A Retinoid-Related Molecule that Does Not Bind to Classical Retinoid Receptors Potently Induces Apoptosis in Human Prostate Cancer Cells through Rapid Caspase Activation

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ABSTRACT

Synthetic retinoid-related molecules, such as N-(4-hydroxyphenyl)retinamide (fenretinide) and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) induce apoptosis in a variety of malignant cells. The mechanism(s) of action of these compounds does not appear to involve retinoic acid receptors (RARs) and retinoid X receptors (RXRs), although some investigators disagree with this view. To clarify whether some retinoid-related molecules can induce apoptosis without involving RARs and/or RXRs, we used 4-[3-(1-heptyl-4,4-dimethyl-2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)-3-oxo-E-propenyl] benzoic acid (AGN193198) that neither binds effectively to RARs and RXRs nor transactivates in RAR- and RXR-mediated reporter assays. AGN193198 potently induced apoptosis in prostate, breast, and gastrointestinal carcinoma cells and in leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells. The mechanism(s) of action of these compounds does not appear to involve retinoic acid receptors (RARs) and retinoid X receptors (RXRs), although some investigators disagree with this view. To clarify whether some retinoid-related molecules can induce apoptosis without involving RARs and/or RXRs, we used 4-[3-(1-heptyl-4,4-dimethyl-2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)-3-oxo-E-propenyl] benzoic acid (AGN193198) that neither binds effectively to RARs and RXRs nor transactivates in RAR- and RXR-mediated reporter assays. AGN193198 potently induced apoptosis in prostate, breast, and gastrointestinal carcinoma cells and in leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells. AGN193198 also abolished growth (by 50% at 130–332 nM) and induced apoptosis in primary cultures established from prostatic leukemia cells.

INTRODUCTION

Recent reviews have highlighted the potential of retinoids as preventive and therapeutic agents for various cancers (1–5). Moreover, some synthetic retinoids are effective in preclinical studies against common carcinomas, such as prostate (reviewed in Ref. 3), lung (6), and breast (7), for which mortality rates have not been substantially decreased by current treatments. Prostate cancer accounts for 13% of cancer deaths in males in the United States, and late-stage disease is incurable after metastasis (8–10). Some synthetic retinoid-related molecules (RRMs; 4) appear to induce apoptosis in malignant cells, including prostate carcinoma cells, through mechanisms that do not involve RARs and RXRs. Compounds that display potential as therapeutic agents include N-(4-hydroxyphenyl)retinamide (fenretinide) and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437). Possible targets have been identified for these agents, but their mechanisms of action, particularly the involvement of RARs, remain unresolved (reviewed in Refs. 2 and 18). Fenretinide binds poorly to RARs in vitro (19), induces apoptosis in, for example, prostate (20, 21) and ovarian carcinoma cells (22–25), and is chemopreventive against oral leukoplakias and ovarian and breast carcinomas (26–28). CD437 was synthesized as an RARγ agonist but also binds to RARβ and to a slight extent to RARα (29, 30), and RAR-independent mechanisms of action have been proposed (31–33). Like fenretinide, CD437 induces apoptosis in a variety of cancers including lung, cervical, breast, and ovarian carcinomas and melanoma (25, 30, 33–39).

Is binding of RRRMs to nuclear retinoid receptors, even if weak, and their transactivation essential for RRMs to induce apoptosis in cancer cells? During the course of screening compound libraries for anticancer activity, we identified a novel chalcone structure-based RRM (Ref. 40; 4-[3-(1-heptyl-4,4-dimethyl-2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)-3-oxo-propenyl] benzoic acid (AGN193198). This compound neither binds effectively to RARs and RXRs nor transactivates in RAR- and RXR-mediated reporter assays but yet was effective in inhibiting the growth of prostate cancer and other malignant cell lines. AGN193198 is an extremely potent inducer of apoptosis in primary cultures established from 13 patients with prostatic carcinoma. The induction of apoptosis by RRMs, such as fenretinide and CD437, involves mitochondrial depolarization and activation of caspase-3 and caspase-9 (25, 36). Lopez-Hernandez et al. (41, 42) have proposed that caspases are the major effectors of RRM action and, together with other workers, have shown caspase-8 activation leading to mitochondrial depolarization. AGN193198 provokes mitochondrial depolarization, activation of caspase-3, -8, -9, and -10 and BID/p15 production, leading ultimately to DNA fragmentation. These processes seem to involve neither RARs nor RXRs, suggesting that RRMs such as AGN193198 that do not bind to these receptors may be useful in the treatment of a variety of malignancies.
MATERIALS AND METHODS

