Resveratrol Augments Paclitaxel Treatment in MDA-MB-231 and Paclitaxel-resistant MDA-MB-231 Breast Cancer Cells

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Abstract. *Background: Resveratrol (RES) inhibits cell growth, induces apoptosis and augments chemotherapeutics in multiple cancer types, although its effects on drug-resistant cancer cells are unknown. Materials and Methods: To study the effects of resveratrol in triple-negative breast cancer cells that are resistant to the common cancer drug, paclitaxel, a novel paclitaxel-resistant cell line was generated from the MDA-MB-231 cell line. Results: The resistant MDA-MB-231/PacR cells exhibited a 12-fold increased resistance to paclitaxel. RES treatment reduced cell proliferation and colony formation and increased senescence and apoptosis in both parental and resistant cells. Importantly, RES augmented the effects of paclitaxel in both cell lines. Up-regulation of the MDR1 and CYP2C8 genes were shown to be potential mechanisms of paclitaxel resistance in the resistant cells. Conclusion: RES, both alone and in combination with paclitaxel, may be useful in the treatment of paclitaxel-sensitive and paclitaxel-resistant triple-negative breast cancer cells.*

The anticancer effects of resveratrol (RES) have been extensively studied in many *in vitro* and *in vivo* human cancer models. RES has been shown to inhibit angiogenesis and cell proliferation, as well as induce cell-cycle arrest and apoptosis in various cancers (1, 2). Although the anticancer effects of RES are seen at high micromolar concentrations *in vitro*, it has been shown that RES can accumulate to these high amounts in tissue (3, 4). In addition, RES is very well tolerated in animals and humans (5). These properties make RES an attractive anticancer agent, particularly for solid tumors. Interestingly, these anticancer effects and their mechanisms seem to be cell-type dependent. For example, the type of cell-cycle arrest caused by RES, if any, is dependent on cancer cell-type (6). Therefore, it is important to study the effects of RES in different cancer models. Notably, RES was shown to inhibit tumor growth and cause cell death in an ectopic mouse model of triple- negative breast cancer (7).

The mechanism of RES-induced apoptosis has been extensively studied, but the pathways are not fully-understood. Sirtuin 1 (SIRT1) can be activated by RES indirectly by dissociation with the negative protein regulator, Deleted in Breast Cancer 1 (DBC1), *via* the cAMP/PKA/AMPK pathway (8, 9). SIRT1 activation by RES has been shown to decrease survivin protein levels in a dose-dependent manner, which allows activated caspase 3 and 7 to induce apoptosis (10-14). To explore the mechanism of resveratrol in paclitaxel-sensitive and –resistant triple-negative breast cancer cells, the effects of RES on survivin, SIRT1, DBC1 and P-AMPK protein levels were investigated.

Recent studies show positive combination effects of RES and irradiation, as well as various chemotherapeutics in multiple human cancers (15, 16). Although paclitaxel is a very successful drug, some tumors are intrinsically resistant and others can acquire resistance to paclitaxel. Approximately one third of paclitaxel-resistant breast cancers are triple-negative (17). Many mechanisms of paclitaxel resistance in breast cancer have been identified such as evading chemotherapeutic-induced cell death by hijacking apoptosis pathways, removing the drug quickly by efflux or metabolism, or altering drug-binding sites (18). Studying these mechanisms is important for developing strategies for overcoming drug resistance. We, therefore, wanted to determine if RES can be used alone or in combination with paclitaxel in triple-negative breast cancer cells and whether RES can re-sensitize paclitaxel-resistant cells to paclitaxel. To study paclitaxel resistance in triple negative breast cancers, we developed a cellular model that mimics acquired drug resistance in tumors.

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Herein we present data showing that RES induces cell proliferation inhibition, senescence and apoptosis in paclitaxel-sensitive and -resistant triple-negative breast cancer cells. Importantly, we show that RES can be used in combination with paclitaxel to not only re-sensitize the resistant cells to paclitaxel but also to augment the effects of paclitaxel in both the parent and resistant cells. The overexpression of both P-glycoprotein (P-gp), a paclitaxel efflux pump, and CYP2C8, a paclitaxel metabolizing enzyme, was found to be involved in the paclitaxel-resistance of our breast cancer cell model, which provides a possible mechanism of RES sensitization in these cells.

Materials and Methods

Cell culture and reagents. MDA-MB-231 cells (American Type Culture Collection (ATCC); Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) media (Corning cellgro; Corning, NY, USA) with Hyclone 10% Cosmic Calf Serum (Thermo Fisher Scientific; Waltham, MA, USA) at 37˚C, 5% CO_2 and 95% humidity. Cells were periodically tested to ensure mycoplasma contamination was not present. RES and paclitaxel (Sigma-Aldrich; St. Louis, MO, USA) were dissolved in DMSO and further diluted with 1x Hank's Balanced Salt Solution (HBSS). SIRT1 (Active Motif; Carlsbad, CA, USA), DBC1, survivin, caspase 7, AMPK, P-AMPK (Cell Signaling; Danvers, MA, USA) antibodies were used according to manufacturer's suggestion. For all experiments, treatments were done after allowing cells to attach to the plate overnight.

Resistant cell line generation. To generate the paclitaxel-resistant line, an intermittent, stepwise method of treatment was used (19). MDA-MB-231 cells were treated with the determined 24-hour 30% inhibitory concentration (IC_{30}) of paclitaxel for 3-4 days. The drug was then removed for 3-4 days before treating again. This interval lasted for 2-4 weeks before the treatment was increased to the IC_{40}. This process was repeated until the cells were growing successfully under IC_{60} treatment of paclitaxel. After these cells were obtained, they were no longer grown in the presence of paclitaxel. Single cell derived clones were obtained by limiting dilution in which cells were serially diluted across a 96-well plate and single clones were expanded.

Methylene blue cell proliferation assay. Cells were fixed in methanol for 15 minutes and then stained with 0.05% methylene blue stain (Ricca Chemical; Arlington, TX, USA) for 10 minutes (20). The plates were washed, dried and de-stained with 0.5 M hydrochloric acid solution. The absorbance was then measured using a plate reader at 610 nm. For IC50 determinations, cells were treated with a serial dilution of paclitaxel or resveratrol. Combination assays were completed using a checkerboard method such that cells plated onto 96-well plates were treated for 5 days with a serial dilution of paclitaxel across the plate horizontally and a dilution of resveratrol (2.5-40 μM) across the plate vertically; the data were analyzed by determining the IC_{50} value for the constant drug ratio curves using the Chou Talalay method (21, 22). For the P-glycoprotein and CYP2C8 inhibitor assays, cells were treated with a serial dilution of paclitaxel either alone or in combination with 1 μM verapamil, 25 μM trimethoprim (Sigma-Aldrich) or both. GraphPad Prism version 4.00 for Windows (GraphPad Software; San Diego, CA, USA) was used to calculate IC_{50} values.

Clonogenic cell survival assay. A clonogenic cell survival assay was conducted as previously described with modification (23). Cells were plated in 6-well plates at 30 cells per well and treated for 72 h. RES was then removed and the cells were allowed to grow undisturbed for 17 days. Plates were then washed with 1X HBSS, fixed with methanol for 15 minutes, stained with crystal violet for 1 hour and washed. Colonies consisting of 50 cells or more were counted and scored.

