The Novel Retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-napthalene Carboxylic Acid Can Trigger Apoptosis through a Mitochondrial Pathway Independent of the Nucleus

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INTRODUCTION

Retinoids have been described as displaying pleiotropic activities including inhibition of growth, induction of differentiation, and apoptosis in a wide variety of tumor cell lines (1). On the basis of these effects, retinoic acid was used with some efficiency in the chemoprevention and treatment of various malignancies (2). The molecular mechanism of action of retinoic acid involves the binding and activation of specific nuclear receptors, RARs and retinoic X receptors, which modulate gene expression (3). During the last few years, synthetic retinoids, specific for different receptor types (RAR or retinoic X receptor) and subtypes (α, β, γ) of RARs have been discovered, and some already seem very promising for the treatment of certain cancers (4).

Among them, one new compound, AHPN/CD437, is particularly interesting. First described as a RARγ selective retinoid (5), this molecule has been found to inhibit growth and promote apoptosis in an extremely wide variety of tumor cell lines (6–19). Moreover, several other synthetic retinoids structurally related to CD437 are also able to induce apoptosis in many human cancer cell lines (20, 21). Compared with retinoic acid and other RARγ selective synthetic retinoids, CD437 is characterized by unique properties. It is active not only in retinoic acid-sensitive cell lines but also in retinoic acid-resistant cells (6–9, 11–14) and in RARγ-negative cells (17). Moreover, its antiproliferative and apoptogenic activities in cancer cells are not inhibited by either RARβ or RARγ but by pan-RAR selective antagonists (11, 14, 19). Structure-activity relationship studies comparing CD437 with various RARγ selective synthetic retinoids did not show any correlation between antiproliferative activator protein activity and RARγ-dependent transactivating activities of the tested drugs (9, 13, 22). A significantly higher concentration of CD437 was required to inhibit cell proliferation and to induce apoptosis than to trigger RARγ-dependent transcriptional activity (5, 6, 8, 11–13, 18). Taken together, these data suggest that the antiproliferative effects of CD437 cannot be related to only its RARγ selectivity but probably involve other, still unknown, mechanisms.

CD437 arrests cells in Go-G1 (8, 18, 23, 24) by increasing the cyclin-dependent kinase inhibitor p21cip1/waf1 (8, 12, 16, 17, 24). This increase has been related to a posttranscriptional stabilization of p21cip1/waf1 mRNA (8, 25). CD437 induces both p53-dependent and -independent apoptosis in human cancer cells (26). Conflicting data have been reported about the effects of CD437 on the c-jun/activator protein pathway (6, 8, 24). Nur77 has also been proposed to play a role in the CD437-induced apoptosis (24), whereas the effects of CD437 on the expression of Bcl-2, Bcl-x, and Bax seemed variable according to the cell model studied (6, 8, 11, 27). However, overexpression of Bcl-2 or Bcl-xL inhibits CD437-triggered apoptosis of cancer cells (18, 23). Recent observations suggest that CD437-induced apoptosis can be dissociated from the effects of CD437 on growth arrest and could involve both caspase-dependent and -independent pathways (18, 19).

From all of these data, it seems that a clear identification of the molecular targets of CD437 that are specifically responsible for its unique proapoptotic activities is still lacking. Surprisingly, whereas...
growing attention is paid to the effects of CD437 on caspase activation (18, 19, 23, 28), very few data have been reported about the involvement of mitochondria in CD437-induced apoptosis.