Retinoids. The following synthetic retinoids and RRM s were synthesized at Allergan Inc. (Irvine, CA): the RRM 4-[3-(1-heptyl-4,4-dimethyl-2-oxo-1,2,3,4-tetrahydroquinolin-6-y1)-3-oxo-propenyl] benzoic acid (AGN193198), the RAR pan-antagonist 4-2(6-2,2-dimethyl-(1H)-4-(4-ethylphenyl)-1-benzothio.pyran)ethynyl] benzoic acid (AGN194310), the RAR β,γ-selective antagonist 3-fluoro-4-[7-(4,4-dimethyl-3,4-dihydro-1-(p-tolyl)-naphthyl)-2-azo] benzoic acid (AGN194431), the RXR pan-antagonist 7-(2-heptyloxy-3,5-diisopropyl-phenyl)-3-methyl-octa-2Z,4E,6E trienoic acid (AGN195393), and the RXR pan-agonist 3,7-dimethyl-6S,7S-methano,7-[1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphth-7-y1]2E,4E heptadienoic acid (AGN194204). Stock solutions of these compounds and ATRA were prepared at 10 mM in 50% ethanol/50% dimethylsulphoxide and stored at -20°C. Retinoids were analyzed as described previously (43) for their ability to competitively inhibit binding of 5 nM [3H]ATRA (for RARs) or 10 nM [3H]9-cis-retinoic acid (for RXRs) to extracts (5–50 μg) of baculovirus/sf9-expressed RARs (α, β, and γ) and RXRs (α, β, and γ), respectively. Receptor transactivation assays were performed using CV-1 cells (at 2500 cells/well in a 96-well plate) that were LipofectAMINE transfected with 100 ng of luciferase reporters and receptor plasmid DNA (for amounts see Fig. 1; Ref. 44). After 5 h, cells were fed with DMEM containing 10% charcoal-treated fetal bovine serum (FRS, Life Technologies, Inc.). Twenty h after transfection, cells were treated with compounds for 16 h before measuring luciferase activity and β-galactosidase activity using the Promega system. Triplicate wells were used, and relative luciferase activity was normalized to β-galactosidase activity. For both agonism and antagonism RXR homodimer transactivation assays, wild-type RXRs were used together with the reporter plasmid pRXRE-ik-Luc that contains five tandem repeats of a 35-bp sequence (DR-1) from the promoter of the mouse CRBP-II gene (45) inserted immediately upstream of tk-luciferase. For RXR transactivation assays, the ERE-ik-Luc that contains the binding site for estrogen receptors was cotransfected with chimeric estrogen receptor-RARs, in which the DNA-binding

Fig. 1. Structures of retinoids and their transactivation properties. A, structures of all-trans-retinoic acid (ATRA), 9-cis-retinoic acid, and AGN193198. B, transactivation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) by AGN193198. CV-1 cells were transfected with a 100-ng RAR reporter and each 4-ng chimeric estrogen receptor-RAR or a 100-ng RXR reporter and each 40-ng RXR and then treated for 16 h with the different doses of ATRA or AGN193198 for activities of RARs (panel 1), and 9-cis-retinoic acid or AGN193198 for activities of RXRs (panel 2). Luciferase activity of transfected and treated samples was measured and normalized to the activity of β-galactosidase. C, antagonism of RARs and RXRs by AGN193198. Panel 1, different doses of AGN194310 (a RAR pan-antagonist as the positive control) and AGN193198 were used to antagonize the transactivation of each 50-ng RAR-progesterone/10-ng RXR heterodimer by 1 nM ATRA. Panel 2, different doses of AGN195393 (a RXR pan-antagonist as the positive control) and AGN193198 were used to antagonize the transactivation of each 40-ng RXR homodimer by 1 nM AGN194204 (a RXR pan-agonist).
domain of RAR was substituted with that of the estrogen receptor. Antagonism of ATRA transactivation of RARs by AGN193198 or AGN194310 was assayed using RARs-progestosterone/RXRα heterodimers, in which the P-box sequence of RAR had been changed into the sequence (GSCKV) of glucocorticoid receptor, so called RARs-progesterone (44). RARs-progesterone/RXRα heterodimers recognize the half-site binding motif of glucocorticoid receptor and the half-site binding motif of RXR.

