Synthesis and Biological Activity of Novel Retinamide and Retinoate Derivatives

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Retinoic acid and its amide derivative, N-(4-hydroxyphenyl)retinamide (4-HPR), have been proposed as chemopreventative and chemotherapeutic agents. However, their low cytotoxic activity and water solubility limit their clinical use. In this study, we synthesized novel retinoid derivatives with improved cytotoxicity against cancer cells and increased hygroscopicity. Our syntheses were preceded by selective O-acylation and N-acylation, which led to the production of retinoate and retinamide derivatives, respectively, in one pot directly from aminophenol derivatives and retinoic acid without protection. Transcription assays in COS-1 cells indicated that the N-acylated derivatives (2A—5A) and 4-HPR (1A) were much weaker ligands for all three subtypes of retinoic acid receptor (RAR) than all-trans retinoic acid (ATRA), although they showed some selectivity for RARβ and RARγ. In contrast, the O-acylated retinoate derivatives (1B—5B) activated all three RAR isotypes without specificity to an extent similar to ATRA. The cytotoxicity was determined using an MTT assay with HCT116 colon cancer cells, and the IC50 of N-acylated retinamide derivative 4A and O-acylated retinoate derivative 5B was 1.67 μM and 0.65 μM, respectively, which are about five and 13-fold better than that of 4-HPR (8.21 μM), a prototype N-acylated derivative. When retinamide derivative 5B was coupled to organic acid salts, the resulting salt derivatives 5C and 5D had RAR activation and cytotoxicity similar to those of 5B. These data may delineate the relationship between the structure and function of retinoate and retinamide derivatives.

Key words retinamide; retinoate; derivative; cytotoxicity; retinoic acid receptor (RAR)

The amide analogue of retinoic acid, N-(4-hydroxyphenyl)retinamide (4-HPR), was developed as a therapeutic agent for various skin conditions. It is currently being assessed as a drug to prevent and treat several cancers.1,2 Unlike all-trans retinoic acid (ATRA), 4-HPR selectively binds to retinoic acid receptors (RAR) β and γ and activates them less strongly than ATRA. These properties make 4-HPR less toxic and substantially less teratogenic than ATRA.3 Therefore 4-HPR has been used as an antitumor agent in animal studies4,5 and as a chemopreventive agent for several cancers in clinical trials.6—8 A number of in vitro biological studies indicated that 4-HPR has marked cytotoxicity in various cancer cells by inducing apoptosis.9,10 However, 4-HPR has a major limitation in its clinical use. The plasma levels in patients receiving 200 mg of 4-HPR daily are less than 1 μM, which is far less than the effective concentration (usually 10 μM) required to induce apoptosis in vitro. Therefore it is necessary to use higher doses of 4-HPR or to synthesize other derivatives with better clinical efficacy. Taking the second approach, several retinamide derivatives have recently been synthesized, which have hydroxyl, carboxy, or methoxy substituions on the terminal phenylamine ring.11,12 Of these derivatives, 3-HPR showed the most active growth inhibition in four bladder cancer cell lines,13 while 2-CPR, a carboxylic derivative, was the most effective in some head and neck, and lung cancer cell lines.12

To develop other potentially potent antitumor agents, we synthesized many retinoid derivatives. Since butyric acid has been implicated as an anticancer agent,13 we introduced aminophenol or its butyryl derivatives into ATRA by selective N-acylation (carboxamide) and O-acylation (carboxyl ester) to produce retinamide (1A—5A) and retinote (1B—5B) derivatives, respectively. To increase their water solubility, a selected derivative (5B) was coupled to organic acid salts. We determined the selectivity of these compounds in the activation of RAR subtypes and measured their cytotoxic activity against HCT116 colon cancer cells. Our data may be useful for delineating the relationship between structure and function in retinoate and retinamide derivatives.

