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BRIEF REPORT

Proteasome Inhibitors Synergize with Tumor Necrosis Factor-Related Apoptosis-Induced Ligand to Induce Anaplastic Thyroid Carcinoma Cell Death

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Context: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive types of cancer characterized by complete refractoriness to multimodal treatment approaches. Therapeutic strategies based on the simultaneous use of proteasome inhibitors and death receptor ligands have been shown to induce apoptosis in several tumor types but have not yet been explored in ATC.

Objective and Methods: The aim of this study was to investigate the ability of the proteasome inhibitor Bortezomib to induce apoptosis in ATC cell lines. Bortezomib was used as a single agent or in combination with TNF-related apoptosis-induced ligand (TRAIL), a member of the TNF family that selectively induces tumor cell apoptosis. The molecular effects of Bortezomib were investigated by analyzing the expression of key regulators of cell cycle and apoptosis and the activation of different apoptotic pathways.

Results: Bortezomib induced apoptosis in ATC cells at doses achieved in the clinical setting, differently from conventional chemotherapeutic agents. Simultaneous treatment with low doses of Bortezomib and TRAIL had a synergistic effect in inducing massive ATC cell apoptosis. Bortezomib increased the expression of cytotoxic TRAIL receptors, p21 (WAF/CIP1) and proapoptotic second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI, and reduced the expression of antiapoptotic mediators such as cellular Fas-associated death domain-like IL-1β converting enzyme inhibitory protein, Bcl-2, Bcl-X_L, and inhibitor of apoptosis-1, thus resulting in cell death induction through the mitochondrial apoptotic pathway.

Conclusions: The combination of proteasome inhibitors and TRAIL synergizes to induce the destruction of chemoresistant neoplastic thyrocytes and could represent a promising therapeutic strategy for the treatment of anaplastic thyroid carcinoma. **(*J Clin Endocrinol Metab* 92: 1938–1942, 2007)**

THE UBIQUITIN-PROTEASOME system represents the main degradation pathway for proteins involved in the regulation of cell survival, proliferation, apoptosis, and other critical cellular functions (1). Recently it has been shown that proteasome inhibitors interfere with key steps of tumor cell regulation, leading to a block of proliferation and death of neoplastic cells both *in vitro* and *in vivo*, thus emerging as a new class of powerful anticancer drugs (2). Accordingly, the proteasome inhibitor Bortezomib (PS-341) has been recently approved for therapeutic use in progressive multiple myeloma (3), and a large-scale phase II trial in a variety of other tumors is currently in progress (4). Bortezomib has been shown to induce apoptosis in several tumor types through activation of the intrinsic cell death pathway, which relies on the release from mitochondria of apoptotic mediators such as cytochrome c and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI (SMAC/Diablo). By contrast, the extrinsic apoptotic pathway is activated by the interaction of TNF family members such as CD95 ligand and TNF-related apoptosis-induced ligand (TRAIL) with death domain-containing receptors and is based on the direct activation of caspases responsible for the cleavage of key cellular substrates.

Anaplastic thyroid carcinoma (ATC) is among the most aggressive human malignancies, being responsible for the majority of thyroid cancer-related deaths. Despite multimodal therapy including surgery, chemotherapy, and radiotherapy, this tumor is rapidly fatal, with a mean survival of 6 months after diagnosis (5). Because proteasome inhibitors may provide a therapeutic opportunity for ATC, we investigated the antitumor effects of Bortezomib on ATC cells, both as a single agent or in combination with the proapoptotic death ligand TRAIL.

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Abbreviations: ATC, Anaplastic thyroid carcinoma; cFLIP, cellular FLIP; FLIP, Fas-associated death domain-like IL-1β converting enzyme inhibitory protein; LZ, leucine zipper; NF-kB, nuclear factor-κB; SMAC/Diablo, second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI; TRAIL, TNF-related apoptosis-induced ligand; TRAIL-R, TRAIL receptor.

