Potent Cytotoxic Effects of Novel Retinamide Derivatives in Ovarian Cancer Cells

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Retinoids act as potential chemopreventive and chemotherapeutic agents by inhibiting uncontrolled cell growth, inducing apoptosis, and promoting the differentiation of cancer cells.1–3 Natural retinoids, such as all-trans retinoic acid (AtRA) and its isomer 9-cis RA, are physiologically metabolites. RA-bound receptors, RAR and RXR, associate with cis-acting RA response elements (RARE) and stimulate the transcription of the responsive genes.4 In addition to receptor activation, RA interferes with the transactivation function of activation protein-1 (AP-1), which is mostly composed of c-Jun and c-Fos. The RA-dependent interference of AP-1 may inhibit the expression of the matrix metalloproteinases collagenase (MMP-1) and stromelysin (MMP-3), the expression of the cytokines IL-1 and TGF-β, and the expression of the HPV-16/18 oncogenes E6 and E7. These functions make RA a promising therapeutic agent for the treatment of tumor invasion, inflammation, and skin aging.5–7

However, RA is frequently teratogenic and causes severe side effects in tissues of the skin, blood vessels, liver, nerves, and bone when large doses are used clinically.8 Thus, the toxicity associated with natural retinoids limits their clinical use. This has led to the development of novel RA derivatives that are currently being tested to evaluate their clinical effectiveness.9 One of the newly developed RA derivatives, 4-hydroxyphenyl retinamide (4-HPR), has demonstrated increased anticancer activity associated with a favorable toxicity profile.10 It has been reported that 4-HPR is cytotoxic against a wide variety of cancer cell lines, including ovarian cancer cells in vitro,11,12 and is effective in reducing the sizes of breast, prostate, and ovarian cancer tumors in animal models.13–16 Currently, 4-HPR is in clinical trials for the treatment of ovarian, breast, bladder, prostate, and lung cancers.17–21 However, the plasma levels in patients receiving 200 mg of 4-HPR daily are under 1 μM, which is far less than the effective concentration (usually 10 μM) required to induce apoptosis in vitro.22 The higher doses of 4-HPR that appear to be required may cause skin irritation and tissue injury. Derivatives with less irritating side effects and better clinical efficacy are needed. Recently, several retinamide derivatives were identified that were effective in inhibiting growth and inducing the apoptosis of several human cancer cells.23,24 These derivatives bear hydroxyl, carboxy, or methoxy substitutions on the terminal phenylamine ring. Of the derivatives developed, 3-HPR showed the most active growth inhibition in the four bladder cancer cell lines,23 and 2-HPR was the most effective in some head and neck cancer and lung cancer cell lines.24

In our studies, we synthesized a series of fatty acid derivatives of 4-HPR by the addition of acetate (compound 1), propionate (2), pyruvate (3), butyrate (4), or stearate (5) to the 4-hydroxyphenyl moiety of 4-HPR. In our initial proliferation assays, we identified compound 3 as the most cytotoxic of the series against four ovarian cancer cell lines (OVCAR-3, PA-1, 2774, and SKOV-3). Dose–response curves yielded IC50 values of 3.75–7.75 μM for AtRA, 2.80–5.50 μM for 9-cis RA, 0.65–4.05 μM for 4-HPR, and 0.25–0.75 μM for compound 3, depending on the cell type treated. Nuclear staining with 4’-6-diamidino-2-phenylindole (DAPI) and DNA fragmentation assays clearly indicated that the antiproliferative effect of compound 3 was mediated by apoptosis. In contrast to natural retinoids, both 4-HPR and compound 3 activated two (RARβ and RARγ) of the three retinoic acid receptor (RAR) subtypes tested, but did not activate any of the three retinoid X receptors (RXRs), as determined by transcription assays in OVCAR-3 cells. However, like natural retinoids, 4-HPR and compound 3 actively suppressed c-Jun transcriptional activity. Thus, compound 3 not only showed more potent antiproliferative activity than any other retinoid derivatives tested, but also effectively inhibited the c-Jun activity that has been implicated in tumor promotion and invasion. These results, together with compound 3’s selectivity for RAR subtypes, suggest that compound 3 could be an effective anticancer drug for ovarian cancer, with less toxicity than RA.

