



Research article

Anticancer activity of RAPTA-EA1 in triple-negative BRCA1 proficient breast cancer cells: single and combined treatment with the PARP inhibitor olaparib

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ABSTRACT

RAPTA-EA1 is a promising glutathione transferase (GSTP-1) inhibitor that has previously been shown to inhibit the growth of various breast cancer cells. We studied the anticancer activity of RAPTA-EA1 on triple-negative BRCA1 competent breast cancer MDA-MB-231 cells. MDA-MB-231 cells are significantly more sensitive to RAPTA-EA1 than MCF-7 cells. Treatment reveals a higher degree of cytotoxicity than cisplatin against both cell lines. Ruthenium accumulation in MDA-MB-231 cells is mainly in the nuclear fraction (43%), followed by the cytoplasm (30%), and the mitochondria (27%). RAPTA-EA1 blocks cell growth at the G2/M phase, leading to nuclear condensation and cell death. The compound slightly inhibits DNA replication of the 3,426-bp fragment of the BRCA1 exon 11 of the cells, with approximately 0.6 lesion per the BRCA1 fragment. The expression of BRCA1 mRNA and its protein in the Ru-treated cells is curtailed by 50–80% compared to the untreated controls. Growth inhibition of the triple-negative BRCA1 wild-type MDA-MB-231 and the sporadic BRCA1 wild-type MCF-7 cells by olaparib (a poly [ADP-ribose] polymerase (PARP) inhibitor) is dose-dependent, with MDA-MB-231 cells being two-fold less susceptible to the drug than MCF-7 cells. Combining olaparib with RAPTA-EA1 results in a combination index (CI) of 0.78 (almost additive) in MDA-MB-231 cells and 0.24 (potent synergy) in the MCF-7 cells. The PARP inhibitor alone differently regulates the expression of BRCA1 mRNA in both cell lines, whereas the olaparib-RAPTA-EA1 combination induces overexpression of BRCA1 mRNA in these cells. However, the expression level of the BRCA1 protein is dramatically reduced after treatment with the combined inhibitors, compared with the untreated controls. This observation highlights the cellular responses of triple-negative BRCA1 proficient breast cancer MDA-MB-231 cells to RAPTA-EA1 through BRCA1 inhibition and provides insights into alternative treatments for breast cancer.

1. Introduction

Breast cancer is a diverse disease and has been classified into biological subtypes with distinct histopathological, genetic and epigenetic characteristics [1]. Based on gene expression profiling, breast cancer can be divided into five major breast tumor subtypes that include, luminal A, luminal B, overexpression of human epidermal growth factor receptor 2 (HER2), basal-like, and normal breast-like. The basal-like subtypes have distinctive histological features, response to chemotherapy, and clinical outcomes. Patients with basal-like cancers have main characteristics

similar to those with the triple-negative breast cancers (TNBC) that are defined as none expression of estrogen receptor (ER), progesterone receptor (PR), and HER2, which are often observed in younger patients (<50 years) and more frequently in African-American women and black ethnicities [1]. TNBC patients exhibit important clinical implications with diverse histology, high grade, and high rate of recurrence and metastases within 5 years of the initial diagnosis. They have a poorer overall survival than other subtypes of breast cancers and do not normally benefit from currently viable targeted therapies, such as endocrine or anti-HER2 agents, and consequently, these patients rely primarily on

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only systemic treatment options, and chemotherapy with standard cytotoxic agents [2]. Currently, there is no preferred standard form of chemotherapy for TNBC. The first-line treatment for TNBC includes a combination of surgery, radiation, and neoadjuvant/adjuvant chemotherapy. In a clinical study, TNBC patients who received platinum-based drugs had a significantly higher pathological response compared to patients treated with non-platinum drugs (64.3 versus 49.2%) [2]. However, platinum-based drugs have several disadvantages such as induced secondary mutations, acquired resistance and high general toxicity [3]. Many non-platinum-based metal complexes have therefore been investigated as new leads that potentially overcome the limitations of platinum compounds. One promising class is ruthenium-based complexes, including arene-ruthenium (II)-complexes containing PTA (1,3,5-triaza-7-phosphaadamantane), often termed RAPTA (Figure 1) [4].

RAPTA compounds such as RAPTA-C and RAPTA-T exhibit anti-metastatic [5] and antiangiogenic [6] properties and are active against various tumor types [7, 8]. It appears that while these compounds can bind to DNA, they preferentially bind to proteins [9, 10]. It has been reported that RAPTA compounds perturb the BRCA1 RING protein domain secondary structure, resulting in the displacement of zinc ions, and leading to loss of BRCA1-mediated E3 ubiquitin ligase activity [11]. A RAPTA compound with ethacrynic acid (EA) tethered to the arene ring, termed RAPTA-EA1, is a strong inhibitor of glutathione-S-transferase P1 (GSTP-1) that inhibits growth of various cancer cells [5, 12]. GSTP-1 represents the most prevalent mammalian isoenzyme of glutathione S-transferases (GSTs) that plays an important role in detoxification and antioxidant defense [12]. It has recently been shown that GSTP-1 inhibition is a promising strategy for TNBC treatment [13]. Previous studies have shown that ruthenium (II)-based compounds exhibited promising anticancer activities to treat BRCA1-defective TNBC [14, 15, 16, 17]. The BRCA1 gene responds to DNA damage through multi-cellular pathways including cell cycle regulation, ubiquitination, DNA repair and transcription [14, 15, 16, 17]. RAPTA-EA1 exhibits different cellular responses for the triple-negative BRCA1-mutant HCC1937 cells and the sporadic BRCA1-wild type MCF-7 cells [18]. Differences in anticancer activity were associated with differential ruthenium uptake into breast cancer cells and subsequent apoptosis. RAPTA-EA1 induced significantly more damage to the BRCA1 gene in the BRCA1-mutant HCC1937 cells than to the BRCA1-wild type MCF-7 cells, and reduced the expression level of the BRCA1 protein in both types of breast cancer cells as well [18].

A combination treatment comprising cisplatin and a PARP inhibitor is one of the therapeutic options used to improve the treatment of BRCA1-deficient breast cancer cells [19]. One of the potential PARP inhibitors is olaparib, enhancing synthetic lethality in BRCA1-deficient cancer cells [20], i.e. the cancer cells harboring the mutated BRCA1 gene are

defective in homologous recombination repair [21]. Recent clinical trials assessing olaparib in combination with platinum-based drugs reveal encouraging efficacies [22, 23]. Using olaparib in combination with other metal-based drugs, i.e., cisplatin or carboplatin has been studied in pre-clinical models as a new targeted therapy against BRCA-deficient cancers [22, 23].

