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Bioorganic & Medicinal Chemistry 14 (2006) 409–417
Bioorganic & Medicinal Chemistry

Anticancer and superoxide scavenging activities of *p*-alkylaminophenols having various length alkyl chains

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Received 3 July 2005; revised 7 August 2005; accepted 9 August 2005
Available online 3 October 2005

Abstract—A series of *p*-alkylaminophenols including **3**, *p*-butylaminophenol; **4**, *p*-hexylaminophenol; **5**, *p*-octylaminophenol; and **6**, *N*-(*p*-methoxybenzyl)aminophenol were synthesized based on the structure of fenretinide, *N*-(4-hydroxyphenyl)retinamide (**1**). This latter agent is a synthetic amide of all-*trans*-retinoic acid (RA), which is a cancer chemopreventive and antiproliferative agent. It was found that elongation of the alkyl chain length in these compounds increased antioxidative activity and inhibition of lipid peroxidation. These findings led us to investigate whether antiproliferative activity against cancer cells was effected by the length of alkyl chains linked to the aminophenol residue. All *p*-alkylaminophenols inhibited growth of HL60 and HL60R cells in a dose-dependent manners. The HL60R line is a resistant clone against RA. Growth of various cancer cell lines (HL60, HL60R, MCF-7, MCF-7/Adr^R, HepG2, and DU-145) was suppressed by *p*-alkylaminophenols in a fashion dependent on the aminophenol alkyl chain length (**5** > **4** > **3** > *p*-methylaminophenol (**2**)), with **5** being the most potent inhibitor of cell growth against HL60R, MCF-7/Adr^R, and DU-145 cells among *p*-alkylaminophenols tested, including **1**. In particular, with the exception of compound **2**, antiproliferative activity against DU-145 cells by these *p*-alkylaminophenols was greater than by **1**. In HL60 cells, growth inhibition was associated with apoptosis. On the other hand, elongation of the alkyl chain length reduced superoxide trapping capability (**2** > **3** > **4** > **5**) in contrast to the effects on inhibition of lipid peroxidation. These results indicate that anticancer activity of *p*-alkylaminophenols correlated with the inhibitory activity of lipid peroxidation, but not with the superoxide scavenging activity.

1. Introduction

N-(4-Hydroxyphenyl)retinamide (**1**, fenretinide) (Fig. 1), a synthetic derivative of all-*trans*-retinoic acid (RA), is an effective chemopreventive and antiproliferative agent,[1–4] which is used against a wide variety of tumor types. Compound **1** currently is in clinical trial for the treatment of breast, bladder, and neuroblastoma malignancies.[4–9] Compound **1** induces apoptosis in HL60 and NB4 human leukemia cell lines, as well as in C33A, a human cervical carcinoma cell line and in neuroblastoma.[9–15] Compound **1** alone is a poor inducer of differentiation of HL60 cells as compared to RA.[16]

The mechanism(s) of **1** action is unclear because **1** binds poorly or not at all to RA nuclear receptors (RARs and RXRs),[16–23] which directly activate transcription of target genes by binding to specific DNA sequences. In addition, the mechanism of action of **1** may involve oxidative pathways, since **1** generates reactive oxygen species (ROS) and apoptosis induced by **1** is decreased by addition of the antioxidants α-tocopherol (vitamin E) and *N*-acetyl-L-cysteine in HL60 cells and MCF-7 human breast cancer cell lines and by pyrrolidine dithiocarbamate (PDTC) and nordihydoroguaiaretic acid in C33A cells.[12,24–26] On the other hand, **1** itself exhibits antioxidative activity equivalent to vitamin E.[26]

To clarify mechanisms of **1** action, we studied biological activities of 4-aminophenol, *p*-methylaminophenol (**2**), and *p*-aminoacetophen, which are derived from the side-chain amido portion of **1** that distinguishes it from RA, a poor inducer of apoptosis. Compound **2**, which lacks partial functionality of **1**, exhibited antioxidant activity. It scavenged DPPH radicals and reduced lipid peroxidation in rat liver microsomes. It also had anticancer activity, suppressing growth of HL60 and HL60R cells to the same extent as **1**, and it induced apoptosis of HL60 cells.[27] Recently, we found that elongation of alkyl chains in *p*-alkylaminophenols increased

Keywords: Aminophenol; Fenretinide; Antioxidant; Anticancer; Retinoic acid.
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doi:10.1016/j.bmc.2005.08.016

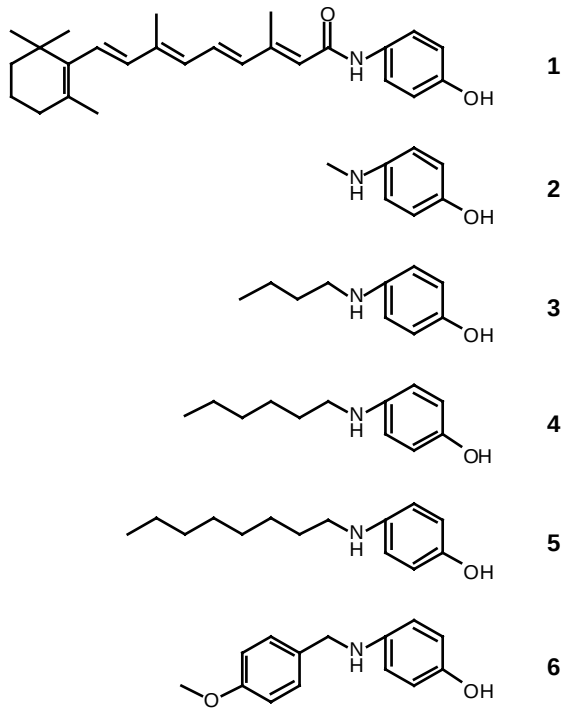

Figure 1. Chemical structures of *p*-alkylaminophenols (**2**, **3**, **4**, **5**, and **6**) and **1**.

antioxidant activity, reducing lipid peroxidation, markedly. These findings raised questions whether the anticancer activity of *p*-alkylaminophenols is dependent on alkyl chain length and whether *p*-alkylaminophenols also scavenge superoxide and oxi-radicals other than hydroxy radicals. In the current study, these questions were evaluated using *p*-alkylaminophenols (Fig. 1) with various lengths of alkyl residues.

