

SYNERGISTIC INDUCTION OF APOPTOSIS OF NEUROBLASTOMA BY FENRETINIDE OR CD437 IN COMBINATION WITH CHEMOTHERAPEUTIC DRUGS

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Retinoic acid therapy improves the survival of children with neuroblastoma and 13-*cis* retinoic acid now forms an important component of treatment for residual disease of stage IV neuroblastoma after chemotherapy. However, although 13-*cis* retinoic acid induces differentiation, other retinoids are effective at inducing apoptosis of neuroblastoma *in vitro*, including the novel compounds fenretinide and CD437 and these may be alternative retinoids for neuroblastoma therapy. The aim of our study was to evaluate the ability of fenretinide, CD437 (6-{3-(1-adamantyl)-4-hydroxyphenyl}-2-naphthalene carboxylic acid) and different retinoic acid isomers to induce apoptosis of neuroblastoma in conjunction with the chemotherapeutic drugs, cisplatin, etoposide and carboplatin. Neuroblastoma cell lines were treated with retinoids prior to treatment with chemotherapeutic agents and flow cytometry used to measure apoptosis and free radical generation. Pre-treatment of neuroblastoma cell lines with fenretinide or CD437 prior to treatment with cisplatin, etoposide or carboplatin synergistically increased apoptosis, an effect not seen with 13-*cis*, all-*trans* or 9-*cis* retinoic acid. Contrary to retinoic acid isomers or chemotherapeutic drugs, apoptosis of neuroblastoma cells induced by fenretinide or CD437 was accompanied by the generation of intracellular free radicals. Quenching of fenretinide- or CD437-induced free radicals with antioxidants abolished the synergistic response seen with the subsequent addition of chemotherapeutic agents. Therefore, the generation of free radicals by fenretinide or CD437 may be the key property of these retinoids leading to synergistic responses with chemotherapeutic drugs. Clearly, these synthetic retinoids provide new opportunities for novel neuroblastoma therapy. *Int. J. Cancer* 88:977–985, 2000.

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Neuroblastoma is the most common malignant disease of infancy and accounts for approximately 15% of all childhood cancer deaths (Maris and Matthay, 1999). Despite intensive treatment, only 25% of children with stage 4 disease over the age of 1 year survive. The chemotherapeutic agents cisplatin, etoposide and carboplatin are important components of the cytotoxic drug regimen used by the United Kingdom Children's Cancer Study Group to treat children with this stage disease. Recently, The Children's Cancer Study Group have shown in a randomised trial that 13-*cis* retinoic acid increases survival when used to treat residual disease after chemotherapy and bone marrow transplantation (Matthay *et al.*, 1999). *In vitro*, retinoic acid and its analogues induce differentiation and apoptosis of neuroblastoma but the response depends upon the particular retinoic acid isomer in use (Lovat *et al.*, 1997a,b; Melino *et al.*, 1994). Recent studies have demonstrated that two particular retinoid analogues, fenretinide and CD437, induce more effective apoptosis of neuroblastoma *in vitro* than either all *trans*, 13-*cis* or 9-*cis* retinoic acid (Maurer *et al.*, 1999; Meister *et al.*, 1998).

The mechanisms of fenretinide-induced cell death are complex and probably involve several overlapping pathways including those involving the retinoic acid receptors (Sun *et al.*, 1999a,b) and

oxidative stress via the induction of free radicals (Shen *et al.*, 1999; Sun *et al.*, 1999a,b). The involvement of several apoptosis-related genes in fenretinide-induced apoptosis of cancer, including components of the Bcl2/Bax pathway and the expression of p21, *c-myc* and *c-jun* in prostate cancer (Shen *et al.*, 1999; Sun *et al.*, 1999b) has also been described. Furthermore, a recent study in neuroblastoma suggested that a p53-independent pathway of fenretinide-induced apoptosis operates through increased intracellular levels of the lipid secondary messenger, ceramide (Maurer *et al.*, 1999).

CD437 (6-{3-(1-adamantyl)-4-hydroxyphenyl}-2-naphthalene carboxylic acid) is a retinoic acid receptor (RAR)- γ selective retinoid that induces growth arrest and rapid apoptosis in non-small lung cancer cell lines (Sun *et al.*, 1999c), prostate cancer cells (Liang *et al.*, 1999) and neuroblastoma cell lines (Meister *et al.*, 1999). However, the mechanism of induction of cell death is uncertain. Both p53-dependent and independent mechanisms have been reported for growth inhibition and apoptosis of non-small-cell lung cancer cell lines (Sun *et al.*, 1999c,d) with the involvement of the p53-regulated genes Killer/DR5 and Bax also being described. Like fenretinide, CD437-induced apoptosis is caspase dependent (Sun *et al.*, 1999d), inducing PARP cleavage at 2 hr in HL60 promyelocytic leukaemia cells (Zhang *et al.*, 1999). Although reported to be a RAR gamma-selective agonist, CD437 can induce apoptosis in gastric cancer cells in a RAR γ -independent manner (Jiang *et al.*, 1999).

Recent studies have suggested that fenretinide may enhance the responses of non-small-cell lung cancer cells and breast cancer cells to chemotherapeutic drugs (Grunt *et al.*, 1998; Kalemkerian and Xialolan, 1999). Chemotherapeutic drugs, particularly cisplatin and etoposide, are the mainstay of chemotherapy for metastatic neuroblastoma and the aim of our study was to ask whether pre-treatment of neuroblastoma cells with retinoic acid or the synthetic retinoids fenretinide and CD437 enhances or abrogates the apoptotic effects of chemotherapeutic drugs on neuroblastoma cells.

Abbreviations: CD437, 6-{3-(1-adamantyl)-4-hydroxyphenyl}-2-naphthalene carboxylic acid; PBS, phosphate-buffered saline; RAR, retinoic acid receptor; DMSO, dimethyl sulphoxide; CI, combination indices.

Grant sponsor: Association for International Cancer Research, UK; Grant sponsor: The Neuroblastoma Association, Genoa; Grant sponsor: AIRC; Grant sponsor: MURST, Italy.

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Received 4 March 2000; Revised 12 July 2000; Accepted 27 July 2000

MATERIAL AND METHODS

Cell culture and treatment with retinoids, chemotherapeutic reagents and antioxidants

Human neuroblastoma cell lines, SH SY 5Y (Biedler *et al.*, 1973), SK N BE (2) (Hanada *et al.*, 1993) and LAN 5 (Sidell *et al.*, 1983) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Life Technologies Ltd, Paisley, UK), supplemented with 10% foetal bovine serum (Sera-Lab, Crawley, UK) (culture medium) at 37°C in a humidified atmosphere of 5% CO₂ in air. For all experiments, cells were seeded into tissue culture flasks or plates and allowed to attach overnight before treatment. The seeding density varied according to the type of experiment: for measurement of free radicals, 1×10^6 cells were seeded into 25 cm² tissue culture flasks (Costar, UK) in 5 ml of culture medium; for time and dose response assays with retinoids or chemotherapeutic reagents where total incubation times did not exceed 4 days, 0.75×10^6 cells were seeded into 6-well tissue-culture plates (Costar, UK) in 2.5 ml of culture medium, and for combination experiments of cytotoxic drugs with retinoids, 0.4×10^6 cells were seeded in tissue-culture grade Petri dishes (100 mm diameter, Costar, UK) in 10 ml of culture medium.

