Antagonism of Cytotoxic Chemotherapy in Neuroblastoma Cell Lines by 13-cis-Retinoic Acid Is Mediated by the Antiapoptotic Bcl-2 Family Proteins

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Abstract

13-cis-Retinoic acid (13-cis-RA) is given at completion of cytotoxic therapy to control minimal residual disease in neuroblastoma. We investigated the effect of combining 13-cis-RA with cytotoxic agents employed in neuroblastoma therapy using a panel of 6 neuroblastoma cell lines. The effect of 13-cis-RA on the mitochondrial apoptotic pathway was studied by flow cytometry, cytotoxicity by DIMSCAN, and protein expression by immunoblotting. Pretreatment and direct combination of 13-cis-RA with etoposide, topotecan, cisplatin, melphalan, or doxorubicin markedly antagonized the cytotoxicity of those agents in 4 out of 6 tested neuroblastoma cell lines, increasing fractional cell survival by 1 to 3 logs. The inhibitory concentration of drugs (IC\textsubscript{50}) increased from clinically achievable levels to nonachievable levels, greater than 5-fold (cisplatin) to greater than 7-fold (etoposide). In SMS-KNCR neuroblastoma cells, 13-cis-RA upregulated expression of Bcl-2 and Bcl-xL RNA and protein, and this was associated with protection from etoposide-mediated apoptosis at the mitochondrial level. A small molecule inhibitor of the Bcl-2 family of proteins (ABT-737) restored mitochondrial membrane potential loss and apoptosis in response to cytotoxic agents in 13-cis-RA treated cells. Prior selection for resistance to RA did not diminish the response to cytotoxic treatment. Thus, combining 13-cis-RA with cytotoxic chemotherapy significantly reduced the cytotoxicity for neuroblastoma \textit{in vitro}, mediated at least in part via the antiapoptotic Bcl-2 family of proteins. Mol Cancer Ther; 9(12); 3164–74. ©2010 AACR.

Introduction

More than 50\% of high-risk neuroblastoma patients develop recurrent progressive disease, often from progression of minimal residual disease remaining after myeloablative therapy (1, 2). Retinoic acid (RA), an agent that induces differentiation and growth arrest of neuroblastoma \textit{in vitro} (1, 3, 4), has been proven clinically active (as 13-cis-retinoic acid) against neuroblastoma minimal residual disease, as evidenced by a significant increase in event-free and overall survival (1–3).

RA, a natural derivative of Vitamin A, is a transcriptional regulator active in embryonic development, causing changes in cell growth and differentiation (4, 5). Many genes relevant to cell proliferation, differentiation, and apoptosis are regulated by the action of RA including \textit{cyclin D} (6), \textit{p21} (7), \textit{p27} (8), \textit{MYCN} (9, 10), \textit{HOX} genes (11), and \textit{Bcl-2} (12, 13). RA exists in several interconvertable isomeric forms: all-\textit{trans}-retinoic acid (ATRA), 9-\textit{cis}-retinoic acid, 11-\textit{cis}-retinoic acid, and 13-\textit{cis}-retinoic acid (13-cis-RA), with ATRA being the predominant natural form \textit{in vivo} (14).

Because of its improved activity against neuroblastoma \textit{in vitro} at clinically achievable concentrations for each agent and superior pharmacological characteristics (15, 16), 13-cis-RA has been employed in neuroblastoma clinical studies. 13-cis-RA has not produced encouraging results against progressive disease, but given at completion of cytotoxic therapy, it can control minimal residual disease, significantly improving event-free survival and overall survival (1, 3, 15), likely because of its ability to induce differentiation and sustained growth arrest of neuroblastoma (10, 17).

In acute promyelocytic leukemia, ATRA was most effective when combined directly with cytotoxic chemotherapy (18), perhaps due to the downregulation of \textit{Bcl-2} by ATRA (14). Therefore, some have advocated direct combination of 13-cis-RA with cytotoxic chemotherapy in neuroblastoma (19). Given the ability of mature neurons to upregulate antiapoptotic molecules (20) and the reports that RA increased \textit{Bcl-2} expression in neuroblastoma and antagonized apoptosis in neuroblastoma (12, 13), we hypothesized that 13-cis-RA might...
antagonize cytotoxic chemotherapy in neuroblastoma cell lines by induction of Bcl-2 family proteins.

