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ORIGINAL ARTICLE

Blockade of TLR4 using TAK-242 (resatorvid) enhances anti-cancer effects of chemotherapeutic agents: a novel synergistic approach for breast and ovarian cancers

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Abstract

It is believed that pathways of the immune system are responsible for eradicating cancer cells; however, their over-activation and also their ectopic expression in tumor cells and microenvironment are major contributors to tumor growth and chemoresistance. Toll-like receptor 4 (TLR4) pathway is an innate immune-related pathway which is usually overexpressed in tumor cells that leads to excessive pro-inflammatory cytokines and eventually results in tumor survival, drug resistance, and metastasis. In this study, we investigated whether TLR4 expression is affected upon the treatment of breast and ovarian cancer cells with common chemotherapeutics (paclitaxel, cisplatin, doxorubicin, and arsenic trioxide) and if TLR4 inhibition using its specific inhibitor TAK-242 could enhance cancer cells' response to the drugs. Both breast (MCF7) and ovarian (2008C13) cancer cells experienced an elevated expression of TLR4 after treatment with the drugs. The expression of this receptor was also upregulated in cisplatin-resistant 2008C13 cells; however, it was significantly higher upon short-term treatment with cisplatin. More importantly, the combination treatment of the drugs with TAK-242 intensified the chemosensitivity of six different breast and ovarian cancer cells to chemotherapeutic drugs. It was also identified that co-treatment of paclitaxel and TAK-242 not only led to enhanced G2/M arrest and apoptosis but also satisfactorily decreased the expression of TLR4 and different interleukins in these cells. Taken together, the results of the present study emphasize that chemotherapy may lead to chemoresistance through inducing TLR4 expression, and therefore inhibiting this receptor using TAK-242 could be a promising approach to improve the outcome of chemotherapy in foreseeable future.

Keywords Toll-like receptor 4 (TLR4) · TAK-242 · Chemotherapy · Combination therapy · Breast and ovarian cancers

Bahareh Kashani and Zahra Zandi contributed equally to this work.

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Introduction

Breast and ovarian cancers are the most prevalent and most fatal carcinomas among women worldwide [1, 2]. The high rate of mortality could be due to late diagnosis, drug resistance, and eventually metastasis [3–5]. It has now been proved that most patients suffering from solid tumors, including breast and ovarian cancers, develop drug-resistant clones [6, 7]; these cells may have existed before the treatment or are formed during chemotherapy [8]. Such clones would lead to treatment failure, relapse, and finally the patient's death. There is a myriad of endeavors undertaken to identify the molecular foundation of drug resistance and lots of key factors have been found that contribute to the creation of a network that drives cells to resistance. The immune system-related pathways and molecules are now known to have inarguable roles in cancer

progression, drug resistance, and metastasis [9]. In fact, stromal and cancer cells secrete different types of cytokines and chemokines into the tumor microenvironment which in turn activate some cellular pathways and lead to cancer progression, regardless of the treatment [10].

Toll-like receptor 4 (TLR4) pathway is one of the most studied pathways involved in drug resistance and metastasis [11]. The normal site of expression for this receptor is on the surface of innate immune system cells, such as macrophages, and is absolutely crucial for pathogen recognition; its main ligand is lipopolysaccharide (LPS) of gram-negative bacteria. Following activation, TLR4 recruits two different chains of proteins inside cells; both chains trigger the activation of NF-κB and a variety of pro-inflammatory cytokines including interleukins such as IL-1, IL-6, and IL-8 [12]. These molecules, especially IL-6, have been proved to be able to induce bone metastasis and drug resistance in a plethora of cancers, particularly breast cancer [13]. Furthermore, it has been reported that TLR4 is usually overexpressed in cancer cells even at the time of diagnosis [14]. In addition to this abnormal expression, paclitaxel, a common chemotherapeutic for breast and ovarian cancers, has been recognized as one of the TLR4 ligands [15]. Besides, the remains of the destructed cells caused by chemotherapy can induce the receptor as well [16], which means a huge expression of TLR4 and as a result, the expression of pro-inflammatory cytokines.

All in all, during recent years, it has been suggested that the inhibition of TLR4, especially in cancers with high expression of this receptor, would be helpful to boost the results of chemotherapy and overcome drug resistance [17]. Among all TLR4 inhibitors, TAK-242 (also known as resatorvid) has recently become popular because of its high affinity and efficiency to inhibit the TLR4 pathway; various studies have shown that this inhibitor has no effect on TLR1, 2, 3, 5, 6, 7, 9, nor does it affect the MD2 pocket of TLR4 and its dimerization [18]. This small molecule binds directly to Cys-747 of the intracellular domain of the TLR4 receptor and interferes with the interaction of the receptor and its downstream adaptors (TIRAP and TRAM proteins), eventually preventing the activation of NF-κB and pro-inflammatory cytokines through two different downstream pathways: MyD88-dependent and MyD88-independent [19]. TAK-242 has been investigated as an anti-inflammatory agent for a broad range of conditions, including septic shock [20], kidney injury [21], and neurological impairments caused by brain edema [22] in animal models. Importantly, our team has previously conducted some pioneer research on the effect of TAK-242 on a panel of breast and ovarian cancer cells to inhibit their proliferation and survival [23, 24]. Moreover, our studies confirmed that this inhibitor can halt epithelial–mesenchymal transition and suppress invasiveness as well [25].

In this present study, it was identified that treatment of two breast and ovarian cancer cells (MCF7 and 2008C13) with four highly used anti-cancer drugs (paclitaxel, cisplatin, doxorubicin, and arsenic trioxide) resulted in considerable overexpression of TLR4. Considering this observation and based on the encouraging results of TAK-242 treatment as a single agent, the combination treatments of these chemotherapeutic drugs with TAK-242 were performed that showed promising synergistic effect in six different breast and ovarian cancer cell lines.

