Mapatumumab and lexatumumab induce apoptosis in TRAIL-R1 and TRAIL-R2 antibody-resistant NSCLC cell lines when treated in combination with bortezomib

Troy A. Luster,[1] Jeffrey A. Carrell,[2] Kathy McCormick,[1] David Sun,[3] and Robin Humphreys[1]

[1]Oncology Research Department, [2]Clinical Immunology Department, and [3]Bioanalytical Department, Human Genome Sciences, Inc., Rockville, Maryland

Abstract

Mapatumumab and lexatumumab are fully human monoclonal antibodies that bind and activate human tumor necrosis factor-related apoptosis-inducing ligand receptors 1 and 2, respectively. These antibodies induce apoptosis in various tumor cell types, although the degree of sensitivity can vary from highly sensitive to completely resistant. Importantly, tumor cells that are partially or completely resistant to mapatumumab or lexatumumab can often be sensitized when treated in combination with chemotherapeutic drugs. In this regard, the proteasome inhibitor bortezomib has recently shown synergistic activity against established lymphoma cell lines and primary lymphomas when combined with mapatumumab and lexatumumab. Here, we report similar findings using a panel of human non-small cell lung cancer (NSCLC) cell lines. Specifically, we show that bortezomib rapidly induces sensitivity to mapatumumab and lexatumumab in NSCLC cell lines that are completely resistant to antibody alone and that bortezomib concentrations as low as 25 nmol/L sensitize NSCLC cells to the antibodies. Furthermore, bortezomib at the tested concentration has minimal effect on its own, indicating the combination generates synergistic cytotoxicity. Combination treatment induces activation of the caspase cascade and the effect of the combination is caspase dependent. Bortezomib treatment increases the intracellular levels of several important apoptosis regulators that may mediate enhanced sensitivity to mapatumumab and lexatumumab. These results suggest future evaluation of mapatumumab or lexatumumab in combination with bortezomib is warranted in NSCLC patients. [Mol Cancer Ther 2009;8(2):292–302]

Received 9/23/09; revised 11/26/09; accepted 12/3/09; published OnlineFirst 01/27/2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Robin Humphreys, Oncology Research Department, Human Genome Sciences, Inc., 14200 Shady Grove Road, Rockville, MD 20850. Phone: 240-314-4400; Fax: 301-309-1321. E-mail: robin_humphreys@hgsi.com

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0918

Introduction

Mapatumumab and lexatumumab (formerly HGS-ETR1 and HGS-ETR2, respectively) are fully human agonistic monoclonal antibodies (mAb) that bind with high affinity to the human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (TRAIL-R1/DR4 and TRAIL-R2/DR5), respectively. These receptors are expressed on a wide variety of tumor cell types and a limited number of normal cell types (1). Accordingly, binding of TRAIL or agonistic TRAIL-R1 and TRAIL-R2 antibodies induces apoptosis in numerous cancer cell types but has little effect on most normal cells. Given the selective toxicity for tumor cells, both recombinant human TRAIL and agonistic TRAIL-R1 and TRAIL-R2 antibodies are currently in various stages of clinical development for the treatment of human cancers (2). Agonistic TRAIL-R1 and TRAIL-R2 antibodies may have greater therapeutic potential than TRAIL due to a prolonged half-life *in vivo* and lack of interaction with so-called ''decoy'' TRAIL receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2, and OPG; ref. 3). Mapatumumab and lexatumumab may also activate Fc-mediated antibody effector functions, such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (4).

TRAIL-R1- and TRAIL-R2-induced apoptosis is mediated through activation of both extrinsic and intrinsic intracellular death signaling pathways. Ligation of death receptors results in formation of the death-inducing signaling complex (DISC), which consists of death receptors, adaptor proteins, and pro-caspase-8, and leads to processing and activation of pro-caspase-8 by an autocatalytic mechanism (5). Activated caspase-8 triggers the extrinsic apoptotic pathway by directly activating effectors such as caspase-3 and caspase-7, resulting in cleavage of downstream targets such as poly(ADP-ribose) polymerase (PARP). Caspase-8 can also initiate the intrinsic apoptotic pathway through cleavage and activation of Bid. Activated Bid induces oligomerization of proapoptotic proteins Bax and Bak, resulting in the release of both cytochrome *c* and Smac/DIABLO from the mitochondria and subsequent activation of caspase-9 (6). Both intrinsic and extrinsic pathways lead to the activation of caspase-3 and eventual apoptotic cell death.

Non-small cell lung cancer (NSCLC) constitutes ~87% of all lung cancer cases in the United States (7). Platinum-based chemotherapy doublets are still considered standard treatment for advanced NSCLC (8). However, the modest gains observed with these regimens are countered by their toxicity profile; therefore, more effective targeted therapies are needed (9). A recent study of stage III NSCLC patients found high TRAIL-R1 expression in 99% of tumor biopsies

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

and high TRAIL-R2 expression in 82% of tumor biopsies (10). A separate study of stage I and II NSCLC patients found high TRAIL-R2 expression in 67% of tumor biopsies (11). Thus, many NSCLC patients may be good candidates for TRAIL-R1 and TRAIL-R2 agonist-mediated therapy, although recent single-agent phase 2 data with mapatumumab suggest that combination with sensitizing agents may be required for maximum efficacy (12).

Mapatumumab and lexatumumab induce apoptosis in various tumor cell types, including NSCLC cell lines, *in vitro* and *in vivo*, although the degree of sensitivity can vary from highly sensitive to completely resistant (13). Importantly, tumor cells that are partially or completely resistant to mapatumumab or lexatumumab can be sensitized by combination treatment with various chemotherapeutic drugs (13–16). The proteasome inhibitor bortezomib has recently been approved as a first-line therapy for multiple myeloma and mantle cell lymphoma and has shown promising antitumor activity in combination with other cytotoxic drugs in patients with NSCLC (17). Bortezomib treatment elicits several cellular changes that may sensitize tumor cells to TRAIL receptor agonists, such as the accumulation of BH3-only proapoptotic Bcl-2 family members (18) and increased TRAIL receptor surface expression (19–21). Accordingly, bortezomib has been shown recently to enhance the sensitivity of NSCLC cell lines to TRAIL (19, 20) and is also known to enhance the sensitivity of other tumor types to mapatumumab and lexatumumab (22–24). The purpose of this study was to examine the effects of treating NSCLC cell lines with mapatumumab or lexatumumab in combination with bortezomib.

Materials and Methods

Cell Culture and Reagents

NSCLC cell lines A549, H441, H460, H596, H2122, and H2347 were obtained from the American Type Culture Collection. All cell lines were cultured in medium recommended by the supplier. The proteasome inhibitor bortezomib (Velcade) was obtained from Millennium Pharmaceuticals. The agonistic TRAIL-R1 and TRAIL-R2 human IgG1 mAbs, mapatumumab and lexatumumab, respectively, were generated and purified as described previously (13). An isotype-matched control mAb (ICmAb) of irrelevant specificity was used for comparison.