Results and Discussion

Chemistry The synthesis of retinoid derivatives 1A—5A and 1B—5B is illustrated in Charts 1 and 2. As shown in Chart 1, the selective N- and O-acylation of ATRA and 4-aminophenol (4-AP) gave either the corresponding carboamide (method A) or carboester (method B). The coupling of ATRA and 4-AP in method A, which uses EDCI/DMAP as a reagent, creates 4-HPR (1A), which contains a carboamide bond. In contrast, the coupling of the same compounds using method B, which uses DCC/DMAP as a reagent, creates 4-APR (1B) with a carboxester bond. As previously reported,14 4-HPR could also be prepared from ATRA via retinyl chloride using method C. Both reactions involve acylation at the carboxy moiety of ATRA. At present, it is not clear how one acylation reaction can give rise to two different compounds. It is generally accepted that the coupling of an acid and amine in the presence of EDCI/DMAP or DCC/DMAP results in a reaction producing a carboamide bond.15—17 In preparing 4-HPR, it is plausible that EDCI/DMAP activates the NH2 group of 4-AP by preferentially shifting a proton to the OH group, making the NH2 group more likely to react with the carboxy moiety of ATRA. How is 4-APR produced? ATRA can be activated by DCC/DMAP, and its activated carboxy moiety reacts with the OH group of 4-AP to form 4-APR. In the presence of DCC/DMAP, it is likely that the NH2 group of 4-AP serves...
as a base to activate the OH group of 4-AP, allowing O-acylation and creating 4-APR as the sole product. Therefore this method enables the efficient preparation of two different types of retinoid derivative via the simple, selective coupling of retinoic acid to the NH₂ or OH group without protection. The structures of 4-HPR and 4-APR were elucidated by IR spectroscopy based on the carbonyl absorption frequencies of 1634 cm⁻¹ for 4-HPR and 1716 cm⁻¹ for 4-APR. Method D was used to introduce a butyryl group into derivatives 1A (4-HPR) and 1B (4-APR), producing derivatives 2A and 2B in yields of 91% and 93%, respectively.

To synthesize other retinamide and retinoate derivatives, we first synthesized target compounds 13, 14, and 15, as shown in Chart 2. To introduce selectively a butyryl group into the ortho-OH (R2) of 4-amino resorcinol 6, a series of reactions was performed. First, the amine of 6 was protected with Cbz–Cl (1.2 eq)/TEA (1.2 eq) to obtain the N-Cbz 7 in 89% yield. Acylation of 7 with butyryl chloride (1.5 eq)/NMM (1.5 eq) according to method D provided the monobutyrated compound 10 in 65% yield. Nitro compounds 11 and 12 were prepared from starting materials 8 and 9 by butyrylation of the OH or NH₂ group (R3) meta to the nitro group, in 61% and 72% yield, respectively. Compounds 10, 11, and 12 were catalytically hydrogenated with 10% Pd/C in MeOH, producing the corresponding compounds 13, 14, and 15 in high yields. Intermediates 13—15 were coupled with ATRA by selective N- or O-acylation according to methods A and B to give final compounds 3A—5A and 3B—5B, respectively, as shown in Table 1. The structures of these compounds were verified by IR spectroscopy.

Consistent with the general notion that most synthetic retinoids are lipophilic, our retinoid derivatives (Fig. 1) were insoluble in aqueous solvents and precipitated in vivo. Unfortunately, this was true for derivative 5B, which has significant biological activity (Table 2). Derivative 5B has a terminal NH₂ group, which can form salts to increase its water solubility. Although the hydrochloride and hydrobromide salts were more hydrophilic, these compounds were very unstable.

<table>
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<tr>
<th>Starting materials</th>
<th>Method A</th>
<th>Method B</th>
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<tbody>
<tr>
<td>4-AP</td>
<td>1A</td>
<td>48</td>
</tr>
<tr>
<td>1A</td>
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<td>14</td>
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a) Method A: EDCI (2.0 eq)/DMAP/CH₂Cl₂, room temperature, 4 h. b) Method B: DCC (2.0 eq)/DMAP/CH₂Cl₂, room temperature, 4 h. c) 2A and 2B were prepared from 1A and 1B, respectively, using Method D: butyryl chloride (1.2 eq)/NMM (1.2 eq)/0 °C, 1 h.
under strongly acidic conditions. Therefore we prepared organic salts $5\text{C} - \text{F}$ by reacting the free base form of $5\text{B}$ with a stoichiometric amount of fumaric, maleic, tartaric, or pamoic acid in THF according to Method E,$^{20}$ as shown in Chart 3.

**Effect of Retinoid Derivatives on RARs** To determine the activity and specificity of our retinoid derivatives on RAR subtypes, a series of conventional transcriptional assays was performed using a retinoid responsive reporter gene in the presence of each receptor. COS-1 cells were cotransfected with a receptor expression vector (RAR$\alpha$, $\beta$, and $\gamma$ in pSG5) and reporter gene [RAR(E)-tk-CAT], and treated with increasing concentrations of ATRA, 4-HPR, or other retinoids. CAT ELISA was used to measure the amount of CAT protein induced in response to the retinoid derivatives. For each retinoid and receptor, a dose–response curve was determined by interpolation of the curves. Each experiment was performed in triplicate and repeated a minimum of three times.

