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TITLE: Repurposing Thymoquinone as Therapy for Metastatic Castration-Resistant Prostate Cancer

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CONTRACTING ORGANIZATION: Morehouse School of Medicine Inc.

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14. ABSTRACT The major goal of this project is to elucidate the mechanism of action of thymoquinone (TQ) against docetaxel (DTX)-resistant metastatic castration-resistant prostate cancer (mCRPC). We formulated a novel TQ nanoparticles (NPs) that are capable of rapid clinical translation; we utilized a highly innovative-patented NP platform, the planetary ball-milling (PBM) platform. In this study, we identified the highly expressed <i>ATP-binding cassette (ABC)</i> transporters, that play a major role in drug efflux activity, in docetaxel resistance cells (C4-2B-R and LNCaP-R). We found that these efflux proteins were downregulated when targeted with PSMA aptamer (A10) conjugated PBM -TQ -NPs and re-sensitizes docetaxel resistant cells towards cell death. In addition, we have also identified PSMA-mediated cellular uptake of PBM NPs in prostate cancer cells and an abundant number of internalized PBM nanoparticle were found through PSMA receptor in PSMA ⁺ cells. Altogether, our engineered PBM NPs displayed, specificity and sensitivity to PSMA receptor, and suggest a novel strategy for treating prostate cancer.					
15. SUBJECT TERMS Prostate cancer, Thymoquinone, Nanoparticle, Aptamer, PSMA, MDR					
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Unpublished data. Please protect it.

1. INTRODUCTION: Metastatic castration-resistant prostate cancer (mCRPC) is lethal and there is no cure. Docetaxel (DTX)-based therapy is the only available chemotherapy with a survival benefit in patients. Unfortunately, the treatment eventually leads to chemoresistance, which remains a major obstacle in the treatment of mCRPC. Thus, it is imperative to develop efficacious therapies to overcome DTX resistance and improve survivorship. Thymoquinone (TQ), a natural compound, was identified as a lead against chemoresistant mCRPC cells. Mechanistically, TQ inhibits the expression of ATP binding cassette (ABC) subfamily B member 1 (ABCB1), a major transmembrane efflux pump responsible for multi-drug resistance, and survivin, a critical anti-apoptotic protein whose overexpression is associated with the progression towards mCRPC and therapeutic resistance. Our discovery of TQ as a potent inhibitor of DTX-resistant mCRPC cells provides a unique opportunity to explore its anti-cancer activity and mechanism of action (MOA) in clinically relevant models. This knowledge will demonstrate the potential of TQ as a novel therapy for lethal mCRPC. We showed in preliminary results that nanoparticle (NP) delivery of TQ could overcome its drug delivery challenges and lower its toxicity. In this project, we will test the central hypothesis that TQ inhibits DTX resistant mCRPC, and can be repurposed as a safe, affordable and effective therapy for lethal mCRPC.

2. KEYWORDS: prostate cancer, thymoquinone, nanoparticle, aptamer, PSMA, MDR

3. KEY RESEARCH ACCOMPLISHMENTS

- **What are the major goals of the project?**

Aim 1: Elucidate the mechanism of action of Thymoquinone against docetaxel-resistant mCRPC.

Aim 2: Validate Thymoquinone as a lead drug against docetaxel-resistant mCRPC.

- **What was accomplished under these goals?**

Below we have described experiments performed and their outcomes during the project duration. Please also see the attached **Appendix I** with research progress marked in the statement of work.

Aim 1.1. To investigate the biologically targeted PBM NP encapsulated with TQ and conjugated with A10 aptamer and CSC antibody.

I. Cell lines and generation of docetaxel resistant cells. The prostate cancer cell lines, C4-2B and LNCaP were first grown and maintained RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), nonessential amino acid, HEPES, 2 mM l-glutamine and penicillin/streptomycin antibiotic solution (Fisher Scientific, Pittsburgh, PA) and then followed to make docetaxel (DTX) resistant cells. DTX resistant cells were prepared from the parental C4-2B and LNCaP cells. The C4-2B resistant cells were generated by gradually increasing the docetaxel concentration starting from 2 nM to 160 nM and were maintained in standard condition incubator for **7 months** at 37 °C and 5% CO₂. Likewise, LNCaP resistant cells were generated by gradually increasing the docetaxel concentration starting from 2 nM to 60 nM and were maintained in standard condition incubator for **7 months** at 37 °C and 5% CO₂. The docetaxel resistant cells were labeled as C4-2B-R and LNCaP-R, and were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), l-glutamine, nonessential amino acid, HEPES, 2 mM l-glutamine and penicillin/streptomycin antibiotic solution (Fisher Scientific, Pittsburgh, PA). The relative growth rate and the representative images from parental and resistant cells are shown in **figure 1**.

II. Cellular toxicity assay (MTT assay). To evaluate the therapeutic potentials of A10-TQ-NP, viability assays were performed for C4-2B-R and LNCaP-R cells. 10,000 cells/well were seeded for overnight and then treated with A10 aptamer-TQ-NPs for 24, 48, or 72 hr at 37°C in a 5% CO₂ incubator. MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; 5mg/ml) was added (20 µL) to each well and were incubated at 37°C for 3-4 hr. The purple formazan crystals were dissolved in 100 µL of dimethyl sulfoxide, and the absorbance was measured at 570 nm with a spectrophotometer. Next, the IC₅₀ (half-maximum inhibitory concentration) values were calculated for both cell lines. Free drug (TQ without encapsulated in PBM NPs) IC₅₀ value was found 170 µM and 161 µM for C4-2B-R and LNCaP-R cells respectively. However, TQ encapsulated PBM NPs treated both cell lines had IC₅₀ values: A10-TQ-NP (33 µM), TQ-NP (42 µM) and IC₅₀ values: A10-TQ-NP (40µM), TQ-NP (90 µM) for C4-2B-R and LNCaP-R cells respectively at 72h.

These results showed that targeted delivery using A10 aptamer conjugated PBM NPs obtained IC₅₀ values were nearly 3-5-fold lower as compared to free drug of TQ.

III. Engineering A10-PBM NP TQ: Below we have described the method of PBM-nanoparticle engineering.

(a) PBM nanoparticle formulation method.

PBM nanoparticles were prepared by using 4% starch (w/v) (FDA approved) solution, Next, thymoquinone (TQ) or docetaxel (DTX) or combination (w/v) at 4°C was added to the starch solution alone with Texas red (for tracking PBM NPs) and stirred for 30 minutes. After centrifugation, the solution was poured into Minispray it forms a dried powder of macroparticles. These macroparticle were then milled using planetary ball milling under controlled temperature (<37°C). PBM particle formulation requires a milling jar, which holds heat absorbent zirconium oxide planetary milling balls rotates about its axis as well as in the opposite direction around a common axis of the chamber wheel. By controlling the centrifugal force with varying the revolution/sec (Ω), jar velocity, the size as well as a number of the zirconium oxide balls,

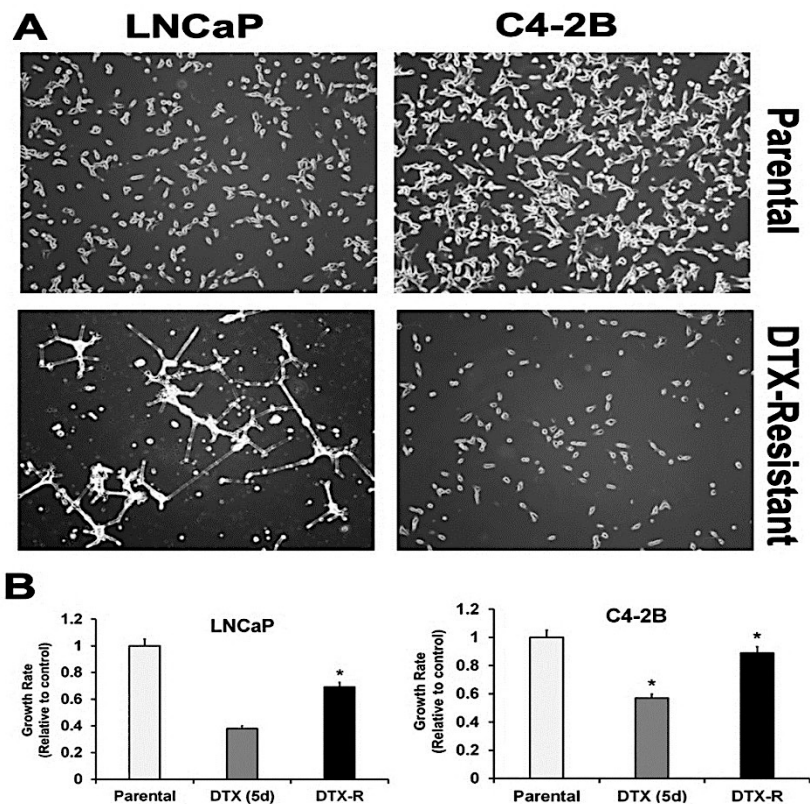


Figure1: Morphology and growth rate to docetaxel of DTX resistant and parental PCa cell lines. DTX resistant cells were prepared from the parental C4-2B and LNCaP cells. The resistant cell lines (C4-2B-R and LNCaP-R) were grown by gradually increasing the docetaxel concentration starting from 2 nM to 160 nM and were maintained in standard condition incubator for **7 months** at 37 °C and 5% CO₂. (A) Phase-contrast images were taken at 40X objective from the parental and docetaxel resistant cells. (B) The relative growth rate of parental and resistant cells treated with DTX (10nM) for 5 days, measured by MTT assay. * indicates p-value < 0.05.

