Primary ovarian cancer cells are sensitive to the proaptotic effects of proteasome inhibitors

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Abstract. Resistance of tumors to cell death signals poses a complex clinical problem. In the present study, we have explored the capacity of proteasome inhibitors to induce cell death of ovarian cancer cells. We explored the sensitivity of primary ovarian cancer cells to a combination of bortezomib (also known as PS-341), a proteasome inhibitor and TRAIL, a death ligand, or mapatumumab or lexatumumab, TRAIL-R1 or TRAIL-R2 targeting agonist monoclonal antibodies, respectively. The results of our study showed that the large majority of primary ovarian cancers are clearly sensitive to the pro-apoptotic action of bortezomib, whose effects are potentiated by the concomitant addition of TRAIL or mapatumumab or lexatumumab. Interestingly, both cisplatin and paclitaxel-chemosensitive and chemoresistant ovarian tumors are equally sensitive to the cytotoxic effect of bortezomib. Bortezomib, combined with TRAIL or TRAIL-R1 or TRAIL-R2 agonist monoclonal antibodies may be a useful treatment for refractory ovarian cancer.

Introduction

Epithelial ovarian cancer derives from the malignant transformation of the epithelial ovarian surface. It accounts for ~90% of all the human ovarian malignancies. Ovarian cancer is the leading cause of cancer-related death in women. In fact, in women, ovarian cancer is the fourth leading cause of deaths related to cancer and represents the most lethal gynecologic malignancy with a 5-year survival of 30-40% (1). Unfortunately, ovarian cancer is often detected only after it has spread throughout the peritoneal cavity. Despite aggressive surgical resection and adjuvant therapy, most patients with advanced-stage ovarian cancer die of their malignancy (1).

Although platinum derivatives, associated with taxanes, are considered the standard first-line chemotherapeutic agents for the treatment of ovarian cancer, chemoresistance remains a major therapeutic problem and the cellular mechanisms involved in the resistance are poorly understood. More effective therapies are needed in the setting of recurrent ovarian cancer to overcome drug resistance.

Among the new agents particularly promising are the proteasome inhibitors. The ubiquitin proteasome pathway is a highly conserved intracellular pathway for the degradation of proteins. The proteasome pathway controls the half-life of short-lived regulatory proteins that play a key role in the regulation of cell proliferation, differentiation, signaling and transcription (2). The ubiquitin proteasome pathway plays an important role in neoplastic growth and metastasis. Particularly, the proteasome is also required for activation of nuclear factor-κB (NF-κB) by degradation of its inhibitory protein, I-κB (3). NF-κB is a transcription factor that upregulates a number of proteins involved in cancer progression, including several proangiogenic factors and anti-apoptotic factors. Many studies have shown that proteasome inhibitors are able to induce apoptosis of tumor cells mainly via inhibition of NF-κB activity (4). In addition, there is evidence in various tumor models that proteasome inhibitors enhance the sensitivity of tumor cells to the death ligand TRAIL (5). Ovarian cancer cell lines displayed a significant sensitivity to the pro-apoptotic effects of proteasome inhibitors (6,7). The analysis of the mechanisms through which proteasome inhibitors induce apoptosis of ovarian cancer cells indicate a main role of mitochondrial damage through stabilization of BH3-only proteins (8).

The analysis of the ubiquitin-proteasome system showed increased levels in malignant ovarian cancer cells (both cell lines and primary tumor cells) compared to their normal counterpart (9). Furthermore, there is evidence that the ubiquitin proteasome system is stressed in ovarian cancer cells

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as supported by the observation that elevated levels of total ubiquitinated proteins and proteasome subunits are observed in ovarian carcinoma tissues, compared to normal surface epithelium (9). The increased expression of proteasome in ovarian cancer cells is associated with a high sensitivity to apoptosis in response to proteasome inhibitors (9).

Importantly, a proteasome inhibitor introduced in clinical practice has been evaluated in phase I studies in ovarian cancer. The initial phase I study showed a significant anti-tumor activity of bortezomib (also known as PS-341 and registered for clinical use as Velcade®), a proteasome inhibitor clinically indicated for the therapy of myeloma and mantle cell lymphoma. More recently, two additional studies have explored the safety profile and the anti-tumor activity of bortezomib when administered together with carboplatin (11) or with paclitaxel (12) to ovarian cancer patients, including patients with platinum- and taxane-resistant disease. Interestingly, some chemoresistant patients responded to treatment with bortezomib.

