

A novel curcumin derivative increases the cytotoxicity of raloxifene in estrogen receptor-negative breast cancer cell lines

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Abstract. There is a need for new, safe and efficacious drug therapies for the treatment of estrogen receptor (ER)-negative breast cancers. Raloxifene and the 2nd generation curcumin derivative 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone (RL91) have been shown to inhibit the growth of ER-negative breast cancer cells *in vitro* and *in vivo*. We investigated whether RL91 could enhance the growth-suppressive effects mediated by raloxifene in MDA-MB-231, MDA-MB-468, Hs578t and SkBr3 human breast cancer cell lines. The cytotoxicity was consistent across the cell lines but RL91 was more potent. EC₅₀ values for RL91 were 1.2-2 μ M while EC₅₀ values for raloxifene were 9.6-11.2 μ M. When the cells were treated with raloxifene (15 μ M), RL91 (1 μ M) or a combination of the two for 6-72 h, the combination treatment consistently elicited significantly greater cytotoxicity compared to all other treatments. In SkBr3 cells the combination treatment caused significantly more cells to undergo G1 arrest compared to raloxifene. In all cell lines apoptosis was synergistically induced by the combination treatment, as shown by both flow cytometry and cleaved caspase-3. Furthermore, the stress kinase p38 was increased and EGFR isoforms were decreased by both raloxifene and raloxifene + RL91. The anti-angiogenic anti-metastatic potential of raloxifene was not increased by RL91, as MDA-MB-231 cell migration and invasion as well as endothelial tube formation by HUVEC cells was not different between raloxifene (10 μ M) and the combination of raloxifene + RL91. Thus, our findings provide evidence that RL91 increases the ability of raloxifene to suppress ER-negative cancer cell growth by increasing the number of apoptotic cells. The broad effect of this drug combination across a range of ER-negative breast cancer cell lines indicates that this drug combination should be explored further in order to find a safe and efficacious therapy for ER-negative breast cancer.

Introduction

Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene are effective treatments for the prevention of estrogen receptor (ER)-positive breast cancer. Raloxifene, a second generation SERM, has been approved for the prevention of osteoporosis and the reduction of the risk of invasive breast cancer in post-menopausal women (1). In breast tissue, SERMs prevent the proliferation of cancer cells by binding competitively to the ER and blocking the mitogenic effect of estradiol (2). Although SERMs are used clinically as an ER antagonist, there are numerous studies showing that they exhibit effects that are not mediated via the ER. For example, SERMs have shown efficacy when used in cancers that do not express ER such as lung (3), brain cancer (4), melanoma (5) and ER-negative breast cancer (6-8). Furthermore, *in vitro*, SERMs trigger multiple signaling pathways that lead to cancer cell death in an ER-independent manner (9). This provides a potential role for these drugs in triple-negative breast cancers (TNBC) that lack the standard drug targets ER α , the progesterone receptor and HER2/neu. Indeed, we have previously shown that the combination of raloxifene and epigallocatechin gallate (EGCG) was cytotoxic toward the aggressive, basal-like (ER-negative/HER2-negative) MDA-MB-231 cell line (10). Additionally, the combination of EGCG and raloxifene elicited a strong apoptotic response, which occurred independently of cell cycle changes (10) and resulted in a significant decrease in the phosphorylation of the EGFR, Akt, mTOR as well as the nuclear localization of NF- κ B compared to raloxifene alone (11). The efficacy of raloxifene was also increased when it was used in combination with 9-*cis* retinoic acid in a rodent model of mammary carcinogenesis induced by nitroso-methylurea (12). Therefore, combination therapy with SERMs may prove to be beneficial in the treatment of TNBC. A prime candidate for combination therapy with raloxifene would be second generation curcumin derivatives because we have previously shown that various derivatives, including 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone, (RL91), 6-bis(pyridin-3-ylmethylene)-cyclohexanone (RL90), 1-methyl-3,5-bis[(E)-4-pyridyl)methylidene]-4-piperidone, (RL66) and 3,5-bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidine-4-one (RL71) all decreased the phosphorylation of the EGFR, Akt and mTOR (13-15) as well as other proteins involved in the regulation of cell proliferation. Furthermore, raloxifene (10 μ M) decreased proliferation of MDA-MB-468

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cells through the endocytosis of the EGFR leading to a decrease in proliferation signaling pathways (8). Based on these earlier findings, we postulated that RL91 would enhance the cytotoxicity of raloxifene in ER-negative breast cancer cell lines and that this effect would be mediated through changes in EGFR signaling. In the present study, we showed that a combination of raloxifene and RL91 promoted cell cycle arrest in G1 phase of the cell cycle and increased the incidence of apoptosis across all the ER-negative breast cancer cell lines. Furthermore, the combination altered the expression of proteins essential for cell proliferation and apoptosis. Finally, we demonstrated that the combination reduced cell migration and invasion and completely abolished tube-like formation of endothelial cells.

Materials and methods

Materials. HUVEC, MDA-MB-231, MDA-MB-468, Hs578t and SKBr3 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Primary antibodies to EGFR, pEGFR, GSK3 α/β , pGSK3 α/β , p38, pp38, NF- κ B, β -catenin, cleaved caspase-3, p27Kip1, HER2, pHER2, AKT, pAKT and β -actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Raloxifene hydrochloride (99% purity), acrylamide, bisacrylamide, sodium dodecylsulfate, sulforhodamine B salt and propidium iodide (PI) were purchased from Sigma-Aldrich (Auckland, New Zealand). Secondary antibodies goat anti-rabbit and goat anti-mouse and PVDF membrane were purchased from Merck Millipore (Auckland, New Zealand). Dulbecco's modified Eagle's medium (DMEM) nutrient mixture Ham's F-12 was purchased from Thermo Fisher Scientific (Auckland, New Zealand). Complete mini EDTA-free protease inhibitor cocktail and Annexin V-Fluos were purchased from Roche Diagnostics Corp. (Auckland, New Zealand). RL91 was prepared as previously described (13). All other chemicals were of the highest purity commercially available.

Cell culture. MDA-MB-231, MDA-MB-468, Hs578t and SKBr3 breast cancer cells were obtained from the American Type Culture Collection. The cells were grown in complete growth media composed of DMEM/Ham's F12 supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin and 2.2 g/l NaHCO₃. HUVEC were seeded in complete HUVEC media (EBM-2 basal media containing FBS and growth supplements) as described by the manufacturer (Lonza, Auckland, New Zealand). For all procedures, cells were harvested using TrypLE Express (Life Technologies, Auckland, New Zealand) and were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Cytotoxicity. ER-negative breast cancer cells were seeded in 96 well plates and incubated for 24 h at 37°C in 5% CO₂ and then treated with 0–30 μ M of raloxifene or RL91. DMSO (0.1%) served as the vehicle control. The cells were incubated for 72 h and were fixed using trichloroacetic acid (TCA) 10% solution. Cell number was determined using the sulforhodamine B (SRB) assay (16). The concentration required to decrease cell number by 50% (EC₅₀) was determined by non-linear regres-

sion using Prism software. For the time-course analysis, cells were treated with RL91 (1 μ M), raloxifene (15 μ M), a combination of the two or vehicle control for 6–72 h. Cell number was then determined by the SRB assay. For all experiments three independent experiments were performed in triplicate. Results are expressed as cell number.

Cell cycle analysis. ER-negative breast cancer cells were seeded in 6-well culture plates in 1.5 ml of complete growth media. Cells were treated with RL91 (1 μ M), raloxifene (15 μ M), a combination of the two or DMSO (0.1%) for 24–48 h. Cell cycle distribution was assessed using propidium iodide staining, as previously described (13). Samples were analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the proportion of cells in each of G1, S and G2/M phases were determined using the CellQuest Pro software (BD Biosciences). Three independent experiments were performed in triplicate. Results are expressed as the number of cells in each phase of the cell cycle as a percent of the total number of cells.

Apoptosis analysis. ER-negative breast cancer cells were seeded in 6-well culture plates in 1.5 ml of complete growth media. Cells were treated with RL91 (1 μ M), raloxifene (15 μ M), a combination of the two or DMSO (0.1%) for 24–48 h. Apoptosis was assessed using Annexin V-Fluos/propidium iodide staining, as previously described (13). Samples were analyzed using a FACScalibur flow cytometer and the proportion of apoptotic cells was determined using CellQuest Pro software. Three independent experiments were performed in triplicate. Results are expressed as the number of apoptotic cells as a percent of the total number of cells.