The antioxidants Vitamin C (ascorbic acid sodium salt) and Vitamin E (α tocopherol) (both from Sigma) were used in experiments to block free radicals and were freshly prepared before use. Vitamin C was diluted in phosphate-buffered saline (PBS; ICN-Flow, High Wycombe, UK) and used at final concentrations of 100 μ M. Vitamin E was diluted in tissue-culture medium, sonicated and used at a final concentration of 1 mM. Where appropriate, antioxidants were added 2 hr prior to treatment with fenretinide, CD437 or vehicle control. Fenretinide or CD437 were added directly to cultures pre-incubated with vitamin E, but added to cultures pre-incubated with vitamin C after washing the cells once with PBS.

The 9-*cis*, 13-*cis* or all-*trans* retinoic acid (Sigma Chemical Co., Poole, UK) or fenretinide (Janssen-Cilag, Ltd., Basserdorf, Switzerland) were added in ethanol at given concentrations and an equal volume of ethanol (< 0.1% of culture volume) was used to treat control cells. CD437 (Dr. U Reichert, Galderma, Sophia Antipolis, France) was added in DMSO, and an equal volume of DMSO used to treat control cells. Fresh stock solutions of cisplatin (100 mM in DMSO) and carboplatin (10 mM in culture medium) (both from Sigma Chemical Co., Poole, UK) were prepared on the day of experiment and further diluted in culture medium to appropriate concentrations. Stock 20 mM aliquots of etoposide (Sigma Chemical Co., Poole, UK) diluted in DMSO were stored at -20°C and diluted in culture medium to appropriate concentrations on the day of experiment.

Measurement of apoptosis and cell viability by flow cytometry

Cells were treated with retinoids or chemotherapeutic reagents for appropriate times; if the incubation period exceeded 2 days the culture medium was changed every other day. Retinoids or retinoid-treated cultures were shielded from light to prevent photodegradation. At each change of culture medium, the existing medium was harvested, centrifuged at 200g for 5 min at 21°C, and the pelleted material, consisting of apoptotic bodies and non-adherent cells, resuspended in fresh culture medium plus the appropriate test reagent. At the end of the experiment, the medium was harvested, and apoptotic bodies and non-adherent cells collected by centrifugation and pooled with cells recovered from the culture vessel by trypsinisation (Lovat *et al.*, 1997a). The resulting sample was

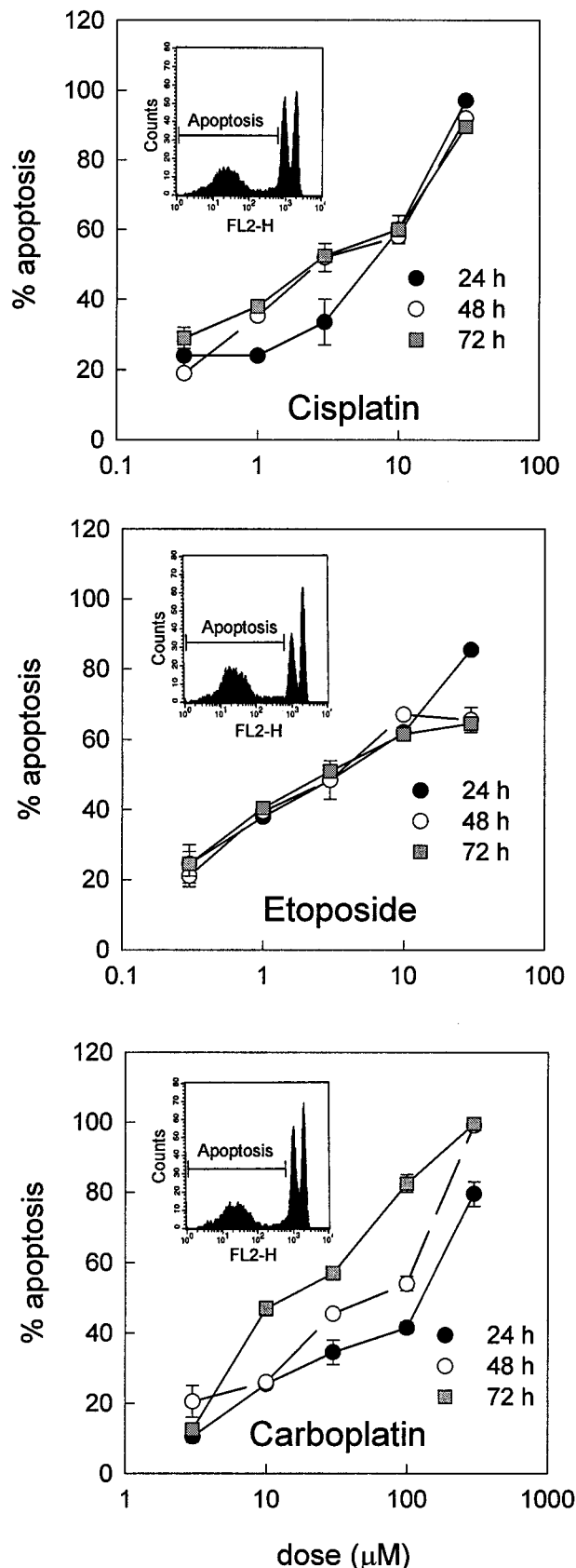


FIGURE 1 – Time and dose response of induction of apoptosis in SH SY 5Y cells by cisplatin, etoposide and carboplatin as determined by flow cytometry of propidium-iodide-stained cells. Each point is the mean \pm range; inserts represent flow cytometry profiles for 3 μ M cisplatin or etoposide or 10 μ M carboplatin, the concentration of chemotherapeutic drug required to induce 50% apoptosis.

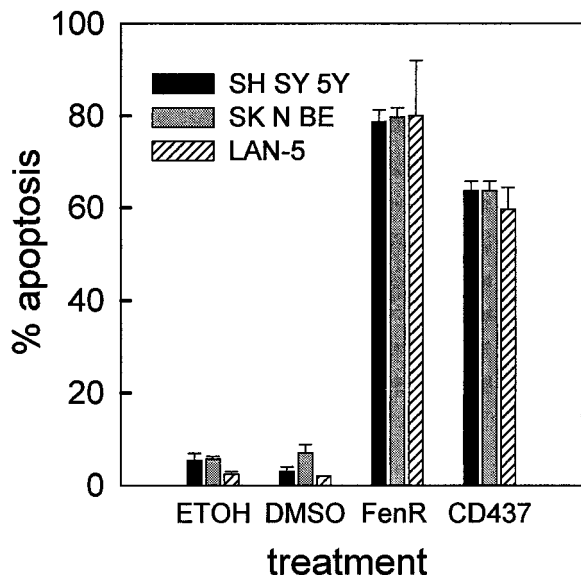


FIGURE 2 – Induction of apoptosis in SH SY 5Y, SK N BE and LAN 5 cells by 1 μM fenretinide or CD437 at 3 days, compared to ethanol and DMSO controls. Each bar represents the mean of 3 experiments ± SD.

resuspended in phosphate-buffered saline (PBS, ICN-Flow, High Wycombe, UK) and then either immediately fixed with an equal volume of cold (–20°C) methanol:acetone (4:1 vol/vol) and stored at 4°C prior to propidium-iodide staining and evaluation by flow cytometry (Lovat *et al.*, 1997a), or divided in 2 where half the sample was fixed and stained as described and the other half was used immediately to evaluate cell viability. Viability was determined by staining cells treated with 100 nM fluorescein diacetate (Sigma Chemical Co.) prior to staining with propidium iodide (2 μg/ml) and analysis by flow cytometry (Aeschbacher *et al.*, 1986). Fluorescence, resulting from excitation at 488 nm with a 15 mW Argon laser, was monitored at 585 nm ± 21. Events were triggered by using a doublet-discriminator parameter to exclude debris; 10,000 events were acquired at 200 cells/sec.