The death receptors of the Tumor Necrosis Factor (TNF) superfamily represent potential targets for promoting apoptosis of cancer cells (13). Among the various TNF members particularly interesting is the TNF-related apoptosis-inducing ligand (TRAIL) for its property to induce apoptotic cell death receptors via both TRAIL-R1 and TRAIL-R2, while sparing normal cells (14,15). These two receptors have a cytoplasmic death domain through which TRAIL can transmit its apoptotic signal by binding as a homodimer. Because of its proposed role in inducing apoptosis in cancer cells, while sparing normal cells, several TRAIL receptor agonists have recently entered the clinic (16,17). Mapatumumab and lexatumumab are fully human monoclonal antibodies that are agonistic to TRAIL-R1 and TRAIL-R2, respectively (18). Preclinical studies in mice and non-human primates, as well as phase I clinical studies in humans have shown the potential utility of recombinant TRAIL and agonistic TRAIL-R1 and TRAIL-R2 antibodies for cancer therapy (reviewed in ref. 19). However, many primary tumor cells, including ovarian cancer cells (8), are resistant to the proapoptotic effects of TRAIL. To bypass TRAIL resistance of primary tumor cells, TRAIL or agonist TRAIL-R antibodies have been combined with other agents (i.e., chemotherapeutic drugs or proteasome inhibitors) able to restore a high sensitivity of these tumor cells to TRAIL (19). In the present study, we have explored the sensitivity of primary ovarian cancer cells *in vitro* to proteasome inhibitors combined with TRAIL or the TRAIL-R1 and TRAIL-R2 agonistic monoclonal antibodies, mapatumumab and lexatumumab.

Materials and methods

Cell culture. Cisplatin-sensitive human ovarian epithelial carcinoma cell line A2780 was obtained from the American Type Culture Collection (ATCC); cisplatin-resistant cell line A2780/DDP and adriamycin-resistant cell line A2780/ADR, derived from its parental ovarian cancer cell line A2780 by applying stepwise increases in concentrations of cisplatin and adriamycin, respectively were obtained from the European Collection of Cell Cultures (ECACC). The A2780/DDP cells were incubated in 30 μM of cisplatin and the A2780/ADR cells in 10 μM adriamycin every 10 passages.

The cells were cultured at 37˚C in an atmosphere of 5% CO_2 in Advanced MEM with 3% fetal bovine serum (FBS, Euroclone, Milan, Italy), 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin and 0.3 μg/ml glutamine. The cells were routinely checked for the presence of mycoplasma.

Isolation and in vitro culture of primary ovarian cancer cells. Intra-operatory biopsies were obtained from 34 ovarian serous adenocarcinoma cancer patients undergoing debulking surgery. Tumor tissue was mechanically dissociated with a scissor and a tumor cell suspension was obtained by digestion in tissue culture medium (RPMI-1640) containing collagenase, deoxyribonuclease I and hyaluronidase. The final tumor cell suspension was checked for the proportion of tumor cells by standard cytology and the percentage of epithelial cells by flow cytometry (determined after staining with Ber-EP4 mAb, Dakopatt, Copenhagen, Denmark). Tumor cell aliquots ($1x10^6$ cells) were plated into 25 cm^3 tissue culture flasks in 10 ml of cell culture medium containing 5% fetal calf serum. After 1 day of *in vitro* culture, non-adherent cells (containing tissue debris and dead cells) were removed and fresh medium was added to the culture and then incubated for additional 24 h either in the absence or in the presence of TRAIL, or bortezomib or both reagents. At 24 h of culture cells were confluent. Tumor cultures contained at least 80% of tumor cells.

In vitro extreme drug resistance. In vitro response to anti-cancer drugs was assessed by the Oncotech EDR assay (20,21). Surgical biopsies (1-2 g) from metastatic sites of stage III ovarian cancer patients (aged between 18 and 70 years) were obtained during primary debulking surgery. Tumor specimens were mechanically disaggregated into suspensions of small tumor clumps. Tissue culture was carried out as previously reported (20,21): malignant cells were suspended in soft agarose and growth media at ~30,000 cells per well in 24-well plates and exposed to various chemotherapeutic agents, including cisplatin, carboplatin, paclitaxel and docetaxel. Treated cell suspensions were incubated for 72 h, at 37˚C in a 5% CO_2 atmosphere with drug and then pulsed with [^{3}H]-thymidine (5 μCi/well) to measure the level of DNA synthesis. After an additional 48 h incubation period, cells were harvested onto glass fiber filters and the incorporated radioactivity was measured by liquid scintillation. Positive and negative controls were performed as previously reported (20,21). EDR score was evaluated, as previously reported (22).

