oxidative Stress in the Absence of Androgen. LNCaP cells in androgen depleted medium were either treated with CPC-200 at a final concentration of 0, 10, 20, or 30µM. The level of oxidative stress was determined through a DCF assay and was normalized to the number of cells in culture using a DNA assay. The data are reported as the mean ± S.D. of 12 replicate wells expressed as a percentage of the mean of the control wells that received no CPC-200 treatments.

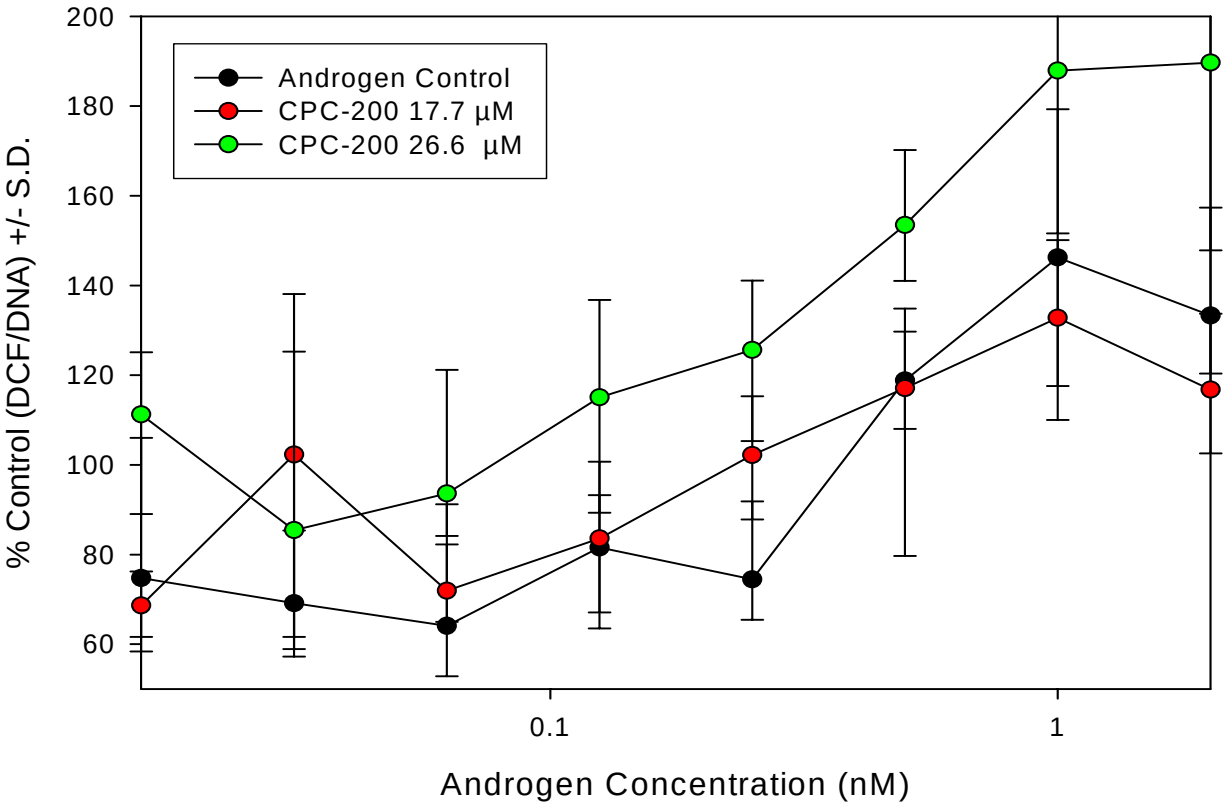


Fig. 4: Efficacy of CPC-200 in Solution for 25 Months at -20 °C. LNCaP cells were treated with a serial dilution of androgen from 2nM to 0nM in androgen depleted medium. 24 hours later the cells were treated with batch 1 CPC-200 that had been in solution for 25 months to a final concentration of 17.7 or 26.6 µM. The androgen control received only androgen. The level of oxidative stress was determined through a DCF assay and was normalized to the number of cells in culture using a DNA assay. The data are reported as the mean ± S.D. of six replicate wells expressed as a percentage of the mean of the control wells that received no androgen or CPC-200 treatments.

