HHS Public Access

Author manuscript

Nutr Cancer. Author manuscript; available in PMC 2018 September 06.

Published in final edited form as:

Nutr Cancer. 2017 ; 69(6): 932–942. doi:10.1080/01635581.2017.1339818.

Hydroxytyrosol Induces Apoptosis, Cell Cycle Arrest and Suppresses Multiple Oncogenic Signaling Pathways in Prostate Cancer Cells

Haseeb Zubair[#1], **Arun Bhardwaj**[#1,*], **Aamir Ahmad**[1], **Sanjeev Kumar Srivastava**[1], **Mohammad Aslam Khan**[1], **Girijesh Kumar Patel**[1], **Seema Singh**[1,2], and **Ajay Pratap Singh**[1,2,*]

[1]Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604, USA;

[2]Department of Biochemistry and Molecular Biology, College of Medicine, University of South Alabama, Mobile, AL 36688, USA

[#] These authors contributed equally to this work.

Abstract

Scope: Hydroxytyrosol (HT), a polyphenol from olives, is a potential anticancer agent. This study was designed to evaluate the anticancer activity of HT against prostate cancer cells, and the mechanism thereof.

Methods and Results: Treatment of LNCaP and C4–2 prostate cancer cells with HT resulted in a dose-dependent inhibition of proliferation. This was in contrast to HT's ineffectiveness against normal prostate epithelial cells RWPE1 and PWLE2, suggesting cancer cells-specific effect. HT induced G1/S cell cycle arrest, with inhibition of cyclins D1/E and cdk2/4, and induction of inhibitory p21/p27. HT also induced apoptosis, as confirmed by flow cytometry, caspase activation, PARP cleavage and BAX/Bcl-2 ratio. It inhibited the phosphorylation of Akt / STAT3, and induced cytoplasmic retention of NF-κB, which may explain its observed effects. Finally, HT inhibited AR expression and the secretion of AR-responsive prostate-specific antigen.

Conclusion: Castration-resistant prostate cancers retain AR signaling and are often marked by activated Akt, NF-κB and STAT3 signaling. Our results establish a pleiotropic activity of HT against these oncogenic signaling pathways. Combined with its non-toxic effects against normal cells, our results support further testing of HT for prostate cancer therapy.

[*]Ajay P. Singh, Ph.D. Department of ncologic Sciences Mitchell Cancer Institute University of South Alabama 1660 Springhill Avenue Mobile, AL 36604-1405, U.S.A. Phone: 251-445-9843; Fax: 251-460-6994 asingh@health.southalabama.edu. [*]Arun Bhardwaj, Ph.D. Department of Oncologic Sciences Mitchell Cancer Institute University of South Alabama 1660 Springhill Avenue Mobile, AL 36604-1405, U.S.A. Phone: 251-445-9843; Fax: 251-460-6994 abhardwaj@health.southalabama.edu.

Author Contributions: APS and AB designed the study; AB and SKS performed cell viability and transcriptional activity assays; HZ, AB, SKS and GKP performed western blot analysis; HZ, AB and MAK designed and analyzed experiments involving flow cytometry analysis; HZ, AB, AA and SKS analyzed and interpreted results, and drafted the manuscript; HZ and AB performed statistical analysis; SS and APS supervised the study, provided resources/infrastructure and edited the manuscript. All authors read the manuscript, provided feedback and approved the submission.

Keywords

Prostate cancer; hydroxytyrosol; apoptosis; cell proliferation; androgen receptor; NF-κB

Introduction

Prostate cancer is the most frequently diagnosed cancer in men in the United States, with a prediction of 180,890 new cases in 2016, accounting for 21% of all cancer diagnoses in the males (1). In recent years there has been a push towards exploring diet-derived factors for their anticancer potential (2). This is largely due to epidemiological studies connecting consumption of certain foods with reduced cancer incidence. In context of prostate cancer, dietary polyphenols have been proposed as promising candidates for future clinical management (3). These non-toxic, well-tolerated compounds are key modulators of several signaling pathways (4) and a renewed interest in them can be attributed to their activity against stem cells, microRNAs as well as the epigenetic changes (3; 5). Epidemiological studies have also suggested a decreased risk of cancer in populations with high olive intake (6) and there has been an interest in evaluating traditional Mediterranean diet, with abundance of olive oil consumption, for possible reduced prostate cancer risk (7). Olive oil has significantly high levels of phenolic acids, particularly the 3,4-dihydroxyphenyl ethanol (Figure 1A), also referred to as hydroxytyrosol (HT). In addition to olive oil, red wines are also a good source of HT (8). Additionally, HT is also produced endogenously as part of dopamine metabolism.

The antitumor activity of HT, mostly *in vitro*, against different human cancers such as breast (9–11), colon (9; 12–14), liver (15; 16), leukemia (14; 17), lung (18) etc. has been reported. However, our understanding of the activity and mechanism of action of HT against prostate cancer cells is rather limited. While an early report (19) suggested generation of reactive oxygen species (ROS) by HT in androgen receptor (AR)-negative prostate cancer cells PC3 and DU145 leading to loss of viability, a more recent one (9) compared prostate cancer cells with breast and colon cancer cells in terms of their ability to scavenge H_2O_2 oxygen species with the resulting effects on cell proliferation.

Clinical management of prostate cancer involves surgery, radiation therapy, chemotherapy or a combination of these options. Although early stage prostate cancers can be treated with androgen depletion therapy by means of chemical or surgical castration; relapse is often observed in the resulting castration-resistant prostate cancers (CRPC). Since these advanced and aggressive prostate cancers attain an active androgen-independent AR signaling which is considered responsible for their relapse and aggressiveness, it is important to fully elucidate the mechanism of HT's anticancer action in AR-expressing cancer cells. Towards achieving this goal, here we report an activity of HT against androgen dependent LNCaP and the androgen independent C4–2 prostate cancer cells, along with an analysis of several signaling factors that are modulated by HT, such as NF-κB (20), Akt (21) and STAT3 (22) that play an important role in progression to CRPC, resulting in efficient induction of apoptosis and cell cycle arrest.

Materials and Methods

Reagents

Roswell park memorial institute (RPMI-1640) medium and Keratinocyte serum free medium were obtained from Thermo Scientific (Logan, UT) and Gelantis (San Diego, CA), respectively. Fetal-bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Penicillin, streptomycin and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). 3,4-dihydroxyphenyl ethanol (DHE/HT) was purchased from LKT Laboratories (St. Paul, MN). FuGENE transfection reagent, phosphatase/protease inhibitors cocktail and cell proliferation reagent WST-1 were procured from Roche Diagnostics (Mannheim, Germany). Propidium iodide/RNAse staining buffer and PE Annexin V apoptosis detection kit were purchased from BD Bioscience (San Diego, CA). Nuclear extract kit was procured from Active Motif, LLC (Carlsbad, CA). Antibodies against PARP1, caspase-7, pBAD, Bcl-2, and Bax (Ser32/36) (rabbit polyclonal), Bcl-xl and NF-κB/p65 (mouse monoclonal) were obtained from Cell Signaling Technology (Beverly, MA). Anti-caspase-3 antibody (rabbit polyclonal) was from GeneTex (Irvine, CA).. Antibodies (rabbit monoclonal) against Akt, p-Akt, androgen-receptor, and PSA were from Epitomics (Burlingame, CA). Antibodies against p21, Cdk4 (mouse monoclonal), p27, cyclin D1, cyclin E, Cdk2 (rabbit polyclonal), and horseradish peroxidase-conjugated secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin (mouse monoclonal-HRP conjugated) antibody was purchased from Sigma-Aldrich (St. Louis, MO). ECL plus western blotting detection kit was procured from Thermo Scientific. pGL4.32 [luc2P/NF-κB -RE/Hygro] plasmid, pRL-TK plasmid, Caspase-Glo 3/7 substrate and Dual Luciferase Assay System kit were from Promega (Madison, WI). Cignal™ AR Androgen Receptor Assay Kit was purchased from SA Biosciences (Frederick, MD).