Our aim was to determine in the laboratory whether 13-cis-RA antagonizes cytotoxic agents used in treating neuroblastoma and whether such antagonism is related to induction of the Bcl-2 family of proteins.

Materials and Methods

Human neuroblastoma cell lines

The 6 human neuroblastoma cell lines used in this study were obtained from patients at various stages of disease; phase of therapy at establishment, and MYCN amplification status have been previously described (21; Supplementary Table 1). None of the patients whose tumors gave rise to the cell lines used in this study had been treated with a retinoid. Two additional cell lines used in our experiments, SMS-LHN 12xRR and SMS-KCNR 12xRR, were selected for RA resistance and exhibit altered MYCN regulation and diminished growth arrest in response to RA (22). The identity of the cell lines was verified by short tandem repeat loci genotyping (23) using the AmpFISTR Identifier PCR Amplification Kit (ABI), SMS-SAN, SMS-KCNR, SMS-LHN, SMS-LHN 12xRR, and SMS-KCNR 12xRR were all cultured in RPMI-1640 (Mediatech) with 10% fetal bovine serum (FBS; Omega Scientific); CHLA-42, CHLA-79, CHLA-90 were cultured in Iscove’s modified Dulbecco’s medium (Mediatech) supplemented with 3 mmol/L of l-glutamine, insulin, transferrin (5 μg/mL each), 5 ng/mL selenious acid (ITS Culture Supplement, Collaborative Biomiocultural Products), and 20% FBS (complete medium). Cell lines were cultured at 37°C in humidified atmospheric oxygen and 5% CO₂ without antibiotics to facilitate mycoplasma detection. Cells were detached from culture vessels with the use of a modified Puck’s saline A + EDTA (24).

As 13-cis-RA is administered in alternating 2-week cycles, and cytotoxic agents are often administered on days 1 to 3 of a 21-day cycle, possible concurrent administrations of these 2 neuroblastoma treatment regiments may involve several days 13-cis-RA treatment prior to the 3-week chemo cycle. Thus, most of our studies employed a short 13-cis-RA pretreatment followed by concurrent treatment with cytotoxic drugs and 13-cis-RA.

Chemicals, drugs, and antibodies

13-cis-RA (Sigma, St. Louis, MO) was dissolved in ethanol. Sytox blue, 5',6',6'-tetrachloro-1',3',3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; CBIC2; ref. 3), and propidium iodide (PI) were from Sigma Chemical Co. Etoposide (ETO) was from Bedford Laboratories; topotecan (TPT), cisplatin (CDDP), melphalan (L-PAM), and doxorubicin hydrochloride (DOX) were from the National Cancer Institute. ABT-737 was kindly provided by Abbott Laboratories and dissolved in dimethyl sulfoxide (DMSO). Chemical structures of drugs used in this study are shown in Fig. 1. All drugs were sterilized by filtration prior to use. Primary antibodies against β-actin, Bcl-2, Bcl-xL, and cytochrome c and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, antibodies against p53, cytochrome c, and p21 were from BD Pharmingen.

Cytotoxicity assay

Cytotoxicity assays with ETOP, TPT, CDDP, L-PAM, and DOX as single agents or with 13-cis-RA were performed in 96-well plates using a semi-automated digital image microscopy (DIMSCAN) system (25). A clinically relevant concentration of 5 μmol/L 13-cis-RA (26) and cytotoxic drug concentrations spanning the clinically achievable plasma levels reported for each drug were employed (21, 27–33). The maximum clinically achievable concentrations considered for this study were 5 μg/mL for ETOP, 60 ng/mL for DOX, 0.1 μg/mL for CDDP, 10 μg/mL for L-PAM, and 100 ng/mL for TPT (21, 34).

Depending on doubling time, 5,000 to 15,000 cells were seeded per well, 12 replicate wells for each drug concentration. Cells settled overnight before the addition of 13-cis-RA or ethanol control. Each cytotoxic drug was added 48 hours after RA treatment. Cytotoxicity was measured 7 days after cytotoxic drug was added by incubating with fluorescein diacetate (10 μg/mL final concentration) for 20 minutes, followed by 30 μL of eosin-Y (0.5% in normal saline) to quench background fluorescence. Total fluorescence (after digital thresholding) was measured using a DIMSCAN system and results were expressed as the fractional survival of treated cells compared to control cells (25). Statistical differences between cytotoxicity responses to drug single agent and its combination with 13-cis-RA were determined using 2-tailed 2-sample t tests. The drug concentrations cytotoxic or inhibiting growth of 99% of cells (IC99) were calculated using the CalcuSyn software (Version 1.1.1 1996, Biosoft).

