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Growth and DNA Damage-Inducible Transcription Factor 153 Mediates Apoptosis in Response to Fenretinide but Not Synergy between Fenretinide and Chemotherapeutic Drugs in Neuroblastoma

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ABSTRACT

Fenretinide induces apoptosis of neuroblastoma cells in vitro and interacts synergistically with the chemotherapeutic drugs cisplatin and etoposide. The stress-inducible transcription factor known as growth and DNA damage (GADD)-inducible transcription factor 153 is induced in response to fenretinide and in other cell types modulates apoptosis via pro- and antiapoptotic members of the BCL2 family. Because BCL2-family proteins are important in apoptosis induced by chemotherapeutic drugs, GADD153 may be a key mediator of synergy between fenretinide and chemotherapeutic drugs. To investigate this, GADD153 cDNA in sense and antisense orientations was stably transfected into SH-SY5Y neuroblastoma cells using a tetracycline-inducible vector. Increased expression of GADD153 raised the background level of apoptosis and increased apoptosis induced by fenretinide or the chemotherapeutic drugs cisplatin and etoposide. However, there was no increase in synergy between fenretinide and chemotherapeutic drugs. Conversely, expression of antisense-GADD153 virtually abolished the induction of apoptosis in response to fenretinide but overall had no significant effect on apoptosis induced by chemotherapeutic drugs. The effect of antisense-GADD153 on synergy between chemotherapeutic drugs and fenretinide varied with the drug used: there was no effect on synergy between fenretinide and cisplatin, but the combination of fenretinide with etoposide became antagonistic. These results suggest that mechanisms mediating synergy between fenretinide and chemotherapeutic drugs lie upstream of GADD153.

Neuroblastoma accounts for approximately 15% of all childhood cancer deaths (Maris and Matthay, 1999), and despite intensive treatment, only 25% of children with stage 4 disease over the age of 1 survive. The chemotherapeutic agents cisplatin and etoposide are used internationally to treat patients with high-risk neuroblastoma. Although retinoic acid inhibits responses to chemotherapeutic drugs (Lasorella et al., 1995), when used to treat residual disease after myeloablative therapy and hemopoietic stem cell rescue, 13-*cis* retinoic acid substantially increases event-free survival (Matthay et al., 1999). Unlike 13-*cis* or all-*trans* retinoic acid, some retinoid analogs may be more effective at inducing apoptosis of neuroblastoma rather than differentiation (Meister et al., 1998; Maurer et al., 1999). This is the case with fenretinide, a retinoid in which the terminal carboxyl group of retinoic acid has been replaced by an amide group. Fenretinide is clinically well tolerated and is currently undergoing clinical trials for neuroblastoma and other cancers (Reynolds and Lemons, 2001). A significant factor in the further development of fenretinide for clinical use is the observation that it interacts synergistically with chemotherapeutic drugs, giving a response greater than the additive effects of fenretinide and chemotherapeutic drugs separately (Lovat et al., 2000b). In this respect, fenretinide shows very different properties to retinoic acid, and these would allow fenretinide to be combined with chemotherapeutic drugs for clinical treatment. Furthermore, identifying the mechanism

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ABBREVIATIONS: RAR, retinoic acid receptor; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; GADD153, growth and DNA damage-inducible transcription factor 153; GLM, general linear model; PBS, phosphate-buffered saline; ROS, reactive oxygen species; Tet, tetracycline; FenR, fenretinide; CDDP, cisplatin; etop, etoposide.

of synergy may reveal new targets for the development of anticancer drugs.

The signaling events of fenretinide-induced apoptosis in neuroblastoma cells involve mitochondria and are caspase-dependent (Lovat et al., 2000a). Current evidence suggests that, in neuroblastoma cells, fenretinide-induced apoptosis is mediated by a retinoic acid receptor (RAR) pathway and a RAR-independent pathway acting together (Lovat et al., 2000a). The latter pathway is dependent on the generation of reactive oxygen species (ROS), involving 12-lipoxygenase and a sustained induction of the growth and DNA damage (GADD)-inducible transcription factor 153 (Lovat et al., 2002). The treatment of some cell types with chemotherapeutic drugs also increases GADD153 levels (Eymin et al., 1997; Los et al., 1999; de las Alas et al., 2000); however, a sustained increase in GADD153 comparable with that achieved with fenretinide has not been observed in SH-SY5Y neuroblastoma cells in response to chemotherapeutic drugs, and we have suggested that fenretinide and chemotherapeutic drugs induce apoptosis in neuroblastoma cells predominantly by p53-independent and -dependent mechanisms, respectively (Lovat et al., 2002).

GADD153 is a member of the CCAAT/enhancer-binding protein family of transcription factors (Wang et al., 1996); under normal physiological conditions, it is either not expressed or expressed at low levels, but it is strongly induced, at a transcriptional level, in response to endoplasmic reticulum (ER) stress (Barone et al., 1994). That GADD153 can play a central role in the induction of apoptosis is supported by the fact that growth arrest and apoptosis results from overexpression of GADD153 (Matsumoto et al., 1996; Gotoh et al., 2002; Oyadomari et al., 2002). In prostrate cancer cells, activation of GADD153 results in the dephosphorylation of the proapoptotic protein Bad, a BH3-domain–containing member of the BCL2 family, and its subsequent translocation to the nucleus and the mitochondria (Tombal et al., 2000). In other cells, GADD153 down-regulates the antiapoptotic protein BCL2, thereby sensitizing the cells to ER stress (McCullough et al., 2001). Clearly, GADD153 displays important regulatory interactions with members of the BCL2 family. Because pro- and antiapoptotic members of this family are central elements in apoptotic pathways, GADD153 may play a key role in the synergism between fenretinide and chemotherapeutic drugs. This idea is supported by studies in gastric cancer cells where overexpression of GADD153 increases sensitivity to cisplatin-induced apoptosis (Kim et al., 1999). Therefore, the aim of this study was to test the hypothesis that GADD153 mediates the synergistic interaction between fenretinide and chemotherapeutic drugs. Two key predictions from the hypothesis are that increasing GADD153 expression will enhance synergy between fenretinide and chemotherapeutic drugs and that blocking GADD153 expression will abrogate this synergism.

