Original Article
WP1130 increases cisplatin sensitivity through inhibition of *usp9x* in estrogen receptor-negative breast cancer cells

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Abstract: Approximately 30% of all breast cancers are caused by a lack of estrogen receptor (ER), which renders the cancer resistant to endocrine-based therapy. Many studies suggest that ubiquitin-specific peptidase 9, X-linked (*usp9x*) regulates multiple cellular behaviors, such as tumor growth, invasion, and resistance to chemotherapeutic agents. This study aimed to evaluate the anti-tumor effects of WP1130, a partially selective inhibitor of deubiquitinating enzymes, in breast cancer cells. We found that WP1130 enhanced cisplatin cytotoxicity in ER-negative tumor cells (MDA-MB-231 and MDA-MB-468), but had little effect in ER-positive Bcap-37 cells. Western blot analysis revealed that usp9x expression was dramatically lower in ER-positive cells compared to that in ER-negative cells. Furthermore, WP1130 treatment suppressed the expression of *usp9x* and *Mcl-1* in ER-negative cells, but not in ER-positive cells. In addition, we found that knockdown of *usp9x* diminished the chemosensitization activity of WP1130 on breast cancer cells in the presence of cisplatin. Taken together, these results demonstrated that combined treatment with WP1130 could increase the cisplatin sensitivity in a usp9x-dependent manner in estrogen receptor-negative breast cancer cells.

Keywords: Breast cancer, estrogen receptor, drug resistance, WP1130, *usp9x*

Introduction

Breast cancer is the leading cause of cancer-related deaths among females worldwide [1]. Estrogen plays a critical role in the growth and differentiation of mammary glands [2]. About 70% of breast cancers are estrogen receptor (ER)-positive, rendering ER an ideal molecular target for hormonal treatment [3]. Nevertheless, approximately 30% of breast cancers fail to express ER protein and are thus resistant to endocrine therapy [4].

Ubiquitination is a covalent modification of cellular proteins at the posttranslational level, which is involved in a wide range of biological processes including cell growth, differentiation, and apoptosis [5, 6]. Deubiquitinating enzymes (DUBs) are a large group of ubiquitin-specific proteases that can cleave ubiquitin from proteins [7, 8]. Currently, many studies report the differential expression or activation of DUBs in non-small cell lung cancer, breast cancer, and pancreatic ductal adenocarcinoma [9-11].

The ubiquitin specific peptidase 9X (*usp9x*), a member of the DUBs family, regulates multiple physiological pathways via targeting a variety of substrates [12]. It has been reported that increased *usp9x* expression predicts poor clinical outcome in patients with esophageal squamous cell carcinoma [13]. In addition, *usp9x* can promote multiple myeloma cell proliferation by stabilizing *myeloid cell leukemia 1* (*Mcl-1*), a member of the pro-survival BCL-2 family [14]. WP1130, a partially selective DUB inhibitor, has been considered a potential chemosensitizer owing to its inhibiting activity on usp9x deubiquitination [15]. In the current study, we investigated the anti-tumor effects of cisplatin-based therapy combined with WP1130 in breast cancer cells.
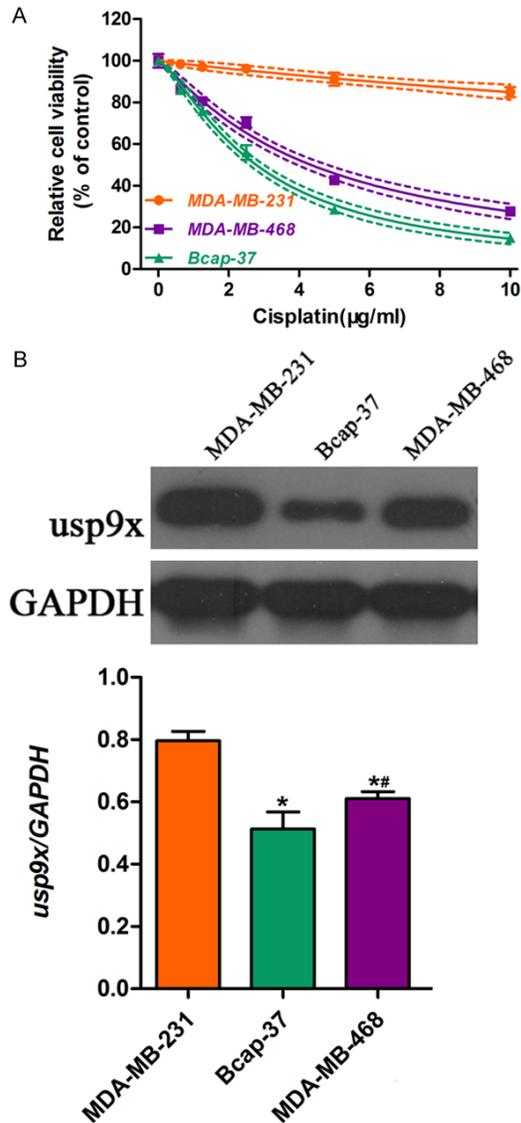
Figure 1. Different cisplatin sensitivity and usp9x expression in breast cancer cells. Breast cancer cell lines including MDA-MB-231, MDA-MB-468, and Bcap-37 were incubated with cisplatin for 48 h. A. CCK-8 assay was performed to measure cell viability of MDA-MB-231, MDA-MB-468, and Bcap-37 cells exposed to cisplatin at different doses (0, 2, 4, 6, 8, 10 μg/mg). B. Western blot was applied to detect the protein expression of usp9x in MDA-MB-231, MDA-MB-468, and Bcap-37 cells. The protein bands were then quantified and normalized to the internal control. * vs. MDA-MB-231 cells, P < 0.05; # vs. Bcap-37 cells, P < 0.05.