The contribution of mitochondria in the execution phase of apoptosis is now well-established (29). Two interspace membrane proteins, cytochrome c and a 57-kDa protein called AIF (30), are released in the cytosol during apoptosis and stimulate pathways of apoptosis. The early stage of apoptosis is characterized by a rupture in the ΔΨm preceding signs of DNA fragmentation (for review, see Ref. 31). This reduction in ΔΨm is thought to result from the opening of megachannels or pores, referred to as the MPT pore. PTPCs are located at the contact sites between the inner and outer mitochondrial membranes. The PTPCs include (a) the ANT in the inner membrane, which cooperates with the proapoptotic molecule Bax to increase mitochondrial membrane permeability and to trigger cell death (32); (b) cyclophilin D in the matrix; (c) porin and the peripheral benzodiazepine receptor in the outer membrane; and (d) possibly other proteins (33). In isolated mitochondria or in intact cells in vitro, MPT is blocked specifically by (a) CsA (a ligand of matrix cyclophilin D); (b) BA (a ligand of ANT); (c) CMX-Ros, which acts on the matrix thiols, (d) phenylglyoxal and 2,3 butanedione, which are thought to modify arginines of inner membrane proteins (34); and (e) trifluoperazine (35), cinnarizine, flunarizine (36), and trimetazidine (37) by acting on yet unknown receptors. CsA (38, 39), BA (40), CMX-Ros (41), trimetazidine (37), and trifluoperazine (35) can also prevent cell death, at least in some models of apoptosis.

We recently demonstrated that the induction of apoptosis by retinoic acid in a human myeloma cell line, RPMI 8226, required the activation of both classes of retinoid receptors, RARs and retinoic X receptors (42). During this previous study, we observed that CD437 had a unique behavior and was able by itself to trigger a fast and massive apoptosis with peculiar morphological and molecular features.

On the basis of the above findings, we decided to evaluate the possibility that CD437 might induce apoptosis via an effect on mitochondria, rather than via an effect on nuclear RARγ receptors.

**MATERIALS AND METHODS**

**Chemicals and Modulation of Apoptosis.** ATRA was purchased from Sigma (St. Louis, MO) and retinoid-derivatives CD437, CD336, CD666, CD2665, and CD3126 were obtained from Galderma Research and Development (Sophia Antipolis, France). Properties of retinoids used in this study are summarized in Table 1. Retinoids were dissolved in DMSO at an initial stock concentration of 10 mM and stored at −20°C in the dark. Subsequent dilutions were performed in PBS or in RPMI 1640. Cells were cultured with these reagents alone or in combination with the following inhibitors of MPT: (a) 1 μM CsA (Sandoz, Hannover, Germany; Ref. 39); (b) 1 μM CMX-Ros (Molecular Probes, Eugene, OR; Ref. 41); (c) 50 μM BA (kindly provided by Dr. Duine, Delft University of Technology, Delft, the Netherlands; Ref. 40); (d) 30 μM trifluoperazine (Sigma; Ref. 35); (e) 30 μM each, cinnarizine and flunarizine (Sigma; Ref. 36); (f) 1 mM Phenylglyoxal (Sigma; Ref. 34); (g) 4 mM 2,3-butanedione (Sigma Chemical Co; Ref. 34); and (h) 2 mg/ml trimetazidine (Servier Laboratories, Neuilly, France; Ref. 37). Fifty μM Z-Asp-Glu-Val-Asp-chloromethyl-ketone (DEVD.cmk; Bachem, Basel, Switzerland) was used as inhibitor of DEVDase activity.

**Cell Lines and Culture Conditions.** RPMI 8226, a human myeloma cell line (CCL-155 American Type Culture Collection), and 2B4.11 T-cell hybridoma cell lines (kindly provided by Jonathan Ashwell, NIH, Bethesda, MD) were routinely cultured in RPMI 1640 supplemented with L-glutamine, antibiotics, and 10% heat-inactivated FCS. Human carcinoma HeLa cells, as well as mouse embryonal carcinoma P19 cells, were cultured in DMEM + 10% FCS.

