Induction of Retinoid X Receptor Activity and Consequent Upregulation of p21WAF1/CIP1 by Indenoisoquinolines in MCF7 Cells

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Abstract
Retinoid X receptor (RXR) has been targeted for the chemoprevention and treatment of cancer. To discover potential agents acting through RXRs, we utilized an RXR response element (RXRE)-luciferase reporter gene assay. Following extensive screening, 3-amino-6-(3-aminopropyl)-5,6-dihydro-5,11-dioxo-11H-indeno[1,2-c]isoquinoline dihydrochloride (AM6-36) was found to induce RXRE-luciferase activities. AM6-36 inhibited COX-2 expression and anchorage-independent growth with 12-O-tetradecanoylphorbol 13-acetate-stimulated JB6 Cl41 cells, induced the expression of CD38 in HL-60 cells, and attenuated the growth of N-methyl-N-nitrosourea–induced mammary tumors in rats. Consistent with other reports describing the antiproliferative effects of RXR agonists in breast cancers, AM6-36 showed growth inhibition with cultured MCF7 breast cancer cells, accompanied by G2/M-phase arrest at lower concentrations and enhanced S-phase arrest at higher concentrations. On the basis of DNA microarray analysis, AM6-36 upregulated the expression of CDKN1A, a target gene of RXR, by 35-fold. In accord with this response, the expression of the corresponding protein, p21WAF1/CIP1, was increased in the presence of AM6-36. Induction of p21 by AM6-36 was abrogated following transient knockdown of RXRα, demonstrating that the effect of AM6-36 on the expression of p21 is closely related to modulation of RXRα transcriptional activity. Intestinal permeability was suggested with Caco-2 cells and limited metabolism resulted when AM6-36 was incubated with human liver microsomes. Oral administration with rats resulted in 0.8 mg/mL, 4.3 mg/g, and 0.3 mg/g in serum, liver, and mammary gland, respectively. In sum, these data suggest that AM6-36 is a promising lead for the treatment or prevention of breast cancer and provide a strong rationale for testing in more advanced antitumor systems. Cancer Prev Res; 4(4); 592–607. ©2011 AACR.

Introduction
Retinoid X receptor (RXR), a member of the nuclear receptor superfamilies, was identified as an orphan receptor with a high sequence homology to retinoic acid receptor (RAR), and a specific responsiveness to vitamin A metabolites (1). Three subtypes of RXRs, RXRα, RXRβ, and RXRγ, have been identified, and essential roles of RXRs have been reported in various physiologic processes including embryonic development, metabolic processes, differentiation, and apoptosis. Similar to other nuclear receptors, RXRs require specific ligands to be functionally activated. Whereas ligand-free RXRs are sequestered by specific nuclear receptor corepressors; on ligand binding, RXRs undergo conformational changes, leading to the dissociation of corepressors and the association of coactivators (2). Consequently, ligand-activated RXR dimers recognize the corresponding cis-acting element in the promoter region of target genes, called hormone response elements (HRE), and are capable of triggering transcription. HREs consist of 2 direct repeats (DR) of the consensus hexamers (AGGTCA) in tandem, separated by 1 to 5 nucleotides, which are designated as DR1 to DR5, respectively. In particular, RXR homodimers are known to interact with DR1, RXR response element (RXRE; ref. 3).

As mentioned above, specific ligands for RXRs generally play key roles in the activation of RXR signaling and several substances are known to function as endogenous ligands including 9-cis-retinoic acid (9-cis-RA), docosahexaenoic acid (DHA), and the endogenous bile acid chenodeoxycholic acid (CDCA). Among RXR-active substances, 9-cis-RA has been known to activate RXRs in the absence of dietary supplementation (4). However, its potential for treating breast cancer remains to be elucidated. In this connection, AM6-36 was identified as a promising lead for the treatment or prevention of breast cancer and an antitumor system.

AM6-36 inhibited COX-2 expression and anchorage-independent growth with 12-O-tetradecanoylphorbol 13-acetate-stimulated JB6 Cl41 cells, induced the expression of CD38 in HL-60 cells, and attenuated the growth of N-methyl-N-nitrosourea–induced mammary tumors in rats. Consistent with other reports describing the antiproliferative effects of RXR agonists in breast cancers, AM6-36 showed growth inhibition with cultured MCF7 breast cancer cells, accompanied by G2/M-phase arrest at lower concentrations and enhanced S-phase arrest at higher concentrations. On the basis of DNA microarray analysis, AM6-36 upregulated the expression of CDKN1A, a target gene of RXR, by 35-fold. In accord with this response, the expression of the corresponding protein, p21WAF1/CIP1, was increased in the presence of AM6-36. Induction of p21 by AM6-36 was abrogated following transient knockdown of RXRα, demonstrating that the effect of AM6-36 on the expression of p21 is closely related to modulation of RXRα transcriptional activity. Intestinal permeability was suggested with Caco-2 cells and limited metabolism resulted when AM6-36 was incubated with human liver microsomes. Oral administration with rats resulted in 0.8 mg/mL, 4.3 mg/g, and 0.3 mg/g in serum, liver, and mammary gland, respectively. In sum, these data suggest that AM6-36 is a promising lead for the treatment or prevention of breast cancer and provide a strong rationale for testing in more advanced antitumor systems. Cancer Prev Res; 4(4); 592–607. ©2011 AACR.
acid, and other unsaturated fatty acids (4). Besides putative endogenous ligands of RXRs, additional compounds have been reported as RXR agonists, so-called rexinoids including AGN194204, CD3254, LG100268, LGD1069, and SR11237. Although the mechanisms underlying their suppression and inhibitory actions against cancerous processes have not been fully clarified, both naturally occurring and synthetic rexinoids have shown promise in the prevention and treatment of cancer (5). For example, 9-cis-RA, LG100268, and LGD1069 have shown efficacy in N-methyl-N-nitrosourea (MN1)-induced breast cancer animal models (6–8). Furthermore, 9-cis-RA and LGD1069 have been approved by the Food and Drug Administration (FDA) for the treatment of Kaposi’s sarcoma and cutaneous T-cell lymphoma, respectively (9, 10).

In searching for novel cancer chemopreventive and therapeutic agents capable of functioning as RXR agonists, we utilized an RXRE-luciferase reporter gene assay. This assay assesses RXR transcriptional activity as well as binding to RXR, since trans-activation occurs only after ligand binding. During the course of testing over 5,000 natural product extracts and compounds, only a single active lead was identified: 3-amino-6-(3-aminopropyl)-5,6-dihydro-5,11-dioxo-1H-indeno[1,2-c]isoquinoline dihydrochloride (AM6-36; Fig. 1). On the basis of the unexpected yet unique ability of AM6-36 to serve as an RXR agonist, 18 structural relatives were tested, but AM6-36 still demonstrated the greatest activity (Table 1).

