RL71, a second-generation curcumin analog, induces apoptosis and downregulates Akt in ER-negative breast cancer cells

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Abstract. There is a need for the development of new, safe and efficacious drug therapies for the treatment of estrogen receptor (ER)-negative breast cancers. RL71 is a second-generation curcumin analog that exhibits potent cytotoxicity towards a variety of ER-negative breast cancer cells. Therefore, we have further examined the mechanism of this anticancer activity in three different ER-negative breast cancer cell lines. The mechanistic studies demonstrated that RL71 (1 μM) induced cell cycle arrest in the G2/M phase of the cell cycle. Moreover, RL71 (1 μM) caused 35% of SKBr3 cells to undergo apoptosis after 48 h and this effect was time-dependent. This correlated with an increase in cleaved caspase-3 as shown by western blotting. RL71 (1 μM) also decreased HER2/neu phosphorylation and increased p27 in SKBr3 cells. While in MDA-MB-231 and MDA-MB-468 cells RL71 (1 μM) significantly decreased Akt phosphorylation and transiently increased the stress kinases JNK1/2 and p38 MAPK. In addition, RL71 exhibited anti-angiogenic potential *in vitro* as it inhibited HUVEC cell migration and the ability of these cells to form tube-like networks. RL71 (8.5 mg/kg) was also orally bioavailable as it produced a peak plasma concentration of 0.405 μg/ml, 5 min after oral drug administration. Thus, our findings provide evidence that RL71 has potent anticancer activity and has potential to be further developed as a drug for the treatment of ER-negative breast cancer.

Introduction

Breast cancer is the most prevalent form of cancer diagnosed in women, and there continues to be limited drug treatment options for the ~30% of patients whose cancer lacks the estrogen receptor (ER) (1,2). ER-negative breast cancer can either express the human epidermal growth factor receptor 2 (Her2, erbb2) or lack this receptor. Triple negative breast cancer (TNBC) is defined by a lack of the expression of the ER, progesterone receptor and HER2. They account for 10-17% of all breast cancers and represent 85% of the basal-like subtype (3), one of the five subgroups of breast cancer categorized in recent years (4). TNBC has an aggressive clinical behavior and is generally associated with poor prognosis, thus chemotherapy remains the only systemic treatment option available for these patients (5).

In the search for effective drugs for ER-negative breast cancer, several lead compounds from natural products such as curcumin (diferuloylmethane), the primary bioactive compound isolated from the rhizome of turmeric (*Curcuma longa* Linn.), have emerged. Curcumin has numerous pharmacological, chemopreventative and chemotherapeutic actions, and *in vivo* studies have demonstrated decreased tumorigenesis of many organs, including the mammary gland (6-11). *In vitro* studies have also demonstrated that curcumin exhibits potent cytoxicity toward numerous cell lines including ER-negative human breast cancer cells (12-18). However, curcumin has shown limited clinical efficacy, due to its low bioavailability and low stability in physiological media (7). Therefore, research groups have concentrated on the synthesis and characterization of curcumin analogs. Cyclohexanone analogs of curcumin as a group have shown enhanced activity and stability in biological medium compared to curcumin (19). Specifically, the cyclohexanone derivative 2,6-bis((3-methoxy-4-hydroxyphenyl)methylene)-cyclohexanone (BMHPC) was cytotoxic towards ER-negative breast cancer cells (IC_{50} of 5.0 μM) (20), although bioavailability and *in vivo* efficacy were still problematic. More recently fluorinated cyclohexanone derivatives have not only shown potent cytotoxicity toward MDA-MB-231 cells (IC_{50} value of 0.8 μM) (21,22), but also induced cell cycle arrest and apoptosis in both breast and prostate cancer cells (22).

Our laboratory has been involved in the search for new drug treatments for ER-negative breast cancer and uses the aggressive triple negative MDA-MB-231 human breast cancer cells, amongst others, to identify new potent drugs. Recently we have performed structure activity relationships to determine the cytotoxic potency of 2nd generation heterocyclic cyclohexanone curcumin analogs. This study demonstrated that 3,5-bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidine-4-one (RL71)(Fig. 1) showed the lowest IC_{50} value (0.3 μM) toward MDA-MB-231 and MDA-MB-468 breast cancer cells (23). It also elicited the strongest apoptotic response, as ~40% of MDA-MB-231 cells underwent apoptosis 18 h following

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RL71 treatment (1 μM) (23). Therefore, this study was designed to comprehensively investigate the *in vitro* activity of this lead compound in various ER-negative breast cancer cell lines in order to determine its potential to be developed into a drug for aggressive breast cancer that lacks the ER.

Materials and methods

Materials. HUVEC, MDA-MB-231, MDA-MB-468 and SKBr3 cells were purchased from American Type Culture Collection (Manassas, VA). Primary antibodies to p38, pp38, NF-κB, JNK, pJNK, cleaved caspase-3, 4EBP1, p4EBP1, p27, mTOR, pmTOR, HER2, pHER2 and β-actin were purchased from Cell Signaling Technology (Danvers, MA). Akt and pAkt primary antibodies were purchased from BD Biosciences (Auckland, NZ). Dulbecco's modified Eagle's medium (DMEM) nutrient mixture Ham's F-12, sulforhodamine B salt, propidium iodide (PI), ammonium persulfate, horseradish peroxidase were purchased from Sigma Aldrich (Auckland, NZ). Acrylamide, bisacrylamide, sodium dodecylsulfate and PVDF membrane were purchased from Bio-Rad Laboratories (Hercules, CA). Complete mini EDTA-free protease inhibitor cocktail and Annexin-V-FLUOS were purchased from Roche Diagnostics Corporation (Mannheim, Germany). RL71 was prepared as described previously (18). All other chemicals were of the highest purity commercially available.

