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

Richard G. Keedwell,1 Yi Zhao,4 Lisette A. Hammond,2 Suofu Qin,4 Kwok-Yin Tsang,4 Armin Reitmair,4 Yanira Molina,4 Yumiko Okawa,4 Larissa I. Atangan,1 Dixie-Lee Shurland,4 Kaisheng Wen,1 D. Michael A. Wallace,3 Roger Bird,1 Roshantha A. S. Chandraratna,4 and Geoffrey Brown1

Divisions of 1Immunity and Infection and of 2Cancer Studies, University of Birmingham Medical School, Edgbaston, Birmingham, United Kingdom; 3Department of Urology, Queen Elisabeth Hospital, Birmingham; and 4Retinoid Research, Departments of Chemistry and Biology, Allergan Inc., Irvine, California

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 of action has 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). Studies of 13-cis-retinoic acid (isoretinoin), with or without IFN-α, in prostate cancer have demonstrated only moderate efficacy (15–17). By contrast to the weak activity of agonists, the RAR pan-agonist 4-2(6-(2,2-dimethyl-(1H)-4-(4-ethylphényl)-1-benzothiopyran)-ethyl-phenyl) benzoic acid (AGN194310) and the RAR β, γ-selective agonist 3-fluoro-4-[7-(4,4-dimethyl-3,4-dihydro-1-(p-tolyl)-naphthyl)-2-azol] benzoic acid (AGN194431) are potent growth inhibitors of prostate carcinoma lines and patients’ cells (3, 14) and inhibit colony formation at ~100 nM. Similarly, the weak RAR antagonist MX781 inhibits the growth of breast carcinoma lines more potently than ATRA (7).

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 RMRs to nuclear retinoid receptors, even if weak, and their transactivation essential for RMRs 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 prostate carcinoma. The induction of apoptosis by RMRs, 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, -10 and BID/p15 production, leading ultimately to DNA fragmentation. These processes seem to involve neither RARs nor RXRs, suggesting that RMRs such as AGN193198 that do not bind to these receptors may be useful in the treatment of a variety of malignancies.

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).

Retinoids bind to and activate the following two classes of receptors: retinoic acid receptors (RARα, β, and γ) and retinoid X receptors (RXRα, β, and γ). All-trans-retinoic acid (ATRA), a natural RAR agonist, and synthetic agonists and antagonists of subtypes of RARs inhibit growth and induce apoptosis in prostate cancer cells. However, the prostate cancer cell lines LNCaP, DU145, and PC3 are relatively insensitive to ATRA and other RAR agonists, with around 1–10 μM needed to inhibit colony formation (11–14). Accordingly, clinical studies of 13-cis-retinoic acid (isoretinoin), with or without IFN-α, in prostate cancer have demonstrated only moderate efficacy (15–17). By contrast to the weak activity of agonists, the RAR pan-agonist 4-2(6-(2,2-dimethyl-(1H)-4-(4-ethylphényl)-1-benzothiopyran)-ethyl-phenyl) benzoic acid (AGN194310) and the RAR β, γ-selective agonist 3-fluoro-4-[7-(4,4-dimethyl-3,4-dihydro-1-(p-tolyl)-naphthyl)-2-azol] benzoic acid (AGN194431) are potent growth inhibitors of prostate carcinoma lines and patients’ cells (3, 14) and inhibit colony formation at ~100 nM. Similarly, the weak RAR antagonist MX781 inhibits the growth of breast carcinoma lines more potently than ATRA (7).

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 RMRs to nuclear retinoid receptors, even if weak, and their transactivation essential for RMRs 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 prostate carcinoma. The induction of apoptosis by RMRs, 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, -10 and BID/p15 production, leading ultimately to DNA fragmentation. These processes seem to involve neither RARs nor RXRs, suggesting that RMRs 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 RRMs were synthesized at Allergan Inc. (Irvine, CA): the RRM 4-[3-(1-heptyl-4,4-dimethyl-2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)-3-oxo-propenyl] benzoic acid (AGN193198), the RAR pan-antagonist 4-(2,2-dimethyl-1(1H)-4-(4-ethylphenyl)-1-benzo-thiopyran)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-yl]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 (FBS, 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.

