N-(4-Hydroxyphenyl)retinamide Induces Apoptosis of Malignant Hemopoietic Cell Lines Including Those Unresponsive to Retinoic Acid

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ABSTRACT

N-(4-Hydroxyphenyl)retinamide (HPR) is a synthetic retinoid of particular clinical interest in cancer chemoprevention. We have examined the in vitro effects of HPR on lymphoid and myeloid malignant cell lines and found that at concentrations between 10^-5 and 3 x 10^-7 M it induces a dose-dependent growth inhibition (the peak plasma concentration in patients treated with HPR is 1 to 2 x 10^-6 M).

The antiproliferative effect of HPR was, in all cell lines except K422, more potent than that induced by an equimolar dose of all-trans retinoic acid (RA). Also, this effect was irreversible on HL60 and DoHH2 cells that had been exposed to HPR (3 x 10^-5 M) for 24 h, but reversible on Raji and DHL4 exposed to the retinoid for 48 and 72 h, respectively.

Time-course growth analysis showed that HPR at 3 x 10^-5 M or below induces a rapid fall of thymidine uptake and viability (>90%), whereas between 10^-6 and 3 x 10^-7 M exhibits cytostatic effects.

Interestingly, the RA-resistant HL-60R and NB306 cells, characterized by a point mutation in the retinoic acid receptor (RAR) and by the loss of the pml/RAR protein, respectively, were, like the parental RA-inducible HL-60 and NB4 cell lines, fully responsive to HPR, thereby suggesting that HPR and RA could act through different receptors or pathways.

DNA flow-cytometric analysis revealed that HPR does not block cells in a specific phase of the cell cycle but triggers programmed cell death or apoptosis. This phenomenon was evidenced both by the visualization, on gel electrophoresis, of fragmented DNA, and by the "in-cell" enzymatic labeling of DNA breaks with fluorescent dUTP. With the latter method, apoptotic cells become detectable by 6 h following exposure to 3 x 10^-7 M HPR. Ultrastructural examination of HPR-treated samples showed cells with chromatin compaction and cytoplasm condensation, characteristic of apoptotic cells.

In conclusion, our study demonstrates that HPR suppresses malignant cell growth and induces apoptosis at pharmacologically relevant doses. The differential responsiveness by a number of cell lines, especially HL-60R and NB306, to HPR and RA indicates that these compounds may act through different receptors. The clinical use of HPR, particularly in retinoic acid-unresponsive acute promyelocytic leukemia patients, is suggested.

INTRODUCTION

Retinoids exert diverse biological effects important in the control of normal growth, differentiation, and fetal development. Oncological investigations have shown that retinoids can suppress epithelial and mesenchymal tumor growth induced by chemicals and radiation in experimental animals (1). Such antineoplastic activity is thought to arise from an antipromotion activity or from potentiation of antitumor immune response by the retinoid (2-4).

HPR is a synthetic retinoid that has been shown to be effective in several animal models. In rats, HPR prevents carcinogen-induced mammary cancer (5-7) and inhibits metastasis of prostate adenocarcinoma (8), and in mice HPR suppresses the growth of transplanted Moloney murine lymphoma, though less effectively than retinoic acid (9).

The in vitro antiproliferative effects of HPR have been demonstrated in the human breast carcinoma cell line MCF-7 (10) and in short-term semisolid cultures of fresh human tumors (11).

The differentiative properties of HPR have been investigated in the myeloid leukemia cell line HL-60 (12), where this compound was shown to be a poorer inducer of granulocytic maturation than an equimolar dose of all-trans RA.

The molecular level of action of HPR is unknown, and whether the biological effects are, like RA, exerted through its binding to a member of the family of RAR or RXR retinoic acid receptors (13) remains to be established, though earlier biochemical studies evidenced lack of binding of HPR to cellular retinol and retinoic acid-binding proteins (14).

Understanding the cellular and molecular level of action of HPR is particularly relevant when considering the clinical interest for this chemopreventive and relatively nontoxic agent (15, 16). Two major clinical trials are ongoing at this Institute to establish the efficacy of HPR to prevent: (a) contralateral primary tumors in women surgically treated for node-negative breast cancer (17); and (b) basal cell carcinomas and oral cavity tumors following leukoplakia, for which promising preliminary results have been reported (18).