Cell maintenance. MDA-MB-231, MDA-MB-468 and SkBr3 cells were maintained in complete growth media composed of DMEM/Ham's F12 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml streptomycin, 250 ng/ml amphotericin B, and 100 units/ml penicillin and 2.2 g/l $NaHCO_3$.

Cytotoxicity. MDA-MB-231, MDA-MB-468 and SKBr3 cells ($95x10^4$ cells/well) were seeded in 12-well plates in 1 ml DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l $NaHCO_3$ and incubated for 24 h at 37˚C. For time course study, cells were treated with RL71 (1 μM) for 6, 12, 24, 36, 48 and 72 h. Vehicle control cells were treated with DMSO (0.1%). Cell number in each well was determined using the sulforhodamine B (SRB) assay (24).

Cell cycle analysis. Flow cytometry was used to analyze DNA content in order to determine cell cycle distribution. MDA-MB-231, MDA-MB-468 and SKBr3 cells were plated and treated with RL71 (1 μM) or 0.1% DMSO as control for 6-48 h in 6-well plates. The cells were harvested, washed with PBS and then fixed in 70% ethanol. Following rehydration with PBS, the cells were stained with PI in the dark at 4˚C as described (25). The samples were analyzed via flow cytometry using a FACScaliber flow cytometer (Becton-Dickinson). The percentage of cells in each phase of cell cycle was determined using Cell Quest Pro software. Results are expressed as percent of cells in each phase of the cell cycle.

Induction of apoptosis. MDA-MB-468, and SKBr3 cells were seeded in 6-well culture plate in 2 ml of DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 μl/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l $NaHCO_3$. The cells were treated with RL71 (1 μM) or vehicle control for 12-48 h. Apoptosis was assessed using Annexin-V-FLUCOS/PI staining, as described (26). The samples were analyzed using a FACScaliber flow cytometer (Becton-Dickinson) and the proportion of apoptotic cells was determined using CellQuest Pro software.

Preparation of cell lysates. MDA-MB-231, MDA-MB-468 and SKBr3 cells were seeded in 10 cm culture dishes at $2.5x10^6$ cells per well in 10 ml of DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l $NaHCO_3$. Cells were treated with RL71 (1 μM) or vehicle control for 0-36 h. At the end of treatment, whole cell lysates were prepared and protein concentration of the lysates was determined using the bicinchoninic acid (BCA) method (25).

Western blot analysis. Cell lysates were resolved by SDS-PAGE (40 μg protein per well) and then the proteins were transferred to a PVDF membrane. Protein levels were analyzed with the desired primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). The digital chemiluminescence images were taken by a Versadoc densitometer (Bio-Rad).

Transwell migration. Transwell migration was performed using 24 well plates containing BioCoat™ Matrigel™ Invasion Chamber inserts (BD Biosciences, Bedford, MA). HUVEC cells (50,000/well) were plated on rehydrated Matrigel coated culture inserts. The bottom chamber contained 500 μl of EGM serum free media. The cells were treated with 0.1% DMSO or RL71 (1 μM) and incubated for 18 h at 37˚C in a humidified 5% CO_2 incubator. After incubation, all contents from well inserts were aspirated and non-migrated cells were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with DiffQuick solution for 1 min and excess stain was washed with water and dried. Cells on the filters were counted using a Zeiss Axioplan camera and compared to the control well insert that contained no Matrigel. Results are expressed as migrated cells as a percent of total cell population.

Endothelial tube formation. The day before performing the tube formation assay, a Matrigel matrix was incubated on ice overnight. On the day of the assay, 125 μl Geltrex Matrigel was transferred into wells of a 24-well plate. The plate was incubated at 37˚C, 5% CO_2 for 30 min. HUVEC cells ($5x10^4$/well) were then loaded into each well, followed by addition of DMSO (0.1%) or RL71 (1 μM). The plate was incubated at 37˚C, 5% CO_2 for 18 h and photographs (200x) were taken by an individual blinded to the treatment groups.

Animals and housing. Female CD-1 mice (6-weeks old) were purchased from the Hercus Taieri Resource Unit (Dunedin, NZ). All procedures were approved by the University of Otago (AEC# 91/07). Mice were housed in pathogen-free conditions with woodchip bedding with access to food (Reliance rodent diet, Dunedin, NZ) and water *ad libitum*. Mice were housed in a 21-24˚C environment on a scheduled 12 h light/dark cycle and acclimatized for 3 days prior to experimentation.

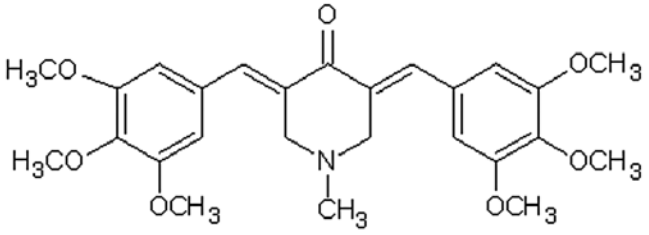

Figure 1. Chemical structure of RL71.