**A** Structures of ATRA, 9-cis-Retinoic Acid, and AGN193198.

[Diagram of ATRA, 9-cis-RA, and AGN193198]

**B** Transactivation of RARs and RXRs by AGN193198.

1: Transactivation of RARs by ATRA and AGN193198.

2: Transactivation of RXRs by 9-cis-RA and AGN193198.

**C** Antagonism of RARs and RXRs by AGN193198.

1: Antagonism of ATRA Transactivation of RARs by AGN194310 and AGN193198.

2: Antagonism of AGN194204 Transactivation of RXRs by AGN194393 and AGN193198.

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 AGN393198 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 AGN194393 (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-progesterone/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 ng/ml), and BSA (0.5 ng/ml) (ITS**⊥**, 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 prostatic 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 Peehl 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 cell line (Cell Signaling Technology), and also analyzed using the GST-c-Jun difluoride membranes for immunostaining. Immunoreactive proteins were visualized by enhanced chemiluminescence.

**Analysis of the Effect of AGN193198 on Cell Growth.** Trypsinized single-cell suspensions of cells were plated into a 96-well microtiter plate at 400 cells/well (100 μl of ITS**⊥** medium (cell lines) or prostate epithelial cell growth medium (primary cells)). Retinoids were added immediately and in day 2 by replacing the medium. Cellular ATP levels were measured at day 5 using the Vialight HS High Sensitivity Cell Proliferation/Cytotoxicity kit and a Berthold LB953 lumimeter (Lumi Tech, Nottingham, United Kingdom). Wells were set up in triplicate, and at least three experiments were performed. Vehiciles, titrated alongside agents, did not substantially affect the growth of any of the cells tested. Anti-Fas antibody 2B4, 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 (10⁻²–10⁻⁶ 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 on 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 effect 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 × 10⁵ 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. Acriflavine 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'-tetramethylindodicarbocyanine 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 dimethylsulphoxide. 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 × 10⁵/ml) were lysed in ice-cold buffer [5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 100 μM NaVO₃, 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 × 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 (JNK) Signaling Pathway.** Eighty percent confluent LNCaP and Jurkat cells were treated with 2 μM AGN193198 (0.5 to 8 h) or 10 μg/ml ansamycin (30 min). Cell lysates were resolved by SDS-PAGE, for immunoblotting with anti-phospho-JNK1/2 antibody (Cell Signaling Technology), and also analyzed using the GST-c-Jun pulling-down and kinase activity assay using the stress-activated protein kinase inhibitor kit (Cell Signaling Technology).

**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 (GCGGAGCTTCCCTC), which was labeled with α-N³-32P-CTP (Klenow fill-in reaction). Nuclei were isolated cells using a NP-40 lysis buffer [0.4% NP-40, 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors] and then lysed [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors]. Five μg of nuclear extract were incubated with probe for 15 min at room temperature in the presence of 1 μg poly(dI:dC) in 20 μl of binding buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, and 10% glycerol]. Protein-DNA complexes were separated on a 5% nondenaturing gel that was dried for autoradiography.

**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; Kᵣ values were as follows: 6.6 μM RARα; >10 μM RARβ; 3.5 μM RARγ; >10 μM RXRα; 5.6 μM RXRβ; and >10 μM RXRγ. 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 efficiencies 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 μM AGN193198.