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Materials and Methods

Cell lines and reagents

Human undifferentiated thyroid carcinoma cell lines 8305C, ARO and KAT4 (DSMZ Cell Lines Bank, Braunschweig, Germany) were grown in RPMI 1640 medium containing 10% fetal bovine serum. Cisplatin, Taxol, and epirubicine were from Sigma-Aldrich (St. Louis, MO), Bortezomib (PS-341) was from Millennium Pharmaceuticals (Cambridge, MA). Human recombinant leucine zipper (LZ)-TRAIL was from Alexis (Lausen, Switzerland) and z-VAD-fmk from Bachem (Bubendorf, Switzerland). Primary antibodies anti-TRAIL-R1 and -TRAIL-R2 were from R&D Systems (Minneapolis, MN), anti-nuclear factor-κB (NF-kB), anti-Smac, anti-Bcl-xL, anti-inhibitor of apoptosis-1, anti-p21 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-cytochrome c and anti-Bcl-2 from PharMingen (San Diego, CA), antiactin from Sigma and anti-Fas-associated death domain-like IL-1β converting enzyme inhibitory protein (FLIP) from Alexis.

Immunohistochemistry and flow cytometry analysis

Immunohistochemical analysis was performed on ATC and colon carcinoma formalin-fixed paraffin embedded sections incubated with anti-NF-kB, TRAIL-R1, and TRAIL-R2 antibodies. Staining was detected using a 3,3′ diaminobenzidine-based substrate (Sigma). To analyze cytochrome c localization, cells were loaded with 100 nM MitoTracker Red CMXRos (Molecular Probes, Leiden, The Netherlands), fixed, stained with anticytochrome c and AlexaFluor 488-conjugated secondary antibodies (Molecular Probes), and then analyzed with an FV-500 laser-scanning confocal inverted microscope (Olympus, Tokyo, Japan). For flow cytometry analysis, cells were incubated with control or specific primary antibodies anti-TRAIL-R1 and -TRAIL-R2 and analyzed with an EPICS XL (Beckman Coulter, Fullerton, CA).

Caspase, cell viability, and cell cycle assays

Caspase activity was determined with the Apo-ONE homogeneous caspase-3/7 kit (Promega, Madison, WI) and analyzed on a plate fluorometer. Cell viability was determined using the CellTiter 96 AQ_{ueous} one solution cell proliferation assay (Promega). Cell cycle fractions were determined by propidium iodide staining (Sigma) and data were collected and analyzed with an EPICS XL (Beckman Coulter).

Retroviral gene transfer

The Bcl-2 cDNAs was cloned into the PINCO retroviral vector carrying the green fluorescent protein as reporter gene and used to transfect the amphotropic packaging cell line Phoenix. ATC cells were subjected to two cycles of spinfection (45 min of centrifugation at 1800 rpm followed by 75 min in the incubator) with the viral supernatant for 2 consecutive days, which yielded greater than 90% of green fluorescent protein-positive cells.

Real-time PCR

Total RNA was extracted using the RNasy minikit (QIAGEN, Hilden, Germany). One microgram of RNA was reverse transcribed by using SuperScript II RT with oligo(dT) as primers (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Real-time PCR was done with ABI Prism 7900HT sequence detection system and all reagents were from Applied Biosystems (Foster City, CA). Assays-on-Demand reagents for TRAIL-R1 and TRAIL-R2 were used (Applied Biosystems).

Statistical analysis

The differences between the various experimental conditions were analyzed with the two-tailed paired *t* test, and a $P < 0.05$ was considered statistically significant. One asterisk indicates $P < 0.01$, two asterisks $P < 0.05$.

Results

Bortezomib induces apoptosis of ATC cells and enhances TRAIL cytotoxicity

To determine the potential cytotoxic activity of Bortezomib on ATC cells, we exposed the 8305C ATC cell line to Bortezomib at doses within the range of clinically achievable concentrations. After 24 h of treatment with higher doses of Bortezomib, ATC cells showed massive cell death, whereas longer exposures significantly decreased ATC cell viability also at low concentrations (Fig. 1A). Similar results were obtained with the ARO and KAT4 ATC cell lines (not shown). We then compared the cytotoxic activity of low-dose Bortezomib (2 logs lower than the mean plasmatic peak) with high concentrations of those chemotherapeutic drugs currently used in the treatment of ATC. Whereas these drugs showed limited efficacy, Bortezomib was able to massively kill ATC cells (Fig. 1B).

