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Anticancer Effects of the Hsp90 Inhibitor 17-Demethoxy-Reblastatin in Human Breast Cancer MDA-MB-231 Cells

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Triple-negative breast cancer (TNBC) possesses a higher rate of distant recurrence and a poorer prognosis than other breast cancer subtypes. Interestingly, most of the heat shock protein 90 (Hsp90) client proteins are oncoproteins, and some are closely related to unfavorable factors of TNBC patients. 17-Demethoxy-reblastatin (17-DR), a novel non-benzoquinone-type geldanamycin analog, exhibited potent Hsp90 ATPase inhibition activity. In this study, the anticancer effects of 17-DR on TNBC MDA-MB-231 cells were investigated. These results showed that 17-DR inhibited cell proliferation, induced apoptosis, and suppressed cell invasion and migration in the MDA-MB-231 cells. Down-regulation of the key Hsp90-dependent tumor-driving molecules, such as RIP1 and MMP-9, by 17-DR may be related to these effects. Taken together, our results suggest that 17-DR has potential as a therapeutic agent for the treatment of TNBC.

Keywords: Hsp90, 17-demethoxy-reblastatin, anticancer effect, TNBC

Introduction

Triple-negative breast cancer (TNBC) accounts for 15% of breast cancers, and for a higher rate of distant recurrence and a poorer prognosis than other breast cancer subtypes. It is, therefore, attracting more and more attention [1, 8]. TNBC is defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) [3]. Clinical studies have shown epidermal growth factor receptor (EGFR) overexpression and activation of the PI3K/Akt pathway in TNBCs to be associated with poor prognosis [26]. Notably, most of the heat shock protein 90 (Hsp90) client proteins are oncoproteins, such as EGFR, Akt, Raf, RIP1, MMPs, *etc* [6, 27].

The molecular chaperone Hsp90 is responsible for maintaining the correct folding and stability of many signaling proteins, and is emerging as an important target in cancer therapeutics [18, 20]. Hsp90 inhibition can trigger proteasomal degradation of multiple oncoproteins, thereby reducing cancer cell proliferation, survival, invasion, and angiogenesis, and can promote apoptosis [5, 17]. Hsp90 is often overexpressed in a range of cancers, including breast cancer. In addition, high levels of Hsp90 also result in poor prognosis for breast cancer patients [4]. In particular, Hsp90 inhibitors display remarkable selectivity for cancer cells, as compared with normal cells [11].

Geldanamycin (GA), produced by *Streptomyces hygroscopicus*,

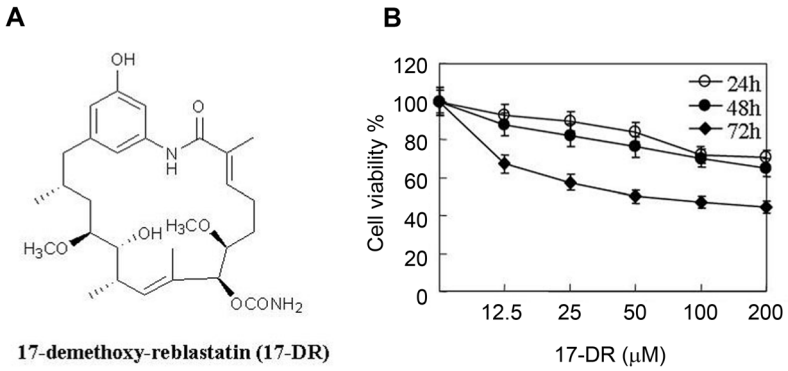

Fig. 1. Inhibitory effect of 17-DR on cell viability in MDA-MB-231 cells.
(**A**) Chemical structure of 17-DR. (**B**) Dose- and time-response curve of the effects of 17-DR on cell viability in MDA-MB-231 cells. Cells were treated with DMSO or various concentrations of 17-DR for 24, 48, and 72 h. The cell viability was measured using a MTT assay.

is a 19-membered macrocyclic lactam that binds to the N-terminal ATP-binding pocket of Hsp90 and inhibits its ATP-dependent chaperone functions [21]. A semisynthetic GA analog, 17-allylamino-17-demethoxyGA (17-AAG), has entered phase III clinical trials, but 17-AAG has several potential limitations, including poor solubility, limited bioavailability, and hepatotoxicity [9,15]. This has led to efforts to identify more effective clinical agents [13, 14, 22, 23]. Previously, we have reported that 17-demethoxy-reblastatin (17-DR) (Fig. 1A) is the main product of a culture using a GA biosynthetic oxidation gene-inactivated strain of *Streptomyces hygroscopicus* JCM4427, a GA producer [10,19]. The 17-DR showed stronger yeast Hsp90 ATPase inhibition activity (IC_{50}, 1.82 μM) compared with the original Hsp90 inhibitor GA (IC_{50}, 3.19 μM) [22]. The aim of this study was to investigate the effects of 17-DR on cell proliferation, apoptosis, invasion, and migration in MDA-MB-231 cells, and to explore its preliminary mechanism.