Senescence staining. Cells were plated onto 24-well plates, treated for 3 days and stained using the Senescence Detection Kit (EMD Millipore; Billerica, MA, USA) per manufacturer's instructions. Positive and negative cells were counted in triplicate for each treatment using a phase-contrast microscope at 20x optical fields.

Cell cycle analysis and Annexin V apoptosis flow cytometry. To analyze the cells' cycle phases following resveratrol treatment, cells were fixed and permeabilized with 70% ethanol and stained with propidium iodide (PI). For apoptosis analyses, cells were stained with FITC-conjugated Annexin V and PI using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences; Franklin Lakes, NJ, USA) according to manufacturer's instructions. Fluorescence was measured using a LSRII 561 nM laser flow cytometer (BD Biosciences; San Jose, CA, USA) and data was analyzed with FlowJo (TreeStar; Ashland, OR, USA).

Immunoblotting. Samples were lysed by sonication in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Hoffmann-La Roche; city, Switzerland) and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and run on 6, 10 or 12% polyacrylamide gels. Polyvinylidene difluoride (PVDF) membranes (Pall Corporation; Port Washington, NY, USA) were used for overnight or 2-h transfer. Blotting was conducted using Pierce ECL Western Blotting Reagent (Thermo Fisher Scientific). ImageJ was used for densitometry quantification.

siRNA knockdown. Caspase 7 and Non-Targeting Dharmacon ON-TARGET*plus* SMARTpool siRNA constructs were used with Dharmacon lipofection transfection reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were transfected for 72-96 h. The transfection media was removed and the cells were then treated with RES for 48-72 h before the cells were collected and analyzed by immunoblotting or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Co-immunoprecipitation. Cells treated with RES for 2 hours were collected and lysed in co-IP lysis buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% NP-40) supplemented with protease inhibitor cocktail (Hoffmann-La Roche). Protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology; Santa Cruz, CA, USA) was used according to manufacturer's instructions and samples analyzed by immunoblotting.

Real-time RT-PCR and PCR array. RNA extraction was performed according to manufacturer's instructions using the RNeasy Mini kit (Qiagen SABiosciences; city, The Netherlands). The Human Cancer

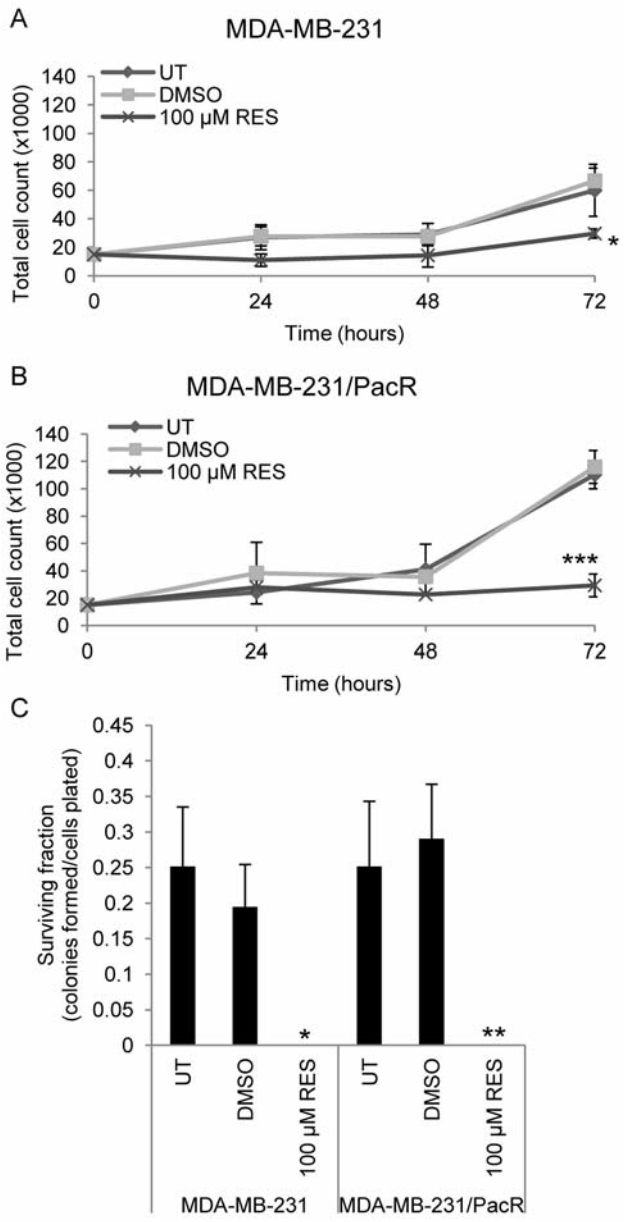

Figure 1. *RES inhibits cell proliferation and colony formation. A) Total cell count from 24-72 h post RES treatment of MDA-MB-231 cells B) MDA-MB-231/PacR (n=3) C) surviving fraction after 72 h of RES treatment and a 17-day incubation (n=3). Error bars signify standard deviation (UT=untreated; *p<0.05; **p<0.01; ***p<0.001; *p-values compare resveratrol treatment to DMSO control).*

Drug Resistance PCR Array (Qiagen SABiosciences) was used according to manufacturer's instructions. Primers for *ABCB1, CYP2C8* and actin (Qiagen SABiosciences) were used in combination with the corresponding RT^2 Primer Assay (Qiagen SABiosciences) reagents and used according to manufacturer's instructions. Real-time PCR was performed with SYBR Green/ROX on an ABI 7500 machine (Supplier, address). P-values and standard deviations (SD) for PCR Array and real-time RT-PCR validation were calculated based on a Student's *t*-test of the replicate $2^{(-\Delta C_T)}$ values for each gene in the control group and treatment groups as suggested by the manufacturer.

Statistics. Student's T-test, two- and one-way ANOVA with Bonferroni correction were used to determine *p*-values where appropriate using GraphPad Prism version 4.00 for Windows (GraphPad Software; San Diego, CA, USA) where $p<0.05$ was considered statistically significant.

Results

Paclitaxel-resistant cell line generation. To provide a cellular model of acquired paclitaxel resistance in MDA-MB-231 breast cancer cells, we developed a paclitaxel-resistant cell line by an intermittent, stepwise treatment with paclitaxel. The limiting dilution cloning method yielded 29 clones that could be successfully established as resistant cell lines. Out of these clones, the 12-fold increased resistance clone, MDA-MB-231/PacR, was selected for further study. The 5-day IC_{50} value of the parent MDA-MB-231cells was 5 nM compared to 62 nM for the MDA-MB-231/PacR cells.

RES inhibits cell proliferation. To determine the effects of RES on cell proliferation of the parent MDA-MB-231 and highly resistant MDA-MB-231/PacR cells, cell counts and a colony formation assay were used. Although the population doubling of the MDA-MB-231/PacR cells was increased to 52 h compared to the MDA-MB-231 cells at 35 h, 100 µM RES significantly inhibited the ability of both cell populations to proliferate (Figure 1A-B). Treatment with 100 µM resveratrol in a clonogenic cell survival assay completely inhibited colony formation in both cell lines, indicating that resveratrol inhibited the ability of a single cell to proliferate (Figure 1C). Treatment with 10 µM resveratrol showed significant proliferation inhibition, but cells were still able to form colonies (data not shown).

