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Biological Activity of *p*-Methylaminophenol, an Essential Structural Component of *N*-(4-Hydroxyphenyl)retinamide, Fenretinide[1]

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Fenretinide, *N*-(4-hydroxyphenyl)retinamide (4-HPR), is a synthetic amide of all-*trans*-retinoic acid (RA), which inhibits cell growth, induces apoptosis, and is an antioxidant, and cancer chemopreventive and antiproliferative agent. These findings led us to investigate which structural component of 4-HPR contributes to these potent activities. Our approach was to examine 4-aminophenol (4-AP), *p*-methylaminophenol (*p*-MAP), and *p*-acetaminophen (*p*-AAP). It was found that vitamin E, 4-AP and *p*-MAP scavenge α,α–diphenyl-β-picrylhydrazyl (DPPH) radicals in a 1:2 ratio, in contrast to 4-HPR and *p*-AAP, for which 1:1 and 1:0.5 ratios were observed relative to DPPH radicals. However, RA was inactive. Lipid peroxidation in rat liver microsomes was reduced by compounds (RA > *p*-MAP = 4-HPR > 4-AP) in a dose-dependent manner, while *p*-AAP was inactive. In addition, both *p*-MAP and 4-HPR are potent inhibitors of cell growth and inducers of apoptosis in HL60 cells. *p*-MAP exhibits the same level of antiproliferative activity as 4-HPR against HL60R cells, which are a resistant clone against RA, and it inhibits the growth of various cancer cell lines (MCF-7, MCF-7/Adr^R, HepG2, and DU-145) to an extent greater than 4-AP and *p*-AAP, but is less potent than 4-HPR. Thus, although the antioxidant activity of *p*-MAP is more potent than that of 4-HPR, *p*-MAP is less potent than 4-HPR in anticancer activity. These results suggest that both the anticancer and antioxidative activities shown by 4-HPR are due to the structure of *p*-MAP. The retinoyl residue or long alkyl chain substituent attached to an aminophenol may be significant for anticancer properties.

Key words: aminophenol, anticancer, antioxidant, fenretinide, retinoic acid.

N-(4-Hydroxyphenyl)retinamide (4-HPR, fenretinide) (Fig. 1), a synthetic derivative of all-*trans*-retinoic acid (RA) produced from β-carotene through retinol, is an effective chemopreventive (*1*) and antiproliferative (*2–4*) agent, which is used against a wide variety of tumor types, including breast, prostate, ovary and bladder cancers. 4-HPR is currently under clinical trial for the treatment of breast (*5–7*), bladder (*8, 9*), and neuroblastoma (*10*) malignancies. While it is generally accepted that the effects of RA and retinoids may be mediated by RA nuclear receptors (RARs and RXRs) (*11*), which directly activate transcription of target genes by binding to specific DNA sequences, the mechanism(s) of the 4-HPR action is unclear. Reports concerning 4-HPR and RA nuclear receptors are conflicting, since 4-HPR binds poorly or not at all to RARs or RXRs (*12–19*).

Previous studies have shown that 4-HPR induces apoptosis in HL60 and NB4 human leukemia cell lines (*20, 21*), as well as C33A, a human cervical carcinoma cell line (*22*), and neuroblastoma (*23–25*), while 4-HPR alone is a poor inducer of differentiation of HL60 cells as compared to RA (*12*). In addition, it has been reported that 4-HPR generates reactive oxygen species (ROS), and that apoptosis induced by 4-HPR is decreased by the addition of 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 nordihydroguaiaretic acid in C33A cells (*22, 26, 27*). On the other hand, 4-HPR itself exhibits antioxidative activity equivalent to that of vitamin E (*28*). Thus, the mechanism of action of 4-HPR may be involved in an oxidative pathway.

In order to clarify the mechanisms of the 4-HPR action, we studied the biological activities of 4-aminophenol (4-AP), p-methylaminophenol (*p*-MAP), and *p*-aminoacetophen (*p*-AAP). The latter are derived from the side chain amido portion of 4-HPR, which distinguishes it from RA, which itself is a poor inducer of apoptosis and a poor antiprofilative agent (Fig. 1). In the present study, we examine which of these structures is critical for the action of 4-HPR as an anticancer agent or antioxidant.

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Abbreviations: RA, retinoic acid; 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; ROS, reactive oxygen species; 4-AP, 4-aminophenol; *p*-MAP, *p*-methylaminophenol; *p*-AAP, *p*-aminoacetophen; 4-HPR, *N*-(4-hydroxyphenyl)retinamide.

