

Bortezomib Sensitizes Human Esophageal Squamous Cell Carcinoma Cells to TRAIL–Mediated Apoptosis via Activation of Both Extrinsic and Intrinsic Apoptosis Pathways

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Abstract

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive human cancers, and novel treatment modalities are required. We investigated the therapeutic potential of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L) in combination with the proteasome inhibitor bortezomib (Velcade) on human ESCC cell lines. Bortezomib enhanced the susceptibility to TRAIL in 12 of the 15 ESCC cell lines tested, although most showed low sensitivity to TRAIL as a single agent. The enhancement of TRAIL-induced apoptosis by bortezomib was caspase dependent. Increased processing of caspase-8 often accompanied enhancement of TRAIL-induced apoptosis by bortezomib. However, the increased cell surface expression of death receptors observed on bortezomib treatment did not seem to be crucial for this effect. For some ESCC, bortezomib treatment resulted in a more efficient recruitment of caspase-8 and the Fas-associated death domain to the death-inducing signaling complex. Additional downregulation of the cellular FLICE-inhibitory protein long isoform [c-FLIP(L)] could cooperate in the activation of the extrinsic pathway in some cases. For other ESCC, the crucial effect of bortezomib treatment seemed to be increased signaling via the intrinsic apoptotic pathway on subsequent exposure to TRAIL. Thus, bortezomib could sensitize ESCC to TRAIL apoptosis by multiple molecular mechanisms of action. Therefore, the combination of bortezomib and TRAIL might be a novel therapeutic strategy for ESCC patients who fail to respond to standard chemoradiotherapy that predominantly targets the mitochondrial apoptotic pathway. *Mol Cancer Ther*; 9(6); 1842–51. ©2010 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the third most common cancer of the digestive tract and the seventh leading cause of cancer-related deaths worldwide. ESCC is one of the most aggressive types of cancer (1, 2). In addition to the many difficulties of curative resection, postoperative recurrences are often resistant to the currently available radiochemotherapies. Hence,

there is a need for a novel treatment modality involving anticancer drugs that selectively target cancer cells and circumvent treatment-resistant pathways for the management of ESCC.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L; refs. 3, 4) is a promising cytokine for anticancer therapy. TRAIL can bind to five receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 [decoy receptor (DcR) 1], and TRAIL-R4 (DcR2), which are located at the cell surface, and the secreted protein osteoprotegerin, which has a lower affinity to the ligand (5). However, apoptosis in cancer cells is only induced by binding to death receptors DR4 and DR5 (5). DR4 and DR5 contain death domain motifs crucial for transmission of an apoptotic signal. DcR1 lacks both cytoplasmic and transmembrane domains, and DcR2 has a truncated death domain, so both are unable to trigger apoptotic death. DcR1 and DcR2 are thought to act as decoys, and their expression is higher in normal tissues than in most cancer cells (6). More recently, DcR2 was proposed to act as a regulatory rather than a decoy receptor (7). The preligand assembly domains in DR5 and DcR2 could permit mixed complex formation as a means to negatively regulate apoptosis induction. These findings led to the proposal that TRAIL might be a novel cancer-targeting drug, and

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doi: 10.1158/1535-7163.MCT-09-0918

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the DcRs could account for the resistance to TRAIL-mediated apoptosis in normal tissues.

Clinical trials to characterize the safety and efficacy of recombinant human TRAIL (rhTRAIL) and receptor-targeted agonist antibodies, both alone and in combination with other cancer therapies, are ongoing (8). The early trial data suggest that rhTRAIL is generally safe and provide preliminary evidence for potential antitumor activity. However, many tumor cell lines are either partially or completely resistant to TRAIL-mediated apoptosis *in vitro* despite the expression of TRAIL death receptors; thus, many studies have explored how this resistance might be overcome to improve the clinical outcome (9). Resistance results from a number of factors including increased Akt activity (10, 11), the overexpression of caspase inhibitors (12, 13), Bcl-2 family members (14), or other molecules (12, 15) in the mitochondrial apoptotic pathway, and the overexpression of cellular FLICE-inhibitory protein (c-FLIP; refs. 12, 16, 17). Combination therapies involving several conventional and novel chemotherapeutic drugs have been reported to increase TRAIL-mediated apoptosis in these resistant cells (9, 12, 18–22).

The inhibition of the proteasome is a novel approach for anticancer therapy. The proteasome inhibitor bortezomib (Velcade) was recently approved for the treatment of patients with multiple myeloma. The treatment of tumor cells with bortezomib results in multiple biological effects, including inhibition of the cell cycle, increased apoptosis, changes in cell adherence, and inhibition of NF- κ B activation. Therefore, bortezomib is currently being tested in clinical trials against a variety of solid tumors and could provide an opportunity for exploring the interaction between proteasome inhibition and other apoptosis-inducing agents *in vitro* (23, 24). Many recent studies have shown that the treatment of tumor cells with proteasome inhibitors overcomes TRAIL resistance in various human solid cancers, including prostate cancer (25), colon cancer (26), glioma (27), non-small cell lung cancer (28, 29), and hepatocellular carcinoma (30, 31). However, the molecular mechanism(s) underlying the effects of the combined treatment is largely unknown. In the current study, we examined the efficacy of combined treatment with bortezomib and TRAIL on apoptosis in a number of human ESCC cell lines and assessed the molecular mechanisms underlying the effects of this combination.