The MDA-MB-231 cell line, isolated at MD Anderson from a pleural effusion of a patient with invasive ductal carcinoma, is commonly used to model late-stage breast cancer, and are a good model of TNBC. TNBC MDA-MB-231 cells are resistant to many anti-cancer agents [24, 25, 26], although they are sensitive to some ruthenium (II)-arene complexes [24, 25, 26]. To address the effect of RAPTA-EA1 on this particular subtype of breast cancer cells, hence, we decided to evaluate the anticancer efficacy of RAPTA-EA1 against triple-negative BRCA1 wild-type breast cancer MDA-MB-231 cells through BRCA1 inhibition. Combination treatment of the PARP inhibitor olaparib with RAPTA-EA1 is also reported, in comparison with the sporadic BRCA1 wild-type MCF-7 cells.

2. Materials and methods

2.1. Materials

RAPTA-EA1 was prepared using a literature method [18]. Cisplatin was obtained from Sigma-Aldrich (USA), and prepared as stock solutions (1–5 mM) in deionized water.

2.2. Cell culture

Human breast cancer cell lines, triple-negative MDA-MB-231 (BRCA1 wild-type) and MCF-7 (sporadic BRCA1 wild-type), were procured from the American Type Culture Collections (ATCC, Rockville, MD). Cells were seeded in 6-well plates with 1 mL of Dulbecco's modified eagle's medium (DMEM) without phenol red, supplemented with fetal bovine serum 10% (FBS), penicillin-streptomycin 1%. Cells were incubated in 5% CO₂ at 37 °C.

2.3. MTT proliferation assay

About 5×10^4 cells (MDA-MB-231 or MCF-7) were plated into each well of a 96-well culture plate and incubated for 48 h at 37 °C in a 5% CO₂ incubator. The culture medium was then removed and 200 μ L of fresh culture medium with various concentrations of RAPTA-EA1 and cisplatin (as a control) was added. The breast cancer cells were further incubated for 48 h. After incubation, each well of the 96-well culture plate was then washed twice with 100 μ L of phosphate-buffered saline (PBS). The solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (0.5 mg/mL) was added to each well and incubated for additional 4 h. The MTT solution was gently removed and dimethylsulfoxide (200 μ L) was added to solubilize the crystals of purple formazan formed. The absorbance of each well was

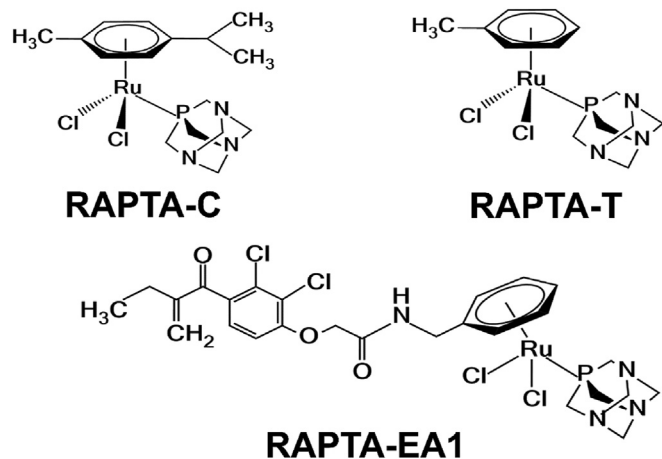


Figure 1. Chemical structure of the arene-ruthenium (II)-complexes containing PTA (1,3,5-triaza-7-phosphaadamantane, often termed RAPTA).

Table 1. Cytotoxicity of MDA-MB-231 and MCF-7 cells induced by IC₅₀ values (μ M) of RAPTA-EA1 and cisplatin using the MTT assay after 48 h of treatment (data reflect the mean and \pm SEM of results from three separate experiments, each performed in triplicate).

Compound	IC ₅₀ (μ M)	
	MDA-MB-231	MCF-7
RAPTA-EA1	10.5 \pm 0.5*,**	20.0 \pm 2.2*,**
cisplatin	128.0 \pm 3.0*,**	42.0 \pm 2.0*,**

Statistically significant differences are indicated by * $p < 0.01$, compared the IC₅₀ values of the same compound on cell lines, and ** $p < 0.01$, compared the IC₅₀ values of the same compounds on each cell line. All experiments were performed in triplicate.