Cell culture and treatments

The human prostate cancer cell lines LNCaP (ATCC, Rockville, MD) and C4–2 (UroCor Inc., Oklahoma City, OK) were maintained in culture as adherent monolayer in RPMI-1640 supplemented with 5% (v/v) FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). The human normal prostate epithelial cell lines RWPE1 and RWPE2 (ATCC) were cultured in keratinocyte serum free medium containing 50 mg/ml gentamycin, 0.05 mg/ml bovine pituitary extract (BPE), and 5 ng/mL epidermal growth factor. Cells were maintained in 5% CO2 humidified incubator at 37^{o}C. Short tandem repeats (STR) genotyping and intermittent testing for androgen-responsiveness (growth and androgen-receptor activity) was used as a way to authenticate the androgen dependent prostate cancer cell lines. For treatment, stock solution of HT (10 mM) was prepared in molecular grade water and stored at −20°C in 50μL aliquot, and diluted with fresh complete medium immediately before use. Cells were treated with various concentrations of HT (as specified in individual figure legends).

Cell growth assay

Cells were seeded overnight in 96 well plates (5000 cells/well). Cell viability in the treated cells was examined after 48 and 72 h by using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene di-sulfonate) assay kit as per manufacturer's instructions with appropriate controls. This assay is based on the conversion of tetrazole to

water-soluble formazan in metabolically active cells. The absorbance of the formazan was measured at a wavelength of 450 nm, with background subtraction at 630, using a Bio-Rad Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). Growth was calculated as percent viability = [(A/B) × 100], where A and B are the absorbance of treated and control cells, respectively.

Cell-cycle analysis

The effect of HT treatment on cell cycle progression was determined by flow cytometry following staining with propidium iodide (PI). In brief, cells (1×10^6 cells/well) were seeded in 6 well plates and synchronized by culturing them in serum free media. After synchronization, medium was replaced with complete medium containing desired concentrations of HT. Floating and attached cells were collected after 24h of treatment and fixed in 70% ethanol overnight at 4°C. The cells were then stained with PI, using PI/RNase staining buffer for 1 h at 37°C. Stained cells were analyzed by flow-cytometry on a BD-FACS Canto™ II (Becton-Dickinson, San Jose, CA) to calculate the percentage of cell population in various phases of cell cycle using Mod Fit LT software.

Apoptosis assay

LNCaP and C4–2 cells (1×10^6 cells/well) were seeded in 6 well plates. At ~60–70% confluence, cells were treated with various concentrations of HT for 48h. Following treatment, cells were harvested, and stained with 7-Amino-Actinomycin (7-AAD) and PE Annexin V, using PE Annexin V Apoptosis Detection Kit I and analyzed by flow cytometry. Percentage of cell population in apoptosis was calculated using Mod Fit LT software (Verity Software House, Topsham, ME).

Caspase activity assay

Activation of caspase 3/7 after treatment of HT was measured using Caspase-Glo assay (Promega, Madison USA), according to the manufacturer's protocol, with appropriate controls. Briefly, cells were grown in 96 well plates and treated with different concentrations of HT. After 48h of treatment, 100 μL of Caspase-Glo substrate was added to each well, mixed gently for 30 seconds and incubated for 2h at room temperature. The luminescence of each sample was measured in a plate-reading luminometer (Thermo Labsystems).

Nuclear and cytoplasmic fractionation

The preparation of cytoplasmic and nuclear extracts was performed using the Nuclear Extract Kit. In brief, cells were washed following treatment with 1 mL ice-cold PBS/phosphatase inhibitors, lysed in 500 μL hypotonic buffer and then centrifuged at 14,000g for 30 s at 4°C. After collecting supernatant (cytoplasmic fraction), pellets were resuspended in 50 μL complete lysis buffer, and centrifuged at 14,000g for 10 min at 4°C, and supernatant (nuclear fraction) were stored at −80°C until further use.

Western blot analysis

Cells were processed for protein extraction and western blotting was done as described earlier (Bhardwaj et al., 2011). Immunodetection was carried out using specific antibodies:

Akt, pAkt, STAT3, p-STAT3, AR, Bcl-xl, Bax (1:1000), PSA (1:2500), p21, p27, cyclin A1, cyclin E (1: 200) and β-actin (1:20000). All secondary antibodies were used at 1:2500 dilutions. Blots were processed with ECL plus Western Blotting detection kit and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan). Densitometry was performed using an AlphaImager (Alpha Innotech Corp., San Leandro, CA).

Androgen receptor and NF-κB transcriptional activity assay

Prostate cancer cells were seeded (5×10^5 cells/well) in 12-well plate, at ~ 60% confluence level, the cells were transiently transfected with 1 μg AR reporter, negative-control and positive-control plasmids (for AR activity); 1μg of NF-κB-luciferase promoter-reporter construct (pGL4.32) and 0.5 μg of control reporter plasmid (pRL-TK), containing *Renilla reniformis* luciferase gene downstream of the TK promoter (for NF-κB activity) using FuGENE transfection reagent as per manufacturer's instructions. After 24 h of transfection, cells were treated with HT as described in figure legend for next 48 h and total protein was isolated in passive lysis buffer. Firefly (for AR and NF-κB activity) and Renilla (for internal normalization) luciferase activities were measured using a Dual-Luciferase assay kit.

Statistical analysis

All the experiments were performed three times, independently. The data obtained were expressed as 'mean ± standard deviation'. Wherever appropriate, the data were also subjected to unpaired two tailed Student's t-test. A value of $p < 0.05$ was considered as significant.

Results

HT selectively decreases the viability of prostate cancer cells

We first examined the dose-dependent effect of HT on cell viability of LNCaP and C4–2 and compared it to its effect on normal human prostate epithelial cells RWPE1 and RWPE2, by WST-1 assay. It was observed that both the prostate cancer cell lines were sensitive to HT treatment, as compared to the normal prostate epithelial cells (Figure 1B). The IC_{50} values of HT against LNCaP (190 and 86.9 μM after 48 and 72h, respectively) and C4–2 (176 and 76.5 μM after 48 and 72h, respectively) were significantly lower than the IC_{50} values against RWPE1 and RWPE2 at both the tested time points. It was observed that the exposure of cells to HT for 48 hours resulted in significant morphological changes, compared to their respective untreated controls when viewed under a light microscope (Figure 1C). With the increasing concentrations of HT, cells became round, shrunken and subsequently detached. Since at 48 hours of HT exposure, the cells exhibited significant reduction in growth at the doses tested, further experiments were carried out at this dose. Thus, our data suggests that HT can selectively inhibit prostate cancer cell lines and has minimal effect of normal prostate epithelial cells.

HT arrests prostate cancer cells in G1/S phase and induces apoptosis

Decrease in viability of a cell population could be due to cell growth inhibition or apoptosis induction. Therefore, we determined the effects of HT on cell cycle progression and apoptosis in prostate cancer cells LNCaP and C4–2. Cell cycle analysis at 48 hours after treatment with increasing concentrations of HT demonstrated an increase in the percentage of cells in the G_1 phase with a concomitant decrease in cells in S-phase in both the cell lines tested, as compared to the untreated cells (Figure 2). The observed maximum fold change was ~2.1 and ~2.3 in LNCaP and C4–2, respectively, suggesting an inhibition of transition of cells from G_1 to S phase.

Since the observed morphological changes in HT-treated cells were similar to cells undergoing apoptosis, we also examined the induction of apoptosis by HT in these prostate cancer cells. A significant increase in apoptosis of HT-treated prostate cancer cells was observed with the increasing concentrations of HT (Figure 3A), as determined by flow cytometry. We then proceeded to examine the molecular events associated with apoptosis. We analyzed the activity of caspase 3/7 and cleavage of PARP1 using luminescence and immunoblot assays, respectively. A dose dependent increase in the activity of caspase 3/7 (Figure 3B), cleavage of caspase 3/7 (Supplementary Figure 1) and PARP1 (Figure 3C) was observed in both cell lines.

