The Unhydrolyzable Fenretinide Analogue 4-Hydroxybenzylretinone Induces the Proapoptotic Genes GADD153 (CHOP) and Bcl-2–Binding Component 3 (PUMA) and Apoptosis that Is Caspase-Dependent and Independent of the Retinoic Acid Receptor

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Abstract

The synthetic retinoid \(N\)-(4-hydroxyphenyl)retinamide (4-HPR) induces apoptosis in a variety of cell lines and has shown promise as an anticancer agent both in vitro and in vivo. The clinical dose of 4-HPR, however, is limited by residual-associated toxicities, indicating a need for a less toxic drug. In this study, we show that 4-hydroxybenzylretinone (4-HBR), the unhydrolyzable analogue of 4-HPR, is effective in producing apoptosis in a variety of 4-HPR–sensitive cell lines, including breast cancer, neuroblastoma, and leukemia cells. We also show through the use of a pan-caspase inhibitor that this 4-HBR–induced apoptosis is dependent, at least in part, on caspase activity. 4-HBR is shown to exhibit binding to the retinoic acid receptors (RAR) at concentrations necessary to induce cell death and induces expression of all-trans-retinoic acid–responsive genes that can be blocked by a RAR pan-antagonist. However, through the use of this RAR pan-antagonist, 4-HBR–induced apoptosis and cell death is shown to be independent of the RAR signaling pathway. To further characterize the mechanism of action of 4-HBR, expression of the endoplasmic reticulum stress–induced genes GADD153 and Bcl-2–binding component 3 was examined. These mRNAs are shown to be rapidly induced in 4-HBR–treated and 4-HPR–treated breast cancer cells, and this up-regulation is also shown to be independent of the RARs. These results suggest that a stress-mediated apoptotic cascade is involved in the mechanism of action of these retinoids. [Cancer Res 2007;67(13):6270–7]

Introduction

Retinoids, both natural and synthetic, are a class of compounds with structural similarity to vitamin A. Vitamin A and its metabolites play essential roles in cellular growth, differentiation, and vision (1). Retinoids have shown promise as anticancer agents, exhibiting antitumor effects in a variety of cancer cell types both in vitro and in vivo (2–4). Although naturally occurring retinoids, such as all-trans-retinoic acid (atRA), are proven inhibitors of cancer cell growth, the therapeutic use of these compounds is limited due to their undesirable side effects, including mucocutaneous irritation, hyperlipidemia, bone toxicity, reduced night vision, and teratogenicity (5). Because of this, a continuing goal in retinoid drug development has been to find new analogues with improved therapeutic indices.

\(N\)-(4-hydroxyphenyl)retinamide (fenretinide, 4-HPR) is a synthetic amide analogue of atRA that was originally developed and studied as a chemopreventive agent (6). More recently, 4-HPR was found to induce apoptosis in a variety of cancer cell lines, including breast, prostate, neuroblastoma, and leukemia (2, 7). Clinically, 4-HPR has been shown to reduce local recurrent and contralateral breast cancer in premenopausal women (8, 9). Although these results are promising, the clinical dose of 4-HPR is limited by residual-associated toxicities, most notably the production of night blindness due to a decrease in serum retinol levels (10).

A new nonhydrolyzable 4-HPR analogue, 4-hydroxybenzylretinone (4-HBR), has been reported (11). Like the parent retinamide, 4-HBR has significant chemotherapeutic activity and has been shown to reduce the size and number of 7,12-dimethylbenz(a)anthracene–induced mammary tumors in rats (12). 4-HBR, like 4-HPR, has been shown to induce apoptosis and cell death in the MCF-7 breast cancer cell line (13). 4-HBR shows an improved therapeutic profile when compared with 4-HPR. Importantly, 4-HBR, unlike 4-HPR, is not hydrolyzed in vivo to liberate atRA, thus eliminating any residual toxicity that might result from generation of the vitamin A acid. Further, 4-HBR given to the animal in an amount that is effective in mammary carcinogenesis does not reduce circulating blood retinol levels, suggesting that this new analogue would not cause night blindness and, thus, could be used safely in the clinic at higher concentrations than the parent 4-HPR.