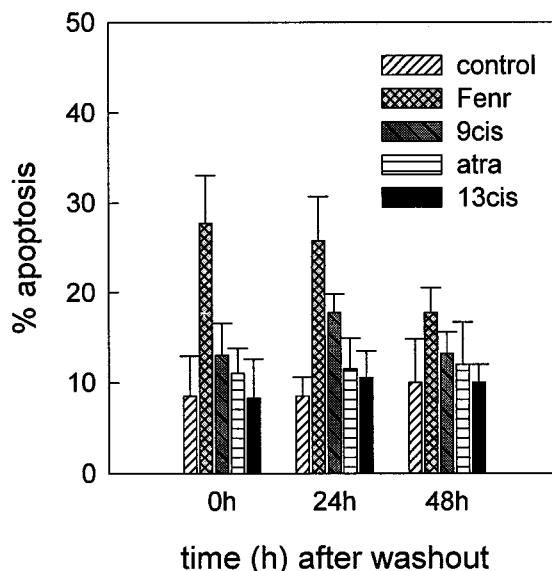
Measurement of free radicals

Free radical induction was measured in SH SY 5Y and SK N BE cells treated for 24 hr with 3 or 1 μM fenretinide, 1 μM CD437, 9-*cis*, 13-*cis* or all-*trans* retinoic acid or in cells treated for 24 hr with 1, 3 or 10 μM cisplatin or etoposide or 10, 30 or 100 μM carboplatin (Possel *et al.*, 1997). At the end of incubation, the cell culture medium was removed and replaced with 20 ml of fresh culture medium containing 2% FCS and 10 μM 2,7-Dihydrodichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, Oregon). After a further 20 min incubation at 37°C, cells were washed twice in PBS before trypsinisation and 10,000 cells acquired immediately for flow cytometry.

Data analysis

For the analysis of synergistic interactions, the CalcuSyn (Elsevier BioSoft) program was used to derive the combination indices (CI) and parameter estimates for the median-effect equation (Chou, 1991; Chou and Talalay, 1984) for both mutually exclusive and mutually non-exclusive models. CI values for both models were similar. Parameter estimates for single treatments were then used to compare drug combination results against the Loewe-additivity surface produced using the CombiTool program described by Dressler *et al.* (1999).

24 h treatment



48 h treatment

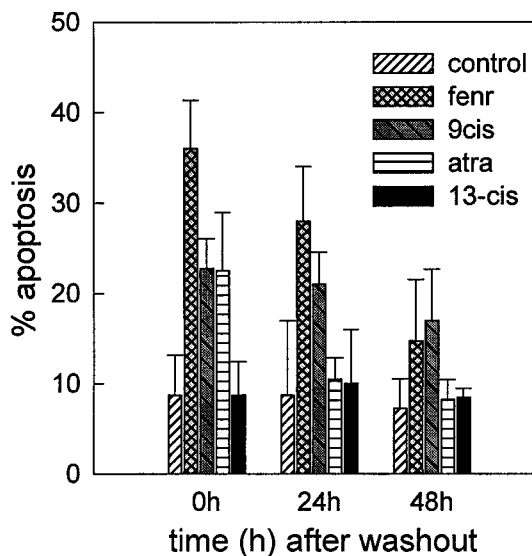


FIGURE 3 – Time course induction of apoptosis of SH SY 5Y cells by 1 μM fenretinide, 9-*cis*, all-*trans* or 13-*cis* retinoic acid treated for 24 hr or 48 hr, followed by incubation in the absence of retinoid for a further 24 hr or 48 hr. Each point is the mean of 4 replicate experiments ± SD.

RESULTS

Apoptotic response of SH SY 5Y cells to cisplatin, etoposide and carboplatin

To define the apoptotic response of SH SY 5Y cells to chemotherapeutic drugs, cells were treated with cisplatin or etoposide at 0.3, 1, 3, 10, and 30 μM for 24 hr (time A); the medium was then changed and cells incubated in the absence of cytotoxic drug for another 24 hr (time B) or 48 hr (time C) prior to analysis. Cells treated with carboplatin were treated in the same way but using final concentrations of 3, 10, 30, 100 and 300 μM. Apoptosis in response to all 3 chemotherapeutic drugs increased in a dose-

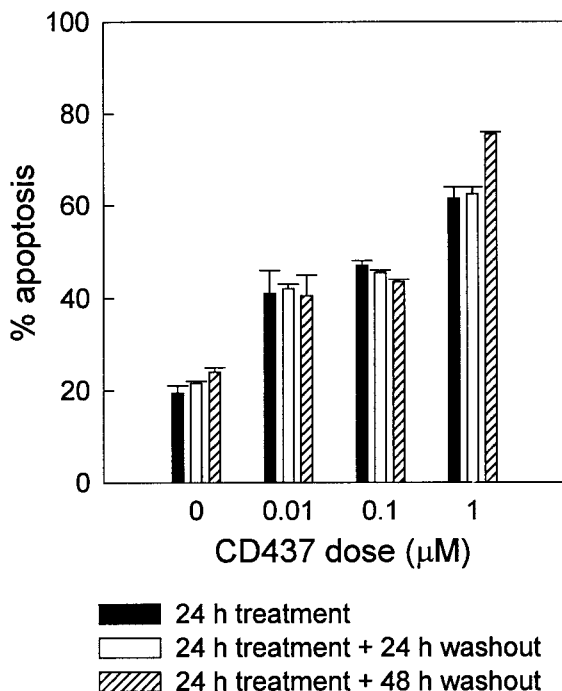


FIGURE 4 – Time and dose response of the induction of apoptosis of SH SY 5Y cells treated with CD437 for 24 hr followed by analysis of apoptosis at 0, 24 and 48 hr.

dependent manner (Fig. 1). There was no increase in apoptosis with increased incubation time (time B or C) for etoposide, but for carboplatin, there was a clear incubation-time-dependent increase at each dose, and for cisplatin 48 or 72 hr total incubation time gave increased levels of apoptosis at the lower doses used. These results were also confirmed by viability assays (data not shown). Similar results were obtained for SK N BE and LAN 5 cells (data not shown).

Induction of apoptosis in SH SY 5Y cells by retinoic acid isomers and the synthetic retinoids fenretinide and CD437

Initial studies of apoptosis induced by micromolar concentrations of either fenretinide or CD437 were performed in SH SY 5Y, LAN 5 and SK N BE cells and confirmed the induction of apoptosis in response to either reagent in all 3 cell lines (Fig. 2) after 3 days incubation. To define an appropriate time of retinoid treatment for combination experiments with chemotherapeutic drugs, SH SY 5Y cells were treated for 24 or 48 hr with 3 µM fenretinide, 1 µM 9-*cis*, all-*trans* or 13-*cis* retinoic acid. Cells were then either harvested and analysed, or incubated in the absence of retinoid for a further 24 or 48 hr. Under these conditions, the maximal response to fenretinide occurred after 48 hr continuous treatment, with levels of apoptosis decreasing after its removal from the culture medium (Fig. 3). Similar results were obtained with 9-*cis* retinoic acid (Fig. 3), but lower levels of apoptosis were induced. All-*trans* retinoic acid also produced low levels of apoptosis in SH SY 5Y cells after 48 hr continuous treatment, but apoptosis decreased to control levels after washout (Fig. 3). Conversely, 13-*cis* retinoic acid did not increase apoptosis of SH SY 5Y cells beyond control levels at any time point (Fig. 3).

