Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 847–853
Bioorganic & Medicinal Chemistry

Potent anticancer activities of novel aminophenol analogues against various cancer cell lines

Toshihiro Ohba,[a] Takayasu Yamauch,[b] Kimio Higashiyama[b] and Noriko Takahashi[a,*]

[a]*Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan*
[b]*Synthetic Organic Chemistry Research Laboratory, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan*

Received 29 September 2006; revised 17 October 2006; accepted 20 October 2006
Available online 25 October 2006

Abstract—Novel aminophenol analogues were synthesized based on the structure of fenretinide (*N*-(4-hydroxyphenyl)retinamide, **5**), which is a potent anticancer agent. Our findings showed that the anticancer activities of **5** were due to the side chain attached to the aminophenol moiety. A *p*-octylaminophenol (*p*-OAP) provided the most potent anticancer activity among *p*-alkylaminophenols examined. In this study, we investigated anticancer activities against various cancer cell lines by the new aminophenols, *p*-dodecylaminophenol (**1**), *p*-decylaminophenol (**2**), *N*-(4-hydroxyphenyl)dodecananamide (**3**), and *N*-(4-hydroxyphenyl)decananamide (**4**), which exhibits a side chain as long as **5**. Cell growth of breast cancer (MCF-7, MCF-7/Adr^R), prostate cancer (DU-145), and leukemia (HL60) cells was suppressed by **1** and **2** in a fashion dependent on the length of the alkyl chain attached to the aminophenol. In contrast, **3** and **4** were extremely weak. Compound **5** was less potent than **1**. Cell growth of liver cancer (HepG2) was not markedly affected by these compounds. In addition, apoptosis of HL60 cells was induced by **1** and **2** in a chain length-dependent manner, but not by **3** and **4**. Incorporation of compounds into HL60 cells was in the order **1** > **2** = **3** > **4**. These results indicated that anticancer activities for **1** and **2** are correlated with their incorporation into cancer cells and their capability to induce apoptosis, but not for **3** and **4**. Compound **1**, a potent anticancer agent with potency strikingly greater than **5**, may potentially be useful in clinic.

1. Introduction

Fenretinide (*N*-(4-hydroxyphenyl)retinamide, **5**) (Fig. 1) is an effective chemopreventive and antiproliferative agent against a wide variety of tumor types,[1–4] which currently is in clinical trial for the treatment of breast, bladder, renal, and neuroblastoma malignancies.[4–10] In addition, **5** induces apoptosis in HL60 and NB4 human

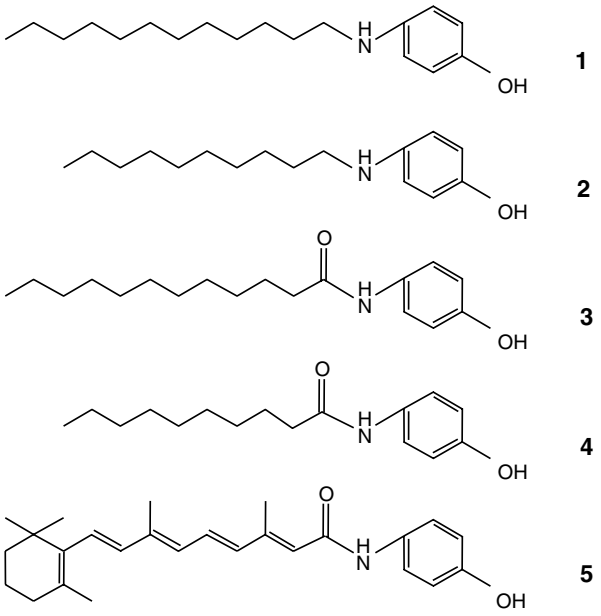

Figure 1. Chemical structures of **1**, **2**, **3**, **4**, and **5**.

Abbreviations: **1**, *p*-dodecylaminophenol, 4-(dodecylamino)phenol; **2**, *p*-decylaminophenol, 4-(decylamino)phenol; **3**, *N*-(4-hydroxyphenyl) dodecananamide, *p*-dodecanoylaminophenol; **4**, *N*-(4-hydroxyphenyl) decananamide, *p*-decanoylaminophenol; **5**, *N*-(4-hydroxyphenyl)retinamide, fenretinide; RA, retinoic acid; *p*-OAP, *p*-octylaminophenol, 4-(octylamino)phenol; *p*-MAP, *p*-methylaminophenol, 4-(methylamino)phenol; 4-HBR, 4-hydroxybenzylretinone; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); RAR, retinoic acid nuclear receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid.

Keywords: Aminophenol; Anticancer; Retinoid; Apoptosis; Cell incorporation.

* Corresponding author. Tel./fax: +81 3 5498 5950; e-mail: t-noriko@hoshi.ac.jp

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.10.042

leukemia cell lines, human cervical carcinoma cell line, meningioma, and neuroblastoma.[8,11–17] On the other hand, **5** exhibits antioxidant activities that included scavenging α,α-diphenyl-β-picrylhydrazyl radicals, and inhibiting linoleic acid lipid peroxidation and lipid peroxidation in rat liver microsomes initiated by hydroxyl radicals.[18,19] Thus, **5** is a biologically superior and active compound.