Quantitative RT-PCR

RNA was isolated with Trizol Reagent (Invitrogen) according to manufacturer’s protocols. Primer and probes were from Integrated DNA Technologies and sequences are available online at http://www.COGcell.org/suppdata. One-step RT-PCR was prepared in 96-well optical reaction plates (ABI) and each assay was repeated at least 3 times. Sample wells were prepared with 1x AmpTaq Gold DNA polymerase mix, 1x RT enzyme mix (Tagman one-step RT-PCR master mix; ABI), 400 nmol/L forward primer, 400 nmol/L reverse Primer, 200 nmol/L probe, 50 ng total RNA, and DEPC-treated water to a total of 25 μL per reaction. An ABI Prism 7700 sequence detector (Applied Biosystems) was employed using 48°C for 30 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute.
for a total of 40 cycles. Results were normalized against expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Significance was determined using 2-sided Student’s t test in Excel software.

Assessment of mitochondrial potential transition

Unless otherwise stated in figure legends, SMS-KCNR cells were seeded overnight and pretreated with 5 or 10 µmol/L of 13-cis-RA or vehicle for 80 hours, followed by treatment with 13-cis-RA or vehicle, 5 µg/mL of ETOP and/or ABT-737 (0.125 µmol/L or 1.25 µmol/L) for 16 hours, pelleted, resuspended in 1 mL of Puck’s EDTA containing 10 µg/mL of JC-1, and incubated at 37°C for 10 minutes. Dead cells were excluded using 1 µmol/L Sytox blue, a nucleic acid stain emitting at 480 nm that penetrates dead but not live cells (35, 36). JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 ± 10 nm) to red (610 ± 10 nm; ref. 37) measured on a BD LSR-II flow cytometer.

Apoptosis

The proportion of cells with sub-G1 DNA content in response to treatment (a generally accepted measure of apoptotic cell death; ref. 37) was assessed using PI staining or by terminal deoxynucleotidyl transferase–mediated dUTP nick end (TUNEL) assay (38). For PI experiments, cells were treated as described earlier in assessing mitochondrial potential transition. Cells were harvested, washed twice, and then fixed in 70% ethanol by slowly adding 700 µL of 100% ice cold EtOH to a 300 µL Pucks EDTA suspension. The samples were rehydrated in PBS, and treated with RNAse A and 50 µg/mL PI. For TUNEL, cells were treated with 10 µmol/L of 13-cis-RA for 48 hours, followed by 64 hours of 5 µg/mL ETOP in the presence of 13-cis-RA. Harvested cells were fixed and labeled using the APO-Direct kit (BD Pharmigen) as per manufacturer’s protocol. Data were collected with a BD LSR-II flow cytometer.
cytometry using a 488 nm laser and a 610 ± 10 nm (PI) or 525 ± 25 nm (FITC) band pass filters. DNA content was analyzed with ModFit LT 3.0.

Subcellular fractionation

Cytoplasmic fractions for Western blot analysis were isolated using a protocol adapted from Adrain et al. (39); cells were harvested, washed and then permeabilized using a Digitonin (Sigma)-based buffer (250 mmol/L sucrose, 70 mmol/L KCl, 137 mmol/L NaCl, 4.3 mmol/L NaN3PO4, 1.4 mmol/L KH2PO4 (pH 7.2), 100 μmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 2 μg/mL aprotinin, containing 200 μg/mL digitonin) on ice for 5 minutes with occasional light vortexing. Cells were then centrifuged at 1,000 g for 5 minutes at 4°C. Supernatants (cytosolic fractions) were