Induction of apoptosis in SH SY 5Y cells in response to CD437 was initially studied with 1 µM concentrations as for the other retinoids. However, at this concentration CD437 induced over 60% apoptosis with no reduction on washout (Fig. 4). Therefore further assays were performed using CD437 at 1, 0.1 and 0.01 µM, treating cells either continuously for 24 hr or for 24 hr followed by 24 or 48 hr washout. Cells treated for 24 hr with 0.01 µM CD437

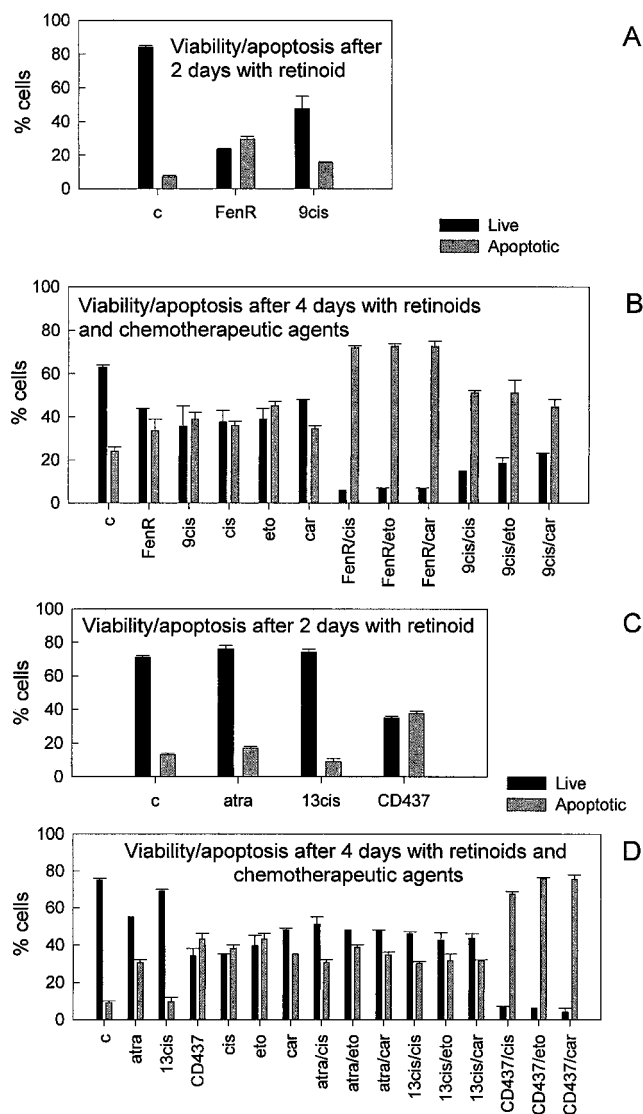


FIGURE 5 – Viability and apoptosis of SH SY 5Y cells in response to pre-treatment with retinoids followed by treatment with 1 µM cisplatin or etoposide or 10 µM carboplatin. (a) Viable and apoptotic cells after 2 days treatment with 1 µM fenretinide or 9-*cis* retinoic acid. (b) Viable and apoptotic cells after pre-treatment for 2 days with fenretinide or 9-*cis* retinoic acid and after subsequent treatment for 2 days (24 hr treatment and 24 hr wash-out) in the presence or absence of chemotherapeutic reagents. (c) Viable and apoptotic cells after 2 days treatment with 1 µM all-*trans* or 13-*cis* retinoic acid or 0.01 µM CD437 (CD437 added for 24 hr followed by washout for 24 hr). (d) Viable and apoptotic cells after pre-treatment with all-*trans* or 13-*cis* retinoic acid or CD437 for 2 days and after subsequent treatment for 2 days in the presence or absence of chemotherapeutic reagents. Each point is the mean ± range.

followed by 24 hr washout induced similar levels of apoptosis to those achieved by 3 µM fenretinide with 48 hr continuous treatment (Fig. 4) and therefore this concentration was used for combination experiments with chemotherapeutic reagents.

Induction of apoptosis in SH SY 5Y cells by pre-treatment with retinoids followed by chemotherapeutic drugs

To study potential additive or synergistic effects of retinoids with chemotherapeutic agents, SH SY 5Y cells were treated with fenretinide at 3 µM or retinoic acid isomers at 1 µM for 48 hr continuously or with CD437 at 0.01 µM for 24 hr followed by 24

hr washout prior to treatment with chemotherapeutic drugs. Apoptosis and viability assays were carried out after pre-treatment with retinoid, and after subsequent treatment with cisplatin, etoposide or carboplatin for 24 hr followed by 24 hr washout. Chemotherapeutic reagents were used at concentrations required to induce approximately 30% apoptosis at these time points. Apoptosis assays on fixed cells stained with propidium iodide confirmed that pre-treatment with fenretinide (3 μM) induced 30–35% apoptosis after 48 hr continuous incubation, with 9-*cis* retinoic acid (1 μM) inducing 15% apoptosis at the same time point (Fig. 5a). The number of viable cells in the duplicate, unfixed population of cells used for viability assay by flow cytometry essentially confirmed the apoptosis data (Fig. 5a). After washout of either fenretinide or 9-*cis* retinoic acid and subsequent addition of 1 μM cisplatin, 1 μM etoposide or 10 μM carboplatin for 24 hr followed by 24 hr washout of the chemotherapeutic drug, viable cell number and apoptosis were again assessed by flow cytometry. Pre-treatment of SH SY 5Y cells with fenretinide followed by treatment with cisplatin, etoposide or carboplatin increased apoptosis to 70% (Fig. 5b). Since, in these experiments, fenretinide or chemotherapeutic drugs on their own induced only 35–40% apoptosis, this suggests an additive or synergistic effect between fenretinide and these cytotoxic drugs.

Conversely, washout of 9-*cis* retinoic acid resulted in an increase of apoptosis to 40%, confirming previous results that apoptosis in response to this retinoic acid isomer increases with time after subsequent washout (Lovat *et al.*, 1997a) (Fig. 5b). However, treatment of cells with chemotherapeutic drugs after 9-*cis* retinoic acid only increased apoptosis to 50% (Fig. 4c). Since these chemotherapeutic drugs on their own induced 35–40% apoptosis, this result suggests that the effects of 9-*cis* pre-treatment followed by cisplatin, etoposide or carboplatin may be less than additive. All-*trans* and 13-*cis* retinoic acid were less effective at inducing apoptosis on their own, and had no apparent effect in combination with the chemotherapeutic drugs (Fig. 5c,d). As with 9-*cis* retinoic acid, levels of apoptosis in response to 0.01 μM CD437 increased after washout of this retinoid (Fig. 5c,d). Treatment of SH SY 5Y cells with etoposide, carboplatin or cisplatin after washout of CD437 resulted in increased levels of apoptosis to around 80% (Fig. 5c).