Key words retinamide derivative; 4-HPR; RAR; c-Jun; ovarian cancer; apoptosis
We synthesized a series of 4-HPR derivatives by the addition of acetate (compound 1), propanoate (2), and propionate (3), butyrate (4), or stearate (5) to the 4-hydroxyphenyl moiety of 4-HPR. All of the products prepared were purified by flash column chromatography on silica gel 60 (Merck, 230—400 mesh). Melting points (mp) were as determined and are uncorrected, using a Buchi 510 capillary apparatus with a system of measurement and temperature control. The 1H- and 13C-NMR spectra were recorded on a JEOL JNM EX-400 using CDCl3 as the solvent. All chemical shifts (δ) are quoted in ppm downfield from TMS, and the coupling constants (J) are given in Hz. Mass spectra were measured on a Shimadzu GCMS-PO 1000 mass spectrometer (EI 70 eV).

**CHEMISTRY**

We synthesized a series of 4-HPR derivatives by the addition of acetate (compound 1), propanoate (2), pyruvate (3), butyrate (4), or stearate (5) to the 4-hydroxyphenyl moiety of 4-HPR. All of the products prepared were purified by flash column chromatography on silica gel 60 (Merck, 230—400 mesh). Melting points (mp) were as determined and are uncorrected, using a Buchi 510 capillary apparatus with a system of measurement and temperature control. The 1H- and 13C-NMR spectra were recorded on a JEOL JNM EX-400 using CDCl3 as the solvent. All chemical shifts (δ) are quoted in ppm downfield from TMS, and the coupling constants (J) are given in Hz. Mass spectra were measured on a Shimadzu GCMS-PO 1000 mass spectrometer (EI 70 eV). All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), if not otherwise specified.

**Synthesis of Fatty Acid Derivatives of 4-HPR**

4-HPR was prepared by coupling all-trans retinoic acid (ATRA) with aminophenol, as described previously.25) To prepare an acetate derivative of 4-HPR, acetic acid (28 μl, 0.51 mmol) was activated in a solution of DCC (105 mg, 0.51 mmol) in dry CH2Cl2 (5 ml). The solution was stirred at room temperature for 0.5 h. To this mixture was then added 4-HPR (100 mg, 0.25 mmol) in dry DMF (2 ml), and DMAP (cat.); the mixture was stirred for 0.5 h. To this mixture was then added 4-HPR (100 mg, 0.25 mmol) in dry DMF (2 ml), and DMAP (cat.); the mixture was stirred for 4 to 5 h. The reaction was quenched with NaH2PO4 (aq.) and extracted with EtOAc (30 ml). The extract was washed with H2O and brine, dried (Na2SO4), and evaporated. The crude product was chromatographed on a silica gel column and eluted with EtOAc–hexane (1:4) to give fine yellow solids. Other derivatives were similarly prepared.

4-{{2,4,6,8-Et4}[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)nona-2,4,6,8-tetraenoylamino]}-phenyl Acetate (1): Yield = 82%, mp = 166—169 °C. 14H-NMR (400 MHz, CDCl3): δ 1.01 (6H, s), 1.45 (2H, m), 1.60 (2H, m), 1.98 (5H, br s), 2.43 (3H, s), 2.59 (s, 3H), 5.78 (1H, s), 6.23 (4H, m), 6.97 (1H, dd, J = 15.1, 11.2 Hz), 7.00 (2H, d, J = 8.8 Hz), 7.33 (1H, s), NH, 7.52 (2H, d, J = 8.7 Hz). 13C-NMR (100 MHz, CDCl3): δ 13.02, 13.83, 19.32, 21.20, 21.84, 29.04, 33.18, 34.33, 39.65, 120.47, 120.49, 120.52, 120.94, 121.81, 128.44, 129.38, 129.82, 130.40, 135.09, 135.87, 137.12, 137.58, 139.22, 150.69, 169.49. MS: m/z (%) = 434 (100, M+), 282 (20).

UV = λmax (dimethyl sulfoxide (DMSO)) nm (ε): 368.

