
**Research Article**

## Liquiritigenin Enhances the Inhibitory Effects of the Cholesterol Biosynthesis Inhibitor RO 48-8071 on Cell Viability in Ovarian-Cancer Cells in Vitro

Yayun Liang and Salman M Hyder\*

### Abstract

Almost 25,000 new cases of epithelial ovarian cancer (EOC) are reported each year in the United States. Cancers of the ovary have poor prognosis due to drug resistance and metastasis, and have the highest mortality rate of all the known gynecological malignancies. Despite concerted efforts to develop new strategies for preventing and treating ovarian cancer, novel and more effective non-toxic therapies for ovarian cancer are urgently needed. Recent observations show that RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate] [RO], a small-molecule inhibitor of the key cholesterol biosynthesis enzyme 2, 3-oxidosqualene cyclase, inhibits breast and prostate cancer cells. RO also induces the tumor-suppressor protein estrogen receptor (ER)  $\beta$  in both breast- and prostate-cancer cells, and also inhibits growth of ovarian-cancer cells both *in vitro* and *in vivo*. Extending upon these earlier studies, here we found that RO also induces ER $\beta$  expression in OVCAR-3 ovarian-cancer cells. Further, we demonstrated that treatment of two ovarian-cancer cell lines (OVCAR-3 and SK-OV-3) with liquiritigenin (LQ), a naturally occurring compound that is an ER $\beta$  agonist, reduced cell viability *in vitro*. When we treated OVCAR-3 and SK-OV-3 cells with RO, LQ, or RO + LQ, we observed that RO + LQ combination treatment synergistically reduced cell viability. Further *in vivo* studies examining the effects of RO + LQ combination treatment on EOC are warranted, as a means of exploring a potential new therapeutic approach to combat ovarian cancers with minimal toxicity.

**Keywords:** Ovarian cancer; Estrogen receptor- $\beta$ ; RO 48-8071; Liquiritigenin

**Abbreviations:** ANOVA: one-way analysis of variance; EOC: Epithelial Ovarian Cancer; ER: Estrogen Receptor; FBS: Fetal Bovine Serum; LQ: Liquiritigenin; OSC: 2, 3-oxidosqualene cyclase; PBS: Phosphate-Buffered Saline; RO: RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]); SEM: Standard Error of the Mean; SRB: Sulforhodamine B

### Introduction

Almost 25,000 new cases of epithelial ovarian cancer (EOC) are diagnosed annually in the United States [1]. EOC is a deadly disease characterized by aggressively growing tumor cells and often results in multifocal intraperitoneal dissemination, accompanied by intense neovascularization [2]. Because EOC tumors are often detected at a late stage, they are generally non-responsive to chemotherapy, have poor prognosis, and demonstrate the highest

### Affiliation:

Dalton Cardiovascular Research Center and Department of Biomedical Sciences University of Missouri, Columbia 65211, United States

### \*Corresponding author:

Dr. Salman M. Hyder, Dalton Cardiovascular Research Center, University of Missouri-Columbia, 134 Research Park Drive, Columbia, MO 65203, United States.

**Email:** hyders@missouri.edu

### Citation:

Yayun Liang, Salman M Hyder. Liquiritigenin Enhances the Inhibitory Effects of the Cholesterol Biosynthesis Inhibitor RO 48-8071 on Cell Viability in Ovarian-Cancer Cells in Vitro. Journal of Cancer Science and Clinical Therapeutics. 7 (2023): 158-163.

**Received:** August 14, 2023

**Accepted:** August 30, 2023

**Published:** September 20, 2023

mortality rate of all the known gynecological malignancies [3]. Standard treatments include systemic high-dose, toxic chemotherapeutic drugs; however, drug resistance almost always occurs, and cancers originating in the ovary often metastasize to distant organs [3,4]. More than half of ovarian-cancer patients die from this resulting metastasis; thus, the outlook for those afflicted with the disease is generally bleak [5]. For these reasons, efforts to develop new strategies for preventing and treating ovarian cancer have been undertaken. Although novel and more effective non-toxic therapies for ovarian cancer are urgently needed, thus far success in this regard has been limited.