**Cytotoxic Activity of Retinoid Derivatives** The cytotoxic potential of the retinoid derivatives was investigated by determining their concentrations required for 50% growth inhibition (IC$_{50}$ value) for HCT116 colon cancer cells. For each retinoid and receptor, a dose–response curve was determined by interpolation of the curves. Each experiment was performed in triplicate and repeated a minimum of three times.

![Chart 3. Synthesis of Organic Acid Salts of Retinoid Derivative 5B](image)

These compounds were prepared according to method E: THF, 45 °C—hexane, 10 °C.

![Table 2. EC$_{50}$ and IC$_{50}$ Values of Retinoid Derivatives for RAR Activation and Cytotoxicity](image)

<table>
<thead>
<tr>
<th>Retinoids</th>
<th>EC$_{50}$ (mM) for RAR</th>
<th>IC$_{50}$ (mM) for HCT116 cells</th>
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<th>$\beta$</th>
<th>$\gamma$</th>
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<tr>
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<td>9.73</td>
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<td>&gt;10$^{-6}$</td>
<td>&gt;20</td>
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<tr>
<td>5B</td>
<td>4.9×10$^{-9}$</td>
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<td>8.7×10$^{-9}$</td>
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<td>0.78</td>
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<td>5D</td>
<td>5.2×10$^{-9}$</td>
<td>6.1×10$^{-9}$</td>
<td>6.3×10$^{-9}$</td>
<td>0.73</td>
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$a$) The dose–response curves were plotted using data obtained from transcription assays with individual RAR subtypes in the presence of increasing concentrations of test retinoid. EC$_{50}$ values, the concentrations required for 50% activation, were determined by interpolation of the curves. $b$) The dose–response curves were plotted by MTT assay as described in Experimental. IC$_{50}$ values, the 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. $c$) $p<0.05$ for all IC$_{50}$ values.
position of the amine group inhibited HCT116 cell growth 50 times more potently than did a derivative with the butyryl in the ortho position. When the butyryl groups of 3A and 5B were removed, the cytotoxic potential was completely abrogated. Furthermore, the number of carbons in the side functional group was also important in both 3A and 5B. A derivative with four carbons at that position, i.e., a butyryl group, was the most potent under our assay conditions.\textsuperscript{24} These overall results suggest that increased cytotoxicity is closely associated with side chain length. Based on the structure–function relationship, we propose that a cellular target protein(s) responds to the four-carbon fatty acid chain by direct association.

**Conclusion**

This paper describes a new synthetic method for retinoid derivatives that involves selectively controlling N-acetylation (carboxamidine) and O-acetylation (carboxyl ester). Our method led to the facile synthesis of various retinoid derivatives in one pot directly from retinoic acid and aminophenol derivatives without protection. In terms of RAR activation, retinamide derivatives 2A—5A functioned like 4-HPR, i.e., they were specific for RAR $\beta$ and $\gamma$, whereas, like ATRA, retinamide derivatives 1B—5B were active for all three RARs without specificity. In cytotoxicity assays using HCT116 cells, we found that retinamide derivatives 3A and 4A and retinamide derivatives 2B, 3B, and 5B were superior to 4-HPR, the parent retinamide. Data derived from derivatives 4B and 5A indicated that RAR activation is not correlated with cytotoxicity. Organic salts 5C and 5D were similar to parent compound 5B in terms of RAR activation and cytotoxicity in HCT116 cells. Although the data were not shown, the increased cytotoxicity of retinoid derivatives appears to be closely associated with the side chain length of the functional group. From these overall results, we conclude that our derivative 5B and its organic salt forms, the most effective of the derivatives tested, are promising cancer prodrugs.

**Experimental**

ATR was purchased from Sigma Chemical Co. (Sigma-Aldrich Korea, Seoul). The dry DMF was stored over 4 Å sieves and degassed before use by bubbling argon through it for at least 1 h. Dry CH$_2$Cl$_2$ was obtained by distillation from CaH$_2$. The other commercially available reagents and solvents were used without further purification. All reactions were conducted under an Ar atmosphere, except for those reactions utilizing water as a solvent. They were monitored by TLC (Merk Kieselgel 60, F254). All the products prepared were purified by flash column chromatography on silica gel 60 (Merck, 230—400 mesh). \(^1\)H- and \(^13\)C-NMR spectra were recorded on a Bruker AC-200F and JEOL JNN EX-400 using CDCl$_3$ and DMSO-d$_6$ as solvents. All chemical shifts ($\delta$) are quoted in ppm downfield from TMS and coupling constants ($J$) are given in Hz. Mass spectra were measured on an Agilent 1100 LC/MS (API-ES) mass spectrometer, Micro MS-autospec/OA ToF (HR-EL-MS). IR spectra were recorded on Perkin Elmer 16FFC FT-IR spectrophotometer and frequencies are given in reciprocal centimeters.

