Identification of the Fenretinide Metabolite 4-Oxo-Fenretinide Present in Human Plasma and Formed in Human Ovarian Carcinoma Cells through Induction of Cytochrome P450 26A1

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ABSTRACT

Purpose: The synthetic retinoid fenretinide (4-HPR) exhibits preventive and therapeutic activity against ovarian tumors. An unidentified polar metabolite was previously found in 4-HPR-treated subjects and in A2780 human ovarian carcinoma cells continuously treated with 4-HPR (A2780/HPR). The metabolite and the enzyme involved in its formation in tumor cells are herein identified.

Experimental Design: The metabolite was identified by mass spectrometry in A2780/HPR cell extracts and in plasma from 11 women participating in a phase III trial and treated with 200 mg/d 4-HPR for 5 years. The expression of proteins involved in retinoid metabolism and transport, cytochrome P450 26A1 (CYP26A1), cellular retinol-binding protein I (CRBP-I), and cellular retinoic acid-binding protein I and II (CRABP-I, CRABP-II) were evaluated in tumor cells by reverse transcription-PCR and Western blot analyses. Overexpression of CYP26A1 and retinoic acid receptors (RARs) in A2780 cells was obtained by cDNAs transfection.

Results: The polar metabolite was 4-oxo-(N-(4-hydroxyphenyl))retinamide (4-oxo-4-HPR) i.e., an oxidized form of 4-HPR with modification in position 4 of the cyclohexene ring. 4-oxo-4-HPR plasma levels were slightly lower (0.52 ± 0.17 μmol/L) than those of the parent drug (0.84 ± 0.53 μmol/L) and of the already identified metabolite N-(4-methoxyphenyl)retinamide (1.13 ± 0.85 μmol/L). In A2780/HPR cells continuously treated with 4-HPR and producing 4-oxo-4-HPR, CYP26A1 and CRBP-I were markedly up-regulated compared with A2780 untreated cells. In A2780 cells, not producing 4-oxo-4-HPR, overexpression of CYP26A1 caused formation of 4-oxo-4-HPR, which was associated with no change in 4-HPR sensitivity. Moreover, the addition of 4-oxo-4-HPR to A2780 cells inhibited cell proliferation. Elevated levels of CYP26A1 protein and metabolism of 4-HPR to 4-oxo-4-HPR were found in A2780 cells transfected with RARβ and to a lesser extent in those transfected with RARγ.

Conclusions: A new metabolite of 4-HPR, 4-oxo-4-HPR, present in human plasma and in tumor cells, has been identified. The formation of this biologically active metabolite in tumor cells was due to CYP26A1 induction and was influenced by RAR expression. Moreover evidence was provided that 4-HPR up-modulates the expression of CRBP-I transcript, which is lost during ovarian carcinogenesis.

INTRODUCTION

N-(4-Hydroxyphenyl)retinamide or fenretinide (4-HPR) is a synthetic amide of all-trans-retinoic acid (RA) that has shown lower toxicity than other retinoids while maintaining preventive and therapeutic activity against various tumor types in animals (1). The retinoid is well tolerated in humans (1) and has shown efficacy in oral leukoplakia (2), lichen planus (3), and actinic keratoses (4) and a potential for preventing breast cancer in premenopausal women (5). Promising preliminary results have suggested that 4-HPR can protect against ovarian cancer (5, 6), and clinical trials are in progress to test the effect of the retinoid against this tumor. Moreover, we have shown that 4-HPR has therapeutic efficacy in ovarian tumors in nude mice and that it enhances cisplatin activity in cisplatin-sensitive (7) and -resistant tumors (8). 4-HPR, by inducing apoptosis, has shown growth-inhibitory effects against a variety of human tumor cell lines, including ovarian tumors (9, 10). A number of mechanisms have been proposed as responsible for the tumor growth-inhibitory effects of 4-HPR. Some studies have suggested that its growth-inhibitory effects are mediated through the nuclear retinoid receptors (RARs and RXRs; refs. 9–11). Other studies have reported that apoptosis in response to 4-HPR occurs by receptor-independent mechanisms, such as generation of reactive oxygen species (12), alteration of mitochondrial membrane potential (13), and increase in ceramide levels (14).

