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Epigallocatechin-3-gallate (EGCG) inhibits PC-3 prostate cancer cell proliferation via MEK-independent ERK1/2 activation

Daniel S. Albrecht [a], Elizabeth A. Clubbs [a], Mario Ferruzzi [b], Joshua A. Bomser [a,*]

[a] *Department of Human Nutrition, The Ohio State University, 1787 Neil Avenue, 325 Campbell Hall, Columbus, OH 43210, United States*
[b] *Department of Food Science, Purdue University, West Lafayette, IN 47907, United States*

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Abstract

Epigallocatechin-3-gallate (EGCG), a tea polyphenol, inhibits the proliferation of many cancer cell lines; however, the antiproliferative mechanism(s) are not well-characterized. The objective of this study is to identify the cellular signaling mechanism(s) responsible for the antiproliferative effects of EGCG in the PC-3 prostate cancer cell line. EGCG inhibited PC-3 cell proliferation in a concentration-dependent manner with an IC_{50} value of 39.0 μM, but had no effect on the proliferation of a nontumorigenic prostate epithelial cell line (RWPE-1). Treatment of PC-3 cells with EGCG (0–50 μM) resulted in time and concentration-dependent activation of the extracellular signal-regulated kinase (ERK1/2) pathway. EGCG treatment did not induce ERK1/2 activity in RWPE-1 cells. The activation of ERK1/2 by EGCG was not inhibited using PD98059, a potent inhibitor of mitogen-activated protein kinase kinase (MEK), the immediate upstream kinase responsible for ERK1/2 activation; suggesting a MEK-independent signaling mechanism. Pretreatment of PC-3 cells with a phosphoinositide-3 kinase (PI3K) inhibitor partially reduced both EGCG-induced ERK1/2 activation and the antiproliferative effects of this polyphenol. These results suggest that ERK1/2 activation via a MEK-independent, PI3-K-dependent signaling pathway is partially responsible for the antiproliferative effects of EGCG in PC-3 cells.

Keywords: Epigallocatechin-3-gallate; Extracellular signal-regulated kinase; Prostate cancer; Green tea

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in men, and the second leading cause of cancer deaths in the United States [1]. Epidemiological and experimental studies suggest that increased consumption of green tea reduces the risk of prostate cancer [2–4]. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, is thought to be responsible, in part, for the chemopreventive properties of this beverage [4,5]. EGCG (Fig. 1) has been shown to inhibit cellular proliferation and induce apoptosis in the androgen-insensitive PC-3 cell line [6–8]; however, the antiproliferative mechanism remains unclear.

The modulation of signal transduction cascades controlling cellular proliferation, apoptosis, differentiation and cell cycle progression by dietary agents has emerged as a potential target for treatment and prevention of prostate cancer. The ERK1/2 signaling cascade is a member of the mitogen-activated protein kinase (MAPK) family and is required for the normal growth, development and survival of the prostate epithelium [9]. Nontumorigenic prostate epithelial cells have high basal levels of ERK1/2 activity, while this activity is often dysregulated and gradually declines

* Corresponding author. Tel.: +1 614 247 6622; fax: +1 614 292 8880.
E-mail address: bomser.1@osu.edu (J.A. Bomser).

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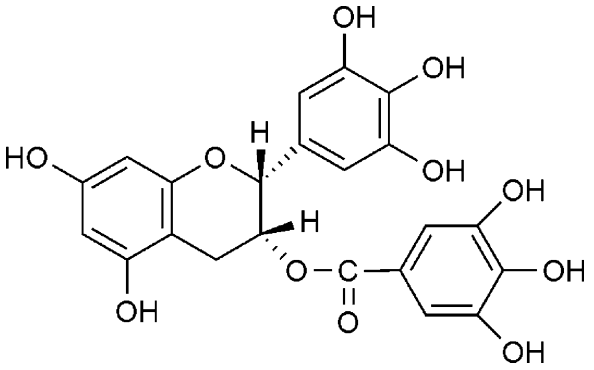

Fig. 1. Chemical structure of the green tea catechin epigallocatechin-3-gallate (EGCG).

during the progression of prostate cancer [10–12]. Therefore, maintaining ERK1/2 activity may represent one strategy for prostate cancer chemoprevention.