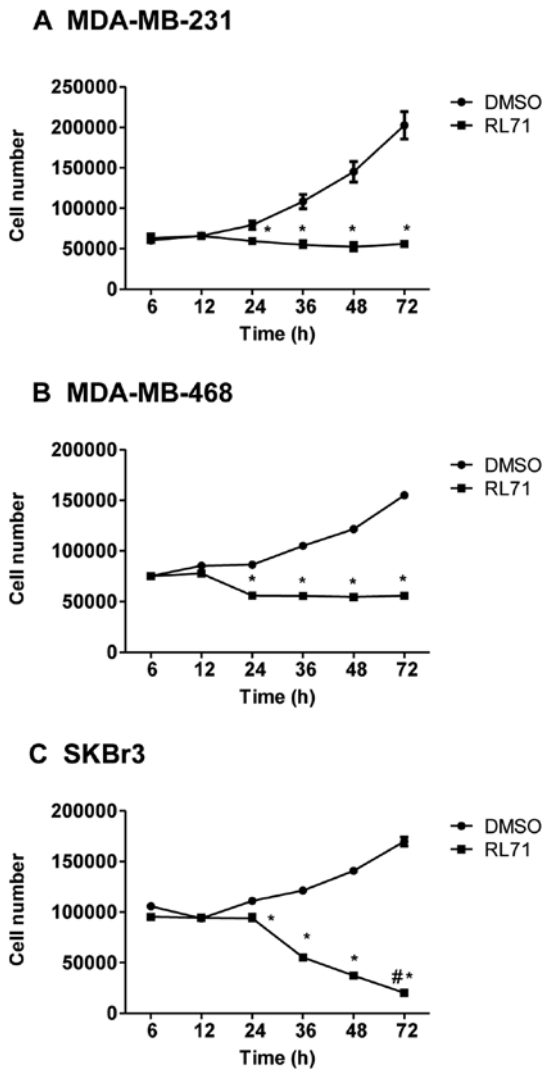

Figure 2. Time-course cytotoxicity of RL71 in (A) MDA-MB-231, (B) MDA-MB-468, and (C) SKBr3 cells. Cells were treated with either RL71 (1 μM) or DMSO (0.1%) for 6-72 h. Cell number was determined using the SRB assay. Each symbol represents the mean ± SEM of 3 independent experiments performed in triplicate. The data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test where asterisk indicates a statistically significant difference compared with the control group, $p<0.001$. #Statistically significant difference compared with all previous time points, $p<0.01$.

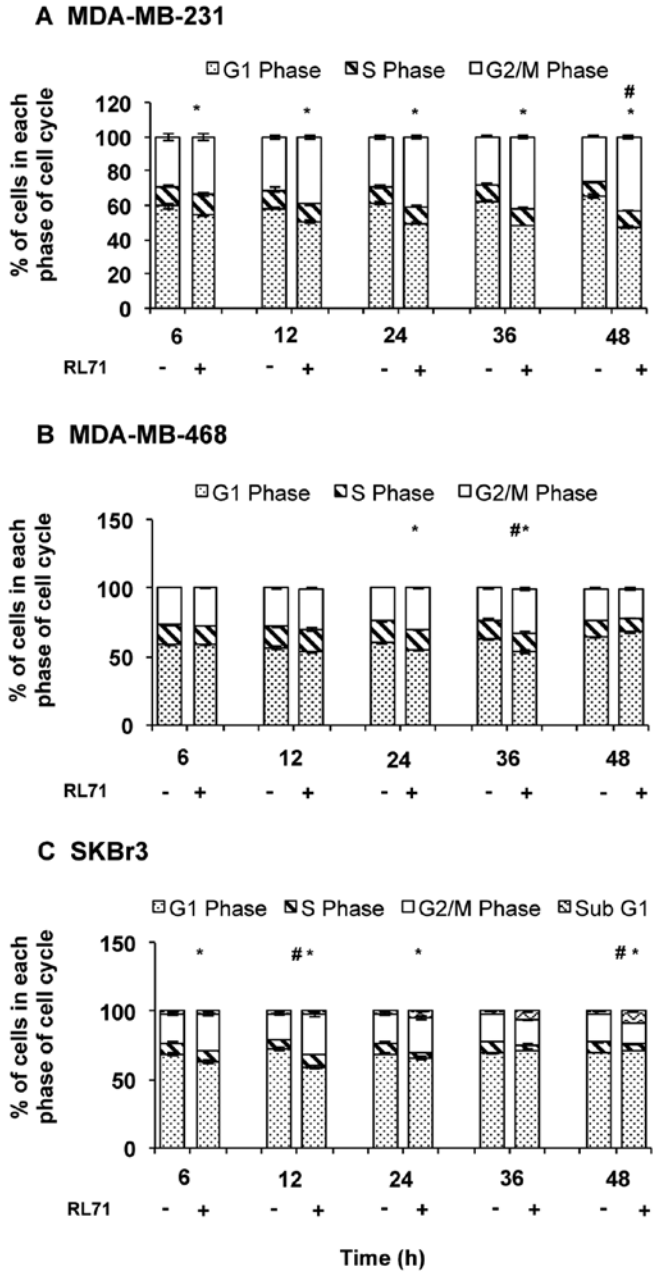

Figure 3. Cell cycle arrest following treatment of ER negative breast cancer cells with RL71 in (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells. Cells were treated with RL71 (1 μM) for 6, 12, 24, 36 and 48 h. Vehicle control cells were treated with 0.1% DMSO. Propidium iodide staining and flow cytometry was used to determine the proportion of cells in the various phases of the cell cycle. Columns represent the mean proportion of cells in each phase of cell cycle (% of total) ± SEM of 3 independent experiments conducted in triplicate. Data were analyzed with a two-way ANOVA coupled with a Bonferroni post-hoc test. Asterisk indicates a statistically significant difference between treatment and control in the number of cells in the G2/M phase of the cell cycle, $p<0.001$. #Statistically significant difference in the number of cells in the G2/M phase of the cell cycle compared with all other time points, $p<0.01$.