Materials and Methods

Reagents and Antibodies

Dulbecco's modified Eagle's medium (DMEM), trypsin, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), cocktail of protease inhibitors, and propidium iodide (PI) were purchased from Sigma (USA). The mitochondrial membrane potential (ΔΨm) assay kit (JC-1) was obtained from Beyotime Institute of Biotechnology (China). The transwell Boyden chamber system was purchased from Corning Life Sciences (NY, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). Anti-caspase-3 monoclonal antibody was obtained from Abcam (Cambridge, UK). Anti-Bcl-2, anti-Bax, anti-MMP-9, and anti-TIMP-1 antibodies were purchased from Biosynthesis Biotechnology Co. (China). Anti-IκBα antibody was purchased from Beyotime Institute of Biotechnology (China). Anti-β-actin, anti-PARP, anti-RIP1, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 17-DR was isolated from *Streptomyces hygroscopicus* JCM4427 by the methods described previously [22]. The purity of 17-DR used in our study was over 98%, detemined by high pressure liquid chromatography (HPLC). The structure of 17-DR was determined on the basis of data obtained using NMR and ESI-MS. ^{1}H-NMR (400 MHz, DMSO-d_6) data for 17-DR: 9.31 (1H, s, -NH), 9.22 (1H, s, 18-OH), 6.59 (1H, s, H-19), 6.24 (1H, s, H-17), 6.19 (1H, s, H-21), 5.68 (1H, brs, H-3), 5.21 (1H, d, J = 10 Hz, H-9), 4.83 (1H, d, J = 7.2 Hz, H-7), 4.30 (1H, brd, 11-OH), 3.39 (1H, m, H-11), 3.30 (3H, s, 6-OCH_3), 3.21 (3H, s, 12-OCH_3), 3.17 (1H, m, H-6), 2.96 (1H, m, H-12), 2.29~1.06 (m), 1.70 (3H, s, H-22), 1.36 (3H, s, H-23), 0.90 (3H, d, J = 6.8 Hz, H-24), (0.71 (3H, d, J = 6.8 Hz, H-25). ESI-MS for 17-DR: m/z $[M+Na]^+$ 541, $[M-H]^-$ 517.

Cell Culture

Human breast cancer MDA-MB-231 cells were obtained from Shanghai Cell Bank (China). The cells were grown in DMEM, supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), and HEPES (25 mM). All cells were maintained in the presence of 5% CO_2 at 37°C.

Cell Viability Assay

The cell viability was determined by the MTT assay [16]. First, 5×10^3 cells were plated in 96-well microtiter plates and treated with various concentrations of 17-DR for 24, 48, and 72 h. At the end of each time point, 15 μl of MTT (5 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h. After 4 h, the MTT solution was removed, and 150 μl of DMSO was added to each well to dissolve the formazan crystals. The plate was further incubated at room temperature for 10 min, and the absorbance (A) of the wells was determined using a plate reader at a test wavelength of 570 nm.

Determination of Apoptotic Cells

MDA-MB-231 cells were seeded at 1×10^5 cells/well in 6-well cell culture plates and allowed to reach exponential growth for 24 h before treatment. Whole-cell lysates from MDA-MB-231 cells were treated with various concentrations of 17-DR for 24 h using PI staining and then evaluated using flow cytometry. Quantitative analysis of subG$_1$ cells was carried out in a BD Accuri C6 flow cytometer using the Cell Quest software.