Previous studies have shown that the structure of the *p*-methylaminophenol (*p*-MAP) moiety derived from the side-chain amido portion of **5** contributes most significantly to the anticancer and antioxidative activities of **5** as compared with 4-aminophenol and *p*-aminoacetophen moieties.[18,20] In addition, the elongation of the methyl chain in *p*-MAP increases antioxidative activities against lipid peroxidation,[21,22] but not superoxide scavenging activity. On the other hand, the growth of various cancer cell lines, including human leukemia cell line, HL60 and HL60R, which is resistant against RA, was suppressed by *p*-alkylaminophenols (C_1–C_8) in an alkyl chain length-dependent manner.[21]

Recently, we synthesized four novel compounds which had lengths of carbon side chains similar to that of **5**, *p*-dodecanoylaminophenol (**1**), *p*-decanoylaminophenol (**2**), *N*-(4-hydroxyphenyl)dodecananamide (**3**), and *N*-(4-hydroxyphenyl)decananamide (**4**) (Fig. 1). We examined the antioxidant activities and antiproliferative properties against leukemia cell lines in these compounds. We found that **1** and **2** exhibit antioxidant activities, inhibition of lipid peroxidation and superoxide scavenging activity, and suppressed leukemia cell growth greater than **5**. These findings raised questions whether **1** and **2** would be effective against other various cancer cell lines as well in addition to leukemias, and whether anticancer activities result from the induction of apoptosis. It was also of question whether anticancer potencies of compounds were correlated with the intensities of cell incorporation. In the current study, these questions were addressed.

2. Results

2.1. Growth suppression of breast cancer cell lines by aminophenol analogues

We examined whether novel aminophenol analogues affect human breast cancer cell growth. Compound **5**, a parent compound, and RA were used as internal standards for the inhibition of cell growth. The % net cell growth in the presence of aminophenol analogues was shown with values adjusted by subtracting the initial cell concentrations of experimental cultures from the initial concentrations of control cultures, which were defined as 100%. As shown in Figure 2, MCF-7 cells having estradiol receptors (ER-positive cells) or MCF-7/Adr^R cells not having estradiol receptors (ER-negative cells) were grown in the presence of aminophenol analogues or retinoids at a concentration of 4 μM.

In MCF-7 cells, cell growth was inhibited approximately 66% by **1**, 57% by **2**, 22% by **3**, 30% by **4**, 62% by **5**, and 74% by RA (Fig. 2A), and in MCF-7/Adr^R cells, approximately 100% by **1** and **2**, 21% by **3**, 23% by **4**, 60% by **5**, and 12% by RA (Fig. 2B). Thus, while **1** showed cell growth inhibitory effects as great as **5** against MCF-7 cells, **1** was more potent than **5** in MCF-7/Adr^R cells, which were resistant to RA (Fig. 2). Growth inhibition by these compounds was dose-dependent (data not shown). In contrast, **3** and **4** were less potent antiproliferative agents than **1** and **2** against both MCF-7 and MCF-7/Adr^R cells (Fig. 2). These results indicate that **1** and **2** were potent growth inhibitors against human breast cancer cell lines, particularly RA-resistant cells. The insertion of carbonyl moieties into **1** and **2** affected their ability to inhibit cell growth greater than did differences in alkyl chain lengths.

2.2. Effects of aminophenol analogues on the growth of prostate and liver cancer cells

Proliferation of DU-145 and HepG2 cells was suppressed by aminophenol analogues to different extents (Fig. 3). At 4 μM concentration, growth inhibition against DU-145 cells was approximately 73% by **1**, 66% by **2**, 41% by **3**, 51% by **4**, 41% by **5**, and 46% by RA (Fig. 3A). These results suggest that **1** and **2** show more potent inhibitory activities than **5**, which is well known as an effective compound against prostate cancer, and that **3** and **4** are as potent as RA and **5**. Antiproliferative activities by **1** and **2** increased depending on the alkyl chain length. Thus, all four new compounds were effective against DU-145 cells.

In contrast, the % growth inhibition against HepG2 cells by aminophenol analogues was much less than that against MCF-7, MCF-7/Adr^R, and DU-145 cells (Figs. 2 and 3A), whereas **4** inhibited HepG2 cell growth as potently as **5** (Fig. 3B). At 4 μM concentration, growth of HepG2 cells was inhibited by approximately 13% for **1**, 17% for **2**, 22% for **3**, 26% for **4**, 27% for **5**, and 13% for RA. The elongation of the alkyl chain decreased cell growth inhibition and replacing the alkyl moiety with an acyl residue resulted in an enhancement of inhibitory activity. Therefore, this functionality may be essential for the action against liver cancer cells.

