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Cholesterol biosynthesis inhibitor RO 48-8071 suppresses growth of TNBC *in vivo* and *in vitro*

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Abstract

Treating TNBC patients is difficult because tumors lack traditional ER, PR and Her-2-neu and targeted therapy approaches are therefore ineffective. The main purpose of this report is to identify alternatives that could be targeted using agents to control TNBC progression without being toxic to the patient. With this in mind, we considered inhibition of the cholesterol biosynthesis pathway as a possible target and conducted studies, both *in vitro* and *in vivo* to examine the effectiveness of such a strategy. RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]; RO), a small-molecule inhibitor of oxidosqualene cyclase (OSC, a key enzyme in cholesterol biosynthesis), potently reduced the viability of MDA-MB-231 and BT-20 TNBC cells, as well as a cell line that expresses Her-2-neu but not ER or PR. Exposure of TNBC cells *in vitro* to pharmacological levels of RO (24 to 48 h), or a dose close to the IC_{50} for OSC (nM) for a week, reduced cell viability significantly. Importantly normal mammary cells were unaffected by RO. FACS analysis showed that RO induced apoptosis of TNBC cells. BT-20 tumor xenografts were generated in nude mice to determine whether RO is effective in reducing TNBC cells *in vivo*. Administration of RO to mice prevented tumor growth, with no apparent toxicity. Examination of cross-sections of RO-treated tumor tissues showed that RO induced TUNEL expression (apoptosis) and suppressed the angiogenic markers VEGF and CD-31. In conclusion, RO is a potent inhibitor of TNBC cell proliferation. The anti-tumor properties of RO appear to be due to induction of apoptosis and suppression of angiogenesis.

Keywords: TNBC; Breast cancer; Tumor progression; Cholesterol biosynthesis inhibitors

Abbreviations: TNBC: Triple-Negative Breast Cancer; ER: Estrogen Receptor; PR: Progesterone Receptor; Her-2-neu, EGFR-like protein; OSC: Oxidosqualene Cyclase; RO: RO 48-8071 ([4'-[6-(Allylmethylamino) hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]); FBS: Fetal Bovine Serum; SRB: Sulforhodamine B; PI: Propidium iodide; SC: subcutaneous; iv: intravenous; PBS: Phosphate-Buffered Saline; ANOVA: Analysis of Variance; SE: Standard Error; FACS: Fluorescence-activated cell sorting

Introduction

Triple-negative breast cancers (TNBC) constitute approximately 15–20% of all detected human breast cancers. TNBC fail to express estrogen receptor; progesterone receptor; and Her-2-neu [1-4], the three molecules commonly targeted chemotherapeutically in hormone receptor-positive tumors. Although aggressive non-targeted chemotherapeutic approaches are typically

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used against TNBC, such protocols are generally ineffective in the long run, making this cancer virtually untreatable. In addition, TNBC tumors frequently become resistant to chemotherapeutic drugs and metastasize, leading to poor patient prognosis and death [5,6]. It is therefore important that we develop better treatment strategies to reduce TNBC-related breast cancer mortality, since for patients with this aggressive type of cancer, the prognosis is not favorable following recurrence.

Enzymes in the cholesterol biosynthetic pathway are attractive therapeutic targets for TNBC because cholesterol is a vital component of cell membranes, which are prone to therapeutic targeting since tumor cells rapidly expand. Statins, which are commonly used to lower cholesterol, inhibit HMG-CoA reductase, an enzyme in the cholesterol biosynthetic pathway; however, certain undesirable side effects limit their long-term use for cancer therapy [7]. 2, 3-oxidosqualene cyclase (OSC) is an enzyme that acts downstream of HMG-CoA reductase to convert 2, 3-monoepoxysqualene to lanosterol (a key step in the biosynthesis of cholesterol) [8-10]. While testing small-molecule inhibitors of OSC, we identified RO 48-8071 ([4'-[6-(Allylmethylamino) hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate] (RO) as a potent suppressor of breast tumor cell viability [11-13]. We previously found that RO effectively blocks the growth of hormone-dependent breast cancer, as well as prostate and ovarian cancer [14-16]. In the present study, we describe the anti-tumor effects of RO on TNBC, both *in vitro* and *in vivo*. We observed that RO induced apoptosis of TNBC *in vitro* and *in vivo* as well as inhibiting markers associated with angiogenesis. Thus, RO exhibits unique anti-tumor properties, making it an exciting candidate compound for clinical management of TNBC.