**Cell Lines and Cell Culture.** Cultures of the carcinoma cells of patients were established and maintained serum-free and, for comparison, cell lines were grown serum-free or in low serum. Serum-free sub-lines of the prostate carcinoma cell lines LNCaP, PC-3, and DU-145 (serum stocks from American Type Culture Collection, Rockville, MD) and of the promyelocytic leukemia cell line HL60 have been described previously (14, 46). These lines are stable because they have been maintained in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) containing antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), insulin (100 µg/ml), transferrin (5.5 µg/ml), selenium dioxide (5 µg/ml), linoleic acid (5 mg/ml), and BSA (0.5 mg/ml) (ITS+1, Sigma, Poole, United Kingdom) for 5 years. The three prostate carcinoma lines are identical in appearance to serum-grown cells, and their doubling times and sensitivities to ATRA (inhibition of colony formation and growth in flask cultures) are similar to those of their serum-grown counterparts (14). The breast carcinoma lines MDA-MB-231 and MCF-7 (provided by Dr. K. Colston, St. Georges Hospital, London) were adapted for growth in serum-free medium (MDA-MB-231) and in RPMI 1640 supplemented with antibiotics and 0.5% FBS (MCF-7) as described previously (14). HeLa cells were grown in DMEM, 10% FBS, and antibiotics, and Jurkat cells were grown in RPMI 1640, 10% FBS, and antibiotics. Cells were grown at 37°C in a humidified atmosphere of 5% CO2 in air, and adherent cells were passaged by trypsinizing with trypsin-EDTA (Life Technologies, Inc.).

**Growth of Primary Carcinoma Cells.** Core biopsies were obtained from patients undergoing investigation for suspected prostate carcinoma. Consent for research use was obtained from the patients and the Local Research Ethics Committee. Histological examination confirmed that the biopsies were tumor material. Cultures were established as described by Peehi et al. (47), using the commercially available serum-free medium prostate epithelial cell growth medium supplemented with SingleQuots (BioWhittaker, Wokingham, United Kingdom). A primary culture was established from a gastro-intestinal carcinoma biopsy in ITS medium. Cells grown from biopsies were subcultured onto plates coated with collagen-1 (Greiner, Stonehouse, United Kingdom), and these plates and prostate epithelial cell growth medium were used for assays.

**Analysis of the Effect of AGN193198 on Cell Growth.** Trypsinized single-cell suspensions of cells were plated into a 96-well microtiter plate at 1000 cells/75 cm² flask, and AGN193198 was added immediately and in day 2 by replacing the medium. Cellular ATP levels were measured at day 5 using the Vivatag HS High Sensitivity Cell Proliferation/Cytotoxicity kit and a Berthold LB953 luminoimeter (Lumi Tech, Nottingham, United Kingdom). Wells were set up in triplicate, and at least three experiments were performed. Vehicles, titrated alongside agents, did not substantially affect the growth of any of the cells tested. Anti-Fas antibody ZB4, used in blocking experiments, was obtained from Upstate (New York).

In colony formation assays, trypsinized single-cell suspensions of cells were plated at 1000 cells/65 mm² dish in 4 ml of serum-free medium. Retinoids (10⁻² to 10⁻⁶ M) were added immediately, and the medium plus retinoids were replaced at day 2. Plates were incubated for 14 days, fixed using 1% formaldehyde in 0.9% NaCl, stained with 1% methylene blue in 0.9% NaCl, and dried. Around 200 colonies were counted in each duplicate plate, and three experiments of each type were performed. Colony formation on agent-treated plates is presented as a percentage (mean ± SE) of the number of colonies on untreated plates. Vehicle alone did not substantially affect colony formation (foritations see Ref. 14).

**Measurements of Apoptotic Events.** All of the experiments were performed in triplicate. LNCaP cells and primary prostate carcinoma cells were seeded at 5 x 10⁴ cells/ 75 cm² flask, and AGN193198 was added immediately. Apoptotic cells were identified, within pooled adherent (trypsinized) and suspension cells, by the terminal deoxynucleotidyltransferase-mediated nick end-labeling assay (48). A FITC-conjugated antibody to BrdUrd (Becton-Dickinson Co., Mountain View, CA) was used, and fluorescence was measured by fluorescence-activated cell sorter analysis at 510–550 nm. Acidine orange (5 µg/ml) was used to stain nuclear material of apoptotic HL60 cells. At least 200 cells were scored using a Zeiss microscope equipped with a mercury burner for incident illumination.

Changes to the mitochondrial membrane potential after treatment of bulk cultures of cells with AGN193198 were measured by incubating harvested cells with 5 µg/ml of the fluorescent probe JC-1 (5,5’,6,6’-tetrachloro-1,1’,3’,3’-tetrabenzimidazolylcarbocyanine iodide; Eastman Kodak Co., Rochester, NY) for 20 min at 37°C in an atmosphere of 5% CO2. After washing twice in PBS for 10 min, cells were analyzed on a Becton-Dickinson fluorescence-activated cell sorter (49).

**Caspase Studies.** Stock solutions of the pan-caspase inhibitor Z-VAD-FMK and of the caspase-8 inhibitor Z-IETD-FMK (R & D Systems, Abingdon, United Kingdom) were prepared in 20 mm dimethyl sulfoxide. Inhibitors were added to bulk cultures of cells 1 h before adding AGN193198, and cells were monitored for changes to the mitochondrial membrane potential and by using the terminal deoxynucleotidyltransferase-mediated nick end-labeling assay.