The biological effects of atRA are mediated through binding to the nuclear retinoic acid receptors (RAR; ref. 14). Several authors have reported that 4-HPR acts, at least in part, through activation of the RAR, whereas others have reported that 4-HPR acts independent of the RAR signaling pathway (2, 15–17). Whether RAR signaling plays a role in 4-HBR action is unknown. Whereas 4-HPR could conceivably activate RAR-mediated gene transcription by acting either directly as an agonist at the receptor binding site or indirectly via hydrolysis to atRA, 4-HBR would be expected to act only directly at the RAR-binding site as it cannot undergo hydrolysis. Both 4-HPR and its nonhydrolyzable analogue exhibit much less avid binding to RARs in vitro than does the natural ligand atRA (12). Whether 4-HBR acts as an agonist at the RAR-binding site and whether this plays a role in its ability to induce apoptosis or tumor cell death remains to be established.

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Most knowledge about the mechanism of 4-HPR action stems from studies focused on the more downstream mediators of the death signal invoked by 4-HPR, including a dependence on caspase activity in several cell lines (18–20). Some reports indicate that 4-HPR acts through the production of reactive oxygen species (21, 22) and/or the induction of the lipid secondary messenger ceramide (23, 24). Still, others report that 4-HPR regulates the cellular levels of proapoptotic and/or antiapoptotic genes (25–29). Whether the downstream signaling pathways for 4-HBR are similar to those of 4-HPR is unknown.

In the work described herein, 4-HBR is shown to be effective in inducing apoptosis and cell death in four different cancer cell lines (MCF-7 and T-47D breast cancer, LAI-15n neuroblastoma, and HL-60 myeloid leukemia), and the necessity for RAR activation in 4-HBR action is studied in detail. The ability of 4-HBR to induce the expression of atRA-responsive genes is reported, and a high-affinity antagonist of RA-induced function at all three RAR subtypes (30) is shown to have no effect on the ability of 4-HBR to induce apoptosis or cell death. Further, the present work shows that the proapoptotic genes GADD153 (CHIP) and Bcl-2-binding component 3 (BBC3; PUMA) are rapidly induced by 4-HBR in all four cell lines and that apoptosis produced by this 4-HPR analogue involves a caspase-dependent mechanism.

Materials and Methods

Chemicals. [20-methyl 3H or 11,12-3H]-all-trans-RA (1H)atRA; 70 or 40.5 Ci/mmol, respectively) and [20-methyl 3H]-9-cis-RA (1H)heisRA; 69.4 Ci/mmol) were obtained from New England Nuclear. atRA was purchased from Spectrum Chemical and Laboratory Products. 4-HPR was a gift from McNeil Pharmaceuticals. 4-HBR was synthesized as described previously (11). AGN193109 and LGD1069 were prepared using published methods (31, 32).

Cell culture. MCF-7, T-47D, and HL-60 cells were obtained from the American Type Culture Collection and LAI-15n cells were a gift from Dr. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). MCF-7 and LAI-15n cells were cultured as described previously (13, 33). HL-60 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, t-glutamine (0.3 g/l), and sodium bicarbonate (1.5 g/L). T-47D cells were cultured in RPMI 1640 containing the same supplements with the addition of bovine pancreas insulin (0.2 unit/ml).

Live cell number and terminal deoxynucleotidyl transferase–mediated duTP nick end labeling assays. For time course assays, cells were plated at 50,000 per well in 12-well plates and assayed at various times after treatment with vehicle (0.2% ethanol) or retinoid (4-HPR or 4-HBR at both 10⁻⁵ and 10⁻³ mol/L, in ethanol). UV irradiation (120 mJ/cm²) for two cycles in the Stratagene UV Stratalinker 2400 and hydrogen peroxide (50 mmol/L; 1 h) treatment served as controls for apoptosis and necrosis, respectively. These conditions were optimized for MCF-7 cells in our laboratory in a series of control experiments. Before harvesting, cells were incubated with 10 μg/ml propidium iodide for 10 min at 37°C to stain necrotic cells. Floating and adherent cells were collected and centrifuged at 250 × g for 5 min at 4°C and resuspended in 1 × PBS. Half of the sample was assayed for total and live cell numbers using fluorescein diacetate (cleaved in metabolically active cells to liberate a fluorescent product), whereas the other half was assayed for apoptosis by terminal deoxynucleotidyl transferase–mediated duTP nick end labeling (TUNEL) staining (In Situ Cell Death Detection kit, Roche Molecular Biochemicals) as described previously (13).