Materials and methods

Cell lines and chemicals

All breast (MCF7, MDA-MB-231, and SKBR3) and ovarian (2008C13, A2780CP, and SKOV3) cancer cell lines were purchased from the National Cell Bank of Iran (NCBI; Tehran, Iran) and preserved in a humidified incubator in 5% CO_2 at 37 °C. Breast cancer cells were cultured in DMEM and ovarian ones were cultured in RPMI 1640, both enriched with 10% FBS (Gibco; Grand Island, USA) and 1% Pen-Strep. Cells were screened for mycoplasma infection regularly. Toll-like receptor 4 inhibitor (TAK-242) was purchased from Calbiochem (San Diego, CA, USA), diluted in 100% dimethyl sulfoxide (DMSO) and preserved at − 20 °C as stocks of 50-mM concentration. Paclitaxel, cisplatin, doxorubicin, and arsenic trioxide (ATO) were all purchased from the pharmacy of Shariati Hospital (Tehran, Iran).

Drug treatments

Cell lines were treated with each drug at the relevant IC_{50} for 48 h. For MCF7, these concentrations were as follows: 5 μg/ml for paclitaxel, 15 μg/ml for cisplatin, 2 μg/ml for doxorubicin, and 3 μM for ATO. Since 2008C13 cells were more sensitive to these drugs, the IC_{50}s were 0.1 μg/ml for paclitaxel, 2.5 μg/ml for cisplatin, 0.1 μg/ml for doxorubicin, and 1 μM for ATO. All control cells were treated with the relevant amount of DMSO.

Chemoresistance induction

2008C13 cells were seeded in a 25-ml culture flask and incubated until they were 70% confluent, then they were treated with 0.5 μg/ml cisplatin and after 48 h, the media was removed and the treatment was repeated. Cells were further incubated until the flask was filled and were passaged. The same steps were repeated with the following concentrations: 0.7 μg/ml, 1 μg/ml (for four passages), 1.2 μg/ml (for two passages), and 1.5 μg/ml (for two passages) during 6 months. An MTT assay with increasing concentrations of paclitaxel and RNA extraction were carried out before and after the

resistance-induction process to identify IC_{50}s and TLR4 and OCT4A expression levels.

MTT assay and CI identification

2.5×10^3 2008C13 cells and 3×10^3 MCF7 cells/well were seeded in 96-well plates. After 24 h of incubation, they were treated with concentrations of 20 μM and 40 μM of TAK-242, increasing concentrations of the desired drug, and their combination. The plates were incubated for 48 h and then the metabolic activity of viable cells was identified using tetrazolium dye and an ELISA-reader (Powerwave XS2-BioTek) at 570 nm wavelength. Final combination indices (CIs) and dose reduction indices (DRIs) were calculated based on Chou and Talalay [26] using CalcuSyn software (Biosoft, Cambridge, UK). CI < 1, CI = 1, and CI > 1 show synergism, additive effects, and antagonism of the drugs, respectively.

Crystal violet staining assay

About 1.5×10^4 cells were seeded in 24-well plates and treated with concentration 40 μM of TAK-242, IC_{25} of paclitaxel (0.1 μg/ml for MCF7 and 0.0025 μg/ml for 2008C13), and their combination. After 48 h of incubation, cells were fixed with ice-cold methanol and then stained with crystal violet (0.5%*w*/*v*). Each well was then photographed under an inverted microscope.

Colony formation assay

Five hundred cells of 2008C13 and 1000 MCF7 cells were seeded per well in 24-well culture plates and treated with aforementioned concentrations of TAK-242 and/or paclitaxel; drug-containing media was removed after 48 h and drug-free media was added. The plates were further incubated until each colony consisted of about 50 cells (7–10 days). After fixation with ice-cold methanol, colonies were stained using crystal violet solution (0.5%*w*/*v*) and photographed. Surviving fractions were calculated as:

SF = (mean colony counts of treated well)/(mean colony count of control well).

Cell cycle analysis using propidium iodide

5×10^4 cells/well were seeded into 12-well plates. Cells were harvested 48 h after treatment with aforementioned concentrations of TAK-242 and/or paclitaxel, washed with ice-cold PBS, and fixed in 70% ethanol. After being preserved at − 20 °C overnight, RNase A (100 μg/mL) (Sigma), PI (propidium iodide, 50 μ g/mL) (Sigma), and 0.05% Triton X-100 were added to cell plates. Cells were further preserved at 4 °C for an hour and then DNA content was detected with FACSCalibur (BD Bioscience, San Jose, CA, USA) flow cytometer equipped with CellQuest Pro software and analyzed using FlowMax software.

Annexin V/PI apoptosis test

6×10^4 cells were seeded into each well of 12-well plates and treated after 24 h. After 48 h, they were harvested and the kit manual protocol was carried out using both Annexin V-FITC and PI dyes (Thermo Fisher Scientific, USA). The percentage of apoptotic cells was measured using the Partec PAS III flow cytometer (Partec GmbH) and analyzed by WindowsTM FloMax software (Partec).

Quantitative real-time PCR

About 10^5 cells per well were seeded in culture plates and harvested 48 h after treatment. RNA was extracted using RNX plus trizol protocol (SinaClon BioScience, Tehran, Iran) and then cDNA was synthesized according to the PrimeScript Reagent Kit protocol (TaKaRa Bio INC, Japan) on Applied Biosystems 96-well thermal cycler. Next, qRT-PCR was performed using SyberGreen and the LightCycler® 96 instrument. The experiment's thermal condition was as follows: an activation step for 15 min at 95 °C followed by 40 cycles of denaturation step (15 s at 95 °C) and a combined annealing/extension step for 1 min at 60 °C. Beta-2-microglobulin (B2M) was used as the housekeeping gene and relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. Table 1 contains a list of the primers used for gene amplification.

Statistical analysis

All data were evaluated in triplicate against untreated control cells and collected from three independent experiments. Data were analyzed using unpaired two-tailed student's *t* test, two-way ANOVA, and Tukey's multiple comparisons test. All data were graphed by GraphPad Prism Software 7.0a and presented as mean ± standard deviation (SD).

