Antioxidant Properties of *N*-(4-Hydroxyphenyl)retinamide (Fenretinide)

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Fenretinide, *N*-(4-hydroxyphenyl)retinamide (4-HPR), is a cancer chemopreventive and antiproliferative agent whose mechanism of action is unknown. 4-HPR is a potent inducer of apoptosis in HL60 human leukemia cells which generates intracellular reactive oxygen species. The structural similarity of retinoic acid (RA), 4-HPR, and α-tocopherol (vitamin E) led us to investigate whether 4-HPR exhibits antioxidant activity. It was found that 4-HPR scavenged α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals in a 1 : 1 ratio in contrast to vitamin E, where a 1 : 2 ratio relative to DPPH radicals was observed. In addition, linoleic acid peroxidation initiated by hydroxyl radicals was decreased by 4-HPR to the same extent as by vitamin E. Furthermore, lipid peroxidation in rat liver microsomes was reduced by 4-HPR to a greater extent than by vitamin E. Based on these results, 4-HPR appears to be an effective antioxidant that may have clinical utility for diseases treated with vitamin E.

Key words fenretinide, antioxidant, vitamin E, lipid peroxidation, α-tocopherol, retinoic acid

N-(4-Hydroxyphenyl)retinamide (4-HPR, fenretinide), a synthetic amide of all-*trans*-retinoic acid (RA) (Fig. 1), is an effective chemopreventive[1] and antiproliferative agent[2—4] used against a wide variety of tumor types, including breast, prostate, ovary, and bladder cancers. 4-HPR currently is in clinical trial for the treatment of breast[5—7] and bladder[8,9] malignancies.

Previous studies have shown that 4-HPR alone is a poor inducer of differentiation of a human myelogenous leukemia HL60 cell line as compared with RA.[10] Additionally, 4-HPR induces apoptosis in HL60 and NB4 human leukemia cell lines,[11,12] as well as in C33A, a human cervical carcinoma cell line.[13] The mechanism of action of 4-HPR is unclear. It is generally accepted that many effects of RA are mediated by RA nuclear receptors (RARs and RXRs)[14] which directly activate transcription of target genes by binding to specific DNA sequences. RARs and RXRs mediate various effects of retinoids in general. However, 4-HPR binds poorly or not at all to RARs or RXRs, and fails to transactivate the RARs or RXR α in chloramphenicol acetyl transferase assays.[10,15,16] Furthermore, 4-HPR showed no repression or activation of genes controlled by the retinoid nuclear receptors.[17,18] In contrast to these findings, 4-HPR is active with both RAR γ and RAR β in transactivation assays, and with RAR α, RAR β, and RXR α in transrepression assays.[19,20]

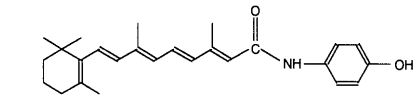
Recently, it was reported that 4-HPR generates reactive oxygen species (ROS), and that apoptosis induced by 4-HPR is decreased by the addition of the antioxidants α-tocopherol (vitamin E) and *N*-acetyl-L-cysteine in HL60 cells and by pyrrolidine dithiocarbamate (PDTC) and nor-dihydroguaiaretic acid in C33A cells.[13,21] These results indicate that the mechanism of action of 4-HPR may involve an oxidative pathway. On the other hand, 4-HPR is a synthetic derivative of RA produced from β-carotene through retinol. Therefore its structure (Fig. 1) is related to β-carotene, retinol, and RA, all of which exhibit antioxidant activities.[22—26] These structural similarities led us to the findings reported in this study that 4-HPR scavenges free radicals.

MATERIALS AND METHODS

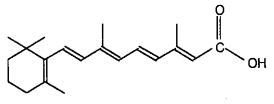
Chemicals RA, α-tocopherol (vitamin E), α,α-diphenyl-β-picrylhydrazyl (DPPH), linoleic acid, adenosine 5′-diphosphate (ADP), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4-HPR was provided by Dr. R. C. Moon, University of Illinois, Chicago, IL, U.S.A. Lubrol PX, dithiothreitol (DTT), and trichloroacetic acid (TCA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ascorbic acid and 2-thiobarbituric acid (TBA) were purchased from Wako Pure Chemicals Industries, Co. Ltd. (Osaka, Japan).