Cell Invasion Assay

The invasion assay was performed using a 24-well cell culture plate with 8.0 μm pore membrane inserts. The membrane undersurface was coated with 50 μl of Matrigel with serum-free medium for 30 min at 37°C. MDA-MB-231 cells were starved in serum-free medium overnight, and 5×10^4 cells were resuspended in 100 μl of serum-free medium and placed in the upper chambers. The lower well of each chamber was filled with 800 μl of DMEM supplemented with 10% FBS and incubated for 36 h. Reagents added to the upper surface of the membrane were removed by cotton buds, and the cells on the lower chamber were incubated with paraformaldehyde in PBS buffer and stained with 0.1% crystal violet. Five visual fields were randomly selected for each insert and photographed under a light microscope at 200× magnification. The number of invasive cells was then counted and analyzed to determine statistically significant differences. Each condition was assayed in triplicate, the experiments were performed independently at least three times, and the results are expressed as the number of cells/field. A one-way analysis of variance was used to determine significant differences.

Cell Migration Assay

The migration assay was performed using a 24-well cell culture plate with 8.0 μm pore membrance inserts without Matrigel. First, 5×10^4 MDA-MB-231 cells were added to the upper wells, and the chambers were incubated for 24 h at 37°C. The lower chamber was filled with 800 μl of 10% FBS as the chemoattractant. After 24 h in normoxic conditions, the cells that had migrated were stained and photographed under a light microscope at 200× magnification. The number of migratory cells was then counted and analyzed to determine statistically significant differences. Each condition was assayed in triplicate, the experiments were performed independently at least three times, and the results are expressed as the number of cells/field. A one-way analysis of variance was used to determine significant differences.

Western Blot Analysis

The cells were plated in a 6-well culture plate at a density of 5×10^5 cells per well. After incubation with drugs, the cell were harvested, washed twice in PBS, and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and a cocktail of protease inhibitors (Sigma)). Lysates were loaded onto 10%~15% SDS-PAGE and transferred to a PVDF membrance. The membranes were blocked with 5% skim milk, and were incubated with primary antibodies overnight at 4°C. Detection was performed using secondary antibody for 1 h at room temperature and an ECL detection system (Bio-Rad, CA, USA).

Statistical Analysis

All experiments were repeated at least three times. Data are presented as the mean ± SD. Statistical comparisons between 17-DR treatment groups and control were carried out using one-way analysis of variance (ANOVA) followed by Dunnett *t*-tests. Differences were considered significant at $p < 0.05$, and herein denoted as *. All statistical analyses were performed using SPSS 19.0 software (Chicago, IL, USA).

Results and Discussion

17-DR Exhibits Antiproliferative Effect in MDA-MB-231 Cells

To understand the anticancer activity of 17-DR on cancer cells, we analyzed the antiproliferative effect of 17-DR on human breast cancer MDA-MB-231 cells after treatment with increasing doses of the compound (0~200 μM) for specified courses (24, 48, and 72 h). As shown in Fig. 1B, 17-DR showed significant antiproliferative activity on MDA-MB-231 cells in a dose- and time-dependent manner, with a IC_{50} value of 74.9 μM at 72 h after treatment. Similar to the MTT assays, the data-analyzed colony formation assays also showed that 17-DR inhibited cell growth at low dose (Fig. S1).

17-DR Induces Apoptosis in MDA-MB-231 Cells

To clarify whether 17-DR induced apoptosis, MDA-MB-231 cells were treated with 17-DR, and the effects on apoptosis of several effectors and mediators were analyzed. According to the flow cytometer apoptosis detection by PI single staining method, the corresponding share of subG1 cells phase ratio represents the apoptosis rate of each group. After 24 h, the rate of apoptosis in the control was 0.9%, while the apoptotic rate was 4.8%, 14.1%, and 22.6% for the cells treated with 10, 50, or 100 μM of 17-DR, respectively (Fig. 2A). One of the early critical events in apoptosis is the loss/disruption of mitochondrial membrane potential ($\Delta\Psi_m$) in the cells, which eventually causes the initiation and activation of apoptotic cascades. We sought to determine whether 17-DR treatment had any effect on the $\Delta\Psi_m$ in MDA-MB-231 cells using JC-1 staining and flow cytometry. The results in Table S1 show that, compared with the control group, the ratio of red and green fluorescence intensity of the cells in the 17-DR treated group decreased gradually as the concentration increased.