Although EGCG has been shown to induce ERK1/2 activity in a number of prostate cancer cell lines [13] it is unclear whether the antiproliferative activity of this compound is dependent upon activation of this signaling pathway. The present study examines the mechanism by which ERK1/2 signaling may mediate the antiproliferative effects of EGCG in PC-3 cells.

2. Materials and methods

2.1. Materials

EGCG was obtained from Sigma–Aldrich (St. Louis, MO, USA). EGCG was prepared as stock solutions in water, diluted in media and sterile filtered before use. Specific chemical inhibitors of phosphoinositide-3 kinase (PI3K, LY294002) and mitogen activated protein kinase kinase (MEK1/2, PD98059) were purchased from Tocris (Ballwin, MO, USA). PD98059 and LY294002 were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were utilized in cell culture media at appropriate dilutions with a final DMSO concentration of 0.1%. Bicinchoninic acid (BCA) Trade Mark protein assay kit and superSignal ECL Trade Mark chemiluminescent substrate kit were obtained from Pierce (Rockford, IL, USA). ECL Western blotting detection reagent was obtained from Amersham Biosciences Corporation (Piscataway, NJ, USA). Polyclonal antibodies for phospho-ERK1/2 kinase and total ERK1/2 kinase were obtained from Cell Signaling-Technology (Beverly, MA, USA). CellTiter 96® Aqueous [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) cell proliferation assay was obtained from Promega (Madison, WI, USA).

2.2. Cell culture

The nontumorigenic human prostate epithelial cell line (RWPE-1) and the metastasized human prostate cancer line (PC-3) were purchased from the American Type Culture Collection (Rockville, MD, USA). RWPE-1 cells were maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 50 mg/L bovine pituitary extract, 5% L-glutamine and 5 μg/L epidermal growth factor (EGF). The PC-3 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Both cell lines were maintained in a humidified incubator (5% CO_2, 95% O_2) at 37 °C.

2.3. Cellular proliferation

PC-3 prostate cancer cells and RWPE-1 prostate epithelial cells were plated in 96-well cell microtiter plates or 48-well cell culture plates at a target density of 6.6×10^4 cells/mL (1×10^4 cells/well in 96-well plate). Cells were allowed to attach overnight in growth medium. Growth medium was aspirated after 24 h. PC-3 and RWPE-1 cells were treated with EGCG (0–50 μM) in serum free or supplement free growth medium, respectively, for 48 h. After incubation, cellular proliferation was measured using the MTS assay and absorbance was measured at 492 nm. For mechanistic studies, cells were treated with 50 μM PD89059 or 20 μM LY294002 1 h prior to treatment with EGCG for 48 h. Cell proliferation was determined by the MTS assay. Proliferation data are presented as means ± S.E.M.

2.4. Immunoblot detection of active and total ERK1/2

RWPE-1 and PC-3 cells were grown in 60 mm dishes for 48 h (90% confluent). Growth media was removed and cells were washed with phosphate-buffered saline (PBS) and incubated in supplement free media for 24 h. Cells were then treated with EGCG alone or in combination with specific inhibitors (LY294002 or PD98059) at appropriate times and concentrations. Following treatments, growth medium was removed and cells were washed with PBS. Crude proteins were isolated and separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed using primary antibodies that recognize active (phospho-) and total ERK1/2 at recommended dilu-

tions in TTBS (1× tris-buffered saline (TBS) with 0.1% Tween-20) overnight at 4 °C. Following incubation with secondary antibodies, protein signals were visualized on autoradiography film using ECL, and quantified by densitometry using Scion imaging software (Frederick, MD, USA). Basal (control) levels of protein expression are reported as 1, and treatment effects are expressed as a fold increase or decrease.

2.5. Statistical analyses

Statistical significance between treatment groups was determined by one-way analysis of variance (ANOVA) with Tukey's post hoc comparisons (Minitab statistical software; State College, PA, USA).