In fluorometric assays for caspase activity, 50 µl of cell lysate and 50 µl of assay buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT] containing 50 µM peptide substrates for caspase-3 (DEVD-afc), caspase-8 (IETD-afc), or caspase-9 (LEHD-afc); all from Biovision Inc., Mountain View, CA) were added to each well of microtiter plates. Backgrounds were measured in wells that contained assay buffer, substrate, and lysis buffer. Fluorescence was measured on a CytoFluor 4000 plate reader (Applied Biosystems) set at 400 nm excitation and 508 nm emission. Caspase activities were calculated as fold increases relative to control wells.

Antibodies to caspase-3 (Stressgen), caspase-8 (Cell Signaling Technology), caspase-9 (Cell Signaling Technology), caspase-10 (MBL, Nagoya, Japan), BID (Cell Signaling Technology), and Bel-2 (Upstate Biotechnology, clone 100) were used to stain blots. Cells (1 x 10⁷/ml) were lysed in ice-cold buffer [5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 100 µg/ml Na3VO4, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 50 mM Tris (pH 7.4)] for 0.5 h with gentle rotation at 4°C. Lysates were clarified (at 16,000 x g for 15 min at 4°C), resolved by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes for immunostaining. Immunoreactive proteins were visualized by enhanced chemiluminescence.

**Analysis of the c-Jun NH2-Terminal Kinase (JKN) Signaling Pathway.** Eighty percent confluent LNCaP and Jurkat cells were treated with 2 µM AGN193198 (0.5 to 8 h) or 10 µg/ml ansomycin (30 min). Cell lysates were resolved by SDS-PAGE, and blots were probed with an antibody to IkBα (Cell Signaling Technology), and also analyzed using the GST-c-Jun pulling-down and kinase activity assay using the stress-activated protein kinases inhibitors kit (Biorad). Analyses of the Nuclear Factor (NF)-κB Signaling Pathway. Interference of AGN193198 with tumor necrosis factor (TNF)-α-driven degradation of inhibitor of nuclear factor-κB (IκBα) was determined as follows. Eighty percent confluent cultures of cells were grown overnight in medium containing 10% charcoal-treated FBS. The cells were then treated for 1 h with either AGN193198 or the proteosomal inhibitor MG132 (Calbiochem), followed by 10 ng/ml TNF-α (R & D Systems) for 10 min. Lysates were resolved by SDS-PAGE, and blots were probed with an antibody to IκBα (Santa Cruz Biotechnology). Levels of NF-κB in nuclear extracts were examined using an electromobility shift assay and a double-stranded oligonucleotide probe containing a consensus NF-κB sequence (GGGGACCTTCCCC), which was labeled with α-32P-CTP (Klenow fill-in reaction). Nuclei were isolated cells using a NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). Nuclear lysates were assayed for IL-1β and TNF-α production by enzyme-linked immunosorbent assay (ELISA) and neutralization with neutralizing antibody.

**RESULTS**

**Binding and Transactivation Properties of AGN193198.** In the studies described below, 1 µM AGN193198 completely inhibited the growth of various malignant cells and induced apoptosis. This amount
of AGN193198 did not bind in any substantial degree to baculovirus-expressed RARs and RXRs; \( K_i \) values were as follows: 6.6 \( \mu M \) RAR\( \alpha \); >10 \( \mu M \) RAR\( \beta \); 3.5 \( \mu M \) RAR\( \gamma \); >10 \( \mu M \) RXR\( \alpha \); 5.6 \( \mu M \) RXR\( \beta \); and >10 \( \mu M \) RXR\( \gamma \). Compared with the transactivation activities of ATRA on RARs or 9-cis-RA on RXRs in cells expressing RARs or RXRs and reporter constructs, very low efficacies were obtained using AGN193198 (Fig. 1B). AGN193198 also showed weak antagonisms of 1 \( nM \) ATRA-driven transactivation of RARs and of 1 \( nM \) AGN194204-driven transactivation of RXRs (Fig. 1C). One \( nM \) of the pan-RAR antagonist AGN194310 blocked ATRA-driven transactivation of RXRs, whereas only partial effects were observed for 1 \( \mu M \) AGN193198.

**AGN193198 Potently Inhibits the Growth of Patients’ Prostate Carcinoma Cells.** Primary cultures of carcinoma cells were established from core biopsies of 13 patients with prostate carcinoma (see Fig. 2 for Gleason scores). After biopsy, the patients received androgen therapy (cyproterone acetate and Zoladex), and have responded as shown by substantial reductions in their serum levels of prostate-specific antigen (Fig. 2). Hence, the cultures were established from androgen-dependent tumors. Cultures had epithelial morphology, confirmed by immunocytochemical staining for cytokeratins and prostate-specific antigen, and contained few fibroblasts.

