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Rosemary (Rosmarinus officinalis L.) extract inhibits prostate cancer cell proliferation and survival by targeting Akt and mTOR

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\textbf{ABSTRACT}

Prostate cancer is the most commonly diagnosed type of cancer in North American men and is typically classified as either androgen receptor positive or negative depending on the expression of the androgen receptor (AR). AR positive prostate cancer can be treated with hormone therapy while AR negative prostate cancer is aggressive and does not respond to hormone therapy. It has been previously reported that rosemary extract (RE) has antioxidant, anti-inflammatory and anti-cancer properties. In the present study, we found that treatment of the androgen-insensitive PC-3 prostate cancer cells with RE resulted in a significant inhibition of proliferation, survival, migration, Akt, and mTOR signaling. In addition, treatment of the androgen-sensitive 22RV1 prostate cancer cells with RE resulted in a significant inhibition of proliferation and survival while RE had no effect on normal prostate epithelial PNT1A cells. These findings suggest that RE has potent effects against prostate cancer and warrants further investigation.

1. Introduction

Prostate cancer accounted for roughly 1.3 million cases and 359,000 deaths globally in 2018, and is the second leading cause of death in North American men despite all available treatment strategies including surgery, radiotherapy, and chemotherapy [1]. Finding novel approaches to prevent and treat prostate cancer effectively is highly desirable. Major disruptions of cellular homeostasis of the prostate gland leads to prostate cancer [2]. The growth of prostate epithelial cells is influenced by growth factors, the expression and function of androgen receptors (AR) and by the hypothalamic-pituitary axis [2,3]. Androgens provide important growth stimuli for prostate cells, and ARs are typically expressed in the stromal and epithelial compartments of the prostate gland [2,4]. Production of luteinizing hormone (LH)-releasing hormone (LHRH) by the hypothalamus induces the production of LH by the pituitary gland [5] leading to increased androgen production. Hypothalamic LHRH and pituitary LH production are regulated by a negative feedback mechanism [5]. Androgen binding to AR leads to transcriptional activation of AR target genes which are involved in various biological processes such as proliferation and apoptosis [2–4]. Overall, AR signaling is directly involved in maintaining normal prostate tissue homeostasis [2].

Cancer cells are characterized by their ability to proliferate uncontrollably and evade apoptosis [6,7]. These characteristics are often acquired as a result of mutations in key proteins involved in the signaling pathways responsible for regulating cellular function and maintaining homeostasis [8–13]. Molecular signaling pathways of growth factor receptors; such as Epidermal Growth Factor (EGF) Receptor (EGFR) initiate signal transduction pathways that lead to increased cell proliferation and survival [8,14–16]. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated by growth factor [17] and androgen receptor [18] signaling and plays a prominent role in prostate cancer. Mutations that result in the overactivation of this cascade, activation of other oncogenes, and/or the inactivation of proteins that serve as tumor suppressors such as p53, p27, and phosphatase and tensin homologue (PTEN) [19,20], contribute to carcinogenesis and the development of prostatic tumors [21–23].

Increased Akt activation is associated with carcinogenesis as well as increased resistance to chemotherapeutic agents such as cisplatin, methotrexate, and paclitaxel [24,25]. Akt expression/activation is often elevated in human prostate cancers [26,27]. Aberrations in the PI3K/Akt pathway have been reported in approximately 40 % of early prostate cancer and 70–100 % of advanced cases [28,29]. Specifically, the loss of PTEN leads to the constitutive activation of the PI3K/Akt pathway.
pathway [4]. Due to the importance of Akt in prostate cancer, several small molecules that target/inhibit Akt are currently in clinical development [30,31].

Mechanistic target of rapamycin (mTOR), a 289 kDa serine/threonine kinase, is a downstream effector of the PI3K/Akt pathway, and is involved in the control of cell growth [32,33]. Twice the levels of total and phosphorylated mTOR have been reported in prostate cancer tissue when compared to normal prostate epithelium [34]. As a result mTOR is an appealing therapeutic target and mTOR inhibitors such as sirolimus, deforolimus, everolimus, and temsirolimus are used as a monotherapy or combined therapy for various types of cancers [24,33,35].

The process of programmed cell death, known as apoptosis, is an essential process in the maintenance of cell homeostasis. The most common signaling cascades involved in regulating cellular apoptosis promote the downstream activation of caspases and Poly (ADP-ribose) polymerase-1 (PARP-1) cleavage to form 89 and 24 kDa fragments [36]. PARP-1 is an enzyme responsible for DNA repair and therefore, plays a role in genomic maintenance [37,38]. Cleaved PARP is an established indicator of apoptosis [36,38].