Figure 2. 13-cis-RA pretreatment antagonized the cytotoxicity of etoposide, alkylating agents, or doxorubicin as single agents. A, dose-response curves to cytotoxic agents as single agents or in combination with 13-cis-RA. Neuroblastoma cell lines were treated with 13-cis-RA for 48 hours and then exposed to ETOP, CDDP, L-PAM, or DOX for another 7 days. The drug concentrations used were as follows: ETOP (μg/mL) 0.1, 0.5, 5, 10; CDDP (μg/mL) 0.1, 1, 3, 6, 10; L-PAM (μg/mL) 1, 3, 6, 10; and DOX (ng/mL) 5, 30, 60, 120. After a total incubation period of 9 days, cytotoxicity was measured using a DIMSCAN system. Results are expressed as the fractional survival of treated cells compared to control cells after 9 days in 5 μmol/L of 13-cis-RA (●), 7 days with the indicated agent (□), or 2 days in 13-cis-RA followed by 7 days with the indicated agent and 13-cis-RA (▲). Error bars indicate SD. Significance was defined as P ≤ 0.05 and significance of the differences between treatments was calculated using 2-tailed 2-sample t tests for the 3 highest drug concentrations. *, statistically significant survival advantage for the combination; (**), significant advantage for the single agent. B, representative dot plots and corresponding percentages showing cells undergoing DNA fragmentation, staining positively by TUNEL stain. SMS-KCNR cells were treated with 13-cis-RA (0.1, 1, 3, 6, 10 μmol/L) and ETOP (5 μmol/L) using 2-tailed Student’s t test. *, significant differences (P ≤ 0.05); 13cRA, 13-cis-RA.
IC99 values (drug concentrations cytotoxic or inhibiting manifested resistance to most of the tested drugs (Table 1).)

CHLA-79, or CHLA-90, likely because these lines already most drugs with the multidrug resistant lines CHLA-42, SMS-KCNR cells for each drug. This was not the case for substantial protective effect on SMS-LHN, SMS-SAN, and achievable concentration range for each agent. As shown of the clinically IC99 higher than the clinically achievable concentration. The protective effect of 13-cis-RA was most dramatic with SMS-KCNR cells, where 13-cis-RA treatment increased IC99 values greater than 5-fold for CDDP, greater than 6-fold for DOX, and greater than 7-fold for ETOP. SMS-LHN, SMS-SAN, and SMS-KCNR cell lines were also tested against TPT (20, 40, 80, 100 ng/mL), giving results similar to those obtained with ETOP (data not shown). A direct combination of ETOP with 13-cis-RA (without 13-cis-RA pretreatment) in SMS-KCNR cells also antagonized the cytotoxic effect of ETOP (Supplementary Fig. 1).

Western blotting

Cell pellets were suspended in 1x RIPA lysis buffer containing 1/100x protease inhibitor cocktail (Sigma) and 1 mmol/L PMSF for 20 minutes on ice. Proteins were fractionated on 4% to 20% or 10% to 20% Tris-Glycine precast gels (Novex), transferred to nitrocellulose membrane (Protran), probed with primary antibodies, then horseradish peroxidase-conjugated secondary antibodies, and were visualized on film using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Western blots were quantified using Un-Scan-It software (Silk Scientific).

Results

13-cis-RA antagonized the cytotoxicity of topoisomerase inhibitors, doxorubicin, and alkylating agents

Neuroblastoma cell lines were pretreated with 5 μmol/L 13-cis-RA for 48 hours followed by a 7-day incubation with topoisomerase inhibitors (etoposide, topotecan), alkylating agents (cisplatin, melphalan), or doxorubicin in the presence of absence of 13-cis-RA. Drug concentrations for the cytotoxicity assays spanned the clinically achievable concentration range for each agent. As shown in Fig. 2A, 13-cis-RA induced statistically significant ($P < 0.05$) shifts in most cytotoxicity curves and had a substantial protective effect on SMS-LHN, SMS-SAN, and SMS-KCNR cells for each drug. This was not the case for most drugs with the multidrug resistant lines CHLA-42, CHLA-79, or CHLA-90, likely because these lines already manifested resistance to most of the tested drugs (Table 1). IC99 values (drug concentrations cytotoxic or inhibiting growth for 99% of cells) with or without 13-cis-RA, (Table 1) increased in the presence of 13-cis-RA from clinically achievable to nonachievable concentrations for 4 out of 6 cell lines.