**Cells and Cell Culture**

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The ovarian cancer cell lines used in our studies are OVCAR-3, PA-1, 2774, and SKOV-3, as described previously.30) These cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) that was previously inactivated at 56°C for 20 min. In transfection experiments, OVCAR-3 cells were grown in DMEM supplemented with charcoal-treated 10% fetal bovine serum (FBS) (HyClone).
mocytometer, and was expressed as the mean value of at least four wells. To determine IC50 values, i.e., the concentrations required for 50% growth inhibition, cells were treated with different concentrations of AtRA, 9-cisRA, 13-cisRA, 4-HPR, or compound 3 (0, 0.01, 0.1, 0.5, 1, 5 μM), and cell numbers were counted after 4 d.

4',6-Diamidino-2-phenylindole (DAPI) Staining Nuclear staining with DAPI was performed as described. Single cell suspensions of treated OVCAR-3 cells were washed with 1× phosphate-buffered saline (PBS), fixed with 70% ethanol for 20 min at room temperature, and washed again with 1× PBS. Cells were then treated with DAPI (1 mg/ml) at a 1 : 1000 dilution, incubated for 12 min, and washed again with 1× PBS for 5 min. After treatment with 50 μl VectaShield (Vector Laboratories, Burlingame, CA, U.S.A.), stained nuclei were visualized under a fluorescence microscope (Provis AX70: Olympus Optical Co., Japan). Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation.

DNA Fragmentation Assay The DNA fragmentation assay, an indication of apoptosis, was performed as described previously. DNA extracts were prepared from OVCAR-3 cells (2×105 cells) that had been treated with AtRA, 4-HPR, or compound 3 at concentrations of 0.01, 0.1, 0.5, and 1 μM for 4 d; the amount of DNA extracted was determined spectrophotometrically. Following electrophoresis on 1.8% agarose gels, DNA was stained with ethidium bromide, and DNA ladders were visualized under UV light.

Transcriptional Activity of RAR/RXR and AP-1 Cells were transfected with a liposome-based method using Lipofectamine (GibcoBRL, Gaithersburg, MD, U.S.A.), as previously reported. Briefly, OVCAR-3 cells (1×106 cells), maintained in DMEM supplemented with charcoal-treated 10% FBS, were plated in 60 mm dishes 5 h before transfection. After overnight transfection with the indicated reporter and expression plasmids (DR5-tk-chloramphenicol acetyl transferase (CAT) for RARs, DR1-tk-CAT for RXRs, and Coll-CAT for AP-1), cells were washed, fed with the complete medium supplemented with 10 nM of AtRA, 9-cisRA, 4-HPR, or compound 3, as indicated in the text, and further incubated for 24 h. Cell extracts were then prepared and β-galactosidase activity was determined for the normalization of transfections. Clear lysates were tested for CAT concentration using the CAT enzyme-linked immunoabsorbent assay (ELISA) according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany). All the data presented in the text represent the means of at least three independent transfections.

Statistical Analysis All experiments were performed in triplicate unless otherwise noted. Results were expressed as the mean±S.D. Statistical significance (p<0.05) was assessed by Student’s t-test.

RESULTS AND DISCUSSION

As extensively reported, 4-HPR is cytotoxic against a variety of cancer cell lines, including ovarian cancer cells in vitro, and is effective in reducing the tumor sizes of certain cancers in animal models. It is currently in clinical trials for the treatment of several cancers. However, the low plasma levels of 4-HPR available in patients have limited clinical trials, and led to a search for derivatives with better efficacy. In this study, we synthesized fatty acid derivatives of 4-HPR by adding acetate (compound 1), propionate (2), pyruvate (3), butyrate (4), or stearate (5) to the 4-hydrox-
ylphenyl moiety of 4-HPR (Fig. 1). The chemical structures of the synthesized compounds were verified by ¹H- and ¹³C-NMR and mass analysis.

Cellular proliferation experiments were performed in four ovarian cell lines (OVCAR-3, PA-1, 2774, and SKOV-3). At 1 μM treatment for 4 d, AtRA did not suppress ovarian cancer cell proliferation in any of the four cell lines as compared to the DMSO control (Fig. 2). However, 4-HPR showed an overall 40—80% growth inhibition of the cells. Of the 4-HPR derivatives, compounds 1 and 5 showed inhibition similar to that of 4-HPR, whereas compounds 2, 3, and 4 suppressed cell growth to a greater extent than did 4-HPR. Specifically, compound 3 showed more than 95% growth inhibition of OVCAR-3 and SKOV-3 cells, 90% inhibition of PA-1 cells, and 70% inhibition of 2774 cells.