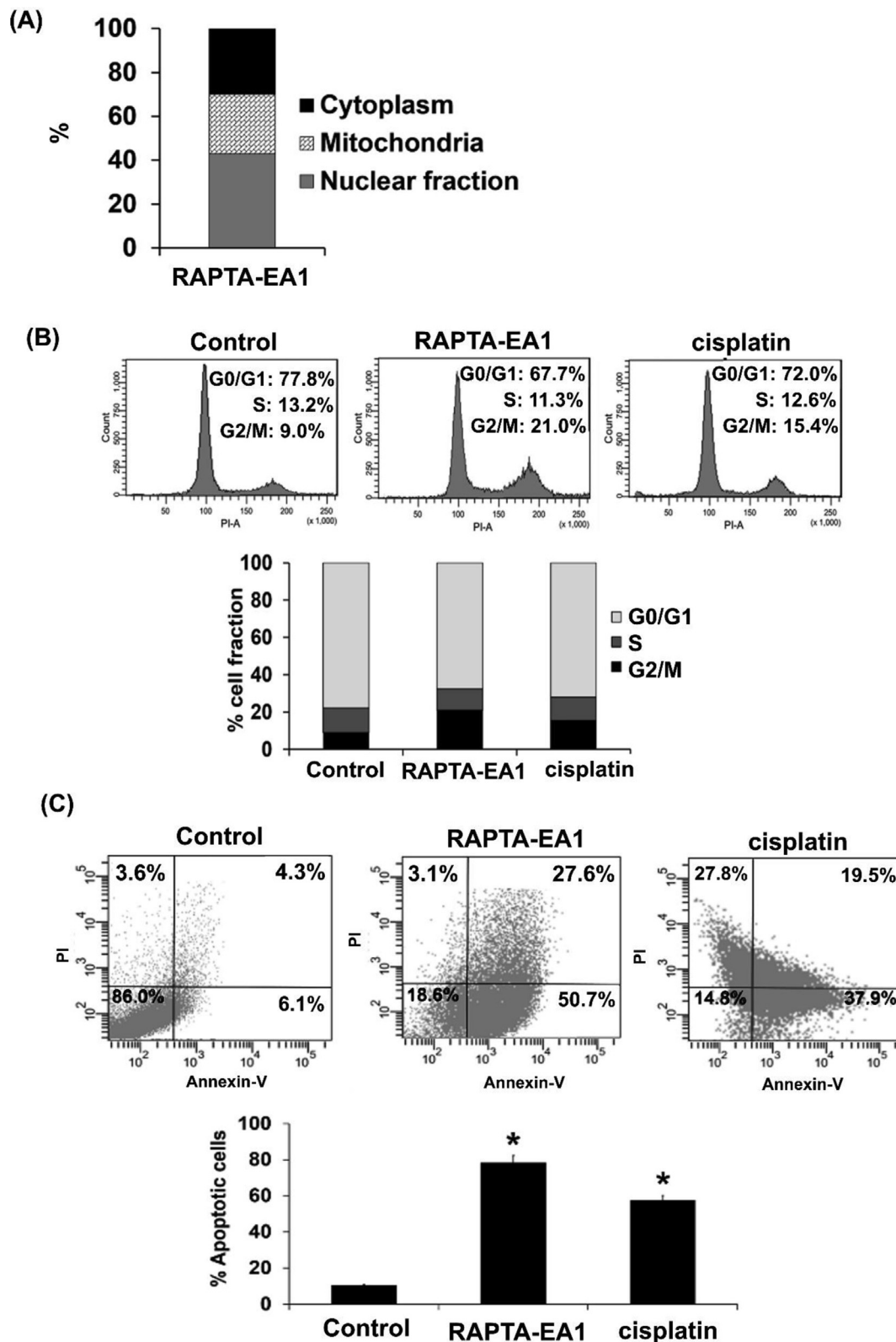


Figure 2. Cellular uptake, cell cycle progression, and apoptosis of RAPTA-EA1-treated MDA-MB-231 cells. (A) Accumulation and distribution of ruthenium in breast cancer cell lines were treated with RAPTA-EA1 at the IC₅₀ concentration (10 μM) at 37 °C for 48 h. The distribution of ruthenium was determined in the nuclear fraction, mitochondria and cytoplasm using ICP-MS. (B) Quantification of DNA by PI staining after treatment of the cells with RAPTA-EA1 (10 μM) or the IC₅₀ concentration of cisplatin (128 μM) (Table 1) at 37 °C for 48 h. FACS (fluorescence-activated cell sorting) was used to investigate the proportion of cells arrested at multifarious phases of the cell cycle. Each experiment used 20,000 cells. (C) Determination of starting apoptotic cells (Annexin V-positive/PI-negative) and late apoptosis cells (Annexin V-positive/PI-positive) following incubation with RAPTA-EA1 (10 μM) or cisplatin (128 μM) at 37 °C for 48 h. Flow cytometric analysis was used to measure cells that bind Annexin V-FITC and PI apoptosis staining. Experiments were performed in triplicate and the bar represents the standard error (*p < 0.01).

spectrophotometrically measured at 570 nm using an automated microplate reader. The cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{absorbance of the Ru-treated cells}}{\text{absorbance of the control (Ru-untreated cells)}} \right) \times 100$$

Percentage cell viability was plotted against various concentrations of the ruthenium compound or cisplatin to determine the 50% inhibitory concentration (IC_{50}), defined as the concentration of the ruthenium compound or cisplatin which inhibited cell viability by at least 50% compared to the control condition [18].

2.4. Cellular accumulation and distribution

About 5×10^6 MDA-MB-231 cells were incubated with RAPTA-EA1 (IC_{50} concentration of 10 μM , Table 1) at 37 °C for 48 h under 5% CO_2 [18]. Cells were washed twice with 5 mL of PBS buffer and the cells were then fractionated, and the ruthenium content distributed in the cytoplasm, mitochondria and nucleus was determined by means of mass spectrometry equipped with inductively coupled plasma (ICP-MS) (Agilent Technologies, USA) [27].

2.5. Cell cycle analysis

Approximately 10^6 MDA-MB-231 cells were added into 6-well culture plates and grown at approximately 80% confluence. The cells were treated with RAPTA-EA1 (IC_{50} concentration of 10 μM) or cisplatin (128 μM) (Table 1) at 37 °C for 48 h under 5% CO_2 . The breast cancer cells were then prepared as described previously [14]. Briefly, cells were trypsinized using 0.25% trypsin, washed with PBS buffer, and then centrifuged at 300 g for 5 min. The cells were collected and fixed with cold ethanol (70%) and stored overnight at 20 °C. The cell pellets were washed and re-suspended in PBS (1 mL) containing RNase (100 $\mu\text{g}/\text{mL}$), PI (50 $\mu\text{g}/\text{mL}$), and Triton-X-100 (0.1%), and then placed in an incubator at 37 °C for 30 min in the dark. Fluorescence was recorded using a FACSCanto flow cytometer. MultiCycle software was used to analyze the cell cycle distribution, which represents the percentages of the cells at the G0/G1, S and G2/M phases.

2.6. Apoptosis detection by Annexin V

Approximately 10^6 MDA-MB-231 cells were added into 6-well plates and grown to approximately 80% confluent. The cells were treated with RAPTA-EA1 (IC_{50} concentration of 10 μM) or cisplatin (128 μM) (Table 1) at 37 °C for 48 h under 5% CO_2 . After incubation, the cells were harvested, washed twice with PBS buffer, and then centrifuged at 300g