2. Results

2.1. Inhibition of HL60 and HL60R cell growth by *p*-alkylaminophenols

HL60 and HL60R cells, which are resistant to RA, were grown in medium containing 10% FBS in the presence of *p*-alkylaminophenols (Fig. 1). Compound **1**, a parent compound, was used as an internal standard for inhibition of cell growth. The percentage of net cell growth in the presence of *p*-alkylaminophenol 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% at each time period. Growth of HL60 and HL60R cells was inhibited by various concentrations of *p*-alkylaminophenols and **1** in dose-dependent manners (Fig. 2A). In HL60 cells, at low concentration in the range of 0.1 μM to **1** μM, **5** was a more potent inhibitor of cell growth than **1**, while in HL60R cells this order was reverse (Fig. 2A). The percentage of net growth of HL60 and HL60R cells in the presence of **4** μM *p*-alkylaminophenol is shown in Figure 2B. Compound **5** (approximately 60% inhibition) was as potent as **1** (approximately 62% inhibition) in HL60 cells. In contrast, **5** (approximately 70% inhibition) was less potent than **1** (approximately 90% inhibition) in HL60R cells. In both HL60 and HL60R cells, elongation of the alkyl chain attached to the aminophenol residue increased antiproliferative activities. Compound **5** inhibited cell growth to a greater extent than **6**, which has a methoxybenzyl residue instead of an alkyl chain. These results indicate that the inhibition of cell growth by both compounds cannot be attributed to cell necrosis, and that leukemia cell growth is suppressed by **5** more strongly as compared with other aminophenols. Compound **5** was the most potent inhibitor among the five *p*-alkylaminophenols examined. This may indicate that hydrophobic properties of an octanyl residue linked to the aminophenol may be significant for antitumor activity.

2.2. Effects on growth of various cancer cell lines

We examined whether *p*-alkylaminophenol or **1** affects breast cancer cell growth. As shown in Figure 3, 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 **2**, **3**, **4**, **5**, **6** or **1** at concentrations of 4 μM. In MCF-7 cells, cell growth was inhibited approximately 75% by **1**, 8% by **2**, 34% by **3**, 37% by **4**, 70% by **5**, and 25% by **6**, and approximately 67% by **1**, 13% by **2**, 49% by **3**, 55% by **4**, 98% by **5**, and 38% by **6** in MCF-7/Adr^R cells (Fig. 3). Thus, while **1** showed cell growth inhibitory effects as great as **5** against MCF-7 cells, **1** was less potent than **5** in MCF-7/Adr^R cells, which are resistant to RA (Fig. 3).[28,29] Growth inhibition by these compounds was carried out in dose-dependent manner (data not shown). These results indicate that **5** is a potent growth inhibitor against human breast cancer cell lines, particularly RA-resistant cells. In addition, the extent of antiproliferative activity against breast cancer cells was also enhanced depending on the length of the alkyl residue.

Proliferation of DU-145 and HepG2 cells was suppressed by *p*-alkylaminophenols and **1** (Fig. 4). At 4 μM concentrations, antiproliferative activities against DU-145 cells were approximately 65% for **1**, 43% for **2**, 75% for **3**, 78% for **4**, 86% for **5**, and 50% for **6** (Fig. 4). These results suggested that **3**, **4**, and **5** showed more potent cell growth inhibitory activities than **1**, which is well known as an effective compound against prostate cancer. Growth inhibitory activity increased depending on the alkyl chain length.

In contrast, the percentage of growth inhibition against HepG2 cells by *p*-alkylaminophenols was much less than against DU-145 cells, whereas **5** was as potent as **1** against HepG2 cells (approximately 45% growth inhibition). At 4 μM concentrations, growth of HepG2 cells was inhibited by approximately 10% for **2**, 12% for **3**, 20% for **4**, and 10% for **6**. These results indicated that elongation of the alkyl chain enhanced cell growth inhibition. Replacing the alkyl moiety with a methoxybenzyl residue resulted in marked loss of inhibitory activity,