To determine the IC₅₀ values, cells were treated with increasing concentrations of AtRA, 9-cisRA, 4-HPR, or compound 3 for four days, dose–response curves were plotted (Fig. 3), and IC₅₀ values were determined from the curves by interpolation. As summarized in Table 1, IC₅₀ values were 3.75—7.75 μM for AtRA, 2.80—5.50 μM for 9-cisRA, 0.65—4.05 μM for 4-HPR, and 0.25—0.75 μM for compound 3, depending on the cell types treated. Compound 3 was 2.6—5.4-fold more cytotoxic than 4-HPR in the four ovarian cancer cell lines. Overall, these results indicate that, among the RA derivatives tested, compound 3 is the most effective growth suppression agent at the lowest concentrations, which suggests that it would be less toxic in clinical use. These data were consistent with microscopic observations (data not shown). OVCAR-3 cells treated with 1 μM of 4-HPR or compound 3 rounded up and detached from the surface, changes indicative of cell death. This appearance was more apparent in compound 3-treated cells.

The proliferation assays demonstrate that compound 3, in which the 4-hydroxyl group of 4-HPR is substituted with pyruvate, is the most cytotoxic against the four ovarian cancer cell lines. To determine whether the cytotoxic effect of

<table>
<thead>
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<th>Cell line</th>
<th>IC₅₀ (μM)ᵃᵇ</th>
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<th>IC₅₀ (μM)ᵇᵇ</th>
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<td>4.05</td>
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<tr>
<td>SKOV-3</td>
<td>3.75</td>
<td>3.15</td>
<td>0.65</td>
<td>0.25</td>
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ᵃ One day after seeding at a density of 3×10³ cells/well, cells were treated with various concentrations of the indicated retinoids for 4 d. The dose-response curves were plotted using the number of viable cells counted with a hemocytometer. IC₅₀ values, i.e., concentrations required for 50% growth inhibition, were determined by interpolation of the curves. Each experiment was performed in triplicate and repeated a minimum of three times. ᵇ p<0.05 for all IC₅₀ values.

Fig. 3. Dose–Response Curves of Retinoid Derivatives in Ovarian Cancer Cell Lines

Cells (3×10³) were treated with increasing concentrations of retinoid derivatives as indicated (0, 0.01, 0.1, 0.5, 1, 5 μM) in 6-well culture plates. After 4 d, the number of viable cells was counted using a hemocytometer and expressed as the % ratio relative to the number of cells in the DMSO control.
compound 3 is due to the induction of apoptosis, two kinds of apoptosis assays, nuclear staining and DNA fragmentation, were performed in OVCAR-3 cells treated with 1 μM compound 3 for 4 d. DMSO (0.01%), AtRA, and 4-HPR were used as controls. When a fluorescent DNA-binding dye, DAPI, was used to examine nuclear morphology, cells treated with 4-HPR or compound 3 displayed condensed and fragmented nuclei (Fig. 4A), which are typical morphologi-

![DAPI staining](image1)

![DNA fragmentation](image2)

Fig. 4. Effect of Compound 3 on Apoptosis

(A) DAPI staining. OVCAR-3 cells (2×10⁵) were treated with DMSO (a), 1 μM of AtRA (b), 4-HPR (c), or compound 3 (d). After 4 d, cells were stained with DAPI, and stained nuclei were visualized under a fluorescence microscope. Magnification, ×400. (B) DNA fragmentation. DNA was prepared from cells treated with increasing concentrations of AtRA, 4-HPR, or compound 3, subjected to agarose gel electrophoresis, and visualized with ethidium bromide staining. M, molecular weight marker (1-kb ladder), purchased from GibcoBRL.