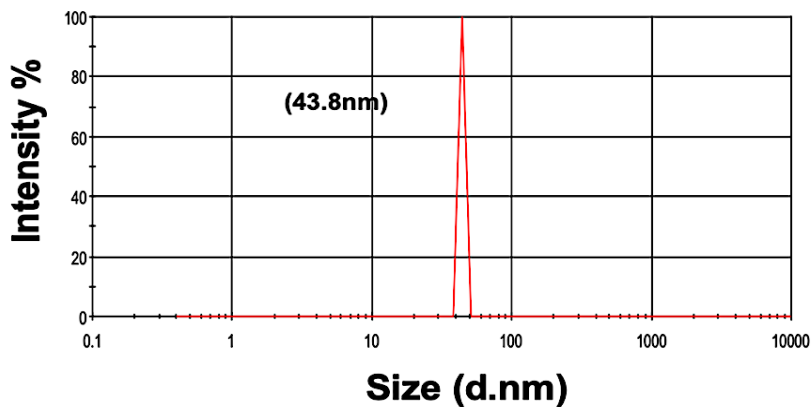


Figure 2: Characterization of prostate cancer- specific membrane (PSMA) aptamer (A10) conjugated TQ encapsulated PBM nanoparticles for size. The size of A10-TQ-PBM NPs (PCL-PEG coated or uncoated) were measured at pH 6.8 using a Malvern Zetasizer ZS instrument at a concentration of 0.1 mg/mL (5% mass, assuming a density of 1 g/cm³) of nanoparticles. In order to the characterization of aggregation or agglomeration, NPs were dispersed in solution and ultrasonication were applied that pulls the liquid apart to form micro voids and overcomes the Vander Wall force holding agglomerates together.

resulting particles were either used alone (i.e., uncoated) or coated with a 5%, 10% or 20% PCL solution (in methylene chloride) by continuous stirring at 1000 rpm. The PCL-coated bio-polymer-PEG particles were finally rinsed with dH₂O, dried and stored as a powder. (*The detailed formulation methods used also see in my patents US #8,231,907, US #8,568,784, US20130045162, and PCT/2012/049988*).

(b) Nanoparticle-aptamer conjugation. To engineer the PBM NP-A10 conjugation, first 20 mg of DSPE-PEG-NHS (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol- N-hydroxy succinimidyl ester; is one of the most commonly used reactive phospholipids to conjugate antibodies, aptamer, proteins, peptides or other substrates to the surface of lipid PEG nanoparticles.) (NANOCOS, Boston, MA, USA) were dissolved in 1:1 ratio (DMSO/water), then NHS activated particles were covalently linked to 200 μ l of NH₂- modified PSMA aptamer (A10-3.2) (GGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCU-spacer-NH₂) (1 μ g/ μ L in nuclease- free water) and mixed overnight at 4°C. Next, the A-10-3.2 aptamer NP conjugates were washed, resuspended, filtered, and then lyophilized and stored as a powder.

(c) Synthesis of TQ encapsulated NPs. TQ-encapsulated starch NPs were prepared by using a planetary ball milling method. Briefly, 20mg NP-Aptamer conjugates and TQ-starch-NP (10mg) were dissolved in methylene chloride (1mL) and then coated with activated polycaprolactone (PCL) 2% (For PCL activation, 2gm PCL and dry dioxane (6mL) mixed and heated in a water bath and then N, N'- disuccinimidyl carbonate and pyridine were added and placed in a shaker to react for 6 hours. Then filter the precipitate with diethyl ether and re-dissolve in acetone and placed to become a dry powder.) and polyethylene glycol (PEG 2%). The NPs were stirred at 4°C overnight, and the remaining organic solvent was removed by evaporation under reduced pressure. The PBM NPs were then washed with deionized water. The size of particle was measured at pH 6.8 using a Malvern Zetasizer ZS instrument at a concentration of 0.1 mg/mL (5% mass, assuming a density of 1 g/cm³) of nanoparticles (**Figure 2**).

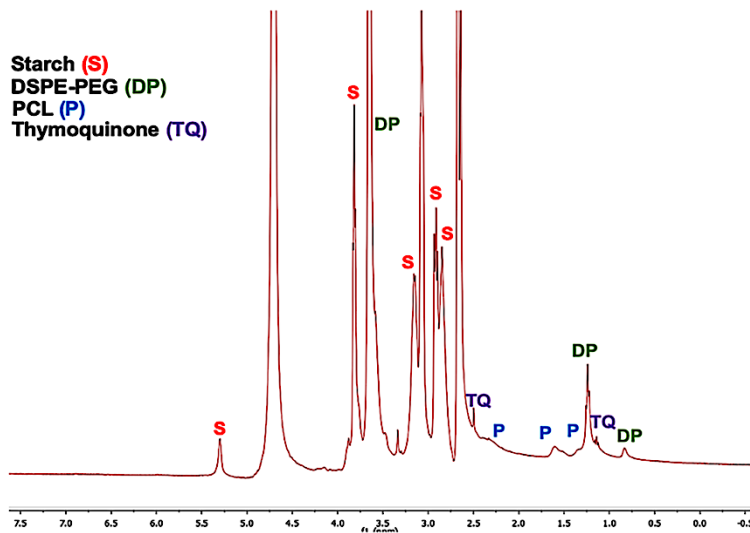


Figure 3: Nuclear Magnetic Resonance. ¹H NMR spectra were recorded on Bruker 400 MHz Spectrometer in D₂O. The peak at 3.6 ppm clearly showed the PEG presence, peaks at 0.8 and 1.2 indicates the presence of DSPE; peaks at 5.3 (anomeric) 3.8, 3.0, 3.1 ppm showed the presence of starch. Peaks at 1.5, 1.6 and at 2.3 show the existence of polycaprolactone, and existence of thymoquinone indicates the peaks at 1.4 and 2.5.

(d) Nuclear Magnetic Resonance. ¹H NMR spectra were recorded on Bruker 400 MHz Spectrometer in D₂O. The peak at 3.6 ppm clearly showed the PEG presence, peaks at 0.8 and 1.2 indicates the presence of DSPE; peaks at 5.3 (anomeric) 3.8, 3.0, 3.1 ppm showed the presence of starch. Peaks at 1.5, 1.6 and at 2.3 show the existence of polycaprolactone (PCL), and the existence of thymoquinone (TQ) indicates the peaks at 1.4 and 2.5. The resulting TQ-PBM-NP was characterized by NMR, shown in the **Figure-3**.

IV. Validation of PSMA-mediated uptake of NPs. To investigate the cellular uptake and effluxes of PBM NPs, we encapsulated a fluorescent dye Texas red (1mg/100mL) in A10-TQ-PBM-NP. Next, PSMA positive C4-2B-R cells (20,000) were grown overnight in 48 well plates, and then treated with Texas red conjugated NPs (A10-TQ-PBM) (33 μ M) and or empty (Starch) PBM- NP. Cells were incubated for two hours at 37°C and washed with PBS three times. Images were captured at 40X by fluorescent microscope with the appropriate filter Texas red. As shown in **Figure-4**, an abundant number of internalized PBM nanoparticles were found through PSMA receptor in PSMA + cells compared to non-conjugated (no aptamer) empty NP.

V. In Vitro comparison of A10-PBM -NP TQ to PBM NP TQ

Validation of ATP-binding cassette (ABC) transporters at the mRNA level in DTX resistant C4-2B-R and LNCaP-R cells. Further, to identify the expression of ABC transporter genes that mediate the drug efflux, RT PCR was conducted in both cell lines. The RNA isolation method was followed to the standard protocol from our lab. Briefly, as described above IC50 value, PCa cells were treated with TQ-NP and A10-TQ-NPs for 72hrs and lysed with Trizol reagent. Finally, RNA was precipitated, quantified and cDNA was synthesized. Primers for ATP-binding cassette transporter markers were synthesized from the National Center for Biotechnology Information (NCBI) gene bank database. The following sequences of the primers (Forward (F) and Reverse (R)) were used;

ABCB1: F- 5'TGACATTTATTCAAAGTTAAAAGCA3' and R-5'TAGACACTTTATGCAAACATTTCAA3';

ABCB2: F-5'AGGGCTGGCTGGCTGCTTTGA3' and R-5'ACGTGGCCCATGGTGTGTTGTTAT3';

ABCB3: F-5'ACGGCTGAGCTCGGATACCAC3' and R-5'CCTCGGCCCCAAAACACTGC3';

ABCB4: F-5'ACCGACTGTCTACGGTCCGAA3' and R-5'TCCATCGGTTTCCACATCAAGG3';

ABCB5: F- 5' TCTGGCCCCCTCAAACCTCACC3' and R- 5'TTTCATACCGCCACTGCCAACTC3';

ABCC1: F-5'AGTGGAACCCCTCTCTGTTTAAG3' and R- 5'CCTGATACGTCTTGGTCTTCATC3';

ABCC2: F-5'TCCTTGCGCAGCTGGATTACAT3' and R-5'TCGCTGAAGTGAGAGTAGATTG3';

ABCC3: F- 5' CAGAGAAGGTGCAGGTGACA3' and R-5' CTAAAGCAGCATAGACGCC3';

ABCC4: F-5'TGATGAGCCGTATGTTTTGC3' and R-5'CTTCGGAACGGACTTGACAT3';

ABCC5: F-5'AGGGGCAAGAAAGAGAAGGTGAGG3' and R-5'GAGGGGGTCCGTCAGGATGTAGAT3';

18S: F-5'GGCCCTGTAATTGGAATGAGTC-3' and R- 5'-CCAAGATCCAACACTACGAGCTT3'.

18S primer was used as endogenous control, and the experiments were repeated three times.

As shown in the **Figure-5**, elevated expression of ABCB1 (MDR1), ABCB3 and ABCC1 (MRP1) were found in DTX resistant C4-2B-R cells. However, upon treatment with TQ-NPs and A10-TQ-NPs, the expression of these markers significantly downregulated. Similarly, in LNCaP-R cells, the expression of ABC markers (ABCB1, ABCB3, ABCB5, ABCC1, and ABCC3) were downregulated after treatment with A10-TQ-NP for 48hrs. These results demonstrate MDR genes were significantly upregulated in drug resistance cell lines while were blocked upon treatment. Finally, our data suggested that PSMA aptamer (A-10) conjugated-TQ-PBM-NP inhibits ATP binding cassette (ABC) markers in DTX resistant PSMA expressing cells, thereby reducing the drug efflux activity.