Oral bioavailability of RL71. Mice (3/group) were orally gavaged with RL71 and blood samples were collected at the following time points 0, 5 min, 10 min, 15 min, 30 min, 1 h, 1.5 h and 2 h). Plasma was separated and stored at -20˚C. The samples for analysis were prepared by addition of methanol to precipitate the proteins, followed by sonication and filtration. The samples were analyzed by HPLC with UV-DAD detection. HPLC analysis was performed using an Agilent HP1100 system at 25˚C on a C18 column (Phenomenex Gemini-NX) 3 μ (110A, 150x2 mm) with a 2x4 mm C18 guard column. RL71 was monitored 390 nm. The mobile phase was acetonitrile

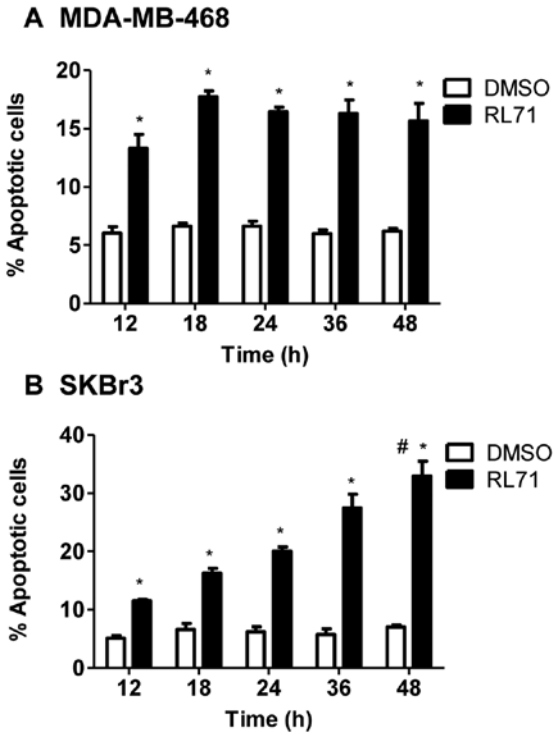

Figure 4. Induction of apoptosis following treatment of (A) MDA-MB-468 and (B) SKBr3 cells with RL71. Cells were treated with RL71 (1 μM) for 12, 18, 24, 36 and 48 h. Vehicle control cells were treated with 0.1% DMSO. Values are expressed as mean number of apoptotic cells as a percentage of the total population ± SEM from 3 independent experiments conducted in triplicate. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. Asterisk indicates a statistically significant difference compared with the control group, $p<0.001$. #Statistically significant difference compared with all previous time points, $p<0.01$.

in water with 0.1% formic acid: t_0=30%, t_{10}=70%, t_{15}=100%, t_{17}=30%, t_{20}=30%. The flow rate was 0.3 ml/min, with an injection volume of 5 μl.

Statistical analysis. When time was a factor data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. Data that did not have time as a facor were analysed using a one-way ANOVA coupled with a Bonferroni post-hoc test. Transwell migration data were analyzed by a Student's t-test. $p<0.05$ was the minimal requirement for a statistically significant difference.

Results

Previously we have shown that treatment for 5 days with RL71 elicited sub micromolar IC_{50} values in three different ER-negative breast cancer cell lines (20). Therefore, the first aim of this study was to examine the cytotoxicity of RL71 toward MDA-MB-231, MDA-MB-468 and SKBr3 cells over a time-course. The results showed that RL71 (1 μM) elicited time-dependent and cell line-dependent cytotoxicity. Specifically, time-dependent cytotoxicity was elicited in SKBr3 cells with significantly increased cytotoxicity at 72 h compared with all other time points (Fig. 2). However, in the two triple negative breast cancer (TNBC) cell lines no further cytotoxicity was elicited after 24 h. Thus, RL71 showed potent cytotoxicity toward SKBr3 cells compared to a cytostatic effect in TNBC cells.

We next examined whether the cytotoxicity of RL71 was due to G2/M phase cell cycle arrest. Treatment of MDA-MB-231, MDA-MB-468, and SKBr3 cells with RL71 (1 μM) produced G2/M phase arrest in all three cell lines. Specifically, at 48 h, RL71 caused an 162% increase in the proportion of MDA-MB-231 cells in G2/M phase over control (Fig. 3A), while in MDA-MB-468 cells, the proportion of cells in G2/M phase increased by 140% over control at 36 h (Fig. 3B). In SKBr3 cells, after 12 h, the proportion of cells undergoing G2/M phase was increased by 153% over control (Fig. 3C). Moreover, there was a significant reduction in the proportion of cells in S phase at 24, 36 and 48 h. SKBr3 cells were the only cell type to show an increase in subG1 cells. The effect in MDA-MB-231 cells was time-dependent as the number of cells undergoing G2/M

