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Resveratrol derivatives increase cytosolic calcium by inhibiting plasma membrane ATPase and inducing calcium release from the endoplasmic reticulum in prostate cancer cells

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ABSTRACT

Resveratrol (RES) is a putative chemotherapeutic naturally found in grapes, peanuts, and Japanese knotweed. Previous studies demonstrate that RES modulates calcium signaling as part of its chemotherapeutic activity. In this study, we determined the chemotherapeutic activity of three RES esters that have been modified at the 4' hydroxyl by the addition of pivalate, butyrate, and isobutyrate. All of the RES derivatives disrupted the calcium signaling in prostate cancer cells more than the parent compound, RES. Further, we demonstrate that the RES derivatives may disrupt the calcium homeostasis by activating calcium release from the endoplasmic reticulum and inhibiting plasma membrane Ca^{2+}-ATPase. The pivalated and butyrated RES derivatives decreased cell viability significantly more than RES. Because pivalated and butyrated RES are more effective than RES at targeting calcium signaling pathways, pivalated and butyrated RES may serve as more effective chemotherapeutics.

1. Introduction

Prostate cancer was diagnosed in 164,690 American men in 2018 [1]. For the majority of these men, the tumor grows slowly and can be effectively treated. However, in 7%, the tumor becomes aggressive and metastatic. In advanced prostate cancer cases, treatments are much less effective and the 5-year mortality rate drops from 98% for all prostate cancer to 30% for advanced prostate cancer [2]. The reason that advanced prostate cancer is so difficult to treat is that the tumors acquire a series of molecular alterations that limit the efficacy of the standard treatments. It is therefore critical to find a treatment that targets unique pathways in the more aggressive tumor that are not targeted with standard chemotherapeutics. One of the pathways that is modulated in prostate cancer is calcium signaling [3,4]. The calcium pathway continues to be modified as the tumor progresses to more advanced forms and may be exploited as a target in advanced tumors without affecting the normal cell counterparts [5][1].

Calcium signaling is the result of changes in the homeostatic calcium concentration in the cell [6]. The size, duration, and subcellular location of changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) determine what calcium binding proteins are activated, and thereby the resulting cell signal. In this study we focus on sustained calcium increases that result in cell death in advanced prostate tumor cells. The $[Ca^{2+}]_i$ is approximately 10,000 fold lower than the $[Ca^{2+}]_i$ outside of the cell and in the endoplasmic reticulum (ER). The cells maintain this concentration through a series of pumps and channels. Calcium pumps require energy to move calcium from low concentration to high concentration whereas calcium channels open and enable calcium to flow down the gradient. Plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endoplasmic reticulum ATPase (SERCA) are calcium pumps that use ATP hydrolysis to push calcium from the cytosol into the extracellular milieu and ER, respectively. Inositol triphosphate receptor (IP_3R) and

Abbreviations: RES, resveratrol; PIV, 4′-pivalate resveratrol; IsoRV, 4′-isobutyrate resveratrol; BuRV, 4′-butyrate resveratrol; $[Ca^{2+}]_i$, cytosolic calcium concentration; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarcoendoplasmic reticular Ca^{2+}-ATPase; Fura-2, Fura-2-Acetoxymethyl ester; TG, thapsigargin; HBSS, Ca^{2+} and Mg^{2+}-free Hank's Balanced Salt Solution; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; AUC, area under the curve; DMEM, Dulbecco's modified Eagle medium; ER, endoplasmic reticulum; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; PIP_2, phosphatidylinositol biphosphate; PLC, phospholipase C; 2-APB, 2-Aminoethyl diphenylborinate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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the ryanodine receptor (RyR) are calcium channels on the ER that open and release calcium from the ER into the cytosol. IP_3R can be activated through a phospholipase C-mediated (PLC) pathway. Voltage gated, store operated, receptor operated, and mechanosensitive channels are all classes of plasma membrane calcium channels that open in response to changes in membrane potential, ER calcium depletion, ligand binding, or changes in pressure, respectively.

