Chemotherapeutic drugs sensitize cancer cells to TRAIL-mediated apoptosis: up-regulation of DR5 and inhibition of Yin Yang 1

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Abstract

Several chemotherapeutic drugs in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) result in reversal of resistance to TRAIL-mediated apoptosis through up-regulation of DR5 expression. The promoter of DR5 has one putative binding site for the transcription repressor Yin Yang 1 (YY1), and thus, we hypothesized that the sensitizing drugs may inhibit YY1. We have found that treatment of tumor cells with various chemotherapeutic drugs inhibited nuclear factor-κB. We examined whether drugs also inhibit YY1 activity and whether YY1 inhibition correlates with up-regulation of DR5 expression and sensitization of cells to TRAIL-induced apoptosis. The TRAIL- and drug-resistant prostate carcinoma PC-3 cell line was treated with CDDP, VP-16, ADR, and vincristine. DR5 luciferase reporter constructs and small interfering RNA against YY1 were used to determine the role of YY1 in DR5 transcription. Pretreatment of PC-3 cells and other tumor cell lines with various chemotherapeutic drugs sensitized the cells to TRAIL-induced apoptosis concurrently with up-regulation of DR5 expression and inhibition of YY1 expression and its DNA-binding activity. The baseline luciferase activity in PC-3 cells transfected with the wild-type DR5 reporter was significantly augmented in cells transfected with DR5 constructs carrying deletions or mutation in the YY1-binding site. Treatment with drug enhanced DR5 wild-type luciferase activity, with no increase in cells transfected with the YY1-deleted or YY1-mutated constructs. Cells transfected with YY1 small interfering RNA showed up-regulation of DR5 expression and sensitization to TRAIL-mediated apoptosis. The findings provide evidence that drug-induced sensitization of tumor cells to TRAIL is mediated, in part, by inhibition of the transcription repressor YY1 and up-regulation of DR5 expression. Hence, YY1 may be a potential therapeutic target to reverse resistance to TRAIL-induced apoptosis. [Mol Cancer Ther 2007;6(4):1387–99]

Introduction

Chemotherapy and γ-radiation are currently used approaches for cancer control and treatment. Most antineoplastic drugs kill cells predominantly by triggering their apoptotic program (1). The cytotoxic mode of action of alkylating agents and anthracyclines has been considered to be mediated through several different mechanisms, including interactions with DNA to form DNA adducts or complexes formation with DNA by intercalating between DNA base pairs (2). These alterations are capable to activate several signal transduction pathways, including those involving ATR, p53, p73, and mitogen-activated protein kinase, and culminate in the activation of apoptosis (2). The mechanism of action of other classes of antitumor drugs, such as Vinca alkaloids, is more related to the inhibition of microtubule formation in the mitotic spindle, resulting in an arrest of the dividing cells at metaphase (3). DNA damage-mediated apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of chemo-based tumor therapies (4, 5). Alternative therapies have been considered, including both antibody- and cell-mediated immunotherapy, with potential antitumor activity that are mediated by various mechanisms, including the tumor necrosis factor (TNF)-α family [TNF-α, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL/APO-2L); ref. 6]. Thus, targeting death receptors and their respective signaling pathways to trigger apoptosis in drug-resistant tumor cells is currently being evaluated for cancer therapy.

TRAIL is a relatively safe and most promising death ligand for clinical application compared with other death ligands of the TNF-α family. It has been shown to exhibit

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potent tumoricidal activity against a variety of human cancer cell lines *in vitro* and *in vivo* with minimal or no toxicity to nonmalignant human cells (7). TRAIL induces apoptosis in tumor cells by binding to death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 (8). These receptors include an intracellular death domain, which triggers the activation of the caspase signaling cascade after association of ligand with the receptor, with or without the involvement of mitochondria (9). Two decoy receptors for TRAIL have been also identified, the DcR1 and DcR2; however, they cannot induce apoptosis due to the presence of mutations or deletions in their death domains (8).

The majority of breast, prostate, ovarian, lung carcinoma, multiple myeloma, and leukemia cells are resistant to apoptosis induced by TRAIL (1). Resistance of tumor cells to TRAIL seems to occur through the modulation of various molecular targets. These may include differential expression of death receptors, such as low expression of DR4 and DR5, increased surface levels of decoy receptors, constitutively active Akt and nuclear factor-κB (NF-κB), overexpression of antiapoptotic molecules, mutations in apoptotic genes, such as *Bax* and *Bak*, defects in caspase signaling, and caspase inhibition in resistant cells (1, 10). The above observations suggest that the use of TRAIL by itself may not be a viable option to treat TRAIL-resistant tumors. Conventional chemotherapeutic and chemopreventive drugs and radiation have been used as sensitizing agents to enhance the therapeutic potential of TRAIL. Besides γ-radiation, genotoxic drugs, such as ADR, VP-16, and CDDP, sensitize cells to TRAIL concomitant with up-regulation of DR5 expression in a p53-dependent or p53-independent manner (11–13). This finding suggests that other transcription factors may also induce or suppress death receptor expression, especially in cells where DR5 expression is p53 independent. By expressing more of the death receptor, TRAIL-resistant cells may become sensitive to TRAIL (12, 14). TRAIL and drugs may activate distinct and complementary apoptotic pathways leading to synergy. However, the mechanism by which most of the chemotherapeutic drugs sensitize tumor cells to TRAIL-mediated apoptosis is not known.

Studies with sodium butyrate treatment of colon tumors have identified a functional Sp1-binding site that is responsible for regulation of DR5 expression (14). We have also identified another binding site for the transcription factor Yin Yang 1 (YY1) in the DR5 promoter region (−804 to −794 bp; ref. 15). YY1 is a ubiquitously expressed zinc finger transcriptional regulator in numerous viral and cellular genes involved in the control of cell growth, development, differentiation, and tumor suppression (16). Through interplay with various basal transcription factors and other transcriptional regulators, YY1 can exert wide activities at target promoters acting either as an activator, or a repressor, or an initiator binding protein (17, 18). In previous findings, we have reported that Fas expression is negatively regulated by the transcription repressor YY1 via binding of YY1 to the silencer region of the Fas promoter (19). We have also reported that inhibition of NF-κB correlated with inhibition of YY1 (20). Because there is a putative binding site for YY1 in the DR5 promoter, we hypothesized that chemotherapeutic drugs may inhibit YY1 expression or its DNA-binding activity, resulting in up-regulation of DR5 expression and sensitization to TRAIL-induced apoptosis.

This study tested the above hypothesis by using, as experimental model, a human androgen-independent and TRAIL-resistant p53-deficient prostate cancer line PC-3, which can be sensitized by chemotherapeutic drugs (CDDP, VP-16, ADR, and vincristine) to TRAIL-induced apoptosis. The followings were investigated. (*a*) Do the sensitizing drugs up-regulate DR5 expression? (*b*) Do the sensitizing drugs inhibit YY1 expression/activity? (*c*) Is the baseline luciferase reporter activity augmented with constructs with a deleted region for YY1-binding site or a mutated YY1-binding site? (*d*) Does the direct inhibition of YY1 by YY1 small interfering RNA (siRNA) up-regulate DR5 expression and sensitize the cells to TRAIL-induced apoptosis? The present findings validated the tested hypothesis and established YY1 as a regulator of response to TRAIL-induced apoptosis.

Materials and Methods

Cell Lines and Culture Conditions

PC-3 is a human prostate, androgen-independent cell line, whereas Ramos and M202 are human non–Hodgkin's B-cell lymphoma and melanoma cell lines, respectively. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Invitrogen Corp., Carlsbad, CA), 50 IU/mL penicillin, and 50 μg/mL streptomycin (all from Cellgro, Herndon, VA). Cell cultures were maintained at 37°C and 5% CO_2 for incubation. Phenotypically, PC-3 is a relatively P-glycoprotein–positive cell line (21, 22).