Materials and Methods

Tumor cell lines

The KE3, KE4, KE5, KE6, KE7, KE8, and KE10 ESCC cell lines were established in our facility from surgical sections taken at Kurume University Hospital (Kurume, Japan), as previously described (32, 33). The TE8, TE9, and TE10 cell lines were a generous gift from T. Nishihira (Tohoku University, Sendai, Japan; refs. 32–34). The YES1, YES2, YES3, YES5, and YES6 cell lines were generously provided by T. Murakami (Yamaguchi University,

Ube, Japan; refs. 33, 35). Cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin; Life Technologies, Invitrogen Co.) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Agents and bortezomib/TRAIL treatment

Soluble rhTRAIL was purchased from Biomol and was cross-linked by adding an anti-6 \times histidine monoclonal antibody (mAb; R&D Systems) at a concentration of 2 μ g antibody/ μ g TRAIL. Bortezomib was obtained from Millennium Pharmaceuticals. To determine the effects of TRAIL, bortezomib, and bortezomib/TRAIL, the cancer cells were plated (at 1×10^4 to 2×10^4 per well in a flat-bottomed 96-well plate, and 2×10^5 to 4×10^5 per well in a six-well plate) and incubated overnight. The cells were either untreated or treated with bortezomib, TRAIL, or both at the indicated concentrations and for the indicated time periods. For the inhibition of NF- κ B or general caspase activity, SN50 (Biomol), Bay11-7085 (Calbiochem), or zVAD-fmk (R&D Systems) was added to the culture with pretreatment for 2 hours at the indicated concentration.

Cell viability assay and detection of apoptosis

After the incubation with bortezomib/TRAIL treatment, the viable cells were quantified by a MTS assay (Promega), as described previously (36). The percentage decrease in cell number was calculated as follows: $1 - [\text{absorbance of treated cells} - \text{absorbance of media}] / [\text{absorbance of untreated cells} - \text{absorbance of media}] \times 100\%$. For the detection of apoptosis, the cells were stained with Hoechst 33342 (Dojon) and/or FITC-coupled Annexin V/propidium iodide using Annexin-V-FLUOS Staining kit (Roche). Images were acquired with an IN Cell Analyzer 1000 (GE Healthcare), and cells with apoptotic morphology of nuclei (condensation/fragmentation) or Annexin V-positive cells were analyzed using the Developer Toolbox software (GE Healthcare). Apoptosis was also evaluated by measuring the enzyme activity of the executioner caspase-3. The caspase-3, caspase-8, and caspase-9 activities were estimated using the Caspase-Glo assay kit (Promega), as previously described (36). Each experiment was carried out in triplicate wells and repeated separately at least thrice.

Flow cytometric analysis of cell surface expression of TRAIL receptors

After the blocking of nonspecific binding with mouse immunoglobulin G (IgG; Caltag), the cells were stained with mouse anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 mAbs (R&D Systems) or isotype-matched mAbs, followed by phycoerythrin-labeled goat anti-mouse IgG polyclonal antibody (BD Pharmingen) according to standard procedures. In the experiment shown in Fig. 5A, mouse anti-human TRAIL-R1 (HS101) and TRAIL-R2 (HS201) mAbs from Alexis were used as the primary antibody. The HS201 mAb showed

higher sensitivity against DR5 than the other mAb from R&D Systems in our staining conditions. Flow cytometric analysis of stained cells was done using the FACSCalibur system (BD Biosciences), and the results were analyzed using CellQuest (BD Biosciences) and FlowJo (TreeStar, Inc.) software. Three independent experiments were done with single samples. The expression levels were evaluated based on the values corresponding to a ratio of the signal of the specific TRAIL receptor antibody and the negative isotype-matched control antibody.

Evaluation of mitochondrial membrane depolarization

Cells were incubated with a serum-free culture medium containing 10 $\mu\text{mol/L}$ 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1) dye (Wako; ref. 36) for 15 minutes. After washing with PBS, the stained cells were observed using an inverted fluorescence microscope (IX81, Olympus). The red and green fluorescence emissions were separately recorded and merged by DP manager software (Olympus).

Western blot analysis

Western blotting was done as described previously (17). mAbs for caspase-8, cleaved caspase-3 (Cell Signaling Technology), c-FLIP (Alexis), Fas-associated death domain (FADD), Bcl-2, Bax, X-linked inhibitor of apoptosis protein (XIAP), p27 (BD Transduction Laboratories), and β -actin (Sigma-Aldrich) or polyclonal antibodies for caspase-9, poly(ADP-ribose) polymerase (PARP), Bcl-XL, Mcl-1 (Cell Signaling Technology), cIAP-1 (R&D Systems), and p21 (Santa Cruz Biotechnology) were used as primary antibodies. These were followed by appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). The antibody-protein complexes were detected using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The signals were analyzed using a LAS-1000 image analyzer and MultiGauge software (Fuji Film).

c-FLIP knockdown by small interfering RNA transfection

A small interfering RNA (siRNA) pool consisting of four siRNAs specific for the c-FLIP gene (siGENOME SMARTpool reagent, M-003772-06) and control siRNA were purchased from Dharmacon. Target sequences of the c-FLIP gene (RefSeq NM_003879) are as follows: AAUAACUUCAGGCUCCAUA, GCUAUGAAGUCCAGAAUU, GAUGUGUCCUCAUAAUUU, and UAAAGAACAUCACAGAAU. The transfection of these siRNAs was conducted at a final concentration of 100 nmol/L using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Each transfection experiment was done at least in triplicate and repeated separately at least thrice.

Immunoprecipitation of death-inducing signaling complex

Death-inducing signaling complex (DISC) analysis was done as previously described with some modifications (19). Briefly, $\sim 10 \times 10^6$ cells were treated with bortezomib or medium for 8 hours, and then His-tagged rhTRAIL cross-linked by an anti-6 \times histidine mAb was added to the cultures at a concentration of 1 $\mu\text{g/mL}$. After 30 minutes of incubation with TRAIL, the cells were intensively washed with cold PBS, and cell lysates were prepared and subjected to immunoprecipitation with a protein G column (Pierce). The precipitated samples were tested for assembled DISC components, which contain protein complex with receptor-ligand (TRAIL) aggregation by Western blot analysis as described above. Horseradish peroxidase-conjugated anti-mouse IgG antibody (True-Blot; eBioscience) was used as the secondary antibody.