Cholesterol is an essential structural and functional component of cellular membranes and cellular metabolism, and is therefore required for tumor growth [6,7]. Endogenous steroid hormones, including estradiol, are derived from the metabolic precursor cholesterol, which itself can be modified to 27-hydroxy cholesterol. 27-hydroxy cholesterol can serve as a selective estrogen-receptor modulator that increases estrogen signaling in some tissues, including in a subset of ovarian-cancer cells. Because ovarian-cancer cells express enzymes involved in the biosynthesis of endogenous cholesterol [8,9], inhibition of cholesterol synthesis may be an effective means by which to arrest tumor-cell proliferation; furthermore, the combined use of cholesterol biosynthesis inhibitors and commonly used chemotherapeutic drugs may provide a novel and hitherto untested approach to combatting the progression of ovarian cancer. Statins have been used to inhibit HMG-CoA reductase, an enzyme that is essential for cholesterol biosynthesis, but statin therapy could cause some undesirable side effects, attributed to reduced levels of isoprenoids, defective post-translational modification of membrane proteins, and impaired membrane structure and function [10]. Thus, alternative approaches to inhibit cholesterol biosynthesis are being considered. 2, 3-oxidosqualene cyclase (OSC), which converts 2, 3-monoepoxysqualene to lanosterol, a key step in the biosynthesis of cholesterol, has recently emerged as a possible new target for inhibiting the cholesterol-biosynthetic pathway [11]. Because OSC functions downstream of HMG-CoA reductase during cholesterol biosynthesis, it is not likely to be associated with the same adverse effects as statins. Potent small-molecule inhibitors of OSC, such as RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]; RO) have been identified [11-14].

We recently showed that RO inhibits growth of ovarian-cancer cells, both *in vitro* and *in vivo* (13). RO also inhibits the growth of other types of cancer, and, in some of these (including breast and prostate cancer), RO induces estrogen receptor- $\beta$  (ER $\beta$ ), which acts as a tumor-suppressor protein in most cancers [15-18]. In this study, we tested whether RO could also induce ER $\beta$  in EOC *in vitro*, and whether

liquiritigenin (LQ), a natural non-toxic plant product and an agonist of ER $\beta$  [19-21], could enhance the inhibitory effects of RO on cell viability in ovarian-cancer cells, as it does in breast-cancer cells [15,16].

## Materials and Methods

### Reagents

Antibody to detect ER $\beta$  was obtained from Santa Cruz Biotechnology (Dallas, TX) (H-150). The  $\beta$ -Actin antibody clone (AC-74), was obtained from Sigma (St Louis, MO). Sulforhodamine B (SRB) was obtained from Sigma (St. Louis, MO). BD Matrigel Matrix was purchased from BD Biosciences (Bedford, MA). RO and LQ were bought from Tocris (Bristol, UK).

### Cell lines and cell culture

Epithelial ovarian cancer cell lines OVCAR-3 (high grade serous) and SK-OV-3 (non-high grade serous) were obtained from ATCC (Manassas, VA, USA). Cell-culture media, phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA were purchased from Invitrogen Corporation & Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was bought from JRH Biosciences (Lenexa, KS). OVCAR-3 cells were maintained in 20% FBS RPMI-1640, while SK-OV-3 cells were maintained in 10% FBS McCoy's 5a medium. All cells were grown in 100  $\times$  20 mm tissue-culture dishes and harvested with 0.05% trypsin-EDTA. During treatment of cells with RO or LQ, the percent FBS was reduced by half for the duration of treatment so that the process of reduction of cell viability and/or apoptosis would not be rescued with serum.

