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Inhibition of Yin Yang 1-Dependent Repressor Activity of DR5 Transcription and Expression by the Novel Proteasome Inhibitor NPI-0052 Contributes to its TRAIL-Enhanced Apoptosis in Cancer Cells

Stavroula Baritaki,§ Eriko Suzuki,‡ Kazuo Umezawa,‡ Demetrios A. Spandidos,§ James Berenson,¶ Tracy R. Daniels,† Manuel L. Penichet,*,† Ali R. Jazirehi,* Michael Palladino,¶ and Benjamin Bonavida**

TRAIL promotes apoptotic tumor cell death; however, TRAIL-resistant tumors need to be sensitized to reverse resistance. Proteasome inhibitors potentiate TRAIL apoptosis in vitro and in vivo and correlate with up-regulation of death receptor 5 (DR5) via an unknown mechanism. We hypothesized that the proteasome inhibitor NPI-0052 inhibits the transcription repressor Yin Yang 1 (YY1) which regulates TRAIL resistance and negatively regulates DR5 transcription. Treatment of PC-3 and Ramos cells with NPI-0052 (≤2.5 nM) and TRAIL sensitizes the tumor cells to TRAIL-induced apoptosis. By comparison to bortezomib, a 400-fold less concentration of NPI-0052 was used. NPI-0052 up-regulated DR5 reporter activity in both surface and total DR5 protein expression. NPI-0052-induced inhibition of NF-κB activity was involved in TRAIL sensitization as corroborated by the use of the NF-κB inhibitor dehydroxymethylepoxyquinocin. NPI-0052 inhibited YY1 promoter activity as well as both YY1 mRNA and protein expression. The direct role of NPI-0052-induced inhibition of YY1 and up-regulation of DR5 in the regulation of TRAIL sensitivity was demonstrated by the use of YY1 small interfering RNA. The N1-0.52-induced sensitization to TRAIL involved activation of the intrinsic apoptotic pathway and dysregulation of genes that regulate apoptosis. The NPI-0052 concentrations used for TRAIL sensitization were not toxic to human hematopoietic stem cells. The present findings demonstrate, for the first time, the potential mechanism by which a proteasome inhibitor, like NPI-0052, inhibits the transcription repressor YY1 involved in TRAIL resistance and DR5 regulation. The findings also suggest the therapeutic application of subtoxic NPI-0052 concentrations in combination with TR/DEP agonists DR4/DR5 mAbs in the treatment of TRAIL-resistant tumors.

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Conventional treatment for the majority of cancers consists of surgery, chemotherapy, radiation, hormonal therapy, and immunotherapy. However, many patients experience recurrences and relapses and develop tumor cross-resistance to the above cytotoxic and apoptotic therapies, and tumor cells often develop mechanisms to evade apoptosis-inducing stimuli. For instance, tumor cells exhibit constitutively hyperactivated cell survival pathways that regulate cell proliferation and several antiapoptotic gene products. The NF-κB signaling pathway regulates cell survival and is activated in many cancers. It regulates the transcription of many apoptotic gene products including an X-linked inhibitor of apoptosis (XIAP), inhibitors of apoptosis proteins (IAPs), and Bcl-2 family members (1). Inhibition of the NF-κB pathway or inhibition of the above antiapoptotic gene products can overcome tumor cell resistance to chemotherapy and immunotherapy and, thus, proteasome inhibitors have been considered as anticancer therapeutic agents.

The 26S proteasome is a multifunctional proteolytic complex that plays critical roles in cell cycle regulation and apoptosis by mediating the degradation of ubiquitinylated target proteins that include p21, p53, members of the Bcl-2 family, and the inhibitor of apoptosis protein (IAP). Inhibition of the NF-κB pathway or inhibition of the above antiapoptotic gene products can overcome tumor cell resistance to chemotherapy and radiation. Bortezomib (PS-341, Velcade; Millennium Pharmaceuticals), a synthetic reversible peptide boronate inhibitor of the proteasome chymotrypsin-like (CT-L) and caspase-like proteolytic activities, was the first proteasome inhibitor evaluated in clinical trials for cancer treatment and the only such agent that has been approved by the Food and Drug Administration for clinical use in multiple myeloma (MM) with objective response

Abbreviations used in this paper: XIAP, X-linked inhibitor of apoptosis; IAP, inhibitor of apoptosis protein; MM, multiple myeloma; CT-L, chymotrypsin-like proteolytic activity; DR, death receptor; CFU-E, CFU-erythroid; BFU-E, burst-forming unit erythroid; CFU-GEMM, CFU granulocyte/erythrocyte/megakaryocyte/megakaryocyte multilineage colony; siRNA, small interfering RNA; 2MAM-A3, 2-methoxyaminoacrylinic acid; DR1, Yin Yang 1; DHMEQ, dehydroxymethylepoxyquinocin; DiOC6, 3,3'-dihexylocarbocyanine; qRT-PCR, quantitative RT-PCR.

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S.B. and E.S. contributed equally to this work.