Apoptosis assessment by Annexin V staining. After drug treatments, cells were resuspended in 200 μl staining solution (containing Annexin V fluorescein and propidium iodide in a Hepes buffer, Annexin V-FITC staining kit, Pharmingen, San Jose, CA, USA). Following incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells

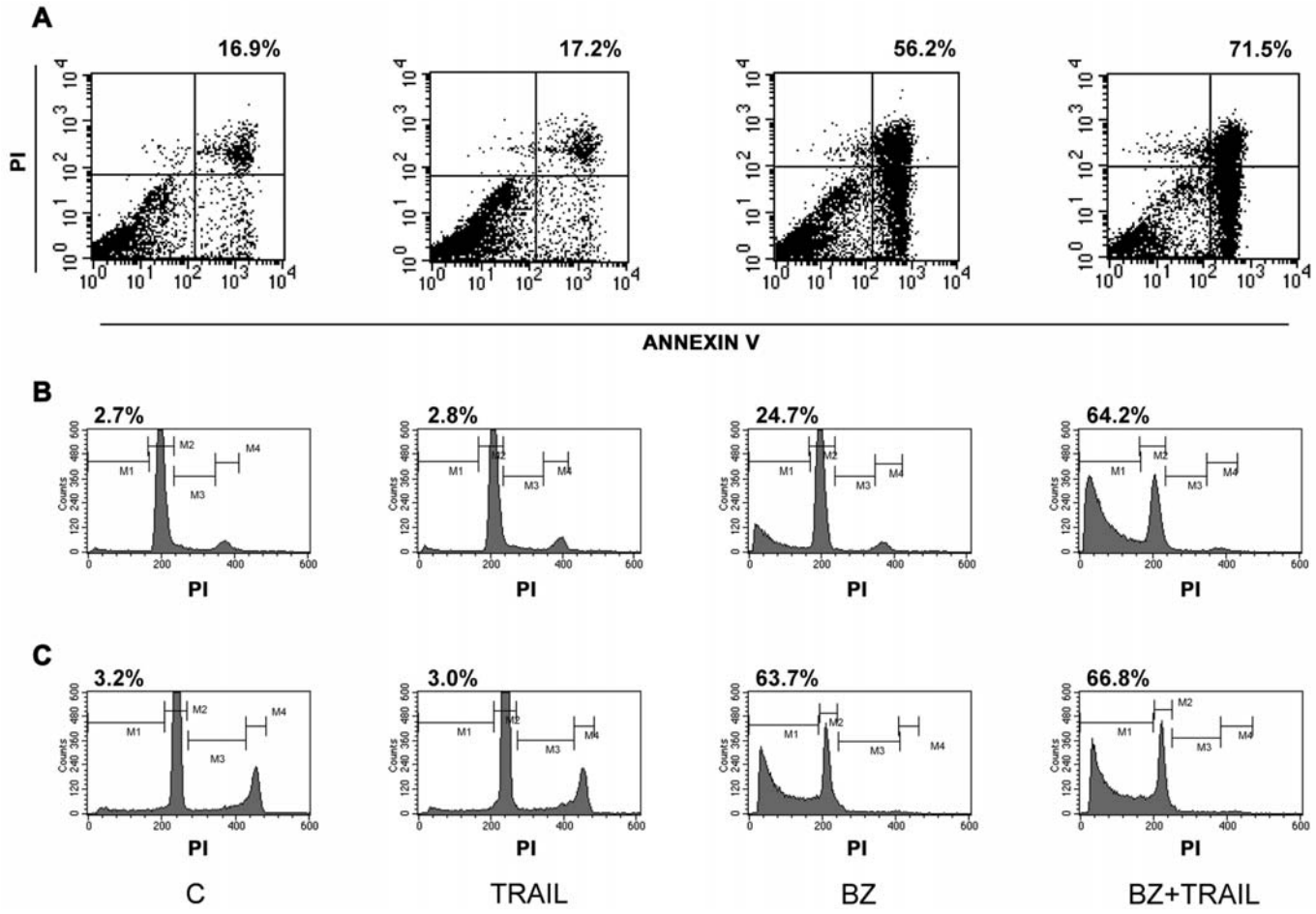

Figure 1. Flow cytometric analysis of Annexin V binding (A) and PI DNA labeling (B and C) of primary ovarian cancer cells treated with either no additives (C) or 50 ng/ml TRAIL or 0.5 μM bortezomib (BZ) or both bortezomib and TRAIL at the above concentrations. (A) Apoptotic cells are present in the top and bottom right squares. (B and C) Hypodiploid, apoptotic cells are included within the M1 gate; M2 corresponds to G_0/G_1 cells, M3 to S-phase cells and M4 to G_2/M-phase cells. In each panel the percentage of apoptotic cells is indicated. B and C correspond to two different patients.

(stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide).

Quantification of apoptosis and cell cycle analysis by propidium iodide/fluorescence activated cell sorting. Cells were harvested with trypsin, washed, fixed and resuspended in 400 μl of propidium iodide (PI) solution (50 μg/ml PI, 0.1% Triton X-100 and 0.1% sodium citrate in PBS) (Cycle Plus DNA staining kit, Becton-Dickinson, USA). The cells were then analyzed by flow cytometry using software dedicated for DNA analysis (ModFit LT software, Verity Software House, Topsham, ME, USA). The cells with subdiploid DNA content were quantified to determine the percentage of cells containing apoptotic, fragmented DNA.

Reagents used to induce apoptosis of tumor cells. Recombinant human TRAIL (rh superkiller TRAIL) was purchased from Alexis Co. (Lausen, Switzerland). In some experiments the cells were preincubated with a pan-caspase inhibitor, N-benzyloxy-carbonyl-Val-Ala-Asp(OMe)-fluoromethyl-ketone (zVADfmk, Sigma, St. Louis, USA) before the addition of the various compounds stimulating apoptosis.