Materials and Methods

Growth of Human Neuroblastoma Cell Lines and Treatment with Retinoids and Chemotherapeutic Reagents. The human neuroblastoma cell line SH-SY5Y (Biedler et al., 1973) or SH-SY5Y cells stably transfected with tetracycline-inducible sense (GADD S 7) or antisense (GADD AS 8) GADD153 cDNA (Lovat et al., 2002) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Invitrogen) (culture medium) in a humidified atmosphere of 5% CO_2 in air. For all experiments, cells were seeded overnight before treatment. The seeding density varied according to the type of experiment; for apoptosis experiments with chemotherapeutic drugs (cisplatin, etoposide, or carboplatin) and fenretinide, 0.4×10^6 cells were seeded into 100-mm tissue culture-grade Petri dishes (Costar, Cambridge, MA) in 10 ml of culture medium, or, for experiments with inducible sense-GADD153 or antisense-GADD153 clones, into 25-cm^2 tissue culture flasks (Costar) in 5 ml of culture medium. Expression of the sense-GADD153 or antisense-GADD153 cDNA was induced by adding tetracycline to 1 μg/ml for 24 h before treatment with fenretinide and or chemotherapeutic agents, as described previously (Lovat et al., 2002). Fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) was added to cultures in ethanol at given concentrations, and an equal volume of ethanol (<0.1% of culture volume) was used to treat control cells. For experiments with wild-type SH-SY5Y cells, fenretinide was added to the culture medium for 48 h, whereas experiments with stably transfected derivatives (SH-SY5Y^{Tet12}, sense-GADD153 and antisense-GADD153) were done by adding fenretinide (in the presence or absence of tetracycline, with or without chemotherapeutic drug as appropriate) for 24 h before measuring apoptosis. Stock solutions of cisplatin (100 mM, freshly prepared in DMSO), carboplatin (10 mM, freshly prepared in culture medium) and etoposide (20 mM in DMSO, stored at −20°C) (all from Sigma Chemical Co., Poole, UK) were diluted in culture medium to appropriate concentrations. Cells were exposed to chemotherapeutic drugs for 24 h; in the case of wild-type SH-SY5Y cells, this was followed by washout and 24-h culture in the absence of drug before adding fenretinide or measuring apoptosis, as appropriate. For all experiments with stably transfected derivatives, apoptosis was measured at the end of 24-h exposure to chemotherapeutic drugs (in the presence or absence of fenretinide, with or without tetracycline, as appropriate to the experiment).

Flow Cytometry. Apoptosis was evaluated by flow cytometry of propidium iodide-stained cells recovered by trypsinization and pooled with apoptotic bodies and nonadherent cells recovered from the culture medium as described previously (Lovat et al., 2000a).

Western Blotting. Total protein (25 μg) extracted from cultured cells was separated by SDS-polyacrylamide gel electrophoresis through 12.5% gels and blotted onto nitrocellulose (Lovat et al., 2000a). GADD153 was identified with a mouse anti-human monoclonal GADD153 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted 1:1000, and detected by chemiluminescence (Lovat et al., 2002) using an affinity-purified goat anti-mouse peroxidase-conjugated IgG (Bio-Rad, Hemel Hempstead, UK).

Statistical Analysis. For the analysis of synergistic interactions, the CalcuSyn program (Biosoft, Cambridge, UK) was used to derive parameter estimates for the median-effect equation with single drug treatments (fenretinide, cisplatin, and etoposide) and combination indices for treatments with fenretinide and etoposide or cisplatin. Because combination indices were estimated in both the presence and absence of tetracycline, the parameter estimates for single drug treatments were obtained using the drug (fenretinide, cisplatin, or etoposide) in the presence and absence of tetracycline, as appropriate. Thus, for example, combination indices for the effects of fenretinide and cisplatin on antisense-GADD153 cells in which antisense-GADD153 was induced by tetracycline were obtained by reference to the single drug treatments also in the presence of tetracycline. Median-effect values (doses producing 50% apoptosis) are reported in the text. Parameter estimates for single drug treatments (median effect equation) were used to calculate the Loewe-additivity surface using the CombiTool program (Dressler et al., 1999) as described previously (Lovat et al., 2000b); this provides a visual interpretation of synergy, antagonism, and additivity. The level of apoptosis in response to different drug treatments with and without tetracycline was analyzed using general linear models (GLMs) or two-way

ANOVA. The responses of SH-SH5Y^{Tet12}, sense-GADD153, and antisense-GADD153 cells to fenretinide, with and without tetracycline, were approximately linear when regressed against $\log_{10}$(fenretinide dose) and when GLM was used for analysis. The same approach was used for analysis of the effects of etoposide and cisplatin and combinations of fenretinide with etoposide and cisplatin on the antisense-GADD153 cells. For the sense-GADD153 clones, responses to combinations of chemotherapeutic drugs with fenretinide were analyzed by multiway ANOVA, because dose-response effects were not linear at the high levels of apoptosis obtained. Two-way ANOVA was used to compare combination indices ($\log_{10}$-transformed) in relation to the combination of fenretinide with either cisplatin or etoposide and tetracycline induction of sense-GADD153 and antisense-GADD153 expression.

Results

Synergistic Induction of Apoptosis by Simultaneous Treatment with Fenretinide and Chemotherapeutic Drugs. Pretreatment of neuroblastoma cells with fenretinide results in the synergistic induction of apoptosis when subsequently treated with cisplatin, etoposide, or carboplatin (Lovat et al., 2000b). To evaluate the effect of abolishing GADD153 expression on the synergistic effects of fenretinide with these chemotherapeutic drugs, we first sought to determine whether fenretinide is also synergistic with chemotherapeutic drugs when cells are treated with fenretinide and chemotherapeutic drugs at the same time or after pretreatment with chemotherapeutic drugs. SH-SY5Y cells were treated either with chemotherapeutic drugs (cisplatin, etoposide, or carboplatin) for 48 h (24 h with drug then 24 h in fresh medium without drug), followed by 3 μM fenretinide for 48 h, or were treated with chemotherapeutic drugs (as above) and fenretinide together for 48 h. Pretreatment with chemotherapeutic drugs followed by fenretinide (data not shown) or treatment with fenretinide and chemotherapeutic drugs at the same time (Fig. 1) both resulted in synergistic induction of apoptosis, comparable with previously published results (Lovat et al., 2000b).