Results

TRAIL sensitization with bortezomib in ESCC cell lines

Most human ESCC cell lines were resistant or showed a low sensitivity to TRAIL-mediated apoptosis. The KE6 cell line was the only one to show >50% reduction of cell number following TRAIL treatment even at the high concentration, 500 ng/mL (Fig. 1A). All human ESCC expressed DR4 and/or DR5 on the cell surface. Although the sensitivities could have been related to the surface expression levels of these TRAIL receptors or the balance between DR4/DR5 and DcR2, there seemed to be no clear direct correlation between level of surface receptor expression and sensitivity to TRAIL (Table 1).

We then tested whether the proteasome inhibitor bortezomib could influence TRAIL sensitivity in 15 human ESCC cell lines. Bortezomib at a concentration of 100 nmol/L was added to the cultures 2 hours before treatment with TRAIL (100 ng/mL) for 16 to 18 hours. All of the cell lines showed decrease in cell numbers when treated with a combination of TRAIL and bortezomib compared with TRAIL alone. Furthermore, in 12 of the cell lines (i.e., all except TE10, YES2, and YES3), this combination resulted in a greater reduction in cell number than additive effects of the individual agents (Fig. 1B). These results indicate that bortezomib could cooperate with TRAIL and increase the susceptibility of ESCC cell lines to TRAIL-mediated apoptosis.

Cooperative effect of bortezomib and TRAIL on caspase activities

To assess the mechanisms underlying the effects of bortezomib on TRAIL sensitization, we further analyzed three cell lines (KE4, TE8, and TE9) that were refractory to TRAIL as a single agent and showed great enhancement of TRAIL sensitivity in the presence of bortezomib. This enhancement was blocked by the pan-caspase inhibitor zVAD-fmk in all three cell lines (Fig. 1C). Figure 1D also shows in TE9 cells that the combined treatment

resulted in a significant induction of apoptotic cell death as determined by Annexin V and nuclear staining, which was completely inhibited by zVAD-fmk, indicating that this process was caspase dependent. Similar profiles were detected in KE4 and TE8 cells (data not shown).

The initial critical step in TRAIL signaling after the aggregation of the receptor with its cognate ligands is thought to be the dimerization and activation of procaspase-8 enzyme activity in the DISC (37), resulting in the subsequent accumulation of the cleaved form of caspase-8 (38, 39). The apoptotic signal can then diverge into two pathways. The first involves the direct activation of caspase-3 without the involvement of mitochondria (termed as extrinsic apoptosis pathway). The second involves perturbations at the mitochondria due to truncated Bid generation, resulting in the formation of apoptosomes and activation of caspase-9 (intrinsic or mitochondrial pathway) with subsequent activation of caspase-3. This

caspase-based signaling was subjected to Western blot analysis (Fig. 2A). With TRAIL alone, caspase-8 showed little activation and processing into cleaved forms (43/41 and 18 kDa), whereas the combined treatment greatly enhanced the processing of caspase-8 in the KE4 and TE9 cell lines. An increased activation of caspase-9 and caspase-3 by the accumulation of cleaved 37/35- and 19/17-kDa forms, respectively, as well as increased PARP cleavage was also observed with the combined treatment. In contrast to the KE4 and TE9 cell lines, the TE8 cell line showed similar cleavage of caspase-8 following treatment with TRAIL alone or the combined treatment. However, caspase-9 and caspase-3 were only significantly processed in TE8 following the combined treatment. Mitochondrial function as assessed by JC-1 dye staining showed that treatment with both drugs together, but not with either drug alone, produced significant depolarization of the mitochondrial membrane in the TE8 cell line. This indicated