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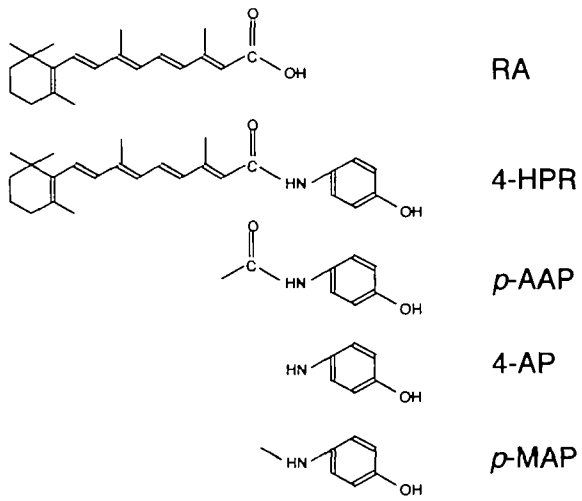

Fig. 1. **Chemical structures of aminophenols (*p*-MAP, 4-AP, and *p*-AAP), 4-HPR, and RA.**

MATERIALS AND METHODS

Chemicals—RA, α-tocopherol (vitamin E), α,α-diphenyl-β-picrylhydrazyl (DPPH), adenosine 5′-diphosphate (ADP), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO, USA). 4-HPR was provided by Dr. R.C. Moon, University of Illinois, Chicago, IL, USA. Dithiothreitol (DTT), trichloroacetic acid (TCA), *p*-methylaminophenol (*p*-MAP), and *p*-aminoacetophen (*p*-AAP) were purchased from Nacalai Tesque (Kyoto). 4-Aminophenol (4-AP) was purchased from Tokyo Kasei Kogyo (Tokyo). Ascorbic acid and 2-thiobarbituric acid (TBA) were purchased from Wako Pure Chemicals Industries (Osaka).

DPPH Radical Analysis—DPPH radical analysis was performed as described previously (*29–31*). Ethanol (2 ml) and a 500 μM DPPH ethanol solution (1 ml) were added to 0.1 M acetic acid buffer, pH 5.5 (2 ml). To this mixture (5 ml) were added 100 μl of a 1 mM solution of vitamin E, RA, 4-HPR, *p*-MAP, 4-AP, or *p*-AAP (Fig. 1) dissolved in DMSO, and cysteine dissolved in 0.1 M acetic acid buffer, pH 5.5. The final concentration of each compound is indicated in Fig. 2 (5–40 μM). After incubation at room temperature for 30 min, absorbance was measured spectrophotometrically at 517 nm (Shimazu UV-1600; Shimazu, Kyoto). Acetic acid buffer or DMSO (100 μl) was used in place of compounds as a blank control.

Preparation of Rat Liver Microsomes—Eight week-old male Sprague-Dawley rats (Slc. SD, 280–300 g) were obtained from Tokyo Laboratory Animals Science (Tokyo). All fractionation and sonication steps were performed at 0–5°C. Livers (5–8 g) were homogenized in 5 volumes (w/v) of cold 0.25 M sucrose containing 100 mM Tris-HCl (pH 7.4), 5 mM DTT, and 10 mM $MgCl_2$. The homogenate was fractionized by means of usual differential centrifugation techniques. Homogenates were centrifuged at 400 ×*g* (10 min), the supernatants were centrifuged at 10,000 ×*g* (10 min), and the resulting supernatants were then centrifuged at 105,000 ×*g* (1 h). The pellets were resuspended in 100 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, 5 mM DTT, and 10 mM $MgCl_2$, and stored at –80°C. Protein concentrations were determined by the method of Bradford (*32*).

Measurement of Microsomal Lipid Peroxidation—Lipid peroxidation in rat liver microsomes was quantitated by measurement of malondialdehyde (MDA) using ADP-chelated ions and ascorbate as described previously (*33*). Reaction mixtures consisting of microsomes (0.5 mg protein/ml) and a compound (vitamin E, RA, 4-HPR, *p*-MAP, 4-AP, or *p*-AAP dissolved in DMSO) in 100 mM Tris-HCl (pH 7.5) containing 15 μM $FeCl_3$ and 4 mM ADP were preincubated at 37°C for 1 min. Reaction mixtures in which ascorbic acid was added to a final concentration of 1 mM were incubated at 37°C for 20 min, and then an equal volume of TBA reagent (0.375% TBA and 15% TCA in 0.25 N hydrochloride) was added. Mixtures were heated in boiling water for 15 min and then centrifuged at 1,000 ×*g* (10 min). Supernatant absorbance was measured spectrophotometrically at 535 nm (ε = 156,000 cm^{-1} M^{-1}).