**Table 1 Structure, binding constants, and transactivating properties of the retinoids used**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>kDaα (nM)</th>
<th>Activity</th>
</tr>
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<td>ATRA</td>
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<td>16</td>
<td>7</td>
</tr>
<tr>
<td>CD336 (Am580)</td>
<td><img src="Image2" alt="Structure" /></td>
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<tr>
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<td>306</td>
</tr>
<tr>
<td>CD3126</td>
<td><img src="Image6" alt="Structure" /></td>
<td>b</td>
<td>b</td>
</tr>
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* kDa values are from Szondy et al. (63).
* No binding (U. Reichert, CIRD Galderma, personal communication).
FCS. L363, another human myeloma cell line (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSM ACC 49), was characterized for the absence of RARγ and controlled by reverse transcription-PCR (data not shown) and cultured in the same condition as RPMI 8226. Thymocytes and hepatocytes were obtained from female BALB/c mice, 6–8 weeks of age. For apoptosis modulation, cells were resuspended at 500,000/mL in RPMI 1640 or DMEM without FCS but supplemented with 1% of insulin-transferrin-selenium (Sigma). Rat-1 fibroblasts, constitutively expressing a Myc-estrogen receptor fusion protein and stably expressing the different versions of Bcl-2, were a gift from David W. Andrews (Department of Medicine, McMaster University, Hamilton, Ontario, Canada). Rat-1/myc cell culture and apoptosis induction by serum starvation have been detailed elsewhere (43).

**Anucleate Cells.** RPMI 8226 cells were enucleated following procedures described previously (44). Briefly, cells (2 × 10⁷/mL) were cultured in RPMI 1640 + 5% FCS in the presence of cytochalasin B (10 μg/mL; Sigma) for 45 min at 37°C and then were subjected to centrifugation on a discontinuous Ficoll (Pharmacia) density gradient (2 mL of 25%, 2 mL of 17%, 0.5 mL of 16%, 0.5 mL of 15%, and 2 mL of 12.5% Ficoll in RPMI 1640 containing 10 μg/mL cytochalasin B preequilibrated 24 h at 37°C + CO₂ 5%). Two mL of the cell suspension were gently applied to the centrifuge and were centriped in a precooled and balanced rotor SW41 Beckman at 35,000 rpm for 1 h at 4°C. Cytoplasts (anucleate cells) were collected from the interface between 15 and 17% Ficoll layers. Then, cytoplasts were washed with RPMI 1640 + 10% FCS and resuspended in RPMI 1640 + 1% ITS (insulin, transferrin, and selenium, Sigma). Control staining with trypan blue revealed >99% viability in this fraction, and microscopy analysis using May-Grünwald-Giemsa dye showed that more than 90% of the cells had lost their nuclei (data not shown).

**Cytosolic Fluorometric Analysis.** To evaluate Δψm and superoxide generation, a procedure described elsewhere was followed (45). Briefly, cells (5 × 10⁶/mL) were incubated for 15 min at 37°C with 40 μM 3′,3′-dihexylxlocarbocyanine iodide [DiOC(6)](3) in PBS (Molecular Probes) and with 2 μM HE in PBS (Molecular Probes). DEVD cleavage was assayed by using Phi-Phi Lux DEVd-Rhodamine substrate according to the manufacturer’s recommendations (Oncoimmunin Inc., College Park, MD) at the final concentration of 10 μM. After incubation, cells were immediately analyzed on a FACSCalibur (Coulter). For determination of the external exposition of phosphatidylserine residues, staining with Annexin-V-FITC (1:20 dilution; PharMingen, San Diego, CA) was performed in binding buffer containing 10 mM HEPES-ME in PBS (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂ and was analyzed by cytometry within 1 h.

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde and 0.19% picric acid in PBS for 60 min and were washed in PBS for 10 min. The cells were further permeabilized with 0.1% SDS in PBS + 1% FCS for 5 min before incubation with 2 μg/mL 6H2-B4 mouse IgG1 anticytochrome c antibody (PharMingen). Nuclei were added to the supernatants from mitochondria at the final concentration of 1 × 10⁷/mL and were incubated for 90 min at 37°C, then stained with 10 μM of DAPI, and examined by fluorescence microscopy.

**Analysis of Nuclear Apoptosis.** The frequency of hypoploid cells (sub-G₁ cells) was assessed by measuring the cell cycle after fixation overnight at 4°C with 70% ice-cold ethanol-PBS followed by propidium iodide (50 μg/mL) staining and analysis on a Coulter XL cytometer-45, DNA fragmentation (1 × 10⁶ cells/lane) was determined by horizontal agarose gel electrophoresis following published methods (46).