3. Results

3.1. Effects of EGCG on PC-3 and RWPE-1 cellular proliferation

The proliferative effects of EGCG on PC-3 prostate cancer cells and RWPE-1 prostate epithelial cells are given in Fig. 2. Cells were treated with 0–50 μM EGCG for 48 h. The rate of PC-3 cellular proliferation upon treatment with 12.5, 25 and 50 μM EGCG was 73 ± 3.9, 63 ± 3.8 and 46 ± 3.8% as compared to untreated control, respectively. The IC_{50} value 48 h after treatment was determined to be 38.95 μM EGCG. Treatment of RWPE-1 cells with EGCG up to 50 μM had no effect on proliferation.

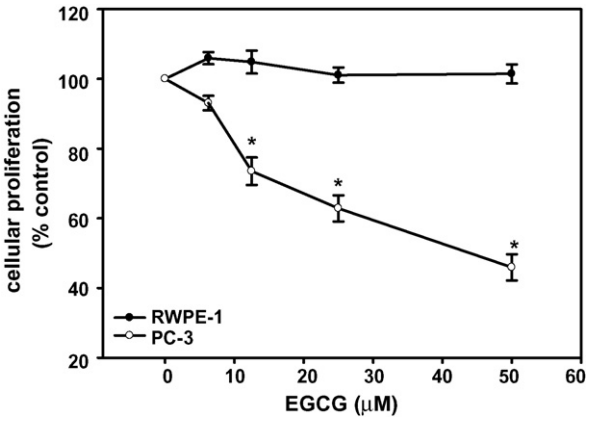

Fig. 2. Proliferation effects of EGCG in nontumorigenic (RWPE-1) and tumorigenic (PC-3) prostate cells. Cells were treated with EGCG (0–50 μM) and incubated for 48 h and proliferation was assessed by MTS assay. Experiments were performed independently at least three times with an $n = 8$. Data are given by percentage of means ± R.S.E. with significant differences from controls indicated by $^{*}p < 0.05$.

3.2. EGCG induces ERK1/2 activity in PC-3 but not RWPE-1 cells

The effect of EGCG on ERK1/2 activity in RWPE-1 and PC-3 cells is given in Fig. 3. PC-3 cells were treated with 0–50 μM EGCG for 2 h (Fig. 3A). ERK1/2 activity strongly correlated with inhibition of proliferation ($r^2 = 0.865$) when PC-3 cells were treated with 0–50 μM EGCG for 2 h (Fig. 3B). EGCG-induced ERK1/2 activity was both dose and time-dependent in PC-3 cells (Fig. 3A and C). EGCG (50 μM, 2 h) induced ERK1/2 activity 12.7 times greater than control ($p < 0.05$). EGCG induced ERK1/2 activity within 30 min yet was strongest at 2 h and remained elevated above control levels through at least 6 h post treatment. EGCG did not induce ERK1/2 activity at concentrations tested in RWPE-1 cells (Fig. 3D). However, epidermal growth factor (EGF) was capable of inducing ERK1/2 activity in this cell line. No changes in total ERK1/2 (*t*-ERK1/2) were observed with any treatment.

3.3. EGCG induces ERK1/2 activity via a MEK-independent, PI3-K-dependent mechanism

In order to determine the mechanism of EGCG-induced ERK1/2 activation, PC-3 cells were treated with EGCG alone (50 μM, 2 h) or in combination with the MEK inhibitor, PD98059 (50 μM, 1 h pretreatment). The effect of MEK inhibition on EGCG-induced ERK1/2 activation in PC-3 cells is given in Fig. 4A. EGCG treatment alone induced ERK1/2 activity, while PD98059 treatment did not alter basal levels of ERK1/2 activity. EGCG-induced ERK1/2 activity was not inhibited upon pretreatment of PC-3 cells with the MEK inhibitor, PD98059.

The effect of PI3K inhibition on EGCG-induced ERK1/2 activity is given in Fig. 4B. PC-3 cells were treated with or without the PI3K inhibitor LY294002 (20 μM) for 1 h prior to treatment with EGCG (50 μM, 2 h). Treatment with EGCG alone caused an ∼10-fold increase in ERK1/2 activation as compared to untreated control. Pretreatment with LY294002 caused a significant decrease in EGCG-induced ERK1/2 activation ($p < 0.05$); however, a 4.6-fold increase in ERK1/2 activity above controls was still observed.