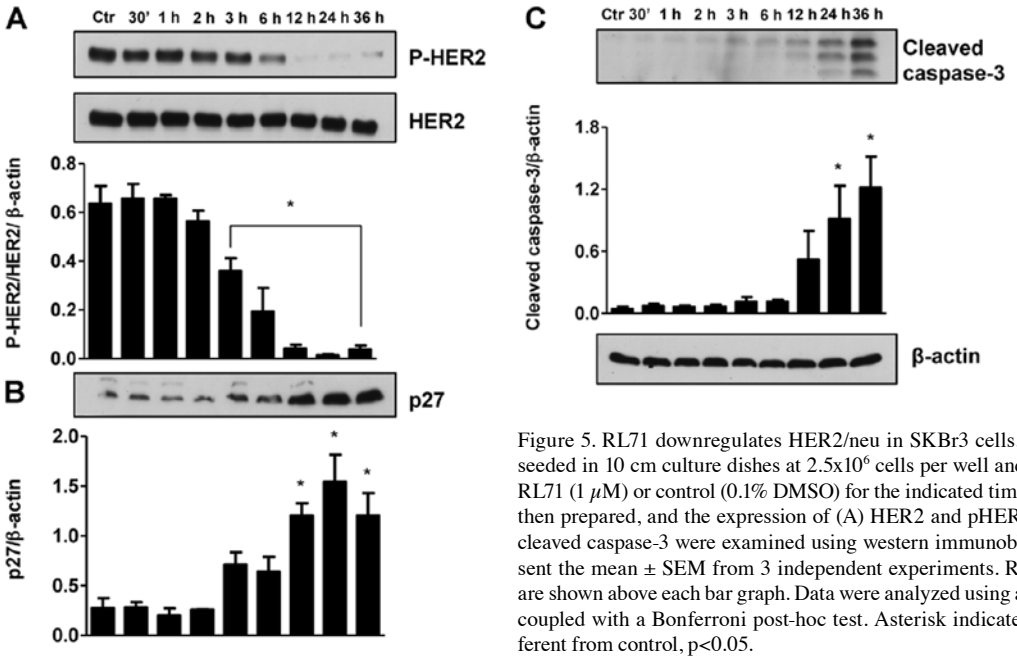

Figure 5. RL71 downregulates HER2/neu in SKBr3 cells. SKBr3 cells were seeded in 10 cm culture dishes at $2.5x10^6$ cells per well and were treated with RL71 (1 μM) or control (0.1% DMSO) for the indicated time. Cell lysates were then prepared, and the expression of (A) HER2 and pHER2, (B) p27 and (C) cleaved caspase-3 were examined using western immunoblotting. Bars represent the mean ± SEM from 3 independent experiments. Representative blots are shown above each bar graph. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. Asterisk indicates significantly different from control, $p<0.05$.

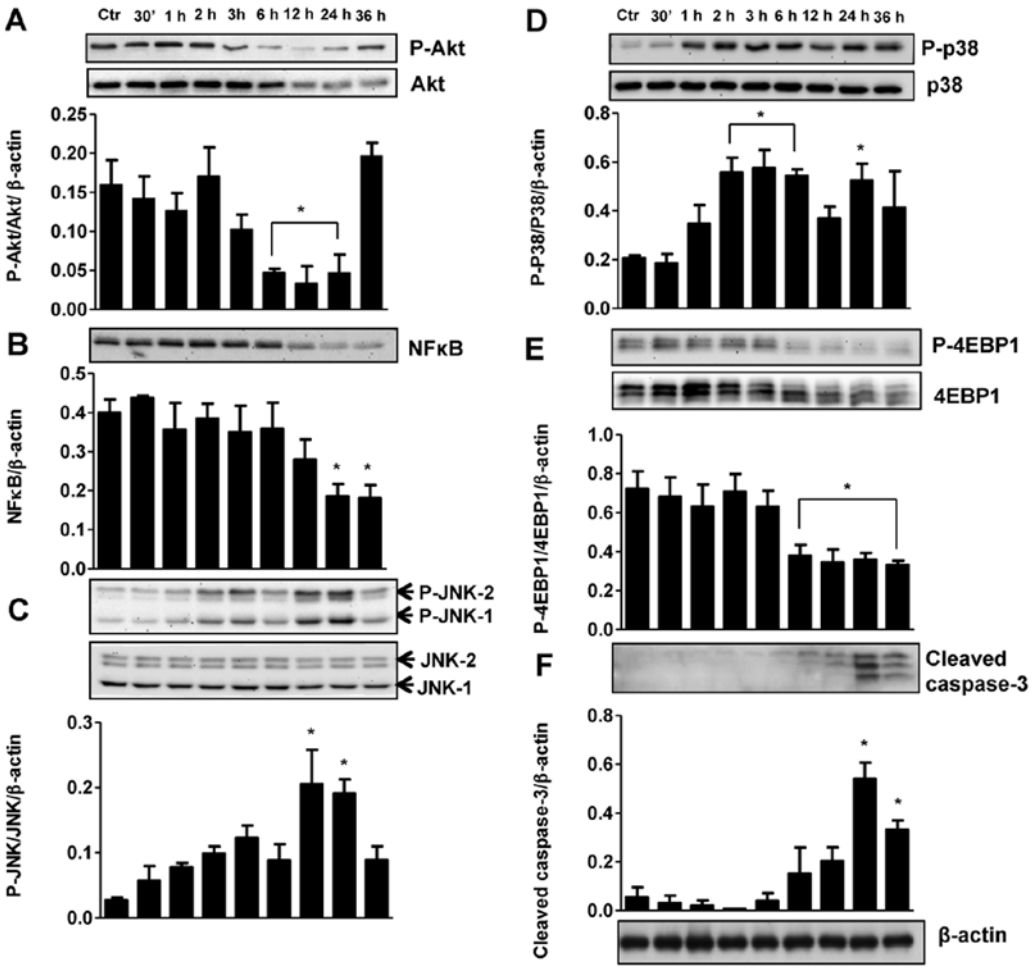

Figure 6. RL71 modulates cell signaling proteins in MDA-MB-231 cells. MDA-MB-231 cells were seeded in 10 cm culture dishes at 2.5x10^6 cells per well and were treated with RL71 (1 μM) or control (0.1% DMSO) for the indicated time. Cell lysates were then prepared, and the expression of (A) Akt and pAkt, (B) NF-κB, (C) JNK1/2 and pJNK1/2, (D) p38 and pp38, (E) 4EBP1 and p4EBP1 and (F) cleaved caspase-3 were examined using western immunoblotting. Bars represent the mean ± SEM from 3 independent experiments. Representative blots are shown above each bar graph. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. Asterisk indicates significantly different from control, $p<0.05$.

phase arrest was signficantly increased at 48 h compared to all other time points.

To determine if cell cycle arrest drives apoptosis, time-dependent changes in apoptosis were examined. RL71 (1 μM) induced apoptosis in MDA-MB-468 and SKBr3 cells (Fig. 4). The effect was time-dependent in SkBr3 cells, as 35% of cells were apoptotic after 48 h and this was significantly elevated compared to all other time points (Fig. 4B). In contrast 14-18% of MDA-MB-468 cells underwent apoptosis and this effect was maintained from 12-48 h (Fig. 4A) indicating the lack of a time-dependent effect. G2/M arrest did not drive apoptosis in MDA-MB-468 cells, as apoptosis was increased at 12 h, which was prior to the increase in G2/M phase arrest. However, the early appearance of G2/M phase arrest at 12 h in SKBr3 cells is a likely reason why these cells show a strong apoptotic response over time. Additionally, our previous work with RL71 in MDA-MB-231 cells indicated that the induction of apoptosis was strongest in this cell line, as 43% of cells underwent apoptosis and this effect was maintained from 18-36 h (23). Thus, the time-dependent increase in G2/M phase arrest leads to the sustained apoptotic effect. It is clear that overall RL71 displayed a more potent cytotoxic effect in SKBr3 cells. To determine if this was due to the inhibition of HER2/neu expression, changes in cell signaling proteins were determined via western blotting.