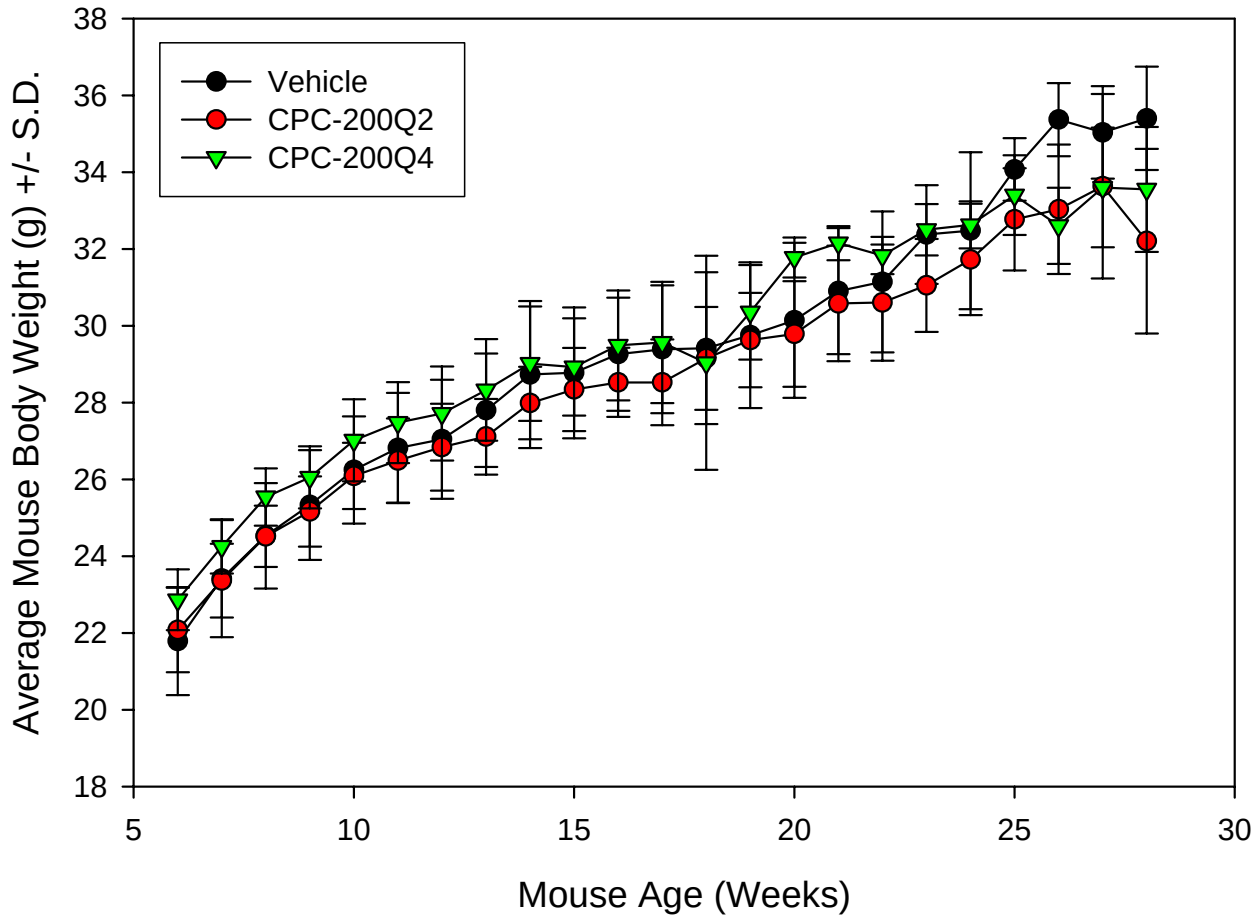