2.3. Growth inhibition and DNA fragmentation in HL60 cells treated with aminophenol analogues

Figures 2 and 3 show that growth of attached cancer cell lines except HepG2 cells was suppressed by **1** and **2** to a greater extent than **3** and **4**. Here, HL60 cells, non-adherent human leukemia cell line, were grown in medium containing 10% FBS in the presence of 4 μM aminophenol analogues under similar conditions as used with other cancer cell lines. Figure 4 shows the % net cell growth after 72 h in the presence of aminophenol analogues. HL60 cell growth was inhibited by aminophenol analogues to a different extent. Compound **1** (approximately 72% inhibition) was more potent than **2** (approximately 66% inhibition), **5** (approximately 55% inhibition), and RA (approximately 42% inhibition). In contrast, **3** and **4** were less potent than other com-

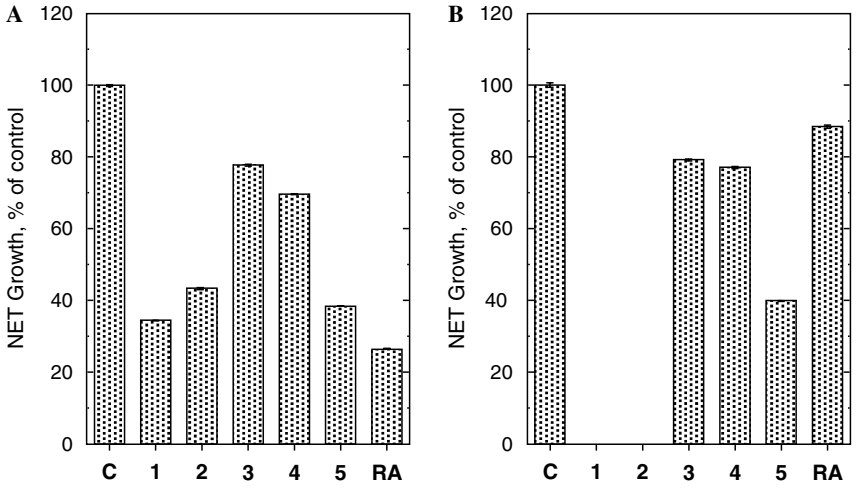

Figure 2. Growth of MCF-7 and MCF-7/Adr^R cells in the presence of **1**, **2**, **3**, **4**, **5** and RA. MCF-7 (A) and MCF-7/Adr^R (B) cells (0.5×10^4 cells/ml) were grown in the presence of **1**, **2**, **3**, **4**, **5**, and RA at 4 μM concentration in medium containing 10% FBS for 68 h. Measurements were made as described in Section 4. Net growth % of control was shown for each compound. Each *point* is the mean of at least three measurements. C: control.

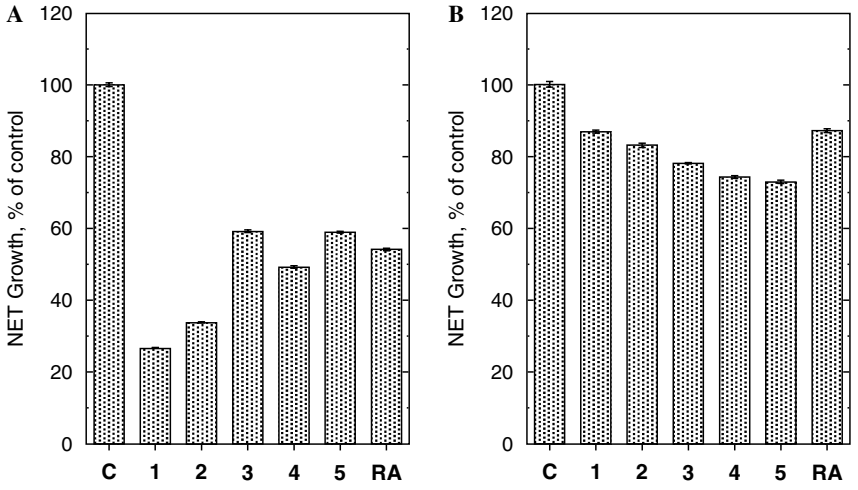

Figure 3. Growth of DU-145 and HepG2 cells in the presence of **1**, **2**, **3**, **4**, **5** and RA. DU-145 (A) and HepG2 (B) cells (0.5×10^4 cells/ml) were grown in the presence of **1**, **2**, **3**, **4**, **5**, and RA at 4 μM concentration in medium containing 10% FBS for 68 h. Measurements were made as described in Section 4. Net growth % of control was shown for each compound. Each *point* is the mean of at least four measurements. C: control.

pounds and inhibited cell growth to a similar extent as each other (approximately 6–8% inhibition). Thus, **1** and **2** inhibited HL60 cell growth greater than **3** and **4**, which had an acyl residue instead of an alkyl chain (Fig. 4). In addition, the extent of antiproliferative activity against leukemia cells was enhanced in an alkyl chain length-dependent manner. These results suggest that growth inhibition for non-adherent cells (Fig. 4) is similar to adherent cells (Figs. 2 and 3). The findings that the length and structure of the side chain residue in the aminophenol analogues affects cell growth inhibitory potency let us to investigate whether antiproliferative activity against cancer cells is due to the induction of cell apoptosis.

In order to evaluate the effect of these aminophenol analogues on the induction of apoptosis, DNA isolated from HL60 cells treated with aminophenol analogues at the concentration of 4 μM for 20 h was visualized on agarose gel. DNA extracted from cells treated with DMSO showed no fragmentation (Fig. 5A). In contrast, cells treated with 4 μM concentrations of **2** (Fig. 5D) and **1** (Fig. 5E) contained fragmented ladder DNA to a greater extent than 4 μM of **5** (Fig. 5F). The intensity of ladder DNA increased depending on the length of the alkyl chain of aminophenol (Fig. 5D and E). Compound **1** is a potent inducer of DNA fragmentation greater than **2**. Aminophenol analogues, **4** and **3**, induced no or little DNA fragmentation (Fig. 5B and C). These results indicate that **1** and **2**, *p*-alkylaminophenols containing 12 and 10 carbon chains, may potentially induce apoptosis of HL60 cells and thereby inhibit cell growth. In addition, in these aminophenol analogues, antiproliferative activity for cancer cells was correlated with capability of apoptosis induction for cancer cells.