Viability Assay

Cell viability was determined using the Cell Titer-Glo assay (Promega). Cells were plated in opaque white 96-well white polystyrene plates (Corning) at a density of 1×10^4 per well in 100 μL culture medium and incubated overnight at 37°C. Culture medium was aspirated and replaced with 100 μL medium containing the indicated concentrations of ICmAb, mapatumumab, lexatumumab, or bortezomib alone or in combination. Cells were then incubated for various periods and viability was measured according to the manufacturer's protocol. Briefly, 100 μL assay reagent was added directly to cells in culture medium and mixed for 10 min at room temperature. Luminescent signal was read using a Wallac Envision 2100 plate reader (Perkin-Elmer). All treatments were done in triplicate. The average and SE were determined and plotted using Prism software (GraphPad Software). Data are relative to the viability of cells treated with medium only. Each viability experiment was done at least twice. For studies using the general caspase inhibitor z-VAD-FMK (BD Biosciences), cells were treated with test compounds as described above in the presence of 10 μmol/L z-VAD-FMK.

Flow Cytometry

Cells were treated with 100 nmol/L bortezomib for various periods before analysis of TRAIL-R1 and TRAIL-R2 expression. Cells were detached from culture flasks using Enzyme-Free Cell Dissociation Buffer (Invitrogen), washed in PBS, and resuspended in FACS buffer (PBS with 0.1% bovine serum albumin). Cell surface expression of TRAIL receptors was determined using commercial mouse anti-TRAIL-R1 or anti-TRAIL-R2 antibodies (eBiosciences). Commercial mouse IgG (eBiosciences) was used as a negative control. Cells were incubated with antibodies for 20 min at room temperature, washed, resuspended in FACS buffer containing 0.5 μg/mL propidium iodide, and analyzed on a FACScan machine (Becton Dickinson).

Western Blot Analysis

Cells (1×10^6) were plated overnight on 100 mm cell culture dishes and then treated with 10 μg/mL lexatumumab or 100 nmol/L bortezomib alone or in combination for 3, 8, or 24 h. Cells were then washed and scraped in PBS, pelleted by centrifugation, and lysed in ice-cold radioimmunoprecipitation assay buffer (25 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitors (Roche Applied Science) and 0.2 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were determined using a modified Bradford assay (Coomassie Plus; Pierce). Proteins were separated by Tris-glycine SDS-PAGE (10-20% Novex pre-cast gel; Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrolon; Invitrogen). The membranes were then probed with antibodies recognizing the pro-forms and cleaved forms of the apoptotic proteins caspase-8 and -10 (MBL) and caspase-2, -3, -6, -7, -9, and -10 and PARP (Cell Signaling Technology). Blots were also probed for Bik, Bim, p21, and PUMA (Cell Signaling), Bax, Bcl-2, Bid, and Mcl-1 (Santa Cruz Biotechnology), Noxa (Calbiochem), and actin (Sigma). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and enhanced chemiluminescence substrate (GE Healthcare). Bortezomib conditions resulting in altered protein levels were repeated at least once to verify the results.

DISC Immunoprecipitation

Immunoprecipitation of the TRAIL-R2-associated DISC with lexatumumab was done as described previously (25). Briefly, 1×10^7 NSCLC cells were suspended in 10 mL culture medium, incubated with 1 μg/mL lexatumumab at

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

4°C for 30 min, transferred to 37°C for 1 h, washed three times in PBS, and then lysed in immunoprecipitation buffer (150 mmol/L NaCl, 20 nmol/L Tris-HCl, 1% Triton X-100) supplemented with complete protease inhibitors (Roche). Antibodies were precipitated with protein A beads (Sigma) overnight at 4°C. Beads were washed three times in immunoprecipitation buffer supplemented with 0.5 mmol/L NaCl and samples were subjected to Western blot analysis as described above.

Results

Bortezomib Enhances Sensitivity of NSCLC Cell Lines to Mapatumumab and Lexatumumab

Several studies have explored the cytotoxic effects of recombinant human TRAIL and bortezomib on NSCLC cell lines (19, 20), but the effects of agonistic TRAIL-R1 and TRAIL-R2 antibodies alone or in combination with bortezomib on NSCLC cell lines have not been studied. Therefore, we treated a panel of NSCLC cell lines with mapatumumab or lexatumumab using a range of concentrations in the absence or presence of bortezomib. The H460 and H2122 lines showed a dose-dependent decrease in cell viability on treatment with either mapatumumab or lexatumumab alone, whereas the A549, H441, H596, and H2347 lines were refractory to treatment with these antibodies (Fig. 1). The differential sensitivity of NSCLC cell lines to TRAIL/TRAIL receptor therapy is consistent with previous reports (19, 20). Addition of 100 nmol/L bortezomib significantly induced or enhanced sensitivity of all NSCLC cell lines to mapatumumab and lexatumumab (Fig. 1). The results are particularly striking for the A549, H441, H596, and H2347 lines, which are refractory to individual treatments but quite sensitive to the combination treatments. The apparent lack of bortezomib activity is due to the relatively early time points analyzed here. Indeed, bortezomib is known to be effective against NSCLC cell lines as a single agent but decreases in viability generally take >24 h to develop (21–23). Thus, the combination of mapatumumab or lexatumumab with bortezomib rapidly generates an additive (H460 and H2122) to synergistic (A549, H596, H441, H2347) increase in cytotoxicity that kills ~100% of NSCLC cells treated with the highest concentration of antibody.

To develop a more detailed time course of sensitivity, cells were incubated with mapatumumab or lexatumumab at 10 μg/mL in the absence or presence of 100 nmol/L bortezomib for 8 to 72 h. Consistent with the findings above, the H2122 and H460 lines were sensitive to mapatumumab and lexatumumab alone, whereas the A549 and H441 lines were refractory (Fig. 2). Interestingly, the H460 cells contain a resistant subpopulation that remain refractory following 72 h of treatment and can be cultured indefinitely in the presence of 10 μg/mL antibody (data not shown). Bortezomib alone caused incremental decreases in viability over time, which were generally more apparent at the later time points (Fig. 2). As mentioned above, the delayed sensitivity of NSCLC cell lines to bortezomib is consistent with literature reports (26–28). Combining mapatumumab or lexatumumab with bortezomib resulted in significant increases in cytotoxicity that occurred at earlier time points relative to the effects generated by individual agents (Fig. 2). The effects of combination treatment are especially striking for the A549 and H441 lines. No single agent reduced the viability of A549 cells by >10% at 16 h, but the combination reduced viability by ~80% at the same time point. Similarly, single agents decreased the viability of H441 cells by <15% at 48 h, but the combination decreased viability by ≥80% at 48 h. Taken together, these data show the combination of mapatumumab or lexatumumab with bortezomib increases the potency of treatment and decreases the time required for cytotoxicity to occur.

The studies described above were done using a fixed bortezomib concentration of 100 nmol/L. To determine the minimum concentration of bortezomib required to sensitize cells to mapatumumab or lexatumumab, H460 and A549 cells were treated with the antibodies in combination with 50, 25, or 10 nmol/L bortezomib. Bortezomib concentrations as low as 25 nmol/L were able to sensitize cells to both mapatumumab and lexatumumab, although this concentration was slightly less effective than 50 nmol/L (Supplementary Fig. S1).[4] These data show that the threshold for bortezomib sensitization of NSCLC cell lines to mapatumumab or lexatumumab is between 10 and 25 nmol/L. This concentration of bortezomib is within the threshold range required to inhibit proteasome activity in NSCLC cell lysates (27, 28), suggesting that the sensitizing effect of bortezomib is due to proteasome inhibition and not off-target effects.