For antagonist assays, cells were plated in triplicate at 50,000 per well in 12-well culture plates and treated with vehicle, atRA, 4-HPR, or 4-HBR alone (in ethanol) or combined with AGN193109 for 8 days (or 5 days; HL-60 and LAI-15n cells).

Caspase inhibition experiments involved a 1-h incubation with vehicle (0.1% DMSO) or 50 μmol/L z-VAD-fmk (Sigma Chemical, Inc.) before dosing with retinoid. MCF-7, T-47D, and LAI-15n cells were treated with vehicle, 10⁻⁵ or 10⁻³ mol/L 4-HPR or 4-HBR, UV irradiation, or hydrogen peroxide. HL-60 cells were treated with vehicle, 10⁻⁵ or 10⁻³ mol/L 4-HPR, UV irradiation, or hydrogen peroxide. Cells were harvested and assayed for total and live cell number and apoptosis as was described previously.

Cell growth inhibition assay. Cells were seeded at 10,000 per well (or 50,000 per well; LAI-15n) in 12-well plates and allowed to attach overnight (except HL-60, which grow in suspension), and wells in triplicate were dosed with vehicle or varying amounts of atRA, 4-HPR, or 4-HBR alone or combined with AGN193109 for 8 days (or 5 days; HL-60 and LAI-15n cells).
The medium was changed and fresh vehicle or retinoid was added daily. Cells were harvested and fluorescein diacetate was used to determine the number of live cells as described previously (13). Using fluorescent microscopy, at least 200 cells per sample were counted with a hemacytometer.

Nuclear retinoid receptor binding. The ability of retinoids to compete with $[^3]H$-retinoic acid (RA) for binding to individual RAR subtypes and with $[^3]H$-9-cisRA for binding to retinoid X receptor (RXR) was determined using recombinant receptors in an in vitro ligand-binding assay as described previously (34, 35). The final concentration of $[^3]H$-RA was 2 or 4 nM, and that of $[^3]H$-9-cisRA was 10 nM.

RNA isolation and quantitative PCR. Cells were harvested and suspended in Trizol reagent (Invitrogen), and total RNA was collected according to the manufacturer’s protocol. Total RNA (1 µg) was reverse transcribed using avian myeloblastosis virus reverse transcriptase (15 units/25 µL) and random hexamers (100 pmol/25 µL; Promega) in the presence of 0.4 mM dNTP, deoxyoligonucleotides, 28 units RNasin, and 1× reverse transcriptase buffer supplied with the enzyme. The LightCycler instrument (Roche Molecular Biochemicals) was used to do real-time quantitative PCR with the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. Values were then expressed as fold change from the vehicle-treated samples. The following primer sets were designed using the cDNA sequences: hRAR, 5′-CCAGGCAAAATCAGGTT-3′ (upstream) and 5′-TCGCCAGGCTAAGGAAGTT-3′ (downstream); hRXR, 5′-CCCTTCTTGGGAAACCTT-3′ (upstream) and 5′-GCCGGCATAATCACCTTCTT-3′ (downstream); GADD153, 5′-CCAGGACAAATCATCAGGGT-3′ (upstream) and 5′-GAGAATAAGTCCCTTCCCTTCC-3′ (downstream); RA was 10 nmol/L. Results

4-HBR induces apoptosis and cell death in human breast cancer, neuroblastoma, and myeloid leukemia cell lines

The ability of 4-HBR to induce cell death was tested in a variety of cancer cell lines known to be sensitive to 4-HPR, including MCF-7 (36), T-47D (37), LAI-15n, and HL-60 (15, 38). In the breast cancer cell lines, 4-HBR at the highest concentration (10−4.5 mol/L) resulted in the death of nearly all cells by 48 h (Fig. 1A and B, top). A 50% reduction in live cell number was detected as early as 8 h in T-47D cells exposed to 4-HBR, whereas MCF-7 cells responded more slowly. Cell death was also induced by 4-HBR in the human neuroblastoma cell line LAI-15n (Fig. 1C, top). An 85% reduction in live cell number was evident at the 10−4.5 mol/L dose as early as 8 h, whereas nearly all cells were dead by 48 h at both the 10−3.5 mol/L and 10−4.5 mol/L doses of 4-HBR. The human myeloid leukemia cell line HL-60 was also very sensitive to the highest dose of 4-HBR, with an 85% reduction in live cell number noted by 16 h (Fig. 1D, top). In general, 4-HBR was ~0.5 log unit less potent than 4-HPR in producing cell death. In summary, 4-HBR at 10−4.5 mol/L is effective in inducing the death of cell types that are also sensitive to 4-HPR.