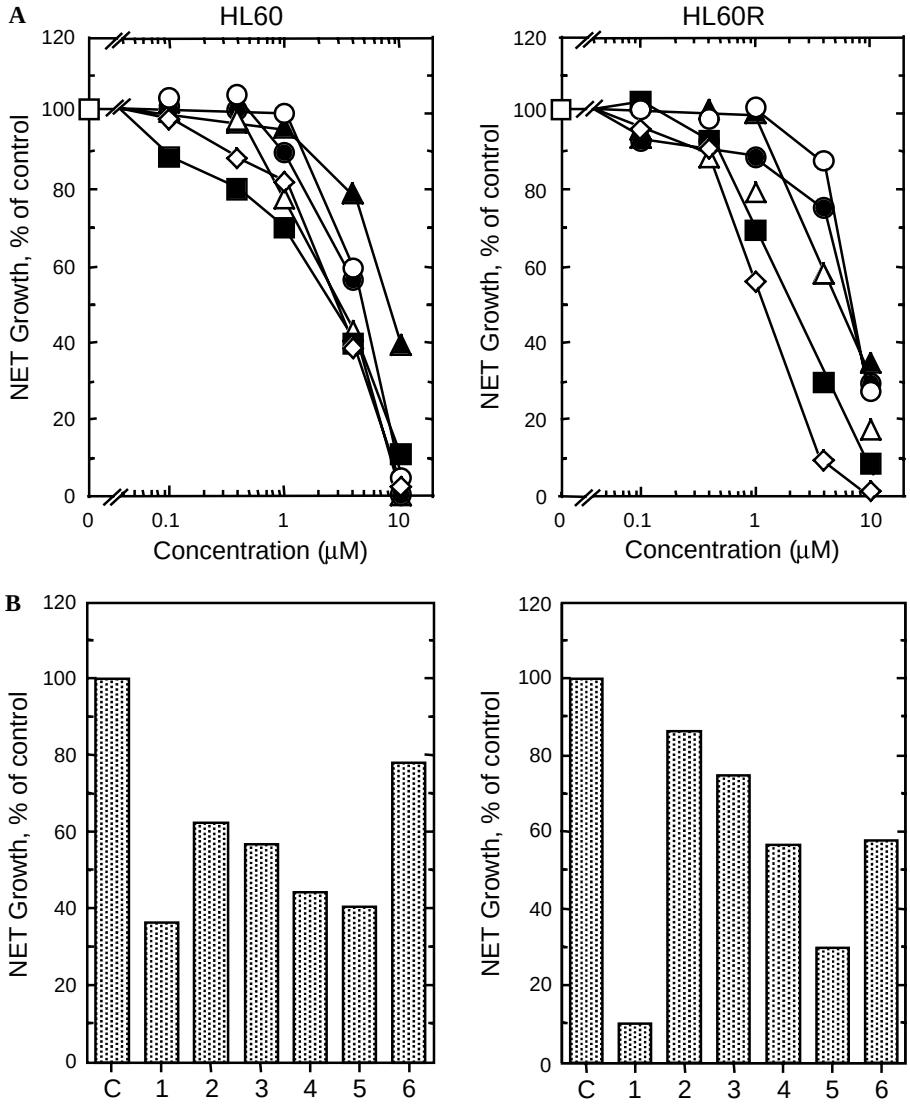

Figure 2. Growth of HL60 and HL60R cells in the presence of *p*-alkylaminophenols and **1**. (A) Cells (2 for HL60 or 1 for HL60R $\times 10^5$ cells/ml) were grown without (□) or with various concentrations of **1** (◊) and *p*-alkylaminophenols (**2** (○), **3** (●), **4** (△), **5** (■), and **6** (▲)) in RPMI medium containing 10% FBS. Growth was measured at 113 h for HL60 or 67 h for HL60R as described under Section 4. (B) Net growth percent of control (C) was shown for each compound at 4 μM concentration. Each point is the mean of at least four measurements. The SD of each point was ≤8% of the mean.

thereby indicating that this functionality is essential for action against cancer cells.

2.3. Agarose gel analysis of DNA fragmentation in HL60 cells

The finding that the length of the alkyl residue in the *p*-alkylaminophenols affects cell growth inhibitory potency led us to investigate the effect of these *p*-alkylaminophenols on induction of apoptosis. DNA isolated from HL60 cells treated with *p*-alkylaminophenols for 48 h was visualized on agarose gel. DNA extracted from cells treated with DMSO showed no fragmentation (Fig. 5A). In contrast, cells treated with 10 μM concentrations of *p*-alkylaminophenols contained fragmented ladder DNA (Fig. 5). The intensity of ladder DNA increased depending on the length of the alkyl chain of aminophenol. These results indicate that *p*-alkylaminophenols may potentially induce apoptosis of HL60 cells and thereby inhibit cell growth.

2.4. Scavenging of superoxides

The superoxide scavenging activity of compounds was measured using the absorbance of blue formazan produced from the yellow dye NBT^{2+}. Blue formazan was formed by superoxide remaining after compounds scavenged superoxide produced by a hypoxanthine/xanthine oxidase system. The absorbance of control was approximately 0.8. At 10 μM concentration, all *p*-alkylaminophenols scavenged superoxides completely (>85%) (Fig. 6), which showed no effects on xanthine oxidase activity (Fig. 7). In contrast, RA and **1** were inactive of both scavenging of superoxides and production of superoxides by the xanthine oxidase system (Fig. 6). In addition, **2**, **3**, **4**, **5**, and **6** eliminated superoxide in

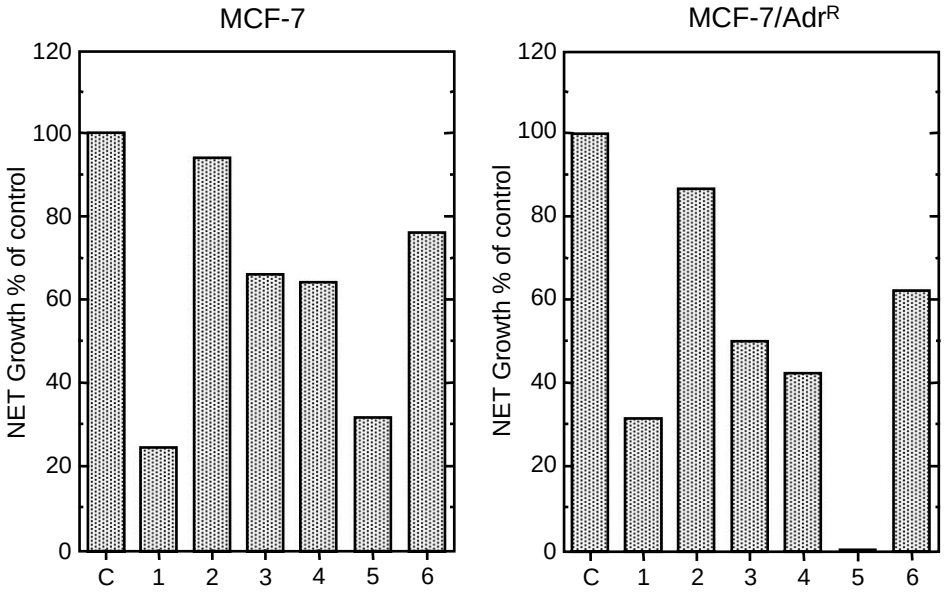

Figure 3. Effects on growth of MCF-7 and MCF-7/Adr^R cells by *p*-alkylaminophenols with various lengths of alkyl chains. MCF-7 and MCF-7/Adr^R cells were grown in the presence of **1** and *p*-alkylaminophenols (**2**, **3**, **4**, **5**, and **6**) at 4 μM concentration or DMSO (Control, C) in medium containing 10% FBS for 68 h. The SD of each data point was ≤8% of the mean.