Materials and methods

Cell culture

Human breast cancer cell lines, including MDA-MB-231, MDA-MB-468, and Bcap-37, were pur-

chased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 incubator.

Cell viability assay

Cell viability was measured by using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) following the manufacturer's protocol. In brief, cells were seeded onto 96-well plates at a density of 3000 cells/well. After treatment with cisplatin for 48 h, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each cell. Three hours later, the absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

EdU incorporation assay

Measurement of inhibitive rate of cell proliferation was carried out using a Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA) following the procedure previously described [16].

Flow cytometry

Breast cancer cells were exposed to cisplatin alone or in combination with WP1130. Cells were trypsinized and centrifuged 48 h post the treatment, and the pellet obtained was washed twice with phosphate buffered saline (PBS). Cells were resuspended and then washed with PBS thrice. Apoptotic cells were detected with annexin V-FITC/PI following the protocol of Annexin V-FITC cell Apoptosis Detection Kit (BD, USA).

siRNA transfection

Cells were transfected with usp9x-small interfering RNA (siRNA) or negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection medium (Opti-MEM, Gibco, USA) was replaced with complete medium 12 h post transfection, and the cells were incubated for 48 h.

Western blot analysis

Cells were lysed in 50 μL cell lysis buffer containing protease inhibitors (Sigma, USA), and the cell lysates were subjected to sodium
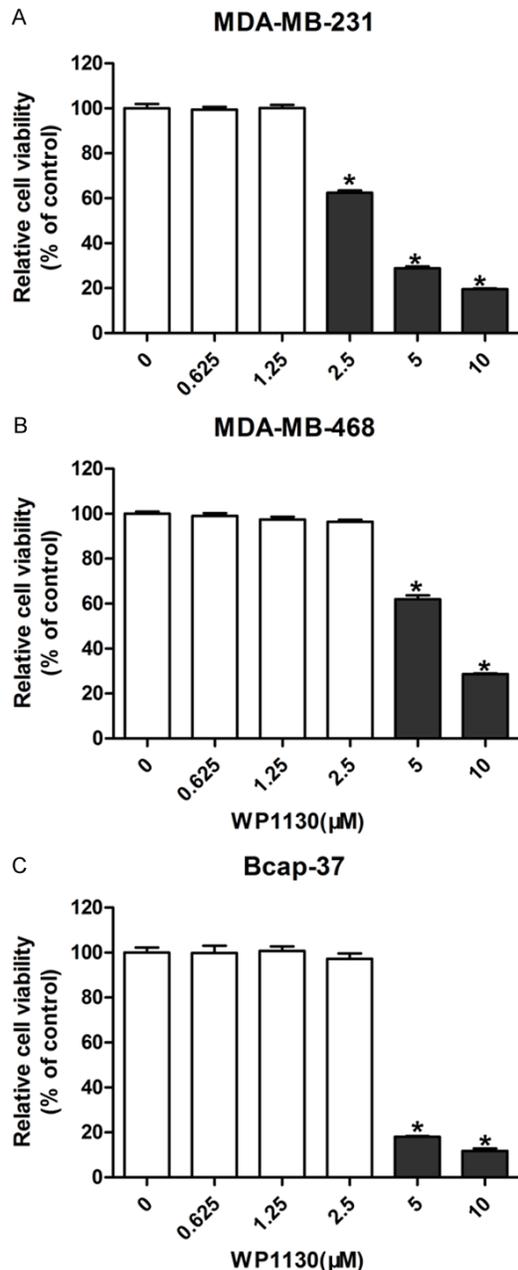
Figure 2. Effects of WP1130 on breast cancer cell viability. Breast cancer cells were exposed to different concentrations of WP1130 (0.625, 1.25, 2.5, 5, 10 μM) for 48 h. CCK-8 assay was performed to measure the cell viability in MDA-MB-231 (A), MDA-MB-468 (B) and Bcap-37 (C). *P < 0.05.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring proteins to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), primary antibodies, including anti-usp9x, anti-Mcl-1, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, Cambridge, MA,