Since the pre-treatment of SH SY 5Y cells with either fenretinide or CD437 followed by treatment with chemotherapeutic drug enhanced the apoptotic response beyond either retinoid or chemotherapeutic drug alone, the induction of apoptosis by these two retinoids was studied in combination with varying doses of cisplatin, etoposide or carboplatin to establish if the effect was additive or synergistic. Since the viability measurements of previous experiments gave similar results to measurement of apoptosis on fixed and propidium-iodide-stained cells, these dose-response experiments were analysed for apoptosis alone. As in previous experiments, fenretinide (3 μM , continuous treatment) and CD437 (0.01 μM , 24 hr treatment followed by 24 hr wash-out) on their own each induced 35% apoptosis (Fig. 6). Induction of apoptosis by each cytotoxic drug (24 hr treatment followed by 24 hr wash-out) increased in a dose-dependent manner (Fig. 6a–c). Pre-treatment with CD437 (0.01 μM , 24 hr treatment then 24 hr wash-out) followed by treatment with either cisplatin, etoposide or carboplatin increased the level of apoptosis induced by all three drugs at all concentrations (Fig. 6a–c). Furthermore the combination indices (CI) for all 3 cytotoxic drugs with CD437 were ≤ 0.1 , indicative of strong to very-strong synergism (for additivity, $\text{CI} \approx 1$). At lower doses, fenretinide in combination with cisplatin or carboplatin was also synergistic ($\text{CI} 0.7\text{--}0.3$) but the synergistic effect increased with higher doses of cisplatin, etoposide or carboplatin ($\text{CI} < 0.3$). The synergistic effects are illustrated in Figure 6d,e, where the effects of CD437 with carboplatin and fenretinide with cisplatin at different doses all lie well above the Loewe-additivity surface defined by the independent dose-response curves for the synthetic retinoids and chemotherapeutic drugs.

The induction of free radicals in response to retinoids and chemotherapeutic drugs in neuroblastoma cells

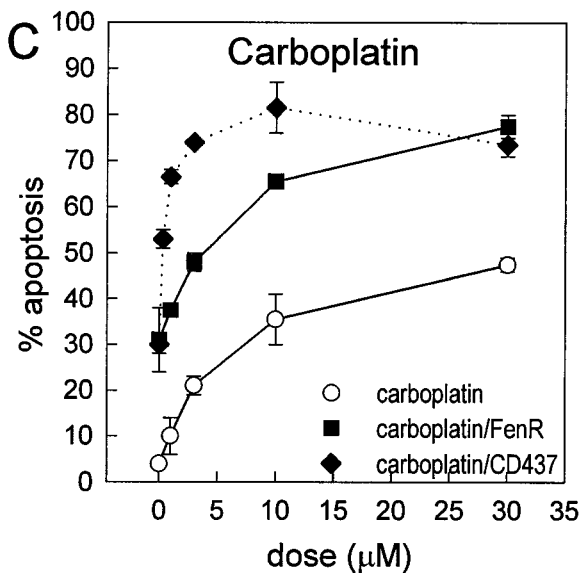
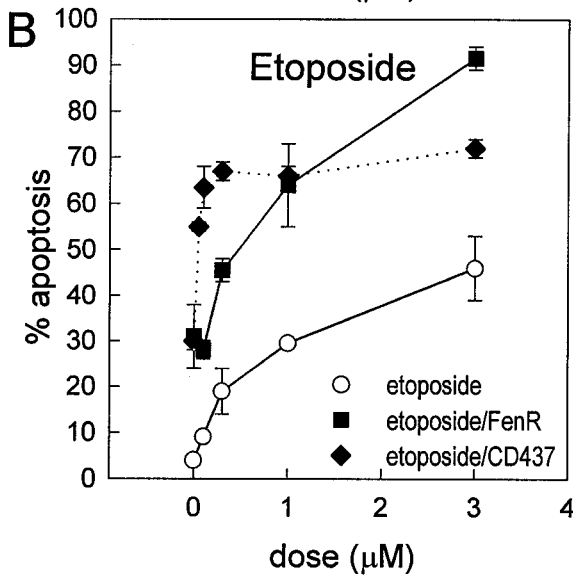
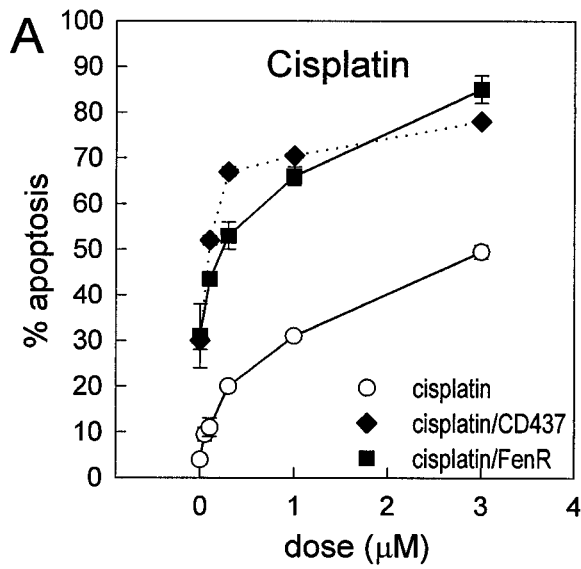
Fenretinide induces free radicals in neuroblastoma cells (Maurer *et al.*, 1999) and other cell types (Delia *et al.*, 1997). Therefore, the apoptotic effects of fenretinide and CD437 compared to retinoic acid isomers may be a result of the ability of these synthetic retinoids to induce free radicals in neuroblastoma cells. To test this idea and to ask whether free radicals are also induced by chemotherapeutic drugs, SH SY 5Y cells were treated with chemotherapeutic drugs or retinoids for 24 hr before assay for intracellular free radicals. Free radicals in SH SY 5Y cells were only marginally induced (up to 1.5-fold) in response to high doses of cisplatin, carboplatin or etoposide (Fig. 7). Conversely, both fenretinide and CD437 induced free radicals in these neuroblastoma cells to 5 or 6 times control levels whereas 9-*cis*, all-*trans* or 13-*cis* retinoic acid had no effect (Fig. 8).

We have previously demonstrated that inhibition of fenretinide-induced free radicals by antioxidants blocks the apoptotic response of neuroblastoma cells (Lovat *et al.*, 2000). To question if antioxidants inhibit the synergistic response seen with subsequent addition of chemotherapeutic agents to fenretinide or CD437-treated cells, SH SY 5Y cells were pre-treated with either vitamin C or E for 2 hr prior to the addition of fenretinide (3 μM), CD437 (0.01 μM) or control vehicle. Retinoids and chemotherapeutic agents (1 μM cisplatin or etoposide, 10 μM carboplatin) were added using the same experimental design as described above. Both vitamin E (Fig. 9) and vitamin C (data not shown) inhibited fenretinide- or CD437-induced apoptosis but not apoptosis induced by chemotherapeutic drugs. However, both antioxidants inhibited the response to fenretinide or CD437 in combination with cisplatin, etoposide or carboplatin to the level achieved by each chemotherapeutic drug alone (Fig. 9). Thus, the synergistic apoptotic response of these retinoids with chemotherapeutic agents was abolished by antioxidants.