Cytotoxicity Induced by Mapatumumab or Lexatumumab Combined with Bortezomib Is Caspase Dependent

TRAIL-R1 and TRAIL-R2 agonists induce receptor multimerization leading to formation of the DISC. This complex initiates the extrinsic apoptotic pathway and subsequent activation of the caspase cascade. To determine whether caspase activation is involved in bortezomib-enhanced sensitivity to mapatumumab and lexatumumab, H460 and A549 cells were treated with mapatumumab or lexatumumab ± bortezomib in the presence of the general caspase inhibitor z-VAD-FMK. H460 cells, which are partially sensitive to mapatumumab and lexatumumab, were completely resistant to the antibodies in the presence of z-VAD-FMK (Fig. 3A). This finding is consistent with the known mechanism of action for these antibodies (29). Similarly, z-VAD-FMK significantly inhibited the bortezomib-enhanced sensitivity of both H460 and A549 cells to mapatumumab and lexatumumab (Fig. 3A), indicating that the cytotoxicity of the combination treatment is mediated primarily through caspase activation.

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

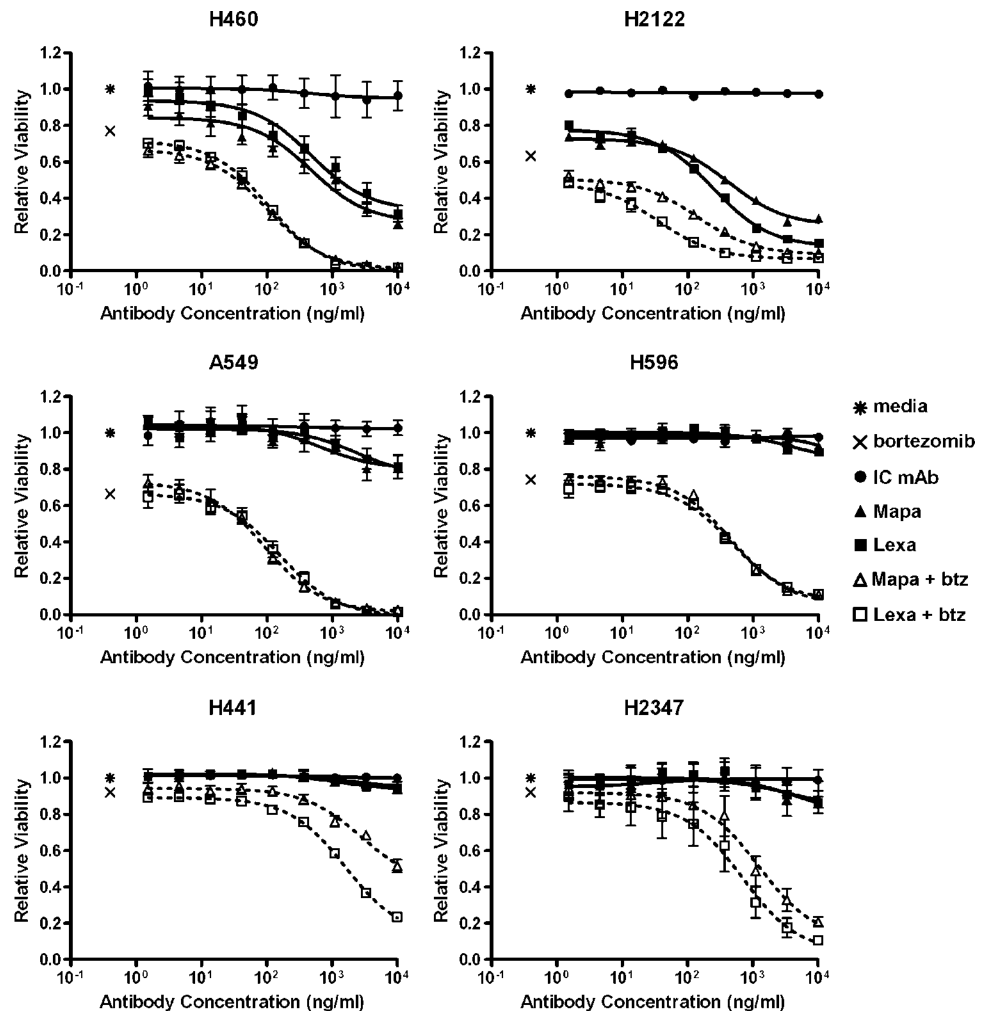

Figure 1. Bortezomib enhances sensitivity of NSCLC cell lines to mapatumumab and lexatumumab. The indicated NSCLC cell lines were treated with a serial dilution of mapatumumab, lexatumumab, or isotype-matched control mAb (*IC mAb*) starting at 10 μg/mL in the absence or presence of bortezomib at a fixed concentration of 100 nmol/L. Cells were also treated with culture medium (*asterisk*) or 100 nmol/L bortezomib alone (*cross*) for comparison. Cell viability was analyzed following 24 h of treatment for all cells lines, except H441, which was analyzed following 48 h of treatment. Data are viability relative to cells treated with medium only.

Combining Lexatumumab with Bortezomib Induces Caspase Activation

To verify caspase activation is involved in lexatumumab + bortezomib-induced cell death, H460, A549, and H441 cells were treated with lexatumumab, bortezomib, or both for various periods. Lysates were prepared and caspase activation was monitored by Western blot. In H460 cells, lexatumumab alone induced cleavage of all caspases analyzed following only 3 h of treatment (Fig. 3B). By 24 h, many of the pro-caspases began to reappear. This is likely due to continued growth of the lexatumumab-resistant subpopulation described above (see Figs. 1 and 2). Bortezomib alone did not activate caspases at 3 or 8 h (Fig. 3B). By 24 h, bortezomib treatment began to activate caspase-9 but none of the other caspases analyzed. Combining lexatumumab and bortezomib activated all caspases within 3 h of treatment (Fig. 3B). By 8 h, many pro-caspases were completely degraded, and by 24 h, most caspases were undetectable due to loss of intact cells. In A549 and H441 cells, lexatumumab stimulated low levels of caspase activation at early time points, suggesting the presence of a very small subpopulation of lexatumumab-sensitive cells. Bortezomib alone had little effect at 3 h but began to activate caspase-9 in H441 cells by 8 h and in A549 cells by 24 h (Fig. 3B). Combining lexatumumab and bortezomib activated all caspases in both lines by 3 h, although the degree of activation was less intense in the H441 cells. In A549 cells, many pro-caspases were completely degraded by 8 h and most caspases were undetectable by 24 h due to loss of intact cells. Meanwhile, caspase activation in H441 cells intensified at 8 and 24 h, but pro-caspases were still detectable as most cells remained viable (see Fig. 2). In general, caspase activation correlated well with the cell viability data described earlier (see Figs. 1 and 2).