USA), were added. Overnight incubation at 4°C was followed by the membranes being incubated with the appropriate HRP-conjugated secondary antibodies. Protein expression was detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and normalized with the internal control (GAPDH).

Statistical analysis

Data were presented as means ± standard deviation (SD) and analyzed using SPSS 16.0. Analysis of variance (ANOVA) was used to analyze differences between groups with the threshold significance level set at P < 0.05.

Results

Different cisplatin sensitivity and usp9x protein expression in breast cancer cells

First, we performed the CCK-8 assay to measure the viability of breast cancer cell lines (MDA-MB-231, MDA-MB-468 and Bcap-37) exposed to cisplatin for 48 h. Results suggested that the cisplatin sensitivity varied among these three cell lines (**Figure 1A**). The ER-positive Bcap-37 cells were most sensitive to cisplatin drug treatment; ER-negative MDA-MB-231 and MDA-MB-468 cells were more resistant to cisplatin drug treatment. Western blot analysis revealed that the level of usp9x protein expression in the breast cancer cells was higher in ER-negative MDA-MB-231 and MDA-MB-468 cells compared with that in ER-positive Bcap-37 cells (**Figure 1B**). These results indicated that the higher expression of *usp9x* in ER-negative cells may be associated with resistance to cisplatin in breast cancer cells.

