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Novel mechanisms of androgen receptor degradation by alpha-tocopherylquinone and curcumin analog 27

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NOVEL MECHANISMS OF ANDROGEN RECEPTOR DEGRADATION BY ALPHA-TOCOPHERYLQUINONE AND CURCUMIN ANALOG 27

BY

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DEDICATION

In loving memory of my Grandparents Fred and Carmen Fajardo and Grandmother Elizabeth Zamora whose love and guidance provided inspiration during this project.

This dissertation is dedicated to my mentors, family, friends and colleagues who encouraged me in the beginning of this journey and have supported me throughout. A special thanks to the UNM College of Pharmacy Department of Pharmaceutical Sciences for their support of this research.

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ABSTRACT

Defining underlying molecular mechanisms exploited by cancer cells in their development and progression provides a necessary foundation for experimental therapeutics. The androgen receptor (AR) is a known therapeutic target for prostate cancer (CaP) given its well-established role in both the development and progression of CaP. The AR is a ligand activated transcription factor that regulates the expression of many genes involved in proliferation and differentiation. Identifying agents that down-regulate AR expression may elucidate mechanism(s) for selectively targeting the AR. Two related agents of the natural products curcumin and vitamin E, curcumin analog 27 (ca27) and alpha-tocopheryl quinone (TQ), respectively were identified that down-regulate AR protein expression in CaP cells. *The purpose of this dissertation project was to identify molecular pathways that contribute to AR down-regulation mediated by ca27 and TQ.* While both ca27 and TQ down-regulate the AR, the kinetics of AR down-regulation was distinct between the two agents. ca27's down-regulation of AR protein expression was observed within hours, while TQ effects were seen after two days. Despite this difference, ca27 and TQ were found to have many similarities in their

mechanism of AR down-regulation. Both ca27 and TQ up-regulate CYP1A1 expression, a known aryl hydrocarbon receptor (AHR) regulated gene. The AHR is a ligand activated transcription factor known to be involved with detoxification and metabolic pathways. However, the AHR itself did not appear to be regulating the observed effects on AR expression mediated by ca27 and TQ. Interestingly, additional data suggests TQ might serve as a ligand for the AHR (Chapter 4). Further, ca27 and TQ down-regulation of AR protein expression was determined to be independent of proteasomal degradation and transcriptional inhibition. Due to chemical structure considerations of ca27 and TQ, their potential to modulate CaP cell reduction/oxidation parameters was examined. Both ca27 and TQ were shown to down-regulate AR protein expression through a cellular redox mechanism, which was attenuated by the presence of the antioxidant N-acetylcysteine (Chapter 2 and 3), respectively. This study identifies pathways critical to the mechanism of action of ca27- and TQ-mediated AR protein down-regulation in human CaP cells and demonstrates that these novel agents act though alterations in cellular redox.

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AHR	…………………………………………	Aryl hydrocarbon receptor
AKR1C1	…………………………………………	Aldoketoreductase 1C1
α-NF	…………………………………………	Alpha-napthoflavone
AR	…………………………………………	Androgen receptor
ARE	…………………………………………	Antioxidant response element
ARNT	…………………………………………	Aryl hydrocarbon nuclear receptor
ARRE	…………………………………………	Androgen receptor response element
AT	…………………………………………	Alpha-tocopherol
ATBC	…………………………………………	Alpha-tocopherol, beta-carotene cancer prevention study
ATF	…………………………………………	Activating transcription factor
B(a)P	…………………………………………	Benzo(a)pyrene
BSA	…………………………………………	Bovine serum albumin
BSO	…………………………………………	Buthionine sulfoximine
ca27	…………………………………………	Cucumin analog 27
CaP	…………………………………………	Prostate cancer
CSS	…………………………………………	Charcoal- stripped serum
CYP1A1	…………………………………………	Cytochrome P450 1A1
DCF	…………………………………………	2’,7’-dichlorofluorescein diacetate
DRE	…………………………………………	Dioxin receptor response element
EGCG	…………………………………………	(-)-epigallocatechin gallate
ER	…………………………………………	Endoplasmic reticulum
GR	…………………………………………	Glucocorticoid receptor

GSH	Glutathione, reduced glutathione
GSSG	Oxidized glutathione
HAH	Halogenated aromatic hydrocarbon
HPLC	High-performance liquid chromatography
IRE1	Inositol-requiring enzyme 1
MAF G	Small MAF G
MMTV-LTR	Mouse mammary tumor virus long terminal repeat
NAC	N-acetylcysteine, N-acetyl-L-cysteine
NQO1	NAD(P)H quinone oxidoreductase
Nrf2	Nuclear factor (erythroid-derived 2) like 2
PAH	Polycyclic aromatic hydrocarbon
PDI	Protein disulfide isomerase
PER	Period
PERK	(PKR) like ER kinase
PSA	Prostate specific antigen
REDOX	Reduction/oxidation
ROS	Reactive oxygen species
RXRα	Retinoid X receptor, alpha
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SIM	Single minded
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TMF	6,2',4'-Trimethoxyflavone
TQ	Alpha-tocopherylquinone

UPR	…………………………………………...	Unfolded protein response
VDR	…………………………………………...	Vitamin D receptor
VE	…………………………………………...	Vitamin e (alpha-tocopherol)
XBP1	…………………………………………...	X-box binding protein 1
XBP 1(s)	…………………………………………...	X-box binding protein 1 spliced
XBP1(u)	…………………………………………...	X-box binding protein 1 unspliced
XRE	…………………………………………...	Xenobiotic response element

CHAPTER 1: INTRODUCTION

Brief Summary

With the high risk of developing prostate cancer (CaP) in men, and the possibility that it will progress to a more advanced disease, development of novel targeted therapeutic strategies for CaP is crucial. The androgen receptor (AR) plays a critical role in CaP growth and progression. However, current strategies for CaP treatment eventually fail to effectively inhibit the contribution of the AR to disease progression. Several natural products have been identified as AR inhibitors *in vitro* but these agents often have limitations for *in vivo* use. Two agents representative of natural products will be the focus for this study, vitamin E (VE) and curcumin as experimental therapeutic agents for CaP. The agents alpha-tocopheryl quinone (TQ) and curcumin analog 27 (ca27) were screened for their potential *in vitro* anti-androgenic activity. Several human androgen-responsive CaP cell lines were utilized in the characterization of TQ and ca27 actions. Both agents were evaluated for their inhibition of cell proliferation and viability, AR activation and AR expression. The focus of this study was to identify TQ and ca27's mechanism(s) of AR protein down-regulation. Several potential mechanisms of TQ and ca27's AR down-regulation were systematically identified and evaluated. These potential mechanisms included, transcriptional inhibition, proteasomal degradation, aryl hydrocarbon receptor (AHR) mediated degradation and pathways involving oxidative stress. This study identifies a potential mechanism of TQ and ca27's AR down-regulation. Inhibiting the expression of the AR may be an effective therapeutic strategy for prostate cancer. TQ's and ca27's actions on AR down-regulation may in part have similar activities, but these two agents will be presented separately in this dissertation. The results of this study may provide insight into therapeutically useful mechanisms of AR protein down-regulation.

The prostate gland, prostate cancer, and the androgen receptor

The prostate is a male sex accessory gland located at the base of the bladder behind the pubic bone just in front of the rectum. The prostate wraps around the urethra, the urethra is a tube that carries urine from the bladder to the penis (1). Its primary physiologic role is the addition of secretions to sperm during ejaculation. Androgens such as testosterone and dihydrotestosterone (DHT) are essential for normal prostate development and function. Both testosterone and DHT exert their effects through their binding of the AR (2). The AR is a transcription factor that regulates genes involved in masculinization during development, reproduction, muscle development and prostate growth (3). The AR is required for normal prostate development and also has a significant role in CaP.

CaP is the second most frequently diagnosed malignancy and the sixth leading cause of cancer death in men world-wide (4). The incidence rates for prostate cancer vary greatly world-wide, with the highest rates recorded for more developed countries (4). In the United States (US), CaP accounts for 12% of cancer incident cases (5). Age, ethnicity and family history are major risk factors for developing CaP. The progression of CaP varies among individuals; while some CaP grow slowly and remain confined to the prostate gland others are more aggressive and can spread quickly. CaP is initially sensitive to androgen deprivation therapy but usually progresses to a castration-resistant disease. This progression can be attributed to the activation and signaling of the androgen receptor (6,7).

The AR or NR3C4 is a member of the steroid hormone receptor family of ligand-dependent nuclear receptors. The activity of the AR is essential for normal prostate development and is an important mediator of CaP growth and development. One of the AR roles is as a transcription factor for several genes involved in the development and differentiation of the prostate (8). The AR is activated by androgens such as testosterone or its more active metabolite, DHT. Most (90-95%) testosterone in men is produced by the Leydig cells of the testies, with additional androgens or androgen precursors produced by the adrenal gland (9). DHT is converted from testosterone by the enzyme 5-α reductase (10,11). Upon ligand binding, the AR releases from chaperone proteins such as heat shock protein 90 (HSP90), homodimerizes, and is phosphorylated. The AR is then free to translocate into the nucleus and bind co-regulators leading to its activity as a transcription factor (8,12). Specific recognition sequences known as androgen receptor response elements (ARREs) in the promoter and enhancer regions of target genes, such as prostate specific antigen (PSA) gene, are recognized by the AR (Fig. 1A) (11). Although inhibition of androgen production and AR activity are currently used as therapeutic targets for CaP, targeting the AR itself may prove to be a more effective therapeutic strategy.

Androgen deprivation therapy targeting the synthesis of testicular androgens such as the use of luteinizing hormone-releasing hormone (LHRH) analogs or surgical castration increases the survival of CaP patients but it is not curative for the disease (13,14). Two possible explanations are that either there is an incomplete ablation of androgen allowing for continued AR activation or the receptor can bypass the androgen depleted environment in an alternative fashion. Both are possible explanations since, after androgen ablative treatment, remaining residual of circulating testosterone and 5α-dihydrotestosterone (DHT) can be detected (15). The importance of the AR function in CaP is evident by the *de novo* autocrine intra-tumoral synthesis of androgens from cholesterol. One of the key enzymes in this production of androgens is CYP17. CYP17 activity can be inhibited by the irreversible inhibitor abiraterone acetate (i.e. Zytiga) or the antifungal agent ketoconazole (Fig.1A) (7). For recurrent disease, the low concentrations of androgens can be sufficient to activate a functional AR. The inhibition of the conversion of testosterone into DHT has been identified as another strategy for CaP. Inhibiting DHT expression can be achieved by inhibition of the enzymes 5-α reductase type 1and 2. Two inhibitors are currently available finasteride, a type 1 5-α reductase inhibitor and dutasteride, a dual 5-α reductase inhibitor (16). However, these inhibitors target the production of DHT thereby inhibiting activation of the AR indirectly. The inclusion of other treatment options such as the nonsteroidal AR antagonists biclutamide (i.e. Casodex) and MDV3100 directly target the AR (Fig. 1A) (7,17). Biclutamide and MDV3100 competitively bind the ligand binding domain of the AR, inhibiting natural ligand binding (7). Both of these treatments inhibit the AR, but they do not down-regulate AR expression. However, studies have demonstrated that most biclutamide resistant CaP still express AR protein (18,19). This insufficient suppression of AR can lead to adaptation such as reduced selectivity for ligands capable of AR activation, increased activation of AR signaling pathways and increased expression of AR mRNA and protein (13,19,20). CaP therapeutics down-regulating AR expression may provide a novel strategy that would bypass adaptive mechanisms and inhibit advancement of the disease.

The human *AR* gene is located on the X-chromosome (Xq11-q12), and is therefore present as a single copy in men. Since there is a single copy of the *AR*, any gene mutations could lead to phenotypic manifestation (21). The AR's first exon codes for the amino-terminal domain that contains several regions of repetitive DNA sequences. These regions code for polyglycine, proline and glutamine stretches, which have different significances in AR function (Fig 1B). For instance, the length of the polyglutamine stretch has been linked to the neurodegenerative disease, named Kennedy's disease, or spinal and bulbar muscular atrophy (SMBA) (21,22). It has been demonstrated that the extended poly-glutamine stretch (greater than 40 glutamines) induces a misfolded confirmation of the AR that leads to the formation of intracellular aggregates (22). The human AR protein contains approximately 919 amino acids resulting in an approximately 110kDa protein. However, this length and size can vary due to poly-glutamine and/or poly-glycine stretches. The AR has a centrally located DNA binding domain (DBD) consisting of two zinc-finger motifs (Fig. 1B) (21). Also, it features a hinge region which connects the DBD to the ligand binding domain (LBD) (Fig. 1B) (23). The importance of the LBD in activation and stability of the AR is through the interaction with ligands and multiple chaperones. It has recently been demonstrated that a truncated AR lacking the LBD was constitutively active (24). The LBD is critical for preventing the non-selective activation of the AR.

The expression and function of the AR can be regulated through multiple cellular pathways. The complexity of targeting the AR requires a broader understanding of the AR's role in normal development, as well as various disease etiologies. The investigation of ca27 and TQ provides a means to identify mechanisms of directly targeting the AR protein itself. The goal of this study was to identify novel mechanisms of AR protein down-regulation that may have relevance in the prevention or treatment of prostate cancer.

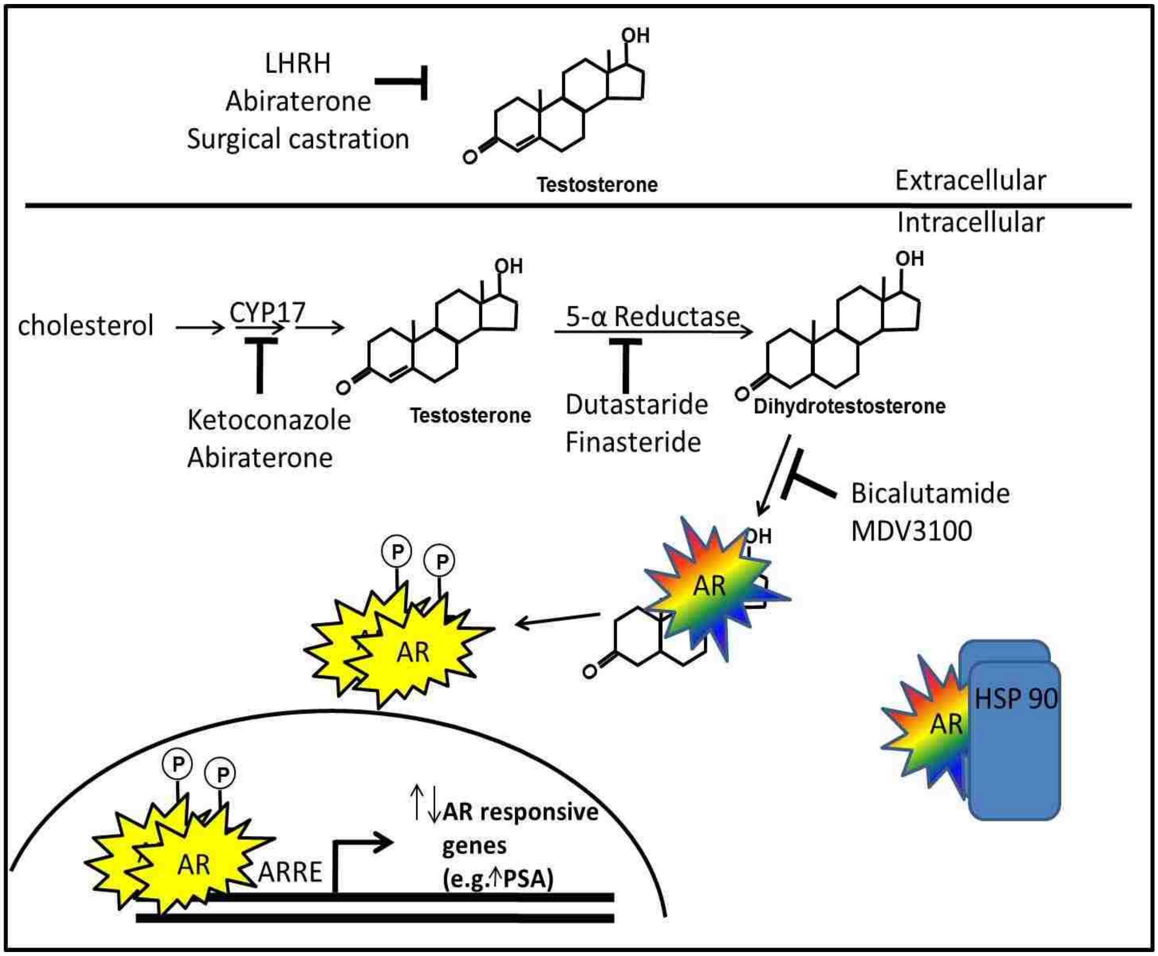


Fig. 1A

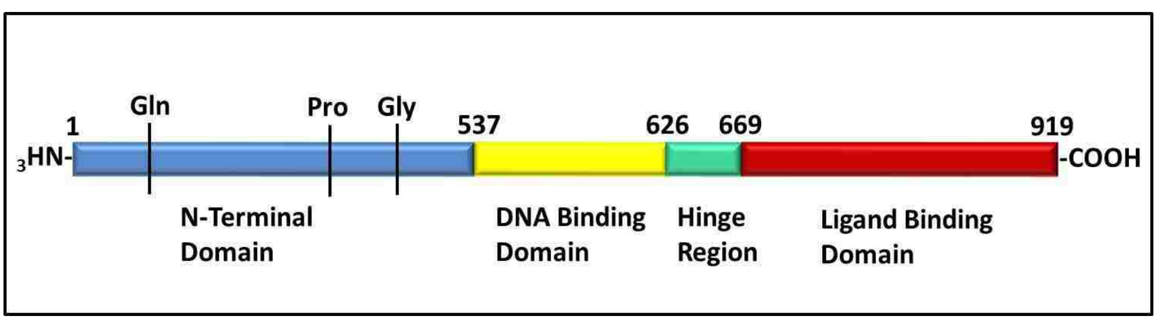


Fig. 1B

Fig. 1: Diagram of the AR activation pathway and AR protein structure. A, illustrates activation of the AR by DHT and demonstrates selective agents that target multiple steps in the AR activation pathway. Fig. 1A, adapted from Ref. 7. B, represents AR protein structure with several domains indicated. Fig.1B Image adapted from Ref. 21.

Curcumin, ca27, and ca27's down-regulation of the AR

Curcumin, (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, a diferuloylmethane compound (Fig. 2), isolated from the plant *Curcuma longa* has been proposed as a cancer chemopreventative agent (25). Previous studies have shown the potential of curcumin to inhibit metastasis, angiogenesis and proliferation in prostate cancer cell lines (26,27). Curcumin has also been shown to reduce cellular proliferation, AR transactivation and inhibit AR expression in CaP cells (28). However, despite the inhibitory actions of curcumin in CaP cells *in vitro*, it has demonstrated limitations *in vivo* due to a low bioavailability, warranting the search for more bioactive analogs (29). Initial screenings of a combinatorial chemical library based on the structure of curcumin, synthesized by Drs. Vander Jagt's (Department of Biochemistry and Molecular Biology, University of New Mexico) and Deck's (Department of Chemistry and Chemical Biology, University of New Mexico) laboratories, identified curcumin analogs that were able to inhibit transcription factors involved in cancer progression such as activator protein-1 (AP-1) and nuclear factor kappa B (NFkB) (30,31). Several analogs of this library were screened for their anti-androgenic activities. The primary screen of these analogs was designed to evaluate the inhibition of CaP cell proliferation and viability. These analogs were further tested for their ability to inhibit AR activity and then validated for their inhibition of AR protein expression. This screening procedure resulted in the identification of curcumin analog 27 (ca27) as a potential lead agent for identifying mechanisms of AR down-regulation. ca27 effectively inhibits AR activity and expression in multiple human prostate cancer cells. Identification of ca27's actions in determining the mechanism of AR inhibition could provide insight into novel approaches for down-regulating the AR.

Several modifications were made to curcumin's chemical structure in the synthesis of ca27 (Fig. 2). ca27 contains an α, β-unsaturated carbonyl, instead of the diketone or enol, the seven carbon linker between the aryl groups of curcumin was reduced to five, the methoxy groups were removed and the phenolic hydroxyl groups of the aromatic moieties were placed at the ortho-positions (Fig. 2). These modifications demonstrated distinct differences in the cellular activity between ca27 and curcumin. ca27 significantly down-regulated AR protein expression within 3 hours, while curcumin did not inhibit AR protein expression in my studies (Chapter 2). ca27 down-regulation of AR protein expression may be through its activity as a pro-oxidant. Agents that induce oxidative stress, such as piperlogumine, have been reported to induce selective cell death in multiple cancer cell lines with little effect, in normal cells (32). To determine if the induction of oxidative stress by ca27 resulted in the down-regulation of AR protein, cells were treated with ca27 and the anti-oxidant, glutathione analog, N-acetylcysteine (NAC) (33,34). NAC significantly prevented AR down-regulation upon ca27 treatment (Chapter 2). Thus, the increase in oxidative stress may be part of ca27's mechanism of AR down-regulation.

Cellular oxidative stress and the generation of reactive oxygen species (ROS) play important roles in the regulation of cell signaling and cell survival. Low to moderate levels of oxidative stress may function as signals to promote cell proliferation and survival. However, sudden or prolonged periods of cellular oxidative stress can induce cell death (35). The transcription factor nuclear factor E2-related protein (Nrf2) regulates the expression of several cytoprotective enzymes including antioxidant and phase II detoxifying enzymes (36). Transcriptional activation of Nrf2 is through the activation of the Keap1/Nrf2/ARE pathway. Keap1 (Kelch-like ECH-associated protein 1) is a repressor protein of Nrf2 transcriptional activity. Keap1 retains Nrf2 within the cytoplasm and promotes its ubiquitination and proteasomal degradation. An accepted explanation of this regulatory mechanism is provided by agents or inducers that react with sulfhydryl groups and modify the highly reactive cysteine residues of Keap1 disrupting its interaction and repression of Nrf2 (37). Upon release from Keap1, Nrf2 drives the transcription of antioxidant or electrophile response regulated genes. The transcriptional activity of Nrf2 can be mediated through pharmacological agents, redox potential and natural products (38). Transcriptional activation of Nrf2 can be monitored as an indirect means of agents that perturb cellular redox homeostasis.

Glutathione is an endogenous antioxidant whose expression can be mediated through the activation of Nrf2. Two genes regulated by Nrf2 are the enzymes required for glutathione synthesis, γ-glutamate cysteine ligase and glutathione synthetase (39). One of the major antioxidant defenses of the cell is endogenous thiols (sulhydryl containing compounds) such as glutathione and thioredoxin (34,35). Glutathione is the primary non-protein thiol in cells and exists in two redox forms, reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG). Cells can excrete GSSG or reduce it back to GSH through the NAD(P)H dependent activity of glutathione reductase (35). The oxidation of glutathione can be catalyzed by the selenoprotein glutathione peroxidase (GPx). GPx detoxifies reactive hydrogen peroxide and other hydroperoxides into molecular oxygen and water by the oxidation of two thiol groups into a disulfide (e.g. GSSG) (35,40). GSH cellular content ranges from 1-10 mM depending on cell type and is critical for redox balance and normal cellular function (33,35). GSSG can be reduced back into GSH by the enzyme glutathione reductase and the cofactor NADPH. GSH synthesis is a two-step enzymatic process catalyzed by γ-glutamate cysteine ligase (γ-glutamylcysteine synthetase) and GSH synthetase. The antioxidant activity of GSH is partially through its role as an endogenous thiol. NAC also contains a thiol group and has been reported to be a precursor of L-cysteine and reduced glutathione (33,34). The antioxidant activity of NAC may in part inhibit the activity of ca27. ca27 evokes cellular redox response pathways and the generation of ROS. ca27's pro-oxidant activity may be required for the down-regulation of the AR. ca27's down-regulation of the AR is attenuated by the presence of NAC.

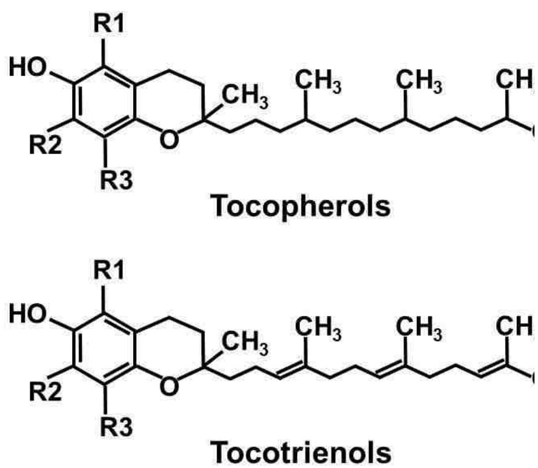
Fig. 2: Structures of curcumin and curcumin analog 27 (ca27).

Vitamin E, α-tocopherylquinone (TQ) and TQ's down-regulation of the AR

Vitamin E (VE) is a family of dietary agents (e.g. α-,β-,γ-,δ-tocopherols and -tocotrienols),which were first described 1922 by Evans and Bishop (41) as an accessory food factor essential for reproduction of rats. VE exists in eight different naturally occurring forms which all feature a chromanol ring with a hydroxyl group and a 16-carbon hydrophobic phytyl side chain (Fig. 3). The α-tocopherol (α-T) isoform is a lipophilic antioxidant that prevents free radical production and lipid peroxidation (42).The chromanol ring moiety is responsible for α-T antioxidant activity and the lipophilic phytyl chain determines it retention in membranes and subcellular distribution (43).

α-T is the most bioactive of the VE isoforms and shown to reduce the incidence and mortality of prostate cancer in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study. This was a large prevention trial conducted in Finnish men, who were randomized to receive 50 mg of DL-α-tocopheryl acetate for 5 to 8 years. The outcome of this trial showed a decrease in CaP incidence (32%) and mortality (41%) in men that were cigarette smokers who received α-T (44). This chemopreventive activity may be unique to α-T since other studies have demonstrated that the intake of the β-, γ-, and δ-tocopherol isoforms are not associated with the inhibition prostate cancer risk (45). α-T's actions as a CaP chemopreventive agent has been highly controversial due in part to the outcome of the Selenium and Vitamin E Cancer Prevention Trial (SELECT). This phase III, randomized, placebo-control trial was initiated in 2001 and was terminated in 2008 due to increases in potentially problematic side effects. The two major problematic trends reported were the increase in type II diabetes mellitus in the selenium cohort and an increase in CaP incidence in the VE cohort; neither of these trends were found to be statistically significant (46). The discrepancy between the outcomes of the ATBC and SELECT trials may be due to the selective cohort of men who were heavy smokers in the ATBC trial compared to the majority of men who were non-smokers in the SELECT trial. In an alternative experimental setting, Wurzel, H et al. (47) conducted an *in vivo* study which exposed rats to chronic cigarette smoke and α-T for 65 weeks. In the experimental group, they found high levels of TQ in the bronchoalveolar lavage fluid demonstrating that smoke-exposed animals generated a larger amount of oxidative products (47). In my studies, there are distinct differences between TQ and α-T actions on CaP cells and on the AR. In contrast to α-T, I found TQ to inhibit AR activity and expression in human CaP cells (Chapter 3).

There is a large degree of variation in the potential cellular actions between the tocopherol forms and their corresponding quinone forms. In a review by David Cornwell and JiyanMa (48), the comparison of γ-tocopherol quinone (γ-TQ), δ-tocopherol quinone (δ-TQ) and α-TQ chemical activities were evaluated from multiple studies. Both γ-TQ and δ-TQ were found to be potent arylating electrophiles leading to Michael adduct formation with nucleophiles such as the thiol group in glutathione (48). However, α-TQ (TQ) was found to be a non-arylating quinone electrophile with distinct cellular and chemical properties from the arylating quinone electrophiles γ-TQ and δ-TQ in their studies. Arylating quinone electrophiles are highly cytotoxic agents that can induce apoptosis and result in cell death. Both γ-TQ and δ-TQ were found to have profound effects on cell viability and morphology in comparison to α-TQ in a human acute lymphoblastic leukemia cell line (49). In a follow-up study, γ-TQ (not α-TQ) was found to induce endoplasmic reticulum stress (ER stress) pathways due to its actions as an arylating electrophile, which may lead to Michael adduct formation with protein disulfide isomerases (50). These studies have evaluated the actions of α-TQ and γ-TQ in a very short treatment time (50µM for 24h) (49,50). In my studies, α-TQ's actions on AR protein down-regulation and induction of ER stress pathways were time-dependent (Chapter 3). Further, TQ induces oxidative stress and down-regulation of the AR that may dependent on its activity as a pro-oxidant. The reactivity of the quinone forms described are very different, but their cellular actions may provide further insight into α-TQ's mechanism of AR down-regulation.



	R1	R2	R3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

Fig. 3: Structures of α-, β-, γ-, δ-tocopherols and -tocotrienols within the VE family. **Table 1:** R-groups represent the indicated group at various positions for the multiple forms. Adapted from Ref. 48.

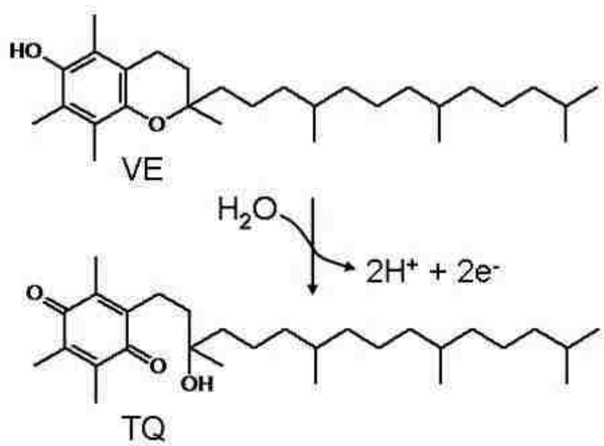


Fig. 4: Oxidative conversion of α-tocopherol (VE) into the metabolite α-tocopheryl quinone (TQ).

The role of the AHR and other agents on AR down-regulation

Environmental toxins such as the polycyclic aromatic hydrocarbon benzo(a)pyrene (B(a)P) exert their toxic effects through the activation of the aryl hydrocarbon receptor (AHR) (51). The AHR is a well-characterized ligand activated transcription factor which belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. The AHR regulates several genes involved in xenobiotic metabolism and detoxification pathways such as cytochrome P450 1A1 (CYP1A1), CYP1B1 and glutathione-S-transferase (52). Over the last several years, studies conducted by Dr. Kato and colleagues (52,53) have elucidated a novel cellular role of the AHR independent from its transcriptional activity. The AHR can act as an adaptor protein for E3 ubiquitin ligases which enhances the proteasomal degradation of steroid hormone receptors such as the estrogen receptor and AR (52,53). This novel action of the AHR may explain some of its toxicological and physiological effects. In this dissertation, ca27 and TQ were evaluated for activation of the AHR and the AHR's potential role in AR protein down-regulation.

The AR's expression is regulated post-translationally by the ubiquitin/proteasome system. Ubiquitylation is based on the attachment of ubiquitin to the lysine residues on a target protein (e.g., AR) and involves the action of three ubiquitin ligases E1, E2 and E3. These three ligases work in a defined order to ubiquitylate the AR, which then becomes degraded by the proteasome (12,54). AR expression can be regulated by 26S proteasomal degradation either in the presence or absence of ligand. The inactive AR is retained in the cytoplasm bound to a multichaperone complex including HSP90: this interaction prevents the degradation of the AR (54). Agents such as genistein or geldanamycin disrupt the AR and chaperone interaction resulting in AR proteasomal degradation (55,56). To identify TQ and ca27's mechanism(s) of AR protein down-regulation I evaluated the role of the AHR on AR down-regulation. In 2004, Lin, et al. (57) demonstrated that the AHR agonist B(a)P could inhibit AR protein expression in the human adenocarcinoma cell line H1355. In elucidating the mechanism of AHR activation and AR down-regulation Ohtake, et al. (53) demonstrated activation of the AHR by the AHR agonist 3-methylcholanthrene (3-MC) which led to the proteasomal degradation of the AR. I showed that treatment of CaP cells with B(a)P led to the proteasomal degradation of AR protein (Chapter 4). However, TQ and ca27's down-regulation of the AR was not attenuated by the knock-down of AHR expression. Although, the AHR was not found to be a critical contributor to TQ or ca27's mechanism of AR down-regulation, the interaction of the AHR and AR may provide further insight into mechanisms of endocrine disruption.

The importance of AR function and expression in CaP has led to the development of multiple strategies that lead to AR down-regulation. The identification of AR inhibitory mechanisms by natural products such as genistein can be utilized in the development of analogs designed to enhance activity or potentially overcome limitations. As discussed previously targeting down-regulation of AR protein expression can be accomplished by the agent genistein which distrupts AR and HSP90 interaction resulting in AR proteasomal degradation (56). This strategy for down-regulating the AR can be utilized in diseases other than CaP where the AR is a target. The genistein analogs, 17-allylamino-17-demethoxygeldanamycin (17-AAG) or 17-(dimethlaminoethlamino)-17-demethoxygeldamycin (17-DMAG) are being investigated for their beneficial role in inhibiting mutant aggregate prone AR found in SBMA (58,59). The recent identification of andrographolide an inhibitor of interleukin-6 has recently been identified to disrupt the binding of HSP90 and AR and promote AR proteasomal degradation (60). These strategies require a functional proteasome and a continued AR/HSP90 complex but disruption of proteasomal function or alternative AR forms could limit the potential of these agents.

Other natural products such as VE have shown potential benefits for CaP prevention but are controversial. VE analogs such as VE succinate have been reported to inhibit CaP cell growth, inhibit PSA expression and down-regulate AR protein expression (61). The green tea polyphenol, (-)-epigallocatechin gallate (EGCG) has been previously reported to inhibit AR activation and AR expression in CaP cells (62). However concerns about bioavailability of VE succinate and ECGC limit the use of these agents. Natural products provide a meaningful foundation for the development of experimental therapeutic agents.

The identification of variant forms of the AR provides potential targets for the inhibition of CaP. In CaP, splice variants of the AR have been identified and their role in CaP development and progression are still being determined (63). The constitutive activation of splice variants lacking domains (i.e. LBD) critical for the HSP90/AR interaction would be resistant to previously mentioned strategies. Therefore alternative approaches for inhibiting AR expression are currently being investigated. Agents such as Nigericin are being investigated for their inhibitory actions of multiple variant AR mRNA expression (64). The strategy of inhibiting AR mRNA expression is also utilized by generation of AR antisense agents. Recently Zhang, Y et al. (65) has demonstrated the use of a locked nucleic acid-based antisense oligonucleotide, EZN-4176. EZN-4176 demonstrates selective down-regulation of AR mRNA in animal models (65). EZN-4176, potential activity *in vivo* is a promising approach but still requires verification this is a deliverable approach in humans. The down-regulation of AR expression is a meaningful target in multiple diseases including CaP. Identifying novel mechanisms regulating AR expression will provide insight and opportunity for the development of therapeutic agents.

Dissertation Objectives:

The purpose of this study was to identify TQ and ca27's mechanism of AR down-regulation. The following objectives outline my investigations of ca27 and TQ.

1. Characterize the anti-androgenic activity of TQ and ca27 in comparison to VE and curcumin

    - **a.** Determine dosage range and time course for TQ and ca27 to effectively inhibit CaP cell proliferation and viability
    - **b.** Determine concentrations of TQ and ca27 that effectively inhibit AR activity as measured by an AR reporter assay and expression of an endogenous AR regulated gene (e.g., PSA)

2. Characterize the effect of ca27 and TQ on AR expression in human CaP cells

- **a.** Determine the inhibitory effects of agents on AR mRNA expression
- **b.** Determine the effects of ca27 and TQ on AR protein levels
- **c.** Determine the kinetics of AR down-regulation by TQ and ca27

3. Identify potential mechanisms of ca27 and TQ's down-regulation of AR protein expression

- **a.** Determine if AR protein down-regulation is due to inhibition of AR mRNA expression
- **b.** Determine if ca27 and TQ induce proteasomal degradation of the AR
- **c.** Determine if activation of AHR activity by ca27 and TQ leads to AR protein down-regulation
- **d.** Determine if TQ and ca27 induce oxidative stress and if this contributes to AR down-regulation

Summary

Men have a one in six risk of developing CaP over their lifetime. While current therapies successfully reduce the progression of CaP for the majority of men, the remainder may receive treatment targeting AR activation. The AR is an important mediator of CaP growth and progression. Therefore, identifying mechanisms to down-regulate AR protein may be useful in developing novel strategies to treat advanced prostate cancer. In the following studies, we investigated two agents that possess anti-androgenic activities in CaP cells. These novel agents (i.e., TQ and ca27) were further investigated for their mechanisms of AR down-regulation and induction of oxidative stress. Overall, the hypothesis is posed that TQ and ca27 are pro-oxidants that this contributes to the down-regulation of AR. Agents capable of down-regulating AR protein will facilitate the elucidation of novel mechanisms of AR inhibition and potentially lead to the development of novel CaP therapies.

References

1. Lepor H. Prostatic Diseases. W.B.Saunders, editor. Philadelphia, PN: W.B.SaundersCompany. 2000; 1-586.
2. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. Semin Oncol 1999; 26(4): 407-421.
3. Grosse A, Bartsch S, Baniahmad A. Androgen receptor-mediated gene repression. Mol Cell Endocrinol 2011 (Ahead of Pub).
4. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians 2011; 61(2): 69-90.
5. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: a cancer journal for clinicians 2010 ;60(5): 277-300.
6. Lamont KR, Tindall DJ. Minireview: Alternative activation pathways for the androgen receptor in prostate cancer. Molecular Endocrinology 2011; 25(6): 897-907.
7. Massard C, Fizazi K. Targeting continued androgen receptor signaling in prostate cancer. Clin Cancer Res 2011; 17(12): 3876-3883.
8. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004; 25(2): 276-308.
9. Ismail M, Ferroni M, Gomella LG. Androgen suppression strategies for prostate cancer: is there an ideal approach? Curr Urol Rep 2011; 12(3): 188-196.
10. Deslypere JP, Young M, Wilson JD, McPhaul MJ. Testosterone and 5 alpha-dihydrotestosterone interact differently with the androgen receptor to enhance

transcription of the MMTV-CAT reporter gene. Mol Cell Endocrinol 1992; 88(1-3): 15-22.

11. Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 2007; 28(7): 778-808.

12. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 2007; 120(4): 719-733.

13. Singh P, Uzgare A, Litvinov I, Denmeade SR, Isaacs JT. Combinatorial androgen receptor targeted therapy for prostate cancer. Endocr Relat Cancer 2006; 13(3): 653-666.

14. Beltran H, Beer TM, Carducci MA, de Bono J, Gleave M, Hussain M, Kelly WK, Saad F, Sternberg C, Tagawa ST, Tannock IF. New therapies for castration-resistant prostate cancer: efficacy and safety. European Urology 2011; 60(2): 279-290.

15. Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS. Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 2007; 67(10): 5033-5041.