Over 50 % of modern chemotherapeutic agents that are used for cancer treatment are derived from natural products [39,40]; for example, the chemotherapeutic drug paclitaxel was originally isolated from the bark of the Pacific yew tree (Taxus brevifolia) and the chemotherapeutic drug docetaxel was originally isolated from the needles of the European yew tree (Taxus baccata) [41].

Scientific interest in finding chemicals in plants with anti-cancer potential continues today. The rosemary (Rosmarinus officinalis L.) plant, native to Mediterranean countries, contains the polyphenols carnosic acid (CA), rosmarinic acid (RA), and carnosol (COH) in high concentrations [42,43]. In recent years, rosemary extract (RE) and RE polyphenols have been reported to have antioxidant, antimicrobial, and anti-cancer properties [44–48]. Limited data exists regarding the effects of rosemary extract in prostate cancer [49–52], and little is known about the underlying signaling mechanisms involved in mediating its pro-apoptotic and anti-proliferative effects.

In the present study, we investigated the effects of rosemary extract in PC-3 and 22RV1 prostate cancer cells, as well as in PNT1A normal prostate epithelial cells.

2. Materials and methods

2.1. Materials

The PC-3 human epithelial prostate cancer cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), 0.25 % trypsin and the antibiotic-antimycotic solution were purchased from Gibco Life Technologies (Burlington, ON, Canada). Akt (#9272) (1:1000 dilution), p-Akt (Ser473) (#9271) (1:1000 dilution), mTOR (#2972) (1:1000 dilution), p-mTOR (Ser2448) (#2971) (1:1000 dilution), PARP (#9542) (1:1000 dilution), β-actin (#8457) (1:1000 dilution), and the media was replaced every 48 h until the cells reached 90 % confluency. When confluency was reached the cells were incubated with 100 % DMSO to yield a stock concentration of 100 mg/mL in cell culture media) and the final concentration of DMSO in the RE-treated cells was less than 0.1 %. Exposure of the cells to DMSO to match the concentration of DMSO seen by cells exposed to RE (vehicle control) did not have any effect on any assays/measurements used in the current study.

2.4. Cell proliferation assay

Cells were seeded (1000 cells/well) in a 96-well plate supplemented with DMEM and treated as indicated in the figures for 72 h. The cells were fixed with 10 % formalin and stained using 0.5 % crystal violet stain. The next day solubilizer solution containing 0.05 M NaH₂PO₄ was added into each well and the absorbance was read at 570 nm using the KC4 microplate reader.

2.5. Clonogenic survival assay

Cells were seeded (1000 cells/well) in six-well plates and allowed to adhere for 24 h followed by treatment as indicated in the figures for seven days. At the end of the treatment, the cells were washed twice with sterile phosphate-buffered saline (PBS) and stained with 0.05 % w/v methylene blue. The next day, colonies greater than 50 cells were counted under the microscope.

2.6. Wound healing assay

The wound healing assay was used to assess cell migration [54,55]. Cells were seeded at a density of 2.5 × 10⁴ cells/mL into a 6-well plate and the media was replaced every 48 h until the cells reached 90–100 % confluency. When confluency was reached the cells were incubated with mitomycin-C (MMC) (1 μg/mL) for 1 h to prevent cell proliferation. After the incubation period, a vertical line was drawn in the centre of each well using a 100 μL pipette tip. The wells were drained of media and washed twice with PBS to get rid of floating cells, followed by treatment as indicated in the figures. Before taking photographs, horizontal lines were drawn underneath the well plates to be used as a reference for future time points. Photos were taken at 0 and 40 -h time points. Wound closure percentage was calculated using the equation

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\text{Wound closure} \times 100
\]

2.7. Immunoblotting

Cell lysate samples containing 20 μg of protein, determined using the Bradford assay [56], were loaded onto 10 % polyacrylamide gel and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred onto a membrane.
polyvinylidene difluoride (PVDF), membrane which was exposed to blocking buffer (5% (w/v) dry milk in Tris-buffered saline) for 1 h and incubated with the primary antibody overnight at 4 °C. The following day the membrane was incubated with horseradish peroxidase (HRP)-linked IgG anti-rabbit secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL), the Bio-Rad clarity western solution, was used to detect the bands corresponding to the proteins of interest. Densitometric analysis was performed using ImageJ software. The data (arbitrary densitometric units) were corrected to β-actin levels and expressed as a percentage of untreated control cells.