3.4. PI3K inhibition partially blocks the antiproliferative effects of EGCG

The effects of partial inhibition of EGCG-induced ERK1/2 activity on PC-3 cellular proliferation were determined using the PI3K inhibitor, LY294002 (Fig. 5).

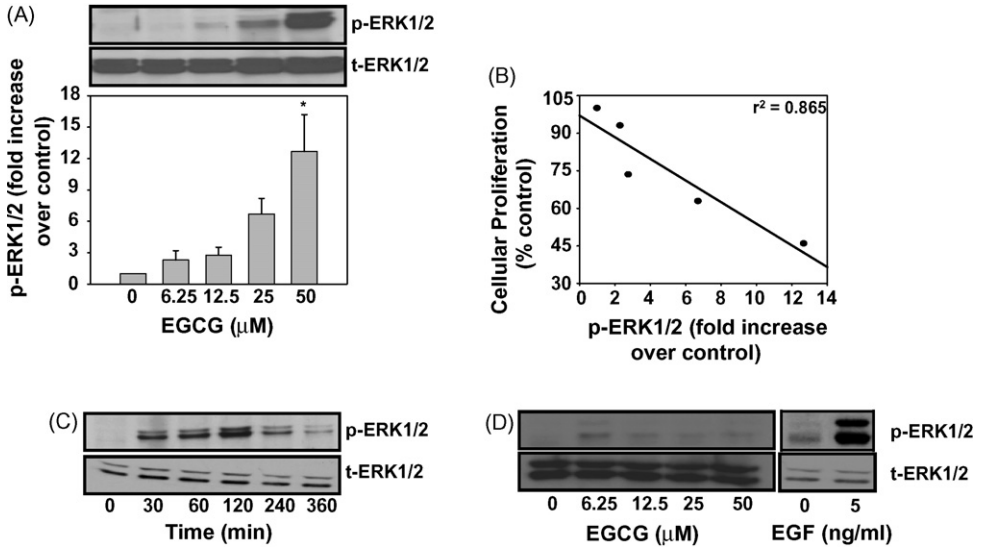

Fig. 3. Concentration and time-dependent induction of ERK1/2 activity by EGCG in RWPE-1 and PC-3 cells. (A) Active ERK1/2 (*p*-ERK1/2) was measured by immunoblot in PC-3 cells following 2 h treatment with EGCG (0–50 μM). Data are given as a representative immunoblot and for quantification. Data are represented as means ± S.E.M. with significant differences from controls indicated by $^{*}p < 0.05$. (B) Active ERK1/2 correlated with inhibition of cellular proliferation in PC-3 cells following 2 h treatment with EGCG (0–50 μM). (C) Time-dependent (0–360 min) activation of ERK1/2 following treatment of PC-3 cells with 50 μM EGCG. (D) Active ERK1/2 (*p*-ERK1/2) was measured by immunoblot in RWPE-1 cells following 2 h treatment with EGCG (0–50 μM) or 10 min with EGF (5 ng/mL). All experiments were performed independently at least three times with an $n = 3$. Total ERK1/2 (*t*-ERK1/2) was used as the loading control.

PC-3 cells were pretreated with or without LY294002 (5 μM, 2 h) prior to treatment with EGCG (0–50 μM, 48 h). Treatment of cells with 6.25, 12.5, 25 and 50 μM EGCG alone resulted in a 7.0 ± 1.0, 12.4 ± 4.3, 37 ± 3.5 and 48 ± 4.1% reduction in proliferation, respectively. Cells treated with LY294002 prior to treatment with EGCG resulted in a 13 and 25% reduction in proliferation at 25 and 50 μM, respectively.

4. Discussion

After water, tea is the most commonly consumed beverage worldwide. Epidemiological evidence suggests that consuming 1–3 cups or more of green tea daily for greater than a year may reduce the risk for prostate cancer incidence by nearly half [3]. Experimental evidence strongly suggests that dietary polyphenols found in tea and other plant based foods may be responsible, in part, for protection against prostate cancer [14] and recent studies have demonstrated the presence of tea polyphenols in prostate tissue after consumption of this beverage [15]. In vivo studies suggest that tea polyphenols inhibit prostate carcinogenesis in the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model by reducing circulating insulin-like growth factor-1 and inducing insulin-like growth factor binding protein 3 [16,17] and the proapoptotic protein clusterin [18].

