Enhanced Anticancer Effect of the Combination of Cisplatin and TRAIL in Triple-Negative Breast Tumor Cells

Liping Xu, Shuping Yin, Sanjeev Banerjee, Fazlul Sarkar, and Kaladhar B. Reddy

Abstract

Women with triple-negative breast cancer (TNBC) have a worse prognosis compared with other breast cancer subtypes. Hormonal or Herceptin-based therapies were found to be ineffective because of the loss of target receptors, such as ER, PR, and HER-2 amplification. Conventional chemo- and/or radiation therapy also seems to have limited efficacy in TNBC patients. We studied the effects of cisplatin plus TRAIL on 1 normal and 2 TNBC cells in vitro. The in vitro studies indicate that cisplatin plus TRAIL significantly enhanced cell death in TNBC cell lines CRL2335 and MDA-MB-468 by approximately 60%–70% compared with approximately 10%–15% in CRL8799 normal breast cell line. Treatment with cisplatin/TRAIL also inhibited the expression of EGFR, p63, survivin, Bcl-2, and Bcl-xL in TNBC cells. Specific inhibition of EGFR and/or p63 protein in TNBC cells by small interfering RNA (siRNA) does not increase TRAIL-induced apoptosis. However, inhibition of survivin by siRNA enhances TRAIL-induced apoptosis. These observations suggested the possibility that survivin played an important role in cisplatin plus TRAIL-induced apoptosis in TNBC cells. In vivo experiments, treatment of mice with cisplatin plus TRAIL resulted in a significant inhibition of CRL2335 xenograft tumors compared with untreated control tumors. Taken together the data suggest that cisplatin plus TRAIL treatment have the potential of providing a new strategy for improving the therapeutic outcome in TNBC patients.

Introduction

Breast cancer continues to be a major health problem worldwide despite recent advances in its diagnosis and treatment. Based on gene expression profiles breast cancer can be the classification of into 5 main groups: (i) luminal A, (ii) luminal B (both are ER+), (iii) basal-like, (iv) HER2, and (v) normal breast-like tumors (1). At present, most treatment in breast cancer is targeted to either to ER or HER-2. Triple-negative breast cancer (TNBC) is characterized by the absence of 3 receptors, viz., estrogen receptor (ER), progesterone receptor (PR), and HER2, hence the term triple-negative. In approximately 60% of cases, these tumor cells express the receptor for epidermal growth factor (EGFR; 2–4) and may also contain mutations in p53 gene (5–7). The disease is diagnosed more frequently in younger and premenopausal women (6, 8–11). It was previously shown that mutations in BRCA1 gene leads to erroneous DNA repair resulting in genomic instability and predisposition to carcinogenesis. In TNBC patient’s loss of ER, PR, and HER-2 prevents targeted therapy. In general TNBC tumors are refractory to commonly used chemotherapeutic agents, as a result it leads to relatively poor prognosis (1, 12). Some reports have shown that BRCA1-associated TNBC tumors are sensitive to cisplatin chemotherapy (13). The use of platinum complexes for the breast cancer therapy is an emerging new treatment modality. Platinum drugs including cisplatin and carboplatin are widely used as anticancer drugs. Cisplatin is currently used to treat tumors of the head, neck, lungs, and ovarian cancer (14–16). However, therapeutic efficiency of cisplatin in TNBC is moderate. Therefore, it is important to identify new treatment modalities for TNBC patients.

Tumor necrosis-factor–related apoptosis-inducing ligand (TRAIL, a member of tumor necrosis factor family of death-receptor ligands) is reported to have a potential use in cancer therapy because of its ability to selectively kill cancer cells without affecting normal cells (17). TRAIL is shown to bind with death receptor 4 (DR4) and death receptor 5 (DR5) leading to the formation of death-inducing signaling complex (DISC) and Fas-associated protein with death domain (FADD). In turn, DISC and FADD recruits caspase-8 (or caspase-10) that plays an important role in inducing apoptosis either by direct activation of downstream effector caspases (caspase-3, caspase-6, and caspase-7) or by cleaving apoptotic molecules (Bcl-2 and Bcl-xL) resulting in further activation of caspase-9 complex (18). Studies in animals have also shown that TRAIL...
regresses cancer xenografts without affecting normal tissues (19).