DPPH Radical Analysis DPPH radical analysis was performed as described previously.[27—29] Ethanol 2 ml and DPPH 500 μM in ethanol solution 1 ml were added to acetic acid buffer 0.1 M, pH 5.5 (2 ml). To this mixture (5 ml) were added 100 μl of a 1 mM solution of vitamin E, RA, or 4-HPR dissolved in DMSO and cysteine dissolved in acetic acid buffer 0.1 M, pH 5.5. The final concentration of each compound was 20 μM. After incubation at room temperature for 30 min, absorbance was measured spectrophotometrically at 517 nm (Shimazu UV-1600, Shimazu, Kyoto, Japan). Acetic acid buffer or DMSO 100 μl were used in place of compounds as blank controls.

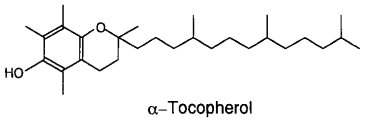
Linoleic Acid Peroxidation Assay Lipid peroxidation was assayed as described previously.[30] Stock solutions of linoleic acid micelles 10 mM (1 ml) were prepared daily by adding linoleic acid 2.8 mg/ml to NaCl 30 mM, pH 7.0. Equal volumes of 0.8% Lubrol PX and linoleic acid micelles 10 mM



N-(4-Hydroxyphenyl)-retinamide



Retinoic Acid



α-Tocopherol

Fig. 1. Chemical Structures of 4-HPR, RA, and α-Tocopherol

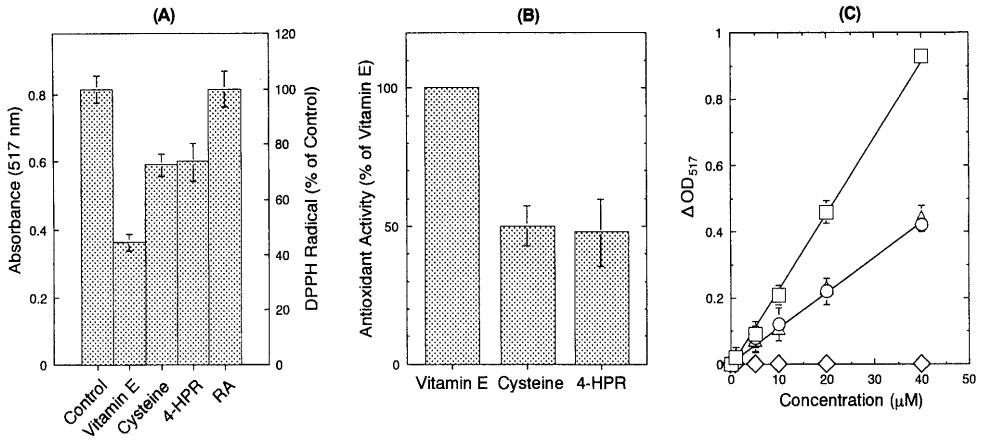


Fig. 2. Effects of 4-HPR and RA on DPPH Radicals

Ethanol 2 ml and a 500 μM solution of DPPH radicals in ethanol 1 ml were added to acetic acid buffer 0.1 M, pH 5.5 (2 ml). Vitamin E, RA, 4-HPR, and cysteine were added, and 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. (A) Absorbance at 517 nm in the presence of vitamin E, RA, 4-HPR, and cysteine at concentrations of 20 μM. (B) Antioxidant activity expressed as % of vitamin E. The % antioxidant activity was calculated according to the following formula: (control absorbance−absorbance in the presence of 20 μM of compound)/(control absorbance−absorbance in the presence of vitamin E 20 μM)×100. (C) Antioxidant activity at various concentrations of 4-HPR (○), vitamin E (□), RA (◇), or cysteine (△) expressed as ΔOD_{517} calculated by the following formula: (OD_{517} of control)−(OD_{517} of compound).

were mixed along with sufficient NaCl 30 mM, pH 7.0 to achieve a concentration of Lubrol PX of 0.08%. The resulting opaque micellular suspension was rendered as an optically clear dispersion by sonication (Branson Ultrasonic, Danbury, CT, U.S.A.). Reaction mixtures containing linoleic acid 1 mM, $FeSO_4$ 30 μM, H_2O_2 0.1 mM, and various concentrations of vitamin E, RA, or 4-HPR dissolved in DMSO were incubated at 37 °C, and conjugated diene formation was monitored spectrophotometrically at 234 nm. DMSO was added as a blank.