16. Tindall DJ, Rittmaster RS. The rationale for inhibiting 5alpha-reductase isoenzymes in the prevention and treatment of prostate cancer. The Journal of Urology 2008; 179(4): 1235-1242.

17. Zhou C, Wu G, Feng Y, Li Q, Su H, Mais DE, Zhu Y, Li N, Deng Y, Yang D, Wang MW. Discovery and biological characterization of a novel series of androgen receptor modulators. Br J Pharmacol 2008; 154(2): 440-450.

18. Masai M, Sumiya H, Akimoto S, Yatani R, Chang CS, Liao SS, Shimazaki J. Immunohistochemical study of androgen receptor in benign hyperplastic and cancerous human prostates. Prostate 1990; 17(4): 293-300.

19. Ichikawa T, Suzuki H, Ueda T, Komiya A, Imamoto T, Kojima S. Hormone treatment for prostate cancer: current issues and future directions. Cancer Chemother Pharmacol 2005; 56 Suppl 1: 58-63.

20. Ghosh PM, Malik SN, Bedolla RG, Wang Y, Mikhailova M, Prihoda TJ, Troyer DA, Kreisberg JI. Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. Endocr Relat Cancer 2005; 12(1): 119-134.

21. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002; 20(13): 3001-3015.

22. Palazzolo I, Gliozzi A, Rusmini P, Sau D, Crippa V, Simonini F, Onesto E, Bolzoni E, Poletti A. The role of the polyglutamine tract in androgen receptor. J Steroid Biochem Mol Biol 2008; 108(3-5): 245-253.

23. Claessens F, Denayer S, Van Tilborgh N, Kerkhofs S, Helsen C, Haelens A. Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal 2008; 6: e008.

24. Dehm SM, Tindall DJ. Alternatively spliced androgen receptor variants. Endocr Relat Cancer 2011; 18(5): R183-196.

25. Khan, N Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid Redox Signal 2008; 10(3): 475-510.

26. Mukhopadhyay A, Banerjee S, Stafford LJ, Xia C, Liu M, Aggarwal BB. Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. Oncogene 2002; 21(57): 8852-8861.

27. Thangapazham RL, Sharma A, Maheshwari RK. Multiple molecular targets in cancer chemoprevention by curcumin. AAPS J 2006; 8(3): E443-449.

28. Nakamura K, Yasunaga Y, Segawa T, Ko D, Moul JW, Srivastava S, Rhim JS. Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines. Int J Oncol 2002; 21(4): 825-830.

29. Shehzad A, Wahid F, Lee YS. Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. Arch Pharm 2010; 343(9): 489-499.

30. Weber WM, Hunsaker LA, Roybal CN, Bobrovnikova-Marjon EV, Abcouwer SF, Royer RE, Deck LM, Vander Jagt DL. Activation of NFkappaB is inhibited by curcumin and related enones. Bioorg Med Chem 2006; 14(7): 2450-2461.

31. Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL. Anti-oxidant activities of curcumin and related enones. Bioorg Med Chem 2005; 13(11): 3811-3820.

32. Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, Tolliday NJ, Golub TR, Carr SA, Shamji AF, Stern AM, Mandinova A, Schreiber SL, Lee SW. Selective

killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 2011; 475(7355): 231-234.

33. Russell J, Spickett CM, Reglinski J, Smith WE, McMurray J, Abdullah IB. Alteration of the erythrocyte glutathione redox balance by N-acetylcysteine, captopril and exogenous glutathione. FEBS Lett 1994; 347(2-3): 215-220.

34. Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 2003; 60(1): 6-20.

35. Biswas SK, Rahman I. Environmental toxicity, redox signaling and lung inflammation: the role of glutathione. Mol Aspects Med 2009; 30(1-2): 60-76.

36. Lee JS, Surh YJ. Nrf2 as a novel molecular target for chemoprevention. Cancer letters 2005; 224(2): 171-184.

37. Dinkova-Kostova AT, Talalay P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Archives of biochemistry and biophysics 2010; 501(1): 116-123.

38. Zhao CR, Gao ZH, Qu XJ. Nrf2-ARE signaling pathway and natural products for cancer chemoprevention. Cancer Epidemiol 2011; 34(5): 523-533.

39. Lu SC. Regulation of glutathione synthesis. Mol Aspects Med 2009; 30(1-2): 42-59.

40. Flohe L, Toppo S, Cozza G, Ursini F. A comparison of thiol peroxidase mechanisms. Antioxidants & redox signaling 2011; 15(3): 763-780.

41. Evans HM, Bishop KS. On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction. Science (New York, NY 1922; 56(1458): 650-651.

42. Wolf G. The discovery of the antioxidant function of vitamin E: the contribution of Henry A. Mattill. J Nutr 2005; 135(3): 363-366.

43. Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annual review of nutrition 1990; 10: 357-382.

44. Heinonen OP, Albanes D, Virtamo J, Taylor PR, Huttunen JK, Hartman AM, Haapakoski J, Malila N, Rautalahti M, Ripatti S, Maenpaa H, Teerenhovi L, Koss L, Virolainen M, Edwards BK. Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. J Natl Cancer Inst 1998; 90(6): 440-446.

45. Wright ME, Weinstein SJ, Lawson KA, Albanes D, Subar AF, Dixon LB, Mouw T, Schatzkin A, Leitzmann MF. Supplemental and dietary vitamin E intakes and risk of prostate cancer in a large prospective study. Cancer Epidemiol Biomarkers Prev 2007; 16(6): 1128-1135.

46. Ledesma MC, Jung-Hynes B, Schmit TL, Kumar R, Mukhtar H, Ahmad N. Selenium and vitamin E for prostate cancer: post-SELECT (Selenium and Vitamin E Cancer Prevention Trial) status. Mol Med 2011; 17(1-2): 134-143.

47. Wurzel H, Yeh CC, Gairola C, Chow CK. Oxidative damage and antioxidant status in the lungs and bronchoalveolar lavage fluid of rats exposed chronically to cigarette smoke. J Biochem Toxicol 1995; 10(1): 11-17.

48. Cornwell DG, Ma J. Studies in vitamin E: biochemistry and molecular biology of tocopherol quinones. Vitam Horm 2007; 76: 99-134.

49. Thornton DE, Jones KH, Jiang Z, Zhang H, Liu G, Cornwell DG. Antioxidant and cytotoxic tocopheryl quinones in normal and cancer cells. Free Radic Biol Med 1995; 18(6): 963-976.

50. Wang X, Thomas B, Sachdeva R, Arterburn L, Frye L, Hatcher PG, Cornwell DG, Ma J. Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. Proc Natl Acad Sci U S A 2006; 103(10): 3604-3609.

51. Chen S, Nguyen N, Tamura K, Karin M, Tukey RH. The role of the Ah receptor and p38 in benzo[a)pyrene-7,8-dihydrodiol and benzo[a)pyrene-7,8-dihydrodiol-9,10-epoxide-induced apoptosis. The Journal of biological chemistry 2003; 278(21): 19526-19533.

52. Ohtake F, Fujii-Kuriyama Y, Kato S. AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions. Biochemical pharmacology 2009; 77(4): 474-484.

53. Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T, Fujii-Kuriyama Y, Kato S. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 2007; 446(7135): 562-566.

54. Jaworski T. Degradation and beyond: control of androgen receptor activity by the proteasome system. Cellular & molecular biology letters 2006;11(1):109-131.

55. Vanaja DK, Mitchell SH, Toft DO, Young CY. Effect of geldanamycin on androgen receptor function and stability. Cell stress & chaperones 2002; 7(1): 55-64.

56. Basak S, Pookot D, Noonan EJ, Dahiya R. Genistein down-regulates androgen receptor by modulating HDAC6-Hsp90 chaperone function. Molecular cancer therapeutics 2008; 7(10): 3195-3202.

57. Lin P, Chang JT, Ko JL, Liao SH, Lo WS. Reduction of androgen receptor expression by benzo[alpha]pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[alpha]pyrene in human lung cells. Biochemical pharmacology 2004; 67(8): 1523-1530.

58. Waza M, Adachi H, Katsuno M, Minamiyama M, Tanaka F, Sobue G. Alleviating neurodegeneration by an anticancer agent: an Hsp90 inhibitor (17-AAG). Annals of the New York Academy of Sciences 2006; 1086: 21-34.

59. Tokui K, Adachi H, Waza M, Katsuno M, Minamiyama M, Doi H, Tanaka K, Hamazaki J, Murata S, Tanaka F, Sobue G. 17-DMAG ameliorates polyglutamine-mediated motor neuron degeneration through well-preserved proteasome function in an SBMA model mouse. Human molecular genetics 2009; 18(5): 898-910.

60. Liu C, Nadiminty N, Tummala R, Chun JY, Lou W, Zhu Y, Sun M, Evans CP, Zhou Q, Gao AC. Andrographolide targets androgen receptor pathway in castration-resistant prostate cancer. Genes & cancer 2011; 2(2): 151-159.

61. Zhang Y, Ni J, Messing E, Chang E, Yang C, Yeh S. Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate specific antigen in prostate cancer cells. Proc Natl Acad Sci USA 2002; 99: 7408-7413.

62. Ren F, Zhang S, Mitchell SH, Butler R, Young CY. Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. Oncogene 2000; 19(15): 1924-1932.

63. Haile S, Sadar MD. Androgen receptor and its splice variants in prostate cancer. Cell Mol Life Sci 2011 (Ahead of Pub).

64. Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. Molecular pharmacology 2010; 78(5): 846-854.

65. Zhang Y, Qu Z, Kim S, Shi V, Liao B, Kraft P, Bandaru R, Wu Y, Greenberger LM, Horak ID. Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection. Gene therapy 2011; 18(4): 326-333.

CHAPTER 2: THE CURCUMIN ANALOG CA27 DOWN-REGULATES ANDROGEN RECEPTOR THROUGH AN OXIDATIVE STRESS MEDIATED MECHANISM IN HUMAN PROSTATE CANCER CELLS

Abstract

Background The androgen receptor (AR) plays a critical role in prostate cancer development and progression. Therefore, the inhibition of AR function is an established therapeutic intervention. Since the expression of the AR is retained and often increased in progressive disease, AR protein down-regulation is a promising therapeutic approach against prostate cancer. We show here that the curcumin analog (ca27) down-regulates AR expression in several prostate cancer cell lines.

Methods ca27 at low micromolar concentrations was tested for its effect on AR expression, AR activation, and induction of oxidative stress in human LNCaP, C4-2 and LAPC-4 prostate cancer cells.

Results ca27 induced the down-regulation of AR protein expression in LNCaP, C4-2 and LAPC-4 cells within 12 hours. Further, ca27 led to the rapid induction of reactive oxygen species (ROS). To further support this finding, ca27 treatment led to the activation of the cellular redox sensor NF-E2-related factor 2 (Nrf2) and the induction of the Nrf2-regulated genes NAD(P)H quinone oxidoreductase 1 and aldoketoreductase 1C1. We show that ROS production preceded AR protein loss and that ca27 mediated down-regulation of the AR was attenuated by the antioxidant, N-acetyl cysteine.

Conclusions ca27induces ROS and mediates AR protein down-regulation through an oxidative stress mechanism of action. Our results suggest that ca27 represents a novel agent for the elucidation of mechanisms of AR down-regulation which could lead to effective new anti-androgenic strategies for the treatment of advanced prostate cancer.

Introduction

The AR is a ligand activated steroid hormone receptor and a key regulator of both normal prostate development and function (1). The AR plays a critical role in both prostate cancer development and progression (2). Consequently, the current therapeutic strategies for prostate cancer intervention, such as androgen ablation therapy (3) target the inhibition of AR function. Such treatment, in its most aggressive form is based on combinations of androgen synthesis suppression and AR inhibition (4). Fortunately, the majority of men undergoing androgen ablation therapy successfully respond to this therapy. However, the median response to androgen ablation is typically less than two years, and patients recur with progressive disease within 12-18 months, developing androgen ablation resistant cancer (5). This advanced stage is characterized by the continuous expression and function of the AR in the presence of low concentrations of androgens (6-7).Under these conditions, the AR supports prostate cancer cell survival, as the down-regulation of AR protein in androgen ablation resistant prostate cancer cells and animal models leads to cell growth inhibition and death (8-9). These findings emphasize the importance of the AR and its signaling axis for all stages of prostate cancer, thus rendering it a prominent and promising target (2,4,10-11). Therefore, the identification of chemical agents that down-regulate AR expression by known or novel mechanisms warrant further investigation for development as a novel prostate cancer therapeutic approach.

We have previously reported the synthesis of an enone analog chemical library of the natural diphenolic product curcumin (diferuloylmethane, or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione) (12-14). In the present study, we report on a compound from this library, curcumin analog 27 (ca27) (14). ca27 belongs to a series of symmetrical diphenolic analogs which in contrast to curcumin feature a shorter 5-carbon unsaturated linker with a single carbonyl group (Fig. 1A)(14). The two phenolic rings of ca27 feature symmetrical ortho-hydroxyl groups. The carbon linker retains the character of an α,β-unsaturated ketone which has properties of a Michael acceptor for strong nucleophilic groups (15). Structure analysis relationship (SAR) studies reported by several other groups indicate that this property is responsible for conferring the anti-proliferative abilities of curcumin analogs (15-16).

In the current study we have demonstrated that ca27 mediates the down-regulation of AR protein expression and activity. We further provide a potential mechanism of action for ca27 on the AR by studying its effect on the redox status in prostate cancer cells. We show that ca27 induced the generation of intracellular reactive oxygen species (ROS) by the 2',7'-dichlorofluorescein diacetate (DCF) assay. In support of this finding, ca27 increased the activation of the cellular redox sensor, NF-E2-related factor 2 (Nrf2), followed by expression of the Nrf2 regulated detoxification genes, NAD(P)H quinone oxidoreductase 1 **(**NQO1**)**and aldoketoreductase 1C1 (AKR1C1). Because the antioxidant (electrophilic) response element regulation is associated with Nrf2 activation the two concepts were used interchangeably and will be referred to as Nrf2 activity. Finally, we show that the antioxidant N-acetyl cysteine (NAC) abrogates ca27 mediated AR down-regulation, which provides further support that ca27 induced AR protein loss is mediated by oxidative stress. Importantly, ca27 and similar curcumin analogs represent a novel class of agents for the elucidation of mechanisms of AR down-regulation in prostate cancer cells which could lead to effective new anti-androgenic strategies for the treatment of advanced prostate cancer.

Materials and Methods

Chemical Reagents

The curcumin analog 27 (ca27) (1,5-Bis(2-hydroxyphenyl)-1,4-pentadien-3-one) was synthesized and characterized as previously described (14). This diphenolic chemical was solubilized in 100% dimethyl sulfoxide (DMSO) stored protected from light at 4°C. The synthetic androgen methyltrienolone (R1881) was from Perkin Elmer/NEN Life Science Products (Boston, MA). MG132, N-acetyl-L-cysteine (NAC) and Actinomycin D (Act D) were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Treatment Protocols

The human prostate cancer cell lines LNCaP (American Type Culture Collection, Manassas, VA), C4-2 (gift from Dr. G.N. Thalmann, University of Bern, Switzerland) and a variant of the LAPC-4 (acquired from Dr. George Wilding, University of Wisconsin Paul P. Carbone Comprehensive Cancer Center) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and streptomycin-penicillin antibiotics (DMEM/FBS). To evaluate androgenic responses cells were cultured in DMEM containing 4% charcoal-stripped FBS and 1% heat-inactivated FBS (DMEM/CSS). All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. ca27 was added to the cells for the indicated lengths of time and final concentrations. Vehicle controls never amounted to a final concentration of>0.1% DMSO.

Cell Proliferation and Viability Assays

Cells were plated in quadruplicate in a 12-well tissue culture plates (Invitrogen, Carlsbad, CA) in DMEM/FBS and treated with ca27 at the indicated final concentrations for 96 hours. After cell detachment in 2.5% Trypsin/EDTA (Invitrogen, Carlsbad, CA), cell proliferation was determined by total cell count in a hemacytometer by light microscopy. Viability was determined by trypan blue dye exclusion (0.4%; Sigma, St. Louis, MO). Results are expressed as percent of vehicle control.

Promoter Activation Assays

Cells were cultured in quadruplicate in 24-well plates (Invitrogen, Carlsbad, CA) in DMEM/CSS. After 48 hours, cells were co-transfected with a reporter plasmid carrying a mouse mammary tumor virus (MMTV) promoter regulating luciferase cDNA expression (17) and a control plasmid carrying a thymidine kinase (TK) promoter regulating *Renilla* luciferase cDNA expression (Promega, Madison, WI) using Lipofectamine 2000 transfection agent (Invitrogen, Carlsbad, CA). Twenty-four hours post-transfection cells were treated with ca27 at the indicated concentrations for 24 hours. After stimulation with 1 nMR1881 for 6 hours, whole cell extracts were generated using Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay Substrate kit (Promega, Madison, WI) and relative luciferase units determined on a Perkin Elmer Victor^{3}V 1420 counter and analyzed using Wallac 1420 software (Perkin Elmer, Turku, Finland). Cells were cultured as described above and co-transfected with a reporter plasmid carrying an antioxidant response element promoter regulating luciferase cDNA expression, pNQO1hARE (18) and the control TK promoter plasmid. Forty eight hours post-transfection, cells were treated with the indicated concentrations of ca27 for

16 hours. Luciferase activity was determined as outlined above. Normalized luciferase expression is expressed as a percent of vehicle control.

AR activation was further measured using the Multifunctional Androgen Receptor Screening (MARS) Assay (19). Androgen independent PC-3human prostate cancer cells were co-transfected with a wild-type AR expressing plasmid and a plasmid carrying an MMTV promoter containing an AR response element driving destabilized enhanced green fluorescent protein (dsEGFP). In this assay, AR activation is stimulated by R1881 at 1 nM. Images of fluorescent cells were captured using an Olympus IX70inverted fluorescent microscope and fluorescence was quantified by ImageJ software (20). The number of fluorescent cells was expressed as percent of control.

Messenger RNA (mRNA) Expression Analysis by Quantitative (Real Time) Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Cells were cultured in quadruplicate in 24-well plates (Invitrogen, Carlsbad, CA) in DMEM/FBS and treated with ca27 for 3 or 12 hours at the indicated concentrations. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA). PCR cycling parameters were 95˚C for 10 minutes followed by 40 cycles of 95˚C for 15 seconds, and 60˚C for 1 minute. Forward and reverse primers for the AR and the normalization control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were available in the QuantiTect Primers Assays from Qiagen (Valencia, CA). Forward and reverse primers for PSA, NQO1, AKR1C1 and MafG were purchased from Integrated DNA Technologies (Coralville, IA). PSA forward primer sequence is 5'-CGCTGGACAGGGGGCAAAA-3' and the reverse primer sequence is 5'-ACAAGTGGGCCCCCAGAATCA-3'. NQO1 forward primer sequence is 5'-TGAGCTCGAGCCCCGGACTGCACCAGA-3' and the reverse primer sequence is 5'-CTACCGCGGCAAGTCAGGGAAGCCTGGAAAGAT-3'. AKR1C1 forward primer sequence is 5'-GATGGCCTAAACAGAAATGTGCGAT-3' and the reverse primer sequence is 5'-GGATAATTAGGGGGGCCAGCAA-3'. MafG forward primer sequence is 5'-GCTGTGCCCCCGGGTTATGA-3' and the reverse primer sequence is 5'-CCGTCAGGCTGGTGCCATTCT-3'. AR, PSA, NQO1, AKR1C1 and MafG mRNA expression levels normalized to GAPDH were determined using the ΔΔCt method and are shown relative to control.

Reactive Oxygen Species(ROS) Detection by DCF

Cells were cultured in 96-well plates (Corning Inc., Corning, NY) in DMEM/FBS for 48 hours and then treated with ca27 for 1 hour at the indicated concentrations. Cells were analyzed for the formation of ROS by use of the fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCF) (Invitrogen, Carlsbad, CA) as described by Basu et al.(21). DCF fluorescent units per well were measured 1 hour after DCF addition. DNA content per well was measured by the Hoechst 33258 dye (Sigma, St. Louis, MO) (22). Fluorescence measurements for both the DCF assay and Hoechst dye were taken using a TECAN plate reader (TECAN Austria GmbH, Salzburg, Austria) and analyzed with Magellan software. Over 12 replicates were used per treatment group. Hoechst dye normalized DCF fluorescent units are shown relative to control.

Protein Expression by Western Blot

Cells were cultured in quadruplicate in 12-well plates (Invitrogen, Carlsbad, CA) in DMEM/FBS and treated with ca27 for 12 hours at the indicated concentrations. Cells were washed in cold phosphate buffered saline (PBS) and whole cell extracts were generated using 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1mg/ml phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, and 10µg/ml aprotinin in PBS. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL). 30 µg of protein were size-separated by SDS polyacryalmide gel electrophoresis (SDS-PAGE) in triplicate in 12.5% gels (BioRad, Hercules, CA) and electro-transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Trizma base (Tris) buffered saline (TBS) containing 5% nonfat dry milk at 4°C and then incubated with mouse anti-AR monoclonal antibody (441; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β-actin monoclonal antibody (A5441; Sigma, St. Louis, MO) at the concentrations indicated by the manufacturers. After washing in TBS, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (Biomeda, Foster City, CA). Bound antibodies were detected using Western Lightening Chemiluminescence Reagent Plus (Boston, MA) on a Kodak Image Station 4000MM (Rochester, NY). Band intensities were determined by densitometric analysis (ratio AR:β-actin) using Kodak Molecular Imaging Software (Rochester, NY). AR expression is shown relative to DMSO control.

Statistical Analysis

Significant differences in values between groups were assessed using the unpaired *t*-test with SigmaStat 3.1 software (Systat Software, San Jose, CA). *P* values of less than 0.05 were used to signify statistical significance.

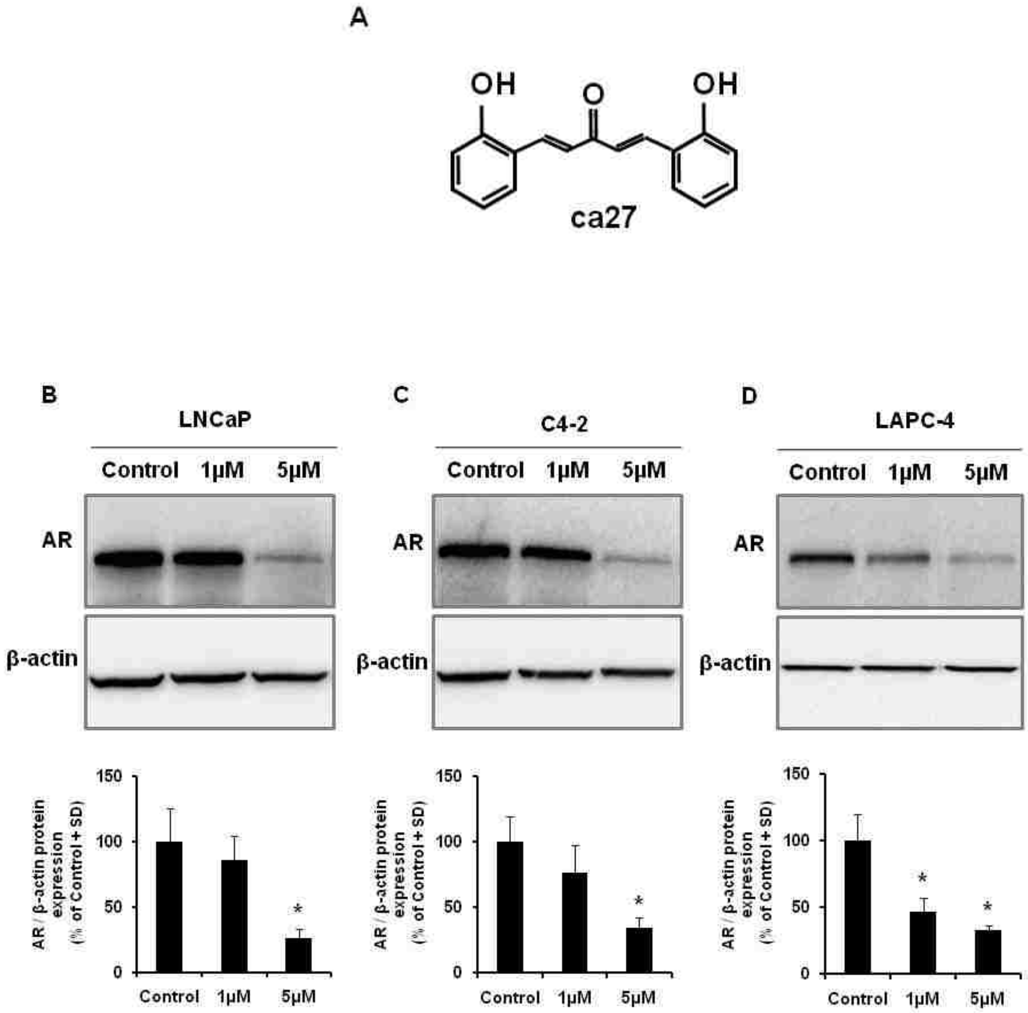
Results

Inhibition of Androgen Receptor Expression by ca27in Human Prostate Cancer Cells

The effects of the synthetic curcumin analog ca27 (Fig. 1A) were first determined on the endogenous AR protein expression in different human prostate cancer cell lines, i.e. LNCaP, C4-2, and LAPC-4. The cells were treated with ca27 for 12 hours at concentrations in the low micromolar range of 1 to 5μM. Western blot analysis and densitometric quantitation revealed a significant decrease in AR protein expression in LNCaP (Fig. 1B), C4-2 (Fig. 1C) and LAPC-4 (Fig. 1D) cells treated with 5 μM ca27. Five μM ca27 led to a significant reduction of AR protein expression to approximately 30% of control within 12 hours for all the cell lines tested. In addition, there was a significant decrease in AR protein expression in LAPC-4 (Fig. 1D) cells treated with 1 μM ca27. Curcumin did not down-regulate the AR in our experimental system, as shown in Figure 1E. C4-2 cells treated with 20 μM ca27 for 72 hours demonstrated a significant loss of AR protein expression, whereas treatment with up to 20 μM curcumin for 72 hours did not inhibit AR protein expression (Fig. 1E). Similar results were observed in LNCaP cells (data not shown). To determine whether proteasomal degradation is involved in AR down-regulation, we used the proteasomal inhibitor MG132. As shown in

Figure 1F, loss of AR protein expression by ca27 is independent of MG132 administration. LNCaP cells pretreated with 10 µM MG132 for 1 hour and then with 5 µM ca27 for 6 hours showed no inhibition of protein down-regulation in the presence of the proteasomal inhibitor (Fig. 1F).Collectively, these data indicate that ca27 mediates the down-regulation of endogenous AR protein in LNCaP, C4-2, and LAPC-4 prostate cancer cells within 3 hours of treatment independent of proteasomal degradation.

To test whether ca27 affects AR protein levels independent of mRNA transcription, we used the transcription inhibitor Actinomycin D (Act D). LNCaP cells were treated with 10 µM Act D or 5 µM ca27 for 3 and 6 hours (Fig. 2). AR protein expression was significantly inhibited by 5 µM ca27 after 3 hours (Fig. 2A). At this time point, AR mRNA and protein expression were unaffected by Act D (Fig. 2A and C). However, Act D significantly inhibited AR mRNA expression after 6 hours (Fig. 2C) but did not inhibit AR protein expression at this time point (Fig 2B). Together, these data indicate that ca27 at least in part down-regulates AR protein levels independent of its effect on AR mRNA transcription.


A
OH
O
OH
ca27
B
LNCaP
Control 1μM 5μM
AR
β-actin
C
C4-2
Control 1μM 5μM
AR
β-actin
D
LAPC-4
Control 1μM 5μM
AR
β-actin
AR / β-actin protein expression (% of Control + SD)
150
100
50
0
Control 1μM 5μM
*

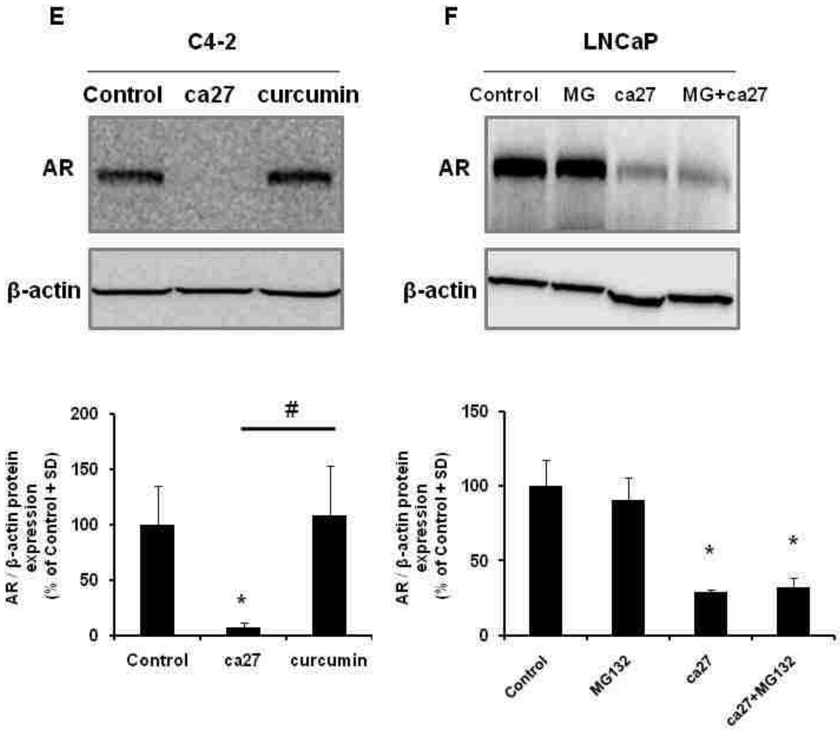


Fig. 1: Structure of the synthetic curcumin analog ca27 and down-regulation of AR protein expression by ca27. ca27 (1,5-Bis(2-hydroxyphenyl)-1,4-pentadien-3-one) consists of two phenolic rings with symmetrical hydroxyl groups on the ortho position of the aryl rings, which are linked by an unsaturated 5-carbon spacer with a single carbonyl (A). The synthesis of ca27 was previously described in Weber et al. 2006 (14).Down-regulation of endogenous AR protein expression by the synthetic curcumin analog ca27 in LNCaP, C4-2 and LAPC-4 cells. LNCaP (B), C4-2 (C) and LAPC-4 (D) cells were treated with 1 and 5 μM ca27. AR protein was measured by western blotting and densitometric analysis (ratio AR:β-actin) after 12 hours. LNCaP (E) cells were treated with 20 µM ca27 or curcumin for 72 hours AR protein expression was measured and quantitated as described above. LNCaP (F) cells were pretreated with 10 µM MG132 for 1 hour before the addition of 5 µM ca27 for 6 hours. One representative western blot is

shown; bars in the graph represent the average of triplicate values + standard deviation.* denote $P< 0.05$ compared to control.

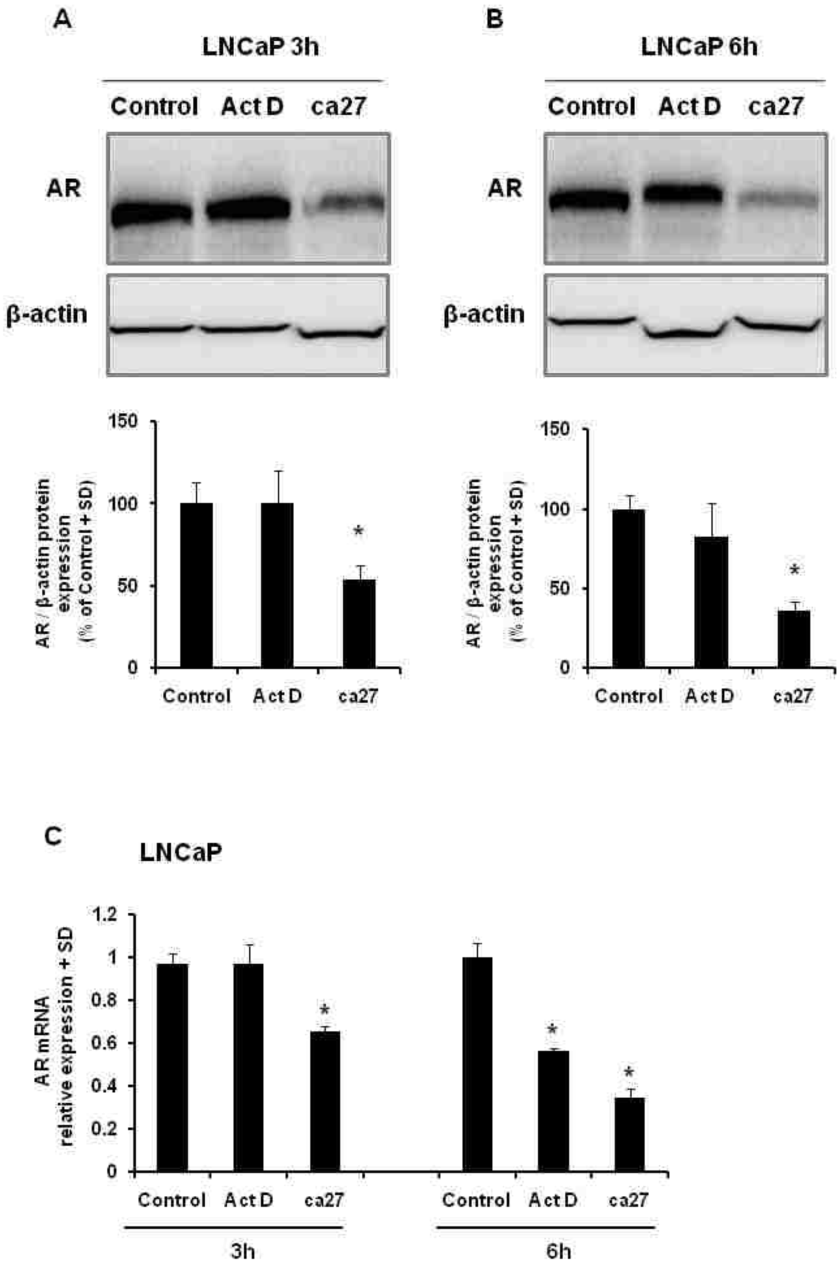


Fig. 2: Down-regulation of endogenous AR protein expression by ca27 in LNCaP cells. LNCaP (A) cells were treated with 10 μM Act D or 5 μM ca27 for 3 hours or 6 hours.AR

protein (A and B) was measured by western blotting and densitometric analysis (ratio AR:β-actin) after the indicated time. One representative western blot is shown; bars in the graph represent the average of quadruplicate values + standard deviation. AR and GAPDH mRNAs (C) were measured by qRT-PCR. Bars represent the average of quadruplicate values + standard deviation. AR expression normalized to GAPDH is shown relative to control. * denote $P < 0.05$ compared to control.

Inhibition of Cell Growth and Induction of Cell Death by ca27 in Human Prostate Cancer Cells

The anti-proliferative effects of ca27 were tested on LNCaP and C4-2 prostate cancer cells. Due to the relatively long doubling time of LNCaP and C4-2 of approximately 48 hours, cell proliferation data was analyzed after 96 hours of treatment. The effect of ca27 on prostate cancer cell growth was determined by cell counts upon treatment with ca27 concentrations between 0.5 μM and 15 μM. As shown in Fig. 3A, ca27 at ≥ 10 μM markedly inhibited growth of both LNCaP and C4-2 cells. Using trypan blue exclusion, we also determined the extent of cell death induced by ca27. As shown in Fig. 3B, the rate of cell death increased extensively and variably at concentrations of > 2.5 μM for C4-2 cells and > 10 μM for LNCaP cells. These data indicate that the synthetic curcumin analog ca27 both inhibited prostate cancer cell growth and induced cell death. Of note, the loss of AR protein expression occurs within a shorter exposure time to ca27 and at lower concentrations (Figs. 1B and 1C), demonstrating that it precedes the effects on cell viability. Nevertheless, the loss of AR expression may contribute to cell growth inhibition and death, although a pleiotropic effect of ca27 acting through additional pathways cannot be excluded.

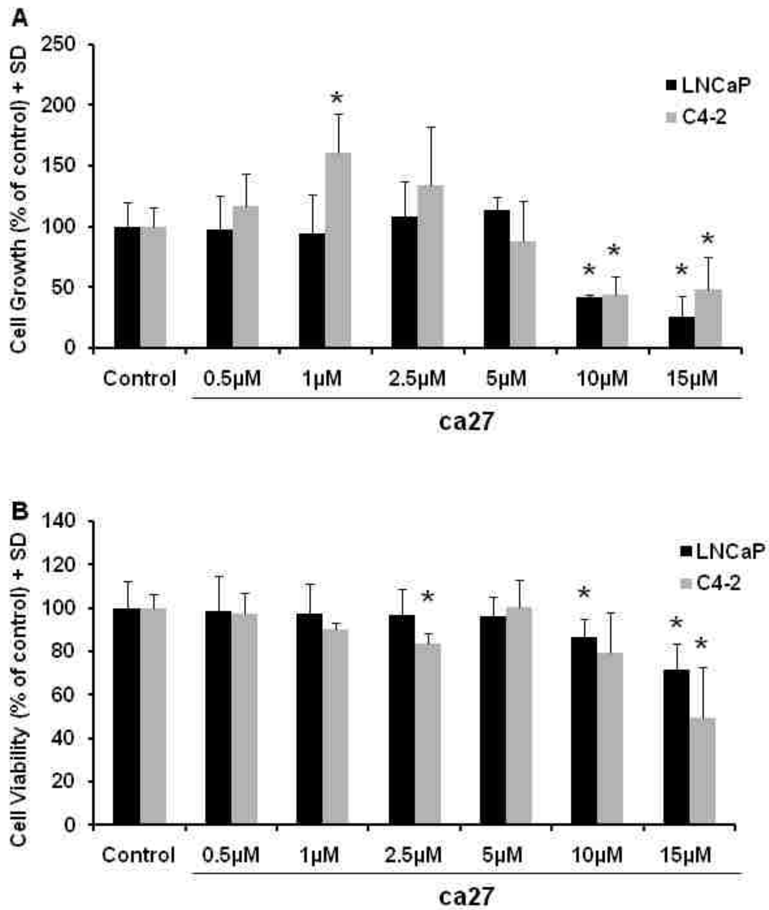


Fig. 3: Growth inhibition and induction of cell death in LNCaP and C4-2 human prostate cancer cells by ca27. Cell growth (A) and death (B) were determined by total cell counts and trypan blue positive cell counts, respectively. Cells were cultured in the presence of 0.5, 1, 2.5, 5, 10, or 15 µM ca27 for 96 hours. Bars represent the average of quadruplicate values + standard deviation. Cell growth and cell viability are expressed as percent of control.* denote P< 0.05 compared to control.