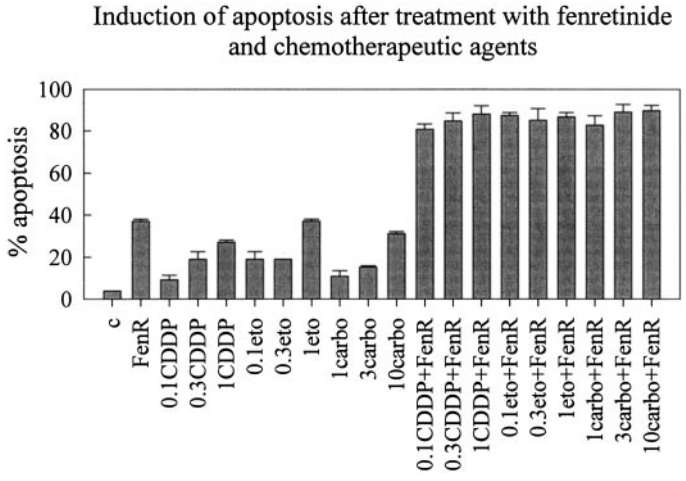


Fig. 1. Induction of apoptosis in wild-type SH-SY5Y cells after treatment at the same time with fenretinide and chemotherapeutic drugs. The percentage apoptosis (ordinate) was determined by propidium-iodide staining and flow cytometry of cells treated for 48 h with 3 μM fenretinide (FenR), the chemotherapeutic drugs cisplatin (CDDP; 0.1, 0.3, or 1 μM), etoposide (eto; 0.1, 0.3, or 1 μM), or carboplatin (carbo; 1, 3, or 10 μM), or each chemotherapeutic drug in combination with 3 μM fenretinide. Each bar is the mean ± S.D.

Controlling GADD153 Expression by Transfection of Sense and Antisense-GADD153 cDNA. Previous studies have suggested that GADD153 is required for fenretinide-induced apoptosis of SH-SY5Y neuroblastoma cells (Lovat et al., 2002). To evaluate the importance of GADD153 in the synergistic induction of apoptosis induced between fenretinide and chemotherapeutic drugs, GADD153 cDNA was cloned into the tetracycline-inducible vector pcDNA4/TO in sense and antisense orientation (Lovat et al., 2002). Stably transfected cells were obtained by transfection of these constructs into SH-SY5Y cells (SH-SY5Y^{Tet12}) that had been previously stably transfected with the pcDNA6/TR plasmid expressing the tetracycline repressor (Lovat et al., 2002). As with the parental SH-SY5Y cells, apoptosis was induced in the recipient SH-SY5Y^{Tet12} cells in response to 24-h treatment with fenretinide (median effect value, 9.4 μM; 95% confidence range, 7.4–12 μM), and there was no detectable effect of tetracycline on fenretinide-induced apoptosis (GLM, $\log_{10}$[fenretinide dose] and tetracycline status, effect of tetracycline, $F_{1,15} = 0.806$; $P > 0.3$). Tetracycline did not increase the level of apoptosis of SH-SY5Y^{Tet12} cells beyond the background control ($t_4 = -2.08$, $P > 0.1$). Similarly, there was no significant effect of tetracycline on apoptosis in response to 24-h treatment with 1, 3, or 10 μM cisplatin (two-way ANOVA; effect of tetracycline, $F_{1,12} = 0.31$; $P > 0.5$) or 0.1, 0.3, and 1 μM etoposide (two-way ANOVA; effect of tetracycline, $F_{1,12} = 2.31$; $P > 0.15$).

Two clones of stably transfected SH-SY5Y^{Tet12} cells, one sense-GADD153 and one antisense-GADD153, were selected for further study. Fenretinide induced apoptosis to a similar extent in both clones: median-effect values after 24-h treatment were 8.4 μM (95% confidence range, 6.6–10.5 μM) for the sense-GADD153 clone and 9.5 μM (95% confidence range, 7.8–11.4 μM) for the antisense-GADD153 clone. Expression of the sense- and antisense-GADD153 cDNAs was confirmed by Western blotting: in cells stably transfected with the sense-GADD153 construct, induction with tetracycline increased GADD153 levels 4.5-fold over the background control and 1.4-fold over the level achieved with 10 μM fenretinide alone (Fig. 2A). In contrast, although fenretinide induced an increase in GADD153 levels in cells stably transfected with the antisense construct, GADD153 protein was undetectable, both in the presence and absence of fenretinide, when expression of the antisense-GADD153 cDNA was induced with tetracycline (Fig. 2A).

Effect on Apoptosis of GADD153 Overexpression. The effect of increased expression of GADD153 on cell death was investigated by treating the sense-GADD153 transfectants with tetracycline for 24 h before adding fenretinide or chemotherapeutic drugs. For these sense-GADD153 transfectants, the induction of GADD153 with tetracycline increased cell death in response to fenretinide (GLM, $\log_{10}$[fenretinide dose]; effect of tetracycline, $F_{1,70} = 114.8$; $P < 10^{-5}$; Fig. 2B). Treatment of these cells with cisplatin or etoposide also induced apoptosis (GLM; effect of cisplatin or etoposide, $F_{1,18} = 158.9$ and 64.5, respectively; $P < 0.001$; Fig. 3, A and C). Overexpression of GADD153 increased apoptosis in the absence of chemotherapeutic drugs and also increased apoptosis in response to cisplatin or etoposide (GLM; effect of tetracycline on response to cisplatin or etoposide, $F_{1,18} = 40.1$ and 8.8, respectively; $P \leq 0.008$; Fig. 3, A and C). The combination of fenretinide with cisplatin or etoposide produced