**In Vitro Tests of MPT and Generation of Mitochondrial Supernatants.** Mitochondria were purified from the livers of female (ages, 6–8 weeks) BALB/c mice on a Percoll gradient (47) and were resuspended on ice in buffer containing 200 mM sucrose, 10 mM Tris/4-morpholinepropanesulfonic acid, 1 mM NaPO₄, 10 μM EDTA, 2 μM rotenone, 5 mM succinate ([pH 7.4]) reagents from Sigma Chemical Co) at the concentration of 0.5 mg protein/ml of buffer before manipulation. For determination of swelling, mitochondria were diluted at 1:10 in the same buffer and adsorption was recorded at 540 nm in a Beckman DU 7400 spectrophotometer, as described previously (48). For cell-free system analysis, mitochondrial supernatants and the resulting pellets containing mitochondria were separated after centrifugation at 150,000 × g for 1 h at 4°C, were aliquoted, and were frozen at −80°C.

**Western Blot Analysis.** From 1 × 10⁷ intact cells for each sample, mitochondrial supernatants (cystolic fraction) and the resulting pellets containing mitochondria (designated as mitochondrial fraction) were aliquoted at −80°C. Twenty-five μg of protein from both fractions for each sample were loaded on a 12% polyacrylamide gel. After electrophoresis, the gels were blotted onto nitrocellulose membrane (AmerHAM Life Science) which was then probed with a mouse monoclonal antibody 7H8.2C12 (1:500; PharMingen) specifically recognizing the denaturated form of cytochrome c. Primary antibody binding was detected with a goat antinimal IgG conjugated with horseradish peroxidase (1:1000; Sigma Chemical Co) and visualized by enhanced chemiluminescence (ECL) following the manufacturer’s instructions (AmerHAM).
The first detectable sign of apoptosis is a loss of HE to the fluorescent product ethidium (HE followed by an enhanced ROS formation detected by the oxidation of D. When compared with the activity was detected 1 h after CD437 exposure, coupled to amino methyl coumarine). The increase in DEVDase activities were assessed by using a specific fluorogenic substrate (DEVD). Agarose gel electrophoresis confirms the apoptotic nature of CD437-induced apoptosis, the appearance of the first signs of nuclear apoptosis (hypoploidy).

DiOC6 (3) in RPMI 8226 cells (increase in the % of DiOC 6 (3) low HE measured by the reduction of the uptake of the potential-sensitive dye SD). Thus, in the CD437-induced apoptotic, the Δψm reduction precedes the downstream events of the apoptotic cascade including caspase-3 activation, ROS generation, and nuclear apoptosis.

CD437 Cytotoxicity Is Observed in the Absence of RARs, Transcription, and Even Nucleus. To explore the role of RAR in the CD437-induced apoptosis, we preincubated RPMI 8226 cells, which express RARα, β, and γ (42), with various synthetic retinoids. Cells preincubated with these specific ligands were then treated with CD437. None of the compounds used for the preincubation step (CD666, a RARγ agonist; CD2665, a RARY antagonist; CD336, a RAR agonist, or CD3126, a transcriptionally inactive methyl ester of CD437), was able to protect RPMI 8226 cells against the apoptotic effect of CD437 (Fig. 3A). This experiment suggests that the proapoptotic effect of CD437 is not influenced by the pharmacological modulation of the functional status of the RARs. An additional series of experiments confirmed that CD437-induced apoptosis can occur in the short incubation time (Fig. 1). We reported previously that the induction of apoptosis by ATRA in RPMI 8226 follows slow kinetics, becoming detectable only after 72 h and becoming maximum after 6 days (42). The lack of activity of CD666 and CD3126 strongly suggests that CD437 acts via a RARγ-independent pathway but requires a free carboxylic group. The effects of CD437 seemed dose-dependent with an ED50 at 3 μm, and, at the indicated times, the percentage of DiOC6(3)low HE−, DiOC6(3)low HE−, and subdiploid cells was determined (SD was <10%). Data are representative of three independent experiments. B, time course of caspase-3 activity. At the indicated interval after CD437 treatment, RPMI 8226 cells were lysed and assayed for DEVDase activity. Data are presented as an average of three determinations of arbitrary fluorescence units/mg of protein (±SD).