Some of the statistically significant changes observed in the survival plots would likely not be clinically significant because they did not result in meaningful IC99 value changes. For example, the CHLA-79 response to DOX single agent was calculated to have a significant advantage over the combination at 30 and 60 ng/mL, but this is not meaningful because both treatments have an IC99 higher than the clinically achievable concentration. The protective effect of 13-cis-RA was most dramatic with SMS-KCNR cells, where 13-cis-RA treatment increased IC99 values greater than 5-fold for CDDP, greater than 6-fold for DOX, and greater than 7-fold for ETOP. SMS-LHN, SMS-SAN, and SMS-KCNR cell lines were also tested against TPT (20, 40, 80, 100 ng/mL), giving results similar to those obtained with ETOP (data not shown). A direct combination of ETOP with 13-cis-RA (without 13-cis-RA pretreatment) in SMS-KCNR cells also antagonized the cytotoxic effect of ETOP (Supplementary Fig. 1).

13-cis-RA decreased etoposide-induced apoptosis

We chose the SMS-KCNR cell line and the antagonism by 13-cis-RA of ETOP cytotoxicity for investigating the molecular mechanism(s) involved in this antagonistic interaction. As ETOP is cytotoxic via the p53 and mitochondrial apoptotic pathways (40), we evaluated perturbation of apoptotic events by 13-cis-RA (Fig. 2B and C). Apoptosis (TUNEL assay) in response to ETOP was present in 68% of the cell population (basal 0.5%), whereas 13-cis-RA protected SMS-KCNR cells from etoposide and decreased apoptosis to 4.5%. Cell viability was measured using a hemocytometer and trypan blue exclusion (Fig. 2C), and the results were consistent with the TUNEL data.

Table 1. Treatment with 13-cis-RA increased the IC99 drug concentrations from clinically achievable to nonachievable levels

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Abbreviations: MCA, maximum clinically achievable; N/D, not done.

*<* and > indicate IC99 concentrations lower and higher than the lowest or highest concentration tested, respectively. All numbers are rounded to the first decimal point.
13-cis-RA treatment increased mRNA expression and protein levels of antiapoptotic members of the Bcl-2 family, and protected against etoposide-mediated mitochondrial depolarization

As ATRA has been shown to upregulate Bcl-2 expression in neuroblastoma cell lines (13), we determined whether Bcl-2 upregulation occurred with 13-cis-RA. SMS-KCNR cells were treated with 13-cis-RA, and mRNA expression of Bcl-2 family members, Bcl-2, Bcl-xL, Bcl-xL, MCL1-v1, and MCL1-v2, was measured by real-time RT-PCR (Fig. 3A), whereas protein levels for Bcl-2 and Bcl-xL were measured by Western blotting (Fig. 3C). Average mRNA expression for target genes was normalized to that of the GAPDH housekeeping gene. Downregulation

**Figure 3.** 13-cis-RA treatment altered the expression of antiapoptotic Bcl-2 family proteins and protected neuroblastoma cells from inner mitochondrial membrane potential (ΔΨm) loss and cytochrome c release. A, effects of 13-cis-RA on mRNA expression of antiapoptotic Bcl-2 family proteins. SMS-KCNR cells were treated with 13-cis-RA, and total RNA was used as a template for real time RT-PCR using intron-spanning PCR primer-probe sets for the genes indicated. At least 3 independent assays were conducted for each RNA sample. Results from replicate wells in each assay were averaged and normalized by GAPDH expression, and by the expression in EtOH controls to determine a normalized expression value. Normalized values from different assays were then averaged to determine the relative mean mRNA expression. The error bars shown indicate the SD of the normalized expression values. Two-tailed Student’s t tests were used to compare the sets of normalized expression values for each treatment to those of the EtOH control (relative mean expression equal to 1). *, significant (P < 0.05) differences from the EtOH control; 13cRA, 13-cis-RA. B, cells were pretreated with 5 µmol/L 13-cis-RA or EtOH vehicle, followed by a 16-hour treatment with 5 µg/mL ETOP; treated cells were stained with JC-1 dye and analyzed by flow. Sytox blue counterstain was not used. The bar graphs show mean percentage of cells exhibiting mitochondrial membrane potential loss, by treatment. Cell line name is indicated inside each graph. 13-cis-RA reduced the percent of cells with ΔΨm loss from 26% to 22% (basal 4%) in SMS-LHN and from 31% to 16% (basal 7%) in SMS-KCNR cells. Triplicates from each treatment were compared using 2-sided Student’s t tests. *, significant differences (P < 0.05). C, Western blotting illustrating the effects of 13-cis-RA treatment on antiapoptotic Bcl-2 family protein levels, p53 pathway activation, and cytochrome c release in response to etoposide. SMS-KCNR cells were pretreated with 13-cis-RA for 48 hours and then treated with 5 µg/mL etoposide for the indicated number of hours in the presence of 13-cis-RA. Ethanol was used as vehicle control. Detection of Bcl-2, Bcl-xL, p53 activation and p21 was done using whole cell lysates. Cytochrome c release was detected in cytoplasmic fractions extracted as described. Percent sample viabilities by trypan blue exclusion are indicated below the blot. D, Western blotting showing a lack of effect of 13-cis-RA treatment on p53 pathway activation and the effect on cytochrome c release from the mitochondria in response to cisplatin. SMS-KCNR cells were pretreated with 10 µmol/L of 13-cis-RA for 48 hours; they were then treated with 1 µg/mL of CDDP for 24 hours in the presence of 13-cis-RA (control samples with 13-cis-RA or vehicle were treated for a total of 72 hours). Accumulation of p53 was detected in whole cell lysates. The cytoplasmic fraction was extracted as described and was used to assay for cytochrome c release. Percent sample viabilities by trypan blue exclusion are indicated below the graph.
of MYCN mRNA in response to 13-cis-RA has been observed previously (22) and was used as an indicator of molecular responsiveness to 13-cis-RA.

