N-(4-Hydroxyphenyl)retinamide (Fenretinide) in Combination with Retinoic Acid Enhances Differentiation and Retinoylation of Proteins

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ABSTRACT
The synthetic retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR; Fenretinide), is a cancer chemopreventive and antiproliferative agent whose mechanism of action is unknown. 4-HPR alone is a poor inducer of differentiation of HL-60 cells compared to all-trans-retinoic acid (RA). Here, we found that combinations of 4-HPR and RA synergistically induced differentiation of HL-60 cells. In addition, 4-HPR increased the level of retinoylation, the covalent binding of RA to proteins. Retinoylation occurs in many eukaryotic cell lines and may be involved in RA-induced differentiation. These results suggest that 4-HPR may be a member of a class of retinoids that are active because they displace RA from extracellular and intracellular sites or because they inhibit RA catabolism. On the basis of these proposed mechanisms, retinoids that do not cause differentiation as sole agents may have utility in the clinic in combination with RA.

INTRODUCTION
4-HPR$^2$ is an effective chemopreventive (1) and antiproliferative agent (2–8) for a wide variety of tumor types including those of the breast, prostate, ovary, and bladder. 4-HPR is in clinical trials for cancers of the breast (9) and the bladder (10).

A mechanism for the chemopreventive and antiproliferative activities of 4-HPR is unknown. 4-HPR and RA compete with retinol for binding to plasma retinol binding protein (11), inhibit the retinol-metabolizing enzymes lecithin-retinol acyltransferase (12), acyl-CoA-dependent retinal reductase (12), and acyl-CoA-retinol acyltransferase (13), and up-regulate RAR-β in normal mammary epithelial cells (14). In addition, 4-HPR impairs the secretion of the retinol-plasma retinol-binding protein complex from liver and other tissues (15). It is unclear whether these effects, either singly or in combination, mediate the anticarcinogenic and antiproliferative activities of 4-HPR.

Although 4-HPR and RA share some activities, there is evidence that they have divergent mechanisms of action. Thus, 4-HPR induces apoptosis in HL-60 and NB4 human leukemia cell lines that are resistant to RA (16), binds poorly or not at all to the RARs or the retinoid X receptors,$^3$ and fails to trans-activate the RARs or the retinoid X receptor α in chloramphenicol acetyltransferase assays.$^5$ It generally is accepted that many effects of RA are mediated by these receptors (17) which directly activate transcription of target genes by binding to specific DNA sequences.

RA induces the differentiation of HL-60 cells to cells with many functional and morphological characteristics of mature granulocytes (18). In addition to the retinoid nuclear receptors, RA acylation (retinoylation) of proteins is a candidate mechanism for the effects of RA on cells (19, 20). We showed that retinoylation involves the formation of a covalent bond with preformed protein. The extent of retinoylation is dependent on the initial concentration of RA in a saturable manner. Furthermore, with intact HL-60 cells the ED₅₀ values for RA-induced differentiation and for retinoylation are similar. These results suggest that retinoylation may be involved in the mechanism by which HL-60 cells differentiate in the presence of RA.

In a previous study, we found that Am80, a synthetic retinoid which is a ligand for the nuclear retinoid receptors, is covalently bound to protein at a very low level compared to RA and does not induce differentiation of HL-60 cells (20). However, Am80 increases the level of retinoylation, and combinations of RA with Am80 synergistically induce differentiation of HL-60 cells. These findings are consistent with a covalent modification of proteins by a retinoid playing a role in differentiation of HL-60 cells.

In the present study, we have extended our earlier study with Am80 by examining the modulation by 4-HPR of RA-induced differentiation and retinoylation in HL-60 cells.

MATERIALS AND METHODS
Cells. Early passage (<30) human myeloid leukemia cell line HL-60 (21) and HL-60(S), an HL-60 variant with enhanced sensitivity to RA (22) (provided by Dr. K. Shudo, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan) were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10 mm 4-(2-hydroxyethyl)-1-piperazinethene sulfonic acid (pH 7.3) and 10% FBS (v/v; GIBCO).

$^2$ The abbreviations used are: 4-HPR, N-(4-hydroxyphenyl)retinamide (Fenretinide); RA, all-trans-retinoic acid; FBS, fetal bovine serum; CI, combination index; RAR, retinoic acid nuclear receptor; Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl) benzoic acid.

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$^3$ A. A. Levin, personal communication.