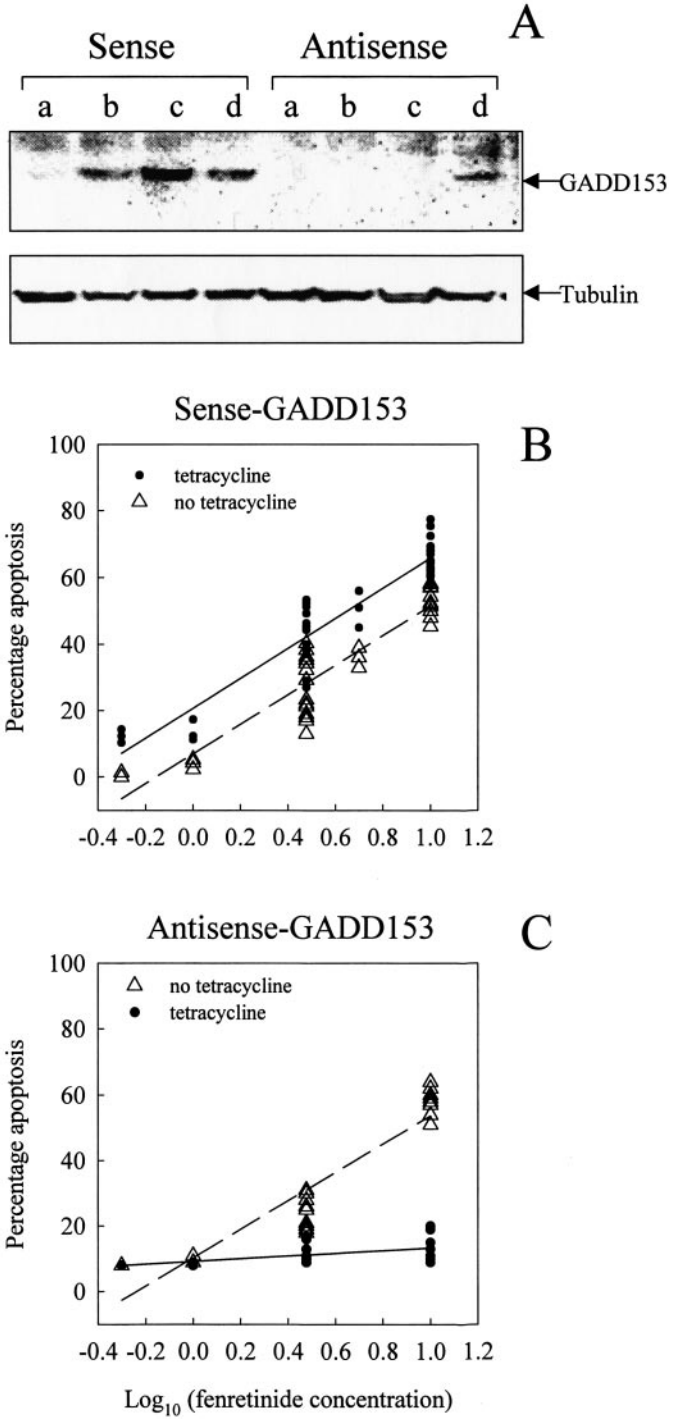
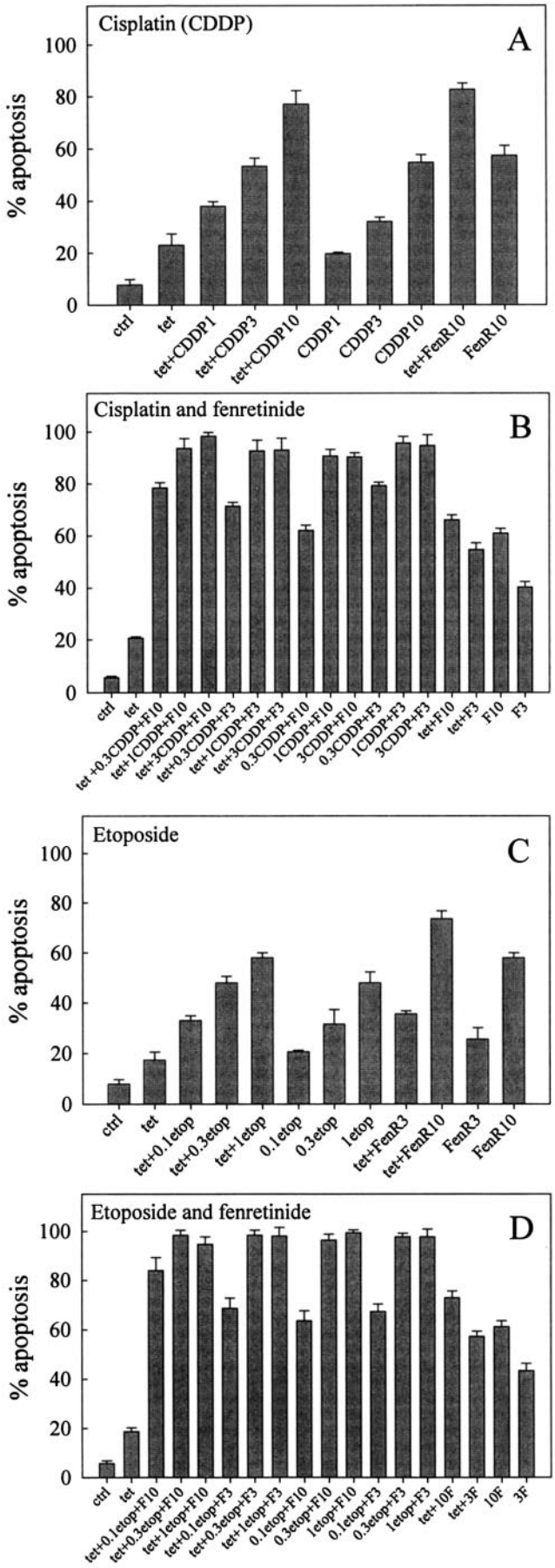


Fig. 2. A, the expression of GADD153 protein in SH-SY5Y^{Tet12} cells stably transfected with tetracycline-inducible sense-GADD153 or antisense-GADD153 cDNA. The Western blot was probed with an antibody to GADD153 and subsequently with an antibody to tubulin as a loading control. Cells were treated with ethanol (a, control), 1 μg/ml tetracycline (b) for 48 h or for 24 h with 1 μg/ml tetracycline followed by addition of 10 μM fenretinide and incubation for a further 24 h (c), or with 10 μM fenretinide alone for 24 h (d). B and C, apoptosis in response to fenretinide of SY-SY5Y^{Tet12} cells stably transfected with sense-GADD153 (B) or antisense-GADD153 (C). Expression of the construct was induced by treating the cells with 1 μg/ml tetracycline (●) or vehicle control (△) for 24 h before adding fenretinide at the doses indicated (abscissa). Ordinate, percentage apoptosis after exposure to fenretinide for 24 h, as measured from flow cytometry profiles.

high levels of apoptosis that were also enhanced by GADD153 overexpression, although this effect was only apparent at lower doses of fenretinide and chemotherapeutic drug, as otherwise the extent of cell death was too high to detect an effect (Fig. 3, B and D).