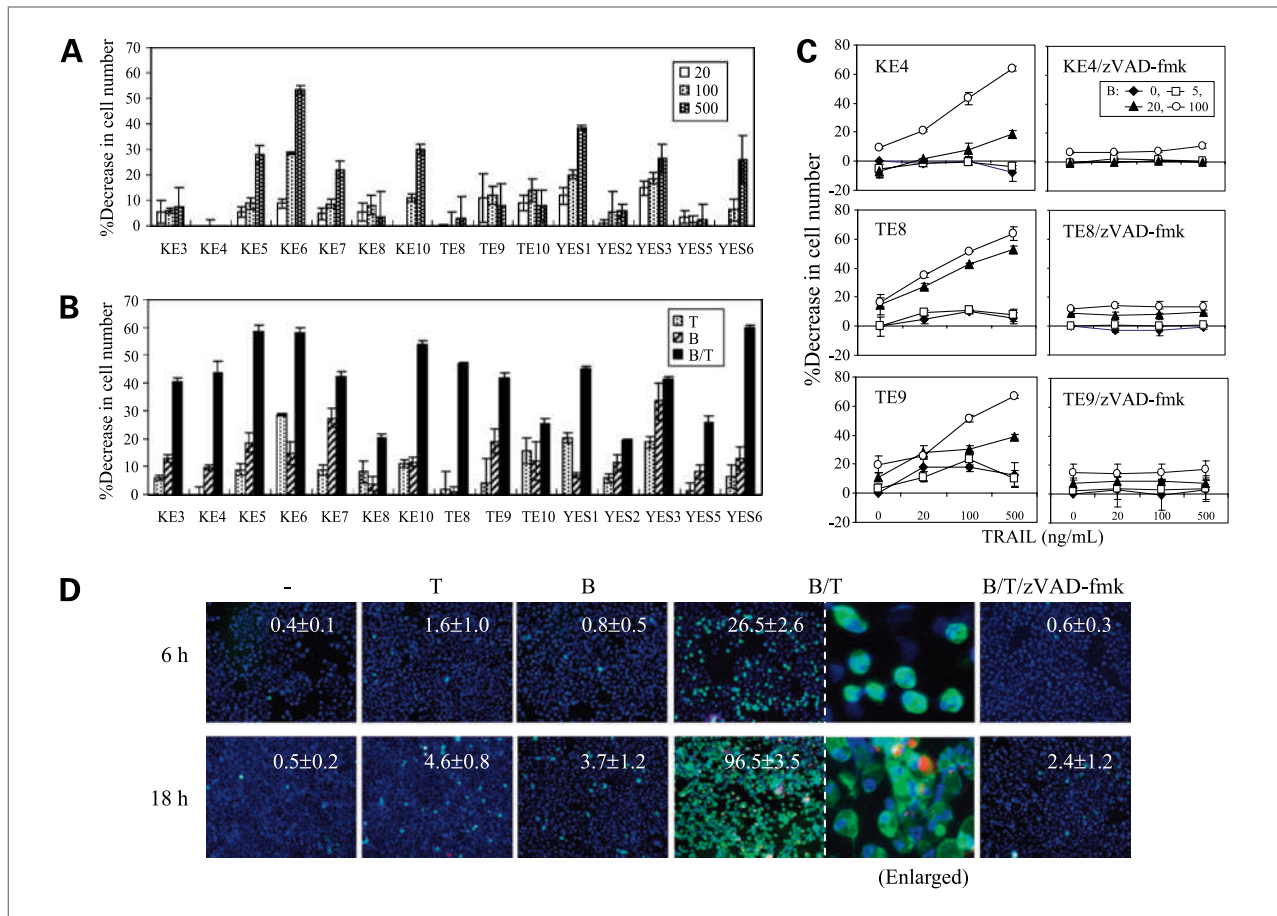


Figure 1. Enhancement of sensitivity to TRAIL in ESCC cell lines. A, 15 ESCC cell lines were seeded on 96-well plates and treated with TRAIL at 20, 100, and 500 ng/mL for 18 h. B, the cells were treated with bortezomib (B; 100 nmol/L) or medium alone for 2 h and subjected to a further 16- to 18-h incubation with or without the addition of TRAIL (T; 100 ng/mL). C, KE4, TE8, and TE9 cells were incubated in the various concentrations of bortezomib (◆, 0 nmol/L; □, 5 nmol/L; ▲, 20 nmol/L; ○, 100 nmol/L) and TRAIL, as described in B, in the presence or absence of zVAD-fmk (100 μmol/L). Cell viability was assessed by an MTS assay, and the percentage decrease in cell number (±SD) compared with the medium control is shown in A to C. D, TE9 cells were also stained by Annexin V-FITC (green), propidium iodide (red), and Hoechst 33342 (blue) at 6 and 18 h after the addition of TRAIL. Representative images with bortezomib (B; 100 nmol/L) and TRAIL (T; 500 ng/mL) are shown with the values (±SD) of % apoptotic cells as described in Materials and Methods.

Table 1. Expression levels of TRAIL receptors

		KE3	KE4	KE5	KE6	KE8	KE10	TE8	TE9	TE10	YES1	YES2	YES5	YES6
DR4	—	3.0	1.3	1.5	3.2	2.1	1.5	3.6	1.3	1.2	1.3	1.3	1.4	2.8
	Bzb	2.9	1.6	N.T.	N.T.	2.4	N.T.	2.5	1.4	1.1	N.T.	1.4	N.T.	N.T.
DR5	—	1.6	1.1	1.5	1.5	1.4	1.6	1.7	1.3	1.0	1.3	1.2	1.3	1.7
	Bzb	2.0	1.3	N.T.	N.T.	3.3	N.T.	2.6	1.7	1.1	N.T.	1.5	N.T.	N.T.
DcR1	—	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Bzb	1.0	1.0	N.T.	N.T.	1.1	N.T.	1.0	1.0	1.0	N.T.	1.0	N.T.	N.T.
DcR2	—	1.2	1.0	1.1	1.0	1.6	1.1	3.2	1.0	1.1	1.5	1.1	1.3	1.4
	Bzb	1.5	1.0	N.T.	N.T.	2.5	N.T.	6.8	1.1	1.4	N.T.	2.1	N.T.	N.T.

NOTE: Constitutive surface expression of TRAIL receptors was examined in 13 ESCC lines. In seven cell lines, effect of bortezomib (100 nmol/L) on the receptor expression was also tested after 18-h treatment. Cells were stained with anti-DR4, anti-DR5, anti-DcR1, and anti-DcR2 mAbs and subjected to flow cytometric analysis. The values indicate the relative surface expression levels of receptors, as assessed by mean channel fluorescence, compared with the negative control (set at 1.0). Representative results from three independent experiments with similar results are shown.

Abbreviations: Bzb, bortezomib; N.T., not tested.

that only the combined treatment could efficiently elicit activation of the mitochondria-dependent apoptosis pathway in TE8 cells (Fig. 2B). A quantitative analysis of caspase enzyme activities on 2-hour TRAIL stimulation after bortezomib pretreatment was consistent with the

Western blot data in all three cell lines (Fig. 2C). Taken together, these findings suggest that amplification of caspase-8 activation might be important to trigger the complementary enhancement of cell death between bortezomib and TRAIL in the KE4 and TE9 cell lines. By

