4-Oxo-Fenretinide, a Recently Identified Fenretinide Metabolite, Induces Marked G<sub>2</sub>-M Cell Cycle Arrest and Apoptosis in Fenretinide-Sensitive and Fenretinide-Resistant Cell Lines

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Abstract

4-Oxo-N-([4-hydroxyphenyl]retinamide (4-oxo-4-HPR) is a recently identified metabolite of fenretinide (4-HPR). We explored the effectiveness of 4-oxo-4-HPR in inducing cell growth inhibition in ovarian, breast, and neuroblastoma tumor cell lines; moreover, we investigated the molecular events mediating this effect in two ovarian carcinoma cell lines, one sensitive (A2780) and one resistant (A2780/HPR) to 4-HPR. 4-Oxo-4-HPR was two to four times more effective than 4-HPR in most cell lines, was effective in both 4-HPR–sensitive and 4-HPR–resistant cells, and, in combination with 4-HPR, caused a synergistic effect. The tumor growth-inhibitory effects of 4-oxo-4-HPR seem to be independent of nuclear retinoid receptors (RAR), as indicated by the failure of RAR antagonists to inhibit its effects and by its poor ability to bind and transactivate RARs. Unlike 4-HPR, which only slightly affected the G<sub>1</sub> phase of the cell cycle, 4-oxo-4-HPR caused a marked accumulation of cells in G<sub>2</sub>-M. This effect was associated with a reduction in the expression of regulatory proteins of G<sub>2</sub>-M (cyclin-dependent kinase 1 and cdc25c) and S (cyclin A) phases, and with an increase in the expression of apoptosis-related proteins, such as p53 and p21. Apoptosis was induced by 4-oxo-4-HPR in both 4-HPR–sensitive and 4-HPR–resistant cells and involved activation of caspase-3 and caspase-9 but not caspase-8. We also showed that 4-oxo-4-HPR, similarly to 4-HPR, increased reactive oxygen species generation and ceramide levels by de novo synthesis. In conclusion, 4-oxo-4-HPR is an effective 4-HPR metabolite that might act as therapeutic agent per se and, when combined with 4-HPR, might improve 4-HPR activity or overcome 4-HPR resistance. (Cancer Res 2006; 66(6): 3238-47)

Introduction

Retinoids are natural and synthetic derivatives of vitamin A (retinol) that play an important role in different cellular processes, including proliferation, differentiation, and apoptosis (1). Retinoids exert their biological effects through the binding and transactivation of retinoic acid receptors (RAR<sub>a</sub>, RAR<sub>β</sub>, and RAR<sub>γ</sub>) and retinoid X receptors (RXR<sub>a</sub>, RXR<sub>β</sub>, and RXR<sub>γ</sub>; ref. 2). However, these activities do not explain all their growth-inhibitory and apoptotic effects (3).

A large number of synthetic retinoids have been investigated in preclinical models, and clinical data have already supported the potential of these compounds as cancer preventive and therapeutic agents (4, 5). One of the major limits in the clinical use of retinoids is their toxicity. N-([4-hydroxyphenyl]retinamide (4-HPR), an amide of all trans-RA is, unlike other retinoids, well tolerated in humans (6–8) and has already shown promising results in preneoplastic and neoplastic conditions. Preneoplastic diseases, including oral leukoplakia (9, 10), lichen planus (11), and actinic keratoses (12), have been successfully treated with this retinoid. In a trial in women operated on for early-stage breast cancer, 4-HPR prevented second breast malignancies in premenopausal women (6), and, during the 5-year intervention period, it reduced the occurrence of ovarian carcinoma (7). Promising preliminary results have recently been obtained with this retinoid in a phase I trial in children with neuroblastoma (8). 4-HPR has been shown to inhibit the growth and induce apoptosis in a variety of human tumor cell lines (13). Although 4-HPR has been shown to be a selective activator of RARα and, to a lesser extent of RARβ (14), the role of RARs in 4-HPR tumor growth-inhibitory effects is still controversial. 4-HPR can induce apoptosis and growth inhibition by both RAR-dependent and RAR-independent pathways (15), and among RAR-independent effects, reactive oxygen species production (16, 17) and increase in ceramide levels (18, 19) have been shown.

To date, two metabolites of 4-HPR have been identified: N-([3-methoxyphenyl]retinamide (4-MPR) and 4-oxo-N-([4-hydroxyphenyl]retinamide (4-oxo-4-HPR). 4-MPR, the most abundant metabolite in human plasma (20), has been found to be ineffective in inducing cell growth inhibition (21, 22). 4-Oxo-4-HPR, a recently identified polar metabolite, is an oxidized form of 4-HPR with modification in position 4 of the cyclohexene ring (20). The compound was found in the plasma of patients treated with 4-HPR and formed in tumor cells through the induction of CYP26A1 enzyme (20).

The present study was designed to evaluate the ability of 4-oxo-4-HPR to induce cell growth inhibition and to explore some aspects of its growth-inhibitory effects. Our results indicate that 4-oxo-4-HPR is more effective than 4-HPR in inhibiting the growth of ovarian, breast, and neuroblastoma cell lines; is not cross-resistant with 4-HPR; and interacts synergistically with 4-HPR. The antiproliferative effect of 4-oxo-4-HPR is due to modulation of cell cycle regulator proteins associated with marked G<sub>2</sub>-M cell cycle

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arrest, induction of apoptosis via activation of the caspase cascade, and enhancement of intracellular ceramide level. Furthermore, we suggest that 4-oxo-4-HPR exerts its effect on cell growth via retinoid receptor–independent mechanisms.