Reagents

CDDP, VP-16, and ADR were purchased from Sigma (St. Louis, MO). Vincristine was obtained from Dr. Mizutani (Kyoto University, Kyoto, Japan). Stock solutions of ADR and vincristine were prepared in PBS buffer, whereas CDDP and VP-16 stocks were prepared in DMSO. DHMEQ was kindly provided by Dr. Umezawa (Keio University, Tokyo, Japan; ref. 23). Soluble recombinant human TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ). The NF-κB inhibitor Bay11-7085 (specific inhibitor of IκBα phosphorylation) and the mouse anti-β-actin monoclonal antibody were obtained from Calbiochem (San Francisco, CA). Rabbit anti-DR5 polyclonal antibody was purchased from Axxora, LLC (San Diego, CA). Monoclonal mouse anti-YY1 and horseradish peroxidase–labeled goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase–conjugated goat anti-rabbit IgG and R-phycoerythrin–labeled goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Caltag (Burlingame, CA).

The monoclonal mouse anti-DR5 and polyclonal rabbit anti-YY1 antibodies were obtained from Biosource (Camarillo, CA) and Active Motif, Inc. (Carlsbad, CA), respectively. FITC-labeled anti–active caspase-3 as well as FITC- and R-phycoerythrin–conjugated IgG isotype controls were obtained from PharMingen (San Diego, CA). SureSilencing YY1 siRNA kit and X-tremeGENE siRNA Transfection Reagent were purchased from SuperArray Bioscience Corp. (Frederick, MD) and Roche Diagnostics (Mannheim, Germany), respectively.

Plasmid Constructs

The DR5 wild-type promoter (pDR5 W/T) luciferase reporter plasmid and the pDR5 construct with the 5′-deletion (−605 bp) that excludes the YY1-binding site (pDR5/−605) have been previously characterized (15). The pDR5 reporter missing active YY1-binding sequence (pDR5/YY1 mutant) was generated by using the QuikChange site-directed mutagenesis method. The NF-κB W/T promoter luciferase reporter plasmid was purchased from Invitrogen (Carlsbad, CA).

Cell Treatment

Adherent cells, such as PC-3 or M202, were plated at a density of 3.5×10^5/mL in 24-well plates and left grown overnight in complete medium. Before treatment, cells were synchronized for 18 h in medium supplemented with 0.1% fetal bovine serum. For protein analysis, drug treatment was done in serum-free conditions for 6, 12, 18, or 24 h. Ramos cells were plated at a density of 2×10^5/mL and treated with drugs in complete medium. For the drug-mediated sensitization assays, cells were initially treated with drugs for 6 h followed by addition of TRAIL and incubation for an additional 18 h.

Determination of Apoptosis

Cells were treated initially with drugs and subsequently with TRAIL as described above. After a total of 24 h of treatment, cells were harvested using, where appropriate (adherent cells), 1 mmol/L EDTA in PBS. After incubation with Cytofix/Cytoperm solution (PharMingen) for 30 min at room temperature, the cells were stained with active caspase-3 antibody as previously described (24) and subjected to flow cytometry analysis. FITC-conjugated IgG isotype control served as negative control. Because ADR is autofluorescent with excitation range overlapping partially the FITC excitation region, apoptosis induced by ADR was determined by DNA staining with propidium iodide (excitation in FL3 region). Briefly, ADR-treated cells were incubated with cold 75% ethanol at −20°C for 1 h, washed with PBS, and resuspended in 150 μL of propidium iodide solution consisting of 50 μg/mL propidium iodide (Sigma) and 0.05 mg/mL RNase A (Amersham, Piscataway, NJ) for 30 min of incubation at 37°C. Both analyses were done on an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). For propidium iodide staining, fluorescence channel 3 was used and region markers were drawn for sub-G_0, G_0-G_1, S, and G_2-M cell populations. The sub-G_0 population represents the cells containing DNA hypoploidy, a characteristic of apoptotic cells undergoing DNA fragmentation.

Protein Analysis by Flow Cytometry

Drug-treated or YY1 siRNA–treated and untreated cells were recovered with 1 mmol/L EDTA in PBS, where appropriate (adherent cells), for determination of surface DR5 and intracellular YY1 protein levels by flow cytometry. Extracellular DR5 staining was done using the mouse anti-DR5 polyclonal antibody as primary antibody and the R-phycoerythrin–conjugated goat anti-mouse IgG as secondary antibody. Cells were incubated with the above antibodies in Perm/Wash buffer solution (PharMingen) for 1 h and 30 min, respectively, at room temperature according to the manufacturer's instructions. Intracellular YY1 staining was done after cell permeabilization with Cytofix/Cytoperm solution for 30 min. Cells were initially incubated with a rabbit anti-YY1 monoclonal antibody for 3 h and subsequently with a secondary R-phycoerythrin–labeled goat anti-rabbit IgG antibody for 30 min at room temperature. All samples were analyzed on an Epics XL flow cytometer. Data were processed using the incorporated System II Software, and the mean fluorescence intensity was recorded.

Western Blot Analysis

Cell lysates were prepared from drug-treated and untreated cells using radioimmunoprecipitation assay buffer (Assay Designs, Inc., Ann Arbor, MI) supplemented with one tablet of protease inhibitor mixture (Roche, Indianapolis, IN). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Total protein lysates (20–30 μg) were subjected to electrophoresis in 10% SDS-PAGE, and the resolved proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL) as described previously (24). YY1 and DR5 protein detection was done by overnight incubation of membranes with 1:1,000 dilution of rabbit anti-DR5 polyclonal antibody and mouse anti-YY1 monoclonal antibody, respectively, at 4°C. Dilution (1:5,000) of horseradish peroxidase–conjugated anti-mouse IgG was used as secondary antibody for 30 min of incubation at room temperature. The immunoblots were probed with enhanced chemiluminescence Western blot detection kit (New England Biolabs, Beverly, MA). Densitometric analysis of digitized autographic images was done, and values were normalized against a β-actin loading control.

Electrophoretic Mobility Shift Assay

For determination of YY1 DNA-binding activity before and after treatment, nuclear extracts from 18 h CDDP-treated cells were prepared and analyzed by electrophoretic mobility shift assay as described previously (20).

Transfection with Plasmids

Transfection of cells with pDR5-Luc or pNF-κB-Luc reporter constructs was done in six-well plates using LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). Plasmids (1 or 2 μg) were mixed with 5 μL of the transfection reagent and incubated with 0.4 mL of serum-free medium for 25 min at room temperature. The resulted liposome-DNA mixture was added to cells with 0.8 mL/well of serum-free medium. Twelve hours after transfection, the transfection medium was removed and

fresh medium containing 10% fetal bovine serum was added to allow the recovery of cells for 6 h. Subsequently, the cells were treated or left untreated with different concentrations of CDDP, DHMEQ, or Bay11-7085 for 4 or 18 h in serum-free conditions. Luciferase activity in protein extracts was measured in an analytic luminescence counter according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA). Data were normalized to protein concentration levels using the Bio-Rad protein assay.