HT alters the survival- and cell cycle-associated proteins

As evident from the above observations, HT affects cell proliferation, cell cycle as well as apoptosis. Therefore, we next investigated the various factors that play an important role in these cellular phenomena. Looking first at the apoptotic factors, we observed HT-induced down-regulation of expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL in a dose-dependent manner with simultaneous induction of Bax, a pro-apoptotic protein (Figure 4A), thereby leading to an increase in the ratio of Bax/Bcl-2 as well as Bax/Bcl-xL (Figure 4B) in both the cancer cell lines tested. The Bcl-2 family is just upstream of irreversible cellular damage and, therefore, a critical checkpoint for apoptotic cell death. As HT inhibits the expression of pro-survival Bcl-2, with concomitant induction of apoptosis-inducing BAX, this tilts the balance in favor of BAX in the cancer cells, marked by increased BAX/Bcl-2 ratio. In the absence of an apoptotic signal, Bcl-2 is relatively in excess and Bcl-2 homodimers or the Bcl-2-BAX heterodimers are present in the cytosol, both of which support survival and proliferation of cells. HT-induced inhibition of Bcl-2 expression / induction of BAX lead to disruption of these associations, resulting in predominantly BAX homodimers, leading to apoptosis. A dose-dependent effect of HT on various cell cycle associated proteins was also observed (Figure 4C). Expression of cyclins (D1 and E) and cyclin-dependent kinases (Cdk2 and Cdk4) decreased while that of cyclin-dependent kinase inhibitors (p21 and p27) increased after HT treatment, in both LNCaP and C4–2 prostate cancer cells. These findings demonstrate that HT can alter expression of proteins involved in the regulation of apoptosis as well as cell cycle to confer its growth inhibitory effect.

Inhibition of Akt, STAT3 and NF-κB pathways by HT in prostate cancer cell lines

Activation of PI3K/Akt and STAT3 signaling pathways is known to be important for survival and proliferation of prostate cancer cells, and these pathways serve as hubs for many

different signaling cascades. As such, their modulation by a potential anticancer agent is therapeutically relevant. Therefore, we examined the effects of HT on these signaling molecules, by immunoblot analysis. We observed a dose-dependent inhibition of Akt and STAT3 activation by HT, as suggested by the decrease in their respective phosphorylations (Figure 5A). No effect, however, was observed on total Akt and STAT3 proteins. NF-κB is another key factor which facilitates tumor growth by targeting genes involved in the apoptosis and cell cycle. Therefore, we examined the effect of HT on transcriptional activity of NF-κB -responsive promoter in a luciferase reporter assay. We detected that the transcriptional activity of NF-κB was considerably decreased, dose-dependently, by HT in both the cell lines (Figure 5B). Since transcriptional activity of NF-κB is directly correlated with its nuclear localization, we examined the cellular distribution of NF-κB after HT-treatment, in cytoplasmic and nuclear fractions, by immunoblot assay. The levels of NF-κB in the nuclear fraction of HT-treated cells decreased, as compared to untreated cells, with a simultaneous increase in the cytoplasmic fraction (Figure 5C).

HT downregulates AR expression

Lastly, we studied an effect of HT on AR signaling because of its importance in prostate cancer. NF-κB expression in prostate cancer cells has been shown to significantly increase AR mRNA and protein levels (23). Since HT was found capable of inactivating NF-κB, and NF-κB inhibition decreases AR expression levels, prostate-specific antigen (PSA) secretion, and proliferation of prostate cancer cells, we were interested in investigating the transactivation activity of AR and the levels of PSA after HT treatment. Using the luciferase assay system, we found a significant inhibition of AR activity by increasing HT concentrations in both the prostate cancer cells (Figure 6A). This correlated with the dose-dependent decrease in total AR as well as PSA expression levels (Figure 6B).

Discussion

HT, an active polyphenolic constituent of olive oil, is known for its potent antioxidative, antibacterial and antiinflammatory properties (24). In addition, it affects multiple signaling pathways resulting in an efficient anticancer action (25). The several reported targets of HT include peroxisome proliferator-activated receptor gamma (PPARγ) (26), G-protein-coupled receptor GPER/GPR30 (11), epidermal growth factor receptor (EGFR) (12), Chemokine C-C motif ligand 5 (CCL5) (10), interleukin-6 (IL-6) (16) and Vascular endothelial growth factor receptor-2 (VEGFR-2) (27). Here, we report an activity of HT against prostate cancer through regulation of Akt, STAT-3, NF-κB signaling and down-regulation of AR (Figure 7). We established such activity of HT using androgen-sensitive LNCaP as well as castration-resistant C4–2 prostate cancer cells.

Akt and STAT3 signaling pathways have long been recognized to mediate cancer cells' proliferation, invasion and metastasis. Our results establish a significant inhibition of not only Akt and STAT3, but also that of NF-κB signaling. Normally, in cancer cells, there is a basal Akt activity resulting in NF-κB activation and translocation. Akt dependent activation of IκB kinase (IKK) leads to the phosphorylation (and subsequent degradation) of IκBα, a protein that keeps NF-κB sequestered in the cytoplasm. The released NF-κB translocates to

the nucleus, resulting in induced transcription of pro-survival and oncogenic genes. Both STAT3 and NF-κB are known to enhance proliferation of cancer cells and control apoptosis. It is possible that the inhibition of one of these pathways can be compensated by routing the pro-survival mechanisms in cancer cells through the alternate pathway. In this context, a simultaneous modulatory effect of HT against several potential targets is a highly desirable attribute. Our results also suggest a cell cycle modulatory effect of HT with dose-dependent inhibition of cell cycle regulatory molecules cyclin D1 and cyclin E. Inhibition of cyclin D1 seems to be an important part of HT and there are reports of cyclin D1 down-regulation by HT in few other cancer models as well, such as colon cancer Caco-2 (28), breast cancer MCF-7 (29)/MDA-MB-231 (10) and thyroid cancer cells (30). Similarly, there is evidence for HT-mediated up-regulation of p21 and p27 (17), confirming our current findings. We also report inhibition of Akt by HT, a mechanism that has been validated in hepatocellular carcinoma cells (15).

One major reported mechanism of HT action is via modulation of ROS production. In one of the earliest reports on the subject, HT was shown to protect against peroxynitrite-dependent nitration of tyrosine and DNA damage (31). More recently (9), HT has been shown to protect peripheral blood mononuclear cells from H_2O_2-induced oxidative DNA damage, and hepatocellular carcinoma Hep G2 against ER-stress (32). In colon cancer cells, HT has been shown to generate ROS leading to apoptotic cell death and mitochondrial dysfunction (33). Even in prostate cancer PC3 cells, there is evidence for ROS generation by HT, leading to induction of apoptosis (19). Interestingly, Odiatou *et al.* (34) determined that sodium bicarbonate, a component of cell culture media, mediates the production of cytotoxic ROS by HT which can result in decreased viability of not only cancer cells such as breast MDA-MB-231 cells, but also the ‘normal’ breast epithelium MCF-10A cells. While this cautions against misinterpretation of cytotoxic and anticancer activity of polyphenols such as HT, and emphasizes a more critical evaluation of assay components, the cancer cells-specific-cytotoxic activity has been demonstrated by several independent groups. The generation of ROS may actually represent another interesting anticancer mechanism of polyphenolic compounds of natural origin that involves their prooxidant action (35; 36).

There is growing realization for the involvement of epigenetic events in tumor progression. The delicate balance between oncogenes and tumor suppressors is fine-tuned through epigenetic regulations. A number of dietary agents have been shown to regulate such epigenetic events (37). A recent work (13) reported on such activity of HT. While the study focused on epigenetic regulation by extra virgin olive oil, the phenolic constituents of olive oil were also investigated. It was shown that *CNR1* gene, which codes for type 1 cannabinoid receptor CB(1) and is down-regulated in colon cancer Caco-2 cells, can be epigenetically up-regulated by HT through reduced promoter methylation. This represents a newly realized activity of HT that has never been reported before. It is conceivable that the differential regulation of several signaling factors by HT, as observed in the present study, as well as reported by others, involves epigenetic changes.