Bortezomib Treatment Increases Surface Expression of TRAIL-R2 but not TRAIL-R1

Bortezomib has been shown recently to increase surface expression of TRAIL-R1 and TRAIL-R2 on NSCLC cell lines, reportedly leading to increased sensitivity to TRAIL (19, 20). To determine whether a bortezomib-induced increase in TRAIL-R1 and TRAIL-R2 surface expression may explain the enhanced sensitivity to mapatumumab and lexatumumab described above, we treated H460, A549,

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

and H441 with 100 nmol/L bortezomib and analyzed TRAIL-R1 and TRAIL-R2 surface expression by flow cytometry. Untreated cells expressed both TRAIL receptors. TRAIL-R2 levels increased in all three lines following bortezomib treatment, whereas TRAIL-R1 levels remained constant or decreased slightly (Table 1). These data indicate that receptor expression on untreated cells does not correlate with sensitivity to mapatumumab or lexatumumab because H460 are partially sensitive to the antibodies, whereas A549 and H441 are insensitive, yet all three lines express both receptors. Increased surface expression of TRAIL-R2 may contribute to bortezomib-enhanced

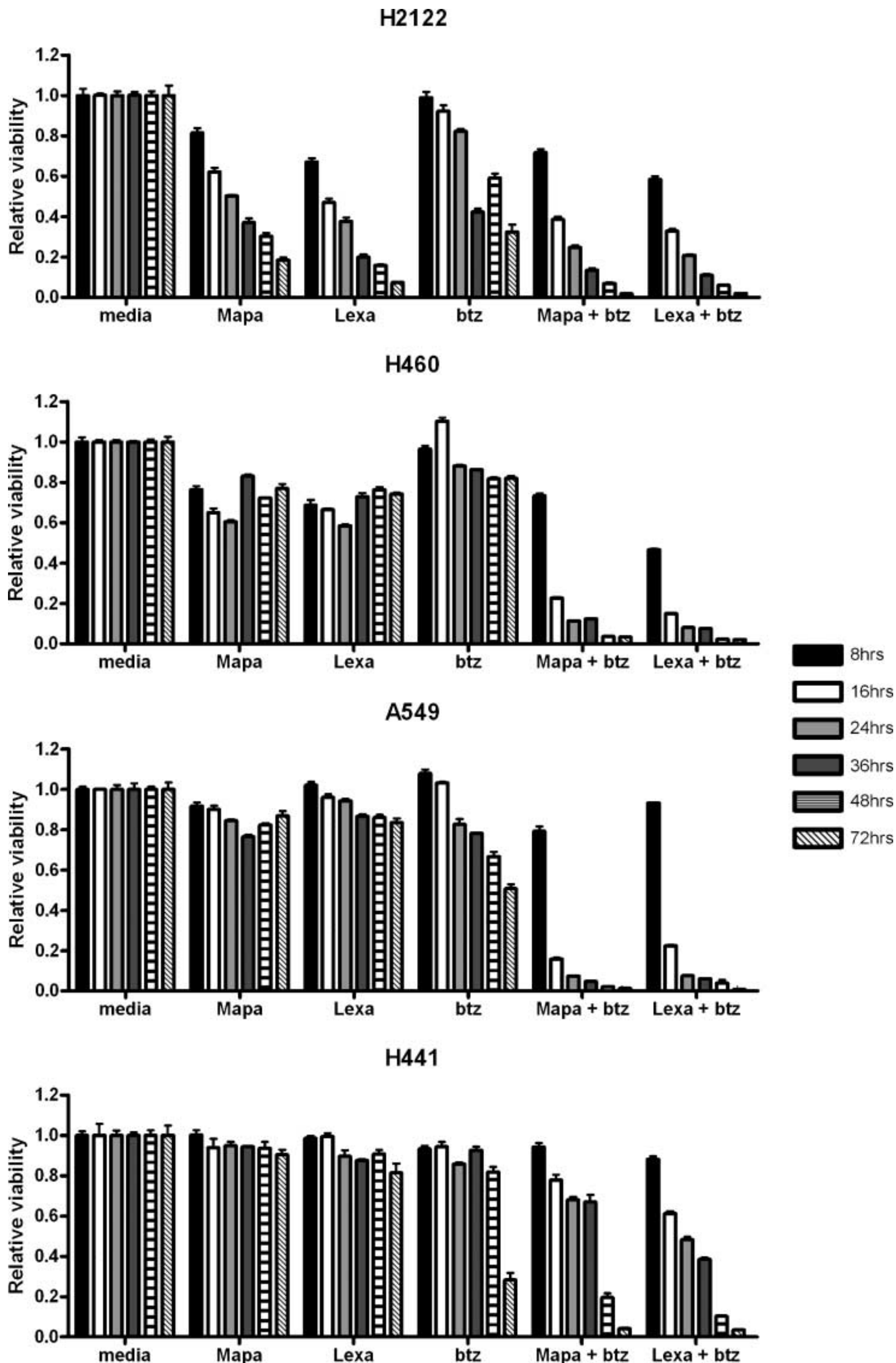

Figure 2. Combination treatment is more potent and rapid than individual treatments. The indicated NSCLC cell lines were treated with mapatumumab or lexatumumab at 10 μg/mL in the absence or presence bortezomib at 100 nmol/L. Cells were also treated with culture medium or 100 nmol/L bortezomib alone. Cell viability was analyzed following 8, 16, 24, 36, 48, and 72 h of treatment. Data are viability relative to cells treated with medium only.

