Resveratrol analogues surprisingly effective against triple-negative breast cancer, independent of ERα

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Abstract. Resveratrol, a plant-derived stilbene compound, has exhibited anticancerous properties, including breast cancer. Stilbenes have a molecular structure highly similar to estrogen and have the ability to bind estrogen receptors and regulate activity. Numerous studies have demonstrated the effectiveness of resveratrol in estrogen receptor-positive (ER-positive) subtypes of breast cancer, yet the effects in ER-negative subtypes, including triple-negative breast cancer (TNBC), have been limited. In the present study, resveratrol and 28 analogues were tested on a panel of ER-positive and TNBC cell lines to determine effects on cell viability. Several compounds exhibited significant impacts on cell viability and suggested changes in cell morphology, with high potency of select compounds compared to resveratrol observed in a dose-dependent manner. Due to the lack of estrogen receptors in TNBC and the estrogenic nature of stilbenes, regulation of breast cancer-associated cellular pathways was assessed for five analogues shown to significantly inhibit cell viability. Top regulated pathways included apoptosis (confirmed by caspase assay) and DNA damage repair. Overall, our results indicated several resveratrol analogues to be active in ER-negative phenotypes, acting through an ER receptor-independent manner, supporting further investigation into their mechanism of action and use as potential chemotherapeutics in higher-risk breast cancer cases.

Introduction

Despite many advancements in treatments and annual screenings, breast cancer remains as one of the leading causes of cancer-related deaths in women, second only to lung cancer. In 2017, there was a projected number of 252,710 new cases of invasive breast cancer occurrence, with increases in breast cancer incidence observed from 2005 to 2014 in Asian/Pacific Islanders, non-Hispanic Black, and Hispanic ethnic groups (1). One factor that may complicate treatment for a patient is the specific subtype of breast cancer diagnosed. Cancers of the breast are diverse and distinct from one another; each subtype possessing its own prognosis and treatment implications. The diversity among subtypes makes treatment and prevention plans differ based on the molecular and receptor status of the cancer, complicating research endeavors for new and effective treatments (2). Subtypes resulting from estrogen receptor-positive (ER-positive) cancer cells, representing ~70% of diagnosed cases, are characterized by expressing both the estrogen receptor (ER) and progesterone (PR) that serve as targetable receptor sites for chemotherapeutic treatments (2,3). Owing to previous study on hormone-signaling pathways as a therapeutic target, the majority of ER-positive breast cancer cases are treated with advanced endocrine therapies that function to block estrogen activity and downregulate tumor growth. Drugs that resemble the molecular structure of estrogens are a common means of therapy due to similarities in chemical composition that allow binding at these receptors to either decrease endogenous estrogen activity or act antagonistically with receptors for estrogen signaling (4,5). As the need for more effective treatment methods arises, the potential use of plant-derived compounds, such as resveratrol, has gained popularity in the treatment of breast cancer due to their phenolic structure and role in endocrine receptor binding (Fig. 1) (5,6). Plant-derived compounds known as stilbenes have been associated with many health benefits including the treatment of metabolic disorders such as diabetes and obesity, as well as increasing cardiovascular health.

Resveratrol (Fig. 1A) has received much attention as a chemotherapeutic agent in recent years for its anticancerous properties exhibited in many types of cancer, including lung, pancreatic, ovarian, and breast. Found naturally in many plants such as peanuts, cocoa, grapes, berries, and red wine, the antioxidant properties of resveratrol are attributed to its polyphenolic stilbene structure (7). Resveratrol and its analogues have also been classified as phytoestrogens since their structural resemblance allows them to bind estrogen

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Abbreviations: ER, estrogen receptor; TNBC, triple-negative breast cancer

Key words: resveratrol, triple-negative breast cancer, TNBC, ER-negative, ER-positive

receptors, enabling them to signal through the associated pathways, inducing alterations of kinase activity, transcription of mRNA, or other key cellular functions (8). In the ER-positive cell line MCF-7, several resveratrol phytoestrogenic analogues have demonstrated the ability to: induce pro-apoptotic events leading to significant inhibitory effects on proliferation through ER-dependent signaling (9,10); induce epigenetic modifications in the regulation of cancer (11); and promote reversal of the epithelial-mesenchymal transition (EMT) (12). While resveratrol has shown great promise as a chemotherapeutic agent, low bioavailability limits its effectiveness in clinical settings as it is metabolized rapidly and has a very low solubility, with an oral bioavailability reported in a study by Walle of <1% (13,14). More resent investigations have unveiled novel analogues of resveratrol that exhibit increased effects in cytotoxicity in addition to increased potency in the treatment of breast cancer cells when compared to resveratrol (13,15).

Tumors resulting from the triple-negative breast cancer (TNBC) subtype lack both ER and PR receptors and are negative for the human epidermal growth factor receptor 2 (HER2) gene. While the TNBC subtype accounts for only 10-17% of breast cancer cases, these are among the most difficult to treat since they lack receptors predominantly targeted and exploited by contemporary therapies, leaving traditional chemotherapy as the primary treatment (2,16). In addition, patients diagnosed with the TNBC subtype often experience early and increased rates of metastasis resulting from induction of the epithelial-mesenchymal transition (EMT) in cells (17). This change in morphology decreases cell adherence to primary tumor location, allowing cells, devoid of contact restrictions, to metastasize (16).

Traditionally, resveratrol analogues were thought to affect breast cancer cells through ER-dependent signaling pathways and reversal of the EMT. However, we have found similar effects of resveratrol and 28 analogues on both ER-positive and TNBC cell lines. The lack of ER and other significant modalities often targeted in breast cancer treatments should make resveratrol analogue treatments of TNBC cells ineffective, according to the previously proposed mode of action; yet we demonstrate significant effects of 8 resveratrol analogues in decreasing cell viability in a dose-dependent manner, indicating the existence of an alternative, ER-independent mechanism. To better understand this activity and the potential for future chemotherapeutic use in the treatment of breast cancer, we investigated modulation of intra- and inter-cellular pathways within TNBCs by these resveratrol analogues. Our results suggest that stilbene compounds 3c and 4a-c (Fig. 2) impede TNBC cell viability by inhibiting cell survival pathways and upregulating cell death pathways, including apoptosis signaling.

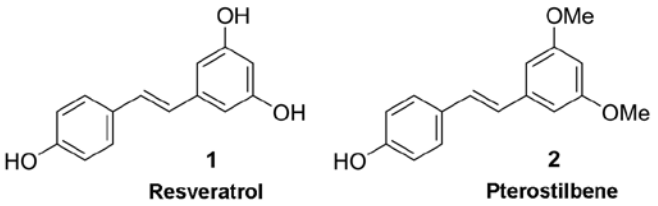

Figure 1. Prototypical plant-derived phytoestrogens. Resveratrol and pterostilbene.

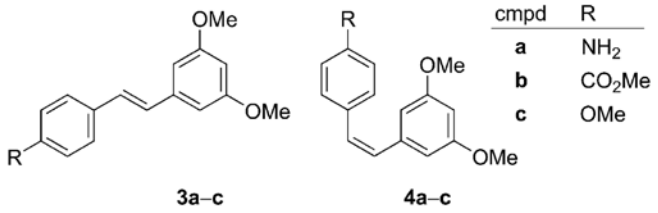

Figure 2. Key resveratrol analogues.