Using MCF7 breast cancer cells as a model, AM6-36 was found to suppress proliferation and this was accompanied by cell-cycle arrest. RXRe transcriptional activity was upregulated with subsequent expression of RXR target gene CDKN1A. Moderate absorption and slow metabolism was demonstrated. Strong indications of cancer chemopreventive potential were observed with HL-60 cells, 12-O-tetradecanoylphorbol 13-acetate (TPA)-treated JB6 Cl41 cells, and rats bearing mammary tumors. Finally, a synthetic procedure for large-scale production is reported that will enable additional antitumor evaluations.

Materials and Methods

Reagents

9-cis-RA, TPA, trichloroacetic acid (TCA), sulforhodamine B (SRB), parabformaldehyde, IgG1-fluorescein isothiocyanate (FITC) isotype control from murine myeloma, and 7-hydroxy-staurosporine (UCN-01) were purchased from Sigma-Aldrich, Inc. Bexarotene was purchased from LC Laboratories. Cell lysis buffer (10×), and Lumiglo chemiluminescent detection kit were from Cell Signaling Biotechnology. Rabbit polyclonal anti-β-actin, rabbit polyclonal anti–COX-2, Cell lysis buffer (10×), and Lumiglo chemiluminescent detection kit were from Cell Signaling Biotechnology. RT2 First Strand Kit (C03) and RT2 Profiler PCR Array related to human cell cycle were purchased from SABiosciences. Cell transformation detection assay kit was purchased from Millipore.

Synthesis of indenoisoquinolines

As outlined in Figure 1, scalable conditions were developed for the preparation of AM6-36 (compound 3) and related indenoisoquinolines. Azidopropyl indenoisoquinoline 2 was obtained following previously described procedures (11, 12). We have previously prepared AM6-36 on small scale from compound 1 according to method A. However, this method was found impractical for the preparation of milligram quantities of the target compound. Therefore, an alternative approach for the reduction of compound 2 or conversion of compound 1 to the desired amount of AM6-36 was necessary. After a series of attempts to reduce both nitro and azido groups of compound 2 stepwise using various conditions, the reduction with iron powder in the presence of ammonium chloride solution was found to be the best method (method B). Tosylated analogues of AM6-36 12–15 were prepared by treatment of the corresponding alkoxy derivatives 21–24 with 4-toluenesulfonyl chloride in dichloromethane in the presence of 4-dimethylaminopyridine and triethylamine. Compound 5 was prepared by condensation of 5-nitrohomophthalic anhydride with N-benzylideneethylamidine, followed by treatment of the product with thionyl chloride, intramolecular Friedel–Crafts reaction, and reduction of the nitro group (11). Compounds 17 and 18 were obtained as the products of Sandmeyer reaction of 5. Compounds 6 to 11 and 21 were prepared as previously described (11–13, 14, 15). Melting points were determined using capillary tubes with a Mel-Temp apparatus and are uncorrected. Proton nuclear magnetic resonance (1H NMR) spectra were recorded using ARX300 300 MHz and DRX500 500 MHz Bruker NMR spectrometers. IR spectra were recorded using a Perkin-Elmer 1600 series FTIR spectrometer. Combustion microanalyses were performed at the Purdue University Microanalysis Laboratory and the reported values were within 0.4% of the calculated values. High-performance liquid chromatography (HPLC) analyses were performed on a Waters 1525 binary HPLC pump/Waters 2487 dual λ absorbance detector system. Analytic thin-layer chromatography was carried out on Baker-flex silica gel IB2-F plates and compounds were
visualized with short-wavelength UV light. Silica gel flash chromatography was performed using 230 to 400 mesh silica gel. Physical and spectral data are reported as Supplementary Method S1.

**Cell culture**

All cell lines were grown in media supplemented with penicillin G (100 units/ml), streptomycin (100 µg/ml), and heat-inactivated FBS at 37°C in 5% CO₂ in a humidiﬁed incubator. COS-1 African green monkey kidney ﬁbroblast cells and MCF7 human breast cancer cells were maintained in DMEM containing 10% FBS. JB6 Cl41 mouse epidermal cells were grown in MEM containing 5% FBS. HL-60 human promyelocytic leukemia cells were grown in RPMI 1640 supplemented with 10% FBS. These cell lines were obtained from the American Type Culture Collection (ATCC). Stock cultures were prepared and aliquots were stored in liquid nitrogen. During the course of the study, cells were used below a passage number of 20 and no signiﬁcant changes were noted in morphology or growth characteristics. The cell lines were not authenticated by the authors.

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**Figure 1.** Synthesis of AM6-36 and structurally related indenoisoquinolines.
RXRE-luciferase reporter gene assay

COS-1 cells (1 × 10^6 cells/well) were plated in a 96-well culture plate and incubated for 24 hours. Then, 100 ng of pRXRE, 50 ng of pHREtI, and 3 ng of pRL were transiently cotransfected into COS-1 cells in each well by using Lipofectamine 2000 according to the manufacturer’s protocol. After 24 hours of transfection, cells were treated with compounds and further incubated for 24 hours. Cells were then lysed and the RXRE transcriotional activities were determined by measuring the reporter luciferase activities using the Dual-Luciferase Reporter Assay System.

Evaluation of antiproliferative potential

MCF7 cells (1 × 10^6 cells/well) were seeded in 96-well plates and incubated with compounds at 37°C in a humidified atmosphere with 5% CO₂ for 24, 48, and 72 hours. After the incubation, cell viability was estimated by the SRB cellular protein staining method as previously described (16).

Analysis of cell-cycle distribution

Cell-cycle distribution was assessed by staining DNA content with NIM-DAPI solution according to the manufacturer’s instructions. Briefly, the media was discarded from MCF7 cells after 24, 48, and 72 hours of incubation with various concentrations of AM6-36. Cells were harvested and stained with NIM-DAPI solution just before the measurement using Cell Lab Quanta SC flow cytometer (Beckman Coulter; ref. 17).

Evaluation of protein expression

Cells were lysed by using 1 × cell lysis buffer diluted from 10 × cell lysis buffer according to the manufacturer’s protocol. Cell lysates were centrifuged at 14,000 × g for 10 minutes at 4°C and the resultant supernatants were collected and stored at −80°C until use. After quantification of protein by the Bradford method (18), equal amounts of total protein in each cell lysate were resolved using 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with 5% skimmed milk in 0.1% Tris-buffered saline (TBS) containing Tween 20 (TBST) for 1 hour at room temperature to block nonspecific protein binding. Then, membranes were incubated overnight at 4°C with corresponding primary antibodies in 3% skimmed milk in TBS followed by the incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using a LumiGLO chemiluminescent detection kit under Galiance 1000 imager (Perkin Elmer, Inc.).