Materials and Methods

Cell culture conditions, drugs, and chemicals. Human ovarian, breast, and neuroblastoma tumor cells were tested for their sensitivity to 4-oxo-4-HPR, 4-HPR and 4-MPR. Ovarian tumor cell lines: A2780 (obtained from Dr. Oxls, Bethesda, MD), A2780/HPR (i.e., A2780 cells made resistant to 4-HPR as previously described; refs. 19, 21), IGROV-1 (obtained from Dr. Bénard, Villejuif, France), OVCAR-432 (obtained from Dr. Knopp, Boston, MA), OAW-42 (obtained from Dr. Britten, Liverpool, United Kingdom), OVCAR-3, TOV-21G, and SKOV-3 (purchased from American Type Culture Collection (ATCC), Manassas, VA). Breast tumor cell lines: T47-D, BT-20, MCF-7, and MDA-MB-231 (obtained from Dr. R. Sutherland, Sidney, Australia). Neuroblastoma cell lines: GI-L-N (obtained from Dr. M. Ponzoni, Genoa, Italy), SK-N-BE, SK-N-MC, and SK-N-SH (ATCC). Cells were cultured in monolayer in the following media: RPMI 1640 (ATCC) containing 10% fetal bovine serum for A2780, A2780/HPR, OVCAR-432, IGROV-1, SKOV-3, SK-N-BE, and GI-L-N; RPMI 1640 containing 10% fetal bovine serum and 0.25 unit/ml of insulin for T47-D, BT-20, MCF-7, and MD-MBA-231; modified RPMI 1640 (ATCC) containing 2% fetal bovine serum for OVCAR-3; E-MEM (ATCC) containing 10% fetal bovine serum for OAW-42, SK-N-MC, and SK-N-SH; MCDB105 and M199 (1:1, respectively; Sigma, St. Louis MO) supplemented with 15% fetal bovine serum for TOV-21G. All cell lines were incubated at 37 °C in 5% CO₂.

4-HPR and 4-MPR (provided by Dr. Crowell, National Cancer Institute, Bethesda, MD) were dissolved at 10 mmol/L in DMSO, whereas 4-oxo-4-HPR, 4-HPR and 4-MPR, synthesized as previously described (20), was dissolved at 10 mmol/L in absolute ethanol. The RARα antagonist CD2503 and the RARβ/γ antagonist CD2848, provided by Galderma (Sophia Antipolis, France), were dissolved at 10 mmol/L in DMSO. All procedures were carried out under subdued light. In each experiment, control cells were treated with the same amount of DMSO or ethanol as treated cells.

Growth inhibition assay. Cells (7 × 10⁴ per well) were seeded onto 96 cluster tissue culture plates and treated the next day with 0.3, 1, 3, 5, and 10 μmol/L 4-oxo-4-HPR, 4-HPR, or 4-MPR. Seventy-two hours after treatment, cell number was estimated by using the sulforhodamine B assay (23). The sulforhodamine B assay was linear in the range of the optical densities values observed for not treated cells (corresponding to 100%) and cells treated with the highest dose (10 μmol/L; corresponding to 1-10% of controls). Because the sulforhodamine B assay is capable of measuring cytotoxicity only over a 2 log range, cell count using a Z2 counter (Beckman Coulter, Fullerton, CA), was used in combination experiments testing the effect of high doses. Analysis of drug interaction was done by a modified method of Dretwinko et al. (24). Dretwinko index (DI) was calculated as follows: SF1 × SF2/SF1-2, where SF1 and SF2 are the surviving fraction of cells exposed to compound 1 and 2, respectively, and SF1-2 is the surviving fraction of cells exposed to compound 1 in combination with compound 2. DI > 1 indicates greater than additive effects (i.e., synergism), DI = 1 indicates additivity, and DI < 1 indicates antagonism.

Cell cycle analysis. For analysis of cell cycle distribution, cells (9 × 10⁴) were plated into 100-mm tissue culture dishes and at ~30% confluence treated with 5 μmol/L 4-oxo-4-HPR or 4-HPR. Twenty-four hours after the treatment, floating and attached cells were collected and washed twice with cold Dulbecco’s PBS, fixed in ice cold 70% ethanol, and stored at ~−20 °C until the use. Subsequently, cells were rinsed with Dulbecco’s PBS and incubated for 1 hour in the dark with PBS containing 20 μg/mL propidium iodide (Sigma) and 1 mg/mL RNase A (Sigma). Cell cycle analysis was done using FACScan flow cytometer (Becton Dickinson, San Jose, CA). The percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME).

Apoptosis assays. Fragmentation of DNA was determined by photometric enzyme immunoassay using Cell Death Detection ELISA™ kit (Roche, Penzberg, Germany) according to the manufacturer's instruction. Briefly, cells (1 × 10⁶ per well) were seeded in 96-well culture cell plates and treated the day after with 5 and 10 μmol/L 4-oxo-4-HPR or 4-HPR for 24 hours. Adherent and floating cells were then lysed and centrifuged and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates and incubated for 2 hours at room temperature with a mixture of anti–histone-biotin and anti–DNA-peroxidase antibodies. Quantitative determination of the amount of nucleosomes by the peroxidase retained in the immunocomplex was determined photometrically with 2′,7′-dichloro-4'-[3-ethylbenz-thiazolino-sulfonate-6-diaminodium salt] as peroxidase substrate. DNA fragmentation in control and treated cells was expressed as absorbance at 405 nm.