Effect on Apoptosis of Blocking GADD153 Expression. The levels of apoptosis in SH-SY5Y^{Tet12} cells stably transfected with antisense-GADD153 cDNA and exposed to tetracycline were similar to the vehicle-treated control cells (Fig. 4). In contrast to the marked effect of GADD153 overexpression in increasing sensitivity to fenretinide, the induction of antisense-GADD153 with tetracycline substantially decreased the level of apoptosis induced by 0.5 to 10 μM fenretinide (GLM; $\log_{10}$[fenretinide dose] and tetracycline treatment; effect of tetracycline, $F_{1,57} = 76.84$; $P < 0.001$; Fig. 2C). Like the recipient SH-SY5Y^{Tet12} cells, the antisense-GADD153–transfected cells were sensitive to the chemotherapeutic drugs cisplatin (1, 3, and 10 μM) and etoposide (0.1, 0.3, and 1 μM; Fig. 4). However, unlike the sense-GADD153 transfectants, there was no significant effect of antisense-GADD153 expression on the amount of cell death in response to cisplatin (GLM on cisplatin and tetracycline treatment; effect of tetracycline, $F_{1,18} = 0.23$; $P = 0.637$; Fig. 4A, inset). Although there was some indication for a slight reduction in apoptosis in response to etoposide when antisense-GADD153 was induced (Fig. 4C, inset), this was not statistically significant (GLM on etoposide and tetracycline treatment; effect of tetracycline, $F_{1,18} = 2.737$; $P = 0.115$). The induction of antisense-GADD153 with tetracycline also had a dramatic effect on reducing the response to the combination of fenretinide with cisplatin or etoposide (GLM; effect of tetracycline, $F_{1,41} = 1000.8$ and $F_{1,32} = 591$ for cisplatin and etoposide, respectively; $P < 10^{-8}$; Fig. 4).

Effect of GADD153 Over- and Underexpression on Synergy between Fenretinide and Chemotherapeutic Drugs. Combination indices were calculated for the response of cells to combinations of fenretinide (1, 3, 5, and 10 μM) with cisplatin (0.3, 1, and 3 μM) or etoposide (0.1, 0.3, and 1 μM) in the sense-GADD153 and antisense-GADD153 clones in the presence and absence of tetracycline (Chou and Talalay, 1984; Chou, 1991). Parameter estimates for the median-effect equation with single drug treatments (fenretinide, cisplatin, or etoposide alone, with and without tetracycline as appropriate) were also used to calculate the Loewe-additivity surface on three-dimensional plots (Dressler et al., 1999) (Fig. 5). For the sense-GADD153 clone, mean combination indices (mean of combination indices for all dose combinations) for fenretinide treatments with cisplatin or etoposide, with and without overexpression of GADD153, ranged from 0.56 to 0.28, indicative of synergy. Although there was no difference in combination indices between cisplatin and etoposide in combination with fenretinide (two-way ANOVA; $F_{1,22} = 0.53$; $P > 0.4$), there was an effect of sense-GADD153 induction in reducing the level of synergy between fenretinide and chemotherapeutic drug (two-way ANOVA; $F_{1,22} = 6.986$; $P = 0.015$; least-squares mean combination index, 0.192 in the absence of tetracycline but 0.446 with tetracycline; Fig. 6). This is also illustrated for cisplatin by Fig. 5, in which the response to the combination of cisplatin and fenretinide in the presence of tetracycline to induce GADD153 was lower across the dose range compared with this drug combination in the absence of tetracycline. Although induc-



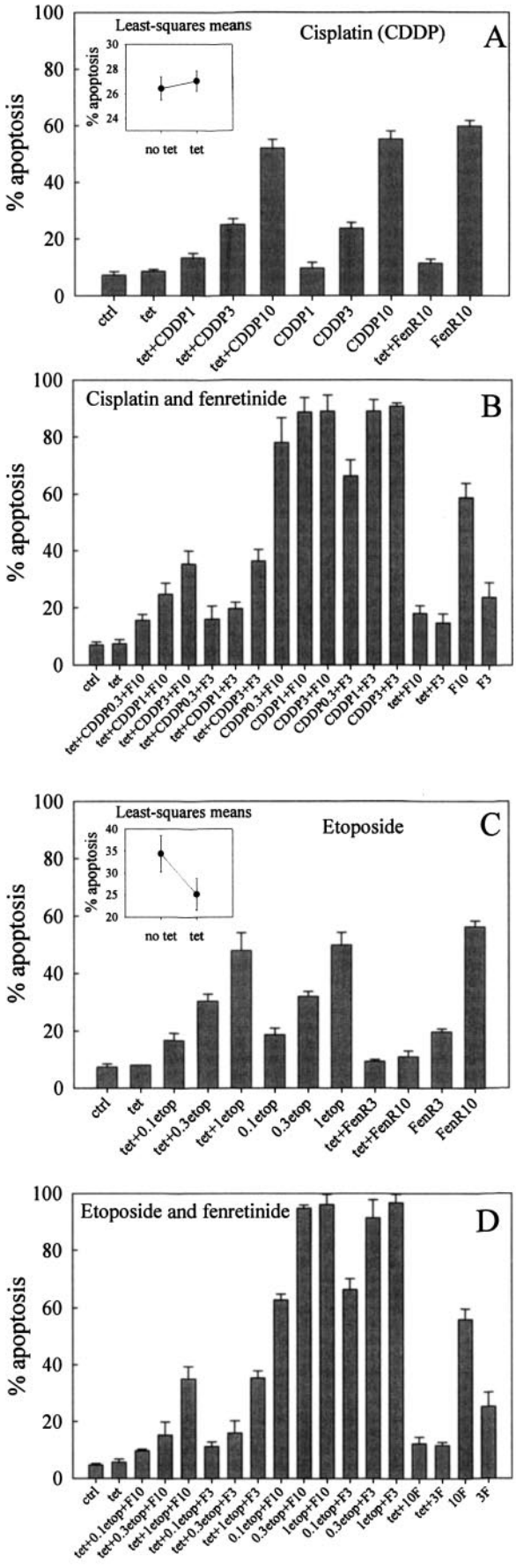
tion of sense-GADD153 with tetracycline did not reduce (but increased) apoptosis induced in response to fenretinide, cisplatin, or etoposide alone, we can not exclude the possibility that tetracycline itself reduced the synergistic interaction between fenretinide and chemotherapeutic drugs in the sense-GADD153 clone. There was no difference between the cisplatin and etoposide treatments with respect to their response to the induction of sense-GADD153 (two-way ANOVA; tetracycline-drug interaction, $F_{1,22} = 0.002$; $P > 0.9$).