Profile of altered gene expression

Total RNA was extracted from cells using Trizol reagent according to the method of Chomczynski and Sacchi (19). Isolated RNA was dissolved in RNase-free water and the quality and quantity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Total RNA (1 μg) was incubated with genomic DNA elimination mixture at 42°C for 5 minutes followed by reverse transcription to cDNA under the condition of 42°C for 15 minutes and 95°C for 5 minutes using an RT² First Strand Kit (C-03) on an ABI 7300 thermocycler (Applied Biosystems Inc.). cDNA was applied to RT² Profiler PCR Array related to human cell-cycle according to a manufacturer’s instructions. Samples were run in quadruplicate to ensure amplification integrity. The expression levels of 84 cell-cycle–related genes in cDNA of each sample were analyzed (20).

siRNA transient transfection

MCF7 cells were plated at 10 × 10^4 cells per well in 6-well culture plates and incubated for 24 hours. Cells in each well were transfected with 50 pmol of human RXRα siRNA, human p53 siRNA, human p21 siRNA, or control siRNA-A using Lipofectamine 2000 for 24 hours followed by sample treatment for additional 24 hours. To examine the expression of RXRα and p21, Western blot analysis was performed as described above.

Expression of COX-2 in TPA-treated JB6 Cl41 cells

JB6 Cl41 cells were seeded in 6-well plates (2 × 10^5 cells/well) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. After incubation, cells were exposed to TPA (10 ng/mL) with vehicle (0.1% dimethylsulfoxide, DMSO) alone or various concentrations of AM6-36. After 15 hours of further incubation, protein of each well was extracted and subjected to Western blot analysis as described in the “Evaluation of protein expression” section.

Anchorage-independent cell transformation

The effect of AM6-36 on TPA-induced neoplastic transformation was investigated with JB6 Cl41 cells cultivated in soft agar according to the manufacturer’s protocol. Briefly, 500 exponentially growing cells were suspended in 50 μL 0.4% agar complete MEM medium over 100 μL 0.8% agar complete MEM medium in a 96-well plate. Cells were exposed to 5 mmol/L TPA, with or without AM6-36, at 37°C in a humidified atmosphere with 5% CO₂ for 28 days. After incubation, cell quantification solution was added to each well and further incubated at 37°C for 4 hours. TPA-treated control wells turned to burgundy-brown. The absorbance was measured at 490 nm and data were expressed as % transformation activity relative to TPA-treated control.

Expression of cell surface marker, CD38

HL-60 cells (2 × 10^5 cells/well) were seeded in 12-well plates and incubated with samples at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. Following the manufacturer’s instructions, cells were harvested and centrifuged at 300 × g for 5 minutes, then washed with phosphate-buffered saline (PBS) once. Cells were incubated with anti–CD38-FITC for 30 minutes in the dark. Similarly, autofluorescence control (cells only) and negative staining control (cells + FITC-conjugated, isotype-matched nonspecific mouse immunoglobulin) were prepared. After the incubation, cells were washed with...
diluents and centrifuged at 500 × g for 10 minutes. Precipitated cells were fixed with 2% cold paraformaldehyde solution and analyzed by flow cytometry.

**Metabolism of AM6-36 by human liver microsomes**

The metabolic stability of AM6-36 was determined using human liver microsomes. Incubations were performed with pooled human liver microsomes containing 0.5 mg/mL of microsomal protein, 10 or 50 μmol/L AM6-36, and 1 mmol/L NADPH in 50 mmol/L phosphate buffer (pH = 7.4), in a total volume of 0.4 mL. The reactions were initiated by addition of NADPH and, after a 5-minute preincubation, were performed at 37°C for 40 minutes. The incubations were terminated by adding 1.6 mL of an ice-cold mixture of acetonitrile-ethanol (1:1, v/v) and processed high resolution liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis as described previously (21).

**Determination of intestinal epithelial monolayer permeability**

To evaluate the absorption of AM6-36 in intestinal epithelial cells, a Caco-2 cell monolayer assay was performed. In preparation for the Caco-2 cell monolayer assays, the cell culture medium was removed from both the apical (AP) and basolateral (BL) chambers. The cells were washed 3 times and preincubated with Hanks’ balanced salt solution (HBSS) containing 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH = 7.4, for 30 minutes at 37°C on a shaker bath at 50 rpm. A 20 mmol/L stock solution of AM6-36 in DMSO was diluted to different concentrations in HBSS/HEPES buffer (the final concentration of DMSO was >1%). These test solutions containing AM6-36 were added to either the apical chambers (for AP → BL measurement) or the basolateral chambers (for BL → AP assay), and blank HBSS/HEPES buffer was added to the other side. As a test of the integrity of the monolayer and as a marker of low permeability, sucrose (50 μmol/L final concentration) was added to the apical chambers. Propranolol (10 μmol/L final concentration) was also added as a marker for compounds that are highly permeable. The total volumes of solution in the apical and basolateral chambers were 1.5 and 2.6 mL, respectively. The solutions in the recipient compartments were removed and replaced with fresh medium. The samples were stored at −20°C until analysis of AM6-36 by LC-MS-MS as described below for the analysis of extracts of serum and tissue. At the end of each experiment, basolateral samples were also analyzed by LC-MS for sucrose and propranolol. The apparent permeability coefficients (Papp, × 10⁻⁶ cm/s ± SD) were then calculated. The Papp of sucrose from the apical to the basolateral side of each well was also measured and remained acceptably low at 0.16 × 10⁻⁶ cm/s (22).