Primary prostate carcinoma cells were tested, at passage 2, for their sensitivity to AGN193198 and to ATRA. As measured using the microtiter plate assay, AGN193198 potently inhibited the growth of 13 of 13 patients’ cells, with concentrations that inhibited growth by 50% (\( IC_{50} \) values) in the range 130–332 \( nM \) (see table in Fig. 2). Complete growth inhibition of each of the patients’ cells was observed at 1 \( \mu M \). Cultures from patients whose carcinomas had high Gleason gradings appeared to be slightly more sensitive to AGN193198 (Fig. 2B), although ANOVA revealed that this relationship was not statistically significant. Whether grading is important to sensitivity will require testing of more patients’ cells. ATRA at 2 \( \mu M \) only partially inhibited the growth of cells from 11 of the patients (values between 5 and 35% and as illustrated in Fig. 2A; see also Ref. 14), and \( IC_{50} \) values of ~1 \( \mu M \) were obtained for PrC2 and PrC6.

**AGN193198 Is Active Against Several Types of Malignant Cells.** AGN193198 potently inhibited the growth of prostate carcinoma cell lines in the microtiter plate assay, with \( IC_{50} \) values of 238 ± 17 \( nM \) for LNCaP cells, 163 ± 26 \( nM \) for DU-145 cells, and 223 ± 18 \( nM \) for PC-3 cells (Fig. 3A). For comparison, Fig. 3C (right panel) shows the relative insensitivity of LNCaP cells to ATRA. Activity of AGN193198 does not relate to an inhibitory effect on cell attachment. Allowing LNCaP cells to adhere to microtiter wells (by 6 h) before adding compound did not displace the dose-response curve, and complete growth inhibition was observed at 1 \( \mu M \) (data not shown).

The pan-specific RAR antagonist AGN194310 potently inhibits the growth of serum-free-grown prostate carcinoma lines, but a component(s) of serum blocks this effect (14). We, therefore, compared the activity of AGN193198 against serum-free-grown versus serum-grown LNCaP cells cultured in microtiter wells. AGN193198 substantially inhibited the growth of serum-grown LNCaP cells at 2 \( \mu M \), as compared with 500 \( nM \) for serum-free-grown cells (Fig. 3C, left panel). The \( IC_{50} \) value obtained for serum-grown cells was 1.2 ± 0.3 \( \mu M \). This 4-fold difference relates to either a slight difference in the sensitivity of serum-free versus serum-grown cells to AGN193198 or binding of this agent to a component(s) of serum.

In the clonal proliferation assay, 100 \( nM \) AGN193198 prevented colony formation by LNCaP and PC-3; \( IC_{50} \) values were 51 ± 4 \( nM \) (\( n = 9 \)) and 50 ± 8 \( nM \) (\( n = 4 \)), respectively (Fig. 3B). For comparison, 1 \( \mu M \) ATRA inhibited colony formation by LNCaP and PC3 cells by ~70%, and \( IC_{50} \) values were 344 ± 32 and 419 ± 129 \( nM \), respectively. That less AGN193198 was needed for inhibition of colony formation (~50 \( nM \)) than for inhibition of growth (~230 \( nM \)) is commensurate with the widely held view that the colony assay is a more sensitive indicator of agent effects.

To investigate the range of malignant cell types sensitive to AGN193198, we measured activity against microtiter well cultures of the breast carcinoma lines MDA-MB-231 and MCF-7, the promyelocytic cell line HL60, and a primary culture of gastrointestinal carcinoma cells. All these cells were as sensitive as the prostate carcinoma lines and patients’ cells to growth inhibition by AGN193198, with \( IC_{50} \) values in the range 110–236 \( nM \) (see table in Fig. 3). HL60 cells treated with AGN193198 underwent apoptosis without differentiating. At day 1, 20% ± 2% of cells had fragmented nuclei, <1% of cells phagocytosed complement-coated yeast and reduced nitroblue tetrazolium, and cell death was complete by day 2 (50).

**AGN193198 Induces Apoptosis in Prostate Carcinoma Cells via Activation of Caspases.** The time course of the effect of 1 \( \mu M \) AGN193198 on the growth and survival of LNCaP cells was determined by growing cells in microtiter wells and measuring ATP levels at intervals. A 65% \( \pm \) 1% (\( n = 4 \)) reduction in the growth/survival of LNCaP cells was observed after 2 days of treatment with AGN193198 (Fig. 4A, left panel), and by day 3, survival was 16% ± 0.2% (\( n = 4 \)) of control cultures. Terminal deoxynucleotidyltransferase end-labeling of DNA strand-breaks and fluorescence-activated cell sorter analysis revealed that cells were undergoing apoptosis. The rate of appearance of DNA fragmentation was commensurate with the loss of cell number/viability (Fig. 4A, compare left and middle panels). By day 2, 69% ± 15% (\( n = 4 \)) of the cells in flask cultures of LNCaP cells treated with 1 \( \mu M \) AGN193198 had undergone apoptosis, as compared with 2% ± 0.5% in control cultures, and apoptotic cells had risen to
81% ± 2% (n = 4) by day 3 (Fig. 4A, middle panel; illustrated in right panel). We treated a bulk culture of early passage cells of the primary prostate carcinoma PrC10 with 2 μM AGN193198. By 24 h, cells had detached from the flask and 48% were apoptotic, as compared with 8% in the control culture (data not shown).