Materials and Methods

Cell lines and culture

Two TNBC cell lines (MDA-MB-231 and BT-20), one Her2-neu positive cell line (Sk-Br-3), and normal mammary cells (184A1) were obtained from the American Type Culture Collection (Manassas, VA, USA), Tumor cell lines were grown in phenol red-free DMEM:F12 medium (Invitrogen Corporation & Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). 184A1 cells were grown in serum free medium supplemented 5 mg/ml transferrin and 1 ng/ml of cholera toxin.

Reagents

RO 48-8071 (RO) was provided by Roche Pharmaceuticals (Basel, Switzerland) for *in vivo* studies, and was synthesized as previously described (8-10). RO for *in vitro* studies was purchased from Sigma-Aldrich (St Louis, MO).

Cell viability assay

The sulforhodamine B (SRB) assay was used to measure cell viability, as previously described previously [15-17].

Cell apoptosis and death assay

Cells were analyzed for apoptosis and cell death using the Annexin V-FITC Apoptosis Detection Kit (Biovision Research Products, Mountain View, CA, USA) as previously described [18].

In vivo breast tumor growth inhibition assays

All animal experiments were approved by the Institutional Review Committee. Female athymic nude mice (nu/nu, Foxn1), 5- to 6-weeks-old and weighing 20-22 g, were purchased from Envigo, Inc. (Indianapolis, IN, USA). Mice were inoculated with BT-20 TNBC cells as previously described by us [19] and tumor volumes were measured over time [19]. Drug treatment was started when tumor volumes reached approximately 80 mm^3. Mice treated with RO received 5 or 10 mg/kg RO by intravenous (iv) injection of 0.1 ml solution into the tail vein daily for five days, followed by an injection every other day for five additional treatments and then a final injection 2 h prior to sacrifice. Control mice received the same volume of phosphate-buffered saline (PBS) on the same schedule. Animals were weighed twice weekly throughout the study.

Tumors were collected following the last injection and processed for immunohistochemical analysis of TUNEL, CD-31 and VEGF as described previously [12,19,20]. Quantitation of VEGF immunolabeled signal was achieved using a morphometric analysis program (FoveaPro 3.0, Reindeer graphics), on images photographed at 20× magnification as described earlier [19,20]. Results are expressed as area in square pixels. TUNEL and CD-31 signals were counted per section for positive cells/section field. 4-5 tumors/group were analyzed (tumors from different animals), and 4-6 representative sections were collected from each tumor. 30 sections were assessed for VEGF and 25 and 18 sections for CD-31 and TUNEL respectively.

Statistical analysis

Differences between groups or among groups were tested, respectively, using one-way analysis of variance (ANOVA) with repeated measures over time. The assumption of the ANOVA was examined, and a nonparametric measure based on ranks was used if needed. Values are reported as mean ± SEM. When ANOVA indicated a significant effect (F-ratio, $P < 0.05$), the Student-Newman-Keuls multi-range test was used to compare the means of the individual groups. Statistical analyses were conducted using SigmaStat software, version 3.5. For immunohistochemical analysis, data were analyzed using Kruskal-Wallis ANOVA, followed by Tukey's procedure as a posthoc test. For all comparisons, $P<0.05$ was

Liang Y, et al., J Cancer Sci Clin Ther 2024
DOI:10.26502/jcsct.5079232

regarded as statistically significant. Values are reported as mean ± SEM. When only two groups were involved student t-test was used.

Results

RO reduces cell viability of TNBC but not normal mammary cells

In a previous study we found the OSC inhibitor RO 48-8071 to be an effective anti-cancer agent against hormone-dependent breast cancer cell lines [13,14]. In this study we extended our investigation to ascertain the effect of RO on MDA-MB-231 and BT-20 TNBC cell lines, as well as a cell line (SK-Br-3) that expresses high levels of Her-2-neu but does not express ER or PR. SK-Br-3 cells represent breast cancer types that form aggressive tumors *in vivo* [21-23]. We examined the ability of OSC inhibitors to reduce cell viability in all three cell lines (Figure 1A). Following exposure for either 24 or 48 h, RO was effective against all three cell lines. Table 1 lists the IC_{50} values for inhibitory effects against the three cell lines, which ranged from approximately 10-18 µM in a 24–48 h SRB assay (Table 1). To determine whether RO specifically reduces cancer cell viability, leaving normal cells unaffected, we conducted studies using normal 184A1 mammary cells. Concentrations of RO up to 10 µM reduced cancer cell viability but had no effect on normal cells (Figure 1B).