EGCG is the most abundant polyphenol in green tea, representing at least 40% of the polyphenolic profile. The chemopreventive effects of EGCG alone have not been well characterized in vivo; however in vitro studies suggest that this polyphenol inhibits proliferation and induces cell death in a number of prostate cancer cell lines, including LNCaP, DU-145 and PC-3 [19–22]. Several studies have previously established that EGCG inhibits PC-3 cell proliferation via cell cycle arrest [7] resulting in increased apoptosis [6–8]. However, the specific cellular mechanisms responsible for these effects are unknown. The present study examined the cellular signaling mechanisms by which EGCG inhibits the proliferation of PC-3 cells.

EGCG inhibited the proliferation of PC-3 cells with an IC_{50} value of approximately 39.0 μM. Others [6,18] have reported IC_{50} values of approximately 100 μM for EGCG in the PC-3 cell line. This concentration discrepancy is most likely due to differences in treatment times.

We hypothesized that activation of the ERK1/2 signaling cascade may mediate some of the antiproliferative effects of EGCG. This hypothesis is based on previous studies demonstrating that EGCG-induced ERK1/2 activation was associated with decreased proliferation of DU-145 and LNCaP prostate cancer cells [13]. Concentration-dependent activation of ERK1/2

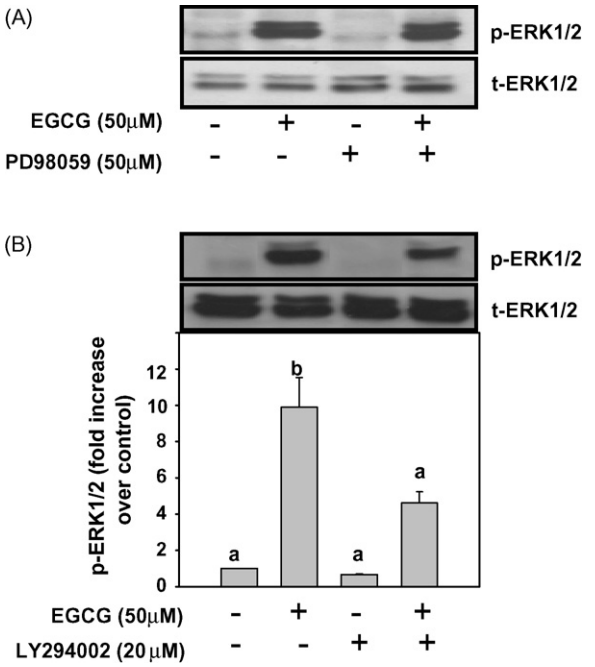

Fig. 4. EGCG-induced ERK1/2 activation is dependent on PI3K and not MEK signaling. (A) Active ERK1/2 (*p*-ERK1/2) was measured by immunoblot in PC-3 cells following treatment with or without PD98059 (50 μM, 1 h) prior to EGCG (50 μM, 2 h). (B) Active ERK1/2 (*p*-ERK1/2) was measured by immunoblot in PC-3 cells following treatment with or without LY294002 (20 μM, 1 h) prior to EGCG (50 μM, 2 h). All experiments were performed independently at least three times with an $n = 3$. Data are given by fold increase over control (means ± S.E.M.). Mean values not sharing common letter superscript differ significantly ($p < 0.05$). Total ERK1/2 (*t*-ERK1/2) was used as the loading control.

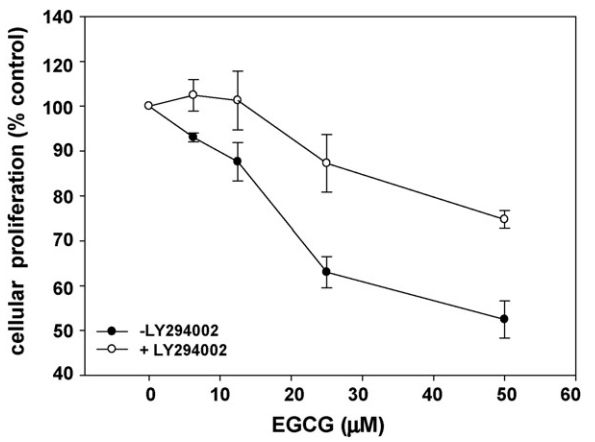

Fig. 5. PI3K inhibition protects PC-3 cells from the antiproliferative effects mediated by EGCG. PC-3 cells were treated with or without LY294002 (5 μM, 2 h) prior to EGCG treatment (0–50 μM) and incubated for 24 h and proliferation was assessed by the MTS assay. Experiments were performed independently at least three times with an $n = 8$. Data are given by percentage of means ± R.S.E.