HER2/neu expression was assessed in SKBr3 cells to determine the role of this protein in the cytotoxic actions of RL71. Treatment of SKBr3 cells with RL71 (1 μM) decreased the ratio of pHER2/HER2 in a time-dependent manner, with an almost complete inhibition following 12 h (Fig. 5A). To link the changes in HER2/neu with cell cycle progression protein changes in the cyclin dependent kinase inhibitor, p27 were determined. The results showed that that decrease in HER2/neu correlated with a significant increase in the expression of p27 (Fig. 5B). Thus the decrease in HER2/neu leads to an increase in p27 leading to the observed G2/M arrest and apoptosis. The presence of apoptosis was also confirmed in SKBr3 cells by the signficant increase in cleaved caspase-3 (Fig. 5C).

To determine the molecular mechanisms responsible for apoptosis and cell cycle arrest in TNBC cells we first examined other isoforms of the EGFR in these cells. RL71 (1 μM) failed to alter the ratio of pEGFR/EGFR protein levels (data not

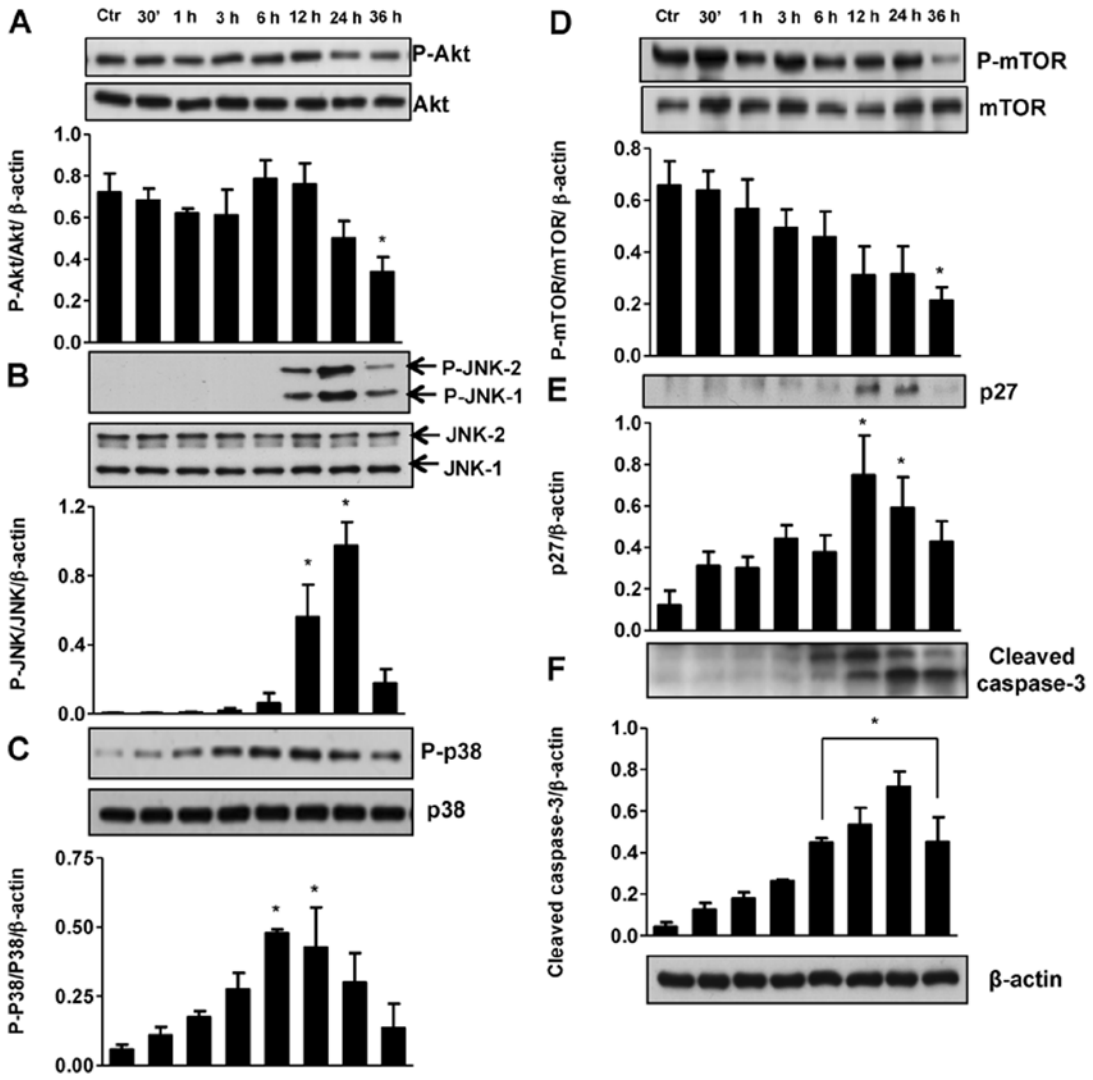

Figure 7. RL71 modulates cell signaling proteins in MDA-MB-468 cells. MDA-MB-468 cells were seeded in 10 cm culture dishes at 2.5x10^6 cells per well and were treated with RL71 (1 μM) or control (0.1% DMSO) for the indicated time. Cell lysates were then prepared, and the expression of (A) Akt and pAkt, (B) JNK1/2 and pJNK1/2, (C) p38 and pp38, (D) mTOR and pmTOR, (E) p27 and (F) cleaved caspase-3 were examined using western immunoblotting. Bars represent the mean ± SEM from 3 independent experiments. Representative blots are shown above each bar graph. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. Asterisk indicates significantly different from control, $p<0.05$.