Inhibition of Androgen Receptor Activation by ca27 in Human Prostate Cancer Cells

Other reports demonstrating that curcumin analogs have inhibitory action against the AR (23-25) prompted us to test the effect of ca27 on AR function. LNCaP and C4-2 cells (Figs. 4A and 4B) were transiently transfected with a reporter plasmid expressing luciferase regulated by the MMTV promoter containing androgen responsive elements (17), cultured in medium containing charcoal stripped serum, and treated for 24 hours with increasing concentrations of ca27. AR activation measured by luciferase activity was determined 6 hours after addition of 1 nM R1881 synthetic androgen. As shown in Fig. 4A, ca27 significantly inhibited AR activation in LNCaP cells at 5 μM. ca27 affected AR activation similarly in C4-2 cells, with more variation and potentially at lower concentrations of 2 μM (Fig. 4B).

The ability of ca27 to inhibit AR activation was confirmed using the multifunctional androgen receptor screening (MARS) assay developed to screen for compounds with antagonistic and agonistic effects on androgenic activity (19). The MARS assay features androgen independent PC-3 human prostate cancer cells transiently co-transfected with an expression vector for the wild-type human AR and a plasmid carrying an androgen-sensitive promoter regulating the expression of destabilized enhanced GFP (19). In this sensitive assay, ca27 inhibited AR activation at low micromolar concentrations. In particular, ca27 above 1 μM proved to be a potent inhibitor of AR activation (Fig. 4C). Collectively, these data indicate that ca27 is a potent inhibitor of AR activation.

Inhibition of Prostate Specific Antigen Expression by ca27in Human Prostate Cancer Cells

To corroborate ca27 mediated AR down-regulation, we analyzed the effect of ca27 on the well-established transcriptional target of the AR, prostate specific antigen (PSA). LNCaP and C4-2 cells were treated with 1 and 5 μM ca27 for 12 hours, followed by assessment of endogenous PSA mRNA expression by qRT-PCR. In agreement with the observations on AR, PSA expression was significantly inhibited by 1 μM ca27 at 12 hours (Figs. 4D and 4E). Further, the effect of ca27 on PSA mRNA expression was tested after 3 hours when AR protein expression was significantly reduced as previously shown in Figures 1 and 2. At this time point ca27 did not reduce PSA mRNA expression in LNCaP or LAPC-4 cells (Figs. 4F and 4G). Together, these data indicate that ca27 is able to rapidly affect a biologically important downstream target of androgenic activity in prostate cancer cells, i.e. PSA. Further, the lack of PSA inhibition after the short exposure time of 3 hours suggests that ca27's effect on PSA is a result of reduced AR activity due to AR down-regulation.

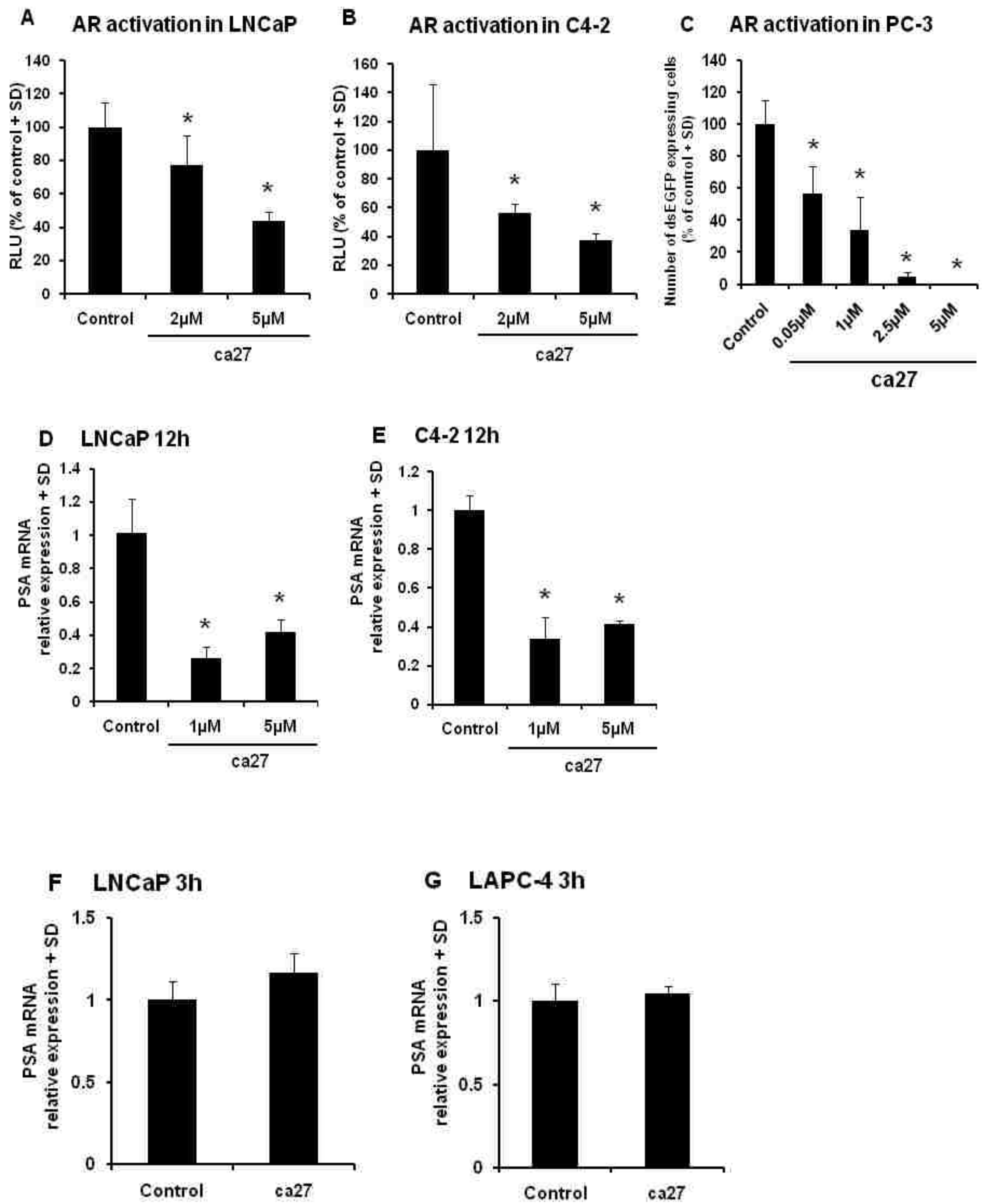


Fig. 4: Inhibition of AR activation and endogenous PSA expression by ca27 in LNCaP, C4-2, and PC-3 cells. (A) and (B): LNCaP (A) and C4-2 (B) cells were co-transfected with AR reporter plasmid driving firefly luciferase and a thymidine kinase reporter plasmid driving *Renilla* luciferase. Cells were treated with ca27 at 2 and 5 μM for 24

hours. Normalized luciferase activity (relative luciferase units, RLU) was determined 6 hours after addition of 1 nM R1881 synthetic androgen. (C) MARS assay (21): AR- and dsEGFP-transfected PC-3 cells were treated with increasing concentrations of ca27 for 24 hours and stimulated with 1 nM R1881. Bars in A-C represent the average of quadruplicate values + standard deviation. AR activation is expressed as percent of control. (D) and (E): LNCaP (D) and C4-2 (E) cells were treated with 1 and 5 μM ca27. PSA and GAPDH mRNAs were measured by qRT-PCR after 12 hours. Bars represent the average of quadruplicate values + standard deviation. PSA expression normalized to GAPDH is shown relative to control. LNCaP (F) and LAPC-4 (G) cells were treated with 5 μM ca27 for 3 hours. Bars represent the average of triplicate values + standard deviation. PSA expression normalized to GAPDH is shown relative to vehicle control.* denote $P< 0.05$ respectively compared to control.

Increased Cellular Oxidative Stress by ca27 Leads to AR Down-Regulation in LNCaP Cells

Given the rapid action of ca27,we evaluated the status of oxidative stress upon ca27 treatment in human prostate cancer cells. LNCaP cells were treated for 1 hour with 1-5 μM ca27 and assayed for the production of reactive oxygen species (ROS) as measured by DCF fluorescence. Treatment of LNCaP cells with 3 μM ca27 led to a significant production of ROS (Fig. 5A). In order to determine if this significant increase in oxidative stress by ca27 induces the down-regulation of AR protein expression, LNCaP (Fig. 5B) and LAPC-4 (Fig. 5C) cells were simultaneously treated with ca27 and the antioxidant N-acetyl-L-cysteine (NAC) for 3 hours. ca27 (5 μM) significantly inhibited

AR protein expression after this short incubation time in both cell lines. Further, NAC prevented ca27 mediated AR protein loss in both LNCaP and LAPC-4 cells. To determine if the down-regulation of AR protein expression could be due to the inhibition of AR mRNA by ca27, LNCaP and LAPC-4 cells were treated with 5 μM ca27 for 3 hours and AR mRNA was measured by qRT-PCR. In agreement with our previous result (Fig. 2C), within this short time period ca27 significantly inhibits AR mRNA expression in both cell lines (Figs. 5D and 5E). Further, AR mRNA expression is recovered when cells are simultaneously treated with ca27 and NAC demonstrating that the alleviation of oxidative stress induced by ca27 prevents the inhibition of AR expression. This result supports the hypothesis that induction of oxidative stress by ca27 mediates the down-regulation of AR expression in human prostate cancer cells.

Activation of Nrf2 and Up-Regulation of Nrf2 Regulated Genes by ca27

A typical downstream effect of cellular oxidative stress is the activation of the critical cellular redox sensor Nrf2. The increased ROS generation by ca27 treatment led us to investigate the activation status of Nrf2. A 5 μM ca27 treatment in LNCaP cells significantly increased Nrf2 activation, as measured by an antioxidant response element promoter driving a luciferase reporter (Fig. 6A). In addition, in LAPC-4 cells there was a significant activation of Nrf2 by 1 μM ca27 (Fig. 6B). This result demonstrates that ca27 leads to increased transcriptional activation of Nrf2. In addition, these concentrations are in agreement with the induction of AR protein down-regulation in the LNCaP and LAPC-4 cells as shown in Figs. 1B and 1D. To further illustrate activation of Nrf2 we evaluated Nrf2 regulated genes such as NQO1, AKR1C1 and MafG. LNCaP cells were treated with

5 µM ca27 for 3 hours and NQO1, AKR1C1 and MafG mRNA expression was measured by qRT-PCR. NQO1, AKR1C1 and MafG mRNA expression were increased ≥ 2 fold by ca27 treatment in comparison to the vehicle control (Fig. 6C). Collectively, these results corroborate the induction of oxidative stress by ca27 by demonstrating the activation of Nrf2 and the increased expression of Nrf2 regulated genes.

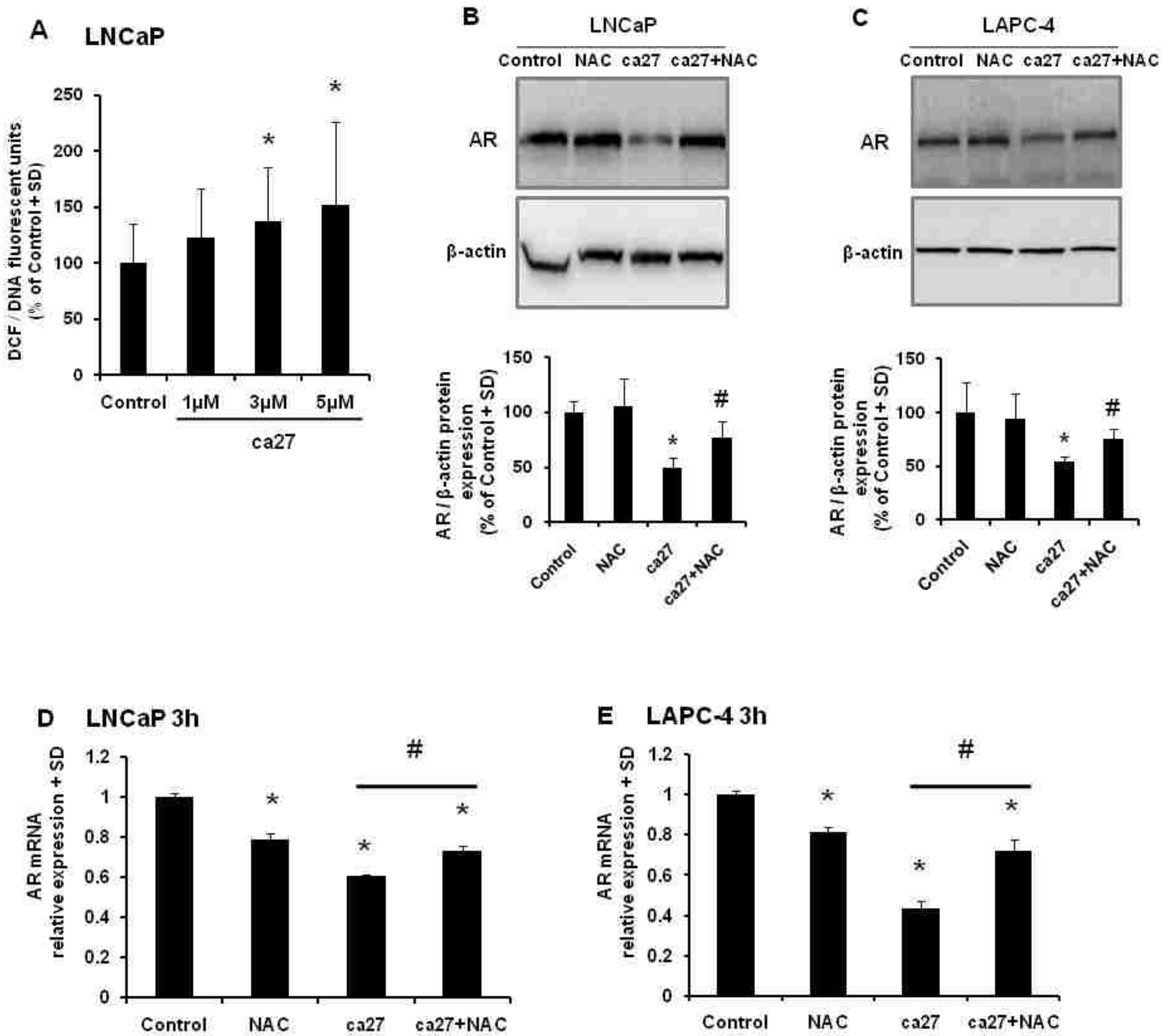


Fig. 5: Increased ROS generation induced by ca27 and prevention of AR down-regulation by antioxidant NAC in LNCaP cells. LNCaP cells were treated with increasing

concentrations (1, 3, and 5 μM) of ca27 for 1 hour. Increased ROS production was measured by DCF fluorescence and normalized to DNA content (A). LNCaP (B) and LAPC-4 (C) cells were treated for 3 hours with or without 5mM NAC in the presence or absence of 5 μM ca27 and assayed for AR protein expression by western blot; one representative western blot is shown. Protein expression was quantitated by densitometry. Bars represent the average of triplicate values + standard deviation. AR expression normalized to β-actin is shown relative to vehicle control. LNCaP (D) and LAPC-4 (E) cells were treated for 3 hours with or without 5 mM NAC in the presence or absence of 5 μM ca27 and assayed for AR mRNA expression and normalized to GAPDH bar graph shown is relative to control.* denote $P < 0.05$ respectively compared to control. # denote $P < 0.05$ respectively compared to ca27 treatment.

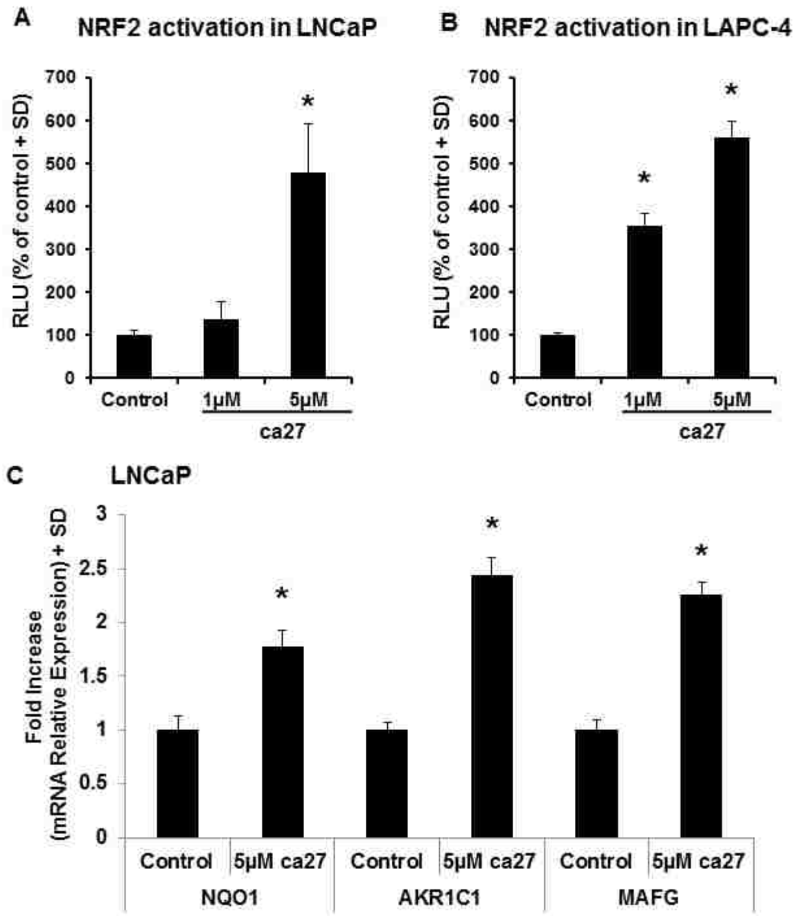


Fig. 6: Nrf2 activation and up-regulation of Nrf2 regulated genes in LNCaP and LAPC-4 cells by ca27. LNCaP (A) and LAPC-4 (B) cells were co-transfected with Nrf2 reporter plasmid driving luciferase and thymidine kinase reporter plasmid driving *Renilla* luciferase. Normalized luciferase activity was determined 16 hours post-treatment with 1 and 5 µM ca27. Bars represent the average of quadruplicate values + standard deviation. Nrf2 activation is expressed as % of control. LNCaP cells (C) were treated with vehicle control or 5 µM ca27 for 3 hours. NQO1, AKR1C1 and MafG mRNA expression was measured by qRT-PCR. Bars represent the average of triplicate values + standard

deviation. NQO1, AKR1C1 and MafG expression normalized to GAPDH is shown relative to control. * denote $P < 0.05$ respectively compared to control.

Discussion

The development of prostate cancer relies initially on androgenic activation of the AR by testosterone and its more active metabolite dihydrotestosterone (DHT) (1-2). While AR activation in normal prostatic tissue represents part of normal physiology and maintains normal differentiation of epithelial cells, in the malignant setting it leads to the expression of target genes that promote tumorigenesis and cancer progression (11,26). Clinically, the persistence of AR expression and function in androgen ablation resistant prostatic tissue is manifested by the successful yet transient application of second line androgen ablation strategies after primary failure, and by symptoms associated with androgen withdrawal (27-29). Furthermore, this stage of disease is characterized by a number of molecular mechanisms supporting the function of the AR in very low or even absent levels of DHT (10,30-31). Importantly, AR function under these conditions is still essential for prostate epithelial cell survival, as targeted AR down-regulation in androgen ablation resistant prostate cancer cell and animal models leads to cell growth inhibition (8-9). Therefore, given the persisting importance of the AR and its signaling axis in advanced prostate cancer, it remains a prominent and promising target for this stage of disease.

The natural product curcumin (diferuloylmethane) has been shown to inhibit many targets in prostate epithelial cells with an importance in cancer formation and progression. Among these targets are transcription factors, receptors, intracellular kinases, cytokines, and growth factors (32). Curcumin's effect on the AR and on its target PSA has been demonstrated by several independent investigators using both endogenously expressed AR in LNCaP cells and ectopically expressed AR in PC-3 cells (33-34). However, in these reports curcumin was used at relatively high concentrations, typically at $\geq$ 20 μM. It has previously been reported that curcumin has poor bioavailability which has been determined in both animal models and humans (35). This limitation has led researchers to generate a variety of synthetic analogs of curcumin and to investigate their capability to affect a number of molecular pathways implicated in tumorigenesis and cancer progression (16,36-39). Typical structure modifications include the introduction of substituents on the biphenyl moieties and modifications of the length of the linker between the biphenyl rings. A specific group of such analogs has been exploited towards their ability to inhibit AR function (23-25), and some of these agents have been shown to down-regulate the expression of AR (24).

Along this line, we report here on the anti-androgenic action of curcumin analog ca27, which originates from our previously reported chemical libraries (12-14). In particular, we have shown that ca27 at concentrations below those typically used for curcumin inhibits the growth of LNCaP and C4-2 human prostate cancer cells. Our data indicate that the observed growth inhibition and cell death of prostate cancer cells by ca27 could be in part mediated by the suppression of AR function. In fact, AR protein expression is significantly down-regulated by ca27 within 3 hours of treatment in various human prostate cancer cell lines. This rapid loss of AR protein expression could be due in part to the initial concomitant loss of AR mRNA expression. However, our investigations using the transcriptional inhibitor actinomycin D at multiple time points indicate an additional post-transcriptional inhibitory effect of ca27 on AR protein. Further, ca27's inhibition of the AR is selective, as ca27 significantly inhibited AR but not PSA mRNA expression in LNCaP and LAPC-4 cells, indicating that PSA inhibition is a result of reduced AR activity due to AR down-regulation.

ca27 induced AR protein down-regulation seems to be mediated by a distinct mechanism. We evaluated the actions of a well-established AR degradation mechanism, the ubiquitin-proteasomal pathway (40-41), and found that ca27 mediated loss of AR expression was not prevented by the proteasomal inhibitor MG132. This indicates an alternative down-regulation pathway for the AR activated by ca27. Accordingly, we show here that a potential mechanism for ca27 mediated AR down-regulation is through the induction of cellular oxidative stress. We demonstrate the pro-oxidant activity of ca27 by the increased ROS generation in human prostate cancer cells. The induction of ROS by ca27 was further demonstrated by the transcriptional activation of a known cellular redox sensor, the transcription factor Nrf2 (42). Further, the expression of Nrf2 regulated detoxification genes, NQO1 and AKR1C1 (42-43), were significantly increased by ca27. This is in agreement with a previous study by Dinkova-Kostova et al. who reported that the identical structure induces NQO1 activity in murine hepatoma and papilloma cells (44). Further, ca27 induced the mRNA expression of the small Maf protein, MafG. MafG is a known heterodimerization partner of Nrf2 and leads to Nrf2 transcriptional activity, and MafG expression has been shown to be regulated by Nrf2 transcriptional activity under oxidative stress conditions (45). Evidence that AR down-regulation is mediated by ca27 induced ROS generation is provided by our data showing that AR loss is attenuated by the addition of the antioxidant NAC. Finally, the generation of cellular oxidative stress by ca27 could partially explain the proteasomal-independent down-regulation of the AR observed in this study, as previous studies have demonstrated that increased cellular oxidative stress can lead to protein aggregates which inhibit the functions of the proteasome (46-47). While the exact mechanism(s) of ca27 mediated AR protein down-regulation is at present unknown, it seems to entail oxidative stress mediated pathways. Our results are in agreement with two recent studies showing that AR mRNA transcription was inhibited in LNCaP and rat hepatoma cells by the pro-oxidant tert-butyl hydroperoxide (TBH) (48), and that the black seed oil ingredient thymoquinone induces oxidative stress and affects AR expression (49).

Conclusions

We conclude that the curcumin analog ca27 represents a lead structure with anti-androgenic activity in human prostate cancer cells, possibly through the induction of oxidative stress. Therefore, ca27 and similar compounds can be exploited as molecular tools to study pathways relevant to AR protein down-regulation. By extension, given the prominent role of the AR in prostate cancer (2, 4, 10-11) and because AR degradation has been recognized as an effective therapeutic strategy (9-10), we propose that ca27 is a potential lead in the development of novel therapeutics for prostate cancer. This is in agreement with recent reports on other compounds derived from natural products with similar anti-androgenic activities mediated by oxidative stress (50), and may represent an emerging theme for novel prostate cancer therapeutics.

Acknowledgements

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References

1. Gao, W., C.E. Bohl, and J.T. Dalton, Chemistry and structural biology of androgen receptor. Chem Rev 2005. 105(9): p. 3352-70.
2. Heinlein, C.A. and C. Chang, Androgen receptor in prostate cancer. Endocr Rev, 2004. 25(2): p. 276-308.
3. Klotz, L., Hormone therapy for patients with prostate carcinoma. Cancer, 2000. 88(12 Suppl): p. 3009-14.
4. Simmons, M.N. and E.A. Klein, Combined androgen blockade revisited: emerging options for the treatment of castration-resistant prostate cancer. Urology, 2009. 73(4): p. 697-705.
5. Fitzpatrick, J.M., Is hormone ablation still the right choice for advanced prostate cancer? BJU Int, 2007. 100 Suppl 2: p. 36-9.
6. Chodak, G.W., et al., Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate. J Urol, 1992. 147(3 Pt 2): p. 798-803.
7. Sadi, M.V., P.C. Walsh, and E.R. Barrack, Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy. Cancer, 1991. 67(12): p. 3057-64.
8. Eder, I.E., et al., Inhibition of LNCaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. Cancer Gene Ther, 2000. 7(7): p. 997-1007.
9. Snoek, R., et al., In vivo knockdown of the androgen receptor results in growth inhibition and regression of well-established, castration-resistant prostate tumors. Clin Cancer Res, 2009. 15(1): p. 39-47.

10. Chen, Y., C.L. Sawyers, and H.I. Scher, Targeting the androgen receptor pathway in prostate cancer. Curr Opin Pharmacol, 2008. 8(4): p. 440-8.

11. Knudsen, K.E. and H.I. Scher, Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer. Clin Cancer Res, 2009. 15(15): p. 4792-8.

12. Weber, W.M., et al., Anti-oxidant activities of curcumin and related enones. Bioorg Med Chem, 2005. 13(11): p. 3811-20.

13. Weber, W.M., et al., TPA-induced up-regulation of activator protein-1 can be inhibited or enhanced by analogs of the natural product curcumin. Biochem Pharmacol, 2006. 72(8): p. 928-40.

14. Weber, W.M., et al., Activation of NFkappaB is inhibited by curcumin and related enones. Bioorg Med Chem, 2006. 14(7): p. 2450-61.

15. Mosley, C.A., D.C. Liotta, and J.P. Snyder, Highly active anticancer curcumin analogues. Adv Exp Med Biol, 2007. 595: p. 77-103.

16. Anand, P., et al., Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. Biochem Pharmacol, 2008. 76(11): p. 1590-611.

17. Thompson, T.A., et al., Transient promoter activity in primary rat mammary epithelial cells evaluated using particle bombardment gene transfer. In Vitro Cell Dev Biol, 1993. 29A(2): p. 165-70.

18. Moinova, H.R. and R.T. Mulcahy, An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gamma-glutamylcysteine

synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. J Biol Chem, 1998. 273(24): p. 14683-9.

19. Dennis, M.K., et al., A multifunctional androgen receptor screening assay using the high-throughput Hypercyt flow cytometry system. Cytometry A, 2008. 73(5): p. 390-9.

20. Abramoff, M.M. and S.J. Ram, Image processing with imagej. Biophotonics International, 2004. 11: p. 36-43.

21. Basu, H.S., et al., A small molecule polyamine oxidase inhibitor blocks androgen-induced oxidative stress and delays prostate cancer progression in the transgenic adenocarcinoma of the mouse prostate model. Cancer Res, 2009. 69(19): p. 7689-95.

22. Ripple, M.O., et al., Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. J Natl Cancer Inst, 1997. 89(1): p. 40-8.

23. Ohtsu, H., et al., Antitumor agents. 217. Curcumin analogues as novel androgen receptor antagonists with potential as anti-prostate cancer agents. J Med Chem, 2002. 45(23): p. 5037-42.

24. Shi, Q., C.C. Shih, and K.H. Lee, Novel Anti-Prostate Cancer Curcumin Analogues that Enhance Androgen Receptor Degradation Activity. Anticancer Agents Med Chem, 2009.

25. Zhou, J., et al., Design and synthesis of androgen receptor antagonists with bulky side chains for overcoming antiandrogen resistance. J Med Chem, 2009. 52(17): p. 5546-50.

26. Kung, H.J. and C.P. Evans, Oncogenic activation of androgen receptor. Urol Oncol, 2009. 27(1): p. 48-52.

27. Lam, J.S., et al., Secondary hormonal therapy for advanced prostate cancer. J Urol, 2006. 175(1): p. 27-34.

28. Muthuramalingam, S.R., K. Patel, and A. Protheroe, Management of patients with hormone refractory prostate cancer. Clin Oncol (R Coll Radiol), 2004. 16(8): p. 505-16.

29. Van Allen, E.M. and C.J. Ryan, Novel secondary hormonal therapy in advanced prostate cancer: an update. Curr Opin Urol, 2009. 19(3): p. 315-21.

30. Edwards, J. and J.M. Bartlett, The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: Androgen-receptor cofactors and bypass pathways. BJU Int, 2005. 95(9): p. 1327-35.

31. Scher, H.I. and C.L. Sawyers, Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol, 2005. 23(32): p. 8253-61.

32. Aggarwal, B.B., Prostate cancer and curcumin: add spice to your life. Cancer Biol Ther, 2008. 7(9): p. 1436-40.

33. Nakamura, K., et al., Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines. Int J Oncol, 2002. 21(4): p. 825-30.

34. Tsui, K.H., et al., Curcumin blocks the activation of androgen and interlukin-6 on prostate-specific antigen expression in human prostatic carcinoma cells. J Androl, 2008. 29(6): p. 661-8.

35. Anand, P., et al., Bioavailability of curcumin: problems and promises. Mol Pharm, 2007. 4(6): p. 807-18.

36. Adams, B.K., et al., Synthesis and biological evaluation of novel curcumin analogs as anti-cancer and anti-angiogenesis agents. Bioorg Med Chem, 2004. 12(14): p. 3871-83.

37. Basile, V., et al., Curcumin derivatives: molecular basis of their anti-cancer activity. Biochem Pharmacol, 2009. 78(10): p. 1305-15.

38. Ishida, J., et al., Antitumor agents. Part 214: synthesis and evaluation of curcumin analogues as cytotoxic agents. Bioorg Med Chem, 2002. 10(11): p. 3481-7.

39. Ohori, H., et al., Synthesis and biological analysis of new curcumin analogues bearing an enhanced potential for the medicinal treatment of cancer. Mol Cancer Ther, 2006. 5(10): p. 2563-71.

40. Lin, H.K., et al., Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. Embo J, 2002. 21(15): p. 4037-48.

41. Sheflin, L., et al., Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. Biochem Biophys Res Commun, 2000. 276(1): p. 144-50.

42. Jaiswal, A.K., Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med, 2004. 36(10): p. 1199-207.

43. Burchiel, S.W., et al., Activation of dioxin response element (DRE)-associated genes by benzo(a)pyrene 3,6-quinone and benzo(a)pyrene 1,6-quinone in MCF-

10A human mammary epithelial cells. Toxicol Appl Pharmacol, 2007. 221(2): p. 203-14.

44. Dinkova-Kostova, A.T., et al., Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc Natl Acad Sci U S A, 2001. 98(6): p. 3404-9.

45. Katsuoka, F., et al., Nrf2 transcriptionally activates the mafG gene through an antioxidant response element. J Biol Chem, 2005. 280(6): p. 4483-90.

46. Breusing, N. and T. Grune, Regulation of proteasome-mediated protein degradation during oxidative stress and aging. Biol Chem, 2008. 389(3): p. 203-9.

47. Szweda, P.A., B. Friguet, and L.I. Szweda, Proteolysis, free radicals, and aging. Free Radic Biol Med, 2002. 33(1): p. 29-36.

48. Shi, L., et al., Loss of androgen receptor in aging and oxidative stress through Myb protooncoprotein-regulated reciprocal chromatin dynamics of p53 and poly(ADP-ribose) polymerase PARP-1. J Biol Chem, 2008. 283(52): p. 36474-85.

49. Koka, P.S., et al., Studies on molecular mechanisms of growth inhibitory effects of thymoquinone against prostate cancer cells: role of reactive oxygen species. Exp Biol Med (Maywood), 2010. 235(6): p. 751-60.

50. Ketola, K., et al., Monensin is a potent inducer of oxidative stress and inhibitor of androgen signaling leading to apoptosis in prostate cancer cells. Mol Cancer Ther. 9(12): p. 3175-85.

CHAPTER 3: ALPHA-TOCOPHERYL QUINONE INHIBITS ANDROGEN RECEPTOR EXPRESSION THROUGH MODULATION OF CELLULAR REDOX

Abstract

Due to discrepancies in results between epidemiological studies, the role of tocopherols in cancer prevention is controversial. This may be due, in part, to assuming equivalency between the biological action of tocopherols and their oxidized forms on cellular functions. In this study, we show that tocopheryl quinone (TQ), the oxidation product of vitamin E (VE), has biological properties that are distinct from VE. TQ, but not VE, was found to have inhibitory activity on both the growth and androgenic activity of human prostate cells. TQ potently inhibited the growth of androgen-sensitive prostate cancer cell lines, but did not affect the growth of androgen-independent prostate cancer cells. Due to the selective growth inhibition observed with androgen-sensitive cells, the anti-androgenic properties of TQ were examined. TQ treatment led to the significant down-regulation of androgen receptor (AR) protein expression. Moreover, TQ treatment inhibited androgen-induced release of prostate specific antigen from androgen-sensitive prostate cells and the TQ-mediated down-regulation of AR resulted in the inhibition of an androgen-responsive reporter system. The anti-androgenic action of TQ was further evidenced by the down-regulation of genes dependent on AR activity for their expression. Further, we identified a potential mechanism of TQ's actions on AR down-regulation may be in part, due to the increase in oxidative stress as measured by glutathione levels and the prevention of AR down-regulation in the presence of

antioxidants. Overall, TQ, but not VE, was shown to be a potent inhibitor of androgenic activity and AR expression in androgen-sensitive human prostate cancer cells suggesting that the actions of TQ may account for some of the biological actions attributed to VE.

Introduction

Prostate cancer is a growing health problem worldwide (1-3), making it an important candidate for the development of preventive measures (4). The use of vitamin E (VE) for prostate cancer prevention has become increasingly controversial following the negative results of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (5-7). The SELECT results contrast those of the alpha-tocopherol, beta-carotene cancer prevention (ATBC) study, where VE was found to reduce both the incidence and mortality of prostate cancer (8). A major difference between these two studies is that the participants of the ATBC trial were all smokers, whereas only a small percentage of participants in the SELECT were smokers. Other studies support that smoking in combination with VE supplementation may be responsible for reduced levels of prostate cancer (9-12). An intriguing explanation for the discrepancies between these studies is that the oxidation product of VE, tocopheryl quinone (TQ), which may be elevated in the oxidative stress environment produced by smoking, is the active factor responsible for the decrease in prostate cancer among smokers taking supplemental VE. To support this hypothesis, VE and TQ should have differential effects on prostate cancer cells. Indeed, in this study, TQ, but not VE, was found to have significant anti-androgenic activity. If TQ is active against prostate cancer development, then men could be supplemented with TQ directly for more effective prostate cancer prevention.

VE is a family of naturally occurring dietary factors (e.g., α-,β-,γ-,δ-tocopherols and -tocotrienols) whose major biologically active form is RRR-α-tocopherol (13,14). Normal blood levels of VE are variable with a mean of approximately 25 μM (15-17). Physiologically, VE is believed to act as an antioxidant, reducing cellular oxidative damage produced by oxidized lipids (13,14). The major oxidation product of VE as α-tocopherol is α-tocopheryl quinone, which is formed by the two-electron oxidation of the chromanol moiety of VE (Fig. 1). TQ has unique chemical properties compared to VE. Although VE has been studied extensively with an interest in reducing disease pathology, to date, the role of VE in preventing cancer development is unclear. However, VE-derivatives are emerging as potentially useful agents to target androgenic activity that may prove effective for prostate cancer prevention (18,19).

The AR is recognized as a key contributor to prostate cancer development and has been suggested as a meaningful target for prostate cancer prevention (4). This is supported by the recognized importance of the AR in prostate cancer progression (20-22) and from the outcome of studies using inhibitors of testosterone metabolism to prevent prostate cancer development (21,23,24). The AR is a member of the steroid hormone/nuclear receptor superfamily (25), which acts as a ligand-activated transcription factor for genes involved in the growth, survival, and differentiation of the prostate (26). In addition, AR activity contributes to the development, progression, and maintenance of prostate cancer (22,27). Down-regulation of AR activation can be achieved either through direct interference of androgen binding to the AR as with AR antagonists, by decreasing dihydrotestosterone production with 5-alpha-reductase inhibitors, or by decreasing the production of testosterone by gonadotropin-releasing hormone agonists (22,27). It should be noted that these strategies do not directly target the expression of AR protein and thus the AR remains functional. A unique strategy for prostate cancer prevention is the identification of agents that down-regulate the expression of AR protein.

Studies on the actions of TQ are limited compared to the more extensive investigations on VE. Importantly, to date, no studies addressing the effect of TQ on prostate cancer cells have been reported. However, down-regulation of AR activity by VE-related chemicals have been reported. The mechanism of androgenic inhibition by these agents may be direct or indirect. For example, we have previously shown that the chromanol moiety of VE blocks androgenic activity by competitive inhibition of androgen binding to the AR (19). Direct inhibition of the AR has been observed with VE succinate, which has been shown to down-regulate AR protein in prostate cancer cells in culture (18). Direct targeting of AR protein may serve as useful strategy for inhibiting the progression of prostate cancer. In this study, we evaluated TQ's effects on prostate cancer cell proliferation, anti-androgenic activity and potential mechanism of AR protein down-regulation. Compared to VE, TQ was found to have distinctive properties on androgen-responsive prostate cancer cell lines with notable actions on the expression of the AR. This study further begins to elucidate the mechanism of TQ's actions on inhibiting AR protein expression may be through its activity as a pro-oxidant.

Materials and Methods

dl-α-tocopheryl quinone was obtained from Research Organics (Cleveland, OH). Methyltrienolone (i.e., R1881) was obtained from Perkin Elmer/NEN Life Science Products (Boston, MA). Bicalutamide was from LKT Laboratories, St. Paul, MN. Vitamin E as dl-α-tocopherol and other chemicals used in these studies were from Sigma Chemical Co (St. Louis, MO).

The LNCaP and DU145 cells used in these studies were acquired from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 5% heat-inactivated fetal calf serum (FCS; Sigma, St. Louis, MO) with streptomycin-penicillin antibiotics (designated DMEM/FCS) in a 5% CO_2 incubator at 37°C. LAPC4 cells adapted to growth in DMEM and 5% FCS were acquired from Dr. George Wilding (University of Wisconsin Paul P. Carbone Comprehensive Cancer Center). For most experiments evaluating androgenic responses, cells were cultured in DMEM containing 4% charcoal-stripped FCS and 1% unstripped FCS (designated DMEM/CSS). Methods were developed to insure that TQ and VE could effectively be delivered to prostate cancer cells in culture. This was achieved using a carrier-based delivery method for TQ and VE dissolved first in ethanol which was added to a 7.5% bovine serum albumin (BSA) solution for a 20-fold concentrated stock. This solution was then added to standard growth medium at a 5% concentration (i.e., a final concentration of 0.4% BSA) to produce concentrations of VE in culture medium ranging from 10 to 40 μM.