Effects of WP1130 on breast cancer cell viability

Tumor cell lines (MDA-MB-231, MDA-MB-468, and Bcap-37) were exposed to different concentrations of WP1130 (0.625, 1.25, 2.5, 5, 10 μM) for 48 h to determine the cytotoxic effects of WP1130. We found that higher doses of WP1130 (5, 10 μM) strongly suppressed the viability of MDA-MB-231 (**Figure 2A**), MDA-MB-468 (**Figure 2B**), and Bcap-37 cells (**Figure 2C**). However, low concentrations of WP1130 (< 2.5 μM) showed negligible cytotoxicity on breast cancer cells. Thus, 2.5 μM WP1130 was used for further co-administration with cisplatin.
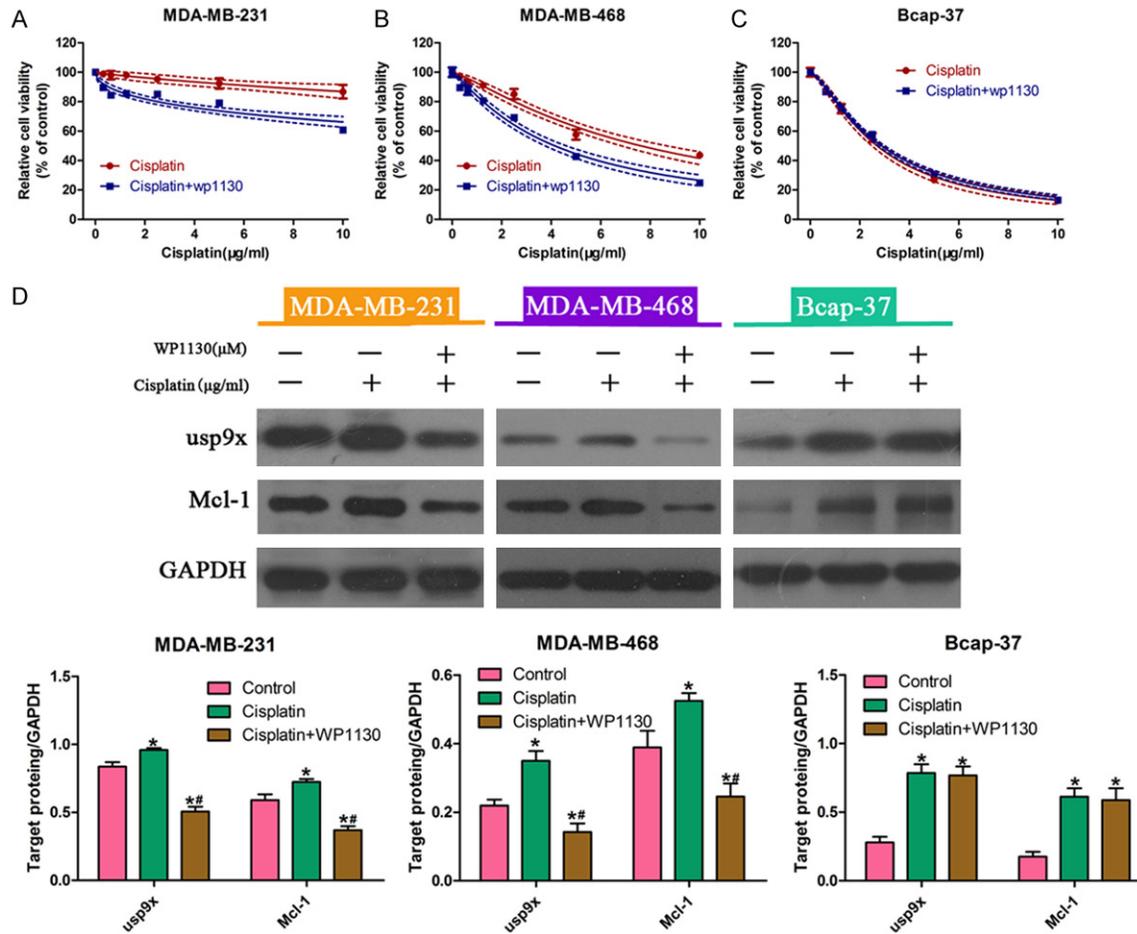
Figure 3. WP1130 increased the cytotoxic effects of cisplatin in breast cancer cells. Breast cancer cells were incubated with cisplatin alone or in combination with WP1130 for 48 h. The CCK-8 assay was performed to determine viability of MDA-MB-231 (A), MDA-MB-468 (B), and Bcap-37 (C) cells. Western blot analysis was performed to measure the protein expression of usp9x and Mcl-1 in MDA-MB-231, MDA-MB-468, and Bcap-37 cells exposed to cisplatin alone or in combination with WP1130 (2.5 μM). The protein bands were then quantified by densitometry with the imaging system (D). * vs. control, P < 0.05; # vs. cisplatin, P < 0.05.

WP1130 increased the cytotoxic effects of cisplatin in ER-negative breast cancer cells

Next, we incubated tumor cells with cisplatin alone or in combination with WP1130. Data from the CCK-8 assay revealed that ER-negative cells (MDA-MB-231 and MDA-MB-468) showed increased sensitivity to cisplatin treatment (**Figure 3A** and **3B**). No significant change in cisplatin cytotoxicity in ER-positive Bcap-37 cells (**Figure 3C**) was observed.

In addition, we investigated the expression of usp9x proteins in cells treated with cisplatin alone or in combination with WP1130. Western blot analysis showed that the protein levels of usp9x and Mcl-1 were significantly reduced in MDA-MB-231 and MDA-MB-468 cells in the

presence of WP1130. However, there was no obvious change in usp9x and Mcl-1 protein expression in Bcap-37 cells (**Figure 3D**). Taken together, these results implied that WP1130 increased cisplatin sensitivity, at least in part, through regulation of *usp9x* in breast cancer cells.

