

Distinct Properties of Fenretinide and CD437 Lead to Synergistic Responses With Chemotherapeutic Reagents

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The RAR β / γ -selective retinoids fenretinide and CD437 induce caspase-dependent apoptosis but generate free radicals independently of caspases. Apoptosis, but not free radical generation, induced by these retinoids is inhibited by RAR β / γ -specific antagonists. Both fenretinide and CD437 induce apoptosis synergistically with cisplatin, carboplatin, or etoposide. However, antioxidants inhibit this synergy to the level obtained with chemotherapeutic drugs alone, and this implies that free radical generation is important in the synergistic re-

sponse. Since apoptosis induced by fenretinide or CD437 is mediated by apoptotic pathways involving RARs and/or mitochondria and differs from mechanisms of chemotherapy-induced apoptosis this may explain the strong synergistic response seen between these synthetic retinoids and chemotherapeutic drugs. These results suggest that fenretinide or CD437 may be useful adjuncts to neuroblastoma therapy. *Med. Pediatr. Oncol.* 35:663–668, 2000.

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Key words: fenretinide; CD437; chemotherapy

INTRODUCTION

Despite intensive treatment, only 25% of children with stage 4 neuroblastoma over the age of 1 year survive [1]. The chemotherapeutic agents cisplatin, etoposide, and carboplatin are important components of the cytotoxic drug regimen used in the United Kingdom. Randomised clinical trials have recently shown that 13-cis-retinoic acid increases survival when used to treat residual disease after chemotherapy and bone marrow transplantation [2]. *In vitro*, retinoic acid and its analogues induce differentiation and apoptosis of neuroblastoma, but the response depends on the particular retinoic acid isomer in use [3,4]. Recent studies have demonstrated that two particular retinoid analogues, fenretinide and CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid), induce more effective apoptosis of neuroblastoma *in vitro* than either all-trans, 13-cis-, or 9-cis-retinoic acid [5–7].

The mechanisms of both fenretinide and CD437-induced cell death are complex and probably involve several overlapping pathways, including those involving the retinoic acid receptors [8,9] and oxidative stress via the induction of free radicals [8–10]. In addition, a recent study in neuroblastoma suggested that a p53-independent pathway of fenretinide-induced apoptosis may operate through increased intracellular levels of the lipid secondary messenger ceramide [6]. However, the precise mechanisms by which these synthetic retinoids induce cell death is uncertain.

Recent studies have suggested that fenretinide may enhance the responses of non-small cell lung cancer cells and breast cancer cells to chemotherapeutic drugs [11,12]. Chemotherapeutic drugs, particularly cisplatin and etoposide, are the mainstay of chemotherapy for metastatic neuroblastoma and the aim of this study was to ask whether pretreatment of neuroblastoma cells with fenretinide or CD437 enhances or abrogates the apoptotic effects of chemotherapeutic drugs on neuroblastoma cells.

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Contract grant sponsor: Association for International Cancer Research, United Kingdom; Contract grant sponsor: The Neuroblastoma Association, Genoa; Contract grant sponsor: AIRC; Contract grant sponsor: MURST, Italy.

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MATERIALS AND METHODS

Cell Culture and Treatment With Retinoids, Chemotherapeutic Reagents, Caspase Inhibitors, RAR Antagonists, and Antioxidants

Human neuroblastoma cell lines SH SY 5Y [13] and SK N BE (2) [14] were grown in 1:1 Dulbecco's modified Eagle's medium:Ham's F12 (Life Technologies Ltd., Paisley, United Kingdom), supplemented with 10% foetal bovine serum (Sera-Lab, Crawley, United Kingdom; culture medium) as described earlier [7]. For all experiments, cells were seeded into 25 cm² tissue culture flasks (1 × 10⁶ cells) or 100 mm tissue culture-grade Petri dishes (Costar, Cambridge, United Kingdom; 0.4 × 10⁶ cells) and allowed to attach overnight before treatment.

Fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) was added in ethanol, and an equal volume of ethanol (<0.1% of culture volume) was used to treat control cells. CD437 and the RARβ/γ antagonist CD2665 (Dr. U. Reichert, Galderma, Sophia Antipolis, France) were added in DMSO, and an equal volume of DMSO was used to treat control cells. Fresh stock solutions of cisplatin (100 mM), carboplatin (10 mM), and etoposide (20 mM; all from Sigma Chemical Co., Poole, United Kingdom) were prepared in DMSO or culture medium on the day of experiment and further diluted in culture medium to appropriate concentrations. Caspase activity was inhibited by the IL-1β-converting enzyme (ICE) inhibitor VI, Z-Val-Ala-Asp-fluoromethylketone (ZVAD; Calbiochem-Novabiochem Ltd., Nottingham, United Kingdom), diluted in DMSO, and used at a final concentration of 25 μM. The antioxidants vitamin C (ascorbic acid sodium salt) and vitamin E (α-tocopherol; both from Sigma) were freshly prepared and added 2 hr prior to treatment with fenretinide, CD437, or vehicle control as previously described [7].

Measurement of Apoptosis by Flow Cytometry

Cells were treated with retinoids and/or chemotherapeutic reagents for appropriate times; when the incubation period exceeded 2 days, the culture medium was changed every other day. At each change of culture medium, the existing medium was harvested and centrifuged at 200 g for 5 min at 21°C, and the pelleted material, consisting of apoptotic bodies and nonadherent cells, was resuspended in fresh culture medium plus the appropriate test reagent. At the end of the experiment, the medium was harvested, and apoptotic bodies and nonadherent cells were collected by centrifugation and pooled with cells recovered from the culture vessel by trypsinisation [15]. The resulting sample was resuspended in phosphate-buffered saline (PBS; ICN-Flow, High Wycombe, United Kingdom), fixed with an equal volume of cold (-20°C) methanol:acetone (4:1 vol/vol), and stored at 4°C prior to evaluation of apoptosis by flow cytometry [7].

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 10 μM fenretinide or 1 μM CD437 [16]. At the end of treatment, cells were incubated in the presence of 10 μM 2,7-dihydrodichlorofluorescein diacetate (Molecular Probes Inc., Eugene, OR) before acquisition for flow cytometry [7].

Data Analysis

For the analysis of synergistic interactions, the CalcuSyn (Elsevier BioSoft) program was used to derive combination indices (CI) and parameter estimates for the median effect equation [17,18]. Parameter estimates for single treatments were then used to compare drug combination results against the Loewe additivity surface produced using the CombiTool program [19].

RESULTS

Inhibition of Fenretinide- and CD437-Induced Apoptosis and Free Radical Generation by the Caspase Inhibitor ZVAD

Fenretinide and CD437 induce caspase-dependent apoptosis of neuroblastoma cells associated with free radical generation and mitochondrial-mediated apoptosis [7]. Conversely, cisplatin, carboplatin, or etoposide do not induce comparable levels of free radicals in neuroblastoma cells (Lovat et al., unpublished results). Free radicals are generated in SH SY 5Y cells in response to fenretinide [7], an effect not seen in response to retinoic acid (data not shown). To examine the role of caspases in free radical generation, SH SY 5Y and SK N BE cells were preincubated for 2 hr with ZVAD before addition of 1 μM CD437 and incubation for a further 22 hr. In both SH SY 5Y (data not shown) and SK N BE cells, ZVAD did not induce free radicals or inhibit the induction of free radicals in response to CD437 (Fig. 1). Similar results have been obtained using fenretinide [7]. These results suggest that, although fenretinide or CD437-induced apoptosis can be inhibited by ZVAD, free radical generation in response to these retinoids is caspase-independent.

Inhibition of Fenretinide- and CD437-Induced Apoptosis by RAR Antagonists

To test the hypothesis that the action of fenretinide or CD437 is mediated by specific RARs, SH SY 5Y cells were treated with 1 μM fenretinide or CD437 for 2 days in the presence or absence of the RARβ/γ antagonist CD2665 (1 μM). Both fenretinide- and CD437-induced apoptosis were inhibited by CD2665 (Fig. 2). These results suggest that RARβ or RARγ or both may be in-