Materials and methods

Chemical methods. Our resveratrol analogue screening library was generously donated by Agnes Rimando at the United States Department of Agriculture (USDA). Larger quantities of select compounds were obtained thus: resveratrol and pterostilbene (Fig. 1) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), and compounds 3a, 3c and 4a-c were synthesized by known methods (18,19); Spectra agreed with the aggregate of previously reported characterizations (18-21). All compounds were analyzed by ^{1}H NMR and GCMS to confirm identity and purity prior to biological analysis. *E/Z* isomers were determined by ^{1}H NMR, by observing *J*=16-16.5 Hz (*E*) and *J*=12-12.5 Hz (*Z*) for the vinyl protons, and isomers were confirmed pure (>99:1 isomeric ratio) by GC (FID). ^{1}H NMR analysis was performed on a JEOL 400 MHz spectrometer in solutions of $CDCl_3$. GC separations were performed on a Restek RTX-5MS column (30 m, 0.25 mm, 0.25 μm); 2 runs, 60˚C/min, 2 ml/min, and 20˚C/min, 3 ml/min. Compound weights were obtained on a Mettler Toledo MS105 micro balance (0.01 mg precision). 4-Hydroxy-tamoxifen and 17β-estradiol were obtained from (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Breast cancer cell culture. ER-positive cell lines ZR-75-1 and MCF-7, and TNBC cell lines MDA-MB-231, MDA-MB-157, and BT-549 were generously provided by Dr Matthew E. Burow of Tulane University [all originally obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA)]. Liquid nitrogen stocks were conducted upon receipt and maintained until the start of each study. Cells were used for no >6 months after being thawed with periodic recording of morphology and doubling times to ensure maintenance of phenotype (22). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA), insulin (24 μg) and 1% each of antibiotic-antimycotic, essential and non-essential amino acids, and sodium pyruvate (Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C in 5% CO_2 and 95% relative humidity, re-fed every three to four days, and passaged as needed.

Proliferation and viability assays. Cell culture plates (96-well) were seeded at a cell density of $1.5x10^3$ cells/well for proliferation and $7.5x10^3$ cells/well for viability at 100 μl/well of 10% DMEM and incubated at 37˚C in 5% humidified CO_2 for attachment overnight. Following incubation, cells were treated

with the corresponding compound suspended in 100 μl 10% DMEM at designated concentrations in internal triplicates. Cells were incubated at 37˚C and harvested after 7 days. For Fig. S1, cells were harvested and fixed after 24 h. Each plate was fixed with 10 μl 25% glutaraldehyde/well for 20 min at room temperature and stained with 50 μl 0.4% crystal violet in 20% methanol solution/well for 20 min at room temperature. Excess crystal violet was removed, and plates were rinsed with water, inverted, and allowed to dry overnight. Cells were lysed with 50 μl 33% acetic acid and rocked for 15 min at room temperature to ensure complete lysing. Lysates were read on plate reader at 490 nm absorbance. Readings were normalized to dimethyl sulfoxide (DMSO) control set to 100%.

RNA isolation, cDNA synthesis and RT^2-PCR assays. To identify effects on gene expression induced by compounds, five compounds were chosen to screen in 88 breast cancer gene arrays through RT-PCR. ER-negative cell line BT-549 were chosen as representative line and treated as outlined above with compounds 1, 3c, and 4a-c and DMSO control at 10 μM for 24 h before harvesting. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) and cDNA was prepared using RT^2 First Strand Kit (Qiagen, Inc.). RT^2 Breast Cancer PCR Array panels were run according to the manufacturers' instructions (Qiagen, Inc.) on the CFX96 Bio-Rad Real-Time PCR thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). CT values were exported to an Excel file to create a table of Cq values. This table was then uploaded onto the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to controls and test groups. Cq values were normalized based on an automatic selection from HKG panel of reference genes. The data analysis web portal calculated fold change/regulation using the ΔΔCq method, where ΔCq is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by ΔΔCq calculations [ΔCq (Test Group) ΔCq (Control Group)]. Fold change was then calculated using $2^{-\Delta\Delta Cq}$ formula (23). The data analysis web portal also created the clustergram heat map. This data analysis report was exported from the Qiagen web portal at GeneGlobe (https://www.qiagen.com/us/shop/genes-and-pathways).

Caspase-3/-7 apoptosis assays. Induction of apoptosis by resveratrol analogues was detected using CellEvent Caspase-3/7 Green Detection reagent as per the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Cells were plated at 5x10^3 cells/well in 96-well cell culture plates and incubated overnight under standard culture conditions. Cells were treated with corresponding analogues at 10 μM final concentration, with 125 μM hydrogen peroxide and DMSO included for positive and negative controls, respectively. Caspase-3/-7 detection reagent was diluted in phosphate-buffered saline (PBS) with 5% FBS to 5 μM stock concentration immediately preceding usage. At 48 h, media was removed from cells and 100 μl of diluted reagent was added to each well. Cells were incubated for 30 min at 37˚C in 5% humidified CO_2. Cells were imaged at the corresponding time-points using an Olympus CKX41 inverted microscope (Olympus Corp., Tokyo, Japan) with fluorescence (X-Cite 120Q; EXFO Inc., Quebec, QC, Canada) absorption/emission at 502/530 nm and a CMS camera (Olympus DP21; Olympus Corp.). All cell images were captured at x40 original magnification. For each condition, the number of caspase-positive (green) was divided by the total number of cells per image (bright field) and multiplied by 100 to determine the percent of positive apoptotic cells/condition.

Statistical analysis. All statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). All treatment groups were compared to control (DMSO) using one-way ANOVA testing with multiple comparisons post hoc tests (Bonferroni). Each trial was plated as internal triplicates with a minimum of three biological replicates for statistical testing.

Results

Analogues of resveratrol reduce cell viability and proliferation in ER-positive cells. To confirm the anticancerous effects of resveratrol reported in previously published studies (12), and to identify additional active analogues and their effects on cell viability, resveratrol and 28 of its analogues, synthesized and obtained from the USDA (18), were screened in two ER-positive, luminal cell lines: MCF-7 and ZR-75-1 (Fig. 3). Effects of our compounds on cell viability were tested against those of tamoxifen, a known anti-estrogen chemotherapeutic agent, as a positive control (24). Of the 29 compounds screened (Figs. 3A and B), we observed significant activity of several compounds on cell viability in both cell lines tested, with minimal variation between active compounds in the two lines. Five of these analogues (3a, 3c, 3r, 4b and 4c, Table I) significantly reduced cell viability in both MCF-7 and ZR-75-1 lines, and an additional two compounds were implicated in the reduction of viability in one of the two cell lines (4a in ZR-75-1 and 4g in MCF-7).