DISCUSSION

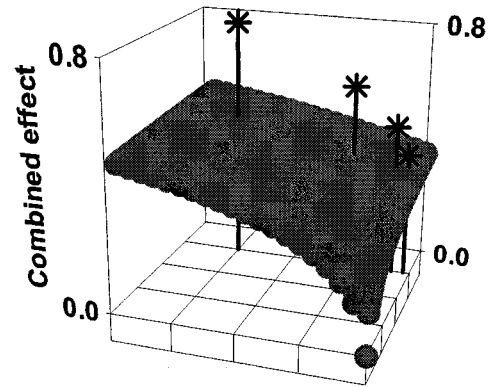
Recent clinical trials of 13-*cis* retinoic acid in the treatment of neuroblastoma after chemotherapy and bone marrow transplantation indicate that retinoids used post-chemotherapy may produce a substantial improvement in survival rates (Matthay *et al.*, 1999). Whether 13-*cis* retinoic acid would also improve survival rates if given before or during chemotherapy is unknown, although observations that neuroblastoma cells treated with retinoic acid may become more resistant to chemotherapeutic drugs (Lasorella *et al.*, 1995) suggest that this would not be beneficial. Resistance to chemotherapeutic drugs may be expected if the main effect of 13-*cis* retinoic acid is to promote differentiation resulting in reduced proliferation and reduced sensitivity to cytotoxic drugs. However, interactions between retinoic acid and chemotherapeutic drugs may be both cell-type and drug-specific, since all-*trans* retinoic acid abrogates the effects of etoposide on head and neck cancer cells (Kim *et al.*, 1989) while enhancing the apoptotic response to cisplatin (Aebi *et al.*, 1997). While we were unable to detect any effect of 13-*cis* or all-*trans* retinoic acid on drug

FIGURE 6 – Apoptosis (measured by flow cytometry) in SH SY 5Y neuroblastoma cells pre-treated with 3 μM fenretinide or 0.01 μM CD437 and then exposed to different concentrations of cisplatin (a), etoposide (b), or carboplatin (c). Parameters for dose-response curves for fenretinide, CD437, cisplatin and carboplatin on their own were estimated for the median-effect equation (Chou, 1991; Chou and Talay, 1984) and used to define the Loewe-additivity surface (Additive Model) shown as three-dimensional graphs in d and e; the observed responses (Response) for fenretinide with cisplatin (d) and CD437 with carboplatin (e) are shown (asterisk) with vertical lines dropping through the surface to the x-y plane. Axes: x, cisplatin (d, 0–4 μM) or carboplatin (e, 0–30 μM), y, fenretinide (d, 0–4 μM) or CD437 (e, 0–0.25 μM), z, combined effect (0–0.8).



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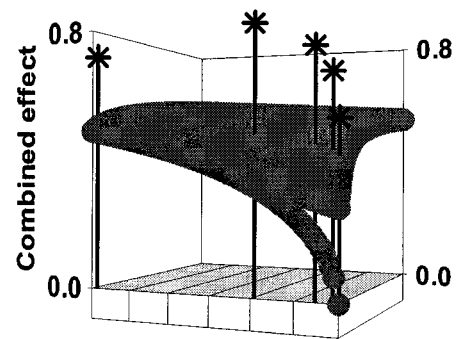
Fenretinide and Cisplatin



* *Response*
● *Additive Model*

E

CD437 and Carboplatin



* *Response*
● *Additive Model*

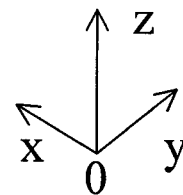


FIGURE 6.

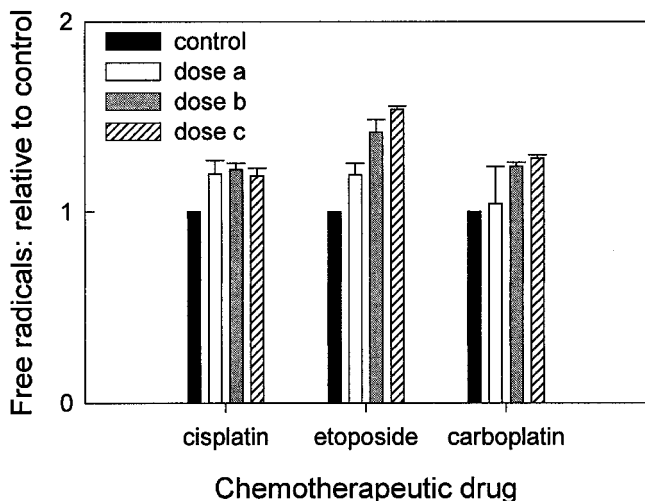


FIGURE 7 – Generation of free radicals in SH SY 5Y cells in response to treatment for 24 hr with 1, 3 or 10 μM cisplatin or etoposide (dose a, dose b and dose c, respectively) or 10, 30 or 100 μM carboplatin (dose a, dose b and dose c, respectively). Each point is the mean \pm range plotted relative to control cells.

sensitivity with the present experimental design, 9-*cis* retinoic acid did show some evidence that its effects with chemotherapeutic drugs were less than additive. Although 9-*cis* retinoic acid can induce apoptosis of N-type neuroblastoma cells *in vitro* under certain conditions (Lovat *et al.*, 1997a), this isomer is a more powerful differentiation-inducing agent than 13-*cis* or all-*trans* retinoic acid (Lovat *et al.*, 1997b).

Unlike 13-*cis* retinoic acid, the retinoid analogues fenretinide and CD437 do not induce morphological differentiation but are

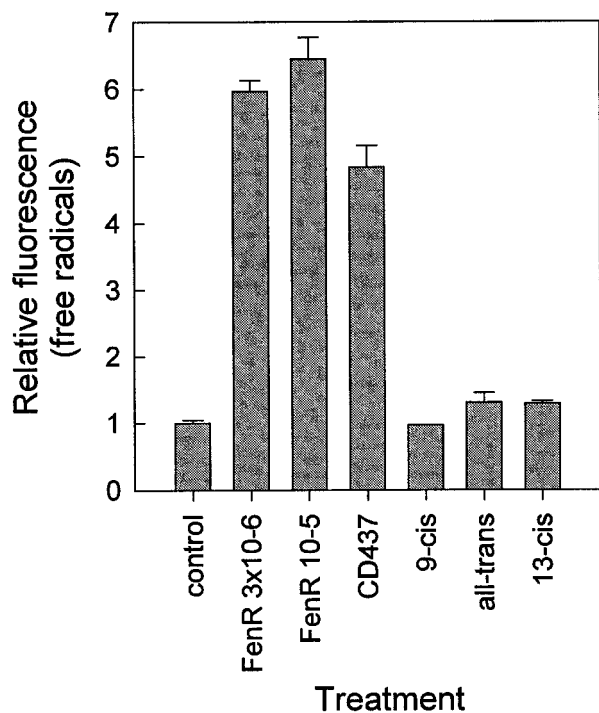


FIGURE 8 – Generation of free radicals in SH SY 5Y cells in response to 24 hr treatment with 3 or 10 μM fenretinide or 1 μM CD437, 9-*cis*, all-*trans* or 13-*cis* retinoic acid. Each point is the mean \pm range plotted relative to control cells.

