Research Article

Oxidosqualene Cyclase Inhibitor RO 48-8071 Increases Functional ERβ Levels in Triple- Negative Breast Cancer Cells

Yayun Liang and Salman M. Hyder*

Abstract

Purpose: In most human breast cancers, tumor cell proliferation can be suppressed with anti-hormones or drugs targeting Her-2-neu. TNBC lack ER, PR and Her-2-neu and thus are not susceptible to traditional targeted therapies. TNBC are therefore treated with toxic chemotherapies and eventually develop resistance that leads to metastasis. Our goal was to identify alternative targets in TNBC that might be regulated to suppress progression of the disease. Previously we found that inhibition of oxidosqualene cyclase, an enzyme involved in cholesterol biosynthesis, by RO 48-8071 reduced proliferation of hormone-dependent breast cancer cells. RO 48-8071 induced anti-proliferative ERβ protein in these cells. We recently reported that RO 48-8071 also blocked progression of TNBC disease. With this in mind, we conducted studies to ascertain whether RO 48-8071 might also elevate ERβ protein in TNBC.

Methods: 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), was used for this study. ERβ and ERα protein expression in whole-cell extracts from TNBC cells treated with RO was determined by Western blotting. ERβ specific ligands were used to determine whether modulation of ERβ activity can control cell viability, as determined by SRB assay. RO treated TNBC BT20 tumor xenografts in nude mice were studied to determine whether levels of ERβ increased *in vivo* concomitant with reduced tumor growth.

Results: *In vitro* or *in vivo* exposure of TNBC to RO induced the anti-proliferative protein ERβ, but not ERα, in TNBC cells. An ERβ antagonist prevented RO-dependent loss of cell viability, while agonists of ERβ increased the antiproliferative effects of RO.

Conclusions: RO is a potent inhibitor of TNBC and the anti-tumor properties of RO appear to be in part due to an off-target effect that increases the level of anti-proliferative protein ERβ in TNBC cells.

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

Abbreviations: E: estrogen; ER: estrogen receptor; PR: progesterone receptor; OSC: oxidosqualene cyclase; RO: RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]); FBS: fetal bovine serum; PBS: phosphate-buffered saline; TBS-T: Tris-buffered saline containing 0.1% Tween 20; ANOVA: analysis of variance; SE: standard error; DPN, 2,3-bis(4-Hydroxyphenyl)-propionitrile;

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PHTPP: 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]
pyrimidin-3-yl]phenol

Introduction

Almost 70% of human breast tumors express both estrogen receptor (ER) and progesterone receptor (PR) and proliferate in response to the respective hormones [1,2]. These proteins can be targeted chemotherapeutically to control tumor progression [3,4]. In contrast, approximately 15-20% of human breast cancers are classified as triple-negative (TNBC), because they are devoid of ER and PR. as well as Her-2-neu (epidermal growth factor receptors) [5-7], the three identifiable molecular targets for chemotherapy. Thus, patients diagnosed with TNBCs are treated with conventional toxic chemotherapy regimens, which are non-specific and almost always lead to resistance and subsequent tumor progression [8]. Estrogens (E) are essential steroid hormones that regulate sexual development and reproductive functions in humans. The diverse biological effects of E are mediated by the specific estrogen receptors (ER) ERα and ERβ [9,10]. While TNBC are devoid of ERα, the anti-proliferative ERβ protein has recently been shown to be present in TNBC cells [11,12]. It is possible that by modulating its activity in TNBC cells, ERβ might be exploited to trigger an anti-proliferative response [13,14].

Enzymes in the cholesterol biosynthetic pathway are attractive therapeutic targets for several different types of cancers [15]. Statins, the most commonly used class of cholesterol-lowering drug, inhibit HMG-CoA reductase, an enzyme in the cholesterol biosynthetic pathway; however, certain undesirable side effects limit their long-term use for cancer therapy [16]. 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) [17-19]. While testing small-molecule inhibitors of OSC, we previously identified RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate] (RO) [13,20] as a potent suppressor of breast tumor cell viability for hormone-dependent breast cancer. In addition, RO down-regulated ERα but up-regulated ERβ [13] in tumor cells. Generally, ERβ is an anti-proliferative protein [21-24]. Since upregulation of ERβ in regressing hormone-dependent breast cancers treated with RO is a contributing mechanism through which cell proliferation is controlled, we sought to determine whether ERβ might also be elevated in TNBC and if so, whether its upregulation also blocks progression of the disease. In the present study, we describe the role of RO in elevating ERβ levels in TNBC and show how controlling ERβ activity with ERβ specific ligands can alter TNBC progression. Based on our findings we contend that RO represents an exciting candidate compound for clinical management of TNBC progression when used as monotherapy. Furthermore, we propose that combination therapies involving RO and ERβ-specific ligands that function as agonists, will enhance the anti-proliferative effects of RO alone.

