Ginsenoside PT reverses MDA-MB-231 paclitaxel resistance by inhibiting the IRAK1/NF-κB and ERK pathways

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Background. Paclitaxel resistance is a major obstacle in the treatment of triple-negative breast cancer (TNBC). Previously, we reported that interleukin-1 receptor-associated kinase 1 (IRAK1) and its downstream pathways are associated with paclitaxel resistance in TNBC cells. In this study, we sought to investigate the combination treatment of ginsenoside panaxatriol (PT) with paclitaxel on viability and apoptosis of MDA-MB-231 paclitaxel resistant (MB231-PR) cells, and explore the role of IRAK1 mediated signaling pathways in the therapeutic effects.

Methods. CellTiter-Glo and colony formation assays were used to assess cell viability. Flow cytometry was used to analyze subG1 and apoptosis. Western blot was used to detect expressions of proteins involved in apoptosis and the IRAK1/NF-κB and ERK pathways. The mRNA expression of inflammatory cytokines, S100A7/8/9, and cancer stem cell (CSC) -related genes were examined by qPCR. Stem cells were identified by tumor sphere assay. Cell invasion ability was examined by transwell assay.

Results. We show that ginsenoside PT inhibits MB231-PR cell viability in a dose dependent manner. When combined with paclitaxel, ginsenoside PT synergistically causes more cell death, induces subG1 accumulation and cell apoptosis. Besides, up-regulation of BAX/BCL-2 ratio, and down-regulation of MCL-1 are also observed. Moreover, this combination inhibit IRAK1, NF-κB and ERK1/2 activation, and lead to down-regulation of inflammatory cytokines (IL6, IL8, CXCL1, CCL2), S100A7/9, and CSC-related genes (OCT4, SOX2, NANOG, ALDH1, CD44) expression. In addition, the combination treatment suppresses tumor sphere growth and cell invasion ability.

Conclusion. Our study demonstrates that ginsenoside PT can resensitize MB231-PR cells to paclitaxel by inhibiting the IRAK1/NF-κB and ERK pathways and reducing stem cell characteristics

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Abstract

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Introduction

Triple-negative breast cancer (TNBC) is a highly invasive subtype of breast cancer with poor prognosis (Foulkes et al. 2010). Because of the lack of hormone receptors and human epidermal growth factor receptor 2 (HER2) amplification, TNBC does not respond to hormone or anti-HER2 treatment, and mainly relies on traditional chemotherapy (Denkert et al. 2017). Paclitaxel-based chemotherapy regimens are the most widely used first-line therapeutic strategies for clinically treatment of TNBC. Although effective in the initial treatment, a subset of patients eventually develops resistance, and leads to disease progression (Mustacchi & De Laurentiis 2015; Schettini et al. 2016). Hence, it is highly necessary to find a solution for paclitaxel resistance in TNBC.

The nuclear factor kappa B (NF-κB) signaling pathway plays an important role in cancer initiation, progression, and resistance, thus making it a good target for cancer treatment (Chaturvedi et al. 2011; Hoesel & Schmid 2013; Taniguchi & Karin 2018). However, despite numerous attempts to develop molecular drugs that specifically target NF-κB, few clinical advancements have been made (Baud & Karin 2009). Previously, by using gain and loss of function methods, we reported that activation of interleukin-1 receptor-associated kinase 1

(IRAK1), an upstream kinase of the NF-κB signaling pathway, is associated with paclitaxel resistance in TNBC cells (Wee et al. 2015). Importantly, together with S100A7, S100A8 and S100A9 (S100A7/8/9), IRAK1 form a druggable circuitry which drives the malignancy of TNBC cells (Goh et al. 2017). These observations prompted us to search for potential candidate drugs that can target IRAK1 and its downstream signaling pathways.

Ginseng and its active ingredient ginsenosides have been widely used in China to treat cancers in the clinic. Moreover, it has been reported that some ginsenosides can inhibit the activation of IRAK1 and its downstream pathways (Joh et al. 2011; Nag et al. 2012; Shaukat et al. 2019). In this study, we investigated the *in vitro* anti-viability of ginsenoside PT in MB231-PR cells, and found that ginsenoside PT can target IRAK1/NF-κB and ERK pathways to overcome MB231-PR resistance.

Materials & Methods

2.1 Chemicals and reagents

Ginsenoside PT was obtained from Must Bio-Technology (Chengdu, China). Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and FBS were bought from Life Technologies (Grand Island, NY, USA). MammoCul medium (human) and supplements were purchased from STEMCELL Technologies (Vancouver, Canada). CellTiter-Glo luminescent cell viability assay kits were purchased from Promega Corporation (Madison, WI, USA). iScript gDNA Clear cDNA Synthesis Kits and iTaq Universal SYBR Green Supermix Kits were purchased from Bio-Rad Laboratories (Hercules, CA USA). p-IRAK1 S376, IRAK1, p-P65 S536, P65, p-ERK1/2, ERK1/2, BAX, BCL-2 and MCL-1 antibodies were supplied by Cell Signaling Technology (Danvers, MA, USA). Beta-actin antibody was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Cell culture and viability assay

MB231 cells were obtained from ATCC. MB231-PR cells were established as previously described (Wee et al. 2015). Cells were cultured in DMEM supplemented with 75 nM paclitaxel, penicillin/streptomycin, and 10 % fetal bovine serum (FBS) at 37 °C with 5% CO_2. For cell viability assay, 1000 cells/well in 90 µl medium were seeded into Costar 96-well white plates.

