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To cite this article: Roxane Tenta, Elizabeth Fragopoulou, Magafoula Tsoukala, Marianna Xanthopoulou, Maria Skyrianou, Harris Pratsinis & Dimitris Kletsas (2017): Antiproliferative Effects of Red and White Wine Extracts in PC-3 Prostate Cancer Cells, Nutrition and Cancer, DOI: 10.1080/01635581.2017.1340489

To link to this article: http://dx.doi.org/10.1080/01635581.2017.1340489

Published online: 25 Jul 2017.

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ABSTRACT
Experimental and epidemiological studies have shown that antioxidant polyphenols can act as chemopreventive agents against prostate cancer. Cabernet Sauvignon and Rombola wine were extracted in order to obtain fractions containing different classes of compounds. All extracts inhibited the androgen-insensitive human prostate cancer cells (PC-3) proliferation in a dose-dependent manner. The most potent compounds were selected to be further tested. Treatment of PC-3 cells with the selected wine extracts marginally increased the cell distribution in S phase, while producing a remarkable induction of autophagy. Finally, the levels of glutathione along with the concentration of hydrogen peroxide and nitrogen oxide were modulated in the treated cells. Herein, we show that red and wine extracts have direct effects on the proliferation, survival, oxidative status, and the induction of autophagy of PC-3 cells. Our data may have important implications for the design of a more effective adjuvant treatment for prostate cancer patients.

Introduction
Prostate cancer is the most common malignancy among men and is the second highest cause of cancer death among men of all races. At the molecular level, development of androgen and chemotherapy refractoriness of prostate cancer is synonymous with the escape of prostate cancer cells from apoptosis induced by androgen ablation and chemotherapy (1). Although the etiology of this disease remains largely unclear, it is suggested that oxidative stress plays a role in prostate carcinogenesis. Among the numerous factors, oxidative stress plays an important role in cancer initiation, promotion, and progression by inducing DNA damage and interfering with the intracellular signal transduction pathways. Oxidative stress can occur through overproduction of reactive oxygen and nitrogen species due to either endogenous or exogenous insults. Reactive oxygen species (ROS), including hydroxyl radicals, superoxide radicals, and singlet oxygen, as well as reactive nitrogen species, are continuously generated in the cell as a result of normal human metabolism and can be harmful, as they can attack biological macromolecules, and cause membrane and DNA damage and enzyme inactivation (2).

Natural dietary agents have demonstrated their potential to prevent the occurrence of cancer in various research studies, either alone or in combination with chemotherapeutic agents. Dietary agents have been shown to modulate cellular processes, exhibit chemopreventive and/or chemotherapeutic effects, and induce apoptosis and autophagy against cancer. During the last decades, several data support the idea that diet could have beneficial effect against diseases, and several patterns of diet or individual foods are highlighted. Among them, the consumption of foods rich in antioxidants, such as fruits, vegetables, and wine, is considered to promote health (3,4).

Wine emerged in the center of scientific attention, and its contribution in promoting health was highlighted after the introduction of the term “French paradox” (5), an epidemiological observation that links moderate wine consumption to low cardiovascular diseases incidence in French population, despite the relatively high intake of saturated fats. It is believed that wine’s protective action against CVD is attributed not only to its bioactive constituents, especially the phenolic ones, but also to the phospho- and/or gluco-glycerol lipids (6). The consumption of alcohol has been identified as one of the top-10

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risks contributing to the worldwide burden of disease. However, there is evidence that moderate wine consumption may decrease the risk of several cancers, including colon, basal cell carcinoma, ovarian, and prostate cancer (7,8).

Phenolic compounds represent a large class of plant-derived molecules with a general chemical structure that acts as potent-free radical scavengers. They have long been recognized to possess several therapeutic activities ranging from antithrombotic and antiinflammatory to antioxidant. The proposed mechanisms for their antioxidant action include 1) direct radical scavenging; 2) inhibition of enzymes such as NO synthase, xanthine oxidase, cyclooxygenase, and lipoxygenase; 3) iron chelation; and 4) direct inhibition of lipid peroxidation (9–11). Phytoestrogens, in particular, are phenolic compounds derived from plants and exert an estrogenic as well as an antiestrogenic effect and also various biological efficacies. Chemopreventive properties of phytoestrogens have emerged from epidemiological observations indicating that the incidence of some cancers including breast and prostate cancers is much lower in Asian people who consume significantly higher amounts of phytoestrogens than Western people (4). Currently, resveratrol (RESV) is recognized as another major phytoestrogen present in grape and red wine and has been studied in many biological studies. RESV is a plant-derived polyphenol reported to extend lifespan in lower organisms through mimicking caloric restriction. As such, RESV has been shown to reduce a variety of age-related diseases in rodents, including obesity, diabetes, cancer, cardiovascular, and neurodegenerative diseases (12). However, the underlying protective mechanisms of RESV remain elusive. Quercetin is the main representative of the flavonol class, and besides having antioxidant and antiinflammatory activities, it has been shown to possess potent antiproliferative effects against various malignant cells, although its molecular mechanism involved in chemoprevention of prostate cancer remains unclear (13).