$^5$ P. G. Felici, unpublished observations cited in Ref. 16.
The population doubling times in medium containing 10% FBS were 17 h for HL-60(S) cells and 36 h for HL-60 cells. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Cell Differentiation.** Cells (2 × 10⁶/ml for HL-60 and 2 × 10⁵/ml for HL-60(S)) were grown in RPMI 1640 medium containing various concentrations of either RA (Sigma Chemical Co., St. Louis, MO) or 4-HPR (gift from Dr. R. C. Moon, Specialized Cancer Center, University of Illinois, Chicago, IL) and either 5% or 10% FBS (serum-containing medium) or 5 μg/ml of both insulin and transferrin (serum-free medium). We estimated cell number on an electronic particle counter (Coulter Electronics, Hialeah, FL), viability by trypan blue dye exclusion, and differentiation by the capacity of the cells to reduce nitroblue tetrazolium (23).

**Analysis of Combined Drug Effects.** Isobologram analysis was the basis for analyzing the combined effects of 4-HPR and RA on differentiation. The interaction of two inducers was quantified by determining a CI value for each fixed concentration ratio according to the classic isobologram equation (24):

\[
CI = [(D)₁/(Dₐ₆)] + [(D)₂/(Dₐ₆)]
\]

where \(Dₐ₆\) is the dose required to produce an effect alone, and \((D)₁\) and \((D)₂\) are the doses of agents 1 and 2 in the mixture that produce the same effect. This analysis generates the combination effect as: summation (additivity or zero interaction) is indicated when \(CI = 1\); synergism is indicated when \(CI < 1\); and antagonism is indicated when \(CI > 1\).

**Retinoylation.** HL-60 cells from exponentially growing cultures were harvested by centrifugation and resuspended in serum-free medium. RA (Sigma) and [1,12-3H]RA (50 Ci/mmol; Du Pont-New England Nuclear, Boston, MA) were dissolved in absolute ethanol and added to the growth medium. The final concentration of ethanol was no higher than 0.1%.

HL-60 cells were grown for 24 h with 10 or 100 nm [³H]RA and various concentrations of radiointact 4-HPR or RA. Cells were harvested by centrifugation (200 × g for 5 min) and washed extensively with phosphate-buffered NaCl solution (1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 136.9 mM NaCl, pH 7.2). The cell pellet was then extracted using the Bligh-Dyer procedure (25) and centrifuged at 10,000 × g for 5 min in a microcentrifuge. This extraction was repeated about five times or until there was <300 cpm/ml in the supernatant fraction. The delipidated pellet was then dried in a centrifugal vacuum device (Savant, Farmingdale, NY) and dissolved in 10% SDS solution. Radioactivity was measured on a liquid scintillation spectrometer.

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

**RESULTS**

**Effects of RA and 4-HPR on Differentiation, Growth, and Viability of HL-60 Cells.** We found that 4-HPR was much less active than RA in inducing differentiation of HL-60 cells (Fig. 1). These results are in agreement with previous reports using either serum-free (26, 27) or serum-containing media (16, 28, 29). The induction of differentiation, decrease in viability, and inhibition of growth by either RA or 4-HPR were greater in serum-free medium than in serum-containing medium (Fig. 1).

Differentiation induced by RA was associated with decreased cell growth in either serum-free or serum-containing media (Fig. 1). We saw marked decreases in the percentage of viable cells only in serum-free medium containing 400 nm or 1 μM RA. At 92 h, these two concentrations of RA induced 100% differentiation, and viabilities were about 60% with 400 nm RA and 55% with 1 μM RA (Fig. 1). At an earlier time (67 h), these two concentrations of RA also induced 100% differentiation but with viabilities of 87% with 400 nm RA and 80% with 1 μM RA that were much higher than at 92 h. These results indicate that nonviable cells originated from a pool of viable mature cells and are consistent with a report that RA-treated HL-60 cells die via apoptosis subsequent to differentiation (30).

Compared to RA, the decreased cell growth and viability by 4-HPR in serum-free medium were not coupled as closely to cell differentiation (Fig. 1). The marked decrease in viability with 4-HPR at concentrations >400 nm in serum-free medium precluded determinations of differentiation with the nitroblue tetrazolium assay, which requires live cells (23).