Effect of WP1130 on cell growth and apoptotic death in breast cancer cells

The DNA synthesis in breast cancer cells were determined using EdU incorporation assay. We found that the proliferation ability of MDA-MB-231, MDA-MB-468, and Bcap-37 cells was significantly reduced after cisplatin treatment. In addition, co-treatment of cells with WP1130 further decreased the DNA synthesis (**Figure**
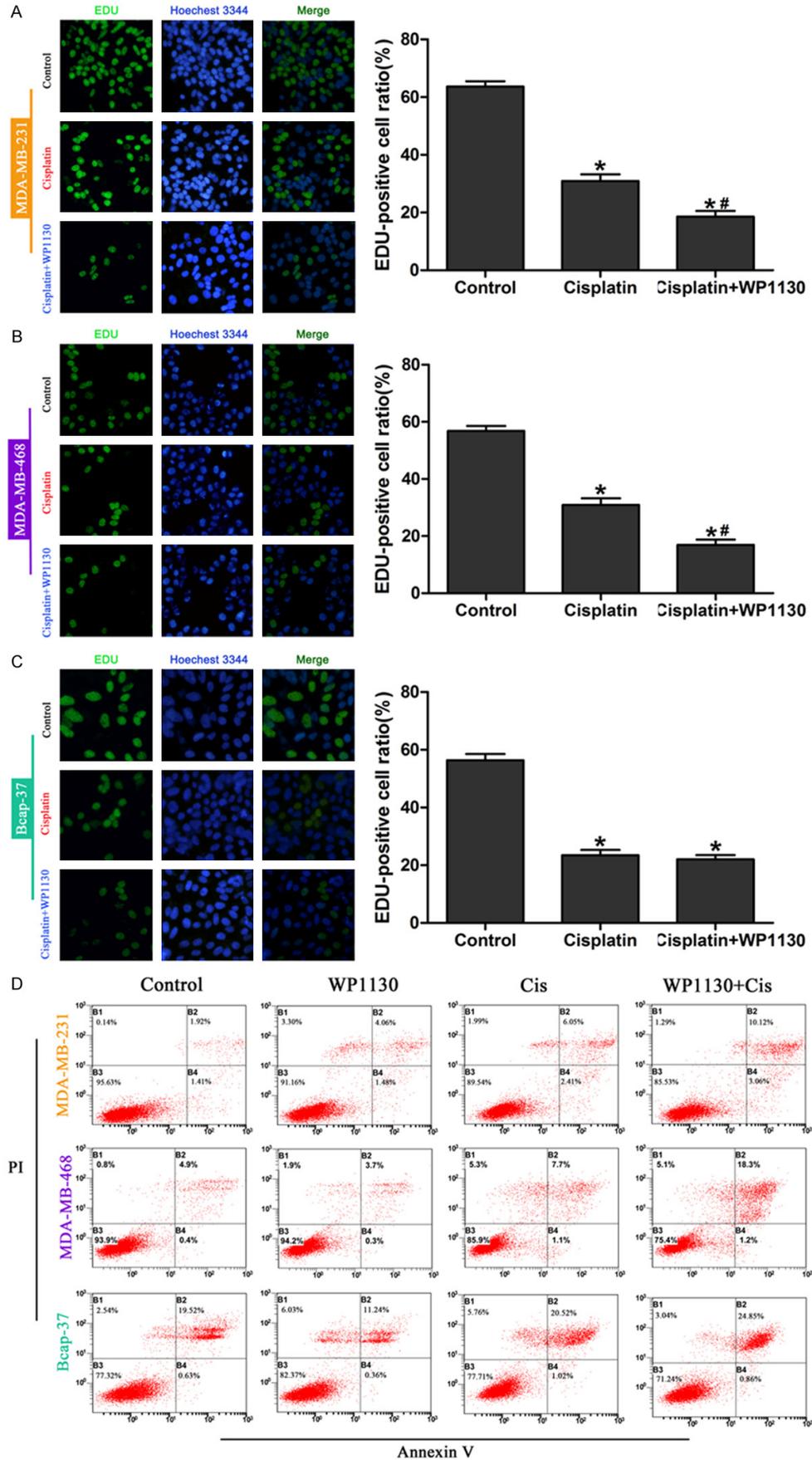
Figure 4. Effect of WP1130 on cell growth and apoptotic death in breast cancer cells. Breast cancer cell lines (MDA-MB-231, MDA-MB-468 and Bcap-37) were incubated with cisplatin alone or in combination with WP1130 for 48 h. Photomicrographs and bar charts depict the EdU staining and relative EdU-positive ratio, respectively, of (A) MDA-MB-231, (B) MDA-MB-468, and (C) Bcap-37 cells after treatment with cisplatin alone or