**Abstract.** Lenalidomide is an immunomodulatory drug and possesses anti-angiogenic and immunomodulatory activities against multiple myeloma. The present study assessed the **in vitro** effect of lenalidomide combined with cisplatin on MDA-MB-231, a triple-negative breast cancer (TNBC) cell line and explored the underlying molecular mechanism of this combination. Cell viability, apoptosis and the protein expression of phosphorylated (p) and total extracellular signal-regulated kinase (ERK), B-cell lymphoma-2 (Bcl-2), caspase-3, cleaved poly-adenosine diphosphate-ribose polymerase (cPARP), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were measured in MDA-MB-231 cells treated with different concentrations of lenalidomide, cisplatin and their combination using different biochemical assays. Lenalidomide demonstrated no significant effect on the cell viability of MDA-MB-231 cells, even at high concentrations, whereas lenalidomide in combination with cisplatin, significantly reduced cisplatin IC$_{50}$ from 7.8 to 3.0 µM in MDA-MB-231 cells. In addition, lenalidomide and cisplatin in combination significantly induced cell apoptosis by 1.6- and 1.38-fold, respectively compared with lenalidomide and cisplatin alone (P<0.05). The expression levels of VEGF, bFGF and Bcl-2 proteins were significantly reduced (P<0.01), whereas caspase-3 and cleaved PARP expression were significantly increased in MDA-MB-231 cells treated with the combination compared to those treated with single agents (P<0.01). Lenalidomide treatment alone significantly reduced the p-ERK level compared with the control (P<0.05) and cisplatin treatment alone significantly increased it (P<0.01), however treatment with them in combination significantly reduced the p-ERK level in MDA-MB-231 cells compared with cisplatin treatment alone (P<0.05). In conclusion, the present study provides the basis for using lenalidomide in combination with cisplatin in TNBC therapy.

**Introduction**

Breast cancer is a significant worldwide health problem in women and accounts for 12% of all cancers with 1.7 million newly diagnosed cases and 6% of all cancer deaths globally in 2012 (https://www.cdc.gov/cancer/international/statistics-hm.htm. Retrieved on March 30, 2017). Fortunately, decades-long advancement in breast cancer treatment and prevention lead to dramatic improvement of breast cancer patient's survival. According to St Gallen Conference 2013, breast cancer can be molecularly classified into different subtypes, including luminal A (ER and PR-positive, low rate of Ki-67 and HER2-negative) and luminal B, luminal B1, HER2-negative (ER-positive, PR <10% or negative, high rate of Ki-67), luminal B2 HER2-positive (ER-positive and PR-negative), HER2-positive non-luminal (ER and PR-negative) and basal-like (ER, PR and HER2-negative) (1). The receptors-positive breast cancer is usually cured with anti-receptor therapy (e.g., tamoxifen treatment for ER+ breast cancer, while Trastuzumab for HER2+ breast cancer (2-4). The basal-like subtype, also known as triple negative breast cancer (TNBC), is commonly negative for ER, PR and HER2. However, TNBC, represents 15% of all breast cancer, often occurs in young women and 80-90% of TNBC is an aggressive invasive ductal carcinoma (5), while 15-30% of TNBC patients will eventually develop tumor brain metastasis (6). To date, there is no effective therapy for TNBC and poor survival is common in TNBC compared to other breast cancer subtypes. Thus, effective therapy that prolongs or improves survival of TNBC patients is needed.

Lenalidomide is a derivative of thalidomide, which is an immunomodulatory drug. Lenalidomide has been reported to possess anti-angiogenic and immunomodulatory effects (7-10) and was approved by the US FDA as the first-line therapy in multiple myeloma (11). Recent clinical trials have demonstrated the antitumor effect of lenalidomide...
in different solid tumors, such as hepatocellular cancer (12) and colorectal cancer (13). In addition to the anti-angiogenic and immunomodulatory properties, lenalidomide has been shown to have cytotoxic effect in tumor cells (13). In clinical trials, lenalidomide was either used as a single agent or in combination with other chemotherapeutic drugs in many solid tumors; for example, Said et al showed that lenalidomide in combination with 5-fluorouracil, leucovorin, and oxaliplatin was able to control advanced colorectal cancer (13), while Safran et al assessed the activity and efficacy of lenalidomide on advanced hepatocellular cancer that was previously treated with sorafenib (14), where six out of 40 patients (15%) achieved a partial response, including two patients (5%) had stable disease for more than 32 months (14). These data indicate that lenalidomide could be more effective in combination with chemotherapeutic drugs, such as gemcitabine or docetaxel (12,15). Furthermore, Brosseau et al (16) demonstrated that lenalidomide was able to inhibit proliferation of MDA-MB-231 cells through the restoration of vitamin D sensitive phenotype. Wu et al (12) also showed that combination of lenalidomide with gemcitabine improved survival of pancreatic cancer patients. Thus, in the present study, we will investigate the therapeutic effect of lenalidomide on TNBC cells in combination with cisplatin, a frequently used chemotherapeutic drug for TNBC. The concentration of drug required to reduce cell viability by 50% (IC50) was calculated using Compusyn software (ComboSyn, Inc., Paramus, NJ, USA) (17). We expected that lenalidomide could reduce of cisplatin, reducing cisplatin IC50 value, inducing tumor cells to undergo apoptosis, and inhibiting angiogenesis in triple-negative breast cancer MDA-MB-231 cells in vitro.