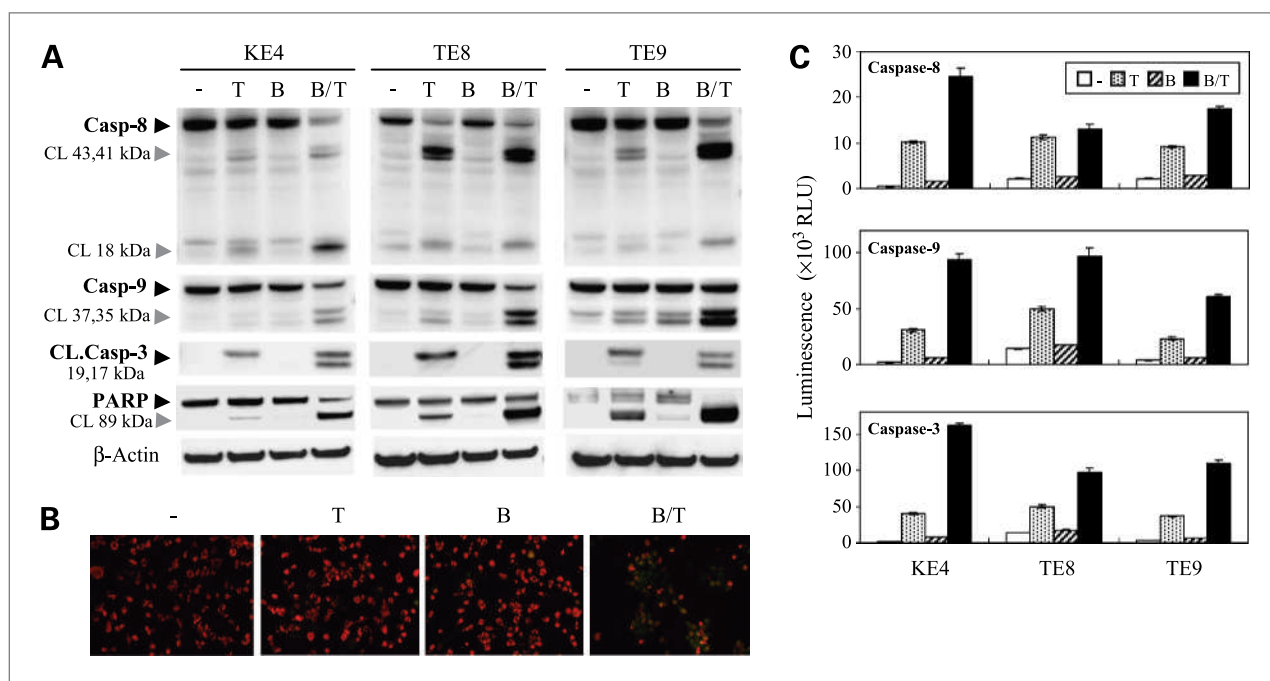


Figure 2. Caspase activities with bortezomib/TRAIL treatment in KE4, TE8, and TE9 cells. Cells were incubated in the presence or absence of bortezomib (100 nmol/L for KE4 and TE9 cells and 20 nmol/L for TE8 cells) for 2 h before the addition of vehicle or TRAIL (100 ng/mL). A, after a further 6 h, cell extracts were prepared, and Western blotting was done with anti-caspase-8, anti-caspase-9, anti-cleaved (CL) caspase-3, anti-PARP, and anti- β -actin antibodies. B, cells were incubated with JC-1 dye during the last 15 min of culture. Mitochondrial depolarization was evaluated as indicated by a decrease in the red-to-green fluorescence intensity ratio. Representative results for TE8 cells are shown. C, cells were incubated in the presence or absence of bortezomib for 6 to 8 h. TRAIL was added for a further 2 h (500 ng/mL), and the enzyme activities of caspase-8, caspase-9, and caspase-3 in the cells were measured as described in Materials and Methods.

contrast, the major molecular mechanism of bortezomib sensitization to TRAIL-mediated apoptosis in TE8 seemed to be further downstream at the mitochondrial level, indicating that multiple molecular mechanism(s) of TRAIL sensitization by bortezomib could occur even among ESCC isolated from different individual patients.

Upregulation of death receptors by treatment with bortezomib and its partial contribution to TRAIL-induced apoptosis

The surface expression levels of TRAIL receptors were tested after overnight treatment with bortezomib in seven of the ESCC cell lines: five cell lines (KE3, KE4, KE8, TE8, and TE9) that showed increased TRAIL sensitivity with bortezomib and two cell lines (TE10 and YES2) that showed no circumvention of TRAIL resistance with bortezomib (Table 1). Increased cell surface levels of DR5 were seen on all of the cell lines following bortezomib treatment, although the upregulation of DR5 in the TE10 cell line was marginal. Four of the cell lines (KE4, KE8, TE9, and YES2) showed increased DR4 expression according to flow cytometric analysis. By contrast, the surface expression of DcR2 was also increased significantly in five cell lines (KE3, KE8, TE8, TE10, and YES2) and marginally in one cell line (TE9). The TE8 cell line showed marked enhancement of TRAIL sensitivity with bortezomib treatment despite an increase of DcR2 expression that was significantly greater than that of DR4 and/or DR5. This finding was consistent with several previous studies that failed to show a good correlation between expression of DcRs and TRAIL sensitivity

(40–42). To further investigate any contribution of death receptor upregulation to TRAIL sensitization by bortezomib, a “wash kill” experiment was done, as previously reported by Koschny et al. (27, 31, 43). KE4 and TE9 cells were pretreated with TRAIL to occupy the TRAIL receptors already present on the cell surface, followed by thorough washing to remove all unbound TRAIL after 1 hour. The cells were then treated with bortezomib either alone or in combination with TRAIL for 12 hours and subjected to an apoptosis assay (Fig. 3A). Pretreatment with TRAIL followed by the addition of bortezomib without further TRAIL, which activated the preexisting but not the upregulated receptors after bortezomib treatment, significantly enhanced the activity of caspase-3, although to a lesser extent than when further TRAIL was added. These findings suggest that the upregulation of DR4 and DR5 partially contributed to, but was not crucial for, the TRAIL sensitization by bortezomib in both KE4 and TE9 cells.

Effects of c-FLIP modification by bortezomib on TRAIL sensitivity

We previously reported that the reduction of c-FLIP expression correlated with TRAIL sensitization in mouse and human renal cancer cell lines (16, 17). Western blot analysis of whole-cell lysates revealed that c-FLIP long isoform [c-FLIP(L)] expression was reduced in the KE4 cell line and increased in the TE8 cell line, with no significant change seen in the TE9 cell line following bortezomib treatment (Fig. 3B). By contrast, the c-FLIP short isoform [c-FLIP(S)] was elevated in all the cell lines tested. The