TQ and VE measurements in tissue culture medium

The addition of TQ and VE to medium was performed as described earlier. Levels of TQ and VE in tissue culture medium were measured using an ESA high-performance liquid chromatography (HPLC) system (ESA, Inc., Chelmsford, MA) with a 250 mm AltechLiChrosorb RP-18 reverse-phase column, an ESA model 582 solvent delivery system, and an ESA CoulArray detector controlled by CoulArray Software for Windows. The mobile phase consisted of 5 mM sodium acetate and 5 mM acetic acid in HPLC grade methanol.

Cell proliferation assays

Relative cell growth changes were determined using DU145, LNCaP, and LAPC4 cells plated in 96-well tissue culture plates. Relative cell numbers with and without TQ and VE treatment were determined using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen), according to kit instructions.

AR protein immunoblot analysis

LNCaP and LAPC4 cells were plated at a density of 1×10^6 cells per 100 mm cell culture plate in 10 ml of DMEM/CSS and maintained in incubators at 37°C in 5% CO_2. For dose-response studies, LNCaP cells were cultured in 6-well plates (BD Biosciences, San Jose, CA) in DMEM containing 5% FBS. After a 4 d treatment with vehicle, VE, or TQ, cells were washed in cold 1× PBS and lysed in a buffer containing 1.0 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 µg/ml aprotinin in 1× PBS. Cell extracts were stored at -80°C until analysis. Sample protein levels were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL), according to kit instructions. Total protein (25 to 30 µg) from cell extracts were electrophoresed on 12.5 % SDS-polyacrylamide gels (BioRad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk at 4°C and then incubated with mouse anti-AR (441) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β-actin antibody (A5441; Sigma). After washing, membranes were incubated with a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (Biomeda, Foster City, CA) and analyzed using Western Lightening Chemiluminescence Reagent Plus (Boston, MA) on a Kodak Image Station 4000MM (Rochester, NY). Band intensities were determined using Kodak Molecular Imaging Software.

Messenger RNA expression analysis

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen) and cDNA was prepared from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed for mRNA levels using an Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA) and QuantiTect Primers Assays (Qiagen Inc., Valencia, CA) for *AR*, *NQO1* and *GAPDH* mRNA. Additional forward and reverse primers used for qPCR are listed in Table 1.

Table 1: Quantitative PCR primer sequences. [1] Listed from 5′ to 3′.

Gene (Abbreviation)	Primer Direction	Primer Sequence[1]
***Prostate Specific Antigen* (PSA)**	Forward	CGCTGGACAGGGGGCAAAA
	Reverse	ACAAGTGGGCCCCCAGAATCA
***Kallikrein 2* (KLK2)**	Forward	CTGGGCTCTGGACAGGTGGTAAA
	Reverse	TACAGACAAGTGGACCCCCAGAAT
***Prostein* (SLC45A3)**	Forward	CCTCCCTCTACCACCGGGAGAA
	Reverse	CCCTCGGTATTTGGGCAGGAA

***Prostatic Acid Phosphatase* (PAP)**	Forward	CTTCTTGCCACTTGACGGAATTGT
	Reverse	GTGCTGCGTCTCATTCCGGTAGTA
***NKX3.1* (NKX3-1)**	Forward	GGCCGAGACGCTGGCAGAGA
	Reverse	GGGCGCCTGAAGTGTTTTCAGAGT
***Prostate Specific Membrane Antigen* (PSMA; FOLH1)**	Forward	TCAGTGAGAGACTCCAGGACTTTGACA
	Reverse	GTTGTGGCTGCTTGGAGCATAGAT
***Retinoid X Receptor alpha* (RXRα)**	Forward	GTGGAGGCGCTGAGGGAGAA
	Reverse	GGCAGGCGGAGCAAGAGCTTA
***Vitamin D Receptor* (VDR)**	Forward	CGGGCAGCCACCTGCTCTA
	Reverse	TGCGCAGGTCGGCTAGCTTCT
***Aldoketoreductase 1C1* (AKR1C1)**	Forward	GATGGCCTAAACAGAAATGTGCGAT
	Reverse	GGATAATTAGGGGGGCCAGCA
***Small MafG* (MafG)**	Forward	GCTGTGCCCCCGGGTTATGA
	Reverse	CCGTCAGGCTGGTGCCATTCT
***X-box protein 1 (XBP-1)* Spliced**	Forward	CCGCAGCAGGTGCAGG
	Reverse	GAGTCAATACCGCCAGAATCCA
$P58^{IPK}$	Forward	GAGGTTTGTGTTGGGATGCAG
	Reverse	GCTCTTCAGCTGACTCAATCA
Activating transcription factor 4 (ATF4)	Forward	TAGGGGCCTCCTACCTTTGT
	Reverse	GTGTCATCCAACGTGGTCAG

Activating transcription factor 6 (ATF6)	Forward	GCCTTTATTGCTTCCAGCAG
	Reverse	TGAGACAGCAAAACCGTCTG
CHOP	Forward	ATGGCAGCTGAGTCATTGCCTTTC
	Reverse	AGAAGCAGGGTCAAGAGTGGTGAA

Prostate specific antigen analysis

LNCaP cells were cultured in 96-well plates at $5x10^3$ cells per well in DMEM/CSS 1 d before treatment. After a 4 d treatment with 50 pM R1881 and TQ or VE, media levels of PSA released from LNCaP cells were measured using a PSA Enzyme Immunoassay Test Kit (BioCheck, Inc., Foster City, CA) according to the kit's instructions. PSA levels were normalized to cell number, which were determined using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) described above.

Promoter activation assay

LNCaP cells were cultured in 12- or 24-well plates (Invitrogen) in DMEM/CSS 2 to 3 d before transfection. Androgen-induced transcriptional activation was determined using a reporter construct with an androgen-sensitive MMTV-LTR that regulates the expression of luciferase (25,28). Cells were transfected using the calcium phosphate precipitation method with the MMTV/luciferase plasmid (28). Twenty-four h after transfection, cells were treated with R1881 with or without test reagents at the specified concentrations. Cell extracts were acquired after treatment in 100 μL of Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay

Substrate (Promega) and relative luciferase units determined on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

Glutathione Assay

LNCaP cells were cultured in 6-well plates in DMEM containing 5% FBS. LNCaP cells were treated for the indicated times and total cell number was determined by a hemacytometer and light microscopy immediately after collection. GSH and GSSG were measured using a modified Tietze et al. (29) protocol of the GSH/GSSG Ratio Assay Kit (Calbiochem, San Diego, CA) in combination with 2-vinyl pyridine and triethanolamine according to instructions from Rhaman et al. (30). GSH_t and GSSG were determined according to the kit's instructions. GSH_t and GSSG were normalized to total cell number.

Statistical analysis

Significant differences in values between groups were assessed using an unpaired *t*-test with SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). *P* values less than 0.05 were used to signify statistical significance. Studies were performed as specified with a minimum of 3 samples (i.e., n $\geq$3).

Results

Validation of TQ and VE dissolution in tissue culture medium

TQ and VE are composed of lipophilic hydrocarbon chains (Fig. 1) that greatly limit their solubility in cell culture medium and, thus, complicates the treatment of cells in culture with these agents. Therefore, for these studies, methods were developed to effectively treat prostate cancer cells in culture with TQ and VE. This was achieved using bovine serum albumin (BSA) as a carrier-based delivery method. BSA was found to be suitable carrier for the administration of TQ and VE at levels up 40 μM. Validation of TQ and VE dissolved in medium were performed using HPLC and electrochemical detection (see *Materials and Methods*). Fig. 2A and Fig. 2B show electrochemical detector output from an HPLC analysis for VE and TQ, respectively. Fig. 2C shows that increasing concentrations of VE in cell culture medium were linear from 1 to 40 μM, which was found to be similar for TQ (data not shown). Because normal blood levels of VE range from 20 to 30 μM (15-17), for most experiments performed in this study, a concentration of 25 μM TQ and VE was used, unless specified otherwise.

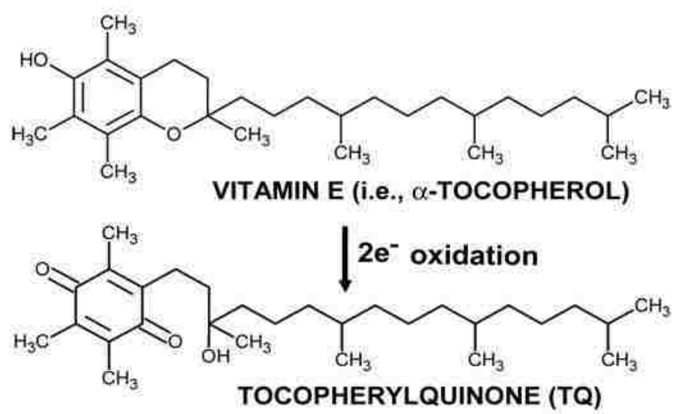


Fig. 1: Tocopherylquinone is produced by the two-electron oxidation of the chromanol moiety of vitamin E (VE).

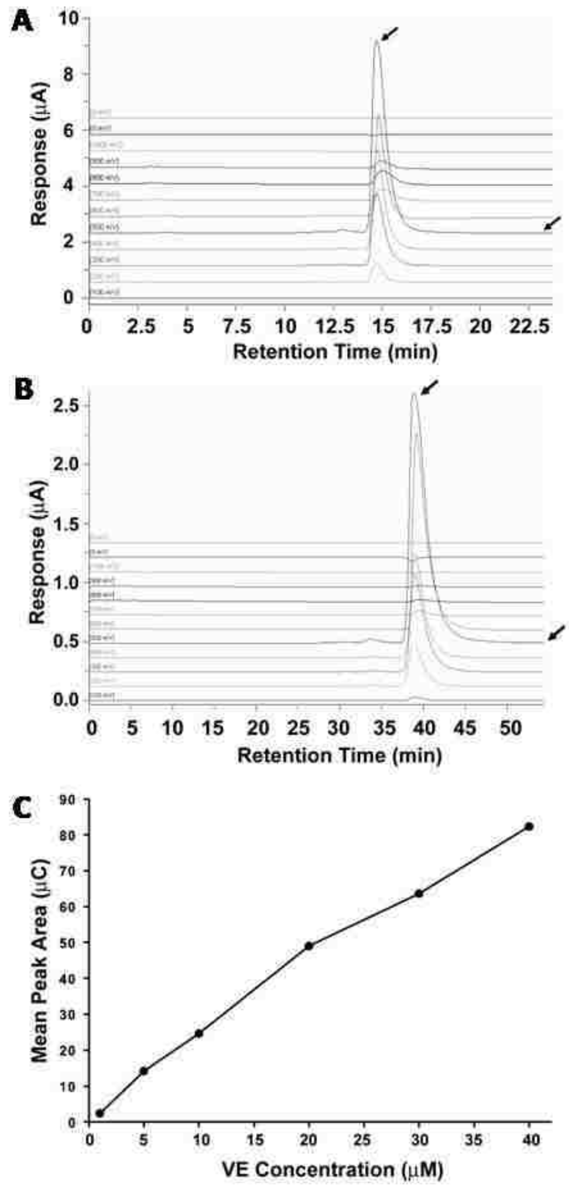


Fig. 2: Analysis of VE and TQ. Retention time determination of VE (A) and TQ (B) analyzed by HPLC using electrochemical detection. The sensitivity of detection was greatest with an array potential of +500 mV for both VE and TQ (A & B; arrows). (**C**) A linear relationship of VE in cell culture media was observed for the concentration range tested of 0 to 40 μM.

Inhibition of prostate cancer cell growth by TQ

Previous studies have demonstrated that ester-conjugated, water soluble VE analogs (e.g., vitamin E succinate) can inhibit prostate cancer cell growth in culture (18,31). Using the methods described in this study to dissolve the free forms of TQ and VE, their ability to inhibit prostate cell growth was determined. TQ treatment inhibited cell proliferation of AR expressing LAPC4 cells but had minimal effect on the androgen-independent DU145 prostate cancer cell line, which does not express the AR, after treatment with concentrations of up to 40 μM TQ (Fig. 3A)**.** In contrast, treatment with TQ produced a dose-dependent decrease in prostate cancer cell growth in LAPC4 (Fig. 3B) and LNCaP (Fig. 3C) androgen-sensitive prostate cancer cells, which was significantly reduced at a low dose of 10 μM TQ. A small, but significant, decrease in LAPC4 cell growth was observed at VE treatment levels equal to or greater than 30 μM (Fig. 3B). In LNCaP cells, treatment with VE up to 40 μM did not significantly decrease growth (Fig. 3C).

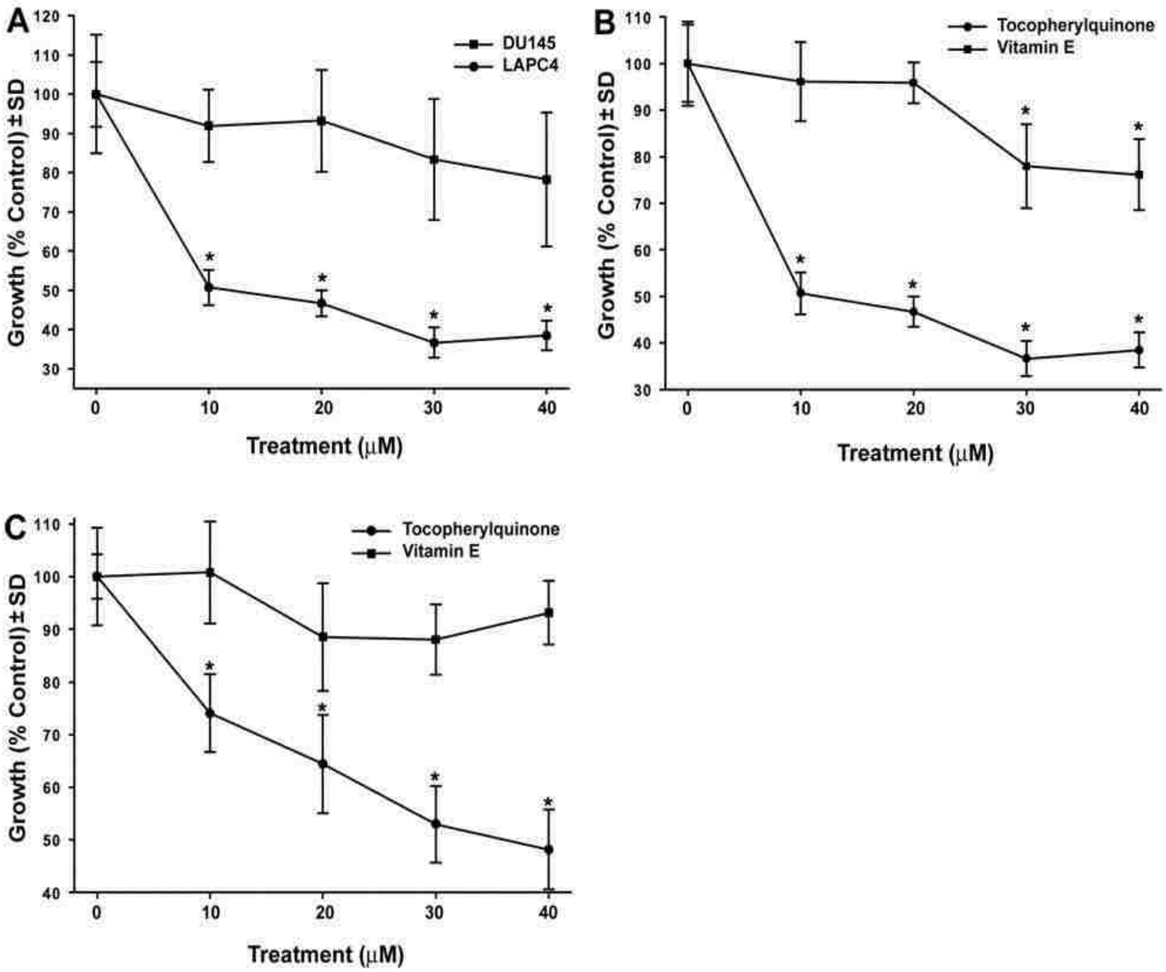


Fig. 3: TQ inhibits prostate cancer cell proliferation in androgen-sensitive prostate cancer cell lines. (A) Comparison of growth changes induced by TQ treatment in androgen-sensitive LAPC4 cells and androgen-independent DU145 prostate cancer cells treated with 10 to 40 μM TQ. (B) The growth of LAPC4 cells treated with either TQ or VE for 4 d, which was significantly decreased after treatment with 10 to 40 μM TQ and ≥ 30 μM VE (*P<0.05). (C) Determination of LNCaP cell growth after treatment with either TQ or VE for 4 d. Cell growth was significantly decreased after treatment with 10 to 40 μM TQ (*P<0.05). In contrast, cell growth in LNCaP cells was not altered by 10 to 40 μM VE treatment.

Down-regulation of AR protein levels in androgen-responsive prostate cancer cells by TQ

To determine the effects of TQ on AR protein in androgen-responsive LNCaP and LAPC4 cells, immunoblots for AR protein were performed. For each immunoblot, AR protein levels were normalized to levels of β–actin protein, which was not affected by TQ. The levels of AR protein were measured in LNCaP cells treated with 4, 12.5, or 25 μM TQ for 4 d in LNCaP cells. Cells treated with TQ showed significantly reduced AR protein levels (Fig 4 A-B). Similar to LNCaP cells, TQ significantly inhibited AR protein levels in LAPC4 cells (Fig. 4 C-D). LAPC4 cells were treated with TQ for 24, 48, 72, and 96 h (Fig. 4D). Twenty-four h treatment with 25 μM TQ significantly inhibited AR protein expression with a time-dependent decrease in AR protein levels up to 96 h (Fig. 4D). Therefore, TQ produced a dose- and time-dependent down-regulation of the AR protein in androgen-sensitive prostate cancer cell lines.

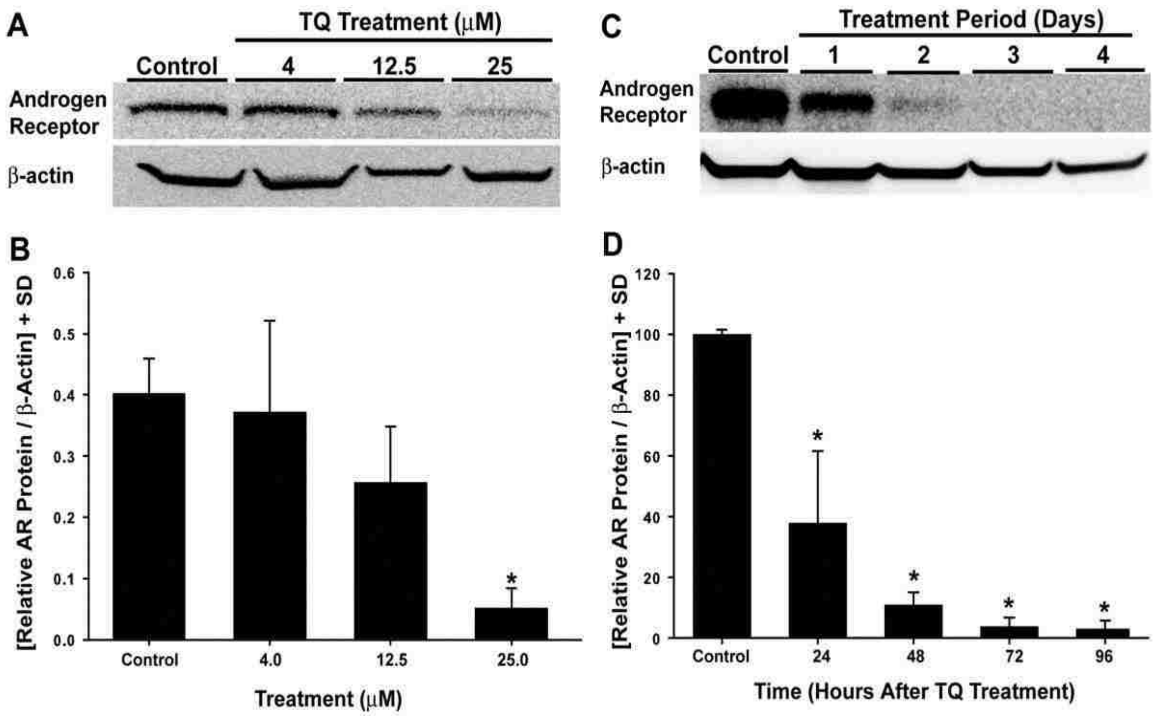


Fig. 4: AR protein levels determined by immunoblot in androgen-sensitive prostate cancer cells treated with TQ and VE. Quantified AR protein levels are present below each blot. (A) Immunoblot analysis of AR protein expression in LNCaP cells treated for 4 d with 25 µM TQ or 25 µM VE compared to vehicle control treated cells. Treatment with 25 µM TQ significantly reduced AR protein expression in comparison to control cells (**P*<0.05). (B) AR protein expression in LAPC4 cells treated with 25 µM TQ or 25 µM VE for 4 d. TQ significantly reduced AR protein expression (**P*<0.05). (C**)** TQ dose-dependent reduction in AR levels in LNCaP cells treated with 4, 12.5, or 25 µM TQ for 4 d. (D) Representative immunoblot of time-dependent changes in AR protein levels from LAPC4 cells treated with 25 µM TQ. AR protein levels were significantly reduced after 24 h in LAPC4 cells, which remained decreased for up to 96 h (**P*<0.05). For all immunoblots, quantification of AR protein levels was normalized to β-actin.

The androgenic response of LNCaP cells is decreased by TQ treatment

Studies to determine if TQ or VE modulated AR activity were initiated using an androgen-sensitive luciferase reporter system. For this study, androgen-sensitive reporter activity was stimulated using the synthetic androgen R1881 and was assessed after treatment with either 30 μM TQ or VE (Fig. 5A). TQ treatment alone did not modulate reporter activity. In contrast, TQ was found to significantly inhibit R1881-induced reporter activation after 2 d in comparison to R1881-stimulated control cells. Surprisingly, 30 μM VE treatment increased androgen-sensitive reporter activity (Fig. 5A). This data supports an inhibitory role for TQ on AR activity in contrast to VE, which did not exhibit antiandrogenic activity.

The release of prostate specific antigen (PSA) from LNCaP cells is recognized as a sensitive indicator of androgenic response in LNCaP cells (32). To further examine TQ's effects on androgenic pathways, the androgen-stimulated release of PSA from LNCaP cells was determined. LNCaP cells treated with TQ showed a dose-dependent reduction in R1881-induced PSA release compared to untreated control cells (Fig. 5B). In contrast, treatment with 10 to 40 μM VE did not affect androgen-induced PSA release from LNCaP cells (Fig. 5B).

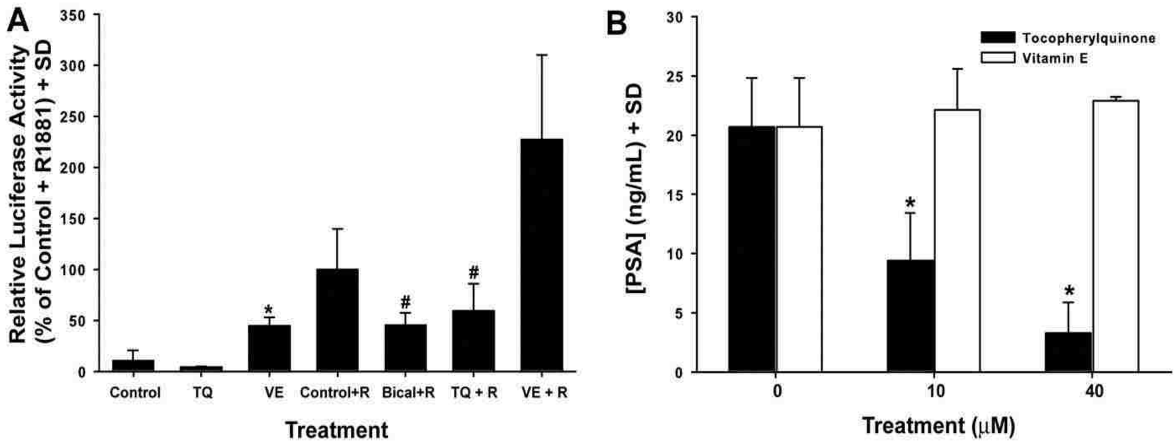


Fig. 5: Inhibition of androgenic responses in LNCaP cells by TQ treatment. (A) Androgen-induced (i.e., R1881 (R)) luciferase expression from an androgen-sensitive promoter measured after TQ or VE treatment for 48 h. VE treatment, but not TQ, increased promoter activity compared to control, untreated LNCaP cells (*P<0.05). In LNCaP cells stimulated with 50 pM R1881 and the established antiandrogen bicalutamide (Bical) or TQ showed decreased promoter activity compared to cells stimulated by exposure to 50 pM R1881 alone (# P<0.05). (B) PSA release was stimulated by 50 pM R1881 exposure in LNCaP cells and measured 4 d after TQ or VE treatment. PSA levels were significantly lower from cells treated with 10 or 40 μM TQ (* P<0.05), but remained unchanged by VE treatment.

TQ, not VE, treatment decreases AR and AR responsive gene mRNA levels

The decrease in PSA release may be due in part to down-regulation of *PSA* gene expression by TQ (Table 2). In addition to *PSA* mRNA levels, other androgen-responsive genes were measured after TQ treatment. As shown in Table 2, the mRNA levels for the AR responsive genes *kallikrein 2*, *prostein*, *prostatic acid phosphatase*, *NKX3.1* and *prostate specific membrane antigen* were reduced in LNCaP cells 4 d after treatment with TQ. In contrast to TQ, VE treatment did not decrease expression of the androgen-sensitive mRNAs (Table 2).

To determine the effects of TQ and VE on AR protein in androgen-responsive LNCaP and LAPC4 cells, immunoblots for AR protein were performed. For each immunoblot, AR protein levels were normalized to levels of β–actin protein, which was not affected by TQ or VE. LNCaP cells treated with TQ showed significantly reduced AR protein levels; whereas VE did not change AR protein levels (Fig. 6A). Similar to LNCaP cells, TQ significantly inhibited AR protein levels in LAPC4 cells and VE did not affect the levels of AR protein (Fig. 6B) after 96 h. We further demonstrate TQ down-regulates *AR* mRNA and this action is distinct from VE, the levels of *AR* mRNA were measured using qPCR after treatment with 25 µM TQ or VE for 96 h. *AR* mRNA levels were decreased 1.4- and 1.7-fold after treatment with 25 µM TQ in LNCaP and LAPC4 cells, respectively (Fig. 6C-D). However, mRNA down-regulation was not an overt action of TQ in prostate cancer cells as neither *retinoid X receptor, alpha* mRNA nor *vitamin D receptor* mRNA levels were decreased (Fig. 6E-F). It is interesting to note that whereas VE treatment did not affect the mRNA levels of androgen-responsive genes, the *AR,* or the *vitamin D receptor*, VE produced a 20% reduction in *retinoid X receptor, alpha*α mRNA levels.

Table 2: Down-regulation of androgen-responsive gene expression in LNCaP cells by TQ.

Gene	Gene Symbol	Fold decrease in (mRNA)[1, 2]	
		TQ	**VE**
***Prostate Specific Antigen* (PSA)**	KLK3	9.1	1.1
Kallikrein 2	KLK2	7.1	1.0
Prostein	SLC45A3	2.6	1.2
***Prostatic Acid Phosphatase* (PAP)**	ACPP	2.4	1.2
NKX3.1	NKX3-1	1.9	1.1
***Prostate Specific Membrane Antigen* (PSMA)**	FOLH1	1.5	1.1

[1] Determined using quantitative PCR (see *Materials and Methods*).

[2] Compared to control, vehicle-treated LNCaP cells.

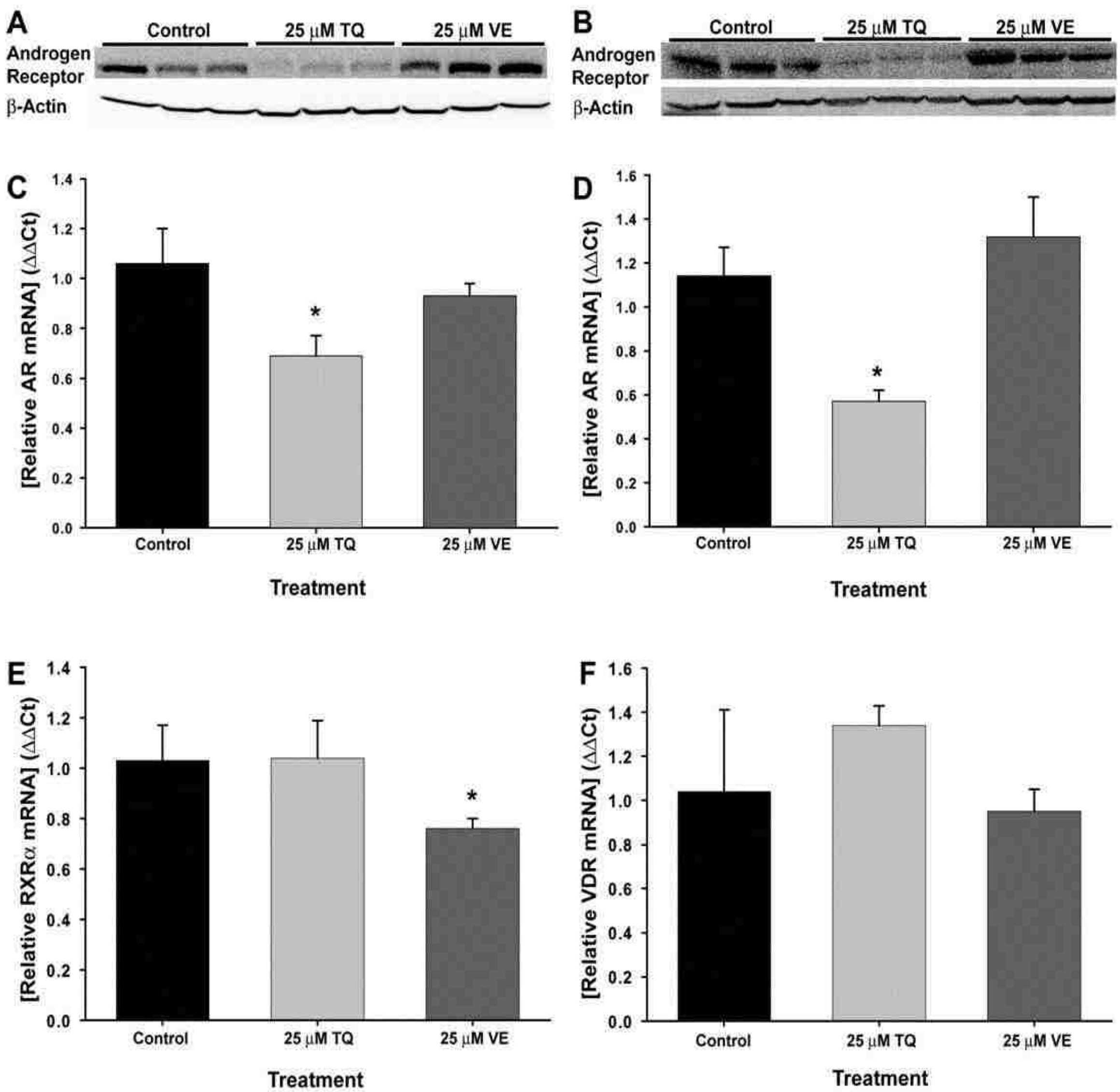


Fig. 6: VE does not alter AR protein or mRNA levels in AR-expressing prostate cancer cells. (A) Immunoblot analysis of AR protein expression in LNCaP cells treated for 4 days with 25 µM TQ or 25 µM VE compared to vehicle control treated cells. (B) AR protein expression in LAPC4 cells treated with 25 µM TQ for 4 days. (C) Quantitative PCR analysis of *AR* mRNA levels in LNCaP cells treated for 4 days with 25 µM TQ or 25 µM VE compared to vehicle control treated cells. (D) *AR* mRNA levels in LAPC4 cells treated for 4 days with 25 µM TQ or 25 µM VE. TQ does not inhibit *RXR*α or *VDR*

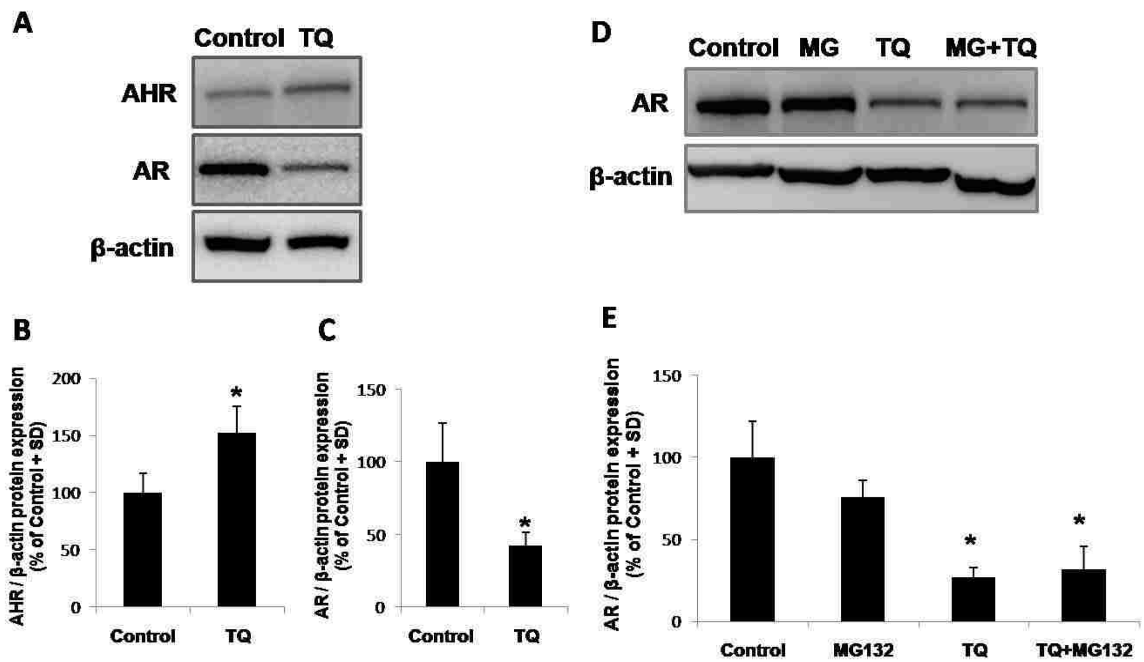
mRNA expression levels. Levels of *RXR*α mRNA (E) and *VDR* mRNA (F) in LNCaP cells treated for 4 days with 25 μM TQ or 25 μM VE. *RXR*α or *VDR* mRNA expression levels were not changed in LNCaP cells treated with 25 μM TQ (* $P<0.05$).

AR protein down-regulation by TQ is selective, independent from proteasomal degradation and independent of mRNA expression

To determine the relative selectivity of TQ's actions on the AR we evaluated the expression of the ligand activated basic helix-loop-helix transcription factor the aryl hydrocarbon receptor (AHR). TQ significantly inhibits AR protein expression, but not AHR protein expression in LNCaP cells after 48h of 25μM treatment (Fig. 7A-C). As shown in Fig. 7A-C TQ significantly inhibited AR protein expression and in contrast significantly increased AHR protein expression.

Degradation of the AR is primarily mediated through the activity of the ubiquitin-proteasome pathway. To determine if TQ increased AR proteasomal degradation LNCaP cells were treated with 10μM of the proteasomal inhibitor MG132 in the presence or absence of TQ. TQ's down-regulation of the AR was not attenuated by the presence of MG132 (Fig. 7D-E). Investigators used multiple concentrations of MG132 and various treatment strategies but the results were consistent in that inhibition of proteasomal degradation did not prevent AR down-regulation by TQ.

The down-regulation of AR protein by TQ may be mediated through the inhibition of AR mRNA. To address this potential mechanism a time course experiment was conducted in which protein and mRNA extracts were collected from the same treatment sample. Although there was a significant inhibition of AR mRNA at 96 h upon TQ treatment, as shown previously in Fig. 6C, this inhibition was not correlated with the down-regulation of AR protein expression (Fig. 7 F-H). In contrast to the significant down-regulation of AR mRNA at 96h by TQ, AR protein was significantly inhibited by 48h in these matched samples.