4-HPR induces cell death via induction of apoptosis (7), and evidence has been presented that 4-HBR also induces apoptosis in MCF-7 cells (13). In the present study, apoptosis was examined in three additional cancer cell lines (T-47D, LAI-15n, and HL-60) exposed to 4-HBR. In all cell lines, an increase in the percentage of cells exhibiting TUNEL labeling was observed over time at the 10−4.5 mol/L concentration of 4-HBR (Fig. 1A–D, bottom). A small amount of TUNEL labeling was observed in T-47D breast cancer cells treated with 4-HBR at 10−4.5 mol/L at 8 h (4%), and this number increased with time, with 70% of cells showing TUNEL labeling at 48 h (Fig. 1B, bottom). Similarly, in MCF-7 cells treated with 4-HBR (10−4.5 mol/L), 2% of cells stained positive for TUNEL at 16 h, increasing to 16% and 61% of cells at 24 and 48 h, respectively (Fig. 1A, bottom). A high percentage of apoptotic cells were present after exposure of both LAI-15n and HL-60 cells to 4-HBR at 10−4.5 mol/L, and the number of labeled cells increased with time (Fig. 1C and D, bottom). Therefore, 4-HBR, like 4-HPR, induces apoptosis and cell death in breast cancer (MCF-7 and T-47D), neuroblastoma (LAI-15n), and myeloid leukemia (HL-60) cells.

In most of the cell lines tested, a significant number of TUNEL-positive cells were observed at the same time that cell death was increased. However, for the T-47D breast cancer cells, a significant reduction in cell number occurred before the time that any appreciable apoptosis could be detected. For example, when T-47D cells were exposed to 4-HPR at 10−4.5 mol/L for 8 h, an 82% reduction was noted in live cell number, whereas only 1% of the total cell population showed TUNEL labeling. Likewise, after exposure of cells to 4-HBR at 10−4.5 mol/L for 8 h, 50% of the cells had died, whereas only 4% of the cells were undergoing apoptosis. This suggests that, although apoptosis may play an important role in the mechanism whereby 4-HBR and fenretinide lead to cell death, other pathways may also be involved.

4-HBR–Induced Apoptosis Is Dependent, at Least in Part, on Caspase Activity

Because the exposure of MCF-7, T-47D, LAI-15n, and HL-60 cells to 4-HBR caused the cells to undergo apoptosis, the pan-caspase inhibitor z-VAD-fmk was used to determine the role of caspases in 4-HBR–induced cell death. Cells were pretreated with caspase inhibitor (or vehicle) for 1 h followed by exposure to the ethanolic...
vehicle (data not shown), 4-HPR, or 4-HBR. In MCF-7 and T-47D breast cancer cells, the presence of z-VAD-fmk caused a 79% and 64% reduction in TUNEL labeling after 24 h in 4-HBR–treated cells, respectively, with similar reductions noted in the 4-HPR treatment group (Fig. 2). LAI-15n cells showed even greater reductions in TUNEL labeling in the presence of z-VAD-fmk, with 93% and 79% reductions, respectively, in the 4-HBR and 4-HPR groups (Fig. 2). The caspase inhibitor was also effective in inhibiting TUNEL labeling by 68% in HL-60 cells exposed to 4-HBR and by 36% in 4-HPR–treated cells (Fig. 2). Thus, the induction of apoptosis by 4-HBR is, at least in large part, caspase mediated. Although the presence of z-VAD-fmk decreased the amount of TUNEL labeling after treatment with retinoid in each cell type, there were still a significant number of cells undergoing apoptosis even in the presence of the pan-caspase inhibitor (data not shown). Although this may have represented incomplete caspase inhibition, it might also be an indication that there was a noncaspase-mediated component to the cell death.