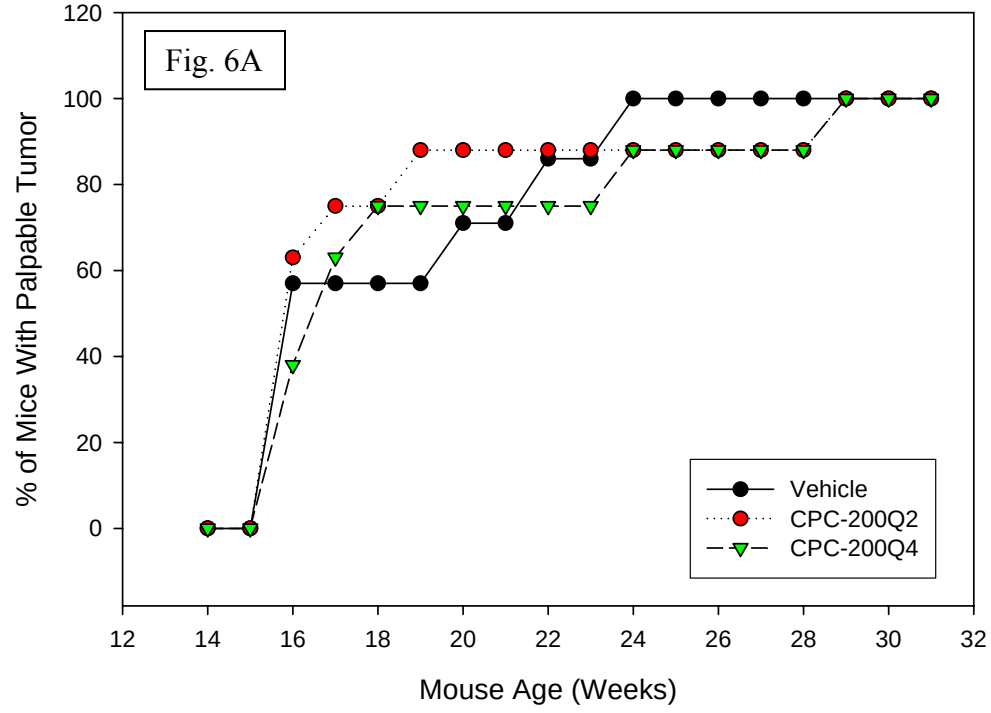