Autophagy is one of the major pathways for degrada-
tion of cellular components in animal cells that control the turnover of long-lived proteins and organelles. Although initially identified as a process induced by cellular starvation, an autophagic pathway is now recognized as the cellular response to a variety of stimuli. Accumulating evidence suggests that autophagy acts in both cancer progression and suppression, while recent studies show that the autophagy and apoptosis pathways are regulated by common factors, share common components, and exert overlapping functions. Dietary compounds can influence the risk of cancer and other diseases through diverse mechanisms that include the activation or inhibition of macroautophagy. Macroau-
tophagy is a catabolic process for the lysosomal degrada-
tion and recycling of cytoplasmic constituents which has been implicated in several pathologies, including cancer and neurodegeneration. In some instances, macroau-
tophagy acts to suppress tumor formation and neural degeneration. Thus, it may be feasible to design diets, supplements, or therapeutics that can alter the level of macroautophagy within cells to prevent or treat disease. In fact, several members of the flavonoid family of phytochemicals can stimulate autophagic vacuolization (10,14,15).

The objective of the present study was the evaluation of the cytostatic action of red and white wine extracts on PC-3 prostate cancer cells. PC-3 is a widely used androgen-insensitive human prostate cancer cell line. Furthermore, the scope of this study was the investigation of the mechanism of action of the most potent representatives of red and white wine extracts in terms of the impact on the autophagic pathways and the identification of the oxidative profile of prostate cancer cells.

Materials and Methods

Extraction Methods

Ambelon (A: white wine, main grape Robola) and Cabernet Sauvignon (C: red wine, main grape Cabernet Sauvignon) wines were extracted with two different methods as previously described (16). In the first method, total lipid fraction and water (W) fraction were obtained according to Bligh-Dyer method (17) and were further separated into polar lipid (PL) and neutral lipid fractions by countercurrent distribution (18), using a successive extraction procedure with petroleum ether/ethanol 87% system. In the second method, several fractions containing different classes of phenolic compounds were obtained (FI: anthocyanins; FII: procyanidins, catechins, and flavonols; FIII: phenolic acids and quercetin 3-O-glucuronide; and FIV: the rest phenolic components). More specifically, removal of alcohol was performed by under-vacuum treatment (at 30°C and 30 mbar) (19). 150 mL of the dealcoholized wine (pH 2.0) was first extracted with ethyl acetate (three times with 100 mL of ethyl acetate each), obtaining an aqueous residue (FI fraction) and an organic phase. The organic phase (ethyl acetate) was evaporated and redissolved in 100 mL of water at pH 7.0, and a further extraction with ethyl acetate (three times with 100 mL of ethyl acetate each) was performed. The ethyl acetate phase is the FII fraction. The aqueous residue from this extraction was adjusted at pH 2.0 and extracted again with ethyl acetate (three times with 100 mL of ethyl acetate each) to obtain the...
Cells were then washed twice with ice-cold PBS and resuspended in 1 mL PBS. After centrifugation, the supernatants were collected and assayed for protein concentration using the Bradford method using bovine serum albumin as the protein standard (22).

**Biochemical Parameters: GSH, NO, and H2O2**

After treatment with the appropriate factors, the PC-3 cells grown in 25-cm² culture flasks, were washed twice with ice-cold PBS and resuspended into chilled PBS with protease inhibitors. The cell pellet was washed once with PBS and then resuspended in 1 mL PBS. After centrifugation, the supernatants were collected and assayed for protein concentration using the Bradford method.

**Glutathione Reductase Recycling Assay**

The cyclic DTNB-glutathione reductase assay was used to determine total glutathione reduced (GSH) and oxidized (GSSG) form in terms of GSH equivalents. The neutralized samples were obtained and the reaction was started by adding 20 μL β-nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt (NADPH) 0.02 mM, in 143 mM phosphate buffer at pH 7.0 containing 200 μL of 0.4 U/mL glutathione reductase per well, 6.3 mM of EDTA, and 0.6 mM of DTNB. The initial rate of 5-thio-2-nitrobenzoic acid production was monitored.
**Nitrogen Oxide Assay**
Nitrogen oxide (NO) concentration was determined by measuring the amount of released NO₂ with modified Griess reagent (Sigma-Aldrich) according to the Griess reaction (23). Briefly, 50 mL of cell culture supernatant was transferred to another 96-well plate and 50 mL of modified Griess reagent was added to a total volume of 100 mL. After 15 min of incubation at room temperature, OD at 540 nm was measured and the concentration of released NO₂ was extrapolated from the NaNO₂ standard curve.