2.4. Cell incorporation of aminophenol analogues

Compound **1** was an excellent antiproliferative agent against various cancer cell lines (MCF-7, DU-145, and

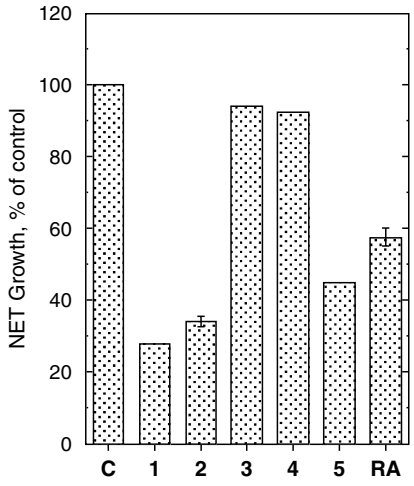

Figure 4. Growth of HL60 cells in the presence of **1**, **2**, **3**, **4**, **5**, and RA. HL60 cells (1×10^5 cells/ml) were grown in the presence of **1**, **2**, **3**, **4**, **5**, and RA at 4 μM concentration in medium containing 10% FBS for 72 h. Measurements were made as described in Section 4. Net growth % of control was shown for each compound. Each *point* is the mean of at least four measurements. C: control.

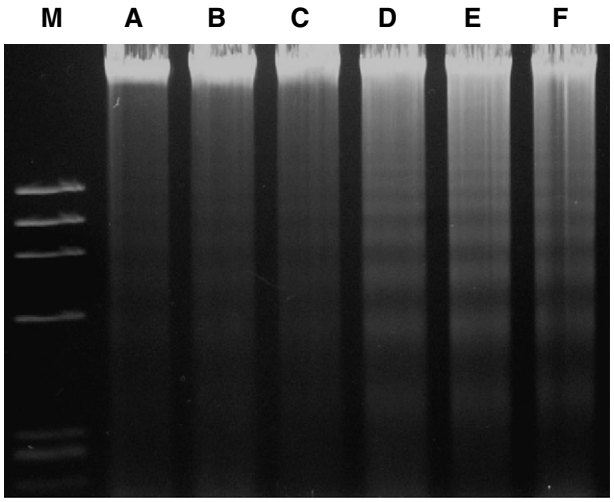

Figure 5. Agarose gel analysis of DNA fragmentation of HL60 cells grown in the presence of **1**, **2**, **3**, and **4**. HL60 cells (2×10^5 cells/ml) were grown in the presence of DMSO (A), **4** (B), **3** (C), **2** (D), **1** (E), and **5** (F) at a concentration of 4 μM in medium containing 10% FBS for 20 h. DNA was extracted from cells and DNA fragmentation was measured by agarose gel electrophoresis as described under Section 4. These experiments were repeated at least four times. M: marker.

HL60) and RA-resistant cancer cell lines (MCF-7/Adr^R and HL60R) except liver cancer cell line (HepG2) (Figs. 2 and 3, Ref. 22) and induced apoptosis of HL60 cells (Fig. 4). Side-chain structures of aminophenol analogues were critical for growth inhibition and apoptosis induction (Figs. 2–5). These results raised the question whether hydrophobicities of compounds affect these activities. We attempted to compare the hydrophobicity of each compound by retention time of reverse-phase C_{18} column using high pressure liquid chromatography (HPLC, mobile phase: 80% MeOH, 20% H_2O, and 10 mM ammonium acetate). Retention times of compounds were 4.45 min for **4**, 7.89 min for **3**, 9.19 min for **2**, and 17.96 min for **1** (data not shown). These results indicate that **1** was the most lipid-soluble compound among four aminophenol analogues (**1** > **2** > **3** > **4**), and that alkyl lengths of *p*-alkylaminophenols (**1** and **2**) affect the hydrophobicities of compounds.

These findings raised the question whether incorporation of aminophenol analogues into cells is affected by their hydrophobicities. Compounds in HL60 cells after the incubation at 37 and 4 °C for given time periods in the presence of 10 μM compounds were extracted and quantitated by HPLC. Cell uptake of all compounds at 4 °C was low level as compared at 37 °C (data not shown). The incorporation of compounds at 37 °C increased along with the prolonging of incubation time and was almost saturated at 37 °C after 60 min except **1**, which decreased (Fig. 6). The amount of **1** in HL60 cells at 30 min was approximately 3-fold higher than **2**. In contrast, the incorporation of **3** into cells was almost as much as **2** (Fig. 6), while **4** showed extremely low uptake levels. These results indicate that cell incorporation of *p*-alkylaminophenols depends on alkyl chain length related to hydrophobicities and correlates with antiproliferative activities. In addition, the insertion of oxygen atoms into the alkyl chain led to the reduction of antiproliferative activity for cancer cell lines except liver cancer. The finding that **3** was almost inactive, even though **3** entered into cells, implied that the carbonyl moiety in **3** is critical for the suppression of anticancer activity.

