Pivotal Roles of Snail Inhibition and RKIP Induction by the Proteasome Inhibitor NPI-0052 in Tumor Cell Chemoimmunosensitization

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Abstract

The novel proteasome inhibitor NPI-0052 has been shown to sensitize tumor cells to apoptosis by various chemotherapeutic drugs and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), although the mechanisms involved are not clear. We hypothesized that NPI-0052–mediated sensitization may result from NF-κB inhibition and downstream modulation of the metastasis inducer Snail and the metastasis suppressor/immunosurveillance cancer gene product Raf-1 kinase inhibitory protein (RKIP). Human prostate cancer cell lines were used as models, as they express different levels of these proteins. We show that NPI-0052 inhibits both NF-κB and Snail and induces RKIP expression, thus resulting in cell sensitization to CDDP and TRAIL. The direct role of NF-κB inhibition in sensitization was corroborated with the NF-κB inhibitor DHMEQ, which mimicked NPI-0052 in sensitization and inhibition of Snail and induction of RKIP. The direct role of Snail inhibition by NPI-0052 in sensitization was shown with Snail small interfering RNA, which reversed resistance and induced RKIP. Likewise, the direct role of RKIP induction in sensitization was revealed by both overexpression of RKIP (mimicking NPI-0052) and RKIP small interfering RNA that inhibited NPI-0052–mediated sensitization. These findings show that NPI-0052 modifies the NF-κB-Snail-RKIP circuitry in tumor cells and results in downstream inhibition of antiapoptotic gene products and chemoimmunosensitization. The findings also identified Snail and RKIP as targets for reversal of resistance. [Cancer Res 2009;69(21):8376–85]

Introduction

A new class of regimens reported to regulate NF-κB and, therefore, tumor cell resistance to therapy, are proteasome inhibitors. Hence, proteasome inhibitors have been developed to target the proteasome with the objective to induce cancer cell cytostasis or cell death when used alone or in combination with other cytotoxics (1). NPI-0052 (salinosporamide A) is a novel member of the proteasome inhibitor family identified from the marine actinomycete Salinispora tropica and is a nonpeptide inhibitor of all three enzymatic activities of the 20S proteasome (2). The antitumor properties of NPI-0052 have been evaluated in a wide range of nonclinical studies including in vitro and in vivo models (3–9). NPI-0052 has shown good sensitizing effects to apoptotic death in tumors resistant to chemotherapy and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; refs. 5, 10). The major biological effect of NPI-0052 in tumors is apoptosis induction and osteoclastogenesis suppression (7, 11).

Yeung and colleagues first identified the suppressing effect of a novel gene product, the Raf-1 kinase inhibitory protein (RKIP), on the activation status of the Raf-1/MEK/ERK and NF-κB survival pathways. RKIP belongs to the phosphatidylinositol-4,5-bisphosphate-binding protein family (12, 13). RKIP loss or depletion has been associated with metastatic disease in an increasing number of solid tumors. RKIP involvement in the reversal of tumor cell resistance to drugs and TRAIL apoptosis has also been reported previously (14–16). A recent study by Beach and colleagues (17) reported that RKIP is under the regulation of the transcription repressor Snail in prostate tumors. Snail is a member of the Snail superfamily of zinc-finger transcription factors with a pivotal role in embryonic development and cell survival (18). Snail has been identified as one of the key modulators for the initiation of epithelial-to-mesenchymal transition during tumor cell metastasis (17, 19–24). Snail is transcriptionally regulated, in part, by NF-κB (25, 26) and by itself via binding to its own promoter and repressing activity (27).

In this study, we hypothesized that one potential mechanism by which NPI-0052 reverses tumor resistance to chemotherapeutic and immunotherapeutic stimuli is via inhibition of NF-κB and, consequently, downregulation of Snail expression and upregulation of RKIP as well as downstream modification of various antiapoptotic gene products. This hypothesis was tested using human tumor cell lines with constitutively active NF-κB as models and with different levels of RKIP and Snail expression. The following questions have been addressed: (a) Does NPI-0052 inhibit Snail transcription and expression? (b) Does NPI-0052–induced Snail suppression correlate with NPI-0052–induced RKIP expression? (c) Is NPI-0052–induced repression of Snail due, in part, to NPI-0052–mediated inhibition of NF-κB activity and whether a specific NF-κB inhibitor, DHMEQ, mimics NPI-0052–induced repression of Snail and induction of RKIP? (d) Does silencing of Snail or overexpression of RKIP have a direct role in drug and/or TRAIL-mediated apoptosis? (e) Does NPI-0052–mediated NF-κB and Snail suppression and RKIP induction result in activation of the mitochondrial apoptotic pathway via...
inhibition of antiapoptotic gene products? The findings reported herein confirm our hypothesis and reveal new insights into the mechanism of tumor cell sensitization to apoptosis by the proteasome inhibitor NPI-0052 and identify gene products as potential therapeutic targets to reverse tumor resistance to conventional therapies.