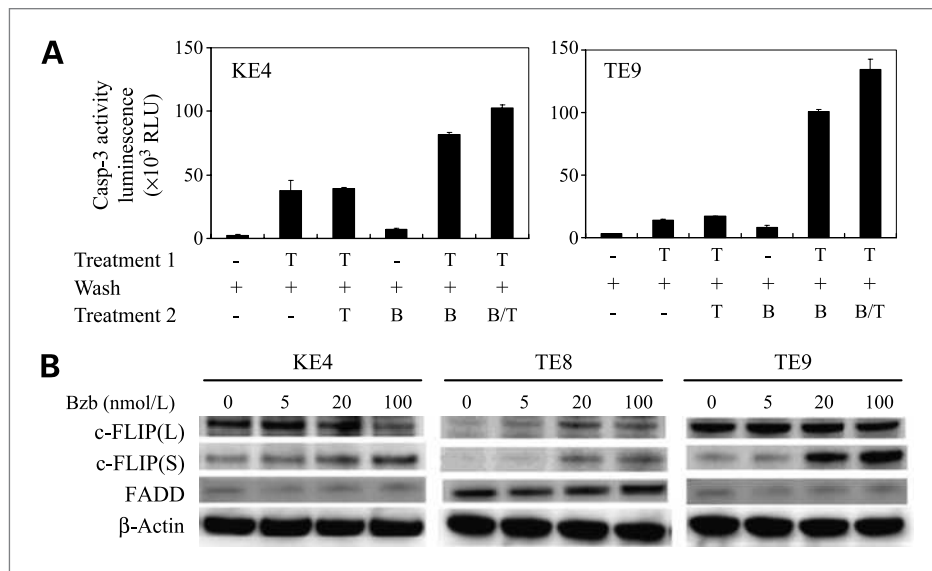


Figure 3. A, contribution of bortezomib-induced upregulation of death receptors to TRAIL-induced apoptosis. KE4 and TE9 cells were incubated with TRAIL at 500 ng/mL for 1 h and then washed five times with medium to remove unbound TRAIL. Cells were left untreated or sensitized with bortezomib (100 nmol/L) for 12 h, either with or without the further addition of TRAIL (500 ng/mL). Apoptotic cells were evaluated by an active caspase-3 assay as described in Materials and Methods. B, expression of c-FLIP and FADD proteins following bortezomib (Bzb) treatment. Cells were incubated in the presence or absence of various concentrations of bortezomib for 18 h. Cell extracts were then prepared, and Western blotting was done with anti-c-FLIP, anti-FADD, and anti-β-actin antibodies.

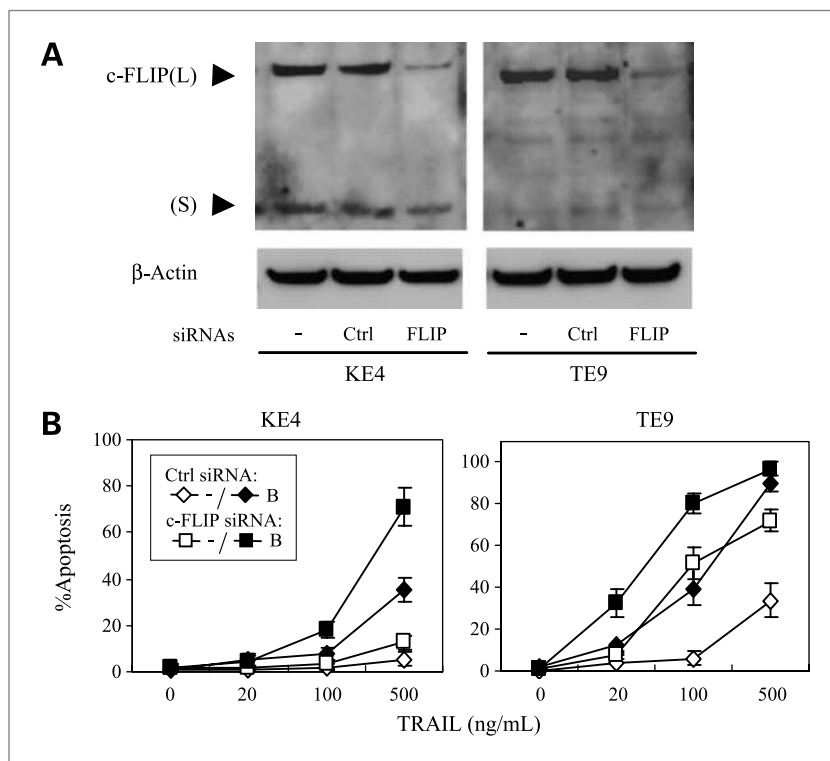


Figure 4. Enhanced susceptibility to TRAIL-induced apoptosis in bortezomib-treated cells following specific downregulation of c-FLIP with siRNAs. KE4 and TE9 cells were transfected with siRNAs targeting c-FLIP or control (Ctrl) siRNAs and cultured for 48 h. A, cells were lysed and analyzed for c-FLIP and β -actin expression by Western blotting. B, transfected cells were incubated in the presence or absence of bortezomib (100 nmol/L) for 2 h and for a further 10 to 12 h with the addition of TRAIL at various concentrations. Percentage apoptotic cells (\pm SD) were assessed after Hoechst 33342 nuclear staining as described in Materials and Methods (control siRNA: \diamond , vehicle; \blacklozenge , bortezomib; c-FLIP siRNA: \square , vehicle; \blacksquare , bortezomib).

total cellular level of FADD expression did not change at the various concentrations of bortezomib tested in all three cell lines. To evaluate the involvement of c-FLIP expression in TRAIL-mediated apoptosis, we used c-FLIP siRNAs to block c-FLIP function. c-FLIP-targeting siRNAs and control siRNAs were transfected into the KE4, TE8, and TE9 cell lines. TE8 had undetectable amounts of both c-FLIP(L) and c-FLIP(S) proteins (data not shown). However, protein levels of c-FLIP(L) were reduced on average (\pm SD) by $73.2 \pm 11.2\%$ in KE4 and $84.1 \pm 9.5\%$ in TE9. By contrast, c-FLIP(S) levels in KE4 and TE9 cells were not obviously changed by the siRNA treatment (Fig. 4A). Interestingly, these reductions of c-FLIP(L) using siRNA showed that c-FLIP(L) could protect the two ESCC lines from TRAIL apoptosis (Fig. 4B). Furthermore, these findings suggest that reduced levels of c-FLIP(L) observed in KE4 cells in response to bortezomib were likely to be of importance for increasing caspase-8 activation on exposure to TRAIL.