PMCA and IP_3R have been identified as putative chemotherapeutic targets in advanced stage prostate cancer or drug resistant prostate cancer [7]. A recent paper has demonstrated that as prostate cancer cells become castration resistant, they produce more PMCA in extracellular vesicles [8]. This paper postulates that PMCA is a key component that modulates the calcium signaling network in castration-resistant prostate cancer. Another study demonstrated that PMCA is more active in advanced prostate tumors than in normal prostate cells [9]. IP_3R are modulated in response to androgen deprivation in LnCAP cells [10]. Additionally, an analysis of the TCGA database revels that IP_3R is altered in 30% of prostate tumors [11]. Together this information indicates that PMCA and IP_3R are good targets for treating advanced, androgen-independent prostate cells. PC3 cells are androgen-independent cells derived from metastatic prostate cancer [12]. It is thus ideal for our studies because it represents the most difficult to treat and drug resistant tumor.

Resveratrol [13] (RES) is a putative chemotherapeutic that has been shown to have chemotherapeutic activity against breast [14], prostate [15], and colon cancers [16]. RES appears to be a promiscuous molecule that binds to many cellular proteins [17], and thereby simultaneously activates several pathways. In tumor cells, RES has been shown to activate intrinsic apoptotic [18], extrinsic apoptotic [19], necrotic [20] and autophagic [21,22] pathways. RES has been shown to modulate many of the calcium pumps and channels resulting in calcium signals that activate apoptotic pathways [23,24]. RES activates a large and sustained calcium signal in tumor cells that results in tumor cell death [23,25]. One of the advantages of RES promiscuity is that it can both activate IP_3R and inhibit PMCA leading to a cytotoxic increase in $[Ca^{2+}]_i$ [26–28]. In this study we are screening for RES derivatives that are more potent than their parent compound at increasing cytoplasmic calcium by inhibiting PMCA and stimulating IP_3R.

2. Materials and methods

The PC-3 prostate cancer cell line was purchased from ATCC (Manassas, VA, USA). The passage numbers of cells used in experiments ranged from 5 to 20. Thapsigargin (10522) (TG), fura-2-acetoxymethyl ester (14591) (Fura-2), and *trans*-resveratrol (70675) (RES) were purchased from Cayman Chemical (Ann Arbor, MI, USA). U-73122 (J62898) and 2-Aminoethyl diphenylborinate (A16606) (2-APB) were purchased from Alfa Aesar (Ward Hill, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (AC158990050) (MTT) was purchased from Acros Organics (Morris Plains, NJ, USA). 4′-Butyrate resveratrol (BuRV), 4′-isobutyrate resveratrol (IsoRV) and 4′-pivalate resveratrol (PIV, Fig. 1) were synthesized by the Andrus lab in the Department of Chemistry and Biochemistry at Brigham Young University [29]. The purity of the compounds was determined to be greater than 98% by NMR and mass spectrometry.

2.1. Cell culture

PC-3 cells (passage numbers 5–20) were cultured in Dulbecco's modified Eagle medium (DMEM). DMEM was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. PC-3 cells were cultured at 37 °C in 5% CO_2.

2.2. Cell viability

Cell viability was determined using an MTT assay as described previously [28]. Briefly, PC-3 cells were plated at 10,000 cells per well in a Greiner Bio-One Cellstar 96-well plate (Greiner Bio-One, Monroe, NC, USA). Cells were grown for 24 h and then treated with RES or RES derivatives at the indicated concentrations using 1.5% dimethyl sulfoxide (DMSO) in cell culture media as vehicle. The cells were treated for 48 h, then 20 µL of 5 mg/mL MTT was added to each well. Cells were then incubated for 3.5 h at 37 °C in 5% CO_2. Media and MTT were decanted, after which 150 µL of MTT solvent was added. Cells were agitated on an orbital shaker at 75 rpm for 15 min before absorbance was read at 590 nm with a reference filter at 620 nm on a BMG LABTECH FLUOstar OPTIMA plate reader (BMG LABTECH Inc., Cary, NC, USA). All experiments were performed in triplicate (n = 3).