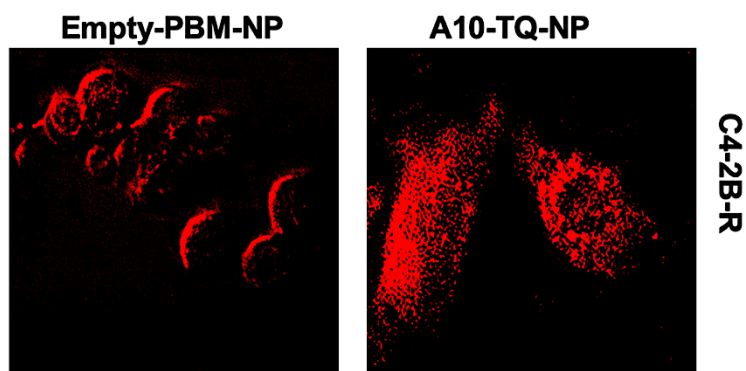


Figure 4: PSMA-mediated Cellular uptake of PBM nanoparticles in prostate cancer cells. To investigate the cellular uptake and effluxes of PBM NPs, C4-2B-R cells (20,000) were grown overnight in 48 well plates, and then treated with texas red conjugated NPs (A10-TQ-PBM) (33 μ M) and empty PBM (Starch) NP. Cells were incubated for two hours at 37°C and washed with PBS three times. Images were captured by a fluorescent microscope with the appropriate filter. Red indicates the PBM nanoparticle within the cell.

VI. To investigate whether Thymoquinone inhibits ABCB1 activity

Aptamer-TQ-NPs induces the modulation of ATP-binding cassette genes in PCa cells. Several accumulating evidences has been reported that ATP- binding transporters genes play a major role in drug efflux. Considering these facts, we validated ABC genes in drug resistant PCa cells. To investigate the expression of ATP- transporters in DTX resistant C4-2B-R and LNCaP-R cells, the protein's isolation method was followed to our lab protocol. To evaluate the effect of NPs, first C4-2B-R and LNCaP-R cells were treated with TQ-NP (C42B-R: 42 μ M; LNCaP-R:90 μ M) and A10-TQ-NPs (C4-2B-R: 33 μ M; LNCaP-R: 40 μ M) for 72 h. The protein concentration of the samples was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL). An equal amount of cell lysate containing 35 μ g of protein was analyzed on 4–12% polyacrylamide gels. Next, immunoblots were probed with primary antibodies overnight at 4°C followed by horseradish peroxidase (HRP) labeled secondary

antibodies (1:2000 dilution) in TBS-T for 2 h at room temperature. The primary and secondary antibody for ATP-transporters (ABCB1 and ABCC1), ZEB1, GAPDH were procured from Cell Signaling Technology (MA, USA).

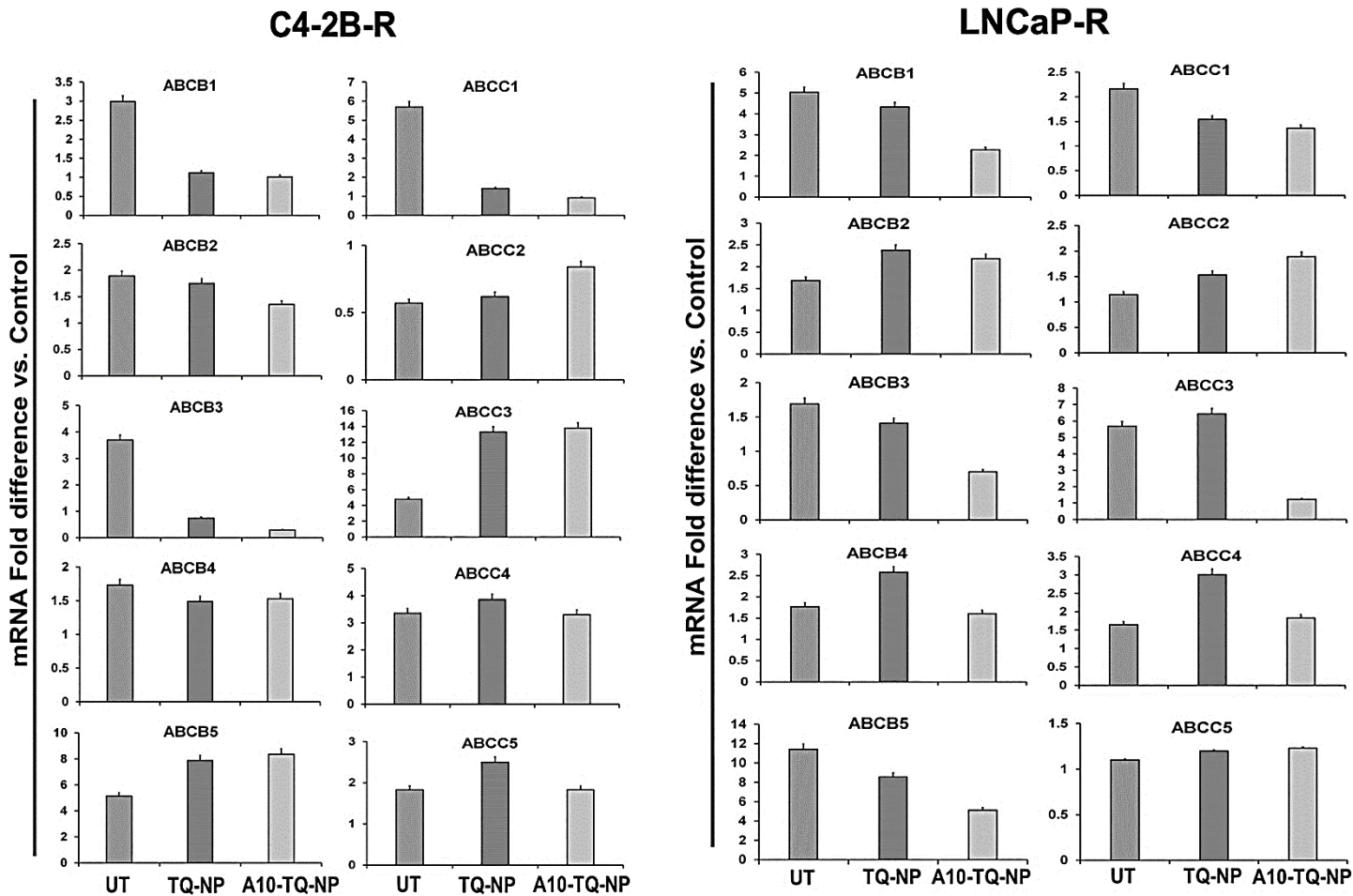


Figure 5: ATP-binding cassette (ABC) transporters expression at the mRNA level in DTX resistant cells. PCa cells were treated with IC50 values, TQ-NP (C42B-R: 42 μ M; LNCaP-R: 90 μ M) and A10-TQ-NPs (C4-2B-R: 33 μ M; LNCaP-R: 40 μ M) for 72hrs and lysed with trizol reagent. The RNA isolation method was followed to the standard protocol and mRNA expression levels of the indicated genes were quantitated by RT-PCR. (A) LNCaP-R (B) C4-2B-R shows the expression level of ABC transporter. Data were normalized for levels of the housekeeping gene 18S rRNA and presented as fold difference in expression ($\pm$ standard error), and the experiments were repeated three times. These data strongly suggested that ABC transporters were significantly upregulated in resistant cells, while were blocked with the PBM-NPs treatment.

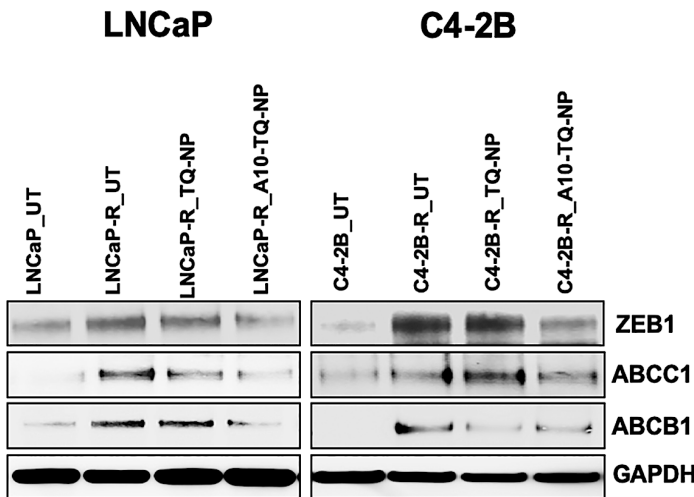


Figure 6: Western blot analysis of aptamer-nanoparticles mediated ABC transporter genes in PCa cells To investigate the expression of ABC-transporters and EMT marker in DTX resistant cells, C4-2B-R and LNCaP-R were grown and treated with TQ-NP (C42B-R: 42 μ M; LNCaP-R:90 μ M), and A10 aptamer conjugated TQ NPs (A10-TQ-NP) (C42B-R: 33 μ M; LNCaP-R:40 μ M) for 72 h. An equal amount of protein (35 μ g) was loaded on 4–12% polyacrylamide gels. Immunoblotting was carried out with primary antibodies against ABC-transporters (ABCB1 and ABCC1), and EMT transcriptional factor markers (ZEB1). As an internal standard for equal loading blots was probed with an anti-GAPDH antibody. Data are representative of three independent experiments. The results from the immunoblots showed that ABCB1, ABCC1, and ZEB1 was dramatically upregulated in both DTX resistant cells in comparison to parental cells while downregulated upon treatment of A10-TQ-NPs.

The signal detection was performed using chemiluminescence (ECL) western blotting detection reagent (Fisher Scientific, Pittsburgh, PA). Protein bands were acquired with Image Quant LAS4000 (GE Healthcare-Biosciences, Pittsburgh, PA).

The results from the western blots showed that ABCB1 and ABCC1 were dramatically upregulated in C4-2B-R control (untreated) cells while was downregulated upon treatment of A10-TQ-NP and TQ-NPs (**Figure-6**). Also, we further observe the expression of the transcriptional factor gene (ZEB1) on western blots, consistent with our previous finding, the ZEB1 protein expression was significantly downregulated with A10-TQ-NP treatment. Similarly, LNCaP-R cells were treated with the individual dosage of TQ-NP and A10-TQ-NP to monitor the drug efflux activity. As expected, the expression of ABCB1, ABCC1, and ZEB1 was markedly decreased compared to TQ-NP treated and control (LNCaP-R untreated) cells. Altogether, these observations further suggested that MDR signaling cascade was hampered by blocking with the aptamer -NPs conjugation.