CD437 induces apoptosis through a retinoic acid nuclear receptor-independent pathway. A, RPMI 8226 cells were either treated with the various retinoids tested alone (left column part of the panel) or preincubated for 2 h with CD666 (3 μm), CD336 (3 μm), CD3126 (3 μm) or CD2665 (3 μm) before the exposure to CD437 (1 μm). After 12 h, cells were analyzed by flow cytometry for their DNA content. Results are representative of two independent experiments.
Absence of functional RARγ. Indeed, we compared the onset of apoptosis in RPMI 8226 and L363 cells, a RARγ negative human myeloma cell line (42). As shown in Fig. 3B, L363 cells—exposed 8 h to 1 μM CD437—exhibited ΔΨm reduction and generation of ROS associated with chromatolysis. Control experiments confirmed that RPMI 8226 cells undergo mitochondrial dysfunction and nuclear apoptosis to the same extent (Fig. 3B).

Inhibition of mRNA or protein synthesis by actinomycin D or cycloheximide, respectively, did not significantly affect the CD437-induced apoptosis, whereas substantial inhibition was observed for the glucocorticoid-induced apoptosis, used as control, in the 2B4.11 mouse T-cells model, both at the mitochondrial and at the nuclear levels (Fig. 4). To further document that the cell nucleus was dispensable for the triggering of apoptosis by CD437, we generated anucleate RPMI 8226 cells. Control cells and cytoplasts behaved similarly.

Fig. 4. mRNA and protein synthesis are dispensable for CD437-induced apoptosis. 2B4.11 T cells were cultivated with 1 μM CD437 or 1 μM dexamethasone in the presence of actinomycin D (30 nM) or cycloheximide (1 μM). After 12 h of culture, mitochondrial parameters were assessed by DiOC₆(3)/HE staining (A), and DNA hypoploidy was measured (B). Results are representative of three independent experiments.

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Annexin V-FITC

Fig. 5. Induction of apoptosis in CD437-treated cytoplasts. RPMI 8226 cells were enucleated as described in “Materials and Methods.” Anucleate cells (cytoplasts) and control cells were incubated for 2 h in the presence or absence of 3 μM CD437, followed by (A) determination of mitochondrial signs of apoptosis using fluorochromes (DiOC₆(3)/HE); (B) detection of DEVDase activity with DEVD-Rhodamine fluorochrome; and (C) determination of the cell surface exposure of phosphatidylserine residues with FITC-Annexin-V. Results are representative of three different experiments.
DEVDase activity. After 4 h of incubation with CD437 (1 μM), RPMI 8226 cells were treated for 4 h with CD437 (1 μM); phenylglyoxal (1 mM); 2,3-butanedione (4 mM); BA (50 μM); CMX-Ros (1 μM); trifluoperazine (30 μM); cinnarizine (30 μM); trimetazidine (2 mg/ml); or flunarizine (30 μM). FK-506 (1 μM)—an immunosuppressive drug devoid of any MPT activity—and DEVD.cmk (50 μM)—a caspase-3 inhibitor—were also used. After 6 h, ΔΨm and sub-G1 cells were determined by flow cytometry using DiOC6(3) and propidium iodide, respectively. Results obtained in samples treated with CD437 and the various compounds tested were compared with those obtained after treatment with CD437 alone (45% ΔΨm loss; 29 ± 3% hypoploid cells) and expressed as the percentage of suppression of this control response (100 (Y − X) ÷ Y, where X and Y correspond to the observed % of ΔΨm or sub-G1 cells in the samples treated with CD437 alone (Y) or in association (X).) Results were corrected for the very low amount of spontaneous apoptosis occurring in untreated cells.