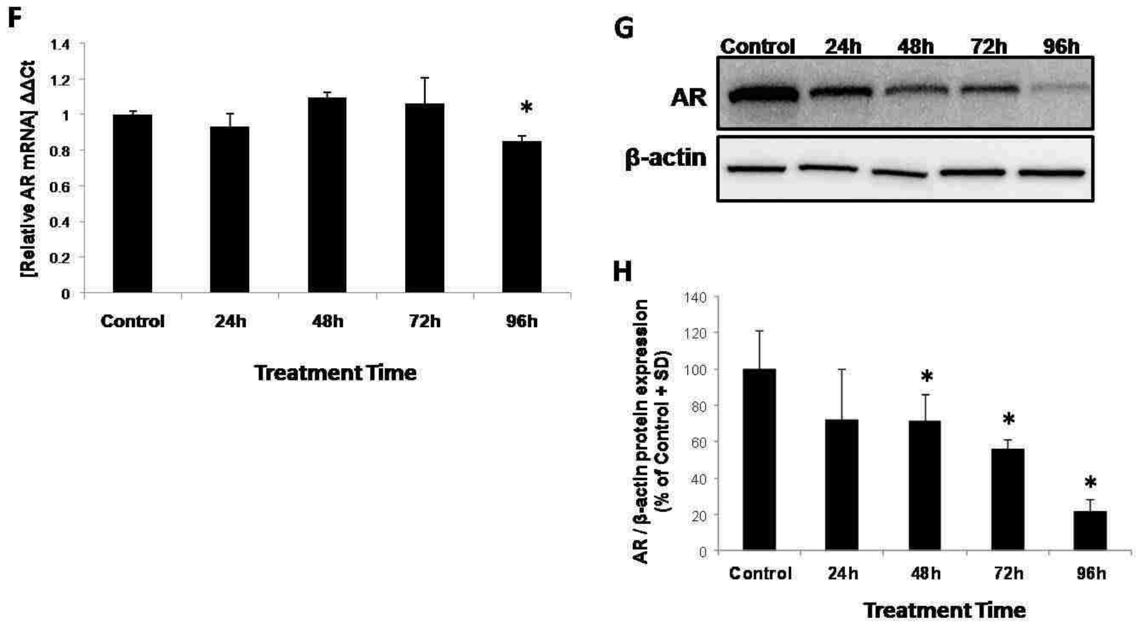
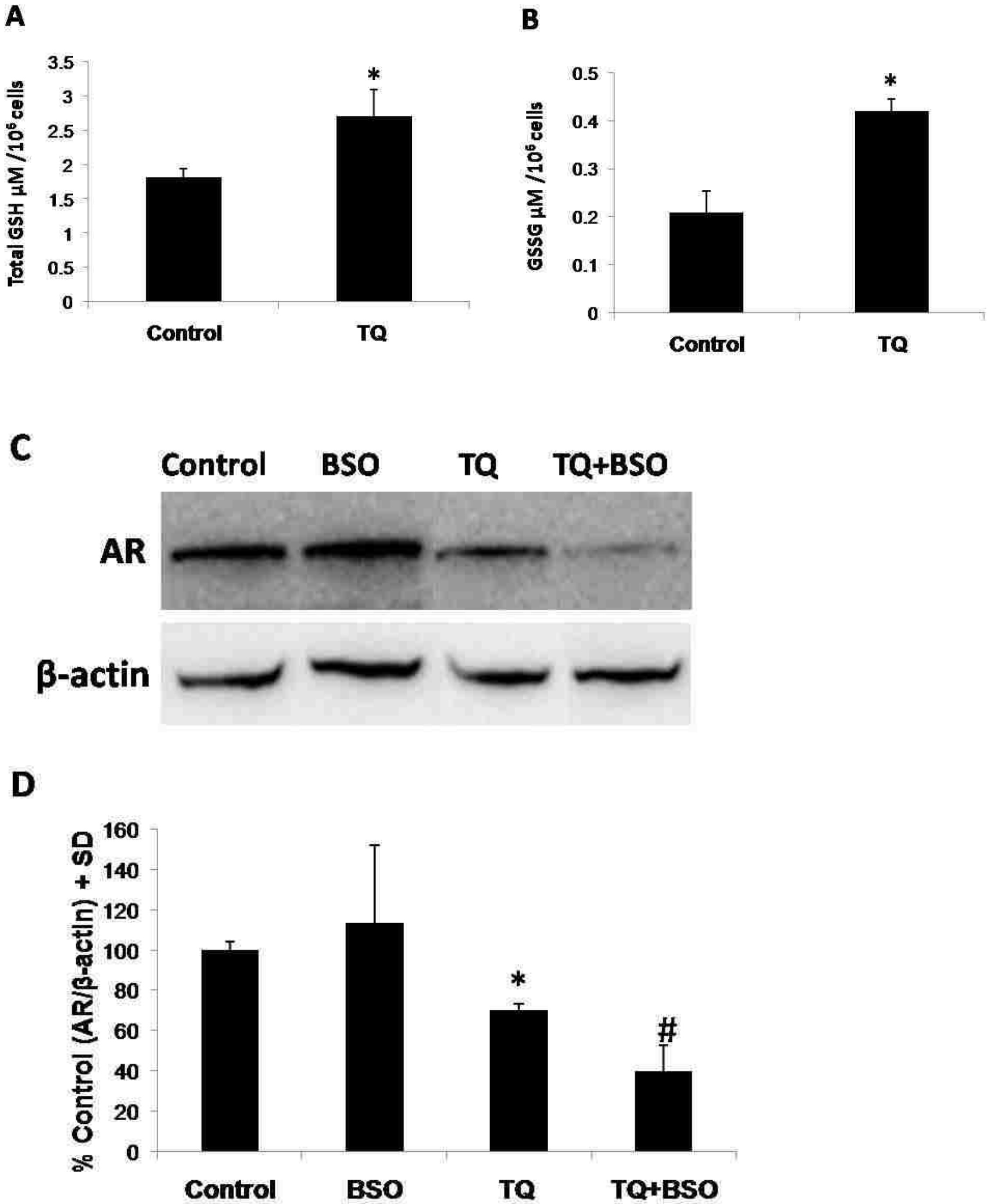


Fig. 7: Selective down-regulation of AR protein expression by TQ in LNCaP cells. (A) Immunoblot analysis of AHR and AR protein expression in LNCaP cells treated for 48 h with 25 µM TQ compared to vehicle control treated cells. (B) Immunoblot analysis of AHR protein expression or (C) AR protein expression after TQ treatment. (D) LNCaP cells were treated with 25 µM TQ for 16 h and then treated with 10µM MG132 for an additional 24 h (D+E) (**P*<0.05 compared to control). (F) Quantitative PCR analysis of AR mRNA levels and (G+H) immunoblot analysis of AR protein expression in LNCaP cells treated for 1-4 d with 25 µM TQ (**P*<0.05 compared to control).

TQ induces cellular oxidative stress

To determine TQ's cellular mechanism of action we measured total glutathione (GSH_t) and oxidized glutathione (GSSG) levels after 96h of treatment (Fig. 8A-B).

To determine if TQ's down-regulation of the AR was potentiated by the depletion of GSH levels, LNCaP cells were treated with TQ and the glutathione ligase (gamma-glutamylcysteine synthetase) inhibitor buthionine sulfoximine (BSO). TQ significantly inhibited AR protein expression and this inhibition was significantly potentiated by the presence of BSO (Fig. 8C-D). In order to confirm if the observed increase in oxidized glutathione levels was due to increased oxidative stress, we evaluated genes regulated by the antioxidant response element (ARE). The ARE is activated upon binding of the cellular redox sensor nuclear factor E2-related protein 2 (Nrf2) and regulated the expression of genes such as, Nadph quinone oxidoreductase 1 (*NQO1*), aldoketoreductase 1C1 (*AKR1C*) and *MafG*. *NQO1*, *AKR1C*1 and *MafG* mRNA expression were significantly increased upon 25µM TQ treatment after 96h (Fig. 8E).


A
Total GSH µM /10⁶ cells
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
TQ
B
GSSG µM /10⁶ cells
0.5
0.4
0.3
0.2
0.1
0
*
Control
TQ
C
Control
BSO
TQ
TQ+BSO
AR
β-actin
D
% Control (AR/β-actin) + SD
160
140
120
100
80
60
40
20
0
*
#
Control
BSO
TQ
TQ+BSO

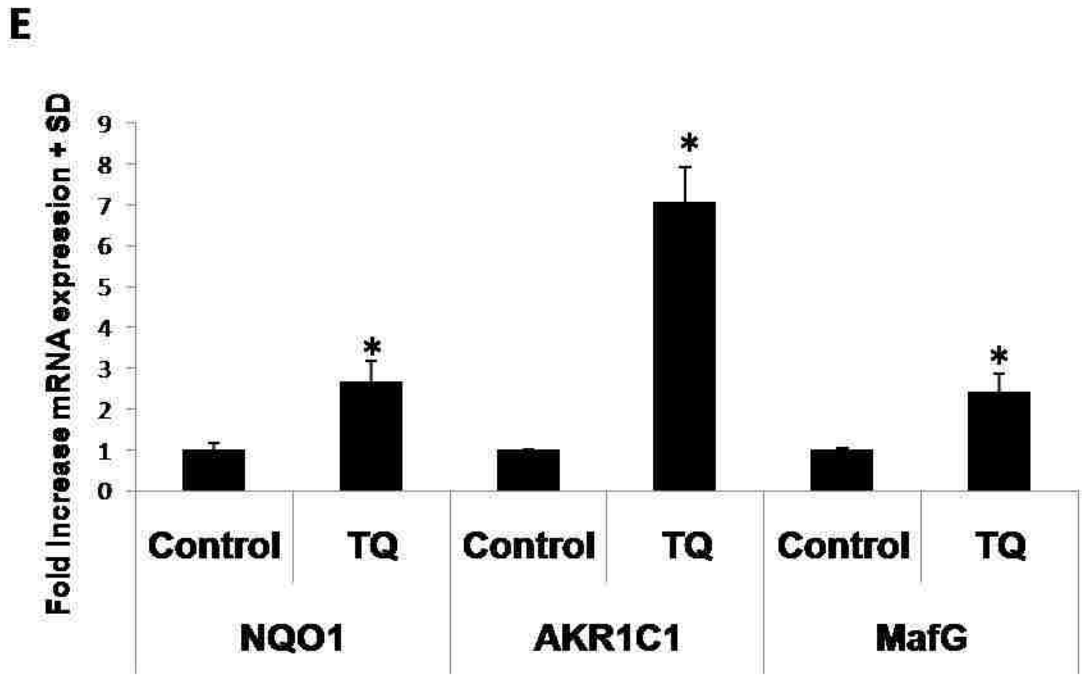


Fig. 8: TQ modifies glutathione expression and inhibition of glutathione production potentiates TQ's down-regulation of AR protein expression. (A) Expression of total glutathione and (B) oxidized glutathione were measured after treatment with 25 µM TQ or vehicle control for 96 h in LNCaP cells. (C+D) Immunoblot analysis of AR protein expression in LNCaP cells. Cells were pretreated with 5mM BSO for 24 h and then treated with 25 µM TQ or vehicle control in the presence or absence of BSO for an additional 48 h. (E) Quantitative PCR analysis of *NQO1*, *AKR1C1* and *Maf G* mRNA levels in LNCaP cells treated for 96 h with 25 µM TQ (**P*<0.05 compared to control).

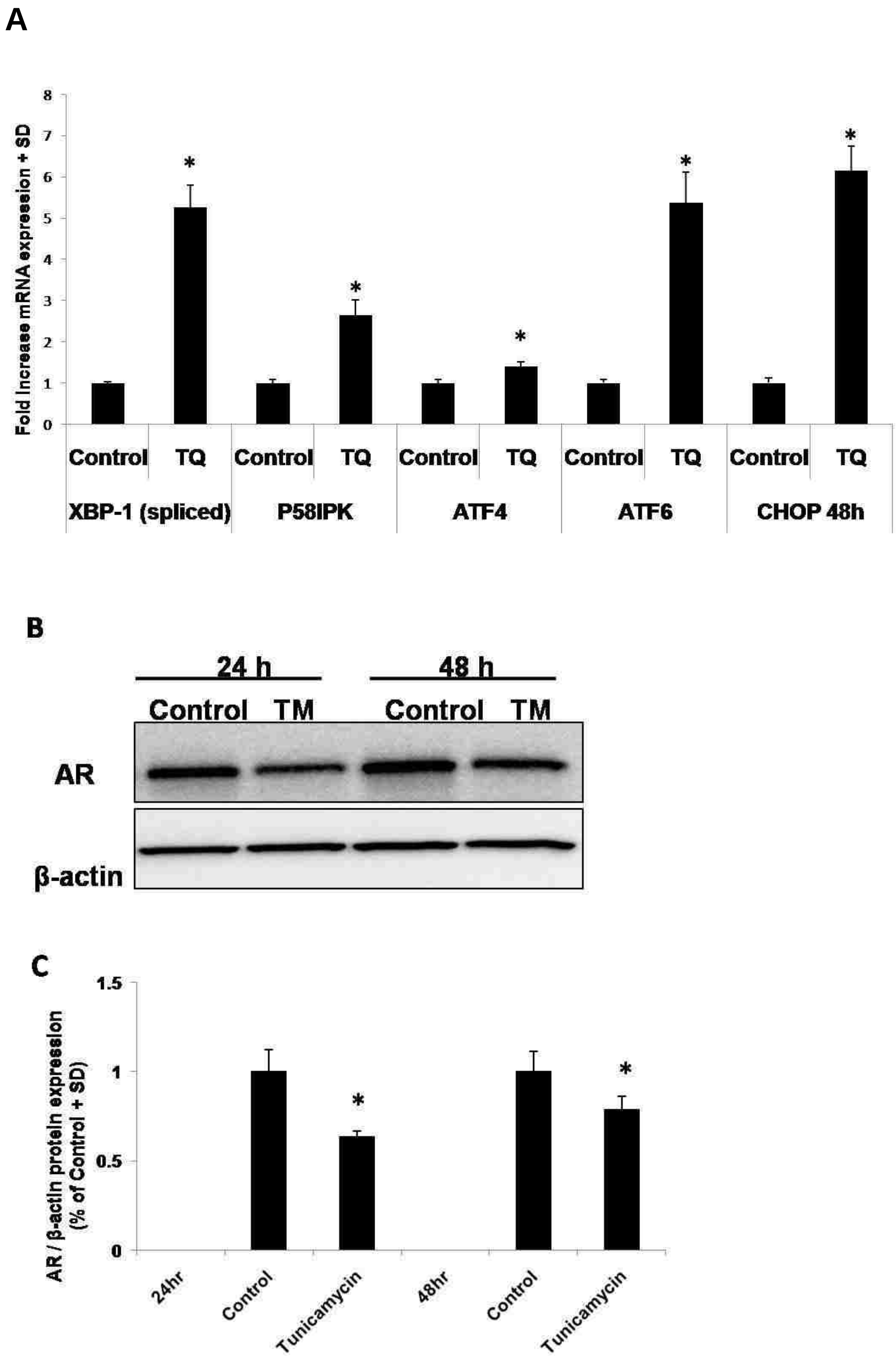

A
Fold Increase mRNA expression + SD
0
1
2
3
4
5
6
7
8
*
Control
TQ
XBP-1 (spliced)
P58IPK
ATF4
ATF6
CHOP 48h
B
24 h
48 h
Control
TM
AR
β-actin
C
AR / β-actin protein expression (% of Control + SD)
0
0.5
1
1.5
24hr
Control
Tunicamycin
48hr

Fig. 9: TQ increases expression of UPR regulated transcripts and activation of UPR by tunicamycin leads to AR down-regulation. (A) Quantitative PCR analysis of *XBP-1* (spliced), *P58*^{IPK}, *ATF4* and *ATF6* mRNA levels in LNCaP cells treated for 96 h with 25 µM TQ. *CHOP* mRNA levels were measured after 25 µM TQ treatment for 48 h. (B+C) Immunoblot analysis of AR protein expression in LNCaP cells. Cells were treated with 2ug/ml tunicamycin (TM) for 24 h or 48 h (**P*<0.05 compared to control).

TQ activates the Unfolded Protein Response and activation of UPR leads to AR down-regulation

With the induction of oxidative stress by TQ treatment and the selective inhibition of AR protein expression investigators addressed if this agent led to activation of the Unfolded Protein Response (UPR). There are three key signaling pathways that are activated in the UPR, PERK, IRE1 and ATF6. Down-stream genes that are increased upon activation of these pathways such as, *ATF4*, *XBP-1* spliced, *ATF6* and *CHOP* were significantly increased upon TQ treatment. LNCaP cells were treated with 25µM TQ for 96h, the transcripts *XBP-1* spliced and *ATF6* were increased 5-fold. There was a small but significant increase in *ATF4* mRNA expression 1.7-fold and a 6-fold increase in *CHOP* in as early as 48 h (Fig. 9A). To further determine if activation of the UPR by the inducer tunicamycin led to the down-regulation of AR protein expression LNCaP cells were treated with 2 µg/ml for 24 and 48 h. There was a significant inhibition of AR protein expression upon tunicamycin treatment at both 24 and 48h (Fig 9B-C).

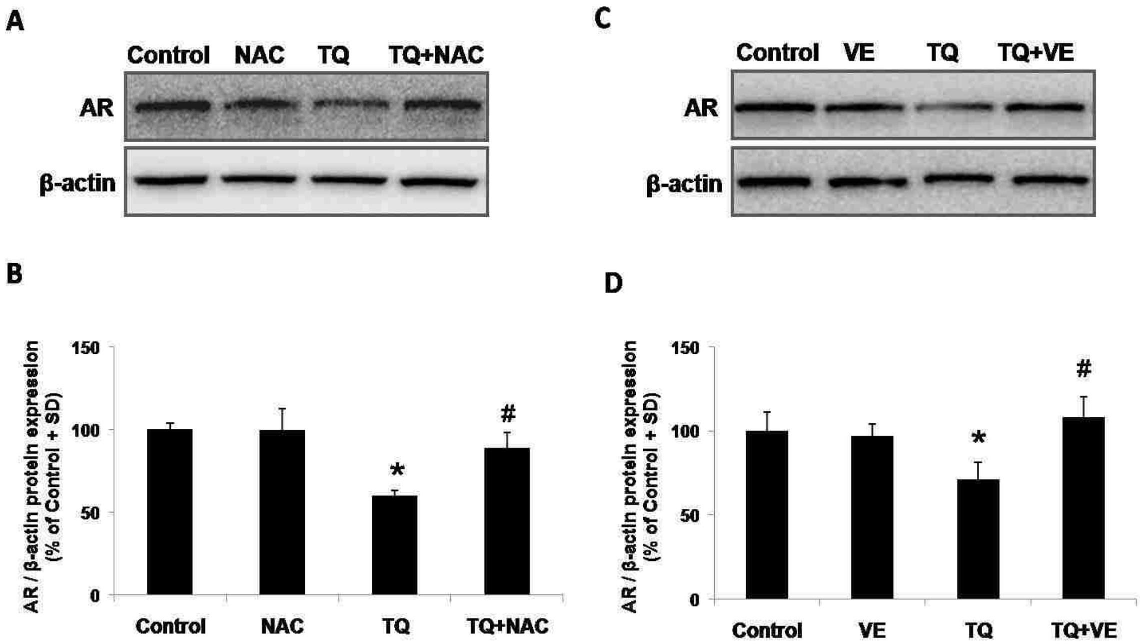


Fig. 10: Inhibition of AR protein expression by TQ is attenuated by antioxidants NAC and VE. (A) Immunoblot analysis of AR protein expression in LNCaP cells pretreated for 24 h with 5 mM NAC and then treated with 25 µM TQ in the presence or absence of 5 mM NAC for 48 h. (B) Immunoblot analysis of AR protein expression in LNCaP cells pretreated for 24 h with 25 µM VE and then treated with 25 µM TQ in the presence or absence of 25 µM VE for an additional 48 h (*P<0.05 compared to control).

TQ's down-regulation of AR expression is attenuated by the presence antioxidants NAC and VE

The antioxidants N-acetylcysteine (NAC) and Vitamin E (VE) were used to determine if a potential mechanism of TQ's down-regulation of AR protein expression is through the increase in oxidative stress (Fig 10 A-D). LNCaP cells were pre-treated with 5mM NAC or 25µM VE for 24h and then treated with 25µM TQ with or without NAC

(Fig 10 A-B) and VE (Fig. 10 C-D) for 48h. TQ's down-regulation of AR protein expression was significantly attenuated by the presence of either antioxidant.

Discussion

Biological actions for TQ, the oxidation product of VE, are largely undefined. Here, we begin to identify TQ's anti-androgenic activity is through its actions as a potential pro-oxidant. VE did not significantly affect either the growth of prostate cancer cells or pathways known to be critical in prostate cancer progression compared to TQ. TQ significantly inhibited AR protein expression, activated antioxidant pathways and induced ER stress pathways. This study begins to identify a novel activity of TQ (α-TQ) as a potential arylating electrophile in human CaP cells. This potential activity is in contrast to previous studies reporting the weak electrophile activity of α-TQ in comparison to γ- or δ-TQ. In addition, we do not observe overt toxicity in the cell lines tested upon TQ treatment. However, in the studies evaluating the activity of TQ (α-TQ) versus γ- or δ-TQ were conducted within a relative short time period, we observe a time-dependent activity of TQ within our system (33). For example, TQ's down-regulation of AR protein expression in LNCaP cells requires 48 h for significant inhibition as does its pro-oxidant activity. We further demonstrate TQ's down-regulation of the AR is attenuated by the antioxidants VE and N-acetylcysteine (NAC). TQ's anti-androgenic actions in prostate cancer cells may be an explanation for the chemopreventive actions of VE in men who smoke (ATBC trial) (10) and the lack of prevention in men who are non-smokers (SELECT trial) (7).

In this study VE did not significantly affect either the growth of prostate cancer cells or pathways known to be critical in prostate cancer progression compared to TQ, which potently inhibited the growth of androgen-sensitive prostate cancer cells. The decrease in cell growth produced by TQ treatment may be AR-dependent as TQ treatment did not have a pronounced effect on the growth of the androgen-independent DU145 human prostate cancer cell line. Importantly, TQ, but not VE, was found to reduce both *AR* mRNA and AR protein levels in prostate cancer cells with a concomitant reduction in androgenic pathways. Several studies have shown that down-regulation of the AR results in decreased cell proliferation in androgen-sensitive prostate cancer cells. For example, decreased AR expression was achieved in LNCaP human prostate cancer cells using siRNA resulting in a decrease in LNCaP growth (34,35). Thus, the decrease in cell growth produced by TQ in androgen-sensitive prostate cancer cell lines may be due at least in part to the action of TQ to down-regulate AR expression.

The AR is a tissue-specific, ligand-activated transcription factor that is known to regulate the expression of genes such as *PSA*, *kallikrein 2*, *prostein*, *prostatic acid phosphatase*, *NKX3.1,* and *prostate specific membrane antigen* in prostate cells (36-40). Because the AR plays a key role in maintenance of the expression of these genes, the reduced expression of these genes would result from down-regulation of the AR. In fact, the expression of several of these genes was reduced after treatment of LNCaP cells with TQ. Additionally, expression from an androgen-sensitive reporter was inhibited by concurrent androgen and TQ treatment. In contrast, VE had minimal effects on the modulation of androgen-responsive genes or gene products. The reduced expression of

AR-responsive genes induced by TQ treatment strongly supports that the AR is a major target of TQ in prostate cancer cells.

The AR is recognized as a major contributor to all stages of prostate cancer from carcinogenesis to castration-resistant disease (22,27,41,42). To date, most interventions against prostate cancer reduce AR activation through inhibiting the production of androgenic ligands, such as testosterone or dihydrotestosterone. These strategies do not affect the AR itself. To modulate AR activity, it is necessary to identify interventions that target down-regulation of AR expression in prostate cells. Here, we show that down-regulation of AR protein and mRNA can be achieved using TQ, the natural oxidation product of VE, with a pronounced impact on androgenic activity in prostate cancer cells. It is noteworthy that VE as α-tocopherol did not inhibit either AR expression or activity in prostate cancer cells. This is important as this is the form of VE that is expected to be physiologically active in contrast to ester conjugated forms, such as vitamin E succinate, that are converted α-tocopherol by esterases in the body. Although VE did not exhibit anti-androgenic properties within our system VE analogs have been reported to affect AR protein expression in prostate cancer cells. For example, Zhang et al. (31) reported that the VE analog, VE succinate, reduces AR activity in androgen-sensitive human prostate cancer cells. Similar to TQ, VE succinate treatment was found to decrease both *AR* mRNA and protein levels in LNCaP cells (31). Importantly, Zhang et al. (31) found that at least part of VE succinate's action is due to a decrease in AR translation. We have previously reported on the anti-androgenic activity of another VE analog, 2,2,5,7,8-Penatmethyl-6-chromonol (PMCol) (19). This antioxidant moiety of VE, PMCol, consists of the chromonal ring structure of VE but lacks the phytyl chain. Thompson et al. (19)

demonstrated PMCol inhibited androgen sensitive prostate cancer cells proliferation, acts as a competitive inhibitor of AR ligand binding and inhibits AR activation. However, PMCol did not inhibit AR expression within in these cells. Identifying the mechanism of TQ's anti-androgenic activity and selective inhibition of AR protein expression may provide insight into novel AR regulatory mechanisms.

Because TQ had pronounced inhibitory effect on markers of AR activity, the AR in androgen-sensitive prostate cancer cell lines was examined. Both AR protein and *AR* mRNA were found to be reduced by TQ treatment. However there was significant reduction of AR protein expression that preceded the inhibition of *AR* mRNA expression. Demonstrating TQ's actions on AR down-regulation may not be entirely due to the inhibition of *AR* mRNA expression. To determine the relative selectivity of TQ's actions on AR protein expression we evaluated the expression of the aryl hydrocarbon receptor. TQ significantly induced AHR protein expression within 48 h. Further, we demonstrate the increase of several different transcripts such as *VDR*, *RXRα*, *NQO1*, *AKR1C1* and *CHOP* in comparison to the significant inhibition of *AR* mRNA expression. We also demonstrate that TQ's down-regulation of AR protein expression in not mediated through proteasomal degradation. To determine the mechanism(s) of action involved in TQ-mediated down-regulation of AR expression in CaP cells we examined TQ's potential actions as a pro-oxidant. TQ was found to increase the levels of total glutathione and oxidized glutathione (GSSG). Glutathione is a major antioxidant redox recycling thiol which plays a major role in cellular defense against oxidative insult (43). GSH and GSSG balance has been reported to be critical regulator in maintaining the proper folding and function of various proteins. Perturbations of the GSH/GSSG ratio within the lumen of the ER can interfere with the activity of protein disulfide isomerases (PDI) which can directly lead to protein misfolding (44). Accumulation of these misfolded proteins within the lumen of the ER leads to ER stress and the activation of the unfolded protein response (UPR). The UPR is mediated through the activation of three ER stress pathways pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (45). We demonstrate the up-regulation of known target genes of the PERK, IRE1 and ATF6 pathways. *ATF4*, *p58*^{IPK}, *XBP*-1 spliced, *ATF6* and *CHOP* mRNA expression are significantly increased upon treatment with TQ.

CHOP is a known death mediator whose expression is increased by all three UPR signaling cascades and although TQ is not overtly toxic by 96 h, longer time points have not been evaluated (46). Further, we demonstrate treatment of LNCaP cells with a known inducer of ER stress tunicamycin, significantly inhibits AR protein expression by 24 h. Tunicamycin is an inhibitor of N-glycosylation which leads to the accumulation of misfolded proteins within the lumen of the ER inducing ER stress and activation of the UPR (47). The UPR activation observed by TQ treatment occurs at later time points than that observed for AR protein down-regulation thus it may not explain the early activity of TQ but provides insight into the mechanism of TQ's actions.

Reports on the biological effects of TQ are limited. This may be due in part to TQ being regarded simply as the product of VE oxidation with limited inherent biological activity. However, TQ is chemically distinct from VE and, therefore, may have unique biological actions compared to VE. The distinct biological actions of TQ and VE are strongly supported by the results on selective AR down-regulation by TQ observed in the current study. A physiological action associated with TQ is anticoagulant activity (48). This is not surprising in that the quinone and phytyl chain structure of TQ is reminiscent of vitamin K, a critical vitamin involved in blood clotting. In general, chemicals possessing quinone structures are found to be toxic. This is largely due to the presence of electrophilic carbon centers present in the quinone structure that may be acted upon by nucleophiles present in cellular constituents. In the current study, TQ was not found to be highly cytotoxic. Interestingly, all electrophilic sites in TQ are blocked by methyl substitutions and thus TQ would be expected to be less reactive than chemicals with unblocked quinone structures. Additionally, TQ has been found to be a potent substrate for the biotransformation enzyme NAD(P)H quinone oxidoreductase 1 (NQO1) (49). The reduction of TQ to the hydroquinone by NQO1 was found to be so efficient it was suggested that TQ may be one of the primary substrates for NQO1's biological activity (49). Results from the current study and others strongly support that TQ has potent biological actions that are distinct from VE.

The actions of VE as a measure for alleviating prostate cancer are controversial. Intriguingly, some epidemiological studies support a role for the prostate cancer preventive actions of supplemental VE when taken by men who smoke, an activity that produces a chronic physiologic oxidative stress. For example, the Finnish α-Tocopherol, β-Carotene Cancer Prevention Study examined men that were heavy smokers (8). In this study, a 32% reduction in prostate cancer incidence and 41% reduction in mortality was observed among smokers taking supplemental VE compared to control groups (8). In the Harvard Health Professionals study, no effect of supplemental VE alone was found on prostate cancer incidence; however it was reported that, "among current smokers and recent quitters, those who consumed at least 100 IU of supplemental VE per day had a relative risk of 0.44 for metastatic or fatal prostate cancer" (9). Two additional studies have found no effect of supplemental VE when taken alone, but did report a reduction in the development of prostate cancers among smokers taking VE supplements (10,11). In contrast to these reports, a recent study has found that VE itself may have activity against the development of advanced prostate cancer (50). This finding conflicts with the results from the Selenium and Vitamin E Cancer Prevention Trial (i.e., SELECT), which failed to find prostate cancer preventive actions of supplemental VE (6,7). Thus, most studies to date suggest that VE itself may not be an effective intervention against prostate cancer. In agreement with these findings, the results from the current study did not find significant effects on prostate cancer cells by VE. However, we have found that TQ, the major oxidation product of VE, is highly effective at reducing both growth and androgenic activity in prostate cancer cell lines. It is intriguing to consider that TQ may be the active derivative of VE involved in prostate cancer prevention among heavy smokers taking supplemental VE, which in possessing a physiologic oxidative stress effectively transforms VE to TQ. The results from the current study strongly support further investigations to determine the efficacy of TQ as a modality for prostate cancer prevention.

In conclusion, we have begun to identify TQ's mechanism of action as a potential pro-oxidant which induces oxidative stress, activation of the UPR and down-regulates AR protein expression in human prostate cancer cells. TQ's down-regulation of AR protein expression was attenuated by the presence of the antioxidants NAC and VE. This study provides insight into how the actions of TQ may be an explanation for the discrepancies found in various chemopreventive trials using VE. Further investigation into TQ's actions can provide insight into novel mechanisms of AR down-regulation as a potential prostate cancer chemopreventive strategy.

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References

1. Quinn M, Babb P. Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part II: individual countries. BJU international 2002;90(2):174-184.

2. Nelen V. Epidemiology of prostate cancer. Recent results in cancer research Fortschritte der Krebsforschung 2007;175:1-8.

3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians 2011;61(2):69-90.

4. Thompson IM, Tangen CM, Goodman PJ, Lucia MS, Klein EA. Chemoprevention of prostate cancer. The Journal of urology 2009;182(2):499-507; discussion 508.

5. Ledesma MC, Jung-Hynes B, Schmit TL, Kumar R, Mukhtar H, Ahmad N. Selenium and vitamin E for prostate cancer: post-SELECT (Selenium and Vitamin E Cancer Prevention Trial) status. Molecular medicine (Cambridge, Mass 2011;17(1-2):134-143.

6. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, Hartline JA, Parsons JK, Bearden JD, 3rd, Crawford ED, Goodman GE, Claudio J, Winquist E, Cook ED, Karp DD, Walther P, Lieber MM, Kristal AR, Darke AK, Arnold KB, Ganz PA, Santella RM, Albanes D, Taylor PR, Probstfield JL, Jagpal TJ, Crowley JJ, Meyskens FL, Jr., Baker LH, Coltman CA, Jr. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Jama 2009;301(1):39-51.

7. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM, Karp DD, Lieber MM, Walther PJ, Klotz L, Parsons JK, Chin JL, Darke AK, Lippman SM, Goodman GE, Meyskens FL, Jr., Baker LH. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Jama 2011;306(14):1549-1556.

8. Heinonen OP, Albanes D, Virtamo J, Taylor PR, Huttunen JK, Hartman AM, Haapakoski J, Malila N, Rautalahti M, Ripatti S, Maenpaa H, Teerenhovi L, Koss L, Virolainen M, Edwards BK. Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. Journal of the National Cancer Institute 1998;90(6):440-446.

9. Chan JM, Stampfer MJ, Ma J, Rimm EB, Willett WC, Giovannucci EL. Supplemental vitamin E intake and prostate cancer risk in a large cohort of men in the United States. Cancer Epidemiol Biomarkers Prev 1999;8(10):893-899.

10. Kirsh VA, Hayes RB, Mayne ST, Chatterjee N, Subar AF, Dixon LB, Albanes D, Andriole GL, Urban DA, Peters U. Supplemental and dietary vitamin E, beta-carotene, and vitamin C intakes and prostate cancer risk. Journal of the National Cancer Institute 2006;98(4):245-254.

11. Rodriguez C, Jacobs EJ, Mondul AM, Calle EE, McCullough ML, Thun MJ. Vitamin E supplements and risk of prostate cancer in U.S. men. Cancer Epidemiol Biomarkers Prev 2004;13(3):378-382.

12. Cheng TY, Barnett MJ, Kristal AR, Ambrosone CB, King IB, Thornquist MD, Goodman GE, Neuhouser ML. Genetic variation in myeloperoxidase modifies the

association of serum alpha-tocopherol with aggressive prostate cancer among current smokers. The Journal of nutrition 2011;141(9):1731-1737.

13. Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13(10):1145-1155.

14. Constantinou C, Papas A, Constantinou AI. Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs. International journal of cancer 2008;123(4):739-752.

15. Borel P, Moussa M, Reboul E, Lyan B, Defoort C, Vincent-Baudry S, Maillot M, Gastaldi M, Darmon M, Portugal H, Planells R, Lairon D. Human plasma levels of vitamin E and carotenoids are associated with genetic polymorphisms in genes involved in lipid metabolism. The Journal of nutrition 2007;137(12):2653-2659.

16. Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annual review of nutrition 1990;10:357-382.

17. Tangney CC, Shekelle RB, Raynor W, Gale M, Betz EP. Intra- and interindividual variation in measurements of beta-carotene, retinol, and tocopherols in diet and plasma. The American journal of clinical nutrition 1987;45(4):764-769.

18. Ni J, Chen M, Zhang Y, Li R, Huang J, Yeh S. Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. Biochemical and biophysical research communications 2003;300(2):357-363.

19. Thompson TA, Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. Molecular cancer therapeutics 2003;2(8):797-803.

20. Dehm SM, Tindall DJ. Androgen receptor structural and functional elements: role and regulation in prostate cancer. Molecular endocrinology (Baltimore, Md 2007;21(12):2855-2863.

21. Scher HI, Buchanan G, Gerald W, Butler LM, Tilley WD. Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. Endocrine-related cancer 2004;11(3):459-476.

22. Wilding G. Endocrine control of prostate cancer. Cancer surveys 1995;23:43-62.

23. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, Lieber MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman CA, Jr. The influence of finasteride on the development of prostate cancer. The New England journal of medicine 2003;349(3):215-224.

24. Vis AN, Schroder FH. Key targets of hormonal treatment of prostate cancer. Part 2: the androgen receptor and 5alpha-reductase. BJU international 2009;104(9):1191-1197.

25. Prins GS. Molecular biology of the androgen receptor. Mayo Clinic proceedings 2000;75 Suppl:S32-35.

26. Zhu ML, Kyprianou N. Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocrine-related cancer 2008;15(4):841-849.

27. Knudsen KE, Scher HI. Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer. Clin Cancer Res 2009;15(15):4792-4798.

28. Thompson TA, Gould MN, Burkholder JK, Yang NS. Transient promoter activity in primary rat mammary epithelial cells evaluated using particle bombardment gene transfer. In Vitro Cell Dev Biol 1993;29A(2):165-170.

29. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Analytical biochemistry 1969;27(3):502-522.

30. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nature protocols 2006;1(6):3159-3165.

31. Zhang Y, Ni J, Messing EM, Chang E, Yang CR, Yeh S. Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. Proceedings of the National Academy of Sciences of the United States of America 2002;99(11):7408-7413.

32. Young CY, Montgomery BT, Andrews PE, Qui SD, Bilhartz DL, Tindall DJ. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer research 1991;51(14):3748-3752.

33. Cornwell DG, Ma J. Studies in vitamin E: biochemistry and molecular biology of tocopherol quinones. Vitamins and hormones 2007;76:99-134.

34. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. The Journal of steroid biochemistry and molecular biology 2005;96(3-4):251-258.

35. Yang Q, Fung KM, Day WV, Kropp BP, Lin HK. Androgen receptor signaling is required for androgen-sensitive human prostate cancer cell proliferation and survival. Cancer cell international 2005;5(1):8.

36. Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, Ricklis RM, Isaacs JT. Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. The Prostate 2003;54(4):249-257.

37. He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, Carter KC. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 1997;43(1):69-77.

38. Heer R, Robson CN, Shenton BK, Leung HY. The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population. Journal of cellular physiology 2007;212(3):572-578.

39. Langeler EG, van Uffelen CJ, Blankenstein MA, van Steenbrugge GJ, Mulder E. Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP. The Prostate 1993;23(3):213-223.

40. Xu J, Kalos M, Stolk JA, Zasloff EJ, Zhang X, Houghton RL, Filho AM, Nolasco M, Badaro R, Reed SG. Identification and characterization of prostein, a novel prostate-specific protein. Cancer research 2001;61(4):1563-1568.

41. Taplin ME. Drug insight: role of the androgen receptor in the development and progression of prostate cancer. Nature clinical practice 2007;4(4):236-244.

42. Yuan X, Balk SP. Mechanisms mediating androgen receptor reactivation after castration. Urologic oncology 2009;27(1):36-41.

43. Biswas S, Chida AS, Rahman I. Redox modifications of protein-thiols: emerging roles in cell signaling. Biochemical pharmacology 2006;71(5):551-564.

44. Schwaller M, Wilkinson B, Gilbert HF. Reduction-reoxidation cycles contribute to catalysis of disulfide isomerization by protein-disulfide isomerase. The Journal of biological chemistry 2003;278(9):7154-7159.

45. Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S, Samali A. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. European journal of pharmacology 2009;625(1-3):234-246.

46. Tiwary R, Yu W, Li J, Park SK, Sanders BG, Kline K. Role of endoplasmic reticulum stress in alpha-TEA mediated TRAIL/DR5 death receptor dependent apoptosis. PloS one;5(7):e11865.

47. Elbein AD. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annual review of biochemistry 1987;56:497-534.

48. Dowd P, Zheng ZB. On the mechanism of the anticlotting action of vitamin E quinone. Proceedings of the National Academy of Sciences of the United States of America 1995;92(18):8171-8175.

49. Siegel D, Bolton EM, Burr JA, Liebler DC, Ross D. The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant. Molecular pharmacology 1997;52(2):300-305.

50. Peters U, Littman AJ, Kristal AR, Patterson RE, Potter JD, White E. Vitamin E and selenium supplementation and risk of prostate cancer in the Vitamins and lifestyle (VITAL) study cohort. Cancer Causes Control 2008;19(1):75-87.

CHAPTER 4: ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY ALPHA-TOCOPHERYLQUINONE AND CURCUMIN ANALOG 27 AND EFFECTS ON THE ANDROGEN RECEPTOR

Abstract

The AHR is a ligand activated transcription factor that regulates the expression of several genes involved in Phase I and II metabolism. The oxidative metabolite of vitamin E, alpha-tocopheryl quinone (TQ) and the curcumin analog 27 (ca27) have significant anti-androgenic effects and down-regulate AR protein expression in human prostate cancer (CaP) cells. In this study, both TQ and ca27 are shown to induce AHR activation and increase the expression of AHR regulated transcript CYP1A1 in CaP cells. However, the effects on AHR expression are different between TQ and ca27. ca27 significantly down-regulates AHR protein expression. In contrast, TQ increased AHR mRNA and protein expression in a time-dependent manner. In examining these agents' mechanism(s) of AHR regulation interactions of AHR and AR in CaP cells were evaluated. TQ and ca27 down-regulate AR protein expression in a dose- and time-dependent manner. The mechanism of AR protein down-regulation by TQ or ca27 was independent of the AHR. However, TQ modulated AHR expression and activity. TQ was shown to induce CYP1A1 expression through an AHR dependent-mechanism. This is the first study demonstrating TQ's activity as an AHR agonist in human CaP cells. Differential effects on AHR expression by TQ and ca27 were observed providing a potential role for the AHR toward these agents' mechanism(s) of AR down-regulation.