Although Bortezomib was able to activate caspase-3 and -7 in ATC cells, caspase activation accounted only partially for the cytotoxic activity of Bortezomib as shown by the limited protective activity of the broad-spectrum caspase inhibitor zVAD-fmk (supplementary Fig. 1, A–C, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org). Bortezomib treatment induced cytochrome c translocation from the cytosol to the mitochondria, indicating it activates mitochondrial apoptotic events in ATC cells (supplementary Fig. 1D). Thyroid cancer cells are relatively resistant to death receptor-induced apoptosis (6). However, Bortezomib has been reported to sensitize several tumors for TRAIL-mediated killing (7–9). Therefore, we preincubated ATC cells with low concentrations of Bortezomib and subsequently treated them with increasing doses of TRAIL. Whereas the treatment with TRAIL as a single agent was ineffective, the combination of Bortezomib and TRAIL showed a synergistic effect in tumor cell killing (Fig. 1C), indicating that Bortezomib treatment renders ATC cells permissive for TRAIL-mediated cytotoxicity. Importantly, freshly isolated normal thyrocytes were scarcely sensitive to Bortezomib, which was not directly cytotoxic nor sensitized the cells to TRAIL-mediated apoptosis (supplementary Fig. 2).

Proteasome inhibitors have been shown to increase TRAIL receptor (TRAIL-R)-1 and TRAIL-R2 expression levels in a variety of cell lines (7, 8, 10, 11). To investigate whether Bortezomib sensitizes ATC cells to TRAIL-induced apoptosis by increasing the levels of death domain-containing TRAIL-Rs, we analyzed the expression of TRAIL-R1 and TRAIL-R2 in ATC cells before and after treatment with Bortezomib by flow cytometry and real-time PCR. Immunohistochemical analysis of TRAIL receptors on paraffin sections of four ATC specimens confirmed the low expression levels of TRAIL-Rs in ATC, in contrast with the high expression detected in epithelial colon carcinoma cells (Fig. 1D). Exposure to Bortezomib significantly increased the surface expression of both TRAIL-R1 and TRAIL-R2 in ATC cell lines (Fig. 1E). Interestingly, Bortezomib-mediated up-regulation of TRAIL-Rs did not strictly correlate with an up-regulation of the corresponding mRNA levels (Fig. 1F), indicating the prevalence of posttranscriptional effects in TRAIL-R modulation by Bortezomib.

Bortezomib modulates the level of apoptotic and cell cycle regulators and induces ATC cell apoptosis through the mitochondrial apoptotic pathway

To elucidate the molecular basis of Bortezomib antitumor effect, we evaluated the expression of key proteins responsible for the regulation of cell growth and survival in ATC treated with Bortezomib. Bortezomib significantly increased the levels