Activation of caspase-3, caspase-9, and caspase-8 proteases was determined using the caspase-3, caspase-9, and caspase-8 colorimetric assay kits, respectively (MBL International, Woburn, MA). Briefly, cells (8 × 10⁴ per well) were plated in six-well cell culture plates and treated the day after with 5 and 10 μmol/L 4-oxo-4-HPR or 4-HPR for 24 hours. Whole-cell lysate from adherent and floating cells was centrifuged and the supernatant assayed for protein concentration. An aliquot of 200 μg proteins was incubated for 1 hour at 37 °C with the p-nitroanilide (pNA)–labeled caspase-3–specific substrate (DEVD/pNA), or pNA-labeled caspase-9–specific substrate (LEHD/pNA), or pNA-labeled caspase-8–specific substrate (IETD/pNA). Cleavage of the caspase substrates by active caspases and the release of pNA were quantified in control and treated cells by measuring absorbance at 405 nm.

Determination of reactive oxygen species. Production of reactive oxygen species (ROS) was detected by use of the oxidation-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Inc., Eugene, OR). CM-H₂DCFDA was prepared freshly each time at 0.5 mmol/L in DMSO. The thiol antioxidant N-acetyl-cysteine (NAC; Sigma) was dissolved at 100 μmol/L in H₂O. A2780 cells (5 × 10⁵ per well) were plated in six-well cell culture plates and incubated for 6 hours in the presence of 10 μmol/L 4-HPR or 4-oxo-4-HPR with or without 1 mmol/L NAC added 30 minutes before retinoid treatment. Medium was discarded and, under low light conditions, replaced with 50 μmol/L CM-H₂DCFDA in whole medium for 20 minutes at 37 °C. Cells were harvested, transferred to foil-wrapped tubes, and analyzed, immediately, by flow cytometry.

Immunoblot analysis. Western immunoblots were prepared to analyze the protein levels of p21WAF1 (Neomarkers, Union City, CA); p53 (Dako, Glostrup, Denmark); cyclin A, cyclin B1, cyclin D1, cyclin E, cc25c, p16, and cyclin-dependent kinase 1 (cdk1; Santa Cruz Biotechnology, Santa Cruz, CA); phosho-cdk1 (New England Biolabs, Ipswich, MA); and actin (Sigma). Briefly, cells were treated with 5 μmol/L 4-oxo-4-HPR or 4-HPR for 24 hours and then lysed in Laemmli sample buffer containing 5% β-mercaptoethanol and boiled for 3 minutes. Aliquots containing 40 μg of total cell proteins were fractionated on SDS-PAGE, and the proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL). Membranes were blocked in 5% nonfat milk powder (w/v) in TBS containing 0.1% Tween 20 for 1 hour at room temperature and then probed using the above-mentioned primary antibodies. After an overnight incubation at 4 °C, membranes were washed in TBS and incubated with the appropriate peroxidase-conjugated secondary antibody. Specific complexes were revealed by chemiluminescence according to the enhanced chemiluminescence Western blotting detection system kit (Amersham).

Nuclear retinoid receptor binding assay. Competition of retinoids with [³H]RA for binding to RARα, RARβ, and RARγ was determined using an in vitro ligand binding assay as previously described (25). Briefly, recombinant human RARα was expressed as a fusion protein in Escherichia coli, and the human RARβ and murine RARγ proteins were expressed in insect cells by infection with baculovirus expression vectors followed by the preparation of nuclear cell extracts. Radiolabeled ligands (10 Ci/mmol) were added to receptor-containing extracts (RAR, 2-5 μg/mL) in the absence and presence of increasing concentrations of competing ligands followed by the separation of ligand bound to receptor from that free in solution using a hydroxyapatite assay.

Measurement of RA responsive element transactivation. A2780 cells, which express all three RAR subtypes (26), were seeded (3.5 × 10⁶ per well) in six-well plates. After 24 hours, cells were cotransfected with (a) pIf-RARE-Luc,
a pGL3-promoter vector (Promega, Madison, WI) containing, upstream the firefly luciferase reporter gene, the nucleotides −87 to −23 bp carrying a RA responsive element (RARE), from the transcriptional start site of RARβ and (b) pRL, a control plasmid constitutively expressing Renilla luciferase (Promega). A mixture of LipofectAMINE 2000 reagent (Life Technologies, Gaithersburg, MD), 3.5 μg of reporter plasmid pβ-RARE-Luc, and 0.5 μg of pRL plasmid was added, and plates were incubated for 6 hours in serum-free medium.

Cells were then exposed to 4-HPR, 4-oxo-4-HPR, or RA for an additional 24 hours. Cells were lysed, and luciferase activity was determined using the Dual Luciferase Reporter Assay (Promega) according to the manufacturer’s instructions. Renilla luciferase activity was used as an internal control to normalize all the results.