The agonistic monoclonal antibodies to TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab) are fully human antibodies of IgG1 isotype (18) and were generously provided by Human Genome Sciences, Inc. (Rockville, MD, USA).

Western blot analysis. Whole cell extracts were obtained lysing the cells in a buffer containing 20 mM HEPES, 50 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.5% NP-40, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 25 mM NaF and 10 mM Na_3VO_4. After incubation for 30 min on ice, the protein lysates were cleared of debris by centrifugation at 10,000 x g for 10 min. The protein concentration in the soluble supernatant, was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, VA, USA).

Antibodies. Anti-caspase-8 was purchased from Upstate (Upstate Biotechnology, Lake Placid, NY, USA).

Results

Primary ovarian cancer cells are sensitive to the proapoptotic effects of bortezomib. In a first set of experiments we evaluated the effect of bortezomib on primary ovarian cancer cells. We then isolated primary ovarian cancer cells from 34 patients undergoing debulking surgery and these cells were

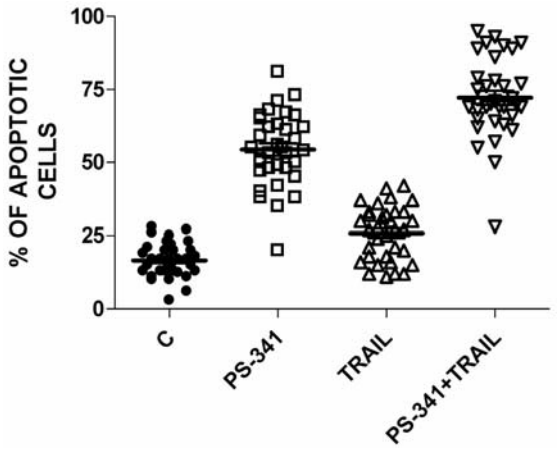

Figure 2. Percentage of apoptotic cells of 34 primary ovarian cancer cells grown for 16 h either in the absence (C) or in the presence of 0.5 μM bortezomib or 50 ng/ml TRAIL or both bortezomib and TRAIL at the above concentrations. The proportion of apoptotic cells was evaluated by Annexin V binding assay. The difference between bortezomib and C and between TRAIL and bortezomib + TRAIL was highly significant ($p<0.001$). The bars correspond to mean values ± SEM.