When comparing the effects of resveratrol analogues to resveratrol itself, surprisingly, we observed that resveratrol exhibited no significant decreases on cell viability for either line tested at 10 μM concentration. However, this could be due in part to the inhibitory dose-dependent effects of resveratrol (25), where our screen identified several analogues that may possess a greater potency than resveratrol, having the capacity to induce these inhibitory effects with greater efficiency than the well-studied parent compound. Of particular interest were the effects of two compounds, 4b and 4c, that induced marked decreases at or below 50% cell viability. Structural comparison of these two compounds reveal various chemical differences between the two analogues and resveratrol, suggesting functional group substitutions alter compound activity.

Notably, when tested under proliferation conditions with the MCF-7 line, no significant effects on cell proliferation were observed for any of the compounds tested. Several compounds were found to possess significant proliferation inhibition, but only in the ZR-75-1 cell line (Fig. 3C and D). The compounds found to be active in suppression of proliferation were unique from those effective at decreasing viability, suggesting differences in mechanism between inhibition of these cell effects. It also indicated that the absolute configuration as well as functional group substitution of the compounds play key roles in determining the mode of inhibition.

Several resveratrol analogues significantly decrease viability in TNBC cells in a dose-dependent manner. A common assump-

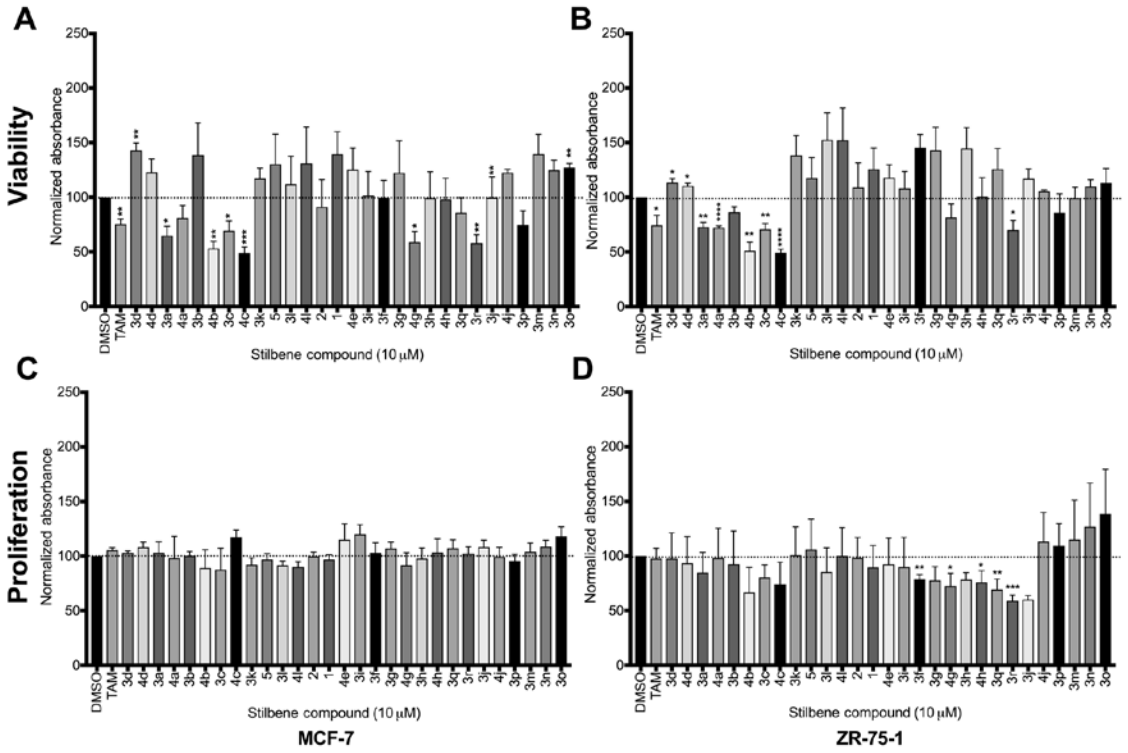

Figure 3. Resveratrol analogues significantly reduce cell viability in estrogen receptor (ER)-positive cell lines. ER-positive breast cancer cell lines (A and C) MCF-7 and (B and D) ZR-75-1 were plated each for (A and B) viability or (C and D) proliferation and quantified as described in Materials and methods. After 24 h to allow cell adherence, wells were treated with 10 μM final concentration of corresponding resveratrol analogues in triplicates with positive and negative controls of tamoxifen (TAM) and dimethyl sulfoxide (DMSO), respectively, for seven days. Bars represent normalized mean ± SEM for biological triplicate experiments. Statistical significance was denoted by *P<0.05, **P<0.01, ***P<0.001, or ****P<0.0001).

tion is that phytoestrogens, including stilbenes, elicit their effects by binding to and regulating estrogen receptor function. As a preliminary probe into the mechanism of action of our resveratrol analogues on the ER-positive cell lines, we screened the 29-compound library in three triple-negative breast cancer (TNBC) cell lines (MDA-MB-157, MDA-MB-231 and BT-549). Our results demonstrated unprecedentedly similar activity of these phytoestrogenic compounds in both ER-positive and ER-negative cell lines; six compounds exhibited significant inhibitory effects in at least one of the three TNBC lines, with 4c being common among all three lines (Fig. 5A-C). In the BT-549 cells, 4c induced a marked decrease in viability of ~70%, whereas in the MDA-MB-157 and MDA-MB-231 lines, the decrease was closer to 25 and 10%, respectively, suggesting even slight genetic variation may significantly affect the effectiveness of a compound. As with our ER-positive cell line screen, neither resveratrol (Fig. 1A) nor pterostilbene (Fig. 1B) exhibited significant inhibition of cell viability. Notably, the resveratrol analogues that exhibited significant effects in the ER-positive lines also exhibited marked decreases on cell viability in the ER-negative lines, with compounds 3a, 3c, 3r and 4a-c significantly decreasing viability in at least one of the three TNBC lines screened (Fig. 5A-C). Although we did not anticipate observing significant effects in the ER-negative cells, these results indicated that select stilbenes may utilize an ER-independent mechanism to induce effects on cell viability.

Further investigation into the six most effective compounds led us to examine the potency of these compounds through a dose-response assay. TNBC cells were treated with increasing doses of each compound as follows: 0.1, 1, 5, 10 and 25 μM final concentration. Resveratrol (Fig. 1A) and pterostilbene (Fig. 1B) were included as our representative parent compounds as proven anticancerous effects in breast cancer cells. Compounds 4a, 4c and 3r exhibited the highest potency with significant inhibition of viability evident at 1 μM and increasing effectiveness as concentration increased in at least 2 out of the 3 cell lines tested (Fig. 5D-F). Consistent with the 10 μM screen, resveratrol (Fig. 1A) and pterostilbene (Fig. 1B) behaved with little effect on cancer cell viability in any of the TNBC lines, with significant decreases observed at only the highest doses tested.