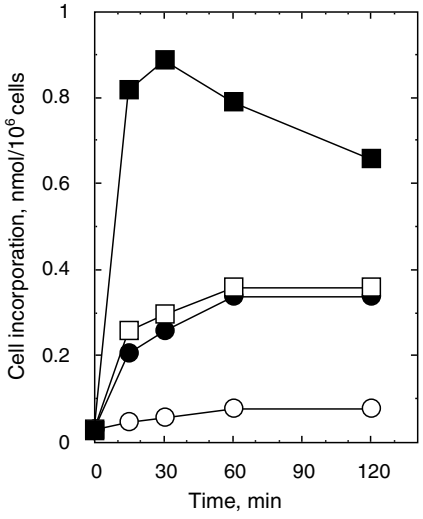

Figure 6. Incorporation of **1**, **2**, **3**, and **4** into HL60 cells. HL60 cells (1×10^6 cells/ml) were incubated in the presence of **1** (■), **2** (□), **3** (●), and **4** (○) at a concentration of 10 μM in medium containing 10% FBS for the indicated time. Cells were harvested and measurements were performed as described under Section 4, and a LUNA® 5 μ C18(2) column (150 × 4.6 mm, Phenomenex) was used to separate compounds. The column was eluted with 80% MeOH, 20% H_2O, and 10 mM ammonium acetate at a flow rate of 1.0 ml/min. The SD of each data point was ⩽8% of the mean. These experiments were repeated at least three times.

3. Discussion

In the current study, **1** and **2** (Fig. 1) were potent anticancer agents against various cancers except liver cancer as compared with **5**, a cancer chemopreventive and antiproliferative agent in clinical study (Figs. 2–4). In particular, **1** inhibited cell growth of a variety of cancer cells most potently among the aminophenol analogues examined. The effects of compound **1** on cell growth were probably caused by inducing cell apoptosis (Fig. 5). Differences of alkyl chain length between **1** and **2** changed their hydrophobic properties and cellular incorporation of the compounds, resulting in the potential variation on growth inhibitory activity (Fig. 6). Both **3** and **4** exhibit structures similar to that of **5**. However, these failed to inhibit cell growth and induction of apoptosis, while hydrophobicity and cell uptake of both compounds were distinct from each other (Figs. 2–6). Compound **1** showed excellent anticancer activity through high cell association, uptake into cell, and induction of apoptosis.

Previous studies have shown that *p*-alkylaminophenols reduce the extent of microsomal lipid peroxidation induced by hydroxy radicals, in a fashion dependent on the length of the alkyl chain appended to the aminophenol residue with positive correlation.[23] The IC_{50} values of *p*-MAP, *p*-butylaminophenol, *p*-hexylaminophenol, and *p*-octylaminophenol (*p*-OAP) were 4.6, 0.3, 0.033, and 0.014 μM, respectively. Recently, we showed that **1** (IC_{50}, 0.0155 μM) and **2** (IC_{50}, 0.015 μM) are potent inhibitors of lipid peroxidation, being approximately 1300- and 400-fold higher potency than **3** (IC_{50}, 20 μM) and **4** (IC_{50}, 6.1 μM), respectively.[22] Thus, **1** and **2** show inhibitory activities against lipid peroxidation to a similar extent as *p*-OAP, while this antioxidant activity gradually increased in going from *p*-MAP to *p*-OAP. In the current study, the hydrophobicities of **1**, **2**, **3**, and **4** were compared, resulting in **1** > **2** > **3** > **4**. Although the hydrophobicities of compounds appeared to be related to the interaction with microsomal membranes, inhibitory activities of lipid peroxidation by these aminophenol analogues required optimum hydrophobicity. These results suggested that tertiary structures of aminophenol analogues may be significant for the suppression of lipid peroxidation by hydroxy radicals. On the other hand, **1** and **2** with high hydrophobicity exhibit superoxide scavenging abilities dependent on the length of the alkyl chains with negative correlation. However, **3** and **4**, which were less hydrophobic, were either inactive or weakly active.[22] The insertion of a carbonyl residue in the *p*-alkylaminophenols diminished superoxide scavenging capabilities as an antioxidant. It would be interesting to examine the differences in the intracellular actions by these aminophenols.

Growth of various cancer cell lines, leukemia (HL60 and HL60R), breast cancer (MCF-7 and MCF-7/Adr^R), and prostate cancer (DU-145), except liver cancer, was markedly suppressed by **1** and **2** (Figs. 2–4, Ref. 22). In these five cell lines, potency of growth inhibition by **1** and **2** increased according to the elongation of the alkyl chain and both were greater than **5**. Their potencies as anticancer agents were abolished by the insertion of one oxygen atom (Figs. 2–4). Compound **5** is a potent anticancer drug in clinical trials for breast, bladder, renal, and neuroblastoma malignancies.[4–10] These results suggest that **1** and **2** may be promising anticancer agents as well as **5**. Further in vivo studies are required to create new drugs. In addition, it would be of interest to design new compounds with further elongated normal carbon chain or branched carbon chain and examine anticancer activity in order to optimize.