Because CD437 induces a rapid disruption of the ΔΨm, an early apoptotic event allowing for the recognition of apoptotic cells and their phagocytosis by adjacent cells (52).

**Inhibition of the MPT Pore Prevents CD437-induced Apoptosis.** Because CD437 induces a rapid disruption of the ΔΨm, we addressed the question of the exact role of MPT in the CD437-provoked apoptosis by pharmacological modulation of this mitochondrial event using well-known inhibitors (for a review, see Ref. 53). As shown in Fig. 6A, inhibitors of MPT were able to efficiently suppress both the mitochondrial depolarization and the nuclear apoptosis induced by CD437. Thus, CsA, the prototype inhibitor of MPT that acts on mitochondrial matrix cyclophilin D, inhibits the mitochondrial effects of CD437. In contrast, the immunosuppressor FK506, which acts via calcineurin but not via cyclophilin D, is totally devoid of any effect on MPT or on the nuclear apoptosis induced by CD437 (Fig. 6A). MPT inhibitors other than CsA were also efficient in preventing CD437 effects. Thus, 2,3-butanedione and phenylglyoxal, two reagents used to modify accessible arginine residues in native proteins and reported to inhibit the MPT of isolated mitochondria (34), impeded the ΔΨm reduction and subsequent nuclear hypoploidy induced by CD437 (Fig. 6A). These effects were also observed with various known agents, recently shown to be effective MPT inhibitors, including trimetazidine (37) trifluoperazine (35), and cinnarizine (36). BA—an inhibitor of ANT, which is located in the inner mitochondrial membrane of the PTPC—and the thiol reactive reagent, CMX-Ros, were also protective against the dysfunction induced by CD437. In contrast, flunarizine, reported to be effective in inhibiting MPT at low concentrations (<0.5 μM; Ref. 36), was totally inefficient in our model. Contrasting with the effects of these MPT inhibitors, DEVD.cmk—an inhibitor of caspase-3 activities—impeded nuclear apoptosis without affecting the ΔΨm suppression (Fig. 6A), which confirms that MPT precedes the appearance of the DEVDase activity, itself upstream of the nuclear events.
Next, we tested the effects of CsA, the reference inhibitor of MPT, on different manifestations of CD437-induced apoptosis, including the release of mitochondrial proapoptotic factors and the activation of caspases. CD437 treatment resulted in cytochrome c release from mitochondria. Immunoblot analysis of subcellular fractions revealed an increase of cytochrome c in the cytosol concomitant to its decrease in the mitochondrial fraction (Fig. 6B). Immunofluorescence detection of cytochrome c in intact cells (Fig. 6C) confirms that control cells showed a bright spotted cytoplasmic staining consistent with a mitochondrial location (Fig. 6C, upper left), whereas the immunostaining of CD437-treated cells was diffuse, which indicated cytochrome c release from mitochondria (Fig. 6C, upper right). Interestingly, CsA prevented the mitochondrial-cytosolic redistribution of cytochrome c induced by CD437 (Fig. 6, B and C). Moreover, CsA blocked the caspase-3-like activation induced by CD437 (Fig. 6D). Taken together, these data indicate that the MPT is mandatory for all of the CD437-induced apoptotic events, including the activation of downstream caspases.

CD437 Provokes the Release of Apoptogenic Factors from Mitochondria in a Cell-free System. To further correlate the direct effect of CD437 on MPT and nuclear apoptosis, we used a cell-free system of apoptosis (54), in which isolated mitochondria were treated with CD437, CD666, CD3126, or ATRA alone or in combination with CsA and then centrifuged. The mitochondrial supernatants were recovered and added to isolated nuclei to determine their effect on chromatin condensation. Proapoptotic activity was detected only in the supernatant of mitochondria treated with the MPT-inducer CD437 but not in the supernatants of mitochondria treated with CD666, CD3126, or ATRA (Fig. 7A). Moreover, the addition of CsA reduced the release of apoptosis-inducing activity by CD437-treated mitochondria. None of the retinoids were able to induce apoptosis in isolated nuclei on its own in the absence of mitochondria (Fig. 7A). Typical morphology of the nuclei submitted to these various conditions is presented in Fig. 7B. These results, which suggested a direct action of CD437 on mitochondrial fractions to induce the release of proapoptotic factors, prompted us to test its ability to induce MPT in isolated mitochondria. MPT gives rise to the colloidosmotic swelling of isolated mitochondria resuspended in a protein-free buffer. This swelling causes a reduction in the absorbance (A540 nm) (33). As shown in Fig. 8, 1 μM CD437 (but neither CD666 nor ATRA) induced the permeability transition-dependent colloidosmotic swelling of mitochondria, and this effect was inhibited by CsA. Hence, CD437 can exert a direct effect on mitochondria that involves MPT.