Resveratrol analogues alter gene expression of cell death and survival signaling pathways. Due to the lack of classical estrogen signaling via ER in TNBC cells, we set out to understand the mechanism of action of stilbenes in the inhibition of cell viability in this system. Investigation into the intracellular pathways was carried out through RT^2-PCR profiling. The effects of four of the most potent representative compounds (3c, 4a-c) and the parent compound resveratrol (Fig. 1A) on breast cancer-associated gene expression were assessed in the BT-549 cell line. The RT^2 Breast Cancer Profiler PCR assay containing specific primers for 88 known breast cancer-associated genes was run for each sample and compared to DMSO-treated control cells. Resveratrol exhibited little change in the BT-549 cell line, only 4 of the 84 genes tested responded >2-fold compared to the control (Fig. 6). Overall, the greatest differences in gene expression for the stilbene compounds tested were observed in genes associated with the cell cycle, apoptosis, and DNA repair. Fig. 7A summarizes the simi-

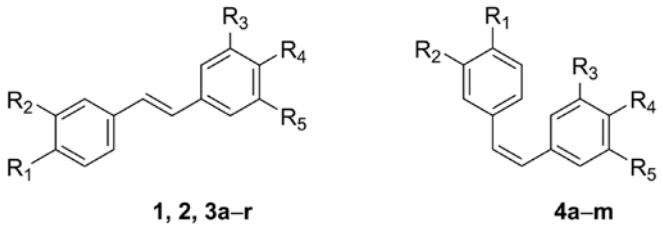

Figure 4. Resveratrol analogue library (see Table I).

Table I. Resveratrol analogue library (Fig. 4).

Compound	R_1	R_2	R_3	R_4	R_5
1	OH	H	OH	H	OH
2	OH	H	OMe	H	OMe
Dihydro-2	OH	H	OMe	H	OMe
a	NH_2	H	OMe	H	OMe
b	CO_2Me	H	OMe	H	OMe
c	OMe	H	OMe	H	OMe
d	NO_2	H	OMe	H	OMe
e[b]	CO_2H	H	OMe	H	OMe
f[a]	F	H	OMe	H	OMe
g	Br	H	OMe	H	OMe
h	CF_3	H	OMe	H	OMe
i[a]	Glucose[c]	H	OMe	H	OMe
j	H	H	OMe	H	OMe
k[a]	OH	H	OH	H	OMe
l	OMe	H	OH	H	OH
m[a]	OH	H	OH	H	OAc
n	OH	H	OAc	H	OAc
o	OAc	H	OAc	H	OAc
p	OH	H	OMe	Me	OMe
q	OH	OMe	OMe	H	OMe
r	OH	OH	OMe	H	OMe

[a]*E*-isomer only; [b]*Z*-isomer only; [c]D-glucose attached as the glucose acetal of compound 2; specific anomer not confirmed.

larities and differences in gene expression changes between compounds 3c, 4a-c for genes exhibiting >2-fold regulation. Of the 22 genes that were commonly regulated by the compounds tested, downregulation of steroid hormone signaling, particularly estrogen (ESR1, ESR2, PgR and RARB), inhibition of growth factors and oncogenes associated with cancer (EGF, IGF1, MUC1, ADAM23, GLI1 and MGMT), and inhibition of EMT and metastasis-related genes (CDH1, CDH13, TWIST1, MMP2, MMP9 and GRB7) were among the top altered. Top pathways commonly regulated by these stilbenes included apoptosis, DNA damage and repair, and, surprisingly, steroid receptor-mediated signaling (Fig. 7B).

Pathway analysis for each compound was conducted using Ingenuity Pathway Analysis. Of all the Disease and Function pathways tested, cell death and survival were the top predicted pathways altered by all stilbenes tested. Upon further investigation, cell death pathways, including apoptosis, necrosis, and anoikis, were the most upregulated pathways predicted for 3c, 4a and 4b, while cell viability and survival were among the most downregulated pathways predicted for 3c and 4a-c (Fig. 7C). Compound 4c presents an interesting case. As the most effective compound on the viability of the cell lines tested, we expected to see broad effects throughout the cell death and survival spectrum. However, the cell death and apoptotic pathways were not upregulated in the 4c-treated cells. Although cell death pathways appear to be activated in cells treated with 3c, 4a and 4b, but not 4c, we did not observe any significant cell death at 24 h via crystal violet staining (Fig. S1). Yet we observe that, collectively, the overall effect from the aforementioned results presented is clearly broad-spectrum downregulation of cancer cell viability. While there are obvious differences in the specific gene regulation induced by each stilbene, these results indicated that select stilbenes induce cell death via apoptosis with simultaneous inhibition of cell survival signaling pathways.

cis-Resveratrol analogues with methoxy functional groups induce apoptosis in TNBC cell lines. To confirm that the effects on cell viability were due to activation of apoptosis as suggested by gene expression array, caspase activation assays were conducted with stilbenes 3a, 3c, 3r and 4a-c, with resveratrol (Fig. 1A) and pterostilbene (Fig. 1B) serving as negative controls and hydrogen peroxide (125 μM) serving as a positive control. Significant induction of apoptosis was observed in 3 of the 8 compounds dosed at 10 μM 48 h post-treatment. While differences in the magnitude of caspase activation were observed across all three TNBC cell lines tested, a clear and consistent induction of apoptosis was revealed. These results confirmed that the decrease in cell viability caused by these compounds was due, at least in part, to activation of programmed cell death pathways. These findings also supported gene expression pathway analysis that indicated increased cell death including apoptotic pathways.

Discussion

With the occurrence of breast cancer on the rise, quickly becoming the most commonly diagnosed cancer in the world, the need for reliable and effective treatments has never been more necessary. With a majority of breast cancer cases being derived from a subtype with targetable modalities, significant advancements in modern endocrine therapies have been made, leaving these patients with hopeful prognosis as they journey to remission (3). In the search of alternative means for the utilization of hormone therapy, such as phytoestrogenic compounds, resveratrol is one that has long been studied in the treatment of ER-positive breast cancer cases. Investigations into the mechanism of stilbenes in ER-positive subtypes has revealed great promise for their use in hormone therapy (3), and more recently, in overcoming therapy-resistant breast cancer cells.

A recent investigation into cisplatin-resistant MCF-7 cells, a widely used chemotherapeutic agent, demonstrated the ability of resveratrol to enhance cisplatin antiproliferation effects, lowering the IC50 in both normal and resistant MCF-7 lines, while causing downregulation of Rad51 protein