Materials and methods

Reagents and cell culture. Lenalidomide was purchased from the AMQUAR Corporation (cat. no. EY0006; Denver, CO, USA), while cisplatin was obtained from MCE Corporation (cat. no. HY-17394; Dublin, CA, USA). Lenalidomide was dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock solution, stored at -20°C, and used within a week. Cisplatin was dissolved in distilled water at 10 mM stock solution and stored at 4°C.

A human triple-negative breast cancer line MDA-MB-231 was obtained from the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco’s modified Eagle’s medium (Thermo-Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (both from Gibco, Gaithersburg, MD, USA) in a humidified incubator with 5% CO2 at 37°C.

Cell viability MTT assay. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) assay. In brief, MDA-MB-231 cells were seeded into 96-well plates at a density of 5x10⁴ per well and cultured overnight. On the next day, cells were treated with various concentrations of cisplatin (0.12-30 µM), lenalidomide (1.25-320 µM) or their combination in 100 µl volumes of growth medium for 72 h. Cells were then collected in ice-cold PBS, and apoptotic cells were detected using FITC Annexin V-FITC/PI Apoptosis Detection kit (cat. no. 4A; Biotech Co., Ltd., Beijing, China) according to the manufacturer’s protocol. The stained cells were then measured with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometric apoptosis assay. To assess cell apoptosis, MDA-MB-231 cells were seeded into 6-well plates at a density of 1x10⁵ per well. After 24 h culture, cells were treated with cisplatin (3 µM), lenalidomide (1 µM), or their combination for 72 h. Cells were then collected in ice-cold PBS, and apoptotic cells were detected using FITC Annexin V-FITC/PI Apoptosis Detection kit (cat. no. 4A; Biotech Co., Ltd., Beijing, China). Protein samples (20 µg) were heated at 100°C for 10 min and then separated in 10% Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidenedifluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). For western blotting, the membranes were blocked for 2 h at room temperature in 5% bovine serum albumin (BSA) and then incubated with primary antibodies against B-cell lymphoma-2 (Bcl-2) (1:1,000), caspase-3 (1:1,000), cleaved poly-adenosine diphosphate-ribose polymerase (cPARP) (1:1,000) (Proteintech Group, Inc., Cambridge, MA, USA), phosphorylated and total extracellular signal-regulated kinase (ERK) (1:1,000; Cell Signaling Technology, Inc., Shanghai, China), basic fibroblast growth factor (bFGF) (1:1,000) or vascular endothelial growth factor (VEGF) (1:1,000) (both from Affinity Biosciences, Cincinnati, OH, USA) for 12 h at 4°C. The membranes were washed with Tris-based saline-Tween-20 solution (TBST) for three times and then incubated with a secondary antibody at a dilution of 1:1,000 (EMD Millipore) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL)-Plus Western blotting detection reagents (Millipore).

Statistical analysis. All experiments were repeated at least three times and the data were expressed as mean ± standard deviation. All statistical analyses were performed by using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Student’s t-test was used to analyze the mean difference between two groups, while comparisons of the means among multiple groups were analyzed using one-way analysis of variance followed by the post-hoc Student-Newman-Keuls test. The IC50 and combination index values were calculated using Compusyn software version 1.0 (ComboSyn, Inc.). A P-value equal to or <0.05 was considered statistically significant.
Results

Lenalidomide enhanced the cytotoxic effect of cisplatin on MDA-MB-231 cells. We first assessed the effects of lenalidomide and cisplatin combination on MDA-MB-231 cell viability using the MTT assay. Our results showed that cisplatin had a dramatic effect on the viability of MDA-MB-231 cells with an IC\textsubscript{50} of 7.8 µM (Fig. 1A); in addition, MDA-MB-231 cells viability was decreased with increasing the incubation time from 24 to 72 h. At 72 h, MDA-MB-231 cell viability was decreased to the minimum (Fig. 1B). However, lenalidomide treatment alone had a minimal effect on MDA-MB-231 cell viability, up to 320 µM (Fig. 1C). The maximum concentration of lenalidomide that could be safely used in patients is 1 µM; thus, we combined lenalidomide at 1 µM with nine concentrations of cisplatin in MDA-MB-231 cells. As a result, cell viability was dramatically reduced in combined group as compared to cisplatin alone (Fig. 1D). The effect of drug combination was assessed by Compusyn software. IC\textsubscript{50} is the major evaluation index in Compusyn software. The IC\textsubscript{50} of cisplatin was reduced to 3.0 µM in combined treatment group. Therefore, this concentration was further applied in apoptosis experiments.