Cells—Early passage (<30) human myeloid leukemia cells, HL60 (*34*) and HL60R, a mutant subclone of HL60 that exhibits relative resistance to RA and that harbors RA receptors with markedly reduced affinity for RA (*35, 36*), 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% (v/v) fetal bovine serum (FBS) (GIBCO).

Human breast cancer cell lines MCF-7 and MCF-7/Adr^R (*37*) were obtained from the American Type Culture Collection, Rockville, MD. Cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO). Human hepatoma cell line HepG2 (*38*) was obtained from RIKEN Cell Bank (Tokyo). Human prostate cancer cell line DU-145 (*39*) was obtained from Dr. Y. Pommier of the National Cancer Institute (Bethesda, MD, USA). 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 under a humidified atmosphere of 5% CO_2 in air. Cell numbers were determined with an electric particle counter (Coulter Electronics, Hialeah, FL), and viability was determined by means of trypan blue dye exclusion.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis—DNA isolation was performed as described previously (*40*). Briefly, HL60 cells (2×10^5 cells/ml) treated with various compounds were harvested and then washed with ice-cold PBS. Cells were suspended in PBS (10^6 cells/ml) containing 0.1 mg/ml proteinase K, 0.1 mg/ml RNase A and 1% SDS, and then incubated at 37°C for 30 min. After the 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), the cells were incubated at 60°C for 15 min and then diluted with an equal volume of isopropanol. Cells were mixed vigorously and then stood at room temperature for 15 min. Mixtures were centrifuged at 10,000 ×*g* for 5 min, and the supernatants were discarded. Precipitated DNA was suspended in 1 ml of 40% isopropanol, vortexed, and then centrifuged at 10,000 ×*g* for 5 min to recover the DNA. Pellets were dried under vacuum and dissolved in sample solution for analysis by agarose gel electrophoresis. Electrophoresis was performed on 2% agarose gels at 100 V/gel. The presence of DNA in the gels was visualized by ethidium bromide.

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Cell Growth—HL60 and HL60R cells (1 and 2 × 10^5/ml, respectively) were grown in RPMI 1640 medium containing 10% FBS and various concentrations of the compounds. Cell numbers were determined with an electric particle counter and viability by means of trypan blue dye exclusion. Percentage net growth is expressed as values adjusted by subtracting the initial cell concentrations of experimental cultures from the initial concentrations of control cultures, which were defined as 100%. Percent net growth values 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 under a humidified atmosphere of 5% CO_2 in air. After one day, various concentrations of the compounds were added to the cultures. Cells were incubated for 72 h, and then the viable cell number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (*41, 42*). Percent net growth values were determined 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.

Presentation of Results—Each experiment was performed at least 3 times, and most experiments were repeated at least 3 times with consistent results.

RESULTS

Scavenging of DPPH Radicals—The DPPH method is a radical-trapping antioxidant parameter assay. The stoichiometries of the reaction of DPPH radicals and antioxidants have been reported (*29–31*). One molecule of cysteine reacts with one DPPH radical, quenching it to yield the non-radical DPPH (*29*). In contrast, vitamin E reacts with two equivalents of DPPH radicals as compared to cysteine, such that each molecule of vitamin E scavenges two DPPH radicals (*31*). The absorbance of the control was approximately 0.99. In the presence of 20 μM vitamin E, cysteine, 4-HPR, *p*-MAP, 4-AP, *p*-AAP, and RA, the absorbance at 517 nm was approximately 0.61, 0.80, 0.79, 0.60, 0.58, 0.89, and 1.0, respectively. Vitamin E, *p*-MAP, and 4-AP caused an approximate 40% decrease in absorbance of DPPH radicals at 517 nm (ΔOD_{517} = 0.38, 0.39, and 0.41) relative to the control, as shown in Fig. 2A. In contrast, cysteine and 4-HPR or *p*-AAP reduced the absorbance by approximately 20 or 10% relative to control (ΔOD_{517} = 0.20 or 0.10). In addition, *p*-MAP and 4-AP exhibited dose-dependent antioxidant activity in the range of 0–40 μM to the same extent as vitamin E (Fig. 2A). When the antioxidant activity of 20 μM vitamin E was defined as 100%, the antioxidant activity level of 4-HPR or cysteine was approximately 50%, or half that of vitamin E (Fig. 2B). *p*-AAP showed approximately 25% of the antioxidant activity of vitamin E (Fig. 2B). RA was inactive, being the same as the control (data not shown).