CD437-induced free radicals in SK N Be cells:
lack of inhibition by ZVAD

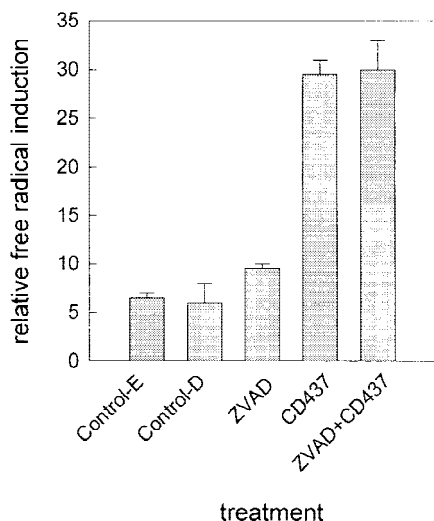


Fig. 1. CD437-induced free radical generation in SK N BE cells: lack of inhibition by ZVAD. Induction of free radicals by control ethanol (E) or DMSO (D) or ZVAD (25 μ M), CD437 (1 μ M), or ZVAD + CD437 when cells were pretreated with ZVAD for 2 hr, followed by 22 hr in the presence or absence of CD437. Each bar shows the mean and range.

involved in fenretinide- and CD437-induced apoptosis of neuroblastoma cells.

Induction of Apoptosis in SH SY 5Y Cells by Pretreatment With CD437 or Fenretinide Followed by Chemotherapeutic Drugs

To study potential additive or synergistic effects of retinoids with chemotherapeutic agents, SH SY 5Y cells were treated with fenretinide (3 μ M) for 48 hr or with CD437 (0.01 μ M) for 24 hr, followed by 24 hr washout, prior to treatment with chemotherapeutic drugs. These doses and times were defined on the basis that in these conditions both CD437 and fenretinide induce 30–40% apoptosis. Evaluation of apoptosis was carried out after pretreatment with retinoid and after subsequent treatment with cisplatin, etoposide, or carboplatin for 24 hr followed by 24 hr washout. Chemotherapeutic reagents were also used at concentrations required to induce approximately 30% apoptosis at these time points. Because the pretreatment 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 (see Fig. 4), the induction of apoptosis by these two retinoids was studied in combination with varying doses of cisplatin, etoposide, or carboplatin to establish whether the effect was additive or synergistic. Evaluation of combination indices (CI) for all three cytotoxic drugs with CD437 or

Inhibition of Fenretinide- and CD437-induced apoptosis of SH SY 5Y cells by an RAR β / γ antagonist

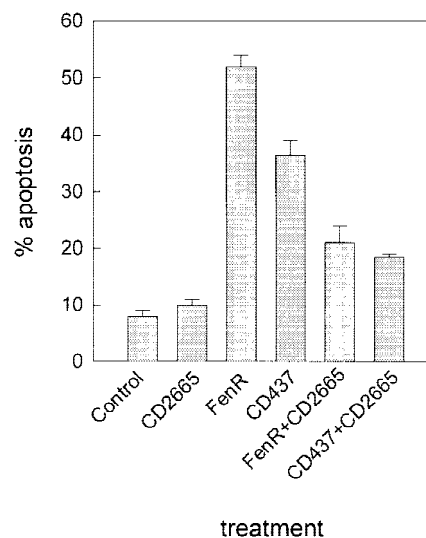


Fig. 2. Inhibition of fenretinide or CD437-induced apoptosis of SH SY 5Y cells at 2 days by the RAR β / γ antagonist CD2665. Induction of apoptosis by control, CD2665 (1 μ M), fenretinide (3 μ M; FenR), CD437 (1 μ M), fenretinide + CD2665, or CD437 + CD2665 at 48 hr. Each bar shows the mean and range.

fenretinide demonstrated synergism in all cases; this is shown as a three-dimensional plot for the combination of fenretinide or CD437 with etoposide in Figure 3.

We have previously demonstrated that inhibition of fenretinide-induced free radicals by antioxidants blocks the apoptotic response of neuroblastoma cells [7]. To determine whether antioxidants inhibit the synergistic response seen with subsequent addition of chemotherapeutic agents to fenretinide- or CD437-treated cells, SH SY 5Y cells were pretreated with either vitamin C or vitamin E for 2 hr prior to the addition of fenretinide (3 μ M), CD437 (0.01 μ M), or control vehicle. Retinoids and chemotherapeutic agents (1 μ M cisplatin or etoposide, 10 μ M carboplatin) were added using the same experimental design as described above. Both vitamin C (Fig. 4) and vitamin E (data not shown) inhibited fenretinide- or CD437-induced apoptosis but not apoptosis induced by chemotherapeutic drugs. The antioxidants inhibited the response to fenretinide or CD437 in combination with cisplatin, etoposide, or carboplatin only to the level achieved by each chemotherapeutic drug alone (Fig. 4). 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