For the antisense-GADD153 clone, there was no difference in mean combination index between cisplatin and etoposide in combination with fenretinide in the absence of tetracycline (two-way ANOVA; hypothesis test, $F_{1,23} = 0.138$; $P > 0.7$). However, induction of antisense-GADD153 had a significant effect on combination indices (two-way ANOVA; effect of tetracycline, $F_{1,23} = 27.21$; $P < 0.0001$), and there was a significant difference between etoposide and cisplatin when antisense-GADD153 was induced (two-way ANOVA; tetracycline-drug interaction, $F_{1,23} = 9.5$; $P = 0.0053$). These effects of antisense-GADD153 and antisense-GADD153-drug interaction were caused by the response to fenretinide in combination with etoposide: after induction of antisense-GADD153, there was a marked increase in mean combination index to 2.355, indicative of a change to antagonism of the fenretinide/etoposide combination (two-way ANOVA; $F_{1,23} = 31.57$; $P = 0.00001$; Fig. 6). In contrast to the effects of antisense-GADD153 in inducing an antagonistic interaction between fenretinide and etoposide, there was no statistically significant effect of antisense-GADD153 induction on the mean combination indices for fenretinide and cisplatin (two-way ANOVA; $F_{1,23} = 2.5$; $P = 0.13$), indicating that although the antisense-GADD153 had knocked out the apoptotic response to fenretinide (Fig. 2C), the synergistic effect of fenretinide and cisplatin was retained (Figs. 5 and 6).

Discussion

This study shows that increasing the level of GADD153 expression in SH-SY5Y neuroblastoma cells increased the

Fig. 3. Overexpression of sense-GADD153: apoptosis in response to fenretinide, cisplatin, or etoposide and combinations of fenretinide and cisplatin or etoposide. A, response to 24-h treatment with CDDP (1, 3, and 10 μM) in the presence and absence of tetracycline (tet; 1 μg/ml) added 24 h before cisplatin to induce expression of the sense-GADD153 construct. For comparison, cells were also treated for 24 h with 10 μM FenR alone in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. B, apoptosis in response to 24-h treatment with FenR (3 and 10 μM) and CDDP (0.3, 1, and 3 μM) in the presence (tet) or absence of tetracycline added 24 h previously to induce expression of the sense-GADD153 construct. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. C, response to 24-h treatment with etoposide (etop; 0.1, 0.3, and 1 μM) in the presence and absence of tet (1 μg/ml) added 24 h previously to induce expression of the sense-GADD153 construct. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. D, apoptosis in response to 24-h treatment with FenR (3 and 10 μM) and etop (0.1, 0.3, and 1 μM) in the presence (tet) or absence of 1 μg/ml tetracycline added 24 h previously to induce expression of the sense-GADD153 construct. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. Bars, mean ± S.D.



level of active cell death or apoptosis, whether in the absence of drugs or in response to treatment with fenretinide or chemotherapeutic drugs. This result is, therefore, similar to the effects of GADD153 overexpression in sensitizing gastric cancer cells to cisplatin-induced cell death (Kim et al., 1999). However, there was no evidence for an increase in synergistic response when cells induced to overexpress GADD153 were treated with fenretinide in combination with a chemotherapeutic drug. In contrast, the mean combination indices, overall, were slightly reduced. With respect to this, we cannot exclude the possibility that the tetracycline used to induce GADD153 expression interfered with the interaction between fenretinide and chemotherapeutic drugs. Although tetracycline at 1 μg/ml (2.25 μM) did not significantly affect the apoptotic response of the parental SH-SY5Y^{Tet12} cells to fenretinide, cisplatin, or etoposide, other studies have shown that tetracyclines can inhibit ROS generation in neutrophils (Gabler and Creamer, 1991) and protect neuronal cells against radiation-induced apoptosis (Tikka et al., 2001) within the concentration range 0.02 to 40 μM. The fact that treatment of the antisense-GADD153 cells with tetracycline (to induce expression of the antisense construct) did not significantly affect the level of apoptosis in response to cisplatin or etoposide indicates that the antibiotic did not inhibit responses to single treatments with chemotherapeutic drugs. This result also indicates that GADD153 is not an important mediator of cell death induced by cisplatin in SH-SY5Y cells, although we argue below that GADD153 may play a part in apoptosis induced by etoposide.

The expression of antisense-GADD153 in SH-SY5Y cells completely abrogated apoptosis induced by fenretinide, and this clearly demonstrates a pivotal role for GADD153 in fenretinide-induced cell death. Considerable evidence indicates that GADD153 mediates cellular responses to oxidant injury (Guyton et al., 1996); however, it is not essential for all apoptotic signaling pathways, because GADD153−/− knockout mice demonstrate normal development and fertility, and they exhibit reduced apoptosis only in response to ER stress (Oyadomari et al., 2002). Indeed, mediating ER stress (Ron

Fig. 4. Overexpression of antisense-GADD153: apoptosis in response to fenretinide, cisplatin, or etoposide and combinations of fenretinide and cisplatin or etoposide. A, response to 24 h treatment with CDDP (1, 3 and 10 μM) in the presence and absence of tet (1 μg/ml) added 24 h previously to induce expression of the antisense-GADD153 construct. For comparison, cells were treated with 10 μM FenR in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. Inset, least-squares means from ANOVA to show the effect of induction of sense-GADD153 on cisplatin-induced apoptosis, corrected for dose variation. B, apoptosis in response to 24-h treatment with FenR (3 and 10 μM) and CDDP (0.3, 1, and 3 μM) in the presence (tet) or absence of tetracycline added 24 h previously to induce expression of antisense-GADD153. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. C, response to 24-h treatment with etop (0.1, 0.3, and 1 μM) in the presence and absence of tet (1 μg/ml) added 24 h previously to induce expression of antisense-GADD153. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. Inset, least-squares means from ANOVA to show the effect of induction of antisense-GADD153 on etoposide-induced apoptosis, corrected for dose variation. D, apoptosis in response to 24-h treatment with FenR (3 and 10 μM) and etop (0.1, 0.3, and 1 μM) in the presence (tet) or absence of 1 μg/ml tetracycline added 24 h previously to induce expression of antisense-GADD153. For comparison, cells were also treated with FenR alone (3 and 10 μM) in the presence and absence of tetracycline. Ctrl, vehicle control; tet, cells treated with tetracycline alone. Bars, mean ± S.D.

and Habener, 1992) may be one of the main functions of GADD153. The induction of GADD153 in response to fenretinide and its requirement for fenretinide-induced cell death suggests, therefore, that fenretinide induces apoptosis as a result of ER stress. Because GADD153 is a transcription factor, the transcriptional activation of other genes, at least in SH-SY5Y neuroblastoma cells, may be key elements in the apoptotic signaling pathway induced by fenretinide.