Materials and Methods

Cell lines and culture

TNBC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and 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). Prior to treatment with RO, serum was reduced to 5%.

Reagents

RO 48-8071 was purchased from Sigma-Aldrich (St Louis, MO). 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a] pyrimidin-3-yl]phenol (PHTPP) were from Tocris Biosciences. Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), RNAZol for RNA isolation was purchased from Molecular Research, Cincinnati, OH, USA). ER alpha (D-12) and ERβ (H-150) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). β-Actin antibody was obtained from Sigma-Aldrich.

Western blots

Whole-cell extracts were prepared with a nuclear extraction TransAm kit (Active Motif, Carlsbad, CA, USA) and Western blotting carried out as previously described [25,26].

Cell viability assay

The sulforhodamine B (SRB) assay was used to measure cell viability, as previously described by us [27,28].

RT-PCR analysis for ERβ

Cells were grown in DME/F12 medium supplemented with 5% dextran-coated, charcoal-treated serum for 24 h. RNA was prepared using UltraSpec (Biotecx, Houston, TX, USA) according to the manufacturer's protocol. Reverse transcription (RT)-PCR was carried out using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) in an Applied Biosystems 9700 thermocycler. PCR parameters were as follows: 60°C for 30 min, 94°C for 2 min, 35 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 60 s, followed by 68°C for 5 min. PCR was performed in triplicate and the end point products were analyzed on a 1.5 % agarose gel containing 1 μg/ml ethidium bromide. Electrophoresis was carried out in 0.5 × TBE (pH 8.0) at 80 V for 2 h. Gels were scanned on a BioRad GelDoc imager. Primer sequences were as follows:

Sequence for ER-beta [29]:

ER-beta-F: 5'- AGA GTC CCT GGT GTG AAG CAA G -3'

ER-beta-R: 5'- GAC AGC GCA GAA GTG AGC ATC -3'

Sequence for GAPDH:

GAPDH-F: 5'- ATG AGA AGT ATG ACA ACA GCC -3'

GAPDH-R: 5'- TGA GTC CTT CCA CGA TAC C - 3'

Primers for ERβ and GAPDH amplification were from Integrated DNA Technologies, Inc. (Coralville, Iowa).

Statistical analysis

Differences among groups were tested 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 regarded as statistically significant. Values are reported as mean ± SEM.

Results

RO induces ERβ but not ERα protein in ER negative cell lines *in vitro*

Based on our previous finding that RO decreases ERα in hormone-dependent breast cancer cell lines, while simultaneously inducing ERβ [13], we evaluated whether ERβ is also expressed in TNBC and other cell lines that do not exhibit ERα but express Her-2-neu (SK-Br-3). As shown in Figure 1, RO induced ERβ, but not ERα in MDA-MB-231, BT-20 as well as in Sk-Br-3 cells. The natural expression of ERβ and ERα in BT-474 cells was used as control (left lane in Figure 1 top and bottom). We found that pharmacological levels of RO induced ERβ in a dose-dependent manner in TNBC cell lines (Figure 2).

RO increases ERβ mRNA levels in ERα negative breast cancer cells

To determine whether ERβ mRNA levels also increased in TNBC cells, we exposed MDA-MB-231 cells to 10- or 25 mM RO for periods between 3 and 24 h (in triplicate) and analyzed mRNA by RT-PCR. The amplified ERβ bands were scanned and normalized with GAPDH signal from each sample. As shown in Figure 3, both concentrations of RO increased ERβ mRNA at 6 h and 12 h post treatment, and mRNA levels remained elevated until 24 h.