The next day, different concentrations of drugs in 10 µl medium were added and incubated for the indicated times. Then, cells were lysed with 50 µl CellTiter-Glo reagent and the chemiluminescent signals were detected with a PerkinElmer VICTOR X4 plate reader.

2.3 Cell cycle and apoptosis assay

Cell cycle and apoptosis analysis was performed by DNA content quantification to quantify the subG1 population, which is a reflective of the extent of cell death. Briefly, floating and adherent cells were harvested together after 24 h and 48 h treatment respectively. Then, cells were fixed by 70 % ethanol at 4 °C overnight. After washing with phosphate buffered saline (PBS), cells were resuspended in 100ul of 100 ug/ml RNase A. 5 min later, 400ul of 50 µg/ml propidium iodide were added, and cells were incubated for 30 min in dark area. Finally, the stained cells were analyzed by FACScalibur and quantified using CellQuest software.

2.4 Colony formation assay

1000 cells/well were seeded into 12-well plates. The next day, drugs were added and incubated for 12 days. Medium was changed every three days. Then, cells were washed with PBS and fixed with methanol for 10 min. Finally, cells were then stained with 0.1 % crystal violet at room temperature for 10 min and photographed.

2.5 Tumor sphere formation assay

1000 cells/well in 180 µl medium were seeded into Corning 96-well spheroid microplates in complete MammoCul medium. The next day, drugs in 20 µl medium were added and incubated for 12 days. Pictures were taken on day 6 and day 12. Finally, on day 12, cells were lysed with 100 µl CellTiter-Glo reagent and the chemiluminescent signal was detected with a PerkinElmer VICTOR X4 plate reader.

2.6 Transwell invasion assay

10000 cells in 100 µl serum-free DMEM containing DMSO, paclitaxel, ginsenoside PT, or combination were added into Corning Transwell polycarbonate membrane inserts coated with Matrigel (300 µg/mL). And medium containing 10 % FBS was added to the bottom chamber.

After 24 h incubation, the cells that remained on the above surface of the insert membrane were scraped off with a cotton swab. The cells that passed through Matrigel to the bottom of the insert were fixed with paraformaldehyde and stained with 0.1 % crystal violet in methanol. The inserts were photographed, and the cells were counted.

2.7 Quantitative-PCR (qPCR) assay

RNA extraction and purification were performed according to the instructions from Zymo Research (R2052). 750 ng RNA was used to synthesize cDNA. And qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system. All primers are listed in table 1. For quantification of mRNA levels, 18S was used as the internal control, and the expression of target genes were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.8 Western blot assay

Western blot was performed using whole-cell extracts in protein lysis buffer with freshly added protease inhibitor cocktail. Proteins were separated on 8 %–10 % SDS polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes. The membrane was blocked with 5% non-fat dry milk in tris-buffered saline (TBS) containing 0.1 % Tween 20 (TBST). The membrane was then incubated with primary antibody (1:1000 dilution) in 5 % bovine serum albumin overnight. After washed three times with TBST, the membrane was incubated with secondary antibody (1:2000 dilution) in 5 % non-fat dry milk at room temperature for 1 h. Then, SuperSignal West Femto Maximum Sensitivity Substrate was added, and images were taken using the ChemiDoc MP System.

2.9 Statistical analysis

Data are shown as mean ± SD. The t test was used to determine whether there are any statistically significant differences between two groups. $P < 0.05$ was considered statistically significant.

Results

3.1 Ginsenoside PT promotes cytotoxicity of paclitaxel in MB231-PR cells

To explore whether ginsenoside PT can promote cytotoxicity of paclitaxel in TNBC resistant cells, MB231-PR was constructed and used as cell model. Firstly, we conducted CellTiter-Glo assay to observe different concentration of ginsenoside PT on cell viability. As shown in Fig. 1A, ginsenoside PT treatment significantly decreased cell viability of MB231-PR cells in a dose dependent manner, with the half maximal inhibitory concentration (IC50) 21.39 μM. Secondly, we combined ginsenoside PT with paclitaxel to check whether they have synergistic effects. Results showed that the combination caused dramatic cell death in a dose and time dependent manner, comparing to either single use group (Fig. 1B). Interestingly, the synergistic effects didn't apply to MB231 parental (MB231-PT) cells, although MB231-PT cells showed more sensitive to ginsenoside PT when treated with the same concentration (Fig. 1C). In addition, colony formation assay confirmed the synergistic cytotoxicity effects of the combination on MB231-PR cells (Fig. 1D).