These results indicated that *p*-MAP and 4-AP were equipotent antioxidants as to vitamin E, and had greater potency than 4-HPR and *p*-AAP. One molecule each of *p*-MAP and 4-AP scavenged two DPPH radical molecules, and one molecule of *p*-AAP scavenged one half DPPH radical molecule. The residue attached to the 4-hydroxyphenyl moiety may change the ability to quench DPPH radicals.

Inhibition of Liver Microsomal Lipid Peroxidation—MDA, which results from the breakdown of polyunsaturated fatty acids, was identified as a product of lipid peroxidation. MDA formation was inhibited in a dose-dependent manner by RA, *p*-MAP, 4-HPR, and 4-AP, but to a very limited extent by *p*-AAP, vitamin E and DMSO in the range of 0–10 μM (Fig. 3). Approximate IC_{50} values observed were 2–3 μM for RA, 4.5 μM for *p*-MAP, and 4-HPR, and 20 μM for 4-AP. In contrast, *p*-AAP was inactive in the range of 0–100 μM (Fig. 3). These results indicate that the residue linked to the aminophenol moiety markedly affects the inhibition of microsomal lipid peroxidation.

Effects on Growth of HL60 Cells—HL60 cells were grown in culture medium containing RA, 4-HPR, *p*-MAP, 4-AP, or *p*-AAP at the concentration of 1 or 10 μM for 94 h. RA was used as an internal standard. As shown in Fig. 4, *p*-MAP at the concentration of 1 μM inhibited approximately 33% of HL60 cell growth, which compared favorably with in the

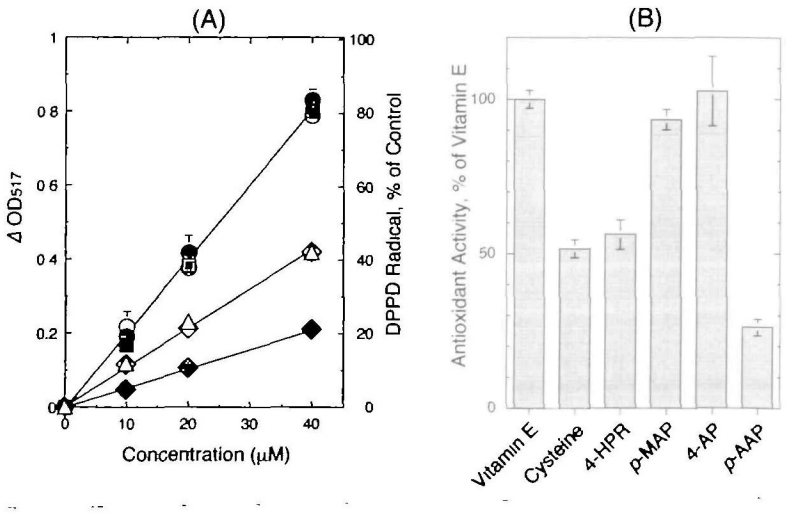

Fig. 2. **Effects of aminophenols (*p*-MAP, 4-AP, and *p*-AAP) and 4-HPR on DPPH radicals.** (A) Ethanol (2 ml) and a 500 μM solution of DPPH radicals in ethanol (1 ml) were added to 0.1 M acetic acid buffer, pH 5.5 (2 ml). *p*-MAP (■), 4-AP (●), *p*-AAP (◆), 4-HPR (△), vitamin E (○), or cysteine (◇) was added at various concentrations, and the mixtures were incubated at room temperature for 30 min as described under "MATERIALS AND METHODS." Absorbance was measured spectrophotometrically at 517 nm using acetic acid buffer as a blank. Antioxidant activity of compounds expressed as ΔOD_{517} was calculated with the following formula: (OD_{517} of control) – (OD_{517} of compound). (B) Antioxidant activity in the presence of compounds at the concentration of 20 μM, expressed as % of vitamin E calculated according to the following formula: (control absorbance -absorbance in the presence of compound)/(control absorbance – absorbance in the presence of vitamin E) × 100. The means ± SD of at least four measurements are shown. Experiments were repeated at least five times.