In this study, we evaluated the molecular mechanism by which cisplatin sensitizes TNBC cell lines to TRAIL-induced apoptosis. Data show that cisplatin and TRAIL downregulates antiapoptotic proteins, survivin, Bcl-2, Bcl-xL and induces activation of caspase-3, -8, and -9 and increases apoptosis. However, in normal breast cell lines combination of cisplatin/TRAIL had a minimal effect on antiapoptotic proteins and shows a marginal increase in apoptosis in comparison with TNBC cells.

Materials and Methods

Cell lines and reagents

The human breast carcinoma cell line CRL8799, CRL2335, and MDA-MB-468 cells were obtained from the American Type Culture Collection (ATCC) and maintained in accordance with ATCC recommended culture media. All cells obtained from ATCC were immediately expanded and frozen down such that all cell lines could be restarted every 3 to 4 months from a frozen vial of the same batch of cells and no additional authentication was done in our laboratory. DR4/TRAIL-R1 monoclonal antibody and DR5/TRAIL-R2 polyclonal antibody was purchased from Imgenex. Monoclonal antibodies of PARP, Bid, actin, caspase-8 were purchased from BD Biosciences. Caspase-3, and caspase-9 antibodies and survivin small interfering RNA (siRNA) were purchased from Cell Signaling Technology. Anti-Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal was obtained from Sigma. Monoclonal antibodies of PARP, Bid, actin, caspase-8 were purchased from BD Biosciences. Caspase-3, and caspase-9 antibodies and survivin small interfering RNA (siRNA) were purchased from Cell Signaling Technology. Anti-Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal was obtained from Trevigen. TRAIL and caspase-3 inhibitors were purchased from R&D systems. Cell death Detection ELISAPLUS was purchased from Roche. Reagents for protein concentration analysis and protein gel electrophoresis were obtained from Bio-Rad p63 siRNA was obtained from Santa Cruz biotechnology. EGFR and random siRNA were obtained from Millipore. All other chemicals, unless otherwise specified, were obtained from Sigma in the highest suitable purities.

MTT assay

In brief, 5 × 10^4 cells were added in 96-well tissue culture plates. After 24 hours, cells were treated with TRAIL (10 ng/mL), cisplatin (10 μg/mL), or combination of TRAIL plus cisplatin for another 24 hours. Following treatments, 100 μL of MTT (1 mg/mL) was added into each sample and incubated for 3 hours under 5% CO2 and 37°C. The cell viability was measured using a standard protocol. In brief, cell extracts were obtained by lysing the cells in RIPA buffer (20 mmol/L Hepes, 100 mmol/L NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 1 mmol/L Na2VO4, 1 mmol/L EGTA, 50 mmol/L NaF, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture). Samples containing 100 μg of total protein were electrophoresed on 10% or 15% SDS-polyacrylamide gels and transferred on to PVDF membrane by electroblotting. Membranes were probed with antibodies as indicated, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham). Anti-G3PDH was used for loading controls.

RNA interference assay

Cells were plated in 6-well tissue culture plates at a density of 3 × 10^5/well in medium containing 10% FBS. After 24 hours, cells were transfected with 100 pmol of siRNA’s from EGFR, and/or p63, or survivin or random siRNA with scrambled sequence according to the manufacturer’s instructions. After 48 hours of transfection, cells were treated with or without TRAIL for additional 24 hours. Cells were then harvested for Western blot analysis.

Apoptosis assay

Cells were treated with cisplatin, TRAIL, or combination of cisplatin plus TRAIL for 16 hours. Cells were harvested and stained with FITC Annexin V and propidium iodide (PI) to identify early apoptotic cells. Apoptosis was determined using FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions.

Crystal violet staining

Crystal violet stain binds to the nuclei of the cells and gives a violet color (an OD_{595} reading) that is proportional to surviving cell. In brief, 5 × 10^3 cells were plated in 12-well tissue culture plates. After 24 hours, cells were treated with TRAIL, cisplatin, or combination of TRAIL plus cisplatin for another 24 hours. Following treatments, the medium was removed and cells were washed with PBS, fixed, and stained with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 30 seconds. Excess crystal violet solution was removed and cells were washed 3 times with PBS. The pictures were taken after the plates completely dried.