**Method A. (E,E,E,E,E,E,E)-3,7-Dimethyl-2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]-phenyl Butanoate (2A)**

Using method D, yield: 91% as a yellow solid, mp 146—148 °C. \(^1\)H-NMR (400 MHz, CDCl$_3$): $\delta$ 7.56 (d, $J=7.84, 2H$), 7.19 (s, 1H), 7.04 (d, $J=8.80, 2H$), 7.00 (dd, $J=15.00, 11.40, 1H$), 6.26 (m, 4H), 5.78 (s, 1H), 2.53 (t, $J=7.34, 2H$), 2.41 (s, 3H), 2.01 (br, s, 5H), 1.78 (m, 2H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.04 (t, $J=7.34, 3H$), 1.02 (s, 6H). \(^1\)C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 172.21, 161.05, 148.56, 135.91, 137.60, 136.17, 135.85, 135.16, 130.30, 129.77, 129.39, 128.39, 121.77, 121.07, 120.61, 115.42, 39.69, 36.26, 34.33, 31.18, 29.03, 21.83, 19.33, 18.54, 13.83, 13.74, 12.96. MS: $m/z$ (%) = 462 (M$^+$, 33), 461 (100), 339 (7), 282 (18). HR-EL-MS m/z 461.2931 (Calcld for C$_{30}$H$_{39}$NO$_3$, 461.2932).
NMR (100 MHz, CDCl3): δ 7.80 (d, J = 7.42, 1H), 7.27 (s, 1H), 7.12 (d, J = 14.90, 11.00, 1H), 6.90 (d, J = 8.70, 1H), 6.28 (m, 1H), 6.00 (s, 1H), 3.72 (brs, 2H), 2.41 (s, 3H), 2.32 (t, J = 7.44, 2H), 2.01 (brs, 5H), 1.72 (s, 3H), 1.70 (m, 2H), 1.64 (m, 2H), 1.46 (m, 2H), 1.02 (s, 6H), 0.98 (t, J = 7.34, 3H). 13C-NMR (100 MHz, CDCl3): δ 175.32, 141.25, 139.37, 138.76, 129.14, 128.50, 127.33, 123.19, 119.31, 113.89, 110.18, 33.51, 32.87, 32.54, 31.34, 28.95, 21.75, 19.18, 18.43, 14.14, 13.64, 12.94. MS: m/z (%) = 478 (M+1), 100 (15), 283 (65). HR-ESI-MS m/z = 477.2878 (Calcd for C30H39NO4, 477.2879).

4-Amino-3-(butyl-4,6,6,8-tetraeylanoyloxy)-1-cyclohexenyl-2,4,6,8-nonatetraenoate (5B): Yield: 43% as a yellow oil.

To a solution of 10% HCl in water (50 ml) was added dropwise slowly into the cooled triethylamine (0.14 ml, 0.99 mmol) and 4-aminophenol (0.072 g, 0.66 mmol) in dry, degassed DMF (2 ml).

The temperature was maintained at 0–15°C during the addition. The dark-colored reaction solution was stirred at room temperature until TLC analysis indicated none remaining (about 2 h).

The reaction was quenched with Na2CO3 (aq.) and extracted with EtOAc. The extracts were washed with H2O and brine, dried (Na2SO4), and evaporated. The residue was purified by column chromatography using hexane/EtOAc (3:1) as the elution grade 1A (0.10 g, 78%) as a yellow solid.

(2,4-Dihydroxyphenyl)-carbanic Acid Benzyolste (7) TEA (trimethyl-amine, 0.72 ml, 741 mmol) was added to a solution of 4-amino resorcinol (1.0g, 6.18 mmol) dissolved in dry DMF (5 ml). To the resulting mixture dicyclohexylcarbodiimide (1.06 ml, 741 mmol) was added dropwise at 0°C and stirred for 2 h.

The reaction mixture was extracted with EtOAc (50 ml) and washed with H2O (2×30 ml). The organic layer was dried over MgSO4 and evaporated in vacuo. The crude product was crystallized from EtOAc/hexane=1/3 to give 7 (1.42 g, 89%), as a white solid, mp 122–124°C. 1H-NMR (100 MHz, CDCl3, DMSO-d6): δ 7.54, 145.93, 136.91, 128.60, 128.40, 128.32, 127.69, 125.89, 122.42, 117.00, 65.27. MS: m/z (%) = 260 (M+, 100), 126 (23), 110 (21).