4-HBR–Induced Apoptosis Is Independent of the RAR

To address whether the RAR pathway contributes to the activity of 4-HBR and/or its parent 4-HPR in inducing apoptosis in MCF-7, T-47D, LAI-15n, or HL-60 cells, a series of experiments were initiated to determine, first, whether these compounds at the concentrations needed to induce apoptosis could bind to the RAR in vitro and, second, whether the retinoids were also capable of inducing RAR-mediated gene transcription in these cells. Using the information gleaned from these experiments, we determined the optimal concentration of a RAR α,β,γ pan-antagonist that would block RAR-mediated gene transcription for each individual cell line (except HL-60 cells for which the changes observed in gene expression in response to RA were less robust) and then verified that the antagonist could block atRA-mediated growth inhibition in all four cell lines. Using this information, we then determined whether this same concentration of antagonist would block the induction of apoptosis or cell death by 4-HBR or the parent 4-HPR.

4-HBR exhibits some binding to RARs at concentrations necessary to induce cell death. The results of competition-binding experiments showed that both 4-HBR and 4-HPR were weak competitors for [3H]atRA binding to all three RAR types (4-HBR: $K_i = 2.5, 4.6,$ and $16 \mu M$ for RARα, RARβ, and RARγ, respectively; Supplementary Fig. S1) but neither showed competition for [3H]9cisRA binding to the RXR (data not shown). As expected, atRA competed well ($K_i$ values in the subnanomolar range) for binding to all three RAR types. Thus, 4-HBR is a much lower affinity ligand for the RAR compared with atRA but is capable of binding to a fraction of receptors when present at concentrations shown to induce apoptosis and cell death ($10^{-5}$ M). A role for the RAR in 4-HBR action, therefore, cannot be ruled out solely based on RAR-binding activity.

4-HBR induces expression of atRA-responsive genes in cancer cell lines and this activity can be blocked by a RAR pan-antagonist. Because 4-HBR at high concentrations can bind to the RAR, the ability of 4-HBR to induce RAR-mediated gene transcription was examined. Preliminary experiments were done using atRA to determine optimal times for measuring induction of selected atRA-responsive genes (RARβ, CYP26A1, and/or CYP26B1) in the three cell lines examined (data not shown). Based on these results, the ability of 4-HBR to induce these mRNAs was examined and the ability of a RAR pan-antagonist to prevent this induction was also studied. AGN193109, a RAR antagonist that binds all three

![Figure 3](https://www.aacrjournals.org/6273/CancerRes2007;67:(13).July1,2007)

**Figure 3.** The induction of RA-responsive genes by 4-HBR is blocked by addition of the RAR pan-antagonist AGN193109 (AGN). Total RNA was isolated from cells treated with retinoid alone or combined with the RAR pan-antagonist at $10^{-5}$ M at 4 h in MCF-7 (A), RAR pan-antagonist at $10^{-6}$ M at 4 h in T-47D (B), or at $10^{-5}$ M at 12 h in LAI-15n cells (C). mRNA was reverse transcribed and assayed in triplicate by real-time quantitative PCR. Values are normalized to GAPDH mRNA expression. The concentration of AGN193109 chosen for use in each cell line was determined in a separate set of control experiments; it is the highest concentration that was shown not to affect the live cell number when added daily for 5 d (LAI-15n) or 8 days (MCF-7 and T-47D) in culture compared with cells exposed for the same time period to the vehicle control (data not shown; see also Fig. 4A).

RAR subtypes with an affinity similar to that of atRA (AGN193109 $K_i = 3.2, 1.1,$ and $1.2 \mu M$; atRA $K_i = 0.8, 0.8,$ and $0.4 \mu M$; at the RARα, RARβ, and RARγ, respectively) but does not bind to the RXR (Supplementary Fig. S2), was chosen for this study (30). The exposure of MCF-7 cells to atRA, 4-HBR, or 4-HPR resulted in
CYP26A1 induction at 4 h (Fig. 3A) and this was completely inhibited by cotreatment with the pan-RAR antagonist (10^{-5} mol/L). In T-47D cells, a robust induction of CYP26A1 by atRA and more modest increases in this mRNA were observed after exposure to 4-HBR or 4-HPR at 10^{-6} and 10^{-4.2} mol/L, and these changes were completely blocked by AGN193109 (Fig. 3B). In LAI-15n cells, atRA, 4-HBR, and 4-HPR all increased RARβ mRNA, and the increase in this mRNA was blocked by coadministration of AGN193109 (Fig. 3C). Thus, it is clear that 4-HBR at concentrations used to induce apoptosis and cell death, like fenretinide, is also active in inducing RAR-mediated gene transcription. Importantly, this work also shows that the RAR signaling induced by 4-HBR, even at the highest concentration of 10^{-4.5} mol/L, can be blocked by cotreatment with the high-affinity pan-RAR antagonist AGN193109.

