

## Exploring the Therapeutic Effects of Bisphenol-A on Prostate Cancer

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ORIGINAL  
ARTICLE



**Abstract:** Prostate cancer (PCa) is the most common cancer in American men and its metastatic spread is responsible for its high mortality rates. Since PCa cells evolve against various treatment approaches, there is an urgent need for new treatment solutions. Bisphenol A (BPA) is an industrial chemical with structural similarities to androgens and estrogens and is postulated to interfere with androgen receptor (AR) and estrogen receptor function by competitive inhibition. Previous studies reported that low concentrations of BPA stimulate the proliferation of cancer cells expressing AR, whereas high concentrations of BPA unexpectedly inhibited the growth of those cells. This prompted the hypothesis that high concentrations of BPA might kill AR-positive, treatment-resistant PCa cells. In this study, the effect of high concentrations of BPA on AR-positive, Enzalutamide (a recent oral AR inhibitor)-resistant PCa cells were evaluated. At high concentrations, BPA was found to reduce cell growth by 90% and to trigger cell death. To explore the underlying molecular mechanisms, the expression and activity of AR were examined; both were decreased upon treatment of high BPA concentrations. In summary, high concentrations of BPA can inhibit cell growth and stimulate cell death of Enzalutamide-resistant PCa by targeting AR signaling. These findings may pave the road for novel approaches for treatment-resistant PCa.

**Keywords:** *Castration-Resistant Prostate Cancer; Bisphenol A; Androgen-AR Pathway*

### I. INTRODUCTION

Prostate cancer (PCa) is currently both the most prevalent and the second deadliest cancer for American men [1]. Hormonal therapy and chemotherapy are used to treat advanced PCa, but frequently can only palliate the disease. Currently, advanced PCa is incurable and there is an urgent need to understand PCa development.

Androgens are a group of male hormones vital for normal prostate cell growth [2]. The androgens bind to and activate the androgen receptor (AR), a transcription factor, in the cytoplasm. The resulting androgen-AR complex then translocates to the nucleus to bind to DNA and trigger a series of AR target gene expression or suppression essential for prostate cell growth. Since Huggins and Hodges' breakthrough study proving that PCa cells heavily rely on androgens for survival and growth, androgen-deprivation therapy (ADT), a hormonal therapy focusing on the reduction of androgen levels through chemical or surgical means, has served as the standard treatment for advanced PCa [3]. The depletion of hormones effectively causes the PCa to regress. However, after 1 to 3 years, PCa inevitably develops a resistance and progresses into castration-resistant PCa (CRPC) [4].

Research has convincingly demonstrated that CRPC has been shown to exhibit active AR signaling despite ADT [5]. Accordingly, scientists have been exploring a variety of approaches to more effectively block AR signaling as PCa cells develop resistance to treatments. The current frontline treatment for advanced PCa is Enzalutamide, an AR antagonist that prevents AR binding to DNA, blocks PCa cell growth, induces cell death, and shows great promise on patients who developed a resistance to earlier generations of AR antagonists [6]. However, most men eventually develop a resistance to Enzalutamide within 4 to 6 months [7]. Therefore, novel approaches are desperately needed in order to design novel approaches for treatment-resistant PCa.

Bisphenol A (BPA) is an industrial chemical that is widely used in everyday products like polycarbonate plastics and epoxy resins. BPA has a chemical structure similar to estrogen. Previous studies have shown that BPA functions as an endocrine disruptor. It binds to and interrupts estrogen receptor signaling [8]. Furthermore, BPA has been reported to similarly interfere with AR function by competitive inhibition of androgen binding [9].

Previous studies reported that while lower concentrations of BPA stimulated the proliferation of cancer cells expressing AR, higher concentrations of BPA unexpectedly inhibited their proliferation [10, 11]. The reason for the differential effects of BPA on cancer cell growth is unknown. Nevertheless, since BPA may act as an AR antagonist at high concentrations, it may continue to interfere with AR activity even after enzalutamide resistance develops. Therefore, the effects of high concentrations of BPA on the AR signaling in advanced PCa and whether BPA can be exploited as a treatment option for treatment-resistant PCa are questions that warrant further research.