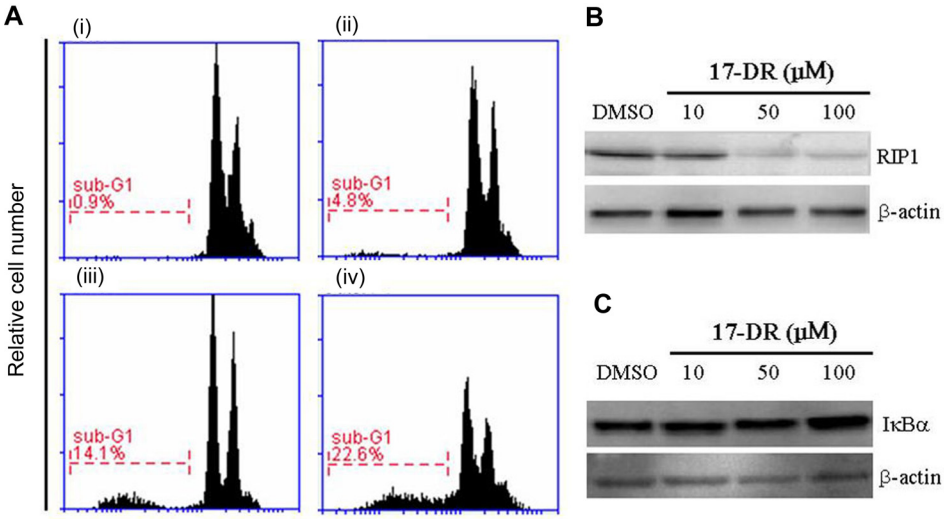

Fig. 2. 17-DR induces apoptosis in MDA-MB-231 cells.
(**A**) Representative flow cytometry histograms of apoptosis. MDA-MB-231 cells were treated with (i) vehicle, (ii) 10 μM of 17-DR, (iii) 50 μM of 17-DR, and (iv) 100 μM of 17-DR for 24 h, respectively. Apoptosis was measured by the propidium iodide method using flow cytometry. (**B**) The 17-DR down-regulated RIP1 levels. Whole-cell lysates from MDA-MB-231 cells treated with vehicle or various concentrations of 17-DR for 24 h were subjected to western blot analysis. (**C**) Effect of 17-DR on the expression of IκBα.

These results confirm that 17-DR induced apoptosis in MDA-MB-231 cells.

We next examined whether apoptosis-related proteins (Bcl-2, Bax, caspase 3, and PARP) were regulated by 17-DR. As shown in Fig. S2, which illustrates with increasing concentrations of 17-DR, antiapoptotic protein Bcl-2 was down-regulated, and proapoptotic protein Bax expression gradually increased. In addition, the caspase 3 protein cleavage fragment became more obvious and resulted in a dose-dependent cleavage of PARP.

17-DR Induces Apoptosis *via* Down-Regulation of RIP1 in MDA-MB-231 Cells

A number of cancer-associated proteins have been identified as Hsp90 clients, including RIP1 and MMP-9 protein, to name but a few [6, 27]. RIP1 is expressed in multiple tissues, as a sensing protein necessary for the integral variety of membrane stress signals inside or outside the cell. It not only activates NF-κB against apoptosis, but also induces cell death, including non-caspase-dependent apoptosis and necrosis. The gradual decrease of RIP1 protein levels was observed after treatment with 17-DR at 50 and 100 μM concentrations (Fig. 2B). The activity of NF-κB is regulated by the endogenous inhibitor IκBα, and interaction with IκBα blocks the nuclear localization of NF-κB [2]. In order to understand the relationship between RIP1 and NF-κB, we detected IκBα expression in the cells by 17-DR treatment. As shown in Fig. 2C, there was no reduction in the expression of IκBα, thus inhibiting the activation of NF-κB. RIP1 is an important upstream signaling protein in the cell death receptor-mediated apoptosis pathway; changes in the level of its expression by 17-DR treatment are, to some extent, involved in the regulation of apoptosis.

17-DR Changes the Expression of Oncoproteins Involved in the Invasion and Migration Potential of MDA-MB-231 Cells

We examined the effects of 17-DR on MDA-MB-231 cell invasion and migration. Low concentrations of 17-DR did not have a significant effect on cell death (Fig. 1B), but suppressed cell invasion and migration. As shown in Fig. 3, with increasing concentrations of 17-DR treatment, invaded cells gradually decreased ($p < 0.05$ *vs* control). Next, we examined the effect of 17-DR on cell migration using the transwell cell migration assay and wound-healing assay. The results showed that with increasing concentrations of 17-DR, the migration inhibition rate also increased (Fig. 4 and Fig. S3).