Introduction

The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix (bHLH-PAS) transcription factors which include Period (Per), AHR nuclear translocator (ARNT) and single minded (SIM) (1). The AHR is a ligand activated transcription factor which heterodimerizes with ARNT to activate gene transcription through a xenobiotic (dioxin) response element (XRE or DRE). Over 400 environmental toxicants and natural compounds have been reported to bind and activate this receptor (2). The AHR is a xenobiotic sensor and regulator of detoxification enzymes. It has additional cellular roles including, but not limited to development, protein regulation and cell cycle control (3). Thus, the AHR is considered to be a master regulator of cellular pathways. Well-characterized AHR ligands include a wide array of environmental contaminants such as halogenated aromatic hydrocarbons (HAH) such as, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAH) such as, benzo(a)pyrene (B(a)P) (4,5). AHR ligands can vary dramatically in their chemical structures and include natural products, endogenous and synthetic agents (6). Importantly, an endogenous ligand for the AHR has not been firmly established. The AHR ligand also influences the regulatory actions of the AHR on multiple cellular pathways (2,7,8). This study evaluated the effects of α-tocopheryl quinone (TQ) and curcumin analog 27 (ca27) on AHR activity and expression. The AHR is a regulator of multiple cellular pathways including AR expression.

The androgen receptor (AR) is a ligand activated nuclear receptor that plays a critical role in male development, fertility, sex accessory organ development and function (9-11).

The AR is activated by androgens such as testosterone and its more active metabolite 5α-dihydrotestosterone (DHT). The AR is required for the development and progression of CaP (12). The activation of the AR is a major target in current prostate cancer therapeutics in which the depletion of androgen and inhibition of AR activation are primary strategies. Unfortunately, resistance to these therapies can occur and the expression of the AR can still be retained and activated (13,14). TQ and ca27 down-regulate AR protein expression and activate the AHR. In an effort to elucidate TQ and ca27 mechanism(s) of inhibition, the potential action of the AHR on AR down-regulation was examined.

Expression of the AHR, and its heterodimer partner ARNT, have been detected in developing fetal prostate and the normal and malignant prostate of adult males (15,16). The AHR has been shown to be an important regulator of prostate development in multiple rodent models. Activation of the AHR by agents such as TCDD demonstrate retardation of fetal and perinatal prostate development (15,17,18). However the role of the AHR is dependent on the stage of development, species, cell-type and AHR ligand. In 2007, Fritz et al. (19) demonstrated that the AHR can act as a tumor suppressor in the CaP developing mouse model, TRAMP. Wild-type, heterozygous and AHR null TRAMP mice were evaluated for prostate cancer incidence, neuroendrocrine differentiation markers and AR expression. Heterozygous and AHR null animals developed malignant prostate tumors more frequently than wild-type (19). Several studies have begun to identify the ligand-specific regulatory role that the AHR may have on AR activation, expression and the role the AR may have on AHR activation. Activation of the AR by DHT repressed AHR transcriptional activation upon treatment with the PAH, 3-

methylcholanthrene (3-MC) (20). In contrast, activation of the AHR by 3-MC demonstrated AHR's novel activity as a ligand activated adaptor protein for E3 ubiquitin ligases which led to proteasomal degradation of the AR (21). The interplay and regulation between the AHR and AR is complex, with multiple components having to be taken into consideration. TQ and ca27's actions as potential AHR agonists may have consequences resulting in AR down-regulation.

TQ and ca27 have previously been reported as anti-androgenic agents in human CaP cells (Chapter 3) (22). TQ is the oxidative metabolite of VE, and has demonstrated unique properties in comparison to VE (Chapter 3). TQ inhibits prostate cancer cell proliferation, AR activation and AR expression. However, VE demonstrated no growth inhibitory effects on CaP cells, AR activation or AR expression (Chapter 3). The curcumin analog ca27 also demonstrated anti-androgenic activities similar to TQ. However, its parent compound curcumin did not inhibit AR expression (Chapter 2) (22). Both TQ and ca27 were found to be potent inhibitors of AR expression in comparison to VE or curcumin. Although, both TQ and ca27 have anti-androgenic activities their kinetics of AR down-regulation and effects on cell viability are very different between the two agents. This study further elucidates TQ and ca27's inhibitory actions on the AR by evaluating their regulation of the AHR.

Materials and Methods

Chemicals, cell culture, and treatment protocols

dl-α-tocopheryl quinone was obtained from Research Organics (Cleveland, OH). Ca27 was synthesized by Drs. Vander Jagt and Deck laboratory (Department of Biochemistry and Molecular Biology and Department of Chemistry, University of New Mexico).

6,2',4'-Trimethoxyflavone (TMF) and α-napthoflavone: 2-phenyl-4*H*-benzo(*h*)chromen-4-one (α-NF) and other chemicals used in these studies were acquired from Sigma Chemical Co (St. Louis, MO).

The LNCaP cells used in these studies were acquired from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 5% heat-inactivated fetal calf serum (FCS; Sigma, St. Louis, MO) with streptomycin-penicillin antibiotics (designated DMEM/FCS) in a 5% CO_2 incubator at 37°C.

Messenger RNA expression analysis

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen) and cDNA was prepared from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed for mRNA levels using an Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA) and QuantiTect Primers Assays (Qiagen Inc., Valencia, CA) *GAPDH* mRNA. Additional forward and reverse primers include *AHR* forward 5'-GCCAGGCCAACAGGCATTTTT-3' and reverse 5'-GGTCTGGCTTCTGACGGATGATGA-3', and *CYP1A1* forward 5'-CCCAAGGGGCGTTGTGTCTTT-3' and reverse 5'-CAGGGGTGAGAAACCGTTCAG-3'.

AR and AHR immunoblot analysis

LNCaP cells were plated at a density of 1×10^6 cells per 100 mm cell culture plate in 10 ml of DMEM/CSS and maintained in incubators at 37°C in 5% CO_2. For dose-response studies, LNCaP cells were cultured in 6-well plates (BD Biosciences, San Jose, CA) in DMEM containing 5% FBS. After a 4 d treatment with vehicle, VE, or TQ, cells were washed in cold 1× PBS and lysed in a buffer containing 1.0 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 µg/ml aprotinin in 1× PBS. Cell extracts were stored at -80°C until analysis. Sample protein levels were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL), according to kit instructions. Total protein (≤ 40 µg) from cell extracts were electrophoresed on 12.5 % SDS-polyacrylamide gels (BioRad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk at 4°C and then incubated with mouse anti-AR (441) monoclonal antibody) or mouse anti-AHR (A-3) (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β-actin antibody (A5441; Sigma). After washing, membranes were incubated with a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (Biomeda, Foster City, CA) and analyzed using Western Lightening Chemiluminescence Reagent Plus (Boston, MA) on a Kodak Image Station 4000MM (Rochester, NY). Band intensities were determined using Kodak Molecular Imaging Software.

Co-immunoprecipitation assays

AHR and AR proteins were isolated by co- immunoprecipitation. Cells were cultured as described above and treated as described in the figure legends. Cells were harvested in lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium vanadate, 1 μg/ml leupeptin and 1mM PMSF), sonicated and centrifuges at 10,000rpm for 5min at 4˚C to remove cellular debris. Protein (500 μg/500μl) was incubated with 5μl of rabbit polyclonal antibody (AR (Ab-2) Thermo-Scientific, Fremont, CA) for at least 1h at 4˚C, then Protein A beads (Invitrogen, Carlsbad, CA) were added in a 1:1 slurry and samples were incubated for an additional 1-2 h at 4˚C. The beads were recovered by centrifugation at 10,000 rpm for 5min at 4˚C and washed five times with 1ml of lysis buffer.

XRE reporter assay

LNCaP cells were cultured in 12- or 24-well plates (Invitrogen) in DMEM/CSS 2 d before transfection. Xenobiotic-induced transcriptional activation was determined using a reporter construct with a xenobiotic response element (XRE) (or DRE dioxin response element) that regulates the expression of luciferase (23). Cells were co-transfected with the XRE-Luciferase reporter plasmid and a control plasmid carrying a thymidine kinase (TK) promoter regulating *Renilla* luciferase cDNA expression (Promega, Madison, WI) using Lipofectamine 2000 transfection agent (Invitrogen, Carlsbad, CA). Fourty-eight h post-transfection cells were treated with the indicated agents for 24 hours. Whole cell extracts were generated using Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay Substrate kit (Promega, Madison, WI) and relative luciferase units determined on a Perkin Elmer Victor^{3}V 1420 counter and analyzed using Wallac 1420 software (Perkin Elmer, Turku, Finland). Normalized luciferase expression is expressed as a percent of vehicle control.

AHR RNAi assays

LNCaP cells were transfected with 20 nM siAHR or scrambled negative control (siNC) (Ambion, Carlsbad, CA) using Hiperfect (Qiagen, Valencia, CA) following manufacture protocol. Cells were transfected for 48 h, then treated with 25 µM TQ or 5 µM ca27 for the indicated times. RNA and protein were isolated and analyzed according to the protocols described above.

Microarray Analysis

LNCaP cells were treated with BSA (vehicle control) or 30 µM TQ for 4 d. Samples were processed following instructions provided by Affymetrix for the Human Genome U1333A Plus 2.0 Gene Chip Array. Arrays were analyzed by UNM Keck-UNM genome facility. Fold ratios were computed for TQ exposed cells compared to BSA controls.

Statistical analysis

Significant differences in values between groups were assessed using an unpaired *t*-test with SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). *P* values less than 0.05 were used to signify statistical significance. Most studies were performed as specified with a minimum of 3 samples (i.e., n ≥3) unless otherwise specified.

Results

TQ and ca27 activate the AHR

In an effort to identify pathways important in the mechanism of TQ's action on CaP cells, microarray studies were performed using Affymetrix Human Genome U1333A Plus 2.0 GeneChip arrays to examine alterations in gene expression induced by TQ. A high ranked pathway modulated by TQ included xenobiotic metabolism pathways. An increase in phase I and II metabolizing enzymes such as CYP1A1, aldoketoreductase 1C1 (AKR1C1), AKR1B10, glutamate-cysteine ligase, and AHR expression was observed (Table 1). TQ demonstrated a time-dependent increase of *AKR1C1* expression upon TQ treatment in LNCaP cells (Fig. 1A). *CYP1A1* was also significantly increased 15-fold upon 25µM TQ after 24 h but there was no significant change upon 25µM VE treatment (Fig. 1B). To determine ca27 effects on AHR-activation *CYP1A1* mRNA expression was measured. One µM ca27 significantly increased *CYP1A1* expression after 12h in LNCaP and C4-2 cells (Fig. 1C-D). Studies to further determine if TQ or ca27 modulated AHR activity were initiated using a xenobiotic-response element (XRE) luciferase reporter system in CaP cells. XRE reporter activity was assessed in PC3 cells after treatment with 25 µM TQ or VE for 24 h. TQ treatment significantly increased AHR activity in contrast to VE, which had no detectable effect (Fig 1E). ca27 also significantly increased AHR activity, but not with 10 µM curcumin after 12 h (Fig. 1F).

Table 1. Up-regulation of AHR regulated genes by TQ.

AHR regulated genes[1]	Gene Symbol	Accession Number	(mRNA) Fold Increase[2]
Aldoketoreductase 1C1	**AKR1C1**	**NM_001353**	**96.0**
Cytochrome P450 1A1	**CYP1A1**	**NM_000499**	**43.1**
Aldoketoreductase 1B10	**AKR1B10**	**NM_020299**	**24.0**
Glutamate-cysteine ligase, m-su	**GCLM**	**NM_002061**	**4.9**
Aryl Hydrocarbon Receptor	**AHR**	**NM_001621**	**2.2**

[1] AHR regulated transcripts identified from pathway profile analysis Microarray studies were performed as described previously (Chapter 3). Arrays were analyzed by the UNM Keck-UNM Resources facility.

[2] Fold increases compared to vehicle control in LNCaP cells

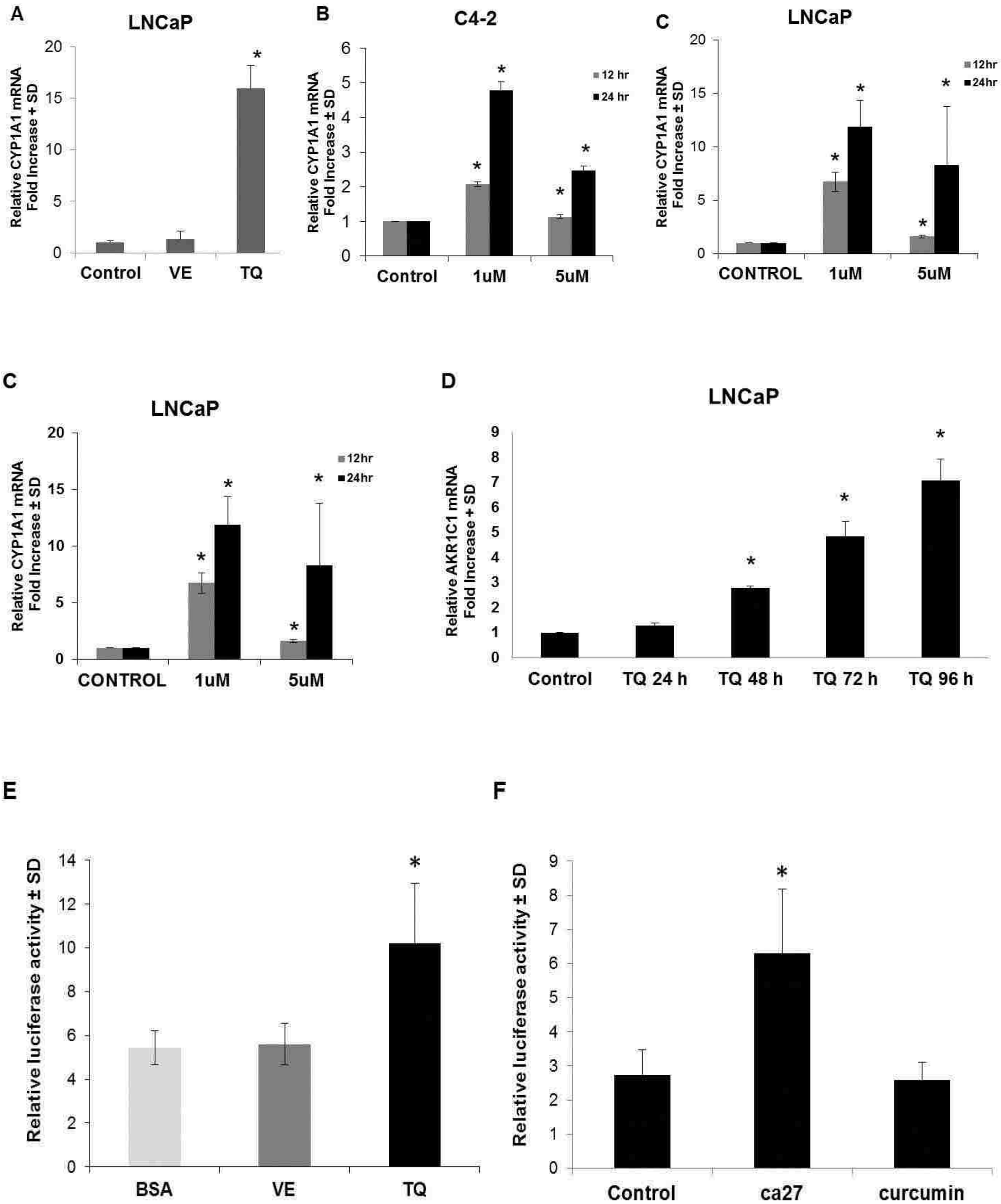


Fig. 1: TQ and ca27 activate the AHR in CaP cells. TQ activates the AHR as measured by the AHR regulated transcripts *CYP1A1* (A) and *AKR1C1* (B) mRNA expression. LNCaP cells were treated with vehicle control, 25uM VE or 25uM TQ for 48h (A) or 1-

4d (B). ca27 induces *CYP1A1* expression in C4-2(C) and LNCaP (D) cells treated with 1 or 5uM ca27 for 12 and 24h. *CYP1A1* and *GAPDH* mRNAs were measured by qRT-PCR. TQ (E) and ca27 (F) activate the AHR, as measured by an AHR reporter assay. Cells were co-transfected using a XRE reporter plasmid driving luciferase and thymidine kinase reporter plasmid driving *Renilla* luciferase. * denotes $P < 0.05$ compared to control.

TQ up-regulation of *CYP1A1* and *AHR* expression is AHR dependent

The observation that TQ treatment induces AHR transcriptional activity, (Fig. 1) led investigators to evaluate the AHR in modulating *AHR* and *CYP1A1* expression. *AHR* mRNA expression was knocked down using siRNA. LNCaP cells were transfected with 20 µM siAHR or siNC for 48 h and then treated with 25 µM TQ for 48 h. Samples treated with siAHR showed a significant inhibition of AHR mRNA expression in comparison to siNC (Fig. 2A). siNC samples treated with TQ showed a significant 8-fold induction of AHR mRNA expression. This increase in expression was significantly inhibited by AHR knock-down (siAHR) at both 48 h and 96 h time points. Twenty-five µM TQ significantly increased CYP1A1 mRNA expression in a time dependent manner in siNC controls. This induction was significantly attenuated by knock-down of AHR expression at both time points tested (Fig. 2B). To further evaluate TQ's induction of AHR expression in LNCaP cells the levels of *AHR* mRNA were measured using qPCR after treatment with 25 µM TQ for 96 h significantly increased AHR expression by 20-fold (Fig. 2C). AHR protein levels were measured by immunoblot and normalized to levels of β–actin protein after 96 h of 25 µM TQ treatment (Fig. 2D).

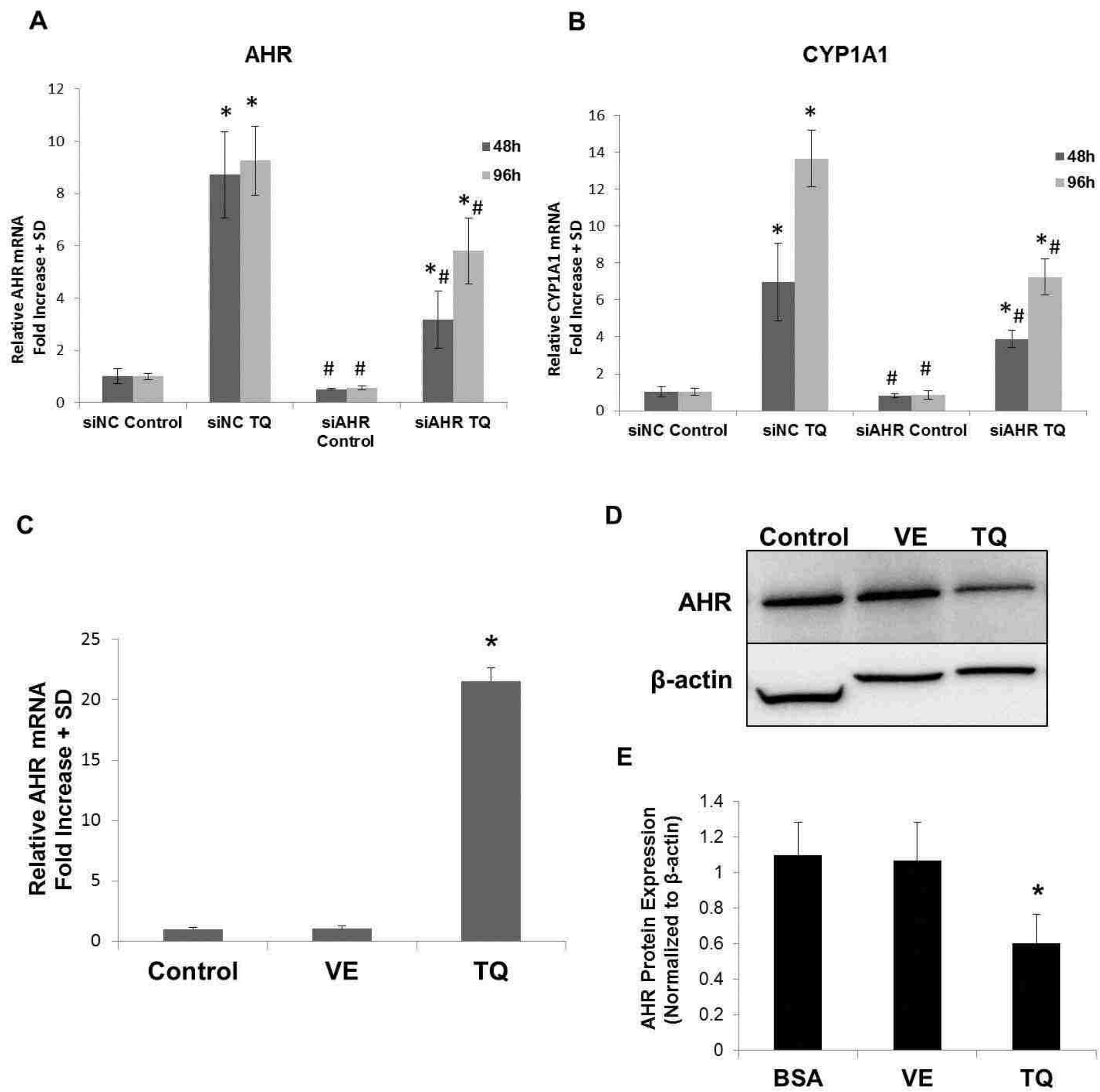


Fig. 2: AHR expression is critical for TQ induction of CYP1A1. AHR expression was knocked down using RNAi. (A+B) LNCaP cells transfected with 20 µM siAHR or scrambled Negative Control (siNC) for 48 h and treated with 25 µM TQ for an additional 48 h, *AHR*, *CYP1A1* and *GAPDH* mRNAs were measured by qRT-PCR. LNCaP cells were also treated with 25 µM TQ or 25 µM VE for 4 d, AHR mRNA (C) and protein expression (D+E). (E) Graph represents values of AHR protein expression normalized to β-actin expression, * denote $P < 0.05$ compared to control.

AHR agonist benzo(a)pyrene inhibits AR protein expression

To determine if AHR activation led to the proteasomal degradation of the AR in human prostate cancer cells, cells were pretreated with 10 µM MG132 for 2 h and the treated with 1 µM B(a)P in the presence of MG132 for 2 h. B(a)P significantly inhibited AR protein expression. This inhibition of AR by B(a)P was significantly inhibited by the proteasomal inhibitor MG132 (Fig. 3A-B). To determine if the AHR inhibited AR protein expression upon activation two AHR antagonists were used, TMF and α-NF (24). LNCaP cells were treated simultaneously with 25µM TQ and 10µM TMF or α-NF for 48 h. AR protein levels were normalized to levels of β–actin protein, which was not affected by the treatments. Graphs represent a n=2, therefore no statistical analysis were performed. LNCaP cells treated with either TMF or α-NF (Fig. 3C-D) did not prevent TQ's down-regulation of the AR. To evaluate AHR inhibition upon ca27 treatment LNCaP cells were treated simultaneously with 5µM ca27 and 10µM TMF or α-NF for 3 h. Neither, TMF or α–NF prevented ca27 down-regulation of AR protein expression (Fig. 3E-F).

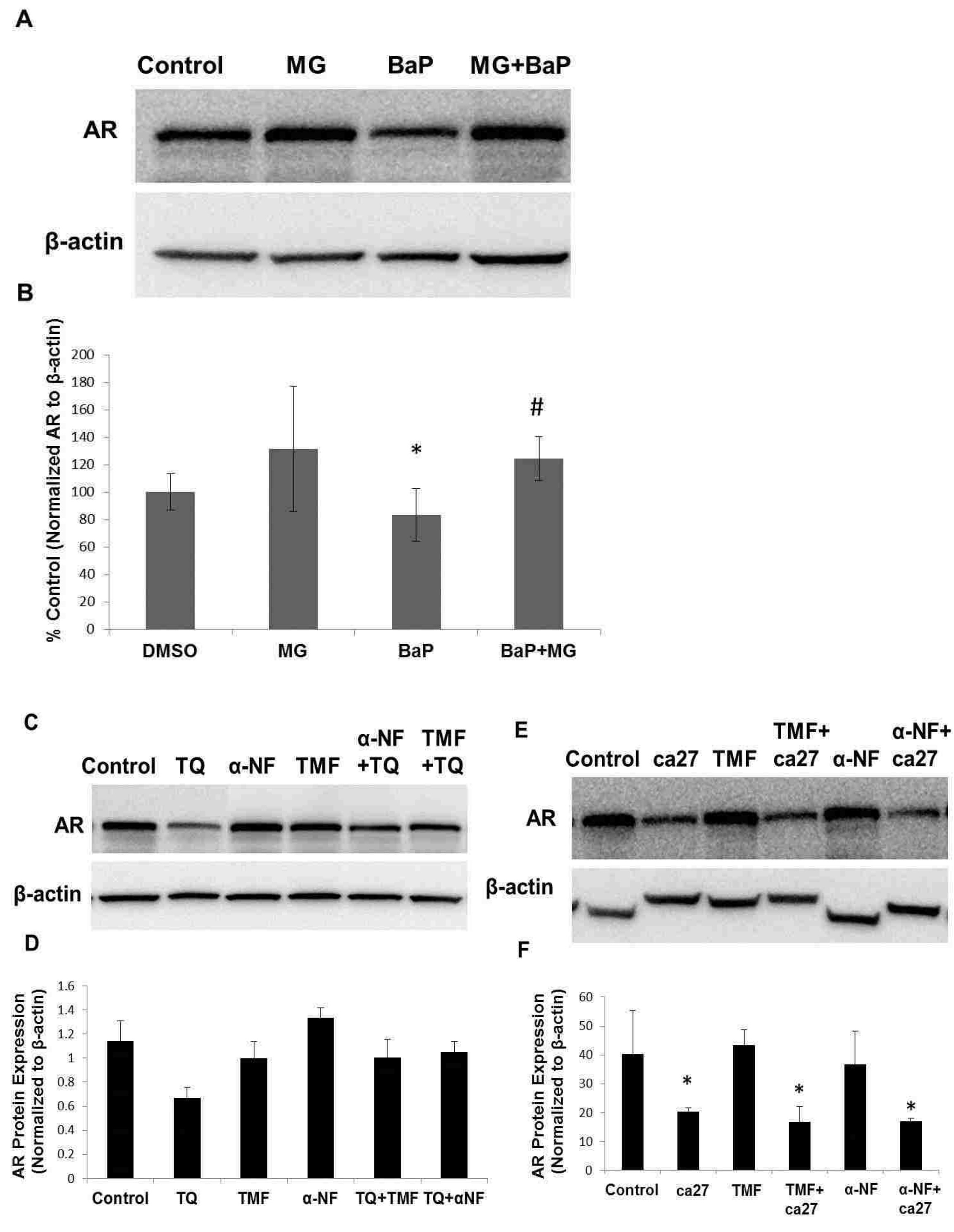


Fig. 3: B(a)P inhibits AR protein expression, AHR antagonist do not rescue AR from TQ or ca27. AHR activation leads to AR down-regulation can be rescued by proteasomal

inhibitor. (A+B) LNCaP cells were pretreated with 10uM MG132 for 2hrs and then 1uM BaP for 2hrs. (C+D) LNCaP cells treated with 25 µM TQ in the presence or absence of 10 µM TMF or α-NF for 48 h. (E-F) LNCaP cells treated with 5µM ca27 and 10µM TMF or α-NF for 3 h, AR immunoblots (C and E), and densitometry analysis (D and F) are represented.* denote $P < 0.05$ compared to control.

AHR expression is not critical for AR down-regulation by TQ or ca27

To determine if TQ or ca27 increased AHR and AR protein interaction co-immunoprecipitation pull down assays were performed. LNCaP cells were treated with 5 µM ca27 or 25 µM TQ for the indicated times. AR and associated proteins were immunoprecipitated using AR-specific antibody following described in the *Materials and Methods*. Immunoblots were probed for AHR protein first, stripped and then probed for AR protein expression. Cell lysates treated with TQ for 6 h demonstrate no difference AHR/AR protein interaction in comparison to control (Fig. 4A-B). Cells treated with ca27 demonstrate no difference in AHR/AR protein interaction after 15-45 min compared to control (Fig. 4C-D).

To determine if TQ and ca27 activation of the AHR induced AR protein down-regulation, AHR protein expression was knocked down using siRNA. LNCaP cells transfected with 20 µM siAHR or scrambled Negative Control (siNC) for 48 h and treated with 25 µM TQ for an additional 48 h. AHR and AR protein were measured by WB and normalized to levels of β–actin protein. Knock-down of AHR protein expression did not rescue AR expression upon TQ treatment (Fig. 5A-C). LNCaP cells were also transfected with 20 µM siAHR and treated with 5uM ca27 for 3h. However, knock-down of AHR protein expression does not rescue AR expression upon ca27 treatment (Fig. 5D-F).

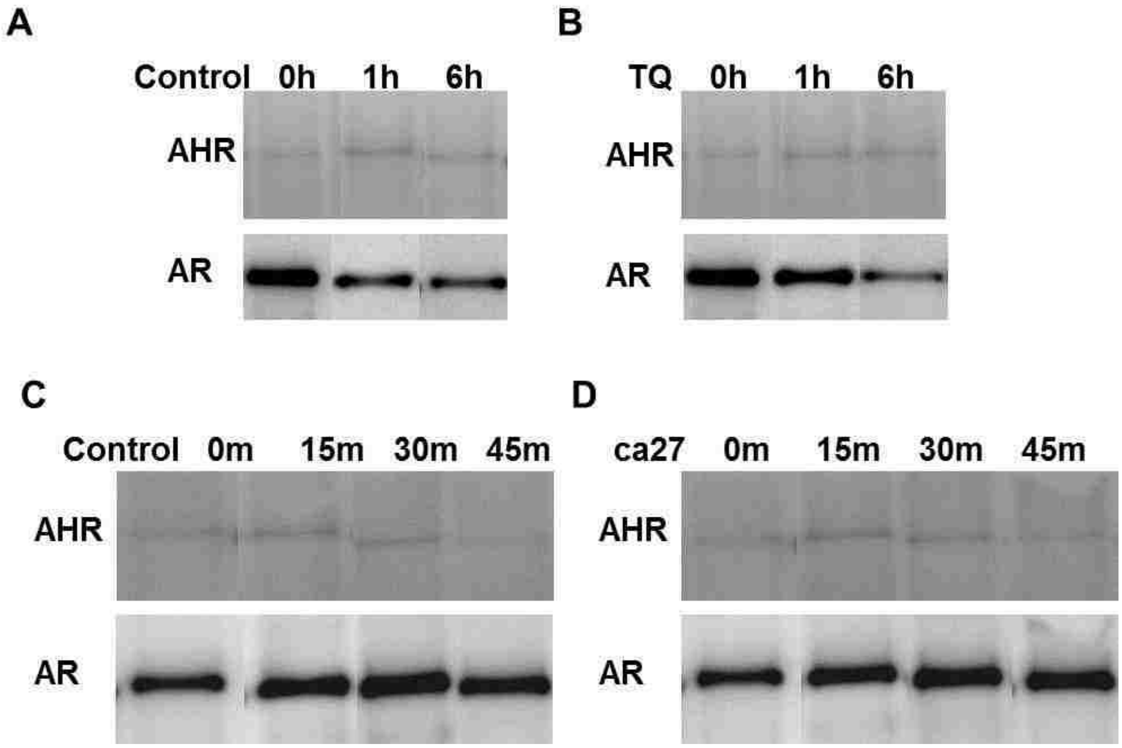


Fig. 4: AHR and AR protein interaction is not modulated by TQ or ca27. LNCaP cells were treated with 25 µM TQ (A-B) or 5 µM ca27 (C-D) or vehicle control for the indicated times. AR and AHR were immunoprecipitated using AR-specific antibody. Immunoblots were probed for AHR protein first, stripped and then probed for AR protein expression, both immunoblots are represented.

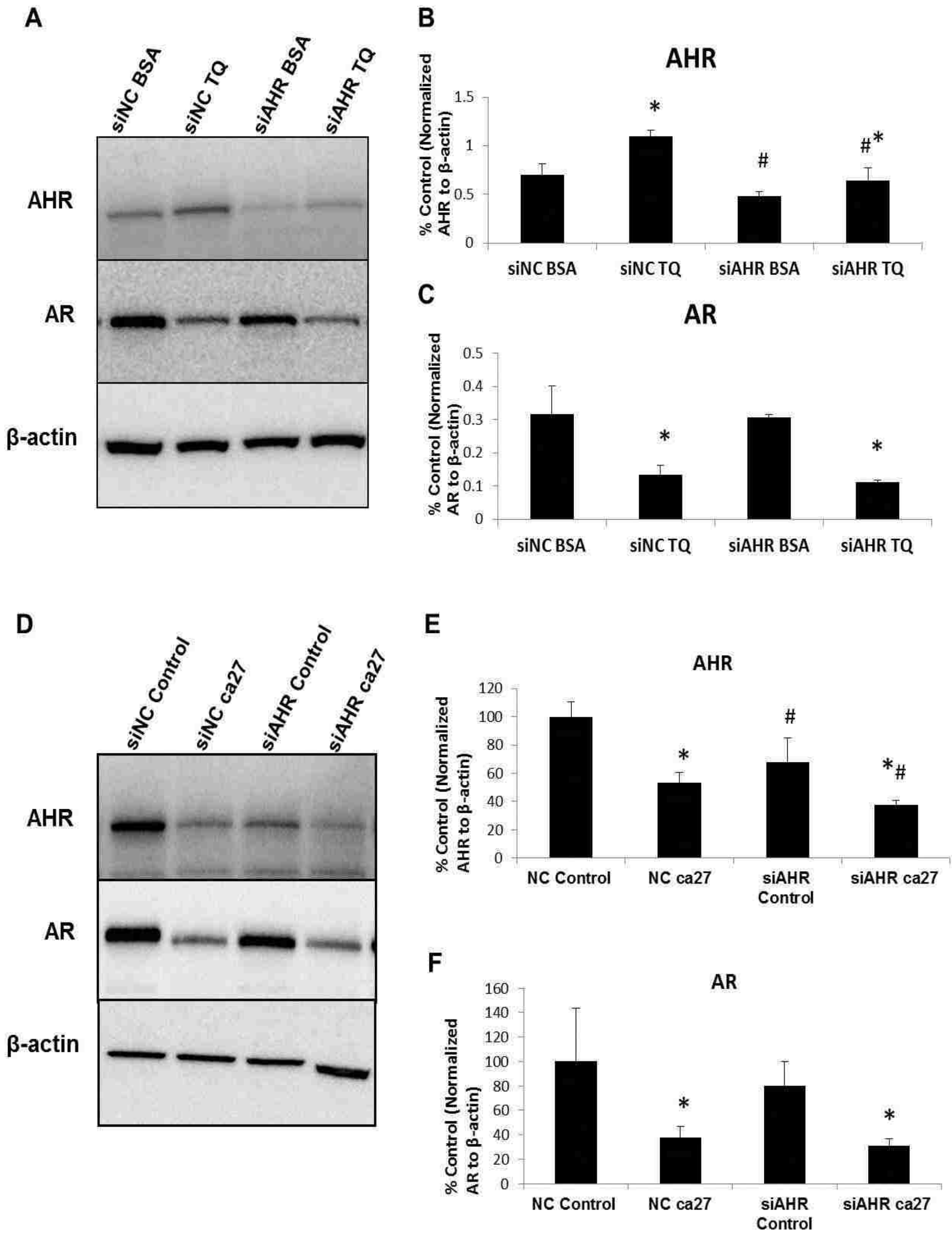


Fig. 5: Knock-down of AHR protein expression does not rescue AR expression upon TQ or ca27 treatment. LNCaP cells were treated with siAHR or siNC (scrambled Negative Control) and treated with 25uM TQ (A-C) for 2 d or with 5 μM ca27 for 3 h. AHR and

AR protein was measured by WB and densitometric analysis (ratio AR:β-actin). * denote $P < 0.05$ compared to control, # denote $P < 0.05$ compared to negative control.

Discussion

In this study we investigated the agents, TQ and ca27 as potential AHR activators by evaluating AHR transcriptional activation and its role in AR down-regulation in CaP cells. To begin identifying potential pathways activated upon TQ treatment, Affymetrix Gene chip arrays were performed. Profile analysis from this study demonstrated modulation of xenobiotic metabolic pathways (Fig. 1A). The AHR is a well-characterized transcription factor that regulates expression of several detoxification and metabolizing enzymes. The AHR may have alternative functions in the cell other than as a ligand activated transcription factor. One alternative function of the AHR previously reported is upon 3-MC activation, to act as adaptor protein for E3 ubiquitin-ligase complex formation targeting the degradation of the AR (21). The increased expression of AHR regulated transcripts and down-regulation of AR expression by TQ led us to further evaluate the potential mechanism of AHR down-regulation of AR.

The AHR regulates the expression of several detoxification and metabolizing enzymes including *CYP1A1* and *AKR1C*1 (23,25). TQ induced the expression of both *AKR1C1* and *CYP1A1* in a time-dependent manner and induced AHR activation as measured by a XRE-reporter assay (Fig. 1). AHR activity was only significantly induced by TQ, while VE demonstrated no effect. To determine if *CYP1A1* induction was through activation of the AHR, AHR expression was knocked down using RNAi. The increase in *CYP1A1* expression was significantly inhibited by AHR knock-down at both time points tested. Although expression of *CYP1A1* was still significantly increased in siAHR treated samples, this could be explained by residual AHR or transcription factor, such as LXR in regulating its expression. The liver X receptor α has recently been demonstrated to be a regulator of *CYP1A1* mRNA expression (26). Regardless, *CYP1A1* mRNA induction by TQ is significantly repressed upon siAHR. This is the first study to demonstrate that TQ can act as potential agonist ligand for the AHR.

The activation of the AHR by TQ led us to investigate ca27's actions on the AHR. ca27 significantly increased *CYP1A1* expression in a time- and dose-dependent manner and AHR activation as measured by a XRE-reporter assay. Although, ca27 induced AHR activation it had differential effects on AHR expression in comparison to TQ. TQ induced AHR mRNA and protein expression after 48 h. However, ca27 significantly inhibited AHR protein expression after only 3 h. The differences between TQ and ca27 are also demonstrated in their inhibition of AR protein expression. ca27 inhibits AR protein expression within 3 h as a opposed to TQ which requires at least 48 h (22) (Chapter3). TQ's regulation of AHR expression is time dependent for both mRNA and protein levels. The significant increase of AHR protein expression after 48 h is selective since AR protein expression is significantly inhibited at this time. The regulation of AHR expression induced by TQ has been demonstrated for specific AHR ligands such as TCDD and 3-MC (27,28). This study provides support for the selectivity of TQ's AR down-regulation and begins to address additional questions regarding TQ's regulation of AHR expression. In this study, we have begun to demonstrate TQ's potential role as a ligand for the AHR.