Since chemotherapy resistant appears partly due to aberrant changes of signaling pathways that endowed cells with the abilities to escape apoptosis, restoring apoptosis is a very important therapeutic strategy for antitumor therapy (Baig et al. 2016; Plati et al. 2008). Therefore, next, we used flow cytometry to measure subG1 changes after the combination treatment, which is marker of apoptosis. Not surprisingly, ginsenoside PT combined with paclitaxel significantly increased subG1 cell accumulation both after 24 h and 48 h (Fig. 1E, *Fig. S1*). Taken together, these results suggested ginsenoside PT as a very effective molecular to reverse paclitaxel resistance in TNBC cells.

3.2 The combination treatment activates mitochondria mediated apoptosis

The alteration of pro-apoptotic proteins and anti-apoptotic proteins play important roles in the determination of cancer cells apoptosis, and are associated with chemoresistance (Campbell & Tait 2018; Warren et al. 2019). Thus, we observed the protein expression of BAX and BCL-2 after treatment, two key mediators of apoptotic response to chemotherapy. As shown in Fig 2a-b, ginsenoside PT combined with paclitaxel significantly increase BAX and decrease BCL-2 expression in a dose and time dependent manner.

Besides BAX and BCL-2, MCL-1 was recently reported to be associated with poor prognosis in TNBC patients and can be used as a therapeutic target (Campbell et al. 2018). Notably, we have shown that IRAK1 inhibitor can decrease MCL-1 expression in MB321-PR cells to induce cell apoptosis. Therefore, we also evaluated the protein expression of MCL-1 after treatment. As shown in Fig. 2A-B, the combination treatment also resulted in down-regulation of MCL-1 expression, contrasts to the changes of BAX to BCL-2 ratio, which together leads to cell apoptosis.

3.3 The combination treatment inhibits IRAK1/NF-κB and ERK pathways

To further clarify the signaling pathways that involved in ginsenoside PT effects, gene expression profiling was conducted in MB231-PR cells treated with DMSO, paclitaxel, ginsenoside PT, and combination, respectively. Results showed that NOD-like receptor signaling pathways played an important part in ginsenoside PT activity in MB231-PR cells (data not shown). Interestingly, through loss and gain of function study, we previously reported that activation of IRAK1, a key kinase of NOD-like receptor signaling pathway, is associated with paclitaxel resistance in TNBC cells (Wee et al. 2015) Moreover, target IRAK1 using pharmacologic inhibitor can induce MB231-PR cells apoptosis, when combined with paclitaxel (Wee et al. 2015) Thus, consideration was given to IRAK1 and its downstream signaling pathways. Results showed that the combination treatment can significantly inhibit the phosphorylation of IRAK1, P65 and ERK1/2 in a dose and time dependent manner (Fig. 2A-B). To additionally characterize the functional effects of IRAK1 mediated pathways, we investigated the mRNA expression of NF-κB target genes by qPCR, including interleukin 6 (IL6), IL8, chemokine (C-X-C motif) ligand 1 (CXCL1), and chemokine (C-C motif) ligand 2 (CCL2). The above cytokines were shown to be distinctly expressed among different group in our gene expression profiling experiment, and were reported to be critical for the anchorage independent growth of TNBC cells (Hartman et al. 2013). As shown in Fig. 2C, (*Table S1*, *Table S3*), compared to DMSO, paclitaxel significantly promoted the expression of IL6, IL8, CXCL1 and CCL2. However, this induction can be significantly attenuated when combined with ginsenoside PT.

Except these target cytokines, we previously published that IRAK1 and S100A7/8/9 form a feedback loop to drive the malignancy of TNBC cells (Goh et al. 2017). Here, we also showed

that the combination treatment significantly decreased S100A7 and S100A9 mRNA expression (Fig. 2C, *Table S1*, *Table S3*), although S100A8 mRNA expression level was too low to detect. These results together suggested that the combination treatment overcome paclitaxel resistance by inhibiting IRAK1 mediated NF-κB and ERK pathways.

3.4 The combination treatment inhibits CSC-related genes expression and impairs tumor sphere growth and invasion ability

Companied with killing cancer cell, paclitaxel treatment has been reported to induce CSC enrichment, another key mechanism suggested to be responsible for chemoresistance and cancer metastasis (Bousquet et al. 2017; Zhang et al. 2019). And drug that can target cancer stemness are proposed as new strategies for clinical cancer treatment (Saygin et al. 2019; Sun et al. 2019). In order to testify the effect of combination therapy on characteristics of CSC, firstly, qPCR was used to check the expression of a group of CSC-related genes. As shown in Fig. 3A, (*Table S2*, *Table S4*, *Table S5*), compared to paclitaxel, the combination treatment significantly lead to down-regulation of octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), NANOG, aldehyde dehydrogenase 1 (ALDH1), and CD44 gene expression. Secondly, tumor sphere and transwell invasion assay were conducted to assess CSC properties. As shown in Fig. 3B and 3C, the combination treatment significantly impaired tumor sphere growth and invasion ability of MB231-PR cells.