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cases of RA (29%) and 4-HPR (28%). At the concentration of 10 μM, *p*-MAP was the most potent antiproliferative agent among the three aminophenols, and was as potent as 4-HPR. Cell growth was inhibited approximately 99.7% for *p*-MAP, 99.6% for 4-HPR, 39% for RA, 21.8% for 4-AP, and 4% for *p*-AAP. These results indicated that HL60 cell growth is suppressed by *p*-MAP more strongly as compared with other aminophenols. It was the most potent inhibitor among the three aminophenols examined, indicating that the methyl residue linked to aminophenol may be significant for antitumor activity.

Agarose Gel Analysis of DNA Fragmentation in HL60 Cells—The finding that *p*-MAP caused marked inhibition of HL60 cell growth led us to investigate the effects of *p*-MAP on the induction of apoptosis. DNA isolated from HL60 cells was treated with *p*-MAP for 24 h and then visualized on an agarose gel. DNA extracted from cells treated with DMSO and 1 μM *p*-MAP showed no fragmentation (Fig. 5, A and B). In contrast, cells treated with *p*-MAP and 4-HPR at 10 μM contained fragmented ladder DNA (Fig. 5, C and D). These results indicate that *p*-MAP may potentially induce apoptosis of HL60 cells, and thereby inhibit cell growth.

Effects on Growth of Various Cancer Cell Lines—We examined whether *p*-MAP, 4-AP, *p*-AAP, or 4-HPR affects HL60R cell growth, which is resistant to RA. As shown in Fig. 6, HL60R cell growth was completely inhibited by *p*-MAP and 4-HPR (>99%) at 10 μM, while RA was inactive. *p*-MAP exhibited greater antiproliferative activity than 4-AP (20%) and *p*-AAP (<1%) (Fig. 6). These results suggest that *p*-MAP shows antiproliferative activity toward both HL60 (Fig. 4) and HL60R (Fig. 6) cells to the same extent as 4-HPR.

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 various concentrations of *p*-MAP, 4-AP, *p*-AAP, and 4-HPR. *p*-MAP, 4-AP, and 4-HPR each inhibited MCF-7 cell growth in a dose-dependent manner (Fig. 7A). At 10 μM, cell growth was inhibited approximately 20% by *p*-MAP, 10% by 4-AP, and 95% by 4-HPR. On the other hand, 4-HPR showed a greater cell growth inhibitory effect than *p*-MAP, 4-AP, and *p*-AAP against MCF-7/Adr^R cells, which are resistant to RA

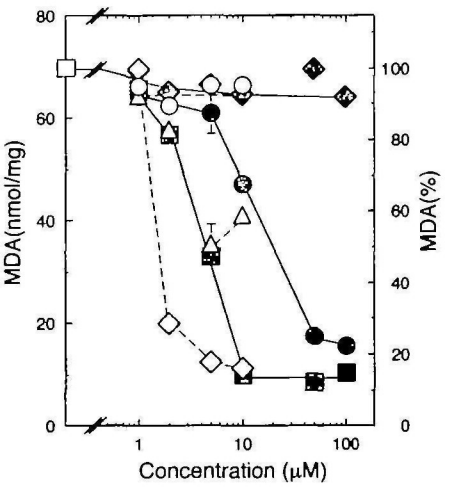

Fig. 3. **Inhibition of ascorbate-dependent lipid peroxidation by aminophenols (*p*-MAP, 4-AP, and *p*-AAP), 4-HPR, vitamin E, and RA.** Microsomes (0.5 mg protein/ml) without (□) or with various concentrations of *p*-MAP (■), 4-AP (●), *p*-AAP (◆), 4-HPR (△), vitamin E (○), or RA (◇) in 100 mM Tris-HCl (pH 7.5) containing 15 μM $FeCl_3$, and 4 mM ADP were preincubated at 37°C for 1 min. To reaction mixtures was added 1 mM ascorbic acid and then incubation was continued at 37°C for 20 min. TBA reagent was added, and then the mixtures were heated in a boiling waterbath for 15 min and centrifuged as described under "MATERIALS AND METHODS." Supernatant absorbance was measured at 535 nm (ε = 156,000 cm^{-1} M^{-1}). The means ± SD of at least four measurements are shown. Experiments were repeated at least four times.