The PAH B(a)P has previously been shown to inhibit AR protein expression in human lung adenocarcinoma cell line (29). The study conducted by Lin et al. (29) tested B(a)P and TCDD mediated down-regulation of the AR. TCDD did not significantly inhibit AR protein expression as opposed to B(a)P (29). In 2007, Ohtake et al. (21) demonstrated 3-MC activated AHR was a component of a ubiquitin ligase complex which regulated AR degradation. To address if activation of the AHR by a known agonist such as B(a)P could induce AR degradation in human CaP cells, cells were treated with the AHR agonist B(a)P in the presence of the proteasomal inhibitor MG132. B(a)P significantly led to AR protein degradation which could be prevented by MG132. The environmental contaminant B(a)P may act as an endocrine disruptor through modulation of AHR activity leading to AR degradation in human CaP cells.

To further study AHR activity on AR protein inhibition, two AHR antagonists TMF and α-NF treated in the presence or absence of TQ or ca27 were used (24,30-32). These antagonists did not significantly prevent AR down-regulation upon ca27 treatment. However, treatment with TMF and α-NF did demonstrate an attenuation of AR down-regulation upon TQ treatment. The potential role of the AHR as an adaptor protein for AR degradation may require an increase in AHR and AR interaction. To determine if TQ and ca27 increased the interaction between AHR and AR co-immunoprecipitations were performed. However, neither agent dramatically increased this interaction at the times tested. To further evaluate the role of the AHR upon AR down-regulation by TQ and ca27, AHR expression was significantly reduced by siRNA. This inhibition of AHR expression did not prevent TQ or ca27's down-regulation of AR protein expression suggested that the AHR is not a critical component of TQ or ca27 mechanism of AR down-regulation. TQ and ca27 regulate the expression of the AHR in distinct ways. ca27 increases AHR activation and inhibits AHR protein expression within 3 h. TQ regulates AHR expression in a time–dependent manner and induces the expression of *CYP1A1* through an AHR mediated mechanism. Although TQ's down-regulation of the AR is not mediated by the AHR, TQ's regulation of the AHR expression demonstrates specificity for AR down-regulation and provides a potential mechanism of TQ's actions on activating xenobiotic metabolism pathways.

We conclude that both TQ and ca27's activation of the AHR is not a major component in their mechanism of AR down-regulation. Further, TQ's induction of *CYP1A1* expression is AHR dependent suggesting that TQ may be an agonist for the AHR and regulator of AHR expression. In comparison to VE which did not induce *CYP1A1* expression, TQ modulates distinct cellular pathways such as activation of the AHR and down-regulation of AR protein expression. Although, the activation of AHR by TQ, may be independent of AR protein down-regulation, this study provides evidence of TQ's actions on AHR activation, AHR expression and AHR-dependent induction of *CYP1A1* in human CaP cells.

References

1. Swanson HI, Bradfield CA. The AH-receptor: genetics, structure and function. Pharmacogenetics 1993;3(5):213-230.
2. Denison MS, Soshilov AA, He G, Degroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. Toxicol Sci 2011;124(1):1-22.
3. Ohtake F, Fujii-Kuriyama Y, Kato S. AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions. Biochemical pharmacology 2009;77(4):474-484.
4. Poland A, Knutson JC. 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annual review of pharmacology and toxicology 1982;22:517-554.
5. Shimizu Y, Nakatsuru Y, Ichinose M, Takahashi Y, Kume H, Mimura J, Fujii-Kuriyama Y, Ishikawa T. Benzo(a)pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proceedings of the National Academy of Sciences of the United States of America 2000;97(2):779-782.
6. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annual review of pharmacology and toxicology 2003;43:309-334.
7. Haarmann-Stemmann T, Bothe H, Abel J. Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways. Biochemical pharmacology 2009;77(4):508-520.

8. Puga A, Ma C, Marlowe JL. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochemical pharmacology 2009;77(4):713-722.

9. Grosse A, Bartsch S, Baniahmad A. Androgen receptor-mediated gene repression. Molecular and cellular endocrinology 2011.

10. Wang RS, Yeh S, Tzeng CR, Chang C. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocrine reviews 2009;30(2):119-132.

11. Callewaert F, Boonen S, Vanderschueren D. Sex steroids and the male skeleton: a tale of two hormones. Trends in endocrinology and metabolism: TEM;21(2):89-95.

12. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. Seminars in oncology 1999;26(4):407-421.

13. Masai M, Sumiya H, Akimoto S, Yatani R, Chang CS, Liao SS, Shimazaki J. Immunohistochemical study of androgen receptor in benign hyperplastic and cancerous human prostates. The Prostate 1990;17(4):293-300.

14. Ichikawa T, Suzuki H, Ueda T, Komiya A, Imamoto T, Kojima S. Hormone treatment for prostate cancer: current issues and future directions. Cancer chemotherapy and pharmacology 2005;56 Suppl 1:58-63.

15. Vezina CM, Lin TM, Peterson RE. AHR signaling in prostate growth, morphogenesis, and disease. Biochemical pharmacology 2009;77(4):566-576.

16. Kashani M, Steiner G, Haitel A, Schaufler K, Thalhammer T, Amann G, Kramer G, Marberger M, Scholler A. Expression of the aryl hydrocarbon receptor (AhR)

and the aryl hydrocarbon receptor nuclear translocator (ARNT) in fetal, benign hyperplastic, and malignant prostate. The Prostate 1998;37(2):98-108.

17. Mably TA, Moore RW, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 1. Effects on androgenic status. Toxicology and applied pharmacology 1992;114(1):97-107.

18. Lin TM, Simanainen U, Moore RW, Peterson RE. Critical windows of vulnerability for effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on prostate and seminal vesicle development in C57BL/6 mice. Toxicol Sci 2002;69(1):202-209.

19. Fritz WA, Lin TM, Cardiff RD, Peterson RE. The aryl hydrocarbon receptor inhibits prostate carcinogenesis in TRAMP mice. Carcinogenesis 2007;28(2):497-505.

20. Sanada N, Gotoh Y, Shimazawa R, Klinge CM, Kizu R. Repression of activated aryl hydrocarbon receptor-induced transcriptional activation by 5alpha-dihydrotestosterone in human prostate cancer LNCaP and human breast cancer T47D cells. Journal of pharmacological sciences 2009;109(3):380-387.

21. Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T, Fujii-Kuriyama Y, Kato S. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 2007;446(7135):562-566.

22. Fajardo AM, Mackenzie DA, Ji M, Deck LM, Jagt DL, Thompson TA, Bisoffi M. The curcumin analog ca27 down-regulates androgen receptor through an oxidative stress mediated mechanism in human prostate cancer cells. The Prostate 2011.

23. Burchiel SW, Thompson TA, Lauer FT, Oprea TI. Activation of dioxin response element (DRE)-associated genes by benzo(a)pyrene 3,6-quinone and benzo(a)pyrene 1,6-quinone in MCF-10A human mammary epithelial cells. Toxicology and applied pharmacology 2007;221(2):203-214.

24. Murray IA, Flaveny CA, DiNatale BC, Chairo CR, Schroeder JC, Kusnadi A, Perdew GH. Antagonism of aryl hydrocarbon receptor signaling by 6,2',4'-trimethoxyflavone. The Journal of pharmacology and experimental therapeutics 2010;332(1):135-144.

25. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. The Journal of biological chemistry 2004;279(23):23847-23850.

26. Shibahara N, Masunaga Y, Iwano S, Yamazaki H, Kiyotani K, Kamataki T. Human Cytochrome P450 1A1 Is a Novel Target Gene of Liver X Receptor alpha. Drug metabolism and pharmacokinetics 2011;26(5):451-457.

27. Brauze D, Widerak M, Cwykiel J, Szyfter K, Baer-Dubowska W. The effect of aryl hydrocarbon receptor ligands on the expression of AhR, AhRR, ARNT, Hif1alpha, CYP1A1 and NQO1 genes in rat liver. Toxicology letters 2006;167(3):212-220.

28. Franc MA, Pohjanvirta R, Tuomisto J, Okey AB. In vivo up-regulation of aryl hydrocarbon receptor expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a dioxin-resistant rat model. Biochemical pharmacology 2001;62(12):1565-1578.

29. Lin P, Chang JT, Ko JL, Liao SH, Lo WS. Reduction of androgen receptor expression by benzo(alpha)pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo(alpha)pyrene in human lung cells. Biochemical pharmacology 2004;67(8):1523-1530.

30. Gasiewicz TA, Rucci G. Alpha-naphthoflavone acts as an antagonist of 2,3,7, 8-tetrachlorodibenzo-p-dioxin by forming an inactive complex with the Ah receptor. Molecular pharmacology 1991;40(5):607-612.

31. Santostefano M, Merchant M, Arellano L, Morrison V, Denison MS, Safe S. alpha-Naphthoflavone-induced CYP1A1 gene expression and cytosolic aryl hydrocarbon receptor transformation. Molecular pharmacology 1993;43(2):200-206.

32. Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS. Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonists. Toxicol Sci 2000;55(1):107-115.

CHAPTER 5: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary

Identifying the actions of agents that inhibit androgen receptor (AR) protein expression may elucidate novel mechanism(s) for targeted therapeutics. The expression and activation of the AR is critical for the development of male characteristics and fertility (1). However, abnormal AR activity and/or expression can also lead to various disease etiologies. For example, the AR plays a critical role in normal prostate development and function. However, it also plays a major role in the development and progression of prostate cancer (2). Therefore, the activation of the AR is a major target in current prostate cancer therapeutics in which the depletion of androgen and inhibition of activation are primary therapeutic strategies. Unfortunately, resistance to androgen ablation therapies can occur and expression of the AR can still be retained and activated in CaP (3,4). In this work, two novel agents, curcumin analog 27 (ca27) and alpha-tocopheryl quinone (TQ) were identified, as potent anti-androgenic agents. Both agents inhibit prostate cancer (CaP) cell proliferation, AR activation (i.e. PSA and ARRE reporter assay), and AR expression. In this dissertation respectively, significant progress was made to identify the mechanism(s) of ca27- and TQ-mediated AR down-regulation. Agents that induce AR reduction not only provide a means to elucidate molecular mechanisms of AR down-regulation, but also significantly contribute to experimental therapeutic strategies for CaP.

ca27 is an analog of the natural product, curcumin (5). In Chapter 2 (6), studies illustrating ca27's anti-androgenic properties were presented. ca27 inhibits CaP cell proliferation, viability and AR protein expression. ca27's chemical structure (Chapter 2, Fig. 1) consists of two phenolic rings with symmetrical *ortho*-hydroxyl groups and a 5-carbon unsaturated linker with a single carbonyl group (Chapter 2, Fig. 1). The carbon linker retains the character of an α,β-unsaturated ketone which has properties of a Michael acceptor, a strong electrophile (7,8). Michael acceptors bind and deplete nucleophilic groups such as free thiols.. The mucolytic agent NAC, is also a thiol and a precursor of reduced glutathione (9-11). ca27 increases ROS production, and electrophilic or antioxidant responsive genes (6). ca27 down-regulates AR protein expression and the addition of NAC attenuates ca27's down-regulation of the AR. Results from my dissertation indicate that ca27's mechanism of AR down-regulation involves pro-oxidant activity.

TQ is the oxidative metabolite of vitamin E (VE) and has distinct properties from VE. TQ is a quinone which induces cellular redox cycling through Michael addition reactions; this activity is in contrast to VE's antioxidant activities. In Chapter 3, results illustrating TQ's anti-androgenic activity and down-regulation of AR protein expression are presented. VE on the other hand, did not inhibit AR activation or AR protein expression. TQ's down-regulation of AR protein expression was independent of mRNA inhibition or proteasomal degradation (Chapter 3). To further elucidate TQ's actions on the AR and the potential activity of TQ as a quinone, oxidative stress pathways were evaluated. TQ significantly increased total and oxidized glutathione levels indicating oxidative stress. Further, TQ increased the expression of antioxidant regulated transcripts, induced ER stress and activated the UPR. The induction of oxidative stress by TQ leads to AR protein down-regulation and the presence of antioxidants such as NAC and VE prevent this down-regulation. TQ's mechanism of AR down-regulation its through is activity as a pro-oxidant. TQ's actions on AR protein expression are distinct from VE and the differences reported begin to provide insight into the differences in their potential mechanism(s) regulating AR expression.

In an attempt to identify TQ and ca27's mechanism(s) of AR protein down-regulation, the role of the AHR was investigated. A microarray conducted on TQ treated LNCaP cells, revealed a profile that the AHR pathway was activated. Ohtake et al. (12) reported that the ligand activated AHR could induce proteasomal degradation of the AR. To determine the AHR's role on the AR, cells treated with the AHR agonist B(a)P had significantly reduced AR protein expression. AR protein down-regulation upon AHR activation was prevented by the proteasomal inhibitor MG132. Although TQ and ca27 induced the activation of the AHR (i.e. using a XRE reporter assay and evaluating *CYP1A1* mRNA expression) the knock-down of AHR expression did not prevent AR down-regulation by either agent. To determine if the induction of *CYP1A1* expression was dependent on the AHR, expression of AHR was knocked-down and upon treatment with TQ there is a significant attenuation of *CYP1A1* expression. This study demonstrates TQ induces AHR transcriptional activation resulting in the increased expression of *CYP1A1*. Both TQ and ca27 induce AHR transcriptional activation; however, AHR activation is not a critical factor in TQ or ca27's mechanism of AR down-regulation.

TQ and ca27 both inhibit CaP cell proliferation, AR activation and AR protein expression. TQ and ca27 down-regulate AR protein expression independent of transcriptional or proteasomal inhibition. Both agents increase AHR activity but this activation is independent of AR down-regulation. Although TQ and ca27 have distinct cellular consequences results from my studies support that both agents down-regulate AR protein expression through an oxidative stress mediated mechanism. The results of these studies may provide insight into the development of AR targeted therapeutic strategies through the identification of TQ and ca27's mechanism(s) of action.

Key accomplishments

- Determined that ca27 dose-dependently inhibits CaP cell proliferation and viability
- Determined that TQ inhibits CaP cell growth but does not inhibit cell viability up to 4 days of treatment
    - Determined kinetic differences of ca27 and TQ inhibition of AR activity
    - ca27 inhibits AR activity in ≤ 24 hours while TQ requires ≥ 48 hours
- Determined kinetics and doses required for down-regulation of AR expression
    - AR mRNA down-regulation is inhibited by ca27 within 3 hours, while TQ requires ≥ 48 hours
    - TQ inhibits AR protein expression ≥ 48 hours versus ca27 that requires 3 hours for AR protein down-regulation
- Evaluated potential mechanism(s) for AR protein down-regulation by ca27 and TQ
    - Determined AR protein down-regulation is independent of AR transcriptional inhibition for TQ and ca27
    - Determined TQ and ca27's AR down-regulation is independent of proteasomal degradation

- Determined both agents induce AHR activation but AR down-regulation is independent of the AHR
- Determined both agents modulate cellular reduction/oxidation parameters
    - ca27 increased ROS generation and induced the expression of antioxidant regulated genes within 1-3 hours
    - TQ increased total and oxidized glutathione levels and induced the expression of antioxidant regulated transcripts
    - TQ induced ER stress leading to activation of UPR signaling cascades by 4 days
- Elucidated a potential mechanisms involved in TQ and ca27's down-regulation of AR protein expression involving cellular reduction/oxidation events
    - ca27 down-regulation of AR protein expression is attenuated by the antioxidant NAC within 3 hours
    - TQ down-regulation of the AR protein expression was attenuated by both NAC and VE within 48 hours
    - TQ down-regulation of AR protein expression was potentiated by the glutathione synthesis inhibitor BSO within 48 hours

- Determined both agents induce AHR activation but this activity was independent of AR down-regulation
    - Demonstrated that ca27 induces AHR activity (i.e. using a XRE reporter assay and evaluating *CYP1A1* mRNA expression)

- Determined that the knock-down of AHR expression did not prevent ca27 down-regulation of AR protein expression
- Demonstrated TQ induces AHR activity (i.e. using a XRE reporter assay and evaluating *CYP1A1* mRNA expression)
- Determined that the knock-down of AHR expression did not prevent TQ down-regulation of AR protein expression
- Demonstrated that TQ induces *CYP1A1* expression in an AHR dependent manner

Conclusions

In these dissertation studies to elucidate pathways involved in ca27 and TQ mechanism of action in human CaP cells, their anti-androgenic and pro-oxidant activities were the focus of investigation (Chapters 2-3) (6). The overriding goal of this project was to identify novel pathways for targeting AR expression. The inhibition of the AR is an established target for CaP therapeutics. ca27 and TQ were found to down-regulate AR protein expression in a time- and dose-dependent manner. Although these agents are similar in down-regulating the AR, their differences in this inhibition and other cellular stress pathways provides insight into mechanisms regulating AR expression. Two of the major differences between ca27 and TQ were potency and kinetics of AR down-regulation. A potential explanation may be the capacity of our agents to act as pro-oxidants. Intriguingly, the results in this dissertation demonstrate a similar mechanism of AR down-regulation mediated by ca27 and TQ. ca27 and TQ down-regulate AR protein expression though modulation of cellular redox. The attenuation of ca27's and TQ's down-regulation of AR expression by antioxidants provides further support that modulation of cellular redox can lead to AR down-regulation in CaP cells. Therefore, I conclude that perturbations in cellular redox by agents such as ca27 or TQ can be an effective means of targeting AR down-regulation.

One of the differences between ca27 and TQ is ca27's rapid down-regulation of AR protein expression (i.e, 3 hours). ca27 was identified as a potential anti-androgenic agent due to its inhibition of CaP cell proliferation, viability, AR activity and AR expression. The potential reactivity of ca27 may be due to the chemical moieties within its structure. ca27's structure consists of a hydroxyl group at the *ortho*-positions on both the aryl rings. Dinkova-Kostova et al. (13) demonstrated the importance of these *ortho*-positioned hydroxyl groups as important moieties for the potent induction of NQO1 enzymatic activity and reactivity with sulfhydryl groups (13). The α,β-unsaturated carbonyl group and the ortho-hydroxyl positioned groups on the aryl rings are highly reactive moieties that are most likely responsible for the rapid pro-oxidant and cytotoxic responses observed by ca27 in these studies. The potential reactivity of ca27's structure as a potent electrophile is supported by the increase in ROS generation and the activation of the Nrf2 pathway (Chapter 2). ca27 treatment increased the expression of antioxidant response element regulated transcripts such as *NQO1.* ca27's down-regulation of AR protein expression was determined to be, for the most part, independent of AR mRNA expression and proteasomal degradation. ca27 also induced activation of the AHR and increased expression of the detoxification enzyme CYP1A1. Although the AHR has previously been reported to regulate AR expression, down-regulation of the AR by ca27 was independent of the AHR. To determine if ca27 induced oxidative stress resulted in

AR protein down-regulation the antioxidant NAC was used. Treatment with NAC attenuated ca27 down-regulation of AR protein expression. Although the induction of oxidative stress is a general cellular response, there is a relative selectivity in ca27's actions on the AR. Pro-oxidants such as hydrogen peroxide did not inhibit AR protein expression (Appendix V). This study demonstrates for the first time ca27's regulation of AR protein, AHR activation and induction oxidative stress (Model 1). ca27 induces ROS generation, the expression of antioxidant response element regulated transcripts and down-regulates AR protein expression through an AHR independent oxidative stress-mediated mechanism. The conclusion is drawn that ca27 down-regulates AR protein in CaP cells through a cellular redox-mediated mechanism.

The anti-androgenic activities of TQ were evaluated in this study. TQ inhibited androgen-responsive CaP cell proliferation, AR activity and AR expression. TQ is the oxidative metabolite of VE; however, VE had no inhibitory effects on CaP cell proliferation or the AR. TQ contains a quinone structure and quinones can undergo redox cycling leading to toxicity. α-TQ has distinct chemical properties that are unique in comparison to other quinones such as γ- and δ-TQ. In general, quinone structures are found to be toxic due to the electrophilic carbon centers present in the quinone structure that are reactive to nucleophiles such as sulfhydryl groups. α-TQ did not have a significant effect on cell viability (Appendix IV). However, TQ increased total glutathione levels and increased oxidized glutathione, indicating oxidative stress. In addition, TQ selectively inhibited AR protein expression in a time- and dose-dependent manner. The inhibition of AR protein expression was at least in part, independent of mRNA expression and proteasomal degradation. TQ treatment also induced the activation of the AHR and regulated AHR expression. Further, TQ increased expression of the AHR regulated transcript CYP1A1 in an AHR dependent manner. Demonstrating TQ may be a novel ligand/agonist of the AHR. Although TQ induced AHR activation this was independent of its mechanism of AR down-regulation. To determine if TQ's pro-oxidant activity was leading to AR down-regulation two antioxidants were used. NAC, a glutathione precursor and the antioxidant VE both attenuated AR down-regulation by TQ. This regulation of glutathione levels by NAC and VE may be a mechanism of attenuating TQ's actions on the AR. VE is believed to act primarily as an antioxidant, reducing cellular oxidative damage produced by oxidized lipids (14,15). There is emerging evidence that VE may be playing an alternative antioxidant role through the regulation of glutathione expression (16,17). In a study conducted by Yamagata, K, et al. (16) VE increased glutathione levels and expression of γ-GCS mRNA expression in rats (16). To determine if the depletion of reduced glutathione levels were responsible for TQ's down–regulation of the AR the glutathione inhibitor BSO was used. BSO potentiated TQ's down-regulation of AR protein expression. Demonstrating TQ's modulation of glutathione homeostasis, at least in part, leads to AR protein down-regulation. This study was the first to elucidate α-TQ pro-oxidant anti-androgenic activity and the contrast in TQ's activity in comparison to VE. And demonstrate TQ's induction of *CYP1A1* expression is through an AHR mediated mechanism. TQ has unique inhibitory activities in CaP cells in comparison to VE, TQ down-regulates AR protein expression potentially through the modulation of reduction potential, and this down-regulation is independent of AHR activation. In conclusion, the identification of TQ's actions provides an explanation for the differences reported between TQ and VE. Additionally, TQ's mechanism of action may be exploited for development of agents selectively targeting AR down-regulation. Therefore, the conclusion is drawn that TQ down-regulates AR protein in CaP cells through a cellular redox-mediated mechanism.

The agents tested, TQ and ca27 inhibit androgen-sensitive prostate cancer cell proliferation, AR activation and AR expression. Determining the mechanism by which our agents inhibit AR protein expression has revealed the importance of cellular redox and has begun to elucidate its role in AR expression. Therefore, these studies provide novel insights into molecular mechanisms regulating AR expression and identify mechanisms to effectively target the AR. The mechanisms identified in these studies provide a foundation for the development of AR targeted therapeutics.

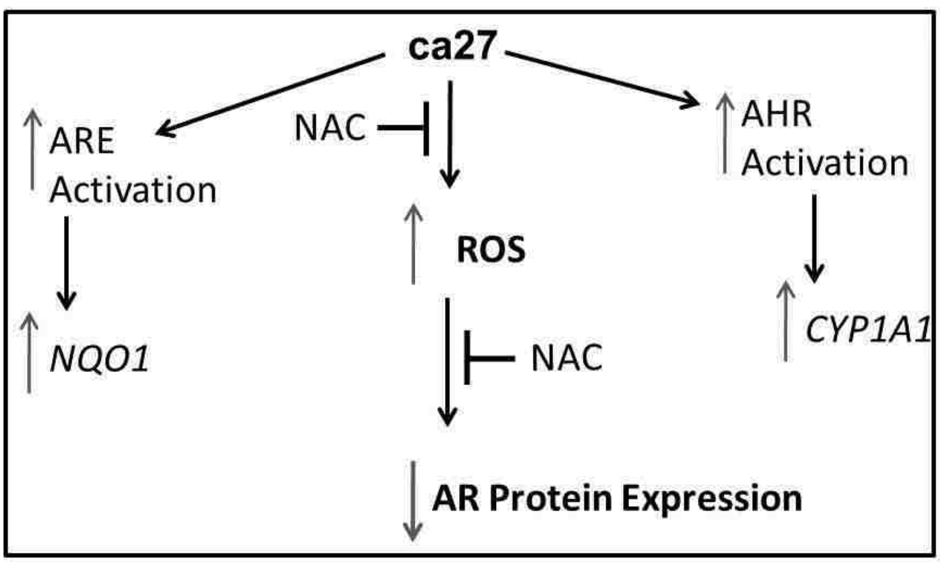


Fig. 1: - Model of ca27's mechanism of AR down-regulation.

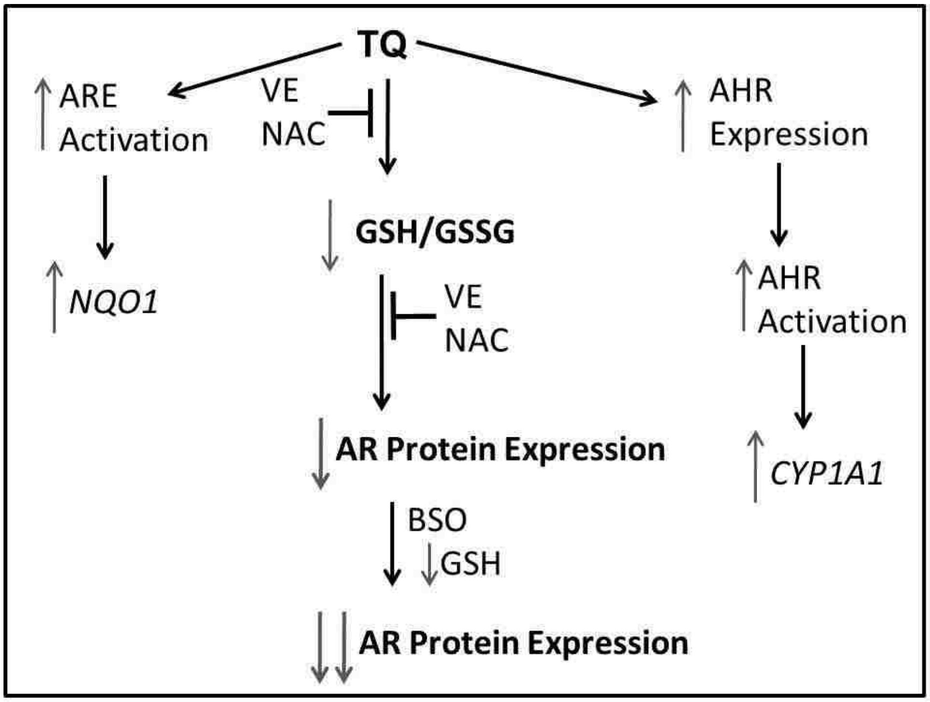


Fig. 2: Model of TQ's mechanism of AR down-regulation.

Future Directions

Our studies begin to address how the agents TQ and ca27 down-regulate AR protein expression in human CaP cells. I have presented that both of these agents induce oxidative stress and that this stress is partially alleviated by the presence of antioxidants such as NAC. In addition, I show that the induction of selective cell stress pathways such as detoxification and ER stress pathways are induced by these agents. This section will focus on additional questions that arose from these studies and provide suggestions for addressing these questions.

The hypothesis driving this study was; AR protein down-regulation by small molecules act through targetable molecular pathways.

To begin addressing the hypothesis, two small molecules were identified, TQ and ca27 through an anti-androgenic activity screening procedure. TQ and ca27 were found to inhibit CaP cell proliferation, AR activation and AR expression. They were found to inhibit both AR mRNA and AR protein expression in CaP cells. To determine the mechanism(s) of TQ and ca27's down-regulation of AR protein expression, several potential pathways were evaluated including AR transcriptional inhibition, proteasomal degradation, and the activation of the AHR leading to AR down-regulation. In brief, the results from these studies demonstrated that AR down-regulation by TQ and ca27 was independent of these mechanisms. Due to the potential reactivity of some of the chemical moieties within the structures of TQ and ca27, studies ensued to determine the effects of TQ and ca27 on cellular redox changes. The results from these studies demonstrated the induction of oxidative stress by TQ and ca27. To determine if the increase in cellular oxidative stress led to the down-regulation of AR protein expression, cells were treated with either TQ or ca27 in the presence of the antioxidant NAC. NAC significantly prevented the down-regulation of AR protein expression by TQ or ca27. These studies demonstrate that TQ and ca27 down-regulate AR protein expression at least in part through the induction of oxidative stress pathways.

Future hypothesis: Molecular oxidative stress pathways have a regulatory role on AR protein maturation and activity.

Both TQ and ca27 increase the expression of the antioxidant (i.e., electrophile) response regulated transcripts. The expression of these transcripts is through the activation of Nrf2. Although I measured Nrf2 activity through a reporter assay and examined known regulated genes, these methods were indirect. Validating the direct interference of Nrf2 transcriptional activity with an electromobility shift assay (EMSA) would be essential in determining if these agents directly lead to Nrf2 transcriptional activity. It would also be important to determine if both TQ and ca27 lead to the increased activity of NQO1. Previous studies support that indeed both our agents induce NQO1 activity (13,18). However, it would be meaningful to determine if their down-regulation of the AR is potentiated by inhibition of NQO1 activity. Targeting NQO1 expression through knock-down experiments would begin to address how important the role of NQO1 is in the activity of these agents.

It is plausible that TQ's actions on the AR are due to its potential activity as an arylating electrophile. It has been reported that all three TQs (α-, γ-, δ-TQ) are redox cycling compounds but only the partially methylated quinones (γ- and δ-TQ) are arylating electrophiles that can lead to Michael adduct formation, which yield covalent bonds with nucleophiles such as cysteinyl thiols (19,20). It would be useful to determine if TQ directly binds to the AR and thus leads to adduct formation; or, if TQ's effects are more general, thus leading to cytotoxicity at later time points. Also, determining if other arylating electrophiles such as, γ-TQ or δ-TQ, inhibit AR protein expression in this series of experiments. In addition, determining if TQ's structure is modified or converted to the more potent γ-TQ through metabolism of α-TQ would provide further understanding of TQ's biological actions.

ca27 and TQ inhibit AR protein expression in a potentially transcriptional and proteasomal independent manner. Determining if ca27 and TQ increase AR protein turn-over by a pulse-chase assay would begin to address their role in AR translation. With the sensitivity of the ER and corresponding chaperones to cellular redox potential, inhibition of proper AR translation and folding may occur upon treatment with these agents. The ER is sensitive to redox transitions within the cell, due to the proper folding required by its retained chaperones. The thiol redox state within the ER has a much lower GSH/GSSG ratio than that found in the cytoplasm (21). This redox potential is optimal for disulfide bond formation and perturbations of this ratio can lead to ER stress. Protein disulfide isomerase (PDI) is essential in catalyzing disulfide bond formation and sensitive to changes in ER thiol redox potential (21,22). Therefore, TQ's pro-oxidant activity and modulation of GSH expression may induce ER stress and this activation may be attenuated by the presence of NAC.

The ER is a subcellular organelle in which secretory and membrane bound proteins are folded, stabilized by disulfide bonds, post-translationally modified (glycosylation), oligomerized and exported (19,23). The ER has a limited capacity to process proteins and the accumulation of misfolded proteins, redox or ionic changes within the ER lumen can lead to ER stress. The biological response to ER stress is activation of the UPR. The UPR mediates its effects through three ER transmembrane stress sensors PERK, IRE1 and ATF6 (23,24). Upon accumulation of misfolded proteins, ER chaperones bind and retain these proteins to prevent their aggregation and formation of large insoluble complexes (25). GRP78 (BiP) a HSP70 family member, represses PERK, IRE1 and ATF6 activity until the accumulation of misfolded proteins and then it releases the three sensors. The activation of these three UPR signaling cascades leads to the time-dependent increased expression of several transcripts including ATF4, $p58^{IPK}$, XBP-1 (spliced), ATF6 and CHOP (Fig 3). I demonstrated that TQ treatment leads to the significant increased expression of these transcripts (Chapter 3). In identifying potential pathways activated upon TQ treatment, gene expression profiles were generated from existing TQ microarray data (Appendix I). These profiles showed all three pathways were activated, but to focus my efforts, I evaluated the potential role the PERK pathway may be playing on TQ's down-regulation of the AR.

In 2008, Ogawa et al. (26) demonstrated γ-TQ's induction of glutathione (GSH) levels was dependent on the activating transcription factor 4 (ATF4). ATF4 is an important basic leucine zipper transcriptional regulator of the eukaryotic initiation factor (eIF2α) kinase pathway (26). PERK is a transmembrane serine/threonine kinase that phosphorylates eIF2α and Nrf2 (27,28). Phosphorylation of eIF2α attenuates translation initiation of most transcripts while increasing translation of select mRNAs such as ATF4 (29). The inhibition of translation has a two-fold cytoprotective function. First, the attenuation of protein synthesis prevents the further accumulation of misfolded or unfolded proteins. Second, this inhibition of protein synthesis results in the decreased consumption of reducing equivalents required for disulfide bond formation. PERK's additional regulation of cellular redox is the phosphorylation of Nrf2, resulting in the increased regulation of detoxifying enzymes (30) and the regulation of ATF4 which can increase GSH levels (26). Interference with this signaling pathway could prevent the downstream antioxidant affects. eIF2α translational inhibition can be inhibited by the selective dephosphorylation inhibitor salubrinal (31). We demonstrate that treatment with TQ in the presence of salubrinal significantly potentiates its down-regulation of AR protein expression (Appendix II). However, knock-down of PERK did not prevent TQ's down-regulation of AR protein expression (Appendix II). Therefore, PERK is not a critical factor in TQ's actions on the AR but may be playing a protective role against TQ's pro-oxidant activities.

One of the most up-regulated transcripts in response to ER stress is GRP78, but I did not observe this increase in my experiments. However, GRP78 is a HSP70 family member, another member HSP70B' is increased ~200 fold upon TQ treatment (Appendix I). Several other chaperones that may be playing a role in AR's proper folding are also increased according to the profiles generated by our microarray data. Hip/p48 (HSC 70 interacting protein and HSP70 co-chaperone) was inhibited upon TQ treatment according to our microarray profile data (Appendix I). Hip plays a major role in the initial stability of the AR with its intermediate chaperone complex for efficient folding (32). The correct folding of steroid hormone receptors into a ligand competent state may occur through an assembly line process that involves specific chaperones, HSP70 (HSC70), HSP40 (Ydj1), HOP (p60), HSP90 and Hip for these initial folding steps (32,33). Several HSP family members are modified in their expression upon TQ treatment, which may reduce AR proper folding. The induction of the PERK signaling cascade by TQ could lead to changes in chaperone gene profiles which prevent the proper folding of AR. TQ's significant down-regulation of the AR occurs within 48 hours, but I do not demonstrate the induction of UPR until later time points. TQ may be exerting its inhibition of existing AR protein through oxidative stress, but it inhibits AR *de novo* synthesis through the induction of ER stress pathways.

TQ and ca27 may serve as lead compounds. Both agents need to be validated *in vivo* for their bioavailability, potential cytotoxicity and down-regulation of AR protein expression. Although, we have begun to identify the mechanism(s) by which these agents inhibit AR protein expression further investigation into their potential reactivity *in vivo* needs to be determined. The potential reactivity of both TQ and ca27 raises concerns for the selectivity of their actions. These agents contain chemical moieties that are electrophilic in nature and determining their relative selectivity of AR down-regulation *in vivo* is required for their advancement.

TQ and ca27's inhibition of AR protein expression through their pro-oxidant activities demonstrates a novel mechanism for targeting the AR. The critical role the AR plays in the etiology of various diseases make it a meaningful target for the prevention and treatment of these diseases. My dissertation studies serve as a paradigm in experimental therapeutics that may provide insight into the development of AR targeted therapeutic strategies and a foundation for future studies in defining TQ and ca27's mechanism(s) of action.

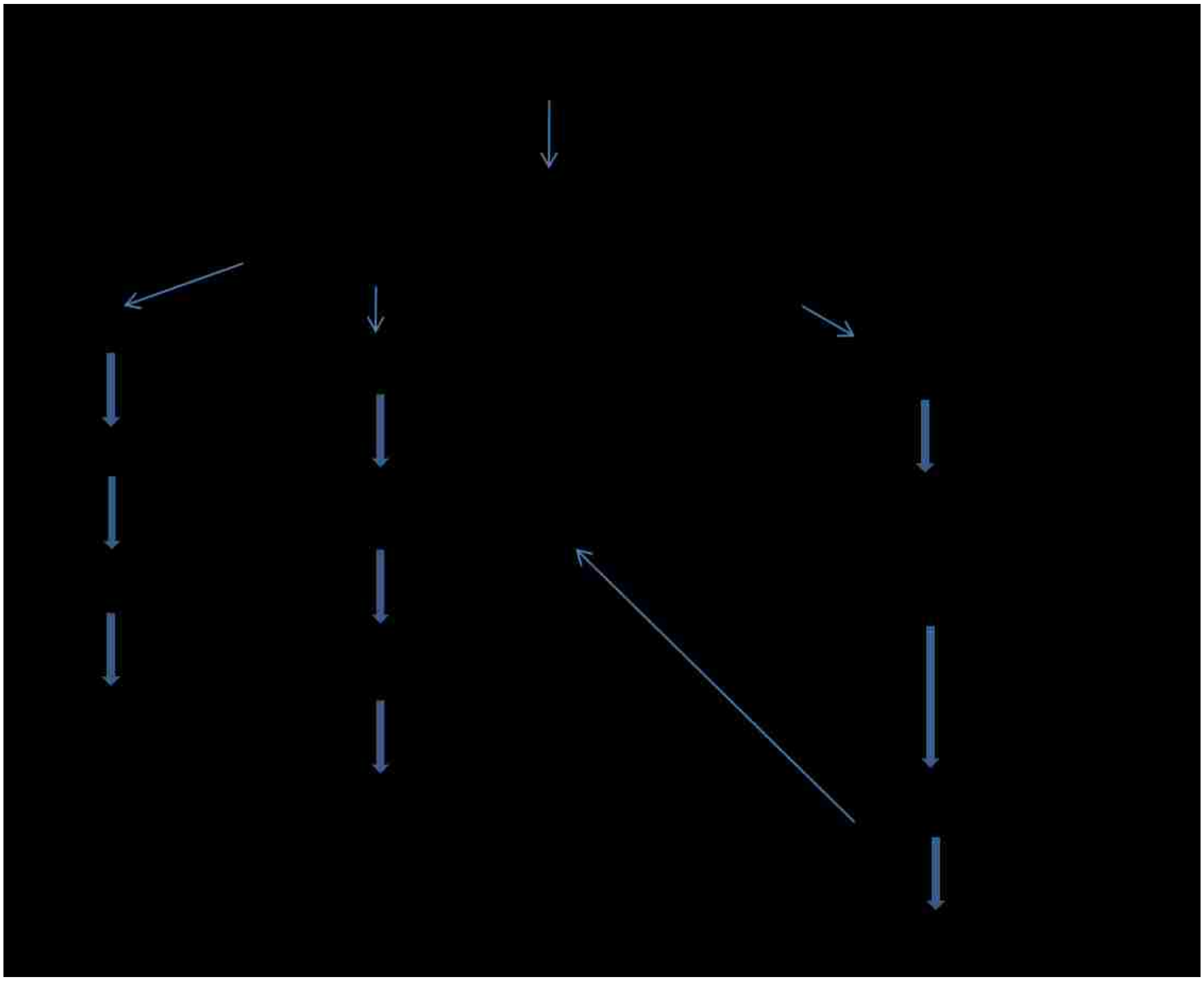


Fig. 3: – The three arms of the UPR signaling cascade; PERK, IRE1 and ATF6. Image adapted from Ref 25.