Fig. 5: Effect of CPC-200 on Average Mouse Body Weight. The CPC-200 treatment groups were given i.p. injections of a 25mg/kg CPC-200-saline mixture every two weeks (CPC-200Q2, n=8) or once monthly (CPC-200Q4, n=8) over 12 weeks. Vehicle mice (n=7) were treated monthly with i.p. injections of the vehicle control solution. Body weight was measured weekly. The values are the mean body weight of all mice in the group ± S.D.



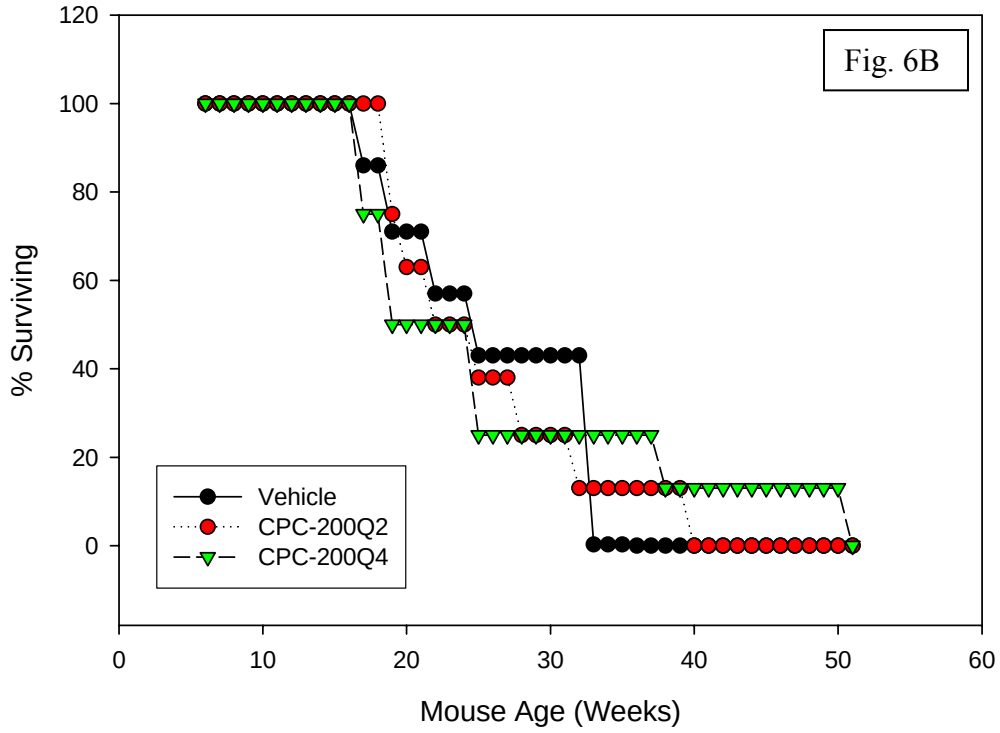


Fig. 6: Effect of CPC-200 on Time to Palpable Tumor (A) and Survival (B). The CPC-200 treatment groups were given i.p. injections of a 25mg/kg CPC-200-saline mixture every two weeks (CPC-200Q2, n=8) or once monthly (CPC-200Q4, n=8) over 12 weeks. Vehicle mice (VEH, n=7) were treated monthly with i.p. injections of the vehicle control solution. The presence of tumor was determined by a weekly palpation of the mouse abdomen. The values are the percentage of mice in the treatment group that had palpable tumors (A). Mice were sacrificed when the diameter of their tumor exceeded 15mm. Values are expressed as a percentage of mice surviving in the treatment group (B).