Analysis of ceramide levels and ceramide de novo synthesis

Analysis of ceramide levels was assessed as previously described (19). Briefly, 24 hours after seeding, cells were incubated in the presence of 5 × 10−3 mol/L [1−3H]sphingosine (2.1 Ci/mmol) for 2 hours (pulse). After steady-state metabolic labeling of cell lipids with [1−3H]sphingosine, at the end of a 24-hour chase, the medium was replaced with medium containing 10 μmol/L 4-HPR or 4-oxo-4-HPR for different times from 2 to 72 hours. After treatment, cells were lyophilized, and lipids were extracted with chloroform/methanol 2:1 by volume. The total lipid extracts were subjected to a two-phase partitioning, resulting in an aqueous phase containing gangliosides and in an organic phase containing all other lipids. Total lipids were separated by monodimensional and two-dimensional high-performance TLC (HPTLC) carried out with the following solvent systems: chloroform/methanol/0.2% aqueous CaCl2/32% NH3, 60:50:9:1 by volume and in an organic phase containing all other lipids.

Results

4-Oxo-4-HPR induces cell growth inhibition in ovarian, breast, and neuroblastoma cancer cell lines more efficiently than 4-HPR. The growth-inhibitory effect of 4-oxo-4-HPR was tested in a

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Figure 1. Effect of 4-oxo-4-HPR, 4-HPR, and 4-MPR on ovarian, breast, and neuroblastoma cancer cell growth. The effect of 4-oxo-4-HPR (C), 4-HPR (A), and 4-MPR (A) on (A) ovarian, (B) breast, and (C) neuroblastoma cancer cell growth was estimated by using the sulforhodamine B assay. Cells were seeded at a density of 7 × 103 per well in 96 cluster tissue culture plates and treated with concentrations ranging from 0.3 to 10 μmol/L 4-oxo-4-HPR, 4-HPR, or 4-MPR for 72 hours. Control cultures received the same amount of vehicle as the treated cultures. Cell survival was estimated from the equation: % cell survival = 100 − A/Ar, where Ar and At are the absorbencies of the sulforhodamine B color reaction in treated and control cultures, respectively. The antiproliferative activity of 4-oxo-4-HPR, 4-HPR, and 4-MPR in each cell line was tested in three independent experiments with four replicate wells for each analysis. Points, average; bars, SD. D, IC50 values of 4-HPR, 4-oxo-4-HPR, and 4-MPR for ovarian, breast, and neuroblastoma cancer cell lines. IC50 values were calculated by interpolation of the sigmoidal dose response curves. R is the ratio between 4-HPR IC50 and 4-oxo-4-HPR IC50.
panel of ovarian, breast, and neuroblastoma cancer cell lines and, compared with that of 4-HPR and 4-MPR, the other known 4-HPR metabolite (Fig. 1A–C). The concentrations required for 50% growth inhibition (IC50) were calculated and reported in Fig. 1D. 4-Oxo-4-HPR was very effective in all the investigated cell lines (Fig. 1A–C) and, notably, it was more potent than the parent drug 4-HPR in most of them (13 of 16), being its IC50 values two to four times lower (Fig. 1D). Another noteworthy feature of 4-oxo-4-HPR was its activity against A2780/HPR, a 4-HPR-resistant cell line (ref. 21; Fig. 1A). Conversely, 4-MPR was substantially ineffective in inhibiting cell proliferation on almost all investigated tumor cell lines. The concentration of 4-MPR required for 50% inhibition of cell growth was ≥10 μmol/L for almost all cell lines. Overall, these results indicate that 4-oxo-4-HPR (a) is a retinoid effective in inhibiting cell proliferation in cancer cell lines with different histotypes; (b) is, in most cases, more potent than 4-HPR; and (c) does not show cross-resistance with the parent drug.

4-Oxo-4-HPR induces G2-M arrest in cell cycle progression and alters cell cycle regulatory protein expression. To determine whether 4-oxo-4-HPR growth-inhibitory effect is associated with cell cycle perturbation, the distribution of cells in different phases of the cell cycle was analyzed. The analyses were conducted on ovarian A2780 and A2780/HPR cells, representative of cells sensitive and resistant to 4-HPR and both sensitive to 4-oxo-4-HPR. 4-Oxo-4-HPR caused a statistically significant increase in G2-M cell population both in A2780 and A2780/HPR cells (Fig. 2A). A concomitant decrease in S as well as G0-G1 cell population was observed. A sub-G0-G1 peak, indicative of apoptosis, was already present at 24 hours in both cell lines. A similar pattern of cell cycle perturbation by 4-oxo-4-HPR was found in a neuroblastoma (SK-N-MC) and a breast tumor (BT-20) cell line (data not shown). In contrast to 4-oxo-4-HPR, 4-HPR induced a moderate increase in G0-G1 cell population, and this occurred only in A2780 and not in A2780/HPR cells.

We determined whether the marked suppression of cell proliferation and the accumulation in G2-M provoked by 4-oxo-4-HPR in contrast to the slight G1 arrest caused by 4-HPR are linked to different alterations in cell cycle regulatory protein expression. As shown in Fig. 2B, the levels of the specific G2-M phase regulatory markers cdc25c and p-cdk1 were decreased after 4-oxo-4-HPR treatment in A2780 and A2780/HPR cells, whereas 4-HPR did not alter their expression. In spite of the down-modulation of p-cdk1, the expression of cdk1 was not affected. Cyclin A expression was also decreased, but this effect was not specific for 4-oxo-4-HPR, because it was observed also after 4-HPR treatment but only in A2780 cells. No alteration in the G1 regulators cyclin B1, cyclin D1, and cyclin E was observed after treatment with both retinoids. The levels of the cdk-inhibitory protein p21 were increased in both 4-oxo-4-HPR- and 4-HPR-treated cells, and this effect was consistent with the increased expression of p53, a p21 regulator. In contrast, p16 levels, which were