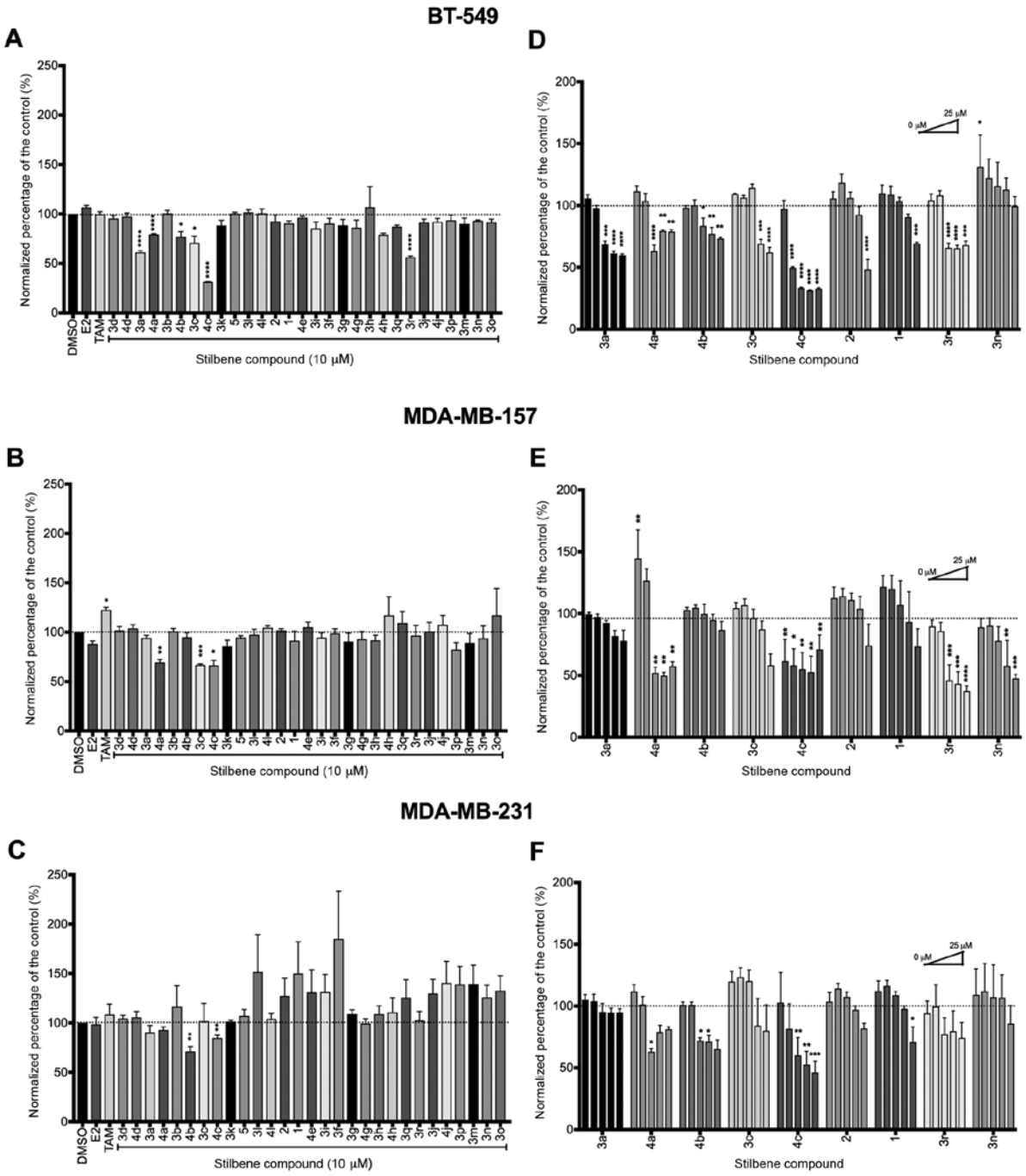

Figure 5. Analogues of resveratrol exhibit significant decreases on cell viability of triple-negative breast cancers (TNBCs) with dose-dependent effects and high potency. Three TNBC cells lines, (A and D) BT-549, (B and E) MDA-MB-157 and (C and F) MDA-MB-231, were treated with stilbene compounds (10 μM final concentration) and examined for viability at a 7-day time-point. Dimethyl sulfoxide (DMSO) served as a vehicle control; 17β-estradiol (1 nM) and 4-hydroxy-tamoxifen (100 nM) were used as additional controls. (D-F) Compounds 1, 2, 3a, 3c, 3n, 3r, and 4a-c were assessed in a dose-response assay. Cells were treated with corresponding compounds at 0.1, 1, 5, 10 or 25 μM and cultured for seven days. Bars represent normalized mean (DMSO control set to 100) ± SEM for biological triplicate experiments. Statistical significance was denoted by *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001).

levels and homologous recombination (HR) complex initiators (26). Increased levels of both Rad51 and HR have been revealed to be associated with genetically predisposed risks for breast cancer contraction and increased development of therapy-resistant cells (27). De Amicis *et al* evaluated the use of resveratrol in cases of tamoxifen-resistant MCF-7 cells, reporting its ability to increase cell-sensitivity by increasing p53 protein expression. They reported that increased p53 resulted in decreased ER expression, leading to downregulation of growth factor pathways and resulting in cross-talk known to arise between pro-survival and hormonal pathways (28). Various studies have highlighted the ability of resveratrol, through downregulation of ERα mRNA transcription, to inhibit expression of major cell cycle and cell proliferation-dependent genes, all initiated by downstream upregulation of p53 and p21 (28-30). While there exists a general consensus on the ability of resveratrol to modulate both ERα and p53 expression in ER-positive breast cancer,