CD437-induced Apoptosis Is Inhibited by Mitochondrial-targeted but not by ER-targeted Bcl-2. Rat-1/myc fibroblasts are a well established model for examining apoptosis triggered by serum deprivation and concomitant treatment with β-estradiol to induce Myc activity (55). We used Rat-1/myc fibroblasts that stably expressed Bcl-2 mutants with restricted subcellular location (43). In the mutated Bcl-2, the COOH-terminal tail has been replaced by heterologous signal peptides specifically targeting Bcl-2 to either the ER (Bcl-cb5...
CD437 can act directly on these organelles. We first confirmed the ability of CD437 to rapidly induce apoptosis in different cell types. Our results document the fact that CD437 is active in cells displaying no, or very low, sensitivity to retinoic acid and to synthetic retinoids, including CD666 (a RARα selective compound) and CD3126 (the methyl ester of CD437). These data are in agreement with structure-activity relationship studies performed by others, which compared CD437 and related compounds to ATRA and synthetic retinoids (7, 9, 13, 20). The importance of structural features like the adamantyl group and the presence of free carboxyl group have already been emphasized. The lack of activity of CD3126 in the four cell lines used (Fig. 1) suggests that there was no significant hydrolysis of the ester bond, in accordance with the recent report demonstrating that such an hydrolysis, yielding free CD437, occurs only in macrophage cell lines (56). The cell death induced by CD437 is particularly rapid when compared with the slow kinetics observed with ATRA (42). The fast kinetics of CD437-induced apoptosis has also been observed in cervical carcinoma, HL-60, and human lymphoma cell lines (11, 17, 18, 28). Kinetic analysis of the apoptotic events triggered by CD437 indicate that MPT preceded the activation of downstream caspases and nuclear apoptosis (Fig. 2). CD 437 has been shown to induce cytochrome c leakage into cytoplasm in different cell types (19, 23). One of the possible consequences of the MPT is the release of intermembrane proteins—AIF and/or cytochrome c—implicated in the activation of downstream caspases. However, in some models of apoptosis, it has been suggested that mitochondrial release of cytochrome c occurs independently of MPT (57). In contrast, we demonstrated in this study that the inhibition of MPT prevents the mitochondrial leakage of cytochrome c and subsequent caspase-3 activation. Moreover, CD437 is also able to release AIF from mitochondria in Rat-1 cells, and this effect is fully prevented by CsA (data not shown). In our model, the identification of MPT as the first step governing the execution of CD437-mediated apoptosis was unambiguously established by the demonstration that MPT inhibitors prevent all of the manifestations of CD437-induced apoptosis including caspase activation. In contrast, the inhibition of downstream caspases prevented nuclear apoptosis without affecting MPT (Fig. 6). The implication of caspases 3 and 7 in the CD437-mediated apoptosis has already been mentioned (18, 19, 28). Interestingly, pro-caspase-3 has been reported to have both a cytosolic and a mitochondrial distribution, the latter being coupled to the Bcl-2-sensitive apoptotic pathway (58).