Materials and Methods

Cell lines. The human prostatic adenocarcinoma cell lines DU-145 and LNCaP were obtained from the American Type Culture Collection. The melanoma cell line M202 was kindly provided by Dr. Ali Jazirehi (University of California). The stable Snail knockdown DU-145 cell line (DU-145 siSnail) as well as the corresponding control (DU-145 siLuc) were generated as described previously (17).

Reagents and plasmid constructs. NPI-0052, MG132, and bortezomib were provided by Nereus Pharmaceuticals, Calbiochem, and Millennium Pharmaceuticals, respectively. Recombinant human TRAIL and CDDP were purchased from PeproTech and Sigma, respectively. DHMEQ was kindly provided by Dr. Kazuo Umezawa (Keio University). Stock solutions and working dilutions of DHMEQ, NPI-0052, MG132, and CDDP were prepared in DMSO, whereas bortezomib and TRAIL were prepared in distilled H2O. Annexin V and propidium iodide were purchased from BD Pharmingen. The Bcl-2 inhibitor 2-methoxyantimycin A3 and the antibodies recognizing human RKIP, Snail, β-actin, X-linked inhibitor of apoptosis, Bcl-xL, active caspase-3, IκBα, and phosphorylated IκBα proteins as well as the small interfering RNAs (siRNA) against RKIP and Snail were obtained from sources described previously (10, 28).

Figure 1. NPI-0052 sensitizes DU-145 prostate and M202 melanoma tumor cell lines to CDDP- and TRAIL-mediated apoptosis and synergy is achieved. DU-145 (A) or M202 (B) cells were pretreated for 6 h with increasing concentrations of NPI-0052 followed by treatment with TRAIL (5 or 10 ng/mL) or CDDP (1–3 μg/mL) for 18 h and apoptosis was determined at 24 h after initial treatment by activation of procaspase-3 as described in Materials and Methods. Mean ± SE of three independent experiments. *P<0.023, cells treated with a single agent (NPI-0052 or TRAIL or CDDP) versus combined treatment (Mann-Whitney U test). Synergy was determined by isobologram analysis. F.I.C., fractional inhibitory concentration.

The CMV-HA-RKIP expression vector and the reporter constructs pNF-κB-Luc and pRKIP-Luc wild-type and pRKIP-Luc mutant (carrying mutations in three of the five E-boxes) have been described previously (14, 16, 17).

Transient transfections. NF-κB and RKIP promoter activities were determined by luciferase assays as described previously (10, 17). The effect of Snail suppression on RKIP promoter activity was determined in DU-145 cells initially transfected with Snail siRNA for 48 h, as described below, followed by transient transfection with the pRKIP-Luc wild-type or pRKIP-Luc mutant constructs for 24 h. RKIP overexpression in DU-145 cells was done as described previously (16).

Application of siRNA. The silencing of RKIP and Snail expression using siRNAs was done according to the manufacturer’s instructions (Santa Cruz Biotechnology; refs. 16, 28).

Determination of apoptosis. Apoptosis was determined by either cleavage of procaspase-3 or Annexin V/propidium iodide staining according to the manufacturer’s instructions (BD Pharmingen).

Quantitative real-time PCR analysis. Total RNA was extracted and purified from 1 × 10⁶ DU-145 cells treated with 50 nmol/L NPI-0052 or 10 μg/mL DHMEQ for different periods using Trizol reagent (Life Technologies). Both RNA isolation and cDNA synthesis were done according to the manufacturer’s instructions (BD Pharmingen).

Western blot analysis. Analysis of the protein expression was done by using Western blot analysis as described previously (10, 16, 28, 29). The expression of β-actin was used as an internal control.
Measurement of mitochondrial membrane depolarization. The mitochondrial membrane potential was determined by using 3,3′-dihexyloxacarbocyanine (Molecular Probes) as described previously (29).

Isobologram analysis. This was done according to Berenbaum (30).

Statistical analyses. Significant differences were determined by the Mann-Whitney *U* and Kruskal-Wallis *H* tests using the SPSS software.