In this paper we show that HPR, at pharmacologically attainable doses, inhibits the growth of human leukemia- and lymphoma-derived cell lines, more strongly than an equimolar concentration of RA. We also demonstrate that 2 RA-resistant cell lines, HL-60R and NB306, retain their responsiveness to HPR, suggesting that these compounds may activate different retinoid receptors.

This finding may have potential clinical implication since it could be exploited in the therapy of APL once the disease becomes unresponsive to retinoic acid treatment (19, 20).

Finally, we show that HPR is a potent inducer of apoptosis or programmed cell death.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cell lines used in this study were: DHL-4 (21), K422 (22), and DoHH2 (23), all carrying the t(14;18) chromosomal translocation typical of follicular lymphomas; the B-lymphoblastoid cell line Raji; the myeloid leukemia cell line HL-60, and its subclone HL-60R, the latter unresponsive to 10^-6 M RA as it carries a mutation in the RAR binding site (24); the t(15;17) acute promyelocytic leukemia cell line NB-4 (25); and the NB-4 variant NB306 (26), whose resistance to RA is associated with altered expression of pml/RARa protein (26). Both NB4 and HL-60 undergo granulocytic differentiation when cultured with RA. All cell lines, Mycoplasma-free, were grown in RPMI 1640 (Flow Laboratories, Irvine, CA)

3 The abbreviations used are: HPR, N-(4-hydroxyphenyl)retinamide; RA, retinoic acid; RAR, retinoic acid receptor; APL, acute promyelocytic leukemia; TdT, terminal deoxyribonucleotidyl transferase; RXR, retinoid X receptor.
supplemented with 10% fetal calf serum (Flow), 100 units/ml penicillin, 100 
µg/ml streptomycin, and 2 mmol/liter glutamine. Cell number was quantitated 
on trypan blue-stained cell suspensions using a hemocytometer. Proliferation 
assays were performed, in triplicate, in 96-well microtiter plates (Costar, Cam-
bridge, MA) by seeding 10,000/well in 200 µl of medium with or without 
addition of retinoid, and incubated for the specified time. Four h before 
harvesting, 20 µCi/ml of [3H]thymidine (Amersham, Amersham, United King-
dom) were added to the cells. Cells were harvested with a Skatron instrument 
(Norway) and radioactivity measured by scintillation counting. All-trans RA 
(Sigma) and HPR (kindly supplied by Johnson Pharmaceutical Research In-
stitute, Spring House, PA) were dissolved in absolute ethanol at a concentra-
tion of 10⁻² M and stored at −20°C. Both compounds were prediluted 1:10 in fetal 
calf serum prior to further dilutions in cell culture medium.

Flow-Cytometric Analysis and DNA Fragmentation Detection. Cell 
cycle analyses were performed as described (27) on samples permeabilized 
with 0.1% saponin (Sigma), treated with 1 mg/ml RNase A (Sigma), and 
stained with 20 µg/ml of propidium iodide (Sigma). Cells were analyzed on an 
EPICS-C instrument (Coulter Electronics, Hialeah, FL). DNA fragmentation 
was measured as reported (28). Briefly, the cells were lysed in 5 mm Tris-HCl, 
pH 8, 10 mm EDTA, 0,5% Triton X-100, kept at 4°C for 30 min, and centri-
fuged for 20 min at 13,000 × g to separate the fragmented (soluble) from intact 
(pellet) DNA. Following treatment with 50 µg/ml DNase-free RNase (Boer-
ingher-Mannheim, Mannheim, Germany) and 100 µg/ml protease K, extrac-
tion with phenol-chloroform and chloroform, and precipitation in 2.5 volumes 
etanol plus 0.15 M NaCl, the DNA was electrophoresed on 1.8% agarose gel. 
ΦX HaelIII DNA markers were included. The DNA on gels was visualized by 
ethidium bromide staining. In situ nuclear DNA fragmentation was measured 
according to a recently described method based on the 3'-OH end labeling of 
DNA breaks with deoxyuridine by the enzyme TdT (29), but modified to 
evidence nucleotide incorporation by fluorescence as follows. Cells, treated or 
untreated, were fixed in 2% paraformaldehyde for 10 min, washed twice with 
0.1 M Tris-buffered saline (pH 7.2), fixed again with acetone for 1 min, washed 
with Tris-buffered saline, and incubated with 0.5 unit/ml TdT (Boeringher-
Mannheim) plus 1.5 µM fluorescein isothiocyanate-dUTP (Boeringher-Mann-
heim) in 1× TdT buffer for 1 h at 37°C. After 2 more washes, the cells were 
analyzed by flow cytometry.