The purpose of this experiment is to determine the effects of BPA on viability of enzalutamide-resistant PCa cells. It was hypothesized that if enzalutamide-resistant LNCaP prostate cells are exposed to higher concentrations of BPA, then cell viability will decrease. Cell viability was measured by MTS assay and cell death was evaluated by Caspase 3 and poly ADP ribose polymerase (PARP) cleavage. To further investigate the underlying mechanisms, the expression and activity of AR were examined by immunoblotting. The control of this study was the group with no BPA treatment.

added to each well. The plate was then incubated for 3 days at 37 °C in an incubator, and then subject to MTS assay.

The enzalutamide-resistant LNCaP cells for immunoblotting were seeded in 3 100-mm petri dishes at a density of 3 x 10<sup>6</sup> cells per dish and incubated at 37 °C in an incubator. After 24 hours of incubation, 3 different concentrations of BPA (0, 50, 100 μM) were added to each petri dish (1 for each concentration). Cells were then incubated for two days at 37 °C in an incubator, and subject to immunoblotting analysis.

## 2.2 MTS Assay

To test the anti-proliferative activity of BPA on treatment-resistant PCa cells, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison WI) was performed. Twenty μL of MTS/PMS solution was added to each well and the cells were incubated for 4 hours. The absorbance of each well was read at 490 nm using an enzyme-linked immunosorbent assay plate reader (Promega, Madison WI) and absorbance of culture medium was subtracted from each well containing cells. The average absorbance of each group was calculated. Viability was calculated by dividing the average absorbance of each experimental group by the average absorbance of the control group.

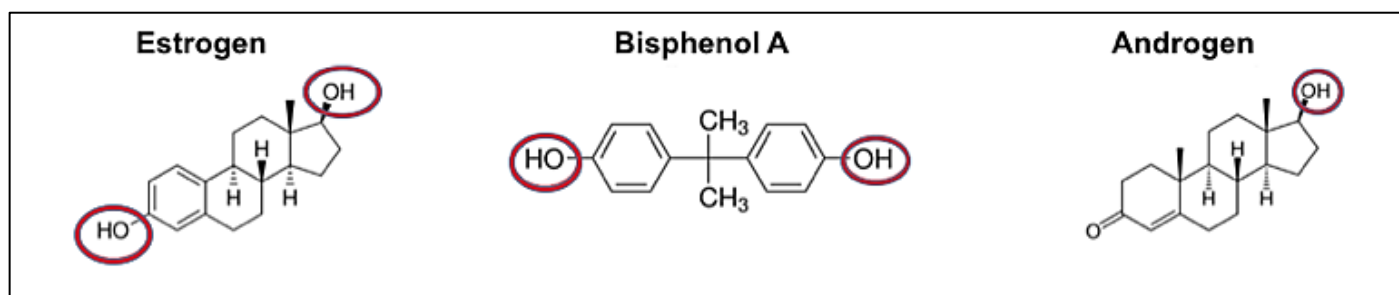


Fig. 1: Structural comparison between estrogen, BPA, and androgen. The hydroxyl groups are a similar distance apart in estrogen and BPA, and BPA and androgen are similar on one end.