shown). However, RL71 did modulate the expression of Akt, JNK1/2, p38 and caspase-3 in MDA-MB-231 and MDA-MB-468 cells and differentially expressed NF-κB, mTOR, 4EBP1 and p27. Specifically, RL71 significantly decreased the ratio of pAkt/Akt from 6-24 h in MDA-MB-231 cells and at 36 h in MDA-MB-468 cells (Fig. 6A and 7A). The stress initiated by the treatment of MDA-MB-231 and MDA-MB-468 cells resulted in a transient increase in both JNK1/2 and p38 MAPK phosphorylation (Fig. 6C and D and Fig. 7B and C). Furthermore, RL71 increased levels of cleaved caspase-3 in a time-dependent manner and this effect was strongest in MDA-MD-468 cells with a significant increase occuring as early as 6 h (Fig. 6F and 7F). MDA-MB-231 cells were the only cell line to show a signficant decrease in NF-κB and the ratio of p4EBP1/4EBP1 following RL71 (Fig. 6B and E), while the ratio of pmTOR/mTOR was signficantly decreased and p27 was signficantly increased by RL71 in MDA-MB-468 cells (Fig. 7D and E).

To determine if RL71 could modulate angiogenesis, *in vitro* assays using HUVEC cells were performed, as the ability of these cells to migrate through Matrigel and form tube-like networks are hallmarks of angiogenesis. We used both quantifiable and visual assys to form a more complete *in vitro* picture. The results showed that RL71 (1 μM) significantly reduced HUVEC cell migration by 46% compared to vehicle control (Fig. 8A) and completely inhibited endothelial tube formation after 18 h (Fig. 8B).

To confirm that RL71 was a new analog that showed improved oral bioavailability, female CD-1 mice were orally gavaged with a single dose of RL71 (8.5 mg/kg) and the resulting plasma concentration was determine by HPLC analysis. The results showed a peak plasma concentration of 0.405 μg/ml, 5 min after oral drug administration. The plasma concentration of RL71 decreased in a time-dependent manner, as 0.15 μg/ml was present after 1 h and by 2 h the plasma concentration was at the limit of detection.

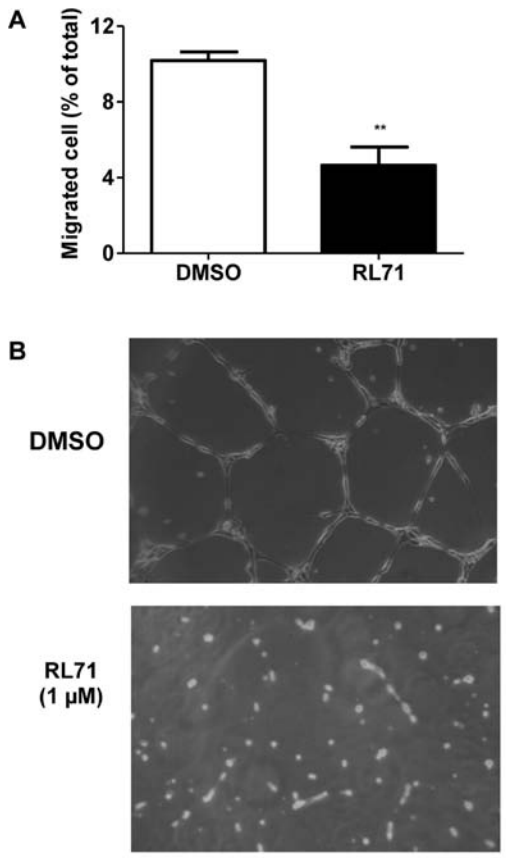

Figure 8. RL71 decreases HUVEC cell migration and tube formation. (A) HUVEC cells (25,000/well) were seeded and treated with either DMSO (0.1%) or RL71 (1 μM) for 18 h. Migrated cells were stained and counted. Bars represent mean ± SEM of 3 independent experiments performed in triplicate. *Significantly different compared to control, $p<0.01$, as determined from a Student's t-test. (B) HUVEC cells (50,000/well) were seeded and treated with DMSO (0.1%) or RL71 (1 μM) for 18 h. Photographs were then taken by an examiner blinded to the treatment groups. Representative photograps from an experiment performed in quadruplicate are shown.

Discussion

We have previously shown that RL71 elicited the most potent cytotoxicity towards ER-negative breast cancer cells compared to all of our previously examined second generation curcumin derivatives (23). Therefore, this study was designed to further characterize this cytotoxic effect *in vitro* in a range of ER-negative breast cancer cell lines. The data presented show that RL71 promoted G2/M cell cycle arrest, induced apoptosis and modulated the Akt-dependent signaling pathway and stress response MAPK pathway. RL71 also downregulated the expression of HER2/neu in SKBr3 cells. In addition, RL71 exhibited anti-angiogenic effects *in vitro* by inhibiting the invasion of HUVEC cells and their ability to form endothelial tube like network. Importantly, RL71 is also orally bioavailable.

Breast cancer patients whose tumors overexpress HER2/neu have a poor prognosis, shorter relapse time and shorter survival time (27). In this study we showed that RL71 inhibited HER2/neu expression in SKBr3 cells and this correlated with a concominant increase in p27 and cleaved caspase-3. Since p27 is a key regulator of G2/M phase arrest and apoptosis (28,29), inhibition of HER2/neu is a key initial mechanism for the apoptotic effect elicited by RL71 in SKBr3 cells. RL71 was more potent than other curcumin analogs at downregulating the expression of HER2/neu, as 4 μM concentrations of RL90 and RL91 and 10 μM concentrations of and FLLL11 and FLLL12, were required to elicit a similar effect (25). This confirms that RL71 is a lead drug candidate for ER-negative/HER2-positive breast cancer.