Results

Cisplatin plus TRAIL enhanced cell death in TNBC cell lines without significantly affecting normal breast cells

We used 3 cell lines, 2 TNBC cell lines, CRL2335 and MDA-MB-468 cells, and an immortalized normal breast cell line CRL8799 to determine the effect of cisplatin,
TRAIL, or combination of cisplatin plus TRAIL on cell death. Western blot analysis indicated the absence of ER, PR, and HER-2 amplification in both CRL2335 and MDA-MB-468 TNBC cells and thus confirmed the triple-negative status of the cells and the cell lines overexpress EGFR (Fig. 1A). HER-2 expressing T47D cells lysate was used as positive control to determine ER, PR, and HER2 immuno-reactivity of antibodies. MTT data indicate that cisplatin or TRAIL treatment induced approximately 10%–30% cell death in TNBC cell lines. However, combination of cisplatin plus TRAIL enhanced cell death to approximately 60%–70% in TNBC cells (Fig. 1B). In contrast, cisplatin plus TRAIL treatment of normal CRL8799 cells had minimal (~10%–15%) effect on cell death (Fig. 1B). The FITC Annexin V and PI staining indicated that the proportion of cells in apoptosis in CRL2335 and MDA-MB-468 TNBC cells treated with cisplatin plus TRAIL was significantly higher (~79% and ~54%, respectively) as compared with those treated with cisplatin or TRAIL alone. Similar treatment of normal CRL8799 cells resulted in a moderate increase in apoptotic cells (~13%; Fig. 1C). These observations were further confirmed in cells stained with crystal violet (Fig. 1D). Thus, taken together, the results suggested that cisplatin plus TRAIL treatment significantly enhanced cell death in both CRL-2335 and MDA-MB-468 TNBC cell lines whereas it had a minimal or moderate effect on normal CRL8799 cells.

Cisplatin plus TRAIL treatment downregulated the expression of EGFR and p63 proteins

Gobson and colleagues (21, 22) reported that EGFR played a dual role in apoptosis: increased expression gave resistance, whereas decreased expression promoted TRAIL-induced apoptosis. Leong and colleagues (23)

Figure 1. Cisplatin plus TRAIL treatment resulted in a significantly enhanced cell death in CRL-2335 and MDA-MB-468 TNBC without affecting CRL8799 normal breast cells. A, whole-cell extracts were prepared and analyzed by Western blotting using antibodies against the estrogen receptor (ER), progesterone receptor (PR), HER-2, and EGFR. Equal protein loading was evaluated by actin. B, cells were treated with cisplatin (10 μg/mL), TRAIL (10 ng/mL), or cisplatin (10 μg/mL) plus TRAIL (10 ng/mL) for 24 hours. Cell viability was then analyzed by MTT method. Cisplatin plus TRAIL treatment significantly inhibited CRL 2335 (P < 0.001) and MDA-MB-468 (P < 0.005) cells. Cells were stained with Annexin V-FITC and PI. Cells undergoing apoptosis were determined using FITC Annexin V apoptosis detection kit. C, cells were treated with cisplatin (10 μg/mL), TRAIL (10 ng/mL), or cisplatin (10 μg/mL) plus TRAIL (10 ng/mL) for 24 hours. Following treatments, the medium was removed and cells were washed with PBS, fixed, and stained with crystal violet.
observed that the p53 family member ΔNp63α isoform promoted the survival of primary breast cancer cells by binding to TAp73 and thus inhibiting its proapoptotic activity. The effect of cisplatin, TRAIL, or cisplatin plus TRAIL on EGFR protein and ΔNp63 was tested in CRL2335 and MDA-MB-468 TNBC cells as well as in CRL8799 cells in order to understand the mechanism involved in cell death. The data in Fig. 2 indicated that in normal CRL8799 cells treatment with cisplatin, TRAIL, or combination of cisplatin plus TRAIL had a minimal effect on both EGFR and p63 proteins compared with untreated control cells. In comparison, CRL2335 TNBC cells expressed both EGFR and p63 proteins, 24-hour treatment with cisplatin resulted in a significant reduction whereas similar treatment with TRAIL had a minimal effect on these proteins. In contrast, cisplatin plus TRAIL treatment resulted in a significant suppression of both proteins. On the other hand, MDA-MB-468 TNBC cells expressed higher level of EGFR and negligible level of p63 protein. Treatments with cisplatin, TRAIL, and cisplatin plus TRAIL for 24 hours resulted in a significant reduction in the expression of EGFR. Taken together, these data suggested that cisplatin plus TRAIL treatment had a significant effect in reducing EGFR and/or p63 protein levels in TNBC cells, whereas similar treatment had a minimal effect in CRL8799 normal breast cancer cells.