RESULTS

Proliferative Analysis. The effect of HPR on cell growth was 
studied and compared to that of RA. HPR was tested at doses between 
10⁻⁵ and 3 × 10⁻⁷ M, whereas RA was tested at 10⁻⁵ and 10⁻⁶ M. It 
was noteworthy that that the peak plasma concentration of HPR in 
patients undergoing chemopreventive trials was 1 to 2 × 10⁻⁶ M (16, 
30).

The 72-h dose-response proliferative results are shown in Fig. 1. 
Treatment with 10⁻⁵ M HPR caused >95% reduction of [³H]thymidine 
uptake by all cell lines except K422. An equal dose of RA, ineffective 
on HL-60, NB306 (as expected), and Raji, suppressed the growth of 
the other cell lines, though less markedly than HPR. K422 was unique 
in its susceptibility to RA, resulting in 99% growth inhibition. At 3 × 
10⁻⁶ M, HPR was almost as effective as the 10⁻⁵ M dose on most cell 
lines. The growth-inhibitory effects were also observed at a 10⁻⁶ M 
concentration.

At 10⁻⁶ M, RA was markedly less inhibitory than an equimolar dose 
of HPR. Importantly, the RA-resistant cell lines HL-60R and NB306 
were, like the parental HL-60 and NB-4 cell lines, highly susceptible to 
HPR.

Trypan blue viability results are shown in Fig. 1. Cytotoxic effects 
by HPR were observed at 10⁻⁵ M on all except K422 cells; at 3 × 10⁻⁶ 
m on all except DHL4, K422, and Raji; and at 10⁻⁶ M on DoHH2 cells 
only. RA was cytostatic on all cell lines.

To examine the kinetics of HPR-induced growth inhibition, time-
course analyses were performed. The results concerning the cell lines 
HL60 and HL-60R are shown in Fig. 2 (top). Treatment with 3 × 10⁻⁶ 
m HPR resulted in a fast growth inhibition of both cell lines, with a 
95% drop in thymidine uptake by 24 h. HPR doses ranging between 
10⁻⁶ and 3 × 10⁻⁷ M were also inhibitory, more effectively in HL-60 
than HL-60R. Growth inhibition by 10⁻⁶ M RA was observed in HL-60 
but not in HL60R, and was less marked than an equimolar dose of 
HPR.
Viable cell counts of retinoid-treated HL-60 and HL-60R cells were determined and compared to those of untreated cells (Fig. 2, bottom). HPR caused, at $3 \times 10^{-6}$ M, a >90% drop in viable cells by 24 h, and at $10^{-6}$ M, a 6- to 10-fold reduction in day 5 cell number. Treatment with $10^{-6}$ M RA, by day 5, resulted in a marked reduction (5-fold) of HL-60 but not HL-60R cell counts.

To determine whether the antiproliferative effects of HPR were reversible upon its removal, a number of time-course experiments were performed. To this aim, the samples were cultured for various intervals of time with $3 \times 10^{-6}$ M HPR, then washed extensively to remove the retinoid, and cultured in fresh medium for a further 48 h before harvesting. The data are shown in Fig. 3. Upon HPR withdrawal, Raji and DHL4 cells acquired a significant proliferative activity, despite exposure to the retinoid for 48 and 72 h, respectively. No growth recovery was observed on NB4 cells when the exposure to HPR exceeded 24 h. A 24-h exposure to the retinoid was sufficient to irreversibly inhibit the growth of DoHH2 and HL60 cells.