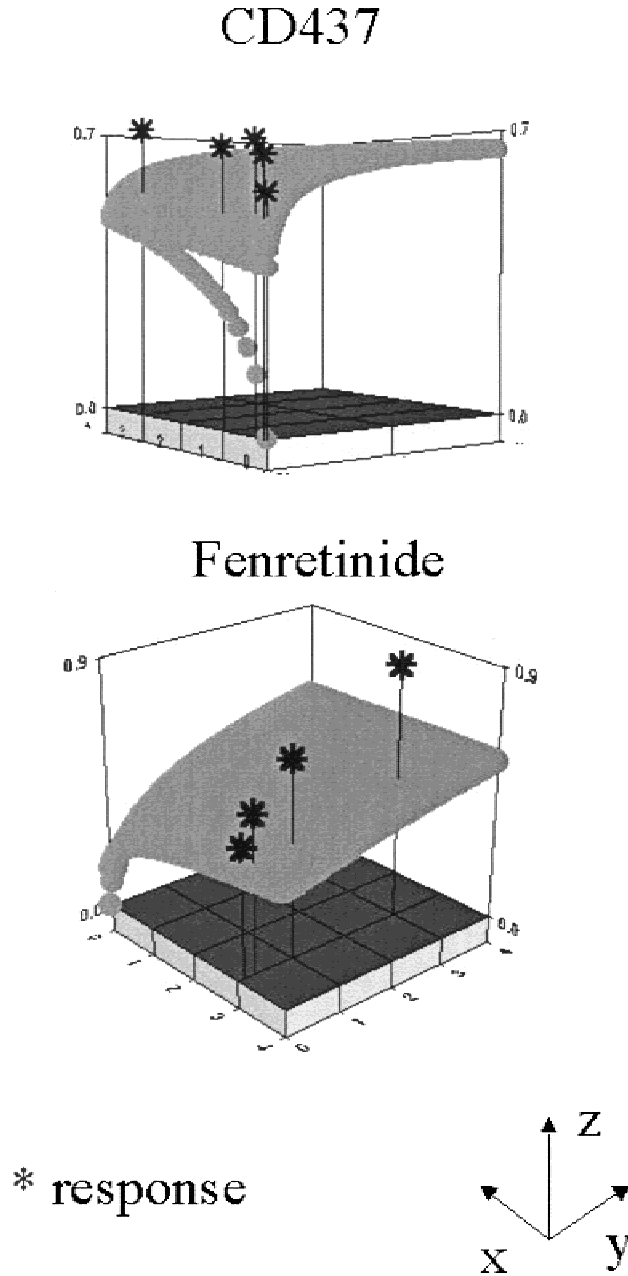


Fig. 3. Synergistic induction of apoptosis between etoposide and fenretinide or CD437 in SH SY 5Y cells. Parameters for dose-response curves for fenretinide, CD437, and etoposide on their own were estimated for the median effect equation [17,18] and used to define the Loewe additivity surface (additive model), shown as three-dimensional graphs for CD437 (upper graph) and fenretinide (lower graph). The observed responses (response) for CD437 or fenretinide with etoposide are shown (asterisks), with vertical lines dropping through the surface to the x-y plane. Axes: x, etoposide (0–4 μ M); y, CD437 (0–0.25 μ M) or fenretinide (0–4 μ M); z, combined effect as a proportion (0 = no apoptosis, 1 = 100% apoptosis).

marrow transplantation indicate that retinoids used post-chemotherapy may produce a substantial improvement in survival rates [2]. Whether 13-cis-retinoic acid would also improve survival rates when given before or during