RO induces ERβ protein in vivo in TNBC xenografts

In order to determine the effects of RO on levels of ERβ protein *in vivo*, we measured its expression in xenografts by immunohistochemically analyzing sections obtained from RO-treated TNBC tumors collected at the end point from a previous study [30] (Figure 4A). Treatment with RO resulted in significant induction of ERβ in TNBC tumor tissue, with no effect on induction of ERα (Figure 4). Significantly elevated levels of ERβ were observed in animals receiving 5 mg/kg and 10 mg/kg RO. Our findings indicate that treatment of TNBC with RO leads to induction of ERβ, but no effect on ERα induction *in vivo*, as was also observed *in vitro* (Figure 1). Thus, loss of ERα and ERβ induction by RO in breast cancer cells appears to be a common off-target effect that occurs in response to treatment with an OSC inhibitor.

Modulation of ERβ activity modifies the anti-proliferative effects of RO on breast cancer cells

ERβ is known to play an anti-proliferative role in breast cancer cells [21-24,31,32]. To determine whether induction of ERβ protein potentiates the anti-proliferative effects of RO, we treated MDA-MB-231 and BT-20 cells with RO in the presence of an ERβ agonist DPN. DPN enhanced the effects of RO by further reducing the viability of breast cancer cells (Figure 5A), suggesting that activation of ERβ is partially responsible for RO-mediated effects on breast cancer cell viability. Incubation of both TNBC cell lines with the ERβ antagonist PHTPP blocked RO-mediated reduction
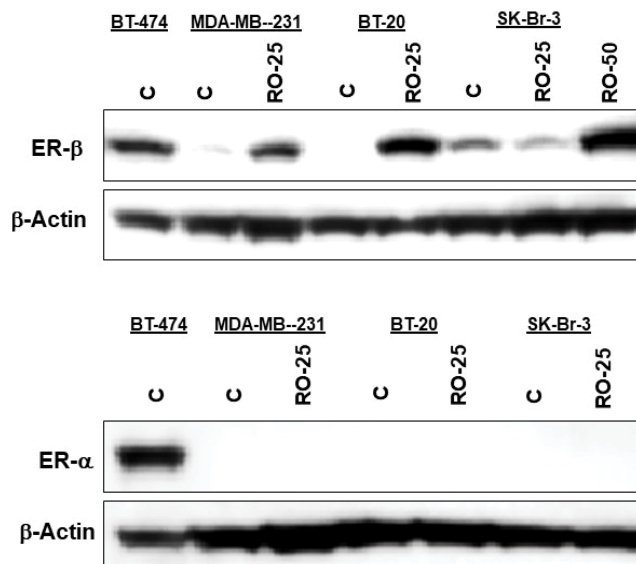