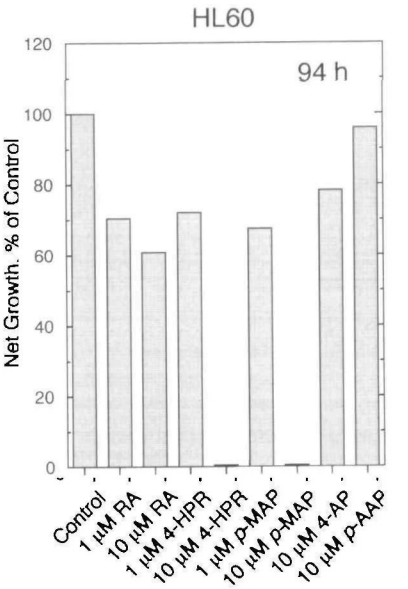

Fig. 4. **Growth of HL60 cells in the presence of aminophenols (*p*-MAP, 4-AP, and *p*-AAP), RA, and 4-HPR.** Cells (2×10^5/ml) were grown without or with an aminophenol (*p*-MAP, 4-AP, or *p*-AAP), RA, or 4-HPR at 1 or 10 μM in medium containing 10% FBS for 94 h. The SD of each data point was ≤8% of the mean. Experiments were repeated at least four times. RA was used as an internal standard for growth inhibition. Percent net growth values were calculated with the following formula: ((cell concentration of experimental culture) − (initial cell concentration))/(cell concentration of control culture) − (initial cell concentration)) × 100.

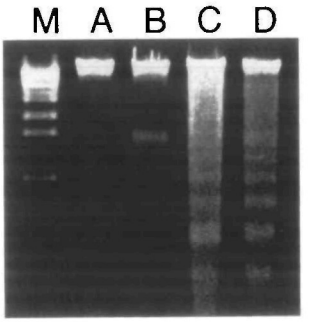

Fig. 5. **Agarose gel analysis of DNA fragmentation of HL60 cells grown in the presence of *p*-MAP and 4-HPR.** Cells (2×10^5/ml) were grown in the presence of DMSO (A), 1 μM *p*-MAP (B), 10 μM *p*-MAP (C), and 10 μM 4-HPR (D) in medium containing 10% FBS for 24 h. DNA was extracted from the cells and DNA fragmentation was measured by agarose gel electrophoresis as described under "MATERIALS AND METHODS." These experiments were repeated at least three times. M: DNA marker.

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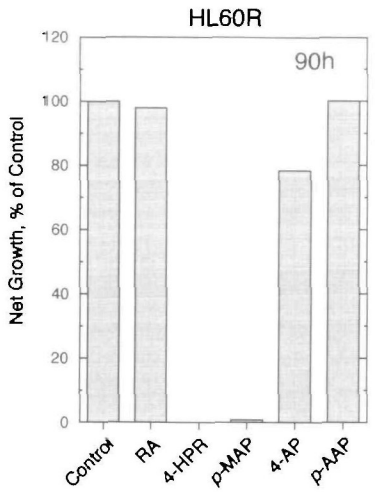

Fig. 6. **Growth of HL60R cells in the presence of aminophenols (*p*-MAP, 4-AP, and *p*-AAP), RA, and 4-HPR.** Cells (1×10^5/ml) were grown without or with RA, 4-HPR, or an aminophenol (*p*-MAP, 4-AP, or *p*-AAP) at 10 μM in medium containing 10% FBS for 90 h. The SD of each data point was ≤8% of the mean. Experiments were repeated at least four times. Percent net growth values were calculated with the following formula: ((cell concentration of experimental culture) − (initial cell concentration)/(cell concentration of control culture) − (initial cell concentration)) × 100.

(Fig. 7B). At 10 μM, *p*-MAP inhibited cell growth approximately 60%, while 4-AP and *p*-AAP were inactive (Fig. 7B). These results indicate that *p*-MAP is a potent growth inhibitor against human breast cancer cell lines, including RA-resistant cells.

The proliferation of HepG2 and DU-145 cells was suppressed in a dose-dependent manner by *p*-MAP, 4-AP, and 4-HPR (Fig. 7, C and D). 4-HPR at 10 μM inhibited the growth of HepG2 and DU-145 cells by approximately 95%. The antiproliferative activity of *p*-MAP toward HepG2 and DU-145 cells was seen at 40 μM, whereas 4-AP and *p*-AAP were inactive. At 100 μM, 4-AP inhibited growth by approximately 55% for HepG2 cells and by approximately 90% for DU-145 cells. In contrast, 100 μM *p*-AAP slightly affected HepG2 and DU-145 cell growth (Fig. 7, C and D). These results suggest that on deletion of their retinoyl moiety, 4-HPR, *p*-MAP, 4-AP, and *p*-AAP lost marked inhibitory activity, thereby indicating that this functionality is essential for the action against cancer cells, particularly attached cancer cells.