RES induces senescence. Due to the inhibition of cellular proliferation observed in both cell lines by RES, we next analyzed the effect of RES on cell-cycle profiles in parental MDA-MB-231 and MDA-MB-231/PacR cells. We chose a 48-hour time point based on the cellular proliferation data which indicates a difference between vehicle-treated and resveratrol-treated is just becoming evident. In addition, a higher concentration, 300 µM, of RES was added to evaluate the effects of RES at concentrations previously used in anti-cancer studies (24, 25) and shown to accumulate in tissues (3). There was no difference of cell-cycle phase between untreated, 10 µM resveratrol or DMSO treatment (Figure 2A); therefore, only DMSO is shown for simplicity. At 48 h, neither 100 nor 300 µM RES treatment caused any accumulation in G_1, G_2/M of S phase compared to vehicle control. Instead, there was accumulation in sub-G_1 phase in

both cell lines, which suggests apoptosis. In the MDA-MB-231 cell line, the sub-G_1 phase increased from 3% in the untreated and DMSO treated to 18% and 49% in the 100 and 300 μM treated cells, respectively. In the MDA-MB-231/PacR cell line, the sub-G_1 phase increased from 8% in the untreated and DMSO-treated to 23% and 34% in the 100 and 300 μM treated cells, respectively. In addition, in both cell lines treated with 300 μM resveratrol, we observed accumulation of a peak with higher DNA content than G_2/M phase, but not enough to denote cell aggregation or mitotic catastrophe. The population increased from 10% and 9% to 24% and 26% in the MDA-MB-231 and MDA-MB-231/PacR cells, respectively. It has been suggested in the literature that micronucleated cells can collect between G_1 and G_2 phase and after G_2/M phase (26). Furthermore, micronuclei were present in both cell populations (data not shown). Therefore, we hypothesize this peak represents a population of micronucleated cells. Because we observed inhibition of cell proliferation without a prominent cell cycle arrest with 100 μM RES treatment, we hypothesized RES treatment was causing senescence in the cells. Due to the greatest cell proliferation difference occurring at 3 days, we used this treatment time to look for senescent cells. We observed 37% and 38% of the MDA-MB-231 and MDA-MB-231/PacR cells, respectively, were senescent by positive β-galactosidase staining (Figure 2B-C).

Resveratrol induces apoptosis. To ensure that the accumulated sub-G_1 phase cells were apoptotic, Annexin V flow cytometry was conducted. After 3 days of 300 μM RES treatment, 96% of MDA-MB-231 cells and 89% of MDA-MB-231/PacR cells had undergone apoptosis. Additionally, after 5 days, 64% of MDA-MB-231 cells and 53% of MDA-MB-231/PacR cells treated with 100 μM RES were apoptotic (Figure 3A-B). Caspase activation was assessed at 48 hs, 3 days and 5 days after RES treatment. After 48 h of treatment with 300 μM RES, procaspase 7 was significantly decreased and the activated, cleaved caspase 7 was significantly increased for both cell lines (Figure 3C-D). Only slight activation of caspase 7 was seen with 100 μM of resveratrol treatment at this time point (Figure 3E). Significant caspase activation was present in the 100 μM but not the 300 μM detected at 3 and 5 days for both cell lines (data not shown). In addition, at 48 h there was slight but significant activation of caspase 3 with 300 μM treatment (data not shown).

RES decreases survivin protein expression at high concentrations. To explore possible mechanisms of resveratrol action, the survivin and SIRT1 pathways were examined as these have been shown to be important in other cancer cells (10). Complete inhibition of survivin protein expression was achieved with 300 μM resveratrol at 48 h and persisted for at least 5 days (Figure 4A-D). However, while we did observe apoptosis occurring at 3 and 5 days with 100 μM treatments, there was no significant difference in survivin protein levels, suggesting that survivin may not be critical in resveratrol-induced apoptosis in these cells at this concentration (Figure 4C-D). Furthermore, the decrease of survivin with 300 μM RES may not be caused by caspase 7 cleavage activity as neither 40% in MDA-MB-231 cells nor 70% of caspase 7-knockdown in MDA-MB-231/PacR cells resulted in any recovery of survivin levels (Figure 4E-F). This result was further supported by the activation of caspase 7 in both cell lines treated with 100 μM RES (Figure 4E) with no resulting decrease in survivin expression (Figure 4B-D). RES showed no effect on the expression of SIRT1 or the protein regulator of SIRT1, DBC1, in these cell lines (data not shown). Further, although 300 μM RES did activate AMPK, which has been shown to cause dissociation of SIRT1 and DBC1 (9), co-immunoprecipitation studies showed little to no association of SIRT1 and DBC1 in these cells (data not shown).

RES augments paclitaxel treatment. To determine whether RES treatment could re-sensitize the resistant cells to paclitaxel, checkerboard combination assays were conducted. The addition of micromolar (2.5-40 μM) amounts of RES simultaneously with paclitaxel resulted in a significant lowering of the IC_{50} value of paclitaxel in both MDA-MB-231and MDA-MB-231/PacR cells without any change in RES IC_{50} (Figure 5A and B). By comparing the effects the same ratios had on the different cell lines, it appears that less RES is required to achieve similar fold changes in IC_{50} in the resistant cells compared to the parent cells. A combination treatment of 1 part paclitaxel to 2,560 parts RES produced the same IC_{50} value in both the MDA-MB-231 and MDA-MB-231/PacR cells; this IC_{50} value was significantly lower than the IC_{50} value of paclitaxel alone in MDA-MB-231 cells. In both cell lines, there were some ratios that caused a decrease of IC_{50} value of both drugs, though more RES was required in the MDA-MB-231 cells compared to the MDA-MB-231/PacR cells to achieve this (Table I). Most combinations did not give a synergistic effect, but rather appear additive or even antagonistic when represented in an isobologram due to the IC_{50} value of resveratrol not decreasing. Some combination ratios resulted in similar IC_{50} values for both cell lines. In contrast, other combination ratios significantly decreased the IC_{50} of the resistant cells but had no effect on the parental cells suggesting that RES may act on two different mechanisms to have a combination effect, one that is specific to the resistant cells and one common to both parental and resistant. Due to these observations, we hypothesized that the mechanism of resistance of the MDA-MB-231/PacR cells was important to the effect of RES in combination with paclitaxel and, therefore, explored the mechanism of resistance in these cells.