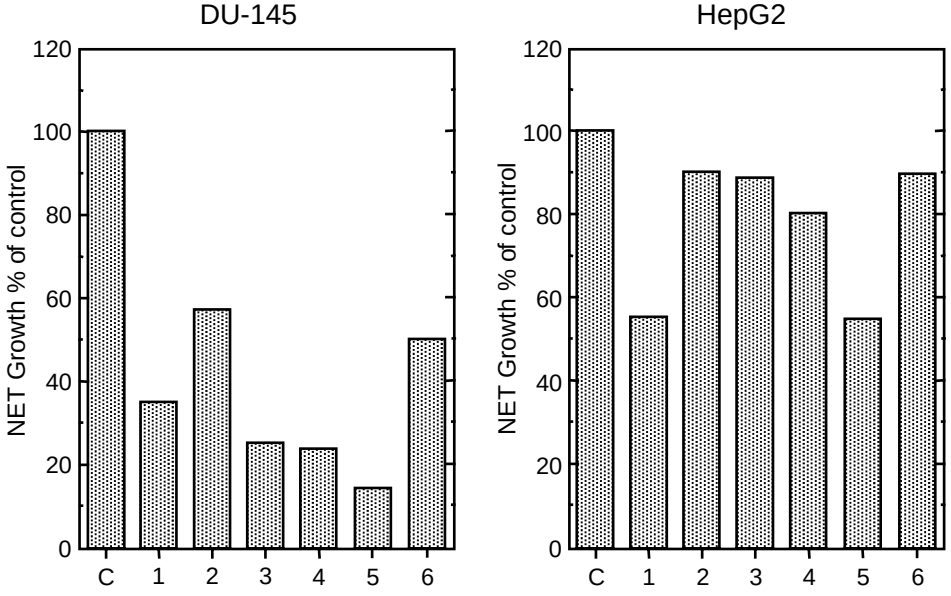

Figure 4. Growth of DU-145 and HepG2 cells in the presence of *p*-alkylaminophenols with various lengths of alkyl chains. DU-145 and HepG2 cells were grown in the presence of **1** and *p*-alkylaminophenols (**2**, **3**, **4**, **5**, and **6**) at 4 μM concentration or DMSO (C) in medium containing 10% FBS for 68 h. The SD of each data point was ≤8% of the mean.

dose-dependent manner (Fig. 8). Surprisingly, **2**, which has the shortest alkyl chain, was the most potent superoxide scavenger among these *p*-alkylaminophenols. At a concentration of 1 μM, the extent of superoxide decreased approximately 72% by **2**, 49% by **3**, 40% by **4**, 38% by **5**, and 34% by **6**. IC_{50} values are approximately 0.6 μM for **2**, 1 μM for **3**, and 1.5 μM for **4**, **5**, and **6**. These results indicate that all *p*-alkylaminophenols exhibited antioxidant activity, scavenging superoxide and that elongation of the alkyl chain reduced the capability to trap superoxide.

3. Discussion

The synthetic retinoid, **1**, is a cancer chemopreventive, antiproliferative agent, and a potent inducer of apoptosis. Its mechanism of action may rely on the generation of ROS. Compound **2**, which lacks partial functionality of **1**, is a more potent antioxidant than **1**, exhibiting antioxidant activity, scavenging DPPH radicals, and reducing lipid peroxidation in rat liver microsomes. In addition, **2** suppresses growth of HL60 and HL60R cells to the same extent as **1** and induces apoptosis in HL60

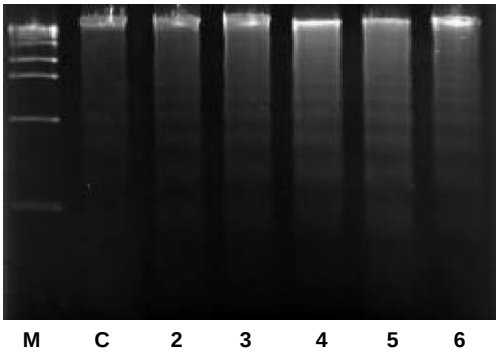

Figure 5. DNA fragmentation of HL60 cells grown in the presence of *p*-alkylaminophenols. HL60 cells (4×10^5 cells/ml) were grown in the presence of **2**, **3**, **4**, **5**, and **6** at the concentration of 10 μM or DMSO (C) in medium containing 10% FBS for 48 h. DNA was extracted from the cells and DNA fragmentation was measured by agarose gel electrophoresis as described under Section 4. M, marker.

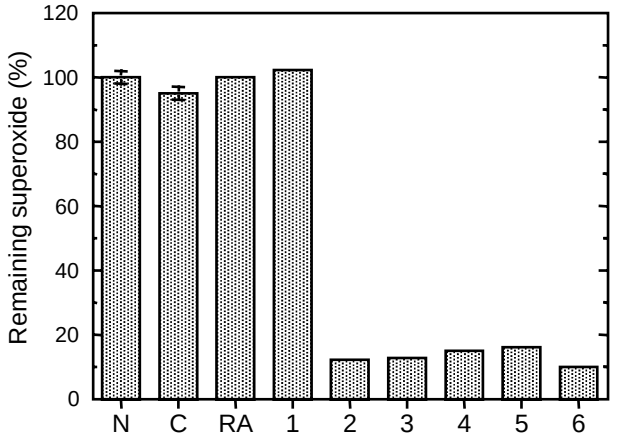

Figure 6. Superoxide scavenging activity by *p*-alkylaminophenols, RA, and **1**. Reaction mixtures containing compounds (*p*-alkylaminophenols, RA, and **1** at the concentration of 10 μM) or DMSO (C) were mixed, and then xanthine oxidase was added as described under Section 4. After the incubation at 25 °C for 20 min, $CuCl_2$ was added to the reaction mixture. The subsequent extent of NBT reduction was determined at 560 nm by spectrophotometer. The SD of each data point was ≤8% of the mean. N, none.