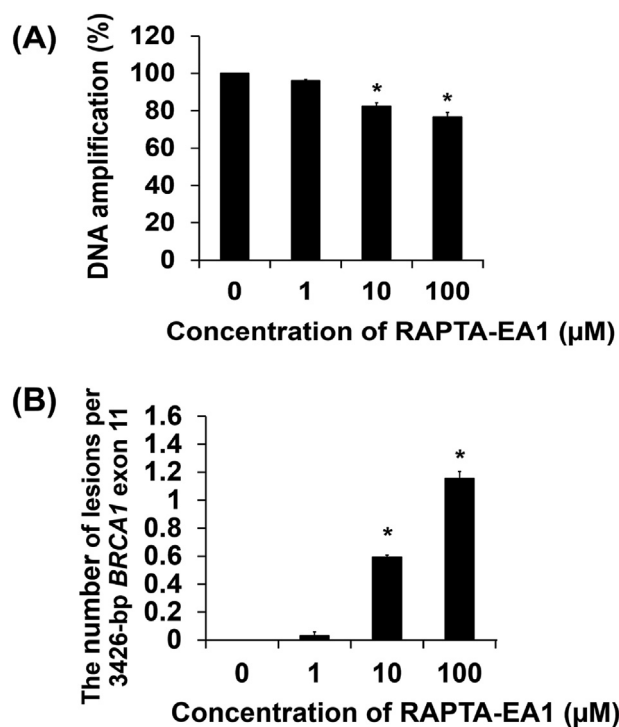


Figure 4. Cellular BRCA1 damage in MDA-MB-231 cells. Cells were incubated with RAPTA-EA1 at different concentrations (0–100 μM) at 37 °C for 48 h. The fragment of BRCA1 was used as the DNA target which comprises 3426 bp located on BRCA1 exon 11. It was isolated from MDA-MB-231 cells, multiplied by PCR then the amplified DNA fragment was electrophoresed on 1% agarose gel. Staining was performed using ethidium bromide and imaged in the presence of ultraviolet light. The band intensity indicates the magnification of products quantified on a Bio-Rad Molecular Imager. The amount of DNA amplification (%) was plotted as a function of concentration (A). RAPTA-EA1-induced damaged BRCA1 fragment (lesions per the 3426-bp fragment) in MDA-MB-231 cells was calculated by the Poisson equation [14] (B). Experiments were performed in triplicate and the bar represents the standard error of experiments. Statistically significant differences from the untreated control cells are indicated by * $p < 0.01$.

for 5 min. One hundred microliter of 1x Annexin-binding buffer was filled to re-suspend the cell pellets. Alexa-Fluor 488 Annexin-V (5 μL), and PI (1 μL) (at 100 $\mu\text{g}/\text{mL}$) were added and the cells were further incubated at room temperature for 15 min. The solution of 1x Annexin-

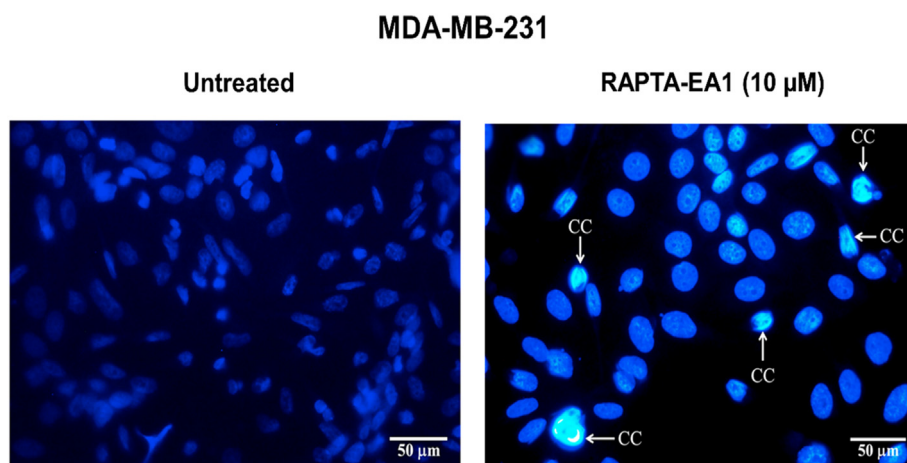


Figure 3. Immunofluorescence assay. Cells treated with RAPTA-EA1 (10 μM) were seeded on coverslips, fixed using 4% paraformaldehyde for 30 min and then permeabilized using 0.2% Triton X-100. The cells were incubated with blocking reagent for 1 h at ambient temperature and then their nuclei were treated with counterstaining, 4', 6-diamidino-2-phenylindole (DAPI; 1 $\mu\text{g}/\text{mL}$). CC stands for chromatin condensation.

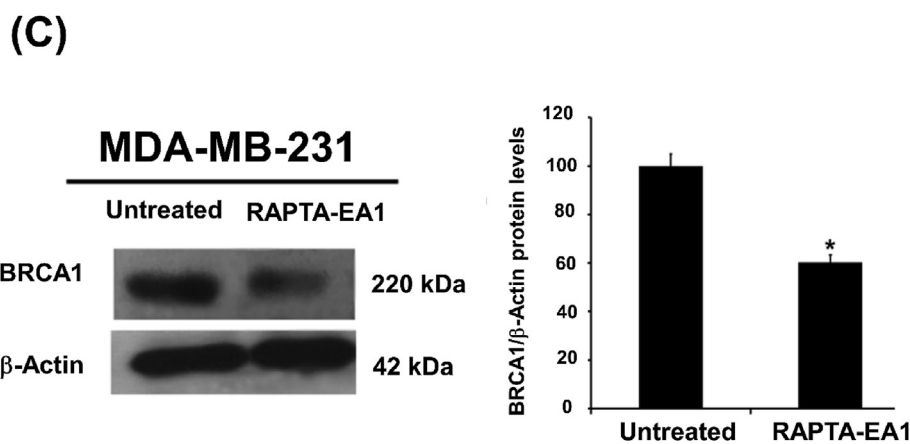
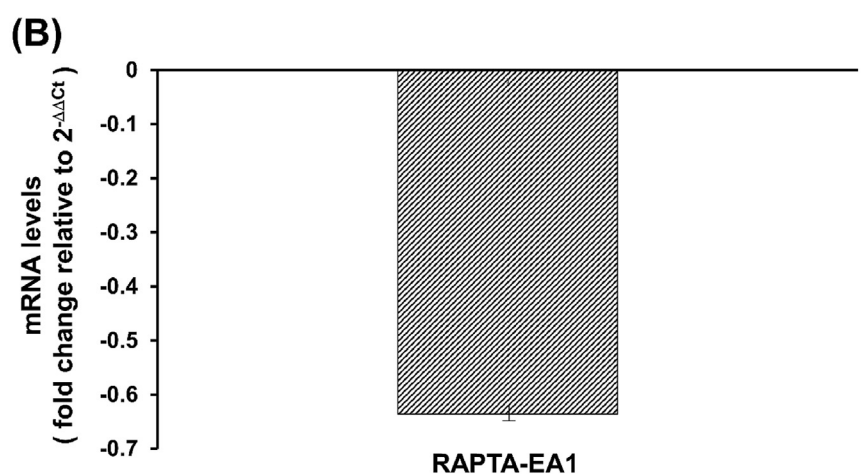
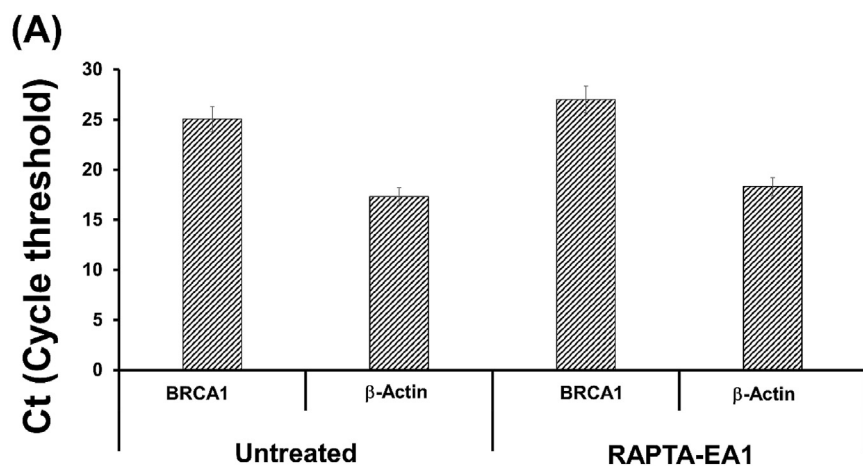