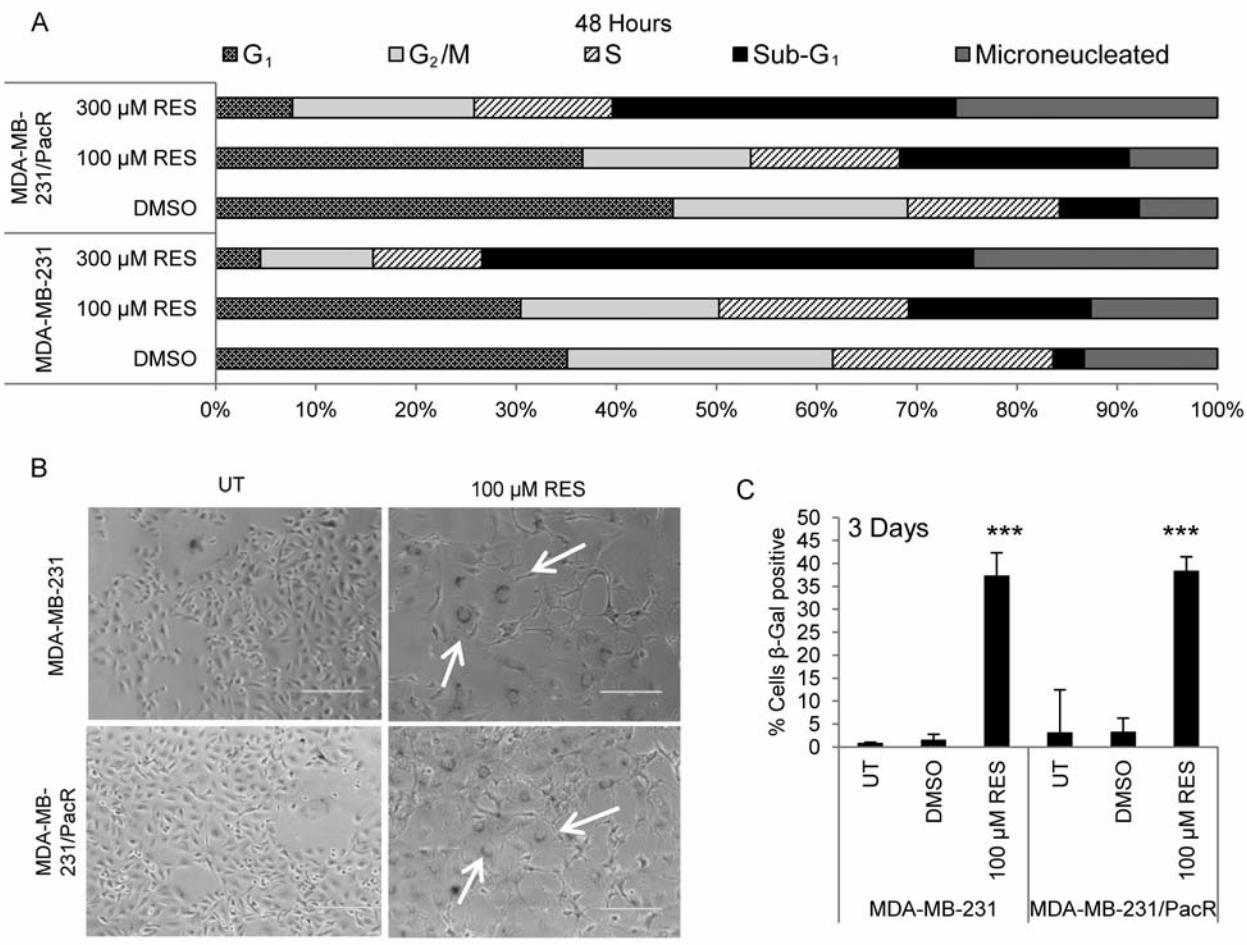

Figure 2. *RES induces sub-G1 accumulation and senescence. A) Cell-cycle analysis showing percent of cells in each phase after 48 h of RES treatment (n=4). The untreated control was omitted for simplicity; there was no difference between untreated and DMSO control. B) β-galactosidase senescence staining after 48 h, representative fields. White arrows point to positive cells. Scale bar represents 200 μm. C) Quantification of percent positive β-galactosidase cells from three representative fields (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to DMSO control).*

P-glycoprotein and CYP2C8 up-regulation contribute to paclitaxel resistance in MDA-MB-231 cells. To determine the mechanism of resistance in these cells, a commercial PCR array for human cancer drug resistance genes was used. Analyses of the PCR array identified two genes to be highly up-regulated in the resistant clone that are known to be factors in paclitaxel resistance, MDR1 (ABCB1) and CYP2C8 (Table II). The over-expression levels of these genes were validated by real-time RT-PCR and the levels were compared between the parent MDA-MB-231 and the MDA-MB-231/PacR cells. ABCB1 was overexpressed in MDA-MB-231/PacR compared to the parent line with a fold increase of 4488.14±0.51 (p=0.026). CYP2C8 was also significantly up-regulated the MDA-MB-231/PacR with a fold increase of 19.09±0.90 (p=0.0013).

Finally, the functional importance of the gene expression data was tested with inhibitors of the protein products. Verapamil was used to inhibit P-glycoprotein, the protein product of *ABCB1*, and trimethoprim was used to selectively inhibit CYP2C8. Inhibition of P-glycoprotein or CYP2C8 alone decreased the resistance of the MDA-MB-231/PacR cells to paclitaxel, as measured by proliferation assays (Figure 6). The paclitaxel IC_{50} for MDA-MB-231/PacR cells alone was 61 nM. After trimethoprim treatment, the IC_{50} decreased to 40 nM. After verapamil treatment the IC_{50} decreased to 8 nM. Simultaneous inhibition of both P-glycoprotein and CYP2C8 did not further lower the IC_{50} value, likely because verapamil-alone lowered the IC_{50} to that of the parent MDA-MB-231 cells. Therefore, it seems that although both P-glycoprotein and CYP2C8 are relevant to the resistance, P-glycoprotein is likely the more important factor.

Table I. *Combination treatment decreases paclitaxel IC_{50}.*

	Paclitaxel: Resveratrol	Paclitaxel IC_{50} (nM)	Fold decrease compared to paclitaxel alone	RES IC_{50} (μM)	Fold decrease compared to resveratrol alone
MDA-MB-231	Alone	4.40±1.49	-	7.79±0.57	-
	1:40960	0.16±0.06***	28.19	6.39±2.58	1.22
	1:20480	0.41±0.09***	10.75	8.38±1.83	0.93
	1:10240	0.80±0.11***	5.53	8.15±1.08	0.96
	1:5120	1.48±0.24***	2.97	7.60±1.22	1.02
	1:2560	2.42±0.38*	1.82	6.20±0.98	1.26
	1:1280	2.91±0.42	1.51	3.73±0.53*	2.09
	1:640	3.34±0.42	1.32	2.14±0.27**	3.64
	1:320	2.41±0.21*	1.82	0.77±0.07***	10.08
MDA-MB-231/PacR	Alone	37.64±13.29	-	9.07±1.57	-
	1:2560	2.52±0.61***	14.93	6.45±1.57	1.41
	1:1280	6.77±1.23***	5.56	8.66±1.58	1.05
	1:640	15.28±2.11*	2.46	9.78±1.35	0.93
	1:320	25.67±4.21	1.47	8.21±1.35	1.10
	1:160	35.96±10.01	1.05	5.75±1.60	1.58
	1:80	25.61±9.37	1.47	2.05±0.75***	4.43
	1:40	2.51±2.57***	15.00	0.10±0.10***	90.41
	1:20	0.16±0.27***	240.95	0.00±0.01***	2904.51

IC_{50} values for paclitaxel and RES and the fold decrease of IC_{50} compared to each drug alone of all combination treatments plus or minus the standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare IC_{50} values of combination treatment to individual drug alone).