Despite the promising anticancer activity of HT, there have been concerns about its poor bioavailability owing to its extensive metabolism (38). It has been suggested that the conjugated forms of HT should also be included in the cytotoxic assays, along with free HT,

because this alone would be truly reflective for the HT in biological systems (38). On a positive note, it was noted that HT is a 'safe' compound and can be administered at higher doses without signs of any genotoxic or mutagenic effects (38; 39). Recently, there has been an interest in synthesis of HT-derivatives with a focus on improving the overall efficacy (40). A novel ester of hydroxytyrosol and alpha-lipoic acid was reported to enhance potency in colorectal cancer cells (41) through the induction of cell cycle arrest. The dodecyl ether derivative of HT was shown to be relatively more cytotoxic than the parent compound as well as more selective for the malignant cells, relative to 'normal' non-malignant cells, in both lung and breast cancer model systems (18). A study performed in leukemia HL60 cells suggested enhanced efficacy of thio derivatives of HT (42). While the derivatives of HT have shown promise *in vitro* with reduced IC50s, it would be interesting to evaluate their bioavailabilities.

In conclusion, HT from olive oil is a promising anticancer agent that has shown potential *in vitro* against clinically relevant models representing benign as well as aggressive prostate cancers. Its ability to modulate multiple signaling pathways, combined with no associated toxicity, makes it an ideal candidate for further testing in pre-clinical prostate cancer models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement:

We acknowledge the funding support from NIH/NCI (CA185490) and USAMCI. We also thank Mr. Steven McClellan, Manager, Flow cytometry core at the USA Mitchell Cancer Institute for his assistance with flow cytometry.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 66, 7–30, 2016. [PubMed: 26742998]
2. Hussain SS, Kumar AP, Ghosh R. Food-based natural products for cancer management: Is the whole greater than the sum of the parts? Semin Cancer Biol 40–41, 233–46, 2016.
3. Lall RK, Syed DN, Adhami VM, Khan MI, Mukhtar H. Dietary polyphenols in prevention and treatment of prostate cancer. International journal of molecular sciences 16, 3350–76, 2015. [PubMed: 25654230]
4. Khan N, Mukhtar H. Modulation of signaling pathways in prostate cancer by green tea polyphenols. Biochemical pharmacology 85, 667–72, 2013. [PubMed: 23041649]
5. Ahmad A, Li Y, Sarkar FH. The bounty of nature for changing the cancer landscape. Molecular nutrition & food research 60, 1251–63, 2016. [PubMed: 26799714]
6. Fabiani R Anti-cancer properties of olive oil secoiridoid phenols: a systematic review of in vivo studies. Food & function 7, 4145–59, 2016. [PubMed: 27713961]
7. Lopez-Guarnido O, Alvarez-Cubero MJ, Saiz M, Lozano D, Rodrigo L, et al. Mediterranean diet adherence and prostate cancer risk. Nutr Hosp 31, 1012–9, 2014. [PubMed: 25726188]
8. Pineiro Z, Cantos-Villar E, Palma M, Puertas B. Direct liquid chromatography method for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines. Journal of agricultural and food chemistry 59, 11683–9, 2011. [PubMed: 21950381]
9. Rosignoli P, Fuccelli R, Sepporta MV, Fabiani R. In vitro chemo-preventive activities of hydroxytyrosol: the main phenolic compound present in extra-virgin olive oil. Food & function 7, 301–7, 2016. [PubMed: 26469183]

10. Sarsour EH, Goswami M, Kalen AL, Lafin JT, Goswami PC. Hydroxytyrosol inhibits chemokine C-C motif ligand 5 mediated aged quiescent fibroblast-induced stimulation of breast cancer cell proliferation. Age (Dordrecht, Netherlands) 36, 9645, 2014.
11. Chimento A, Casaburi I, Rosano C, Avena P, De Luca A, et al. Oleuropein and hydroxytyrosol activate GPER/ GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells. Molecular nutrition & food research 58, 478–89, 2014. [PubMed: 24019118]
12. Terzuoli E, Giachetti A, Ziche M, Donnini S. Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation. Molecular nutrition & food research 60, 519–29, 2016. [PubMed: 26577496]
13. Di Francesco A, Falconi A, Di Germanio C, Micioni Di Bonaventura MV, Costa A, et al. Extravirgin olive oil up-regulates CB(1) tumor suppressor gene in human colon cancer cells and in rat colon via epigenetic mechanisms. The Journal of nutritional biochemistry 26, 250–8, 2015. [PubMed: 25533906]
14. Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP) 11, 351–8, 2002.
15. Zhao B, Ma Y, Xu Z, Wang J, Wang F, et al. Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via inactivating AKT and nuclear factor-kappa B pathways. Cancer letters 347, 79–87, 2014. [PubMed: 24486741]
16. Tutino V, Caruso MG, Messa C, Perri E, Notarnicola M. Antiproliferative, antioxidant and anti-inflammatory effects of hydroxytyrosol on human hepatoma HepG2 and Hep3B cell lines. Anticancer research 32, 5371–7, 2012. [PubMed: 23225439]
17. Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Morozzi G. Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors p21(WAF1/Cip1) and p27(Kip1) and with induction of differentiation in HL60 cells. The Journal of nutrition 138, 42–8, 2008. [PubMed: 18156402]
18. Calderon-Montano JM, Madrona A, Burgos-Moron E, Orta ML, Mateos S, et al. Selective cytotoxic activity of new lipophilic hydroxytyrosol alkyl ether derivatives. Journal of agricultural and food chemistry 61, 5046–53, 2013. [PubMed: 23638972]
19. Luo C, Li Y, Wang H, Cui Y, Feng Z, et al. Hydroxytyrosol promotes superoxide production and defects in autophagy leading to anti-proliferation and apoptosis on human prostate cancer cells. Current cancer drug targets 13, 625–39, 2013. [PubMed: 23597197]
20. Jin R, Yamashita H, Yu X, Wang J, Franco OE, et al. Inhibition of NF-kappa B signaling restores responsiveness of castrate-resistant prostate cancer cells to anti-androgen treatment by decreasing androgen receptor-variant expression. Oncogene 34, 3700–10, 2015. [PubMed: 25220414]
21. Das TP, Suman S, Alatassi H, Ankem MK, Damodaran C. Inhibition of AKT promotes FOXO3a-dependent apoptosis in prostate cancer. Cell Death Dis 7, e2111, 2016. [PubMed: 26913603]
22. Bishop JL, Thaper D, Zoubeidi A. The Multifaceted Roles of STAT3 Signaling in the Progression of Prostate Cancer. Cancers (Basel) 6, 829–59, 2014. [PubMed: 24722453]
23. Zhang L, Altuwaijri S, Deng F, Chen L, Lal P, et al. NF-kappaB regulates androgen receptor expression and prostate cancer growth. Am J Pathol 175, 489–99, 2009. [PubMed: 19628766]
24. Bulotta S, Celano M, Lepore SM, Montalcini T, Pujia A, Russo D. Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: focus on protection against cardiovascular and metabolic diseases. J Transl Med 12, 219, 2014.
25. Bernini R, Merendino N, Romani A, Velotti F. Naturally occurring hydroxytyrosol: synthesis and anticancer potential. Current medicinal chemistry 20, 655–70, 2013. [PubMed: 23244583]
26. Cardeno A, Sanchez-Hidalgo M, Rosillo MA, Alarcon de la Lastra C. Oleuropein, a secoiridoid derived from olive tree, inhibits the proliferation of human colorectal cancer cell through downregulation of HIF-1alpha. Nutrition and cancer 65, 147–56, 2013. [PubMed: 23368925]
27. Lamy S, Ouanouki A, Beliveau R, Desrosiers RR. Olive oil compounds inhibit vascular endothelial growth factor receptor-2 phosphorylation. Experimental cell research 322, 89–98, 2014. [PubMed: 24326154]