following treatment with EGCG was correlated with decreased PC-3 cell proliferation ($r^2 = 0.865$). Interestingly, this correlation was not observed in the RWPE-1 cell line; suggesting that the antiproliferative effects of this polyphenol may be both cell line and cancer-stage specific.

Activation of the ERK1/2 signaling cascade is commonly associated with increased cellular proliferation and its upregulation is observed in approximately 30% of all tumors [23]. Prostate carcinogenesis is unique in that a gradual decline in ERK1/2 signaling is observed with the progression of this disease [12,13]. It is hypothesized that restoring ERK1/2 signaling may inhibit prostate cancer progression [10,24] perhaps by inducing cell cycle arrest and apoptosis [25–28]. However, it is important to emphasize that the duration of the ERK1/2 signal is critically important in determining cellular outcomes. For example, transient ERK1/2 activation, mediated by epidermal growth factor, induces a proliferative response in the PC-3 cell line (data not shown). In contrast, strong and sustained ERK1/2 activation is commonly associated with induced cell cycle arrest [26–28], and has been shown to induce apoptosis in the PC-3 cell line [25]. The strong and sustained ERK1/2 activation mediated by EGCG suggests a potential signaling mechanism by which this polyphenol inhibits cellular proliferation. To further test this hypothesis, EGCG-induced ERK activation was blocked using several chemical inhibitors.

Typically, ERK1/2 activation involves transmission of signals in the form of sequential phosphorylation and activation of the Raf-MEK-ERK 3-tier kinase cascade. Interestingly, inhibition of MEK, the upstream kinase usually responsible for the activation of ERK1/2, did not block EGCG-induced ERK1/2 activation. This suggests that EGCG may activate ERK1/2 via an atypical signaling pathway not involving MEK. Atypical and sustained ERK1/2 activation has been shown to be mediated, in part, via the PI3K pathway in mouse lymphocytes [29]. Therefore, we examined the potential role of PI3K-induced ERK1/2 activation in the PC-3 cell line. Treatment of PC-3 cells with the PI3K inhibitor, LY294002, results in a reduction in activity of PI3K and the downstream target AKT [30,31]. In the present study, chemical inhibition of the PI3K activity partially inhibited EGCG-induced ERK1/2 activation and also reduced the antiproliferative effects of EGCG. The PI3K signaling cascade is a pro-survival pathway that is constitutively active in the PC-3 cell line and is associated with prostate cancer progression [30,32]. The prosurvival activity of PI3K is mediated, in part, via activation of AKT and subsequent inhibition of ERK signaling [33]. We and others hypothesis that treatment of PC-3

cells with EGCG may disrupt this prosurvivial pathway, leading to sustained ERK1/2 activation and subsequent reduced PC-3 cell proliferation [13]. However, the exact role of the PI3K signaling pathway in mediating the antiproliferative effects of EGCG is unclear.

Data presented in this study characterizes a possible MEK-independent mechanism by which the tea catechin, EGCG, induces sustained ERK1/2 activation with subsequent reduction in cellular proliferation in the androgen-independent PC-3 prostate cancer cell line. Androgen-independent prostate cancer is an aggressive, late stage tumor that is highly resistant to chemotherapeutic agents. Metastasized prostate cancer has an overall 5-year survival rate of only 33%, while the 5-year survival rate of localize prostate cancer is 100% [1]. Approximately 60% of these advanced human prostate cancers exhibit a loss of expression of the PTEN protein resulting in constitutively activation of PI3K signaling [34]. Therefore, the cell line used in this study is an applicable model for advanced stage prostate cancer. The discovery of agents that specifically target these chemoresistant cells may aid with adjuvant therapy.

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