Fig. 4B shows that mitochondrial depolarization, as measured by the fluorescent probe JC-1, preceded DNA fragmentation in AGN193198-treated LNCaP cells. Doses of 0.75 and 1 μM resulted in 71% ± 11% and 92% ± 2% (n = 3), respectively, of cells depolarizing their mitochondria (Fig. 4B, left panel). Time course studies of 1 μM AGN193198-treated cultures showed that this occurred quickly (Fig. 4B, middle panel, experimental data illustrated in right panel). There was an increase at 6 h, from 10% ± 4% in untreated cultures to 18% ± 5% (n = 5, P = 0.05) in AGN193198-treated cultures, and about 90% of cells had undergone mitochondrial depolarization by 18 h.

AGN193198-stimulated DNA fragmentation and mitochondrial depolarization are both influenced by caspases. As shown in Fig. 4C, pretreatment of LNCaP cells for 1 h with the pan-caspase inhibitor Z-VAD-FMK before the addition of 1 μM AGN193198 abolished the subsequent appearance of DNA strand-breaks. The proportion of apoptotic cells at 72 h in cultures treated with AGN193198 and the inhibitor was 15% ± 6% as compared with 78% ± 7% in cultures treated with AGN193198 alone (n = 5, P = 0.0004). Pretreatment with the inhibitor also reduced the proportion of cells showing mitochondrial depolarization in response to AGN193198, from 85% ± 5% to 30% ± 4% (n = 4, P = 0.0001).

We used the selective caspase-8 inhibitor Z-IETD-FMK to investigate whether caspase-8 activation lies upstream or downstream of mitochondrial depolarization in LNCaP cells treated with AGN193198. At 72 h, the percentage of apoptotic cells was reduced from 95 ± 0.5 to 61 ± 12 (n = 3, P = 0.05) by the inhibitor, but there was no change in the proportion of cells at 42 h with depolarized mitochondria (Fig. 4C). We re-examined the effect of the caspase-8 inhibitor AGN193198-induced depolarization at 12 h, and there was also no effect (80% ± 4% versus 77% ± 9% in the presence of inhibitor, P = 0.4).

AGN193198 Provokes Activation of Caspases-3, -8, -9, and -10 in LNCaP and Jurkat Cells. We used Jurkat cells for time course studies of activities of individual caspases as suspension cells are more amenable to rapid harvesting. Jurkat T cells, like other malignant cells we tested, were growth-inhibited by 1 μM AGN193198 (Fig. 5A, left panel), their mitochondria became depolarized (29% ± 9% by 12 h) and, as in LNCaP cells, this was unaffected by the caspase-8 inhibitor (also 26% ± 7%, P = 0.9). Activities of caspase-3 and -9, measured using specific substrates, were increased 10- and 3-fold, respectively, in extracts from Jurkat cells treated with 1 μM AGN193198 (see Fig. 5A, middle and right panels).

As shown in Fig. 5C, treatment of LNCaP and Jurkat cells with 2 μM AGN193198 led to the appearance of truncated (active) forms of caspase-3, -8, and -9 within around 4–6 h, and maximals at between 6–8 h. Parallel time course analyses of enzyme activity within Jurkat cells showed that activities of caspase-3, -8, and -9 increased 25-, 4-, and 8-fold, respectively, within 6 h (Fig. 5B). Cleavages of procaspase-10 were detected at 4 to 6 h in LNCaP and Jurkat cells treated
with 2 \( \mu M \) AGN193198 by immunoblotting of extracts (data not shown).

Fig. 6 shows that treatment of Jurkat cells with the caspase-8 inhibitor Z-IETD-FMK (1 or 5 \( \mu M \)) for 1 h before adding 2 \( \mu M \) AGN193198 (for 3 or 6 h) prevented activation of caspases-8, -3 and -9. Similarly, Z-IETD-FMK (5 \( \mu M \)) prevented the appearance of cleaved forms of caspase-9 and caspase-8 (which can cleave itself) in response to AGN193198. A cleaved form of caspase-3 was observed in the inhibitor/AGN193198-treated cells, but this has a higher molecular weight than the forms generated in the presence of AGN193198 alone.