Address correspondence and reprint requests to Dr. Benjamin Bonavida, Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095; E-mail address: bbnavida@mednet.ucla.edu

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rates up to 35% (2, 5). This was the result, in part, of bortezomib-mediated inhibition of NF-κB and expression of genes involved in cancer cell survival such as Bcl-2 family members (2).

NPI-0052 (salinosporamide A), is a novel nonpeptide, marine-derived proteasome inhibitor shown to display irreversible inhibition of all three enzymatic activities (CT-L, trypsin-like, and caspase-like) of the 20S proteasome core (6, 7). NPI-0052 targets CT-L and trypsin-like proteolytic activity at lower concentrations than bortezomib; however, higher concentrations are required for inhibition of C-L which is predominantly affected by bortezomib (8). Recent findings demonstrate that NPI-0052 is a potent, orally active proteasome inhibitor with unique pharmacogenic properties that can achieve high levels of proteasome inhibition in vivo and is also well tolerated (8). It is also an effective anticancer agent that synergizes with various drugs in the treatment of various tumors such as colon cancer in a preclinical animal model (9).

TRAIL (Apo-2L) is a type II transmembrane protein and induces cell death by apoptosis in a variety of tumor cell lines, but fails to induce apoptosis in nontransformed normal cells (10). TRAIL induces apoptosis by interacting with two death receptors, death receptor (DR) 4 and DR5 (10). This has led to the potential of TRAIL as an effective anticancer therapy (11). In addition, Abs directed against TRAIL death receptors DR4 and DR5 are in clinical trials for a variety of cancers (12). There are several reports indicating a synergistic apoptotic response achieved by the combination of TRAIL with chemotherapeutic drugs (13, 14). Increased apoptotic rates in a variety of cancer cell lines have also been reported after the combination of TRAIL with proteasome inhibitors resulting by augmentation of DR5 protein levels (15–23). Although the role of DR5 up-regulation and involvement in TRAIL-induced sensitization to apoptosis by proteasome inhibitors is well documented, the mechanism by which DR5 is up-regulated is not known and is the subject of the present investigation.

In this study, we examined the mechanism of NPI-0052-induced reversal of tumor resistance to TRAIL and the concomitant up-regulation of DR5 expression. Our recent findings demonstrate that inhibition of NF-κB by various chemotherapeutic drugs or by the NO donor DETANONOate resulted in the sensitization of TRAIL-resistant tumor cells to TRAIL-mediated apoptosis (24, 25). The inhibition of NF-κB was concomitant with the inhibition of the transcription repressor Yin Yang 1 (YY1), which has been shown to be regulated by NF-κB (26) and it negatively regulates DR5 transcription and expression. Because proteasome inhibitors inhibit NF-κB, we hypothesized that treatment of TRAIL-resistant...
The findings, herein, establish for the first time a novel mechanism by which NPI-0052 sensitizes tumor cells to TRAIL apoptosis, which is mediated by NPI-0052-induced inhibition of the DR5 transcription repressor YY1, and leads to up-regulation of DR5 expression and activation of the mitochondrial apoptotic pathway.

Materials and Methods

Cell lines/reagents

The cancer cell lines PC-3 (metastatic bone-derived human androgen-independent human prostatic adenocarcinoma) and Ramos (human non-Hodgkin’s B cell lymphoma (B-NHL)) were obtained from the American Type Culture Collection and cultured as previously described (24). Frozen human bone marrow mononuclear cells were purchased from Stem Cell Technologies and thawed according to the manufacturer’s instructions. The proteasome inhibitors NPI-0052 and bortezomib were obtained from Nereus Pharmaceuticals and Millennium Pharmaceuticals, respectively. The monoclonal anti-DcR1, anti-DR4, anti-YY1, and anti-Bcl-xL Abs and the polyclonal anti-DcR2, anti-DR4, anti-YY1, and anti-Bcl-xL Abs were obtained from BD Pharmingen. The polyclonal anti-DcR1, anti-DR2, anti-DR4, anti-YY1, and anti-Bcl-xL Abs and the corresponding IgG1 isotype controls were obtained from Axxora and Chemicon International, respectively. The monoclonal anti-DcR1, anti-DR2, anti-DR4, anti-YY1, and anti-Bcl-xL Abs and the polyclonal anti-Bax and HRP-labeled anti-mouse IgG Abs were obtained from Santa Cruz.
Biotinylated polyA(T) oligonucleotides containing the YY1 binding site were synthesized as previously described (24, 27). The pNF-κB-Luc plasmid was purchased from Invitrogen. Transfections were performed using the Transfecto Transfection Kit (Gene Choice). Transfection solutions consisting of 1 ml/dish of transfection buffer supplemented with 12.5 ng of pYY1-Luc or 12.5 ng of pDR5-Luc DNA plasmids were prepared according to the manufacturer’s instructions. The transfection mix was added to the cells with 4 ml of antibiotic-free cell culture medium for 5 h of incubation followed by replacement with fresh complete growth medium containing various concentrations of NPI-0052 or 10 μg/ml DHMEQ for a further incubation of 20 h. Luciferase activity in protein extracts was measured in an analytical luminescence counter according to the manufacturer’s protocol (BD Biosciences). Data were normalized to protein concentration levels using the Bio-Rad protein assay.