grown either in the absence or in the presence of bortezomib or TRAIL or both agents. To evaluate the effect of these molecules on the apoptosis of ovarian cancer cells we assessed after 16-20 h of incubation the percentage of cells that bind Annexin V (an early marker of apoptosis) (Fig. 1A) and after 36-48 h the percentage of hypodiploid cells (cells with reduced DNA content due to its degradation, a late marker of apoptosis), both by flow cytometry (Fig. 1B and C). The results of these studies showed that: i) bortezomib elicited a pronounced proapoptotic effect on ovarian cancer cells ($p<0.001$, when compared to C); ii) TRAIL when added alone elicited only a weak cytotoxic effect ($p<0.05$, when compared to C); iii) TRAIL, when added in combination with bortezomib, potentiated the proapoptotic effects of bortezomib ($p<0.001$, when bortezomib + TRAIL was compared to bortezomib alone) (Fig. 2).

The inspection of individual cases showed that: i) bortezomib was able to markedly enhance the proportion of apoptotic cells in 33/34 cases ii) the cytotoxic response to bortezomib was heterogeneous ranging from 22 to 80%; iii) bortezomib + TRAIL increased the proportion of apoptotic cells compared to bortezomib alone in 34/34 cases (Fig. 2).

Ovarian cancer cells resistant to cisplatin or paclitaxel are sensitive to bortezomib. The cells of the 34 ovarian cancer patients evaluated for their sensitivity to bortezomib and TRAIL were assessed also for the *in vitro* sensitivity to cisplatin and paclitaxel using the EDR assay. The results of this assay showed that 7/34 and 8/34 cases were extremely resistant to cisplatin and paclitaxel, respectively (Fig. 3). The analysis of the sensitivity of cisplatin-resistant and paclitaxel-resistant cases, compared to sensitive cases