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

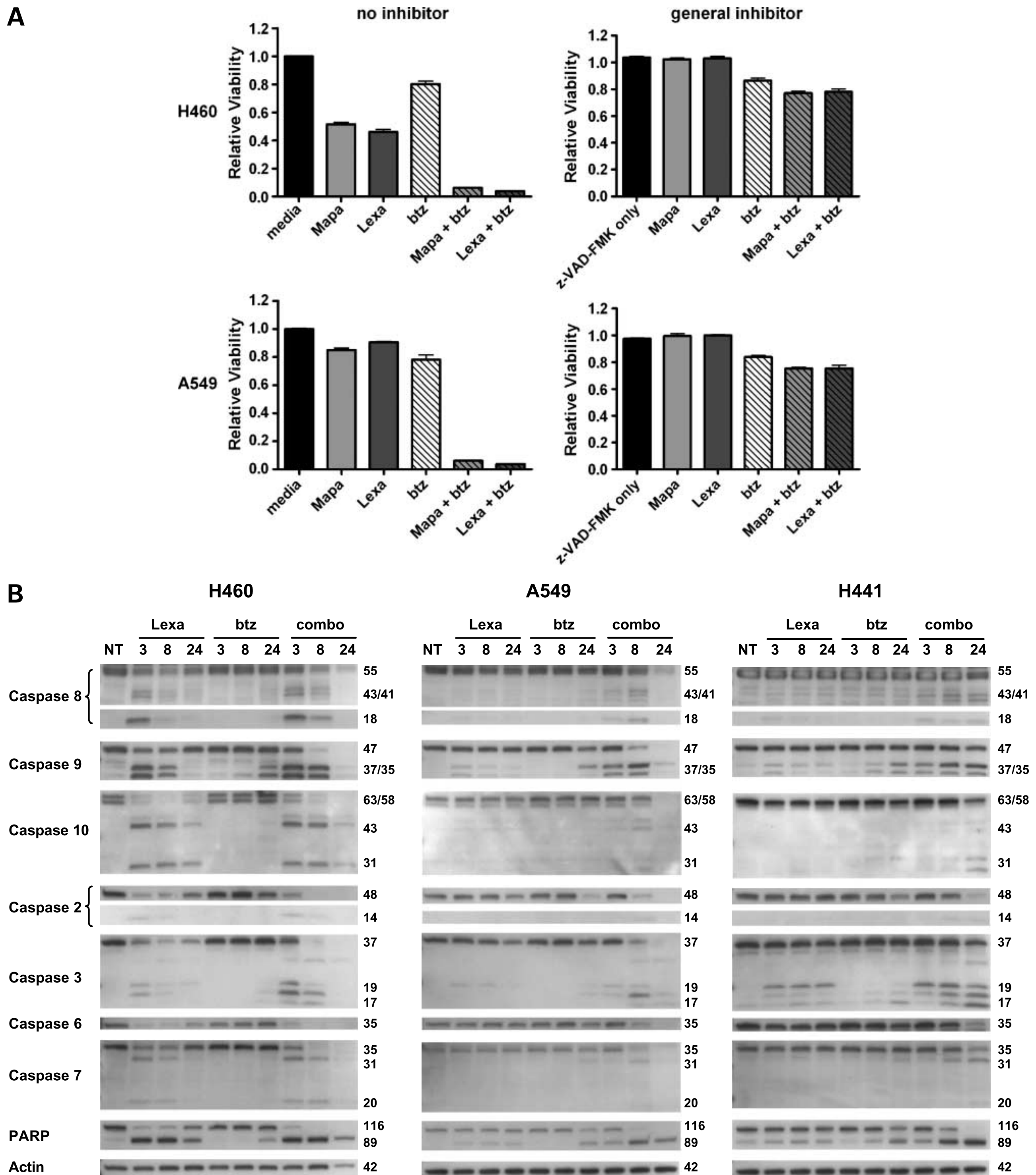

Figure 3. Combination treatment is caspase dependent and enhances caspase activation. **A,** bortezomib-enhanced sensitivity of NSCLC cell lines to mapatumumab and lexatumumab is caspase dependent. H460 and A549 cells were treated with mapatumumab or lexatumumab at 10 μg/mL in the absence or presence bortezomib at 100 nmol/L. In addition, cells were treated in the absence or presence of a general caspase inhibitor (z-VAD-FMK) at 10 μmol/L. Cell viability was analyzed following 24 h of treatment. Data are viability relative to cells treated with medium only. **B,** enhanced caspase activation following treatment with lexatumumab + bortezomib. H460, A549, and H441 cells were treated with lexatumumab at 10 μg/mL, bortezomib at 100 nmol/L, or both for 3, 8, or 24 h before collection of whole-cell lysates for Western blot analysis. Blots were probed using caspase-specific antibodies, most of which detect the pro-forms and cleaved forms of activated caspases. PARP was probed as a further measure of caspase activation and actin was probed as a loading control. *Right,* approximate molecular weights (kDa).

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

Table 1. TRAIL-R1 and TRAIL-R2 surface expression following bortezomib treatment

Cell line	Time (h)	TRAIL-R1 expression*	TRAIL-R2 expression*
H460	0	5.09	15.29
	2	4.11	17.31
	4	3.93	23.86
	6	3.17	29.27
	24	N/A	N/A
A549	0	11.16	20.57
	2	5.77	16.48
	4	6.73	33.98
	6	5.77	47.04
	24	N/A	N/A
H441	0	3.53	14.04
	2	3.90	16.32
	4	3.35	22.39
	6	3.83	23.60
	24	3.99	35.74

*TRAIL-R1 and TRAIL-R2 expression was determined by flow cytometry following treatment with 100 nmol/L bortezomib for the indicated period. Data are the difference in mean fluorescent signal of commercial mouse TRAIL-R1 or TRAIL-R2 antibody and the isotype control antibody. Results of one experiment that was repeated with similar results.

sensitivity to lexatumumab, but this is not the case for mapatumumab because bortezomib appears to have no effect on TRAIL-R1 surface expression in the cell lines tested.

DISC Formation Occurs in Both Lexatumumab-Sensitive and Lexatumumab-Resistant NSCLC Cell Lines

To determine whether aberrant DISC formation could explain the lack of sensitivity to mapatumumab or lexatumumab in resistant NSCLC cell lines, DISC formation was analyzed. The NSCLC cell lines H2122, H460, A549, and H441 were treated with lexatumumab and lysed, and DISC formation was examined by immunoprecipitation and Western blot. FADD and activated caspase-8 were found to be associated with TRAIL-R2 in all four lines (Fig. 4A). Interestingly, only the mature 40 kDa form of TRAIL-R2 was recovered from the H441 cells, whereas both the premature 48 kDa and mature forms were recovered from the other three lines. In addition, the 18 kDa active form of caspase-8 was not detectable in the DISC of A549 or H441 cells. Importantly, this was not due to decreased cellular levels of caspase-8 in A549 or H441 cells (Fig. 4B). These data suggest that incomplete activation of caspase-8 may contribute to resistance in these lexatumumab insensitive lines (see Figs. 1 and 2).

Bortezomib Treatment Causes Accumulation of Several Important Regulators of Apoptosis

Activation of the caspase cascade is generally considered an irreversible commitment to complete the apoptotic process. To prevent unwanted apoptotic cell death, caspase activation is highly regulated by numerous proapoptotic and antiapoptotic factors. Loss of antiapoptotic factors or gain of proapoptotic factors can induce apoptosis or render cells more sensitive to proapoptotic signals (30, 31). In this regard, inhibition of protein degradation by bortezomib is known to cause the accumulation of several proapoptotic factors (18). In some cases, bortezomib has also been reported to cause a loss of key antiapoptotic factors, such as Bcl-2 (32). Therefore, we examined a panel of proapoptotic and antiapoptotic factors in H460, A549, and H441 cells at various time points following treatment with lexatumumab, bortezomib, or both in an attempt to identify factors that may be responsible for bortezomib-induced sensitivity to lexatumumab. Proapoptotic Bcl-2 family members Bid, Bik, Bim, Bax, PUMA, and Noxa as well as the antiapoptotic protein Mcl-1 and cell cycle control protein p21 have all been reported to increase following bortezomib treatment (19, 26–28). Bcl-2, Bax, and Bik levels did not change significantly in any cell line (Fig. 5), although Bik expression was lost in H460 and A549 cells following 24 h of combination treatment due to loss of intact cells (see Fig. 2). Bid and PUMA levels dropped following treatment with lexatumumab in H460 cells or lexatumumab + bortezomib in all three lines (Fig. 5). The decreases