- **What opportunities for training and professional development has the project provided?**

“Nothing to report”

- **How were the results disseminated to communities of interest?**

“Nothing to report”

- **What do you plan to do during the next reporting period to accomplish the goals?**

Subtask 2: (ongoing)

- *To investigate whether thymoquinone counteracts the transporter-mediated chemoresistance in mCRPC cells.*
- *To investigate whether ABCB1 is a direct target of thymoquinone in mCRPC cells.*

Subtask 3: To investigate whether thymoquinone inhibits the transcription of ABCB1 through a ZEB1-dependent mechanism.

- To investigate whether ZEB1 is required to mediate the inhibitory effect of thymoquinone on ABCB1 transcription.
- To investigate the role of EGFR-ERK signaling in mediating the effect of thymoquinone on ZEB1 and ABCB1.

Subtask 4: Elucidate the mechanism by which thymoquinone inhibits survivin expression in mCRPC cells.

- To investigate whether survivin is required to mediate thymoquinone inhibition on mCRPC cell survival.
- To investigate the role of NFκB in mediating thymoquinone inhibition of survivin.
- To investigate the role of EGFR-Akt signaling in thymoquinone inhibition of survivin.

4. IMPACT

- **What was the impact on the development of the principle discipline (s) of the project?**

In this application, we propose to take an innovative approach by developing a novel chemosensitizer to improve the therapeutic ratio of docetaxel. our engineered nanoparticle (NP) formulations of Thymoquinone (TQ), a known sensitizer, and evaluate their toxicity and efficacy as chemosensitizers and novel therapeutic agents for mCRPC. We believe our strategy has a high potential for making a clinical impact on the treatment of mCRPC. Our PBM NP TQ chemosensitizer can potentially target both prostate cancer epithelial cell (PCEC) as well as prostate cancer stem cells (PCSC) to improve the efficacy as well as the response rates of docetaxel chemotherapy.

- **What was the impact on other disciplines?**

We believe TQ NP will have a very low toxicity profile as a chemosensitizer, and its use will increase the therapeutic ratio of chemotherapy. **In the short-term**, the success of our work can result in the clinical development of nanoparticle TQ. **In the long-term**, PBM NP TQ can become part of the docetaxel chemotherapy regimen in the management of mCRPC. Our work can also facilitate the adoption of chemosensitization as a novel strategy to improve systemic chemotherapy treatment. The success of PBM TQ NP as chemosensitizer has the potential to improve survival and quality of life for patients with mCRPC. More broadly, we believe our strategy can be applied to other diseases and lead to other novel nanoparticle and biological treatments for cancer.

- **What was the impact on technology transfer?**

“Nothing to report”

- **What was the impact on society beyond science and technology?**

“Nothing to report”

5. CHANGES/PROBLEMS

“Nothing to report”

6. PRODUCTS

- **Publications, conference papers, and presentation**

1. S. K. Singh, T. Apata, J. B. Gordetsky, and R. Singh 2019. Docetaxel combined with thymoquinone induces apoptosis in prostate cancer cells via inhibition of the PI3K/AKT signaling pathway. *Cancers* (*Accepted*)

- **Website(s) or other Internet site(s)**

“Nothing to report”

- **Technologies or techniques**

“Nothing to report”

- **Inventions, Patent applications, and/or Licenses**

“Nothing to report”

- **Other Products**

“Nothing to report”

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

○ What Individuals have worked on this project?

Following individual worked during the project:

1. Name: Rajesh Singh

Project Role: Principal investigator

Researcher Identifier (ORCID ID): 0000-0002-0397-3794

Nearest person month worked: 3 months

Contribution to project: Performed experiments, data analyses, manuscript writing

Funding Support: None

2. Name: Santosh Kumar Singh

Project Role: Research Associate

Nearest person month worked: 12 months

Contribution to project: Engineered PBM nanoparticles, generated docetaxel resistant cell lines, performed experiment.

Funding Support: None

○ Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

“Nothing to report”

○ What other organizations were involved as partners?

“Nothing to report”

8. SPECIAL REPORTING REQUIREMENTS

“Nothing to report”

9. APPENDICES

I. Statement of work with research progress is noted.

II. Copy of research article currently accepted for publication.

Statement of Work

Specific Aim 1 (specified in proposal)	Timeline	Progress	Comments
Major Task 1 Elucidate the mechanism of action of thymoquinone against docetaxel-resistant mCRPC	Months (1-24)		
Subtask 1 To investigate the biologically targeted PBM NP encapsulated with TQ and conjugated with A10 aptamer and CSC antibody •Engineering A10-PBM NP TQ •Validation of PSMA-mediated uptake of NPs •In vitro comparison of A10-PBM NP TQ to PBM NP TQ	1-7	X	We generated docetaxel resistant cell lines. It takes 7 months to generate stable cell lines. In the meantime, we performed the effect of TQ and DTX combination induces apoptosis in prostate cancer cells via inhibition of the PI3K/AKT signaling pathway. (Accepted Manuscript)
Subtask 2 <i>To investigate whether thymoquinone directly inhibits ABCB1 transporter activity in mCRPC cells.</i> • <i>Engineering of PBM NPs containing both docetaxel and TQ.</i> • <i>To investigate whether thymoquinone inhibits ABCB1 activity.</i> • <i>To investigate whether thymoquinone counteracts the transporter-mediated chemoresistance in mCRPC cells.</i> • <i>To investigate whether ABCB1 is a direct target of thymoquinone in mCRPC cells.</i>	8-14	X	This experiment is ongoing. We generated an engineered PBM nanoparticle and characterized for future experiments.
Subtask 3 To investigate whether thymoquinone inhibits the transcription of ABCB1 through a ZEB1-dependent mechanism. •To investigate whether ZEB1 is required to mediate the inhibitory effect of thymoquinone on ABCB1 transcription. •To investigate the role of EGFR-ERK signaling in mediating the effect of thymoquinone on ZEB1 and ABCB1.	15-20		
Subtask 4 Elucidate the mechanism by which thymoquinone inhibits survivin expression in mCRPC cells. •To investigate whether survivin is required to mediate thymoquinone inhibition on mCRPC cell survival. •To investigate the role of NFκB in mediating thymoquinone inhibition of survivin. •To investigate the role of EGFR-Akt signaling in thymoquinone inhibition of survivin.	21-24		
Milestone(s) Achieved Mechanism of action of thymoquinone against docetaxel-resistant mCRPC is the important milestone in proposed study.			
Local IRB/IACUC Approval	Approved		

	Protocol # 18-07		
Milestone Achieved: HRPO/ACURO Approval	4		
Specific Aim 2			
Major Task 2 Validate thymoquinone as a lead drug against docetaxel-resistant mCRPC	25-36		
Subtask 1 To investigate the pharmacokinetics and Pharmacodynamics of thymoquinone and docetaxel.	25- 28		
Subtask 2 To investigate the in vivo efficacy of thymoquinone in C4-2B-Tax-R or LNCaP-DTX-R xenografts. •Engineering A10-PBM NP TQ. A10 will be conjugated to DSPE-PEG. A10-PBM NP TQ will be formulated. •Validation of PSMA-mediated uptake of NPs. A10-PBM NPs containing fluorescent dye will be incubated with PSMA+ cells and PSMA- cells. PSMA-specific uptake will be confirmed by microscopy.	27- 30		
Subtask 3 To investigate the in vivo efficacy of thymoquinone in PDX models.	31-36		
Milestone(s) Achieved: Proof of concept and the Specific Aims proposed in this study are important milestones towards the end goal of moving this experimental therapy to the bedside.			

Article

Docetaxel Combined with Thymoquinone Induces Apoptosis in Prostate Cancer Cells via Inhibition of the PI3K/AKT Signaling Pathway

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Abstract: Toxicity and the development of resistance by cancer cells are impediments for docetaxel (DTX), a primary drug for treating prostate cancer (PCa). Since the combination of DTX with natural compounds can increase its effectiveness by reducing its toxic concentrations, we evaluated a combination of thymoquinone (TQ) with DTX and determined its cytotoxicity against PCa cells (DU145 and C4-2B). This combination, in a concentration-dependent manner, resulted in synergistic cytotoxicity and apoptosis in comparison to either DTX or TQ alone. In addition, inhibition of cell survival pathways by PI3K/AKT inhibitors conferred sensitivity of DU145 and C4-2B cells to the combination as compared to the individual drugs. Moreover, the combined drugs (DTX+TQ) with inhibitors of PI3K/AKT increased the expression of pro-apoptotic markers (BAX and BID) along with caspase-3, PARP and decreased expression of the anti-apoptotic marker, BCL-XL. These data show that, for PCa cells, the cytotoxic effect of the DTX and TQ combination correlates with a block of the PI3K/AKT signaling pathway. These findings indicate that the combination of DTX and TQ, by blocking of the PI3K/AKT pathway, will improve the survival rate and quality of life of PCa patients.

Keywords: thymoquinone; docetaxel; PI3K/AKT; apoptosis; prostate cancer

1. Introduction

Prostate cancer (PCa) is common in men, causing 250,000 deaths each year [1]. In 90% of cases, PCa is diagnosed as organ-confined or locally advanced, based on clinical stage and levels of prostate-specific antigen. Although PCa can be diagnosed at early stages, some patients develop metastases after local treatment with surgery/radiotherapy [2]. With these considerations, decisions are made whether to opt for active surveillance, or definitive therapy [3]. With the currently available agents, responses are transient; however, secondary hormonal therapy is an option for some patients. PCa that has advanced despite the achievement of castrate levels of androgens are considered as castration-resistant (CRPCs). High levels of prostate-specific antigen, substantive pain, and bone and lymph node metastases are associated with patient morbidity [4].

New drugs, drug sequences, and drug combinations, recently used for treatment of PCa, have improved the outcomes for PCa patients. Among these is taxane-based chemotherapy [3]. Until 2010, the taxol-derived drug, docetaxel (DTX), which prevents microtubule de-polymerization and mitotic division, was the standard chemotherapeutic treatment for PCa patients [5]. DTX combined with androgen deprivation therapy improves the outcomes for men with metastases at the first presentation [6–8]. Despite being effective in treating PCa, DTX resistance due to overexpression of ABC transporter efflux proteins, which limit the intracellular concentrations of the drug, is an impediment

for treating PCa [9]. In addition, DTX treatment induces adverse events, including febrile neutropenia, fatigue, pneumonitis, infusion reactions, fluid retention, cutaneous and nail toxicity, gastrointestinal complications, and neuropathies [10,11]. To circumvent the negative effects and benefit from the useful effects of DTX, a combination of DTX with natural anticancer agents is a relevant approach for the treatment of PCa [12,13].