2.8. Statistical analysis

The data are the mean ± standard error mean (SEM) of the indicated number of independent experiments. Analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test was used to determine the significance of the differences between groups. Significance was assumed at p < 0.05. Statistical tests were performed using GraphPad Prism 8 software.

3. Results

3.1. Inhibition of PC-3 prostate cancer cell proliferation by rosemary extract

The antiproliferative effects of RE were evaluated in the androgen receptor negative PC-3 prostate cancer cells. PC-3 cells were exposed to 5, 10, 25, 50, 75, 100, or 150 μg/mL RE for 72 h and cell proliferation was assessed using the crystal violet assay. The RE powder was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution (100 mg/mL). This solution was diluted using cell culture media to create a working stock (400 μg/mL) that was used to treat the cells. Treatment with RE resulted in a dose-dependent inhibition of cell proliferation (Fig. 1A). A significant inhibition (80.25 ± 4.44 % of control, p < 0.01) was seen with 10 μg/mL RE while maximum inhibition (26.51 ± 2.95 % of control, p < 0.0001) was seen with 50 μg/mL RE (Fig. 1A-C). Higher RE concentrations (75, 100 and 150 μg/mL) did not result in a statistically greater inhibition of cell proliferation compared to 50 μg/mL (Fig. 1A). The data from Fig. 1A were plotted on a log scale (Fig. 1B) and the calculated RE concentration for the half maximal inhibition (IC_{50}) of cell proliferation was 19.72 μg/mL. Docetaxel (DTX), derived from Taxus baccata and paclitaxel (PTX), derived from Taxus brevifolia, are established medications used clinically in the treatment of prostate cancer [57] and we used them in the present study to compare the effects of RE to their effects. We used two different concentrations (5 and 10 nM) of PTX and DTX based on other in vitro studies [58,59]. Treatment of the cells with 5 nM PTX did not result in any significant inhibition of cell proliferation (91.27 ± 2.79 % of control, p > 0.05) (Fig. 1C), while treatment with 10 nM of paclitaxel showed significant inhibition of cell proliferation (67.63 ± 4.24 % of control, p < 0.01) (Fig. 1C). Treatment of the cells with 5 and 10 nM DTX resulted in significant inhibition of cell proliferation (38.11 ± 1.16, 32.08 ± 0.84 % of control respectively, both p < 0.0001) (Fig. 1C). The inhibition of cell proliferation seen with 50 μg/mL of rosemary extract (26.51 ± 2.95 % of control, p < 0.0001) was greater than that seen with 10 nM PTX treatment and at the same level achieved with DTX treatment.
3.2. Inhibition of PC-3 prostate cancer cell survival by rosemary extract

The ability of cancer cells to survive and form colonies was also assessed through a clonogenic survival assay. Exposure of PC-3 cells to 0.5, 1, 2.5, 5, or 10 μg/mL of RE resulted in a concentration-dependent inhibition of survival (Fig. 2A) with a significant inhibition (80.20 ± 4.60 % of control, p < 0.01) seen with 0.5 μg/mL RE. The greatest inhibition (15.27 ± 3.80 % of control, p < 0.0001) of cell survival was seen at 10 μg/mL RE (Fig. 2A and 2C). The data from Fig. 2A were plotted on a log scale and the calculated RE concentration for the half maximal inhibition (IC₅₀) of cell survival was 2.43 μg/mL (Fig. 2B).

Exposure of the cells to 0.5 nM (64.12 ± 6.94 % of control, p < 0.05) and 5 nM paclitaxel (23.81 ± 11.92 % of control, p < 0.0001) both resulted in a significant inhibition of cell survival (Fig. 2C). In addition, treatment with both 0.5 nM (59.18 ± 9.45 % of control, p < 0.01) and 5 nM docetaxel (8.50 ± 8.50 % of control, p < 0.0001) resulted in a significant inhibition of cell survival.

3.3. Inhibition of Akt signaling in PC-3 prostate cancer cells by rosemary extract

We also examined the effects of RE treatment on Akt and measured the levels of total Akt and Akt phosphorylation on the serine 473 residue, an established indicator of Akt activity [60]. Treatment of PC-3 prostate cancer cells with 50 μg/mL RE for 24 and 48 h both significantly reduced Akt phosphorylation/activation (60.65 ± 11.84 % of control, p < 0.001) and (36.46 ± 4.79 % of control, p < 0.0001), respectively (Fig. 3A and B). The total Akt levels were significantly reduced by 48 h (69.93 ± 4.66 % of control, p < 0.01) but not 24 h (87.52 ± 9.50 % of control, p > 0.05) RE treatment.