28. Corona G, Deiana M, Incani A, Vauzour D, Dessi MA, Spencer JP. Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. Molecular nutrition & food research 53, 897–903, 2009. [PubMed: 19685549]
29. Bouallagui Z, Han J, Isoda H, Sayadi S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49, 179–84, 2011.
30. Toteda G, Lupinacci S, Vizza D, Bonofiglio R, Perri E, et al. High doses of hydroxytyrosol induce apoptosis in papillary and follicular thyroid cancer cells. Journal of endocrinological investigation, 2016.
31. Deiana M, Aruoma OI, Bianchi ML, Spencer JP, Kaur H, et al. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. Free radical biology & medicine 26, 762–9, 1999. [PubMed: 10218666]
32. Giordano E, Davalos A, Nicod N, Visioli F. Hydroxytyrosol attenuates tunicamycin-induced endoplasmic reticulum stress in human hepatocarcinoma cells. Molecular nutrition & food research 58, 954–62, 2014. [PubMed: 24347345]
33. Sun L, Luo C, Liu J. Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation. Food & function 5, 1909–14, 2014. [PubMed: 24953710]
34. Odiatou EM, Skaltsounis AL, Constantinou AI. Identification of the factors responsible for the in vitro pro-oxidant and cytotoxic activities of the olive polyphenols oleuropein and hydroxytyrosol. Cancer letters 330, 113–21, 2013. [PubMed: 23201137]
35. Khan HY, Zubair H, Faisal M, Ullah MF, Farhan M, et al. Plant polyphenol induced cell death in human cancer cells involves mobilization of intracellular copper ions and reactive oxygen species generation: a mechanism for cancer chemopreventive action. Molecular nutrition & food research 58, 437–46, 2014. [PubMed: 24123728]
36. Zubair H, Azim S, Khan HY, Ullah MF, Wu D, et al. Mobilization of Intracellular Copper by Gossypol and Apogossypolone Leads to Reactive Oxygen Species-Mediated Cell Death: Putative Anticancer Mechanism. International journal of molecular sciences 17, 2016.
37. Ahmad A, Li Y, Bao B, Kong D, Sarkar FH. Epigenetic regulation of miRNA-cancer stem cells nexus by nutraceuticals. Molecular nutrition & food research 58, 79–86, 2014. [PubMed: 24272883]
38. Rodriguez-Morato J, Boronat A, Kotronoulas A, Pujadas M, Pastor A, et al. Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. Drug metabolism reviews 48, 218–36, 2016. [PubMed: 27186796]
39. Aunon-Calles D, Giordano E, Bohnenberger S, Visioli F. Hydroxytyrosol is not genotoxic in vitro. Pharmacol Res 74, 87–93, 2013. [PubMed: 23769711]
40. Bernini R, Gilardini Montani MS, Merendino N, Romani A, Velotti F. Hydroxytyrosol-Derived Compounds: A Basis for the Creation of New Pharmacological Agents for Cancer Prevention and Therapy. Journal of medicinal chemistry 58, 9089–107, 2015. [PubMed: 26225717]
41. Bernini R, Crisante F, Merendino N, Molinari R, Soldatelli MC, Velotti F. Synthesis of a novel ester of hydroxytyrosol and alpha-lipoic acid exhibiting an antiproliferative effect on human colon cancer HT-29 cells. European journal of medicinal chemistry 46, 439–46, 2011. [PubMed: 21111517]
42. Sepporta MV, Lopez-Garcia MA, Fabiani R, Maya I, Fernandez-Bolanos JG. Enhanced chemopreventive activity of hydroxytyrosol on HL60 and HL60R cells by chemical conversion into thio derivatives. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 48, 790–8, 2013.

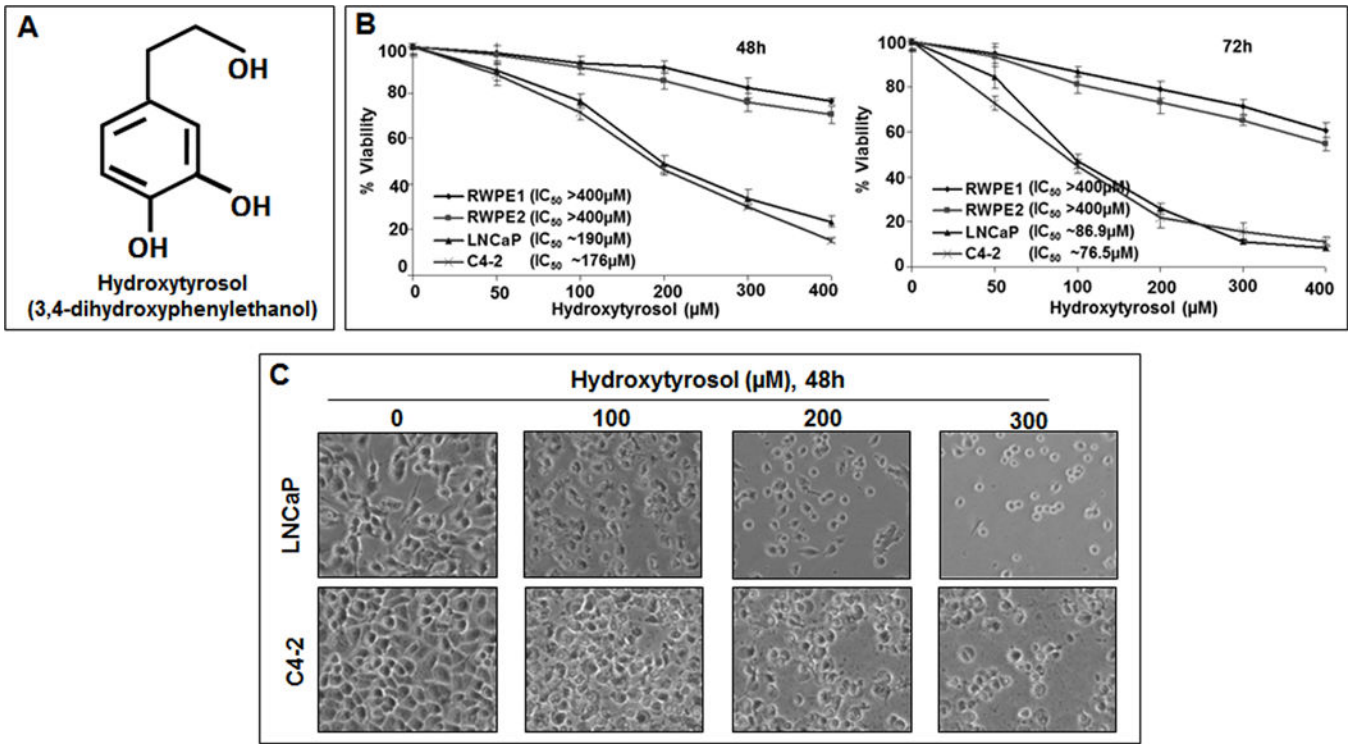
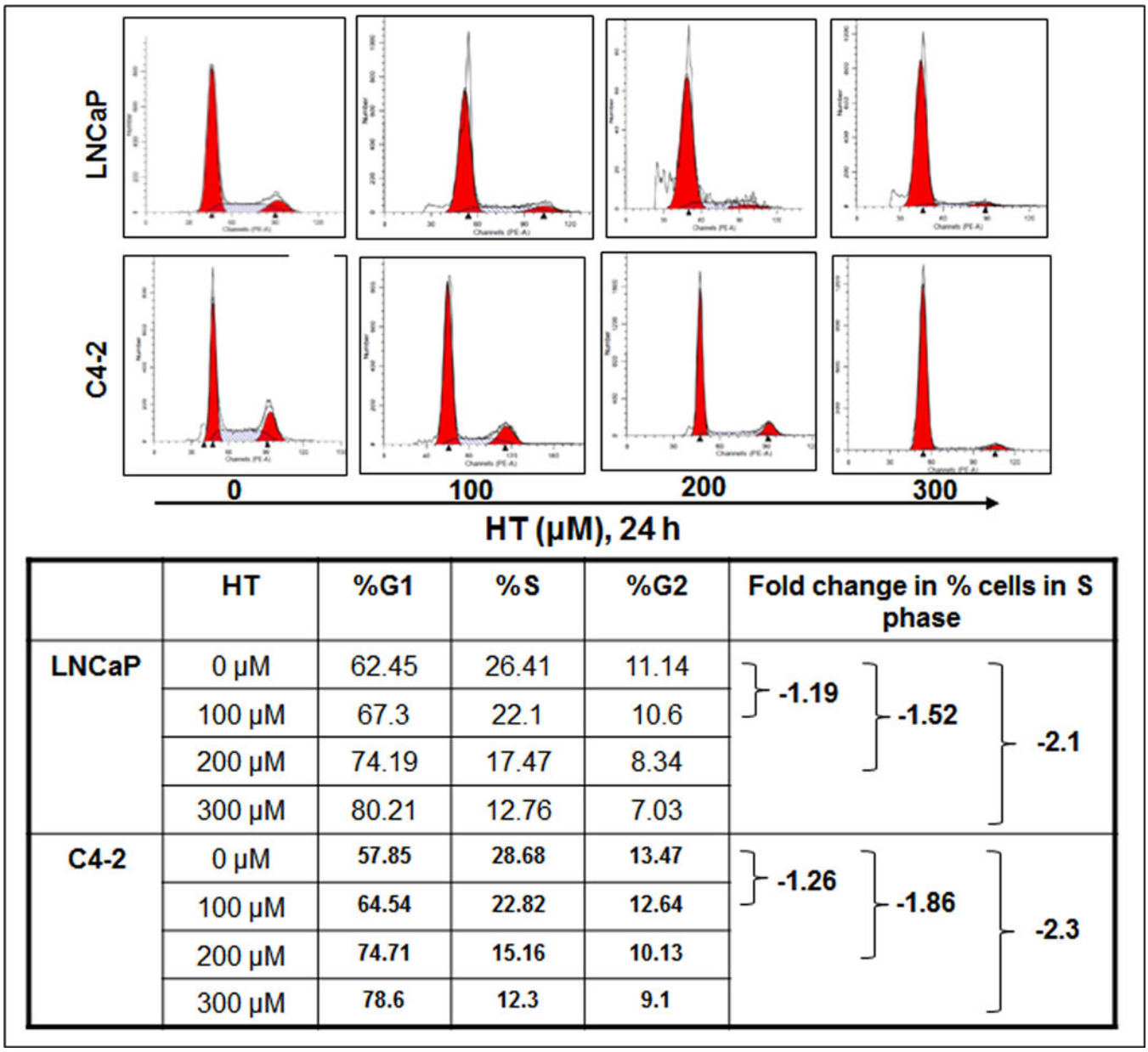