Results

Chemotherapeutic drugs increase the expression levels of TLR4 and OCT4A

A growing amount of evidence suggests that TLR4 expression plays a pivotal role in cancer progression and chemoresistance [27, 28]. There have also been studies that suggest paclitaxel therapy leads to overexpression of this receptor in different cancer cells [15]. To investigate whether this is a common phenomenon of different drugs, the selected cell lines of breast and ovarian cancers

Table 1 List of the primers used for gene amplification in qRT-PCR

Gene	Accession number	Forward primer (5′→3′)	Reverse primer (3′→5′)	Size (bp)
B2M	NM_004048.3	GATGAGTATGCCTGCCGTGT	CTGCTTACATGTCTCGATCCCA	79
TLR4	NM_138557.2	AGACCTGTCCCTGAACCCTAT	CGATGGACTTCTAAACCAGCCA	147
OCT4A	NM_001285987.1	GTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC	164
IL-6	XM_005249745.5	ACTCACCTCTTCAGAACGAA TTG	CCATCTTTGGAAGGTTCAGG TTG	149
IL-6R	NM_181359	CCCCTCAGCAATGTTGTTTGT	CTCCGGGACTGCTAACTGG	171
IL-1β	NM_000576.3	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT	73

(MCF7 and 2008C13 respectively) were treated with highly used chemotherapy agents (paclitaxel, cisplatin, doxorubicin, and arsenic trioxide (ATO)) at IC_{50} concentrations. Our results indicated that both cell lines showed intensified TLR4 expression due to 48-h treatments with almost all the drugs. Interestingly, we also observed upregulated OCT4A expression, a known marker of chemoresistance and stemness [29], which is in consistence with previous studies that suggested a strong relationship between TLR4 and stemness [30]. As shown in Fig. 1a, breast cancer cell line was affected the most by paclitaxel with a 41-fold change in TLR4 and 105 times

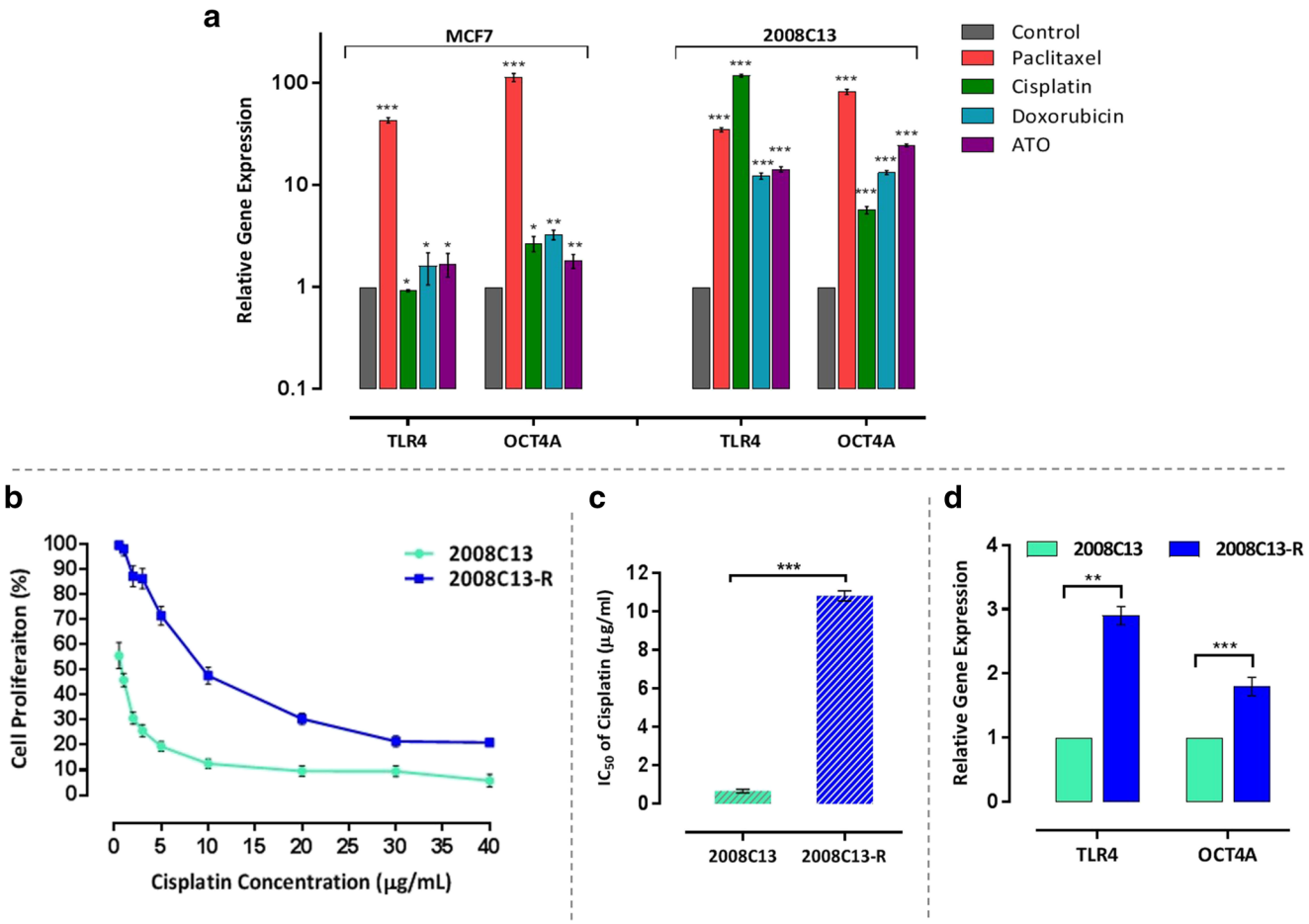

Fig. 1 TLR4 significantly contributes to chemoresistance in breast and ovarian cancer cells. **a** After the treatment of MCF7 and 2008C13 cells with four different chemotherapeutic agents (paclitaxel, cisplatin, doxorubicin, and ATO) at the IC_{50} concentrations, the expression of TLR4 and OCT4A was noticeably increased. **b**, **c** Cisplatin resistance was induced in 2008C13 cells and the results of MTT assay showed that the resistant cells (2008C13-R) were less sensitive to the same concentrations of cisplatin and the IC_{50} became 15 times higher compared with the original cells. **d** The expression of TLR4 and OCT4A in 2008C13 and 2008C13-R cells was assessed using qRT-PCR which showed almost 3 and 2 times increase. Data are shown as mean ± SD from three independent experiments. Statistically significant values of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are determined compared with untreated control group

increase in OCT4A expression; ovarian cancer cell line was most responsive to cisplatin with a fold change of 114 and 5 for TLR4 and OCT4A, respectively.