![RAR Subtypes](image3)

![RXR Subtypes](image4)

![c-Jun](image5)

Fig. 5. Effects of Compound 3 on the Transcriptional Activity of RARs (A), RXRs (B), and c-Jun (C)

OVCAR-3 cells were cotransfected with the DR5-tk-CAT (for RARs) or DR1-tk-CAT (for RXRs) reporter gene and the plasmids expressing each subtype of RARs or RXRs, as described. Similarly, cells were transfected with the Coll-CAT reporter gene in the absence and presence of c-Jun expression. The effects of DMSO (a), AtRA (b; for RARs), 9-cisRA (b; for RXR), 4-HPR (c), or compound 3 (d) were examined. For RAR and RXR assays, 10 nM of retinoids were used. For the c-Jun assay, 1 μM of retinoids were used to reach maximal repression. Transcriptional activities were analyzed using CAT ELISA and expressed as a relative activity compared with that of the DMSO control.
cal features of apoptotic cells. A DNA fragmentation assay performed on cells treated with increasing concentrations of 4-HPR or compound 3 revealed a DNA ladder indicative of apoptosis (Fig. 4B). As expected, the formation of the DNA ladder was more evident in cells treated with compound 3 than in those treated with 4-HPR. These results suggest that the growth-suppressing activity of compound 3 in ovarian cancer cells is due primarily to the induction of apoptosis.

To determine whether compound 3, like RA and 4-HPR, can function as a direct activator of the nuclear retinoid receptor, we examined its ability to activate a retinoid-responsive reporter gene in the presence of retinoid receptors. For that purpose, OVCAR-3 cells were cotransfected with a receptor expression vector (RARα, β, γ, and RXRα in PSG5) and a reporter gene (DRβ5-tk-CAT for RARs or DR1-tk-CAT for RXR), then treated with 10 nM of retinoids. As shown in Fig. 5A, AtRA, a physiological ligand for RARs, activated for RXR), then treated with 10 nM of retinoids. As shown in Fig. 5A, AtRA, a physiological ligand for RARs, activated all three RARs. However, compound 3, like 4-HPR, was not active for RARα, but did activate RARβ and RARγ. The receptor specificity of 4-HPR was consistent with reported data.27 As expected, neither 4-HPR nor compound 3 were active for any of the three RXR subtypes, whereas 9-cisRA, a genuine ligand for RXRs, was active (Fig. 5B). Although compound 3 was similar to 4-HPR in its specificities for RARs and RXRs, compound 3 was more potent in inhibiting growth and inducing apoptosis of ovarian cancer cells. This suggests that the increased cytotoxicity of compound 3 may not be correlated to the activation potential of RARs.

It is well known that AP-1, composed of c-Jun and c-Fos, is a key transactivator for the expression of several genes, including MMP-1 and MMP-3, implicated in tumor promotion and invasion, and that the expression of these genes is repressed by AtRA.5—7 Therefore, the AP-1 transrepressing activity of the synthetic retinoids was further investigated by similar transfection assays using the AP-1-responsive Coll-CAT reporter plasmid. As shown in Fig. 5C, both 4-HPR and compound 3 repressed c-Jun activity to an extent similar to that of AtRA, regardless of additional c-Jun expression. The transrepression activity of compound 3 on AP-1 function was further confirmed by the reduced expression of MMP-1 and MMP-3, as determined by RT-PCR analysis (data not shown). Taken together, these results suggest that, like AtRA and 4-HPR, compound 3 targets AP-1 for MMP down regulation and thus may prevent the invasion of cancer cells.

Our results suggest that the number of carbons in the functional group is critical for growth suppression. Compounds 1 and 5 contain two and eighteen carbons, respectively, in their functional groups; compounds 2 and 3 contain three carbons, and compound 4 contains four carbons. Derivatives with either three or four carbons were more cytotoxic than 4-HPR and other derivatives. Therefore, the increased cytotoxicity of compound 3 appears to be closely associated with fatty acid chain length. On the basis of structure–activity relationships, we propose the presence of cellular target protein(s) that may be responsive to the three- or four-carbon fatty acid chains by direct association. To further investigate this relationship, we are currently preparing related compounds and searching for the responsive cellular protein(s).

In summary, compound 3 not only exhibited stronger inhibition of cell growth than the other derivatives and parent 4-HPR, but also effectively inhibited the c-Jun activity that has been implicated in tumor promotion and invasion. These results, together with compound 3’s selectivity for RAR subtypes, suggest that compound 3 could be an effective anticancer drug for ovarian cancer, with less toxicity than RA.

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REFERENCES