In clinical study, patients treated with **5** were affected with night blindness due to decreasing serum retinol levels.[24,25] This side effect may be due to displacement of retinol from serum retinol binding protein, decreasing the delivery of retinol to the eye.[24,26,27] Compound **5** exhibited extremely poor binding to the nuclear retinoid receptors and showed anticancer activity against RA-resistant cells. It is apparent that **5** may act via pathways which are independent of the nuclear retinoid receptors RARs and RXRs, and act on cells directly rather than through hydrolysis to free RA. Recently, in order to overcome the side effects of **5**, several nonhydrolyzable compounds were designed and evaluated. 4-Hydroxybenzylretinone (4-HBR), a stable C-linked analog of **5** and **5**-C glucuronide (**5**CG), a C-linked analog of **5**-glucuronide, were synthesized and shown to exhibit antitumor effects without side effects of night blindness.[25,28–30] However, 4-HBR and **5**CG contained the structure of the same cyclohexene ring as retinol, which is recognized by retinol binding protein. In the current study, **1** and **2** without cyclohexene rings were potent antiproliferative agents against various cancers as compared with **5**. Particularly, **1** was the most potent antiproliferative and apoptosis-inducing agent against various cancer cell lines, including adherent and non-adherent cells. Therefore, it is possible that **1** may be an effective anticancer drug without the side effects observed by **5**. It would be interesting to examine further the anticancer activity of **1** in vivo.

Compound **1** inhibited cell growth of HL60R, a RA-resistant cancer cell line and MCF-7/Adr^R, a multidrug-resistant cancer cell line. It was more potent than **5** (Fig. 2B and 3B, Ref. 22). In the clinic, cancer patients become resistant to many drugs in chemotherapy of various cancers including breast cancer. The clinical outcome of patients treated with anticancer drugs may be modified by administration of **1**. Studies are presently underway to determine the toxicity of **1** in animals and to measure the effectiveness of **1** on tumors growing in animals.

4. Experimental

4.1. Chemicals

RA, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA, fraction V), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Compound **5** was provided by Dr. R. C. Moon, University of Illinois, Chicago, IL, USA. All other chemicals were of reagent grade. *N*-(4-Hydroxyphenyl)dodecananamide (**3**, *p*-dodecanoylaminophenol), *N*-(4-hydroxyphenyl)decananamide (**4**,

p-decanoylaminophenol), *p*-dodecylaminophenol (**1**), and *p*-decylaminophenol (**2**) (Fig. 1) were synthesized as described previously.[21–23]

4.2. Cell

Human breast cancer cell lines, MCF-7 and MCF-7/Adr^R, were obtained from the American Type Culture Collection (ATCC), Rockville, MD.[31] Human hepatoma cell line, HepG2, was obtained from RIKEN cell bank (Tokyo, Japan).[32] Human prostate cancer cell line, DU-145, was obtained from Dr. Y. Pommier of the National Cancer Institute (Bethesda, MD).[33] MCF-7, MCF-7/Adr^R, HepG2, and DU-145 cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.3, and 10% fetal bovine serum (FBS)(GIBCO), and subcultured every week.

Early passage (<30) human myeloid leukemia cell line, HL60, was maintained in RPMI medium containing 10% FBS.[34–36]

All cells described above were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell number was estimated using on an electric particle counter (Coulter Electronics, Hialeah, FL) and viability was determined by trypan blue dye exclusion.

4.3. Cell growth

MCF-7, MCF-7/Adr^R, HepG2, and DU-145 cells were trypsinized and suspended in RPMI 1640 medium containing 10% FBS. Cells (0.5×10^4 cells/ml) were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 1 day, 4 μM compounds were added to the cultures. Cells were incubated for 68 h, and then viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[37,38] The percentage of net growth is shown with values adjusted by subtracting the initial cell concentration of experimental cultures from the initial concentrations of control cultures which were defined as 100%. Values for percent net growth were calculated with the following formula: [(absorbance of experimental cell concentration) − (absorbance of initial cell concentration)/(absorbance of control cell concentration) − (absorbance of initial cell concentration)] × 100.

HL60 cells (1×10^5 cells/ml) were grown in RPMI 1640 medium containing 10% FBS with 4 μM compounds for 72 h. Cell number was estimated by an electric particle counter and viability by trypan blue dye exclusion. Values for percent net cell growth were calculated with the following formula: [(cell concentration of experimental culture) − (initial cell concentration)/(cell concentration of control culture) − (initial cell concentration)] × 100.

4.4. Analysis of DNA fragmentation by agarose gel electrophoresis

DNA isolation was performed as described previously.[39] Briefly, HL60 cells (2×10^5 cells/ml) treated with various compounds were harvested and washed with ice-cold phosphate-buffered saline (PBS) (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 136.9 mM NaCl, pH 7.2). Cells were suspended in PBS (2.5×10^6 cells/ml) containing 0.5 mg/ml Proteinase K, 0.5 mg/ml RNase A, and 1% SDS, and were incubated at 37 °C for 30 min. After addition of a NaI solution (6 M NaI, 13 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, 10 mg/ml glycogen as a carrier, and 26 mM Tris–HCl, pH 8.0), cells were incubated at 60 °C for 15 min and diluted with an equal volume of isopropanol. Cells were mixed vigorously and placed at room temperature for 15 min. Mixtures were centrifuged at 10,000*g* for 15 min, and supernatants were discarded. Precipitated DNA was suspended in 1 ml of 50% isopropanol, vortexed, and centrifuged at 10,000*g* for 15 min to recover DNA. Pellets after washing with 100% isopropanol were dried under vacuum and dissolved in sample solution for analysis by agarose gel electrophoresis. Electrophoresis was performed on 2% agarose gel at 100 V/gel. The presence of DNAs in the gels was visualized by ethidium bromide.