Table II. *Genes identified by PCR array up-or down-regulated in MDA-MB-231/PacR-Hi cells compared to MDA-MB-231 cells.*

Symbol	Description	Function	Fold change	*t*-Test	Fold up- or down-regulation
			MDA-MB-231/PacR-Hi/MDA-MB-231	*p*-Value[a]	MDA-MB-231/PacR-Hi/MDA-MB-231
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Xenobiotic metabolism	181.99	0.21	181.99
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	Xenobiotic metabolism	12.51	0.16	12.51
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Xenobiotic metabolism	5.06	0.16	5.06
ATM	Ataxia telangiectasia mutated	S/T protein kinase	3.35	0.01	3.35
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a	NFKB activator	3.11	0.00	3.11
APC	Adenomatous polyposis coli	Tumor repressor	3.11	0.05	3.11
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	Xenobiotic metabolism	0.03	0.01	–31.07
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	Receptor tyrosine kinase	0.15	0.02	–6.71
AR	Androgen receptor	Androgen receptor	0.21	0.04	–4.80
ABCC3	ATP-binding cassette, sub-family C, member 3	Xenobiotic metabolism	0.23	0.14	–4.27
ABCG2	ATP-binding cassette, sub-family G, member 2	Xenobiotic metabolism	0.24	0.11	–4.11
FOS	FBJ murine osteosarcoma viral oncogene homolog	Transcription factor	0.32	0.06	–3.16

[a]*p*-Values were calculated with a Student's *t*-test of the replicate 2^(- ΔC_T) values for each gene.

Discussion

A novel, paclitaxel-resistant triple-negative breast cancer cell line was successfully developed with a 12-fold resistance to paclitaxel compared to parental cells. Clinically-relevant acquired drug resistance spanning from 2- to 12-fold increased resistance compared to cells from tumors prior to treatment (27). Therefore, this cellular model is closer to a relevant level of acquired resistance than many other laboratory models, which are often very highly resistant (27). Importantly, this clinically-relevant resistance is maintained unchanged though the cells are no longer cultured in

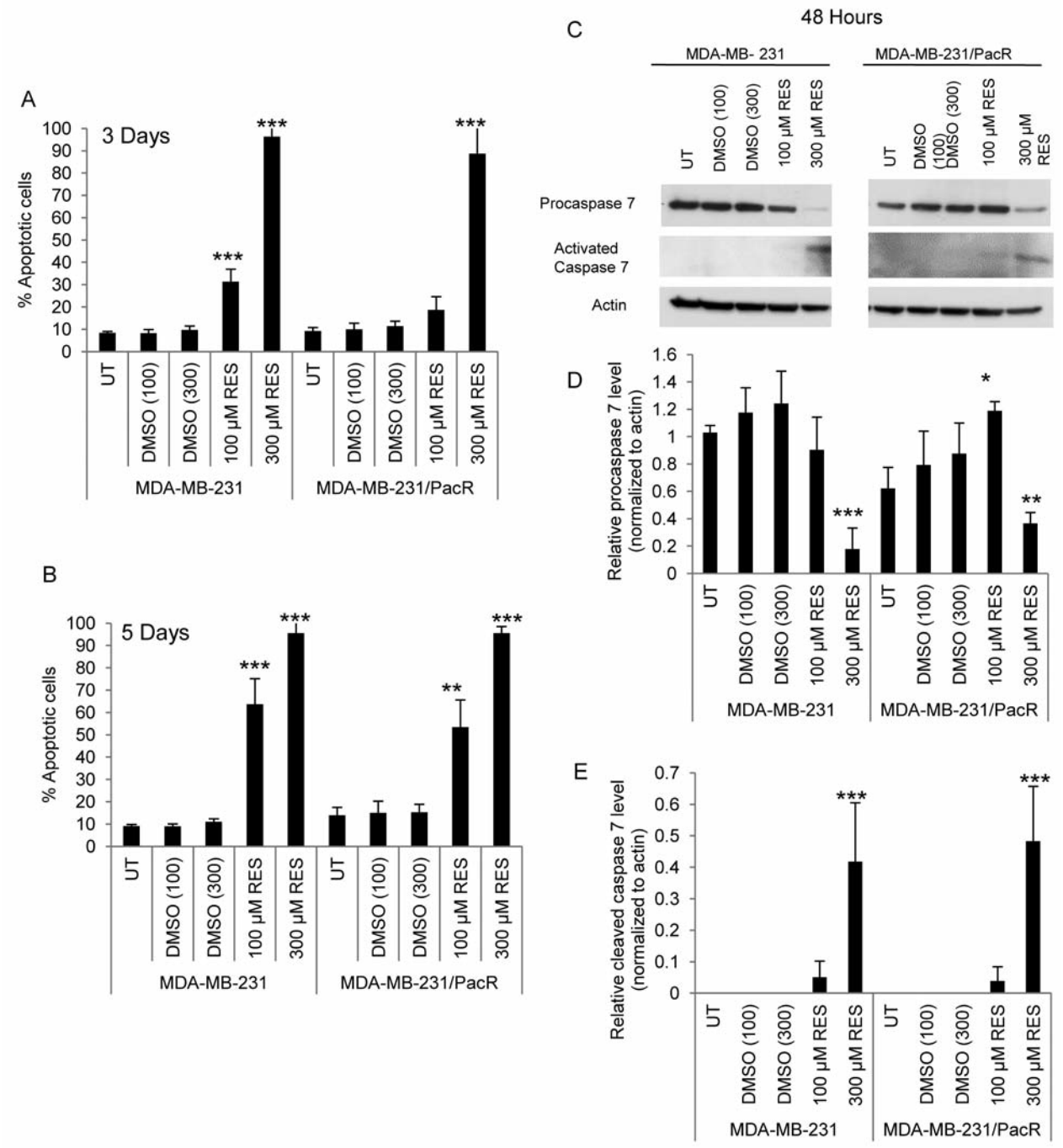

Figure 3. *RES induces apoptosis and activates caspase 7. A) Percent of cells undergoing apoptosis measured by Annexin V flow cytometry after 3 days of RES treatment and B) after 5 days of resveratrol treatment (n=3). C) Representative immunoblot for caspase 7 at 48 h after resveratrol treatment. D) Densitometry quantification normalized to actin of procaspase 7 and E) cleaved caspase 7 (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).*

paclitaxel unlike other clinically-relevant cell lines that must be continuously grown in the presence of the drug to maintain resistance (27).