Since the highest TLR4 expression was observed in cisplatin-treated 2008C13 cells, we decided to make these cells resistant to the mentioned drug and investigate the changes in the expression level of the receptor. As shown in Fig. 1b, 2008C13-R cells were less responsive to the same concentrations of the drug compared with the parental cells. The IC_{50} was 0.7 μg/ml for 2008C13 cells which increased to 10.34 μg/ml during the 6-month process of inducing resistance (Fig. 1c). As shown in Fig. 1d, TLR4 expression was also increased significantly which further highlighted the fact that this gene may contribute to drug resistance in cancer cells. However, the increase observed in long-term exposure of the cells to cisplatin (2.8 times) was considerably lower than that of short-term treatment (114 times). The same results were observed in OCT4A expression. Of note is the fact that the 2008C13 cell line is the platinum-resistant form of 2008 cells (which may explain the noticeable TLR4 level in these cells), yet the level of cisplatin resistance and TLR4 expression were still significantly intensified in this study.

TAK-242 has a synergistic effect with chemotherapeutics

Given the overexpression of TLR4 after treatment with common chemotherapeutic agents, it was tempting to investigate the effect of TLR4 inhibition on the efficiency of the drugs. To do so, TAK-242, a highly specific inhibitor of TLR4, was used in combination with the mentioned chemotherapeutics for both ovarian and breast cancer cell lines. Figure 2 shows that co-treatment of TAK-242 and these drugs led to more significant proliferation inhibition compared with each drug individually. To ascertain that this effect is not cell-type specific, we then performed the same treatments with two more breast (MDA-MB-231 and SKBR3) and two ovarian (A2780CP and SKOV3) cancer cell lines for which the combination therapies seemed to be similarly effective (Supplementary Fig. 1). The panel of cell lines was investigated in our former studies and was chosen as such to contain cells with different expressions of TLR4 so that the effects of TAK-242 could be thoroughly studied. Our results demonstrated that although with different effects and CIs, the efficiency of all the chemotherapeutics could be improved in all cell lines when used in combination with TAK-242. The CI and DRI values of all combinations for all the mentioned cell lines at fraction affected of 0.5 (Fa0.5) are shown in Table 2. The MTT results are also shown as EC_{50} shift graphs in Fig. 3 (MCF7 and 2008C13) and Supplementary Fig. 2 indicating the changes in the EC_{50} values of each drug individually compared with the combination.

The co-treatment of TAK-242 and paclitaxel significantly decreases cell viability and proliferation

As a common chemotherapeutic agent that is used for both breast and ovarian cancers and showed the most synergistic effect with TAK-242 in both MCF7 and 2008C13 cells, we selected paclitaxel for further investigations. First, the crystal violet staining assay was performed and indicated the same results as the MTT assay and also showed morphologic changes of the cells (Fig. 4a). Colony formation assay further showed that this co-treatment significantly suppressed colonization which is a vital step of metastasis and cancer progression (Fig. 4b, c).

As the next step and to shed light on the mechanism of action, a PI staining for cell cycle analysis was done which showed that although TAK-242 did not change cell distribution at this concentration, its co-treatment with paclitaxel increased G2/M arrest in both breast and ovarian cancer cell lines (Fig. 5a). Moreover, annexin V/PI apoptosis test demonstrated that this co-treatment could augment the apoptotic effects of paclitaxel and resulted in better cytotoxic effects in both breast and ovarian cancer cell lines (Fig. 5b).

Interestingly, qRT-PCR further showed that this co-treatment significantly reduced the elevated levels of TLR4, IL-6 and IL-1β (major pro-inflammatory cytokines known to cause chemoresistance), and IL-6R in both MCF7 and 2008C13 cells (Fig. 5c). The expression of TLR4 in combination was 8 and 2.5 times lower compared with those of paclitaxel in MCF7 and 2008C13, respectively. Furthermore, the expression levels of IL-6, IL-6R, and IL-1β were also significantly reduced after the co-treatment. This data could suggest that not only does TAK-242 improve the efficiency of paclitaxel, it could also prevent the activation of inflammatory pathways and the consequent resistance during the chemotherapy process.

Discussion

The immune system is considered a double-edged sword in the concept of cancer; despite its role in eliminating tumor cells, it may help them grow and advance as well [31]. In fact, cytokines and chemokines secreted from tumor cells themselves and their adjacent stromal cells are key factors that effectively contribute to tumor progression, drug resistance, and metastasis [32]. Consistently, studies have shown that during disease progression, carcinoma cells evolve to gain the privilege of