Discussion

Treatment of TNBC has been challenging, due to lack of target therapy options and constantly acquired resistance. In this study, we investigated the combination treatment of ginsenoside PT and paclitaxel on viability and apoptosis of MB231-PR cells, and clarified the signaling pathways underlies. Our data show that the combination can synergistically inhibit MB231-PR cell viability, induce subG1 accumulation and trigger the mitochondrial mediated apoptosis. Our data further suggest that the combination can inhibit IRAK1/NF-κB and ERK signaling pathways, resulted in down-regulation of inflammatory factors and S100A7/9 expression, which are the main cytokines in tumor microenvironment contributed to CSC phenotype and function. In addition, we show that combination can inhibit CSC-related genes expression and impair tumor sphere growth and invasion ability.

It is suggested that the BCL-2 family are key mediators of anti-cancer therapeutics, and abnormal expression of apoptotic proteins contributed to chemoresistance (Hata et al. 2015). In addition to other members, decreased BAX/BCL-2 ratio and elevated MCL-1 expression were reported to be closely related with paclitaxel resistance in breast cancer (Lee et al. 2017; Sharifi et al. 2014). Drugs which can inhibit the activity of these proteins are believed to improve the efficacy of chemotherapeutic agents. Interestingly, our data show that ginsenoside PT augments the effects of paclitaxel by up-regulating BAX/BCL-2 ratio and down-regulating MCL-1 expression.

The results in this study are consistent with our previous published papers, showing that pharmacologic inhibition of IRAK1 phosphorylation and downstream signaling pathways activation can overcome TNBC paclitaxel resistance. Notably, other group recently reported that the expression of IRAK1 was positively correlated with tumor size following neoadjuvant chemotherapy (NCT) (Yang et al. 2019)Breast cancer patients, with higher expression of IRAK1 both before and after NCT, had a shorter survival period (Yang et al. 2019)These results together highlight the role of IRAK1 in chemoresistance and clinical application of IRAK1 inhibitors.

Another our major finding is that inhibition of IRAK1/NF-κB and ERK pathways by Ginsenoside PT reduces stem cell characteristics. CSCs have been reported as one of the determining reasons for chemoresistance and subsequent cancer relapse. And one of the mechanisms that CSCs are acquired is taking advantage of paclitaxel treatment induced inflammation cytokines and S100 protein family in tumor microenvironment.

In our experiment, decreased expression of inflammation cytokines (IL-6, IL-8, CXCL1 and CCL2) can be noticed. The above cytokines are reported to be NF-κB targets, and the expression are induced following NF-κB activation after chemo treatment (Jia et al. 2017). In turn, these factors activate inflammation related signaling pathways such as NF-κB and signal transducer and activator of transcription 3 (STAT3) (Wang et al. 2018; Wong et al. 2015; Yue et al. 2006), which further promote cell survival through regulating apoptosis proteins and promote the formation of CSC through regulating CSC related genes (Rhyasen et al. 2013). Importantly, in accordance to IL-8 inhibitor, anti-IL6 antibody, anti-CXCL1 antibody, or anti-CCL2 antibody, here we showed that target IRAK1 mediated pathways by ginsenoside PPT can effectively down-regulate these cytokines and disrupt this process (Dey et al. 2019; Heo et al. 2016; Miyake et al. 2019; Teng et al. 2017).

Besides, we also identify that S100A7/9 are down-regulated after combination treatment. S100A7/9 are members of the S100 protein family, which are closely related to tumorigenesis and progression (Cancemi et al. 2018; Chen et al. 2014). In addition, S100A7/8/9 can be regulated by NF-κB and STAT3, which in turn can activate NF-κB and ERK (Hermani et al. 2006; Liu et al. 2013; Nemeth et al. 2009). S100A8/9 and CXCL1/2, or S100A7/8/9 and IRAK1, form a feedback loop to cause cancer chemoresistance and drive breast cancer tumor sphere growth (Acharyya et al. 2012; Goh et al. 2017). Collectively, our data suggest that ginsenoside PT can disrupt this feedback loop to inhibit CSC characteristics.

As to molecular phenotype in breast cancer, CSCs showed CD44+/CD24- and high ALDH1 activity. In parallel, other characters include overexpression of transcription factors OCT4, SOX2 and NANOG, which are associated with high-grade stage and poor clinical outcome in TNBC. In this part, we demonstrate that ginsenoside PT combined with paclitaxel can inhibit CSCs related gene expression, impair tumor sphere growth and invasion ability.