Figure 5. Effect of RAPTA-EA1 on the expression of BRCA1 mRNA and its protein in MDA-MB-231 cells. The transcribed BRCA1 gene was quantified by RT-PCR, normalized to the reference gene β -Actin and treatment-free control cells (A, B). Data were calculated using the $2^{-\Delta\Delta Ct}$ method [14]. Protein expression was performed by Western blot analysis (C and SI-Fig. 1). Experiments were performed in triplicate and the bar represents the standard experimental errors. Statistically significant differences from the untreated control cells are indicated by * $p < 0.01$.

binding buffer (400 μ L) was subsequently added. Analysis of Annexin-V binding was performed using a FACSCanto flow cytometer with an excitation wavelength of 488 nm [8, 18].

2.7. Immunofluorescence assay

MDA-MB-231 cells treated with RAPTA-EA1 (10 μ M) were incubated at 37 $^{\circ}$ C for 48 h were seeded on coverslips, fixed with 4% paraformaldehyde for 30 min, and then permeabilized with 0.2% Triton X-

100. The cells were incubated with blocking reagent [(3% bovine serum albumin in Tris buffered saline with 0.1% Tween x20 (TBST)] for 1 h at room temperature and then their nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 1 μ g/mL).

2.8. Utilization of QPCR for cellular BRCA1 damage

RAPTA-EA1 at a various concentration (0–100 μ M) was incubated with MDA-MB-231 cells at 37 $^{\circ}$ C for 48 h under CO₂ 5%. The genomic

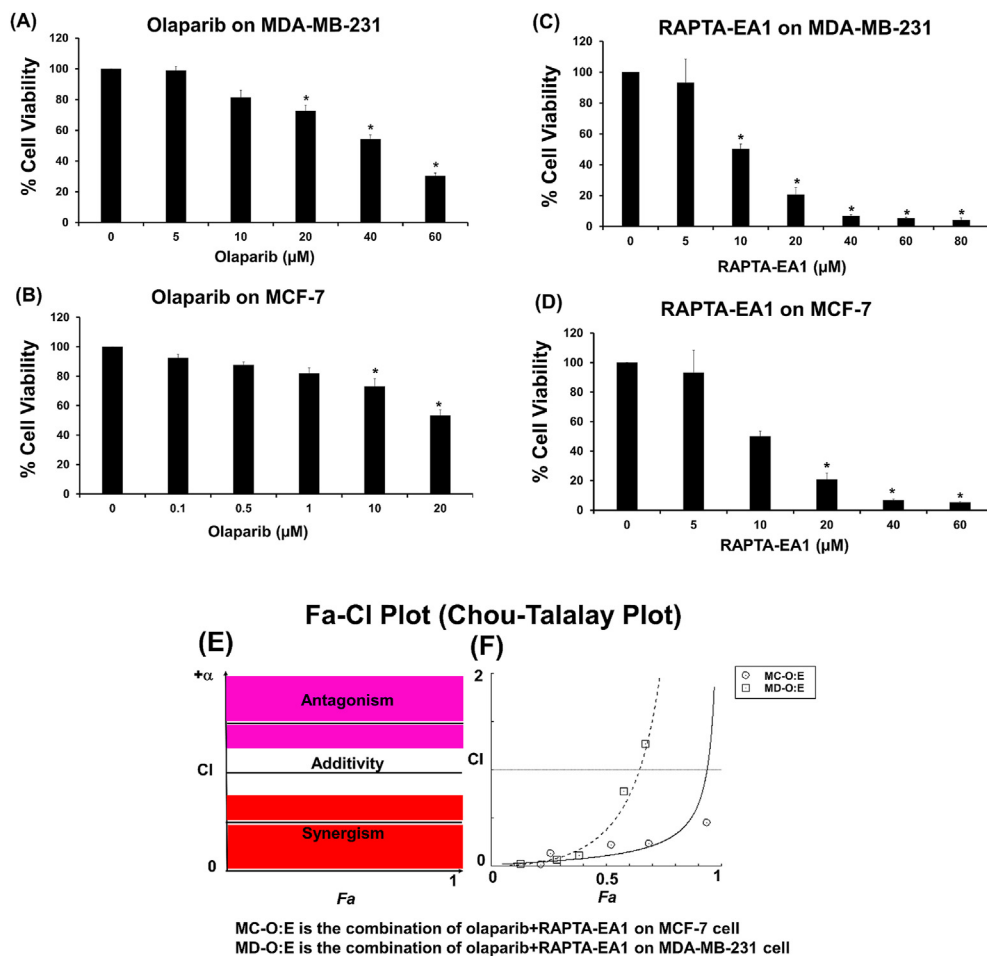


Figure 6. The cell growth inhibitory effect of olaparib alone and in combination with RAPTA-EA1. MDA-MB-231 and MCF-7 cells were treated with various concentrations of olaparib (A, B), RAPTA-EA1 (C, D) or olaparib in combination with RAPTA-EA1 (E, F) for 48 h at 37 °C. Experiments were performed in triplicate and the bar represents the standard experimental errors. Statistically significant differences were considered $p < 0.01$. Entering a series of "dose (D) and effect (Fa)" into computer for each drug alone and their combinations, the software will automatically simulates the CI values at different Fa levels in seconds, based on the CI algorithm. This plot is called the Fa-CI plot or the Chou-Talalay plot, calculated by CompuSyn program (ComboSyn, Inc.) (E, F).