Results

NPI-0052 sensitizes TRAIL- and CDDP-resistant prostate carcinoma cells to TRAIL- and CDDP-mediated apoptosis. The toxicity profile of NPI-0052 in DU-145, LNCaP, and M202 cells was done by the trypan blue dye exclusion assay. The NPI-0052 concentration of 50 nmol/L was determined as the optimal subtoxic concentration and was used in the majority of the subsequent experiments. Pretreatment of DU-145 and M202 cells with NPI-0052 (5-50 nmol/L) for 6 h followed by treatment with TRAIL (5 and 10 ng/mL) or CDDP (1-3 μg/mL for DU-145 and 5-20 μg/mL for M202 cells) for up to 24 h resulted in significant potentiation of apoptosis and synergy was achieved as determined by isobologram analysis. In contrast, single treatment of cells with NPI-0052 or CDDP or TRAIL did not reveal any significant induction of apoptosis in both cell lines (Fig. 1A and B). The apoptosis determined in DU-145 cells by activation of procaspase-3 was corroborated by Annexin V/propiodium iodide staining (data not shown). We have also performed sensitization assays using two other proteasome inhibitors, bortezomib and MG132. Both of these inhibitors sensitized DU-145 cells to TRAIL- and CDDP-mediated apoptosis. However, compared with NPI-0052, higher concentrations (5- to 20-fold) were needed to achieve the same level of apoptosis (Supplementary Fig. S1).

![Figure 2](cancerres.aacrjournals.org) NPI-0052 inhibits transcription and expression of NF-κB and Snail and induces RKIP. A, NPI-0052 inhibits NF-κB promoter activity. NF-κB promoter activity was assessed in DU-145 cells treated with various concentrations of NPI-0052 using a NF-κB-Luc reporter plasmid. Mean ± SE of three independent experiments, calculated based on the control value set at 100%. Transfected cells treated with 10 μg/mL of the NF-κB inhibitor DHMEQ served as a positive inhibition control of the NF-κB promoter activity. *P < 0.013, treated versus untreated cells (Mann-Whitney *U* test). RLU, relative light units. B, NPI-0052 prevents phosphorylated IkBα degradation. DU-145 cells were treated with 50 nmol/L NPI-0052 for the indicated time points and Western blot analysis was done for detection of both phosphorylated and total IkBα levels. Bottom, densitometric analysis of band intensity. Bar graphs show the ratios between the intensities of IkBα or phosphorylated IkBα and actin. C, time kinetic analysis of Snail and RKIP mRNA expression in DU-145 cells treated with 50 nmol/L NPI-0052. Snail and RKIP transcript levels were determined by quantitative real-time PCR for each time point tested. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was used as a normalizing control. *P, untreated versus treated cells at the indicated time points (Mann-Whitney *U* test). D, NPI-0052 inhibits Snail and upregulates RKIP protein expression. Cell lysates from untreated and NPI-0052 (50 nmol/L)-treated DU-145 cells were harvested at the indicated time points and subjected to Western blot analysis for protein determination. Actin was used as an internal control for all loadings.
data show that the combination treatment with NPI-0052 and TRAIL or CDDP results in the sensitization of resistant DU-145 and M202 cells to TRAIL- and CDDP-mediated apoptosis. NPI-0052 inhibits the transcription factors NF-κB and Snail and upregulates the metastasis suppressor gene products, RKIP. Increasing concentrations of NPI-0052 significantly inhibited NF-κB promoter activity in DU-145 cells as shown in Fig. 2A. DHMEQ was used as an internal positive control. Cell treatment with NPI-0052 also resulted in significant increase of phosphorylated IκBα levels (up to 7-fold at 12 h) due to lack of degradation by the proteasome (Fig. 2B). These findings confirm the suppressive effect of NPI-0052 on NF-κB activity in DU-145 cells. Because Snail is partially under the positive regulation of NF-κB (25) and Snail acts as transcriptional suppressor of RKIP (17), we anticipated that NPI-0052–mediated inhibition of NF-κB will affect both Snail and RKIP expression profiles in an inverse relationship. As shown in Fig. 2C and D, the baseline RKIP and Snail mRNA and protein levels in DU-145 cells were inversely correlated, with Snail expression to be dominant over RKIP, as expected, according to the high metastatic potential of this cell line. In contrast, Snail mRNA levels in cells treated with 50 nmol/L NPI-0052 for increasing incubation periods were significantly reduced beginning at 0.5 h posttreatment and lasting for 8 h (Fig. 2C). Concomitantly, Snail protein levels were found significantly reduced at ≥4 h posttreatment with NPI-0052 (Fig. 2D). Conversely, RKIP mRNA expression showed a constant increase starting at 0.5 h posttreatment up to the final time point of 18 h (Fig. 2C). Similarly, RKIP protein levels (Fig. 2D) followed a time-dependent significant increase in the presence of NPI-0052. Preliminary data also show that other proteasome inhibitors such as bortezomib and MG132 have comparable effects on RKIP and Snail expression (data not shown). These results show that NPI-0052–mediated NF-κB suppression correlated with inhibition of Snail and the induction of RKIP expression.