Figure 1: Hydroxytyrosol (HT) specifically inhibits the growth of prostate cancer cells.

(A) Chemical structure of HT/3,4-dihydroxyphenylethanol (HT). (B) Normal prostate epithelium cells, RWPE1 and RWPE2, and prostate cancer cells, C4–2 and LNCaP, grown in 96-well plates, were treated with increasing dose of HT. Percent viability of these cells was measured using the WST-1 assay after 48 or 72 h of HT treatment. (C) Dose dependent HT treatment of LNCaP and C4–2 cell lines significantly altered cell morphology and the cells became round and shrunken in a dose-dependent manner, as examined under phase-contrast microscope. Representative images are shown.



	HT	%G1	%S	%G2	Fold change in % cells in S phase
LNCaP	0 μM	62.45	26.41	11.14	
	100 μM	67.3	22.1	10.6	-1.19
	200 μM	74.19	17.47	8.34	-1.52
	300 μM	80.21	12.76	7.03	-2.1
C4-2	0 μM	57.85	28.68	13.47	
	100 μM	64.54	22.82	12.64	-1.26
	200 μM	74.71	15.16	10.13	-1.86
	300 μM	78.6	12.3	9.1	-2.3

Figure 2: HT treatment of prostate cancer cell lines induces G1-S phase arrest.

LNCaP and C4–2 cells were treated with increasing concentration of HT for 24 h and cell cycle phases were analyzed by propidium iodide (PI) staining using flow cytometry. An enhanced dose-dependent accumulation of cells in the G_1 phase of the cell cycle upon HT treatment was observed.

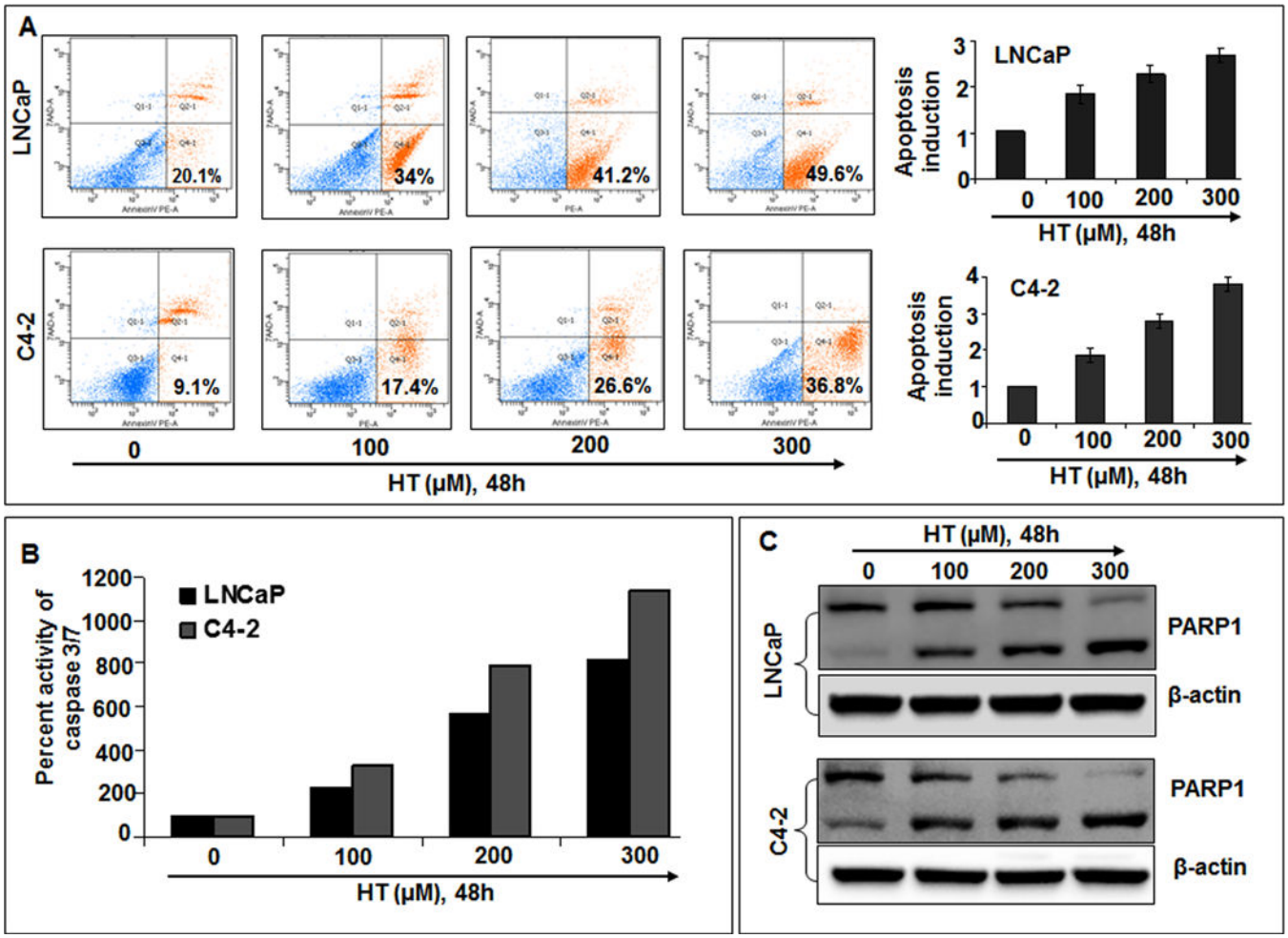


Figure 3: HT treatment induces apoptosis of prostate cancer cells.

(A) Prostate cancer cell lines grown in 6-well plates were treated with increasing concentration of HT. After 48 h, cells were collected and subsequently stained with 7-AAD and PE Annexin V followed by flow cytometry analysis. A dose-dependent increase in the number of apoptotic cells after HT treatment indicates the apoptosis induction potential of HT. (B) A dose-dependent increase in caspase-3/7 activity was also observed in prostate cancer cells treated with HT as evidenced by the increase in luminescence with increase in dose. (C) Whole cell lysate of cells treated with increasing dose of HT were collected and immunoblotted for cleaved PARP1, a marker of apoptosis. HT treatment increased cleaved PARP1 levels in a dose-dependent manner. β-actin was used as loading control.