DNA of MDA-MB-231 cells was collected as previously described [18]. The BRCA1 fragment (exon 11, 3426 bp) was used as the DNA target and then amplified by PCR [14]. The reaction mixture (50 μL) consisted of a rutenated genomic-DNA template at 400 ng, the forward primer (5'-GCCAGTTGGTTGATTCCACC-3') and reverse primer (5'-GTAAAATGTGCTCCCAAAAGC-3') was kept as 0.5 μM , MgCl_2 (300 μM) 1xPhusion™ GC Buffer, and Phusion Hot-Start DNA-polymerase 2 units. Amplification of BRCA1 was performed using PCR following initial denaturation at 94 °C for 3 min; 30 cycles of 30 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C, and a final extension for 7 min at 72 °C. PCR products were electrophoresed on 1% agarose gels at 100 V. Gel staining was done with ethidium bromide and imaged in the presence of ultraviolet light. The band intensity indicated the magnification of products quantified on a Bio-Rad Molecular Imager Densitometer along with the Molecular Dynamics program (version 1.0.2. Bio-Rad, Hercules, CA, USA, 1994). The amount of DNA amplification (%) was plotted as a function of concentration. RAPTA-EA1-induced BRCA1 fragment (lesions per the 3426-bp fragment) in MDA-MB-231 cells was calculated using the Poisson equation [14]. Experiments were conducted in triplicate.

2.9. Quantitative analysis of expression of BRCA1 mRNA following RAPTA-EA1 treatment using real-time quantitative RT-PCR

MDA-MB-231 cells treated with RAPTA-EA1 (10 μM) were incubated at 37 °C for 48 h. The cells were harvested, and the total cellular RNA was isolated using an RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA (1 μg) was used for the first strand synthesis of complimentary-DNA through QuantiTech® Reverse-

Transcription (Qiagen, Germany) following the manufacturer's instructions. Specific primers for DNA amplification; BRCA1 forward: 5'-GCCAGTTGGTTGATTCCACC-3'; BRCA1 reverse: 5'-GTCAAATGTGCTCCCAAAAGC-3'; β -Actin forward: 5'-CCGTAAAGACCTCTATGCCAACA-3'; β -Actin reverse: 5'-CGGACTCATCGTACTCCTGCT-3'. Real-time PCR reactions were then performed in a total volume of 25 μl including 100 ng of the cDNA template, 12.5 μl of QuantiFast SYBR green PCR master mix, and the final concentration of primers of 0.5 μM . The PCR conditions were as follows: an initial step at 95 °C for 5 min, followed by 35 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Fluorescence intensity was measured during the annealing step on an ABI-Prism 7300 analytical thermal cycler (Applied Biosystems). The RT-PCR data were analyzed according to the $2^{-\Delta\Delta C_T}$ method [14], and normalized by β -Actin mRNA expression in each sample. Experiments were performed in triplicate.

2.10. Western blot analysis

MDA-MB-231 cells treated with RAPTA-EA1 (10 μM) were incubated at 37 °C for 48 h. The cells were collected, and the entire cell extract was separated on a SDS-polyacrylamide gel electrophoresis (6% SDS-PAGE) and subsequently subjected to Western blotting as described previously [6]. In brief, the chemiluminescent HRP substrate (Clarity™ Western ECL substrate, BioRad) was used for the development of the bands using a high-performance chemiluminescent film (Amersham Hyperfilm™ ECL). For BRCA1, the primary antibody was a mouse monoclonal IgG1 antibody (BRCA1 (Ab1) Mouse (MS110)) (Calbiochem, EMD Millipore) at 1:1000 dilution. The secondary antibody was a HRP conjugated, goat anti-mouse IgG (HAF, R&D System) at 1:5000 dilution.