Method D - 2-Benzylcarbonylamino-5-(hydroxyphenyl) (10) Compound 7 (10.1 g, 3.85 mmol) was dissolved in dry CHCl3 (15 ml) and N-methyl morpholine (0.62 ml, 5.77 mmol) was added. To the resulting mixture butyl chloride (0.56 ml, 5.77 mmol) was added dropwise at 0°C, and stirred for 30 min and the reaction was quenched with 5% NaHCO3 solution and washed with 15% HCl and H2O. The organic layer was dried over MgSO4 and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexane=1/5) to give 0.83 g (65%) of 10 as a white solid, mp 112–114°C. 1H-NMR (200 MHz, CDCl3): δ 8.75 (d, J = 7.24, 1H), 7.37 (m, 3H), 6.65 (d, J = 7.24, 1H), 6.56 (s, 1H), 6.38 (s, 1H), 5.17 (s, 2H), 2.40 (t, J = 7.35, 2H), 1.72 (m, 2H), 1.00 (t, J = 7.34, 3H). 13C-NMR (100 MHz, CDCl3): δ 171.65, 155.01, 145.93, 135.87, 128.59, 128.38, 128.25, 127.95, 122.72, 121.73, 119.57, 113.62, 109.77, 67.33, 36.00, 18.39. MS: m/z (%) = 494 (M+, 100), 391 (13), 283 (21).
2.03 (brs, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.94 (t, J = 7.40, 3H). 13C-NMR (100 MHz, CDCl3): δ 7.89 (brs, 1H), 7.51 (brs, 1H), 7.09 (dd, J = 14.90, 11.40, 1H), 6.86 (d, J = 8.70, 1H), 6.24 (m, 2H), 6.02 (s, 1H), 4.52 (s, 2H), 2.39 (s, 3H), 2.32 (t, J = 7.38, 2H), 2.03 (brs, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.94 (t, J = 7.40, 3H). 13C-NMR (100 MHz, CDCl3): δ 173.12, 170.98, 164.01, 153.68, 146.21, 139.77, 137.26, 136.91, 135.04, 131.98, 130.66, 129.87, 129.68, 128.25, 122.75, 118.00, 109.83, 109.27, 72.16, 39.00, 38.87, 33.88, 33.36, 28.80, 21.53, 18.73, 18.70, 13.68, 13.50, 12.69. MS (API-ES, Neg, scan): m/z (%) = 625 [(M–1), 8], 476 (16), 475 (42), 406 (18), 405 (100), 283 (36), 255 (41), 149 (21).

Pamoate Salt of 4-Amino-2-(butylallylamino)phenyl]2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8,8-pentanetraenoate (5E) Yield: 95% as a yellow solid, mp 131—135 °C. [1H-NMR (200 MHz, CDCl3, +DMSO-d6): δ 7.89 (brs, 1H), 7.51 (brs, 1H), 7.09 (dd, J = 14.90, 11.40, 1H), 6.86 (d, J = 8.70, 1H), 6.24 (m, 2H), 6.02 (s, 1H), 4.52 (s, 2H), 2.39 (s, 3H), 2.32 (t, J = 7.38, 2H), 2.03 (brs, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.94 (t, J = 7.40, 3H). 13C-NMR (100 MHz, CDCl3): δ 173.12, 170.98, 164.01, 153.68, 146.21, 139.77, 137.26, 136.91, 135.04, 131.98, 130.66, 129.87, 129.68, 128.25, 122.75, 118.00, 109.83, 109.27, 72.16, 39.00, 38.87, 33.88, 33.36, 28.80, 21.53, 18.73, 18.70, 13.68, 13.50, 12.69. MS (API-ES, Neg, scan): m/z (%) = 625 [(M–1), 8], 476 (16), 475 (42), 406 (18), 405 (100), 283 (36), 255 (41), 149 (21).

Cytotoxicity Assay The antiproliferative effect of retinoid derivatives was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). HCT116 colon cancer cells were seeded at an initial density of 3000 cells/well in 96-well plates and treated with medium containing various concentrations of retinoid derivatives. DMSO controls (0.01%) did not affect cell proliferation. After 48 h, 50 μl of MTT solution (2 mg/ml in PBS) was added to the culture medium and the reaction mixture was incubated at 37 °C in a 5% CO2 atmosphere for 4 h. The MTT solution was aspirated and 150 μl of DMSO was added. The optical density was measured spectrophotometrically at 550 nm. Each experiment was performed in triplicate and repeated a minimum of three times.

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