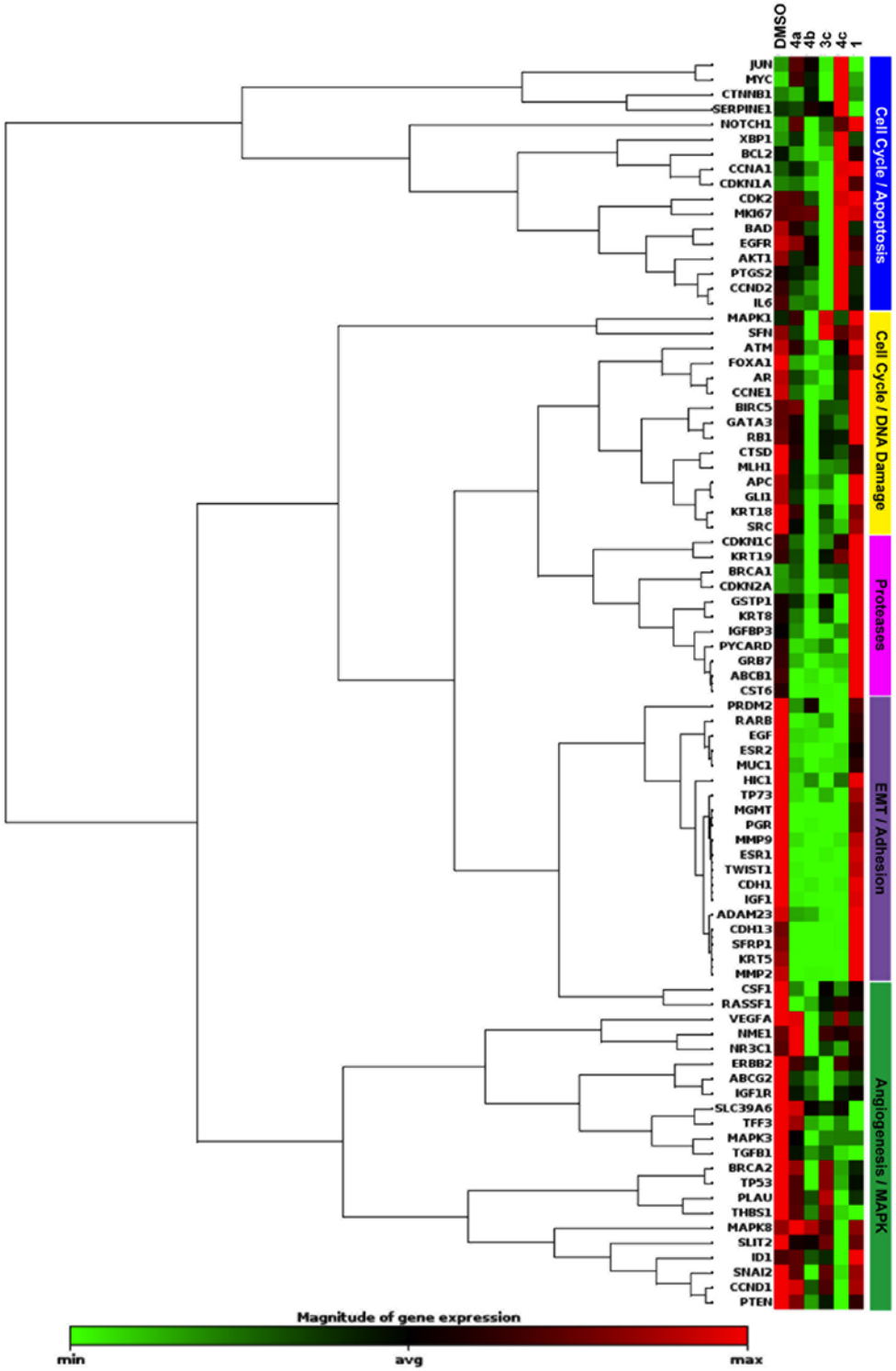

Figure 6. Gene regulation of select stilbenes on breast cancer-related genes. Gene expression of BT-549 cells treated with stilbene compounds (10 μM) for 24 h was examined by RT^2-PCR array. Heatmap represents a non-supervised hierarchical clustering of the entire dataset to display co-regulated genes across groups and individual samples.

a recent study investigated the relationship between this tumor-suppressor protein and hormonal signaling receptor, suggesting an alternate pathway in which resveratrol induces p53 expression to promote anti-survival effects, due to the variable, dose-dependent actions of resveratrol reported (25).

One alternative mechanism recently proposed is of particular interest as it has been observed in both ER-positive and ER-negative breast cancers. A membrane receptor site for resveratrol on an integrin has been uncovered in both subtypes of breast cancer, where p53-dependent apoptosis induction has been confirmed upon resveratrol binding (25,31). Further investigation of binding site affinity for resveratrol and analogues has uncovered an interesting interaction with the integrin. Hsieh *et al* evaluated the binding of resveratrol and *trans*-3,4',5-triacetylstilbene (compound 3o from our library) in MDA-MB-231 cells, revealing significant increases in both p53 and p21 expression

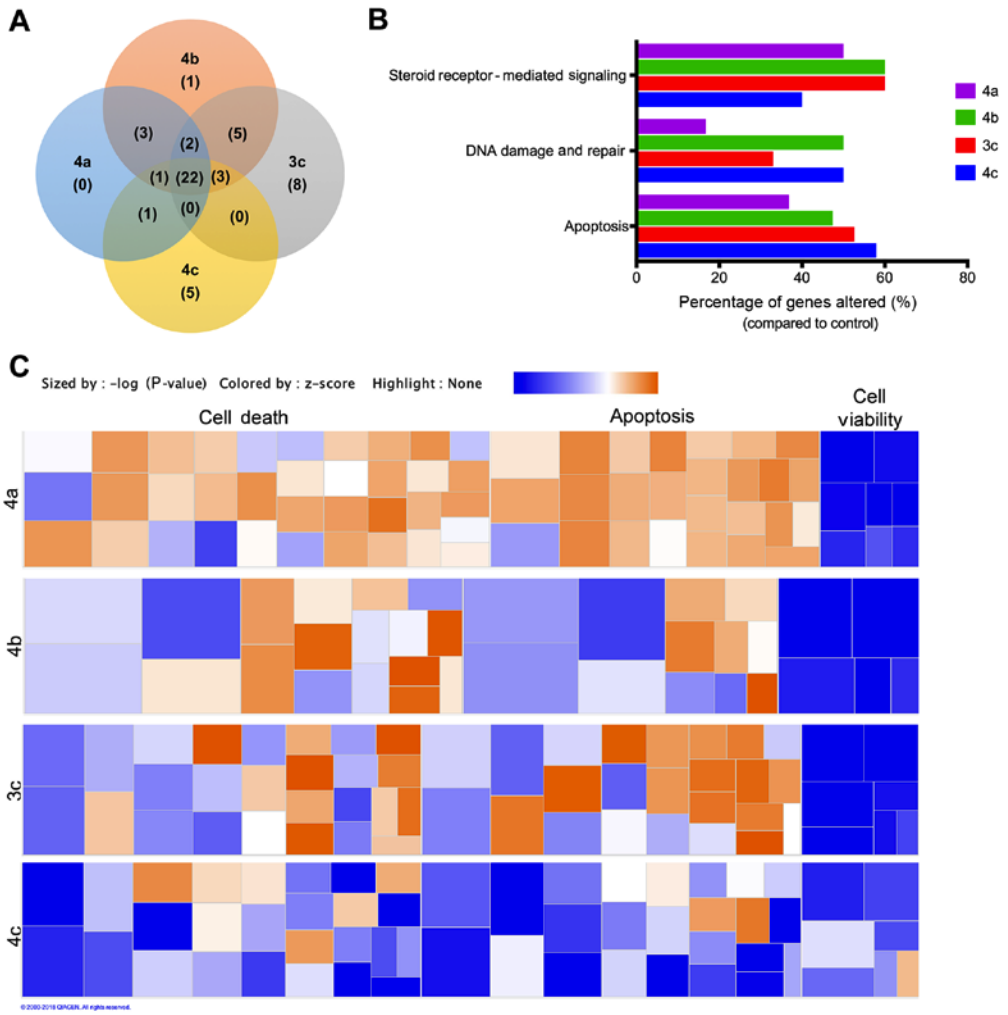

Figure 7. Resveratrol analogues induce gene expression changes associated with decreased cell viability and increased cell death. RT^2-PCR pathway analysis of BT-549 cells treated with stilbenes 3c and 4a-c (10 μM). (A) Venn diagram illustrating common and unique genes regulated >2-fold by each stilbene tested. (B) Changes in the number of genes altered for the apoptosis, DNA damage and repair, and the steroid receptor-mediated signaling pathways are represented as the percent of genes altered for each given pathway. Percent was determined by dividing the number of genes altered in the pathway by the total number of genes assessed for that pathway and converted into a percentage. (C) Heatmap results for cell death pathways including cell death, apoptosis, cell viability, necrosis, fragmentation, and anoikis. Box size represents -log (P-value) and color indicates fold regulation according to z-score. Blue, decreased expression; orange, increased expression as compared to the dimethyl sulfoxide (DMSO)-treated control.