Human colony-forming assay

The human colony-forming assay was performed in MethoCult medium as instructed by StemCell Technologies. Cells were plated in quadruplicate (at a density of 20,000 cells per 35-mm dish) in MethoCult GF H4434 (complete methylcellulose medium with recombinant cytokines and erythropoietin). NPI-0052 was tested at 2.5 nM along with either 10 or 5 ng/ml TRAIL. Both compounds were incubated with the bone marrow mononuclear cells simultaneously for the full 14-day incubation period. Nontreated TRAIL. Both compounds were incubated with the bone marrow mononuclear cells simultaneously for the full 14-day incubation period. Nontreated (upper panel) and Ramos (lower panel) cells were treated with various concentrations of TRAIL in the presence or absence of various concentrations of DHMEQ and apoptosis was assessed. **p values: single cell treatment with DHMEQ or TRAIL vs combinational treatment (Mann-Whitney U test). RLU, Relative light units.

Application of siRNA

Application of siRNA against YY1 in PC-3 and Ramos cells was done as previously described (24, 28).

Determination of apoptosis

Apoptosis was assessed in PC-3 and Ramos cells pretreated for 6 h with different concentrations of either NPI-0052 or bortezomib or DHMEQ (5 or 10 μg/ml) or 2MAM-A3 (15 μg/ml), followed by an 18-h treatment with

"FIGURE 3. Treatment of tumor cells with NPI-0052 inhibits NF-κB activity and the NF-κB inhibitor DHMEQ sensitizes tumor cells to TRAIL-mediated apoptosis. A, NPI-0052 inhibits NF-κB promoter activity. NF-κB promoter activity was assessed in NPI-0052-treated PC-3 (left panel) and Ramos cells (right panel) using a NF-κB-Luc reporter plasmid as described in Transient transfections. Values represent the mean ± SEM of three independent experiments and were calculated based on the control value set at 100% (control: untreated cells). Cells treated with 10 μg/ml DHMEQ, a chemical inhibitor of NF-κB, served as a positive control for NF-κB promoter activity inhibition. **p; treated vs untreated cells (Mann-Whitney U test). B, NPI-0052 inhibits p65 phosphorylation and prevents phospho-p65 degradation. Ramos and PC-3 cells were treated with 2.5 nM NPI-0052 for the indicated time points and Western blot analysis was performed to whole cell lysates for detection of phospho-p65 and total p65 and IκBα levels. Actin expression was used as an internal loading control. C, Sensitization of tumor cells to TRAIL-mediated apoptosis by the NF-κB inhibitor DHMEQ. PC-3 (upper panel) and Ramos (lower panel) cells were treated with various concentrations of TRAIL in the presence or absence of various concentrations of DHMEQ and apoptosis was assessed. ** p values: single cell treatment with DHMEQ or TRAIL vs combinational treatment (Mann-Whitney U test). RLU, Relative light units."
various concentrations of TRAIL. Apoptosis was determined by cleavage of procaspase 3 using flow cytometry as previously described (24).

Tryptic blue exclusion assay

The toxicity of various concentrations of NPI-0052 ranging from 1 to 20 nM was tested on PC-3 and Ramos cells using the tryptic blue exclusion assay. The viability of 24 h treated cells was examined after tryptic blue staining under the microscope. The viability of the untreated cells was set at 100%. Subtoxic NPI-0052 concentrations were considered the concentrations giving >80% cell viability.

Flow cytometry for evaluation of DR5 expression

PC-3 and Ramos cells treated for 24 h with 1, 2.5, or 5 nM NPI-0052 or cells after transfection and treatment with YY1 siRNA and TRAIL, respectively, were subjected to flow cytometric evaluation of DR5 expression as it has been described previously (24).