Figure 1: RO increases ERβ protein levels in TNBC cells. BT-20, MDA-MB-231 and Sk-Br-3 cells were grown in DME/F12/10% FBS; (1.5×10^5 cells/well in 6-well plates) for 24h. After washing and replacing with media containing 5% FBS, cells were treated with 25 μM RO for 6 h, then whole-cell extracts prepared and analyzed by Western blot for expression of ERα and ERβ. β-actin was used as a loading control. Extract from BT-474 cells was used to provide positive controls for ERα and ERβ. For all panels, whole-cell extracts were subjected to Western blotting to analyze protein expression, and levels of β-actin were assessed as a protein loading control. All experiments were conducted at least twice.
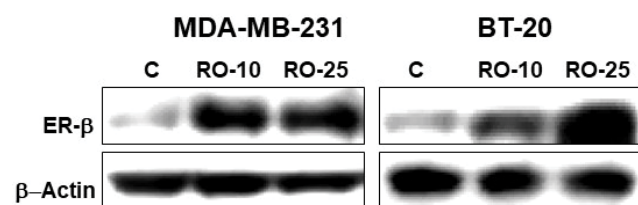
Figure 2: RO increases ERβ protein levels in TNBC cells in a dose dependent manner. BT-20 and MDA-MB-231 cells were grown in DME/F12/10% FBS. At 50% confluence, cells were treated with 10- or 25 µM RO for 6 h in DME/F12/5% FBS, then whole-cell extracts prepared and analyzed by Western blot for expression of ERβ. β-actin was used as a loading control.
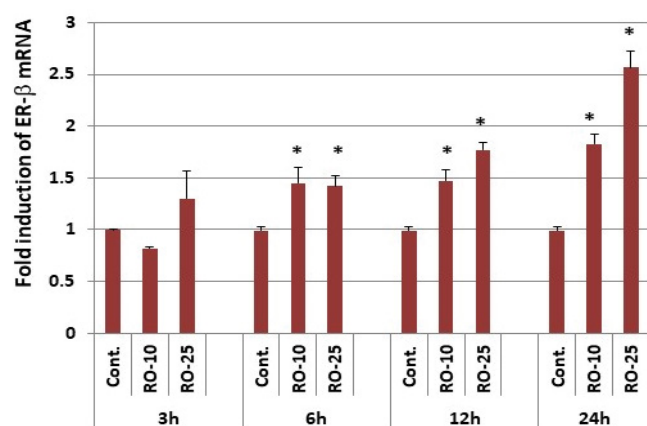
Figure 3: RO increases ERβ mRNA level in TNBC cell. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA isolated from TNBC cells treated with RO for various times. RNA was subjected to RT-PCR analysis as described in Methods. *, significantly different from control (p<0.05 ANOVA).
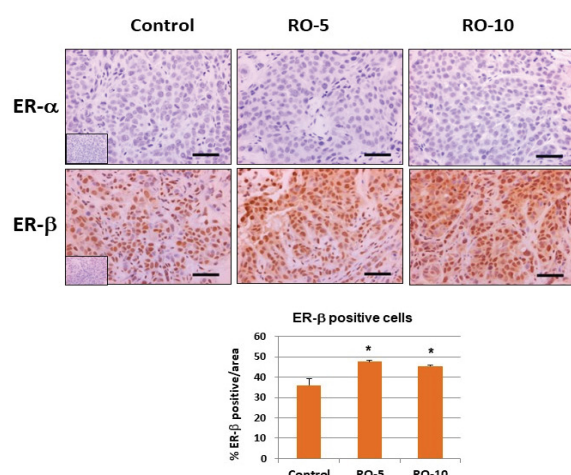
Figure 4: RO treatment increases ERβ levels in BT-20 xenografts in nude mice. Xenograft tumor sections obtained at the end of the experiment shown in Figure 3 of our previously published paper [30] were analyzed for expression of ERβ by immunohistochemistry. Inset represents no antibody control. (magnification × 20). Quantitation for ERβ expression is shown as bar graph below IHC. There was no expression of ERα with or without treatment with RO. Bar represents 50 µm. *, significantly different from control (p<0.05 ANOVA).
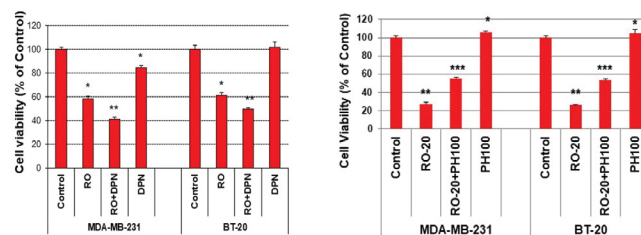
Figure 5: Modulation of ERβ activity influences RO-mediated effects on breast cancer cell viability (A) MDA-MB-231 and BT-20 cells were treated with 10 µM RO ± 1 µM ERβ agonist DPN or with 1 µM DPN alone (dose taken from ref 33) for 48 h. Cell viability was determined by SRB assay. Values represent mean ± SEM (n=6). * Significantly different from control; ** significantly different from RO-treatment and DPN-treatment groups (P < 0.001, ANOVA). (B) Cells were treated with 20 µM RO ± 100 nM ERβ antagonist PHTPP (PH) for 24 h. Cell viability was determined by SRB assay. Values represent mean ± SEM (n=6). * Significantly increased compared with control (set at 100%); ** significantly decreased relative to control group; *** significantly different from RO-treatment group.

of cell viability (Figure 5B), providing further evidence that ERβ plays a role in mediating the effects of RO on breast cancer cells. Interestingly, exposure of cells to PHTPP alone also increased cell viability, slightly but significantly.