Matrix metalloproteinases, especially MMP-9, as well as its inhibitor, play an important role in tumor invasion and metastasis. The imbalance between MMPs and their inhibitors may facilitate tumor progression [7]. The tissue inhibitors of MMPs (TIMPs) provide a negative control for

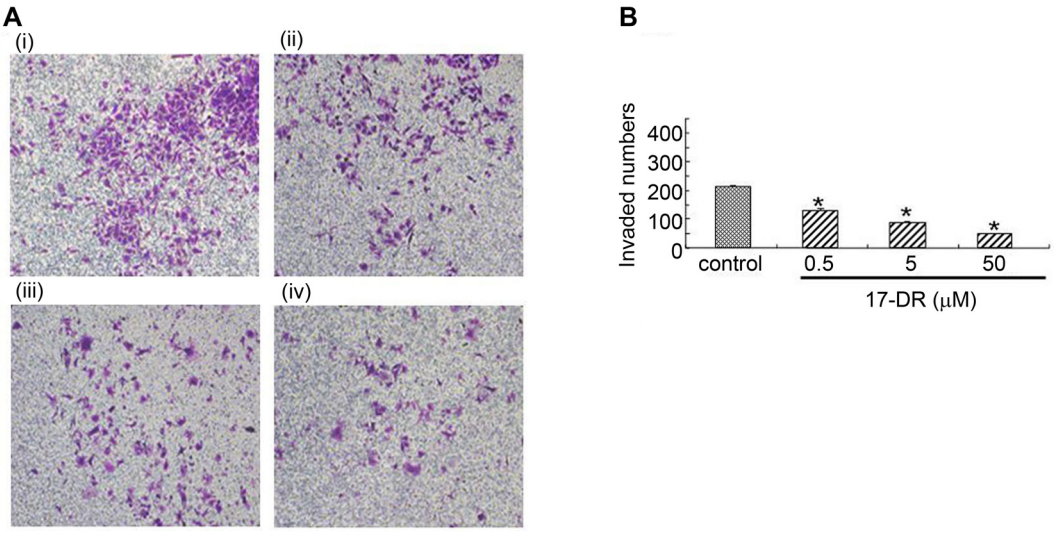

Fig. 3. 17-DR suppressed the invasion of MDA-MB-231 cells.
(**A**) The invasion of MDA-MB-231 cells was suppressed following exposure to vehicle or various concentrations of 17-DR. Using 17-DR-untreated cells as the controls, five visual fields were randomly selected for each insert and photographed under a light microscope at 200× magnification. (**B**) Quantification of invasion of MDA-MB-231 cells inhibited by 17-DR. *$p < 0.05$ compared with the control.

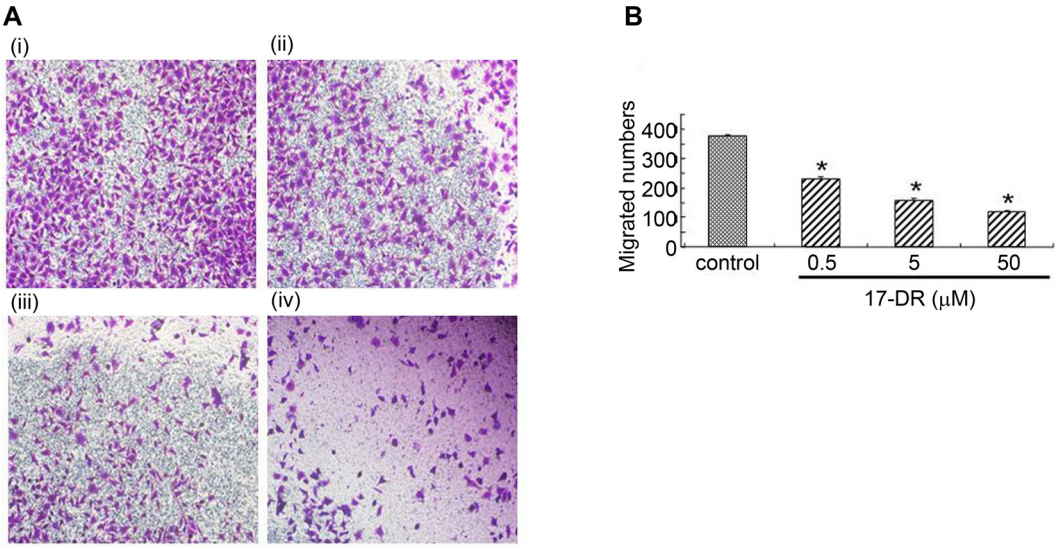

Fig. 4. 17-DR suppressed the migration of MDA-MB-231 cells.
(**A**) The migration of MDA-MB-231 cells was suppressed following exposure to vehicle or various concentrations of 17-DR. Using 17-DR-untreated cells as the controls, five visual fields were randomly selected for each insert and photographed under a light microscope at 200× magnification. (**B**) Quantification of migration of MDA-MB-231 cells inhibited by 17-DR. *$p < 0.05$ compared with the control.