4.5. Incorporation of *p*-aminophenols into cells

HL60 cells were grown in RPMI 1640 medium containing 10% FBS and harvested by the centrifugation at 250*g* for 10 min. Cell number was estimated by an electric particle counter and viability by trypan blue dye exclusion. Cells were washed with RPMI 1640 medium containing 10% FBS three times and suspended in the same medium. Then, cells (1×10^6 cells/ml) treated with compounds at the concentration of 10 μM were incubated for the indicated time at 37 or 4 °C. After the centrifugation at 250*g* for 10 min, cells were washed three times with ice-cold PBS (1 ml) and then suspended in 100 μl ice-cold PBS. Compounds associated with HL60 cells were determined and quantified by high pressure liquid chromatography (HPLC). Compounds incorporated into cells (100 μl) were extracted with 200 μl of ethyl acetoacetate by vortexing for 1 min. The organic layer (150 μl) obtained after centrifugation (10,000*g*, 5 min) was dried under vacuum. The residue was dissolved in 50 μl of the HPLC mobile phase solvent (80% MeOH, 20% H_2O, and 10 mM ammonium acetate), and then its aliquot (20 μl) of the supernatant was analyzed by HPLC using a Shimadzu LC-6A high pressure pump, Shimadzu CTO-10AS column oven including injector, and an SPD-6A UV spectrophotometric detector (Shimadzu Co. Ltd, Kyoto, Japan). A LUNA® 5 μ C18(2) column (150 × 4.6 mm, Phenomenex, Rancho Palos Verdes, CA) was used to separate compounds. The column was eluted with 80% MeOH, 20% H_2O, and 10 mM ammonium acetate at a flow rate of 1.0 ml/min. Compounds were detected with UV monitoring at 240 nm which wavelengths were maximum absorbance for compounds. Measurements were made using the ratio of peak areas to internal standards. Values for cell incorporation were calculated with the extraction efficiencies of each compound.

Acknowledgments

We thank Dr. Terrence Burke, Jr. for helpful comments. This investigation was supported in part by the Ministry of Education, Science, Sports and Culture of Japan.