Western blotting. ER-negative breast cancer cells were seeded in 12-well culture plates in 1 ml of complete growth media and incubated with RL91 (1 μ M), raloxifene (15 μ M), a combination of the two or DMSO (0.1%) for 24–48 h. Cells were then lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100, 1% SDS, 1 mM NaF, 200 μ M sodium orthovanadate and protease inhibitors (1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000 \times g for 10 min and boiled in Laemmli buffer. Cell lysates were resolved by SDS-PAGE (40 μ g protein/well) and then transferred to a PVDF membrane. Protein levels were analyzed with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (Merk Millipore). The film was scanned by GS-710 densitometer (Bio-Rad Laboratories). Three independent experiments were performed in triplicate. The resulting protein density is expressed as a percentage of the β -actin loading control.

Invasion assay. MDA-MB-231 cells (5 \times 10⁵ cells/ml) were seeded onto growth factor-reduced Matrigel invasion chambers (8- μ m pore; BD Biosciences) and treated with raloxifene (10 μ M), RL91 (1 μ M), a combination of the two or vehicle control for 20 h. Lower chambers contained DMEM/Ham's F12 supplemented with 5% FBS, as a chemoattractant. Filters were fixed in methanol and stained using Diff-Quick staining solutions. Cells were counted in four fields of each well under

Table I. EC₅₀ values for raloxifene and RL91 in ER⁻ breast cancer cell lines.

Cell line	Receptor status	EC ₅₀ (μM)	
		Raloxifene	RL91
MDA-MB-231	ER ⁻ , PR ⁻ , HER2 ⁻	11	1.9
MDA-MB-468	ER ⁻ , PR ⁻ , HER2 ⁻	11.2	1.9
Hs578t	ER ⁻ , PR ⁻ , HER2 ⁻	11	2
SkBr3	ER ⁻ , PR ⁻ , HER2 ⁺	10.6	1.2

an inverted microscope at x20 magnification by an examiner blinded to the treatment groups. Their migration towards FBS was calculated as a percentage of control.

Endothelial tube formation assay. Tube formation was carried out using HUVEC cells. Briefly, Geltrex (Invitrogen, Auckland, New Zealand) was allowed to thaw on ice at 4°C overnight, then 40 μl was pipetted into a 96-well plate and kept for 30 min at 37°C for a gel to form. HUVEC cells were seeded in triplicate on the top of Geltrex layer at a density of 1.5x10⁴ cells/well. Raloxifene (10 μM), RL91 (1 μM), a combination of the two or DMSO (0.1%) were added into the wells and incubated for 20 h at 37°C in 5% CO₂ atmosphere. Images were taken at time zero and 20 h by an examiner blinded to the treatment groups. Three independent experiments were performed in triplicate.

Cell migration. Migration of MDA-MB-231 cells was measured using the *in vitro* cell scratch assay. Once the cells in 6-well plates had reached confluence, a scratch was made with a pipette tip followed by extensive washing with serum-free medium to remove cell debris. Raloxifene (10 μM), RL91 (1 μM), a combination of the two or vehicle control were then added. Cells were allowed to migrate into the scratch area for 20 h at 37°C. At the indicated times, cells were photographed by an examiner blinded to the treatment groups.

Statistical analysis. When time was a factor, data was analyzed with a two-way ANOVA coupled with a Tukey's post hoc test, where P<0.05 was required for statistical significance. When time was not a factor, data were analyzed using a one-way ANOVA coupled with a Tukey's post hoc test, where P<0.05 was required for statistical significance.

Results

To provide a frame of reference for the potency of raloxifene and RL91 in the various ER-negative breast cancer cell lines, EC₅₀ curves were initially produced. Both compounds showed a consistent cytotoxicity across the cell lines but RL91 was more potent. Specifically, EC₅₀ values for RL91 ranged between 1.2 and 2 μM while the values for raloxifene ranged between 9.6 and 11.2 μM (Table I). To determine if a low concentration of RL91 would potentiate the effect of raloxifene, the drugs were evaluated in combination, and time-course experiments were performed in each cell line. All cell lines were treated with

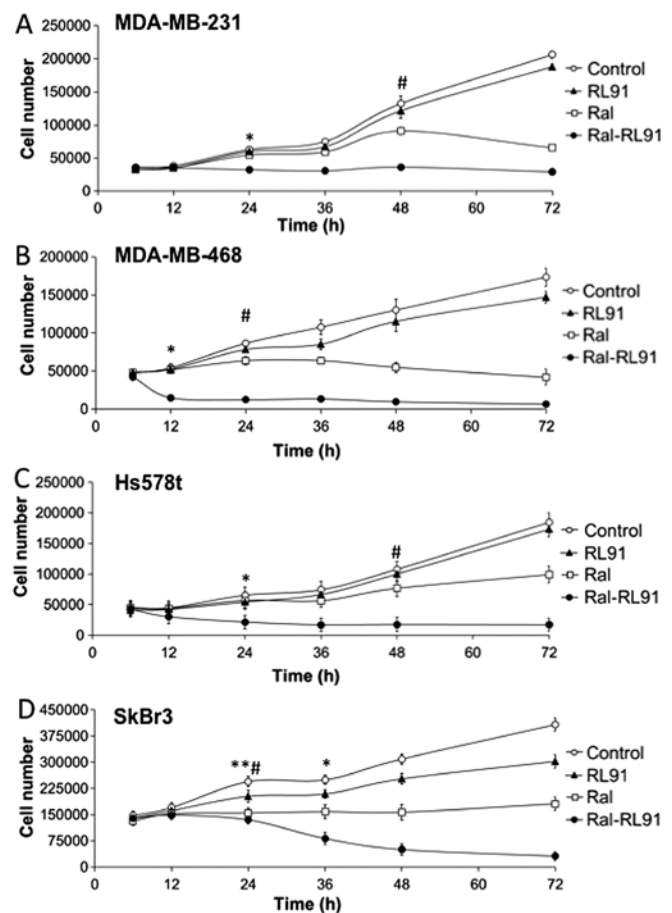


Figure 1. Raloxifene + RL91 elicits synergistic cytotoxicity towards several ER-negative breast cancer cell lines. (A) MDA-MB-231, (B) MDA-MB-468 (C) Hs578t and (D) SkBr3 cells were seeded in 24-well culture plates at 3.5x10⁴ cells/well and were treated either with raloxifene (15 μM), RL91 (1 μM), the combination of the two or vehicle control (0.1% DMSO) for 6-72 h. At the specified time, cells were fixed and cell number was determined using the SRB assay. Results are expressed as total cell number and were obtained from three independent experiments performed in triplicate. Data were analyzed using a two-way ANOVA coupled with a Tukey's post-hoc test. *P<0.05, combination treatment is significantly decreased compared to all other treatments at this point and all further time-points. **P<0.05, RL91 is significantly decreased from control at this point and all further time-points; #P<0.05, raloxifene is significantly decreased from control at this point and all further time-points.

raloxifene (15 μM), RL91 (1 μM) or a combination of the two for 6-72 h. SkBr3 was the only cell line to respond to RL91 (1 μM), which had a growth suppressive effect. Raloxifene (15 μM) significantly suppressed growth in all four cell lines and after 72 h was the most effective in MDA-MB-231 and MDA-MB-468 cells. The combination treatment elicited significantly greater cytotoxicity compared to all other treatments (Fig. 1). In MDA-MB-231 cells the combination treatment was cytostatic, while in the three other cell lines the combination treatment was cytotoxic.