### Cell-viability assay

An SRB assay was used to measure cell viability, as previously described [12-14]. Briefly, cells were grown to 70% confluence, then, following trypsin treatment, cells were seeded into 96-well plates in 100  $\mu$ l growth medium and incubated overnight at 37°C with 5% CO<sub>2</sub>. Cells were washed once with media and incubated for 24 or 48 h in 100  $\mu$ l medium with 10% FBS (OVCAR-3 cells) or 5% FBS (SK-OV-3 cells) in their respective media, in the presence of RO (0–10  $\mu$ M), LQ (0–600  $\mu$ M), or RO (0–10  $\mu$ M) + LQ (0–300  $\mu$ M). At the end of the incubation, surviving or adherent cells were fixed *in situ* by adding 100  $\mu$ l 50% cold trichloroacetic acid for 1 h at 4°C. Cells were washed with ice water, dried, and stained with 50  $\mu$ l 4% SRB for 8 min at room temperature. Unbound stain was removed by washing cells 5X with cold 1% acetic acid, then the cells were dried at room temperature. Cell-bound stain was solubilized in 150  $\mu$ l 10 mM Tris buffer and quantified at 520 nm using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). Six wells were used for each dose/experimental condition and each experiment was performed at least twice.

### Western blots

Whole-cell extracts were prepared with a nuclear extraction TransAm kit (Active Motif, Carlsbad, CA) and Western blotting performed as previously described [15,16].

### Statistical analysis

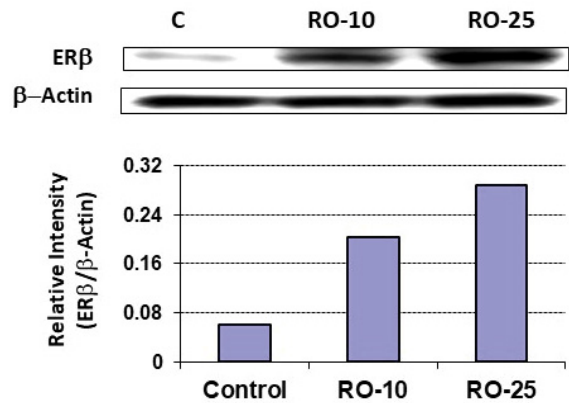
Statistical significance was measured using one-way analysis of variance (ANOVA) with repeated measure over time. When necessary, it was assumed that ANOVA was non-parametric. Values are reported as mean ± standard error of the mean (SEM). For samples with significant F-ratio ( $p < 0.05$ ), the Student-Newman-Keuls multirange test was employed (SigmaStat).

### Results

Because the cholesterol-biosynthesis inhibitor RO has been shown to induce the anti-proliferative protein ERβ in both breast- and prostate-cancer cells and to reduce their viability [15,16], we conducted studies to ascertain whether exposure to RO could also induce ERβ in ovarian-cancer cells. Using OVCAR-3, an aggressive ovarian-cancer cell line, we found that ERβ expression was induced by both 10 μM and 25 μM RO (Figure 1). In order to determine whether an ERβ agonist will enhance the anti-proliferative effect of RO, we tested LQ, a natural ligand that serves as an agonist of ERβ selectively [17,18]. When we examined the ability of high doses of the ERβ agonist LQ to inhibit the viability of ovarian-cancer cells, we found that 24-h or 48-h exposure to LQ reduced the viability of both OVCAR-3 and SK-OV-3 ovarian-cancer cells (Figure 2). The IC<sub>50</sub> values for OVCAR-3 cells and SK-OV-3 cells were  $296 \pm 7 \mu\text{M}$  and  $501 \pm 13 \mu\text{M}$  respectively, in a 48-h SRB assay, demonstrating that SK-OV-3 cells were less sensitive than OVCAR-3 cells to LQ treatment.