Figure 2. Induction of cell cycle arrest and modulation of cell cycle regulatory protein expression. Exponentially growing A2780 and A2780/HPR cells were treated with 5 μmol/L 4-HPR or 4-oxo-4-HPR for 24 hours, and their cell cycle distribution was determined by flow cytometry after propidium iodide staining. Control cells were treated with vehicle alone. A, numbers of cells in sub G0-G1, G0-G1, S, and G2-M phases were calculated as a percentage of the total cell population. Columns, mean of two independent experiments each done in triplicate; bars, SD. Statistical significance of difference between control and treated group was determined by the Student’s t test. *P < 0.05; **P < 0.01. B, effect of 4-oxo-4-HPR and 4-HPR treatment on cell cycle regulatory protein expression was evaluated by Western blot analysis. A2780 and A2780/HPR cells were treated with 5 μmol/L 4-oxo-4-HPR and 4-HPR, and cell lysates were harvested after 24 hours. Control cells were treated with vehicle alone. Specific antibodies were used for each indicated protein. Actin levels were monitored as a control for gel load (top). Expression of each protein was quantified and presented (bottom) as ratio between the levels in 4-HPR-treated (gray columns) or 4-oxo-4-HPR-treated (black columns) cells and the levels in control cells (white columns). Three experiments with similar results were done. Representative experiment.
constitutively lower in A2780/HPR cells than in parental A2780 cells, were not affected by the two retinoids.

**4-Oxo-4-HPR induces apoptosis through caspase-9 but not caspase-8 activation.** To evaluate whether the antiproliferative effect of 4-oxo-4-HPR was associated with apoptosis induction, we assessed the ability of the compound to induce DNA fragmentation and activation of caspase-3. In A2780 cells, 4-oxo-4-HPR, similarly to 4-HPR, caused an increase in the amount of cytoplasmic nucleosomal DNA fragments (Fig. 3A) and in caspase-3 activity (Fig. 3D). 4-Oxo-4-HPR caused similar effects in A2780/HPR cells, after both 5 and 10 μmol/L doses. Conversely, in these cells, 4-HPR had a slight effect only after the highest 10 μmol/L dose.

We further investigated the effect of 4-oxo-4-HPR on the activity of the upstream activators caspase-9 and caspase-8. Similarly to that observed with caspase-3 activity, caspase-9 activity was induced in A2780 cells by both 4-oxo-4-HPR and 4-HPR, whereas, in A2780/HPR cells, this effect occurred, even if at a lower extent, only after 4-oxo-4-HPR treatment (Fig. 3C). No increase in caspase-8 activity could be detected in both A2780 and A2780/HPR cells after treatment with 4-oxo-4-HPR or 4-HPR (Fig. 3D). Time course analysis at 6 and 48 hours confirmed the lack of caspase-8 induction (data not shown). Lack of caspase-8 induction by 4-oxo-4-HPR and 4-HPR was also observed in IGROV-1, SKOV-3, and OVCAR-3 ovarian cancer cell lines (data not shown).

**4-Oxo-4-HPR increases intracellular ceramide levels and ROS.** We have previously shown (19) that 4-HPR–induced apoptosis was accompanied by elevation of ceramide levels in A2780 cells, and that resistance in A2780/HPR cells resulted in lack of ceramide induction. The possible involvement of ceramide levels modification in apoptosis induced by 4-oxo-4-HPR was therefore investigated. Cell sphingolipids were metabolically labeled at steady state with [1-³H]sphingosine, and, after labeling, A2780 and A2780/HPR cells were treated with 4-HPR or 4-oxo-4-HPR for different times (from 2 to 72 hours). Treatment of A2780 cells with 4-HPR or 4-oxo-4-HPR increased the production of radioactive ceramide in adherent (Fig. 4A) and in floating cells (Fig. 4B) only at 48 and 72 hours. In A2780/HPR cells (Fig. 4C and D), the incorporation of [1-³H]sphingosine into ceramide was not modified by 4-HPR treatment, whereas in cells treated with 4-oxo-4-HPR, there was a statistically significant increase, which was, however, lower than that observed in A2780 cells.

To ascertain whether 4-HPR and 4-oxo-4-HPR are responsible for the early activation of ceramide de novo synthesis in A2780 cells, we did pulse labeling experiments with L-[³H]serine (Fig. 4E). Treatment of A2780 cells with 4-HPR or 4-oxo-4-HPR increased the incorporation of L-[³H]serine into ceramide with a peak after 4 hours. The increase induced by 4-oxo-4-HPR was higher than that obtained with 4-HPR.

4-HPR is known to generate ROS in certain tumor cell types (16, 28). To determine whether 4-oxo-4-HPR increased ROS levels in ovarian tumor cells, A2780 cells were exposed for 6 hours to 10 μmol/L 4-oxo-4-HPR or 4-HPR, and ROS were measured using CM-H₂DCFDA. 4-HPR and 4-oxo-4-HPR increased the mean CM-H₂DCFDA fluorescence 2.1- and 3.0-fold, respectively, over controls. The addition of 1 mmol/L NAC to 4-HPR and 4-oxo-4-HPR reduced ROS generation (mean fluorescence, 1.4- and 1.8-fold over controls, respectively; Fig. 4F). However, when the effect of 1 mmol/L NAC was tested on the growth-inhibitory effects of 5 and 10 μmol/L 4-HPR and 4-oxo-4-HPR in A2780 cells, no appreciable effect was observed (data not shown).