We next determined how the combination treatment influence cell cycle progression, as raloxifene has promoted an accumulation of cells in G1 phase in MDA-MB-231 cells (10) and RL91 has elicited G2/M phase arrest (13) in numerous ER-negative breast cancer cell lines. In all cell lines raloxifene (15 μM) and the combination of raloxifene and RL91 (1 μM) promoted the accumulation of cells in the G1 phase of the

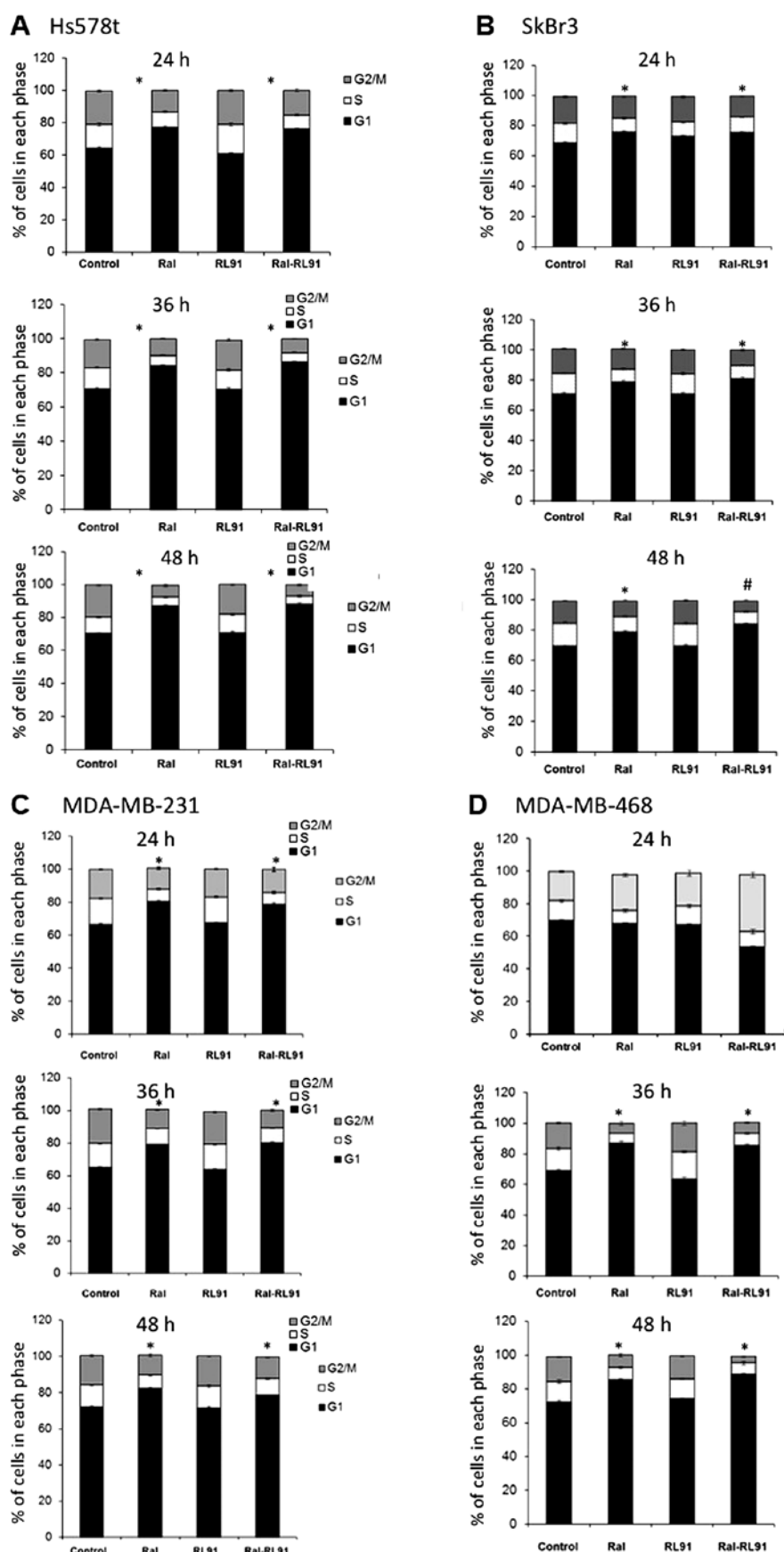


Figure 2. Raloxifene + RL91 arrests ER-negative breast cancer cells in the G1 phase of the cell cycle. Cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24–48 h. Propidium iodide staining and flow cytometry were used to determine the proportion of cells in the various phases of the cell cycle. Bars represent the mean proportion of cells in each phase of cell cycle (% of total) \pm SEM of 3 independent experiments conducted in triplicate. (A) Hs578t, (B) SKBr3, (C) MDA-MB-231 and (D) MDA-MB-468 cells. Data were analyzed using a two-way ANOVA coupled with a Tukey's post-hoc test. * $P < 0.05$, number of cells in the G1 phase of the cell cycle is significantly increased from control; # $P < 0.05$, number of cells in the G1 phase of the cell cycle are significantly increased from raloxifene.

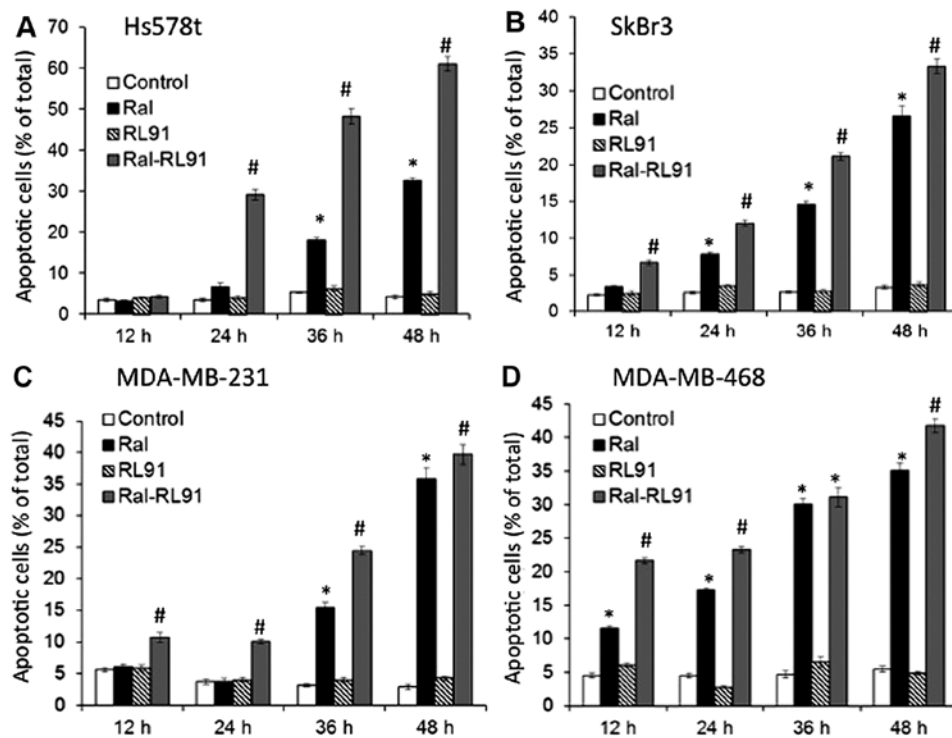


Figure 3. Raloxifene + RL91 synergistically induced apoptosis in ER-negative breast cancer cells. Cell lines were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 12-48 h. Propidium iodide/Annexin V-FITC staining coupled with flow cytometry was used to determine the proportion of cells undergoing apoptosis. Bar represent number of apoptotic cells as a % of the total number of cells \pm SEM from 3 independent experiments performed in triplicate. (A) Hs578t, (B) SKBr3, (C) MDA-MB-231 and (D) MDA-MB-468 cells. Data were analyzed using a two-way ANOVA coupled with a Tukey's post-hoc test. * $P < 0.05$ significantly increased from control; # $P < 0.05$ significantly increased compared to raloxifene.

cell cycle with the concomitant decrease of the percentage of cells in the S and G2/M phases (Fig. 2). In Hs578t, SKBr3 and MDA-MB-231 cells the increase in G1 phase cells started at 24 h and was maintained throughout the treatment period, while in MDA-MB-468 cells, treatment initially potentiated the accumulation of cells in G2/M phase, before triggering G1 phase accumulation at 36 h (Fig. 2D). SKBr3 cells were the only cell line where the combination treatment caused a time-dependent accumulation of the cells in G1 phase (Fig. 2B).