As shown in Fig. 6B, treatment of Jurkat cells for 6 h with 2 \( \mu M \) AGN193198 led to the production of BID/p15, and this was completely blocked by pretreating cells with the caspase-8 inhibitor.

We examined whether Fas signal transduction, JNK activation leading to c-Jun phosphorylation, and changes to the level of Bcl-2 play roles in AGN193198-provoked apoptosis. In the ATP end point plate assay, 250 ng/ml anti-Fas antibody ZB4 blocks agonistic anti-Fas-driven apoptosis of Jurkat cells. ZB4 antibody, at concentrations 62.5–1,000 ng/ml, did not affect the growth of LNCaP cells in the plate assay nor interfere with the growth inhibitory effects of 250 nM, 500 nM, and 1 \( \mu M \) AGN193198 (data not shown). Fig. 6B (middle panel) shows that the level of phosphorylated JNK was increased substantially in LNCaP cells treated with the known JNK activator anisomycin (51), and had increased to a much lesser extent in cells treated with 2 \( \mu M \) AGN193198. Comparable results were obtained for the levels of phosphorylated c-Jun. This weak activation of JNK by AGN193198 is later than the onset of mitochondrial depolarization (see time course in Fig. 4), and is, therefore, unlikely to mediate AGN193198-driven mitochondrial depolarization. Results from similar experiments using Jurkat cells confirmed this finding (data not shown). The level of Bcl-2 was not affected by treatment of
LNCaP and Jurkat cells for up to 48 h with 1 μM AGN193198 (Fig. 6B, bottom panel).

**AGN193198 Is Not an Inhibitor of the NF-κB Signaling Pathway.** The RRM MX781 induces caspase-dependent apoptosis by binding to and inhibiting activity of the inhibitor of nuclear factor B kinase (IKK)α and IKKβ kinases that phosphorylate inhibitor of nuclear factor B, and this, in turn, leads to reduced activity of the transcription factor NF-κB (52). Nonphosphorylated inhibitor of nuclear factor B sequesters NF-κB in the cytoplasm, whereas phosphorylated inhibitor of nuclear factor B is polyubiquitinated and degraded, by the proteosome-dependent pathway, allowing NF-κB to translocate to the nucleus.

We examined whether AGN193198 interfered with IKK-activated degradation of IκBα when IKK activity was stimulated in HeLa, LNCaP, and Jurkat cells by TNF-α (see Fig. 7). As shown in Fig. 7A, treatment of HeLa cells with 10 or 100 ng/ml TNF-α led to complete degradation of IκBα within 10 min, and its concentration returned to that of control cells by 2 h. Fig. 7B (left panel) shows that treatment of HeLa cells for 1 h with up to 2 μM AGN193198 before the addition of 10 ng/ml TNF-α for 10 min did not influence TNF-α-stimulated

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**Fig. 5. AGN193198 activates caspase-3, -8, and -9.** A, Jurkat cells substantially growth inhibited by treatment with 1 μM AGN193198 for 5 days. Cell lysates from AGN193198-treated (4 h) Jurkat cells showed increased activities of caspase-3 and caspase-9, as measured using specific substrates (middle and right panels). **P < 0.01 versus controls (without treatment).** B, time courses obtained for increased enzyme activities of caspase-8, -3 and -9 post-treatment of Jurkat cells with 2 μM AGN193198. C, cleavage of caspase-3, -8 and -9 in 2 μM AGN193198-treated LNCaP and Jurkat cells, as revealed by immunostaining blots of cell extracts.
degradation of IκBα. Micromolar amounts of the proteosomal inhibitor MG132 did block this process (Fig. 7B, right panel). Similar experiments using LNCaP cells and Jurkat T cells confirmed that doses of AGN193198 up to 2 μM had no effect on the activity of IKK (Fig. 7B).

NF-κB is partially active in untreated LNCaP cells (53). We examined levels of NF-κB in nuclear extracts of LNCaP cells that were treated with TNF-α, with or without a 1 h prior exposure to AGN193198. Moderate and similar levels of binding of NF-κB to the NF-κB-specific oligonucleotide probe were observed (electromobility...
shift assay) for extracts from untreated LNCaP cells and from cells pretreated for 1 h with 1 \textmu M AGN193198. Stimulation of the level of \(\text{NF-kB}\) in nuclear extracts by 7.5 ng/ml TNF-\(\alpha\) was also not affected by pretreatment of cells for 1 h with AGN193198 (data not shown).