**Hydrogen Peroxide Assay**
The release of hydrogen peroxide (H₂O₂) was determined by transferring 50 μL of supernatant into a new 96-well plate and adding 50 μL of 0.01% peroxide and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (diluted with distilled water 1:1) as described by supplier (Pierce, Rockford, USA).

**Statistical Analysis**
Results are presented as mean ± SEM. The data were compared using analysis of variance (one-way analysis of variance). Statistical analysis was performed in triplicate determinations at \( P < 0.05 \).

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**Results**

**Cytotoxicity/MTT Assay**
The screening of four standard [RESV, quercetin, gallic acid (1–35 μg/mL), and tyrosol (2–70 μg/mL)] (Fig. 1A), and six different classes of phenolic compounds of red (C) (Fig. 1B) and white (A) (Fig. 1C) wine [FI, FII, FIII, FIV, PI, and W (15–1000 μg/mL)], respectively, showed inhibition of PC-3 cells proliferation by 96-h exposure in a dose-dependent manner. The most potent compounds of each group [RESV, FIIC, and FIIA] were selected to be further tested. Results are expressed as percentage of controls.

**Cell Cycle Analysis**
The cell cycle distribution revealed that treatment of the PC-3 cells with 150 μg/mL of FIIC for 24 and 48 h or FIIA for 72 h marginally (4–7%) increased the cell distribution in S phase, while RESV (15 μg/mL) increased (10%) the distribution of cells in G0/G1 phase (Fig. 2). The extracts tested did not produce evidence of apoptosis. Finally, treatment of the PC-3 cells with 100 nM of adriamycin showed the expected G2/M phase arrest (data not shown).

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**Figure 1.** A: Dose–response curves for the continuous administration (96 h) of standard compounds [RESV, quercetin, gallic acid (1–35 μg/mL), and tyrosol (2–70 μg/mL)] on PC-3 cells, as assessed by the MTT assay. Results are expressed as percentage of controls. Statistical analysis was performed in triplicate (\( P < 0.05 \)). B: Dose–response curves for the continuous administration (96 h) of red wine extracts FIC, FIIC, FIIIC, FIVC, PLC, and CW (15–1000 μg/mL) on PC-3 cells, as assessed by the MTT assay. Results are expressed as percentage of controls. Statistical analysis was performed in triplicate (\( P < 0.05 \)). C: Dose–response curves for the continuous administration (96 h) of white wine extracts FIA, FIIA, FIIIA, FIVA, PLA, and AW (15–1000 μg/mL) on PC-3 cells, as assessed by the MTT assay. Results are expressed as percentage of controls. Statistical analysis was performed in triplicate (\( P < 0.05 \)).
Figure 1. (Continued).
Autophagy Imaging

The immunofluorescence experiments revealed that both selected wine extracts (FIIA and FIIC) exhibited the characteristic hallmarks of autophagy (punctuate localization of LC3) on PC-3 cells. More specifically, treatment of PC-3 cells with the FIIA extract resulted in a remarkable induction of autophagy in both doses tested. As this is a qualitative rather than a quantitative method, we cannot assume that higher intensity is present in FIIA- (300 μg/mL) rather than in FIIA- (150 μg/mL) treated cells. In the case of FIIC, the induction of autophagy was observed in a dose-dependent manner, and therefore, strong evidence of autophagy was monitored when cells were treated with 300 μg/mL of FIIC extract. RESV-treated cells exhibited a pronounced autophagic signal in a dose-dependent manner as well (Fig. 3).

Biochemical Assays

By measuring the levels of GSH, it was found that GSH levels were significantly decreased in the FIIC extract and RESV (Fig. 4A). As for the levels of H₂O₂ and NO, the levels of both parameters were significantly increased in the FIIC-treated cells (Fig. 4B and C).