Direct role of NPI-0052–induced NF-κB inhibition in tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis. The direct role of NPI-0052–mediated NF-κB inhibition in the regulation of Snail/RKIP crosstalk and induction of apoptosis was examined in DU-145 cells treated with the NF-κB inhibitor DHMEQ (31). DHMEQ treatment resulted in a time-dependent inhibition of Snail mRNA expression and induction of RKIP transcript levels (Fig. 3A). Concomitantly, DHMEQ had suppressive effects on Snail and Bcl-xL protein levels, whereas it induced RKIP protein expression (Fig. 3B). In addition, as shown in the representative flow charts in Fig. 3C, DHMEQ had a significant potentiating effect on apoptosis induction in combination with CDDP or TRAIL and the apoptotic levels achieved were in

Figure 3. Role of NPI-0052–induced NF-κB inhibition in tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis. A, DHMEQ, a NF-κB inhibitor, mimics NPI-0052 in terms of Snail inhibition and induction of RKIP transcription. Real-time PCR was done in cDNAs derived from DHMEQ (10 μg/mL)–treated cells for various periods. *, P, untreated versus treated cells at the indicated time points (Mann-Whitney U test). B, NF-κB inhibition by DHMEQ results in inhibition of Bcl-xL and induction of RKIP protein expression. DU-145 cells were treated with 5 or 10 μg/mL DHMEQ and protein lysates were harvested 24 h after treatment for RKIP and Snail protein determination. C, DHMEQ mimics NPI-0052 in the sensitization of DU-145 to TRAIL- and CDDP-mediated apoptosis. Charts are representative of three independent experiments and show percentages of apoptotic cells. The caspase-3–positive plot area was set according to isotype control (left). Mean ± SE apoptotic values for each treatment condition from all the performed experiments (right). *, P < 0.009, cells treated with a single agent (DHMEQ or TRAIL or CDDP) versus combined treatment (Mann-Whitney U test).
the same range as those obtained by NPI-0052 (Fig. 1A). These findings suggest that NPI-0052 mimics DHMEQ and facilitates tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis through suppression of NF-κB and its antiapoptotic targets such as Bcl-xL.

Direct role of NPI-0052–induced RKIP upregulation in tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis. The direct effect of RKIP induction by NPI-0052 in the regulation of tumor cell sensitivity to both CDDP- and TRAIL–apoptosis was examined by ectopic expression of RKIP in DU-145 cells. Cells overexpressing RKIP were more sensitive to induction of apoptosis after addition of CDDP or TRAIL compared with untransfected cells (Fig. 4A) and the percentage of apoptosis was similar to that achieved by NPI-0052 in Fig. 1A. Overexpression of RKIP was also associated with downregulation of Bcl-xL (Fig. 4A). Cells transfected with the control CMV empty vector did not show any significant differences in either potentialization of apoptosis or Snail or Bcl-xL expression compared with untransfected cells.

To further confirm that the observed sensitization of tumor cells to TRAIL- or CDDP-mediated apoptosis by NPI-0052 was the consequence of NPI-0052–induced RKIP overexpression, we examined whether the inhibition of RKIP expression could reverse the sensitization. The nonmetastatic prostate tumor LNCaP cells express higher RKIP levels than DU-145. LNCaP showed a significant RKIP upregulation starting as early as 4 h posttreatment with NPI-0052 (Fig. 4B). LNCaP cells were first transfected with RKIP siRNA for 24 h followed by treatment with NPI-0052 for 6 h and TRAIL or CDDP treatment for an additional 18 h. Apoptosis was determined at 48 h posttransfection. The combination of NPI-0052 with CDDP or TRAIL sensitized the cells to apoptosis, whereas RKIP siRNA treatment significantly reversed this sensitization (Fig. 4C). Cells transfected with control siRNA retained their sensitization pattern, indicating that RKIP has