Thymoquinone (TQ), a phytochemical commonly found in black cumin (*Nigella sativa*), has a long history of medicinal use [14,15]. Both oil and seeds from *Nigella* plants have anticancer, antidiabetic, antihypertensive, and antimicrobial effects [16,17]. In cancer research, TQ shows promising activity in cell culture and animal models [18], and it has anti-proliferative effects for ovary, colon, larynx, breast, and lung cancer cells and for myeloblastic leukemia and osteosarcoma cells [19]. In treated cells, TQ induces apoptosis, chromatin condensation, DNA fragmentation, and translocation of phosphatidylserine across the plasma membrane [18]. In addition to its anti-cancer properties, TQ strengthens the immune system, protects normal cells from oxidative damage, and prevents toxic side effects [19].

In PCa, various growth and survival, promoting pathways interact. The role of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) is being studied, and treatments involving a single inhibitor or a combination of agents who interfere with the androgen receptor are being investigated in clinical studies [20]. For PCa, the activated PI3K/AKT pathway is associated with progression, resistance, and metastasis of cancer cells. Aggressive cancers are characterized by the survival, growth, metabolic, and metastatic functions signaled through this pathway. High grade and progression of PCa correlate with its activation [21]. Although inhibitors of this pathway show antitumor activity in animals [22,23], initial clinical studies of these agents have shown only limited efficacy [3]. Thus, there is a need for developing new agents to target this pathway in PCa. In the present investigation, we used DTX and TQ in combination for treating PCa cells. By activating pro-apoptotic proteins, the combination showed a cytotoxic effect on PCa cells. In addition, in the presence of PI3K/AKT inhibitors, the combination of DTX and TQ activated apoptosis and inhibited expression of an anti-apoptotic gene.

2. Results

2.1. Effects of TQ, DTX, and their Combination on Cell Viability, Proliferation, and Cytotoxicity of PCa Cells

To determine the therapeutic potentials of TQ, DTX, and their combination, viability assays were performed for C4-2B and DU145 cells. In addition, the toxicity of TQ and DTX combined was determined in the presence of the PI3Ki and AKTi. Three-time points (24, 48, and 72 h) were used to determine the IC₅₀ values for each individual drug and the combined drugs. Among the three time points, a concentration-dependent apoptotic response was found at the 48-h time point. DU-145 cells had IC₅₀ values of 60 µM for TQ, 20 nM for DTX, and 50 µM + 10 nM for their combination. In comparison, C4-2B cells had IC₅₀ values for TQ, DTX, and their combination of 54 µM, 20 nM, and 35 µM + 10 nM, respectively (Figure 1A). The combination index (CI) value was found to be 0.41 (CI = 0.41) and 0.32 (CI = 0.32) in DU145 and C4-2B cells when treated with combined drug (TQ + DTX). These results showed that DU-145 cells had higher tolerance to TQ compared to C4-2B cells, in contrast the synergistic effect of combined drug was found more efficient in C4-2B compared to DU145 cells. Although TQ and DTX were individually toxic to PCa cells, their combination had a higher apoptosis-inducing effect, with lower concentrations of both agents being required. In addition to the combined effect of TQ and DTX against PCa cells, their action on cell survival was assessed in the presence of PI3Ki (1.4 µM) and AKTi (625 nM) using a survival assay. There was lower survival of PCa cells with a combination of TQ and DTX along with PI3Ki and AKTi (Figure 1B). There were similar results with a cell viability test in which stained cells were represented by blue and green colors for nuclei of live and dead cells, respectively. More dead cells were evident when they were treated with PI3Ki and AKTi combined with TQ and DTX compared to individual drugs or their combination

of TQ and DTX without the inhibitors (Figure 1C). C4-2B cells had more dead nuclei compared to DU145 cells, showing some drug tolerance of DU145 cells. These results suggest that, for PCa cells, TQ synergizes with DTX and enhances the efficacy of DTX.

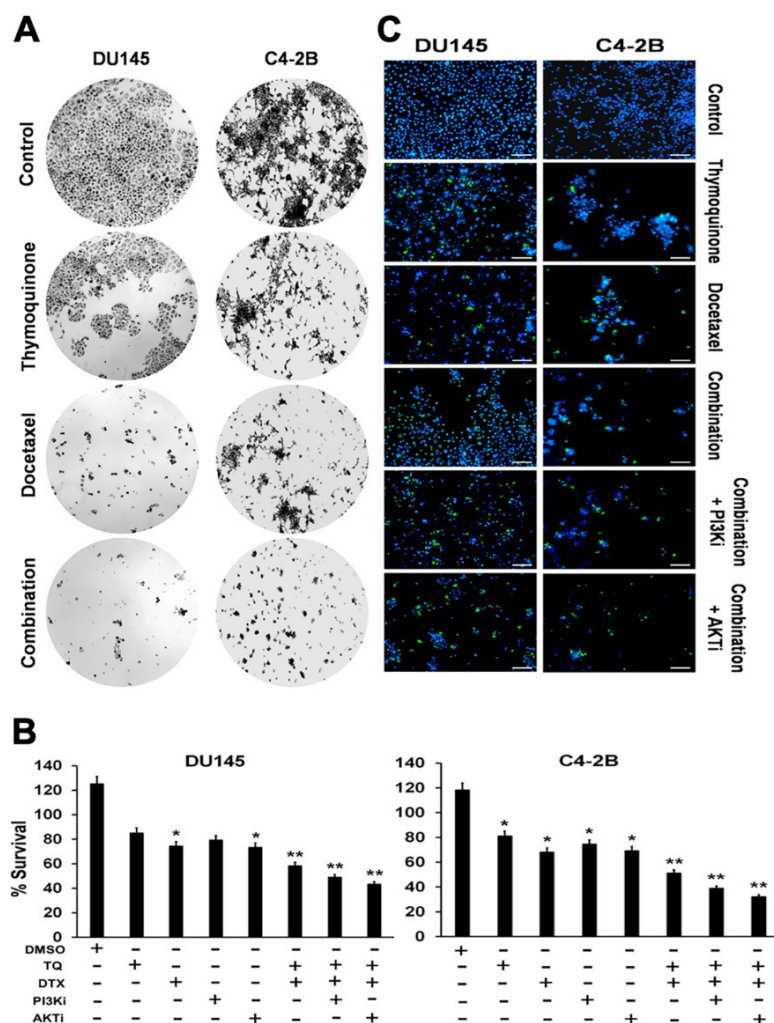


Figure 1. Effect of TQ and DTX on cell viability and cytotoxicity to PCa cells. PCa cells were treated with sequential concentrations of TQ, DTX, or a combination of TQ and DTX for 48 h, and viability was measured by MTT assays for (A) DU145 and C4-2B cells. DU145 cells were treated with TQ (60 μ M), DTX (20 nM), or their combination (TQ+DTX, 50 μ M + 10 nM); C4-2B cells were treated with TQ (54 μ M), DTX (20 nM), or their combination (TQ + DTX, 35 μ M + 10 nM) for 48 h. (B) shows the percentage survival of DU145 and C4-2B cells after treatment with TQ, DTX, PI3Ki, AKTi or their combination with or without PI3Ki (1.4 μ M) and AKTi (625 nM) for 48 h. Sign “+” or “-” represents the presence or absence of the drugs. Data are presented as means $\pm$ standard error of the means of at least three independent experiments and were analyzed by an unpaired t-test. ** and * indicate *p* values of ≤ 0.01 and 0.05, respectively. (C) shows the DU145 and C4-2B cells treated with TQ, DTX, or their combination with or without PI3Ki and AKTi for 48 h and were processed for immunofluorescent staining. More live nuclei (blue) were found in the control group compared to any of the treatment groups. More dead nuclei (green) were found in those treated with the combination in presence or absence of PI3Ki and AKTi compared to treatment with individual drugs. Nuclei of live and dead cells, detected by DAPI staining and a GFP filter, appear as blue or green, respectively.

2.2. The DTX and TQ Combination Induces Apoptosis in PCa Cells

To investigate the extent of apoptotic induction by the TQ and DTX combination in comparison to DTX or TQ alone, we evaluated the cell death effect on DU145 and C4-2B cells by TQ alone, DTX alone, or in combination for 48 h. After treatment, cells were stained by using an apoptosis kit (with Annexin V-FITC and PI) and analyzed by flow cytometry. Quadrants Q1–Q4 represent necrotic, late apoptotic, viable, and early apoptotic cells, respectively (Figure 2). For DU145 cells treated with TQ alone, DTX alone, or their combination, values for the total (early and late) apoptotic cells were 54.9%, 70%, and 89%, respectively, compared to the control (10.1%). However, C4-2B cells had values for early and late apoptotic cells for TQ (35%), DTX (60%), and the combination (90%), respectively. The TQ + DTX combination increased the extent of apoptosis compared with either drug alone. These results confirm the cytotoxicity and efficacy of TQ when given in combination with DTX to treat PCa cells.