Conclusions

Our study demonstrates that ginsenoside PT can resensitize MB231-PR cells to paclitaxel treatment by inhibiting the IRAK1/NF-κB and ERK pathways, reducing stem cell characteristics, thus provide it as a novel molecular for clinic use.

Figures

Fig 1

Ginsenoside PT combined with paclitaxel inhibit MB231-PR cell viability and induce cell apoptosis (A) Single treatment of ginsenoside PT on MB231-PR cell viability. Cells were treated with different concentration of drug for 4 days. (B) Combination treatment of ginsenoside PT and paclitaxel on MB231-PR cell viability. 75 nM paclitaxel was used in this part experiment, and the concentration of ginsenoside PT in single use group was 10 μM. (C) Combination treatment of ginsenoside PT and paclitaxel on MB231-PT cell viability. 1nM paclitaxel was used in this part experiment, and the concentration of ginsenoside PT in single use group was 10 μM. (D) Representative images of colony formation assay. MB321-PR cells were treated for 15 days with DMSO, 75 nM paclitaxel, 10 μM ginsenoside PT or combination, respectively. (E) Flow

cytometry detection of cell cycle after treatment for 24 h and 48 h. **P < 0.001, ****P < 0.0001. P-values were calculated with t test.

Fig 2

The combination treatment activates apoptosis pathway and inhibits IRAK1/NF-κB, ERK pathways in MB231-PR cells. (A) Western blot analysis of proteins expression after cells treated with indicated concentrations of ginsenoside PT for 24 h. (B) Western blot analysis of proteins expression after cells treated with 10 μM ginsenoside PT for indicated hours. (C) qPCR analysis of IRAK1/NF-κB downstream inflammatory cytokines and S100A7/9 gene expression after cells treated with 10 μM ginsenoside PT for indicated hours.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. P-values were calculated with t test.

Fig 3

The combination treatment inhibits inflammatory cytokines expression, tumor sphere growth and cell invasion ability. (A) qPCR analysis of CSC-related genes expression after cells treated with 10 μM ginsenoside PT for indicated hours. (B) Representative images of tumor sphere assays. Cells were seeded into Corning 96-well spheroid microplates and cultured with MammoCul medium. Tumor sphere was observed after treated with 10 μM ginsenoside PT or/ and 75 nM paclitaxel for 12 days. (C) Transwell invasion assay of MB231-PR cells after drug treatment. Cells were seeded into Corning transwell polycarbonate membrane inserts coated with Matrigel (300 μg/mL) and cultured for 24 h. *P < 0.05, **P < 0.01, ***P < 0.001. P-values were calculated with t test.

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Figure 1

Ginsenoside PT combined with paclitaxel inhibit MB231-PR cell viability and induce cell apoptosis

(A) Single treatment of ginsenoside PT on MB231-PR cell viability. Cells were treated with different concentration of drug for 4 days. (B) Combination treatment of ginsenoside PT and paclitaxel on MB231-PR cell viability. 75 nM paclitaxel was used in this part experiment, and the concentration of ginsenoside PT in single use group was 10 μM. (C) Combination treatment of ginsenoside PT and paclitaxel on MB231-PT cell viability. 1nM paclitaxel was used in this part experiment, and the concentration of ginsenoside PT in single use group was 10 μM. (D) Representative images of colony formation assay. MB321-PR cells were treated for 15 days with DMSO, 75 nM paclitaxel, 10 μM ginsenoside PT or combination, respectively. (E) Flow cytometry detection of cell cycle after treatment for 24 h and 48 h. **P < 0.001, ****P < 0.0001. P-values were calculated with t test.