Cell Cycle and DNA Analysis. The effect of retinoids on the cell cycle was examined by flow cytometry. Shown in Fig. 4 are the histograms obtained from the analysis of HL-60 cells treated for 72 h. The fraction of S/G2 M cells, accounting for about 45% in control cultures, declined to 17% following RA treatment (Fig. 4A). However, HPR caused striking changes in the total DNA distribution, particularly at $3 \times 10^{-6}$ and $10^{-6}$ M doses (Fig. 4A), evidenced by the appearance of an additional peak left of G1 generated by cells (22 to 54%) whose DNA content was lower than that of G1 cells. It has been previously reported that cells with these features are those dying of apoptosis (31). By performing the flow-cytometric analysis on cells electronically gated according to the level of forward light scattering, the discrimination and quantification of cells with normal (Fig. 4B, bitmap 2) and reduced, apoptotic DNA (Fig. 4B, bitmap 1) was markedly improved. This analysis demonstrated that, after 72-h treatment with $3 \times 10^{-6}$, $10^{-6}$, and $3 \times 10^{-7}$ M HPR, the fraction of cells with normal DNA content accounted for 1, 11, and 55%, respectively. The DNA histograms of HPR-treated samples (Fig. 4C) showed, in addition, up to 50% reduction of S/G2 M cells and allowed to exclude that HPR arrests cells in a specific phase of the cell cycle.

Fig. 4. Effects of HPR and RA on cell cycle. HL60 cells were cultured with the specified dose of retinoid for 72 h, permeabilized with saponin, stained with propidium iodide as described in “Materials and Methods,” and analyzed by flow cytometry. A, DNA histograms generated from the analysis of the total cell population. The percentage of apoptotic cells is indicated. B, bivariate dot-plots (DNA content, ordinate; logarithmic forward light scattering, abscissa) showing 2 fractions, one with normal (bitmap 2) and one with reduced (bitmap 1) DNA content. C, univariate DNA distributions of cells electronically gated to bitmap 2. Numbers are percentages.
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DNA Fragmentation. Apoptosis is an active process which ultimately leads to the activation of endonucleases and cleavage of DNA into fragments of about 185 base pairs. Two approaches were used to examine DNA fragmentation, the first based on the visualization of DNA separated on agarose gels, the second on the in situ enzymatic labeling of nuclei containing fragmented chromatin with a fluorescent nucleotide (see "Materials and Methods"). The latter technique allows detection of DNA breaks on single cells and by flow cytofluorimetry. The data obtained with both methods are shown in Fig. 6.

On agarose gel, the DNA extracted from control cells showed no fragmentation (Fig. 6A); by contrast, HPR-treated cells contained fragmented DNA, mostly present in the lysate's supernatant fraction.

The flow-cytometric analysis demonstrated, on control cells, 4 to 8% dUTP-positive cells (Fig. 6B). However, after incubation with $3 \times 10^{-6}$ M HPR, the fraction of dUTP-labeled cells accounted for 20 and 70% at 6 and 24 h, respectively, indicating that DNA fragmentation starts just before 6 h. Interestingly, since the cell cycle analysis (Fig. 5) failed to show apoptosis on samples exposed for 8 h to HPR, it is suggested that the fluorescein isothiocyanate-dUTP technique is more sensitive in detecting early DNA fragmentation events.

Ultrastructural Findings. Apoptotic cells exhibit distinctive morphological features such as nuclear chromatin compaction and condensation of cytoplasm, that can be evidenced by electron microscopy (32).

We have analyzed DoHH2 cells treated with $3 \times 10^{-6}$ M HPR for 24 h and found clear features of apoptotic cell death in about 30% of cells (Fig. 7).

DISCUSSION

In this paper we provide a number of in vitro data indicating that the synthetic retinoid HPR is, at pharmacologically relevant doses, a strong inhibitor of leukemia and lymphoma cell growth as well as an inducer of apoptotic cell death. In addition, our findings suggest that the mechanism of action of HPR, while unknown, may differ from that of RA, since the biological effects of these compounds in different cell lines were not superimposable.
Regarding the antiproliferative activity, HPR was earlier shown to inhibit the growth of murine mammary tumor virus-infected tumor cells (9) of the human breast cancer cell line MCF-7 by 25% at the dose of 10^{-6} M (10), and to suppress colony formation of human tumors exposed to 5 \times 10^{-6} M HPR (11). The effects of HPR on hematopoietic cell lines have been poorly investigated. Studies on HL-60 cells showed that HPR has little differentiating activity as compared to RA (12). We have obtained similar results on HL-60 as well as on NB-4 (80 to 94% and 2 to 4% of differentiated cells after 5 days of treatment with 10^{-5} M RA and 10^{-6} M HPR, respectively) (data not shown). Additionally, HPR was also ineffective in inducing the differentiation of the RA-resistant clones HL60R and NB306 (data not shown).