DISCUSSION

The synthetic retinoid 4-HPR is a cancer chemopreventive and antiproliferative agent, and a potent inducer of apopto-

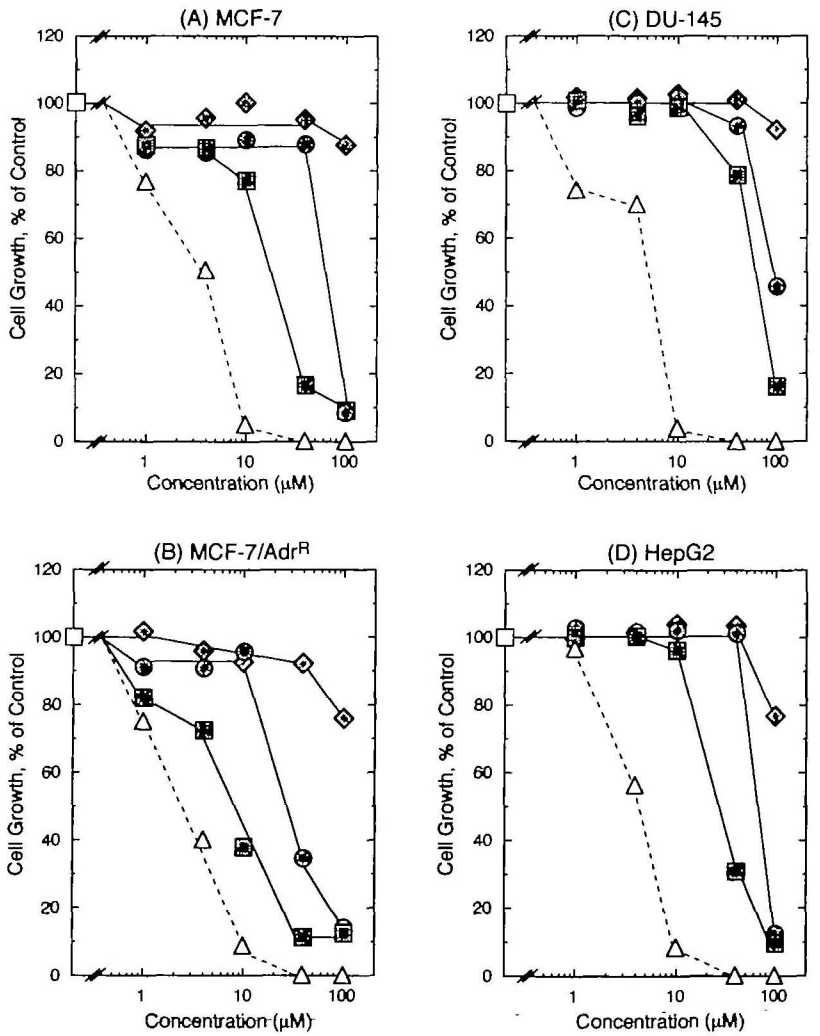

Fig. 7. **Growth of MCF-7, MCF-7/Adr^R, DU-145, and HepG2 cells in the presence of aminophenols (*p*-MAP, 4-AP, and *p*-AAP), and 4-HPR.** Cells were grown without (□) or with various concentrations of 4-HPR (△) and aminophenols [*p*-MAP (■), 4-AP (●), and *p*-AAP (◆)] in medium containing 10% FBS. Growth was measured at 72 h as described under "MATERIALS AND METHODS." Each *point* is the mean of at least four measurements. The SD of each point was ≤8% of the mean. Experiments were repeated at least three times.

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sis, whose mechanism of action may rely on the generation of ROS. In the current study, *p*-MAP, which lacks a partial functionality of 4-HPR, exhibited antioxidant activity, scavenging DPPH radicals and reducing lipid peroxidation in rat liver microsomes. It was a more potent antioxidant than 4-HPR. Additionally, *p*-MAP suppressed the growth of HL60 and HL60R cells to the same extent as 4-HPR, and induced apoptosis of HL60 cells. In contrast, *p*-MAP was a more effective antiproliferative agent against various cancer cells than 4-AP and *p*-AAP, but less potent than 4-HPR.