Figure 4. Direct role of NPI-0052–induced RKIP expression in tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis. A, RKIP overexpression sensitizes DU-145 to TRAIL- and CDDP-mediated apoptosis. DU-145 cells were transfected for 24 h with a CMV-HA-RKIP construct or the relevant CMV-HA-CNTR empty vector followed by treatment with 3 μg/mL CDDP or 5 ng/mL TRAIL. At 48 h, cells were harvested and an aliquot was used to prepare lysates for Western blot analysis and the remaining cells were subjected to flow cytometry analysis for determination of apoptosis. Top left, representative flow charts with the percentages of apoptotic cells of each experimental condition. The caspase-3–positive plot area was set according to an isotype control. Top right, RKIP and Bcl-xL protein levels were assessed before and after transfection in total cell lysates by Western blot analysis. Representative of three independent and reproducible experiments. Bottom, mean ± SE apoptotic values for each treatment condition from all the performed experiment. *, P < 0.003, single treatment or transfection versus transfection with CMV-RKIP and treatment (Mann-Whitney U test); B, NPI-0052 induces RKIP expression in the nonmetastatic LNCaP cells. RKIP levels were screened in untreated and NPI-0052 (50 nmol/L)–treated lysates for the indicated time points. C, reversal of NPI-0052–induced sensitization of LNCaP cells to TRAIL- or CDDP-mediated apoptosis by RKIP siRNA. LNCaP cells were transfected with 0.5 μg RKIP siRNA or an equivalent amount of control siRNA. Forty-eight hours posttransfection, cells were treated with 50 nmol/L NPI-0052 for 6 h followed by TRAIL (5 ng/mL) or CDDP (3 μg/mL) treatment for an additional 18 h. Apoptosis was determined at 72 h by flow cytometry. Mean ± SE of three independent experiments. *, P < 0.023, single treatment versus combined treatment; **, P < 0.011, double treatment versus double treatment after cell transfection with Snail siRNA (Mann-Whitney U test). Whole-cell lysates were prepared at the same time from identical cell treatments and examined for RKIP and Snail expression by Western blot analysis. Actin was used as internal control.
a specific biological role in TRAIL- and CDDP-mediated apoptosis. Moreover, RKIP silencing resulted in augmentation of Snail expression most likely via preventing NF-κB inhibition (13). The above findings show that RKIP is one of the gene products through which NPI-0052 regulates tumor cell sensitivity to CDDP- or TRAIL-mediated apoptosis.

**Direct role of NPI-0052–induced inhibition of Snail expression in tumor cell sensitization to CDDP- and TRAIL-mediated apoptosis.** The direct involvement of NPI-0052–mediated Snail inhibition in tumor cell sensitization to apoptosis was examined in DU-145 cells (high basal levels of Snail) transfected with Snail siRNA. Snail siRNA sensitizes the cells to TRAIL- or CDDP-mediated apoptosis. Forty-eight hours posttransfection with Snail siRNA, DU-145 cells were treated or left untreated with TRAIL or CDDP for 24 more hours and apoptosis was determined at 72 h. Mean ± SE apoptotic values from three independent experiments. *, P < 0.05, single treatment or transfection versus transfection with Snail siRNA and treatment with CDDP or TRAIL (Mann-Whitney U test). Total cell lysates were also harvested from the same experimental setting and subjected to Western blot analysis for determination of RKIP and Snail protein expression. Actin served as an internal control. Representative of three independent and reproducible experiments. B, double Snail and RKIP knockdown DU-145 cells lose their siSnail-mediated chemosensitization. Wild-type DU-145 cells or Snail siRNA stably transfected cells were further transfected with RKIP or control siRNAs for 48 h and then exposed to CDDP (3 μg/mL) for 24 h. Apoptosis was determined by cleavage of procaspase-3. *, P, RKIP siRNA plus CDDP–treated cells versus single CDDP treatment (Mann-Whitney U test). Lysates from the same experimental setting were subjected to Western blot analysis for the determination of RKIP and Snail protein expression. C, regulation of RKIP promoter activity by Snail. DU-145 cells were cotransfected with Snail siRNA or the relative control siRNA and the pRKIP-Luc wild-type (pRKIP-Luc w/t) or the pRKIP-Luc mutant (pRKIP-Luc mut) promoter construct, which has mutated three of five E-boxes. *, P = 0.02, transfected versus transfected and treated with Snail siRNA.