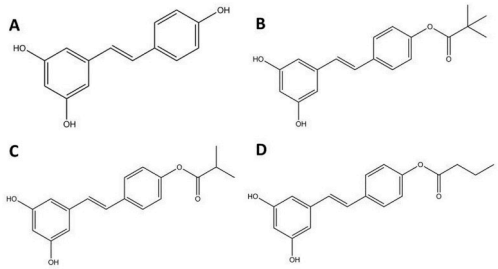

Fig. 1. Structures.
Structures of (A) *trans*-resveratrol, (B) 4′-pivalate *trans*-resveratrol, (C) 4′-isobutyrate *trans*-resveratrol, and (D) 4′-butyrate *trans*-resveratrol.

2.3. Intracellular calcium imaging

Fura-2 loading and experimental protocols performed were similar to those performed as described previously [28]. Briefly, cells were plated and grown in an 8-well chamber, washed twice with 1:1 PBS:FBS solution, and incubated at 37 °C in 5% CO_2 for 30 min in 3 µM Fura-2 in Ringer's solution (NaCl 150 mM, glucose 10 mM, HEPES 5 mM, KCl 5 mM, MgCl2 1 mM, CaCl2 2 mM, pH 7.4). The cells were washed with fresh Ringer's solution, and cells were allowed to equilibrate for 30 min prior to imaging. For experiments studying plasma membrane Ca^{2+}-ATPase (PMCA) inhibition, fresh Ringer's solution was removed and replaced with a Ca^{2+}-free buffer, Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS). The PC-3 cells were imaged with an Olympus IX51 inverted microscope. Treatments were added manually in 100 µL volumes as outlined in the results section of this paper.

Images acquired were analyzed using CellSens software (version number 1.11, Olympus, Tokyo, Japan) from Olympus. At least 10 cells per experiment were chosen and analyzed in order to measure changes in relative $[Ca^{2+}]_i$. The results of the analysis were normalized for each treatment and used to find the mean change in relative $[Ca^{2+}]_i$ for each experiment. Each experiment was performed in triplicate ($n \geq 3$). The calcium traces were quantified by determining the area under the curve (AUC) or total calcium response after treatment addition using GraphPad Prism 7 (La Jolla, CA, USA).

2.4. Statistical analysis

Statistical significance was determined by a two-way analysis of variance (ANOVA) with Bonferroni correction. Statistically significant differences between RES or RES derivative treatments and the vehicle control are indicated by * if $p < 0.05$ and ** if $p < 0.01$. Statistically significant differences between RES and RES derivative treatments are indicated by # if $p < 0.05$ and ## if $p < 0.01$. In experiments where PLC or IP_3R were inhibited, statistically significant differences between the inhibited cells and uninhibited cells (but still treated with the same RES derivative) are represented by * if $p < 0.05$ and ** if $p < 0.01$.

3. Results

3.1. RES derivatives

Modifications to RES may help increase efficacy of RES as a chemotherapeutic by increasing the activity on pathways specific to chemotherapeutic activity and identify structural components that are important for RES chemotherapeutic activity. Previous studies have demonstrated that modifying the 4′ hydroxyl group on resveratrol increased its chemotherapeutic activity [28]. Here we have esterified the 4' hydroxyl with a butyrate (BuRV), isobutyrate (IsoRV), and pivalate (PIV) group (Fig. 1) and measured how these molecules modulate cytosolic calcium concentrations and cell viability in PC-3 prostate cancer cells.