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

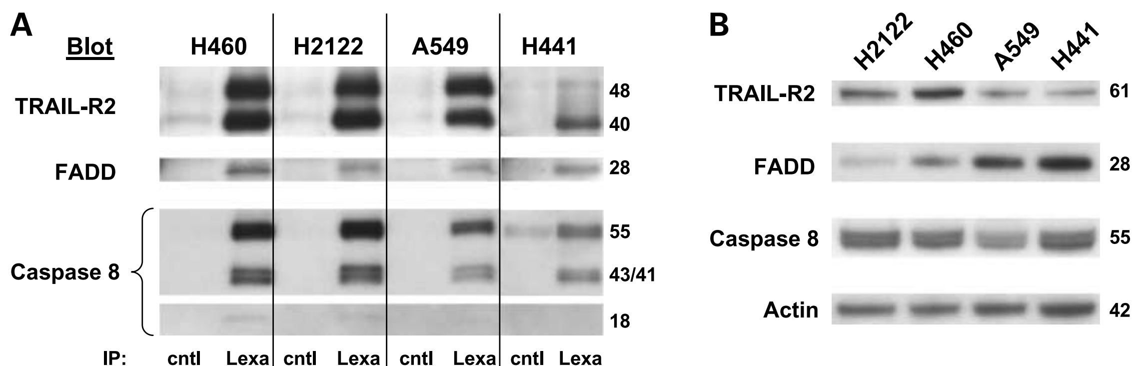

Figure 4. DISC formation following treatment with lexatumumab. **A,** indicated cell lines were incubated with lexatumumab or ICmAb to analyze DISC formation by immunoprecipitation as described in Materials and Methods. Recruitment of FADD and caspase-8 to TRAIL-R2 was assessed by Western blot analysis. **B,** lysates from untreated cells were probed for protein expression as described in Materials and Methods. Actin was probed as a loading control. *Right,* approximate molecular weights (kDa).

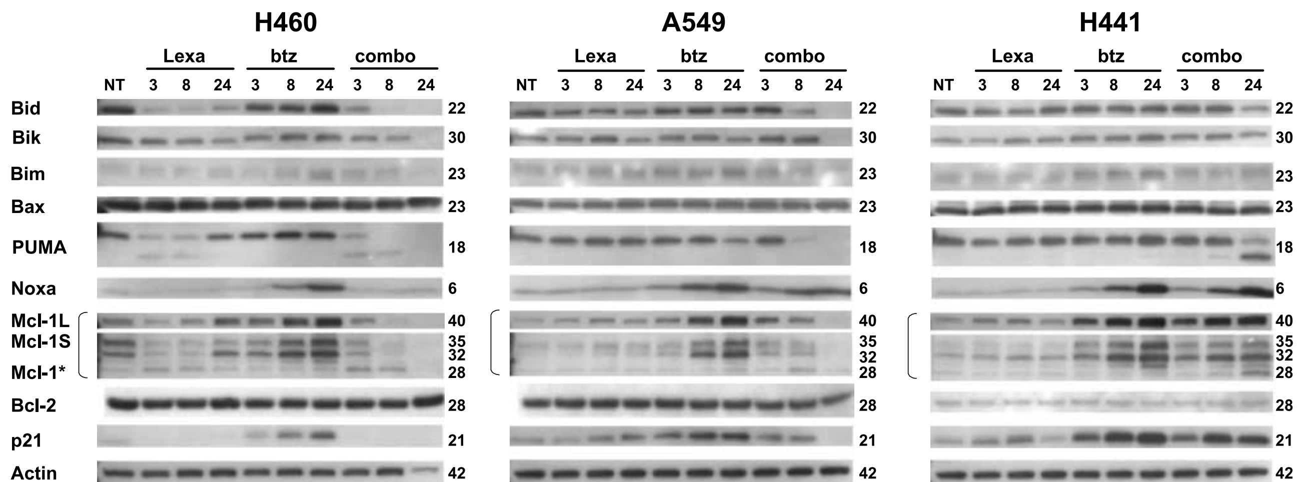

Figure 5. Bortezomib alters expression levels of several important regulators of apoptosis. H460, A549, and H441 cells were treated with lexatumumab at 10 μg/mL, bortezomib at 100 nmol/L, or both for 3, 8, or 24 h before collection of whole-cell lysates for Western blot analysis. Blots were probed using antibodies specific for regulators of apoptosis known to be affected by bortezomib treatment. Actin was probed as a loading control. *Right,* approximate molecular weights (kDa). *Asterisk,* cleaved Mcl-1.

in protein correlate well with sensitivity to treatment and caspase activation (see Figs. 1, 2, and 5). Bortezomib alone slightly increased Bid and PUMA levels in H460 cells but not A549 or H441 cells and dramatically increased Mcl-1L and Mcl-1S levels in all three lines (Fig. 5). Similarly, Noxa and p21 levels were nearly undetectable or quite low in untreated cells but increased dramatically in all three lines on bortezomib treatment. In H460 and A549 cells, the Mcl-1, Noxa, and p21 increases are less pronounced in the combination groups likely due to the loss of intact cells by 24 h (see Fig. 2). Taken together, these data show that bortezomib treatment causes the accumulation of several regulators of apoptosis, which could potentially be responsible for enhancing the sensitivity of these NSCLC cell lines to lexatumumab.

Discussion

Death receptors TRAIL-R1 and TRAIL-R2 are expressed on a wide variety of tumor cell types (1). Agonistic TRAIL-R1 and TRAIL-R2 antibodies mapatumumab and lexatumumab, respectively, can induce apoptosis in many cancer cell types; however, some cancer cell lines are refractory to these agents (33). Importantly, tumor cells that are partially or completely resistant to mapatumumab or lexatumumab can be sensitized by treatment with various chemotherapeutic drugs (13–16). The objective of the current study was to determine whether the proteasome inhibitor bortezomib could enhance the cytotoxicity of mapatumumab or lexatumumab in human NSCLC cell lines. Several recent studies support examination of this drug combination in NSCLC. Specifically, bortezomib is known to enhance the sensitivity of NSCLC cell lines to TRAIL (19, 20) and to enhance the sensitivity of other tumor types to mapatumumab and lexatumumab (22–24). In addition, a recent report indicates that bortezomib in combination with the agonistic mouse TRAIL-R2-specific antibody MD5-1 can inhibit lung metastases and prolong survival of tumor-bearing mice (34). In this study, two of six NSCLC cell lines tested were partially sensitive to treatment with mapatumumab or lexatumumab alone, whereas the remainder of the tested lines was resistant to antibody treatment. However, combined treatment with bortezomib markedly increased the sensitivity of all NSCLC cell lines to mapatumumab and lexatumumab. The increased cytotoxicity was not due to bortezomib alone, which had only moderate activity as a single agent at the time points tested. Thus, in most cases, the combination rapidly caused a more than additive or synergistic increase in cytotoxicity.

The increased sensitivity of NSCLC cells to combination treatment appears to be due primarily to enhanced activation of caspases. This was shown by treating the H460 and A549 lines in the presence of the general caspase inhibitor z-VAD-FMK, which significantly inhibited the cytotoxicity of combination treatment. Western blot analysis indicates early activation of all three initiator caspases as well as executioner caspase-3, -6, and -7 following combination treatment. PARP cleavage was also enhanced by combination treatment. Importantly, caspase activation and PARP cleavage in the H460, A549, and H441 lines correlate well with sensitivity to treatment. For example, the A549 line is resistant to lexatumumab and bortezomib treatment but is sensitive to the combination. Accordingly, significant caspase activation and PARP cleavage occur only following combination treatment. Taken together, the data indicate that the effectiveness of combination therapy results from enhanced activation of the caspase cascade.