However, transfection with RKIP siRNA abolished the apoptotic effect of CDDP on those cells (Fig. 5B). Protein analysis also revealed that Snail knockdown DU-145 cells have much higher RKIP levels compared with siLuc and wild-type DU-145 cells (Fig. 5B).

The derived RKIP induction and apoptosis by Snail siRNA were also corroborated by cotransfecting DU-145 cells with Snail siRNA and the RKIP full promoter constructs with wild-type E-boxes (pRKIP-Luc wild-type) or carrying mutations in three of the five E-boxes present in the full promoter sequence (pRKIP-Luc mutant). Figure 5C shows that the pRKIP-Luc wild-type promoter activity is significantly lower in DU-145 cells when compared with the pRKIP-Luc mutant activity, indicating that the lack of the three functional E-boxes in the mutated construct could be responsible for promoter activation (17). In contrast, silencing of Snail by Snail siRNA gives a significant boost in wild-type RKIP promoter activation without affecting significantly the activity of the mutated promoter. Cells transfected with the relative control siRNA gave the same trend of response as untransfected cells. These findings strongly suggest that Snail inhibition and derepression of RKIP transcription and expression by NPI-0052 are involved in the reversal of tumor cell resistance.
Role of NPI-0052-induced mitochondrial membrane potential depolarization and inhibition of antiapoptotic gene products (Bcl-2 family) in tumor cell sensitization to apoptosis. We further examined the effect of NPI-0052 treatment on the regulation of the different apoptotic pathways. DU-145 cells were either treated with NPI-0052 (50 nmol/L) or CDDP (3 μg/mL) or TRAIL (5 ng/mL) as single agents or the combinations and the mitochondrial membrane potential as well as the expression of different caspases and antiapoptotic gene products were assessed. As shown in Fig. 6A, cells exhibited increased mitochondrial membrane depolarization in both combined treatments and in single treatments with NPI-0052. In contrast, only the combination and not the single treatment activated significantly the caspase-8. NPI-0052 induced also some cleavage of caspase-9; however, the combination treatment resulted in a more significant cleavage of caspase-9. Both Bcl-xL and X-linked inhibitor of apoptosis expressions were inhibited by NPI-0052, whereas neither CDDP nor TRAIL had any effect (Fig. 6B). These findings suggest that NPI-0052-induced tumor cell sensitization to TRAIL- or CDDP-mediated apoptosis promotes the activation of type II apoptotic pathways.

Bcl-2 and Bcl-xL have been reported, by us and others, as dominant factors responsible for the acquired resistance of certain tumor cells to chemotherapy and immunotherapy including TRAIL.

Figure 6. NPI-0052 sensitizes tumor cells to apoptosis via activation of the intrinsic apoptotic pathway and inhibition of Bcl-xL expression. A, depolarization of mitochondrial membrane potential by NPI-0052. DU-145 cells were treated either with 50 nmol/L NPI-0052, 3 μg/mL CDDP, or 5 ng/mL TRAIL or the combinations for 24 h using the 3,3′-dihexyloxacarbocyanine dye. Changes in the mitochondrial membrane potential were detected by flow cytometry. Values are expressed as mean fluorescent intensity (MFI) of DiOC₆ incorporation. Mean ± SE of at least three independent experiments. *, P < 0.038, treated versus untreated cells. B, protein lysates derived from DU-145 cells treated as described above were screened by Western blot analysis for caspase-8, caspase-9, Bcl-xL, and X-linked inhibitor of apoptosis (XIAP) expression. Actin served as an internal control. Representative of three independent experiments. The percentages of apoptotic cells derived from each treatment are also presented. C, role of antiapoptotic Bcl-2 family inhibition in DU-145 cells in the sensitization to TRAIL- and CDDP-mediated apoptosis. DU-145 cells were treated with the Bcl-xL inhibitor 2-methoxyantimycin A₃ (2MAM-A₃) for 6 h and then treated with 5 ng/mL TRAIL or 3 μg/mL CDDP for 18 h and analyzed for apoptosis. Left, representative flow charts with the percentages of apoptotic cells under each experimental condition. The caspase-3–positive plot area was set according to isotype control. Right, mean ± SE apoptotic values for each treatment condition. *, P < 0.001, single-cell treatment with CDDP or TRAIL versus combined treatment with 2-methoxyantimycin A₃. All statistical analyses were done using the Mann-Whitney U test. D, schematic diagram representing the role of the NF-κB-Snail-RKIP circuitry in the regulation of tumor cell sensitivity to TRAIL- and CDDP-induced apoptosis by NPI-0052. The constitutively active NF-κB pathway induces high levels of Snail and antiapoptotic gene products and represses RKIP expression, thus conferring to tumor chemoinmunoresistance. NPI-0052 regulates cell survival and apoptosis via inhibition of phosphorylated IκBα degradation and consequently NF-κB inactivation. NPI-0052–mediated NF-κB inhibition leads to the induction of RKIP through downregulation of its transcriptional repressor Snail. NPI-0052–induced RKIP upregulation potentiates further NF-κB inhibition and suppression of NF-κB-regulated antiapoptotic gene targets, thus leading to tumor chemoinmunosensitization.
(15, 29, 32, 33). Thus, because Bcl-xL is inhibited by NPI-0052 (Fig. 6B) and directly by RKIP overexpression (Fig. 4A) or by DHMEQ (Fig. 3A) and all of such treatments sensitize the cells to apoptosis, we hypothesized that the direct inhibition of Bcl-2 family members will mimic NPI-0052 and RKIP for tumor cell sensitization. Indeed, treatment of DU-145 cells with the family inhibitor 2-methoxyantimycin A3 mimicked NPI-0052 and resulted in significant cell sensitivity to TRAIL- and CDDP-mediated apoptosis as shown in Fig. 6C.