**AGN193198 Potently Inhibits the Growth of Patients’ Prostate Carcinoma Cells.** Primary cultures of carcinoma cells were established from biopsies of 13 patients with prostatic carcinoma (see Fig. 2 for Gleason scores). After biopsy, the patients received antiandrogen 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% (IC₅₀ 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 μM. 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 μM 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 IC₅₀ values of ∼1 μM 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 IC₅₀ 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 μM (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 in microtiter wells. AGN193198 substantially inhibited the growth of serum-grown LNCaP cells at 2 μM, as compared with 500 nM for serum-free-grown cells (Fig. 3C, left panel). The IC₅₀ value obtained for serum-grown cells was 1.2 ± 0.3 μM. 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; IC₅₀ values were 51 ± 4 nM (n = 9) and 50 ± 8 nM (n = 4), respectively (Fig. 3B). For comparison, 1 μM ATRA inhibited colony formation by LNCaP and PC3 cells by ~70%, and IC₅₀ 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 IC₅₀ 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 μM 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% decline in ATP (measured after 10 days on 1 μM AGN193198) and fluorescence-activated cell sorter analysis revealed that cells were undergoing apoptosis. The rate of appearance of DNA fragments was determined by adding 1 μM AGN193198 to LNCaP cells and measuring apoptotic nuclei (see Fig. 3B and 4C, left panel). The rate of appearance of DNA fragments was determined by adding 1 μM AGN193198 to LNCaP cells and measuring apoptotic nuclei (see Fig. 3B and 4C, left panel). The rate of appearance of DNA fragments was determined by adding 1 μM AGN193198 to LNCaP cells and measuring apoptotic nuclei (see Fig. 3B and 4C, left panel). The rate of appearance of DNA fragments was determined by adding 1 μM AGN193198 to LNCaP cells and measuring apoptotic nuclei (see Fig. 3B and 4C, left panel). The rate of appearance of DNA fragments was determined by adding 1 μM AGN193198 to LNCaP cells and measuring apoptotic nuclei (see Fig. 3B and 4C, left panel).
AGN193198 is active against a variety of cell types. A, the activity of AGN193198 against the prostate carcinoma lines LNCaP (○), DU-145 (□), and PC-3 (△) as determined by seeding cells in microtiter wells, treating with agent immediately and at day 2, and measuring cellular ATP levels at day 5. B, the ability of AGN193198 to inhibit colony formation in plates by LNCaP (○) and PC-3 (△). Plated cells were treated with agent immediately and by replacing medium plus agent at day 2. Colonies were enumerated at day 14. IC50 values shown in the table were obtained using the microtiter well assay.

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 in 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 pro-caspase-10 were detected at 4 to 6 h in LNCaP and Jurkat cells.
Fig. 4. AGN193198-induced apoptosis of LNCaP cells involves depolarization of the mitochondrial membrane potential. A, left, the time course for the effect of 1 μM AGN193198 on the survival of LNCaP cells in microtiter wells as determined by measuring levels of cellular ATP. A, middle, shows the time course for DNA fragmentation in 1 μM AGN193198-treated cells (○) and vehicle-treated cells (●). Right panels illustrate these data. B, left, the dose-response curve obtained for AGN193198 for the induction of mitochondrial membrane depolarization (measured using the JC-1 probe). B, middle, the time course for this event when cells were treated with 1 μM AGN193198 (●) and vehicle alone (○). Right panels illustrate these data. C, the effects of pre-treatments for 1 h with the pan-caspase inhibitor Z-VAD-FMK (at 50 μM) and of the caspase-8 inhibitor Z-IETD-FMK (at 5 μM) on the capacity of 1 μM AGN193198 to induce depolarization of the mitochondrial membrane potential (left) and apoptosis (strand breaks, right panel) in LNCaP cells.

with 2 μM 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 μM) for 1 h before adding 2 μM AGN193198 (for 3 or 6 h) prevented activation of caspases-8, -3 and -9. Similarly, Z-IETD-FMK (5 μM) 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 μM 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 μM 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 μM 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
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...
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 μM AGN193198. Stimulation of the level of NF-κB in nuclear extracts by 7.5 ng/ml TNF-α was also not affected by pretreatment of cells for 1 h with AGN193198 (data not shown).

**DISCUSSION**

If RRMs are to be used as therapeutic agents for multiple cancers, an important question is how RRMs 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 μM in several assays used to measure various activities, and there were substantial effects at lower doses. One μM 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 Ki of 2–5 nM and agonise and antagonise at nM amounts (14).