Fig. 2. Inhibition of PC-3 prostate cancer cell survival by rosemary extract. PC-3 cells were seeded (1000 cells/well) in six-well plates and exposed to 0.5, 1, 2.5, 5, or 10 μg/mL of rosemary extract (RE) (A, B, C), 0.5, 5 nM paclitaxel (PTX) (C), or 0.5, 5 nM docetaxel (DTX) (C) for 7 days followed by fixing and staining with 0.05 % methylene blue. Colonies of more than 50 cells were counted. Data are expressed as percent of control, untreated cells. Data are the mean ± SEM of 6 independent experiments. *p < 0.05, **p < 0.01, ****p < 0.0001.

3.4. Inhibition of mTOR signaling in PC-3 prostate cancer cells by rosemary extract

Next, we examined the effects of RE on mTOR activation by utilizing an antibody that recognizes phosphorylation of the serine 2448 residue, an established marker of mTOR activation [61]. Treatment of PC-3 cells with RE for 24 (49.41 ± 6.60 % of control, p < 0.0001) or 48 h (38.82 ± 6.69 % of control, p < 0.0001) resulted in a significant decrease in mTOR phosphorylation (Fig. 4A and B). Treatment with RE for 24 and 48 h also showed a significant decrease of total mTOR levels (71.74 ± 10.38 % of control, p < 0.01) and (60.55 ± 9.02 % of control, p < 0.001), respectively.

3.5. Increased apoptosis of PC-3 prostate cancer cells by rosemary extract

The effect of RE on cell apoptosis was examined by measuring the levels of cleaved PARP, an established indicator of apoptosis [37]. Exposing PC-3 prostate cancer cells to rosemary extract (50 μg/mL) for 24 h resulted in a significant increase in cleaved PARP (177.9 ± 14.50 % of control, p < 0.0001).
of control, $p < 0.01$) relative to the control, indicating enhanced apoptosis (Fig. 5A and B).

We routinely examined microscopically the morphology of cells before and after treatments. Fig. 6 shows a representative image of RE- and DTX-treated PC-3 cells compared to the control untreated cells. No changes in cell morphology were observed with any of the treatments. It is important to note that the same number of cells were seeded in all wells (six-well plates were used). As it can be seen from Fig. 6, treatment with 50 $\mu$g/mL of RE for 24 or 48 h resulted in a substantially reduced cell density relative to the control untreated group. Treatment with DTX for 24 or 48 h showed a reduction in cell density that was not as great as the reduction seen with RE.

### 3.6. Inhibition of PC-3 prostate cancer cell migration by rosemary extract

The wound healing assay was used to assess the ability of prostate cancer cells to migrate. The cells were seeded at a density of $2.5 \times 10^5$ cells/mL into a 6-well plate and grown until the cells reached 90–100% confluency. PC-3 cells were exposed to 1 $\mu$g/mL of mitomycin-C (MMC) for 1 h to inhibit cell proliferation. After the MMC was removed a wound was established by drawing a vertical line in the centre of each well using a 100 $\mu$L pipette tip. The cells were then treated without (control) or with either 50 $\mu$g/mL RE or 10 nM docetaxel for 40 h. Treatment with rosemary extract was shown to significantly inhibit wound closure ($56.14 \pm 3.48\%$ of control, $p < 0.0001$) indicating properties against
cell migration (Fig. 7 B). A significant inhibition of cell migration was also seen when treating the cells with 10 nM docetaxel (70.92 ± 2.35 % of control, p < 0.001), (Fig. 7 A and B).

### 3.7. Inhibition of 22RV1 prostate cancer cell proliferation and survival by rosemary extract

We also examined the effects of RE on the androgen receptor positive 22RV1 prostate cancer cells. A significant inhibition of cell proliferation was seen with 25 μg/mL RE (86.20 ± 4.53 % of control, p < 0.01) while the highest level of inhibition was seen with 150 μg/mL RE (49.80 ± 2.289 % of control, p < 0.0001), (Fig. 8A). The IC_{50} value of RE for cell proliferation, calculated by graphing the data from Fig. 8A on a log scale, was 43.41 μg/mL (Fig. 8B). The effects of RE on 22RV1 prostate cancer cell survival was investigated by treating the cells with 2.5, 5, 10, 15, and 20 μg/mL RE for 7 days (Fig. 8C). A dose-dependent inhibition of cell survival was seen. The calculated RE concentration for the half maximal inhibition (IC_{50}) of cell survival calculated using the data from Fig. 8C and graphing it on a log scale was 4.17 μg/mL (Fig. 8D).