To determine if the changes in cell cycle progression triggered programmed cell death, we measured apoptosis at different times from 12 up to 48 h. The low concentration of RL91 as a single agent did not induce apoptosis, while raloxifene elicited a time-dependent increase of apoptosis across all cell lines. However, this was cell line-dependent, namely 12 h for MDA-MB-468, 24 h for SKBr3 and 36 h for MDA-MB-231 and Hs578t cells. However, after 48 h ~30-35% of the cell population was apoptotic across in all cell lines (Fig. 3). However, in all cell lines the combination of the two drugs caused a significantly higher amount of the cell population to become apoptotic but more importantly the combination triggered an early onset of apoptosis. In Hs578t cells, the number of apoptotic cells increased by 7-fold when compared to other treatment after 24 h (Fig. 3A). In addition, the percentage of cells undergoing apoptosis increased from 33 to 61% following 48 h of treatment with raloxifene and combination, respectively (Fig. 3A). In SKBr3 cells, the combination promoted a higher level of apoptosis at 12 h and increased up to 48 h (Fig. 3B). In MDA-MB-231 cells, the combination treatment doubled the number of apoptotic cells at 12 and 24 h compared to other

treatments and was significantly higher than raloxifene at 36 and 48 h (Fig. 3C). In MDA-MB-468 cells, raloxifene elicited a rapid increase in apoptosis and the combination potentiated this effect, doubling the number of apoptotic cells after 12 h (Fig. 3D). In all cell lines, the combination increased apoptosis independently of changes in the cell cycle.

We have demonstrated that the suppression of MDA-MB-468 xenograft tumor growth following a daily oral dosing with raloxifene was associated with a decreased EGFR and NF- κ B protein expression (8). Therefore, we postulated that the combination treatment will improve the effect of raloxifene and alter the expression of several proteins essential for either cell survival or apoptosis in all ER-negative cell lines. Notably, in all the cell lines raloxifene and combination enhanced the phosphorylation of members of the mitogen-activated protein kinases (MAPKs) family such as dual specificity mitogen-activated protein kinase kinase-1 (MEK1), extracellular signal-regulated kinase 1/2 (ERK1/2) and stress kinase p38 MAPK (Fig. 4). ERK1/2 is a kinase that phosphorylates a large number of proteins implicated in multiple cellular processes including apoptosis (17). p38 MAPK is activated in response to cellular stresses and depending on the cell line may affect multiple cellular processes including cell cycle arrest and apoptosis. Across all the cell lines, the different treatments did not alter the total level of expression of these kinases. Furthermore, the phosphorylation of these kinases varied upon the treatment, time and cell lines. In MDA-MB-231 cells, the basal level of MEK1 phosphorylation is high and its amplification following raloxifene and combination treatment is observed only after 36 h. The enhancement of MEK phosphorylation

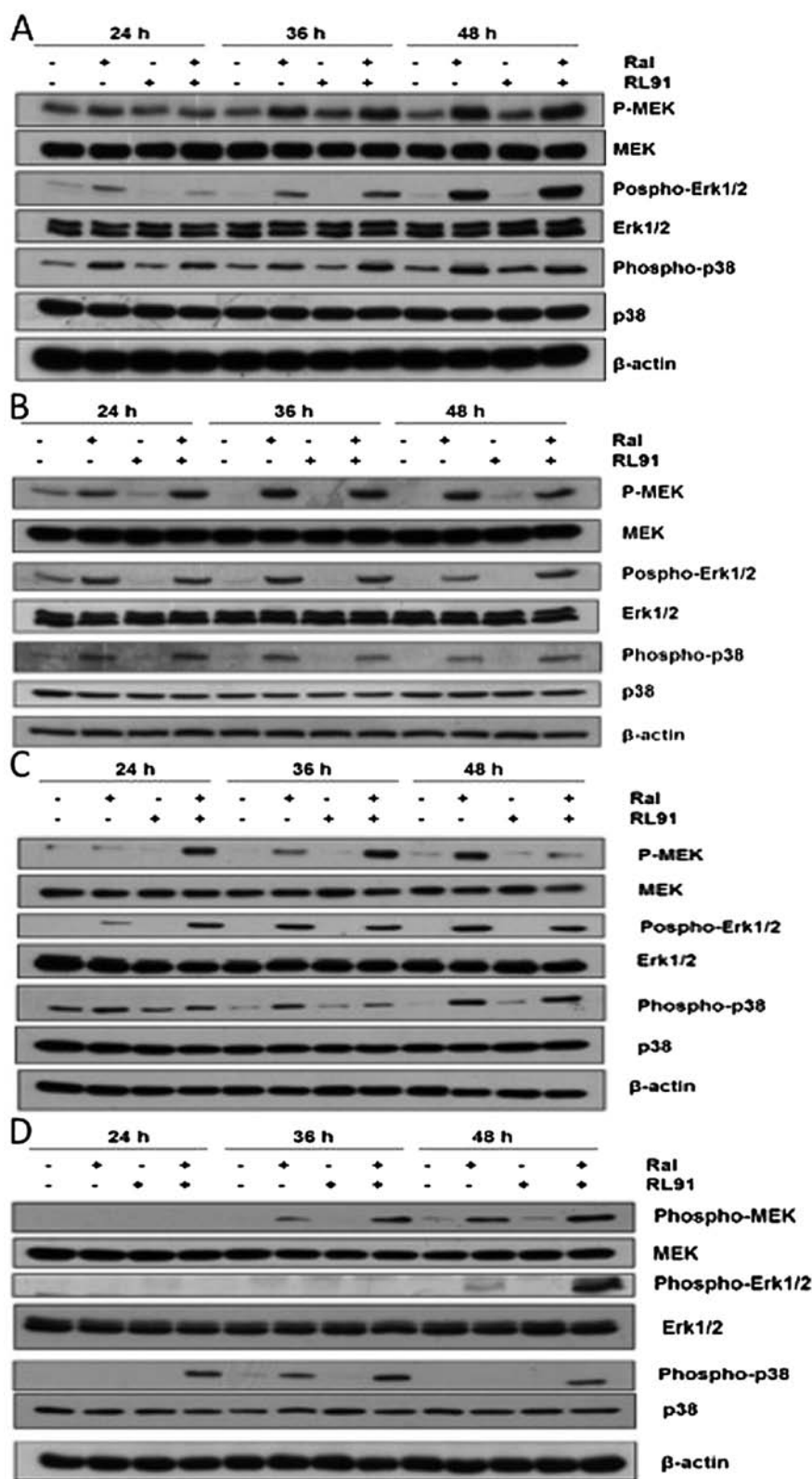


Figure 4. Raloxifene + RL91 modulates the expression of mitogen-activated protein kinases in ER-negative breast cancer cells. ER-negative breast cancer cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24–48 h. Total cell lysates were analyzed by western blotting with the specific antibodies and β -actin served as the loading control. Representative blots from $n=3$ performed in triplicate are shown. (A) MDA-MB-231 cells, (B) MDA-MB-468 cells, (C) Hs578t cells and (D) SkBr3 cells.

triggers phosphorylation of ERK1/2, downstream of MEK1 in the MAPK signaling cascade. Furthermore, this effect was enhanced by the combination with RL91. In addition, p38 MAPK follows the phosphorylation pattern of MEK, where

combination treatment amplified p38 MAPK phosphorylation after 36 and 48 h (Fig. 4A). In MDA-MB-468 cells, raloxifene and combination increased MEK phosphorylation up to 48 h. However, the combination triggered a higher increase of MEK