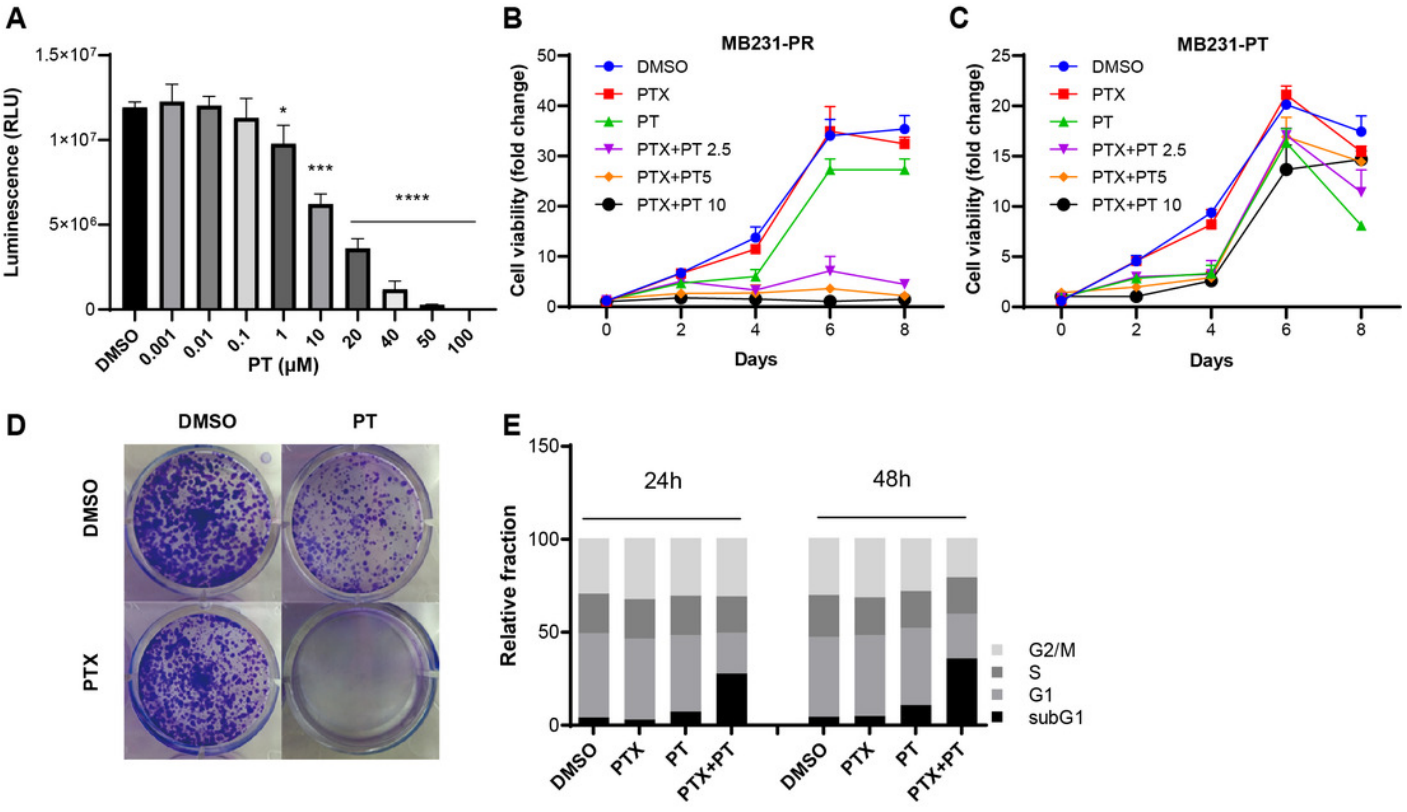
A
Luminescence (RLU)
1.5×10⁷
1×10⁷
5×10⁶
0
*
***
****
DMSO
0.001
0.01
0.1
1
10
20
40
50
100
PT (μM)
B
MB231-PR
Cell viability (fold change)
DMSO
PTX
PT
PTX+PT 2.5
PTX+PT5
PTX+PT 10
Days
C
MB231-PT
Cell viability (fold change)
DMSO
PTX
PT
PTX+PT 2.5
PTX+PT5
PTX+PT 10
Days
D
DMSO
PT
DMSO
PTX
E
Relative fraction
24h
48h
G2/M
S
G1
subG1
DMSO
PTX
PT
PTX+PT
DMSO
PTX
PT
PTX+PT

Figure 2

The combination treatment activates apoptosis pathway and inhibits IRAK1/NF-κB, ERK pathways in MB231-PR cells.

(A) Western blot analysis of proteins expression after cells treated with indicated concentrations of ginsenoside PT for 24 h. (B) Western blot analysis of proteins expression after cells treated with 10 μM ginsenoside PT for indicated hours. (C) qPCR analysis of IRAK1/NF-κB downstream inflammatory cytokines and S100A7/9 gene expression after cells treated with 10 μM ginsenoside PT for indicated hours.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. P-values were calculated with t test.