### 3.8. Effect of rosemary extract on PNT1A normal prostate epithelial cell proliferation

PNT1A prostate epithelial cells represent normal healthy prostate epithelium. Treatment of PNT1A cells with 5, 10, 25, 50, 75, 100, or 150 μg/mL RE for 72 h did not result in any significant changes in cell proliferation (p > 0.05) (Fig. 9).

### 4. Discussion

The current treatment strategies for prostate cancer include surgery, radiotherapy, and chemotherapy [62]. Patients with localized prostate cancer are most often treated with radical prostatectomy or radical radiotherapy, however advanced and metastatic prostate cancer is treated with hormonal therapy [62]. Common hormonal therapies often use androgen-receptor inhibitors or LHRH agonists (such as leuprolide, goserelin, buserelin, or nafarelin) that initially increase testosterone production, but with prolonged exposure downregulate the LHRH receptor and inhibit testosterone production [5]. LHRH antagonists (such as cetrorelix, abarelix, or orgalutran) directly inhibit LHRH, which decreases testosterone production [5]. Surgical castration can also decrease testosterone levels by removing the source of production. For patients that do not respond to androgen therapies, cytotoxic chemotherapeutic agents, such as etoposide, doxorubicin, paclitaxel, and docetaxel are used [5] but resistance often develops, indicating a need for novel therapeutics to be used alone or in combination with existing drugs to treat prostate cancer and improve patient outcome.

Plant extracts have been used traditionally for medicinal purposes and more than half of all available chemotherapy agents used in cancer treatment are derived from plants with paclitaxel and docetaxel representing two such chemotherapeutics [39–41]. Finding chemicals in plants with anti-cancer potential is the focus of many research labs, including ours. In recent years a few studies provided evidence of anticancer properties of rosemary extract [42–48]. In the present study we found a dose-dependent inhibition of PC-3 androgen-independent and 22RV1 androgen-dependent prostate cancer cell proliferation with rosemary extract treatment (Figs. 1, 8, and 10). Similar to our findings, in other studies, treatment with RE dose-dependently inhibited the viability of 22RV1 and LNCaP prostate cancer cells [51]. In addition, a
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A dose-dependent decrease in proliferation and viability of PC-3, DU145, and LNCaP prostate cancer cells was seen by RE treatment [49,52]. Based on our data, we calculated the RE concentration for half maximal inhibition (IC_{50}) of cell proliferation and comparing these IC_{50} values it appears that the androgen-independent PC-3 cells (19.72 μg/mL) are more sensitive to RE treatment than the androgen-dependent 22RV1 cells (43.41 μg/mL). Importantly, treatment of the PNT1A normal prostate epithelial cells with RE did not significantly affect their rate of proliferation (Fig. 9). Similarly to our findings, Petiwala et al. [51], as mentioned above, found a significant inhibition of 22RV1 and LNCaP prostate cancer cell viability but not an effect on normal prostate epithelial cells derived from two different patients undergoing radical prostatectomy [51]. These data indicate that RE is able to discriminate and preferentially target prostate cancer cells while sparing normal healthy prostate epithelial cells.

Apart from cell proliferation, treatment with RE resulted in a dose-dependent inhibition of cell survival with IC_{50} values of 2.43 μg/mL and 4.17 μg/mL for PC-3 and 22RV1 cells, respectively (Figs. 2, 8, and 10). These data indicate a higher sensitivity of the androgen-independent PC-3 cells than the androgen-dependent 22RV1 cells to RE treatment and are in agreement with our proliferation data.

The inhibition of prostate cancer cell (PC-3) proliferation and survival seen with RE treatment was robust and comparable to the inhibition seen with docetaxel (DTX) and paclitaxel (PTX) both routinely used in the treatment of prostate cancer [57]. Cleaved PARP is an established indicator of apoptosis [36,38], and our data showed an increase in cleaved PARP levels in PC-3 cells treated with RE indicating an effect of RE to induce apoptosis (Fig. 5). Similar to our findings, treatment of 22RV1 and LNCaP prostate cancer cell with RE resulted in a significant increase in apoptosis [51]. In addition, treatment of PC-3 cells with the RE polyphenol carnosic acid [63] and PC-3 and DU145 cells with the RE polyphenol rosmarinic acid [64] induced apoptosis as indicated by the increased levels of cleaved PARP.