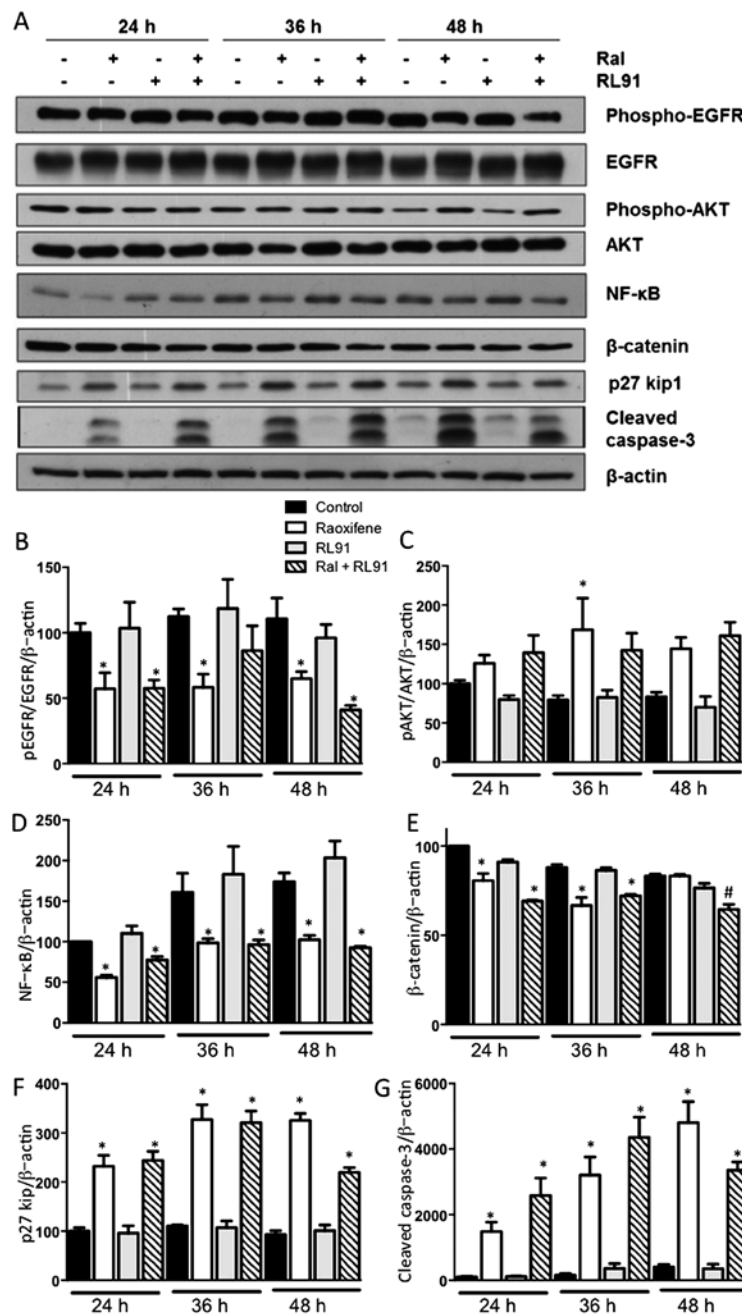


Figure 5. Raloxifene + RL91 modulates the expression of proteins essential for cancer cell proliferation, protein synthesis and apoptosis in MDA-MB-468 cells. MDA-MB-468 cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24-48 h. Total cell lysates were analyzed by western blotting with the specific antibodies and β -actin served as the loading control. (A) Representative blots, scanning densitometry for (B) EGFR, (C) AKT, (D) NF- κ B, (E) β -catenin, (F) p27kip1 and (G) cleaved caspase-3. Bars represent the mean \pm SEM of 3 independent experiments conducted in triplicate. *P<0.05 significantly different from control; #P<0.05 significantly different compared to all other treatments.

phosphorylation at 24 h. ERK1/2 and p38 MAPK follow an identical phosphorylation pattern where raloxifene and combination promote and sustain their phosphorylation (Fig. 4B). In Hs578t cells, the phosphorylation of MEK is more transient and amplified by the combination treatment at 24 and 36 h while being reduced at 48 h. The effect of raloxifene treatment on MEK phosphorylation was more progressive and peaked at 48 h. As a consequence, ERK1/2 phosphorylation was rapidly increased after 24 h and plateaued up to 48 h following combination treatment while raloxifene triggered a more progressive increment of ERK1/2 phosphorylation. Phosphorylation of p38 MAPK was sustained following raloxifene treatment from 24

to 48 h when compared to the combination (Fig. 4C). Finally, in SkBr3 cells the phosphorylation of MEK was not enhanced by combination treatment until 36 h but was increased further at 48 h. Raloxifene promotes only a modest increase at 36 and 48 h. Phosphorylation of p38 was more transient and was only induced by the combination at 24 h, raloxifene induced a transient p38 MAPK phosphorylation at 36 h. Overall, the levels of phosphorylation of MEK, ERK1/2 or p38 MAPK poorly correlate with the accumulation of the cells in G1 of the cell cycle or apoptosis but coincide with the activation of caspase-3 (Fig. 5). Furthermore, the combination treatment of raloxifene with an inhibitor of MEK (U0126) or p38 MAPK (SB203580)

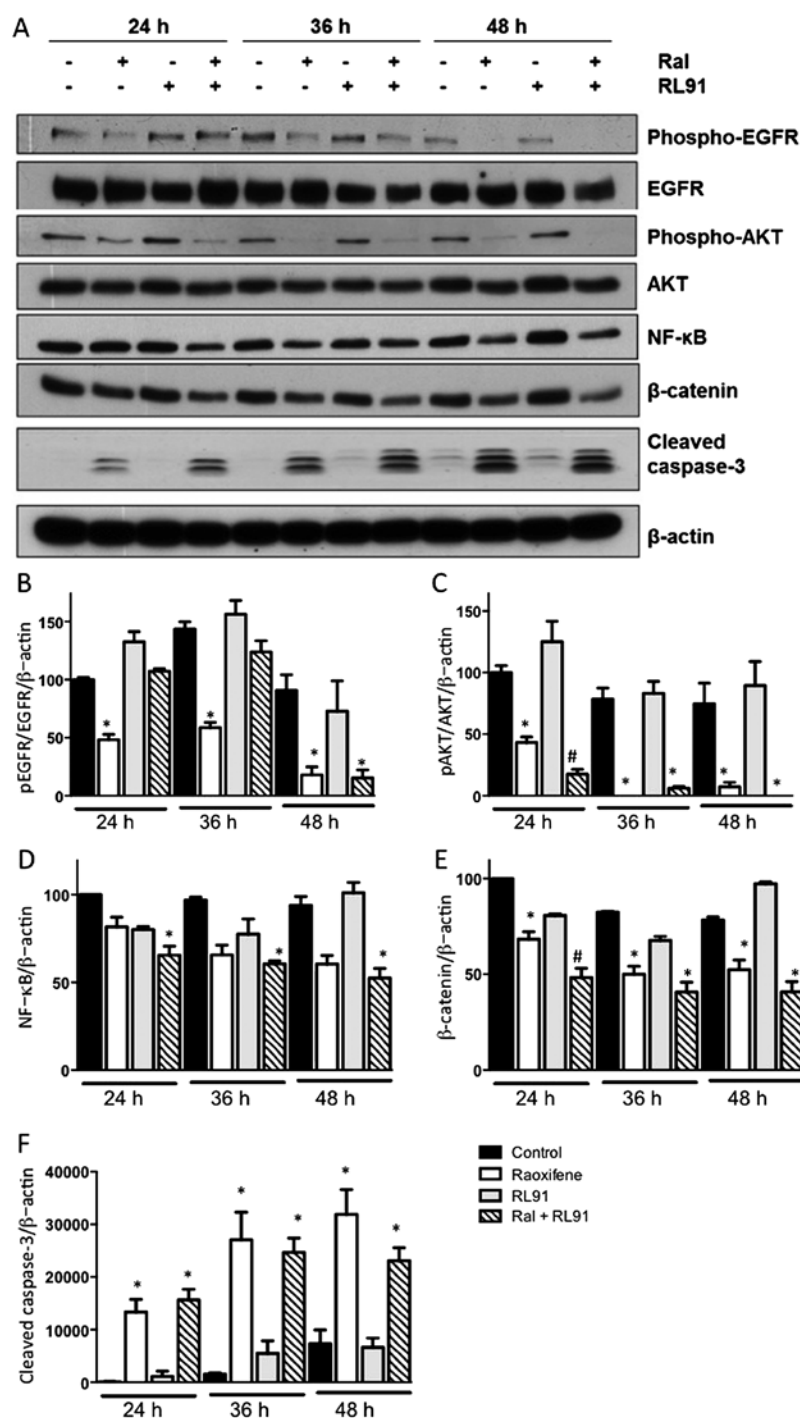


Figure 6. Raloxifene + RL91 modulates the expression of proteins essential for cancer cell proliferation, protein synthesis and apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24–48 h. Total cell lysates were analyzed by western blotting with the specific antibodies and β -actin served as the loading control. (A) Representative blots, (B) scanning densitometry for EGFR, (C) AKT, (D) NF- κ B, (E) β -catenin and (F) cleaved caspase-3. Bars represent the mean \pm SEM of 3 independent experiments conducted in triplicate. * P <0.05 significantly different from control; # P <0.05 significantly different compared to all other treatments.

in MDA-MB-231, HS578t and MDA-MB-468 cells did not reduce the cytotoxicity of raloxifene (data not shown).