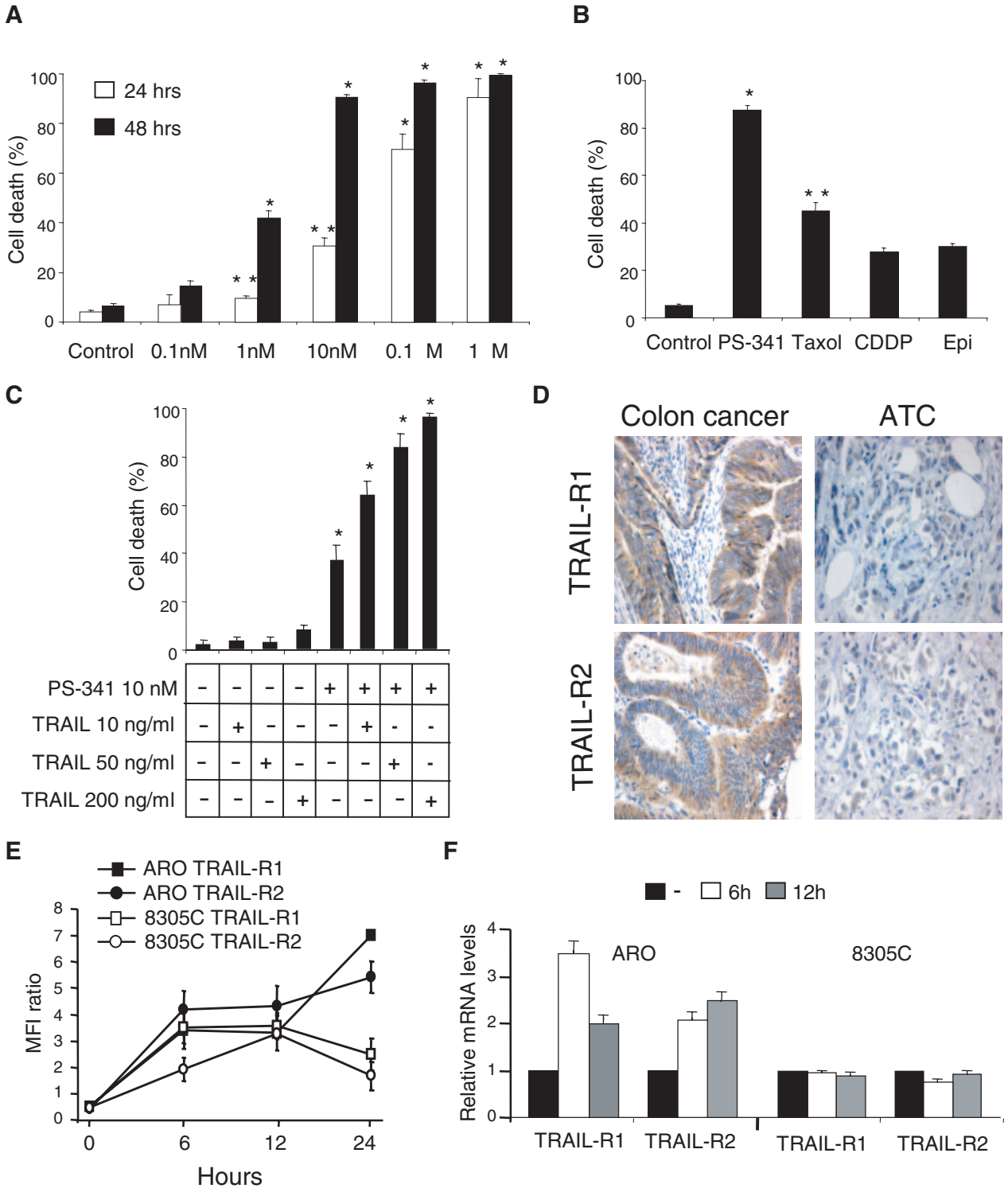

FIG. 1. ATC cells are highly sensitive to apoptosis induced by Bortezomib alone or in combination with TRAIL. A, Apoptosis induced by treating 8305C ATC cells for 24 and 48 h with increasing doses of Bortezomib. *Asterisks* indicate the statistical significance of each treated sample *vs.* untreated (control) cells. B, 8305C cells were treated for 48 h with 0.01 μM Bortezomib (PS-341), 5 μM Taxol, 300 ng/ml cisplatin (CDDP), and 5 μM epirubicin (Epi). *Asterisks* indicate the statistical significance of treated samples *vs.* untreated (control) cells. C, 8305C ATC cells were incubated with 10 nM Bortezomib for 3 h before the addition of LZ-TRAIL 10, 50, and 200 ng/ml. Cell death was evaluated 48 h later. The values represent the mean ± SD of three independent experiments. *Asterisks* indicate the statistical significance of treated samples *vs.* untreated (control) cells. D, Immunohistochemical analysis of TRAIL-R1 and TRAIL-R2 on paraffin sections of ATC and colon cancer specimens. The results are representative of four independent experiments with samples from different ATC patients. Original magnification, ×40. E, Flow cytometry analysis of TRAIL-R1 and –R2 in ARO and 8305C cell lines treated with 0.1 μM Bortezomib for 6, 12, and 24 h. Data are expressed as the mean fluorescence intensity (MFI) ratio between specific and control staining. F, Real-time PCR analysis of TRAIL-R1 and TRAIL-R2 in ARO and 8305C cell lines untreated (−) or treated for 6 and 12 h with 100 nM Bortezomib.