Because the affinity of RO for OSC is in the nM range [8-10], we examined whether a range of low doses of RO would affect cell viability over an extended period (7-day assay) in a manner similar to the effects observed with higher doses over a short 24-h period. We found that RO concentrations as low as 1-10 nM effectively reduced MDA-MB-231 and BT-20 cell viability in 7-day assays (Figure 1C).

RO induces apoptosis and cell death in breast cancer cells

To determine the mechanism by which RO reduced breast cancer cell viability, we treated MDA-MB-231 and BT-20 cells for 24 h with 5, 10, or 20 µM RO. Cells were then collected, and levels of apoptosis and cell death determined by FACS analysis. RO significantly induced apoptosis and cell death in both cell lines in a dose-dependent manner (Figure 2A and B).

Table 1: IC_{50}: RO 48-8071 treatment of Breast Cancer Cell lines.

Cell lines	IC_{50} (µM) (24 hours)	IC_{50} (µM) (48 hours)
MDA-MB-231	14.98±0.48	10.73±0.28
BT-20	15.80±0.34	12.67±0.18
SK-Br-3	17.46±0.88	12.09±0.35

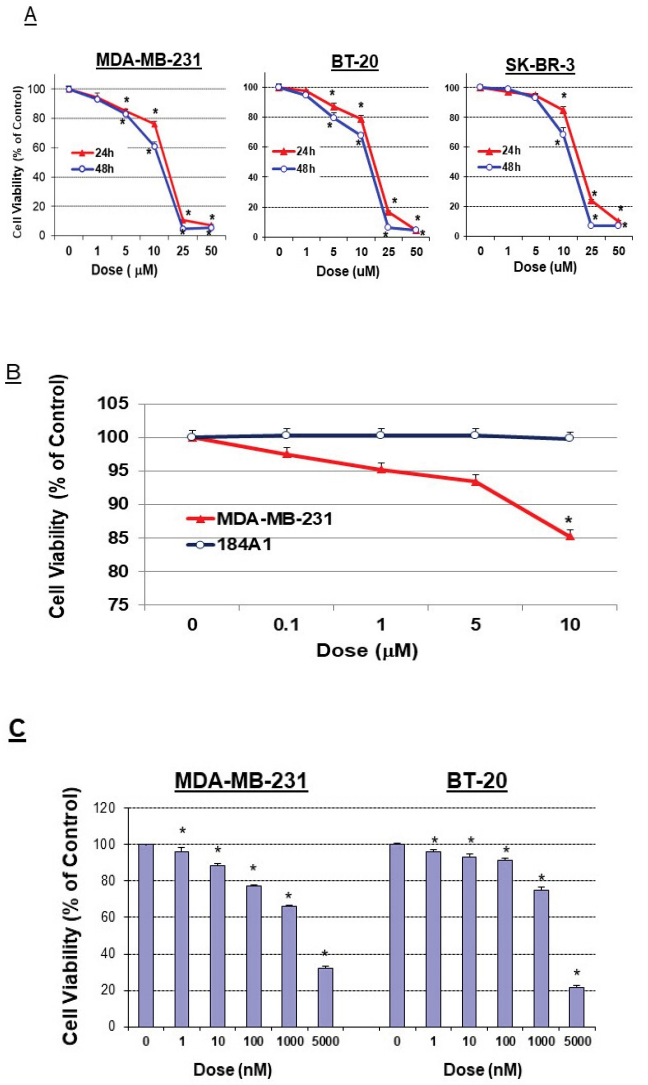

Figure 1: RO reduces the viability of TNBC cells, as well as a cell line representing elevated levels of Her-2-neu, but not normal mammary cells (A) Breast cancer cells (4-, 6-, and 8 X 10^3/well for MDA-MB-231, BT-20 and Sk-Br-3 respectively) were seeded in 96 well plates in DMEM/F12 media +10%FBS. After 24 h media was changed to DME/F12 + 5% FBS and cells were incubated with pharmacological doses of RO for 24 or 48h. (B) Normal mammary cells (184A1) and MDA-MB-231 cells were treated with pharmacological doses of RO for 24 h. (C) Breast cancer cells were incubated with low-dose (1-5000 nM) RO for 7 days. Cell viability was determined by SRB assay. Values represent mean ± SEM (n=6). * Significantly different from control (set at 100%) ($P < 0.05$ using ANOVA or t-test for Figure 1B).