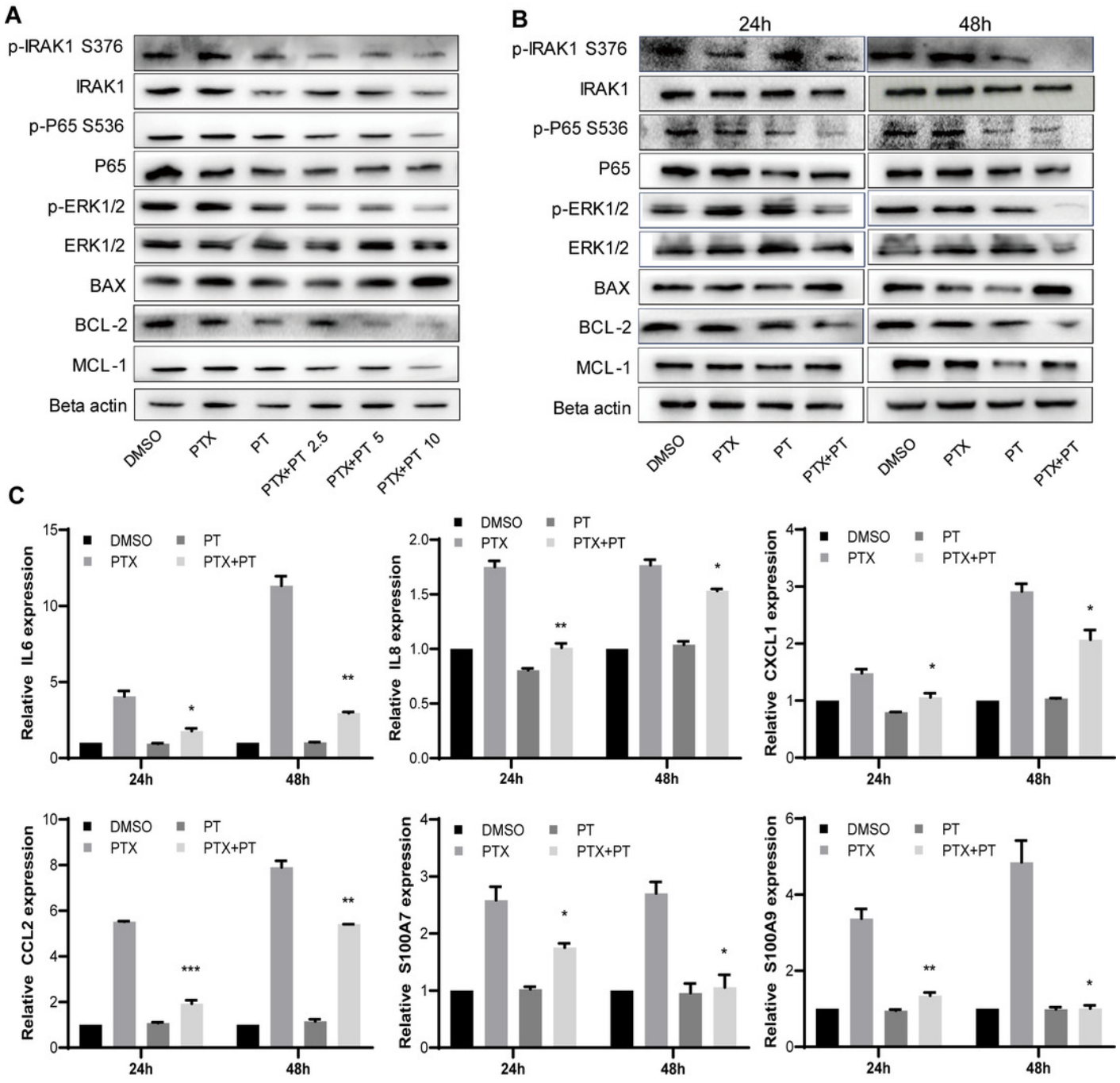
A
p-IRAK1 S376
IRAK1
p-P65 S536
P65
p-ERK1/2
ERK1/2
BAX
BCL-2
MCL-1
Beta actin
DMSO
PTX
PT
PTX+PT 2.5
PTX+PT 5
PTX+PT 10
B
24h
48h
p-IRAK1 S376
IRAK1
p-P65 S536
P65
p-ERK1/2
ERK1/2
BAX
BCL-2
MCL-1
Beta actin
DMSO
PTX
PT
PTX+PT
C
Relative IL6 expression
Relative IL8 expression
Relative CXCL1 expression
Relative CCL2 expression
Relative S100A7 expression
Relative S100A9 expression
DMSO
PTX
PT
PTX+PT
24h
48h

Figure 3

The combination treatment inhibits inflammatory cytokines expression, tumor sphere growth and cell invasion ability

(A) qPCR analysis of CSC-related genes expression after cells treated with 10 μM ginsenoside PT for indicated hours. (B) Representative images of tumor sphere assays. Cells were seeded into Corning 96-well spheroid microplates and cultured with MammoCul medium. Tumor sphere was observed after treated with 10 μM ginsenoside PT or/ and 75 nM paclitaxel for 12 days. (C) Transwell invasion assay of MB231-PR cells after drug treatment. Cells were seeded into Corning transwell polycarbonate membrane inserts coated with Matrigel (300 μg/mL) and cultured for 24 h. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. P-values were calculated with t test.