Discussion

Prostate cancer represents a major concern in human oncology. In order to overcome advanced prostate cancer, the commonly used treatment to date is the inhibition of androgen production and/or androgen function. Although most patients respond initially, the therapeutic effects often last only for a short period. Thereafter, the tumor cells gradually develop to be androgen-independent and resume proliferation even in an androgen-deprived environment. Accumulating evidence indicates that the in vivo response to anticancer therapies is mostly influenced by the toxicity and side effects that the chemotherapeutic schemes may confer (24). Experimental and epidemiological studies have shown that antioxidant polyphenols, present in food rich in fruits vegetables and seeds, can act as chemopreventive agents against numerous diseases including prostate cancer (25).

Herein, the antiproliferative effect of red and white wine extracts and their potent mechanism of action on prostate cancer cells are studied. In the past, the development of anticancer drugs has traditionally relied on in vitro tests aimed almost exclusively at assessing the potential of direct killing or growth inhibiting of cancer cell lines (26). These studies have until now focused on the induction of apoptosis, while the present study addresses the effect of wine, in the field of prostate cancer, with regard to the more novel mechanism of autophagy. Because the characteristics of PC-3 cells consent with many of the clinical aspects of the hormone refractory prostatic cancer, this cell line has been widely used as a model of prostate cancer progression (20).

Phenolic compounds could have dual action depending on their concentration but also by the kind of target cell. More specifically, they could act as antioxidants inhibiting oxidative damage but also could reveal cytotoxic effects, by acting as pro-oxidants, and promote apoptosis of cancer cells. They react with cellular peroxidases and/or cellular thiols leading to the formation of reactive phenoxyl radicals and GSH depletion (27). The extent of their actions is dependent on their chemical structure and the number and/or position of hydroxyl groups. RESV has been reported to have pro-oxidant effects and to promote ROS production by directly forming oxy-radicals, NADPH-dependent ROS production, or by affecting mitochondrial function and ATP synthesis (28). However, the possibility that the complex mixture of phytochemicals in foods may contribute to their protecting effects has been increased (29). In this
concept, it is possible for multiple compounds to act through complimentary or synergistic mechanisms to present a more intense biologic effect than that achieved by any individual component.

This study demonstrates that red and white wine extracts inhibit growth and proliferation of PC-3 cells prostate cancer cells in a dose-dependent manner. The results of the fractions obtained from the first method of

Figure 3. Representative images of the expression of LC3 (autophagic marker) in PC-3 cells in response to FIIA (150 and 300 μg/mL), FIIC (150 and 300 μg/mL), and RESV (15 and 45 μg/mL) for 24 h, by immunofluorescence, as described in “Materials and Methods” section. All treated samples enhanced the expression of LC3 as compared to control cells. Note the punctuate localization of LC3 in treated cells. Negative control indicates the absence of first antibody in order to check for nonspecific binding (magnification: × 60).
extraction revealed that in both wines, the PL fraction is more potent than the water-soluble one and revealed 50% cell death at 300–500 mg/ml. This is in accordance with our previous results indicating that PL fraction is more potent in platelet aggregation and also in antioxidant activity (16,30). It is known that the two wines contain different types of phenolic compounds. Concerning Robola, the predominant phenolic acids reported are caftaric acid, coumaric acid, 2-S-glutathionyl-caftaric acid, fentaric acid, cinnamic acid, and caffeic acid, while the amount of gallic acid is very small (31). Concerning Cabernet Sauvignon wine, the main anthocyanins reported are malvidin, petunidin, and delphinidin glucosides (32), the main procyanidins are the dimers B1, B2, B3, and B4, and it also contains (+)-catechin and (−)-epicatechin, while the main phenolic acids are gallic, caffeic, and caftaric (33). For this reason, a second method of extraction was used in order to obtain fractions with different classes of phenolic compounds. The most potent fraction in both wines was the FII (procyanidins, catechins, and flavonols), which revealed a 50% cell death at 150 μg/ml. Concerning phenolic standards, RESV was by far the most potent, inducing 50% cell death at a concentration lower than 5 μg/ml.

Therefore, the most potent ones were selected in order to perform further experiments. Cell distribution was slightly altered following treatment with the selected red and white wine extracts, although none produced evidence of apoptosis. Furthermore, the fact that there was neither significant alteration of the cell cycle distribution nor significant sub-G1 phase accumulation at the doses tested, which would indicate the induction of apoptosis, led the authors to investigate the implication of another intracellular molecular mechanism, autophagy. Indeed, the results show that both, red and white, wine extracts tested produce a remarkable induction of autophagy, while RESV induces autophagy only in high concentration, almost 200 μM (45 μg/ml), supporting the opinion that a mixture of phytochemicals is more potent than an individual component. Although autophagy has been proposed as a cell death process, the role of autophagy in cancer cell death is still in dispute. Previous studies have shown that when tumor cells are deprived of growth/survival factors, autophagy is increased to prevent the cells from dying. In addition, when autophagy is prevented under these conditions, the cells undergo apoptosis (34). Therefore, autophagy seems to play a role in preventing cellular apoptosis from nutritional stress in cancer cells. However, it is unclear whether autophagy is the mechanism for cell death or a reactive mechanism by which the cell is trying to survive the chemotherapy. Adding specific bioactive dietary constituents present in natural products to cell cultures can also induce autophagy, in addition to that classically observed following nutrient deprivation. RESV for instance, is found to induce autophagy by directly inhibiting mTOR through ATP competition (35). Whereas a variety of food components including vitamin D, selenium, curcumin, RESV, and