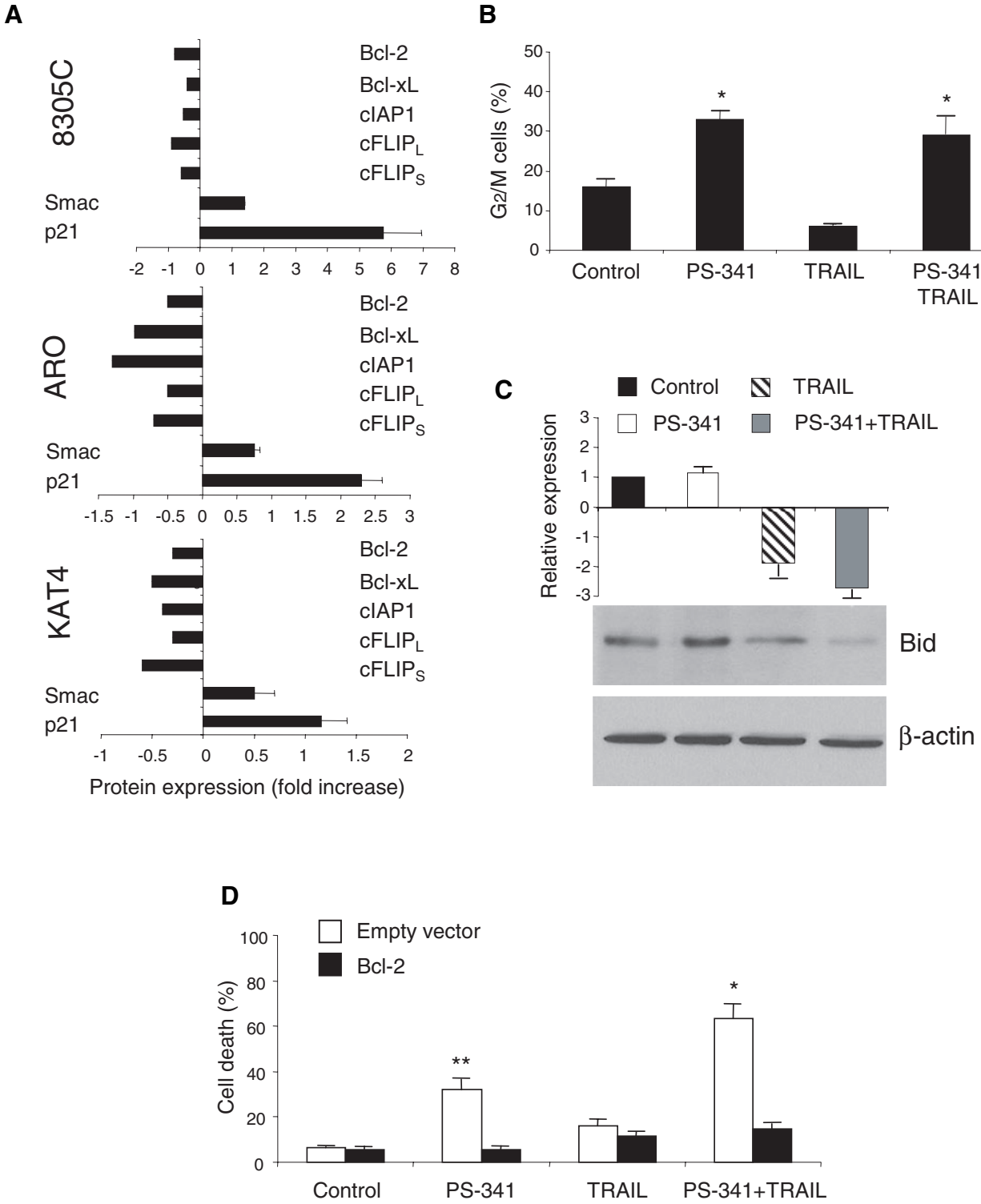

FIG. 2. Bortezomib modifies the expression of cell cycle and apoptosis regulators and induces ATC cell death through the mitochondrial apoptotic pathway. A, Densitometric analysis of p21, Smac, Bcl-2, Bcl-xL, cIAP1, cFLIP$_L$, and cFLIP$_S$ levels in 8305C, ARO, and KAT4 ATC cell lines treated for 24 h with 0.1 μM Bortezomib. Protein levels shown are the mean ± SD of three independent Western blotting experiments. B, Cell cycle status of 8305C cells incubated for 24 h with 0.1 μM Bortezomib (PS-341), 200 ng/ml LZ-TRAIL (TRAIL), or both and stained with propidium iodide for flow cytometric analysis. Results are expressed as mean ± SD of three independent experiments. *Asterisks* indicate the statistical significance of treated samples *vs.* untreated (control) cells. C, Caspase-mediated cleavage of Bid in ARO cells treated for 24 h with 0.1 μM Bortezomib (PS-341), 200 ng/ml LZ-TRAIL (TRAIL), or both. The densitometric analysis of protein expression showed in the *upper panel* refers to the results of three independent Western blotting experiments. D, 8305C cells retrovirally transduced with the empty vector or Bcl-2 were treated with 0.1 μM Bortezomib (PS-341), 200 ng/ml LZ-TRAIL (TRAIL), or both. Cell death was assessed 24 h later. The results are the mean ± SD of three independent experiments. *Asterisks* indicate the statistical significance of empty vector-transduced cells *vs.* Bcl-2-transduced cells within each treatment.