**Evaluation of absorption and distribution in rats**

A preliminary evaluation was performed to assess the absorption and distribution of AM6-36. Female Sprague-Dawley rats were obtained at 4 weeks of age (Harlan, Inc.; virus-free colony 202A) and placed on Teklad diet. At 77 days of age, the rats were treated with AM6-36 [40 mg/kg; 0.5 mL; ethanol:polyethylene glycol (PEG) 400; 10:90, v/v] by gavage, and the treatment was continued on a daily basis for 3 days. Blood samples were collected (jugular vein) 3 hours after the first treatment. Three hours after the final treatment, the animals were sacrificed, and blood, mammary tissue, liver, and perirenal fat were collected. After clotting, the blood samples were centrifuged at 100 × g for 20 minutes. Serum and tissue samples were stored at −80°C until analysis. Rat liver was homogenized in 0.05 mol/L phosphate buffer (pH = 7.4) and mammary gland and perirenal fat tissues were homogenized in a mixture (50:50, v/v) of phosphate buffer and methanol to give homogenates containing 0.2 g tissue per mL. As an internal standard for quantitative analysis, UCN-01 at 1 ng/mL was added to each homogenate and serum sample. Proteins in the homogenized tissue and serum samples were precipitated with 4 volumes of ice-cold acetonitrile. After centrifugation at 10,000 × g for 5 minutes, each supernatant was removed, evaporated to dryness, and reconstituted in 100 μL of methanol/water (50:50; v/v) for analysis using LC-MS-MS (23). The injection volume was 10 μL. Briefly, LC-MS-MS analyses were carried out using a Shimadzu LC-20AD prominence UFLC pump and SIL-20AC HT prominence autosampler interfaced to an Applied Biosystems API 4000 triple quadrupole mass spectrometer. A Waters X Terra MS C18 column (2.1 mm × 100 mm, 3.5 μm) was used for HPLC separation with a 13-minute linear gradient from 10% to 80% acetonitrile in 0.1% aqueous formic acid at a flow rate of 0.25 mL/min. Positive ion electrospray was used for ionization and product ion mass spectra were recorded using collision-induced dissociation and selected reaction monitoring (SRM) of the transition of m/z 320.4 to 303.2 and m/z 483.2 to 130.0 (300 ms per transition) for measurement of AM6-36 and internal standard, respectively. Calibration curves (linear from 0.5–500 ng/mL AM6-36; R² = 0.996) were prepared using blank serum or homogenized tissue from control rats.

**Effect of AM6-36 on MNU-induced mammary tumors**

A preliminary study was performed as described previously (24). Female Sprague Dawley rats at 28 days of age were obtained from Harlan Sprague Dawley and acclimated under the controlled animal quarters maintained at a 12-hour light or 12-hour dark cycle and 22°C ± 2°C. MNU (National Cancer Institute Chemical Carcinogen Repository) was dissolved in sterile-acidified saline (pH = 5.0) and injected via the jugular vein of rats at 50 days of age with a dosage of 75 mg/kg body weight. When an animal developed a palpable mammary tumor (approximately 150–200 mm³), treatment with AM6-36 (10 mg/kg body weight/d; ethanol:PEG 400, 10:90, v/v) or vehicle was initiated (10 rats/group). Over a period of 6 weeks, the largest diameter of the cancer was measured and this value was multiplied by the perpendicular diameter (size expressed as mm²).
Measurements were performed twice a week during the period of the study, the average tumor size in the control and treatment groups were plotted as a function of time, and data were analyzed using the Mann–Whitney test.

**Statistical analyses**

Results are presented as means ± SD. Unless otherwise indicated, statistical comparisons were performed by the use of Student’s t test and the level of P < 0.05 was considered as significantly different.

**Results**

**AM6-36 induced the RXRE transcriptional activity in COS-1 cells and MCF7 cells**

To investigate the effect of samples on the functional role of RXRα protein as a transcriptional activator on RXRE cis-acting element in the promoter region of target gene, an RXRE-luciferase reporter gene assay (RXRE assay) was performed. As described above, ligand-bound RXR homodimer interacts with DR1 and initiates the transcription of target genes. To discover RXR modulators, COS-1 cells were transiently transfected with pRXRE. At the same time, due to the poor expression of endogenous RXRs in COS-1 cells, pRXRα was cotransfected (Fig. 2A). pRL was used as an internal control for normalization. As shown in Figure 2B, 9-cis-RA induced the relative luciferase activity up to about 20-fold in cells cotransfected including pRXRα, whereas there was no marked increase in cells without pRXRα transfection. Therefore, the expression of RXRα is necessary for the RXRE-mediated transcriptional activation by rexinoids, and it is required to enhance the expression of RXRα in COS-1 cell line-based assay.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effect of 9-cis-RA and AM6-36 on RXR transcriptional activity. A, MCF7 cells were transfected with empty vector (control) or pRXRα (pRXRα-TF) for 24 and 48 hours. Total protein lysate (35 µg) of each sample was separated on 12% SDS-PAGE and immunoblotted with anti-RXRα antibody or anti-GAPDH antibody. The mean ± SD of the band density of RXRα relative to internal control GAPDH of each group (n = 3) is shown as a bar graph. Representative Western blot data are presented under the graph. B–D, cells were transiently transfected with plasmids for 24 hours, then, further incubated with 9-cis-RA or AM6-36 for 24 hours. Cells were lysed and dual luciferase activity was measured. Results were expressed as mean ± SD (n = 4). Each mean value was calculated by fold change over control after normalizing ratios of relative light units (RLU) produced by firefly luciferase to RLU produced by Renilla luciferase (firefly Luc/Renilla Luc). B, COS-1 cells cotransfected with pRXRE (100 ng/well) and pRL (5 ng/well), or pRXRE (100 ng/well), pRXRα (50 ng/well), and pRL (7.5 ng/well) were treated with various concentrations of 9-cis-RA. C, COS-1 cells cotransfected with pRXRE (100 ng/well), pRXRα (50 ng/well), and pRL (7.5 ng/well) were treated with various concentrations of 9-cis-RA or AM6-36. D, MCF7 cells cotransfected with pRXRE (100 ng/well) and pRL (5 ng/well) were incubated with the indicated concentrations of 9-cis-RA or AM6-36. *, value of P < 0.05 was considered statistically significant.
Next, we tested several thousand natural products and synthetic compounds. Through the process, only one active lead was found, AM6-36. As illustrated in Figure 2C, AM6-36 greatly induced relative luciferase activities in a dose-dependent manner. Although the compound was less potent than 9-cis-RA, prominent induction was observed at higher concentrations (10 \( \mu \text{mol/L} \); 15 \( \mu \text{mol/L} \), 31.1-fold; 20 \( \mu \text{mol/L} \), 133.2-fold). In comparison, bexarotene, a RXR-specific agonist, increased relative luciferase activities by 13.2 \( \pm \) 2.8-, 16.8 \( \pm \) 2.9-, 25.7 \( \pm \) 3.5-, and 48.9 \( \pm \) 17.6-fold at concentrations of 12.5, 25, 50, and 100 \( \mu \text{mol/L} \), respectively.