RO inhibits growth of TNBC cells *in vivo*

Having demonstrated the effectiveness of RO in suppressing TNBC cell growth and promoting apoptosis *in vitro*, we conducted studies to establish whether it had the same effect *in vivo*. We established BT-20 tumor xenografts in nude mice and began treatment with RO when tumor volumes were approximately 80 mm^3. Compared with controls, the

Liang Y, et al., J Cancer Sci Clin Ther 2024
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tumor burden of animals administered RO was significantly reduced (Figure 3A). Furthermore, animal weights were unaffected by RO treatment, indicating that the compound was nontoxic at the dose administered (Figure 3B).

RO induces TUNEL expression *in vivo*

Since RO induced apoptosis *in vitro* in TNBC cells, we used IHC to determine if the same occurred *in vivo* (Figure 3C). Cross sections of tumor tissue were labeled for TUNEL signaling. Significant induction of apoptosis was observed with both doses of RO compared with controls (2- and 4-fold higher), correlating with the reduction of tumor volume shown in Figure 3A.

RO suppresses angiogenesis in regressing tumors

Since reduction of tumor growth is associated with anti-angiogenic effects of drugs, we assessed the levels of VEGF and CD-31 (blood vessels) in tumor sections. IHC showed that both VEGF and CD-31 were reduced in tumor sections obtained from RO treated animals (Figure 4). VEGF was reduced by 80% in response to both doses of RO, while levels of CD-31 (blood vessels) were reduced by 50 and 70% respectively with 5 and 10 mg/kg RO. A possible explanation for RO-associated loss of VEGF could be a reduction in HIF1α, a major transcription factor that controls VEGF production. We found by Western blot that RO reduced HIF1a in both TNBC cell lines (data not shown).

Discussion

Increased cholesterol biosynthesis has been associated with progression of various cancers and is therefore a viable target to suppress tumor growth [24-27]. While conducting studies to determine the anti-proliferative capacity of cholesterol biosynthesis inhibitors on breast cancer cells, we discovered that RO, a class of compound that blocks OSC activity, and has been shown to block the growth of hormone dependent breast cancer cells [13,14], is also effective against TNBC cells. RO slowed the growth of TNBC cells, induced apoptosis, and blocked the process of angiogenesis as discussed below.