References and notes

1. Abou-Issa, H.; Webb, T. E.; Minton, J. P.; Moeschberger, M. *J. Natl. Cancer Inst.* **1989**, *81*, 1820–1823.
2. Meyskens, F., Jr.; Alberts, D. S.; Salmon, S. E. *Int. J. Cancer* **1983**, *32*, 295–299.
3. Moon, R. C.; Metha, R. G.; Rao, K. V. N. In *The Retinoids: Biology, Chemistry, and Medicine*; Sporn, M. B., Roberts, A. B., Goodmans, D. S., Eds.; Raven Press Ltd: New York, 1994; Vol. 2, pp 573–595.
4. Pienta, K. J.; Nguyen, N. M.; Lehr, J. E. *Cancer Res.* **1993**, *53*, 224–226.
5. Decensi, A.; Bruno, S.; Costantini, M.; Torrisi, R.; Curotto, A.; Gatteschi, B.; Nicolo, G.; Polizzi, A.; Perloff, M.; Malone, W. F., et al. *J. Natl. Cancer Inst.* **1994**, *86*, 138–140.
6. Decensi, A.; Fontana, V.; Fioretto, M.; Rondanina, G.; Torrisi, R.; Orengo, M. A.; Costa, A. *Eur. J. Cancer* **1997**, *33*, 80–84.
7. Rotmensz, N.; De Palo, G.; Formelli, F.; Costa, A.; Marubini, E.; Campa, T.; Crippa, A.; Danesini, G. M.; Delle Grottaglie, M.; Di Mauro, M. G., et al. *Eur. J. Cancer* **1991**, *27*, 1127–1131.
8. Reynolds, C. P. *Curr. Oncol. Rep.* **2000**, *2*, 511–518.
9. Veronesi, U.; De Palo, G.; Costa, A.; Formelli, F.; Decensi, A. *IARC Sci. Publ.* **1996**, *136*, 87–94.
10. Vaishampayan, U.; Heilbrun, L. K.; Parchment, R. E.; Jain, V.; Zwiebel, J.; Boinpally, R. R.; LoRusso, P.; Hussain, M. *Invest. New Drugs* **2005**, *23*, 179–185.
11. Puduvalli, V. K.; Li, J. T.; Chen, L.; McCutcheon, I. E. *Cancer Res.* **2005**, *65*, 1547–1553.
12. Lovat, P. E.; Ranalli, M.; Bernassola, F.; Tilby, M.; Malcolm, A. J.; Pearson, A. D.; Piacentini, M.; Melino, G.; Redfern, C. P. *Int. J. Cancer* **2000**, *88*, 977–985.
13. Lovat, P. E.; Ranalli, M.; Bernassola, F.; Tilby, M.; Malcolm, A. J.; Pearson, A. D.; Piacentini, M.; Melino, G.; Redfern, C. P. *Med. Pediatr. Oncol.* **2000**, *35*, 663–668.
14. Lovat, P. E.; Ranalli, M.; Annichiarrico-Petruzzelli, M.; Bernassola, F.; Piacentini, M.; Malcolm, A. J.; Pearson, A. D.; Melino, G.; Redfern, C. P. *Exp. Cell Res.* **2000**, *260*, 50–60.
15. Oridate, N.; Suzuki, S.; Higuchi, M.; Mitchell, M. F.; Hong, W. K.; Lotan, R. *J. Natl. Cancer Inst.* **1997**, *89*, 1191–1198.
16. Delia, D.; Aiello, A.; Lombardi, L.; Pelicci, P. G.; Grignani, F.; Grignani, F.; Formelli, F.; Menard, S.; Costa, A.; Veronesi, U., et al. *Cancer Res.* **1993**, *53*, 6036–6041.
17. Delia, D.; Aiello, A.; Formelli, F.; Fontanella, E.; Costa, A.; Miyashita, T.; Reed, J. C.; Pierotti, M. A. *Blood* **1995**, *85*, 359–367.
18. Takahashi, N. *Biol. Pharm. Bull.* **2000**, *23*, 222–225.
19. Takahashi, N.; Sausville, E. A.; Breitman, T. R. *Clin. Cancer Res.* **1995**, *1*, 637–642.
20. Takahashi, N.; Ohba, T.; Togashi, S.; Fukui, T. *J. Biochem.* **2002**, *132*, 767–774.
21. Takahashi, N.; Honda, T.; Ohba, T. *Bioorg. Med. Chem.* **2006**, *14*, 409–417.
22. Takahashi, N.; Ohba, T.; Yamauchi, T.; Higashiyama, K. *Bioorg. Med. Chem.* **2006**, *14*, 6089–6096.
23. Takahashi, N.; Tamagawa, K.; Kubo, Y.; Fukui, T.; Wakabayashi, H.; Honda, T. *Bioorg. Med. Chem.* **2003**, *11*, 3255–3260.
24. Formelli, F.; Clerici, M.; Campa, T.; Di Mauro, M. G.; Magni, A.; Mascotti, G.; Moglia, D.; De Palo, G.; Costa, A.; Veronesi, U. *J. Clin. Oncol.* **1993**, *11*, 2036–2042.
25. Abou-Issa, H.; Curley, R. W., Jr.; Alshafie, G. A.; Weiss, K. L.; Clagett-Dame, M.; Chapman, J. S.; Mershon, S. M. *Anticancer Res.* **2001**, *21*, 3839–3844.
26. Costa, A.; Malone, W.; Perloff, M.; Buranelli, F.; Campa, T.; Dossena, G.; Magni, A.; Pizzichetta, M.; Andreoli, C.; Del Vecchio, M., et al. *Eur. J. Cancer Clin. Oncol.* **1989**, *25*, 805–808.
27. Kaiser-Kupfer, M. I.; Peck, G. L.; Caruso, R. C.; Jaffe, M. J.; DiGiovanna, J. J.; Gross, E. G. *Arch. Ophthalmol.* **1986**, *104*, 69–70.
28. Weiss, K. L.; Alshafie, G.; Chapman, J. S.; Mershon, S. M.; Abou-Issa, H.; Clagett-Dame, M.; Curley, R., Jr. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1583–1586.
29. Walker, J. R.; Alshafie, G.; Nieves, N.; Ahrens, J.; Clagett-Dame, M.; Abou-Issa, H.; Curley, R., Jr. *Bioorg. Med. Chem.* **2006**, *14*, 3038–3048.
30. Alshafie, G. A.; Walker, J. R.; Curley, R., Jr.; Clagett-Dame, M.; Highland, M. A.; Nieves, N. J.; Stonerock, L. A.; Abou-Issa, H. *Anticancer Res.* **2005**, *25*, 2391–3298.
31. Batist, G.; Tulpule, A.; Sinha, B. K.; Katki, A. G.; Myers, C. E.; Cowan, K. H. *J. Biol. Chem.* **1986**, *261*, 15544–15549.
32. Cohen, L. H.; Griffioen, M.; Havekes, L.; Schouten, D.; van Hinsbergh, V.; Kempen, H. J. *Biochem. J.* **1984**, *222*, 35–39.
33. Stone, K. R.; Mickey, D. D.; Wunderli, H.; Mickey, G. H.; Paulson, D. F. *Int. J. Cancer* **1978**, *21*, 274–281.
34. Collins, S. J.; Gallo, R. C.; Gallagher, R. E. *Nature* **1977**, *270*, 347–349.
35. Robertson, K. A.; Emami, B.; Collins, S. J. *Blood* **1992**, *80*, 1885–1889.
36. Robertson, K. A.; Emami, B.; Mueller, L.; Collins, S. J. *Mol. Cell. Biol.* **1992**, *12*, 3743–3749.
37. Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589–601.
38. Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936–942.
39. Ishizawa, M.; Kobayashi, Y.; Miyamura, T.; Matsuura, S. *Nucl. Acids Res.* **1991**, *19*, 5792.