**4-Oxo-4-HPR and 4-HPR growth-inhibitory effects are RAR independent.** We investigated whether 4-oxo-4-HPR and 4-HPR require RARs for their antiproliferative effects. The binding affinity of RA, 4-HPR, and its two metabolites 4-oxo-4-HPR and 4-MPR for RARs was tested (Fig. 5A). As expected, RA was very effective at competing with [³H]RA for binding to the RARα, RARβ, and RARγ (Ki = 0.2, 0.6, and 0.7 nmol/L, respectively), whereas 4-HPR showed only weak competition. Likewise, 4-oxo-4-HPR was >1,700 to 5,600 times less potent than RA in binding to the three RAR types. 4-MPR did not compete for [³H]RA binding to any of the RARs. Neither the parent drug 4-HPR nor its metabolites 4-oxo-4-HPR and 4-MPR showed any appreciable binding to RARs, and the RARi CD2503 or CD2848 were tested at concentrations similar and five times less potent than RA in binding to the three RAR types. 4-MPR did not compete for [³H]RA binding to any of the RARs. Neither the parent drug 4-HPR nor its metabolites 4-oxo-4-HPR and 4-MPR showed any appreciable binding to RARs, and the RARi CD2503 or CD2848 were tested at concentrations similar and five times less potent than RA in binding to the three RAR types. 4-MPR did not compete for [³H]RA binding to any of the RARs. Neither the parent drug 4-HPR nor its metabolites 4-oxo-4-HPR and 4-MPR showed any appreciable binding to RARs, and the RARi CD2503 or CD2848 were tested at concentrations similar and five times less potent than RA in binding to the three RAR types.
4-HPR and 4-oxo-4-HPR alone produced a 53% and 75% reduction in A2780 cell number, respectively (Fig. 5B), whereas CD2503 and CD2848 were ineffective. Treatment with the two antagonists did not reverse the reduction in cell number caused by 4-oxo-4-HPR and 4-HPR. To further investigate the hypothesis that the 4-oxo-4-HPR-mediated response is retinoid receptor independent, we measured its ability to activate via a RAR mechanism the transcription of a reporter gene. A2780 cells were transiently transfected with a plasmid containing a β-RARE–controlled luciferase reporter gene, and 24 hours later, they were treated with the indicated retinoid for an additional 24 hours (Fig. 5C). Although RA strongly activated the endogenous retinoid receptors, 4-oxo-4-HPR showed only a marginal increase in luciferase activity, whereas 4-HPR had no effect.

4-Oxo-4-HPR and 4-HPR combined treatment has a synergistic effect on cell growth inhibition. 4-Oxo-4-HPR concentrations in plasma are slightly lower than those of the parent drug and of 4-MPR (20). To study the possible influence of the two metabolites on 4-HPR activity, we investigated the effect of the combined treatment of 4-HPR with 4-oxo-4-HPR or 4-MPR on ovarian cancer cells. 4-HPR and 4-oxo-4-HPR were tested at concentrations with minimal activity (IC = 0.25% for each cell line). To mimic the in vivo situation, 4-MPR, which has no growth-inhibitory effect, was tested at concentrations similar to those of the parent drug (Fig. 6A). A synergistic effect (corresponding to a DI >1) was observed in all tested cell lines when 4-oxo-4-HPR was present together with 4-HPR. On the contrary, 4-MPR did not affect 4-HPR activity. The effect of the combination of 4-HPR and 4-oxo-4-HPR at higher concentrations was also explored by counting the cell number surviving at a 3-day treatment. Figure 6B shows that the combination of 10 μmol/L 4-HPR, a clinically achievable concentration (8), and 10 μmol/L 4-oxo-4-HPR produced, in A2780 cells, a potentiation of the cytotoxic activity of the single agents. Each single agent alone caused less than a two-log reduction in cell number, whereas the combination of the two agents resulted in more than two logs of cell killing.

**Discussion**

4-HPR is one of the most promising synthetic retinoids tested in the clinic. It is well tolerated (13), and it has shown preventive efficacy against breast cancer in young women (6), a potential protective effect against ovarian cancer (7), and promising effects in a phase I trial in children with neuroblastoma (8). Considerable attention has been devoted at investigating the molecular targets implicated in the growth-inhibitory effects of this retinoid. By contrast, little attention has been directed towards an important aspect that may contribute to its activity (i.e., its metabolism). Previous studies have shown that in vitro, cells do not metabolize 4-HPR appreciably (22), whereas, in vivo, 4-HPR is extensively metabolized to a less polar compound, identified as 4-MPR (30–32) and to more polar compounds, one of which was recently identified by us as 4-oxo-4-HPR (20). 4-Oxo-4-HPR is present in human plasma and can also be formed in tumor cells through induction of CYP26A1 (20). In the present study, we show that 4-oxo-4-HPR is a biologically active metabolite with very interesting features, such as higher tumor growth-inhibitory effects than the parent drug, lack of cross-resistance, and synergistic interaction with the parent drug. We also present evidence that 4-oxo-4-HPR causes dramatic cell cycle arrest in G2-M phase and induces apoptosis and report on molecular mechanisms associated with these effects.
When the effects of 4-oxo-4-HPR were tested on a panel of ovarian, breast, and neuroblastoma cell lines, we found that all cell lines were sensitive to the growth-inhibitory effects of the metabolite. Previous studies reported that the oxidized metabolites of retinol (33) and RA (34), 4-oxo-retinol and 4-oxo-RA, respectively, also exhibit strong biological activities. Thus, it seems that the formation of polar metabolites may be an activation step for retinoids. 4-Oxo-4-HPR, besides being effective in all the tested cell lines, seemed more efficient than 4-HPR, because doses two to four times lower caused the same growth-inhibitory effect. The concentrations of 4-oxo-4-HPR in patients treated with 4-HPR have been found, on average, the same growth-inhibitory effect. The concentrations of 4-oxo-4-HPR can be biologically effective at pharmacologic concentrations and thus might contribute to 4-HPR activity. Moreover, the results suggest that differences in 4-HPR metabolic rates may be involved in differences in 4-HPR response.