The expression of EGFR and p63 protein in CRL-2335 TNBC cells were knocked down using EGFR and/or p63 siRNA to determine whether inhibition of these proteins is essential for TRAIL-induced apoptosis. The results presented in Fig. 3A and B suggested that the inhibition of both proteins by siRNA did not increase cell death; the percentages of surviving cells were not significantly different than in control cells treated with random siRNA (Fig. 3B). Similar results were obtained in MDA-MB-468 TNBC cells when EGFR expression was knocked down using EGFR siRNA (which expressed EGFR but negligible level of p63 protein as shown in Fig. 2) and treated with TRAIL. There was no increased cell death and the percentage of surviving cells were not significantly different than in control cells treated with random siRNA (Fig. 3D). These results indicated that inhibition of EGFR and/or p63 may not be sufficient to increase TRAIL-induced apoptosis in TNBC cells. Perhaps, combined treatment with cisplatin may be affecting other death proteins to enhance TRAIL-induced apoptosis.

### Cisplatin and TRAIL enhanced caspase activation and suppressed antiapoptotic proteins in TNBC cells without affecting normal cells

Caspases are reported to play an important role in TRAIL-induced apoptosis (24). Therefore, the effect of cisplatin, TRAIL, and cisplatin plus TRAIL treatment on activation of caspases -8, -3, and -9, Bid and cleavage of PARP was investigated in CRL8799 normal cells and CRL2335 and MDA-MB-468 TNBC cells. The data in Fig. 4 indicated that the activities of caspases -8, -3, and -9 were increased in TNBC cells by cisplatin treatment leading to enhanced Bid and PARP cleavage. However, the maximum effect was observed when the same cells were treated with cisplatin plus TRAIL. In contrast, similar treatments in CRL8799 normal cells had a minimal effect on activity of caspases -8, -3, and -9, Bid and PARP cleavage.

The effect of cisplatin, TRAIL, cisplatin plus TRAIL treatment was also examined on antiapoptotic protein survivin (25), Bcl-xL and Bcl-2 in CRL8799 normal cells and CRL2335, MDA-MB-468 TNBC cells. The results in Fig. 5A showed maximum inhibition of survivin as well as Bcl-xL and Bcl-2 in TNBC cells treated with cisplatin plus TRAIL compared with cisplatin or TRAIL alone. These observations provided support to the data presented in Fig. 1B where CRL2335 and MDA-MB-468 TNBC cells treated with cisplatin plus TRAIL showed significantly decreased percentage of surviving cells. It is interesting to note that similar treatments had no effect or, at best, a minimal effect in CRL8799 normal cells.

Previous reports have suggested that survivin significantly reduces apoptosis by inhibiting caspases (26–28). If downregulation of survivin sensitizes CRL-2335 TNBC cells to TRAIL-induced apoptosis were examined by inhibiting survivin by siRNA in the presence of TRAIL. Indeed, CRL2335 TNBC cells treated with survivin siRNA and TRAIL exhibited higher...
activation of caspases -3 and -8 (Fig. 5B) with a concomitant and significant increase TRAIL-induced cell death (Fig. 5C) in comparison with the cells treated with random siRNA. These results provide support that survivin plays an important role in TRAIL-induced apoptosis in TNBC cells. It is interesting to note that random siRNA treatment had no effect on cell survival in CRL8799 normal cells.

Figure 3. Effect of siRNA knockdown of EGFR and/or p63 in TNBC cells. CRL2335 TNBC cells were transfected with both EGFR siRNA and p63 siRNA and MDA-MB-468 cells were transfected with EGFR siRNA alone or random siRNA. After 48 hours of transfection, cells were treated with or without TRAIL for additional 24 hours. Whole-cell extracts were prepared and analyzed for EGFR, p63 by Western blotting (A, C). Equal protein loading was compared with that of GAPDH. B, D, under similar conditions cell viability was assessed using trypan blue exclusion method.

Figure 4. CRL8799 normal breast cancer cells, CRL2335, MDA-MB-468 TNBC cells were treated with cisplatin, TRAIL, or cisplatin plus TRAIL as shown in Fig. 2. Whole-cell extracts were prepared and analyzed by Western blotting. Equal protein loading was compared with that of GAPDH. Cisplatin plus TRAIL treatment increased the activation of caspases -8, -3, and -9 in TNBC cells and had no effect on CRL8799 normal cells.
Efficacy of cisplatin plus TRAIL treatment on the growth of TNBC cells in vivo

The inhibition of CRL2335 TNBC cell xenografts in mice treated with cisplatin plus TRAIL was assessed in vivo in mice. The results in Fig. 6 indicated that there was a gradual regression of tumor xenografts as indicated by reduction in tumor size/volume in mice treated with cisplatin plus TRAIL as compared with control mice that were injected with vehicle. Also, the mice tolerated cisplatin plus TRAIL treatment with no signs of toxicity or weight loss during the observation period (data not shown). These findings suggest that cisplatin plus TRAIL therapy enhanced antitumor effect in vivo and, these observations were consistent with results obtained in vitro.