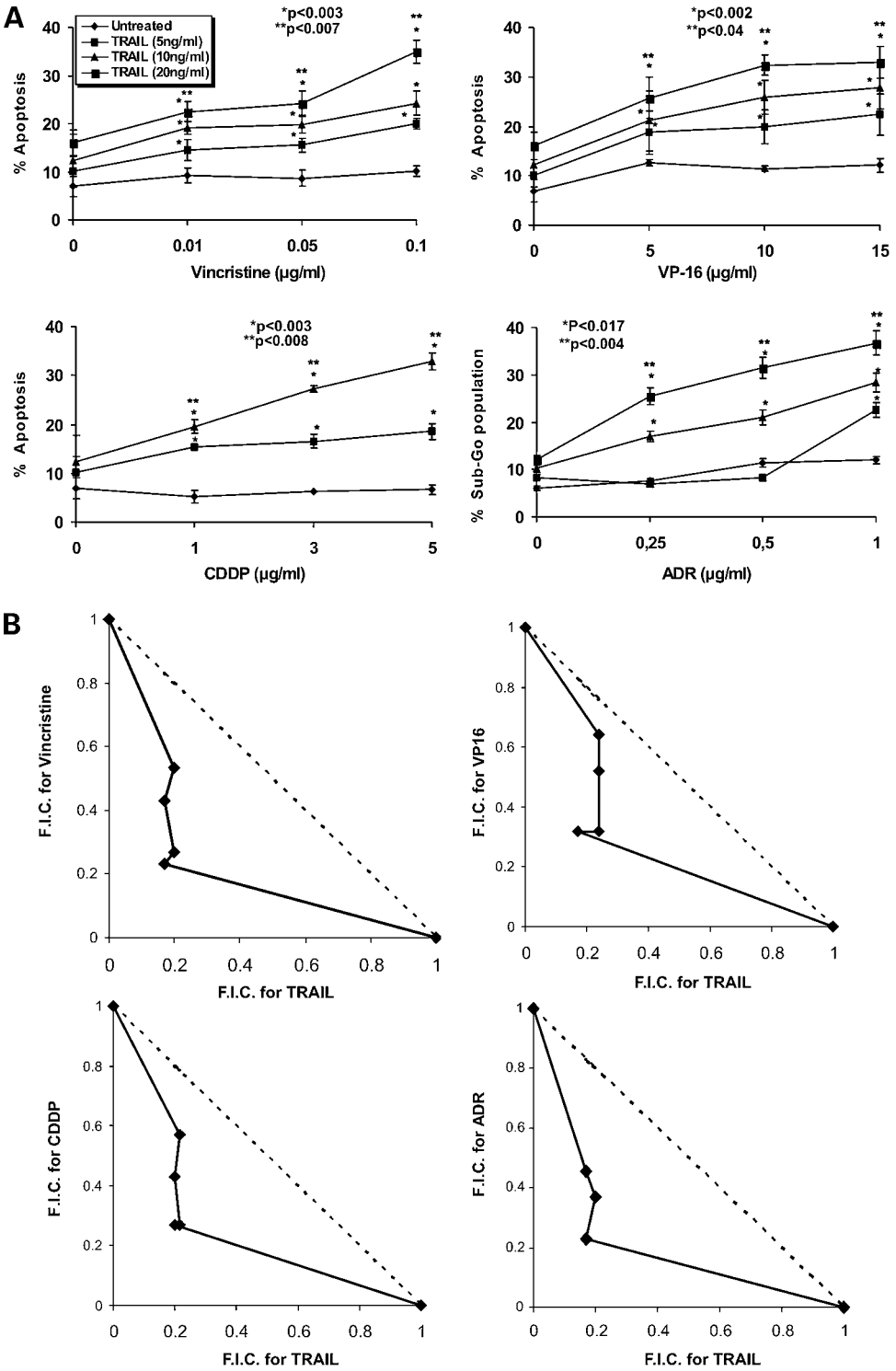

Figure 1. Drug-mediated sensitization of PC-3 cells to TRAIL-induced apoptosis. PC-3 cells were seeded in 24-well plates and subjected to single drug treatment for 6 h followed by 18 h of treatment with 5, 10, and 20 ng/mL of TRAIL. **A,** treated samples were further subjected to flow cytometry for assessment of either active caspase-3 (vincristine-, VP-16–, and CDDP-treated cells) or propidium iodide–based DNA fragmentation (ADR-treated cells). Apoptosis was determined either as % of cells expressing active caspase-3 or as % of sub-G_0 population assessed after propidium iodide staining. **B,** the combination treatment resulted in significant potentiation of apoptosis and synergy was achieved for all the drugs used as indicated by the isobologram analysis. The values on the *Y axis* correspond to apoptotic values obtained without treatment (control) or after single treatment with increasing concentrations of TRAIL. *Points,* mean of at least three independent experiments; *bars,* SEM. *, *P* value: control or single drug or TRAIL treatment versus combined treatment; **, *P* value: TRAIL concentration-dependent increase in apoptosis for each drug concentration used. These *P* values were derived using the Mann Whitney *U* and Kruskal-Wallis H statistical tests, respectively. *F.I.C.,* fractional inhibitory concentration.

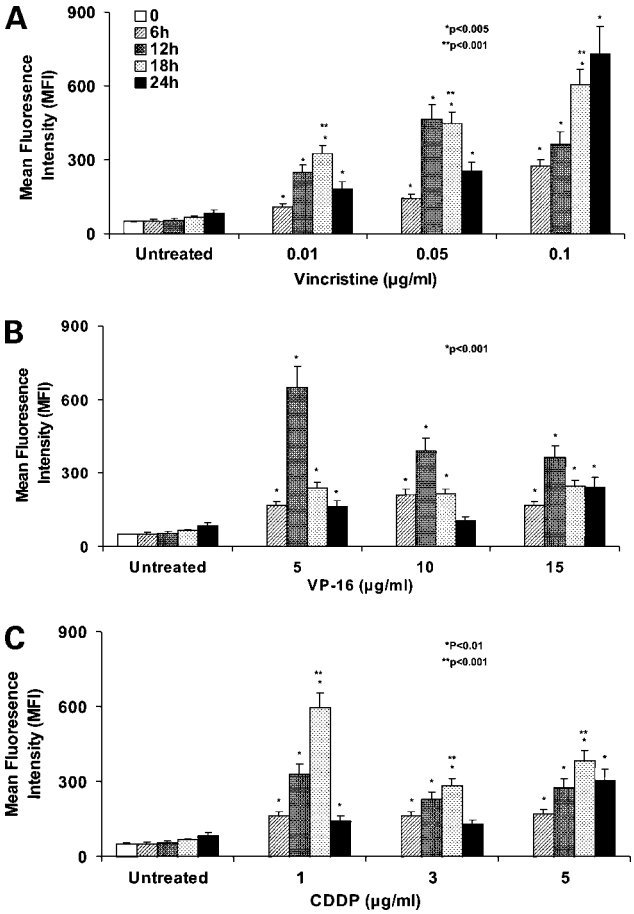

Figure 2. Time kinetic analysis of surface DR5 expression in PC-3 cells treated with drugs. PC-3 cells were incubated with the indicated concentrations of vincristine (**A**), VP-16 (**B**), or CDDP (**C**) for 6, 12, 18, and 24 h. DR5 surface expression was assessed using flow cytometry analysis for each time point. *Columns,* mean fluorescence intensity (*MFI*); *bars,* SEM. *, *P* value: untreated versus drug-treated cells for each time point; **, *P* value: time-dependent (6 – 18 h) increase in DR5 expression for each drug concentration used. *P* values were derived using the Mann Whitney *U* and Kruskal-Wallis H statistical tests, respectively.

Transfection with siRNA

PC-3 cells (0.5×10^4 per well) were plated in a 24-well plate 24 h before transfection. YY1 siRNA (3 μL) or a negative control of siRNA solution was mixed with 6 μL of transfection reagent in reduced serum medium Opti-MEM I (Life Technologies, Invitrogen, Carlsbad, CA), and transfection was done according to the manufacturer's protocol. Surface DR5 expression and YY1 protein levels were detected 36 h after transfection using flow cytometry as described above. For determination of cell sensitization to TRAIL-mediated apoptosis, 24 h after transfection, transfected or untransfected cells were treated for 18 h with TRAIL, CDDP, or the combination in serum-free conditions. Cells were then subjected to anti–active caspase-3-FITC staining by flow cytometry as described above.