Unlike the 4-oxo-4-HPR, 4-MPR was ineffective in most cell lines even at high (10 µmol/L) concentrations. We have previously shown that 4-MPR had no antitumor effect in mice bearing a human ovarian carcinoma and did not increase 4-HPR activity (35). Even if in vitro growth-inhibitory effects of 4-MPR have been reported (36), most in vitro data support our findings of lack of activity of 4-MPR even at 20 µmol/L concentrations (14). Therefore, the contribution of 4-MPR to the in vivo efficacy of 4-HPR seems to be unlikely.

An important finding with potential therapeutic implications was the lack of cross-resistance between 4-oxo-4-HPR and the parent drug. In A2780/HPR cells, resistance to 4-HPR was not associated with differences in sensitivity to 4-oxo-4-HPR. This clearly indicates that 4-oxo-4-HPR exerts its growth-inhibitory effects through mechanisms of action different than those of 4-HPR. Similarly, we had previously shown that A2780/HPR cells do not display any resistance to another apoptotic inducing retinoid, CD437 (21), whose apoptotic-inducing effects diverge from those of 4-HPR (37). We thought that taking advantage of the lack of cross-resistance of 4-oxo-4-HPR with 4-HPR, we could have provided information on molecular pathways involved in 4-HPR resistance. All the investigated molecular pathways were similarly modulated by 4-oxo-4-HPR in A2780 and A2780/HPR cells. As A2780/HPR cells are not only sensitive to CD437 but also to apoptotic-inducing chemotherapeutic agents, like cisplatin and taxol (data not shown), our data suggest that resistance to 4-HPR in A2780/HPR cells do not involve primary alterations in the function of general pro-apoptotic factors but rather modifications in the specific 4-HPR-apoptogenetic pathway. The results of the combination of 4-HPR and 4-oxo-4-HPR in four ovarian carcinoma cell lines suggest that

**Figure 5.** 4-Oxo-4-HPR– and 4-HPR–dependent cell growth inhibition is RAR independent. A, competition of 4-HPR, 4-oxo-4-HPR, 4-MPR, and RA with [3H]RA for binding to RARα, RARβ, and RARγ. K_i values indicate the equilibrium inhibition constant for the interactions of the three retinoids with the RAR subtypes. ***,** taken from Repa et al. (49). ***,** approximate as baseline competition was not achieved. ***,** NC, no competition when tested at a competing concentration of up to 10^-5 mol/L. B, A2780 cells were seeded at a density of 7 x 10^5 per well in 96 cluster tissue culture plates and, the next day, were treated with 1 µmol/L 4-oxo-4-HPR or 4-HPR alone or in the presence of 1 and 5 µmol/L CD2503 or CD2848. Survival was evaluated 72 hours after treatment by using the sulforhodamine B assay. Cell survival was estimated from the equation: % cell survival = 100 x A_t/A_0, where A_t and A_0 are the absorbencies of the sulforhodamine B color reaction in treated and control cultures, respectively. The antiproliferative activity was tested in two independent experiments with four replicate wells for each analysis. Columns, averages; bars, SD. C, A2780 cells were plated onto six-well plates at a density of 3.5 x 10^5 per well and, the next day, were transfected with p-RARE-Luc (3.5 µg/well) coding for firefly luciferase. After 24 hours, cells were treated with 5 and 10 µmol/L 4-oxo-4-HPR or 4-HPR or 5 µmol/L RA, as positive control, for 24 hours. Cells were then harvested and analyzed for luciferase activity. The data were normalized to the activity of the Renilla luciferase coded by the cotransfected pRL plasmid (0.5 µg/well). Columns, mean of two experiments with two replicate wells for each analysis. Bars, SD.
the presence of 4-oxo-4-HPR might not only increase 4-HPR activity in 4-HPR-sensitive cells but also overcome 4-HPR resistance. Similar to our findings, in cells resistant to 4-HPR and sensitive to RA, the combination of the two retinoids resulted in increased effect (38). These data indicate that the combination of retinoids with different mechanisms of action might be an effective method to improve the efficacy of these compounds.