The data presented herein show that RES induces cell proliferation inhibition, senescence and apoptosis in paclitaxel-sensitive and -resistant triple-negative breast

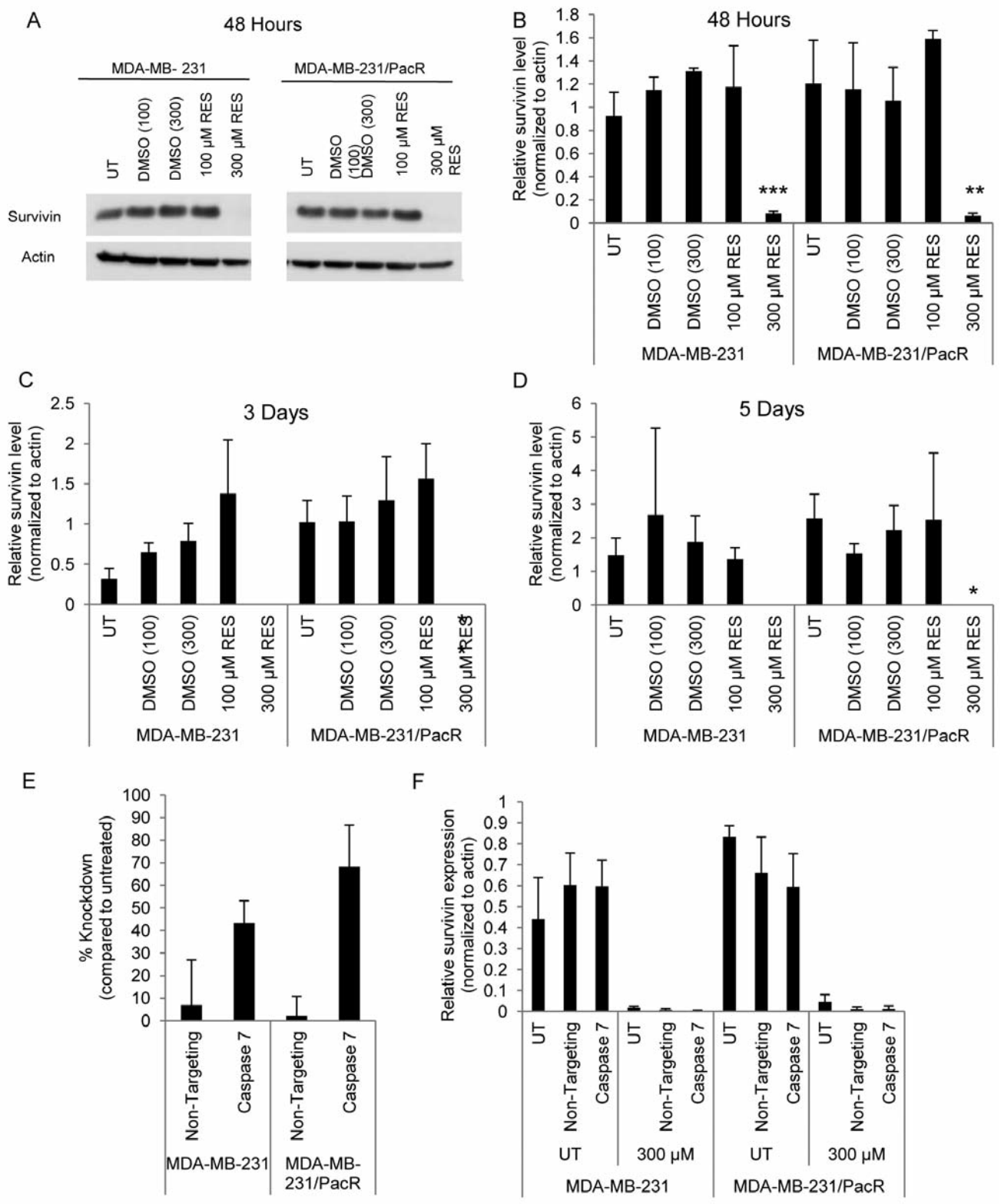

Figure 4. *RES inhibits survivin expression at high concentration. A) Representative immunoblot of survivin 48 h after RES treatment. B) Densitometry quantification normalized to actin of survivin after 48 h. C) 3 days and D) 5 days (n=3). E) Knockdown achieved with caspase 7 siRNAs (n=3). F) Densitometry quantification normalized to actin of survivin expression following siRNA treatment (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare treatment to respective DMSO control).*