AGN193198 (at 1 μM) killed all 13 of the primary cultures established from prostatic carcinoma. Moreover, AGN193198, like other RRMs 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. By contrast, the pan-caspase inhibitor 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.
retinoic acid and retinoid X receptors in mammalian cells and yeast. Correlation with
protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells
inducers and effective inhibitors of human lung cancer cells in vivo. Nat Med 1997;
6:686–90.
Lu X-P, Fanjul A, Picard N, Shroott B, Pfahl M, A selective retinoid with high activity
receptors (RARs) are potent growth inhibitors of prostate carcinoma cells. Br J
prostate-specific antigen levels in patients with recurrent prostate cancer after radical
in patients treated with differentiation agents: an experience of retinoids in prostate
18. Sun SY, Yue P, Lotan R. Implication of multiple mechanisms in apoptosis induced by
19. Sheikh MS, Shao ZM, Li XS, et al. Fontana JA. N-(4-hydroxyphenyl) retinamide (4-HPR)-mediated biological activities involve retinoid receptor-independent pathways
20. Pienta JK, Nguyen NM, Lehr JE. Treatment of prostate cancer in the rat with the
21. Heish TC, Wu JM. Effects of fenretinide (4-HPR) on prostatic LNCaP cell growth,
22. Basi RC Jr, Boyer CM, Xu FJ, et al. Molecular approaches to prevention and
in human ovarian carcinoma cells and its association with retinoid acid receptor
24. Sabichi AL, Hendricks DT, Bober MA, Birrer MJ. Retinoic acid β expression and
growth inhibition of gynaecological cancer cells by the synthetic retinoid N-(4-hy-
of apoptosis in ovarian carcinoma cells by the conformationally restricted synthetic
retinoids CD347 and 4-HPR. J Cell Biochem 2003;89:262–78.
localizations of oral leukoplakias with the synthetic retiniferene (4-HPR).
262–70.
long-term results from a controlled clinical trial for the prevention of contralateral
with selectivity for human retinoid acid receptor γ. Biochem Biophys Res Comm
retinoids and an apoptosis inducing retinoid on the adherent growth of the NIH
31. Hsu CA, Rishi AK, Li X-S, et al. Retinoid induced apoptosis in leukemias through
32. Adachi H, Preston G, Harvat B, Dawson MI, Jetten A. Inhibition of cell proliferation
and induction of apoptosis by the retinoid AHPN in human lung carcinoma cells.
34. Orudate N, Higuchi M, Suzuki S, Shroott B, Hong WK, Lotan R. Rapid induction of
apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-
adamantyl) hydroxyphenyl]-2-naphthalene carboxylic acid (CD347). Int J Cancer 1997;
70:484–7.
35. Shao Z-M, Dawson MI, Li X-S, et al. P53 independent G1/G0 arrest and apoptosis
504.
36. Piedrafita FJ, Pfahl M. Retinoid-induced apoptosis and Sp1 cleavage occur independ-
activation of retinoic acid receptors (RARs) and retinoid X receptors reveals a unique
role for RARγ in induction of differentiation and apoptosis of S91 melanoma cells.
growth arrest and apoptosis in human lung cancer cell lines. Mol Cell Biol 1998;18:
2536–47.
39. Wu S, Zhang D, Donigan A, Dawson MI, Soprano DR, Soprano KJ. Effects of
conformationally restricted synthetic retinoids on ovarian tumor cell growth. J Cell
40. Kagechika H, Kawachi E, Hashimoto Y, Shudo K. Retinobenzoic acids. 2. Structure-
activity relationships of cyclic-4-carboxylic acids and flavone-4-carboxylic acids.
41. Lopez-Hernandez FJ, Ortiz MA, Bayon Y, Piedrafita FJ. Z-FRA-fink inhibits effector
caspases but not initiator caspases 8 and 10, and demonstrates that novel anticancer
retinoid-related molecules induce apoptosis by the intrinsic pathway. Mol Cancer
42. You KR, Shin MN, Park RK, Lee SO, Kim DG. Activation of caspase-8 during
N-(4-hydroxyphenyl) retinamide-induced apoptosis in Fas-defective hepatoma cells.
43. Allegretto EA, McClurg MR, Lazarchik SB, et al. Transactivation properties of
retinoic acid and retinoid X receptors in mammalian cells and yeast. Correlation with

REFERENCES

1. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev
from 9-cis-retinoic acid and retinoid X receptors in mammalian cells and yeast. Correlation with
protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells
inducers and effective inhibitors of human lung cancer cells in vivo. Nat Med 1997;
6:686–90.
receptors (RARs) are potent growth inhibitors of prostate carcinoma cells. Br J


46. Bunce CM, Wallington LA, Harrison P, Williams GR, Brown G. Treatment of HL60 cells with various combination of retinoids and 1α, 25 dihydroxyvitamin D₃ results in differentiation towards neutrophils or monocytes or a failure to differentiate and apoptosis. Leukemia 1995;9:410–8.


49. Salvieri S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DIC6(3) or rhodamine 123, is a reliable fluorescent probe to assess ΔΨ in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.


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


Cancer Res 2004;64:3302-3312.