Measurement of mitochondrial membrane depolarization

The mitochondria-specific dye 3,3′-dihexyloxacarbocyanine (DiOC6, Molecular Probes) was used to measure the mitochondrial membrane potential in both cells before and after 24 h of treatment with 1 and 2.5 nM NPI-0052, as previously reported (25).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted and purified from 1 × 10⁶ PC-3 or Ramos cells treated with 2.5 nM NPI-0052 for 0, 3, 6, 9, 12, and 24 h. Untreated cells served as control. One microgram of total RNA was reverse-transcribed to first-strand cDNA for 1 h at 42°C with 200 U of AMV reverse transcriptase and 20 μM random hexamer primers according to the manufacturer’s instructions (Promega). Transcribed products were subjected to real-time PCR assay with SYBR Green in a programmable thermal controller apparatus (Bio-Rad) for determination of DcR1, DcR2, DR4, DR5, and YY1 mRNA expression. For each target gene, 1.5 μl of 1/5 diluted cDNA was amplified in a total volume of 25 μl containing 1× homemade SYBR Green QPCR Master Mix supplemented with 2.5 mM MgCl₂, 0.4 mM dNTPs mix, 50 mM ROX as passive reference dye, and 200 nM of each primer set. GAPDH was used as internal control to normalize target gene mRNA expression levels. All primer pairs were designed to span at least one intron to avoid amplification of contaminating genomic DNA along with cDNA. Primer sequences were as follows: Dr4 forward, GCCGCGGTCCTGCTGTTG and DcR2 reverse, CGGACCGGCAGCAGAAC (product size: 86 bp); DR5 reverse, GGGGCTGGAACCAGA and DcR2 reverse, CGGAGCGGCGCACGAAC (product size: 82 bp); DcR2 forward, ACAGCGTCGGCCCAAGACCCTAAAGTTCG and DcR2 reverse, CGGGCGAGTGGTG (product size: 127 bp); and GAPDH forward, GGAATACGAGACTCTCGC and GAPDH reverse, GTCATTGATTGGCAACAATATCCACT (product size: 101 bp). All reactions were performed in triplicate. The samples were subjected to 42 cycles of amplification comprised of denaturation at 95°C for 30 s, annealing for 30 s at 60°C, and elongation at 72°C for 30 s. After amplification, standard curves were constructed, from samples used in a series of consecutive dilutions, for both the gene of interest and the internal control (GAPDH). Dissociation curves were also performed for all of the tested genes to exclude the presence of by-products or primer-dimer formation. Data were collected during melting curve analysis. The correct PCR products were further confirmed by analysis on 2% ethidium bromide-stained agarose gels. For all tested samples, the mRNA expression of both the target and the housekeeping gene (GAPDH) was
results
NPI-0052 sensitizes TRAIL-resistant Ramos B-NHL and PC-3 prostate carcinoma cells to TRAIL-induced apoptosis

The prostate carcinoma cell line PC-3 and the Ramos B-NHL cells were treated with various concentrations of NPI-0052 for 24 h, and cell viability was measured by the trypan blue dye exclusion assay. As shown in Fig. 1A, 2.5 nM NPI-0052 was determined, for both cell lines, as the optimal subtoxic concentration for use in the subsequent experiments. Cotreatment of tumor cells with NPI-0052 and various concentrations of TRAIL resulted in significant sensitization of PC-3 and Ramos cells to TRAIL-mediated apoptosis 24 h posttreatment; the intensity of which was a function of the TRAIL concentration used and synergy was achieved (Fig. 1B). In contrast, a single treatment of cells with NPI-0052 or TRAIL did not reveal any significant apoptosis induction. These data indicate that the combination treatment results in significant potentiation of apoptosis. Comparison of PC-3 cell sensitization to TRAIL after treatment with NPI-0052 or bortezomib revealed that a 400-fold less concentration of NPI-0052 than bortezomib was able to induce the same level of TRAIL-mediated apoptosis (Fig. 1C). This finding suggests that NPI-0052 is a more effective agent than bortezomib used at concentrations <5 nM in terms of sensitization of prostate tumor cells to TRAIL apoptosis.

Treatment of tumor cells with NPI-0052 up-regulates DR5 transcription and expression

Our recent findings showed that inhibition of NF-κB and the transcription repressor of DR5, YY1, sensitized the cells to TRAIL apoptosis (24). We, therefore, hypothesized that suppression of NF-κB by NPI-0052 might result in inhibition of YY1 leading to up-regulation of DR5 expression and an increase of tumor cell sensitivity to TRAIL. Quantitative RT-PCR and Western blot analyses (Fig. 2A) performed for DR5 protein and mRNA determination, respectively, confirmed the overexpression of both DR5 transcript and total protein levels. Increased DR5 transcriptional activity was observed as early as 6 or 9 h after NPI-0052 treatment.
in Ramos and PC-3 cells, respectively, while the peak of DR5 protein overexpression was observed 18 and 24 h after treatment for both cell lines. In contrast, no significant change was detected in PC-3 cells in the expression profiles of other TRAIL receptors, such as DR4, DcR1, and DcR2, as a function of time of NPI-0052 treatment. In Ramos cells, treatment with NPI-0052 resulted not only in up-regulation of DR5 transcript levels but also in a slight significant increase of DcR2 mRNA expression; however, the elevated DcR2 transcript levels were not accompanied by increase in total DcR2 protein expression. Similar to PC-3, the expression of all of the other TRAIL receptors tested in Ramos cells remained not significantly changed. Our findings related to the increased DR5 transcript levels after cell treatment with NPI-0052 were corroborated by the elevated DR5 promoter activity in PC-3 cells transfected with a DR5 luciferase reporter construct and treated with different concentrations of NPI-0052 (1–5 nM; Fig. 2B). The NF-κB inhibitor DHMEQ was used as an internal positive control. A significant up-regulation of DR5 surface protein levels after treatment with NPI-0052 was also observed in a concentration-dependent manner (Fig. 2C).