Apoptosis and cell death in cells exposed to 4-HBR is independent of the RAR signaling pathway. We next verified that the RAR pan-antagonist did not, on its own, inhibit cell growth but that growth inhibition induced by atRA could be completely blocked by the antagonist. As shown in Fig. 4A, AGN193109 at concentrations shown to inhibit RAR-mediated gene transcription did not disrupt cell growth. Further, in all four cell lines, the addition of the RAR pan-antagonist was able to prevent the growth-inhibitory effects of atRA, an event known to be dependent on RAR signaling (Fig. 4A). Importantly, cells treated with a dose of 4-HBR (10^{-5} mol/L) that, after 48 h of treatment, was shown previously to not be fully effective in producing apoptosis or cell death were largely dead at the end of the 4- to 8-day assay, and this cell death was not prevented by the addition of AGN193109.

Next, we examined whether the induction of apoptosis by 4-HBR was impaired in the presence of the AGN193109. The results in Fig. 4B show that the extent of TUNEL labeling produced by 4-HBR was unchanged when the RAR pan-antagonist was present, and similar results were obtained with 4-HPR. Thus, a concentration of RAR pan-antagonist that blocks atRA-mediated gene expression does not inhibit the induction of apoptosis by 4-HBR in MCF-7, T-47D, LAI-15n, or HL-60 cells. Taken together, these results show that the induction of apoptosis and cell death by 4-HBR and 4-HPR in these cell lines (MCF-7, T-47D, LAI-15n, and HL-60) is independent of the RAR.

4-HBR Causes a Rapid Increase in GADD153 and BBC3 mRNAs that Is Independent of the RAR

It has been reported previously that 4-HPR causes increased expression of the proapoptotic gene GADD153 (CHOP; refs. 25, 39) in hepatoma and neuroblastoma cells and, more recently, BBC3 (PUMA) in SH-SY5Y neuroblastoma cells (29). It is not known whether 4-HBR affects the expression of either of these mRNAs nor is much known about the expression of these genes in response to fenretinide in other cell types. For this reason, we examined GADD153 and BBC3 mRNA expression using reverse transcription-PCR (RT-PCR) in atRA, 4-HBR–treated, and 4-HPR–treated MCF-7, T-47D, LAI-15n, and HL-60 cells. The expression of both GADD153 and BBC3 mRNAs was increased at 12 h in MCF-7 cells exposed either to 4-HBR (8.2- and 2.6-fold, respectively, at 10^{-4.5} mol/L) or 4-HPR (22- and 3.6-fold, respectively, at 10^{-4.5} mol/L; Fig. 5, left). atRA did not produce any increase in either mRNA. To determine whether the increase in GADD153 or BBC3 mRNAs required binding to the RARs, the assay was done in the presence and absence of AGN193109. The presence of the pan-RAR antagonist did not decrease either GADD153 or BBC3 mRNA expression in response to either retinoid, indicating that the increased expression of these proapoptotic genes is independent of RAR signaling (Fig. 5, left). Both the GADD153 and BBC3 mRNAs were also induced by 4-HBR and fenretinide in T-47D (4 h), LAI-15n (12 h), and HL-60 cells (4 h; data not shown). A time course showed that the increase in the GADD153 and BBC3 mRNAs was detected as

Figure 4. Apoptosis and cell death in 4-HBR–treated cells do not require RAR signaling. A, live cell number after exposure to vehicle, atRA, 4-HBR, or 4-HPR in the presence and absence of the RAR pan-antagonist (10^{-6} mol/L for T-47D and HL-60; 10^{-5} mol/L for MCF-7 and LAI-15n) for 5 d (HL-60 and LAI-15n) or 8 d (MCF-7 or T-47D). Medium and dosing solutions were replenished daily. B, TUNEL labeling induced by retinoids in the presence and absence of the RAR pan-antagonist. The % TUNEL labeling was assessed at 24 h in cells exposed to 4-HBR or 4-HPR in the presence and absence of the RAR pan-antagonist. Data are representative of two independent experiments for each cell line. Columns, mean; bars, SE.
4-HBR induces GADD153, BBC3, and apoptosis