Discussion
This study establishes for the first time the NF-κB-Snail-RKIP circuitry as a crucial regulator of tumor cell response to apoptotic stimuli and introduces this loop as a novel target for therapeutic intervention. One such therapeutic agent studied herein is the proteasome inhibitor NPI-0052. Our findings show that the inhibition of Snail and the induction of RKIP are pivotal factors in the NPI-0052-mediated reversal of tumor cell resistance to both CDDP- and TRAIL-induced apoptosis. Both Snail inhibition and RKIP induction triggered by NPI-0052 were a consequence of NF-κB inhibition and, consequently, led to the inhibition of antiapoptotic gene products and sensitizing the tumor cells to apoptosis.

Although proteasome inhibitors such as bortezomib, carfilzomib, and NPI-0052 exert broad effects on cancer cells, including sensitization of resistant clones to apoptotic stimuli (4, 34–37), the underlying mechanisms of sensitization are not totally clear. NPI-0052 as a single agent has shown advantages compared with conventional proteasome inhibitors such as bortezomib and MG132 with regards to a more rapid onset and longer duration of action, wider spectrum of inhibitory effects of the 20S proteasome, greater suppressive effect on NF-κB activation in many tumor cell models, potent apoptotic activity at low concentrations, and ability to reverse tumor resistance to bortezomib in vitro and in vivo (4, 6, 38). In preclinical studies, NPI-0052 was also able to reverse tumor resistance to chemotherapy and TRAIL apoptosis by inhibiting NF-κB (10, 39). This study investigated the underlying mechanism by which NPI-0052 reverses tumor cell resistance to both TRAIL and CDDP.

The direct role of NPI-0052–induced inhibition of NF-κB activity in the reversal of prostate tumor cell resistance to TRAIL- and CDDP-mediated apoptosis was tested with the use of a specific NF-κB inhibitor, DHMEQ, which inhibits the translocation of active NF-κB from the cytoplasm to the nucleus (31). NPI-0052 mimicked DHMEQ in terms of cell sensitization to CDDP and TRAIL apoptosis through suppression of antiapoptotic proteins such as Bcl-xL (40). The constitutive hyperactivation of NF-κB has been widely investigated as a crucial factor in tumor chemoresistance and immunoresistance in several basic and clinical studies (41–43). Direct inhibition of NF-κB by pharmacologic inhibitors or inhibition of pathways that regulate NF-κB have provided significant chemosensitizing effects in vitro and in vivo in various tumor models mainly through inhibition of prosurvival signals (44–46).

Part of the NF-κB antiapoptotic network was recently reported to be the metastasis inducer transcription factor Snail. NF-κB has been identified as a putative transcription activator of Snail through binding to a region between −194 and −78 bp in the human Snail promoter (25). Our findings show that inhibition of NF-κB by DHMEQ in tumor cells resulted in Snail suppression, whereas treatment with NPI-0052 mimicked DHMEQ and led to Snail down-regulation at the mRNA and protein levels. In addition, cell treatment with Snail siRNA sensitized DU-145 cells to CDDP and TRAIL apoptosis, concomitantly with the decrease of Bcl-xL expression, suggesting the direct role of Snail inhibition in NPI-0052–mediated reversal of tumor resistance to apoptosis.