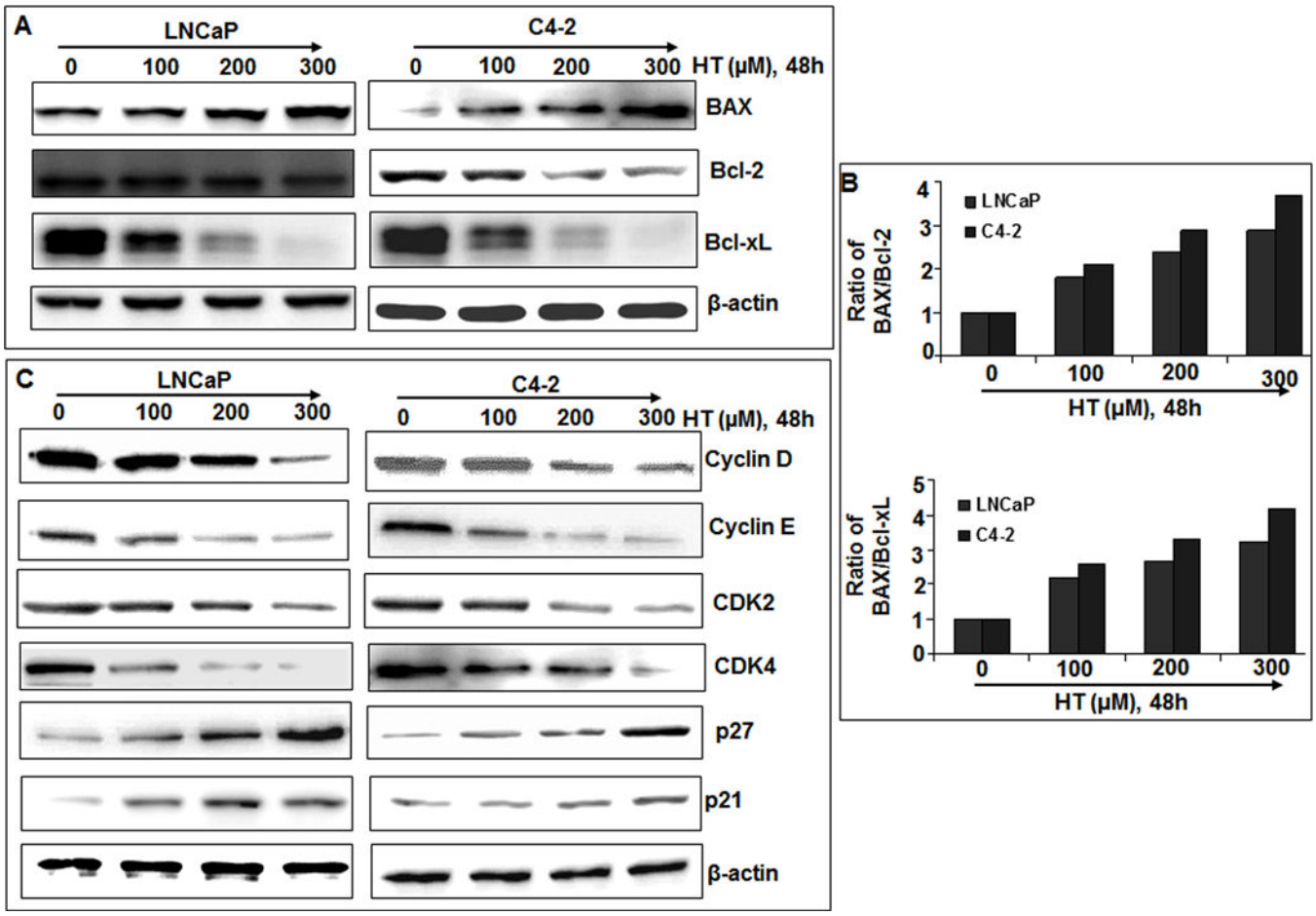


Figure 4: HT treatment modulates the level of apoptosis-related and cell cycle-associated protein.

(A) Total protein of prostate cancer cells treated with increasing dose of HT for 48 h was subjected to immunoblot and subsequent densitometric analysis for apoptosis markers (BAX, Bcl-2, Bcl-xL). (B) Bar diagram indicates an increase in the ratio of BAX/Bcl-2 and BAX/Bcl-xL based on the densitrometric analysis of immunoblots in a dose-dependent manner upon HT-treatment. (C) The whole cell lysates of HT-treated cells were also probed for cell cycle-associated proteins. β-actin was used as loading control.

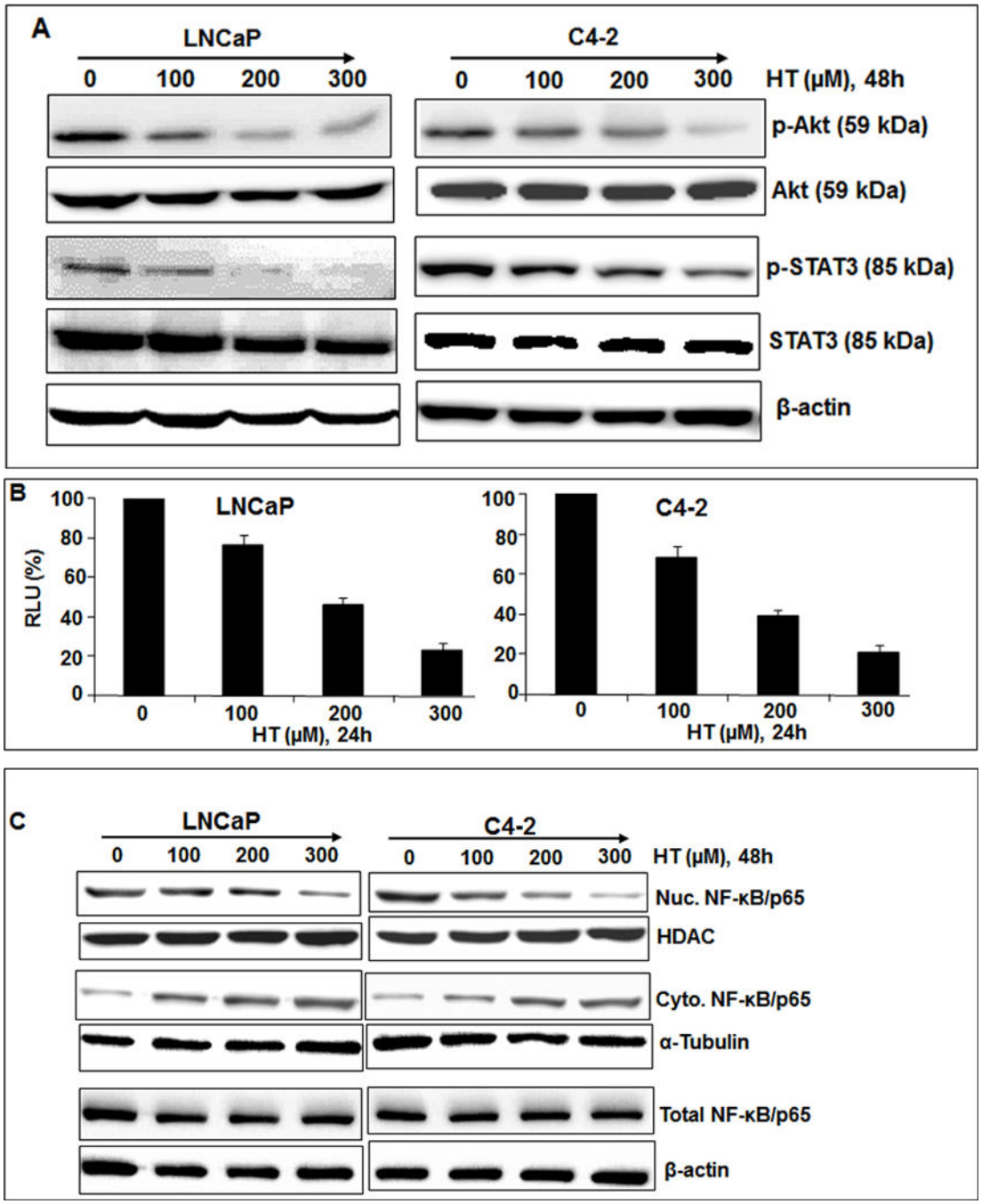


Figure 5: HT-treatment attenuates activity of multiple oncogenic pathways.