Given our finding here that RO induces ERβ in ovarian-cancer cells, and our previous findings that RO reduces cell viability of breast-cancer cells to some extent at 5 and 10 μM in an *in vitro* assay [13-15], we sought to determine whether LQ could enhance the effects of RO and lower the concentrations of RO needed to reduce cell viability in ovarian-cancer cells. To examine this possibility, we examined the viability of OVCAR-3 and SK-OV-3 cells exposed to 5 or 10 μM RO together with 100, 200, or 300 μM LQ. Both cell lines were also incubated with RO or LQ alone. In all cases, cells were pre-treated with either 5 or 10 μM RO for 6-7 h to increase the levels of ERβ (as shown in Figure 1). Exposure of OVCAR-3 cells to either RO (5 or 10 μM) or LQ (100–300 μM) alone resulted in a modest reduction of cell viability (Figure 3). However, RO + LQ combination treatment reduced cell viability synergistically in a 20-h assay. For example, OVCAR-3 viability was reduced by approximately 10% in response to exposure to either 5 μM RO or 100 μM LQ alone, while a combination of RO + LQ at these concentrations reduced viability by approximately 40%.

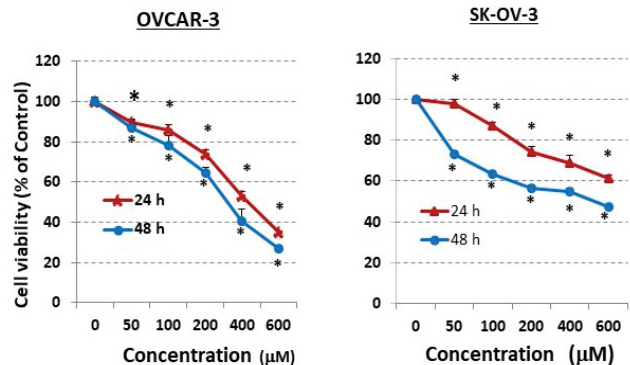
### OVCAR-3



**Figure 1:** RO increases ERβ expression in OVCAR-3 ovarian-cancer cells.

OVCAR-3 cells were treated with the indicated concentrations of RO in 10% FBS RPMI-1640 medium for 7 h. Control cells (C) were treated with vehicle (10% FBS RPMI-1640 + PBS). Cell extracts were prepared, and 50 μg protein/sample was loaded onto 10% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA), electrophoresed, then transferred to PVDF membrane (BioRad, Hercules, CA). Membranes were probed with anti-ERβ antibody (1:200 dilution), followed by anti-rabbit secondary antibody (1:3000 dilution). Immunoreactive bands were visualized using the ECL Plus detection kit (Amersham, Arlington Heights, IL). Beta-Actin expression was probed on same blots for demonstrating equal loading of protein. The experiment was performed twice.

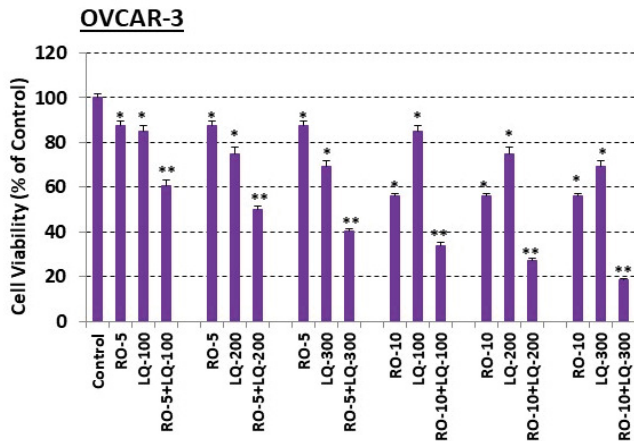
### Liquiritigenin



**Figure 2:** Liquiritigenin (LQ) reduces ovarian-cancer cell viability *in vitro*.

Ovarian-cancer cells (OVCAR-3:  $6 \times 10^3$ /well and SK-OV-3:  $5 \times 10^3$ /well) were seeded into 96-well plates overnight. The next day, cells were washed and treated with pharmacological doses of LQ (μM) for 24 or 48 h in 10% FBS RPMI-1640 medium (OVCAR-3) or in 5% FBS McCoy's 5a medium (SK-OV-3). Control cells (0 μM) were incubated with vehicle (same medium + PBS). Cell viability was determined by SRB assay. Values represent means ± SEM (n=6). \* Significant difference compared with control group (set at 100%) ( $p < 0.05$  using ANOVA).