Statistical Analyses

All experimental values were first evaluated by the one-sample Kolmogorov-Smirnov goodness of fit test to determine whether they follow a normal distribution pattern. Depending on the results, multiple associations with categorical data were examined using one-way ANOVA or Student's *t* test (after examining for equality of variances with Levene's test) or its nonparametric equivalents Mann-Whitney *U* and Kruskal-Wallis H tests. Significant differences were considered for probabilities <5% ($P < 0.05$). The statistical analysis was done using the software Statistical Package for the Social Sciences.

Synergy

To establish whether the cytotoxic effect of the combination of TRAIL and drug was more than additive, isobologram analysis was done according to Berenbaum (25) using a battery of combinations of TRAIL used in different concentrations with various concentrations of drugs. Cytotoxicity obtained by combinational treatment was plotted as percentage of single agent alone that resulted in the same percentage of cytotoxicity (fractional inhibitory concentration: concentration of each agent in combination/concentration of each agent alone). When the sum of this fraction (fractional inhibitory concentration) is 1, the combination is additive and the graph is geometrically expressed as a straight line; when the sum is <1, the combination is synergistic and the graph appears as concave shape; and when the sum is >1, the combination is antagonistic and the graph is geometrically represented as convex shape.

Results

Cytotoxic Drugs Sensitize PC-3 Cells to TRAIL-Mediated Apoptosis

Human prostate PC-3 cancer cells were used as a model and treated with subtoxic concentrations of either vincristine (0.01, 0.05, and 0.1 μg/mL), VP-16 (5, 10, and 15 μg/mL), CDDP (1, 3, and 5 μg/mL), or ADR (0.25, 0.5, and 1 μg/mL), used as single agents or in combination with various concentrations of recombinant TRAIL (5, 10, and 20 ng/mL) for 24 h. The cells were then examined for apoptosis by assessing caspase-3 activation or DNA fragmentation by propidium iodide staining for ADR using flow cytometry as described in Materials and Methods. The findings show that, whereas single agents showed moderate cytotoxicity, the combination treatment resulted in significant potentiation of apoptosis (Fig. 1A). The extent of apoptosis was a function of the TRAIL and drug concentrations used. Synergy was achieved with all four drugs as determined by isobologram analysis (Fig. 1B). Several experiments described below were done to investigate the mechanism of synergy achieved with drugs and TRAIL.

Drug-Induced Up-regulation of DR5 Expression. The cytotoxic agents used above have been reported to up-regulate DR5 expression in various cell lines *in vitro* (12, 13, 26–28). Hence, we examined PC-3 cells treated for 6, 12, 18, and 24 h with various concentrations of vincristine, VP-16, and CDDP for determination of the surface and total DR5 protein levels using flow cytometry and

Western blot analyses, respectively. All drug-treated cells showed a statistically significant increase in DR5 surface expression (determined as increase in the mean fluorescence intensity) for all the concentrations and incubation periods used (Fig. 2). Time kinetic analyses revealed a progressive augmentation of DR5 surface expression between 6 and 18 h of incubation with vincristine (Fig. 2A) or CDDP ($P < 0.001$; Fig. 2C), whereas for VP-16 DR5 expression peaked 12 h after treatment (Fig. 2B). Furthermore, significant concentration-dependent increase in DR5 expression was observed for vincristine ($P < 0.001$). The data presented in Fig. 2 show differences in the expression of surface DR5 as a function of time and drug concentration used. The differences among the drugs used may reflect differences in their intracellular activities and regulation of various gene products.

The total DR5 protein levels were also found to be significantly elevated after treatment of PC-3 with the above drugs (Fig. 3). Time kinetic analysis for each drug showed a time-dependent increase in DR5 expression, peaking mainly 18 to 24 h after treatment with vincristine (Fig. 3A), CDDP (Fig. 3C), or ADR (Fig. 3D), findings similar to those observed for DR5 surface expression. The data in Fig. 3 are representative of one experiment. The mean DR5 protein levels, expressed as mean arbitrary units ± SEM of three independent experiments, are summarized in Table 1. The findings observed corroborate the findings presented in Fig. 3. In contrast to DR5, assessment of DR4 surface levels in PC-3 cells before and after drug treatment did not reveal any significant changes (data not shown). Additionally, examination of DR4 promoter by computer-based transcription search (Transcription Element Search Software) analysis revealed the absence of YY1 putative binding sites clustered within the promoter region (−1,000 bp from the start codon). Overall, the above findings show drug-mediated up-regulation of surface and total DR5 expression in PC-3 cells.

Drug-Induced Inhibition of Both YY1 Expression and DNA-Binding Activity. The observed up-regulation of DR5 expression by the above sensitizing drugs suggested that such agents may inhibit a transcription repressor of the DR5 promoter. We identified one binding site for the transcription factor YY1 in the basic structure of the DR5 promoter (15). We therefore hypothesized that YY1 may negatively regulate DR5 transcription and YY1 activity may be inhibited by the chemosensitizing agents. Thus, the protein levels of YY1 in PC-3 cells were examined following treatment with different concentrations of ADR (Fig. 4A) or CDDP (Fig. 4B) for 6, 12, 18, and 24 h. There was a significant decrease in YY1 levels induced by both drugs. The lowest YY1 protein levels were observed with the highest drug concentration used. The YY1 inhibition was detected as early as 6 h following treatment with the drugs with the higher reduction observed 12 h after treatment for both CDDP and ADR. These findings correlated with ADR- and CDDP-induced DR5 up-regulation, which was detected mostly 18 to 24 h after treatment. The data in Fig. 4 are representative of one experiment. The mean YY1 levels, expressed as mean arbitrary units ± SEM of three independent experiments, are summarized in Table 1. The findings obtained are consistent with the findings presented in Fig. 4.

The effect of CDDP on the YY1 DNA-binding activity was examined by electrophoretic mobility shift assay. YY1 DNA-binding activity was inhibited as a result of cell treatment with different concentrations of CDDP

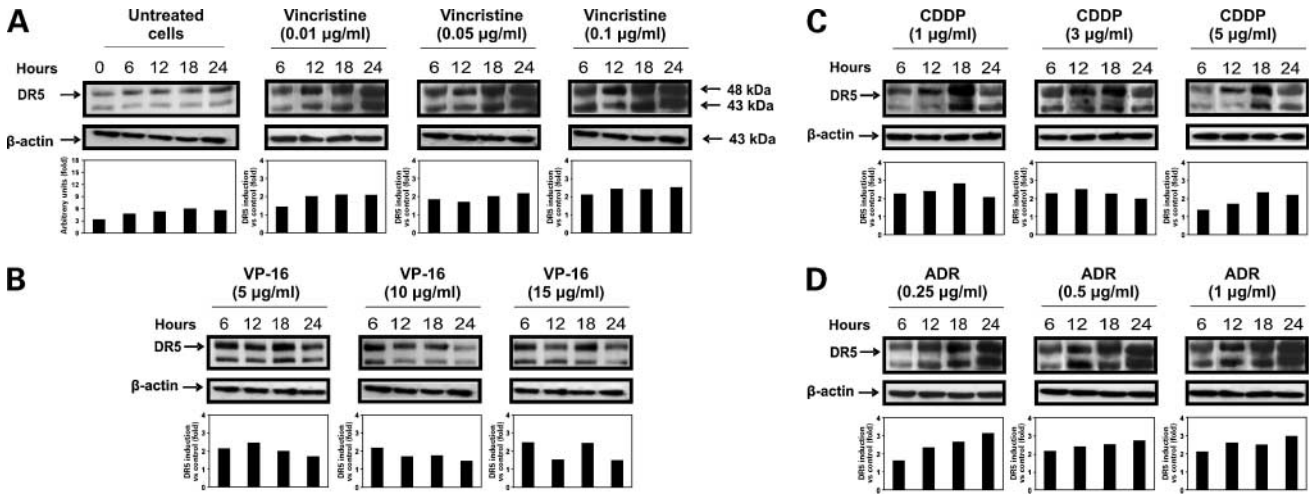

Figure 3. Time kinetic analysis of DR5 protein expression in PC-3 cells treated with drugs. The time course analysis of drug-induced DR5 protein expression was assessed by Western blot. PC-3 cells were treated or left untreated with different concentrations of vincristine (**A**), VP-16 (**B**), CDDP (**C**), or ADR (**D**). Total cellular protein was extracted, separated by SDS-PAGE, and transferred onto nitrocellulose membrane as described in Materials and Methods. The membranes were stained with anti-DR5 monoclonal antibody. Levels of β-actin were used to normalize the protein expression by densitometric analysis. Plots indicate the relative normalized DR5 overexpression compared with normalized untreated control. The 48- and 43-kDa bands correspond to long and short DR5 isoforms, respectively. Blots represent one of three independent experiments.