Combination of lenalidomide and cisplatin increased cell apoptosis in MDA-MB-231 cells. We then treated MDA-MB-231 cells with 1 µM lenalidomide, 3.0 µM cisplatin, or combination and cell apoptosis was measured using FITC Annexin V-FITC/PI apoptosis detection kit. Our results demonstrated that lenalidomide and cisplatin alone induced cell apoptosis (P<0.05). In addition, their combination significantly increased cell apoptosis by 1.60 and 1.38-folds compared to lenalidomide and cisplatin single drug treatment (P<0.01) (Fig. 2). The combination treatment increased the rate of early apoptosis by 1.41 and 1.27-folds as well as the rate of later apoptosis by 1.80 and 1.51-folds compared to lenalidomide and cisplatin treatments, respectively. This suggested that lenalidomide and cisplatin combination reduces MDA-MB-231 cell viability through the induction of cell apoptosis.

Changes in protein expression after lenalidomide, cisplatin, or their combination treatment of TNBC cells in vitro. We further assessed the modulation in protein expression in MDA-MB-231 cells upon treating with lenalidomide and cisplatin combination. We found that lenalidomide treatment alone was able to reduce the level of phosphorylated (p)-ERK, which is known to regulate cell proliferation, in MDA-MB-231 cells, whereas p-ERK level was induced by cisplatin treatment alone (Fig. 3). Their combination significantly reduced the p-ERK level in MDA-MB-231 cells compared to cisplatin alone. Furthermore, lenalidomide and cisplatin alone down-regulated the expression of anti-apoptotic protein Bcl-2 but upregulated expression of pro-apoptotic protein caspase-3 and PARP in MDA-MB-231 cells (Fig. 3). However, the combination further inhibited Bcl-2 expression and upregulated caspase-3 and cleaved PARP expression compared to single treatment (Fig. 3).

Lenalidomide plays a key role in tumor progression through the inhibition of angiogenesis. VEGF and bFGF are the most potent mediators in angiogenesis in human cells. We,
Figure 2. Effect of lenalidomide and cisplatin on MDA-MB-231 cell apoptosis in vitro. MDA-MB-231 cells were grown and treated with lenalidomide, cisplatin, or their combination for 72 h and apoptotic cells were detected using FITC Annexin V-FITC/PI apoptosis detection kit. The bar graph is a quantitative presentation of the flow cytometric data. *P<0.05 and **P<0.01. NS, no significant difference; NC, negative control; Len, lenalidomide; Cis, cisplatin; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control.

Figure 3. Effect of lenalidomide and cisplatin on modulating the different protein expression in MDA-MB-231 cells. MDA-MB-231 cells were grown and treated with lenalidomide, cisplatin, or their combination for 72 h and then subjected to western blot analysis. The bar graphs are a quantitative presentation of the western blot analyses. *P<0.05 and **P<0.01. ERK, extracellular signal-regulated kinase; p-, phosphorylated; Bcl-2, B-cell lymphoma-2; cPARP, cleaved poly-adenosine diphosphate-ribose polymerase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; NC, negative control.
therefore, assessed their levels in MDA-MB-231 cells after treating with lenalidomide, cisplatin or combination. Our data showed that lenalidomide or cisplatin alone was able to reduce the levels of VEGF and bFGF proteins in MDA-MB-231 cells, which were further reduced with the combination (Fig. 3).

Discussion

To date, surgery, chemotherapy, and radiotherapy are the main therapeutic strategies in TNBC. However, a considerable number of TNBC patients are diagnosed at advanced stages of the disease, leading to curable surgery inaccessible because TNBC occurs more often in young women and is prone to early metastasis. Thus, present selections for TNBC treatment are limited and novel therapeutic strategies could help medical oncologists to cure TNBC or improve and prolong survival rates in TNBC patients. The present study assessed the in vitro antitumor activity of lenalidomide and cisplatin combination in TNBC cells. Our data showed that cisplatin alone showed inhibitory effect on MDA-MB-231 cell viability, whereas lenalidomide alone had only minimal effect, even at very high concentrations that are far beyond the clinically achievable dose in humans (16). Moreover, cell apoptosis was significantly induced in MDA-MB-231 cells upon treating with lenalidomide and cisplatin combination. Due to the potential significance of lenalidomide and cisplatin combination in MDA-MB-231, it was also necessary to explore the mechanism of their action in TNBC cells. We found that the combination dramatically reduced the levels of p-ERK, VEGF, bFGF and Bcl-2 proteins (P<0.05), and upregulated caspase-3 activity and cleaved PARP expression. This study, therefore, provides preliminary data to support lenalidomide combination with cisplatin in TNBC treatment.