On the basis of these data, further studies were performed with MCF7 cells, since RXR\( \alpha \) has been shown to have a role in the inhibition of cell proliferation and induction of apoptosis in breast cancer cells (25). We further determined the effect of AM6-36 on RXR–RXRE transcriptional regulation. In MCF7 cells, it is not necessary to cotransfect phRXR\( \alpha \), owing to the expression of RXRs (25). As shown in Figure 2D, treatment of transiently transfected MCF7 cells with 9-cis-RA increased the relative luciferase activity, in a dose-dependent manner, with 3.5-, 3.7-, and 6.1-fold induction at 40, 120, and 400 \( \mu \text{mol/L} \), respectively. Likewise, AM6-36 induced relative luciferase activities by 1.5-, 3.2-, and 16.8-fold at 312.5, 625, and 1,250 \( \mu \text{mol/L} \), respectively. Therefore, with MCF7 cells, AM6-36 clearly increased RXR transcriptional activity. Nonetheless, to confirm the effect of AM6-36 on RXR protein–RXRE DNA sequence binding, an electrophoretic mobility shift assay was performed, similar to 9-cis-RA, AM6-36 enhanced the binding of RXRs to RXRE in MCF7 cells (Supplementary Fig. S2).

Induction of RXRE transcriptional activity and inhibition of MCF7 cell proliferation by AM6-36 derivatives

On the basis of the response observed with AM6-36, additional indenoisouquinolines were assessed to determine if a superior lead could be easily generated, and to learn something about structure–activity relationships. As summarized in Table 1, a small collection of structural derivatives were evaluated in the RXRE assay with COS-1 cells and the SRB assay with MCF7 cells. AM6-36 mediated the strongest induction of RXRE transcriptional activity, as well as the strongest growth inhibitory effect with MCF7 cells. The only other test compounds showing enhanced transcriptional activity were 5 and 6, and these responses correlated with growth inhibition. In detail, compound 5 substituted with an amino group at R\( \beta \) and a methyl group at the R\( \beta \) position and compound 6 substituted with a nitro group at R\( \beta \) and a dimethylaminopropyl group at R\( \beta \), exhibited 3.0- and 3.3-fold induction, respectively. Even though compounds 5 and 6 were less active than AM6-36, they were capable of exerting inductive effects on RXRE activity in a dose-dependent manner (data not shown). Along with RXRE activity, compounds 5 and 6 showed significant antiproliferative effects with MCF7 cells, yielding IC\( _{50} \) values of 0.47 and 0.34 \( \mu \text{mol/L} \), respectively.

AM6-36 inhibited proliferation in MCF7 cells by cell-cycle arrest in the G\( _{2}/\text{M} \) phase at lower concentrations and S phase at higher concentrations concomitant with increased expression of CDKN1A

The potential of AM6-36 to inhibit the proliferation of MCF7 cells was examined. As shown in Figure 3A, vehicle (0.1% DMSO)-treated control cells exhibited over a 4-fold increase in cell proliferation, compared with that of starting time point (0 hours). However, in the presence of AM6-36, MCF7 cell proliferation was reduced in a concentration-dependent manner, yielding IC\( _{50} \) values of 1.09, 0.85, and 0.42 \( \mu \text{mol/L} \) at 24, 48, and 72 hours, respectively. At these respective incubation times, bexarotene inhibited the cell proliferation with IC\( _{50} \) values of 82.3, 55.2, and 43.4 \( \mu \text{mol/L} \).

To better understand the antiproliferative effect of AM6-36, DNA content and cell-cycle distribution were analyzed by flow cytometric analysis. As shown in Figure 3B, treatment with 0.625 \( \mu \text{mol/L} \) AM6-36 caused cells to accumulate in the G\( _{2}/\text{M} \) phase, whereas treatment with higher concentrations, such as 2.5 \( \mu \text{mol/L} \), induced S-phase arrest. This was confirmed by treatment with 0.3125 \( \mu \text{mol/L} \) or 2.5 \( \mu \text{mol/L} \) AM6-36 for 24 hours, as shown in Figure 3C. To further elucidate the mechanism underlying the antiproliferation and cell-cycle arrest induced by AM6-36, a cDNA microarray was employed. For this analysis, MCF7 cells were incubated in the presence or absence of AM6-36 (1 \( \mu \text{mol/L} \); IC\( _{50} \) value after 24 hours of treatment), then RNA was isolated and reverse transcribed to cDNA. The same amount of cDNA was subjected to the microarray plate for real-time PCR reaction. The results were analyzed and genes which were up- or downregulated with a value of \( P<0.05 \) in comparison with control were considered as significantly altered by the treatment (Supplementary Table S1). As shown in Figure 3D, the expression of 26 of 84 genes was altered by treatment of 1 \( \mu \text{mol/L} \) AM6-36. Specifically, the expression of 7 genes, including CCG2, CDC34, CDK5RAP1, CDKN1A, GADD45A, RAD1, and SERTAD1, were significantly increased, whereas those of 19 genes, including BIRC5, BRC2A2, CCNB2, CCNF, CDC2, CDC20, CDK2, CDKN3, CHEK1, DDX11, GTSE1, MAD2L1, MCM2, MCM3, MCM5, MKI67, PCNA, RBL1, and SKP2, were significantly downregulated. Among these, CDKN1A and MKI67 were most highly up- and downregulated, respectively.

Bexarotene (100 \( \mu \text{mol/L} \); approximate IC\( _{50} \) value after 24 hours of treatment) was tested in parallel experiments. Consequently, the expression of 9 of 84 genes was altered significantly (upregulated genes: CDKN1A, CDKN2B, and GADD45A; downregulated genes: BCL2, BIRC5, CDK2, GTSE1, MCM2, and PCNA). In particular, BIRC5, CDK4, CDKN1A, GADD45A, GTSE1, MCM2, and PCNA were altered by both AM6-36 and bexarotene. Of the 2 genes
### Table 1. Effects of indenoisoquinolines in RXRE assay and cell survival

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>R&lt;sub&gt;4&lt;/sub&gt;</th>
<th>Biological activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00 ± 0.20</td>
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<tr>
<td>1</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>H</td>
<td>H</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>H</td>
<td>H</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>3</td>
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<td></td>
<td>H</td>
<td>H</td>
<td>5.49 ± 0.34</td>
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<tr>
<td>4</td>
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<td>H</td>
<td>0.88 ± 0.15</td>
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<td>H</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>H</td>
<td>H</td>
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<td>9</td>
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<td>F</td>
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<tr>
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<td></td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H</td>
<td>0.93 ± 0.54</td>
</tr>
<tr>
<td>18</td>
<td>I</td>
<td></td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H</td>
<td>0.59 ± 0.47</td>
</tr>
</tbody>
</table>

9-cis-RA<sup>a</sup> 14.61 ± 2.10
Bexarotene<sup>b</sup> 48.91 ± 17.55
Camptothecin<sup>c</sup> 1.41 (nmol/L)

<sup>a</sup>COS-1 cells were transiently transfected with pRXRE, pRXRα, and pRL for 24 hours. Then cells were treated with 40 μmol/L indenoisoquinolines (compounds 1-18) for 24 hours and subjected to the dual luciferase assay. Results were expressed as relative values calculated by fold increase over vehicle (0.5% DMSO)-treated controls after normalizing ratios of relative light units (RLU) produced by firefly lucerase activity to RLU produced by R. reniformis lucerase activity (firefly Luc/Renilla Luc).