Furthermore, RE treated PC-3 cells showed a significant inhibition of cell migration (56.14 ± 3.48 % of control, p < 0.0001) that was comparable to the response seen with docetaxel (70.92 ± 2.35 % of control, p < 0.001) (Fig. 7). No other studies examining the anti-migratory or anti-metastatic effects of RE in prostate cancer cells currently exist. We previously found a significant inhibition of MDA-MB-231 breast cancer cell migration by RE treatment [65]. In a study by Pérez-Sánchez et al.,
treatment with RE inhibited the migration of HGUE-C-1, HT-29, and SW480 human colon cells [66]. In prostate cancer metastasis, cells migrate away from the primary tumor to other tissues. Once in other tissues, their invasiveness depends on their clonogenic survival. Treatment with RE significantly reduced both, the cell migration, as assessed by the wound-healing assay, and the clonogenic survival. Our data indicate that RE has the potential to reduce prostate cancer cell proliferation and tumor growth as well as reduce their migration and invasion capabilities.

The expression and activation of the serine/threonine kinase Akt is often elevated in human prostate cancer. Approximately 40% of early cases and 70–100% of advanced cases of prostate cancer have aberrations in the PI3K/Akt signaling [26–29]. This is often due to mutations on PI3K [4], Akt [67] as well as the loss of the tumor suppressor gene PTEN [68], all of which lead to overactivation of Akt resulting in enhanced proliferation and survival. Our study showed a significant inhibition of Akt phosphorylation/activation with RE treatment (Fig. 3). A search of the literature revealed that no other studied have examined the effects of RE treatment on Akt in prostate cancer cells. It should be noted that a significant inhibition of Akt phosphorylation/activation was seen in PC-3 prostate cancer cells treated with the RE polyphenol carnosic acid [63] and carnosol [69]. In previous studies by our lab, we found a significant inhibition of Akt phosphorylation/activation by RE treatment of A549 lung cancer [53] and MDA-MB-231 breast cancer cells [65].

It has been reported that the levels of total and phosphorylated mTOR are twice as great in prostate cancer tissue when compared to normal prostate epithelium [34]. mTOR is a downstream target of Akt and its activation leads to increased protein synthesis and cell proliferation [70]. Our study is the first to show a significant inhibition of mTOR in prostate cancer cells with RE treatment (Fig. 4). Similar to our study, a significant inhibition of mTOR phosphorylation/activation was seen in PC-3 prostate cancer cells treated with the RE polyphenol carnosol [69].
The mechanisms involved in the RE-induced inhibition of Akt and mTOR (Fig. 10) is not known. It is possible that components in RE act as allosteric inhibitors of Akt and/or mTOR or they act on a step upstream of Akt. Another possibility is that components in RE increase the activity of Akt and/or mTOR specific phosphatases [71,72] resulting in their reduced phosphorylation/activation. It is also possible that the inhibition of mTOR is due to Akt inhibition. Future studies should examine these possibilities and elucidate the mechanisms involved in these inhibitory effects of RE.

PC-3 cells, contrary to 22RV1, contain PTEN mutations leading to enhanced Akt activation [73]. It is possible that the increased activation of the PI3K/Akt/mTOR cascade in PC-3 cells may explain their higher sensitivity to RE treatment compared to 22RV1 cells. The notion that RE targets prostate cancer cells characterized by increased Akt-mTOR signaling should be explored in future studies to further define RE’s potential as a therapeutic agent.

Apart from a reduction in phosphorylated/activated Akt and mTOR levels, our study shows a reduction in the total levels of these proteins with RE treatment (Figs. 3 and 4), which may be due to the inhibition of gene transcription, inhibition of protein synthesis, upregulation of
mTOR pathway in prostate cancer is associated with increased proliferation and survival, resistance to treatment, and overall poor prognosis. Our data provide strong evidence that treatment with RE targets this pathway, but it is not known whether this is true in vivo. Future studies using animal models xenografted with prostate cancer cells should be performed to investigate this possibility as well as further examine the anti-cancer properties of RE. Future studies should also examine the exact polyphenolic constituent(s) of rosemary extract that contribute to its anti-cancer effects.

Author contributions

E.T conceived and designed the experiments and contributed to data interpretation and manuscript writing. A.J performed the majority of the experiments, analyzed the data, prepared the figures and contributed to manuscript writing. D.T performed the experiments using 22Rv1 and PNT1A cells. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

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