We then assessed the effect of raloxifene and its combination with RL91 on the expression of EGFR and its downstream effectors. In MDA-MB-468 cells, raloxifene (15 μ M) significantly decreased EGFR phosphorylation at amino acid Tyr1148 but this was not enhanced by co-treatment with RL91 (Fig. 5B). EGFR is phosphorylated at multiple sites and Tyr1148 is generally associated with the activation

of MAP kinase signaling pathway. Contrary to the other cell lines, MDA-MB-468 cells are characterized by the lack of PTEN expression, a key regulator of AKT activity (18), we observed that the treatment with raloxifene and combination did not affect total AKT expression but raloxifene increased phosphorylation of AKT at 36 h (Fig. 5C). The enhanced phosphorylation suggests that raloxifene affects the feedback loop regulation of AKT and promoted cumulative phosphorylation. The increased AKT phosphorylation may be responsible for

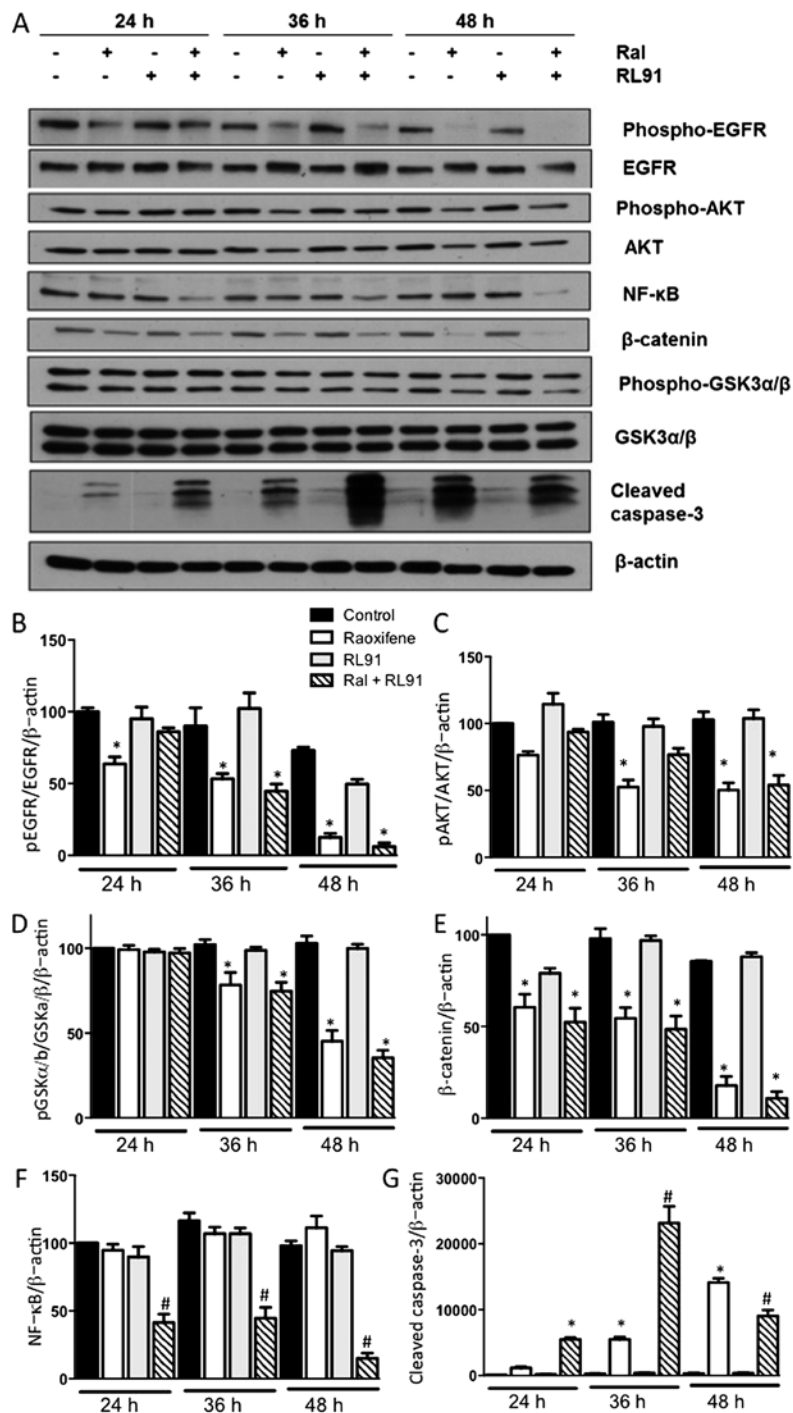


Figure 7. Raloxifene + RL91 modulates the expression of proteins essential for cancer cell proliferation, protein synthesis and apoptosis in HS578t cells. HS578t cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24-48 h. Total cell lysates were analyzed by western blotting with the specified antibodies and β -actin served as a loading control. (A) Representative blots, scanning densitometry for (B) EGFR, (C) AKT, (D) GSK3 α / β , (E) β -catenin, (F) NF- κ B and (G) cleaved caspase-3. Bars represent the mean \pm SEM of 3 independent experiments conducted in triplicate. * P <0.05 significantly different from control; # P <0.05 significantly different compared to all other treatments.

the stabilization of β -catenin protein, but did not prevent the decrease of NF- κ B expression following raloxifene and combination treatment from 24 h (Fig. 5D). In addition, both raloxifene and combination increased expression of p27Kip1 after 24 h (Fig. 5F). The protein is an inhibitor of cell cycle progression and promotes accumulation of the cells in G1 phase. The enhanced expression of p27Kip1 was not observed in the other cell lines. Finally, the activation of caspase-3, a hallmark of the apoptotic process, was observed as soon as

12 h following raloxifene treatment and potentiated by its combination with RL91 (Fig. 5G).

In MDA-MB-231 cells, raloxifene and combination treatment decreased the phosphorylation of EGFR from 24-48 h without significantly affecting total EGFR expression (Fig. 6B). AKT expression and phosphorylation was reduced following raloxifene treatment while combination of raloxifene with RL91 potentiated the reduction of its phosphorylation at 24 h (Fig. 6C). Expressions of NF- κ B and β -catenin, proteins regu-