## II. METHODS AND MATERIALS

### 2.1 Cell Culture and Treatment

Enzalutamide-resistant LNCaP cells were seeded in a 96-well plate at a density of 5,000 cells per well, with six MTS assay trials per group and 9 groups in total. After 24 hours of incubation at 37 °C, 9 different concentrations of BPA (0, 2.5, 5, 10, 20, 40, 80, 160, 320 μM; Sigma, St. Louis MO) were

### 2.3 Immunoblotting

To determine the expression of cleaved Caspase 3 and PARP, AR, and cyclin A, immunoblotting was performed with the concentrations of BPA that led to moderate and severe cytotoxicity. Cells were collected, washed with ice-cold phosphate buffered saline (PBS) twice, and then lysed in Radio Immuno Precipitation Assay (RIPA) buffer (1% NP-40, 0.1% SDS, 0.5% Na-DOC, 20 mM HEPES buffer, pH 7.5, 150 mM NaCl, and 1 tablet of Roche protease inhibitor

cocktail) for 20 min at 4 °C. The cells were centrifuged in a micro centrifuge for 20 minutes at 12,000 rpm and 4 °C. The supernatants were aspirated and placed in fresh tubes. Fifty µg of each cell lysate was mixed with 4 x sodium dodecyl sulfate (SDS) loading buffer and loaded to SDS-PAGE gel, along with a molecular weight marker. The gel was run for 1–2 hours at 100V.

The proteins were transferred from the gel to a nitrocellular membrane using Mini Trans-Blot cell. The membrane was blocked for 1 hour at room temperature using Tris-buffered saline (TBST) containing 0.1% Tween 20 and 5% nonfat dry milk. The blocking buffer was removed and replaced with diluted primary antibodies (anti-cleaved Caspase 3 (Cell signaling, Danvers MA), anti-PARP (Cell Signaling, Danvers MA), anti-AR (Santa Cruz, Dallas, TX), anti-cyclin A (Santa Cruz, Dallas, TX), and anti-β-actin (Santa Cruz, Dallas, TX) in blocking buffer. The membrane was incubated with primary antibodies for overnight at 4 °C. The membrane was washed with TBST 3 times (5 min each), and then incubated with secondary antibody at room temperature for 1 hour. The membrane was washed with TBST 3 times (5 min each). The membrane was covered with transparent plastic wrap and the chemiluminescent signal was detected using an enhanced chemiluminescence (ECL) detection system (Biorad, Hercules, CA) and compared among different groups.

### III. RESULTS

#### 3.1 Cytotoxic effect of BPA on enzalutamide-resistant LNCaP cells

MTS assay was used to evaluate the anti-proliferative activity of BPA on enzalutamide resistant LNCaP PCa cells. MTS assay is a colorimetric method for accessing cell mitochondrial metabolic activity, which indirectly reflects viable cell numbers. Catalyzed by NAD(P)H-dependent cellular oxidoreductase) existing only in living cells, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), in the presence of phenazine methosulfate (PMS), produces a formazan product that has an absorbance maximum at 490 nm on PBS. Enzalutamide-resistant LNCaP cells were seeded in a 96-well plate and treated with various concentrations of BPA (0, 2.5, 5, 10, 20, 40, 80, 160, 320 µM) for 3 days. MTS assay was then carried out to measure viable cells upon each treatment. The absorbance at 490 nm of the control group was set as 100% and the other groups were normalized to the control group based on their average MTS assay absorbance. The results were summarized and presented in Figure 2. The data clearly shows an exponential decay trend, with more than 90% reduction in relative cell viability for the 160 and 320 µM groups. A linear regression t-test performed on the logarithmic relative cell viability at a 0.005 level of significance produced a calculated t value of 5.8922 that was

higher than the table t value of 4.03214, so there is a significant difference between the groups. In addition, a high r-squared value of 0.8322 indicates a strong negative correlation between BPA concentration and cell viability.

MTS assay measures viable cells, but the anti-proliferative activity demonstrated by such an assay could result from either cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence). Cleavage of Caspase 3 and PARP is a pre-requisite for cells who undergo final stage of cell death and is often used as the apoptotic markers. To verify that high concentrations of BPA have cytotoxic effects on enzalutamide-resistant LNCaP cells, the cleavage of Caspase 3 and PARP in those cells treated with 0, 50, or 100 µM of BPA was analyzed by Western blotting, using anti-cleaved Caspase 3 and anti-PARP antibodies. Upon the treatment of 100 µM of BPA, cleaved Caspase 3 and PARP were strongly detected by Western blot (Figure 3), which indicates that high concentrations of BPA killed enzalutamide-resistant LNCaP cells.