3.2. RES derivatives induce an increase in cytosolic calcium

Previous studies have demonstrated that one of RES's chemotherapeutic mechanisms is a prolonged increase in cytoplasmic calcium that can activate apoptosis [24]. To determine the effect of these RES derivatives on prostate cancer cells, live-cell microscopy was performed on PC-3 cells loaded with the calcium-sensitive dye, Fura-2. After collecting the initial baseline for 1 min, the Fura-2 loaded PC-3 cells were then treated with 150 μM RES (dotted, black), BuRV (dash, black), IsoRV (dash, gray), or PIV (solid, gray) (Fig. 2). Changes in $[Ca^{2+}]_i$ are shown in two ways: first (Fig. 2A), the traces of changes in $[Ca^{2+}]_i$ over time relative to a control (solid, black) that was treated with vehicle only (3 separate trials of 10 cells each), and second (Fig. 2B), the quantification of the traces demonstrated in Fig. 2A as determined by measuring the area under the curve (AUC). The 150 μM concentration of RES and the derivatives is high and not likely clinically relevant, however we used a high concentration to make sure that we could detect any differences in the efficacy of RES derivatives relative to the native compound. All four compounds stimulated an increase in $[Ca^{2+}]_i$ of at least 100-fold relative to the vehicle control. All three of the RES derivatives (PIV, IsoRV, and BuRV) stimulated an increase in $[Ca^{2+}]_i$ that was 3.2, 1.6, and 1.6-fold higher, respectively, than that stimulated by RES. The overall shape of the change in $[Ca^{2+}]_i$ for each compound is similar, with an initial rapid rise in $[Ca^{2+}]_i$ followed by a leveling off of $[Ca^{2+}]_i$ occurring 4–5 min after addition of treatment. There is significant noise in the calcium trace after treatment with BuRV and it is unclear what lead to such a noisy response.

3.3. RES derivatives indirectly activate IP_3R in advanced prostate cancer cells

Subsequent to determining that RES derivatives increase $[Ca^{2+}]_i$ in a manner similar to RES, we sought to determine if the RES derivatives were increasing $[Ca^{2+}]_I$ through known RES targets. We focused on two calcium targets that RES has previously been shown to modulate in cancer cells: ER calcium release through IP_3R and PMCA inhibition [28,30]. First, to determine if the RES derivatives are stimulating calcium release from the ER, we inhibited steps in the ER calcium release pathway. PLC hydrolyzes PIP_2 to form IP_3, which binds to and opens the calcium channel, IP_3R, thus allowing calcium to flow from the ER to the cytosol. We used 5 μM U-73122 and 100 μM 2-APB, inhibitors of PLC and IP_3R, respectively. The PC-3 cells were loaded with Fura-2, pretreated with the inhibitor for 10 min, and then 150 μM RES or RES derivative was added and the change in $[Ca^{2+}]_i$ was measured (Fig. 3). When PLC activity was inhibited in PC-3 cells by U-73122, there was no significant change in the calcium trace for RES and IsoRV relative to the uninhibited cells. PIV-induced and BuRV-induced increases in $[Ca^{2+}]_i$ following PLC inhibition were significantly lower than increases in $[Ca^{2+}]_i$ caused by the uninhibited treatment of cells with PIV and BuRV. This difference in $[Ca^{2+}]_i$ change between PLC-inhibited and uninhibited cells indicates that PIV and BuRV are activating calcium release from the ER via a PLC-mediated pathway.

Next, we inhibited IP_3R directly with 2-APB. Inhibiting IP_3R (Fig. 3) caused significantly lower increases in $[Ca^{2+}]_i$ for all of the compounds in comparison to the uninhibited samples. The PIV-induced $[Ca^{2+}]_i$ increase is almost completely eliminated without IP_3R activity. The PIV does retain a signal that increases much more slowly than the uninhibited PIV signal. This is likely due to a secondary action of PIV on calcium signaling similar to the biphasic increase in $[Ca^{2+}]_i$ caused by PIV as seen in Fig. 2. It is important to note that 2-APB is a promiscuous calcium channel inhibitor that has been shown to inhibit calcium-release activated channels (CRAC) in addition to IP_3R at the concentrations used in this study. Therefore, we cannot distinguish which channel is being inhibited in this study. However, the combination of the 2-APB and U-73122 data indicates that the IP_3R is being activated.