in combination with WP1130 (2.5 μM). Symbols: * vs. control, $P < 0.05$; # vs. cisplatin, $P < 0.05$. (D) Flow cytometry was performed to detect the apoptosis rate of MDA-MB-231, MDA-MB-468, and Bcap-37 cells treated with cisplatin alone or combined with WP1130 (2.5 μM).
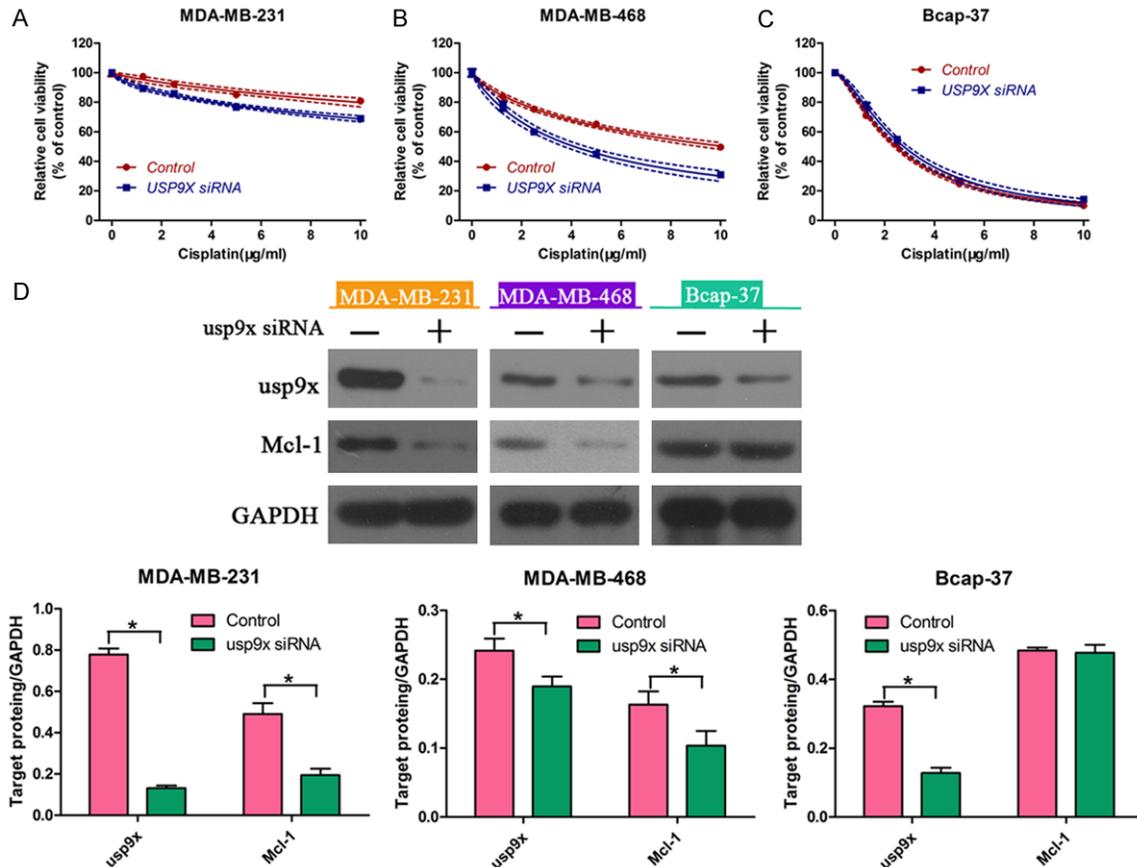
Figure 5. Downregulation of *usp9x* increased cisplatin sensitivity in breast cancer cells. After transfection with usp9x siRNA or negative control, breast cancer cell lines (MDA-MB-231, MDA-MB-468 and Bcap-37) were exposed to cisplatin at different concentrations (0, 2, 4, 6, 8, 10 μg/mg). Viability of (A) MDA-MB-231, (B) MDA-MB-468, and (C) Bcap-37 cells was determined using CCK-8 assay. (D) Western blot was performed to measure the protein expression of usp9x and Mcl-1 in MDA-MB-231, MDA-MB-468, and Bcap-37 cells. * vs. control, $P < 0.05$.