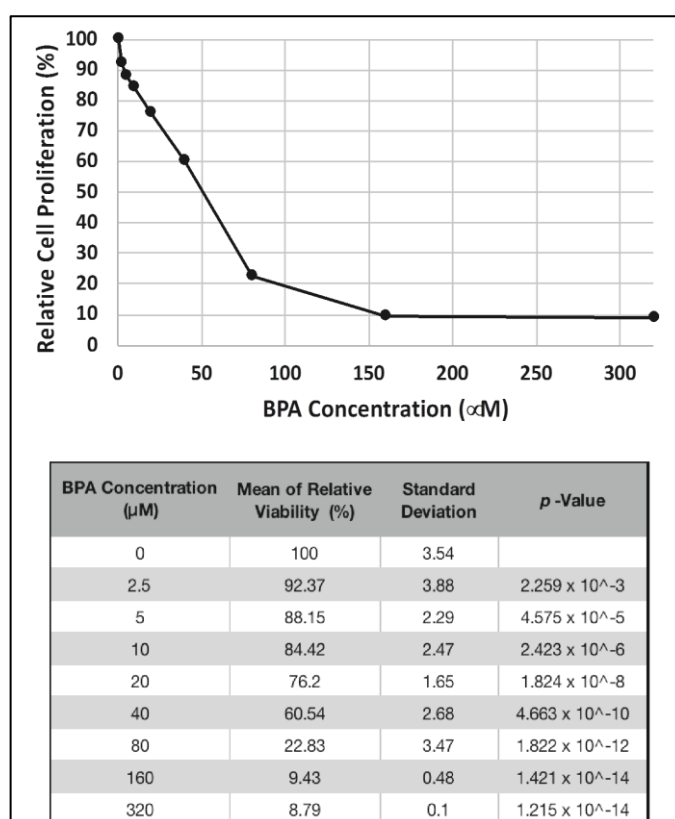


Fig. 2: The effect of various concentrations of BPA on the relative viability of enzalutamide-resistant LNCaP Cells. Enzalutamide-resistant LNCaP Cells were treated with different concentrations of BPA for 3 days, and then subject to MTS assay as described in "Materials and Methods" section. The data are expressed as a percentage of viability in relation to the control group. The control group of 0 µM BPA was set as 100%.

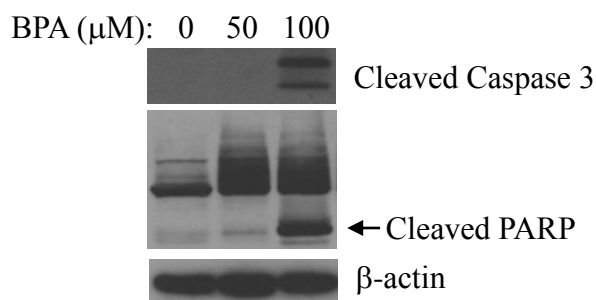


Fig. 3: Cleavage of Caspase 3 and PARP was analyzed by Western blot in enzalutamide-resistant LNCaP cells treated with high concentrations of BPA. Cells were treated with BPA (0, 50, or 100 μM) for 2 days, and then subject to Western blot analysis, using anti-cleaved Caspase 3 and anti-PARP antibodies. β-actin was used as a loading control.