Enhancement of DISC formation on TRAIL stimulation by bortezomib pretreatment

Because our results suggested that increased caspase-8 activation might be crucial for the combined treatment of the KE4 and TE9 cell lines, we analyzed the effects of bortezomib on DISC assembly after TRAIL stimulation in KE4 and TE9 cells. After 8 hours of pretreatment with bortezomib, the cell surface levels of expression of DR4, as assessed by changes in mean channel fluorescence, were only marginally increased (KE4, $11.4 \pm 1.4\%$; TE9,

$13.0 \pm 1.8\%$). In addition, only slight increases in cell surface levels of DR5 (KE4, $24.8 \pm 3.0\%$; TE9, $35.2 \pm 2.7\%$) were observed (Fig. 5A). Following immunoprecipitation, DISC components were subjected to Western blot analysis. As shown in Fig. 5B, significantly enhanced recruitment of FADD at the DISC was observed in both the KE4 and TE9 cell lines following bortezomib pretreatment (fold increase in densitometric analysis: KE4, 3.13 ± 0.77 ; TE9, 3.40 ± 0.23). In addition, caspase-8 was significantly recruited at the DISC in these cells. Although bortezomib treatment did seem to also result in increases of c-FLIP at the DISC, which might be due in part to increased expression level of c-FLIP(S) with bortezomib treatment (Fig. 3B), these changes did not counteract the increased activation of caspase-8. These findings suggest that bortezomib preferentially favored recruitment of FADD and procaspase-8 to this DISC, and this increase may be sufficient to subsequently increased triggering of the extrinsic apoptotic pathway.

Bortezomib-induced changes in other proteins involved in apoptosis

Because in the TE8 cell line bortezomib did not enhance caspase-8 activation in response to TRAIL, effects on the intrinsic apoptotic pathway could be involved in enhanced TRAIL-induced apoptosis in this case. Numerous proteins have been reported to block or promote the apoptotic cascade of TRAIL (9). Interestingly, inhibitors of NF- κ B other than bortezomib did not sensitize ESCC to TRAIL apoptosis (data not shown), in agreement with

previous reports showing that blocking NF- κ B activation is not always critical for the sensitization of tumor cells to TRAIL (17, 30, 44, 45). We therefore tested the expression of apoptosis-related proteins by Western blotting after bortezomib treatment of the KE4, TE8, and TE9 cell lines (Fig. 6). There were no major changes in the levels of Bcl-2, Bcl-XL, Bax, or XIAP in any of the three cell lines. Interestingly, levels of cIAP-1 were reduced to some extent in the TE8 cell line, which would be consistent with bortezomib affecting downstream apoptotic signaling in this particular ESCC. However, Mcl-1, p21, and p27 were increased following bortezomib treatment in all ESCC tested. This would be expected because the levels of all of these proteins are tightly controlled by the proteasome. Because Mcl-1 is reported to be an inhibitor of mitochondrial-mediated TRAIL apoptosis (15), the observed increases could argue against a crucial contribution of intrinsic apoptotic signaling following bortezomib exposure for ESCC. Bortezomib-induced increases in p21 and p27 could be accompanied with cell cycle arrest at G₂-M

phase in ESCC (data not shown) that may also contribute to TRAIL sensitization as previously described (25). However, increases in p21 and p27 also occurred in ESCCs that remain resistant to bortezomib and TRAIL apoptosis (data not shown). Therefore, the importance of cell cycle inhibition by bortezomib on TRAIL sensitization should be investigated in future studies.

Discussion

The effects of TRAIL have not been studied as intensively in ESCC as in other cancers (20–22, 46, 47). However, the relative resistance of ESCC to TRAIL-mediated apoptosis seen in the current study was consistent with a previous report by Kondo et al. (20). Several previous reports showed that combination treatment with agents such as cisplatin, gefitinib, and taurolidine could overcome TRAIL resistance in ESCC (20–22, 46). However, to our knowledge, the effects of proteasome inhibitors on TRAIL sensitivity of ESCC have not been previously reported. Our data clearly indicated that bortezomib could enhance the susceptibility to TRAIL in most ESCC lines tested.

Interestingly, the molecular mechanism(s) underlying bortezomib sensitization to TRAIL seemed to differ among individual ESCC. Thus, in the KE4 and TE9 cell lines, bortezomib treatment resulted in increased caspase-8 activation following TRAIL exposure as described in many previous reports (25, 28, 29, 36), and this amplified caspase-8 activation seemed crucial for the bortezomib sensitization. Liu et al. (28) reported that bortezomib alone induced apoptosis with caspase-8 activation in human non-small cell lung cancer cells. However, no activation of caspase-8 following bortezomib treatment alone was observed in our current study with ESCC. The activation of caspase-8 might have been partially due to an increase in the surface expression of DR4 and/or DR5. However, our wash kill experiments indicated that proteasome inhibitor-mediated upregulation of DR4 and DR5 was not crucial for TRAIL sensitization, consistent with previous reports for leukemias, gliomas, and hepatocellular carcinomas (27, 30, 31, 43).