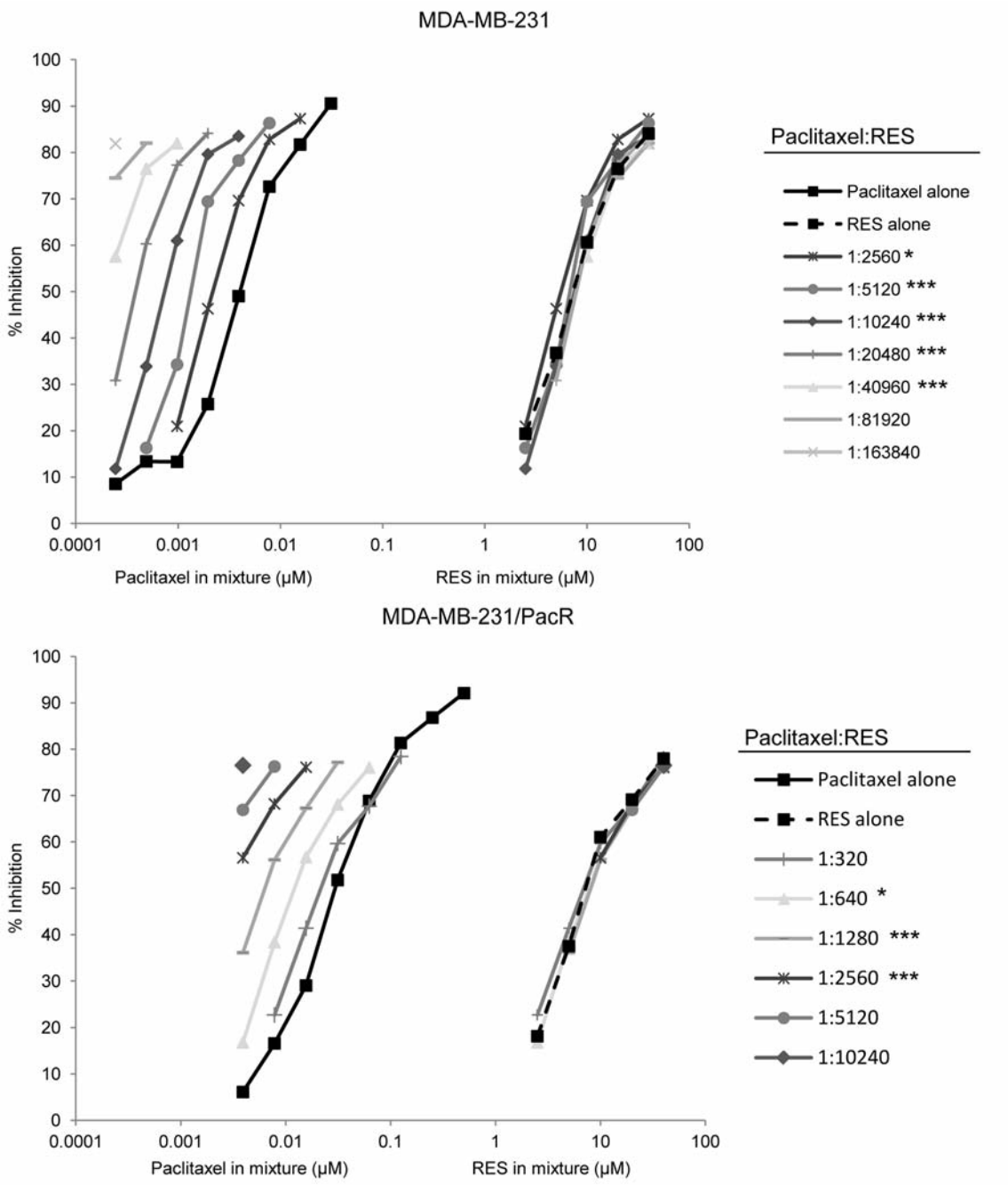

Figure 5. *RES augments paclitaxel-mediated growth inhibition in MDA-MB-231 and MDA-MB-231/PacR cells. Dose-response curves of the constant ratios from the checkerboard combination assay in A) MDA-MB-231 and B) MDA-MB-231/PacR cells (n=3). Each drug ratio has two graphs; one representing the % inhibition versus the amount of paclitaxel in the mixture, as well as one representing the % inhibition versus the amount of RES in the mixture. (*p<0.05; **p<0.01; ***p<0.001; *p-values compare combination treatment IC_{50} values to the paclitaxel alone treatment IC_{50} value).*

cancer cells. RES did not induce cell-cycle arrest in the cells used in this study, which has also been observed in multiple other cancer cell lines (6). Senescence and cells undergoing apoptosis account for almost all of the cell proliferation inhibition in both cell lines, though there is a small gap. Cell-cycle arrest at different time points or an induction of autophagy, which has been reported with resveratrol treatment in other cells (28), may explain this gap. Importantly, the effects of RES were not significantly different between the paclitaxel-resistant and -sensitive cells,

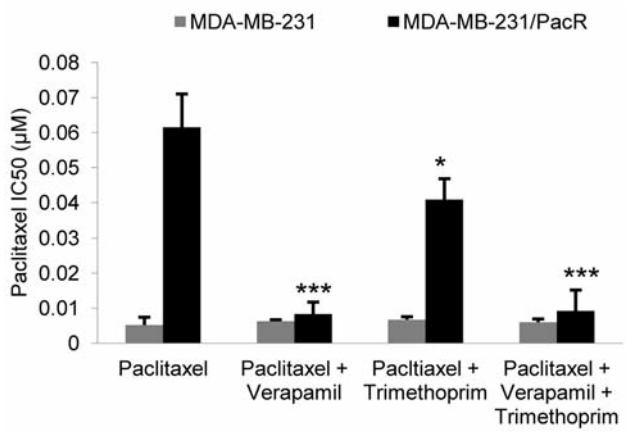

Figure 6. *Inhibition of p-glycoprotein and CYP2C8 re-sensitize MDA-MB-231/PacR cells to paclitaxel. The 5-day IC_{50} values for MDA-MB-231 and MDA-MB-231/PacR cells with paclitaxel alone or in simultaneous combination with the p-glycoprotein inhibitor, verapamil (1 μM), the CYP2C8 inhibitor, trimethoprim (25 μM), or both (n=3). Error bars signify standard deviation (*p<0.05; **p<0.01; ***p<0.001; *p-values compare inhibitor treatment to paclitaxel-alone treatment).*

suggesting that RES may be useful in treating triple-negative breast cancers regardless of paclitaxel sensitivity.

Based on our results, the mechanism of RES-induced apoptosis in this model does not appear to be solely dependent on the decrease of survivin expression. Indeed, only high concentrations of RES caused any decrease in survivin protein levels contrary to what has been observed in other human cancer cell lines (10-13). Therefore, we hypothesized survivin may only play a role at high concentrations of RES treatment or may be an off-target effect of resveratrol in these cells. RES did not affect protein levels of SIRT1 or DBC1 but did activate AMPK, as expected if resveratrol were activating SIRT1 by interrupting the binding of SIRT1 and DBC1. However, we observed little to no binding between SIRT1 and DBC1, which is in accordance with a previous study (29). Together these data suggest the mechanism of RES-induced apoptosis in these cells does not seem to be dependent of survivin depression, changes in SIRT1 and DBC1 levels, nor the binding of SIRT1 and DBC1. Yet, it is possible that RES acts through SIRT1 activation in these cells by allosteric activation (30) or altering SIRT1 localization (31).

Importantly, our data show that resveratrol can augment the effects of paclitaxel when used in simultaneous combination, contrary to a previous report showing RES to attenuate paclitaxel treatment in MDA-MB-231 cells (32). Interestingly, Fukui *et al*. (32) used a 48-h simultaneous treatment, and we also observed similar attenuation of paclitaxel treatment with RES at this time point (data not shown). The present study suggested at a 5-day time point, RES enhances paclitaxel treatment. The IC_{50} values of paclitaxel for both the parent and the resistant cells were lowered significantly with the addition of RES. Notably, addition of RES decreased the paclitaxel IC_{50} value of the resistant MDA-MB-231/PacR cells to the IC_{50} value of the parent line. Moreover, the IC_{50} value could be lowered below that of the parent line with more RES. A trend emerged in these data suggesting that the more RES that there is in the combination solution, the lower the resulting paclitaxel IC_{50} value. This was true in both the parental and the resistant cells. It is most likely that higher amounts of RES in the MDA-MB-231/PacR cells could lower the IC_{50} of paclitaxel further down to similar levels as the combination treatments achieved in the MDA-MB-231 cells. In addition, lower concentrations of resveratrol in the combination decreased the IC_{50} value of both drugs compared to the single drugs alone. Necessary amounts of resveratrol are attainable and well tolerated in humans. Both high concentrations of RES decreasing the paclitaxel IC_{50} with no change in RES IC_{50} and lower concentrations of RES decreasing both paclitaxel and RES IC_{50} could be useful clinical tools. RES has been shown to inhibit P-glycoprotein gene expression (33-35) and decrease the formation of the less active paclitaxel metabolite formed by CYP2C8 (36). Therefore, up-regulation of P-gp and CYP2C8 may partially explain the resensitizing ability of resveratrol to paclitaxel in the resistant cells. However, the inhibitors used in this study did not affect the paclitaxel IC_{50} in the parent cells suggesting there is an alternate resveratrol-paclitaxel combination effect mechanism. These data suggest, for the first time, RES could be used to treat triple-negative breast cancers in combination with paclitaxel to sensitize paclitaxel-resistant cancers and decrease the dose of paclitaxel needed without changing the efficacy of treatment. This proposed regimen could both improve outcomes for patients with paclitaxel-resistant cancers and decrease the general toxicity caused by paclitaxel in patients with both paclitaxel-resistant and -sensitive cancers, though *in vivo* animal studies and clinical trials need to be conducted to validate this in animals and humans.

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References

1 Aluyen JK, Ton QN, Tran T, Yang AE, Gottlieb HB and Bellanger RA: Resveratrol: potential as anticancer agent. J Diet Suppl *9*: 45-56, 2012.