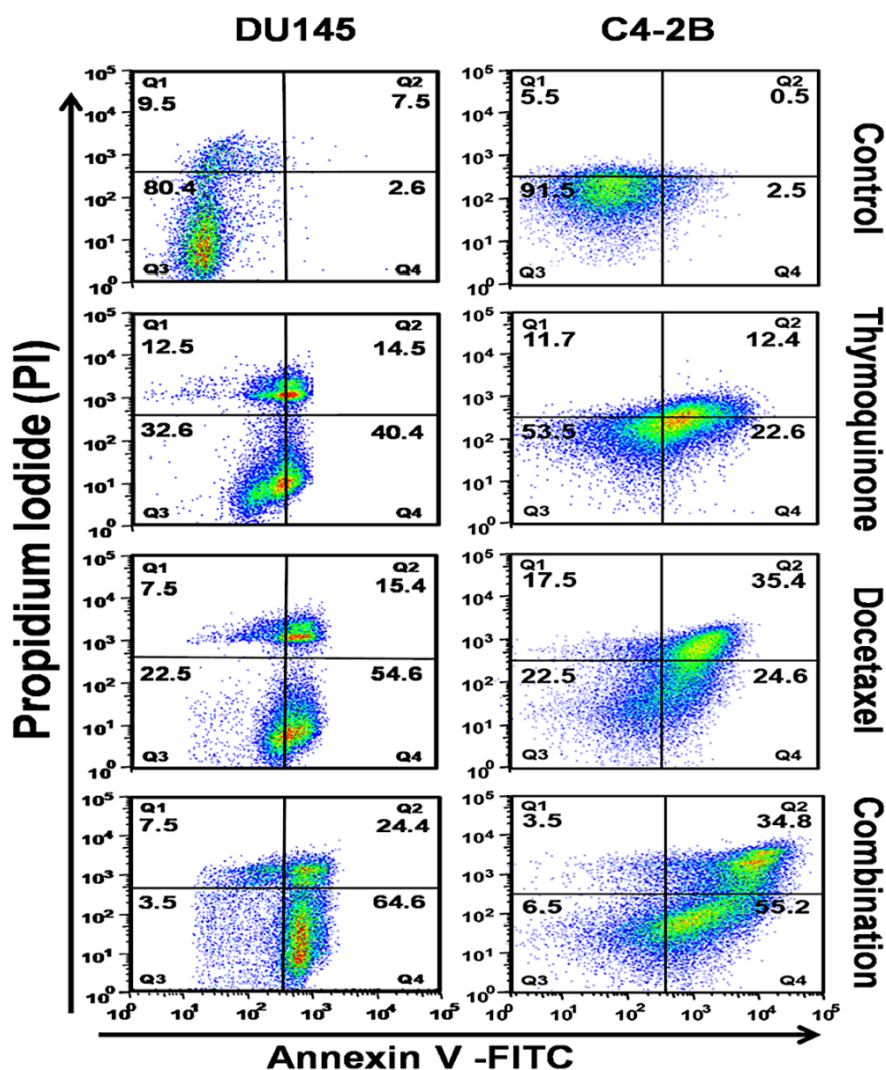


Figure 2. TQ induces DTX-mediated apoptosis in PCa cells. DU145 and C4-2B cells were treated with TQ, DTX, or their combination for 48 h, and apoptosis was assessed by staining with Annexin V-FITC and PI followed by flow cytometry. The percentages of early (Annexin(+)/(PI(-)), late (Annexin(+)/(PI(+)), necrotic (Annexin(-)/(PI(+)), and viable (Annexin(-)/(PI(-)) cells are shown in bold numbers in Q4, Q2, Q1, and Q3, respectively. For both DU145 and C4-2B cells, the combination of TQ + DTX showed high numbers of apoptotic cells in the (early) or (late) quadrants compared to individual drugs alone. Data shown are the means $\pm$ SEM from three independent experiments.

2.3. Effect of PI3K/AKT Inhibition on PCa Cells

To determine if the PI3K/AKT pathway was involved in the proliferation and progression of PCa cells, we evaluated its expression in DU145 and C4-2B cells. Based on their IC₅₀ values, PCa cells were treated with TQ or DTX or with the combination. Upon treatment, cells were exposed to PI3Ki (1.4 μ M) and AKTi (625 nM) for 48 h with a combination of TQ + DTX. DU145 cells showed a lower expression of PI3K/AKT when treated with the combination in presence of inhibitors compared to control (untreated), individual drugs, or their combination. Similarly, in C4-2B cells, the expression was low upon blocking of PI3K/AKT (Figure 3A,B). These results suggesting that reduced phosphorylation of PI3K/AKT inhibits the downstream signaling effectors that lead to cell apoptosis.

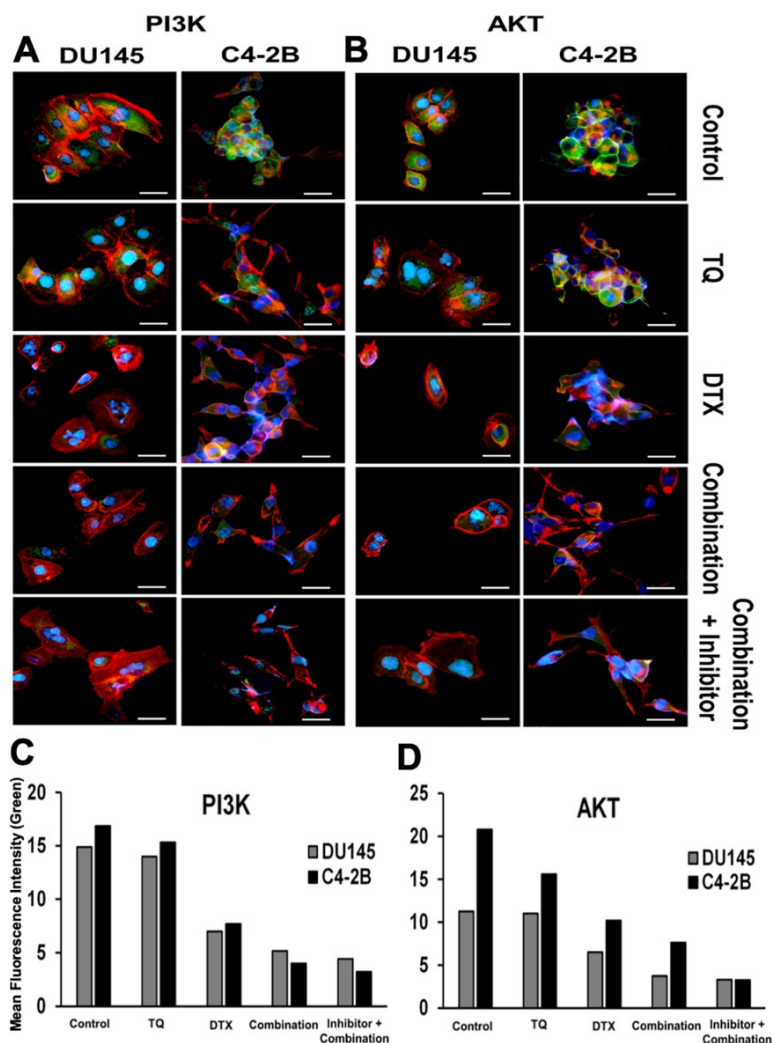


Figure 3. Immunofluorescent analysis of PI3K/AKT expression in PCa cells. DU145 and C4-2B cells, cultured overnight, were treated with TQ, DTX, or their combination with or without PI3Ki and AKTi for 48 h and were processed for immunofluorescent staining. For (A) PI3K, and (B) Akt expression, cells were stained with primary and Alexa Fluor 488-conjugated secondary antibodies and examined with a fluorescent microscope. (C,D) represent the mean fluorescence intensity of PI3K or AKT (green) for DU145 and C4-2B cells respectively. Green indicates the expression of PI3K or Akt; red indicates Phalloidin staining. Nuclei were stained with DAPI, and images were captured at 40 $\times$ magnification.

2.4. The TQ and DTX Combination induced Modulation of Pro- and Anti-Apoptotic Molecules via Inhibition of the PI3K/AKT Pathway in PCa Cells

Cellular signals regulate cancer cell proliferation and apoptosis, and inhibition of these signals can be helpful in eliminating cancer [24]. Considering these facts, we validated the expression of apoptosis-specific genes at the protein level by treating DU145 and C4-2B cells with TQ, DTX, or their combination in the presence and absence of PI3Ki and AKTi. The expressions of pro-apoptotic genes (BID and BAX) were upregulated in DU145 cells treated with the combination (TQ + DTX, 50 μ M + 10 nM) and in the presence of PI3Ki (1.4 μ M) and AKTi (625 nM) for 48 h; in turn, the expression of BCL-XL was downregulated (Figure 4A). Although the individual drugs were effective, their action on apoptotic genes were minimal. Subsequently, C4-2B cells were treated with TQ (54 μ M), DTX (20 nM), or their combination (TQ+DTX, 35 μ M +10 nM) with or without PI3Ki (1.4 μ M) and AKTi (625 nM) to monitor cell death (Figure 4B). The expressions of pro-apoptotic proteins (BID and BAX) were upregulated, and the anti-apoptotic protein, BCL-XL, was downregulated upon treatment of cells with the inhibitors, confirming the effectiveness of TQ and DTX in PCa cells. In addition to effects on pro- and anti-apoptotic genes, there was, in both cell lines at 48 h after treatment, upregulation of pro-caspase 3. Moreover, in respect to DNA damage caused by the combination with inhibitors, poly(ADP-ribose) polymerase (PARP)-1 expression was elevated compared to expression in the control cells, and in cells exposed to the individual drugs (not in the case of DTX alone in C4-2B cells). The protein band intensity for DU145 and C4-2B cells are shown in Figures S1 and S2, respectively. Thus, the combination of TQ and DTX promoted cell death compared to TQ or DTX alone.

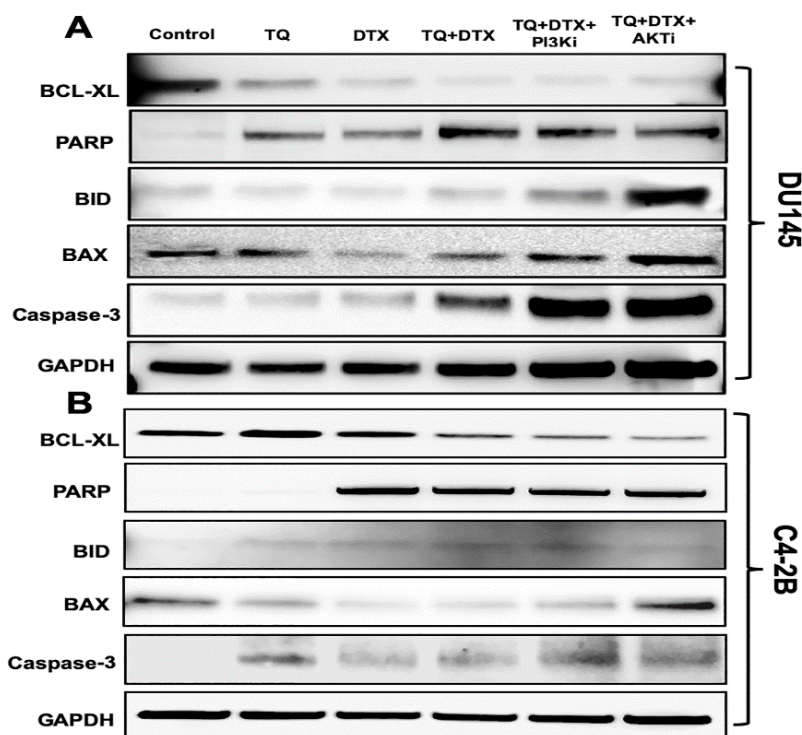


Figure 4. TQ in combination with DTX induces the expression of pro- and anti-apoptotic markers in PCa cells. Immunoblots of (A) DU145 and (B) C4-2B cells showing the expression of pro- and anti-apoptotic genes after treatment with TQ, DTX, or their combination in the presence or absence of PI3Ki and AKTi inhibitor. After 48 h of treatment with the combination, the pro-apoptotic genes, BAX and BID, and caspase-3 and PARP were upregulated relative to effects of the individual drugs. However, for both cells, the anti-apoptotic gene, BCL-XL, was downregulated by the combination. As an internal standard for equal loading, blots were probed with a GAPDH antibody.