In serum-containing medium, we saw about 40% differentiation with 4 μM 4-HPR (Fig. 1). The percentage of viable cells...
Fig. 2  HL-60 cell differentiation in serum-free medium induced by increasing concentrations of 4-HPR in the presence of fixed concentrations of RA. Cells (2 × 10⁶/ml) were grown for 92 h in serum-free medium with the indicated concentrations of RA and 4-HPR. Each point is the mean of at least four measurements. The SE of each data point was ≤8% of the mean. The isoboles (inset) are for combinations of 4-HPR with RA that are isoeffective (ED₅₀) for differentiation of HL-60 cells. When there is additivity, the experimental values for a combination fall on the dashed line connecting the values for each agent alone. The EDᵢₐ value for 4-HPR alone was estimated by extrapolation. This value could not be determined experimentally because of low viability (see "Results"). The viabilities were >85% for each of the other isoeffective conditions shown in the isobologram.

was about 80%, and cell growth was inhibited about 50%. These latter results are not in agreement with those of Delia et al. (16), who found that during growth of HL-60 cells with 3 μM 4-HPR for 5 days there is no increase, and instead a decrease, in the concentration of viable cells. At 300 nM 4-HPR the level of growth inhibition seen by these workers was similar to what we saw with 4 μM 4-HPR. Thus, one explanation for the apparent differences in results is that the concentrations of 4-HPR used by Delia et al. (16) are much higher than they report. Additional support for this explanation is that clonal proliferation of HL-60 cells is not inhibited by 1 μM 4-HPR (29).

Induction of Differentiation of HL-60 Cells by Combinations of RA and 4-HPR. Our findings that 4-HPR was a poor inducer of HL-60 cell differentiation (Fig. 1) prompted an examination of whether 4-HPR affected RA-induced differentiation.

In serum-free medium the dose-effect curves for 4-HPR were displaced to lower concentrations as a function of an increase in the fixed concentration of RA (Fig. 2). The interaction between 4-HPR and RA was visualized by constructing an isobole (31) (Fig. 2, inset) and quantified by calculating a CI value (24). As shown in Fig. 2, inset, the two isoeffective (ED₅₀) combinations of 4-HPR with RA were below and to the left of the summation (additive or zero interaction) isobole displayed by the dashed line. The CI values for these combinations were about 0.6, indicating moderately strong synergism.

We examined also the induction of differentiation of parental HL-60 and HL-60(S) cells by combinations of RA and 4-HPR in medium containing serum (Fig. 3). HL-60(S) is about 10-fold more sensitive to RA for differentiation than HL-60 cells (20). We found that 4-HPR was more toxic to HL-60(S) than to HL-60 cells. Viability was 97% for HL-60(S) cultures exposed to 1 μM 4-HPR for 74 h and <1% for cultures exposed to 4 μM 4-HPR. This toxicity limited to 1 μM the maximum concentration of 4-HPR that we could study. At this concentration, 4-HPR did not induce differentiation of HL-60(S) cells (Fig. 3).

With either HL-60 or HL-60(S) cells the dose-effect curves for RA were displaced to lower concentrations as a function of increases in the fixed concentrations of 4-HPR (Fig. 3). The isoboles drawn from the data in Fig. 3 show that isoeffective combinations of RA and 4-HPR were below and to the left of the summation isoboles (Fig. 4). The extent of this synergy was estimated by determining CI values. The CI values at the ED₅₀ effect level for HL-60 cells were about 0.7. The CI values for HL-60(S) cells at the ED₅₀ effect level were about 0.5. Viability
Enhancement of Differentiation and Retinoylation by 4-HPR

Our findings that combinations of 4-HPR and RA synergistically induced differentiation of HL-60 cell lines (Figs. 2–4) prompted us to see whether these increased levels of differentiation correlated with changes in the level of retinoylation. The levels of retinoylation by two fixed concentrations of [3H]RA increased as a function of the concentration of 4-HPR (Fig. 5). We did not see any cytotoxicity during this relatively short 24-h exposure. In contrast to 4-HPR, the covalent binding of [3H]RA to protein decreased, because of isotope dilution, as a function of the concentration of radioinert RA (Fig. 5).

DISCUSSION

Although 4-HPR is an effective cancer chemopreventive agent and cell growth inhibitor for a wide variety of tumor types, its mechanism of action is still elusive. Patients taking 4-HPR have markedly decreased levels of plasma retinol-binding protein and retinol (32), and many show effects associated with hypovitaminosis A (33). The ability of 4-HPR to compete with retinol for binding to plasma retinol-binding protein (11), to inhibit both lecithin-retinol acyltransferase and acyl-CoA-dependent retinal reductase (12), and to impair the secretion of the retinol-plasma retinol-binding protein complex from liver and other tissues (15) may singly or in combination be the basis for the side effects in vivo. It is unclear whether one or more of these specific effects is involved in the therapeutic efficacy of 4-HPR.

The accumulating evidence that 4-HPR does not bind to the nuclear retinoid receptors\(^3\)–\(^5\) prompted us to examine whether 4-HPR influenced the ability of RA to differentiate HL-60 cells. Previously, we found that the synthetic retinoid Am80 does not induce differentiation of HL-60 cells in serum-free medium, although it is a ligand for nuclear retinoid receptors (20). However, Am80 increases the level of retinoylation and potentiates RA-induced differentiation of HL-60 cells (20).