As a single agent, cisplatin is one of the common chemotherapeutic drugs used in various human cancers, especially TNBC (18) and has been shown to inhibit tumor cell proliferation. In the present study, cisplatin significantly reduced MDA-MB-231 cell viability at an IC₅₀ value of 7.8 µM. On the other hand, it is controversy and debatable whether lenalidomide can be used to control solid tumors (14,16), although lenalidomide significantly inhibited growth of the chronic lymphocytic leukemia cells (19). In the present study, we further confirmed that lenalidomide alone had only a minimal effect on MDA-MB-231 cell viability, even at a very high dose of 320 µM, far beyond the clinically achievable concentration in humans (20). Thus, it is suggested that lenalidomide alone does not have much anti-proliferative activity in solid tumors, especially in TNBC cells. However, the combination of cisplatin with lenalidomide (at 1 µM) showed a synergistic inhibitory effect on MDA-MB-231 cells as compared to cisplatin alone. Furthermore, cisplatin IC₅₀ value was reduced from 7.8 µM to 3 µM when combined with 1 µM lenalidomide. To achieve the same inhibitory effect on MDA-MB-231 cell, cisplatin dose was significantly reduced when combined with lenalidomide and, thus, cisplatin side effects were also reduced.

Furthermore, we examined the ability of cisplatin, lenalidomide, and their combination in the induction of MDA-MB-231 cell apoptosis in vitro. Czarnomysy et al. (21) reported that cisplatin at concentrations 25, 50 and 100 µM was found to induce apoptosis of MDA-MB-231 cells after 24 h incubation. In the present study, we investigated whether cisplatin at a lower concentration 3 µM could induce MDA-MB-231 cell apoptosis. It is supportive that lenalidomide at the 1 µM dose was also able to induce apoptosis of MAD-MB-231 cells or other cancer cell lines (16,22). However, to date, there was no study reporting the effect of combined lenalidomide with cisplatin on TNBC cells. In the present study, we investigated the significant increase in apoptosis after combined treatment, compared with cisplatin and lenalidomide alone. However, further investigation using different TNBC cell lines is required to confirm our current data.

In addition, the present study also illustrated the underlying molecular events on lenalidomide and cisplatin modulation of different cell growth, apoptosis and angiogenesis-related proteins. For example, the ERK1/2 pathway is the classic cell growth signaling of the mitogen-activated protein kinase (MAPK) pathway. Activated ERK1/2 will promote cell survival and proliferation by phosphorylating many nuclear transcription factors (23). The present study showed that lenalidomide alone or in combination with cisplatin could reduce p-ERK levels in TNBC cells, although cisplatin alone induced p-ERK level. Indeed, Fryer et al. (24) also reported that lenalidomide significantly reduced level of p-ERK protein in pancreatic cancer cell lines, while Chen et al. (25) demonstrated that cisplatin had no effect on p-ERK protein expression in hepatocellular carcinoma cells. Furthermore, it is well established that a decrease in cell apoptosis could contribute to human carcinogenesis (26). The B-cell/leukemia-2 (Bcl-2), cleaved PARP and caspase-3 proteins were reported as the key proteins in regulating cell apoptosis. Decreased Bcl-2 or increased cleaved PARP and caspase-3 will promote cell apoptosis (27). In the present study, we found that lenalidomide and cisplatin induces caspases-3 activity and cleaved PARP expression, while reduces Bcl-2 expression. Our findings are consistent with a previous study (28). However, this study does have some limitations; for example, we only analyzed the expression of caspase-3 and cleaved poly-adenosine diphosphate-ribose polymerase (cPARP) and whether this combination may affect other apoptosis-related genes is not clear and needs further analysis. In addition, inhibition of tumor angiogenesis was one of the main mechanisms by which lenalidomide mediate its effect in multiple myeloma (7). Lenalidomide is a potent inhibitor of VEGF and bFGF (29), which is confirmed by the present study. VEGF and bFGF are the core factors in promoting tumor angiogenesis.

In conclusion, the combination of lenalidomide and cisplatin demonstrated a synergetic antitumor effect on MDA-MB-231 cells in vitro. This effect was at least partially due to induction of tumor cell apoptosis. Moreover, the inhibition of VEGF and bFGF by lenalidomide could partially contribute to the synergetic effect of lenalidomide and cisplatin combination, suggesting the potential strategy of lenalidomide and cisplatin combination in TNBC treatment.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
JL and XWW conceived and designed the experiments. LLY and QHL performed the experiments. LLY and XWW analyzed the data. LLY and XWW wrote the manuscript.

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Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors confirm that they have no competing interests.

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