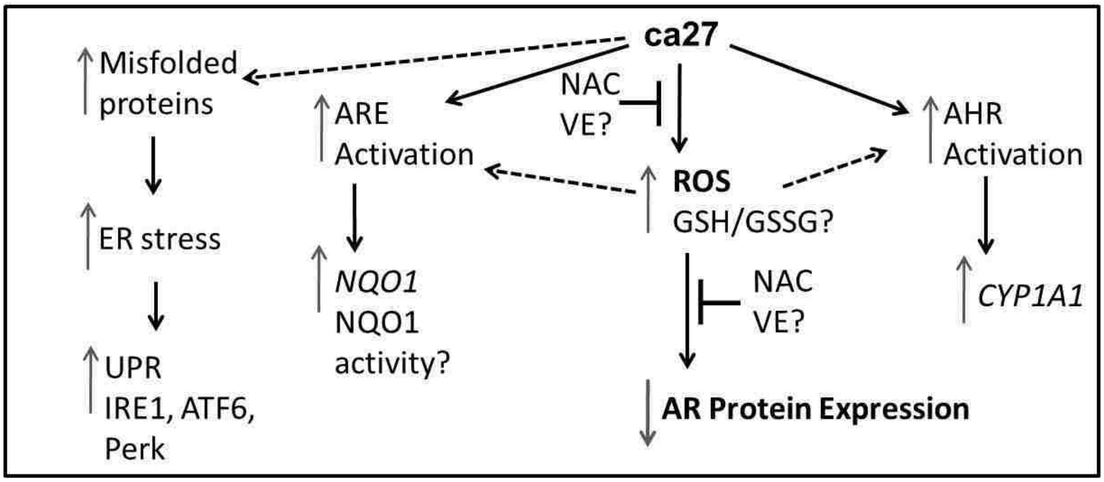


Fig. 4: Model for future directions identifying mechanisms of ca27's down-regulation of AR.

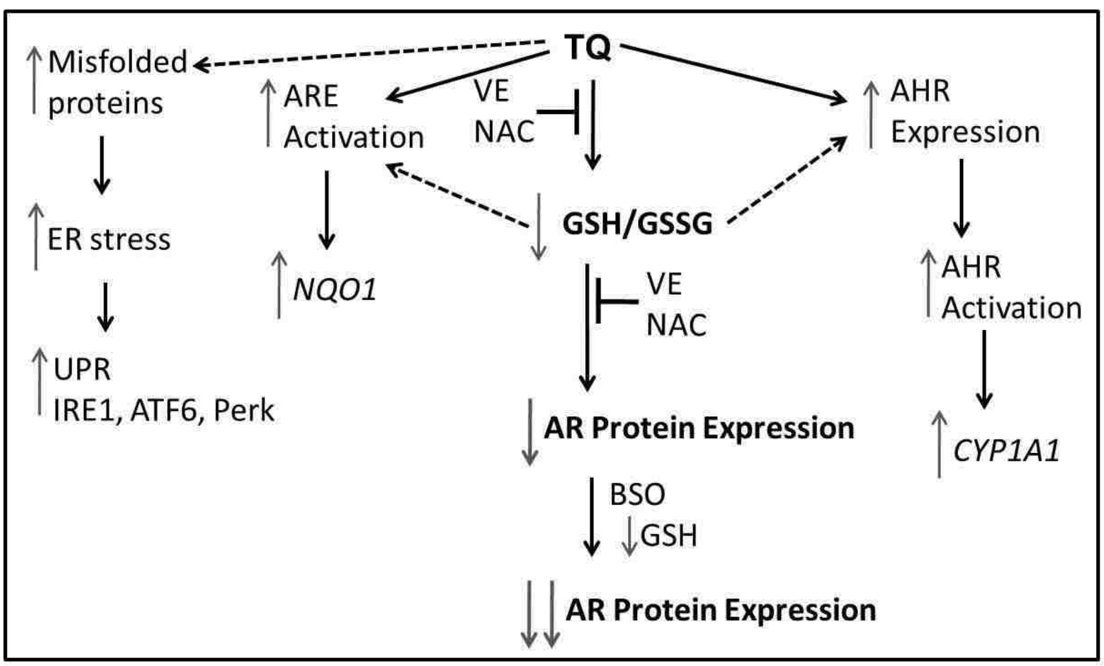


Fig. 5: Model for future directions identifying mechanisms of TQ's down-regulation of AR.

References

1. Grosse A, Bartsch S, Baniahmad A. Androgen receptor-mediated gene repression. Molecular and cellular endocrinology.
2. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. Semin Oncol 1999;26(4):407-421.
3. Ichikawa T, Suzuki H, Ueda T, Komiya A, Imamoto T, Kojima S. Hormone treatment for prostate cancer: current issues and future directions. Cancer chemotherapy and pharmacology 2005;56 Suppl 1:58-63.
4. Masai M, Sumiya H, Akimoto S, Yatani R, Chang CS, Liao SS, Shimazaki J. Immunohistochemical study of androgen receptor in benign hyperplastic and cancerous human prostates. The Prostate 1990;17(4):293-300.
5. Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL. Anti-oxidant activities of curcumin and related enones. Bioorg Med Chem 2005;13(11):3811-3820.
6. Fajardo AM, Mackenzie DA, Ji M, Deck LM, Vander Jagt DL, Thompson TA, Bisoffi M. The curcumin analog ca27 down-regulates androgen receptor through an oxidative stress mediated mechanism in human prostate cancer cells. The Prostate 2011.
7. Mosley CA, Liotta DC, Snyder JP. Highly active anticancer curcumin analogues. Advances in experimental medicine and biology 2007;595:77-103.
8. Weber WM, Hunsaker LA, Roybal CN, Bobrovnikova-Marjon EV, Abcouwer SF, Royer RE, Deck LM, Vander Jagt DL. Activation of NFkappaB is inhibited

by curcumin and related enones. Bioorganic & medicinal chemistry 2006;14(7):2450-2461.

9. Sies H. Glutathione and its role in cellular functions. Free radical biology & medicine 1999;27(9-10):916-921.

10. Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 2003;60(1):6-20.

11. Russell J, Spickett CM, Reglinski J, Smith WE, McMurray J, Abdullah IB. Alteration of the erythrocyte glutathione redox balance by N-acetylcysteine, captopril and exogenous glutathione. FEBS letters 1994;347(2-3):215-220.

12. Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T, Fujii-Kuriyama Y, Kato S. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 2007;446(7135):562-566.

13. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proceedings of the National Academy of Sciences of the United States of America 2001;98(6):3404-3409.

14. Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13(10):1145-1155.

15. Constantinou C, Papas A, Constantinou AI. Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs. International journal of cancer 2008;123(4):739-752.

16. Yamagata K, Ichinose S, Tagawa C, Tagami M. Vitamin E Regulates SMase Activity GSH levels, and Inhibits Neuronal Death in Stroke-Prone Spontaneously Hypertensive Rats during Hypoxia and Reoxygenaton. J Exp Stroke Transl Med 2009;2(2):41-48.

17. Tahan G, Aytac E, Aytekin H, Gunduz F, Dogusoy G, Aydin S, Tahan V, Uzun H. Vitamin E has a dual effect of anti-inflammatory and antioxidant activities in acetic acid-induced ulcerative colitis in rats. Canadian journal of surgery 2011;54(5):333-338.

18. Siegel D, Bolton EM, Burr JA, Liebler DC, Ross D. The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant. Molecular pharmacology 1997;52(2):300-305.

19. Wang X, Thomas B, Sachdeva R, Arterburn L, Frye L, Hatcher PG, Cornwell DG, Ma J. Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. Proceedings of the National Academy of Sciences of the United States of America 2006;103(10):3604-3609.

20. Sachdeva R, Thomas B, Wang X, Ma J, Jones KH, Hatcher PG, Cornwell DG. Tocopherol metabolism using thermochemolysis: chemical and biological properties of gamma-tocopherol, gamma-carboxyethyl-hydroxychroman, and their quinones. Chemical research in toxicology 2005;18(6):1018-1025.

21. Hwang C, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. Science (New York, NY 1992;257(5076):1496-1502.

22. Townsend DM. S-glutathionylation: indicator of cell stress and regulator of the unfolded protein response. Molecular interventions 2007;7(6):313-324.

23. Maattanen P, Gehring K, Bergeron JJ, Thomas DY. Protein quality control in the ER: the recognition of misfolded proteins. Seminars in cell & developmental biology 2010;21(5):500-511.

24. Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S, Samali A. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. European journal of pharmacology 2009;625(1-3):234-246.

25. Lai CW, Aronson DE, Snapp EL. BiP availability distinguishes states of homeostasis and stress in the endoplasmic reticulum of living cells. Molecular biology of the cell 2010;21(12):1909-1921.

26. Ogawa Y, Saito Y, Nishio K, Yoshida Y, Ashida H, Niki E. Gamma-tocopheryl quinone, not alpha-tocopheryl quinone, induces adaptive response through up-regulation of cellular glutathione and cysteine availability via activation of ATF4. Free radical research 2008;42(7):674-687.

27. Bobrovnikova-Marjon E, Grigoriadou C, Pytel D, Zhang F, Ye J, Koumenis C, Cavener D, Diehl JA. PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. Oncogene 2010;29(27):3881-3895.

28. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Molecular and cellular biology 2003;23(20):7198-7209.

29. Raven JF, Koromilas AE. PERK and PKR: old kinases learn new tricks. Cell cycle (Georgetown, Tex 2008;7(9):1146-1150.

30. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochemical and biophysical research communications 1997;236(2):313-322.

31. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science (New York, NY 2005;307(5711):935-939.

32. Prescott J, Coetzee GA. Molecular chaperones throughout the life cycle of the androgen receptor. Cancer letters 2006;231(1):12-19.

33. Langer T, Lu C, Echols H, Flanagan J, Hayer MK, Hartl FU. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. Nature 1992;356(6371):683-689.

APPENDICES

APPENDIX I: TQ Induces Activation of the Unfolded Protein Response Signaling Cascade

Table 1. TQ induces the signaling cascades of the unfolded protein response (UPR)

UPR regulated genes[1]	Expression Level (Fold Change)[2]	Accession Number	Function
IRE1	**11.47**	**NM_001433**	**endoplasmic reticulum to nucleus signalling 1**
ATF6	**4.579**	**NM_007348**	**activating transcription factor 6**
CHOP	**5.637**	**BC003637**	**DNA-damage-inducible transcript 3**
GADD34	**3.519**	**NM_014330**	**protein phosphatase 1, regulatory (inhibitor) subunit 15A**
EDEM	**2.755**	**AW139300**	**ER degradation enhancing alpha mannosidase-like**
ERdj4	**3.148**	**NM_012328**	**DnaJ (Hsp40) homolog, subfamily B, member 9**
ERdj5	**3.482**	**BG168666**	**ER-resident protein ERdj5**
ATF3	**17.78**	**AB078026**	**activating transcription factor 3**

1 UPR regulated transcripts identified from pathway profile analysis Microarray studies were performed as described previously (Chapter 3). Arrays were analyzed by the UNM Keck-UNM Resources facility.

2 Compared to vehicle control in LNCaP cells

Table 2 TQ induces XBP-1 regulated transcripts. XBP-1 transcriptional activation is regulated by the IRE1 pathway.

XBP-1 Regulated Genes[1]	Expression Level[2]	Accession Number	Function
ERdj4	**3.148**	**NM_012328**	**DnaJ (Hsp40) homolog, subfamily B, member 9**
$P58^{IPK}$	**2.882**	**NM_006260**	**DnaJ (Hsp40) homolog, subfamily C, member 3**
Herp2	**9.374**	**NM_012258**	**hairy/enhancer-of-split related with YRPW motif 1**
EDEM	**2.755**	**AW139300**	**ER degradation enhancing alpha mannosidase-like**

[1] XBP-1 regulated transcripts identified from pathway profile analysis Microarray studies were performed as described previously (Chapter 3). Arrays were analyzed by the UNM Keck-UNM Resources facility.

[2] Compared to vehicle control in LNCaP cells

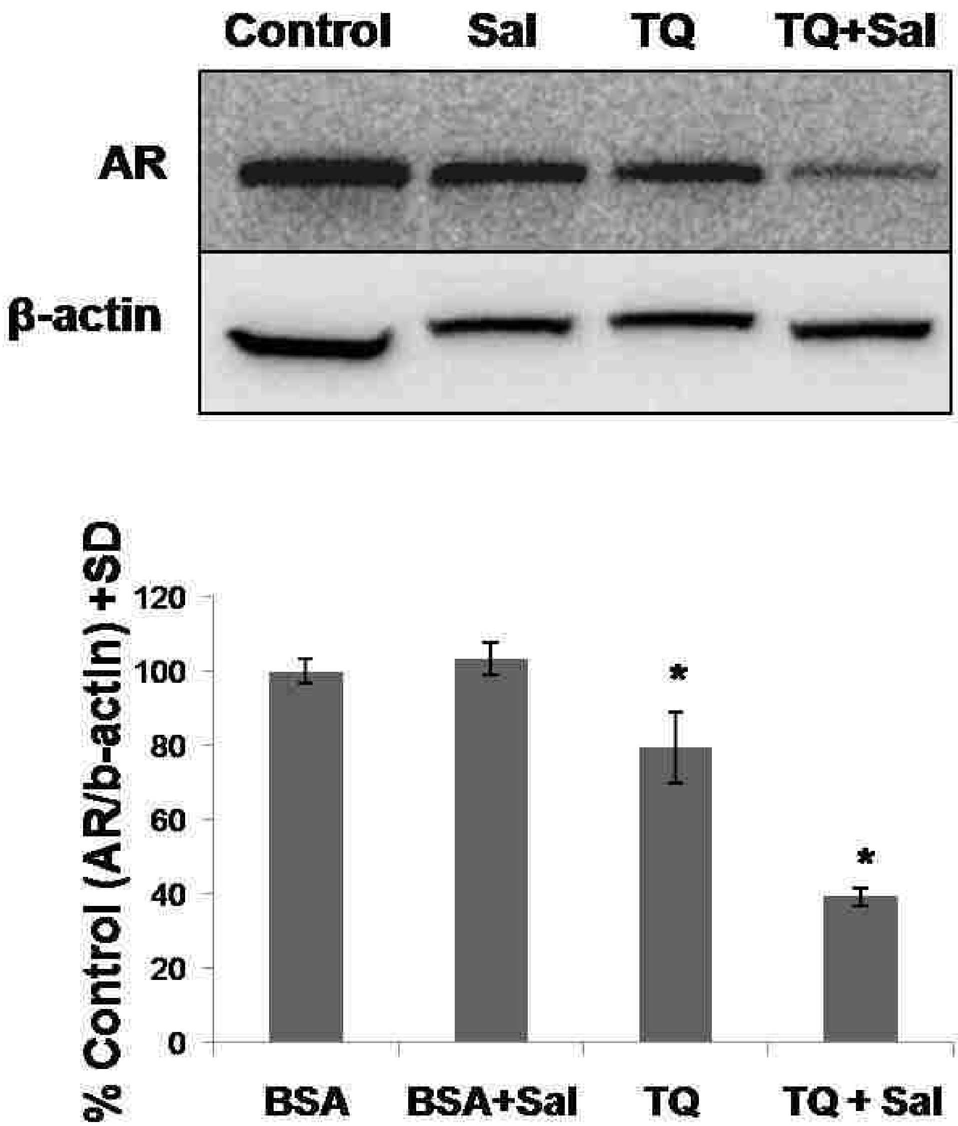
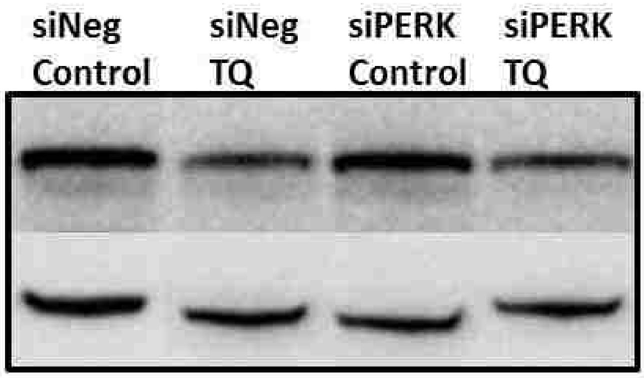


Fig. 1. TQ's down-regulation of AR protein expression is potentiated by the eIF2α inhibitor salubrinal. LNCaP cells were treated for 48 h with 25 µM TQ or vehicle control (BSA) in the presence or absence of 10 µM salubrinal. Top panel represents immunoblots of AR and β-actin expression. Graph represents values of AR protein expression normalized to β-actin expression, * denote $P < 0.05$ compared to control.



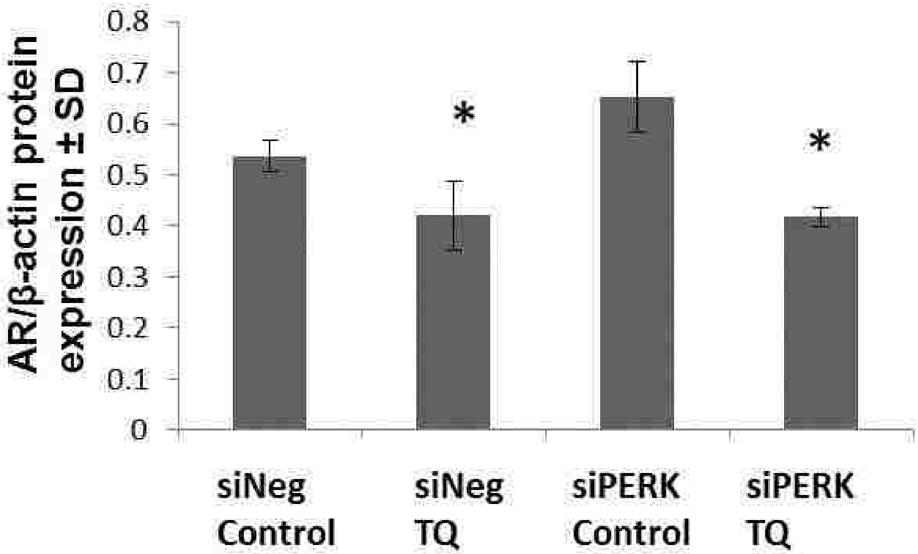


Fig. 2. Knock-down of PERK expression does not prevent TQ's down-regulation of AR protein expression. PERK expression was knocked down using RNAi. LNCaP cells transfected with 40 µM siPERK or scrambled Negative Control (siNC) for 48 h and treated with 25 µM TQ for an additional 36 h, AR protein expression was determined by immunoblot. Top panel represents immunoblots of AR and β-actin expression. Graph represents values of AR protein expression normalized to β-actin expression, * denote $P < 0.05$ compared to control.

APPENDIX II: TQ Modulates Various Chaperones Expression

Table 1. TQ modulates expression of multiple chaperone transcripts.

Chaperone Genes[1]	Expression Level (Fold Change)[2]	Accession Number	Function
HSPA6	386.7	NM_002155	heat shock 70kDa protein 6 (HSP70B')
HSP70B	75.78	X51757cds	Human heat-shock protein HSP70B' gene
MDG1; ERdj4; MST049; MSTP049	3.148	NM_012328	DnaJ (Hsp40) homolog, subfamily B, member 9
P58; HP58; PRKRI; P58IPK	2.882	NM_006260	DnaJ (Hsp40) homolog, subfamily C, member 3
GBP; FLJ20539; HSPA5BP1	0.354	NM_017870	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) binding protein 1
HIP1	0.497	AU145049	Huntingtin interacting protein 1

[1] Protein chaperone transcripts identified from pathway profile analysis Microarray studies were performed as described previously (Chapter 3). Arrays were analyzed by the UNM Keck-UNM Resources facility.

[2] Compared to vehicle control in LNCaP cells

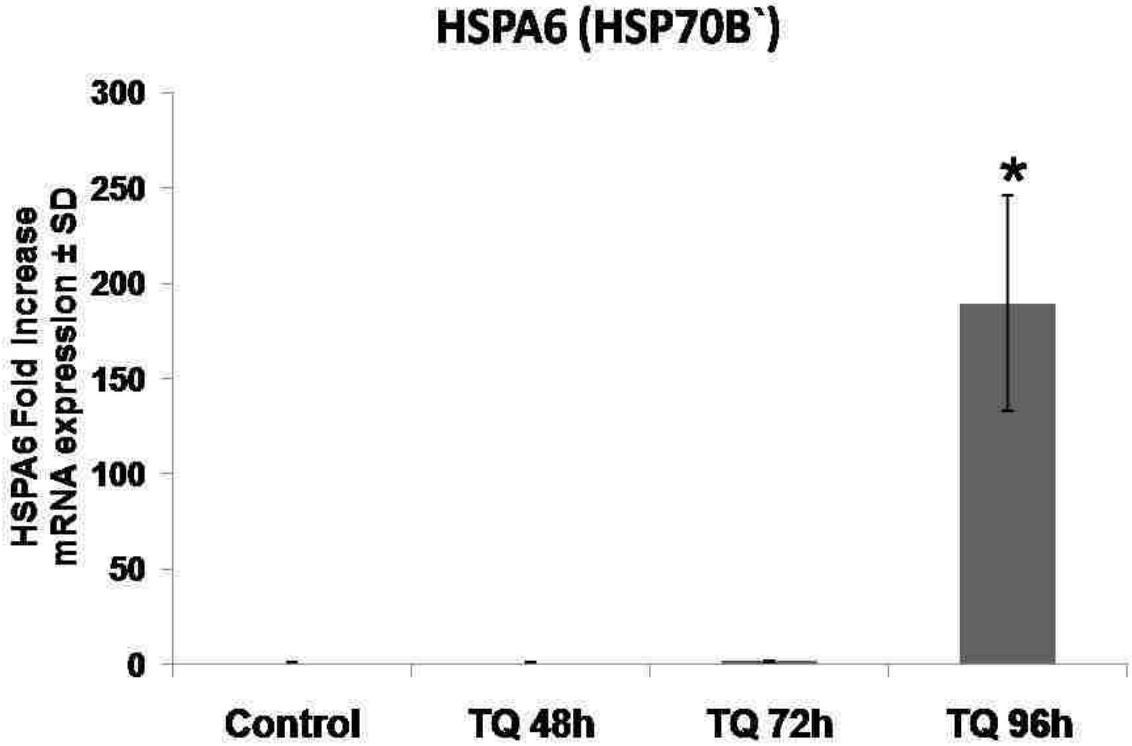


Fig. 1. TQ induces the expression of HSPA6 (HSP70B'). LNCaP cells were treated with 25 µM TQ or vehicle control for 48-96 h. *HSPA6* and *GAPDH* mRNAs were measured by qRT-PCR. Graph represents values of *HSPA6* mRNA expression normalized to *GAPDH* expression. * denote $P < 0.05$ compared to control.

APPENDIX III: TQ Does Not Reduce Cell Viability in Androgen Responsive Prostate Cancer Cells

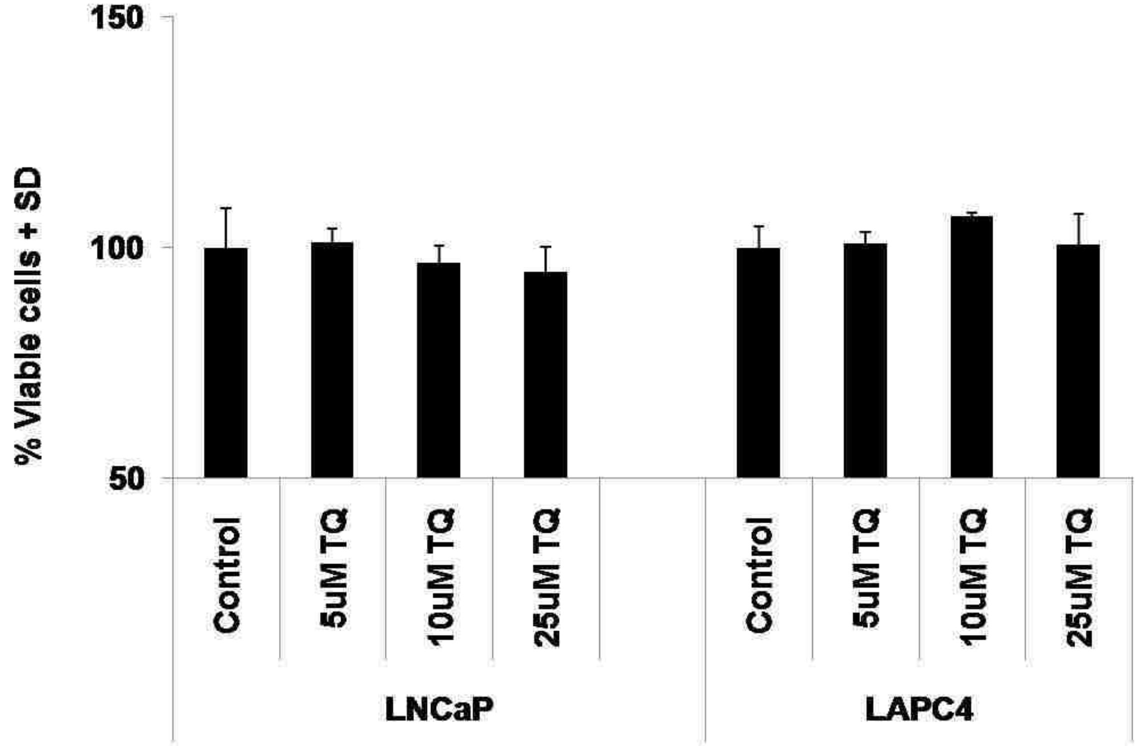


Fig. 1. TQ does not inhibit cell viability in LNCaP and LAPC4 cells after 96 h. LNCaP and LAPC4 cells were treated with 5-25 µM TQ or vehicle control for 96 h. Cells viability was determined by total cell counts and trypan blue positive cell counts respectively. Cell viability is expressed as percent of control.

APPENDIX IV: Reducing Agent DTT or Pro-oxidant H_2O_2 Do Not Inhibit Androgen Receptor Protein Expression in Prostate Cancer Cells

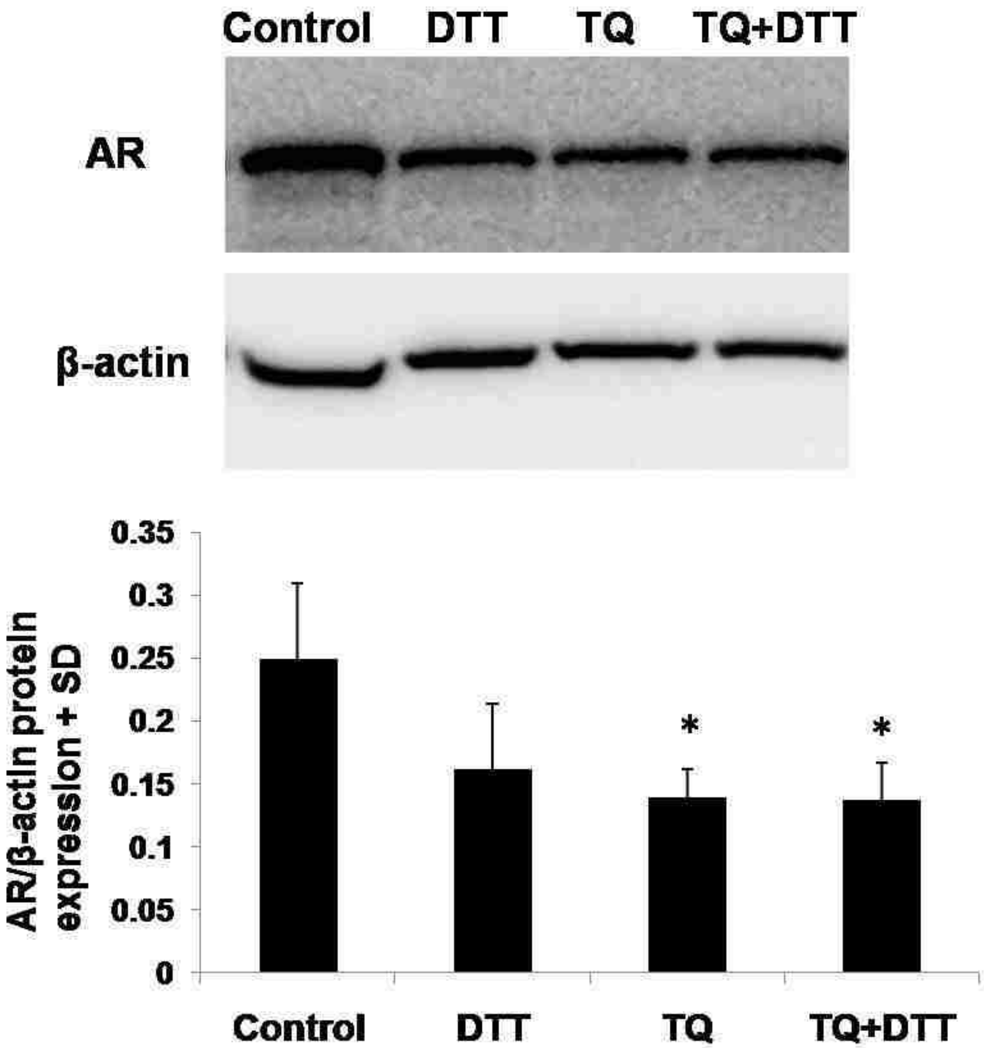


Fig. 1. TQ's down-regulation of AR protein expression is not attenuated by DTT. LNCaP cells were pretreated with 1 mM DTT for 24 h and then treated with 25 µM TQ or vehicle in the presence or absence of 1 mM DTT for an additional 48 h. Top panel represents immunoblots of AR and β-actin expression. Graph represents values of AR protein expression normalized to β-actin expression, * denote $P < 0.05$ compared to control.

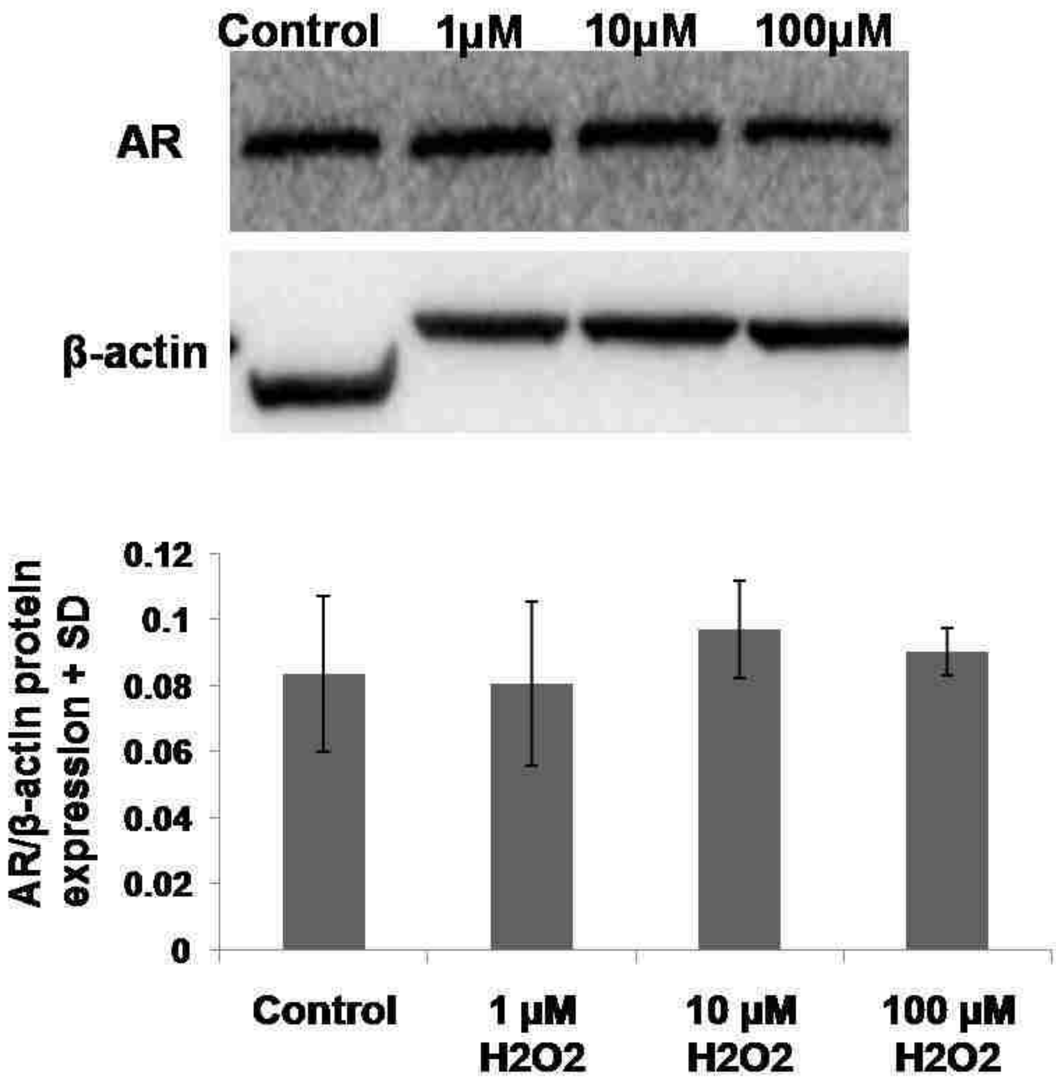


Fig. 2. H_2O_2 does not down-regulate AR protein expression at 100 μM for 3 h. LAPC4 cells were treated with 1, 10, 100 μM or vehicle control for 3 h. Top panel represents immunoblots of AR and β-actin expression. Graph represents values of AR protein expression normalized to β-actin expression, * denote $P < 0.05$ compared to control.

APPENDIX V: ca27 Induces Glucocorticoid Receptor mRNA Expression in Prostate Cancer Cells

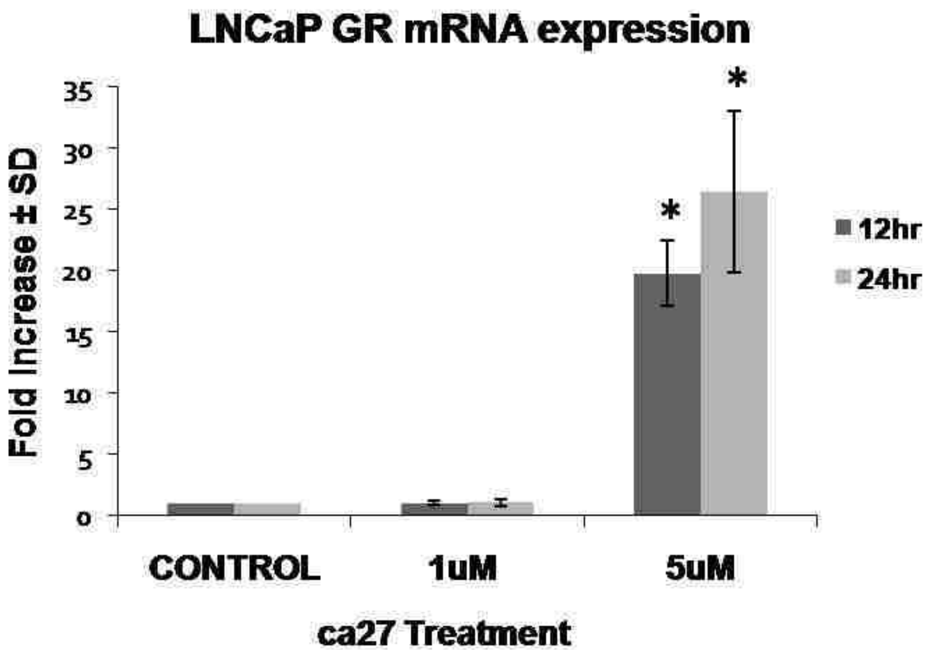


Fig. 1. ca27 induces glucocorticoid receptor (GR) mRNA expression in LNCaP cells. LNCaP human CaP cells were treated with vehicle control of 1 µM or 5 µM ca27 for 12 and 24 h. *GR* and *GAPDH* mRNAs were measured by qRT-PCR. Graph represents values of *HSPA6* mRNA expression normalized to *GAPDH* expression. * denote $P < 0.05$ compared to control.

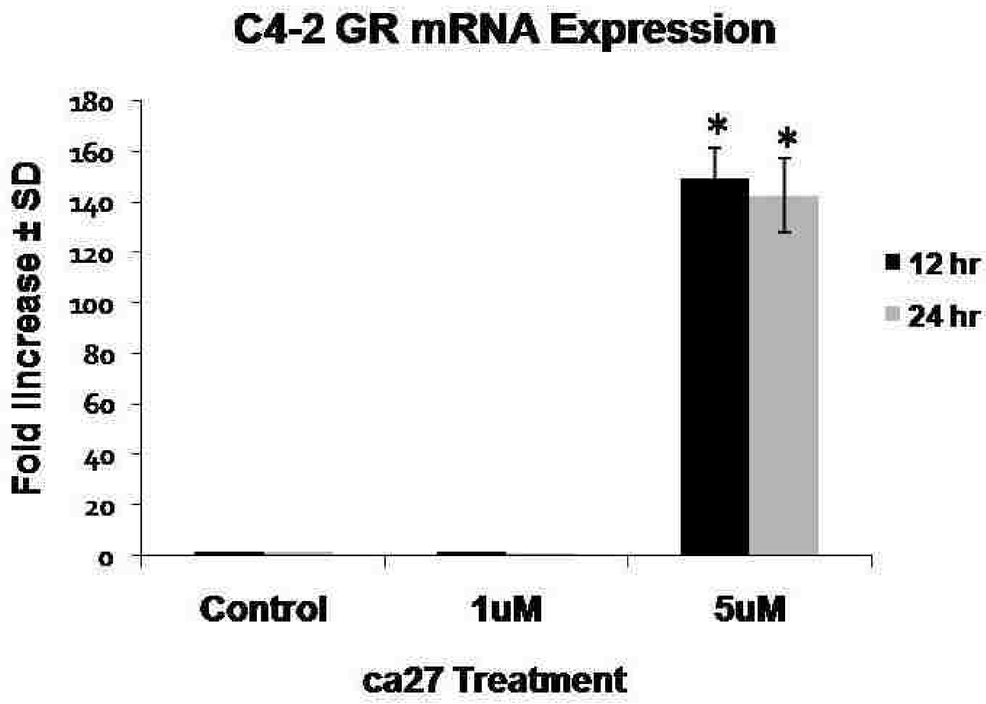


Fig. 2. ca27 induces glucocorticoid receptor (GR) mRNA expression in C4-2. C4-2 human CaP cells were treated with vehicle control of 1 µM or 5 µM ca27 for 12 and 24 h. *GR* and *GAPDH* mRNAs were measured by qRT-PCR. Graph represents values of *HSPA6* mRNA expression normalized to *GAPDH* expression. * denote $P < 0.05$ compared to control.