NPI-0052 inhibits NF-κB promoter activity, p65 phosphorylation, and phospho-IκBα degradation: role of NF-κB inhibition in tumor cell sensitization to TRAIL-mediated apoptosis

The effect of NPI-0052 treatment on NF-κB inhibition has been previously reported. To confirm this observation in our cell systems, we tested the NF-κB promoter activity as well as the IκBα and p65 protein levels after cell treatment with NPI-0052. Our findings revealed that NF-κB reporter activity was suppressed by NPI-0052 in a concentration-dependent manner in both of the cell lines tested (Fig. 3A). Western blot analysis in cell lysates derived from both cell lines treated with 2.5 nM NPI-0052 for 3, 6, and 9 h showed decreased phospho-p65 levels and accumulation of phospho-IκBα protein; however, no significant change was observed in total protein levels (Fig. 3B). The link between NF-κB inhibition and enhancement of tumor cell sensitivity to TRAIL-mediated apoptosis was confirmed by treating Ramos and PC-3 cells with different concentrations of the NF-κB chemical inhibitor DHMEQ and with increasing concentrations of TRAIL. As shown in Fig. 3C, DHMEQ showed a significant potentiating effect in combination with TRAIL in the induction of apoptosis in both PC-3 and Ramos cells. These findings suggest that NPI-0052, as an NF-κB inhibitor, mimics DHMEQ (by acting as a suppressor of the NF-κB survival pathway), facilitating increased sensitization of tumor cells to TRAIL-mediated apoptosis.

Inhibition of YY1 by NPI-0052 sensitizes tumor cells to TRAIL-mediated apoptosis

Recent findings demonstrated that YY1 is under the positive regulation of NF-κB (24, 26, 29); therefore, suppression of NF-κB by NPI-0052 might result in inhibition of YY1 and DR5 overexpression. To test this hypothesis, we examined the YY1 mRNA and protein expression in Ramos and PC-3 cells before and after treatment with NPI-0052 for several time periods. As
shown in Fig. 4A, YY1 transcript levels were significantly reduced as early as 3 or 6 h posttreatment in PC-3 and Ramos cells, respectively, while the peaks of YY1 protein down-regulation were observed at least after 9 h posttreatment for both cell lines. The decreased YY1 mRNA levels after cell treatment with NPI-0052 were corroborated with significant suppression of YY1 promoter activity as assessed in PC-3 and Ramos cells by using a YY1-Luc reporter construct (Fig. 4B). These results demonstrate that NPI-0052 inhibits YY1 transcription and expression and these correlated with NPI-induced up-regulation of DR5 and inhibition of NF-κB.

The direct involvement of YY1 inhibition by NPI-0052 in tumor cell sensitization to TRAIL by up-regulating DR5 was examined by transfecting cells with siRNA against YY1 mRNA and tested the cells for sensitivity to TRAIL. We observed that transfection of both cell lines tested with YY1 siRNA for 72 h inhibited YY1 expression (Fig. 5A) and sensitized the cells to TRAIL-mediated apoptosis in a concentration-dependent manner (Fig. 5A, PC-3 and Ramos, upper and lower panels, respectively). Furthermore, as assessed by flow cytometry, 72 h posttransfection the surface DR5 protein levels were found significantly elevated in both PC-3 and Ramos cells (Fig. 5B). These findings demonstrate that YY1 plays a major role in TRAIL-mediated apoptosis and suggest that NPI-0052 enhances cell sensitivity to TRAIL by enhancing DR5 expression through YY1 inhibition.

The inhibition of antiapoptotic gene products, several of which are regulated by NF-κB such as Bcl-xL, IAPs, and XIAP (30, 31) and/or up-regulation of proapoptotic proteins such as Bax, are known to contribute to mitochondrial membrane depolarization and release of cytochrome c and Smac/DIABLO leading to apoptosis induction (32). We hypothesized that NPI-0052-induced inhibition of the NF-κB pathway may modulate the ratios between pro- and antiapoptotic gene products in favor of proapoptotic activity, thus influencing the mitochondrial membrane potential and promoting apoptosis.

PC-3 and Ramos cells were treated with increasing concentrations of NPI-0052 for 24 h and the mitochondrial membrane potential was assessed by flow cytometry. As shown in Fig. 6A, both cell lines treated with 1 nM NPI-0052 exhibited increased mitochondrial membrane depolarization. Cell lysates extracted from PC-3 and Ramos cells treated with 2.5 nM NPI-0052 for various time periods were subjected to Western blot analysis for determination of the protein expression of the anti- and proapoptotic gene products, including Bcl-xL, survivin, IAPs, XIAP, Bax, caspase 8, and FLIP. As shown in Fig. 6B, treatment of PC-3 cells with NPI-0052 resulted in time-dependent reduction in the

NPI-0052 inhibits NF-κB-regulated antiapoptotic gene products and induces mitochondrial membrane depolarization: role of Bcl-xL down-regulation in TRAIL sensitization