3.4. RES may inhibit PMCA in advanced prostate cancer cells

We tested the effect of RES and RES derivatives on PMCA activity by using the method developed by Baggaley et al. [31] PC-3 cells in Ca^{2+}-free media release ER calcium by adding thapsigargin (TG), a SERCA inhibitor. When this is done, the likely way to decrease an elevated

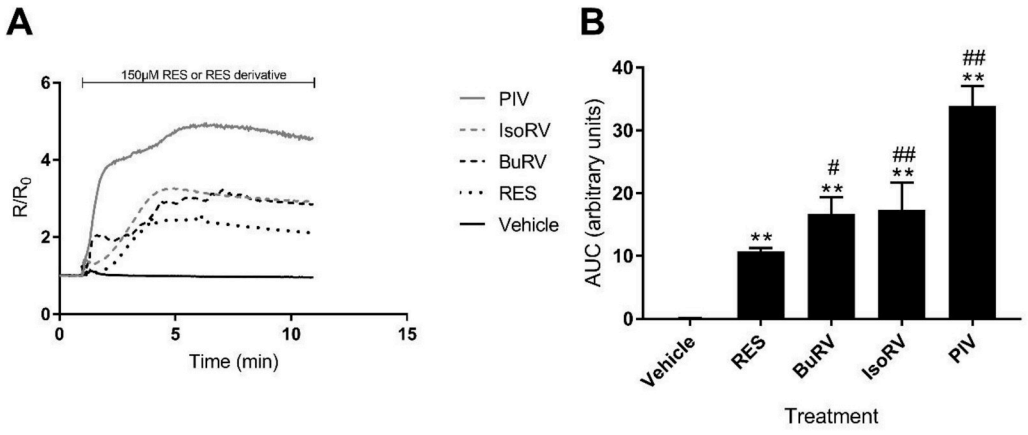

Fig. 2. RES and RES derivatives induce increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in PC-3 prostate cancer cells.
Cells were loaded with the [Ca2+]i detection dye, Fura-2. The cells were treated with 150 μM RES (dotted, black), BuRV (dash, black), IsoRV (dash, gray), PIV (solid, gray), and a vehicle control (solid, black) after 1 min baseline collection. The change in [Ca2+]i was observed for 10 min. Traces (the mean of experiments performed in triplicate, with n ≥ 10 cells analyzed per field) for each compound tested (A) and the mean area under the curve (AUC) quantified from the traces (B) are shown. **indicates $p < 0.01$ compared to vehicle treated control, # indicates $p < 0.05$ compared to RES, ## indicates $p < 0.01$ compared to RES.