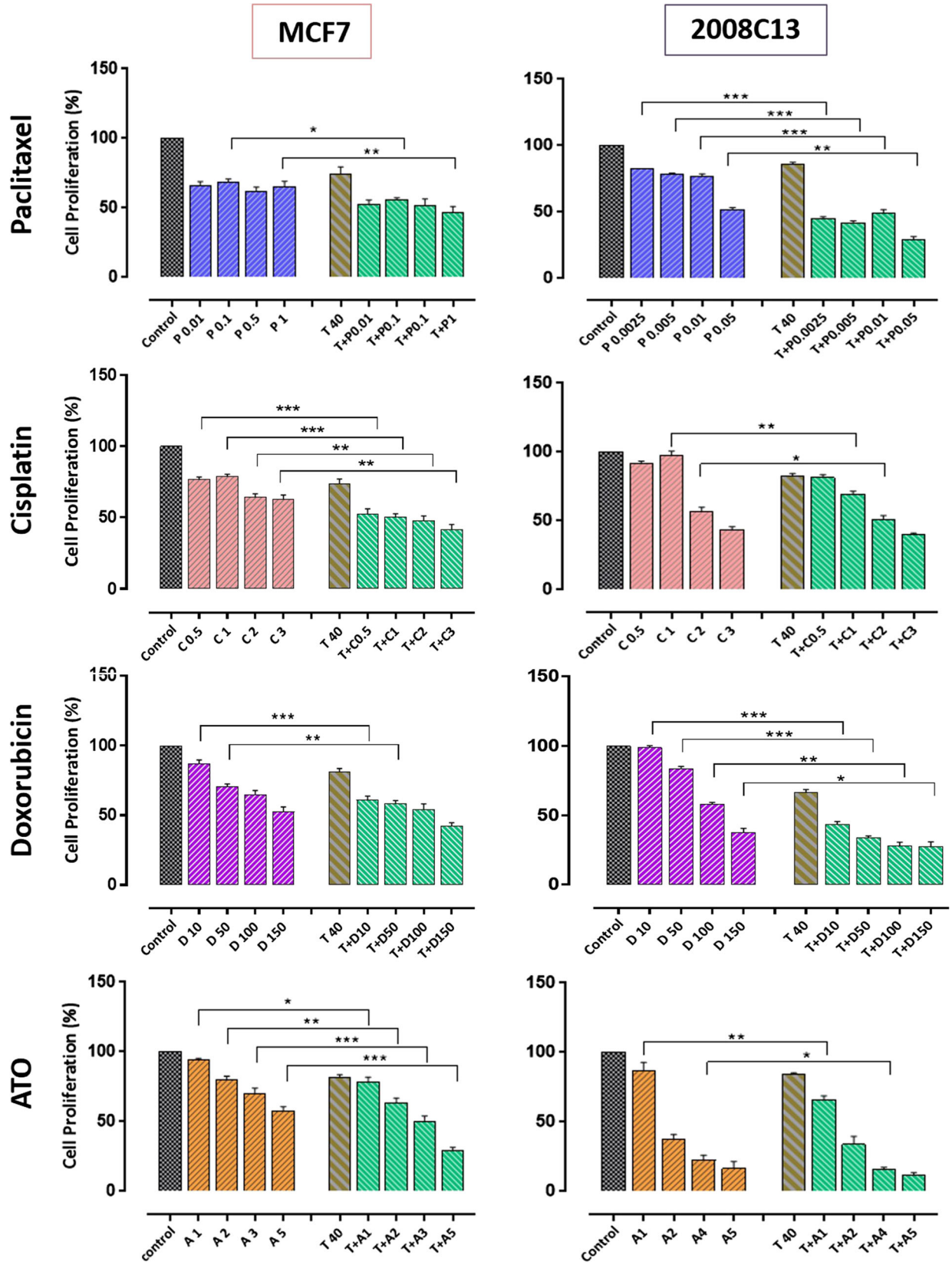

Fig. 2 TAK-242 intensifies the cytotoxic effect of chemotherapeutic drugs in MCF7 and 2008C13 cells. The results of MTT assays indicated that co-treatment of TAK-242 and paclitaxel, cisplatin, doxorubicin, and ATO noticeably inhibited cell proliferation of both cell lines compared with the chemotherapeutic drug. Data are shown as mean ± SD from three independent experiments. Statistically significant values are determined as: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

Table 2 Combination indices (CIs) and dose reduction indices (DRIs) of combination therapy of TAK-242 and chemotherapeutics in six breast and ovarian cancer cell lines

Cell Line/Drug	CI	DRI	
		TAK-242	Drug
MCF7			
Paclitaxel	0.022	1.82E + 04	46.595
Cisplatin	0.731	1.605	6.093
Doxorubicin	0.583	1.576	10.331
ATO	0.583	3.03E + 09	1.715
MDA-231			
Paclitaxel	0.413	2.441	317.175
Cisplatin	0.892	1.444	5.026
Doxorubicin	0.404	162.954	2.516
ATO	0.49	4.38E+04	2.041
SKBR3			
Paclitaxel	0.129	8.55	147.244
Cisplatin	0.44	12.291	2.785
Doxorubicin	0.416	13.976	2.9
ATO	1.184	1.523	1.895
2008C13			
Paclitaxel	0.048	1.8e + 0075	16.815
Cisplatin	0.734	0.393	1.400
Doxorubicin	0.906	1.181	16.781
ATO	0.774	57.353	1.321
A2780CP			
Paclitaxel	0.436	7.267	3.348
Cisplatin	0.641	2.503	4.149
Doxorubicin	0.414	21.568	2.720
ATO	1.765	2.490	0.733
SKOV3			
Paclitaxel	0.331	7.158	5.221
Cisplatin	0.857	4.438	1.584
Doxorubicin	1.075	1.370	2.894
ATO	1.168	2.545	1.290

the overexpression of some immune-related pathways which are normally expressed in innate immune cells [33]. The toll-like receptor 4 pathway, which has previously been considered a key driver of sepsis shock [34] and other immune-related disorders [35], has recently drawn scientists' attention mostly due to its significant role in drug resistance and metastasis [36]. The positive correlation between TLR4 and chemoresistance in ovarian cancer patients was identified in a study conducted by Lou et al. [37]. Moreover, it has been reported that triggering TLR4 in human head and neck squamous cell carcinoma resulted in tumor development and protecting tumor cells against the immune system [38]. Besides, the upregulation of TLR4 was reported to have a huge significance in lymph node metastasis and depth of invasion in esophageal squamous cell carcinoma [39]. Based on these facts and the well-studied roles of TLR4, we decided to investigate whether the inhibition of this receptor using TAK-242 could augment the cytotoxic effects of several chemotherapeutic agents in breast and ovarian cancer cell lines.