Despite the fact that antisense-GADD153 blocked the apoptotic response to fenretinide, there was still a synergistic effect of fenretinide in combination with cisplatin, although the overall level of apoptosis was reduced because an apoptotic effect of fenretinide was lacking. This suggests that some upstream element of the fenretinide response, such as the generation of ROS, is responsible for synergy between cisplatin and fenretinide. Thus, the addition of fenretinide to the treatment of cells with cisplatin results in two additional processes: an increased (additive) level of apoptosis caused by the induction of GADD153 by fenretinide and an enhancement of the effects of cisplatin because of elements of a

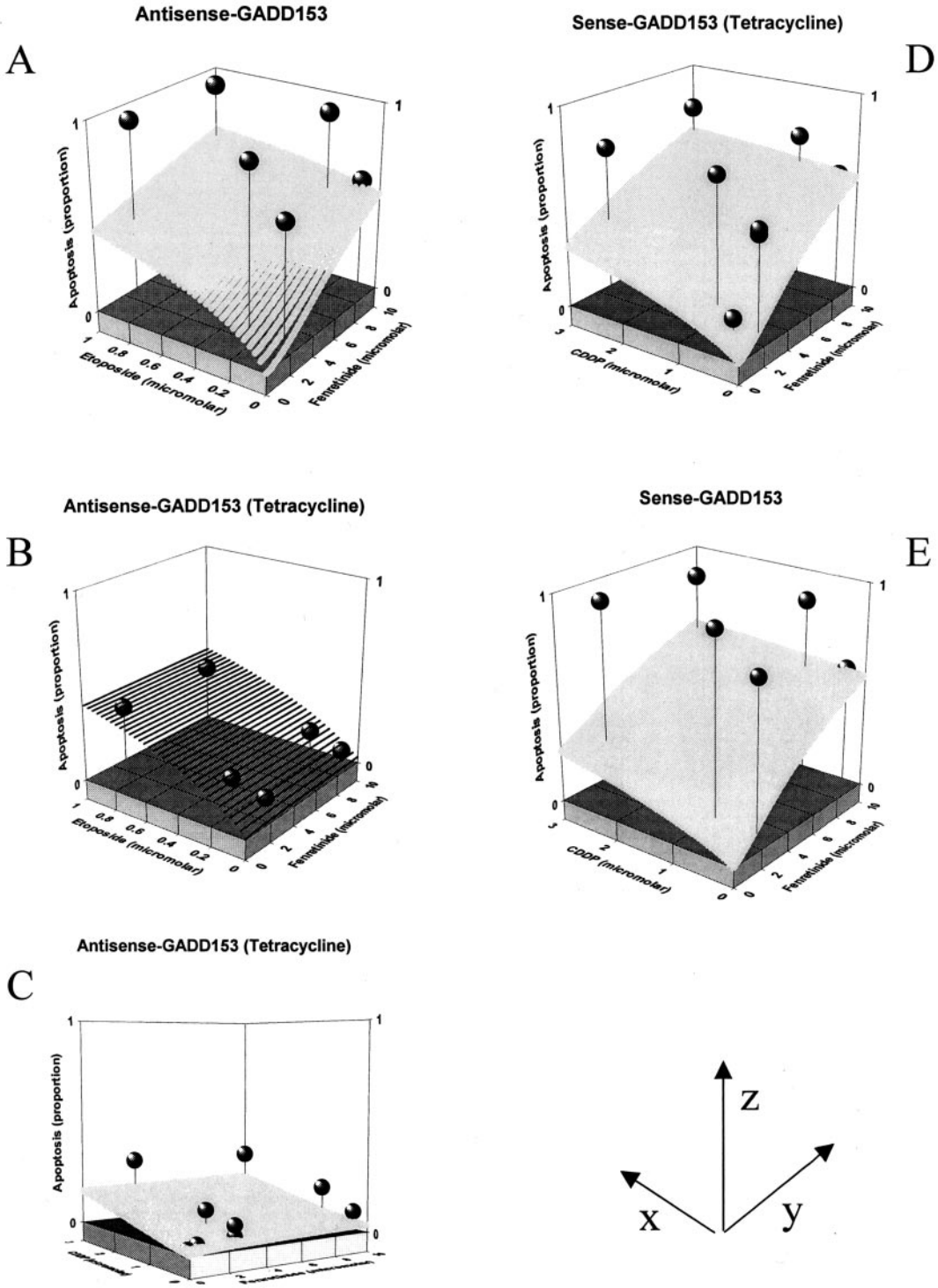


Fig. 5. Apoptosis (measured by flow cytometry) in SY-SY5Y^{Tet12} cells stably transfected with antisense-GADD153 (A–C) or sense-GADD153 (D and E) and treated for 24 h with fenretinide and different concentrations of etoposide (A and B) or CDDP (C–E), either without tetracycline (A and E) or in the presence of 1 μg/ml tetracycline (B–D). Parameters for dose-response curves for 24-h treatment with fenretinide, etoposide, and cisplatin on their own (either without tetracycline or in the presence of 1 μg/ml tetracycline, as appropriate) were estimated for the median effect equation (Chou and Talalay, 1984; Chou, 1991) and used to define the Loewe-additivity surface (additive model) shown as three-dimensional graphs (Dressler et al., 1999). The observed responses for fenretinide with etoposide (A and B) or with cisplatin (C–E), either without tetracycline (A and E) or in the presence of 1 μg/ml tetracycline (B–D), are shown as three-dimensional balls with vertical lines dropping through the surface to the x-y plane. Axes: x, CDDP (0–3 μM) or etoposide (0–1 μM); y, fenretinide (0–10 μM); and z, response (0–1). The plot for antisense-GADD153 with cisplatin was similar to that for antisense-GADD153 with etoposide (A), and the plots for sense-GADD153 with etoposide in the presence or absence of tetracycline were similar to the equivalent plots for sense-GADD153 with cisplatin (D and E) and are therefore not shown.

cellular response to fenretinide upstream of GADD153 induction. Therefore, according to this model, GADD153 does not mediate synergy between fenretinide and cisplatin. Although it is possible that a RAR-mediated function of fenretinide is responsible for synergy, this is perhaps unlikely, because retinoic acid inhibits the response of neuroblastoma cells to chemotherapeutic drugs (Lasorella et al., 1995).