p-MAP and 4-AP showed the same level of DPPH radical scavenging activity as vitamin E. In contrast, 4-HPR and *p*-AAP exhibited one half and one fourth of the scavenging activity of vitamin E, respectively, and RA was inactive. While the antioxidant activity seemed to be due to the hydroxy residue of aminophenol, the retinoyl residue in 4-HPR and the acetyl residues in *p*-AAP, which are linked to the aminophenol, suppressed (changed) the antioxidant activity. Previously, RA and retinoids were reported to act as antioxidants in quenching DPPH radicals (*28, 43*). For all retinoids except 4-HPR, no quenching effect on DPPH radicals was observed at concentrations below 500 μM. However, 13-*cis* RA and a furyl analog of RA scavenged 19% of DPPH radicals at the concentration of 2.5 mM (*43*). This is in agreement with finding that 5–40 μM RA does not scavenge DPPH radicals. In contrast, at the same concentrations, *p*-MAP and 4-AP quenched approximately two-fold more DPPH radicals as compared with 4-HPR (Fig. 2) (*28*). Thus, the antioxidant action of *p*-MAP and 4-AP against DPPH radicals is much greater than that of 4-HPR and other retinoids.

Previous reports have shown that MDA formation in rat brain mitochondria induced by ascorbic acid is inhibited by retinol, retinol acetate, RA, retinol palmitate and retinal at concentrations between 100 μM and 10 mM (*44*). RA and vitamin E at 100 μM inhibit 89 and 37% of total MDA formation, respectively. These results are very similar to ours, in that RA inhibits MDA formation almost completely, while vitamin E causes approximately 10% inhibition of MDA formation at 10 μM (Fig. 3). It should be noted that lipid peroxidation was measured using microsomal lipids rather than mitochondria, and at 10 μM rather than 100 μM. On the other hand, microsomal lipid peroxidation induced by ascorbic acid was suppressed to the extent of 97% by 13-*cis*-RA, with an IC_{50} value of 10 μM (*33*). As can be seen in Fig. 3, 5 μM *p*-MAP inhibited microsomal lipid peroxidation by approximately 53%, which was the same as with 5 μM 4-HPR. The IC_{50} values of *p*-MAP and 4-HPR were both approximately 4.5 μM. Inhibition of lipid peroxidation by 4-AP was 35% at 10 μM, 75% at 40 μM, and 80% at 100 μM, which was lower than with RA, *p*-MAP, or 4-HPR. The level of MDA generation was not markedly affected by vitamin E or *p*-AAP at 100 μM. These results indicate that *p*-MAP is a more effective antioxidant than 4-HPR and vitamin E, and that *p*-MAP is less potent than RA in preventing ascorbic acid-microsomal lipid peroxidation.

As described above, the antioxidant activity of *p*-MAP is more potent than that of 4-HPR. In contrast, growth inhibition of various cancer cells by 4-HPR was greater than that by *p*-MAP. Whether the mechanism(s) behind these two different actions is attributable the same functionality within their structures is becoming clearer. The results of the current study indicate that antioxidant activity and anticancer activity may be due to both the phenolic hydroxyl and alkyl functionality attached to an aminophenol. The alkyl residue may be particularly significant for cell-drug contact and anticancer actions. It would be interesting to examine further the activities of other aminophenols linked to alkyl chains of various lengths.

Recently, it was reported that 4-hydroxybenzylretinone (4-HBR), a stable C-linked analog of *N*-4-HPR, exhibits similar antitumor effects to 4-HPR (*45, 46*). Since both 4-HPR and 4-HBR bind very poorly to nuclear retinoid receptors RARs and RXRs, they appear to act on cells directly rather than through hydrolysis to free RA. 4-HPR treatment had side effects, decreasing the serum retinol level and causing night blindness (*46*). However, 4-HBR did not exhibit such effects (*46*). In the current study, we showed that structural features of *p*-MAP are key to the 4-HPR action. *p*-MAP, which has a markedly different structure than retinol, may be an anticancer drug without side effects observed with 4-HPR.

Certain forms of cancer, cardiovascular and cerebrovascular diseases, and ischemia/reperfusion injuries are also considered to involve free radicals. Antioxidants, including certain vitamins and flavonoids, prevent these diseases (*47–50*). In the current study, *p*-MAP inhibited the growth of RA-resistant cancer cell lines HL60R and MCF-7/Adr^R (Figs. 6 and 7B). The antioxidant and antiproliferative activities of *p*-MAP may influence many factors, including immunostimulation, enhancement of cell communication, and inhibition of metabolic activation. Therefore, *p*-MAP may potentially be useful as a lead compound for the development of therapeutics against a variety of diseases that are currently being treated with antioxidants and 4-HPR.

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