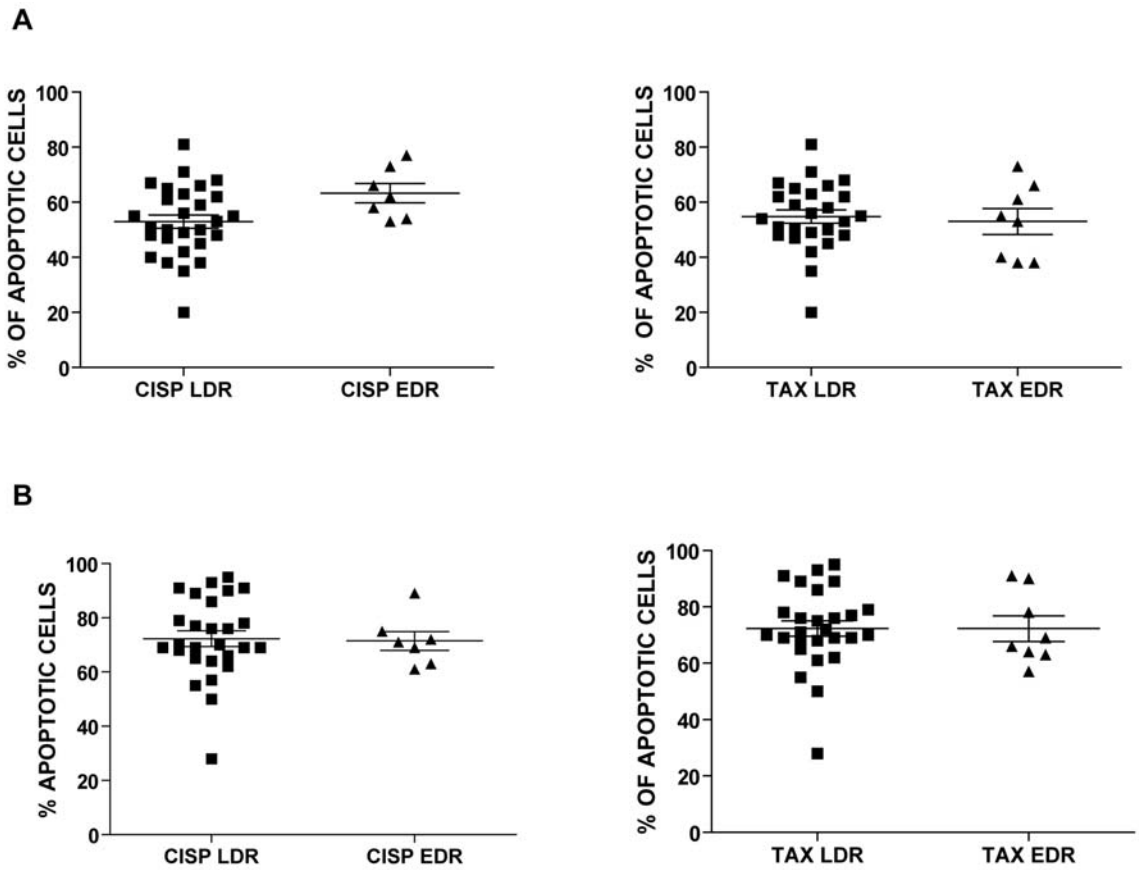

Figure 3. Percentage of apoptotic cells observed in 34 primary ovarian cancer cells subdivided into cisplatin low resistant (CISP LDR) and cisplatin extremely resistant (CISP EDR) or paclitaxel low resistant (TAX LDR) and paclitaxel extremely resistant (TAX EDR). The data are reported for cells incubated with bortezomib alone (A) or in combination with TRAIL (B). The differences between CISP LDR and CISP EDR or TAX LDR and TAX EDR are not significant ($p>0.1$). The bars reported in the graphs represent the mean value ± SEM.

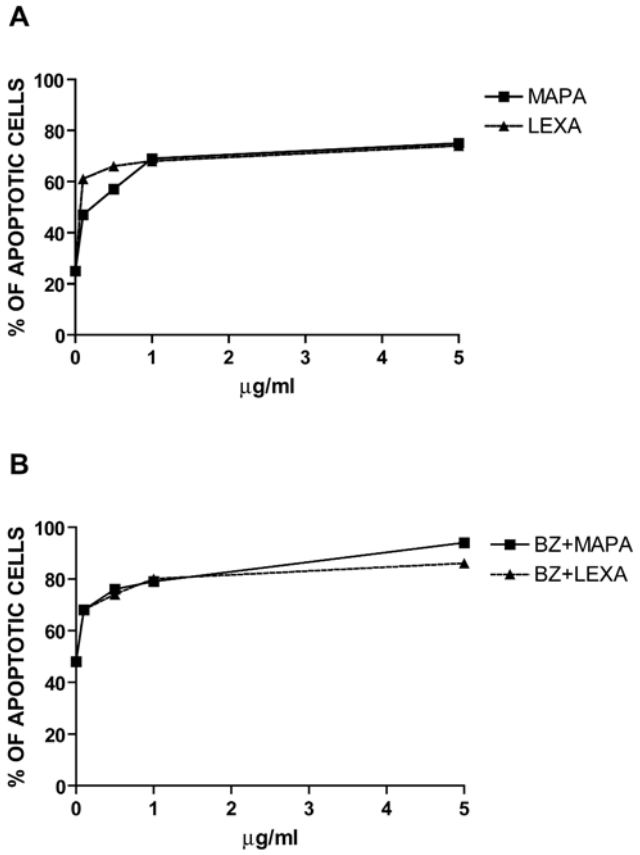

Figure 4. Percentage of apoptotic cells observed in one representative case of primary ovarian cancer cells incubated for 24 h in the presence of increasing concentrations of either mapatumumab (mapa) or lexatumumab (lexa). In the experiments reported in A the antibodies were added alone, while in the experiments reported in B were added in combination with a fixed dose of 0.5 μM bortezomib.

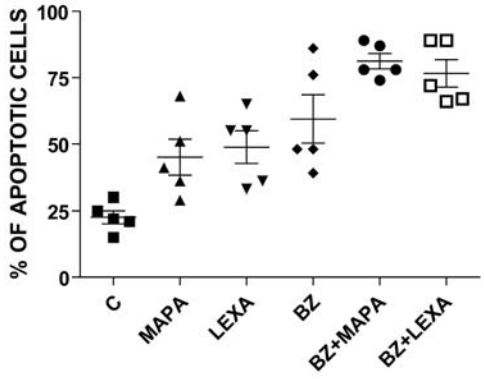

Figure 5. Percentage of apoptotic cells of 5 cases of primary ovarian cancer cells grown for 16 h either in the absence (C) or in the presence of 1 μg/ml mapatumumab (mapa) or 1 μg/ml lexatumumab (lexa) or 0.5 μM bortezomib or mapatumumb + bortezomib or lexatumumab + bortezomib. The proportion of apoptotic cells was determined by Annexin V binding assay.

showed comparable levels of sensitivity to either bortezomib (Fig. 3A) or bortezomib + TRAIL (Fig. 3B).