Preparation of Rat Liver Microsomes Eight-week-old male Sprague-Dawley rats (Slc. SD, 280—300 g) were obtained from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All fractionated and sonication steps were performed at 0—5 °C. Livers (5—8 g) were homogenized in 5 volumes (w/v) of cold sucrose 0.25 M containing Tris–HCl 100 mM (pH 7.4), DTT 5 mM, and $MgCl_2$ 10 mM. The homogenate was fractionized by the usual differential centrifugation techniques. Homogenates were centrifuged at 400×***g*** (10 min), then the supernatant was centrifuged at 10000×***g*** (10 min), and the resulting supernatant was then centrifuged at 105000×***g*** (1 h). Pellets were resuspended in Tris–HCl 100 mM (pH 7.4) containing sucrose 0.25 M, DTT 5 mM and $MgCl_2$ 10 mM, and stored at −80 °C. Protein concentrations were determined by the method of Bradford.[31]

Measurement of Microsomal Lipid Peroxidation Lipid peroxidation in rat liver microsomes was quantitated by measurement of malondialdehyde (MDA) using ADP-chelated ion and ascorbate as described previously.[26] Reaction mixtures consisting of microsomes (0.5 mg protein/ml) and compounds (vitamin E, RA, or 4-HPR dissolved in DMSO) in Tris–HCl 100 mM (pH 7.5) containing $FeCl_3$ 15 μM, and ADP 4 mM were preincubated at 37 °C for 1 min. Reaction mixtures in which ascorbic acid was added at a final concentration of 1 mM were incubated at 37 °C for 20 min, then equal volumes of TBA reagent (0.375% TBA and 15% TCA in hydrochloride 0.25 N) were introduced. Mixtures were heated in boiling water for 15 min, and centrifuged at 1000×***g*** (10 min). Supernatant absorbance was measured spectrophotometrically at 535 nm (ε=156000 $cm^{-1}M^{-1}$).

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

RESULTS

Scavenging of DPPH Radicals by Retinoids Previous reports have shown the stoichiometry of the reaction of DPPH radicals and antioxidants.[27—29] One molecule of cysteine reacts with one DPPH radical, quenching it to the non-radical DPPH.[27] 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.[29] Based on these results, the stable DPPH free radical was incubated with 4-HPR and RA at a concentration of 20 μM, and absorbance was measured spectrophotometrically at 517 nm. Vitamin E and cysteine were used as internal standards for scavenging DPPH radicals, with DMSO added as a control. As shown in Fig. 2A, the absorbance of control was approximately 0.82. In the presence of vitamin E, cysteine, 4-HPR, and RA at concentrations of 20 μM, absorbances at 517 nm were approximately 0.36, 0.59, 0.60, and 0.82, respectively. Vitamin E showed an approximate 56% decrease in the absorbance of DPPH radicals at 517 nm (ΔOD_{517}= 0.46) relative to the control. In contrast, cysteine and 4-HPR reduced absorbance approximately 27% relative to the control (ΔOD_{517}=0.22), with RA being the same as the control. As shown in Fig. 2B, 4-HPR and cysteine exhibited approximately 50% of the antioxidant activity relative to vitamin E. In addition, 4-HPR, cysteine, and vitamin E each exhibited antioxidant activity (ΔOD_{517}) in a dose-dependent manner in the range of 0—40 μM (Fig. 2C), and the level of antioxidant activity of 4-HPR or cysteine was approximately half that of vitamin E at all concentrations (Fig. 2C). These results indicate that 4-HPR is an antioxidant as potent as cysteine, such that one molecule of 4-HPR scavenges one DPPH radical, and that the 4-hydroxyphenylamide residue may be critical for the antioxidant activity of DPPH radicals.