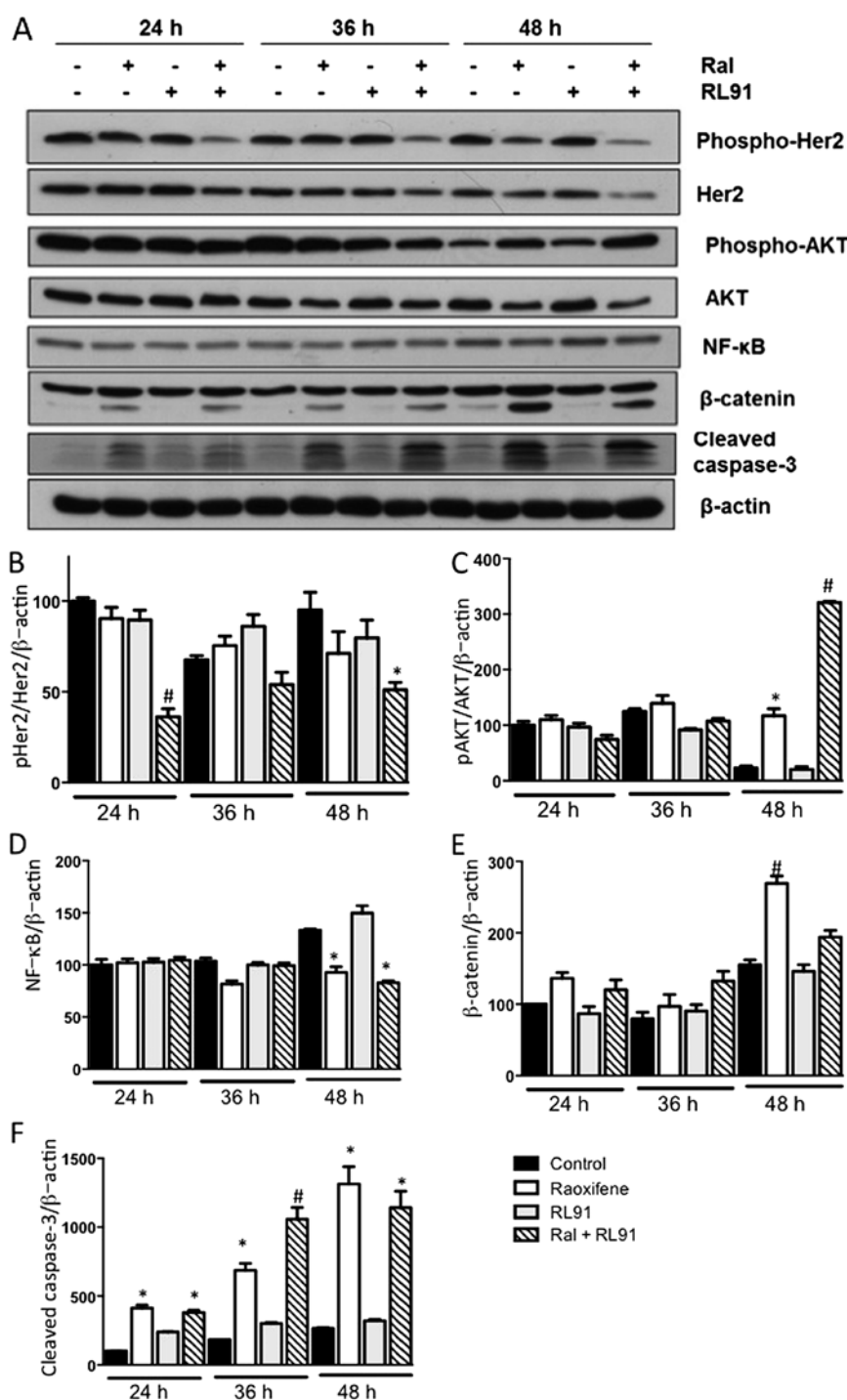


Figure 8. Raloxifene + RL91 modulates the expression of proteins essential for cancer cell proliferation, protein synthesis and apoptosis in SKBr3 cells. SKBr3 cells were treated with raloxifene (15 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO for 24–48 h. Total cell lysates were analyzed by western blotting with the specified antibodies and β -actin served as a loading control. (A) Representative blots, (B) Her2, (C) AKT, (D) NF- κ B, (E) β -catenin and (F) cleaved caspase-3. Bars represent the mean \pm SEM of 3 independent experiments conducted in triplicate. * P <0.05 significantly different from control; # P <0.05 significantly different compared to all other treatments.

lated by AKT, were reduced by raloxifene after 36 and 48 h (Fig. 6DE), while the combination decreased the expression at 24 h. Similar to MDA-MB-468 cells, cleaved caspase-3 was induced following raloxifene and potentiated by the combination at 24 h (Fig. 6F).

In Hs578t cells, the combination treatment also significantly decreased the phosphorylation and total EGFR (Fig. 7B) and AKT (Fig. 7C) expression to a similar extent as raloxifene

alone. Notably, phosphorylation of GSK3 α/β , whose level is regulated by AKT, was reduced following raloxifene and combination treatments (Fig. 7D). Dephosphorylation of GSK3 α/β is generally associated with increased activity of the kinase and is responsible for the phosphorylation and degradation of β -catenin. The expression of β -catenin decreased following raloxifene and combination treatments (Fig. 7E), corresponding to the dephosphorylation profile of GSK3 α/β .

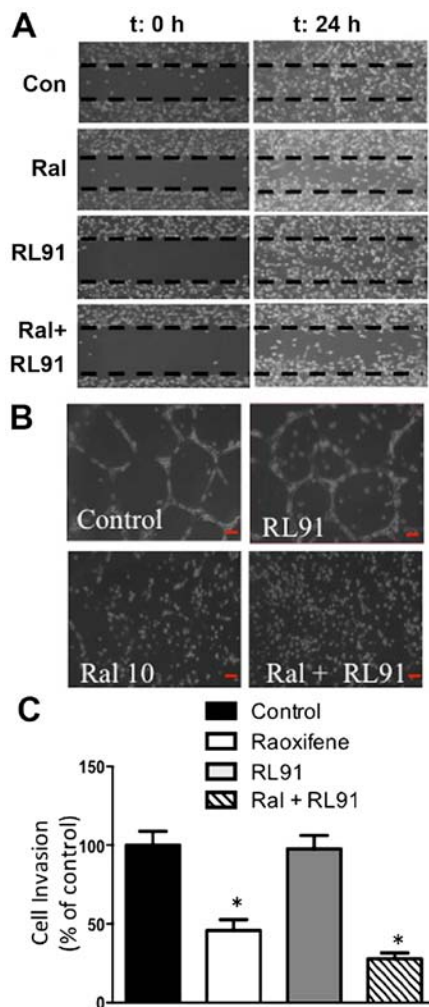


Figure 9. Raloxifene + RL91 inhibits cell migration, endothelial tube formation and invasion *in vitro*. (A) Scratch assay for cell migration. A confluent MDA-MB-231 cell monolayer was wounded and incubated for 20 h with raloxifene (10 μ M), RL91 (1 μ M) or a combination of the two or DMSO (0.1%). Representative monolayer images from three independent experiments performed in triplicate are shown at 0 and 20 h. Cell invasion assay. (B) Tube formation of HUVEC cells. HUVEC cells (50,000/well) were seeded and treated with raloxifene (10 μ M), RL91 (1 μ M), a combination of the two or DMSO (0.1%) for 18 h. Images were taken by an examiner blinded to the treatment groups. Representative images from three independent experiments performed in triplicate are shown. (C) MDA-MB-231 cells were seeded onto filters precoated with Matrigel on the upper surface in Transwell chambers and treated with raloxifene (10 μ M), RL91 (1 μ M), a combination of the two or 0.1% DMSO. The bottom chamber contained growth media with 5% FBS as the chemoattractant. After 20 h the cells migrating to the lower surface were fixed, stained with Diff Quick and counted. The bars represent the mean \pm SEM of three independent experiments performed in triplicate. * P <0.05 significantly different to vehicle control.

NF- κ B expression was only reduced by the combination treatment (Fig. 7F). As in the other cell lines, the activation of caspase-3 was also potentiated by the combination when compared to raloxifene treatment at 24 h (Fig. 7G).

SkBr3 cells are characterized by the overexpression of HER2, an isoform of EGFR. In these cells, the combination treatment significantly decreased the phosphorylation and HER2 protein expression at 24 h, while raloxifene decreased its expression at 48 h (Fig. 8B). Raloxifene and combination treatments reduced the expression of AKT while increasing its phosphorylation at 48 h (Fig. 8C). The deregulation of AKT

phosphorylation was not associated with a lack or mutation of PTEN protein as these cells expressed the wild-type protein. We also observed decreased NF- κ B expression following raloxifene and combination treatments (Fig. 8D). Interestingly, raloxifene and combination did not reduce the expression of β -catenin but triggered the appearance of a truncated form of the protein (Fig. 8E). Additionally, cleaved caspase-3 was increased after 24 h following both raloxifene and combination. The activation of caspase-3 was enhanced by the combination when compared to raloxifene after 36 to 48 h (Fig. 8F).

To determine if the combination treatment could potentially alter metastasis, two different *in vitro* assays were performed. Specifically, MDA-MB-231 cell migration was examined using the scratch assay while MDA-MB-231 cell invasion was determined using transwell cell invasion. All of these assays when combined give an indication of how well a drug may modulate metastasis *in vivo*. In these assays RL91 (1 μ M) was ineffective, while raloxifene (10 μ M) decreased scratch closure and decreased the number of invasive cells by 54 \pm 7% (Fig. 9A and B). The combination suppressed cell migration and decreased invasion by 18% compared to raloxifene but this was not statistically significant. Finally, we assessed the effect of the combination on angiogenesis using an endothelial cell tube-like formation assay. RL91 has no effect on the endothelial tube formation while raloxifene and combination treatment abolished endothelial cell extension (Fig. 9C).