Figure 4. Analysis of redox state in PC-3 cells, after 48 h of exposure to FIIc (150 μg/mL), FIIa (150 μg/mL), and RESV (15 μg/mL). GSH and GSSG production was significantly decreased in the FIIc extract and in RESV (A), whereas the levels of H2O2 and NO were significantly increased in the FIIc-treated cells (B and C). Statistical analysis was performed in triplicate (*P < 0.05).
genistein have been shown to stimulate autophagy vacuolization, it is often difficult to determine if this is a protumorigenic or antitumorigenic response (36).

Nevertheless, growth in a hostile environment, inefficient utilization of glucose, and defective autophagy predict that prostate cancers may be particularly sensitive to therapies that inflict metabolic stress (14). Accordingly, the antiproliferative and autophagic effects of nontoxic dietary agents could be of additional significance for the prevention, control, and management of prostate cancer, specifically that at an advanced and an androgen-independent stage of the malignancy. Additional studies are needed to examine dose and duration of exposures and tissue specificity in response to bioactive food components to resolve the physiologic implications of the autophagy process (36).

Recent biochemical data suggest that human prostate cancer cell lines show a redox imbalance (oxidizing) compared with benign primary prostate epithelial cells. Jorgenson et al. investigated the possible role of reduction/oxidation (redox) state in cancer as it is hypothesized that many modifications in cellular macromolecules, observed in cancer progression, may be caused by redox imbalance. It is also noteworthy that human prostate cancer cell lines of varying degrees of aggressive behavior have distinct redox properties and that each cell type shows distinct cytotoxic responses to low-molecular-weight redox-modulating compounds. Excess intracellular ROS beyond a threshold can induce apoptosis in cancer cells. However, the signal pathways that can augment the proapoptotic function of ROS remain largely unknown. Also, autophagic activities need to be highly regulated to sense intracellular stress, through mechanisms involving cellular redox signaling (37).

Autophagy proteins and tumor suppressors contain cysteine residues that are prone to oxidation. Analysis of the redox state in PC-3 cells showed that the levels of GSH were significantly decreased in the red wine extract (FIIC) which contains procyanidins, catechins, and flavonols, as well as in RESV, whereas the levels of hydrogen peroxide and nitrogen oxide were significantly increased in PC-3 cells treated with the FIIC extract. Intracellular GSH is a crucial factor in modulating NO reactivity. It is reported in neuronal cells that when GSH levels decline, NO availability is increased and may trigger protein oxidation, in terms of S-nitrosylation of cysteines and formation of 3-nitrotyrosine on protein residues. These events result in thiol redox imbalance leading to activation of crucial proteins involved in autophagy induction and execution (38). However, only red wine extract modulates redox status, while both wines induce autophagy. This result indicates that white wine microconstituents interfere with autophagy mechanisms independently from cell redox status. It is possible to speculate that white wine induces apoptosis by inhibiting, for instance, the mTOR pathway through ATP competition, but further experiments are needed toward this direction. Finally, Shin et al. (39) have recently shown that Vitisin A, derived from wine grapes, with known antiadipogenic, antiinflammatory, and antioxidant effects, can be used in conjunction with TRAIL as a potent TRAIL sensitizer for production of ROS in PC-3 prostate cancer cells.

In conclusion, we show herein that red and white wine extracts have direct effects on the proliferation, survival, oxidative status, and the induction of autophagy of PC-3 cells. The above results could highlight a potential role of red and white wine polyphenols in prostate cancer prevention and/or treatment. Because wine is a natural product that is anyway consumed in everyday life, it is regarded as a very promising alternative for the design of a more effective adjuvant treatment for prostate cancer patients.

Acknowledgments

The study was supported through a research funding from the Graduate Program of the Department of Nutrition and Dietetics, Harokopio University.

Disclosures

The authors declare that no conflict of interest or any financial disclosure exists.

References