Of particular interest is the fact that 4-oxo-4-HPR antiproliferative effect was associated with induction of cell cycle arrest different from 4-HPR. Cells treated with 4-oxo-4-HPR showed a marked accumulation in the G2-M phase of the cell cycle, whereas in those treated with 4-HPR, this regulation occurred, as reported in other cell lines (27), in the G1 phase. The effect of 4-oxo-4-HPR on cell cycle seems to be mediated through modulation of the G2-M regulators cdc25c and cdk1. The levels of cdc25c and the phosphorylation of cdk1 were reduced only by 4-oxo-4-HPR. It is known that the active, dephosphorylated form of cdk1/cyclin B1 is devoid of kinase activity when associated with the cdk inhibitor p21 (39). The concomitant reduced phosphorylation of cdk1 and the increase of p21 levels meditated by 4-oxo-4-HPR may explain the G2-M arrest caused by the retinoid. In addition to the regulation of molecules specifically involved in G2-M progression, 4-oxo-4-HPR modulated, similarly to 4-HPR, the expression of other cell cycle–related proteins. Both retinoids induced the expression of p53. p21, whose expression is usually induced by p53, was also up-modulated, and cyclin A, whose expression can be transcriptionally repressed by p53, was down-modulated by both retinoids. The effect of 4-HPR on p53 expression seems to differ according to the cell line. The expression of p53 was not affected by 4-HPR in many systems (28, 40, 41). However, there are just as many systems in which p53 was up-modulated following 4-HPR treatment, and the expression of p21 was also increased (42–44) as we observed in A2780 cells. Other cell cycle–related proteins, such as cyclin B1, cyclin D, cyclin E, and p16, were modulated neither by 4-oxo-4-HPR nor by 4-HPR, suggesting that they are not directly involved in the effect of the two retinoids on cell cycle.

Unlike natural retinoids that often induce differentiation, a number of synthetic retinoids, including 4-HPR, cause tumor cell growth suppression through induction of apoptosis (3). We assayed whether 4-oxo-4-HPR–dependent cell death was associated with induction of apoptosis and whether this occurred through caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation. In A2780 cells, caspase-3 activity was induced by both 4-oxo-4-HPR and 4-HPR. By investigating which upstream caspase activation.
(46), including A2780 cells (19). We have previously reported that 4-HPR treatment increased ceramide levels in A2780 cells, whereas in A2780/HPR cells, ceramide generation was not detected (19). Here, we present evidence that also apoptosis induced by 4-oxo-4-HPR is accompanied in its terminal stage (after 48-72 hours) by increased ceramide levels, probably deriving by hydrolysis of complex sphingolipids (i.e., sphingomyelin). In pulse-labeling experiments with [3H]serine, 4-HPR and 4-oxo-4-HPR caused, at early times of treatment (2-6 hours), an increased incorporation of radioactivity into ceramide, likely reflecting the activation of serine palmitoyltransferase, the key enzyme of sphingolipid de novo biosynthesis. This observation is in agreement with previous reports on the ability of 4-HPR to stimulate ceramide biosynthesis in neuroblastoma and prostate cancer cells (46) and suggests that serine palmitoyltransferase might represent a common point in the mechanism of action of 4-HPR and 4-oxo-4-HPR.

It has been reported that 4-HPR increases ROS, and that antioxidants inhibit 4-HPR-induced apoptosis in some tumor cell systems (16, 28, 47). In A2780 cells, both 4-oxo-4-HPR and 4-HPR increased the rate of ROS generation. However, their cytotoxicity was not antagonized by the antioxidant NAC, suggesting that in this cell system, signaling pathways leading to the production of ROS are not required for the apoptosis induced by the two retinoids. Investigations on the mechanisms responsible for retinoids effects on cell growth have suggested that natural retinoids, for the most part, act through activation of RARs and RXRs (3), and, by contrast, most synthetic retinoids engage other signaling pathways (3, 37). Because 4-HPR has been shown to act through both RAR-dependent (14, 15) and RAR-independent (15) mechanisms, it was important to determine the role of RARs in mediating the growth-inhibitory effect of 4-oxo-4-HPR. In A2780 cells, the mechanism by which 4-oxo-4-HPR induces growth inhibition seems to be independent of nuclear retinoid receptors. High-affinity RARα (CD2503) and RARβ/RARγ (CD2848) antagonists did not inhibit the effect of 4-oxo-4-HPR and 4-HPR on cell survival. Consistent with a retinoid-independent effect, 4-oxo-4-HPR and 4-HPR were found to be weak ligands (thousands times weaker than RA) for all three subtypes of RAR and poor activators of the A2780 endogenous RARs (more than six times weaker than RA). Similarly to our findings, 4-HPR failed to transactivate RARs in CV-1 cells, which constitutively expressed them. However, such an effect was detected in the same cells when transfected with RARs (14). Moreover, it has to be pointed out that in A2780 cells, the same cell line tested here, 4-HPR was able to modulate RA target genes, such as RARβ2 (48), CYP26A1, and CRBP-I (20), thus suggesting that 4-HPR can have RAR-dependent effects, which, however, do not seem to be responsible for its growth-inhibitory effects.

In conclusion, the 4-HPR metabolite 4-oxo-4-HPR is a retinoid endowed with potent antiproliferative and proapoptotic effects in ovarian, breast, and neuroblastoma tumor cells; therefore, it might contribute to the in vivo activity of 4-HPR. As achieving increased biological potency is an important clinical goal, the results also suggest that 4-oxo-4-HPR might be proposed as new agent for cancer therapy and support further preclinical investigations. Furthermore, the demonstration of activation of different pathways and of the synergistic interaction between 4-oxo-4-HPR and 4-HPR represent the basis for a potential therapeutic use of the combination of the two retinoids to improve 4-HPR activity and/or to overcome 4-HPR resistance.

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References

4-Oxo-Fenretinide, a Recently Identified Fenretinide Metabolite, Induces Marked G2-M Cell Cycle Arrest and Apoptosis in Fenretinide-Sensitive and Fenretinide-Resistant Cell Lines

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