Mapatumumab and lexatumumab improve the proapoptotic effect of bortezomib on ovarian cancer cells. Mapatumamab and lexatumumab are fully human agonistic monoclonal

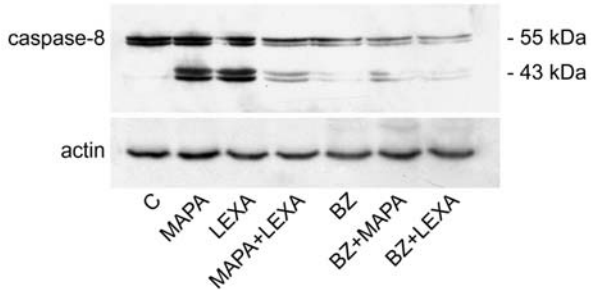

Figure 6. Bortezomib-induced sensitization to the extrinsic TRAIL receptor-mediated pathway of apoptosis in primary ovarian cancer cells. Immunoblots of caspase-8 expression in whole lysates of a case of primary ovarian cancer cells that were treated with media (C) or treated for 20 h with 1 μg/ml mapatumumab, 1 μg/ml lexatumumab or 1 μg/ml of both. Additional treatments included 0.5 μM bortezomib alone or in combination with + 1 μg/ml of mapatumumab or lexatumumab. A representative blot from three different experiments is shown.

antibodies specific for TRAIL-R1 and TRAIL-R2; respectively, which induce TRAIL-R apoptotic signaling. We have therefore evaluated the biologic effects induced by these two antibodies added alone or in combination with bortezomib.

In a first set of experiments we added different doses of mapatumumab and lexatumumab (from 0.1 to 5 μg/ml) of bortezomib alone (Fig. 4A) or in combination with 0.5 μM bortezomib (Fig. 4B) to primary ovarian cancer cells. Interestingly, both mapatumumab and lexatumumab induced a significant pro-apoptotic effect, reaching their maximal effect at 1 μg/ml (Fig. 4A and B). Importantly, the effect of these agonistic TRAIL-R mAbs was greater than the effect of TRAIL alone (see Fig. 2).

In a second set of experiments we evaluated the effects of a fixed amount of either mapatumumab or lexatumumab (1 μg/ml) added alone or in combination with bortezomib on the induction of apoptosis of 5 cases of primary ovarian cancer. The results of this analysis showed that: i) both mapatumumab (mapa) and lexatumumab (lexa) induced, when added alone, a moderate, but significant increase in the proportion of apoptotic cells (mapa-treated cells vs. control, $p<0.01$; lexa-treated cells vs. control, $p<0.01$); ii) both mapatumumab and lexatumumab significantly increased the rate of apoptotic cells induced by bortezomib and uniformly affected all patient samples (mapa + bortezomib-treated cells vs. bortezomib-treated cells, $p<0.05$; lexa + bortezomib-treated cells vs. bortezomib-treated cells $p<0.05$) (Fig. 5).

A critical step in TRAIL signalling after receptor aggregation is thought to be the dimerization of pro-caspase-8 in the death-inducing signalling complex (23), which confers a conformational change that is required for its enzymatic activity (24). Increased enzymatic activation of caspase-8 and accumulation of the cleaved form (43 kDa), associated with a decline of pro-caspase-8 (55 kDa) was observed in primary ovarian cancer cells treated with mapatumumab or lexatumumab, particularly when added in combination with bortezomib (Fig. 6): it was noted that the accumulation of the cleaved 43 kDa bands is particularly evident in cells treated with mapatumumab or lexatumumab, while in samples treated with bortezomib alone or in combination with the two TRAIL-R mAbs the decline of pro-caspase-8 was very pronounced (Fig. 6).