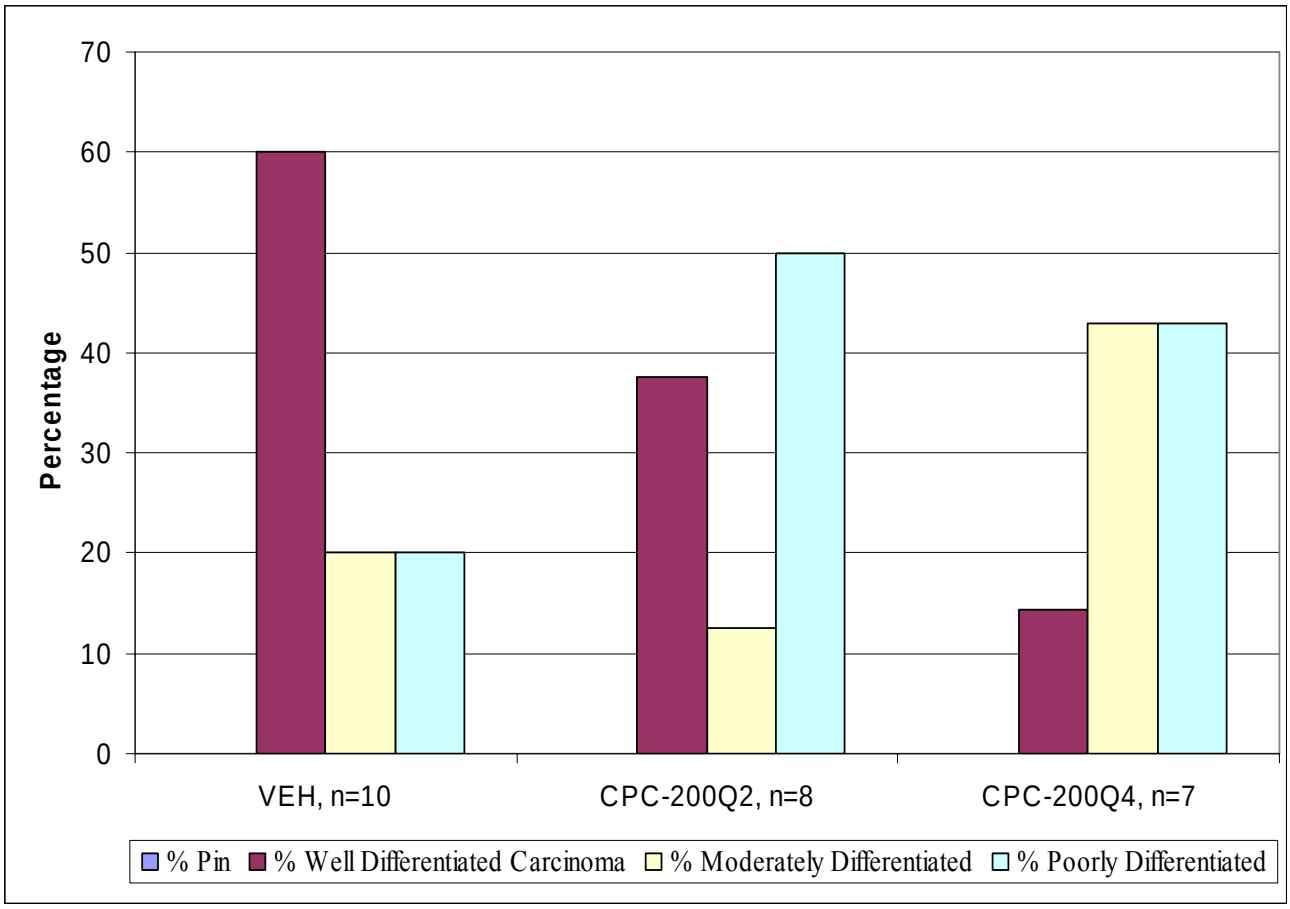


Fig. 7: Effect of CPC-200 on Tissue Pathology in the Timed Harvest Efficacy Study. The CPC-200 treatment groups were given i.p. injections of a 25mg/kg CPC-200-saline mixture every two weeks (CPC-200Q2, n=8) or once monthly (CPC-200Q4, n=7) over 12 weeks. Vehicle mice (VEH, n=10) were treated monthly with i.p. injections of the vehicle control solution over 12 weeks. In week 14 of the study, all mice were sacrificed. The prostate tissue, seminal vesicles, kidney, and tumor (if present) were formalin fixed, paraffin blocked, microtome sectioned, H&E stained and observed under the microscope by a pathologist. In some cases, multiple stages of cancer were found within the same animal. Here, we report only the worst stage of growth. Values are expressed as a percentage of the animals with the indicated stage of cancer in the treatment group.