<sup>b</sup>MCF7 breast cancer cells were diluted at a density of 5 × 10⁴ cells/mL and 190 μL of cell suspension were plated into each well of 96-well cell culture plates which contained 10 μL of diluted compounds and incubated for 72 hours. Cell proliferation was assessed by the SRB assay and the percentage of survival of sample-treated cells relative to vehicle (0.5% DMSO)-treated control cells was calculated. Results were presented as IC₅₀ (μmol/L) values.

<sup>c</sup>9-cis-RA (100 nmol/L) and bexarotene (100 μmol/L) were used as positive controls in the RXRE assay.

<sup>d</sup>Camptothecin was used as a positive control in the SRB assay.
Figure 3. Induction of RXRE-luciferase activity and inhibition of cell proliferation accompanied with cell-cycle arrest by indenoisoquinolines accompanied by significant changes in cell-cycle regulatory genes by AM6-36 treatment in MCF7 breast cancer cells. A, the proliferative state of cells was evaluated with the SRB assay. Cells were incubated for 24, 48, and 72 hours. Results are presented as fold increases of absorbance at 515 nm over control at a starting point (0 hour). B and C, MCF7 cells were seeded at a density of 5000 cells in a 10-cm cell culture dish and incubated for 48 hours. After incubation with AM6-36 for each time period, 10,000 of cells were analyzed for DNA content and the distribution of cells in each phase was estimated by using ModFit LT (Verity Software House). D, MCF7 cells were seeded at a density of 5000 cells in a 10-cm cell culture dish and incubated for 48 hours. Cells were treated with vehicle (DMSO) or serially diluted AM6-36 for 24 hours. RNA was extracted from cells and reverse transcribed to cDNA. Then, cDNA was mixed with RT² qPCR Master Mix and 25 µL were placed in each well of the array plate. Real-time quantitative PCR was performed using SyBR Green detection system. The thermal cycling condition was at 95°C for 10 minutes and then 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. On the basis of the DD_Ct method, the fold changes between the control group and sample-treated groups were analyzed using the Web-based software provided by SABiosciences (RT² Profiler PCR Array Data Analysis). Fold changes of 84 genes induced by AM6-36 or bexarotene were determined and presented as heatmaps (Red: upregulation, green: downregulation).
significantly altered by bexarotene but not AM6-36 (BCL2, downregulated; CDKN2B, upregulated). There was clearly a similar trend of modulation by both compounds. Of the 14 genes significantly downregulated by AM6-36 but not bexarotene, 9 were also downregulated by bexarotene at the \( P \leq 0.083 \) level.

Supplementary Figure S1 illustrates the connectivity of the main genes used in this array that were affected by AM6-36 or bexarotene. Notably, expression levels of several genes, which are transcriptionally activated by p53 (the protein of the TP53 gene), were significantly upregulated by AM6-36 and bexarotene. Prominent examples are CDKN1A (\( P = 0.00029, \) AM6-36; \( P = 0.00014, \) bexarotene) and GADD45A (\( P = 0.00031, \) AM6-36; \( P = 0.00011, \) bexarotene).

**p53 and p21 were increased after AM6-36 treatment and RXRα protein knockdown by RXRα siRNA abrogated the effect of AM6-36 on the induction of p21 expression**

Although the expression of TP53 was not modulated by AM6-36 with MCF7 cells, the potential effect on protein level was examined by Western blot analyses. As shown in Figure 4A, treatment with AM6-36 caused an increase of p53 at 8 hours, followed by decreases at 24 and 48 hours. Even though the protein level of p53 was diminished at later time points, it still remained at higher levels than the control. Also, since CDKN1A levels were most highly elevated by treatment with AM6-36, its protein (p21\(^{CIP1/WAF1} \)) levels were determined. Expression of p21 in control cells was low at all time points, whereas treatment of AM6-36 caused the moderate increase at 8 hours, and incremental expression was observed at 24 and 48 hours. To establish a direct correlation between the transcriptional activity of RXRα and the induction of p21 expression, the expression of RXRα protein was transiently depleted in MCF7 cells by introducing RXRα siRNA into the cells. As shown in Figure 4B, siRNA targeted to RXRα markedly reduced RXRα expression, and consequently affected the upregulating effect of AM6-36 on the protein expression of p21. Also, transfection of p53 siRNA resulted in the abrogation of the p21 expression induced by AM6-36 (Fig. 4C). However, p53 knockdown did not influence the expression of RXRα (Fig. 4D). These data suggested that AM6-36 was not able to induce p21 expression in the absence of RXRα or p53 (i.e., RXRα and p53 are required for AM6-36–mediated mRNA upregulation of p21).

**Effects of AM6-36 on JB6 Cl41 cells**

To begin assessment of the chemopreventive potential of AM6-36, studies were performed with TPA-treated JB6 Cl41 cells. As shown in Figure 5A, the expression of COX-2 was induced after exposure to TPA (10 ng/mL) for 15 hours. In contrast to control, however, cotreatment with AM6-36 attenuated COX-2 protein expression in a concentration-dependent manner. Next, anchorage-independent cell transformation assay was performed. After 28 days of incubation in the presence or absence of AM6-36, cells were stained and quantified. As shown in Figure 5B, as judged by growth in soft agar, treatment with AM6-36 reduced cell transformation by approximately 70%.

**Expression of cell surface marker, CD38**

As an additional test of chemopreventive potential, the ability of AM6-36 to affect the expression of CD38 was assessed with HL-60 cells. As shown in Figure 5C, with this model, treatment with 9-cis-RA can induce the expression of CD38 in about 95% of the cell population at higher concentrations (2,000, 200, and 20 nmol/L). At a concentration of 2 nmol/L, the 9-cis-RA–treated cell population moved to the left with a decreased percentage of FL-1–positive cells (86.7%), and at 0.2 nmol/L, further reduction in expression was observed. When treated with 2,000 or 200 nmol/L, AM6-36, cells exhibited a 72% or 58% FL-1–positive response (CD38-positive), respectively; although, the fluorescence intensity was lower in comparison with 2,000 nmol/L 9-cis-RA–treated cells.