To begin with, we evaluated the expression of TLR4 after treatment with paclitaxel, an important chemotherapeutic agent, in two breast and ovarian cancer cell lines (MCF7 and 2008C13 respectively). Interestingly, the results showed a huge overexpression of the receptor in both cell lines. Our results were in consistence with another study that declared paclitaxel activates TLR4 in the same way as its specific ligands [40]. To expand the domain of this study, we investigated whether other therapeutic agents could exert the same effect as paclitaxel. Intriguingly, both cell lines showed higher expression of TLR4 after treatment with cisplatin, doxorubicin, and arsenic trioxide (ATO). These results suggest that the overexpression of TLR4 after treatment with paclitaxel is not only due to its ability to bind to MD-2 pocket of TLR4, as believed before [41], but it can be a general phenomenon caused by massive destruction of cells and also the secretion of some of the TLR4 ligands such as HMGB1 [42] and heat shock proteins [43] to the tumor microenvironment during chemotherapy. Finally, the expression of OCT4A gene, a stemness marker, was also assessed after the treatments and showed an upregulation similar to TLR4, which might be as a consequence of TLR4 overexpression. In fact, the interaction between TLR4 and stemness has always been appealing to scientists. For example, Zhou et al. showed that TLR4 is responsible for stemness induction and increasing the risk of relapse in human hepatocellular carcinoma and suggested that the inhibition of TLR4 may decrease the chance of relapse [44].

To show the role of TLR4 in drug resistance, we further compared the expression of this receptor in the 2008C13 cell line with its cisplatin-induced resistant form (2008C13-R). The results showed a significant upregulation of TLR4 and OCT4A in resistant cells, which might have occurred during the process of exposure to cisplatin or might be due to the fact that the survived cells are mostly cancer stem cells with higher expression of TLR4. In consistence, in a previous study, our team showed that sensitive ovarian cancer cell line (A2780S) showed no significant expression of TLR4, whereas its resistant form (A2780CP) highly expressed it [25]. Taken together, our results

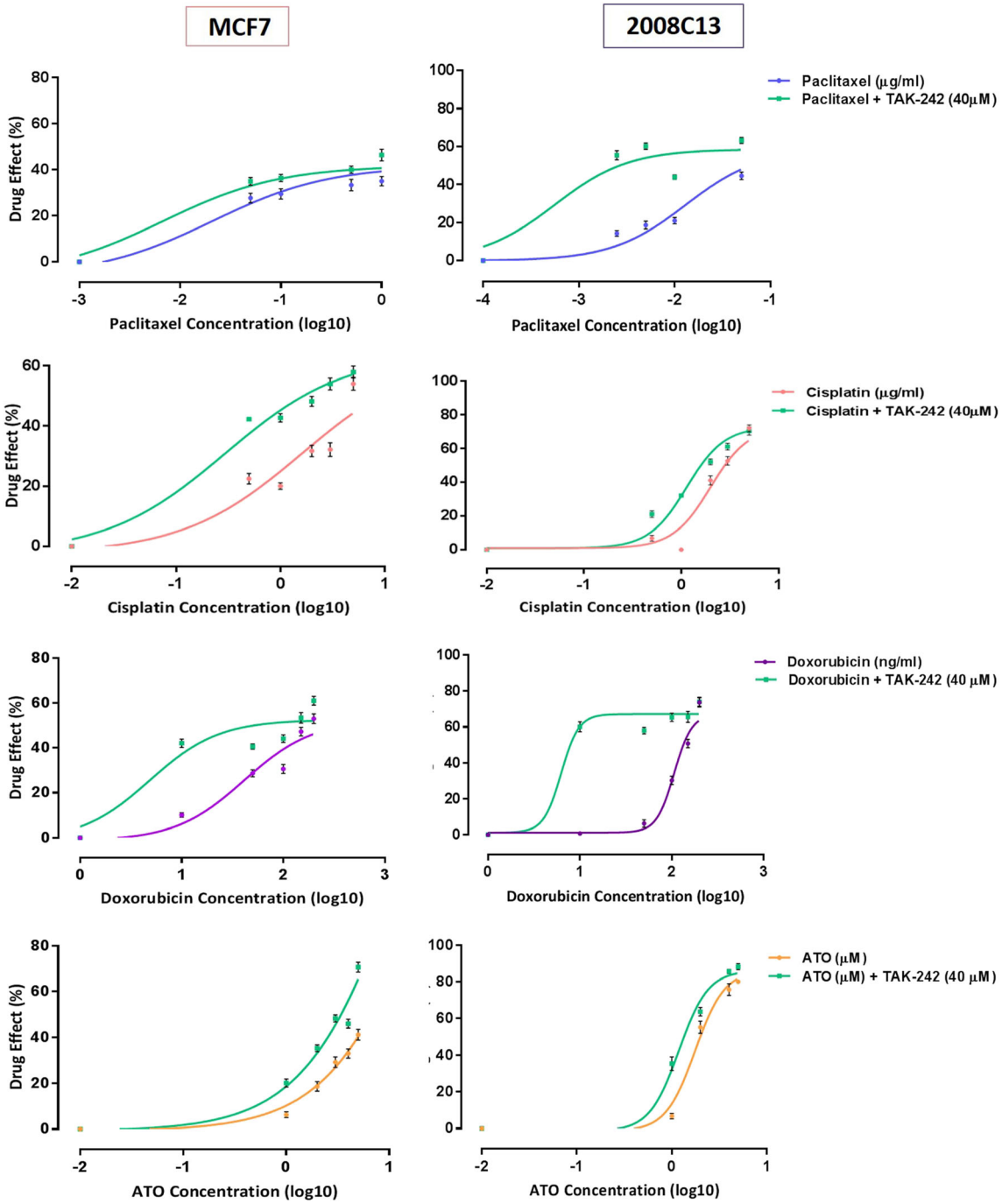

Fig. 3 Co-treatment of TAK-242 with common chemotherapeutics results in EC_{50} shifts in MCF7 and 2008C13 cells. Based on the results of MTT assays, the EC_{50} shift graphs were provided which showed the significant changes in EC_{50} values compared with the drug individually with various values for different drugs in each cell line. Data are shown as mean ± SD from three independent experiments

showed that the treatment of breast and ovarian cancer cells with chemotherapeutics, either in a short- or long-term condition, resulted in an increased expression of TLR4; however, the fold change in short-term treatment was significantly higher than that of long term.