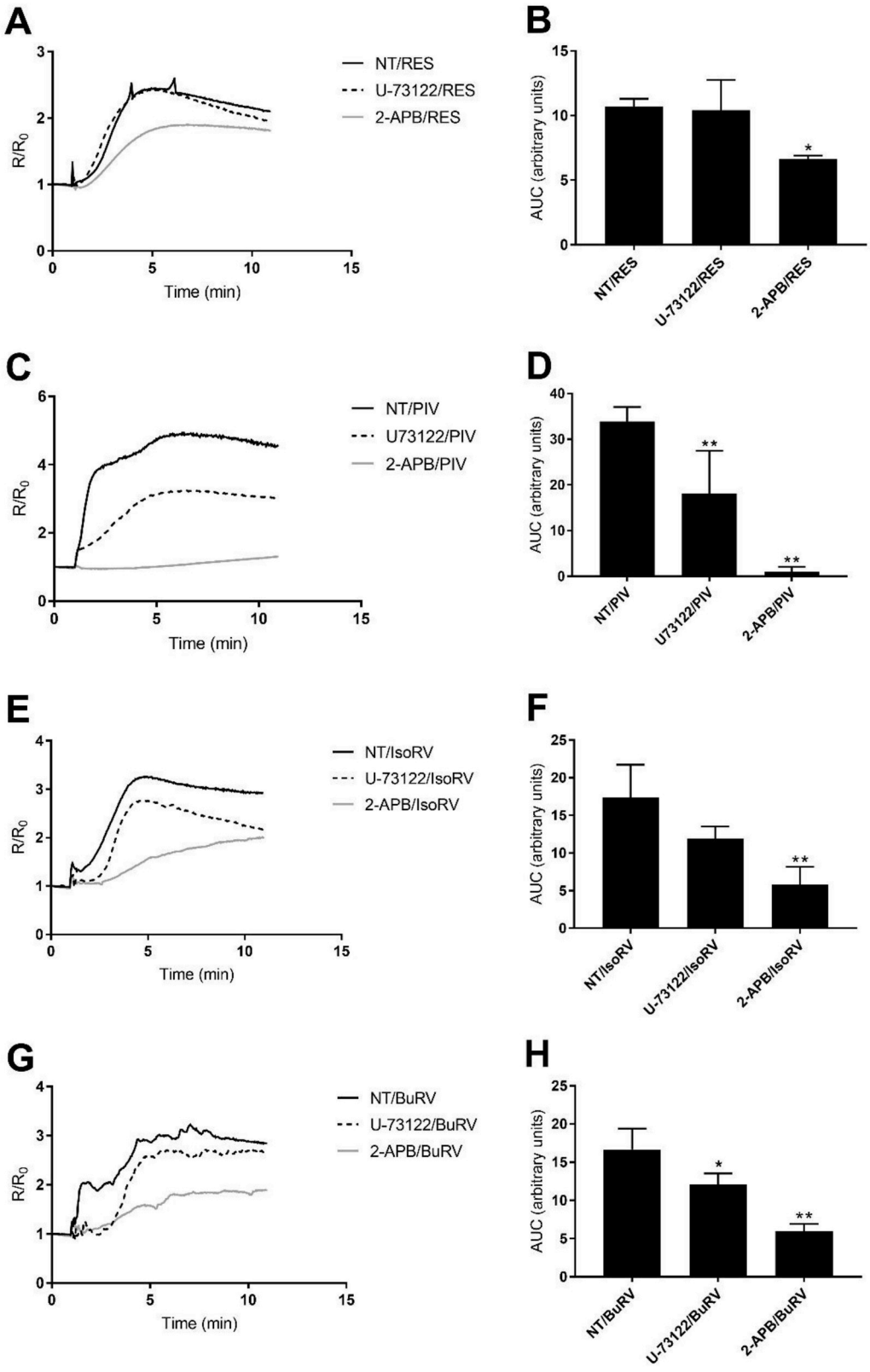

Fig. 3. RES and RES derivatives activate calcium release from the ER through PLC and IP_3R.
PC-3 prostate cancer cells were pretreated with a PLC inhibitor (5 μM U-73122) or an IP3R inhibitor (100 μM 2-APB) for 10 min after which they were treated with 150 μM RES, PIV, IsoRV, or BuRV. The change in [Ca2+]i is shown for 10 min following the addition of RES or RES derivatives (1 min of baseline collection is also displayed for each trace). Traces (the mean of experiments performed in triplicate, with n ≥ 10 cells analyzed per field) for RES (A), PIV (C), IsoRV (E), and BuRV (G). The average area under the curve (AUC) for RES (B), PIV (D), IsoRV (F), and BuRV (H). *indicates $p < 0.05$ compared to uninhibited control, ** indicates $p < 0.01$ compared to uninhibited control.

$[Ca^{2+}]_i$ (leaving the ER) is through PMCA activity; therefore, compounds that inhibit PMCA activity will increase the $[Ca^{2+}]_i$ in this assay. Although, there may be some effect from mitochondrial calcium buffering as well. RES and RES derivatives inhibited PMCA activity significantly relative to the vehicle only negative control (Fig. 4). All of the RES derivatives inhibited PMCA activity significantly more than RES alone. Because mitochondria could also be buffering the calcium we cannot say difinatively that RES and the RES derivatives are inhibiting PMCA. Future studies could use purified PMCA in vessicles to directly measure the RES effect on PMCA.