Table 1. Mean DR5 and YY1 protein expression in PC-3 cells

	Hours of treatment			
	6	12	18	24
A. DR5				
Untreated	4.8 ± 0.15	5.33 ± 0.21	6.07 ± 0.2	5.59 ± 0.24
Vincristine (0.05 μg/mL)	8.88 ± 0.4	9.14 ± 0.22	12.14 ± 0.25	12.24 ± 0.33
*P**	0.02	0.02	0.02	0.019
Untreated	4.8 ± 0.15	5.33 ± 0.21	6.07 ± 0.2	5.59 ± 0.24
VP-16 (5 μg/mL)	10.25 ± 0.31	13.16 ± 0.31	12.18 ± 0.41	9.57 ± 0.21
*P**	0.02	0.021	0.02	0.023
Untreated	4.8 ± 0.15	5.33 ± 0.21	6.07 ± 0.2	5.59 ± 0.24
CDDP (1 μg/mL)	10.82 ± 0.42	12.87 ± 0.41	17.12 ± 1.32	11.58 ± 0.55
*P**	0.021	0.021	0.018	0.02
Untreated	4.8 ± 0.15	5.33 ± 0.21	6.07 ± 0.2	5.59 ± 0.24
ADR (1 μg/mL)	10.19 ± 0.25	13.89 ± 1.31	15.2 ± 0.3	16.65 ± 0.2
*P**	0.021	0.021	0.023	0.017
B. YY1				
Untreated	16.65 ± 0.44	20.67 ± 0.84	14.43 ± 0.82	14.8 ± 0.41
ADR (0.05 μg/mL)	3.41 ± 0.8	4.3 ± 0.85	5.35 ± 0.62	6.9 ± 0.84
*P**	<0.02	<0.02	<0.02	<0.02
Untreated	16.65 ± 0.44	20.67 ± 0.84	14.43 ± 0.82	14.8 ± 0.41
CDDP (1 μg/mL)	7.58 ± 0.42	6.84 ± 0.84	9.71 ± 0.83	8.01 ± 0.81
*P**	0.021	0.021	0.029	0.021

NOTE: Protein determination was assessed by Western blot analysis as described in Materials and Methods. Actin was used as internal control. Values represent the mean arbitrary units ± SEM derived by three independent experiments.
*Asymptomatic significance (two tailed), nonparametric Mann Whitney *U* test.

(Fig. 4C). These observations were corroborated by the use of a YY1 luciferase reporter plasmid. Treatment of transfected PC-3 cells with drugs, such as VP-16, vincristine, CDDP, and ADR, resulted in significant concentration-dependent reduction of YY1 transcriptional activity (data not shown).

The findings observed above with PC-3 were corroborated with two additional model systems (i.e., the non–Hodgkin's lymphoma cell line Ramos and the melanoma cell line M202). In both of these cell lines, we show that treatment with chemotherapeutic drugs sensitized the tumor cells to TRAIL-induced apoptosis. In addition, treatment with the drugs resulted in significant up-regulation of DR5 surface expression and inhibition of YY1 protein levels. The findings are summarized in Table 2.

Negative Regulation of DR5 Expression by YY1. The negative transcriptional regulation of DR5 by YY1 was tested by examining a pDR5 W/T luciferase reporter construct and plasmids, in which the YY1-binding site was either deleted (pDR5/−605) or mutated (pDR5/YY1 mutant). PC-3 cells transfected with pDR5 W/T plasmid expressed a basal luciferase activity, and treatment with CDDP significantly augmented the basal luciferase activity in a concentration-dependent manner. PC-3 cells transfected with the pDR5/−605 or pDR5/YY1 mutant constructs resulted in significant potentiation of the basal luciferase activity in the absence of CDDP; treatment of these transfectants with CDDP did not reveal any statistically significant enhancement of luciferase activity compared with nontreated transfectants (Fig. 5A). These findings suggest that YY1 is implicated in the negative regulation of DR5 transcription.

Correlation between Inhibition of YY1 and Both Up-regulation of DR5 Expression and Sensitization to TRAIL-Induced Apoptosis. It has been shown that inhibition of NF-κB also inhibited YY1 expression (24, 29); thus, inhibition of NF-κB activity may mimic drug-induced inhibition of YY1 and subsequent up-regulation of DR5 transcription. Indeed, treatment of the cells with the specific NF-κB inhibitor DHMEQ resulted in significant augmentation of the DR5 promoter activity comparable with CDDP-mediated activity (Fig. 5A). We then examined if CDDP inhibits NF-κB activity and, hence, YY1, and consequently, if inhibition of YY1 sensitizes the cells to TRAIL-induced apoptosis. Using a wild-type NF-κB promoter luciferase reporter assay, CDDP reduced the NF-κB promoter activity in transfected PC-3 cells in a concentration-dependent manner. Significant inhibition of NF-κB activity was already established after only 4 h of incubation of the cells with increasing concentrations of CDDP (Fig. 5B). The direct role of NF-κB inhibition in CDDP-mediated DR5 up-regulation was corroborated by the use of the NF-κB chemical inhibitor Bay11-7085, which specifically inhibited the promoter activity in our reporter system (Fig. 5B). Other chemotherapeutic drugs tested, such as VP-16 and vincristine, also inhibited the NF-κB promoter activity after 18 h of treatment, and the inhibition was a function of drug concentration used (data not shown).