In contrast to cisplatin, antisense-GADD153 had the reverse effect on the combination of fenretinide with etoposide, which was antagonistic. Clearly, the mechanism of apoptosis induced by etoposide is different from that of cisplatin. Both cisplatin and etoposide are DNA-damage-inducing agents, and the action of these drugs on SH-SY5Y neuroblastoma cells is, at least in part, p53-dependent; this is in contrast to the effects of fenretinide, which do not apparently involve p53 (Lovat et al., 2002). Etoposide and cisplatin may also function independently of p53, but the p53-independent mechanisms of action by these two drugs differ. Etoposide may function through a c-Jun N-terminal kinase pathway (Vivo et al., 2003), whereas cisplatin may induce p53-independent ER stress involving the downstream action of the proapoptotic BCL2-family member, Bak (Mandic et al., 2003). Although neither drug induces ROS to a level comparable with fenretinide in SH-SY5Y cells, there is a slight dose-dependent increase in ROS with etoposide but not cisplatin (Lovat et al., 2000b), and other studies in these cells have shown that etoposide increases oxidative stress (Bernardini et al., 2002).

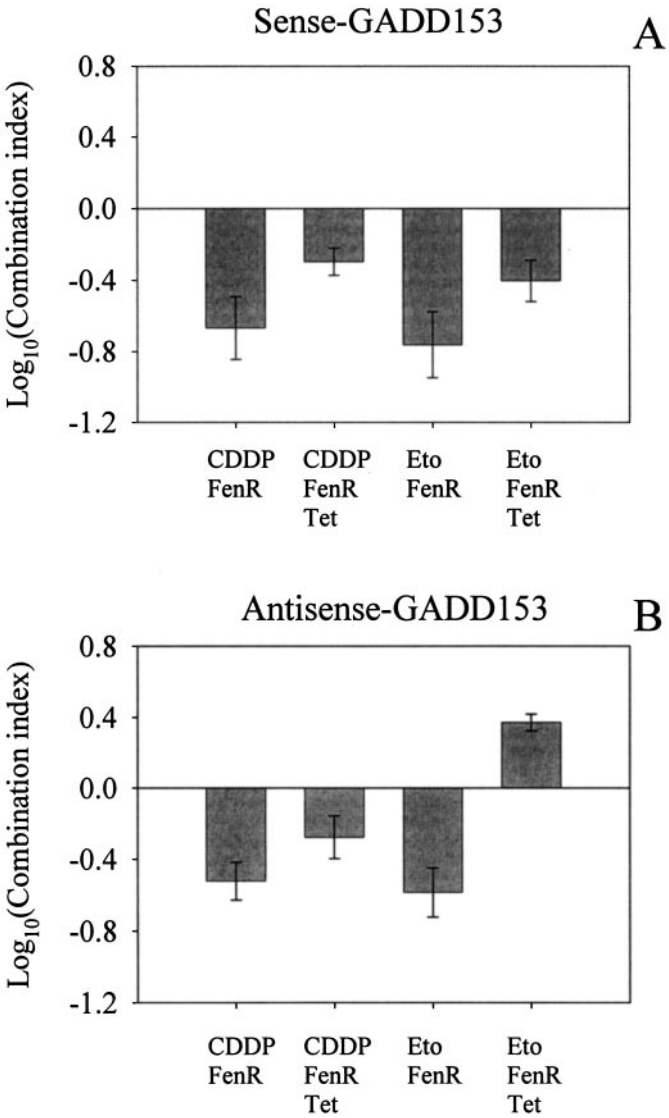


Fig. 6. Combination indices (expressed on a $\log_{10}$ scale, ordinate) for apoptosis in the sense-GADD153 (A) and antisense-GADD153 SH-SY5Y^{Tet12} cell clones in response to combinations of FenR with the chemotherapeutic drugs CDDP or etoposide (Eto) for 24 h. Expression of the sense- and antisense-GADD153 cDNA was induced by adding tetracycline to 1 μg/ml (Tet) 24 h before adding fenretinide and chemotherapeutic drugs. Error bars are ± S.E.M. Negative values indicate synergy, values around 0 indicate additivity, and positive values indicate antagonism.

Etoposide can have both pro- and antioxidant effects (Kagan et al., 2001); the former are caused by phenoxyl radicals, which can reduce endogenous thiols and deplete GSH levels (Tyurina et al., 1995). In SH-SY5Y cells, etoposide induces oligomerization of glutathione *S*-transferase-π (Bernardini et al., 2002), and this may lead to activation of the c-Jun N-terminal kinase pathway (Adler et al., 1999) in which GADD153 has been implicated as a downstream element (Brenner et al., 1997). GADD153 levels increase in response to etoposide in human leukemia cells, (Eymin et al., 1997; Bjorling-Poulsen and Issinger, 2003), and this seems to reflect a dual property of this drug, in which early effects result from a stimulation of apoptotic signaling pathways, whereas later effects result from DNA damage and withdrawal from the cell cycle (Eymin et al., 1997). Although we have not observed an increase in GADD153 expression in SH-SY5Y neuroblastoma cells in response to etoposide (Lovat et al., 2002), in leukemia cells, the etoposide-induced increase in GADD153 is transient and would not have been observed at the single time point used for our earlier study on SH-SY5Y neuroblastoma cells (Lovat et al., 2002). Clearly, GADD153 may mediate, at least in part, early signaling events of the apoptotic response to etoposide. This suggestion is borne out by the effects of antisense-GADD153 induction on the response to etoposide compared with cisplatin, which, although not statistically significant, become more relevant in the context of the drug combination experiments and evidence of a different mechanism of action compared with cisplatin. With respect to synergy, it is unlikely that GADD153 plays a role in synergistic responses with etoposide, and the observed antagonism of the combination between fenretinide and etoposide in the antisense-GADD153 cells may be a result of abrogation of a GADD153-mediated element of etoposide-induced apoptosis.

In conclusion, this study shows that although GADD153 is a key element of fenretinide-mediated cell death, it does not mediate the synergy observed between fenretinide and chemotherapeutic drugs. The results suggest that synergy may result from events upstream of GADD153, and this will narrow the scope of future work to elucidate the mechanism of synergy.

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