4A-C). Moreover, flow cytometry showed no obvious change in the apoptotic rates of tumor cells (**Figure 4D**). Collectively, these data suggested that combined treatment with WP1130 increased cisplatin cytotoxicity mainly through suppression of cell growth.

Down-regulation of usp9x increased cisplatin sensitivity in ER-negative breast cancer cells

RNA interference (RNAi) was used to knock-down the expression of *usp9x* in the three breast cancer cells to confirm the regulatory role of *usp9x* on cellular response to cisplatin.

Western blot analysis indicated that usp9x protein expression was down-regulated in MDA-MB-231, MDA-MB-468, and Bcap-37 cell lines (**Figure 5D**). Moreover, reduced protein expression of Mcl-1 was observed in MDA-MB-231 and MDA-MB-468 cells, but not in Bcap-37 cells (**Figure 5D**). As a result, the usp9x siRNA transfection increased the cisplatin cytotoxicity in MDA-MB-231 (**Figure 5A**), MDA-MB-468 cells (**Figure 5B**). Meanwhile, the cell viability in ER-positive Bcap-37 cells exhibited no significant changes regardless of *usp9x* status (**Figure 5C**). These data suggested that *usp9x*
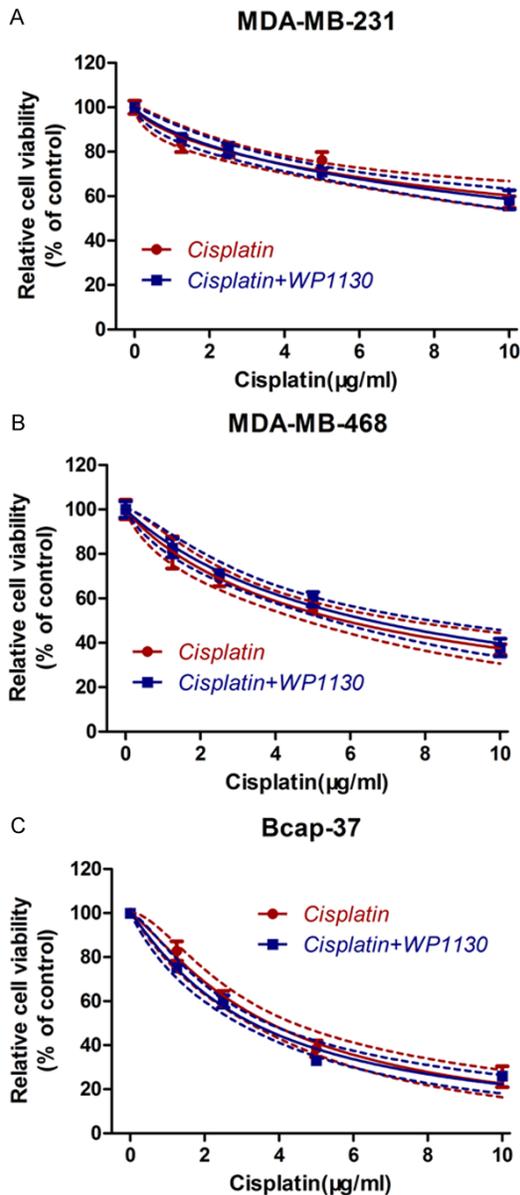
Figure 6. Usp9x knockdown diminished the chemosensitization role of WP1130. After transfection with usp9x siRNA or negative control, breast cancer cell lines (MDA-MB-231, MDA-MB-468 and Bcap-37) were exposed to cisplatin alone (0, 2, 4, 6, 8, 10 µg/mg) or in combination with WP1130 (2.5 µM). Viability of (A) MDA-MB-231, (B) MDA-MB-468, and (C) Bcap-37 cells was determined using CCK-8 assay.

played an important role in chemoresistance of breast cancer cells.