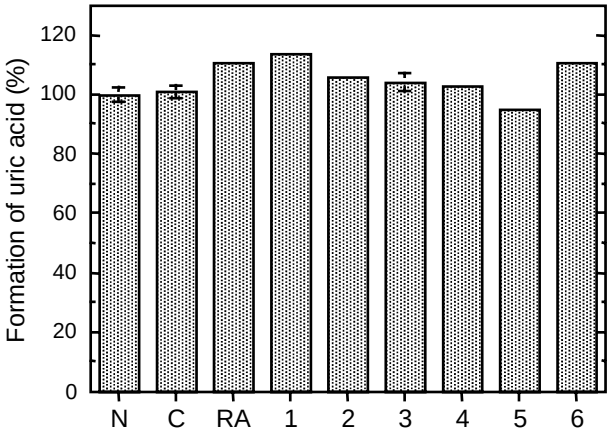

Figure 7. Effects of the formation of uric acid by *p*-alkylaminophenols, RA, and **1**. Reaction mixtures containing compounds (*p*-alkylaminophenols, RA, and **1** at the concentration of 10 μM) or DMSO (C) without NBT solution were mixed, and then xanthine oxidase was added as described under Section 4. After the incubation at 25 °C for 20 min, $CuCl_2$ was added to the reaction mixture. Absorbances of uric acid were determined at 290 nm by spectrophotometer. The SD of each data point was ≤8% of the mean.

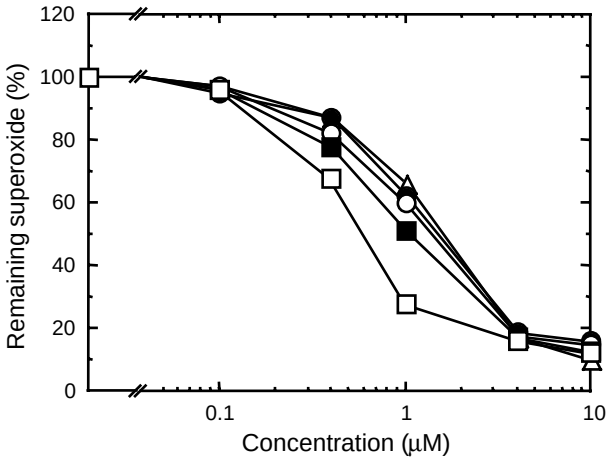

Figure 8. Superoxide scavenging activity by various concentrations of *p*-alkylaminophenols. Reaction mixtures containing various concentrations of *p*-alkylaminophenols (**2** (□), **3** (■), **4** (○), **5** (●), and **6** (△)) were mixed, and then xanthine oxidase was added as described under Section 4. After the incubation at 25 °C for 20 min, $CuCl_2$ was added to the reaction mixture. The subsequent extent of NBT reduction was determined at 560 nm by spectrophotometer. The SD of each data point was ≤8% of the mean.

cells. In contrast, **2** is an effective antiproliferative agent against various other cancer cells, but is less potent than **1**. In the current study, elongation of alkyl chain length in *p*-alkylaminophenols based on the structure of **1** increased antiproliferative activities against several cancer cell lines (HL60, HL60R, MCF-7, MCF-7/Adr^R, HepG2, and DU-145 cells). Analog **5** was the most potent inhibitor of cell growth in MCF-7/Adr^R, and DU-145 cells among *p*-alkylaminophenols as well as **1**. Antiproliferative activities of *p*-alkylaminophenols against DU-145 cells were greater than by **1** with the exception of **2**. Growth inhibition by *p*-alkylaminophenols was also associated with apoptosis in HL60 cells. In contrast, **2** was the most potent scavenger of superoxide.

Previous studies have shown that*p*-alkylaminophenols (**2**, **3**, **4**, **5**, and **6**) exhibit DPPH radical quenching activity in 1:2 ratios, whereas **1** exhibits a 1:1 ratio, and RA was inactive. The extent of reduction of lipid peroxidation by *p*-alkylaminophenols depends on the length of the alkyl chain bound to the aminophenol. Compound **5** is the most potent inhibitor of lipid peroxidation, being approximately 350-fold higher than **2** or **1** and 220 more potent than RA. In the current study, **2** was the most potent inhibitor of superoxide radical scavenging activity, which is another antioxidant activity. The hydrophobic nature of the compounds was not related to Superoxide trapping.

As described above, the superoxide scavenging activity of **2** is more potent than **5** and **1**. Inhibition of lipid

peroxidation by **5** is more potent than **2** and **1**. In contrast, growth inhibition of various cancer cell lines by **5** and **1** is greater than by **2**. Whether the mechanism(s) behind these two different actions is attributable, the same functionality is becoming clearer. The results of the current study indicate that superoxide scavenging activity may be due to the phenolic hydroxyl functionality. In contrast, inhibition of lipid peroxidation may be due to the three-dimensional structure formed by phenolic hydroxyl and alkyl residues. Anticancer action may be attributed to phenolic hydroxyl and alkyl functionality attached to the aminophenol. The alkyl residue may be particularly significant for cell–drug contact and anticancer action. It would be interesting to examine further the activity of other aminophenols linked to various alkyl chain lengths longer than **5**.

Treatment of patients with **1** was accompanied by side effects of night blindness brought on by decreasing serum retinol levels.[23] Since **1** binds to the nuclear retinoid receptors RARs and RXRs very poorly, the mechanism of **1** action is still unclear. However, **1** appears to act on cells directly rather than through hydrolysis to free RA. Recently, it was revealed that 4-hydroxybenzylretinone (4-HBR), a stable C-linked analog of **1**, exhibits antitumor effects to an extent similar to **1**.[23,30] However, it was reported that 4-HBR did not exhibit side effects of night blindness,[23] even though both **1** and 4-HBR contain the same aromatic ring as retinol. In the current study, we showed that **5** which has a markedly different structure than retinol is the most potent antiproliferative agent among *p*-alkylaminophenols against various cancer cell lines. Therefore, it is possible that **5** may be an anticancer drug without the side effects observed in **1**.

Certain forms of cancer, cardiovascular and cerebrovascular diseases, and ischemia/reperfusion injuries are considered to occur by free radicals. Antioxidants, including vitamins and flavonoids, prevent these diseases.[31–34] Our study indicates that **2** may be effective in diseases involving superoxide and that **5** affects diseases concerned with hydroxy radicals. The antioxidant and antiproliferative activities of **2** may influence many physiological processes, including immunostimulation, enhancement of cell communication, and inhibition of metabolic activation. Therefore, *p*-alkylaminophenols may potentially be useful as lead compounds for development of therapeutics against a variety of diseases, which are currently being treated using antioxidants, and **1**.