In an effort to determine how combination treatment enhances activation of the caspase cascade, we first examined surface expression of TRAIL-R1 and TRAIL-R2 before and after bortezomib treatment. Importantly, bortezomib and other chemotherapeutic agents are known to increase

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

surface expression of TRAIL-R2 on a variety of tumor cells types, including NSCLC cell lines (19, 20). However, whether the level of TRAIL receptor surface expression correlates with sensitivity to TRAIL or agonistic TRAIL receptor monoclonal antibodies is unclear (1). In the current study, untreated H460, A549, and H441 lines all expressed TRAIL-R1 and TRAIL-R2 on their surface, yet only the H460 cells were sensitive to mapatumumab or lexatumumab as single agents, indicating that receptor expression alone does not dictate sensitivity to these antibodies. Following bortezomib treatment, surface expression of TRAIL-R2 increased on all three lines, whereas expression of TRAIL-R1 remained steady or decreased slightly. If each individual cell line requires a threshold amount of TRAIL-R2 surface expression to respond to lexatumumab, and the levels on the resistant cells are below this threshold, a bortezomib-mediated increase in TRAIL-R2 levels could be responsible for increased sensitivity to lexatumumab. However, no bortezomib-mediated change in TRAIL-R1 surface expression was observed; therefore, increased TRAIL-R1 receptor expression is unable to explain the increase in sensitivity to mapatumumab, unless TRAIL-R1-mediated sensitivity to this antibody is tied to TRAIL-R2 surface expression levels. To date, there is no convincing evidence to suggest that TRAIL-R1/TRAIL-R2 heterotrimers form under physiologic conditions (1), making it unlikely that TRAIL-R2 levels control sensitivity to TRAIL-R1 agonists.

Inhibition of protein degradation by bortezomib is known to cause the accumulation of several apoptotic regulators (35). Of the regulators analyzed in this study, bortezomib treatment caused marked accumulation of Noxa, Mcl-1, and p21. Noxa is a proapoptotic BH3-only member of the Bcl-2 family of apoptotic regulators, which is dramatically up-regulated in several tumor cell types following bortezomib treatment (19, 28). This occurs due to a concomitant increase in c-Myc levels, which increases transcription of the Noxa gene (36). Several studies indicate that preventing bortezomib-mediated increase in Noxa levels inhibits sensitivity to bortezomib, suggesting that Noxa up-regulation is at least partially responsible for bortezomib-induced cytotoxicity (37, 38). Similarly, bortezomib-mediated Noxa up-regulation may also partly be responsible for sensitizing NSCLC cell lines to mapatumumab and lexatumumab.

Mcl-1 is an antiapoptotic member of the Bcl-2 family and was originally identified in myeloid cells as an important survival factor (39). Full-length Mcl-1 (Mcl-1L) contains BH1, BH2, and BH3 as well as a PEST domain that mediates rapid turnover by the ubiquitin proteasome pathway (40, 41). Accordingly, we show here that Mcl-1L accumulates in NSCLC cells following bortezomib treatment, which is consistent with published reports (27, 28). Recently, shorter Mcl-1 splice variants (Mcl-1S) have been identified (42, 43). These variants retain the PEST and BH3 domains but lack the COOH-terminal domains, generating proapoptotic BH3-only proteins. Mcl-1L can also be cleaved by activated caspase-3 within the PEST domain, generating a BH3-only fragment with proapoptotic function (44, 45). Importantly, mapatumumab and lexatumumab are known to induce caspase-dependent cleavage of Mcl-1L in myeloma cells (29). Similarly, we show here that Mcl-1L is cleaved in the lexatumumab-sensitive H460 cells and in the lexatumumab-resistant A549 and H441 cells when used in combination with bortezomib. Furthermore, we show that bortezomib treatment causes accumulation of Mcl-1S splice variants. Thus, the combined effect of Mcl-1S accumulation and Mcl-1L cleavage may also contribute to the effectiveness of the combination therapy.

The cell cycle plays an important role in determining sensitivity to chemotherapeutic agents. There are numerous intracellular regulators of the cell cycle, some of which are known to accumulate following bortezomib treatment. Increased p21 levels following bortezomib treatment contribute to cellular growth arrest (46) and can enhance the sensitivity of cancer cells to TRAIL (47, 48). Here, we show that p21 accumulates in NSCLC cells following bortezomib treatment. This finding is consistent with previous studies using NSCLC cell lines (26) and may be at least partially responsible for enhanced sensitivity of the NSCLC cell lines to the combination of bortezomib + mapatumumab or lexatumumab.

Bortezomib has been approved recently as a first-line therapy for multiple myeloma and mantle cell lymphoma and has shown promising antitumor activity in combination with other cytotoxic drugs in patients with NSCLC (17). A recent phase 2 trial of mapatumumab in NSCLC patients indicates that the antibody is safe and well tolerated, although no objective single-agent activity was shown (12). Preclinical studies indicate that the maximal activity of mapatumumab occurs when used in combination with chemotherapeutic agents that sensitize tumor cells to proapoptotic stimuli (13). Bortezomib treatment causes several cellular changes that may sensitize tumor cells to TRAIL receptor agonists (49), and we show here that mapatumumab therapy may be significantly enhanced by combination with bortezomib. Bortezomib concentrations of 3.4 μmol/L have been recorded in the plasma of patients following a single injection of 1.3 mg/m^2 (50), which is >100-fold higher than minimum concentration required to sensitize NSCLC cell lines to mapatumumab and lexatumumab. The sensitizing activity of 25 nmol/L bortezomib in the NSCLC cell lines is consistent with the minimum bortezomib concentration required to sensitize liver, colon, pancreatic, renal, and breast cancer cells to TRAIL (21, 34). Moreover, our findings suggest that mapatumumab and lexatumumab may substantially lower the effective dose of bortezomib required to treat cancer patients, likely reducing the risk of side effects. Mapatumumab is currently being tested in combination with bortezomib in multiple myeloma patients and with gemcitabine and cisplatin in NSCLC patients in separate phase 2 trials. Based on the findings presented here, future evaluation of mapatumumab or lexatumumab in combination with bortezomib in NSCLC patients is also warranted.

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

Disclosure of Potential Conflicts of Interest

All authors are employees of, supported by, and declare ownership interest in Human Genome Sciences.

Acknowledgments

We thank Ipsita Mukherjee for outstanding technical assistance.

References

1. Humphreys RC, Halpern W. TRAIL receptors: targets for cancer therapy. Adv Exp Med Biol 2008;615:127 – 58.

2. Ashkenazi A, Herbst RS. To kill a tumor cell: the potential of proapoptotic receptor agonists. J Clin Invest 2008;118:1979 – 90.

3. Duiker EW, Mom CH, de Jong S, et al. The clinical trail of TRAIL. Eur J Cancer 2006;42:2233 – 40.

4. Maddipatla S, Hernandez-Ilizaliturri FJ, Knight J, Czuczman MS. Augmented antitumor activity against B-cell lymphoma by a combination of monoclonal antibodies targeting TRAIL-R1 and CD20. Clin Cancer Res 2007;13:4556 – 64.

5. Sprick MR, Weigand MA, Rieser E, et al. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 2000;12:599 – 609.

6. Ozoren N, El-Deiry WS. Cell surface death receptor signaling in normal and cancer cells. Semin Cancer Biol 2003;13:135 – 47.