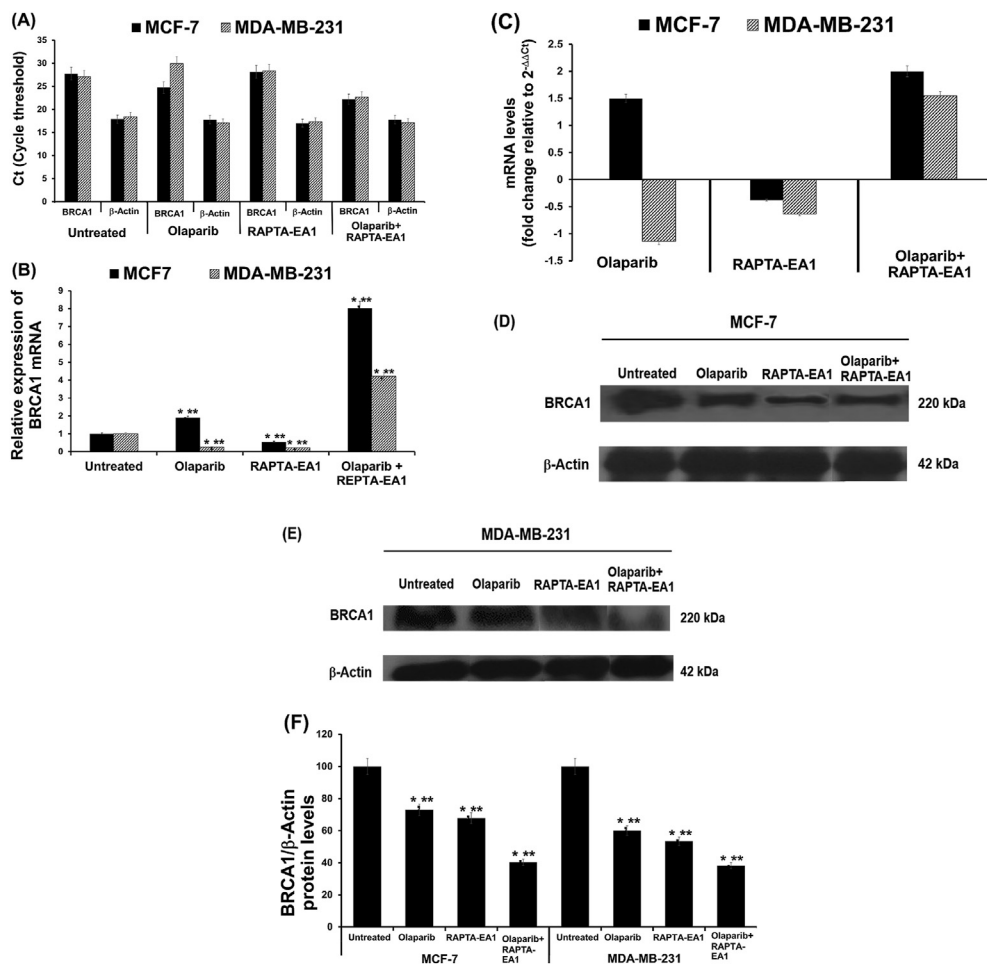


Figure 7. Differential expression of the BRCA1 mRNA and its protein. The amount of transcribed BRCA1 genes was determined quantitatively using RT-PCR, β -Actin expression was used as the normalized gene, compared to the untreated control cells. Data were analyzed according to the $2^{-\Delta\Delta C_t}$ method [14]. Experiments were performed in triplicate. A: cycle threshold (Ct) for each sample, B: relative expression of BRCA1 mRNA, C: fold change for BRCA1 mRNA between MCF-7 and MDA-MB-231 sample. D-E (SI-Fig. 2), and F: Western-blot analysis of BRCA1 protein expression in cells treated with olaparib, RAPTA-EA1 and olaparib-RAPTA-EA1 combination. In all experiments cells were harvested after 48 h treatment. * $p < 0.01$, compared the expression of the BRCA1 mRNA or its protein on each cell line treated by single or combination treatment in untreated control cells, and ** $p < 0.01$, compared the expression of the BRCA1 mRNA or its protein treated by the same.

2.11. Combination treatment of RAPTA-EA1 with olaparib

The cytotoxic effects of RAPTA-EA1 and olaparib either alone at various concentrations (0–60 μ M) or in combination (0.01–20 μ M, at a constant ratio of 1:1) were examined in MDA-MB-231 and MCF-7 cells using the MTT assay [18]. Additive, antagonistic or synergistic interactions were assessed using the combination index (CI) with the Chou-Talalay method [28], calculated by CompuSyn program (ComboSyn, Inc.).

2.12. Statistical analysis

SPSS version 22.0 was used for statistical analysis. All values are expressed as mean \pm standard error of the mean (SEM) comparisons between two groups conducted by One-way ANOVA. Group differences resulting in * $p < 0.01$ were considered statistically significant.

3. Results

3.1. Cytotoxic effect of RAPTA-EA1 on MDA-MB-231 breast cancer cells

The cytotoxicity effect of RAPTA-EA1 on MDA-MB-231 breast cancer cells was performed by a MTT assays, using cisplatin as a control. The results of the MTT assays are shown in Table 1. MDA-MB-231 cells were significantly more sensitive to RAPTA-EA1 than MCF-7 cells. The cytotoxicity of RAPTA-EA1 was found to be much higher ($IC_{50} = 10.5 \pm 0.5$) than that of cisplatin ($IC_{50} = 128.0 \pm 3.0$) in MDA-MB-231 cells. Therefore, we used the concentration of RAPTA-EA1 at its approximate IC_{50} value (10 μ M) to perform all further experiments.

3.2. RAPTA-EA1 uptake into MDA-MB-231 cells blocks G2/M cell cycle and induces nuclear condensation leading to apoptosis

The ruthenium content in the MDA-MB-231 cells following treatment with RAPTA-EA1 (10 μ M) for 48 h was determined using Inductive Coupled Plasma Mass Spectrometry (ICP-MS). The ruthenium was distributed in the nuclear fraction (43%), cytoplasm (30%), and mitochondria (27%) of the MDA-MB-231 cells (Figure 2A). In a previous study RAPTA-EA1 was shown to accumulate in the nuclear fraction and cytoplasm of MCF-7 cells and in the cytoplasm and mitochondrial fraction of HCC1937 cells [18].

The effect of RAPTA-EA1 on cell cycle progression was also studied with the compound inducing a high level of G2/M phase compared with cisplatin and the control (Figure 2B). Therefore, further analysis using Annexin-V flow cytometry was conducted to determine the cell injury, with RAPTA-EA1 inducing apoptosis to a greater extent than in cisplatin-treated cells (78.3 vs. 57.4%), compared to the untreated control cells (10.4%) (Figure 2C). This data closely agrees with a previous study that showed RAPTA-EA1 efficiently induces apoptosis in triple-negative, BRCA1-defective, HCC1937 cells [14]. RAPTA-EA1 induces a rippled nuclear contour and partial chromatin condensation, which are characteristics of nuclear condensation stage I in MDA-MB-231 cells (Figure 3), and is indicative of apoptosis.

3.3. Cellular BRCA1 damage, expression of BRCA1 mRNA and its protein in MDA-MB-231 cells

To assess cellular BRCA1 damage, MDA-MB-231 cells were treated with RAPTA-EA1 over a wide concentration range (0–100 μ M) and following incubation for 48 h their genomic DNA was extracted. A PCR-based assay

was used to quantitate the lesion frequencies induced by RAPTA-EA1 in the BRCA1 gene (3,426-bp fragment of the BRCA1 exon 11) of the MDA-MB-231 cells. The PCR products of ruthenated DNA indicate the polymerization of DNA in the specified BRCA1 [14]. The percentage of BRCA1 amplification as a function of RAPTA-EA1 concentration is shown in Figure 4A, with the growth of the BRCA1 gene decreasing as the RAPTA-EA1 concentration increases. The amplification of the BRCA1 gene decreased by 20% in the concentration range 10–100 μM (Figure 4A). Induction of lesions with the BRCA1 exon 11 were calculated by assessing an irregular (Poisson) spread of injury [29], and the measure of lesions for each BRCA1 fragment were estimated using the Poisson equation [29, 30], with approximately one lesion per BRCA1 fragment in the cells at 20% inhibition of DNA amplification, see Figure 4B.