In the current study, **5** inhibited the growth of the RA-resistant cancer cell lines, HL60R and MCF-7/Adr^R, more potently than the RA-sensitive cancer cell lines, HL60 and MCF-7 (Figs. 2 and 3). In cytodifferentiation therapy, a high percentage of patients in complete remission induced by RA alone relapse within a few months.[35–37] and most relapsed patients are resistant to further treatment with RA[37–39] The clinical outcome of patients treated with RA may be modified by administration of **5**. Studies are presently underway to determine the toxicity of **5** in animals and to measure the effectiveness of **5** on tumors growing in animals.

4. Experimental

4.1. General

RA, nitroblue tetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA, fraction V), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO, USA). Compound **1** was provided by Dr. R. C. Moon, University of Illinois, Chicago, IL, USA. Compound **2** and dithiothreitol (DTT) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

4.2. Cells

Early passage (<30) human myeloid leukemia cell lines, HL60 and HL60R, a mutant subclone of HL60 that exhibits relative resistance to RA and that harbors RA receptors with markedly reduced affinity for RA, were maintained 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).[40–42] Human breast cancer cell lines, MCF-7 and MCF-7/Adr^R, were obtained from the American Type Culture Collection, Rockville, MD.[43] Human hepatoma cell line, HepG2, was obtained from RIKEN cell bank (Tokyo, Japan).[44] Human prostate cancer cell line, DU-145, was obtained from Dr. Y. Pommier of the National Cancer Institute (Bethesda, MD, USA).[45] MCF-7, MCF-7/Adr^R, HepG2, and DU-145 cells were grown in RPMI medium containing 10% FBS and subcultured every week. Attached cells were removed from the tissue culture flask surface with trypsin–EDTA (0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}, Gibco).

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

HL60 and HL60R cells (1 or 2×10^5/ml, respectively) were grown in RPMI 1640 medium containing 10% FBS and various concentrations of compounds. Cell number was estimated by an electric particle counter and viability by trypan blue dye exclusion. 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: [(cell concentration of experimental culture) − (initial cell concentration)/(cell concentration of control culture) − (initial cell concentration)] × 100.

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 or 1×10^4/ml) were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

After one day, various concentrations of compounds were added to the cultures. Cells were incubated for 72 h, and then viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described previously.[46,47] 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.

4.4. Analysis of DNA fragmentation by agarose gel electrophoresis

DNA isolation was performed as described previously.[48] Briefly, HL60 cells (2×10^5 cells/ml) treated with various compounds were harvested and washed with ice-cold PBS. Cells were suspended in PBS (1×10^6 cells/ml) containing 0.1 mg/ml proteinase K, 0.1 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*-laurylsarcosine/10 μg glycogen as a carrier/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 5 min, and supernatants were discarded. Precipitated DNA was suspended in 1 ml of 40% isopropanol, vortexed, and centrifuged at 10,000*g* for 5 min to recover DNA. Pellets 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. Measurement of superoxide anion scavenging activity

The superoxide radicals were generated in vitro by the hypoxanthine/xanthine oxidase system. The scavenging activities of compounds were determined by the NBT reduction method. In this method, $O_2^{\cdot -}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the purple NBT formation.

The capacity of compounds to scavenge the superoxide radical was measured by a partial modified method of previous assay as described.[49] Briefly, a reaction mixture was prepared with a final volume of 2.6 ml/tube. Sodium carbonate buffer (50 mM, pH 10.2) (2.0 ml), 3 mM EDTA (0.1 ml), 1.5 mg/ml BSA (0.1 ml), 0.75 mM NBT (0.1 ml), 3 mM xanthine (0.1 ml), and 2.4 μl compound were mixed, and then xanthin oxidase (0.1 ml, one unit) was added. After the incubation at 25 °C for 20 min, 6 mM $CuCl_2$ (0.1 ml) was added to the reaction mixture. The subsequent extent of NBT reduction was determined at 560 nm by spectrophotometer.

To determine effects on xanthine oxidase by compounds, the formation of uric acid was measured. Reaction mixtures without NBT solution above were incubated at 25 °C for 20 min, and 6 mM $CuCl_2$ (0.1 ml) was added to the reaction mixture. Absorbances of uric acid were determined at 290 nm by spectrophotometer.

The solutions were prepared daily and kept from light. The results are expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without sample (buffer only).

4.6. Presentation of results

Each experiment was performed at least 4 times, and most experiments were repeated at least 3 times with consistent results.

4.7. Synthesis of *p*-alkylaminophenols

Compounds **3**, **4**, **5**, or **6** was synthesized and reported as described previously.[27,50–59] Typical experimental procedures were as follows: to a stirred solution of *n*-butyraldehyde (0.72 g, 10 mmol) in absolute tetrahydrofuran (10 ml) were successively added molecular sieves 3A (4 g) and 4-aminophenol (1.1 g, 10.1 mmol), and the resulting mixture was stirred for 4 h at 5 °C. After addition of sodium borohydride (0.76 g, 20 mmol), the mixture was stirred for an additional 30 min at the same temperature. The mixture was treated with H_2O (50 ml) and the organic solvent was removed under reduced pressure. The aqueous solution was extracted with dichloromethane (3× 30 ml). The combined extracts were dried (Na_2SO_4), and evaporated in vacuo to give a residue, which was purified by column chromatography on silica gel (hexane/ethyl acetate = 6:1) to afford pure **3** (200 mg, 12%). Compound **3** was recrystallized with hexane/ethyl acetate solvent.

4.8. *p*-Butylaminophenol (3)

^{1}H NMR δ: 0.94 (3H, t, J = 7.3 Hz, 4-CH_3), 1.40 (2H, m, 3-CH_2), 1.57 (2H, m, 2-CH_2), 3.05 (2H, t, J = 7.1 Hz, 1-CH_2), 3.80–5.10 (1H, br, NH), 6.54 (2H, d, J = 8.8 Hz, Ar-H), 6.68 (2H, d, J = 8.8 Hz, Ar-H); ^{13}C NMR δ: 13.8, 20.2, 31.5, 45.4, 115.3, 116.3, 141.8, 148.5; EI-MS *m*/*z* (%): 165 (M^+) (84), 122 (100), 108 (28); HRMS Calcd for $C_{10}H_{15}NO$ (M^+) 165.1154. Found 165.1168.