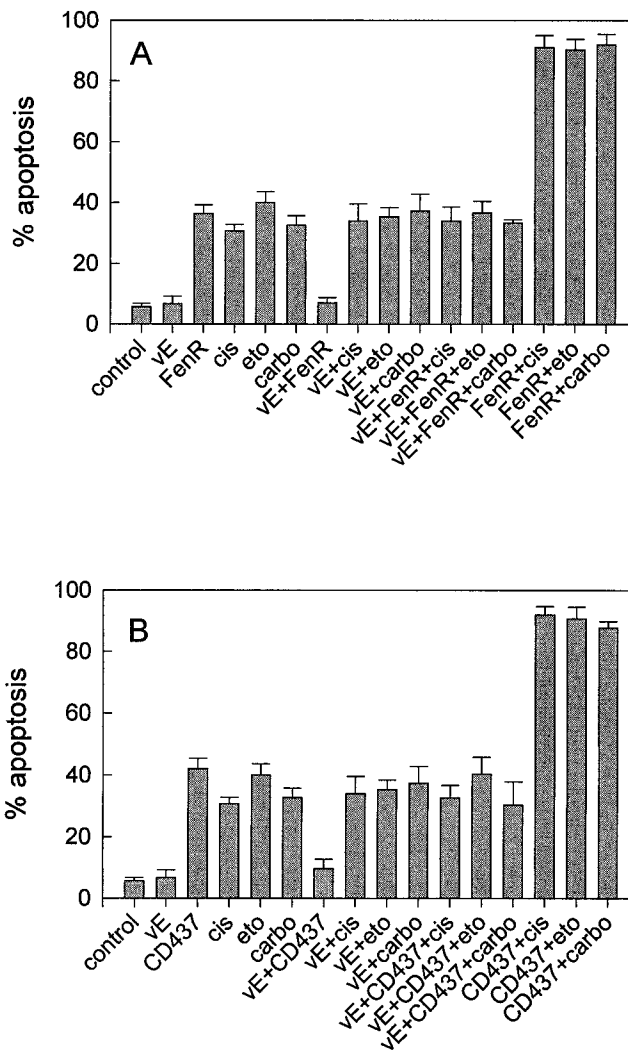


FIGURE 9 – Vitamin E inhibition of synergistic apoptosis induced by fenretinide or CD437 and chemotherapeutic agents. Apoptosis of SH SY 5Y cells after pre-treatment for 2 hr with or without 1 mM vitamin E (VE) before 48 h incubation with either 3 μM fenretinide (FenR, a) or 24 hr treatment and 24 hr wash-out with 0.01 μM CD437 (b) and subsequent treatment (24 hr treatment, followed by 24 hr washout) in the presence or absence of either 1 μM cisplatin (cis), 1 μM etoposide (eto) or 10 μM carboplatin (carbo). Each bar is the mean \pm SD of 3 replicates.

more effective at directly inducing apoptosis of neuroblastoma cells *in vitro* and may therefore be more beneficial *in vivo* if combined with conventional chemotherapy. In contrast to retinoic acid, both CD437 and fenretinide produced synergistic effects with chemotherapeutic drugs, particularly CD437. Similar studies with small-cell lung carcinoma cells *in vitro* have concluded that fenretinide acts synergistically with cisplatin or etoposide to arrest growth (Kalemkerian and Xialolan, 1999) and pre-treatment of breast cancer cells with fenretinide also increases growth inhibition in response to cisplatin (Grunt *et al.*, 1998). Clearly, both fenretinide and CD437 offer considerable potential as new agents for neuroblastoma therapy, which might increase the efficacy of existing chemotherapeutic drugs or facilitate their use at lower concentrations with possible reduction in treatment-associated morbidity. However, studies with animal models will be required to assess whether fenretinide or CD437 in combination with chemotherapeutic drugs has greater dose-limiting toxicity in young patients.

The mechanism of synergy between fenretinide or CD437 and chemotherapeutic drugs is unclear. Although, the synthetic retinoids and chemotherapeutic drugs used for this study induce apoptosis, marked increases in free radicals were only produced by the retinoids fenretinide and CD437. This confirms previous data reporting free-radical induction by fenretinide in neuroblastoma (Lovat *et al.*, 2000) and other cell types (Delia *et al.*, 1997). In addition, like fenretinide (Lovat *et al.*, 2000), CD437 also mediates apoptosis via a mitochondrial mechanism (Marchetti *et al.*, 1999). Since the synergistic apoptotic response between fenretinide or CD437 and chemotherapeutic agents was abolished by antioxidants, this suggests that free radical generation is important for synergy between these synthetic retinoids and chemotherapeutic drugs. Although the biochemical pathway(s) resulting in free radical generation in response to fenretinide or CD437 are unknown, this mechanism is caspase independent and may be an early step in apoptosis (Lovat *et al.*, 2000). Chemotherapeutic drugs do not induce comparable levels of free radicals but are known to mediate apoptosis either by p53-dependent mechanisms (Lauricella *et al.*, 1998; Siemer *et al.*, 1999) or by the activation of death-receptor pathways, such as CD95/Fas (Fulda *et al.*, 1997; Gibson *et al.*,

2000). However, treatment of SH SY 5Y cells with fenretinide does not change levels of CD95/Fas or Fas ligand (Lovat and Redfern, unpublished data). Therefore, synergy between fenretinide or CD437 and chemotherapeutic drugs may result from the priming of neuroblastoma cells to a state of 'apoptotic readiness' or as a result of the activation of alternative apoptotic pathways. These alternative mechanisms are not mutually exclusive and it is important to establish if synergism occurs in response to any agent that induces free radicals in neuroblastoma cells or if it is specific to fenretinide and CD437. In addition, synergism with chemotherapeutic drugs may not be simply a consequence of free radical generation but may depend on other properties of these synthetic retinoids such as their ability to interact with retinoic acid receptors. Clearly, these synthetic retinoids provide new opportunities for novel neuroblastoma therapy.

ACKNOWLEDGEMENTS

The authors thank Janssen-Cilag, Ltd. for supplying the fenretinide and Dr. U. Reichert, Galderma, for CD437.