7. American Cancer Society. Cancer facts & figures 2007. Atlanta: American Cancer Society; 2007.

8. De Petris L, Crino L, Scagliotti GV, et al. Treatment of advanced non-small cell lung cancer. Ann Oncol 2006;17 Suppl 2:ii36 – 41.

9. Socinski MA. Cytotoxic chemotherapy in advanced non-small cell lung cancer: a review of standard treatment paradigms. Clin Cancer Res 2004; 10:4210 – 4s.

10. Spierings DC, de Vries EG, Timens W, Groen HJ, Boezen HM, de Jong S. Expression of TRAIL and TRAIL death receptors in stage III non-small cell lung cancer tumors. Clin Cancer Res 2003;9:3397 – 405.

11. Cooper WA, Kohonen-Corish MR, Zhuang L, et al. Role and prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand death receptor DR5 in nonsmall-cell lung cancer and precursor lesions. Cancer 2008;113:135 – 42.

12. Greco FA, Bonomi P, Crawford J, et al. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008;61:82 – 90.

13. Pukac L, Kanakaraj P, Humphreys R, et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types *in vitro* and *in vivo*. Br J Cancer 2005;92:1430 – 41.

14. Rowinsky EK. Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. J Clin Oncol 2005;23: 9394 – 407.

15. Zhang X, Li W, Olumi AF. Low-dose 12-*O*-tetradecanoylphorbol-13-acetate enhances tumor necrosis factor related apoptosis-inducing ligand induced apoptosis in prostate cancer cells. Clin Cancer Res 2007; 13:7181 – 90.

16. Belyanskaya LL, Marti TM, Hopkins-Donaldson S, Kurtz S, Felley-Bosco E, Stahel RA. Human agonistic TRAIL receptor antibodies mapatumumab and lexatumumab induce apoptosis in malignant mesothelioma and act synergistically with cisplatin. Mol Cancer 2007;6:66.

17. Davies AM, Lara PN, Jr., Mack PC, Gandara DR. Incorporating bortezomib into the treatment of lung cancer. Clin Cancer Res 2007;13: s4647 – 51.

18. Fennell DA, Chacko A, Mutti L. BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. Oncogene 2008;27:1189 – 97.

19. Voortman J, Resende TP, Abou El Hassan MA, Giaccone G, Kruyt FA. TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor-mediated apoptosis by proteasome inhibitor bortezomib. Mol Cancer Ther 2007;6:2103 – 12.

20. Liu X, Yue P, Chen S, et al. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. Cancer Res 2007;67:4981 – 8.

21. Koschny R, Ganten TM, Sykora J, et al. TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. Hepatology 2007;45:649 – 58.

22. Smith MR, Jin F, Joshi I. Bortezomib sensitizes non-Hodgkin's lymphoma cells to apoptosis induced by antibodies to tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1 and TRAIL-R2. Clin Cancer Res 2007;13:5528 – 34s.

23. Georgakis GV, Li Y, Humphreys R, et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br J Haematol 2005;130:501 – 10.

24. Saulle E, Petronelli A, Pasquini L, et al. Proteasome inhibitors sensitize ovarian cancer cells to TRAIL induced apoptosis. Apoptosis 2007;12: 635 – 55.

25. Bin L, Thorburn J, Thomas LR, Clark PE, Humphreys R, Thorburn A. Tumor-derived mutations in the TRAIL receptor DR5 inhibit TRAIL signaling through the DR4 receptor by competing for ligand binding. J Biol Chem 2007;282:28189 – 94.

26. Ling YH, Liebes L, Jiang JD, et al. Mechanisms of proteasome inhibitor PS-341-induced G(2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. Clin Cancer Res 2003;9:1145 – 54.

27. Voortman J, Checinska A, Giaccone G. The proteasomal and apoptotic phenotype determine bortezomib sensitivity of non-small cell lung cancer cells. Mol Cancer 2007;6:73.

28. Voortman J, Checinska A, Giaccone G, Rodriguez JA, Kruyt FA. Bortezomib, but not cisplatin, induces mitochondria-dependent apoptosis accompanied by up-regulation of Noxa in the non-small cell lung cancer cell line NCI-H460. Mol Cancer Ther 2007;6:1046 – 53.

29. Menoret E, Gomez-Bougie P, Geffroy-Luseau A, et al. Mcl-1L cleavage is involved in TRAIL-R1- and TRAIL-R2-mediated apoptosis induced by HGS-ETR1 and HGS-ETR2 human mAbs in myeloma cells. Blood 2006;108:1346 – 52.

30. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647 – 56.

31. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007;26:1324 – 37.

32. Mortenson MM, Schlieman MG, Virudachalam S, et al. Reduction in BCL-2 levels by 26S proteasome inhibition with bortezomib is associated with induction of apoptosis in small cell lung cancer. Lung Cancer 2005; 49:163 – 70.

33. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005;12:228 – 37.

34. Shanker A, Brooks AD, Tristan CA, et al. Treating metastatic solid tumors with bortezomib and a tumor necrosis factor-related apoptosis-inducing ligand receptor agonist antibody. J Natl Cancer Inst 2008;100: 649 – 62.

35. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004;4:349 – 60.

36. Nikiforov MA, Riblett M, Tang WH, et al. Tumor cell-selective regulation of NOXA by c-MYC in response to proteasome inhibition. Proc Natl Acad Sci U S A 2007;104:19488 – 93.

37. Qin JZ, Ziffra J, Stennett L, et al. Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. Cancer Res 2005;65: 6282 – 93.

38. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, et al. Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. Cancer Res 2007;67:5418 – 24.

39. Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A 1993;90:3516 – 20.

40. Nijhawan D, Fang M, Traer E, et al. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 2003;17:1475 – 86.

41. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005;121:1085 – 95.

42. Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MK. Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. J Biol Chem 2000;275:22136 – 46.

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024

43. Marriott HM, Bingle CD, Read RC, et al. Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. J Clin Invest 2005;115:359 – 68.

44. Michels J, O'Neill JW, Dallman CL, et al. Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. Oncogene 2004;23:4818 – 27.

45. Weng C, Li Y, Xu D, Shi Y, Tang H. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. J Biol Chem 2005; 280:10491 – 500.

46. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999; 59:2615 – 22.

47. Lashinger LM, Zhu K, Williams SA, Shrader M, Dinney CP, McConkey DJ. Bortezomib abolishes tumor necrosis factor-related apoptosis-inducing ligand resistance via a p21-dependent mechanism in human bladder and prostate cancer cells. Cancer Res 2005;65:4902 – 8.

48. Conticello C, Adamo L, Giuffrida R, et al. Proteasome inhibitors synergize with tumor necrosis factor-related apoptosis-induced ligand to induce anaplastic thyroid carcinoma cell death. J Clin Endocrinol Metab 2007;92:1938 – 42.

49. Sayers TJ, Murphy WJ. Combining proteasome inhibition with TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) for cancer therapy. Cancer Immunol Immunother 2006;55:76 – 84.

50. Stanford BL, Zondor SD. Bortezomib treatment for multiple myeloma. Ann Pharmacother 2003;37:1825 – 30.

Downloaded from http://aacrjournals.org/mct/article-pdf/8/2/292/1882624/292.pdf by guest on 23 April 2024