**Figure 3:** Liquiritigenin (LQ) enhances RO effects on OVCAR-3 ovarian-cancer cell viability.

OVCAR-3 cells ( $5 \times 10^3$ /well) were seeded into 96-well plates overnight. The next day, cells were washed and treated with 5  $\mu$ M or 10  $\mu$ M RO for 7 h. Cells were then washed and treated with 5  $\mu$ M or 10  $\mu$ M RO, 100, 200, or 300  $\mu$ M LQ, or 5 or 10  $\mu$ M RO in combination with 100, 200, or 300  $\mu$ M LQ for another 20 h in 10% FBS RPMI-1640 medium. Control cells were incubated with vehicle (same medium + PBS). Cell viability was determined by SRB assay. Values represent means  $\pm$  SEM (n=6). \* Significant difference compared with control group (set at 100%) ( $p < 0.05$  using ANOVA). \*\* Significant difference compared with control, RO alone, and LQ alone groups ( $p < 0.05$  using ANOVA).

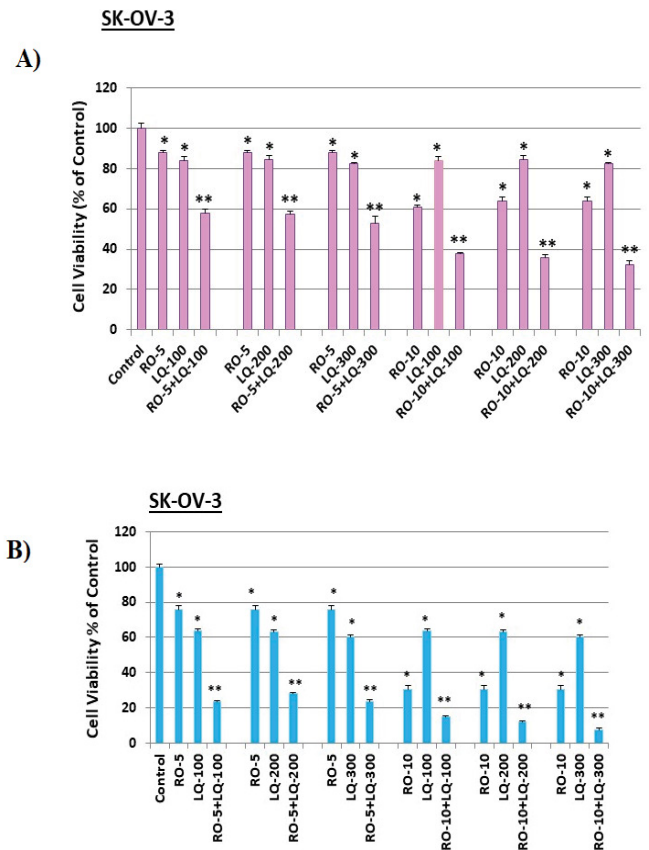
When we conducted this same analysis in SK-OV-3 cells, as we observed with OVCAR-3 cells, viability was reduced by RO alone, while RO + LQ acted synergistically to reduce cell viability in the short-term (18 h) assay (Figure 4A). To determine whether exposure of ovarian-cancer cells to RO and LQ for prolonged periods might cause an even greater effect on viability, we exposed SK-OV-3 cells to the same concentrations of the two compounds used in the studies presented in Figure 4A, but for a longer time (44 h). Under these conditions, a combination of a higher concentration of RO (10  $\mu$ M) and 100–300  $\mu$ M LQ resulted in an approximately 90–95% loss of cell viability, while cells subjected to single-agent treatment exhibited viability reduced by approximately 70% and 40%, respectively, with RO and LQ alone (Figure 4B).