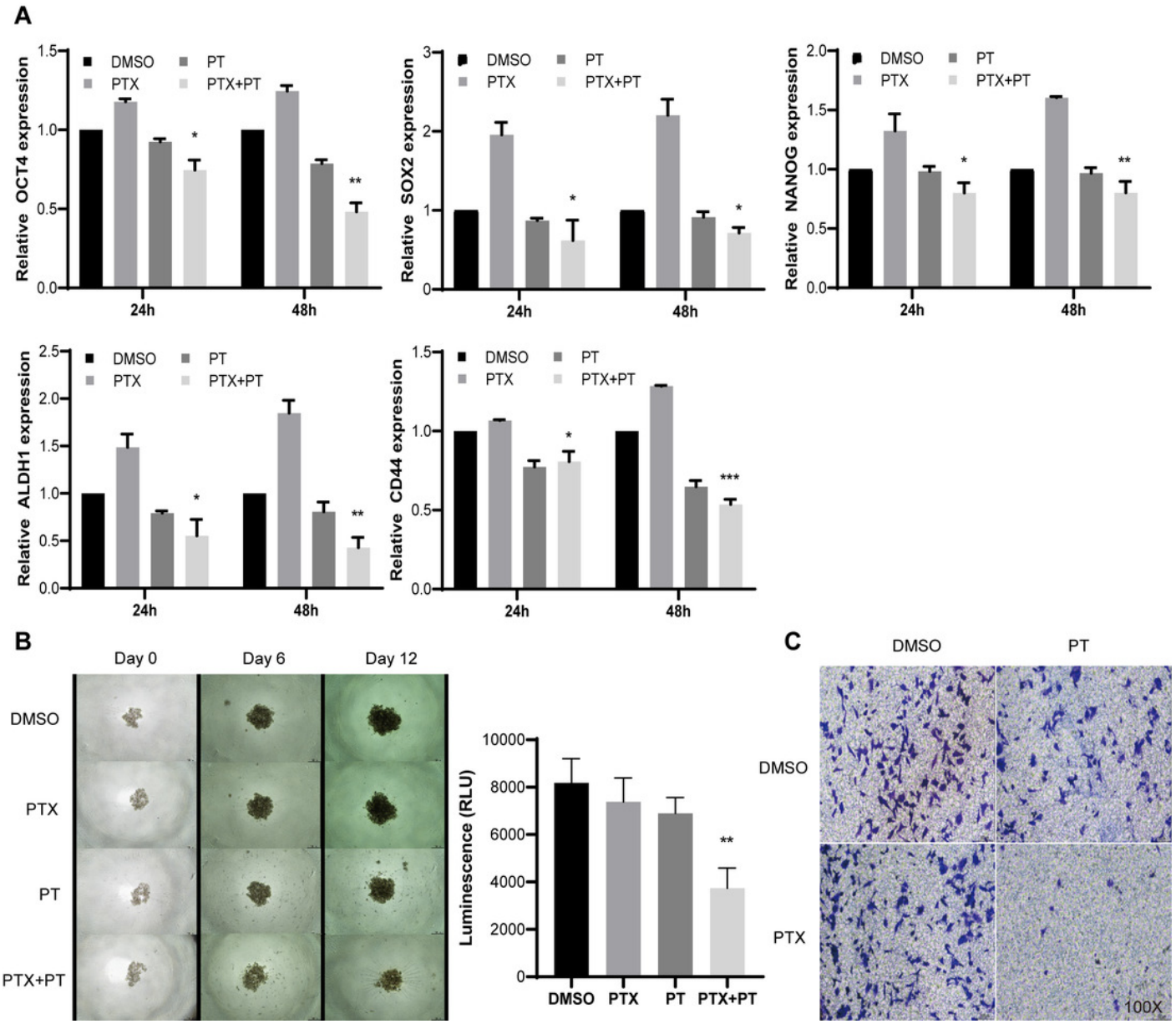
A
DMSO
PT
PTX
PTX+PT
Relative OCT4 expression
Relative SOX2 expression
Relative NANOG expression
Relative ALDH1 expression
Relative CD44 expression
24h
48h
B
Day 0
Day 6
Day 12
DMSO
PTX
PT
PTX+PT
Luminescence (RLU)
C
DMSO
PT
PTX
100X

Table 1(on next page)

qPCR primers

Table 1 qPCR primers

Gene	forward (5’-3’)	reverse (5’-3’)
IL6	AGTTCCTGCAGAAAAAGGCAAAG	AAAGCTGCGCAGAATGAGAT
IL8	ACCGGAAGGAACCATCTCAC	GGCAAAACTGCACCTTCACAC
CXCL1	CCAGCTCTTCCGCTCCTC	CACGGACGCTCCTGCTG
CCL2	CCCAAAGAAGCTGTGATCTTCA	TCTGGGGAAAGCTAGGGGAA
S100A7	GACAAGATTGAGAAGCCAAGCC	TGTGCCCTTTTTGTCACAGG
S100A8	TGCCGTCTACAGGGATGAC	TCTGCACCCTTTTTCCTGATATAC
S100A9	TCCTCGGCTTTGACAGAGTG	TGGTCTCTATGTTGCGTTCCA
OCT4	CTGGGTTGATCCTCGGACCT	CCATCGGAGTTGCTCTCCA
SOX2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT
NANOG	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
ALDH1	CTGCTGGCGACAATGGAGT	GTCAGCCCAACCTGCACAG
CD44	TGCCGCTTTGCAGGTGTATT	CCGATGCTCAGAGCTTTCTCC
18S	CGAACGTCTGCCCTATCAACTT	ACCCGTGGTCACCATGGTA

1