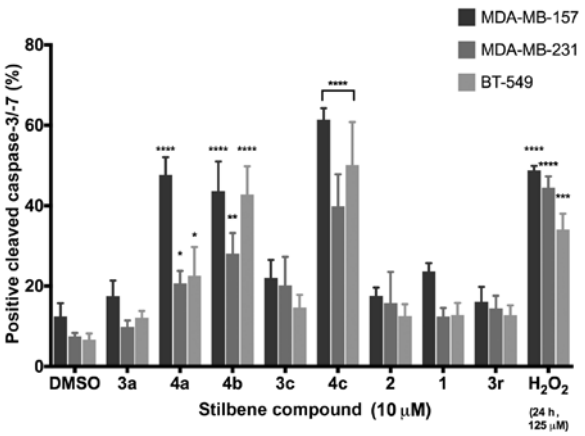

Figure 8. Caspase activation in triple-negative breast cancer (TNBC) by select stilbene compounds. MDA-MB-157, MDA-MB-231 and BT-549 cells were treated with stilbene compounds (10 μM) or dimethyl sulfoxide (DMSO) control for 48 h. H_2O_2 (125 μM) served as a positive control as assessed at 24 h of treatment. Bars represent the percent of cells positive for caspase-3/-7 activation mean ± SEM for each condition. N=4. Statistical significance was denoted by *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001.

and resultant induction of p53-dependent apoptosis at treatment concentrations ranging from 10 to 50 μM (9). While we did not observe these reductions in TNBC viability at 10 μM doses of either resveratrol (1) or 3o, some significant inhibition was observed for resveratrol and 3n, a diacetyl-substituted analogue, at 25 μM (Fig. 5D-F) in select TNBC lines. Our results again support that dose-dependent proliferation verses growth-inhibitory effects that resveratrol and related compounds may exhibit, with doses >20 μM more commonly be associated with antiproliferation effects (25). Where H-donating functional groups have been confirmed to interact with high affinity for the integrin receptor site, analogues substituted with methoxy groups exhibit little interaction with the protein (9). Of greater significance were our results of several resveratrol analogues which increased the amount of apoptosis under the same conditions (Fig. 8). Notably our results elucidate the anticancerous effects of these methoxy resveratrol analogues at low-dose treatment concentrations (0.1-25 μM), supporting various alternative mechanisms that these unique stilbenes may function through in TNBC (9).

Caspase-3/-7 quantification revealed three compounds identified in our screen (4a-c) to significantly decrease TNBC viability, suggesting differential, pro-apoptotic signaling pathways not yet explored in ER-negative cells. When comparing the molecular structures of compounds 4a-c, we have identified several common elements that we believe may play a major role in their apoptotic activity. First, these compounds are all in the cis-confirmation that has been a well-established factor in increasing both the cytotoxic and apoptotic functionality of various stilbene compounds (20,32). Additionally, these three compounds all have the 3,5-dimethoxystyryl structure and are thus closer analogues of pterostilbene, a well-studied *trans*-analogue of resveratrol. Studies into the substitution of the hydroxyl groups for methoxy groups on resveratrol have revealed marked increases in the anticancerous effects (33,34). In one investigation, Hong *et al* compared the activity of resveratrol and trimethoxy-substituted resveratrol analogue (3c and 4c) in pancreatic and breast cancer cells, revealing significantly increased potency of the Z-analogue, as demonstrated though increased inhibition of proliferation, increased cell cycle arrest, and overall increased induction of apoptosis (35). These pro-apoptotic events resulting in increased cellular toxicity in both MCF-7 and MDA-MB-231 cell lines was attributed to the blocking of tubulin formation by stilbenes, resulting in cell cycle arrest (35). Since the blocking of α- and β-tubulin interaction is not dependent upon estrogen signaling, this further supports our hypothesis that some of our compounds are functioning in an ER-independent manner. Compound 4c contains an additional methoxy group on the second benzene ring that may be responsible for its consistently high levels of cellular toxicity observed across our studies and supports future investigation into the intracellular pathways being regulated.

While TNBC cells lack ERα expression, the primary estrogen receptor, they many retain expression of other non-dominant estrogen receptors such as ERβ and GPR30. Our results clearly indicated an increase in apoptosis, and pathway analyses also suggested inhibition of ER-dependent signaling. Even though several of our stilbene compounds elicited anti-viability and/or anti-proliferative effects in ER-positive breast cancer cell lines, in agreement with recent similarly conducted studies, more significantly they elicit greater anti-viability and pro-apoptotic effects in ER-negative TNBC cell lines, a phenomenon that has not been fully explored to understand these compounds potential for therapeutic use. While our gene expression studies raise the possibility of suppression of potentially metastatic pathways (decreased expression of TWIST, MMP2, MMP9 and TGFB), the goal of the present study was to determine general activity of compounds. Our current and future studies will explore the mechanisms regulating their anticancer activity as well as any potential anti-migration or -invasion activity.

Although there has been much success with resveratrol as an anticancer compound in laboratory studies, when taken by patients, its bioavailability continues to limit its effectiveness in clinical settings (14). Thus, the investigation into the activity of analogues of this 'miracle' compound may be a pivotal step in overcoming this biological barrier in the use of resveratrol use as a therapeutic agent. Through our investigation, we have uncovered previously unknown actions of multiple synthetically derived analogues of resveratrol that not only possess increased effects on inhibition of TNBC growth compared to the parent compound, but also increased potency.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XJH planned and conducted the experiments, analyzed the data and drafted the manuscript. HT and EB conducted the experiments and analyzed the data. DHP synthesized the analogues, conducted the characterization/purity studies, and contributed to the drafting and editing of the manuscript. LVR conceptualized the study, planned the experiments, conducted data analysis and statistics, prepared the figures, and assisted in drafting and editing of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no financial or conflicts of interests of any kind.

References

1. DeSantis CE, Ma J, Goding Sauer A, Newman LA and Jemal A: Breast cancer statistics, 2017, racial disparity in mortality by state. CA Cancer J Clin 67: 439-448, 2017.