## Discussion

Extending on our previous reports that the cholesterol biosynthesis inhibitor RO induces ER $\beta$  in prostate- and breast-cancer cells [15,16], here we show that RO also induces ER $\beta$  in ovarian-cancer cells *in vitro*. Although the mechanism by which this ER $\beta$  induction occurs within cancer cells remains unclear, the potential therapeutic significance of this finding is enormous, given that ER $\beta$  is generally regarded as a tumor suppressor in most cell types [17-19]. Our studies reported

here also show that, in accord with observations in a variety of cancer-cell lines, pharmacological levels of LQ effectively reduced the viability of OVCAR-3 and SK-OV-3 ovarian-cancer cells, both of which are difficult to treat with current drug regimens. However, the reduction in viability observed for both of these cell lines increased dramatically when the cells were exposed to a combination of LQ and the ER $\beta$  inducer RO. For this reason, RO + LQ combination therapies may represent an attractive option for treating patients with ovarian cancer.

ER $\beta$  has previously been shown to reduce the effective dose of certain drugs, though the exact mechanism for such an effect is not known at present [22]. For example, it can be



**Figure 4:** Liquiritigenin (LQ) enhances RO effects on SK-OV-3 ovarian-cancer cell viability.

SK-OV-3 cells ( $4 \times 10^3$ /well) were seeded into 96-well plates overnight. The next day, cells were washed and treated with 5  $\mu$ M or 10  $\mu$ M RO for 6 h. Cells were then washed and treated with 5  $\mu$ M or 10  $\mu$ M RO, 100, 200, or 300  $\mu$ M LQ, or 5 or 10  $\mu$ M RO in combination with 100, 200, or 300  $\mu$ M LQ for another 18 h (A) or 44 h (B) in 5% FBS McCoy's 5a medium. Control cells were incubated with vehicle (same medium + PBS). Cell viability was determined by SRB assay. Values represent means  $\pm$  SEM (n=6). \* Significant difference compared with control group (set at 100%) ( $p < 0.05$  using ANOVA). \*\* Significant difference compared with control, RO alone, and LQ alone groups ( $p < 0.05$  using ANOVA).

envisioned that ER $\beta$  not only induces a different complement of genes than its proliferative counterpart ER $\alpha$ , but that it may also inhibit the proliferative signal-transduction pathways, although this possibility needs to be fully investigated. Further, ER $\beta$  has also been shown to inhibit cholesterol biosynthesis [23,24], and thus could synergize with RO in this manner. If utilized, combination RO + LQ treatments could effectively inhibit ovarian-cancer progression and might also reduce the need for potentially toxic chemotherapeutic regimens such as those currently employed, increasing the quality of life of those suffering from this disease. Although our observations regarding the effectiveness of RO + LQ are based on cell-culture studies, we expect that the effects we observed *in vitro* will be translatable to *in vivo* models, given that we have already demonstrated that RO on its own quite effectively inhibits the *in vivo* growth of EOC xenografts in an animal model [13].

Treatment of ovarian cancer with anti-cancer drugs such as cisplatin is somewhat successful in arresting tumor progression, but is accompanied by extreme side effects and the development of drug resistance [25]. Our research explored the effectiveness of modulating estrogen signaling due to induction of ER $\beta$  by the OSC inhibitor RO in ovarian-cancer cells. We demonstrated that treatment of ovarian-cancer cells with RO increased their sensitivity to the natural ER $\beta$  agonist LQ, which binds to ER $\beta$ , thereby reducing their viability. Based on our observations, we propose that low-dose combinations of RO and LQ should be further explored as a potential new therapy for combatting ovarian cancer. Our findings contribute to ongoing efforts to identify novel, effective treatments for primary and metastatic ovarian cancer that employ new non-toxic drugs.

## Acknowledgements

This research was supported by a Faculty COR award from the College of Veterinary Medicine, University of Missouri-Columbia. SMH is the Zalk Missouri Professor of Tumor Angiogenesis.

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