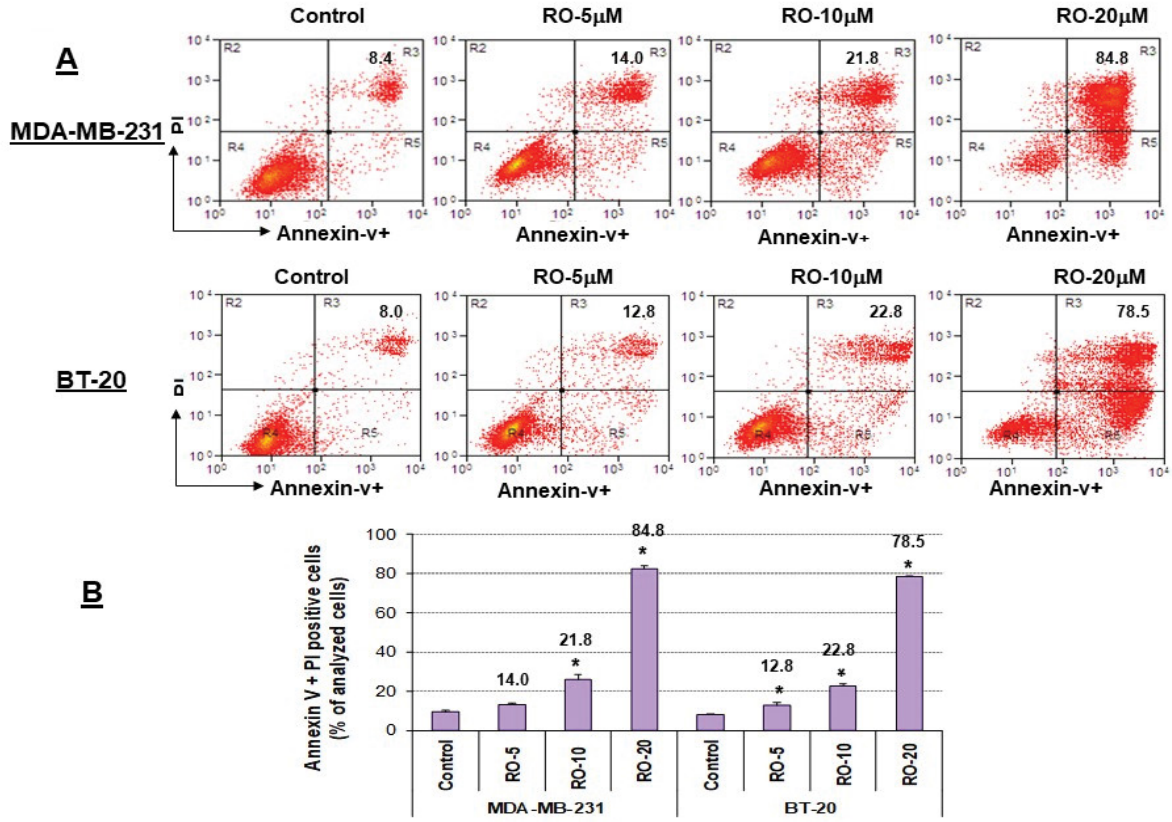

Figure 2: RO induces apoptosis and cell death in TNBC cancer cells. (A) MDA-MB-231 and BT-20 cells were seeded in 6-well plates (1.5×10^5 cells/well) overnight in 10% FBS DMEM:F12. After washing and replacing with media containing 5% FBS, cells were treated with 5, 10, or 20 μM RO or vehicle alone (control) for 24 h. Following treatment, cells were harvested and stained with annexin V-FITC and propidium iodide (PI). Fluorescence-activated cell sorting (FACS) analysis of 10,000 cells/sample was conducted. Quadrant R5 (bottom right) shows annexin V-positive (apoptotic) cells, and quadrant R3 (top right) shows annexin V-positive/PI-positive (dead) cells. (B) Quantitative data from FACS analysis. Values represent mean ± SEM (n=3). * Significantly different from control ($P < 0.05$ using ANOVA).

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Liang Y, et al., J Cancer Sci Clin Ther 2024
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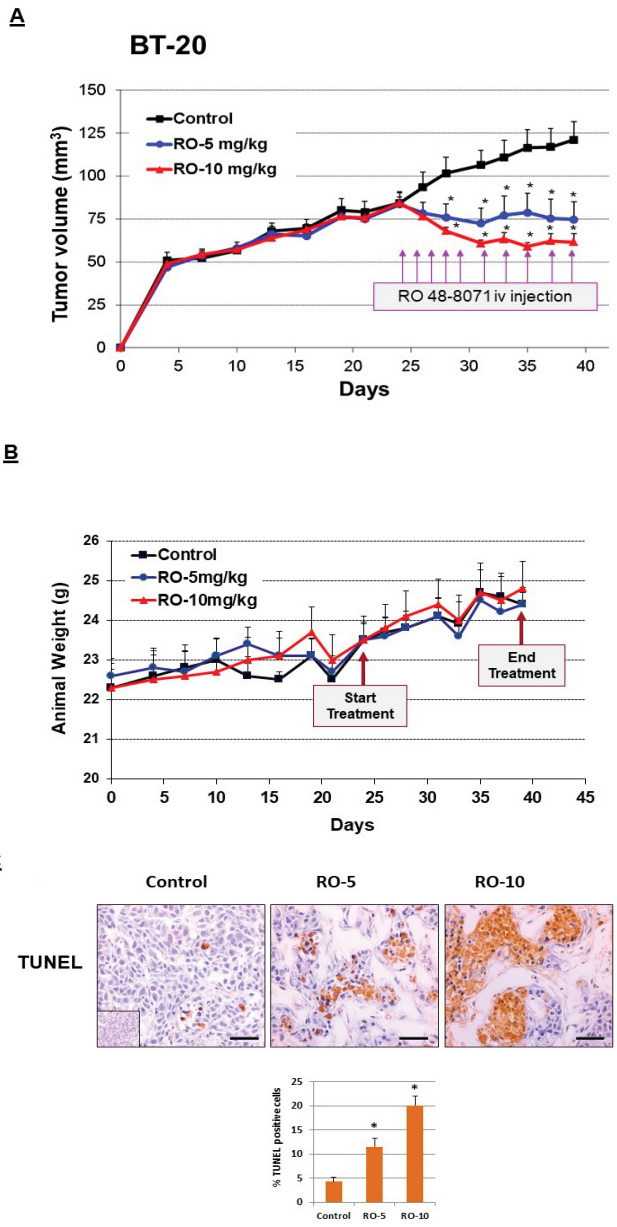