**Evaluation of AM6-36 permeability and metabolic stability**

Judged by the Caco-2 cell permeability model, the apparent permeability coefficient for AM6-36 in the AP → BL, 2.75 × 10\(^{-6}\) cm/second, was found to be 10-fold slower than that of the high permeability standard, propranolol, but 17-fold faster than the low permeability standard, sucrose (Table 2). These measurements indicate that the intestinal absorption of AM6-36 should proceed at a moderate rate following oral administration. Since the apparent permeability coefficients in the AP → BL and in the BL → AP were not significantly different, AM6-36 does not appear to be a substrate for efflux proteins that might reduce its bioavailability. During incubation of AM6-36 with human liver microsomes, 1 abundant and 3 minor metabolites were observed. In the absence of either NADPH or microsomes, no metabolites were detected. Therefore, we assume that all 4 metabolites are produced in a cytochrome P450- and NADPH-dependent manner. The structures of these metabolites are currently under investigation and will be reported later.

**Preliminary studies conducted with rats**

To assess absorption and metabolism, AM6-36 (40 mg/kg body weight) was administered to rats, by gavage, over a period of 3 days. Blood was collected on days 1 and 3, and tissues were collected at the end of the study. As summarized in Table 3, the concentration of AM6-36 in the rat serum was approximately 0.83 μg/mL. The concentration in liver (4.28 μg/g) was around 5 times higher. Applicable quantities were also found in the mammary gland (0.29 μg/g) and perirenal fat (0.28 μg/g). These data are consistent with moderate oral absorption and slow metabolism/excretion.

Finally, to obtain an early indication of chemopreventive potential, a short-term mammary tumor model was used.
Figure 4. Increased protein expression of p53 and p21 by AM6-36, and altered expression of p21 by knockdown of RXRα or p53 in MCF7 cells. A, MCF7 cells were seeded at a density of 10^4 cells in 6-well culture plates and incubated for 24 hours. Cells were treated with 1 µmol/L AM6-36 for the indicated time points. Cell lysates were prepared and subjected to Western blot analysis. B–D, after transient transfection with the indicated siRNAs, MCF7 cells were treated with vehicle (0.1% DMSO), AM6-36 (1 µmol/L, B and C; 0.625, 1, or 1.25 µmol/L, D) or 9-cis-RA (1 µmol/L, B and C) for an additional 24 hours. Cell lysates were prepared and subjected to Western blot analysis.
As shown in Figure 5D, at a dose of 10 mg/kg body weight per day, AM6-36 clearly attenuated tumor growth ($P = 0.1$). It will now be necessary to evaluate larger numbers of animals with various treatment regimens to establish statistical significance, but the trend demonstrates promising antitumor activity.

**Discussion**

RXR agonists have shown promise for the treatment or prevention of cancer. 9-cis-RA and LGD1069 are key examples of agents in clinical use. In addition, unlike other nuclear receptors, RXRs are expressed in human carcinomas and overexpression of RXRα has enhanced the transcriptional response after binding of ligands (26). On the basis of these considerations, we introduced a cell line–based RXRE-luciferase reporter gene assay to evaluate the RXRα transcriptional activity of various test substances. As the result of an extensive search, AM6-36 was found to be active. Since the COS-1 cells used for this discovery were transiently transfected with pRXRE and pHRXRα, a response is expected to be due to the interaction of a test substance with RXR–RXR homodimers. Further investigation is required to determine the potential of any lead, including AM6-36, to interact with other nuclear receptors, such as RXR–PPAR (27). The goal of this work, however, was to investigate the biological ramifications of the highly unique response mediated by AM6-36 with this model system.

Indenoisoquinolines were first synthesized in 1978 (28) and studied as topoisomerase I inhibitors with advantageous properties compared with camptothecins, known as topoisomerase I inhibitors. Additional activities are also known, such as induction of cell-cycle arrest, overcoming...
could be further enhanced. As shown in Table 1, relatives were evaluated to determine whether activity response induced by AM6-36, a small group of structural ferase activity was highly upregulated. On the basis of the reaction (data not shown), but unexpectedly, RXRE-lucmediated only modest inhibition of the DNA religation induction-leading activity of retinoids (29–31). AM6-36 multidrug-resistance, and enhancement of the differentiation-inducing activity of retinoids (29–31). AM6-36 mediated only modest inhibition of the DNA religation reaction (data not shown), but unexpectedly, RXRE-luciferase activity was highly upregulated. On the basis of the response induced by AM6-36, a small group of structural relatives were evaluated to determine whether activity could be further enhanced. As shown in Table 1, compounds 5 and 6 induced the RXR transcriptional activities as well as antiproliferative effects toward MCF7 cells, but AM6-36 was still the most active compound.

The indenoisoquinoline AM6-36 is an atypical rexinoid since it does not contain a carboxylic acid and it does not structurally resemble a retinoic acid. In comparison with known rexinoids, most of which contain carboxylic acids (32–34), the indenoisoquinoline rexinoids constitute a fundamentally different type of RXR ligand. The fact that indenoisoquinolines are structurally unique rexinoids offers the possibility of them having unique pharmacologic properties that could be clinically useful. Considering these factors as a whole, AM6-36 was selected for further evaluation.

As shown in Figure 1, a synthetic procedure was devised that is suitable for production of the lead compound in milligram quantities. Since RXR agonists showed apoptotic effects in RXRα-expressed MCF7 cells (25), we examined the effect of AM6-36 using this cell line. As expected, when MCF7 cells were treated with AM6-36, we observed increased RXRα transcriptional activity, which correlated with the response observed with transiently transfected COS-1 cells. All of these data suggest that MCF7 cells should be a useful model for exploring the action of AM6-36 related to RXRα. Cell-cycle analysis revealed G2/M arrest at lower concentrations and S-phase arrest at higher concentrations (Fig. 4C). A similar dose-related dual effect has been reported when MCF7 cells were treated with adriamycin (35), and other DNA damaging agents showed partial G1- and G2/M-phase cell-cycle arrest at lower doses, whereas they exerted partial or total S-phase cell-cycle arrest at higher doses (36). Analogously, the response induced by lower doses of AM6-36 may be related to effects on DNA.