Taken together with the proliferative data, these results suggest that the HPR-induced growth arrest is, unlike that induced by RA, uncoupled from differentiation.

We found that HPR triggers apoptosis at doses between 10^{-5} and 10^{-6} M. Apoptosis or programmed cell death is a physiological phenomenon particularly evident during embryogenesis and morphogenesis, and, in adult life, in the maintenance of homeostasis of most tissues by hormonal regulation (33). Apoptosis can be induced in vitro by a wide range of compounds, including antineoplastic agents (34). The molecular pathway leading to apoptosis is largely unknown, although a number of genes such as c-myc, p53, and bel-2 appear to play a critical role in the regulation of the apoptotic process (28, 35, 36).

Although RA can induce apoptosis, this event appears secondary to the induction of terminal differentiation, as revealed by studies on the HL-60 cell line (37). As shown here, HPR causes apoptosis very rapidly, at a time when no differentiation is detected, which raises the possibility that the pharmacological effectiveness of HPR as an antineoplastic compound in vivo may reflect its ability to promote apoptosis rather than differentiation.

The experiments on mutant cell lines indicate that the effects of HPR are uncoupled from the expression of functional RARs. Thus, despite the fact that HL-60 is responsive to RA whereas its variant subclone HL-60R is not, as it carries a point mutation in the ligand binding domain of RARs (41) (24), both cell lines are equally well growth-inhibited by HPR. Likewise, the RA-inducible promyelocytic cell line NB4 and its RA-resistant derivative NB-306, whose resistance to RA is associated with the loss of pml/RARs protein expression (26), are both responsive to HPR. Incidentally, we have recently produced an NB4 subclone resistant to HPR but still capable of differentiating with RA.4

Hence, the differential effects of HPR and RA on these myeloid cells as well as on the lymphoid cells, particularly K422, underlie a distinct mechanism of action for these drugs. This possibility is supported by earlier biochemical data showing that neither HPR nor its metabolites interact with the cellular retinol- and retinoic acid-binding proteins (14), and by the activity of HPR on HL60R cells, which, in addition to bearing a mutated RARs, are negative for RARB, RARs or RXRs mRNA expression (38). Additionally, HPR fails to trans-activate, on chloramphenicol acetyl-transferase assays, RARs, RARB, RARs, and RXRs.5

These findings may be clinically relevant, particularly in acute promyelocytic leukemia with the t(15;17) chromosomal translocation, where, although RA therapy can induce complete remission (19, 20), the relapse rate is relatively high despite continued RA therapy. Our experimental data, particularly those on the t(15;17) promyelocytic leukemia cell line NB-4 and its RA-resistant variant NB306, both responsive to HPR, suggest that HPR could be a good candidate for the treatment of RA-resistant APL in relapse. Pharmacological findings would also support this use, in particular the fact that continuous administration of RA in APL patients is associated with progressive reduction in plasma drug concentration (39), below the therapeutic levels, which provides an explanation for the clinical resistance to RA. In marked contrast, the HPR plasma concentrations have been found, at least in breast cancer patients, almost unchanged within a 1-year follow up, despite daily administration of the drug (30).

The substantial information concerning pharmacokinetics and toxicity in humans (16), the cancer chemopreventive features in animals (5, 6), and the ongoing clinical trials in patients for chemoprevention of breast cancer recurrence (17) and of oral leukoplakia (18) should encourage clinical studies to establish the efficacy of HPR in the therapy of APL as well as of other hemopoietic cancers.

In conclusion, our in vitro data indicate that HPR suppresses cell growth of hematopoietic malignant cell lines and induces apoptosis. Since the biological responses to HPR and RA differ, it is conceivable that these compounds act through different pathways, an issue which we are currently investigating.

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