Next, to scrutinize whether the inhibition of TLR4 could intensify the cytotoxicity of the drugs, TAK-242 as a specific inhibitor of TLR4, was used in combination with four important anti-cancer drugs (paclitaxel, cisplatin, doxorubicin, and ATO). Satisfactorily, almost all the combinations were synergistic in both cell lines, which is in harmony with the studies that indicated the role of TLR4 in chemoresistance and its importance in the results of chemotherapy. Notably, investigating the same co-treatments in the 2008C13-R cells showed very few synergistic concentrations (data not shown), which may be

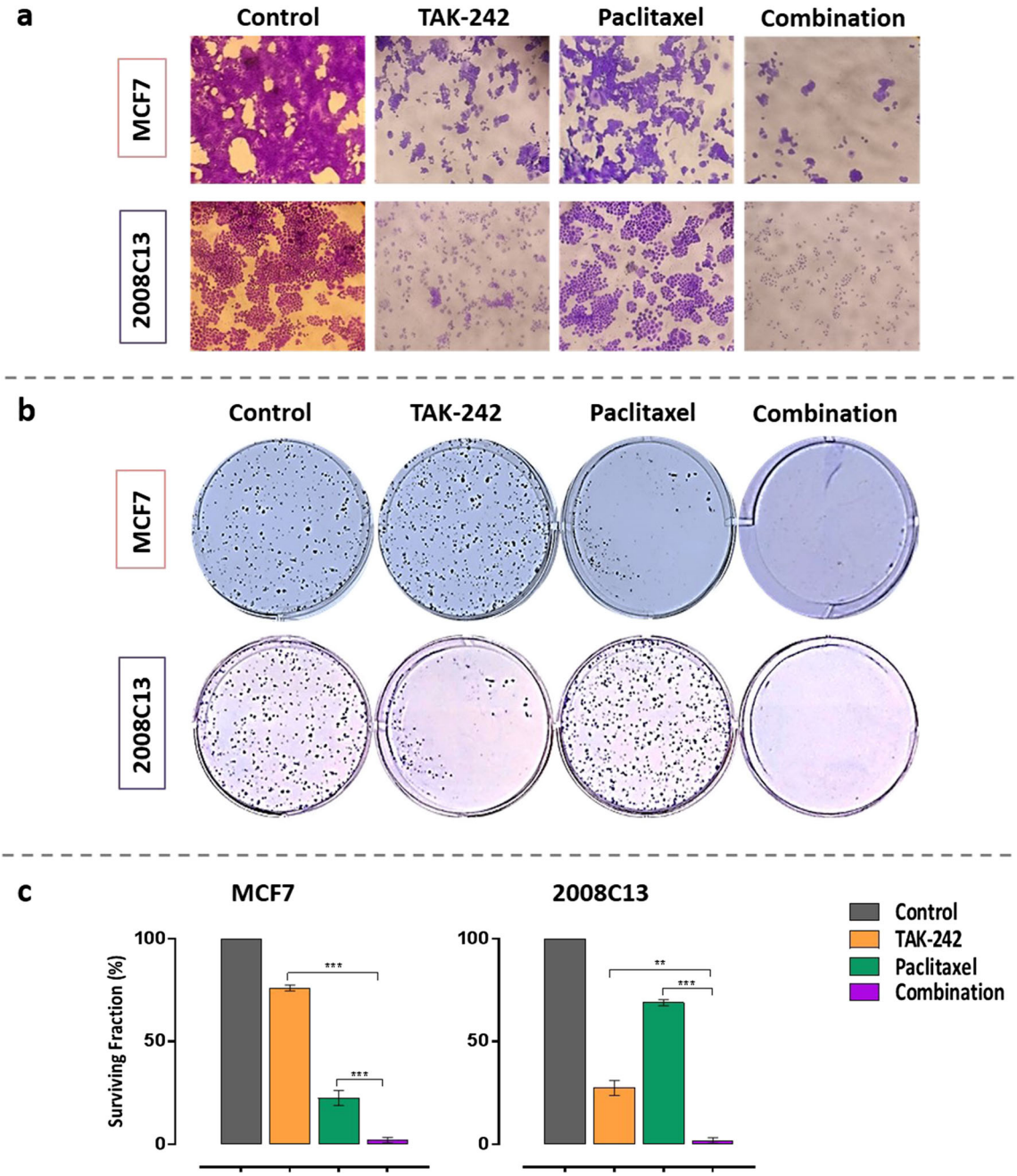

Fig. 4 The combination therapy of TAK-242 and paclitaxel enhances the cytotoxicity of paclitaxel in MCF7 and 2008C13 cell lines. **a** The results of the crystal violet staining assay showed that TAK-242 and paclitaxel co-treatment caused better cytotoxic outcomes in both cell lines compared with paclitaxel individually. **b**, **c** The colony formation abilities of both cell lines subsided substantially in combination treatment. Data are shown as mean ± SD from three independent experiments. Statistically significant values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ are determined compared with each drug individually

due to the lower dependency of these resistant cells to the TLR4 pathway since long-term exposure to the drug may have caused genetic variation in the cells that led to the activation of many other bypass pathways, rather than TLR4.

The correlation between TLR4 inhibition and drug response has mostly been investigated for paclitaxel and taxane-based drugs [45]. These studies maintain that the result of chemotherapy with paclitaxel, especially in breast and ovarian cancers, widely depends on the tumor's TLR4 status [46, 47]. Despite its efficiency in killing cells, paclitaxel therapy shows negative results in TLR4-positive cells and leads to bone and pulmonary metastasis [48]; such results suggest that the inhibition of TLR4 could reverse paclitaxel resistance in such cells. Therefore, we carried out a cell cycle analysis for combination-treated cells to clarify how TAK-242 boosts