Together with significant growth inhibition, genes known as conventional proliferation markers and used in the diagnosis of breast cancer, including MKI67 and PCNA (37), were downregulated by treatment with AM6-36. However, a known target gene of RXR, CDKN1A (38), was the most intensively upregulated. Correspondingly, p21 protein levels were increased (Fig. 4B). Treatment of cells with retinoic acid can lead to elevated p21 levels (39), as well as agents such as 2-[3-(2,3-dichlorophenoxy)propyl]amino]ethanol (40) and IFN-α (41). Interestingly, similar to AM6-36, these agents arrest cells in the S phase of the cycle. In the RXRE assay, AM6-36 showed higher induction in RXRα transcriptional activity than 9-cis-RA. The response may be augmented by enhanced expression of CADD45A, a RXRa coactivator (42). It is further noted from the DNA microarray that SKIP2 is greatly downregulated by treatment with AM6-36. This might give rise to the

Table 2. Intestinal permeability, metabolic stability, and distribution of AM6-36: apparent permeability coefficients of AM6-36 through Caco-2 monolayers

<table>
<thead>
<tr>
<th>AM6-36, µmol/L</th>
<th>P_app (×10⁻⁶ cm/s ± SD)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.75 ± 0.78</td>
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<tr>
<td>10</td>
<td>5.52 ± 0.29</td>
</tr>
<tr>
<td>25</td>
<td>4.45 ± 0.71</td>
</tr>
</tbody>
</table>

²P_app corresponds to the apparent permeability coefficients and is expressed as cm/s (×10⁻⁶).

Table 3. Intestinal permeability, metabolic stability, and distribution of AM6-36 in the rat

<table>
<thead>
<tr>
<th>AM6-36 (40 mg/kg)</th>
<th>Serum 1 d, µg/mL (n = 3)</th>
<th>Serum 3 d, µg/mL (n = 3)</th>
<th>Liver, µg/g (n = 3)</th>
<th>Mammary gland, µg/g (pooled n = 3)</th>
<th>Fat, µg/g (pooled n = 3)</th>
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<tbody>
<tr>
<td>Rat 1</td>
<td>0.84 ± 0.008 (2.14 µmol/L)</td>
<td>0.82 ± 0.003 (2.09 µmol/L)</td>
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<td>–</td>
</tr>
<tr>
<td>Rat 2</td>
<td>0.84 ± 0.01 (2.14 µmol/L)</td>
<td>0.84 ± 0.007 (2.14 µmol/L)</td>
<td>5.03 ± 0.23</td>
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<td>–</td>
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<tr>
<td>Rat 3</td>
<td>0.82 ± 0.004 (2.09 µmol/L)</td>
<td>0.82 ± 0.002 (2.09 µmol/L)</td>
<td>3.75 ± 0.13</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Average</td>
<td>0.83 ± 0.01 (2.12 µmol/L)</td>
<td>0.83 ± 0.01 (2.12 µmol/L)</td>
<td>4.28 ± 0.65</td>
<td>0.29 ± 0.006</td>
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</tr>
</tbody>
</table>

²See text for experimental details.
increased protein expression of p21, since Skp2 is involved in the degradation of p21 (43).

The role of p53 in these processes remains to be further explored. Although p53 gene expression was not increased by AM6-36, other effects, such as modified protein expression and transcriptional activity, may come into play. In addition to upregulation of CDKN1A, other genes associated with p53 transcriptional activity (e.g., GADD45A, CCNG2; ref. 44) showed increases in expression to some extent. Furthermore, the expression of GTSE1, which has been reported to downregulate the level and activity of the p53 tumor suppressor protein (45), was decreased by AM6-36. Thus, although additional studies are warranted, this might explain the increase in p53 protein level.

In any case, enhanced RXRα activity leading to highly elevated expression of p21, an inhibitor of CDKs which negatively regulates cell-cycle progression (46), appears to play a dominant role in the cellular responses orchestrated by AM6-36 treatment. A direct relationship between the activation of RXRα and p21 expression was further established by transiently blocking the expression of RXRα. In the absence of RXRα, the expression of p21 was abrogated (Fig. 4B). The crystal structure of AM6-36 bound to RXRα has yet to be determined, but a hypothetical structure was generated by GOLD (47) docking the indenoisoquinoline into the X-ray structure of RXR-α (1FBY) (48) using the centroid (15.2492, 28.4959, 48.2618) of bound 9-cis-RA. As illustrated in Figure 6, AM6-36 can readily interact with the binding site of 9-cis-RA. The ligand pose with the highest GOLD score and the most favorable binding energy was used for further calculations. The energy of the ligand and a surrounding spherical subset of atoms having a radius of 6 Å was minimized by the conjugate gradient method using the MMFF94s force field and MMFF94 charges (SYBYL 8.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA). The calculation was terminated at a gradient of 0.05 kcal/(mol·Å).

Rexinoids are of interest due to cancer chemopreventive potential (6–8), and, of course, a question of utmost importance is the chemopreventive potential of AM6-36. Some early studies have been completed. As normal cells undergo neoplastic transformation, they gain the ability to demonstrate “anchorage independence” and grow in soft agar (49). Currently, we demonstrated the potential of AM6-36 to suppress TPA-induced neoplastic transformation of JB6 Cl41 cells. Furthermore, during neoplastic transformation stimulated by TPA, JB6 Cl41 cells express...
COX-2, which plays pivotal roles not only in inflammation but also in carcinogenesis. AM6-36 inhibits the expression of COX-2 in this model system.

An additional strategy for cancer chemoprevention is the induction of differentiation (50). All-trans-retinoic acid (ATRA) is known to induce CD38 expression through activation of RAR and RXR in myeloid progenitor cells, which modulates cell differentiation (51, 52). As a further induction of cancer chemopreventive potential, we currently demonstrated AM6-36–treated HL-60 cells express CD38 on membrane surfaces.

Taken together, these data suggest the potential of AM6-36 to serve as a chemopreventive agent. Preliminary results were also obtained with the Caco-2 cell permeability model, suggesting that the compound would be orally absorbed. In addition, incubation with human liver microsomes indicated moderate metabolism. These data were corroborated by studies involving oral administration with rats. Serum and tissue levels were readily achieved that should be of physiologic relevance, based on in vitro response data. In support of this notion, a preliminary evaluation demonstrated attenuation of MNU-induced tumorgenesis in female Sprague Dawley rats. Clearly, additional work is required to assess the effect of various dose regimens with larger numbers of animals representative of different tumor models, but this is completely feasible, since AM6-36 can readily be produced on a multigram scale. AM6-36 is a surprising and unique molecule, representing a promising lead that functions through a novel mechanism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study was supported by NIH under NCI grants P01 CA48112 and U01 CA88566. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 12, 2010; revised January 10, 2011; accepted January 18, 2011; published online April 4, 2011.

References