2 Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y and Bonavida B: Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. Curr Med Chem Anticancer Agents *3*: 77-93, 2003.

3 Patel KR, Andreadi C, Britton RG, Horner-Glister E, Karmokar A, Sale S, Brown VA, Brenner DE, Singh R, Steward WP, Gescher AJ and Brown K: Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. Sci Transl Med *5*: 205ra133, 2013.

4 Patel KR, Brown VA, Jones DJ, Britton RG, Hemingway D, Miller AS, West KP, Booth TD, Perloff M, Crowell JA, Brenner DE, Steward WP, Gescher AJ and Brown K: Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. Cancer Res *70*: 7392-7399, 2010.

5 Patel KR, Scott E, Brown VA, Gescher AJ, Steward WP and Brown K: Clinical trials of resveratrol. Ann NY Acad Sci *1215*: 161-169, 2011.

6 Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S and Takada Y: Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res *24*: 2783-2840, 2004.

7 Garvin S, Ollinger K and Dabrosin C: Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts *in vivo*. Cancer Lett *231*: 113-122, 2006.

8 Kim JE, Chen J and Lou Z: DBC1 is a negative regulator of SIRT1. Nature *451*: 583-586, 2008.

9 Nin V, Escande C, Chini CC, Giri S, Camacho-Pereira J, Matalonga J, Lou Z and Chini EN: Role of deleted in breast cancer 1 (DBC1) in SIRT1 activation induced by protein kinase A and AMP activated protein kinase. J Biol Chem *287*: 23489-234501, 2012.

10 Wang RH, Zheng Y, Kim HS, Xu X, Cao L, Luhasen T, Lee MH, Xiao C, Vassilopoulos A, Chen W, Gardner K, Man YG, Hung MC, Finkel T and Deng CX: Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis. Mol Cell *32*: 11-20, 2008.

11 Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, Nair AS, Shishodia S and Aggarwal BB: Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. Blood *109*: 2293-2302, 2007.

12 Fulda S and Debatin KM: Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol. Oncogene *23*: 6702-6711, 2004.

13 Hayashibara T, Yamada Y, Nakayama S, Harasawa H, Tsuruda K, Sugahara K, Miyanishi T, Kamihira S, Tomonaga M and Maita T: Resveratrol induces downregulation in survivin expression and apoptosis in HTLV-1-infected cell lines: a prospective agent for adult T cell leukemia chemotherapy. Nutr Cancer *44*: 193-201, 2002.

14 Fujie Y, Yamamoto H, Ngan CY, Takagi A, Hayashi T, Suzuki R, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N and Monden M: Oxaliplatin, a potent inhibitor of survivin, enhances paclitaxel-induced apoptosis and mitotic catastrophe in colon cancer cells. Jpn J Clin Oncol *35*: 453-463, 2005.

15 Gupta SC, Kannappan R, Reuter S, Kim JH and Aggarwal BB: Chemosensitization of tumors by resveratrol. Ann NY Acad Sci *1215*: 150-160, 2011.

16 Kma L: Synergistic Effect of Resveratrol and Radiotherapy in Control of Cancers. Asian Pac J Cancer Prev *14*: 6197-6208, 2013.

17 Wysocki PJ, Korski K, Lamperska K, Zaluski J and Mackiewicz A: Primary resistance to docetaxel-based chemotherapy in metastatic breast cancer patients correlates with a high frequency of BRCA1 mutations. Med Sci Monit *14*: SC7-10, 2008.

18 McGrogan BT, Gilmartin B, Carney DN and McCann A: Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta *1785*: 96-132, 2008.

19 Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC and Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci USA *89*: 3070-3074, 1992.

20 Oliver MH, Harrison NK, Bishop JE, Cole PJ and Laurent GJ: A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J Cell Sci *92(Pt 3)*: 513-518, 1989.

21 Chou TC: Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res *70*: 440-446, 2010.

22 Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*: 27-55, 1984.

23 Munshi A, Hobbs M and Meyn RE: Clonogenic cell survival assay. Methods Mol Med *110*: 21-28, 2005.

24 Joe AK, Liu H, Suzui M, Vural ME, Xiao D and Weinstein IB: Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res *8*: 893-903, 2002.

25 Juan ME, Wenzel U, Daniel H and Planas JM: Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells. J Agric Food Chem *56*: 4813-4818, 2008.

26 Frackowiak S, Labidi B, Hernandez-Verdun D and Bouteille M: Preservation of chromosome integrity during micronucleation induced by colchicine in PtK1 cells. Chromosoma *94*: 468-474, 1986.

27 McDermott M, Eustace AJ, Busschots S, Breen L, Crown J, Clynes M, O'Donovan N and Stordal B: *in vitro* Development of Chemotherapy and Targeted Therapy Drug-Resistant Cancer Cell Lines: A Practical Guide with Case Studies. Front Oncol *4*: 40, 2014.

28 Delmas D, Solary E and Latruffe N: Resveratrol, a phytochemical inducer of multiple cell death pathways: apoptosis, autophagy and mitotic catastrophe. Curr Med Chem *18*: 1100-1121, 2011.

29 Sung JY, Kim R, Kim JE and Lee J: Balance between SIRT1 and DBC1 expression is lost in breast cancer. Cancer Sci *101*: 1738-1744, 2010.

30 Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, Riera TV, Lee JE, E SY, Lamming DW, Pentelute BL, Schuman ER, Stevens LA, Ling AJ, Armour SM, Michan S, Zhao H, Jiang Y, Sweitzer SM, Blum CA, Disch JS, Ng PY, Howitz KT, Rolo AP, Hamuro Y, Moss J, Perni RB, Ellis JL, Vlasuk GP and Sinclair DA: Evidence for a common mechanism of SIRT1 regulation by allosteric activators. Science *339*: 1216-1219, 2013.

31 Jin Q, Yan T, Ge X, Sun C, Shi X and Zhai Q: Cytoplasm-localized SIRT1 enhances apoptosis. J Cell Physiol *213*: 88-97, 2007.

32 Fukui M, Yamabe N and Zhu BT: Resveratrol attenuates the anticancer efficacy of paclitaxel in human breast cancer cells *in vitro* and *in vivo*. Eur J Cancer *46*: 1882-1891, 2010.

33 Al-Abd AM, Mahmoud AM, El-Sherbiny GA, El-Moselhy MA, Nofal SM, El-Latif HA, El-Eraky WI and El-Shemy HA: Resveratrol enhances the cytotoxic profile of docetaxel and doxorubicin in solid tumour cell lines *in vitro*. Cell Prolif *44*: 591-601, 2011.

34 Nabekura T, Kamiyama S and Kitagawa S: Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. Biochem Biophys Res Commun *327*: 866-870, 2005.

35 Quan F, Pan C, Ma Q, Zhang S and Yan L: Reversal effect of resveratrol on multidrug resistance in KBv200 cell line. Biomed Pharmacother *62*: 622-629, 2008.

36 Vaclavikova R, Horsky S, Simek P and Gut I: Paclitaxel metabolism in rat and human liver microsomes is inhibited by phenolic antioxidants. Naunyn Schmiedebergs Arch Pharmacol *368*: 200-209, 2003.

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