Discussion

The ubiquitin-proteasome pathway is involved in intracellular protein turnover and its regulated control is essential for cell homeostasis. The studies carried out in the last 10 years have shown that several proteasome inhibitors are potent anti-cancer drugs for their property to induce programmed cell death preferentially in transformed cells. These studies have contributed to introduce bortezomib into the clinic. It is registered for the treatment of multiple myeloma and mantle cell lymphoma. This drug, when administered with established anti-cancer agents, elicited impressive results in the treatment of multiple myeloma (25). *In vitro* and *in vivo* studies have in part elucidated the mechanism of actions of this drug, the toxicity profile and clinical efficacy and have also lead to the identification of biochemical pathways involved in the resistance to this agent (reviewed in refs. 26 and 27).

On the basis of the results obtained in multiple myeloma clinical studies with bortezomib have been carried out in other tumors, including ovarian cancer. The studies carried out in this cancer are at present limited to phase I studies aimed to define the clinical toxicity profile of bortezomib when administered either with cisplatin or paclitaxel (10-12). In the present study we have explored the sensitivity of a panel of primary ovarian cancer to bortezomib added alone or in combination with TRAIL. Our results showed that the large majority of these tumors were sensitive to bortezomib and, particularly, to bortezomib + TRAIL.

We have included in our study only ovarian cancer patients at disease presentation. The analysis of *in vitro* chemoresistance through the EDR assay showed that 19% of patients are extremely drug resistant for cisplatin and 24% for paclitaxel. Previous studies have shown that the EDR assay was accurate (99%) in predicting *in vivo* chemoresistance (20-22). Interestingly, these chemoresistant patients displayed a bortezomib sensitivity comparable to that observed for non-chemoresistant patients. This observation suggests that the bortezomib + TRAIL treatment could be of value in the treatment of chemoresistant ovarian cancer disease.

Our results further support previous studies, mainly based on the analysis of ovarian cancer cell lines, showing a significant sensitivity of ovarian cancer cells to the proapoptotic effects of bortezomib. Proteasome inhibitors induce cell death of ovarian cancer cells through different molecular mechanisms involving BH3-only ptotein stabilization (8), endoplasmic reticulum stress response (28) and oxidative stress (8).

Our results showed also that the cytotoxic effects of bortezomib on ovarian cancer cells were potentiated by the death ligand TRAIL or by agonistic TRAIL-R1 and TRAIL-R2 mAbs. This observation confirms and extends previous studies, mainly based on the analysis of ovarian cancer cell lines and showing that bortezomib greatly potentiates the proapoptotic effect of TRAIL (8,28,29). It is of interest to note that recent preclinical studies have provided both a molecular basis and a translationally relevant proof of principle for the therapeutic combination of the proteasome inhibitor bortezomib with death receptor agonist monoclonal antibodies or recombinant TRAIL *in vivo* to efficiently promote tumor cell apoptosis (30). These observations are of interest in view of future clinical applications. In fact, as above mentioned, fully human TRAIL- R1 and TRAIL-R2 mAbs have been developed and introduced into clinic and tested in phase I/II clinical studies in various tumors, including ovarian cancer (31). In one of these studies 9 ovarian cancer patients with advanced disease were treated with mapatumumab and 3 of them experienced a long-lasting stable disease (31). Importantly, phase I studies also indicate that mapatumumab and lexatumumab are safe and have acceptable toxicity profiles (31,32). On the other hand, an improved recombinant form of TRAIL, which exerts no toxicity in normal human cells, is being currently evaluated in clinical trials (33).

Biochemical studies carried out in primary ovarian cancer cells (the present study) and in ovarian cancer cell lines (8) suggest that bortezomib-induced amplification of caspase-8 activation in response to TRAIL is important for enhanced apoptosis of ovarian cancer cells treated with bortezomib and TRAIL. This crucial role of caspase-8 activation is in agreement with previous studies on bortezomib sensitization of human hepatoma cells to TRAIL (34). Furthermore, we observed that the overexpression of c-FLIP, an antagonist of caspase-8, restores a low sensitivity to the proapoptotic effects of bortezomib + TRAIL.

In conclusion, the findings of the present study suggest that bortezomib combined with TRAIL or TRAIL-R1 and TRAIL-R2 agonistic monoclonal antibodies may be a useful treatment for ovarian cancer.

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