Early as 6 h after exposure of MCF-7 cells to either 4-HBR or 4-HPR and as early as 1 h (GADD153) or 2 h (BBC3) after exposure of T-47D cells (Fig. 5, right; data not shown). Thus, both 4-HPR and 4-HBR treatment of a variety of tumor cell types increased both GADD153 and BBC3 mRNAs in a manner that was independent of binding to the RARs. Furthermore, at least in MCF-7 and T-47D cells, the increase in these mRNAs occurred before the time that any significant apoptosis was detected. This suggests that these gene changes could mediate early events in the mechanism whereby 4-HBR and 4-HPR induce apoptosis and cell death.

Discussion

In the present report, we show that 4-HBR, like its parent compound, produces apoptosis and cell death in several cancer cell types, including breast cancer (MCF-7 and T-47D), neuroblastoma (LAI-15n), and a myeloid leukemia (HL-60) cell line. 4-HBR represents a promising new drug in the arsenal of chemotherapeutic agents and has an advantage over the parent 4-HPR molecule as it cannot be hydrolyzed to atRA (13) and does not alter blood retinol levels when fed to animals at chemotherapeutic levels (12). Although clinical trials of fenretinide in breast cancer and chemoprevention of recurrence in head and neck cancers have shown some promise (9, 40), the overall results are not as dramatic as would be predicted from cell culture and animal studies. Although plasma fenretinide levels of 1 μmol/L were achieved in humans treated orally at 200 mg/d (10), it is possible that the blood and tissue levels of the retinamide were still below optimal to produce greater positive clinical effects. In cell culture studies, doses of 4-HPR of 10 μmol/L are often needed to produce significant apoptosis and cell death. The major clinical dose-limiting side effect of fenretinide is night blindness that results from a decrease in circulating retinol levels. Thus, it may be possible to overcome the dose-limiting effects of fenretinide with higher doses of the novel unhydrolyzable nontoxic 4-HBR analogue.

The chemotherapeutic effect of the 4-HPR molecule has been proposed to occur via a mechanism independent of RAR activity based on its ability to inhibit the growth of atRA-resistant cell lines (15, 17). However, other studies have suggested a role for the RAR in mediating growth inhibition by 4-HPR (41, 42). 4-HPR has been shown to undergo limited hydrolysis to atRA when fed to vitamin A–deficient rats (13), suggesting a means whereby 4-HPR might act as a prodrug to liberate the RAR activator atRA. However, others have reported that atRA could not be detected in 4-HPR–treated MCF-7 cells (17), suggesting that, unlike in the whole animal, little atRA is liberated from this retinamide in cell culture. In the present study, we show that the unhydrolyzable 4-HBR analogue can, at high concentrations, compete with [3H]atRA for binding to all three RAR subtypes. Furthermore, we find that 4-HBR regulates endogenous RAR-mediated gene expression using the native complement of receptors present in two human mammary cancer lines and in a neuroblastoma cell line. Thus, 4-HBR, like fenretinide, is capable of stimulating RAR-mediated gene expression in these cell lines.

To determine whether RAR binding plays any role in the apoptosis or cell death that occurs in cells exposed to 4-HBR, a RAR pan-antagonist was used to block RAR binding and downstream signaling. Using a concentration of antagonist that effectively blocks all atRA-inducible gene expression, we show that the induction of apoptosis by 4-HBR is unaltered. A different RAR pan-antagonist (BMS-204701) was also reported to be ineffective in inhibiting 4-HPR–induced apoptosis in human embryonal carcinoma cells (16). In a study of a different human neuroblastoma cell line (SH-SY5Y), several RAR antagonists were unable to block the 4-HPR induction of Bak, a proapoptotic Bcl-2–related gene (26). However, in contrast to our findings, this group also reported that a RARβ/RARγ antagonist could inhibit apoptosis (43). Thus, the ability of 4-HPR and 4-HBR to induce apoptosis, at least in MCF-7, T-47D, LAI-15n, and HL-60 cells, does not seem to be dependent on the RARs. This agrees with studies in many cell systems indicating that apoptosis in response to 4-HPR seems to be predominantly RAR independent (2, 7).