Bcl-2 and other members of the family are predominantly localized in the outer mitochondrial membrane but also are found in the nuclear membrane and the ER (59). Recently, it has been found that Bcl-2 and Bax interact with the adenine nucleotide translocator of the inner membrane, one of the proteins contained in the PTPC (32). Experiments involving purified PTPC indicate that at least part of the function of Bax, Bcl-2, and Bcl-xL is to facilitate or inhibit MPT. In our model, the identification of MPT as the first step governing the execution of CD437-mediated apoptosis was unambiguously established by the demonstration that MPT inhibitors prevent all of the manifestations of CD437-induced apoptosis including caspase activation. In contrast, the inhibition of downstream caspases prevented nuclear apoptosis without affecting MPT (Fig. 6). The implication of caspases 3 and 7 in the CD437-mediated apoptosis has already been mentioned (18, 19, 28). Interestingly, pro-caspase-3 has been reported to have both a cytosolic and a mitochondrial distribution, the latter being coupled to the Bcl-2-sensitive apoptotic pathway (58).

**DISCUSSION**

In the present report, we have demonstrated the pivotal role of mitochondria in CD437-induced apoptosis and have established that
subcellular location of Bcl-2 in mitochondria for any anti-CD347 effect. Interestingly, it was recently shown that PK 111–95, a specific ligand of the peripheral benzodiazepine receptor (60), one of the PTPC components, was able to prevent the protective action of Bcl-2 against apoptosis (61). Similarly, CD347, by acting on PTCP, could induce a perturbation of the mitochondrial Bcl-2/Bax complexes and alter the efficiency of Bcl-2 to prevent apoptosis.

Previous reports have documented that CD347 is able to induce apoptosis in retinoic acid-resistant and RARγ-negative cells, and that this effect is not inhibited by antiretinoins acting at the RARs level (see “Introduction”). Here, we not only confirmed this observation in our model, but we also extended it to the mitochondrial events of apoptosis and demonstrated that the CD347-mediated apoptosis occurred in the presence of inhibitors of transcription and protein synthesis (Figs. 3 and 4). This observation prompted us to investigate for a proapoptotic effect of CD347 on cytoplasts (Fig. 5) and to explore the possibility of CD347 to act on mitochondria. For that purpose, we have developed subcellular fractionation and a cell-free system of apoptosis in vitro. First, we have shown that CD347 is able to induce MPT of purified mitochondria at the same concentration used for intact cells. Secondly, only supernatants from supernatants from mitochondria that have undergone MPT display apoptogenic activity in a cell-free system. Here again, CD347 was the only active compound, whereas ATRA, CD665, and CD3126 remained inactive. These results confirm the relevance of the cell-free system and identify mitochondria as a direct target of CD347. Interestingly, other chemotherapeutic agents have also been reported to act on mitochondria and to induce MPT in cancer cells (62).

An important point to be stressed is that the effects of CD347 on apoptosis and on isolated mitochondria in this study were observed at rather high concentrations of CD347, i.e., in the micromolar range. This is in accordance with most of the data reported to date about the proapoptotic activity of CD347 in cancer cells (6, 8, 11–13) with the exception of HL-60 cells (17) and normal mouse thymocytes, which are sensitive to low-CD347 concentrations. In addition, normal mouse thymocytes undergo apoptosis in a RARγ-dependent way (63).

Furthermore, CD347 and related compounds have been demonstrated to be able to inhibit the growth of xenografts of retinoic acid-resistant human tumors in nude mice in vivo (6, 10, 20). Here again, high doses—in the 25-μmole/kg-range—were necessary.

Taken together, our results suggest that a significant part of the proapoptotic effects of CD347 reported in the literature could be explained by a direct action of CD347 on the mitochondria of target cells. However, according to the cell context, RARγ and other factors like p21, p53, Bcl-2, and related proteins could also be involved in a variable manner. However, this finding suggests that CD347 may be a particularly useful cancer-cell death-inducer when “classical” retinoids requiring the nuclear action of specific receptors fail to act as therapeutic agents.

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The Novel Retinoid
6-[(3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene Carboxylic Acid Can Trigger Apoptosis through a Mitochondrial Pathway Independent of the Nucleus

Philippe Marchetti, Naoufal Zamzami, Bertrand Joseph, et al.