MAPK signaling which includes activation of JNK and p38 has been shown to be involved in the regulation of the cell cycle and induction of apoptosis in breast cancer cells (30). Various cytotoxic agents induce apoptotic cell death via activation of MAPK signaling and induction of caspase-3 (31-33). Our studies showed that RL71 treatment induced JNK1/2 and p38 MAPK in MDA-MB-231 and MDA-MB-468 cells. Anticancer agents such as curcumin, which causes activation of p38, JNK1/2 and caspase-3, also induce similar apoptotic events (34,35). The MAPK pathway may also upregulate cell cycle regulatory protein, p27 in breast cancer cells (36). Our results demonstrated that in MDA-MB-468 RL71 enhanced the expression of p27 which would contribute to the observed G2/M cell cycle arrest.

We further studied the effect of RL71 on the PI3K/Akt/mTOR pathway. Akt is an important oncoprotein which is constitutively active in breast cancer cells and has been implicated in a myriad of regulatory mechanisms involving protein synthesis, cell cycle progression and inhibition of apoptosis (37,38). Our results showed that RL71 decreased the phosphorylation of Akt on Ser-473 in a cell line and time-dependent manner. Specifically, in MDA-MB-468 cells, RL71 decreased Akt phosphorylation fully whereas in MDA-MB-231 cells the phosphorylation of Akt was partially decreased. However, RL71 was more potent than the analogs RL90 and RL91, which did not decrease the ratio of pAkt/Akt at concentrations of 4 μM (25). The decreased activity of Akt led to decreased activation of its substrate mTOR in MDA-MB-468 cells. In contrast, in MDA-MB-231 cells there was no change in the expression of mTOR but the expression of 4-EBP1 which is downstream of mTOR was fully downregulated. RL71 failed to alter the expression of PI3K (data not shown) and this suggests that RL71 directly targets downstream events of PI3K/Akt signaling in MDA-MB-231 cells and increased phosphorylation of Akt could be due to a feedback loop mechanism.

Akt contributes to the activity of NF-κB by controling its translocation to the nucleus (39) and a decrease in Akt activity may affect the stability and level of NF-κB (40). NF-κB belongs to a family of transcription factors which has been associated with inhibition of apoptosis by promoting the expression of anti-apoptotic proteins such as Bcl-xL, c-Myb and caspase inhibitors (41,42). RL71 downregulated the expression of NF-κB in MDA-MB-231 cells. However, higher concentrations were required to downregulate NF-κB in MDA-MB-468 cells (data not shown). Curcumin has also been shown to interfere with the functions of Akt and MAPKs and further inhibit its downstream target NF-κB (43,44) and thus RL71 retains many of the same actions as curcumin.

To determine if RL71 exhibited anti-angiogenic properties, we examined the effect of RL71 in multiple *in vitro* angiogenesis assays. Angiogenesis is essential for tumor growth and metastasis and inhibition of metastasis is critical as it is cancer spread that leads to mortality. Importantly, RL71 had an inhibitory effect on endothelial cell migration and tube formation

demonstrating an anti-angiogenic effect from this compound. The mechanism of this effect will be studied further using *in vivo* live imaging.

RL71 showed potent cytotoxicity in ER-negative breast cancer cells compared to other cyclohexanone curcumin analogs (21,23). Moreover, it had superior cytotoxicity compared with other curcumin analogs such as 3,5-bis(flurobenzylidene) piperidin-4-one (EF24) (45) 5-bis (4-hydroxy-3-methoxy-benzylidnen)-N-methyl-4-piperidone (PAC) (46) and GO-Y030 (47) in MDA-MB-231 cells. Specifically IC_{50} values of 1.2, 1 and 0.3 μM were reported for EF24, GO-Y030 and RL71, respectively (23,45,47). While EF24 induced G2/M phase arrest and apoptosis in MDA-MB-231 cells (22) and inhibited the NF-κB pathway in a TNFα-dependent manner (45), it has not been examined in other breast cancer cells. Additionally, RL71 has a stronger ability to induce apoptosis compared to the analog 4-hydroxy-3-methoxybenzoic acid methyl ester (HM-BME), where 25 μM was required to cause 37% of LNCaP prostate cancer cells to undergo apoptosis after 24 h (48). The curcumin analogs FLLL11 and FLLL12 were equally potent as RL71 in MDA-MB-468 cells with similar IC_{50} values (0.3 μM). However, this did not translate to other breast cancer cell types as these analogs had IC_{50s} of 2-5 μM in MDA-MB-231 and SkBr3 cells (42). These analogs also down-regulated Akt phosphorylation and HER2/neu expression in SKBr3 breast cancer cells but at concentrations of 10 μM, 10-fold greater than RL71 (49). While other curcumin analogs appear to have a similar mechanism of action as RL71, RL71 is more potent as all of its anti-cancer actions were elicited at concentrations of 1 μM or less. Importantly RL71 is orally available following a single oral dose of 8.5 mg/kg and this is a significant improvement on both curcumin (50) and other first generation analogs (19).

In summary, we showed that RL71 causes cell cycle arrest and induces apoptosis in ER-negative breast cancer cells and also modulates a variety of signaling pathways that culminate in potent cytotoxicity. Specifically, inhibition of Akt pathway and the activation of p38/JNK pathway may contribute to the anti-cancer activity of RL71 in TNBC cells, while inhibition of HER2/neu and induction of p27 are key mechanisms in SKBr3 cells. Thus, RL71 shows potential as a new drug therapy for ER-negative/Her-2-positive breast cancer. Therefore, RL71 is a novel 2nd generation curcumin derivative that warrants further investigation as a potential therapy for ER-negative breast cancer.

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