of proteins involved in apoptosis and cell cycle arrest such as SMAC/Diablo and the cyclin-dependent kinase inhibitor p21 (CIP/WAF-1), respectively. By contrast, Bortezomib decreased the expression of critical antiapoptotic factors such as cellular FLIP (cFLIP), Bcl-2, Bcl-xL, and inhibitor of apoptosis-1 in all three ATC cell lines tested (Fig. 2A). Proteasome regulates p21 expression in a cell cycle-dependent manner. Thus, exposure to Bortezomib may result in proliferation blockade (12, 13). Accordingly, treatment with Bortezomib increased the percentage of ATC cells at the G_2/M phase of the cell cycle, likely contributing to tumor cell destabilization (Fig. 2B). We then investigated the role of the extrinsic and intrinsic apoptotic pathways in the antitumor effect of Bortezomib and TRAIL. First, we analyzed caspase-mediated cleavage of Bid, which is involved in the amplification of death receptor-mediated apoptotic signals. Bid cleavage could not be detected aftertreatment with Bortezomib alone but was induced by TRAIL and further enhanced by combination of the two agents (Fig. 2C), in line with their synergistic apoptotic effect on ATC cells. To evaluate the involvement of the mitochondrial apoptotic pathway in Bortezomib- and TRAIL-mediated cytotoxicity against ATC, we retrovirally transduced 8305C cells with the Bcl-2 gene, which is able to inhibit mitochondrial apoptotic events. Whereas control cells were efficiently killed by Bortezomib, the presence of Bcl-2 inhibited almost completely apoptosis induced by Bortezomib alone or in combination with TRAIL (Fig. 2D). Therefore, Bortezomib acts through the mitochondrial apoptotic pathway to induce apoptosis of ATC cells.

Discussion

The results presented in this study show that proteasome inhibitors are able to induce apoptosis in undifferentiated thyroid carcinoma cells resistant to conventional chemotherapeutic agents. These observations are in accordance with those recently reported by Mitsiades *et al.* (14) on Bortezomib-induced apoptosis of thyroid medullary and anaplastic cell lines. We also show that the cytotoxic effect of Bortezomib can be potentiated through combination with the death ligand TRAIL, predictably allowing the clinical usage of lower doses of both compounds. We have shown that Bortezomib activates the intrinsic (mitochondrial) apoptotic pathway in ATC cells, as demonstrated by apoptosis inhibition mediated by exogenously expressed Bcl-2. Because TRAIL activates the extrinsic (death receptor mediated) apoptotic pathway in sensitive target cells, it is conceivable that the combination of Bortezomib and TRAIL overcomes mechanisms of death resistance in ATC cells by simultaneously activating both apoptotic pathways. The mechanism through which Bortezomib activates mitochondria-mediated apoptosis in ATC cells can be explained by the down-regulation of critical survival factors such as Bcl-2 and Bcl-X_L, and the up-regulation of p21 and SMAC/Diablo, resulting in growth arrest and activation of executioner caspases. At the same time, Bortezomib sensitizes ATC cells to TRAIL-induced apoptosis through the up-regulation of cytotoxic TRAIL-Rs and the down-regulation of cFLIP. Notably, Bortezomib-mediated up-regulation of TRAIL-Rs occurs also in the context of nonfunctional p53 (ARO cells), indicating that TRAIL-Rs accumulation is achieved through a block of the ubiquitine-proteasome pathway rather than through a p53-dependent mechanism. Proteasome inhibition is known to block NF-kB translocation to the nucleus in tumor cells through the stabilization of inhibitory-κB. We observed that NF-kB expression was mostly cytoplasmic in the ATC cell lines examined (supplementary Fig. 3), suggesting that the inhibition of this pathway could be dispensable for Bortezomib-induced cytotoxicity in ATC cells. In conclusion, our results demonstrate that Bortezomib has a strong cytotoxic effect on ATC cells that is further potentiated by combination with TRAIL. Although preclinical studies are required to confirm the antitumor effect of Bortezomib and TRAIL on ATC *in vivo*, the high cytotoxic activity and the good *in vivo* tolerability of the two molecules holds promise for a future use in the treatment of ATC patients.

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