REFERENCES

- AEBI S., KRONING R., CENNI B., SHARMA A., FINK D., LOS G., WEISMAN R., HOWELL S.B. and CHRISTEN R.D., All-trans retinoic acid enhances cisplatin-induced apoptosis in human ovarian adenocarcinoma and in squamous head and neck cancer. *Clin. Cancer Res.*, **3**, 2033–2038 (1997).
- AESCHBACHER M., REINHARDT C.A. and ZBINDEN G.A., A rapid cell membrane permeability test using fluorescent dyes and flow cytometry. *Cell Biol. Toxicol.*, **2**, 247–255 (1986).
- BIEDLER J.L., HELSAN L. and SPENGLER, B.A., Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.*, **33**, 2643–2652 (1973).
- CHOU T.-C., The median-effect principle and the combination index for quantification of synergism and antagonism. In: T.-C. CHOU, and D.C. RIDEOUT, editors. *Synergism and antagonism in chemotherapy*. Academic Press, San Diego p 61–102 (1991).
- CHOU T.C. and TALALAY P., Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.*, **22**, 27–55 (1984).
- DELIA D., AIELLO A., MERONI L., NICOLINI M., REED J.C. and PIEROTTI M.A., Role of antioxidants and intracellular free radicals in retinamide-induced cell death. *Carcinogenesis*, **18**, 943–948 (1997).
- DRESSLER V., MULLER G. and SUHNEL J., CombiTool: a new computer program for analysing combination experiments with biologically active agents. *Comput. Biomed. Res.*, **32**, 145–160 (1999).
- FULDA S., SIEVERTS H. and FRIESEN C., The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res.*, **57**, 3823–3829 (1997).
- GIBSON S.B., OYER R., SPALDING A.C., ANDERSON S.M. and JOHNSON G.L., Increased expression of death receptors 4 and 5 synergises the apoptosis response to combined treatment with etoposide and TRAIL. *Mol. Cell Biol.*, **20**, 205–212 (2000).
- GRUNT T.W., DITTRICH E., OFFTERDINGER M., SCHNEIDER S.M., DITTRICH C.H. and HUBER, H., Effects of retinoic acid and fenretinide on the c-erbB-2 expression, growth and cisplatin sensitivity of breast cancer cells. *Brit. J. Cancer*, **78**, 70–87 (1998).
- HANADA M., KRAJEWSKI S., TANAKA S., CAZALSHATEM D., SPENGLER B.A., ROSS R.A., BIEDLER J.L. and REED J.C., Regulation of Bcl-2 oncoprotein levels with differentiation of human neuroblastoma cells. *Cancer Res.*, **53**, 4978–4986 (1993).
- JIANG S.Y., LIN D.Y., SHYU R.Y., REICHERT U. and YEH M.Y., The RAR gamma selective agonist CD437 inhibits gastric cell growth through the mechanism of apoptosis. *Cancer Lett.*, **137**, 217–225 (1999).
- KALEMKERIAN G.P. and XIALOLAN O., Activity of fenretinide plus chemotherapeutic agents in small lung cancer cell lines. *Cancer Chemother. Pharmacol.*, **43**, 145–150 (1999).
- KIM H.K., ZWELLING L.A., SACKS P.G., HONG W.K., CHAN D., SILBERMAN L. and GLISSON B.S., Effect of retinoic acid on DNA cleavage and cytotoxicity of topoisomerase II-reactive drugs in a head and neck cancer cell line. *Cancer Res.*, **49**, 1197 (1989).
- LASORELLA A., IAVARONE A. and ISRAEL M.A., Differentiation of neuroblastoma enhances Bcl-2 expression and induces alterations of apoptosis and drug resistance. *Cancer Res.*, **55**, 4711–4716 (1995).
- LAURICELLA, M., GIULIANO, M., EMANUELE, S., VENTO, R. and TESORIERE, G., Apoptotic effects of different drugs on cultured retinoblastoma Y79 cells. *Tumour Biol.*, **19**, 356–363 (1998).
- LIANG J.Y., FONTANA J.A., RAO J.N., ORDONEZ J.V., DAWSON M.I., SHROOT B., WILBER J.F. and FENG P., Synthetic retinoid CD437 induces S-phase arrest and apoptosis in human prostate cancer cells LNCaP and PC-3. *Prostate*, **38**, 228–236 (1999).
- LOVAT P.E., IRVING H., ANNICCHIARICO-PETRUZZELLI M., BERNASSOLA F., MALCOLM A., PEARSON A.D.J., MELINO G. and REDFERN C.P.F., Apoptosis of neuroblastoma cells after differentiation with 9-cis retinoic acid and subsequent washout. *J. nat. Cancer Inst.*, **89**, 446–452 (1997a).
- LOVAT, P.E., IRVING, H., MALCOLM, A.J., PEARSON A.D.J. and REDFERN C.P.F., 9-cis retinoic acid: a better retinoid for the modulation of differentiation, proliferation and gene expression in human neuroblastoma. *J. Neuro-oncol.*, **31**, 85–91 (1997b).
- LOVAT P.E., RANALLI M., ANNICHIARRICO-PETRUZZELLI M., BERNASSOLA F.M.P., MALCOLM A.J., PEARSON A.D.J., MELIN G. and REDFER C.P.F., Effector mechanisms of fenretinide-induced apoptosis in neuroblastoma. *Exp. Cell Res.*, **260**, 50–60 (2000).
- MARCHETT P., ZAMZAM N., JOSEP B., SCHRAEN-MASCHK S., MEREAU-RICHAR C., COSTANTIN P., METIVIE D., SUSI S., KROEME G. and FORMSTECHE P., The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. *Cancer Res.*, **59**, 6257–6266 (1999).
- MARIS J.M. and MATTHAY K.K., Molecular Biology of Neuroblastoma. *J. Clin. Oncol.*, **17**, 2264–2279 (1999).
- MATTHAY K.K., VILLABLANCA J.G., SEEGER R.C., STRAM D.O., HARRIS R.E., RAMSY N.K., SWIFT P., SHIMADA H., BLACK C.T., BRODEUR G.M., GERBERG, R.B. and REYNOLDS, C.P., Treatment of high risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis retinoic acid. *N. Engl. J. Med.*, **341**, 1165–1173 (1999).
- MAURER B.J., METELITSA L.S., SEEGER R.C., CABOT M.C. and REYNOLDS C.P., Increase of ceramide and induction of mixed apoptosis/necrosis by N-(4-hydroxyphenyl)-retinamide in neuroblastoma cell lines. *J. nat. Cancer Inst.*, **91**, 1138–1146 (1999).
- MEISTER B., FINK F.M., HITTMAIR A., MARTH C. and WIDSCHWENDTER M., Antiproliferative activity and apoptosis induced by retinoic acid receptor- γ selectively binding retinoids in neuroblastoma. *Anticancer Res.*, **18**, 1777–1786 (1998).
- MELINO G., ANNICCHIARICO-PETRUZZELLI M., PIREDDA L., CANDI E., GENTILE V., DAVIES, P.J.A. and PIACENTINI M., Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. *Mol. Cell Biol.*, **14**, 6584–6596 (1994).
- POSSEL H., NOACK H., AUGUSTIN W., KEILHOFF G. and WOLF G., 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker of peroxytrite formation. *FEBS Lett.*, **416**, 175–178 (1997).
- SHEN J.C., WANG T.T., CHANG S. and HURSTING S.D., Mechanistic studies

- of the effects of the retinoid N-(4-hydroxyphenyl) retinamide on prostate cancer cell growth and apoptosis. *Molec. Carcinog.*, **3**, 160–168 (1999).
- SIDELL N., ALTMAN A., HAUSSLER M.R. and SEEGER R.C., Effects of retinoic acid (RA) on the growth and phenotypic-expression of several human neuroblastoma cell lines. *Exp. Cell Res.*, **148**, 21–30 (1983).
- SIEMER S., ORNSKOV D., GUERRA B., BOLDYREFF, B. and ISSINGER, O.G., Determination of mRNA, and protein levels of p53, MDM2 and protein kinase CK2 subunits in F9 cells after treatment with the apoptosis-inducing drugs cisplatin and carboplatin. *Int. J. Biochem. Cell Biol.*, **31**, 661–670 (1999).
- SUN S.Y., LI W., LIPPMAN S.M., HONG W.K. and LOTAN R., Mediation of N-(4-hydroxyphenyl)retinamide-induced apoptosis in human cancer cells by different mechanisms. *Cancer Res.*, **59**, 2493–2498 (1999a).
- SUN S.Y., YUE P. and LOTAN R., Induction of apoptosis by N-(4-hydroxyphenyl)retinamide and its association with reactive oxygen species, nuclear retinoic acid receptors, and apoptosis-related genes in human prostate carcinoma cells. *Mol. Pharmacol.*, **55**, 403–410 (1999b).
- SUN S.Y., YUE P., WU G.S., EL-DEIRY W.S., SHROOT B., HONG W.K. and LOTAN R., Implication of p53 in growth arrest and apoptosis induced by the synthetic retinoid CD437 in human lung cancer cells. *Cancer Res.*, **59**, 2829–2833 (1999c).
- SUN S.Y., YUE P., WU G.S., SHROOT B., HONG W.K. and LOTAN R., Mechanisms of apoptosis induced by the synthetic retinoid CD437 in human non-small cell lung carcinoma cells. *Oncogene*, **18**, 2357–2365 (1999d).
- ZHANG Y., HUANG Y., RISHI A.K., SHEIKH M.S., SHROOT B., REICHERT U., DAWSON M., POIRER G. and FONTANA J.A., Activation of the p38 and JNK/SAPK mitogen-activated protein kinase pathways during apoptosis is mediated by a novel retinoid. *Exp. Cell Res.*, **247**, 233–240 (1999).