Effects of Retinoids on Linoleic Acid Peroxidation Initi-

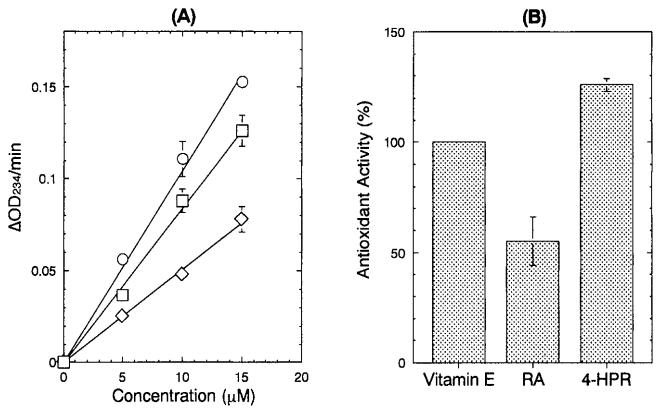


Fig. 3. Inhibition of Linoleic Acid Peroxidation by 4-HPR, Vitamin E, and RA

(A) Reaction mixtures containing NaCl 30 mM, pH 7.0, linoleic acid 1 mM, 0.08% Lubrol PX, H_2O_2 100 μM, $FeSO_4$ 30 μM, and various concentrations of 4-HPR (○), vitamin E (□), or RA (◇) were incubated at 37 °C, and absorbance was measured at 234 nm as described in "Materials and Methods." Antioxidant activity was calculated by the following formula: (OD_{234}/min of control)−(OD_{234}/min of compound)=ΔOD_{234}/min. (B) % antioxidant activity relative to vitamin E for compounds at 10 μM concentration was based on the antioxidant activity of vitamin E at 10 μM concentration being defined as 100%. The means±S.D. of at least four measurements are shown. Experiments were repeated at least four times.

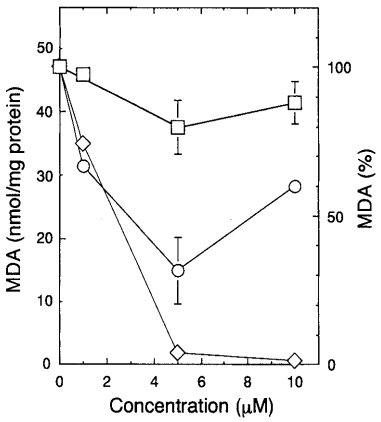


Fig. 4. Inhibition of Ascorbate-Dependent Lipid Peroxidation by 4-HPR, Vitamin E, and RA

Microsomes (0.5 mg protein/ml) and various concentrations of 4-HPR (○), vitamin E (□), or RA (◇) in Tris–HCl 100 mM (pH 7.5) containing $FeCl_3$ 15 μM and ADP 4 mM were preincubated at 37 °C for 1 min. To reaction mixtures was added ascorbic acid 1 mM and incubation was continued at 37 °C for 20 min. TBA reagent was added, the mixtures were heated in a boiling waterbath for 15 min, and centrifuged as described in "Materials and Methods." Supernatant absorbance was measured at 535 nm (ε= 156000 $cm^{-1}M^{-1}$). The means±S.D. of at least four measurements are shown. Experiments were repeated at least five times.

ated by Hydroxyl Radicals Free ferrous iron reacts with H_2O_2 to produce hydroxyl radicals by the Fenton reaction. Formation of hydroxyl radicals causes the rapid oxidation of linoleic acid, as determined by the extent of conjugated diene formation, which results in a rapid increase in absorbance at 234 nm. The initial rate of diene formation (OD_{234}/min) was affected by 4-HPR, RA, and vitamin E. High concentrations (100 μM) produced an immediate decrease in the rate of conjugated diene formation (data not shown). Antioxidant activity (ΔOD_{234}/min) that inhibited diene formation was calculated by the following formula: (OD_{234}/min of control)−(OD_{234}/min of compound). 4-HPR, RA, and vitamin E each exhibited antioxidant activity in a dose-dependent manner (Fig. 3A). Linear dependencies (Fig. 3A) were seen to 15 μM. Antioxidant activity by 4-HPR and vitamin E began to show saturation at 25 μM (data not shown). Of the three, 4-HPR was the strongest antioxidant at all concentrations (Fig. 3A). To compare the antioxidant activity of retinoids at 10 μM concentrations with vitamin E 10 μM, the antioxidant activity of vitamin E was set at 100% and the other values adjusted accordingly. In Fig. 3B, 4-HPR and RA showed approximately 126% and 55% antioxidant activity, respectively, relative to vitamin E. The difference between vitamin E and 4-HPR was significant ($p<0.05$). These results indicate that 4-HPR is a potent antioxidant among those tested in suppressing conjugated diene formation caused by hydroxyl radicals.

Inhibition of Liver Microsomal Lipid Peroxidation by Retinoids Incubation of 4-HPR, RA, or vitamin E with microsomes at 10 μM concentrations decreased ascorbate-dependent lipid peroxidation as measured by the extent of MDA, resulting from the breakdown of polyunsaturated fatty acids. MDA has been identified as the product of lipid peroxidation which reacts with TBA, giving a red species absorbing at 535 nm. A reaction mixture containing the vehicle DMSO was identical to a control mixture in the absence of compounds. MDA formation was inhibited by 4-HPR, RA, and vitamin E, but not by DMSO. As shown in Fig. 4, 4-HPR, RA, and vitamin E inhibited lipid peroxidation in a dose-dependent manner in the range of 0—5 μM, with RA exhibiting an IC_{50} value of between 2 μM to 3 μM. At concentrations higher than 5 μM, 4-HPR and vitamin E significantly increased MDA formation, while RA 10 μM inhibited lipid peroxidation completely (Fig. 4). These results indicate that RA and 4-HPR decreased microsomal lipid peroxidation more efficiently than vitamin E.