Fig. 8A

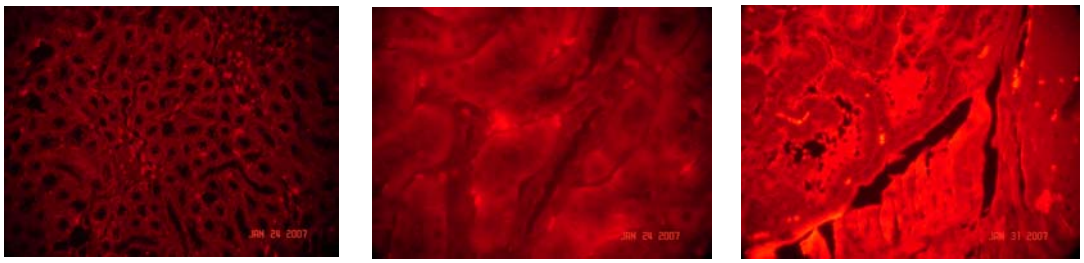


Fig. 8B

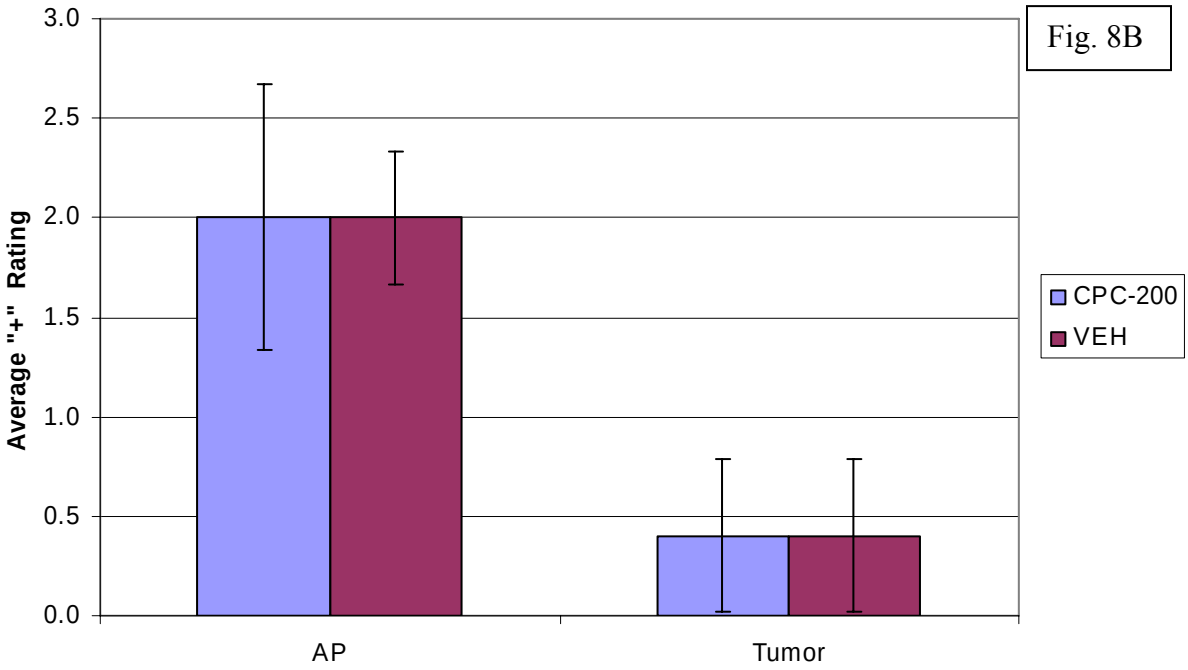


Fig 8: Representation of Relative Scoring Used for HEt Analysis (A) and Relative Oxidative State of Prostate Tissues in CPC-200 vs. Vehicle Treated Mice (B). The CPC-200 treatment group was given i.p. injections of a 25mg/kg CPC-200-saline mixture every two weeks (CPC-200, n=3) over 12 weeks. The vehicle treatment group (VEH, n=3) was given an i.p. injection of a saline control mixture once monthly over 12 weeks. Two weeks after the end of the treatment, the animals were given one injection of HEt at 8mg/kg. One hour after injection with HEt, the animals were sacrificed and a portion of their tumor and anterior prostate (AP) were collected and fixed in paraffin blocks following a published procedure *(19).* Fluorescence microscopy was conducted and the fluorescence of the tissues was scored based the relative "+" system developed in our lab. These three slides (A) were chosen as standard measurements of low levels of fluorescence (+), medium fluorescence (++), or high fluorescence (+++). The + slide is from tumor, while the ++, and +++ slides are both from anterior prostate. All other slides were given a score that was relative to these standards. The values are expressed as the mean ± S.D. of the three mice in each treatment group (B).