Discussion

We have demonstrated that in the absence of ER- α expression, raloxifene promoted cell cycle arrest in the G1 phase, increased apoptosis and internalization of plasma membrane associated EGFR into cytoplasmic vesicles *in vitro* (8). We also showed that raloxifene elicits the reduction of xenograft tumor growth which was associated with a decrease in EGFR expression, an increase apoptosis, and reduction in angiogenesis *in vivo* (8). Triple-negative breast cancers, represented in this study by MDA-MB-231, MDA-MB-468 and Hs578t cells, are characterized by their lack of responsiveness to multiple ligands (19) and, therefore, respond poorly to targeted therapies. Our laboratory has developed a range of second-generation curcumin derivatives that exhibit potent cytotoxicity (13,20) in ER-negative breast cancer cell lines and target multiple signaling pathways *in vitro* (13-15). Therefore, the present study was designed to determine if a low concentration of RL91 could enhance the anticancer properties of raloxifene in a range of ER-negative breast cancer cell lines. Our results showed that RL91 enhanced the cytotoxicity elicited by raloxifene in all ER-negative breast cancer cell lines. Importantly, in all cell lines cytotoxicity and apoptosis were always produced either earlier or amplified by the combination treatment compared to raloxifene alone, as shown by both flow cytometry and cleaved caspase-3 activation. The effects of the different treatments on cell cycle progression are independent from the commitment of the cell to the apoptotic pathway. In Hs578t and MDA-MB-231 cells, raloxifene increased apoptosis only after 36 h while G1 accumulation was observed after 24 h. Similar to results seen with other 2nd generation curcumin derivatives (14,15,17) and combination therapy with tamoxifen and raloxifene (10,11,21), cell cycle arrest did not

drive apoptosis. In fact, significant apoptosis developed regardless of G2 arrest, elicited by curcumin derivatives (13-15), or G1 arrest, elicited by raloxifene (10), or by the combination of raloxifene and RL91.

The cellular stress triggered by the cytotoxic drugs may promote cell cycle arrest in the G1 phase. In all cell lines, the treatment with raloxifene and its combination with RL91 promote the activation of p38 MAPK. The p38 MAPK is activated by a broad range of stress stimuli (22) and has been associated with the inhibition of cell cycle progression and induction of apoptosis (9,23). The activation of p38 MAPK was observed in all cell lines as early as 24 h by raloxifene and the combination treatments, excepted in SkBr3 cells where phosphorylation of p38 MAPK induced by raloxifene treatment was transient. The inhibition of p38 MAPK by the specific inhibitor SB203580, did not alter the cytotoxic profile of raloxifene (data not shown), suggesting that the activation p38 MAPK essentially promotes the cell cycle arrest. In MDA-MB-468 cells only, the treatment by raloxifene and combination enhanced the expression of p27Kip1, a negative regulator of the G1 to S phase transition (24).

The overexpression or mutation of EGFR has been identified in multiple cancer types. In ER-negative breast cancer, the EGFR is especially important, as it is overexpressed in cell lines (25,26) and 50% of tumors (27-29). Previous studies showing that both raloxifene and RL91 modulate the EGFR expression and downstream cell signaling proteins formed the basis of the molecular mechanisms examined in the present study. Furthermore, EGFR controls many basic cellular functions including cell proliferation, survival, mobility and migration, and also promotes the establishment of new blood vessels (angiogenesis) that provide oxygen and nutrients to the tumor (30). The Ras/Raf/MEK/ERK1/2 and Ras/PI3K/AKT signaling cascades are critical to these physiological processes.

The activation of ERK1/2 is generally associated with cell proliferation and resistance to apoptosis. However, in all cell lines, raloxifene treatment increased phosphorylation of MEK and ERK1/2, the effect of raloxifene was enhanced by the combination with RL91. The activation of ERK1/2 can also contribute to the enhancement of cell death through activation of caspase-3 and suppression of AKT signaling (31). Several therapeutic agents have also been associated with an increase of ERK1/2 phosphorylation such as Taxol (32), doxorubicin (33), resveratrol (34) and quercetin (35). The implication of ERK1/2 in the apoptotic process may be the result of the change in the amplitude, duration and frequency of its phosphorylation or even localization (36). In all the triple-negative breast cancer cell lines used in the present study, the increase in the phosphorylation of ERK1/2 coincided with the onset of apoptosis. However, in SkBr3 cells, the increased ERK1/2 phosphorylation occurs at a later time point suggesting that additional factors contribute to the increase of apoptosis following raloxifene and combinations treatments. Furthermore, co-treatment of triple-negative breast cancer cell lines of raloxifene with the MEK inhibitor U0126, failed to decrease the cytotoxicity of raloxifene (data not shown).

The treatment with raloxifene and combination also affected the AKT signaling cascade. In MDA-MB-231 cells, RL91 enhanced the ability of raloxifene to decrease the

downstream effector of EGFR signaling pathway such as AKT implicated in the regulation of β -catenin and NF- κ B. AKT has been implicated in a myriad of regulatory mechanisms involving protein synthesis, cell cycle progression and inhibition of apoptosis (37,38) by promoting the expression of anti-apoptotic proteins such as Bcl-xL, c-Myb and caspase inhibitors (39,40). Recent studies have also suggested that a decrease in the level of AKT could lead to higher activity of p38 MAPK (41) and this protein was increased in response to raloxifene and combination from 24 h onwards.

This trend was examined in the other ER-negative cell lines. In MDA-MB-468 and SkBr3 cells, AKT phosphorylation was not decreased at 48 h, while in control and RL91 treated cells the phosphorylation of AKT was reduced. MDA-MB-468 cells lack the expression of the PTEN protein, a tumor suppressor protein which negatively regulates the AKT pathway (42). In addition, SkBr3 cells are characterized by their low PTEN expression (43). Hs578t cells responded the best to combination treatment as EGFR was decreased 94% at 48 h, NF- κ B was significantly decreased compared to all other treatments at all time points and β -catenin was decreased to the largest extent (94%) at 48 h. Additionally, GSK3 α/β was decreased by 65% following combination treatment after 48 h. GSK3 α/β kinase is negatively regulated by AKT and plays a pivotal role in the stability of β -catenin (44). The decreased phosphorylation is associated with the increased activity of the kinase promoting β -catenin phosphorylation and degradation (44).

We also examined the effect of the combination therapy in SkBr3 cells because breast cancer patients whose tumors overexpress HER2/neu have a poor prognosis, shorter relapse time and shorter survival time (27). Additionally, our curcumin derivatives have shown potent suppression of HER2 in SkBr3 cells (13-15). The results showed that the combination reduced HER2 expression and phosphorylation earlier than raloxifene alone (24 vs. 48 h). The decrease of HER2 expression was also time-dependent. While the combination therapy decreased HER2 by 64% at 24 h, it was not more potent than other curcumin analogs at downregulating the expression of HER2, as 1 μ M of RL71 (15) and 10 μ M concentrations of FLLL11 and FLLL12 (45) decreased HER2 ~92% at 24 h. However, since RL91 at 4 μ M also decreased HER2 99% in SkBr3 cells (13) a higher concentration of RL91 may elicit a stronger response in combination with raloxifene.

To determine if RL91 could enhance the anti-metastatic and anti-angiogenic properties of raloxifene, we examined the effect of the drug combination in multiple *in vitro* assays. Importantly, combination treatment had a significant inhibitory effect on MDA-MB-231 cell migration and invasion as well as endothelial tube formation demonstrating an anti-metastatic and anti-angiogenic effect of the combination treatment. This is not that surprising because in a xenograft model of ER-negative breast cancer raloxifene not only decreased tumor growth but also decreased the number of CD105-positive cells in the tumors, a marker of endothelial cells and neoangiogenesis (8). Overall our results show that RL91 enhances the cytotoxicity and growth suppressive effects of raloxifene and causes an increase in the number of apoptotic cells. The combination targeted multiple proteins across all the cell lines and appears to reduce the metastatic potential of cancer cell lines and may

be sufficient to reduce the formation of new blood vessels. A combination with RL91 warrants further examination in *in vivo* models of ER-negative breast cancer, as it may be a better strategy to address intratumoral heterogeneity.

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