Usp9x knockdown diminished the sensitization role of WP1130 in breast cancer cells

Given that *usp9x* was involved in drug resistance, we hypothesized that the sensitization effects of WP1130 may be dependent on usp9x protein levels. To prove this hypothesis, we transfected usp9x-siRNA into MDA-MB-231 (**Figure 6A**), MDA-MB-468 (**Figure 6B**) and Bcap-37 (**Figure 6C**) cells exposed to cisplatin alone or in combination with WP1130. Consequently, no significant changes in cell viability were observed in cisplatin-treated cells and cisplatin plus WP1130-treated cells. These results suggested that WP1130 increased cellular responses to cisplatin via *usp9x* in breast cancer cells.

Discussion

ERs play a critical role in both normal breast development and in breast cancer progression [17]. The presence of ER is considered an effective predictive biomarker for disease-free survival in patients diagnosed with breast cancer. As such, the prognosis is poor for ER-negative patients who typically have a shorter survival time [18]. Currently, it has been recognized that drug resistance to traditional chemotherapeutics greatly contributes to failure in breast cancer therapy [19]. In the current study, we found that WP1130 greatly increased the cisplatin cytotoxicity on ER-negative breast cancer cells.

Protein ubiquitination has been demonstrated as a versatile posttranslational modification, which regulates a wide range of cellular behaviors via the ubiquitylating/deubiquitylating process. The gene *usp9x*, a member of the DUB family, has been implicated in many biological processes. In pancreatic cells, *usp9x* plays a role in pro-survival pathway through regulation of autophagy [20]. It has been documented that *usp9x* promotes tumor cell survival and suppresses apoptosis via deubiquitinating and stabilizing Mcl-1 [14]. Elevated expression of *usp9x* has been shown to be associated with poor prognosis for patients with multiple myeloma [14]. In addition, RNAi-mediated inhibition of *usp9x* reduced the tamoxifen sensitivity in breast cancer cells, indicating the association between *usp9x* and drug resistance [21]. In our study, we found that ER-positive breast cancer cells were more sensitive to cisplatin compared with ER-negative tumor cells. Intriguingly, usp9x protein express was lower in ER-positive cells compared with ER-negative cells, suggesting that increased usp9x protein expression may render breast cancer cells resistant to cisplatin.

WP1130, initially discovered as the inhibitor of JAK and STAT signals, recently has been shown to inhibit the activity of deubiquitinase [22]. It has been reported that WP1130-mediated inhibition of deubiquitinase triggers aggresome formation and promotes tumor cell apoptosis [23]. A recent study has revealed that WP1130 enhances the doxorubicin sensitivity in hepatocellular carcinoma cells through usp9x-dependent p53 degradation [24]. Our study showed that combined treatment with WP1130 increased cisplatin sensitivity in ER-negative cells (MDA-MB-231 and MDA-MB-468), but not in ER-positive Bcap-37 cells. Moreover, molecular investigation revealed WP1130-induced down-regulation of *usp9x* and *Mcl-1* in ER-negative cells but not in ER-positive cells. We also found that WP1130-cotreatment increased cisplatin sensitivity mainly through suppression of tumor cell growth. Collectively, these data suggest that WP1130 could increase the sensitivity of breast cancer cells to cisplatin, partly via *usp9x*, in ER-negative breast cancer cells.

Elevated expression of usp9x protein has been observed in several types of human cancers, including non-small cell lung cancer, esophageal squamous cell carcinoma, colon cancer, and follicular lymphoma [25-27]. On the molecular level, high levels of usp9x protein have been implicated in tumor growth, invasion, and metastasis through regulation of certain genes including *TGF-β*, *Mcl-1*, and β-catenin. It has been shown that down-regulation of *usp9x* increases the sensitivity of tumor cells to chemotherapies via degradation of Mcl-1 proteins [14]. Currently, we interfered with *usp9x* expression by using RNAi in three breast cancer cell lines. Consequently, the cisplatin sensitivity was increased in ER-negative cells, at least in part, through degradation of Mcl-1 via usp9x inhibition. In addition, we found that knock-down of *usp9x* diminished the sensitization role of WP1130 on breast cancer cells exposed to cisplatin, suggesting that WP1130 enhanced cisplatin cytotoxicity in a usp9x-dependent way.

In conclusion, the present study demonstrates that combined treatment with WP1130 could sensitize breast cancer cells to cisplatin dependent on *usp9x*. Thus, our study suggests that combination therapy with WP1130 may improve survival outcomes in patients with breast tumors lacking estrogen receptors.

Disclosure of conflict of interest

None.

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