**DISCUSSION**

If RRM s are to be used as therapeutic agents for multiple cancers, an important question is how RRM s work and, particularly, whether RARs play any role in their induction of apoptosis. AGN193198 potently induces apoptosis in many types of malignant cells, and it seems very unlikely that the mechanism(s) of induction involves RARs or RXRs. AGN193198 elicited maximum effects at 1 \textmu M in the several assays used to measure various activities, and there were substantial effects at lower doses. One \textmu M AGN193198 shows little binding to RARs or RXRs and does not effectively transactivate in RAR- and RXR-mediated reporter assays. AGN193198 is also not an effective antagonist of RARs and RXRs. By contrast, effective RAR agonists and antagonists generally bind with a \(K_i\) of 2–5 nM and agonise and antagonise at nM amounts (14).

AGN193198 (at 1 \textmu M) killed all 13 of the primary cultures established from prostate carcinoma. Moreover, AGN193198, like other RRM s such as fenretinide and CD437, induces apoptosis in diverse cancer-derived cell lines. Of particular interest is that CD437 induces apoptosis in lung cancer cell lines and malignant human epidermal keratinocytes whereas sparing normal cells (6, 54, 55), although it does arrest the growth of normal lung epithelial cells (6). These differential effects led Sun et al. to advocate the application of CD437 for cancer treatment (6).

Induction of apoptosis by AGN193198 involves mitochondrial depolarization leading to DNA fragmentation. Apoptosis was induced quickly in AGN193198-treated cells: e.g., mitochondria started to depolarize within ~6 h, and most cells showed mitochondrial depolarization by 12 h. In parallel, caspase-3, -8, -9, and -10 were activated. Activated caspase-8 might be needed for activation of caspase-3 and -9, and indeed, blocking caspase-8 activity in AGN193198-treated Jurkat cells prevented activation of caspase-3 and -9. BID/p15 was produced around the same period as caspases were activated, and the caspase-8 inhibitor also blocked the induced appearance of BID/p15. These results led us to consider a sequential pathway involving caspase-8 activation, BID/p15 production, mitochondrial depolarization, cytochrome \(c\) release, activation of caspase-3 and -9, and finally DNA fragmentation, as described for surface death receptors (56, 57). However, the antagonistic Fas antibody did not block the effect of AGN193198. Also, mitochondrial depolarization in AGN193198-treated LNCaP and Jurkat cells was unaffected by the caspase-8 inhibitor, and DNA fragmentation occurred even when the caspase-8 inhibitor was used to block caspase-8-mediated cleavage of caspase-3 and -9. By contrast, the pan-caspase inhibitor did prevent AGN193198-induced mitochondrial depolarization and DNA strand-breaks, confirming the involvement of at least some caspase-related protease in AGN193198-stimulated mitochondrial depolarization and DNA fragmentation. Caspase-10 is activated by AGN193198, although not driven via Fas signaling. Whether AGN193198 generally activates caspases or there is a particular activation sequence, the importance of individual caspases remains to be determined.

It is widely accepted that mitochondrial depolarization and activation of caspase-3 and -9 are involved in apoptosis induction by CD437 or fenretinide (58–62). Moreover, inhibiting mitochondrial depolarization with bongkrekic acid blocks activation of caspase-3 and -9 (25). The timing of involvement of other caspases in RRM-induced apoptosis may be cell type and/or agent specific. Fen-
retinide-stimulated apoptosis in Fas-defective hepatoma cells involves activation of caspase-8 that precedes release of cytochrome c from mitochondria and slight caspase-9 activation (42). By contrast, mitochondrial depolarization is upstream of the activation of caspase-3, -8, and -9 during fenretinide-induced apoptosis in squamous cells (63). However, other workers have suggested that caspase-8 activity is not necessary for either CD437 or fenretinide induction of apoptosis (25). Other caspases are also likely to be important to RRM-induced apoptosis as, for example, caspase-6 plays a role in CD437-induced apoptosis in lung cancer cells (6). What is the immediate molecular target for the action of AGN193198? Mitochondrial depolarization and activation of caspases are well underway within 4–6 h, but we have not yet identified the earlier events. Bayon et al. (52) have offered evidence that MX781 induces apoptosis by inhibiting IKK and have argued that RRM structures serve as a lead for the development of IKK inhibitors. However, concentrations of AGN193198 that trigger apoptosis do not inhibit IKK, so interference with IKK activity is very unlikely to play any role in AGN193198-induced apoptosis. Another recently identified putative target for RRM is the orphan nuclear receptor TR3. CD437, but not fenretinide, induces translocation of TR3 to the cytosol and to mitochondria (25). Whether CD437, fenretinide, MX781, and AGN193198 initially use different pathways to induce mitochondrial depolarization or share a common, but unidentified target, remains a question for the future.

In summary, we have described a novel RRM that is potent in inducing apoptosis in a variety of malignant cell types, apparently without any involvement of retinoid nuclear receptors. Because RRM, such as AGN193198, show promise as therapeutic agents in cancer, identification of their molecular targets is an important aim for future studies.

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