Discussion

TNBC represents 15-20% of detected breast cancers. Initially TNBCs are typically treated using toxic chemotherapeutic approaches, but tumors almost always become drug resistant [6,7]. Drug-resistant stem cells are believed to lead to tumor metastasis and poor prognosis. Consequently, studies are ongoing to identify targets in TNBC that might be exploited chemotherapeutically to control the disease in a more specific manner, reduce toxicity and control disease progression.

We and others previously discovered that the cholesterol biosynthesis inhibitor RO, which blocks OSC activity in the cholesterol biosynthetic pathway, effectively controlled the progression of various types of cancer, including hormone-dependent breast cancers, prostate and ovarian cancers [13,15,20]. Interestingly, RO also up-regulated the druggable anti-proliferative protein ERβ in many cancer types [13,14]. Since ERβ has been shown to inhibit the growth of many different types of tumor cells, including TNBC [13,14], we examined the effect of RO on expression of ERβ in TNBC and observed that RO elevated levels of ERβ protein in TNBC (Figure 1). Importantly, we found that pharmacological concentrations of RO increased ERβ levels in vitro in TNBC cells without influencing expression of ERα, which remained silent, making possible the targeting of ERβ in conjunction with RO to control TNBC progression via a drug combination approach. Interestingly RO also increased ERβ activity in

Her-2-neu positive cells, which are also difficult to treat. However, this was beyond the scope of the present study and warrants further investigation.

RO increased ERβ mRNA levels over time, however its effect on direct induction or stabilization of message to influence ERβ mRNA, and hence production of protein, remains unknown. Apart from studying mRNA stability in response to RO exposure, mechanisms such as promoter methylation will be informative in this respect. The effect of RO on protein stability of ERβ also merits further study.

In order to determine whether RO also induced ERβ *in vivo*, we utilized tumor tissue from a previous study in which RO reduced TNBC growth [30], determining that RO also increased ERβ in these regressing tumors. While induction of ERβ was significant *in vitro*, its upregulation was not as robust *in vivo*. This was most likely due to tumors being collected at the end point of the study, several days after nude mice bearing BT-20 xenografts were initially treated. It is therefore likely that we missed the higher levels of ERβ which were subsequently lost when cells expressing elevated ERβ levels underwent apoptosis. However, this requires confirmation by collection of tumors a few days after initial treatment with RO and assessment of ERβ induction. The consistent *in vitro* induction of ERβ in various cell lines was most likely due to short term exposure to the drug.

We confirmed the important role played by ERβ in reducing cell viability of TNBC cells by exposing cancer cells to DPN, an ERβ-specific agonist [33]. When administered individually, both RO and DPN inhibited breast cancer cell viability. However, when a combination of the two compounds was given, their inhibitory effect was additive, an outcome that may be due to increased cellular levels of ERβ in response to RO. To further confirm that ERβ is at least partially responsible for loss of TNBC cell viability, we blocked receptor activity using PHTPP, a selective ERβ antagonist [34]. PHTPP suppressed the anti-proliferative activity of RO. It therefore appears likely that drugs that increase ERβ activity in breast cancer cells could be made even more effective when administered in conjunction with RO. The development of therapeutic regimens using a combination of two agents might make it possible to manage disease using lower levels of both, reducing the likelihood of toxic side effects that result from current therapeutic modalities.

In summary, the data presented in the current report strongly suggest that, in addition to its ability to suppress cholesterol biosynthesis, the OSC inhibitor RO exerts a powerful anti-tumor effect by the off-target induction of ERβ in TNBC. The role of ERβ in TNBC as an antiproliferative protein is now well established [35-38]. Importantly it is recognized that the presence of ERβ in TNBC leads to a favorable prognosis and that ligand-dependent activation of ERβ in TNBC leads to tumor cell death. In addition, *in vitro* data shows that ERβ promotes some of the anti-tumor properties of RO in TNBC. Thus, we propose that ERβ is at least partially responsible for the observed suppression of TNBC cell viability and suggest therefore that combination therapy using inhibitors of cholesterol biosynthesis (such as RO), together with commonly used chemotherapeutic drugs, or those designed to target ERβ activity, could prove beneficial as a means by which to suppress TNBC progression. We are currently conducting studies to determine the effectiveness of such combination therapies.

Acknowledgments

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