2. Chavez KJ, Garimella SV and Lipkowitz S: Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. Breast Dis 32: 35-48, 2010.
3. Rajappa S, Bajpai J, Basade M, Ganvir M, Goswami C, Murali A, Rathi AK, Kaushal V, Jain S, Parikh PM, *et al*: Practical consensus recommendations regarding the use of hormonal therapy in metastatic breast cancer. South Asian J Cancer 7: 137-141, 2018.
4. Alfarsi L, Johnston S, Liu DX, Rakha E and Green AR: Current issues with luminal subtype classification in terms of prediction of benefit from endocrine therapy in early breast cancer. Histopathology 73: 545-558, 2018.
5. Bowers JL, Tyulmenkov VV, Jernigan SC and Klinge CM: Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β. Endocrinology 141: 3657-3667, 2000.
6. Lu R and Serrero G: Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol 179: 297-304, 1999.
7. Sirerol JA, Rodríguez ML, Mena S, Asensi MA, Estrela JM and Ortega AL: Role of natural stilbenes in the prevention of cancer. Oxid Med Cell Longev 2016: 3128951, 2016.
8. Ko JH, Sethi G, Um JY, Shanmugam MK, Arfuso F, Kumar AP, Bishayee A and Ahn KS: The role of resveratrol in cancer therapy. Int J Mol Sci 18: 2589, 2017.
9. Hsieh TC, Wong C, John Bennett D and Wu JM: Regulation of p53 and cell proliferation by resveratrol and its derivatives in breast cancer cells: An in silico and biochemical approach targeting integrin αvβ3. Int J Cancer 129: 2732-2743, 2011.
10. Yenugonda VM, Kong Y, Deb TB, Yang Y, Riggins RB and Brown ML: *Trans*-resveratrol boronic acid exhibits enhanced anti-proliferative activity on estrogen-dependent MCF-7 breast cancer cells. Cancer Biol Ther 13: 925-934, 2012.
11. Lee PS, Chiou YS, Ho CT and Pan MH: Chemoprevention by resveratrol and pterostilbene: Targeting on epigenetic regulation. Biofactors 44: 26-35, 2018.
12. Chin YT, Hsieh MT, Yang SH, Tsai PW, Wang SH, Wang CC, Lee YS, Cheng GY, HuangFu WC, London D, *et al*: Anti-proliferative and gene expression actions of resveratrol in breast cancer cells in vitro. Oncotarget 5: 12891-12907, 2014.
13. Liu Y, Liu Y, Chen H, Yao X, Xiao Y, Zeng X, Zheng Q, Wei Y, Song C, Zhang Y, *et al*: Synthetic resveratrol derivatives and their biological activities: A review. Open J Med Chem 05: 97-105, 2015.
14. Walle T: Bioavailability of resveratrol. Ann NY Acad Sci 1215: 9-15, 2011.
15. Licznerska B, Szaefer H, Wierzchowski M, Mikstacka R, Papierska K and Baer-Dubowska W: Evaluation of the effect of the new methoxy-stilbenes on expression of receptors and enzymes involved in estrogen synthesis in cancer breast cells. Mol Cell Biochem 444: 53-62, 2018.
16. Jo SJ, Park PG, Cha HR, Ahn SG, Kim MJ, Kim H, Koo JS, Jeong J, Park JH, Dong SM, *et al*: Cellular inhibitor of apoptosis protein 2 promotes the epithelial-mesenchymal transition in triple-negative breast cancer cells through activation of the AKT signaling pathway. Oncotarget 8: 78781-78795, 2017.
17. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428, 2009.
18. Paul S, Mizuno CS, Lee HJ, Zheng X, Chajkowisk S, Rimoldi JM, Conney A, Suh N and Rimando AM: In vitro and in vivo studies on stilbene analogs as potential treatment agents for colon cancer. Eur J Med Chem 45: 3702-3708, 2010.
19. Mizuno CS, Ma G, Khan S, Patny A, Avery MA and Rimando AM: Design, synthesis, biological evaluation and docking studies of pterostilbene analogs inside PPARalpha. Bioorg Med Chem 16: 3800-3808, 2008.
20. Roberti M, Pizzirani D, Simoni D, Rondanin R, Baruchello R, Bonora C, Buscemi F, Grimaudo S and Tolomeo M: Synthesis and biological evaluation of resveratrol and analogues as apoptosis-inducing agents. J Med Chem 46: 3546-3554, 2003.
21. Pettit GR, Grealish MP, Jung MK, Hamel E, Pettit RK, Chapuis JC and Schmidt JM: Antineoplastic agents. 465. Structural modification of resveratrol: Sodium resverastatin phosphate. J Med Chem 45: 2534-2542, 2002.
22. Tate CR, Rhodes LV, Segar HC, Driver JL, Pounder FN, Burow ME and Collins-Burow BM: Targeting triple-negative breast cancer cells with the histone deacetylase inhibitor panobinostat. Breast Cancer Res 14: R79, 2012.
23. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408, 2001.
24. Sauter ER: Breast cancer prevention: Current approaches and future directions. Eur J Breast Health 14: 64-71, 2018.
25. Saluzzo J, Hallman KM, Aleck K, Dwyer B, Quigley M, Mladenovik V, Siebert AE and Dinda S: The regulation of tumor suppressor protein, p53, and estrogen receptor (ERα) by resveratrol in breast cancer cells. Genes Cancer 7: 414-425, 2016.
26. Leon-Galicia I, Diaz-Chavez J, Albino-Sanchez ME, Garcia-Villa E, Bermudez-Cruz R, Garcia-Mena J, Herrera LA, García-Carrancá A and Gariglio P: Resveratrol decreases Rad51 expression and sensitizes cisplatin-resistant MCF-7 breast cancer cells. Oncol Rep 39: 3025-3033, 2018.
27. Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR and Bishop DK: The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J Biol Chem 275: 23899-23903, 2000.
28. De Amicis F, Giordano F, Vivacqua A, Pellegrino M, Panno ML, Tramontano D, Fuqua SA and Andò S: Resveratrol, through NF-Y/p53/Sin3/HDAC1 complex phosphorylation, inhibits estrogen receptor alpha gene expression via $p38^{MAPK}$/CK2 signaling in human breast cancer cells. FASEB J 25: 3695-3707, 2011.
29. Ronghe A, Chatterjee A, Bhat NK, Padhye S and Bhat HK: Tamoxifen synergizes with 4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1,2-diol} and 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol}, novel azaresveratrol analogs, in inhibiting the proliferation of breast cancer cells. Oncotarget 7: 51747-51762, 2016.
30. Ronghe A, Chatterjee A, Singh B, Dandawate P, Murphy L, Bhat NK, Padhye S and Bhat HK: Differential regulation of estrogen receptors α and β by 4-(E)-{(4-hydroxyphenylimino) -methylbenzene,1,2-diol}, a novel resveratrol analog. J Steroid Biochem Mol Biol 144: 500-512, 2014.
31. Chin YT, Yang SH, Chang TC, Changou CA, Lai HY, Fu E, HuangFu WC, Davis PJ, Lin HY and Liu LF: Mechanisms of dihydrotestosterone action on resveratrol-induced antiproliferation in breast cancer cells with different ERα status. Oncotarget 6: 35866-35879, 2015.
32. Simoni D, Roberti M, Invidiata FP, Rondanin R, Baruchello R, Malagutti C, Mazzali A, Rossi M, Grimaudo S, Capone F, *et al*: Heterocycle-containing retinoids. Discovery of a novel isoxazole arotinoid possessing potent apoptotic activity in multidrug and drug-induced apoptosis-resistant cells. J Med Chem 44: 2308-2318, 2001.
33. Kumar A, Rimando AM and Levenson AS: Resveratrol and pterostilbene as a microRNA-mediated chemopreventive and therapeutic strategy in prostate cancer. Ann NY Acad Sci 1403: 15-26, 2017.
34. Moon D, McCormack D, McDonald D and McFadden D: Pterostilbene induces mitochondrially derived apoptosis in breast cancer cells in vitro. J Surg Res 180: 208-215, 2013.
35. Hong YB, Kang HJ, Kim HJ, Rosen EM, Dakshanamurthy S, Rondanin R, Baruchello R, Grisolia G, Daniele S and Bae I: Inhibition of cell proliferation by a resveratrol analog in human pancreatic and breast cancer cells. Exp Mol Med 41: 151-160, 2009.