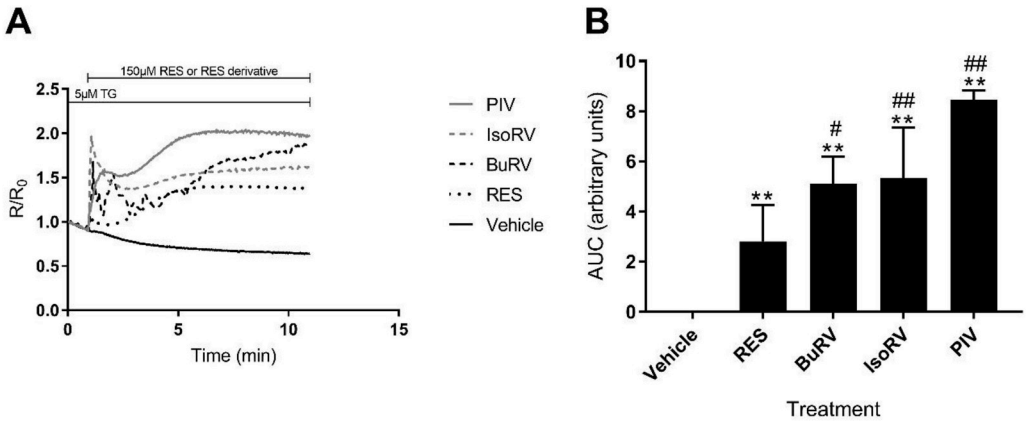

Fig. 4. RES and RES derivatives inhibit PMCA activity.
PC-3 prostate cancer cells were pretreated with thapsigargin (TG) in a calcium-free media for 5 min, after which they were treated with 150 μM RES, PIV, IsoRV, BuRV or a vehicle control. Due to Ca2+-free extracellular conditions and SERCA inhibition by TG. Traces (the mean of experiments performed in triplicate, with n ≥ 10 cells analyzed per field) for RES (dotted, black), BuRV (dash, black), IsoRV (dash, gray), PIV (solid, gray), and a vehicle control (solid, black) (A) and the mean AUC for all compounds tested (B). An increase in [Ca2+]i indicates PMCA inhibition. **indicates $p < 0.01$ compared to vehicle treated control, # indicates $p < 0.05$ compared to RES, ## indicates $p < 0.01$ compared to RES.

3.5. Prostate cancer cell viability

Previous studies demonstrated a link between RES-induced increase in $[Ca^{2+}]_i$ in cancer cells and a decrease in cancer cell viability [15]. PC-3 cells were treated with 100 μM RES or RES derivatives for 48 h and assayed for cell viability with the MTT assay (Fig. 5). This treatment resulted in a 50% ± 9.1%, 74% ± 9.9%, 58% ± 8.8%, 89% ± 5.9% decrease in cell viability for PC-3 cells treated with RES, BuRV, IsoRV, and PIV, respectively. IsoRV was statistically indistiguishable from RES, whereas BuRV and PIV both decreased cell viability significantly more than RES.

4. Discussion

Treating advanced prostate cancer requires novel chemotherapeutics that target unique molecular pathways. Intracellular calcium homeostasis is modified in advanced prostate cancer and targeted by RES. Although RES has low toxicity and high tumor specificity in cell and animal models, these results have not translated to an effective treatment in humans [32]. The apparent low efficacy of RES is likely due to low bioavailability. In this study, we are interested in identifying RES derivatives that increase $[Ca^{2+}]_i$ through mechanisms like PMCA inhibition and/or IP_3R activation more effectively than their parent compound RES. We hope that this screen is the first step in identifying more effective treatments for advance prostate cancer. Calcium signaling is one of the unique chemotherapeutic targets of RES [28,30,33]. We made the three derivatives of RES by esterification at the 4′ hydroxyl with three four-carbon acids in order to find compounds that increase RES efficacy. The effect of the derivatization of RES suggests that having a hydrophobic group attached the 4' hydroxyl group on RES increases the affinity for the protein or proteins that lead to an increase in cytoplasmic calcium levels.