2.5. The TQ and DTX Combination Regulated Gene Expression at the mRNA Level in PCa Cells

Inhibition of the PI3K/AKT signaling pathway in cancer cells is effective in inducing apoptosis [25]. Combinations of drugs have been investigated for tumor cells prevention and treatment. However, since there is no established chemotherapeutic cure for PCa patients, a new strategy or drug target for PCa treatment is needed. Here, we studied the expression of genes in PCa cells in response to TQ, a natural compound, and DTX and to blocking of the PI3K/AKT signaling pathways. For DU145 cells, pro-apoptotic (BAX and BID genes) and PARP expression were upregulated after blocking the PI3K/AKT pathways with PI3Ki and AKTi compared to the effects of the single drugs alone or in combination (Figure 5A). There was an increase in the apoptotic gene, caspase 3, after 48 h of treatment with TQ, DTX, or their combination. To assess the anti-apoptotic genes in DU145 cells, we tested the marker, BCL-XL; there was no appreciable difference for their combination treatment with or without inhibitors.

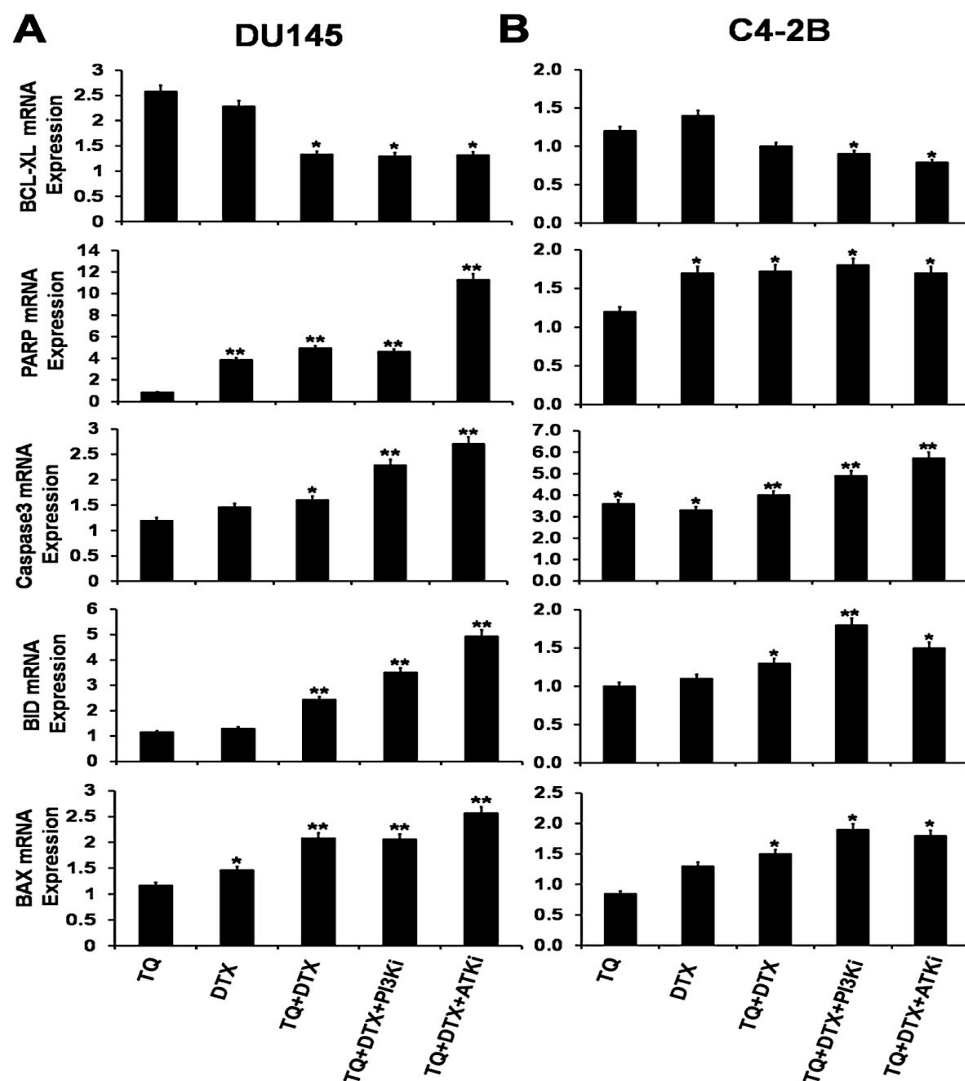


Figure 5. mRNA expression of pro- and anti-apoptotic genes in PCa cells. (A) DU145 and (B) C4-2B cells were treated with various concentrations of TQ, DTX, or their combination with or without PI3Ki and AKTi for 48 h and quantified by RT-PCR. The expression levels of pro-apoptotic factors (BAX, and BID), PARP, caspase 3, and the anti-apoptotic factor (BCL-XL) are presented as fold changes relative to the control cells. Data were normalized to the levels of expression of the housekeeping gene (18 S), and the experiments were repeated three times. Data are presented as means $\pm$ SEM; asterisks indicate significance as determined by Student *t*-tests. * and ** indicates *p* value $\leq$ 0.01 and 0.05, respectively.

Further, to validate the pro- and anti-apoptotic markers in C4-2B cells, these cells were treated for 48 h based on their IC₅₀ values. The pro-apoptotic BAX and BID genes at their mRNA levels were elevated in the presence of blocking agents (Figure 5B). In line with that, the expression of caspase 3 was upregulated when the pathways were blocked with PI3Ki and AKTi inhibitors. PARP expression was upregulated, but there was no appreciable difference between the effects of the combination and with the inhibitors. BCL-XL was suppressed by the combination. Together, these observations suggest that, for their combination of TQ and DTX, the PI3k/AKT signaling cascade is blocked, which causes apoptosis in PCa cells.

3. Discussion

Despite improvements in hormone therapy, many men with PCa develop metastatic CRPC and require chemotherapy. DTX is widely used for treating PCa and is a primary agent for treating other cancers. To reduce the side effects of DTX and improve its efficacy, it has been administered with other drugs. Several epithelial cancers have been treated with combinations containing taxane-based chemotherapeutic agents, but PCa remains as an exception [26]. Although a recent study suggested that histone deacetylase inhibitors, in combination with DTX, enhance its anti-proliferative effect in CRPC cells [27], previous efforts in combination treatment show no additional benefit or increased side effects such as thromboembolic events [1]. In addition, phase III trials in treating CRPC have failed to improve on DTX efficacy [1]. Hence, combination strategies that improve the outcomes for PCa are needed.

With a long history of use of natural products/compounds from plants, microorganisms and fungi against human diseases, they have now been tested against cancer, and some have shown promising results [28]. Among these is TQ, which, by acting on various pathways, shows promising effects for humans [19]. Although, several randomized control trials (RCTs) have been investigated the therapeutic effects of TQ in the prevention and the treatment of various diseases in humans [29]. In the present effort, we investigated the combination of TQ with DTX against PCa cell viability, proliferation, and cytotoxicity. Apoptosis, a physiological form of cell death and a pathway for regulating homeostasis and morphogenesis of cells, is associated with various diseases, in particular, cancer [30]. Apoptosis is described as the organized collapse of cells by membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation [31]. Studies on the mechanism of action of TQ show that it induces apoptosis of cancer cells [31]. Taxanes have a broad spectrum of activity and induce apoptosis of cancer cells in varying degrees [32]. In the present investigation, analysis of changes in apoptosis using Annexin V in the multidomain spectrum of flow cytometry showed that TQ and DTX individually cause apoptosis of PCa cells. However, the extent of apoptosis was higher when these anticancer agents were combined in low concentrations. These results show that TQ can reduce the side effects of DTX and lead to an enhancement of apoptosis of cancer cells.

DTX induces apoptosis in cancer cells, as demonstrated by associated changes in the mitochondrial membrane potential and overexpression of BCL2 [32]. Although the activation of caspase 8 and BID seems to be a late event, apoptosis is induced by the activation of caspase 2 [32]. For PCa cells, TQ activates apoptosis-inducing factor-1 and the DNA damage-inducible gene, GADD45 alpha, and down-regulates the expression of Bcl2, Bcl2L1, BAG-1, Bcl2A1, and BID proteins [19]. The present investigation showed induced expression, at mRNA and protein levels, of pro-apoptotic genes and downregulation of anti-apoptotic genes. As individual compounds, TQ and DTX did not have a profound effect on the expression of BCL-XL, but the combination caused a pronounced decrease. For their combination, there was a reverse trend for pro-apoptotic genes, BAX and BID, as well as high expression of pro-caspase 3 protein. These results show that the combination of the two agents, acting by two mechanisms (inhibiting PI3K/AKT pathway and inducing apoptosis), facilitate apoptosis and that the combination can be used to treat PCa.

PI3K/AKT signaling appears to be necessary for PCa cell survival and proliferation [33]. Due to the aggressive nature of the PI3K/AKT pathway and its frequent presence in metastatic PCa, this pathway

has been used as a biomarker for progressive PCa [21]. To combat PCa, combination therapy with a PI3K/AKT inhibitor can be regarded as horizontal blockade [34]. In breast [35] and gastric cancers [36], TQ inhibits the PI3K/AKT pathway by downregulating the phosphorylation of AKT and upregulating PTEN. In addition, DTX is a candidate for inhibition of the PI3K/AKT pathway. In the current study, we interrogated the effect of PI3K/AKT inhibitors and DTX and TQ individually in affecting the PI3K/AKT pathway and compared the effects with the combination of DTX and TQ and along with PI3K/AKT inhibitors. The combination inhibits the PI3K/AKT pathway, which induces death of PCa cells.