DISCUSSION

4-HPR, a synthetic retinoid, is a cancer chemopreventive, antiproliferative agent, and a potent inducer of HL60 cell apoptosis, whose mechanism of action may rely on the generation of intracellular ROS. In the current study, 4-HPR exhibited antioxidant activity in opposition to the production of ROS. 4-HPR scavenged DPPH radicals, inhibited linoleic acid peroxidation initiated by hydroxyl radicals, and reduced lipid peroxidation in rat liver microsomes to the same extent or more than vitamin E.

Previous studies have shown that 4-HPR suppresses growth, induces apoptosis, and produces intracellular ROS in HL60 cells and cervical carcinoma cells.[11—13,21] The mechanism by which ROS are produced by 4-HPR is not well known. In the current study, 4-HPR, while being a stimulator of ROS production in cells, was also a potent antioxidant. Similar effects have been seen with curcumin, gallic acid, and RA.[12,32,33] It will be of interest to investigate how these two different effects are regulated in cells.

RA and retinoids have been reported to act as antioxidants in quenching DPPH radicals.[34] For all retinoids, no quenching effect on DPPH radicals was observed at concentrations below 500 μM. However, 13-*cis* RA and a furyl analogue of RA scavenged 19.3% of DPPH radicals at 2.5 mM concentrations.[34] This is in agreement with results shown in Fig. 2, which indicate that RA 20 μM did not scavenge DPPH radicals. In contrast, 4-HPR at the same concentration quenched approximately 27% of DPPH radicals (Fig. 2). Thus the antioxidant action of 4-HPR against DPPH radicals is much

greater than that of other retinoids.

The extent of lipid peroxidation *in vitro* was measured by MDA production derived from lipids. MDA formation in rat brain mitochondria induced by ascorbic acid has been reported to be inhibited by retinol, retinol acetate, RA, retinol palmitate, and retinal at concentrations between 0.1 mM and 10 mM.[24] RA and vitamin E at 100 μM concentration inhibited 89% and 37% of total MDA formation, respectively. These results are very similar to the present ones in that RA inhibited MDA formation almost completely, while vitamin E showed approximately 10% inhibition of MDA formation at 10 μM concentration (Fig. 4). It should be noted that in this study lipid peroxidation was measured using microsomal lipids instead of mitochondria and at 10 μM concentrations rather than 100 μM. On the other hand, microsomal lipid peroxidation induced by ascorbic acid was suppressed 97% by 13-*cis*-RA which reacted with peroxyl radicals, and the IC_{50} was 10 μM.[26] Figure 4 shows that 4-HPR inhibited microsomal lipid peroxidation to the same extent (30%) as RA at 1 μM concentration, with inhibition of MDA formation by 4-HPR 5 μM being approximately 70%. The level of MDA generation was not markedly altered by vitamin E at concentrations between 1 μM and 10 μM. These results indicate that 4-HPR is a more effective antioxidant than vitamin E, and is as potent or slightly less potent than RA against ascorbic acid-microsomal lipid peroxidation.

Previously, it has been reported that 4-HPR in combination with RA enhances differentiation and retinoylation of proteins in the HL60 human myeloid leukemia cell line and the human acute promyelocytic leukemia cell line NB4.[10,35] NB4 cells convert RA to 4-hydroxy-RA, 4-oxo-RA, and more polar retinoids, and the level of RA in culture is increased in the presence of 4-HPR, with RA catabolism being inhibited by 4-HPR in a dose-dependent manner. The mechanism of the antioxidant activity of 4-HPR is not yet clear. It will be of interest to determine whether 4-HPR inhibits the oxidation of RA directly, and experiments to confirm this possibility are in progress.

Cardiovascular and cerebrovascular diseases, certain forms of cancer, and ischemia/reperfusion injuries are considered to involve free radicals, with antioxidants such as some vitamins and flavonoids preventing these diseases.[36—39] The antioxidant activities of 4-HPR may influence many factors, including immunostimulation, enhancement of cell communication, and inhibition of metabolic activation. It is possible that 4-HPR may be clinically useful for various diseases for which antioxidants are administered.

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