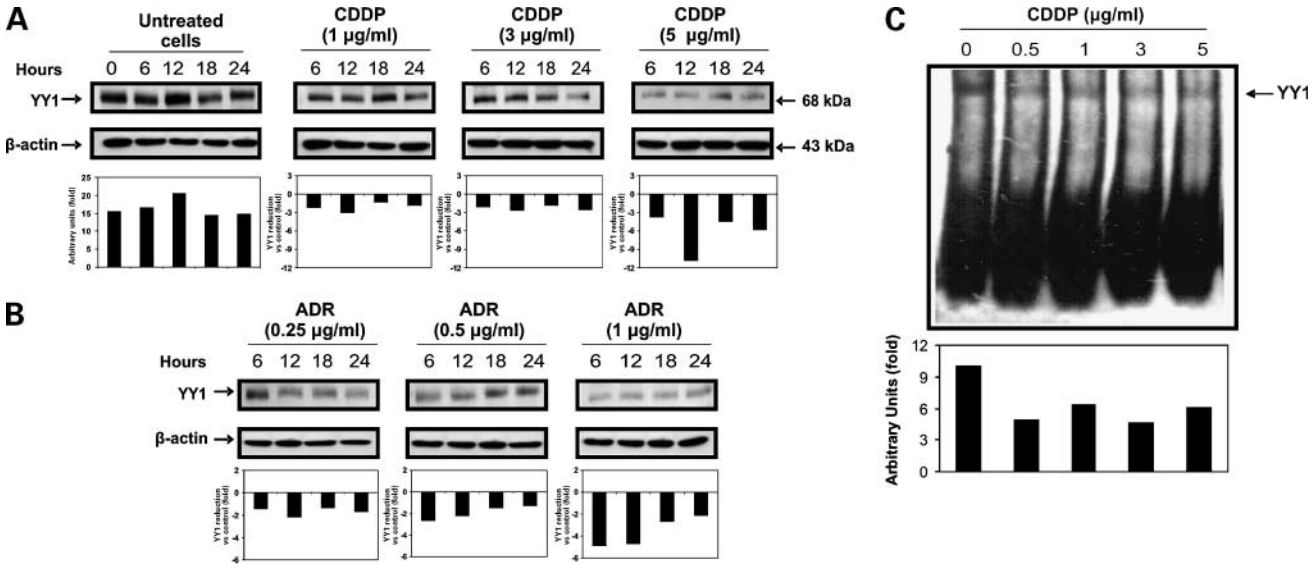

Figure 4. Inhibition of YY1 expression and DNA-binding activity by drugs. Treatment of cells with CDDP (**A**) or ADR (**B**) resulted in YY1 protein down-regulation. Cells were incubated with the indicated drug concentrations for 6, 12, 18, and 24 h, and the extracted protein lysates were subjected to Western blot analysis for YY1 protein determination. Levels of β-actin were used to normalize the protein expression by densitometric analysis. Plots indicate the relative normalized YY1 protein reduction compared with normalized untreated control. **C,** treatment of cells with CDDP also results in reduced YY1 DNA-binding activity. Nuclear extracts were prepared from cells treated with different concentrations of CDDP for 24 h and subjected to electrophoretic mobility shift assay analysis. Extracts from untreated cells served as control. Relative YY1 DNA-binding activity was determined by densitometry and expressed in arbitrary units compared with untreated control. All blots are representative of one of three separate experiments.

The involvement of YY1 in the regulation of DR5 expression and PC-3 sensitivity to TRAIL-induced apoptosis was further examined. PC-3 cells were transfected with a predetermined concentration of siRNA against YY1 mRNA. To confirm the transfection efficiency, we did flow cytometry analysis for determination of YY1 protein levels 36 h after transfection. YY1 protein was found significantly decreased, indicating the siRNA-induced inhibition of YY1 expression ($P < 0.001$; Fig. 5C). Transfection with siRNA control has no effect, thus establishing specificity of the siRNA for YY1. DR5 surface levels were also assessed by flow cytometry 36 h after transfection. There was a significant increase in DR5 expression following inhibition of YY1 by siRNA ($P = 0.001$) compared with nontransfected cells or cells transfected with control siRNA YY1 (Fig. 5D). These findings further confirm the interplay between YY1 suppression and DR5 up-regulation.

To examine the role of YY1 in the sensitization of PC-3 to TRAIL-mediated apoptosis, cells transfected with YY1 siRNA were incubated with TRAIL for 18 h and subjected to flow cytometry for apoptosis determination (Fig. 5E). The findings show a statistically significant augmentation of apoptosis after treatment with a combination of TRAIL and YY1 siRNA compared with treatment with TRAIL alone or YY1 siRNA treatment alone. These findings support the inhibitory role of YY1 in TRAIL-induced apoptosis.

Discussion

The present study provides evidence that treatment of tumor cells with DNA-damaging chemotherapeutic drugs, such as CDDP, ADR, or etoposide, as well as the inhibitor of microtubule formation, vincristine, elicits increased expression of DR5. This correlated with inhibition of the transcription repressor YY1 and sensitization of the cells to TRAIL-induced apoptosis. The combination treatment resulted in synergy in cell death by apoptosis. The observed DR5 up-regulation by the drugs was paralleled by inhibition of the transcription repressor YY1. YY1 protein level and YY1 DNA-binding activity were significantly decreased after treatment of tumor cells with various drugs. The involvement of YY1 in the negative DR5 transcriptional regulation was supported by findings showing significant augmentation of reporter activity using a DR5 promoter luciferase reporter system whereby the YY1 putative binding domain (15) was either mutated or deleted. The findings suggest that drugs inhibit YY1 activity because treatment with drugs also induced enhanced DR5 promoter activity only in wild-type transfectants. Drug-induced inhibition of YY1 may be the result of drug-induced inhibition of NF-κB activity. Like drugs, inhibition of NF-κB by the specific DHMEQ chemical inhibitor inhibited YY1 and up-regulated DR5 promoter activity in agreement with previous findings showing that the NF-κB inhibitor Bay11-7085 sensitizes cells to TRAIL-induced apoptosis (24). The role of YY1 in drug-mediated effect was corroborated by the use of siRNA for YY1, which mimicked drugs and resulted in up-regulation of DR5 and cell sensitization to TRAIL. The present studies introduce YY1 as a new therapeutic target whose modification can sensitize resistant tumor cells to TRAIL-induced apoptosis.

The above findings suggest possible mechanisms underlying the drug-induced sensitization of tumor cells to TRAIL-mediated apoptosis. These mechanisms are consistent with either the direct inhibition of NF-κB activity and function by genotoxic drugs or the direct inhibitory effects of drugs on YY1 expression and DNA-binding activity. These two mechanisms may lead to DR5 up-regulation and apoptosis induction by TRAIL. Alternatively, the drug-induced inhibition of NF-κB may result in inhibition of YY1, as a NF-κB–dependent gene, which in turn results in DR5 overexpression and cell sensitization to TRAIL-induced apoptosis.

Despite the absence of direct evidence in the literature for the direct transcriptional regulation of YY1 by NF-κB, we have previously shown that inhibition of NF-κB by different agents or chemical inhibitors results in suppression of YY1 DNA-binding activity, decreased YY1 protein levels, and reversal of chemoresistance or immunoresistance (19, 22). This study supports these findings and shows that drugs, such as CDDP, VP-16, ADR, and vincristine, inhibit both NF-κB and YY1. Consistent with our findings here, Palayoor et al. (30) have reported that NF-κB is constitutively activated in the hormone-refractory prostate cancer cell lines PC-3 and DU145. The suppression of NF-κB survival signaling by various agents has been shown to sensitize different neoplasms, including prostate tumors, to the antitumor effects of TRAIL (20, 31) via several mechanisms (24, 32, 33). However, many antineoplastic agents, including anthracyclines, paclitaxel, and Vinca alkaloids, have been shown to induce NF-κB activation in human lung adenocarcinoma and other cell lines mainly via activation of protein kinase C, resulting in IκBα degradation (34–36).