Discussion

TNBC is the most aggressive and difficult to treat form of cancer. TNBC patients do not benefit from hormonal or herceptin-based therapies due to loss of target receptors such as ER, PR, and HER-2. The results from the present investigations clearly have shown that CRL2335 and MDA-MB-468 TNBC cells with cisplatin plus TRAIL exhibited significantly enhanced apoptosis (~60%–70% cell death) as compared with untreated cells. Also, similar treatment had a minimal effect on CRL8799 normal breast cancer cells (~10%–15% cell death; Fig. 1). In addition, the results from the current study also provided evidence that cisplatin exerted its influence on TRAIL-induced TNBC cell death by simultaneous activation of caspases and inhibition of antiapoptotic protein survivin. Furthermore, suppression of survivin seems to have played a major role in TRAIL-induced cell death in TNBC cells. These observations gain support from Altier (25) report that increased survivin expression enhanced tumor resistance to various apoptotic stimuli, primarily through caspase-dependent mechanism and antagonizing Survivin-induced apoptosis in tumor cells. Bcl-2 and Bcl-xl reside within the mitochondrial membrane where they act by inhibiting adaptor molecules needed for the activation of effector caspases.

The results from this study have suggested that treatment with cisplatin alone induced approximately 20%–30% and approximately 8%–12% cell death in CRL2335 and MDB-MB-468 TNBC cells, which express high and low levels of p63, respectively (Fig. 1, 2). These observations are consistent with recently published clinical
Data are mean ± SE. The difference between treated and control group was P < 0.05.

Figure 6. Regression of CRL2335 xenografts in mice treated in vivo with cisplatin plus TRAIL. Six scid mice were injected with 5 × 10⁶ CRL2335 TNBC cells. When the tumors were palpable and approximately 50 mm³ in size, 3 mice were treated with cisplatin plus TRAIL (4 mg/kg + 15 mg/kg, respectively, intraperitoneal injection, once a week for 3 weeks). The other 3 animals were used as controls and injected with vehicle (1 × PBS). Day 0 indicates the first injection. All mice were healthy and active. Treated mice exhibited no significant weight loss after 2 injections. Tumor volume was determined at the indicated times after the onset of treatment. Data are mean ± SE. The difference between treated and control group was P < 0.05.

Approximately 60% of TNBC cells show expression of EGFR and, and drugs that target EGFR could be beneficial to treat TNBC patients. Although Carey and colleagues (31) reported that cetuximab, a chimeric monoclonal antibody which targets EGFR, elicited little or no response from patients with advanced TNBC, the results from phase II clinical trial using cetuximab+carboplatin suggested favorable response (18%) and overall therapeutic gain (27%) in 102 patients with advanced TNBC (32). The observations from previous phase II clinical trials have also indicated an overall response rate of approximately 32% when unselected breast cancer patients were treated with cisplatin plus carboplatin (33–35). In more recent studies, platinum-based chemotherapy increased the response rate in TNBC tumors, with a trend toward improved survival in TNBC patients (36). A number of phase III clinical trials are in progress in which advanced TNBC patients were randomized and treated first with carboplatin or docetaxel, with crossover on progression (37). A non-randomized phase II study using cisplatin to treat TNBC patients is also under way in the USA (38).

In conclusion, the results from the present investigations suggested that treatment of CRL2335 and MDA-MB-468 TNBC cells with cisplatin can enhance TRAIL-induced apoptosis by activating caspases along with simultaneous reduction of antiapoptotic proteins, such as survivin, Bcl-xL, and Bcl-2 expression. Comparatively, similar treatment of immortalized CRL8999 normal breast cells had a minimal effect. Individually, cisplatin and TRIAL are approved by FDA to treat other forms of cancer. As TNBC patients do not benefit from hormonal or herceptin-based therapies as well as other such as surgery, chemo- and radiation-therapy, cisplatin plus TRIAL treatment is suggested as a potential new cancer treatment strategy in TNBC patients.

Disclosure of Potential Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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