The inhibition of antiapoptotic gene products, several of which are regulated by NF-κB such as Bcl-xL, IAPs, and XIAP (30, 31) and/or up-regulation of proapoptotic proteins such as Bax, are known to contribute to mitochondrial membrane depolarization and release of cytochrome c and Smac/DIABLO leading to apoptosis induction (32). We hypothesized that NPI-0052-induced inhibition of the NF-κB pathway may modulate the ratios between pro- and antiapoptotic gene products in favor of proapoptotic activity, thus influencing the mitochondrial membrane potential and promoting apoptosis.
levels of Bcl-xL, survivin, IAPs, and XIAP. Maximum inhibitory effects were observed 18 and 24 h after treatment for most of the antiapoptotic gene products. In contrast, the expression of the pro-apoptotic protein Bax was found elevated as early as 3 h after treatment. The same patterns were also observed in Ramos cells after treatment with 2.5 nM NPI-0052 for 24 h (Fig. 6B). However, in both cell lines tested, there was no significant change observed in FLIP expression, while minimal caspase 8 activation was monitored. These findings suggest that NPI-0052 sensitizes tumor cells to TRAIL-mediated apoptosis, at least in part, by decreasing the ratio of antiapoptotic gene products over proapoptotic gene products and thus inducing mitochondrial membrane depolarization and activation of at least the type II apoptotic pathway.

Bcl-xL has been reported, by us and others, as one dominant factor responsible for the acquired resistance of certain tumor cells to chemo- and immunotherapy, including TRAIL (25, 29, 31, 33, 34). Since Bcl-xL is inhibited by NPI-0052, we hypothesized that direct inhibition of Bcl-xL will mimic NPI-0052. Indeed, treatment of PC-3 and Ramos cells with the Bcl-2 inhibitor 2MAM-A3 resulted in significant cell sensitivity to TRAIL-mediated apoptosis, and the extent was a function of the TRAIL concentration used (Fig. 6C). These findings indicate that Bcl-xL inhibition by NPI-0052 via NF-κB inactivation contributes to NPI-0052-induced tumor cell sensitization to TRAIL-mediated apoptosis.

**Toxicity profiles of NPI-0052 and TRAIL combinations treatment of human progenitor cells**

The human colony-forming assay was used to determine the toxicity of NPI-0052 on normal hemopoiesis by evaluating in ability to block colony formation. Each colony formed is the result of cell division and differentiation of a single progenitor cell over time. NPI-0052 was tested at 2.5 nM along with either 5 or 10 ng/ml TRAIL. Both compounds were incubated with bone marrow mononuclear cells simultaneously for 10 days. Untreated and DMSO-treated cells were used as controls. Colony counts of CFU-E, BFU-E, CFU-GM, and CFU-GEMM were determined on day 14 of incubation. NPI-0052 or TRAIL (at both concentrations used) as single agent treatment demonstrated no toxicity to the progenitor cell of any lineage as evidenced by the number of the various colony types compared with untreated controls. However, both combination treatments demonstrated inhibition of colony formation of all types, although they did not block colony formation completely, allowing 40–70% of the colonies to grow and differentiate (Fig. 7).

**Discussion**

In the present study, we have examined the mechanisms by which proteasome inhibitors up-regulate DR5 expression and sensitize tumor cells to TRAIL apoptosis. Our findings demonstrate that NPI-0052 sensitizes both prostate and B-NHL cell lines to TRAIL-mediated apoptosis via a mechanism which involves inhibition of the TRAIL-resistant factor, the transcription repressor YY1, and consequently up-regulation of DR5. Inhibition of YY1 by NPI-0052 is mediated, in part, by inactivation of its positive regulator NF-κB (26), as confirmed by down-regulation of the phosphorylated p65 protein levels and accumulation of phosphorylated IκBα. In addition to YY1 inhibition and DR5 induction, NPI-0052 was shown to promote activation of the mitochondrion-related apoptotic pathway through depolarization of the mitochondrial membrane, inhibition of XIAP, IAPs, and Bcl-xL antiapoptotic gene products, and induction of Bax. Cancer cells, including prostate and B-NHL tumors, have been shown to have constitutive NF-κB activity which regulates the transcription of many gene products involved in cell survival and antiapoptotic pathways (1, 34). Since the above antiapoptotic gene products are under the direct regulation of NF-κB and their elevated expression has been shown to confer resistance of prostate and B-NHL tumors to immunotherapy (25, 29, 32), their inhibition by NPI-0052 in combination with the inhibition of NF-κB and YY1 and DR5 overexpression contribute to resensitization of those tumors to TRAIL-mediated apoptosis.

Several mechanisms have been proposed to elucidate the antitumor activity of proteasome inhibitors in different cancer models. Among such mechanisms, of particular interest is the reduced IκBα degradation, leading to decreased NF-κB-dependent synthesis of antiapoptotic factors resulting in a shift in the balance between pro- and anti-apoptotic Bcl-2 family members and induction of apoptosis (35). Moreover, stabilization and accumulation of p53 (2), generation of reactive oxygen species and oxidative stress, JNK stabilization, increased c-Jun phosphorylation, and AP-1 DNA-binding activity (17, 36, 37), have also been correlated with the use of proteasome inhibitors in cancer therapy. The effect of proteasome inhibitors in tumor cell sensitization to TRAIL has been studied in different experimental models in vitro. Proteasome inhibitors, like, bortezomib (PS-341) and MG-132 have been shown as single agents to induce modest apoptosis in several types of tumor cells; however, low doses in combination with TRAIL result in synergy (17, 23, 35, 40). Some of the proposed underlying mechanisms include proteasome inhibitor-mediated p21 and Bax induction followed by Sp1/ΔNp63α release and activation of proapoptotic c-jun gene (23, 35, 40) and inhibition of c-FLIP, Bcl-2, Bcl-xL, and IAPs antiapoptotic gene products (17, 37) via inactivation of NF-κB.