Although Snail has been well established as a key element in the induction of epithelial-to-mesenchymal transition during tumor metastasis, its involvement in tumor resistance to apoptosis is less studied. Snail has been reported to regulate components of the early- to late G1 transition and the G1-S checkpoint and to promote cell survival after genotoxic stress in breast, lung, and pancreatic adenocarcinomas via regulation of several gene products and pathways (47–52). In all cases, depletion of endogenous Snail by RNA interference led to increased sensitivity to DNA damage accompanied by increased expression of the proapoptotic factors identified as targets of Snail. However, the molecular basis of the protective prosurvival function of Snail superfamily members was found to diverge dramatically among different biological contexts, suggesting that there are tissue-specific regulatory mechanisms (49). The above observations are in agreement with our present findings, supporting the inhibition of Snail by NPI-0052 in the reversal of resistance to both CDDP- and TRAIL-induced apoptosis.

As mentioned above, an additional target of Snail is the metastasis suppressor gene product RKIP shown previously to play a role in tumor cell sensitivity to apoptotic stimuli (14, 16, 46). Beach and colleagues (17) have reported that ectopic expression of Snail in the nonmetastatic cancer cell line, LNCaP, significantly decreased RKIP expression, whereas chromatin immunoprecipitation analysis confirmed the suppressive effect of Snail on the RKIP promoter activity. These data are corroborated by our findings here that RKIP levels were increased in DU-145 when the expression of Snail was knocked down either by Snail-specific siRNA, NPI-0052 or DHMEQ. Concomitantly, cell treatment with Snail siRNA resulted in upregulation of the wild-type pRKIP-Luc activity, but there was no enhancement of the mutated pRKIP-Luc activity above the baseline as expected. RKIP overexpression mimicked Snail siRNA or NPI-0052 treatment in the sensitization of tumor cells to CDDP- and TRAIL-mediated apoptosis and in the decrease of Bcl-xL levels. These effects were reversed by the use of RKIP siRNA, thus confirming the direct role of RKIP expression in the regulation of cell sensitivity to apoptotic stimuli. The present findings are in agreement with previous reports by Chatterjee and colleagues (14) and by us (16), which have shown the reversal of prostate tumor cell resistance to drug- and TRAIL-mediated apoptosis by RKIP overexpression, respectively.

The inhibition of caspase-8 and -9 as well as X-linked inhibitor of apoptosis and Bcl-xL after single or combined treatment with NPI-0052 reveals the involvement of type II apoptotic pathways in NPI-0052–induced apoptotic death. The mitochondrial type II pathway appears to be dominant, because the modified expression of the antiapoptotic gene product Bcl-xL has been shown to regulate resistance (53). The findings show that the Bcl-2 family inhibitor 2-methoxyantimycin A3 mimicked NPI-0052 in the cell sensitization to CDDP and TRAIL apoptosis. Thus, the direct suppressive effect of RKIP overexpression or Snail siRNA or DHMEQ on Bcl-xL expression supports the involvement of Bcl-xL inhibition as part of the mechanism of NPI-0052–mediated reversal of resistance.

Collectively, our findings established that the NF-κB-Snail-RKIP circuitry in tumor cells regulates tumor cell resistance to...
apoptotic stimuli. The interregulatory activities of each of the components of this circuitry are schematically depicted in Fig.6D. We show that NPI-0052 inhibits NF-κB and Snail and induces RKIP and each of these gene products regulates tumor cell survival. Overall, the NF-κB-Snail-RKIP feedback loop is established in cancer cells with a dominant net effect in cell survival, resistance, and metastasis and each of its components separately (43, 54–56), and combined, has also an important role in prognosis. Based on the poor toxicity of NPI-0052 on normal cells (peripheral blood mononuclear cells and normal bone marrow; refs. 3, 4), the present findings establish the NPI-0052–mediated dysfunction of the NF-κB-Snail-RKIP circuitry and propose its potential therapeutic application in the reversal of tumor cell resistance to conventional anticancer regimens.

**References**


**Disclosure of Potential Conflicts of Interest**

M. Palladino is an employee of Nereus Pharmaceuticals, Inc. The other authors disclosed no potential conflicts of interest.

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Pivotal Roles of Snail Inhibition and RKIP Induction by the Proteasome Inhibitor NPI-0052 in Tumor Cell Chemoimmunosensitization

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