4.9. *p*-Hexylaminophenol (4)

^{1}H NMR δ: 0.89 (3H, t, J = 6.7 Hz, 6-CH_3), 1.25–1.45 (7H, m, 3-, 4-, 5-CH_2 and OH), 1.54–1.64 (2H, m, 2-CH_2), 3.04 (2H, t, J = 7.0 Hz, 1-CH_2), 6.54 (2H, d, J = 8.6 Hz, Ar-H), 6.69 (2H, d, J = 8.6 Hz, Ar-H); ^{13}C NMR δ: 14.0, 22.6, 26.8, 29.6, 31.6, 45.2, 114.4, 116.2, 142.7, 147.7; EI-MS *m*/*z* (%): 193 (M^+) (30), 171 (15), 149 (27), 133 (15), 122 (100), 113 (16), 99 (12); HRMS Calcd for $C_{12}H_{19}NO$ (M^+) 193.1467. Found 193.1442.

4.10. *p*-Octylaminophenol (5)

^{1}H NMR δ: 0.88 (3H, t, J = 6.7 Hz, 8-CH_3), 1.25–1.45 (10H, m, 3-, 4-, 5-, 6-, 7-CH_2), 1.54–1.64 (2H, m, 2-CH_2), 3.04 (2H, t, J = 7.1 Hz, 1-CH_2), 3.50–4.60 (1H, br, NH), 6.54 (2H, d, J = 8.7 Hz, Ar-H), 6.68 (2H, d,

J = 8.7 Hz, Ar-H); ^{13}C NMR δ: 14.1, 22.6, 27.2, 29.2, 29.4, 29.6, 31.8, 45.3, 114.6, 116.2, 142.5, 147.8; EI-MS m/z (%): 221 (M^+) (35), 213 (25), 189 (6), 171 (22), 167 (18), 157 (14), 149 (75), 122 (100); HRMS Calcd for $C_{14}H_{23}NO$ (M^+) 211.1780. Found 221.1750.

4.11. *N*-(*p*-Methoxybenzyl)aminophenol (6)

^{1}H NMR δ: 3.80 (3H, s, OMe), 4.19 (2H, s, $NHCH_2$), 6.55 (2H, ddd, J = 2.2, 3.4, and 8.7 Hz, Ar-H), 6.68 (2H, ddd, J = 2.1, 3.5, and 8.7 Hz, Ar-H), 6.87 (2H, ddd, J = 2.0, 3.0, and 8.6 Hz, Ar-H), 7.28 (2H, brd, J = 8.6 Hz, Ar-H); ^{13}C NMR δ: 48.8, 55.3, 114.0, 114.4, 116.2, 128.8, 131.6, 142.4, 147.8, 158.8; EI-MS m/z (%): 229 (M^+) (25), 184 (2), 167 (43), 150 (12), 149 (100), 132 (5), 122 (11), 121 (82), 113 (11), 104 (11); HRMS Calcd for $C_{14}H_{15}NO_2$ (M^+) 229.1103. Found 229.1117.