The induction of TRAIL receptors DR4 and/or DR5 by PS-341 resulting in potentiating of TRAIL-induced apoptosis has been shown in certain types of cancer cells, including colon, prostate, bladder and ovarian cancer, chronic lymphocytic leukemia, non-small-cell lung cancer, and glioblastoma (19–21, 39, 40). The role of proteasome inhibitor-induced DR5 expression in sensitization to TRAIL is well documented by the use of siRNA DR5 in which the transfected cells show reduced levels of apoptosis to TRAIL (20). Our findings agree with those of Liu et al. (20) and those of Kabore et al. (21) for DR5, although unlike Liu et al. (20) we could not demonstrate up-regulation of DR4 in our cell lines in both mRNA and protein level. We also did not detect any changes in the expression profiles of other TRAIL receptors such as DcR1 and DcR2 in PC-3 cells; however, in Ramos cells, a slight significant increase in DcR2 transcript levels was not accompanied with a similar increase in protein level, indicating that among the TRAIL receptors the main target of NPI-0052 is DR5. Nothing is known about the mechanism by which proteasome inhibitors regulate DR5 expression. It is well known that DR5 expression is regulated through p53-dependent or p53-independent mechanisms (41, 42). It was suggested that JNK could possibly regulate DR5 expression (43, 44); however, inhibition of JNK did not affect PS-341-induced sensitization to TRAIL in human non-small-cell lung carcinoma. In contrast, PS-341 induced up-regulation of the CHOP/GADD153 transcriptional factor that regulates DR5 expression (23, 45).

Our findings demonstrate, for the first time, that proteasome inhibitor-induced up-regulation of DR5 expression is mediated, in part, by transcriptional and posttranscriptional inhibition of the transcription repressor YY1 that we have shown to negatively regulate DR5 transcription and expression (24). YY1 can exert wide activities at target promotors acting either as an activator, a repressor, or as an initiator binding protein (46, 47). In addition to previous reports indicating directly or indirectly positive regulation of YY1 by NF-κB (24, 26, 29), here we show that NPI-0052-induced...
YF inhibitor and the subsequent cell sensitization to TRAIL-mediated apoptosis results from inactivation of NF-κB. NF-κB inhibition by NPI-0052 in our cell systems seems to result mainly by progressive accumulation of phospho-IκBα since NF-κB is constitutively active in the tested cell lines and the pIkBα is continuously degraded by the proteasome. However, no significant increase was observed in total IkBα protein levels. Our findings are consistent with data recently reported by Ahn et al. (48) in cell lines treated with NPI-0052 and TNF-α. Similar to IkBα, we did not observe any change in total p65 protein levels after cell treatment with NPI-0052; however, phospho-p65 levels were significantly decreased as a function of time and exposure. Those were expected findings because we used total protein cell extracts for our analysis. These findings are in agreement with the findings reported by Ahn et al. (48).

NPI-0052-induced NF-κB inhibition was consistent with the increased sensitivity of tumor cells to TRAIL-induced apoptosis mediated by DR5 up-regulation after cell treatment with the NF-κB inhibitor DHMEQ or YY1 siRNA. We also show that several NF-κB-dependent antiapoptotic gene products including Bcl-xL, IAPs, and, to a lesser extent, XIAP are down-regulated by NPI-0052 while it promotes the overexpression of proapoptotic gene products such as Bax. Bcl-xL, IAPs, and XIAP have been reported to confer resistance to TRAIL in several tumor models, including prostate cancer cells, and their inhibition by different agents promote tumor sensitization to immuno- or chemotherapy (29, 32, 34). The NPI-0052-induced inhibition of Bcl-xL seems to be a key component in resensitization to immuno- or chemotherapy (29, 32, 34). The NPI-0052 is distinct from bortezomib not only as its chemical structure, but also in the irreversible fashion that affects the three proteolytic activities of the 26S proteasome core as well as the mechanism of action and toxicity profile against normal cells (57). In vitro findings have shown that NPI-0052 induces apoptosis in MM resistant to conventional and bortezomib therapies; however, the first human trial is currently ongoing (5). Moreover, a recent study by Cusack et al. (49) reported that NPI-0052 is well tolerated in mice and enhances tumor responses to conventional cancer therapy in a colon cancer model. A comparable study report by Ruiz et al. (57) demonstrated that NPI-0052 is a more potent apoptotic inducer than bortezomib in lymphocytes from patients with chronic lymphocytic leukemia.