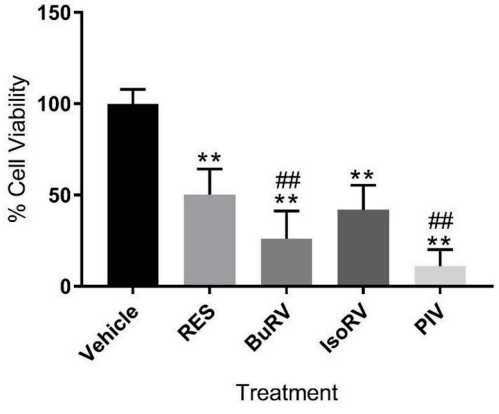

Fig. 5. RES and RES derivatives decrease prostate cancer cell viability.
PC-3 cells were treated with 1.5% DMSO (vehicle control) or 100 μM of RES, BuRV, IsoRV, and PIV for 48 hours. The values were normalized to the vehicle control. **indicates $p < 0.01$ compared to vehicle treated control. ## indicates $p < 0.01$ compared to RES treatment.

The RES derivatives induce an increase in $[Ca^{2+}]_i$ and decrease cell viability in prostate cancer. All three derivatives tested increased $[Ca^{2+}]_i$ more than RES. BuRV and PIV decreased cell viability significantly more than RES. PIV, BuRV, and IsoRV increase $[Ca^{2+}]_i$ possibly by activating calcium release from the ER and inhibiting PMCA. The PIV-induced calcium signal seems especially dependent on activating the IP_3R as we saw a significant decrease in the $[Ca^{2+}]_i$ subsequent to IP_3R inhibition. Additionally, the RES-derivatives may activate plasma membrane calcium channels which should be addressed more fully in future studies. The results for these derivatives are similar to what we previously reported in breast cancer cells [28], indicating that these derivatives, especially PIV, could be a more effective chemotherapeutic than RES.

Cell-based cancer models are an excellent way to identify novel chemotherapeutic pathways and determine molecular mechanisms, however cell-based models are limited in that they do not provide data on metabolism, absorption, and specificity for cancer. We demonstrate that PIV and BuRV derivatives have more chemotherapeutic activity than RES in a cellular model, which may indicate that these compounds would be more effective in humans. However, data is needed to determine the pharmacokinetics, toxicity, and specificity of these compounds in animal models. Because PIV and BuRV have a higher chemotherapeutic activity, we propose that this may translate into a cancer treatment that is more effective in humans than RES.

PIV was consistently the most effective RES derivative; thus, new PIV derivatives could result in even more effective compounds. We have also identified several parts of the calcium signaling pathway that may be modulated by RES and RES derivatives. However, binding partners for RES and the RES derivatives need to be identified and characterized. Additionally, the esterified RES derivatives could be hydrolyzed in the cells by endogenous esterase activity. As a result, the increased chemotherapeutic activity could be due to better drug delivery as the PIV might more readily passively diffuse though the membrane because it is more hydrophobic. Alternately, PIV may have a higher affinity to the protein targets of RES. These compounds should

be tested in animal models to further determine their chemotherapeutic potential.

5. Conclusion

We have demonstrated that esterifying RES at the 4' hydroxyl with 4 carbon acids results in RES derivatives that increase $[Ca^{2+}]_i$ more than RES. These compounds may increase $[Ca^{2+}]_i$ by inhibiting PMCA and activating calcium release from the ER. Further, these compounds are more effective than RES, save IsoRV, at decreasing prostate cancer cell viability.

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100667.

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