### 3.2 Effect of BPA on the expression and activity of AR in enzalutamide-resistant LNCaP cells

Next, the mechanism by which high concentrations of BPA killed enzalutamide-resistant LNCaP cells was studied. Since BPA is an AR antagonist and PCa cells, including treatment-resistant PCa cells, rely on AR function for their survival and proliferation, the integrity of AR signaling was evaluated. The protein level of AR was examined in enzalutamide-resistant LNCaP cells treated with 0, 50, 100 M of BPA by immunoblotting (Figure 4). The protein level of Cyclin A was also examined in order to evaluate AR activity since its expression is controlled by AR activity. In both 50 and 100 M of BPA groups, there is a clear reduction in AR and Cyclin A protein levels when compared to the control. These results suggest that high concentrations of BPA antagonize AR signaling in enzalutamide-resistant LNCaP cells.

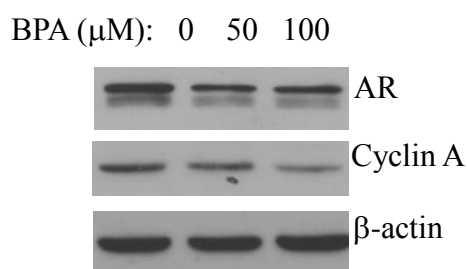


Figure 4. The effect of high concentrations of BPA on AR signaling in enzalutamide-resistant LNCaP cells. Cells were treated with BPA (0, 50, or 100 μM) for 2 days, and then subject to Western blot analysis, using anti-AR (for AR expression) and anti-cyclin A (for AR activity) antibodies. β-actin was used as an internal control for loading.

## IV. DISCUSSION

The main purpose of this experiment was to determine the potential therapeutic effects of BPA on enzalutamide-resistant PCa. It was hypothesized that high concentrations of BPA would decrease the viability of AR-positive, treatment-resistant PCa cells. The results of the MTS assay show that, when compared to the control, all 8 concentrations of BPA exhibited a decrease in cell viability, with the 160 and 320 μM groups experiencing a more than 90% decrease in cell viability. Caspase 3 and PARP cleavage verified the cytotoxic effect of high concentrations of BPA on the enzalutamide-resistant LNCaP cells. Furthermore, high concentrations of BPA function as an antagonist of AR signaling, as indicated by the reduced AR expression and activity. These results strongly support the research hypothesis.

To determine the relationship between BPA concentration and relative cell viability, a linear regression t-test was performed and proved that there is a strong negative correlation and a statistically significant relationship between the two variables.

Other researchers have explored the effects of BPA on the AR pathway. Xu's team identified BPA as an AR antagonist [12]. Teng's group recently studied the binding of BPA and its analogues on the androgen receptor and determined that BPA is capable of binding to the AR, but not its analogues [13]. Interestingly, a recent ISEF winning project reported that BPA has differential effect on the growth of AR-positive cells; high concentrations of BPA inhibited the growth of cancer cells expressing AR whereas low concentrations of BPA stimulated the proliferation of those cells [11]. However, there has not been any studies linking BPA and the AR in enzalutamide-resistant PCa up until this point.

Despite the clinical success of enzalutamide for CRPC patients, resistance to enzalutamide eventually develops. Several mechanisms of the development of such a resistance have been proposed, including mutations in AR that could cause structural changes and make the AR unrecognizable to enzalutamide. Given the observations that BPA is able to bind to the AR and that high concentrations of BPA killed enzalutamide-resistant PCa cells and reduced AR expression and activity, a possible explanation for the results of this experiment is that BPA is capable of binding to the mutated AR that is caused by enzalutamide treatment, and thereby interferes with AR signaling in enzalutamide-resistant PCa cells, which eventually leads to cell death of those treatment-resistance cells. In future studies, we plan to elucidate the molecular action of BPA in enzalutamide-resistant PCa in greater detail. The results from this study suggest that BPA is a promising candidate for CRPC treatment, and its chemical properties can be further analyzed and modified to develop

and optimize AR antagonists, which may pave the road for designing novel approaches for treatment-resistant PCa.

## V. DECLARATION

The author has no conflicts of interest and the study was self-funded.

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