Both NPI-0052 and bortezomib have been reported to exhibit time and concentration-dependent inhibition of the proteasome in vitro based on their different kinetics and pharmacologic profiles (58). With respect to the efficacy of each agent to induce tumor cell sensitization to TRAIL, bortezomib has been shown to be effective at concentrations ranging from 0.5 μM up to 10 μM in various tumor models including ovarian, thyroid, colon, and pancreatic carcinomas (38–40, 59). The concentrations of NPI-0052 used in our experimental models to achieve high tumor sensitization rates to TRAIL apoptosis were significantly lower (1–5 nM) compared with those used for bortezomib in the previous studies. Comparison between bortezomib and NPI-0052 in terms of the concentrations used for sensitization of PC-3 cells to TRAIL revealed that 5 nM NPI-0052 was able to give the same net tumor response to TRAIL as 2 μM bortezomib. This indicates a 400-fold higher efficiency of NPI-0052 to induce TRAIL-mediated apoptosis at such low concentrations than bortezomib, at least in prostate tumor cells. Moreover, by testing the effect of the combination treatment (TRAIL and NPI-0052) on hematopoietic progenitor colony formation, we showed that despite some toxicity observed, the majority of the colonies from all types (40–70%) were still able to grow and differentiate under the combination treatment. These findings are in accordance with reported data on the toxicity profiles of other proteasome inhibitors, such as PS-341, used at higher concentrations than NPI-0052, when combined with TRAIL in normal cells (21).

In summary, our findings here demonstrate, for the first time, that the novel proteasome inhibitor NPI-0052 is able to break the resistance of prostate and B-NHL cell lines to TRAIL via a mechanism which involves inhibition of the constitutively expressed DR5 transcriptional repressor YY1. Inhibition of YY1 by NPI-0052 is the result of inhibition of NF-κB activity which regulates,
activity for YY1 and many other antiapoptotic gene products such as XIAP, IAPs, and Bcl-xL. Thus, treatment of tumor cells with NPI-0052 results in the up-regulation of DR5 and down-regulation of antiapoptotic gene products. In addition, NPI-0052 induces mitochondrial membrane depolarization and the combination of NPI-0052 and TRAIL through the activation of type II apoptotic pathway results in synergy in apoptosis as shown in Fig. 8. The inverse correlation between DR5 and YY1 expression following treatment of the mice with the chemotherapeutic drug cis-diammine dichloroplatinum or the NO donor DETA/NONOate (data not shown). Our data suggest that NPI-0052 can effectively break the tumor cell resistance to TRAIL in very low nontoxic to normal cell concentrations and could thereby support the clinical applicability of a combination of TRAIL and/or receptor agonist Abs with NPI-0052 in the treatment of drug/TRAIL-resistant tumors.

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Disclosures
M.P. is an employee of Nereus Pharmaceuticals, Inc.

References

FIGURE 8. Schematic diagram representing the role of the proteasome inhibitor NPI-0052 in the regulation of tumor cell sensitivity to TRAIL-induced apoptosis. Treatment with NPI-0052 results in the up-regulation of several genes regulating the apoptotic pathways. NPI-0052 inhibits the NF-κB pathway via inhibition of IκBα and p65 phosphorylation. NF-κB inhibition by NPI-0052 leads to the down-regulation of Bel-xL and the induction of Bax (Fig. 6) contributing to the mitochondria-mediated depolarization. Furthermore, NPI-0052 inhibits the transcription repression for YY1, leading to up-regulation of DR5 (Figs. 4 and 2, respectively). Thus, the combination of NPI-0052 and TRAIL results in the activation of the mitochondrial apoptotic pathway, inhibition of antiapoptotic gene products, activation of procaspases 9 and 7, formation of the apoptosome, and altogether downstream activation of the effector caspase 3 resulting in apoptosis.


Letter of Retraction

A request was received from the Acting Research Integrity Officer of the University of California, Los Angeles (UCLA), to retract this article: “Inhibition of Yin Yang 1-Dependent Repressor Activity of DR5 Transcription and Expression by the Novel Proteasome Inhibitor NPI-0052 Contributes to its TRAIL-Enhanced Apoptosis in Cancer Cells” by Stavroula Baritaki, Eriko Suzuki, Kazuo Umezawa, Demetrios A. Spandidos, James Berenson, Tracy R. Daniels, Manuel L. Penichet, Ali R. Jazirehi, Michael Palladino, and Benjamin Bonavida, The Journal of Immunology, 2008, 180: 6199–6210.

The Editor-in-Chief of The Journal of Immunology was informed that UCLA had conducted a review of the work and concluded that data used in some of the figures in this article could not be supported. In particular, issues involving duplication of images were identified in Figs. 2A, 3B, and 6B. The article is therefore retracted.

It is noted that not all coauthors of this article worked on the data presented in the figures identified by UCLA. In particular, Manuel L. Penichet has advised the Editor-in-Chief that his contributions and those of Tracy R. Daniels were confined to data in Figure 7.