Figure 3: RO suppresses growth of TNBC xenografts in nude mice (A) Six-week-old nude mice (n=6-7/group) were injected with 5 × 10^6 BT-20 breast cancer cells in Matrigel:DMEM/F12 (4:1; [v/v]) into each flank/mouse. When tumor volumes reached approximately 80 mm^3, animals were treated with RO (5- or 10 mg/kg) or the same volume of PBS (control) daily for five days, then every other day for five additional treatments by iv tail-vein injection; mice were given a final RO treatment 2 h before they were sacrificed. Values represent mean ± SEM (n=12-14 tumors in each group).* Significantly different from control ($P < 0.05$ using ANOVA). (B) Animal weight was monitored throughout the experiment. Arrows indicate duration of RO treatment. (C) Tumors were collected at end point as shown in Fig 3A and processed for immunohistochemistry and data analysis as described in Methods. Insets represent negative controls and bars represent 50 µm. RO induced TUNEL staining within tumors (top) and quantification is shown below. * indicates $p < 0.05$ compared with controls.

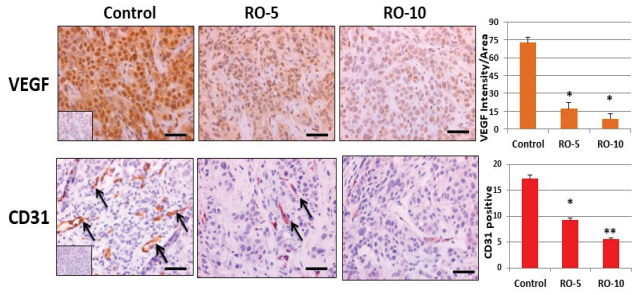

Figure 4: RO decreases VEGF and CD-31 expression in TNBC xenografts. Tumors were collected at end point as shown in Fig 3A and processed for immunohistochemistry and data analysis as described in Methods. Insets represent negative controls and bars represent 50 µm. RO reduced VEGF staining (top) and CD-31 staining (bottom) in tumors. Quantitation is shown on the sides. *indicates $p < 0.05$ compared with controls, ** denotes significant difference compared with control and treatment with 5 mg/kg RO.

We examined the effects of RO on two TNBC cell lines (MDA-MB-231 and BT-20), as well as SK-Br-3 cells, a cell line that express Her-2-neu but not ER and PR; these types of tumors are aggressive with poor prognosis [21-23]. In short term assays, RO effectively reduced the viability of all three cell lines (IC_{50} values between 10-18 µM; SRB 48-h) (Figure 1A; Table 1). This is slightly higher than previously published observations from ER/PR positive cell lines. Lower concentrations (nM) of RO significantly suppressed the viability of TNBC tumor cells in longer-term (7 day) assays (Figure 1C). We also observed that concentrations of RO up to 10 µM had no effect on the viability of normal mammary cells, suggesting that the *in vitro* effects of RO are specific to TNBC cells. Consequently, we propose that since RO appears to be non-toxic to normal cells, it might be used to target tumors with little risk of patient toxicity.

FACS analysis show that *in vitro* RO induces apoptosis in TNBC in a dose-dependent manner. Subsequent *in vivo* studies provide further evidence for induction of apoptosis in regressing tumors following RO treatment (TUNEL Assay). Furthermore, RO reduced levels of VEGF as well as causing a reduction in blood vessels (CD-31) in regressing tumors, indicating that RO has anti-angiogenic properties. We cannot determine whether RO also affects blood vessels directly *in vivo,* or whether vessels decrease due to inhibition of VEGF production. Ascertaining which is the case will be the subject of future studies. A possible explanation for VEGF loss could be that RO reduces levels of HIF1α (data not shown), a transcription factor that controls VEGF synthesis [28,29]. Thus, reduced levels of VEGF could occur through an indirect effect of RO that involves suppression of HIF1α.

Our studies support the use of RO as a therapeutic agent for TNBC with little or no risk for toxic side effects. It will be necessary to expand these studies to include additional TNBC cell lines or organoids to firmly establish the anti-

cancer properties of RO. Studies are currently underway to determine whether administration of higher levels of RO than used in the current report might promote complete xenograft regression without toxicity.

In summary, the data presented in this manuscript strongly suggest that, in addition to its ability to suppress cholesterol biosynthesis, the OSC inhibitor RO exerts a powerful anti-tumor effect on TNBC by inducing cell death and reducing angiogenesis. Further investigation into the molecular effects of RO in TNBC, as well as in tumors and cell lines that express Her-2-neu are warranted.

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