(A) Total protein of LNCaP and C4–2 prostate cancer cells treated with increasing doses of HT for 48 h was collected and probed for phosphorylated and total levels of Akt and STAT3 by immunoblot analysis. (B) LNCaP and C4–2 cells seeded in 12-well plate at 60% confluence were co-transfected with NF-κB luciferase reporter and TK-Renilla luciferase (control) plasmids and 24 h post-transfection treated with increasing doses of HT for 24 h. Luciferase (Fire-fly; test and Renilla, transfection efficiency control) activity in protein lysates was assessed using a dual-luciferase assay system. Relative luciferase unit (RLU)

with respect to control has been plotted. (C) To assess the change in cellular distribution of NF-κB/p65 upon HT-treatment, total, nuclear and cytoplasmic extracts were prepared from HT-treated cells treated and expression of NF-κB/p65 determined by Western blot analysis. β-actin was used as a loading control for whole cell lysates; HDAC and α-tubulin served as loading control of nuclear and cytoplasmic fractions, respectively.

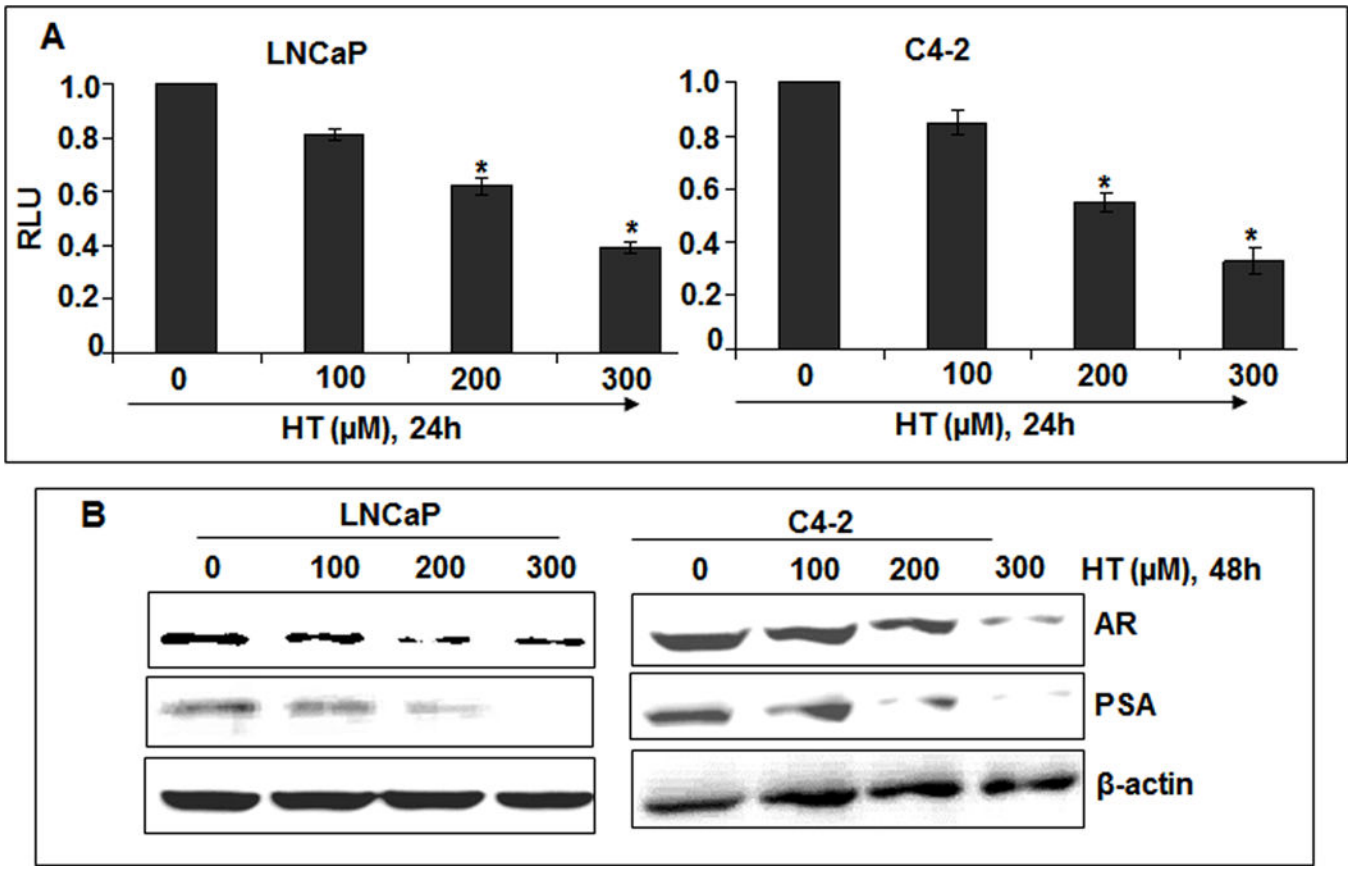


Figure 6: HT-treatment reduces activity and expression of AR.

(A) LNCaP and C4–2 cells seeded in 12-well plate at 60% confluence were transfected with AR-responsive plasmid according to the manufacturer's protocol and treated with increasing concentration of HT after 24 h. Luciferase activity in protein lysates collected post 24 h HT treatment was assessed using a dual-luciferase assay system. (B) Expression of AR and an AR-responsive protein, PSA, was also tested in whole cell lysates of HT-treated cells by immunoblot assay. β-actin was used as the loading control.

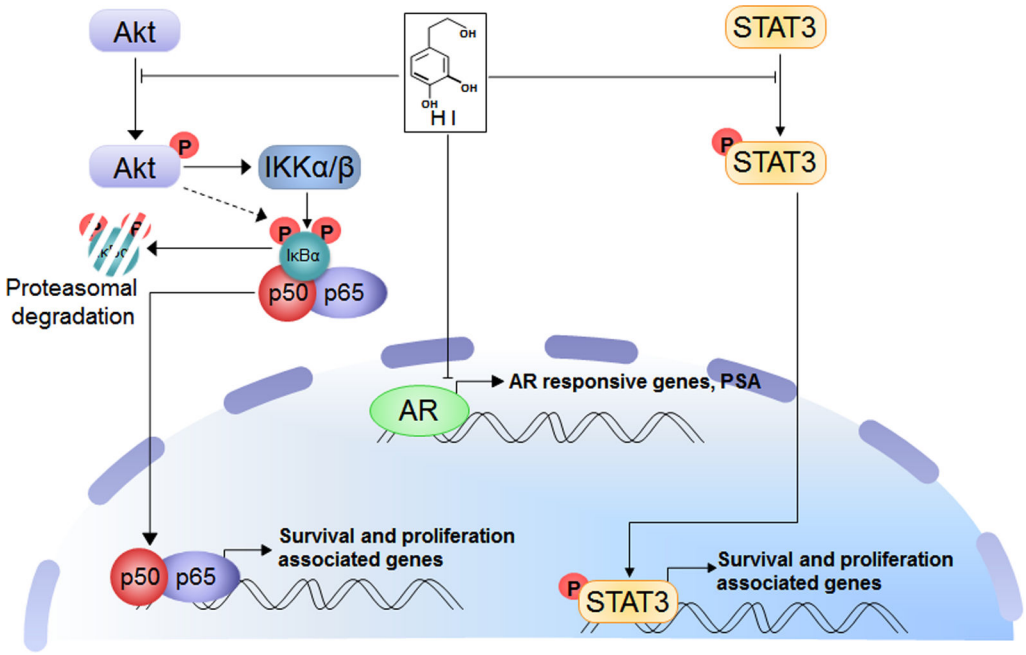


Figure 7: Schematic representation for mechanism of action of HT in prostate cancer cells.

HT inhibits activation of Akt and reduces NF-κB level in the nuclear fractions. Inhibition of NF-κB modulates the expression of cell survival and proliferation related molecules in the HT treated prostate cancer cells. HT prevents constitutive STAT3 activation which may be further be involved in the inhibition of growth and survival of prostate cancer cells. HT also inhibits endogenously expressed androgen receptor (AR) and its target, prostate specific antigen (PSA) in prostate cancer cells.