MMPs activity. Among them, TIMP-1 has been characterized most extensively. In addition, MMP-9 levels in tumor tissue, as well as in serum, plasma, and urine, are significantly elevated in patients with breast cancer [25, 28]. To determine whether MMP-9 or TIMP-1 is changed in 17-DR-treated cells, we investigated the expression of these proteins. As shown in Fig. 5, MMP-9 protein expression was gradually down-regulated with increasing concentrations of 17-DR; meanwhile, TIMP-1 protein gradually increased.

Moreover, Kim *et al*. [12] showed that 17-AAG inhibited IκBα kinase-dependent IκBα phosphorylation/degradation, NF-κB activation, and MMP-9 expression. This leads to the

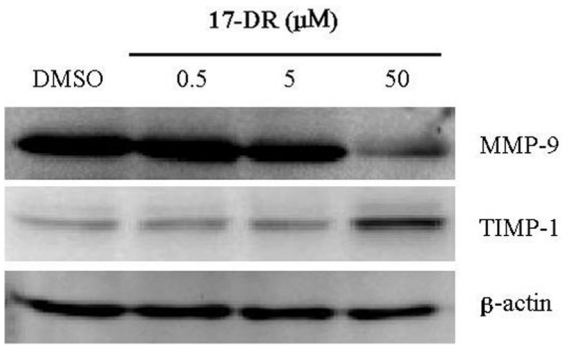

Fig. 5. Effects of 17-DR on the expression of MMP-9 and TIMP-1. Whole-cell lysates from MDA-MB-231 cells treated with vehicle or various concentrations of 17-DR for 24 h were subjected to western blot analysis.

suppression of hyaluronic acid-induced highly invasive glioma cell migration and invasion. NF-κB can activate transcription of its target genes, including MMPs [2]. We have shown that there was no reduction in the expression of IκBα by 17-DR, inhibiting NF-κB activation. The experiments confirmed that 17-DR can significantly suppress the invasion and migration of MDA-MB-231 cells. Its mechanism may be related to the down-regulation of MMP-9 protein, whilst increasing the expression of TIMP-1 protein, thereby suppressing cell invasion and migration.

GA and its benzoquinone analogs conjugate with glutathione, leading to cellular depletion. This conjugation with sulfur-containing nucleophiles may contribute to the dose-limiting hepatotoxicity of these benzoquinone-containing compounds. For these reasons, non-benzoquinone GA analogs with improved pharmacological profiles are needed. Recently, related natural products that lack the problematic quinone have been discovered, synthesized, and demonstrated to also have potent Hsp90-inhibitory profiles (*i.e.*, reblastatin), albeit harboring molecular features that likely impart a similarly suboptimal solubility profile [24].

The non-benzoquinone GA analog 17-DR also showed stronger yeast Hsp90 ATPase inhibition activity (IC_{50}, 1.82 μM) compared with the original Hsp90 inhibitor GA (IC_{50}, 3.19 μM). The concentrations (5 and 50 μM) of 17-DR applied in the invasion and migration assays were much larger than the IC_{50} of Hsp90 ATPase inhibition in this experiment. Taking into account the human and yeast Hsp90 homology, these concentrations can inhibit the chaperone function mediated by Hsp90. Taken together, 17-DR as a non-quinone GA analog could improve binding properties to Hsp90.

In conclusion, this study demonstrated the anticancer effects of the Hsp90 inhibitor 17-DR on breast cancer MDA-MB-231 cells. It is associated with potent reduction in the proliferative, antiapoptotic, invasion, and migration potential of the cells. The down-regulation by 17-DR of multiple key Hsp90-dependent tumor-driving molecules, such as RIP1 and MMP-9 protein, may be related to this effect. In summary, this study supports the proposal that 17-DR has considerable anticancer activity, and deserves further investigation; 17-DR may provide the basis for the development of rational drug combinations for treating TNBC.

Acknowledgments

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