As mentioned above, the inhibition of NF-κB by chemical inhibitors or drug inhibitors resulted in inhibition of YY1, up-regulation of DR5 expression, and sensitization to TRAIL-induced apoptosis. Previous findings in ovarian tumor showed that Fas expression was under the negative regulation of NF-κB via the transcription repressor YY1 (19). Putative DNA-binding sites for both NF-κB and YY1 have been identified in the DR5 promoter region (15), suggesting their role in DR5 regulation. Recent studies have proposed the crucial role of the RelA (p65) and c-Rel expression patterns in the DR5 regulation and TRAIL signaling (37, 38). The exact mechanism of NF-κB–mediated DR5 regulation is, however, unknown. It has been shown that the etoposide-induced DR5 expression requires the first intronic region of the *DR5* gene, and mutation of a putative NF-κB–binding site in this intron eliminates DR5 promoter activity, suggesting a direct

Table 2. TRAIL-mediated apoptosis induced by several drugs in Ramos and M202 melanoma cells

		Apoptosis (%)		*P**	DR5 expression	YY1 expression
		TRAIL (ng/mL)				
		0	10			
Ramos	Untreated	6.3 ± 0.9	16.1 ± 0.4		231.5 ± 29.6	633.6 ± 35
	CDDP (1 μg/mL)	12 ± 1.1	42.3 ± 1.1	0.02	550.2 ± 18.7	567.2 ± 41.4
	CDDP (2 μg/mL)	18.1 ± 0.4	47.6 ± 1.7	0.021	602.4 ± 41.5	374 ± 62.4
	P †		0.01		0.018	0.026
	Untreated	6.3 ± 0.9	16.1 ± 2.2		231.5 ± 29.6	633.6 ± 34.9
	ADR (0.1 μg/mL)	10.1 ± 1.2	36.4 ± 1.7	0.021	350 ± 36.3	539.7 ± 22
	ADR (0.5 μg/mL)	14.2 ± 1.4	42.2 ± 2.74	0.021	464.5 ± 41	420 ± 39.7
	P †		0.014		0.017	0.020
M202	Untreated	8.3 ± 1.2	10.3 ± 0.9		170.9 ± 11.8	540.1 ± 25
	VP-16 (5 μg/mL)	15.6 ± 0.8	47.3 ± 3.2	0.02	340.4 ± 39.3	227.88 ± 40.8
	VP-16 (15 μg/mL)	21.3 ± 1.5	48.6 ± 2.1	0.02	420.2 ± 80.1	123.44 ± 20.7
	P †		0.018		0.023	0.012
	Untreated	8.3 ± 1.2	10.3 ± 0.9		170.9 ± 11.8	540.1 ± 25
	CDDP (1 μg/mL)	8.9 ± 1.1	15.8 ± 1.6	0.035	241.96 ± 25.1	446.25 ± 35.3
	CDDP (5 μg/mL)	12.7 ± 0.7	19.2 ± 0.8	0.04	255 ± 4	401.22 ± 37.5
	P †		0.019		0.045	0.019
	Untreated	8.3 ± 1.2	10.3 ± 0.9		170.9 ± 11.8	540.1 ± 2
	Vincristine (0.01 μg/mL)	11.2 ± 1.7	23 ± 1.7	0.021	265 ± 24.7	386.64 ± 24.5
	Vincristine (0.1 μg/mL)	15.8 ± 2.2	28.5 ± 2.2	0.025	281.77 ± 40.9	312.39 ± 32.8
	P †		0.024		0.036	0.015

NOTE: Treatment of Ramos and M202 with the various drugs and TRAIL was done as described in Materials and Methods for PC-3. In addition, DR5 surface expression and intracytoplasmic YY1 protein determination were done following treatment for 18 h by flow cytometry. Apoptosis was determined by active caspase-3 or propidium iodide–based detection methods as described in Materials and Methods. Values represent either mean arbitrary units ± SE for apoptosis determination or mean fluorescence intensity ± SEM for protein expression.

*Kruskal-Wallis H test, degrees of freedom = 2.

NF-κB involvement in DR5 transcriptional regulation (39). Furthermore, an involvement of histone deacetylase 1 in the differential regulation of DR5 by NF-κB has also been proposed (39, 40).

Our findings show that YY1 negatively regulates DR5 transcription. YY1 has been shown to regulate the transcriptional activity of a series of gene promoters, acting either as activator or repressor (16, 41, 42). Negative regulation of death receptors by YY1 has been reported for Fas (19), whereas a putative YY1-binding site, with still unknown function, has also been identified in the mouse p75 TNF receptor promoter (43). Based on our findings with the various DR5 reporter constructs, we provide for the first time evidence suggesting the direct implication of YY1 in the negative regulation of DR5 promoter by presumably binding to the corresponding region in the DR5 promoter, which confers resistance to TRAIL resistance. However, more direct experiments, such as chromatin immunoprecipitation, are needed to show YY1 binding to the DR5 promoter. Inhibition of YY1 protein level and