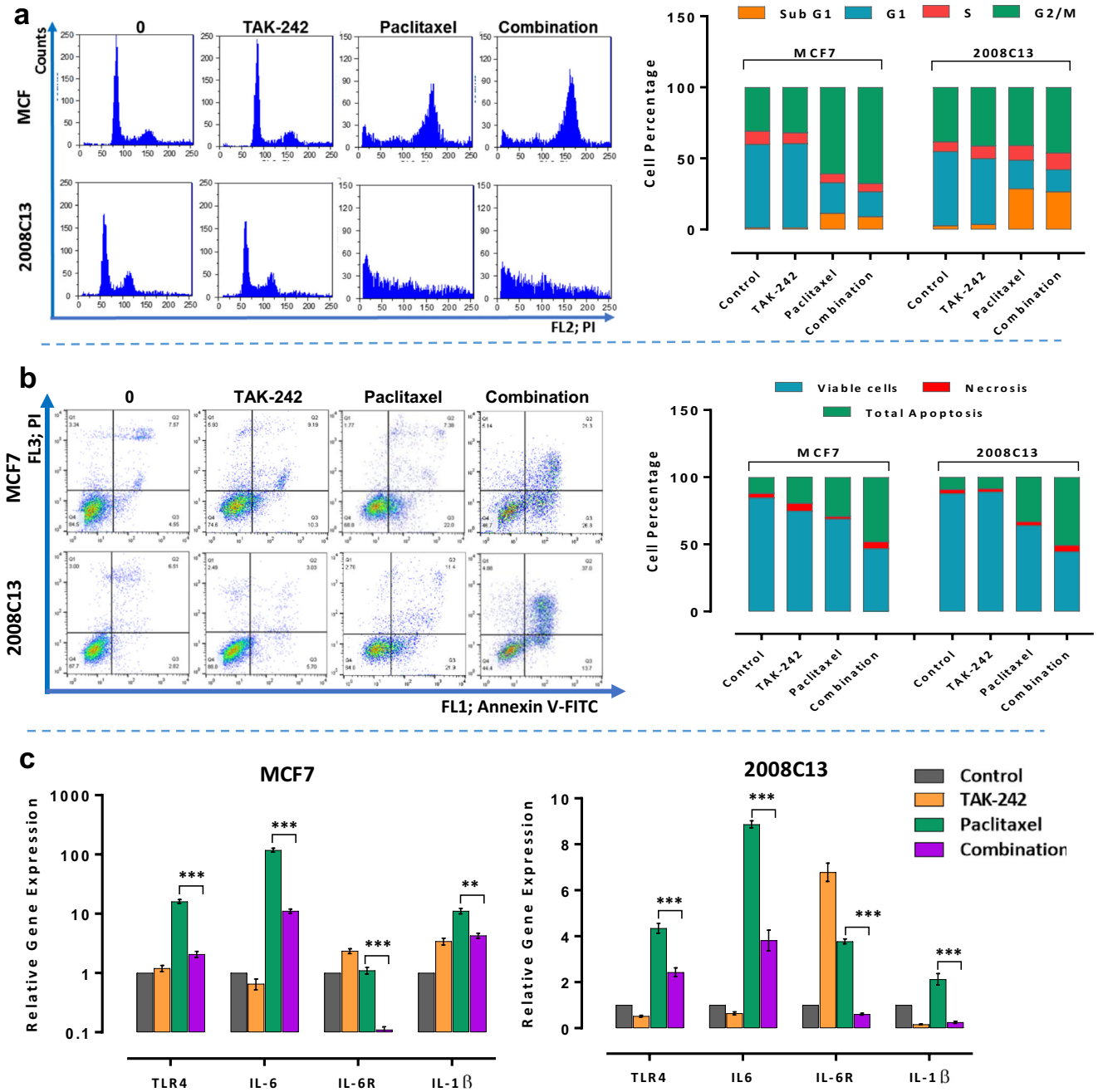

Fig. 5 Co-treatment of TAK-242 and paclitaxel alters cell cycle distribution and gene expression and also increases apoptosis in MCF7 and 2008C13 cell lines. **a** The results of PI staining for cell cycle analysis indicated that cells treated with both TAK-242 and paclitaxel showed higher G2/M arrest in both cell lines. **b** Annexin V/PI assay showed that this co-treatment led to an increased number of apoptotic cells in both MCF7 and 2008C13. **c** Analyzing the expression of TLR4, IL-6, IL-6R, and IL-1β using qRT-PCR showed that this combination therapy could noticeably suppress the TLR4 pathway induced by paclitaxel therapy in both breast and ovarian cancer cell lines. Data are shown as mean ± SD from three independent experiments. Statistically significant values of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are determined compared with paclitaxel-treated cells

paclitaxel effects; the results indicated decreased G1 and increased G2/M phases in comparison with cells treated with either paclitaxel or TAK-242. Furthermore, analyzing the percentage of apoptotic cells after this co-treatment showed that the apoptosis induction level of paclitaxel was significantly improved in both cell lines, mostly as of late apoptosis, which is in consistence with our former studies which showed that TAK-242 could induce both G2/M arrest and apoptosis in breast and ovarian cancer cells [23, 24]. This combination therapy also reduced the colonization ability of cancer cells

that is a vital step in the formation of metastatic sites. Interestingly, qRT-PCR revealed that although paclitaxel increased the expression of inflammatory cytokines, its combination with the TLR4 inhibitor could suppress their expressions. To shed more light on the possible mechanisms by which TLR4 inhibition intensifies the cytotoxic effect of taxane-based drugs (e.g. paclitaxel), multiple explanations may be proposed. Sun et al. showed that the co-treatment of taxol and TLR4 inhibitors could suppress the expression of ABCB1 which serves as a multi-drug resistance protein [49]. In addition, it was shown that the inhibition of TLR4 combined with paclitaxel resulted in enhanced apoptosis through either downregulation of survivin [50] or upregulation of caspase 3/7 activity [47]. All in all, these mechanisms may explain, at least partly, how TAK242 could augment paclitaxel cytotoxicity.

Taking everything into account, this study provides evidence that chemotherapy may lead to chemoresistance and cancer progression through inducing TLR4 expression; therefore, inhibiting this receptor using TAK-242 during the process of chemotherapy, could be a promising method to improve the outcome of chemotherapy for breast and ovarian cancer patients in foreseeable future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent None.

Research involving human participants and/or animals Not applicable

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