The present study shows that both 4-HBR and the parent 4-HPR induce apoptosis and cell death that is dependent, at least in part, on caspase activity. The initial evidence for the involvement of caspases in 4-HPR action included the activation of the effector caspase-3 and resultant cleavage of poly(ADP-ribose) polymerase.
A role for caspase-8 and caspase-9 in 4-HPR action has also been reported (19, 20). In human neuroblastoma and MCF-7 cells, pan-caspase inhibitors (BOC-d-fmk and z-VAD-fmk, respectively) were shown to significantly reduce apoptosis after 4-HPR treatment (24, 44). In Fas-defective hepatoma cells, the general caspase inhibitor z-VAD-fmk completely blocked apoptosis in response to 4-HPR (20). Thus, consistent with previous findings for fenretinide, 4-HBR acts, at least in part, via a caspase-dependent mechanism.

Our results show that the proapoptotic molecule GADD153 is very rapidly up-regulated by 4-HR and 4-HPR in breast cancer cells, suggesting that endoplasmic reticulum (ER) stress could be an important event initiating apoptosis by both of these retinoids. ER stress has recently been implicated in 4-HPR–mediated apoptosis in glioma cells (45). GADD153 has also been reported to be increased in response to 4-HPR treatment in both SH-SY5Y neuroblastoma and Hep3B hepatoma cells (25, 26, 39) and more recently was reported to be induced by 4-HPR in a microarray analysis of T-47D cells treated for 24 or 48 h (46). The present study shows that GADD153 mRNA is very rapidly induced in breast cancer cells (within 1 to 6 h) exposed to 4-HBR or fenretinide. Whether this induction of GADD153 is due to a signal emanating from the ER or results from a pathway distinct from that of the ER stress signaling cascade (47) remains to be established.

Members of the Bcl-2 family of proteins are also key regulators of apoptotic pathways, and they are influenced by ER stress and, conversely, can regulate both mitochondrial-mediated and ER stress–induced apoptosis (48). The BH3-only, proapoptotic Bcl family member protein BBC3, also known as PUMA, was first identified as a Bcl-2–binding protein in a yeast two-hybrid screen (51). In 2005, Wei et al. reported early induction of BBC3 mRNA and protein levels in 4-HPR–treated SH-SY5Y cells. RT-PCR analysis of BBC3 mRNA levels in our studies reveals both a time- and dose-dependent up-regulation of BBC3 mRNA in breast cancer cells, neuroblastoma, and human myeloid leukemia cells. Interestingly, in breast cancer cells, 4-HPR treatment has also been reported to result in a decrease in the antiapoptotic Bcl-2 mRNA (28). Thus, an increase in proapoptotic and a decrease in antiapoptotic Bcl-2 family members could contribute to 4-HBR–induced and fenretinide-induced apoptosis, at least in some cell types. We also report that the up-regulation of both GADD153 and BBC3 by 4-HR and 4-HPR is independent of RAR signaling. Thus, these results suggest that changes in GADD153 and BBC3 could play an important role in both 4-HPR– and 4-HBR–induced apoptosis and suggest that an ER stress–mediated apoptotic cascade is involved in the mechanism of action of these drugs.

In summary, we have shown that the unhydrolyzable 4-HPR analogue, 4-HBR, induces apoptosis and cell death in a caspase-dependent fashion in several different cell lines, including breast, neuroblastoma, and leukemia. 4-HR as well as the parent fenretinide rapidly induce the expression of both GADD153 and BBC3 mRNAs. The induction of these mRNAs by 4-HBR and fenretinide is not altered by the presence of a RAR α,β,γ antagonist nor is the induction of apoptosis in these cell lines (MCF-7, T-47D, LAI-15n, and HL-60) dependent on the RAR. Future experiments will be aimed at identifying key events that lie upstream of this stress response and apoptotic cascade to further elucidate the mechanism of 4-HBR action.

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The Unhydrolyzable Fenretinide Analogue 4-Hydroxybenzylretinone Induces the Proapoptotic Genes GADD153 (CHOP) and Bcl-2–Binding Component 3 (PUMA) and Apoptosis that Is Caspase-Dependent and Independent of the Retinoic Acid Receptor


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