The mRNA expression of Bcl-2, Bcl-xl, Mcl1-v1, and Mcl1-v2 was significantly ($P \leq 0.05$) increased after 48 or 80 hours of 13-cis-RA treatment. RT-PCR data for Bcl-2 and Bcl-xl expressions were confirmed at the protein level by Western blot in cells treated with 13-cis-RA for 72 hours. These 2 proteins were also found to be highly expressed when cells were subsequently treated with 5 $\mu$g/mL ETOP in the presence of 13-cis-RA (Fig. 3C), although Bcl-xl upregulation was both more consistent and pronounced.

Increased Bcl-2 family expression was associated with protection against ETOP-mediated inner mitochondrial membrane potential ($\Delta \Psi_{m}$) loss as indicated by JC-1 flow cytometric analysis (Fig. 3B). A 48-hour pretreatment with 5 $\mu$mol/L of 13-cis-RA reduced the percent cells with $\Delta \Psi_{m}$ loss in response to ETOP from 26% to 22% (basal 4%) in SMS-LHN and from 31% to 16% (basal 7%) in SMS-KCNR cells.

Cells pretreated with 13-cis-RA activated the p53 pathway in response to etoposide or cisplatin but did not release cytochrome c from the mitochondria

We evaluated p53 pathway activation and mitochondrial cytochrome c release in response to ETOP or CDDP. 13-cis-RA did not affect p53 activation in response to either drug, as indicated by p53 levels and p21 accumulation (Fig. 3C and D; Supplementary Figs. 2 and 3). However, 13-cis-RA inhibited cytochrome c release in response to both ETOP and CDDP. Cell viability (trypan blue exclusion) inversely correlated with cytochrome c release.

The protective effect of 13-cis-RA is lost when cells were treated with the Bcl-2 family inhibitor ABT-737

Given that 13-cis-RA inhibits $\Delta \Psi_{m}$ loss in response to etoposide (Fig. 3B), we determined whether the effect was reversible by the small-molecule Bcl-2 family inhibitor ABT-737. SMS-KCNR cells were treated with 10 $\mu$mol/L of 13-cis-RA for 80 hours (to maximally stimulate the cells), incubated for 16 hours with ETOP and/or ABT-737 in the presence or absence of 13-cis-RA, stained with JC-1, and analyzed by flow cytometry. 13-cis-RA decreased the percentage of cells exhibiting mitochondrial depolarization in response to ETOP from 38% to 8% (basal level = 12%), but 1.25 $\mu$mol/L of ABT-737 restored it to 52% (Fig. 4A and B). Similar responses were noted with 0.125 $\mu$mol/L of ABT-737 (data not shown). To exclude the possibility that 13-cis-RA mediates additional apoptotic blocks downstream of the mitochondria, we also measured the accumulation of cells with sub-G$_1$ DNA content in aliquots of the above samples (Fig. 4C and D). 13-cis-RA decreased apoptotic (sub-G$_1$) cells in response to ETOP from 58% to 18% (basal 17%), whereas ABT-737 treatment reversed the antagonistic effect of 13-cis-RA, resulting in 74% apoptosis. These findings suggest that...
13-cis-RA antagonized apoptotic cell death via the mitochondrial pathway.