Recently, Ganten et al. (30) reported that caspase-8 and FADD were more efficiently recruited to the DISC of hepatocellular carcinoma cells pretreated with another proteasome inhibitor, MG132, despite the presence of fewer death receptors and more c-FLIP in the DISC. We also observed significantly enhanced recruitment of caspase-8 and FADD to the DISC in both the KE4 and TE9 cell lines. In previous studies, bortezomib has also been reported to reduce cellular levels of c-FLIP (17, 27, 31) in some cancer cells, whereas others have not observed changes in c-FLIP (29, 48). In our current study, the effect of bortezomib on c-FLIP(L) expression varied between individual ESCC lines. Because bortezomib could facilitate a greatly enhanced recruitment of caspase-8 and FADD to the DISC, this may be its dominant effect in promoting apoptotic signaling, thus overriding any concomitant inhibitory effects of c-FLIP. Recent findings suggest that

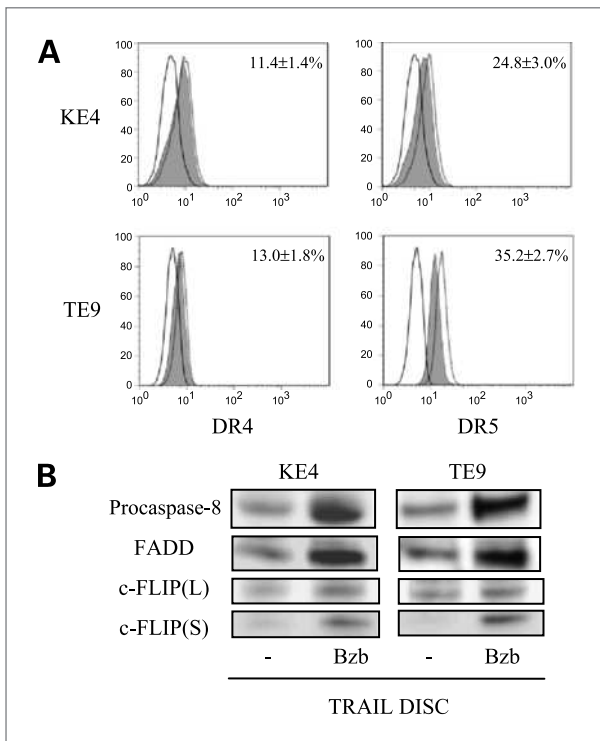


Figure 5. Effects of bortezomib on TRAIL-induced DISC formation. KE4 and TE9 cells were incubated with or without bortezomib (100 nmol/L) for 8 h. A, surface expression levels of DR4 and DR5 were analyzed by flow cytometry. Representative histograms from three independent experiments with similar results are shown (solid line, isotype control; shaded area, untreated; dashed line, bortezomib treated). The percentage increase (average \pm SD) of expression with bortezomib is presented as described in Materials and Methods. B, cells were further incubated with His-tagged rhTRAIL cross-linked by anti-6 \times histidine mAb (1 μ g/mL) for 30 min. The cells were lysed and the precipitated DISC was analyzed by Western blotting. Representative images from three independent experiments are shown.

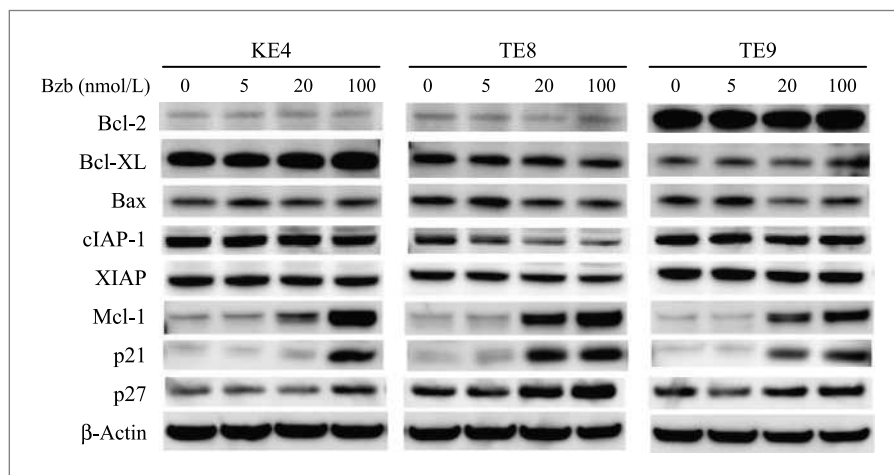


Figure 6. Effects of bortezomib on the expression of various proteins involved in apoptosis or cell cycle arrest. KE4, TE8, and TE9 cells were treated with various concentrations of bortezomib (Bzb) for 18 h, and the cell extracts were then prepared. The given proteins were detected using Western blot analysis.

the glycosylation state of the TRAIL receptors can influence their ability to activate caspase-8 (49). Furthermore, the ubiquitination of caspase-8 can also influence the strength of the apoptotic signal (50). Therefore, the effects of bortezomib on these processes would certainly be of interest for future studies.

Proteasome inhibition has also been reported to induce multiple molecular changes influencing components of the mitochondrial apoptosis signaling pathway, thus promoting TRAIL-mediated apoptosis (23, 24, 26, 44). However, others have questioned the importance of these effects for TRAIL-mediated apoptosis (36). Our findings indicate that the relative importance of bortezomib effects on components of the mitochondrial pathway for promoting TRAIL-mediated apoptosis of ESCC can vary even between individual patient tumor samples. Taken together, our findings indicate that the mechanism of TRAIL sensitization with bortezomib typically involved increased activation of both caspase-8 and caspase-9. This amplified apoptotic signaling could override any bortezomib-induced increase in antiapoptotic proteins such as Mcl-1 or c-FLIP. In addition, the p53 gene is mutated in KE4 and TE8 (33), and a truncated form of p53 is expressed in TE9 as a result of a frameshift accompanied by functional inactivation of the p53 protein (34). Therefore, bortezomib sensitization to TRAIL-mediated apoptosis is independent of p53 in these ESCCs.

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In conclusion, TRAIL-mediated apoptosis was efficiently promoted in ESCC by combined therapy with rhTRAIL and bortezomib, which could activate both the extrinsic and the intrinsic pathways of apoptosis. Combined TRAIL therapy could therefore be a novel therapeutic strategy for use in ESCC patients who fail to respond to standard chemoradiotherapy that mainly triggers the intrinsic apoptotic pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and “High-Tech Research Center” Project for Private Universities: matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology. This project has been funded in whole or in part with federal funds from the National Cancer Institute, NIH, under contract N01-CO-12400. This research was supported (in part) by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

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Received 10/05/2009; revised 04/16/2010; accepted 04/27/2010; published OnlineFirst 06/01/2010.

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