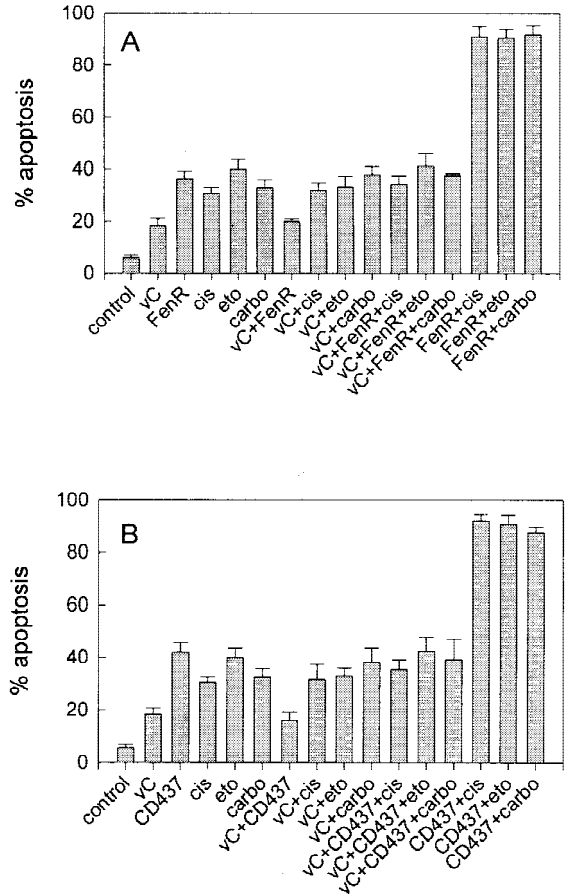


Fig. 4. Vitamin C inhibition of synergistic apoptosis induced by fenretinide or CD437 and chemotherapeutic agents. Apoptosis of SH SY 5Y cells after pretreatment for 2 hr with or without 100 μ M vitamin C (vC) before 48 hr of incubation with either 3 μ M fenretinide (FenR; **A**) or 24 hr of treatment and 24 hr of washout with 0.01 μ M CD437 (**B**) and subsequent treatment (24 hr treatment, followed by 24 hr washout) in the presence or absence of either 1 μ M cisplatin (cis), 1 μ M etoposide (eto), or 10 μ M carboplatin (carbo). Each bar shows the mean \pm SD of three replicates.

chemotherapy is unknown, although observations that neuroblastoma cells treated with retinoic acid may become more resistant to chemotherapeutic drugs [20] 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, in that all-trans-retinoic acid abrogates the effects of etoposide on head and neck cancer cells [21] while enhancing the apoptotic response to cisplatin [22].

Unlike 13-cis-retinoic acid, the retinoid analogues fenretinide and CD437 do not induce morphological differentiation but are more effective at directly inducing apoptosis of neuroblastoma cells in vitro and may there-

fore be more beneficial *in vivo* when combined with conventional chemotherapy. In contrast to retinoic acid, both CD437 and fenretinide produced synergistic effects with chemotherapeutic drugs. Similar studies with small cell lung carcinoma cells *in vitro* have led to the conclusion that fenretinide acts synergistically with cisplatin or etoposide to arrest growth [12] and that pretreatment of breast cancer cells with fenretinide also increases growth inhibition in response to cisplatin [11]. Clearly, both fenretinide and CD437 offer considerable potential as new agents for neuroblastoma therapy that might increase the efficacy of existing chemotherapeutic drugs. 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. Both fenretinide [7] and CD437 [23,24] can mediate apoptosis via mitochondrial mechanisms, although the mechanism of action on mitochondria may differ between fenretinide and CD437; unlike CD437 [24], fenretinide induces cytochrome c release independently of changes in the mitochondrial membrane permeability transition [7]. The fact that the synergistic apoptotic response between fenretinide or CD437 and chemotherapeutic agents was abolished by antioxidants 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 is unknown, this mechanism is caspase-independent and may be an early step in apoptosis [7].

Chemotherapeutic drugs do not induce comparable levels of free radicals, but are known to mediate apoptosis either by p53-dependent mechanisms [25,26] or by the activation of death-receptor pathways, such as CD95/Fas [27,28]. 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 whether synergism occurs in response to any agent that induces free radicals in neuroblastoma cells or whether it is specific to fenretinide and CD437. In addition, synergism with chemotherapeutic drugs may not simply be 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.

ACKNOWLEDGMENTS

The authors thank Janssen-Cilag Ltd. for supplying the fenretinide and Dr. U. Reichert, Galderma, for CD437 and CD2665.

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