**13-cis-RA protected retinoic acid-resistant cell lines from cytotoxic agents**

We determined whether neuroblastoma cells selected for RA resistance (22) differ in their response to cytotoxic treatment in the presence or absence of 13-cis-RA by conducting DIMSCAN cytotoxicity assays with SMS-LHN 12xRR and SMS-KCNR 12xRR cells. Cells were treated with 5 µmol/L 13-cis-RA for 48 hours followed by a 7-day treatment with ETOP, CDDP, or L-PAM in the presence of 13-cis-RA. Cell survival was significantly ($P < 0.05$) increased when treatment included 13-cis-RA (Fig. 5A). This protective effect was also associated with Bcl-2 and Bcl-xL protein upregulation, following treatment with 5 µmol/L 13-cis-RA (Fig. 5B; Supplementary Fig. 4). It should be noted that SMS-LHN 12xRR, which showed greater increase in Bcl-2 and Bcl-xL protein expression than the parental cells, also showed a greater shift in cytotoxicity curves in response to RA (Figs. 2A and 5).

**Resistance to 13-cis-RA did not induce resistance to etoposide, cisplatin, or melphalan**

To determine whether prior RA treatment provided a sustained protection against subsequent treatment with cytotoxic chemotherapy, we compared cytotoxicity of ETOP, CDDP, or L-PAM for SMS-LHN and SMS-KCNR versus cell line variants selected for RA resistance (SMS-LHN 12xRR and SMS-KCNR 12xRR) (Fig. 6). Neuroblastoma cells selected for resistance to RA did not manifest cross-resistance to any of the cytotoxic agents tested. Moreover, for SMS-LHN cells, resistance to RA resulted in a collateral, statistically significant ($P < 0.05$), increase in sensitivity to the agents tested.

![Figure 5](https://example.com/f5.png)

**Figure 5.** 13-cis-RA protects RA-resistant cell lines from etoposide, cisplatin, or melphalan as single agents; the effect correlates with upregulation of Bcl-2 and Bcl-xL. A, dose-response curves in response to cytotoxic agents as single agents or in combination with 13-cis-RA. Neuroblastoma cell lines selected for RA resistance were treated with 13-cis-RA for 48 hours and then exposed to one of ETOP, CDDP, or L-PAM for an additional 7 days. The drug concentrations used were as follows: ETOP ($\mu$g/mL) 1.25, 2.5, 5, 10; CDDP ($\mu$g/mL) 0.1, 2, 5, 10; and L-PAM ($\mu$g/mL) 1, 3, 6, 9. After a total incubation period of 9 days, cytotoxicity was measured using a DIMSCAN system. Results are expressed as the fractional survival of treated cells compared to untreated controls after 9 days in 5 µmol/L of 13-cis-RA (*), 7 days with the indicated agent (○), or 2 days in 13-cis-RA followed by 7 days with the indicated agent and 13-cis-RA (▲). Error bars indicate SD. We used 2-tailed t tests to determine significant differences ($P < 0.05$) between responses for the 3 highest drug concentrations with or without 13-cis-RA. *, significant differences between treatments. B, cells were treated with 5 µmol/L 13-cis-RA for 72 hours; whole cell lysates were used to detect Bcl-2 and Bcl-xL by Western blotting. Cell line names are indicated above the blots.
**Description**

Figure 6. Resistance to RA does not protect against etoposide, cisplatin, or melphalan cytotoxicity. SMS-LHN and SMS-KCNR cell lines and the corresponding cell lines selected for RA resistance (SMS-LHN 12xRR, SMS-KCNR 12xRR) were treated with ETOP, CDDP, or L-PAM for 7 days. The drug concentrations used were as follows: ETOP (μg/mL) 1.25, 2.5, 5, 10; CDDP (μg/mL) 0.1, 2, 5, 10; and L-PAM (μg/mL) 1, 3, 6, 9. Cytotoxicity was measured using a DIMSCAN system. Results are expressed as the fractional survival of treated cells compared to control cells. Parental cell lines are indicated by (●). Error bars indicate SD. Two-tailed t tests were used to determine significant differences (P < 0.05) between plots for the 3 highest drug concentrations. *: significant differences between responses.