4. Materials and Methods

4.1. Cell Culture

The PCa cell lines, DU-145 (HTB-81) and C4-2B (CRL-3315), were procured from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). DU-145 cells were grown in Eagle's Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS), and 10,000 U/mL penicillin/10,000 µg/mL streptomycin antibiotic solution (Fisher Scientific, Pittsburgh, PA, USA). C4-2B cells were maintained in DMEM plus F12 Medium (Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% heat-inactivated FBS, insulin, triiodo-L-thyronine, transferrin, D-biotin, adenine (Millipore Sigma, St. Louis, MO, USA), and 10,000 U/mL penicillin/10,000 µg/mL streptomycin antibiotic solution (Fisher Scientific, Pittsburgh, PA, USA). Both cell lines were maintained in an incubator at 37 °C and with 5% CO₂.

4.2. Cell Proliferation Assay

Growing cells were trypsinized (0.25% Trypsin-EDTA, Fisher Scientific, Pittsburgh, PA, USA) and seeded in 96-well plates at 10,000 cells/well. Cells were treated with DTX (0, 5, 10, or 50 nM), TQ (0, 5, 10, 15, 20, 25, or 50 µM) (Fisher Scientific, Pittsburgh, PA, USA), and a combination of the drugs with an Akt inhibitor (AKTi) (0.5, 1, 2, 5, or 10 nM) and a PI3K inhibitor (PI3Ki, LY294002, 0.5, 1, 2, 5, or 10 µM) (Millipore Sigma, St. Louis, MO, USA). The cells were incubated for 24, 48, or 72 h at 37 °C in a 5% CO₂ incubator. Following our previous protocol [12], 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) 5 mg/mL, (Fisher Scientific, Pittsburgh, PA, USA) was added to each well. The cells were incubated at 37 °C for 3 h. The purple formazan crystals were dissolved in 100 µL of dimethyl sulfoxide, and the absorbance was measured at 570 nm with a spectrophotometer (Spectramax M5, Molecular Devices, Sunnyvale, CA, USA). Next, the IC₅₀ (half-maximum inhibitory concentration) values were calculated for DU145 and C4-2B cells. To evaluate possible additive (0.9 < CI < 1.1), synergistic (CI < 0.9), or antagonistic effects (CI > 1.1), the combination index (CI) method was used [37], and the relative growth rates were calculated for both cell lines.

4.3. Live/Dead Cell Assay

PCa cells (2 × 10⁴) were seeded in 48-well plates overnight and then treated with TQ, DTX, or a combination of TQ+DTX with or without PI3Ki and AKTi for 48 h. Cells were then washed with PBS and incubated with 2 drops/mL of cell viability imaging solution (Thermo Fisher Scientific, Carlsbad, CA, USA) for 15 min at 37 °C according to the manufacturer's protocol. Fluorescence images were acquired after staining by using a DAPI, and FITC/GFP filter and an EVOS FL microscope (Thermo Fisher Scientific, Carlsbad, CA, USA).

4.4. Apoptosis Assay

DU145 and C4-2B cells were seeded in six-well plates and treated with TQ, DTX, or their combination for 48 h. Following our previous protocol [12], the cells were washed, harvested, and counted with a hemocytometer (Countess II FL, Life Technology, Carlsbad, CA, USA). Equal numbers (1 × 10⁵) of DU145 and C4-2B cells were washed twice with cell staining buffer

(BioLegend, San Diego, CA, USA). Subsequently, cells were stained with FITC-Annexin V and propidium iodide (PI) according to the manufacturer's instructions (BioLegend, San Diego, CA, USA). Further, cells were washed and suspended in Annexin V Binding Buffer. The apoptotic cells were processed with a Guava flow cytometer (EMD Millipore, Billerica, MA, USA) and analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

4.5. Immunofluorescence Assay

To determine expression of the intracellular signaling pathway (PI3K/Akt), DU145 and C4-2B cells were seeded in 48-well plates overnight at 37 °C and 5% CO₂ and then treated with TQ, DTX, or with TQ+DTX with or without PI3Ki and AKTi for 48 h. The method for cell staining followed that described in our previous publication [38]. Briefly, cells were washed with cold PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.05% saponin for 10 min. Next, the cells were washed, blocked with 3% bovine serum albumin, and stained with anti-PI3K and anti-Akt primary antibodies at 4 °C overnight, then incubated with FITC-conjugated fluorescent secondary antibody (Cell Signaling, Danvers, MA, USA) for 1 h. For visualization of the F-actin cytoskeleton, cells were stained with Phalloidin™ Red 594 solution (1:50, BioLegend, San Diego, CA, USA) for 20 min at room temperature. Cell nuclei were counterstained with DAPI (Invitrogen, Carlsbad, CA, USA). Immunofluorescence images were taken with a fluorescent microscope with DAPI, FITC/GFP, and RFP filters using an EVOS FL microscope (Thermo Fisher Scientific, Carlsbad, CA, USA).

4.6. Western Blot Analysis

To determine the expression of apoptotic markers, DU145 and C4-2B cells were treated with TQ, DTX, or their combination with or without PI3Ki and AKTi for 48 h. Cells were then harvested and lysed with RIPA buffer containing 1× protease and protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA) and centrifuged for 10 min at 10,000 rpm to collect the supernatants. Following our previous protocol for protein isolation [9], total protein was determined using BCA protein assay kits (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were prepared by using bromophenol blue dye and RIPA buffer and boiled for 10 min. Next, equal amounts of protein (30 µg) were fractionated on 4–12% polyacrylamide gels (Life Technologies, Carlsbad, CA, USA) and transferred to polyvinylidene fluoride membranes, which were blocked in 5% non-fat dry milk (Biorad, Hercules, CA, USA) for 30 min prepared with TBS-T (20 mM TRIS-HCl pH 7.6, 150 mM NaCl, 0.1% Tween 20), (Fisher Scientific, Pittsburgh, PA, USA). After blocking, blots were probed with primary antibodies overnight at 4 °C, followed by exposure to secondary antibodies (1:2000 dilution) for 1 h at room temperature. The primary antibodies, including those for pro-apoptotic (BAX and BID), anti-apoptotic (BCL-XL), and apoptotic (pro-caspase-3; BioLegend, San Diego, CA, USA) and poly[adenosine diphosphate]-ribose polymerase [PARP] factors, and secondary antibodies were procured from Cell Signaling Technology (Danvers, MA, USA). The resulting protein bands were visualized by chemiluminescence with Western detection reagent (Fisher Scientific, Pittsburgh, PA, USA), and images were acquired with an ImageQuant LAS4000 instrument (GE Healthcare-Biosciences, Pittsburgh, PA, USA).

4.7. RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

PCa cells treated with TQ, DTX, or their combination and with PI3Ki and AKTi for 48 h were lysed with Trizol reagent (Invitrogen, Paisley, UK) and extracted following the standard protocol for RNA. Following our previous protocol [12], briefly, RNA was precipitated, suspended in nuclease-free water, and quantified. Total RNA (1.0 µg) and reverse transcription Superscript for RT-qPCR were used to generate cDNA following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Primers for pro-apoptotic (BAX and BID), anti-apoptotic (BCL-XL), and apoptotic factor (PARP and caspase-3) genes were synthesized with data from the National Center for Biotechnology Information (NCBI) gene bank and used for mRNA expression. 18 S primers (5'-GGCCCTGTAATTGGAATGAGTC-3' and 5'-CCAAGATCCAACTACGAGCTT-3')

were used as an endogenous control. The following sequences of primers were used: BAX: 5'-AAACTGGT GCTCAAGGCC-3' and 5'-CTTCAGTGACTCGGCCAGG-3'; BID: 5'-AGCACAGTGC GGATTCTGTC-3' and 5'-ACCGTTGTTGACCTCACAGT-3'; PARP: 5'-GCTTCAGCCTCCTTGCTACA-3' and 5'-TTCGCCACTTCATCCACTCC-3'; caspase 3: 5'-CTCTGGTTTTTCGGTGGGTGT-3' and 5'-CGCTTCCATGTATGATCTTTGGTT-3'; and BCL-XL: 5'-CCTGCCTGCCTTTGCCTAA-3' and 5'-TGGGCTCAACCAGTCCATTG-3. RT-PCR was performed using SYBR[®] Green PCR master mix reagents (Bio-Rad, Hercules, CA, USA), and gene expression was analyzed by CFX-Manager software (CFX96 Real-Time System, Bio-Rad, Hercules, CA, USA). The experiments were repeated three times.

4.8. Statistical Analysis

All experimental data are presented as standard errors of means ($\pm$ SEM) for at least three independent experiments. The level of significance was determined by one-way analysis of means (ANOVA), and p -values < 0.05 were considered statistically significant.

5. Conclusions

Despite improvements in anti-hormone therapy for PCa, many men develop CRPC and require chemotherapy. Although, for treating PCa, combinations containing DTX show potential in terms of tolerability and increased efficacy compared to DTX alone, no additional benefits have been observed. In the present investigation, we found that the combination of DTX with TQ had a greater cytotoxic effect against PCa cells, allowing decreases in the concentrations of both. Further, DTX and TQ were inducers of apoptosis; the extent of apoptosis was greater, even when they were used in lower concentrations. As many patients develop CRPCs, clinical trials are now evaluating the combination of PI3K/AKT inhibitors along with anticancer drugs. Thus, the combination of DTX and TQ in addition to PI3K/AKT inhibitors may improve the survival rate and quality of life of PCa patients.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/11/9/1390/s1>, Figure S1: Densitometry Readings/intensity Ratio (DU145), Figure S2: Densitometry Readings/intensity Ratio (C42B). Western blot densitometry is available in supplementary materials (Figures S1 and S2).

Author Contributions: S.K.S. performed experiments, analyzed data, and wrote the manuscript. T.A. helped in biological assays and reviewed the manuscript. J.B.G. critically reviewed the manuscript. R.S. conceived and designed the experiments, did a critical review of the manuscript, and provided infrastructure and research facility to performed aforementioned experiments.

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