In this study, we saw qualitatively similar results with 4-HPR to those seen with Am80. Combinations of 4-HPR and RA synergistically induced differentiation of HL-60 cells (Figs. 2–4), and 4-HPR increased the levels of retinoylation (Fig. 5). We saw these effects of 4-HPR in the absence of serum (Figs. 2 and 5). Thus, retinol and retinol-binding protein, which are in serum, are not obligatory for some of the effects of 4-HPR seen in this study.

On a cellular basis our results could be explained if 4-HPR acted by a mechanism that increased the availability of RA for either transcriptional regulation of RA responsive genes or for retinoylation of target proteins. In cells exposed to both RA and 4-HPR, 4-HPR may displace RA from sites where it is sequestered. Another possibility is that 4-HPR alters the efficiency of the cytochrome P450-mediated catabolism of RA to 4-hydroxyretinoic acid. This hydroxylation occurs in HL-60 cells (34). However, we are not aware of any report that 4-HPR affects this reaction. Experiments to distinguish these possibilities are in progress.

Another explanation for the increased protein retinoylation seen with 4-HPR is an elevation in the levels of one or more of the proteins that are substrates for retinoylation. The regulatory subunit of cyclic AMP-dependent protein type II is one of the major retinoylated proteins in HL-60 and MCF-7 cells (35, 36), and its level increases about 3-fold in mammary tumors growing in animals treated with 4-HPR (37). Thus, the increased levels of retinoylation in the presence of 4-HPR may, in part, reflect an increase in the amount of this regulatory protein.

The inability of 4-HPR to inhibit retinoylation (Fig. 5) suggests strongly that 4-HPR may not compete with RA in the covalent modification of protein. We could not determine directly whether 4-HPR covalently modifies proteins because of the unavailability of highly radioactive 4-HPR. Taken together with our previous observations with Am80, these results offer additional evidence that covalent modification of proteins by a retinoid may play a role in inducing differentiation of HL-60 cells.

Our results showing synergism in the induction of differentiation of HL-60 cells by combinations of 4-HPR with RA may have clinical utility. Plasma concentrations of about 1 \(\mu M\) are seen in patients receiving p.o. doses of either 4-HPR (32) or RA (38). These values indicate that combinations of 4-HPR and RA that we found to be synergistic are obtainable in patients. It may also be noteworthy that we saw synergistic effects with combinations of 4-HPR and RA at concentrations of RA that are close to the physiological range of about 10 nM (39, 40). Thus, our results raise the possibility that biological effects of 4-HPR in vivo may in part derive from influences on the effective biological availability of endogenous RA. This further raises the prospect that the spectrum of cancers responding to 4-HPR may be greater if RA were combined with 4-HPR. In particular, 4-HPR may be useful in the treatment of acute promyelocytic leukemia or squamous neoplasms. Although cytodifferentiation therapy by RA for acute promyelocytic leukemia patients is...
encouraging, there are several limitations preventing better clinical outcomes. A high percentage of patients in complete remission induced by RA alone relapse within a few months (41–43). Most relapsed patients are resistant to further treatment with RA, although their leukemia cells may respond to RA in vitro (43–45).

One explanation for the resistance of relapsing patients to RA treatment is an increased systemic cytochrome P450-mediated metabolism of RA. The rapid catabolism of RA makes it very difficult to maintain effective plasma levels of RA (44). Another explanation for RA resistance is at the cellular level. Leukemic cells from patients treated with RA have increased levels of cellular RA-binding protein (45, 46). High levels of this protein are associated with alterations in RA metabolism (47–49) and reduced cell differentiation (45, 50–52). Whatever the reason (or reasons) for relapse, treatment with combinations of RA and 4-HPR may be one approach to lower the plasma levels of RA needed for clinical effects. It is likely that decreasing the therapeutic concentrations of RA will also reduce the induction of cytochrome P450-mediated catabolism of RA and of cellular RA-binding protein.

The experiments in this article lead to the hypothesis that clinical outcome in patients treated with RA may be modified by coadministration of a retinoid which alters the intracellular concentration or distribution of RA. This alteration results in an increased biological effect of RA, mediated either by nuclear retinoid receptors or, as shown here, possibly by an increase in the retinoylation of cellular target proteins. On the basis of these proposed mechanisms, many retinoids that are relatively macronutrient-specific for differentiation as sole agents may have utility in the clinic in combination with RA.

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Enhancement of Differentiation and Retinoylation by 4-HPR


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