**Discussion**

Improved survival of high-risk neuroblastoma, with 13-cis-RA treatment (1, 3, 15), has raised the question of whether combining 13-cis-RA with cytotoxic chemotherapy would be beneficial. This study addresses this highly relevant question. Using the DIMSCAN cytotoxicity assay, we were able to show that after a pretreatment with 13-cis-RA, concomitant exposure to 13-cis-RA significantly antagonized the cytotoxicity of ETOP, TPT, CDDP, L-PAM, or DOX, and adversely affected IC50 values in 4 of 6 tested neuroblastoma cell lines. This same antagonism was observed when ETOP was directly combined with 13-cis-RA in SMS-KCNR cells.

We also showed that treatment with 13-cis-RA increased the expression of antiapoptotic Bcl-2 family proteins, which was associated with blocked apoptotic cell death at the mitochondrial level. Changes in Bcl-xL expression were the most consistent and pronounced in response to 13-cis-RA. The antagonism of the cytotoxic agents by 13-cis-RA was reversed by ABT-737 (41), a small molecule inhibitor of Bcl-2 and Bcl-xL.

Induction of apoptotic cell death is a major mechanism by which cytotoxic agents (such as DNA damaging drugs) kill tumor cells (42, 43), and upregulation of antiapoptotic Bcl-2 members by 13-cis-RA likely contributes to decreased sensitivity to cytotoxic treatment by inhibiting the mitochondrial apoptotic pathway (44).

Our observation that 13-cis-RA increased Bcl-2 family expression is consistent with a previous report on neuroblastoma response to ATRA (12), and could be a biological feature of normal neuronal development. Apoptosis of neuroblasts that fail to achieve synaptic connections is integral to viable neural network development (45), whereas mature neurons must last a lifetime and, therefore, have an inherently high threshold for apoptotic death. Neuronal survival is promoted by upregulation of antiapoptotic Bcl-2 family proteins (20).

Our experiments focused on 13-cis-RA, the retinoid used clinically in neuroblastoma, and showed antagonism of activity for several cytotoxic drugs used in neuroblastoma treatment. It is important to note that the antagonism of cytotoxicity by 13-cis-RA was also observed with cell lines selected for RA resistance in vitro and with more than 1 class of compounds: topoisomerase inhibitors, alkylating agents, and an anthracycline.

Our in vitro data strongly suggest that directly combining the differentiating agent 13-cis-RA with cytotoxic drugs in the clinic could have an adverse effect on tumor response to the cytotoxic agents. As there is no reason to expect that the antagonism observed in vitro would not also be operative in vivo, and as showing significant antagonism requires studying dose–response curves, further studies of the observed antagonism using in vivo models would not be informative or justified.

Even though our data indicate antagonism of conventional cytotoxic chemotherapy by an overlapping treatment of 13-cis-RA with cytotoxic agents, prior treatment with 13-cis-RA in which a substantial interval exists between 13-cis-RA exposure and treatment with cytotoxic agents does not appear to compromise subsequent cytotoxic therapy. For example, our data from in vitro-selected RA-resistant cell lines show that resistance to RA does not result in cross-resistance to cytotoxic chemotherapy. In fact 1 line selected for RA resistance (SMS-LHN RR) exhibited increased sensitivity to the cytotoxic agents tested, a response similar to the effect of the cytotoxic retinoid fenretinide on RA-resistant cell lines (22).
In conclusion, our data indicate that overlapping treatments with 13-cis-retinoic acid and cytotoxic agents could diminish tumor response to cytotoxic drugs. Although not investigated in this study, these data also suggest that overlapping treatment of 13-cis RA with radiation should also be avoided unless data are obtained ruling out an antagonistic interaction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Antagonism of Cytotoxic Chemotherapy in Neuroblastoma Cell Lines by 13- cis-Retinoic Acid Is Mediated by the Antiapoptotic Bcl-2 Family Proteins

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