The expression of BRCA1 mRNA and its protein in RAPTA-EA1-treated MDA-MB-231 cells was determined using real-time quantitative RT-PCR and Western blot analysis. RAPTA-EA1 suppresses the expression of BRCA1 mRNA in MDA-MB-231 cells (Figure 5A and B). Notably, BRCA1 expression was reduced by almost 50 % compared to the untreated control cells (Figure 5C).

3.4. Combination treatment of olaparib and RAPTA-EA1

MDA-MB-231 cells are approximately 2-fold less susceptible to olaparib compared to MCF-7 cells (Figure 6A and B), while RAPTA-EA1 is perfectly suppressed the viability of both cells at the same concentration (Figure 6C and D). The combination of olaparib (10 μM) and RAPTA-EA1 (10 μM) results in a combination index (CI) of 0.24 (potent synergy) in MCF-7 cells and 0.78 (almost additive) in MDA-MB-231 cells (Figure 6E and F). Olaparib downregulates the expression of BRCA1 mRNA in MDA-MB-231 cells, whereas it is upregulated in MCF-7 cells. However, the combined inhibitors induce overexpression of BRCA1 mRNA in both cells (Figure 7A, B and C). The expression level of the BRCA1 protein is dramatically reduced after treatment with the olaparib-RAPTA-EA1 combination, compared with the untreated controls (Figure 7D, E and F).

4. Discussion

This study provides further mechanistic insights on the mode of action of RAPTA-EA1, a ruthenium-based GSTP-1 inhibitor, for the treatment of triple-negative BRCA1-competent MDA-MB-231 breast cancer cells. Following cellular uptake, RAPTA-EA1 accumulates mostly in the nuclear fraction of MDA-MB-231 cells. Moreover, RAPTA-EA1 induces an irreversible, anomalous state of condensed chromatin, ruthenium-adducts in the specific nucleosome core, which blocks the binding of a key protein for nuclear factor association and chromatin condensation inducing alterations in chromatin fiber folding [31]. This association generates substantial of mitotic cell death at the G2/M phase of the cell cycle progression [32]. The evidence suggests that cellular DNA damage triggers the inactivation of phosphorylation to cyclin-dependent kinase CDK1-cyclin B1 complex, which leads to cell death through G2/M checkpoint deregulation [32].

It has previously been shown that RAPTA-EA1 triggers multi-mechanistic pathways including programmed cell death and regulation of JNK signaling by GSTp [33, 34]. Some evidence indicates that ruthenium-based complexes induce DNA destruction that halts the DNA replication as well as transcription with a similar kinetic to cisplatin [35]. Therefore, we analyzed the DNA destruction *in vitro* on the BRCA1 gene treated with RAPTA-EA1 and found that the compound damages BRCA1 and subsequently decreases BRCA1 replication in MDA-MB-231 cells. These findings are similar to those previously obtained in triple-negative, BRCA1-mutant HCC1937 cells and sporadic BRCA1-wild type MCF-7 cells [18]. RAPTA-EA1 inhibits BRCA1 replication in HCC1937 cells to a greater extent than in MCF-7 cells [18]. However, the level of inhibition of BRCA1 replication by RAPTA-EA1 in MDA-MB-231 cells was considerably lower. This may be, in part, due to mutual interaction between

RAPTA-EA1 and nucleophilic proteins [34] or the histone-associated DNA rendering some protection [36].

From a curative point of view, the level of BRCA1 expression is a key indicator of the response of chemotherapy and a predictive marker of drug sensitivity in breast cancer treatment [8, 18, 37]. We found a correlation between BRCA1 mRNA and its respective protein, with their expression reduced in RAPTA-EA1-treated cells. Note that previously it was shown that RAPTA-EA1 inhibits the expression of the BRCA1 protein in triple-negative BRCA1-deficient breast cancer HCC1937 cells and BRCA1-proficient breast cancer MCF-7 cells [18].

Olaparib, a PARP inhibitor, applied alone can suppress the expression of BRCA1 mRNA in MDA-MB-231 cells but not in sporadic breast cancer MCF-7 cells. A decrease in the expression level of BRCA1 mRNA reflects the chemosensitivity of the cells to olaparib [38]. Note that an increase in the expression level of BRCA1 mRNA infers chemoresistance [39]. Our findings reveal that the combination of olaparib and RAPTA-EA1 actually increases the level of BRCA1 mRNA expression in both cells, but suppresses BRCA1 protein expression. This can be explained for the normally poor relationships reported between mRNA and protein levels [40]. The inverse correlation between BRCA1 mRNA and its protein expression could be caused from BRCA1 mRNA transcription and decay, translation processes, delayed protein synthesis or protein degradation via the ubiquitin-proteasome pathway [40, 41, 42, 43]. It has previously been demonstrated that BRCA1 mRNA expression is useful as an indicator of chemotherapeutic response in breast cancer treatment, however, it does not always reflect the expression level of its protein as was the case for the HCC1937 cells [18]. Moreover, the influence of microRNAs could be the vital factors in upregulating or downregulating the expression of the BRCA1 protein in breast cancer cells [44, 45, 46, 47, 48, 49].

Importantly, the combination of the PARP inhibitor, olaparib, with the GSTP-1 inhibitor, RAPTA-EA1, inhibits the expression of BRCA1 in the two kinds of breast cancer cells to a greater degree than a sum of the single agents alone, suggesting that assessing other PARP/GSTP-1 inhibitor combinations for the treatment of breast cancer may be warranted. It seems that drug susceptibility in triple-negative BRCA1 wild-type breast cancer MDA-MB-231 cells is linked to the reduced expression of the BRCA1 protein in repairing DNA damage after RAPTA-EA1 exposure. Olaparib targets PARP1, a DNA repair protein, leading to cell death via synthetic lethality [50] when applied in combination in RAPTA-EA1.

Declarations

Author contribution statement

Khwanjira Hongthong: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tidarat Nhugeaw, Pornvichai Temboot: Performed the experiments; Analyzed and interpreted the data.

Paul J. Dyson: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Adisorn Ratanaphan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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