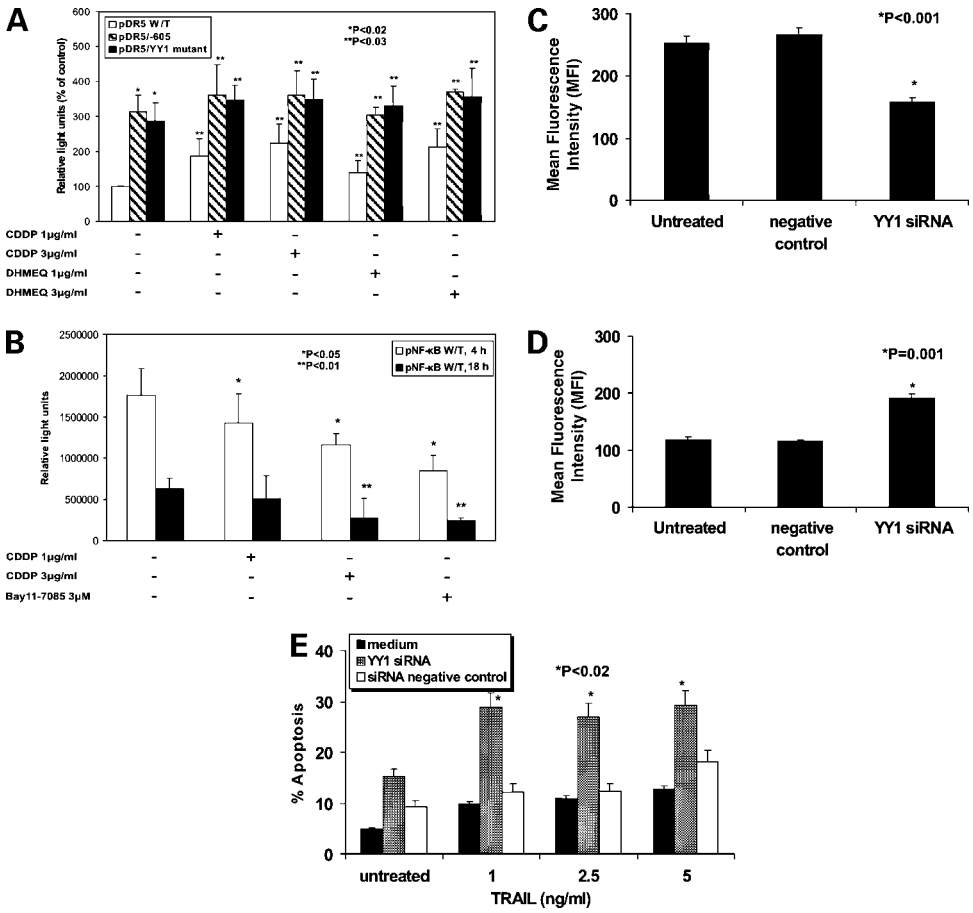

Figure 5. Direct role of YY1 in the negative regulation of DR5 transcription and in resistance to TRAIL-mediated apoptosis. **A,** negative regulation of DR5 transcription by YY1 and CDDP-induced up-regulation of DR5 promoter activity. PC-3 cells were transfected with 1 μg of pDR5 luciferase reporter plasmids (pDR5 W/T, pDR5/YY1 mutant, or pDR5/−605) for 24 h. After transfection, the cells were treated or left untreated with 1 or 3 μg/mL of CDPP or the NF-κB inhibitor DHMEQ for 18 h. The promoter activity was determined by assessment of luciferase activation expressed as relative light units. Values represent the % of control (untreated pDR5 W/T luciferase activity). *, *P* value: control versus pDR5/−605 or pDR5/YY1 mutant; **, *P* value: control versus treated transfectants. **B,** CDDP-induced inhibition of NF-κB promoter activity. Cells were transfected with the pNF-κB W/T-Luc construct for 24 h followed by treatment with CDDP (1 or 3 μg/mL) or Bay11-7085 (3 μg/mL) for an additional 4 or 18 h. Untreated cells served as control. The findings reveal a statistical significant CCDP-induced inhibition of NF-κB promoter activity mainly observed after 4 h treatment with CDDP. Bay11-7085 was used as positive control of NF-κB inhibition. *P* values (* and **): transfected cells versus cells transfected and treated for 4 or 18 h, respectively. **C,** inhibition of YY1 by YY1 siRNA. PC-3 cells were transfected or left untransfected with 3 μL of YY1 siRNA or control siRNA. Thirty-six hours after transfection, YY1 protein levels were found significantly decreased compared with untransfected control (*, *P* value) as assessed by flow cytometry. **D,** DR5 overexpression induced by YY1 siRNA. Thirty-six hours after transfection with YY1 siRNA, DR5 surface overexpression was assessed by flow cytometry. *, *P* value: untreated versus YY1 siRNA treated cultures. Values on **C** and **D** are expressed as mean fluorescence intensity. **E,** determination of cell sensitization to TRAIL-mediated apoptosis after transfection with YY1 siRNA. Twenty-four hours after transfection with YY1 siRNA, PC-3 cells were treated or left untreated with 1, 2.5, or 5 ng/mL of recombinant TRAIL for 18 h. Thereafter, the cells were stained with FITC-labeled anti–active caspase-3 and apoptosis was assessed by flow cytometry. A statistically significant increase in caspase-3 activation after combination treatment of cells with TRAIL and YY1 siRNA was observed. *, *P* value: single TRAIL or YY1 siRNA treatment versus combined treatment. *Columns,* mean of at least three independent experiments; *bars,* SEM. All statistical analyses were done by using one-way ANOVA and confirmed by Mann Whitney *U* test.

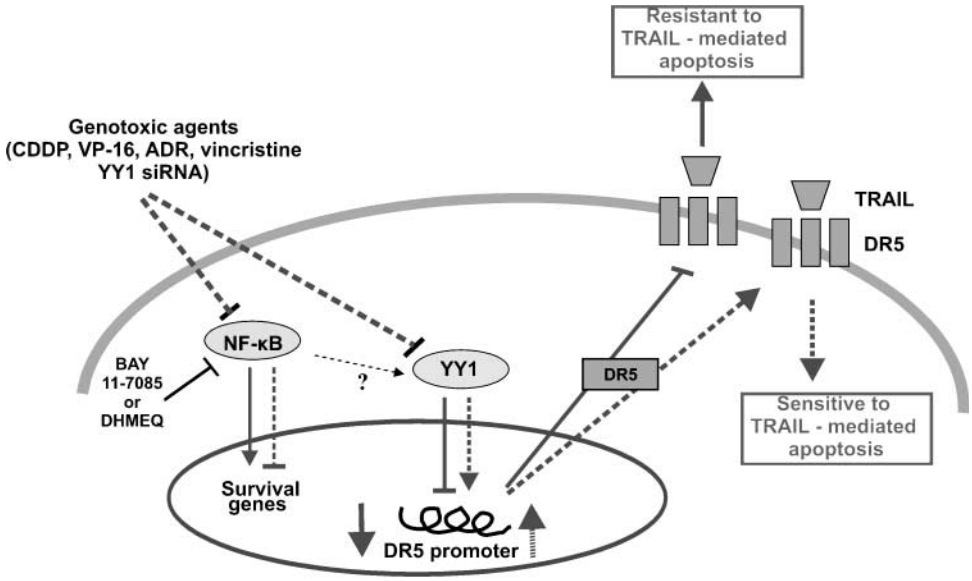

Figure 6. Schematic diagram illustrating the regulation of DR5 expression and the sensitivity to TRAIL-induced apoptosis of tumor cells following sensitization with chemotherapeutic drugs. This diagram illustrates that treatment of tumor cells with drugs results in inhibition of the transcription factors NF-κB and YY1, which in turn lead to up-regulation of DR5 expression and sensitization to TRAIL-induced apoptosis. Findings similar to drugs are achieved following treatment of tumor cells with NF-κB inhibitors, such as Bay11-7085 and DHMEQ. *Solid lines,* untreated tumor cells; *broken lines,* treated tumor cells.

DNA-binding activity following NF-κB suppression results in DR5 up-regulation and TRAIL sensitization. Thus, these findings not only support the notion that YY1 is involved in the transcriptional regulation of a variety of gene promoters but also suggest its direct association with apoptosis. It has been found that, in response to various apoptotic stimuli, including activation of Fas and stimulation by TNF-α or staurosporine and etoposide, YY1 is cleaved by caspases *in vitro* and *in vivo* giving fragments that have lost their transactivation domains; however, they retain their DNA-binding domains and enhance Fas-induced apoptosis via a positive feedback loop mechanism (44). Similar mechanisms could also be activated in TRAIL-mediated apoptosis; thus, the DR5 up-regulation resulting from drug-mediated YY1 inhibition and subsequent induction of apoptosis in the presence of TRAIL could be amplified by the positive implication of the produced YY1 fragments after the initiation of the apoptotic process.

The mechanisms by which different chemotherapeutic drugs or other agents may directly influence YY1 function are not well defined. Recently, we showed that nitric oxide mediates tumor cell sensitization to Fas-mediated apoptosis via inhibition of YY1 by S-nitrosylation (45). Inhibition of YY1 DNA-binding activity has been shown to occur in prostate (24) and ovarian (19) tumor cell lines after treatment with nitric oxide donors or in B-cell non–Hodgkin's lymphoma cell lines after treatment with the chimeric anti-CD20 antibody rituximab (20). The present findings provide additional data supporting the effect of multiple chemotherapeutic drugs on inhibition of YY1 DNA-binding activity and protein levels and the role of YY1 in the mechanism of drug-induced TRAIL-mediated apoptosis. Recently, it was shown that inactivation of endogenous YY1 enhances the accumulation of p53 as well as the expression of p53 target genes in response to DNA damage and sensitizes cells to DNA damage-induced apoptosis (46). This mechanism is unlikely in p53-deficient PC-3 cells. Thus, suppression of YY1 activity could result in tumor cell chemosensitization or immunosensitization to apoptosis via different mechanisms and pathways.

Several lines of evidence imply that YY1 expression and/or activation are associated with tumorigenesis, in addition to its regulatory role in normal biological processes. YY1 overexpression is considered to be an important malignant marker, as it has been found in several tumors, including prostate (47), multiple myeloma, brain, and uterus cervix.[5] As mentioned above, overexpression of YY1 may also regulate resistance and inhibit tumor cell destruction by the host immune system, which may lead to tumor progression. Accordingly, our findings support the notion that YY1 may not only be a useful diagnostic and prognostic marker but also play a role in the regulation of resistance to TRAIL-induced apoptosis. Hence, YY1 could be considered as a new therapeutic target whose modification can reverse resistance.

In summary, we propose a novel mechanism by which chemotherapeutic drugs may sensitize tumor cells to TRAIL-mediated apoptosis through inhibition of the transcription repressor YY1 and up-regulation of DR5 expression. Figure 6 schematically illustrates the drug-induced up-regulation of DR5 and sensitization to TRAIL. The involvement of NF-κB and YY1 as independent or cooperative regulators of DR5 expression seems to play an important role in the mechanism of drug-induced cell response to TRAIL-mediated apoptotic signaling in tumor cells. Thus, we suggest that YY1 and/or gene products

[5] Unpublished data.

implicated in YY1-dependent pathways will become part of a profile of proteins that may be clinically useful for reversal of tumor resistance to chemotherapy or immunotherapy.

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Chemotherapeutic drugs sensitize cancer cells to TRAIL-mediated apoptosis: up-regulation of DR5 and inhibition of Yin Yang 1

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