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. 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: New York, 1994; Vol. 2, pp 573–595.
2. Abou-Issa, H.; Webb, T. E.; Minton, J. P.; Moeschberger, M. *J. Natl. Cancer. Inst.* **1989**, *81*, 1820–1823.
3. Meyskens, F., Jr.; Alberts, D. S.; Salmon, S. E. *Int. J. Cancer* **1983**, *32*, 295–299.
4. Pienta, K. J.; Nguyen, N. M.; Lehr, J. E. *Cancer Res.* **1993**, *53*, 224–226.
5. Decensi, A.; Fontana, V.; Fioretto, M.; Rondanina, G.; Torrisi, R.; Orengo, M. A.; Costa, A. *Eur. J. Cancer* **1997**, *33*, 80–84.
6. 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.
7. Veronesi, U.; De Palo, G.; Costa, A.; Formelli, F.; Decensi, A. *IARC Sci. Publ.* **1996**, *136*, 87–94.
8. 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.
9. Reynolds, C. P. *Curr. Oncol. Rep.* **2000**, *2*, 511–518.
10. 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.
11. Delia, D.; Aiello, A.; Formelli, F.; Fontanella, E.; Costa, A.; Miyashita, T.; Reed, J. C.; Pierotti, M. A. *Blood* **1995**, *85*, 359–367.
12. Oridate, N.; Suzuki, S.; Higuchi, M.; Mitchell, M. F.; Hong, W. K.; Lotan, R. *J. Natl. Cancer Inst.* **1997**, *89*, 1191–1198.
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. 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.
16. Takahashi, N.; Sausville, E. A.; Breitman, T. R. *Clin. Cancer Res.* **1995**, *1*, 637–642.
17. Sani, B. P.; Shealy, Y. F.; Hill, D. L. *Carcinogenesis* **1995**, *16*, 2531–2534.
18. Sheikh, M. S.; Shao, Z. M.; Li, X. S.; Ordonez, J. V.; Conley, B. A.; Wu, S.; Dawson, M. I.; Han, Q. X.; Chao, W. R.; Quick, T., et al. *Carcinogenesis* **1995**, *16*, 2477–2486.
19. Zitnik, R. J.; Kotloff, R. M.; Latifpour, J.; Zheng, T.; Whiting, N. L.; Schwalb, J.; Elias, J. A. *J. Immunol.* **1994**, *152*, 1419–1427.
20. Benedetti, L.; Grignani, F.; Scicchitano, B. M.; Jetten, A. M.; Diverio, D.; Lo Coco, F.; Avvisati, G.; Gambacorti-Passerini, C.; Adamo, S.; Levin, A. A.; Pelicci, P. G.; Nervi, C. *Blood* **1996**, *87*, 1939–1950.
21. Kazmi, S. M.; Plante, R. K.; Visconti, V.; Lau, C. Y. *Cancer Res.* **1996**, *56*, 1056–1062.
22. Fanjul, A. N.; Delia, D.; Pierotti, M. A.; Rideout, D.; Qiu, J.; Pfahl, M. *J. Biol. Chem.* **1996**, *271*, 22441–22446.
23. 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.
24. Delia, D.; Aiello, A.; Meroni, L.; Nicolini, M.; Reed, J. C.; Pierotti, M. A. *Carcinogenesis* **1997**, *18*, 943–948.
25. Hursting, S. D.; Shen, J. C.; Sun, X. Y.; Wang, T. T.; Phang, J. M.; Perkins, S. N. *Mol. Carcinog.* **2002**, *33*, 16–24.
26. Takahashi, N. *Biol. Pharm. Bull* **2000**, *23*, 222–225.
27. Takahashi, N.; Tamagawa, K.; Kubo, Y.; Fukui, T.; Wakabayashi, H.; Honda, T. *Bioorg. Med. Chem.* **2003**, *11*, 3255–3260.
28. Takahashi, N.; Breitman, T. R. *J. Steroid Biochem. Mol. Biol.* **1992**, *43*, 489–497.
29. Tamagawa, K.; Shimizu, K.; Ebine, T.; Maitani, Y.; Fukui, T.; Kawai, K.; Takahashi, N. *Clin. Cancer Res.* **2001**, *7*, 3551–3558.
30. 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.
31. Shklar, G. *Oral Oncol.* **1998**, *34*, 24–29.
32. van Poppel, G.; van den Berg, H. *Cancer Lett.* **1997**, *114*, 195–202.
33. Nagel, E.; Meyer zu Vilsendorf, A.; Bartels, M.; Pichlmayr, R. *Int. J. Vitam. Nutr. Res.* **1997**, *67*, 298–306.
34. Diplock, A. T. *Nutr. Health* **1993**, *9*, 37–42.
35. Castaigne, S.; Chomienne, C.; Daniel, M. T.; Ballerini, P.; Berger, R.; Fenaux, P.; Degos, L. *Blood* **1990**, *76*, 1704–1709.
36. Warrell, R. P., Jr.; Frankel, S. R.; Miller, W. H., Jr.; Scheinberg, D. A.; Itri, L. M.; Hittelman, W. N.; Vyas, R.; Andreeff, M.; Tafuri, A.; Jakubowski, A.; Gabrilove, J.; Gordon, M. S.; Dmitrovsky, E. *N. Engl. J. Med.* **1991**, *324*, 1385–1393.
37. Chen, Z. X.; Xue, Y. Q.; Zhang, R.; Tao, R. F.; Xia, X. M.; Li, C.; Wang, W.; Zu, W. Y.; Yao, X. Z.; Ling, B. J. *Blood* **1991**, *78*, 1413–1419.
38. Muindi, J.; Frankel, S. R.; Miller, W. H. J.; Jakubowski, A.; Scheinberg, D. A.; Young, C. W.; Dmitrovsky, E.; Warrell, R. P. J. *Blood* **1992**, *79*, 299–303.
39. Delva, L.; Cornic, M.; Balitrand, N.; Guidez, F.; Miclea, J. M.; Delmer, A.; Teillet, F.; Fenaux, P.; Castaigne, S.; Degos, L.; Chomienne, C. *Blood* **1993**, *82*, 2175–2181.

40. Collins, S. J.; Gallo, R. C.; Gallagher, R. E. *Nature* **1977**, *270*, 347–349.
41. Robertson, K. A.; Emami, B.; Collins, S. J. *Blood* **1992**, *80*, 1885–1889.
42. Robertson, K. A.; Emami, B.; Mueller, L.; Collins, S. *J. Mol. Cell. Biol.* **1992**, *12*, 3743–3749.
43. Batist, G.; Tulpule, A.; Sinha, B. K.; Katki, A. G.; Myers, C. E.; Cowan, K. H. *J. Biol. Chem.* **1986**, *261*, 15544–15549.
44. Cohen, L. H.; Griffioen, M.; Havekes, L.; Schouten, D.; van Hinsbergh, V.; Kempen, H. J. *Biochem. J.* **1984**, *222*, 35–39.
45. Stone, K. R.; Mickey, D. D.; Wunderli, H.; Mickey, G. H.; Paulson, D. F. *Int. J. Cancer* **1978**, *21*, 274–281.
46. 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.
47. Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936–942.
48. Ishizawa, M.; Kobayashi, Y.; Miyamura, T.; Matsuura, S. *Nucleic Acids Res.* **1991**, *19*, 5792.
49. Beauchamp, C.; Fridovich, I. *Anal. Biochem.* **1971**, *44*, 276–287.
50. Bickoff, E. M. *J. Am. Oil Chem. Soc.* **1951**, *28*, 65–68.
51. Gaylor, V. F.; Conrad, A. L.; Landerl, J. H. *Anal. Chem.* **1957**, *29*, 228–232.
52. Gaylor, V. F.; Conrad, A. L.; Landerl, J. H. *Anal. Chem.* **1957**, *29*, 224–228.
53. Gonchar, N. R.; Kevroleva, K. M.; Treskina, M. N. *Izv. Toms. Poli. Inst.* **1971**, *204*, 27–33.
54. Huneck, S.; Schreiber, K.; Grimmecke, H. D. *J. Plant Growth Regul.* **1984**, *3*, 75–84.
55. Lipnick, R. L.; Bickings, C. K.; Johnson, D. E.; Eastmond, D. A. *ASTM Spe. Tech. Publ.* **1985**, *891*, 153–176.
56. Politzer, P.; Murray, S. J.; Flodmark, P. *J. Phys. Chem.* **1996**, *100*, 5538–5540.
57. Shen, Q.; Shekhar, S.; Stambuli, J. P.; Hartwig, J. F. *Angew. Chem. Int. Edit.* **2005**, *44*, 1371–1375.
58. Strickland, B. R. *Anal. Chem.* **1948**, *20*, 55–56.
59. Varma, R. S.; Dahiya, R. *Tetrahedron* **1998**, *54*, 6293–6298.