An important aspect that may contribute to the biological effects of 4-HPR and that has been poorly investigated is its metabolism. Very little information is available on 4-HPR me-
metabolism in vivo, and nothing is known about 4-HPR metabolism in tumor cells. It is not known whether 4-HPR is metabolized at the tumor level, whether its metabolism plays a role in the growth-inhibitory effects, and which enzymes are involved. To date, the only identified metabolite of 4-HPR is N-(4-methoxyphenyl)retinamide (4-MPR), which has been found in vivo, in rodents and in humans (15–18). In addition to 4-MPR, an unidentified polar metabolite was found in tissues of rodents (15) and in plasma, mammary glands, and tumors of 4-HPR-treated patients (16–18). A polar metabolite was also found in human ovarian carcinoma cells (A2780) when continuously exposed to 4-HPR (A2780/HPR; ref. 19). These cells, compared with parental cells, were less sensitive (10-fold) to the drug, had reduced intracellular 4-HPR levels, and showed changes in marker expression, suggestive of a more differentiated phenotype, including a marked increase in RARβ expression (19). The purposes of this study were to identify the polar metabolite of 4-HPR found in human plasma and in A2780/HPR cells and to elucidate the metabolic pathway involved in its formation. As far as the metabolism of retinoids is concerned, it is known that the natural retinoid RA has autoregulatory mechanisms involved in its own metabolism through modulation of a number of genes. RA induces a cytochrome P450 gene, CYP26A1, which is responsible for its oxidation to polar metabolites and which is constitutively expressed in numerous embryonic and adult tissues and in some human tumor cell lines (20, 21). Moreover, RA modulates the expression of cellular retinoic acid-binding proteins (CRABP) I and II and the cellular retinol-binding protein (CRBP) I, which are supposed to be involved in RA metabolism through regulation of intracellular RA concentration and transport (22).

Our results show that 4-HPR is metabolized in humans to the biologically active derivative 4-oxo-4-HPR, which is formed in tumor cells through induction of CYP26A1 expression. Moreover, we show that CRBP-I, which is lost during ovarian carcinogenesis, is up-regulated by 4-HPR chronic treatment.

MATERIALS AND METHODS

Subjects, Cell Culture Conditions, and Drugs. The subjects, whose plasma samples were herein analyzed for identification of the polar metabolite and for evaluation of its concentrations, were women participating in a Phase III breast cancer prevention trial, whose results have been previously reported (5). The study had received Institutional Review Board approval, and all subjects have signed a written informed consent form. The design and all study features of the prevention trial have been previously described (5). Briefly, the trial enrolled women who met the following criteria: age between 30 and 70 years, breast cancer (T1–T3 or N0) or ductal carcinoma in situ treated with curative surgery within the previous 10 years, no adjuvant treatment, and no evidence of disease at study entry. Women were randomly assigned to receive no treatment or 4-HPR 200 mg orally daily for 5 years (two capsules at dinner) with a monthly 3-day drug holiday. Eleven patients were chosen as the last ones to be treated and whose blood was collected at intervals from last daily drug intake ≤24 hours. Blood samples were collected, as already described (17, 18), in heparinized tubes wrapped in aluminum foil, and all of the procedures were carried out in the dark to prevent exposure to light. After centrifugation, plasma samples were collected and kept frozen at −80°C until drug analysis.

The human ovarian carcinoma cell line A2780, obtained from Dr. Ozols (National Cancer Institute, Bethesda, MD), was grown in monolayer in RPMI 1640 containing 10% fetal bovine serum in 5% CO2 at 37°C. A2780/HPR cells were obtained by exposure of A2780 cells to increasing concentrations of 4-HPR and were maintained in the presence of 5 μmol/L 4-HPR as described previously (14, 19). 4-HPR was kindly provided by Dr. J. A. Crowell (National Cancer Institute). RA was purchased from Sigma (St. Louis, MO) and 4-oxo-4-HPR was synthesized as described in one of the following sections. 4-HPR and RA were dissolved in DMSO, and 4-oxo-4-HPR was dissolved in EtOH, at a concentration of 10 mmol/L, and were stored at −80°C before further dilution in culture medium. In all of the experiments, cells were treated 24 hours after seeding with the concentrations of the retinoids and for the times indicated in the Results section. Control cultures received the same amount of DMSO or EtOH as the treated cultures (0.1%). For high-performance liquid chromatography (HPLC) analysis, cells were treated with 5 μmol/L 4-HPR and, at the indicated times after 4-HPR addition, medium was collected and cells were scraped and washed three times in 0.9% NaCl. The pellets and the media were then frozen and kept at −80°C until drug analysis. For growth-inhibitory assays, the cell number was determined by trypanized cell count with a ZBI Counter (Coulter, Luton, United Kingdom). All of the procedures were carried out with the samples protected from light.

HPLC and Atmospheric Pressure Chemical Ionization Mass Spectrometry Analyses. The presence of 4-HPR and its metabolites in tumor cell extracts, in culture medium, and in plasma samples was evaluated by HPLC as described previously (18, 19). Briefly, cell pellets were first resuspended in 1 ml of distilled water containing 125 μg/ml butylated hydroxytoluene (BHT; Sigma) as antioxidant and then sonicated. An aliquot of 200 μL of each cell, medium, and plasma sample was added to 400 μL of CH3CN containing 125 μg/ml BHT, and the mixture was vortexed and centrifuged to pellet the precipitated proteins. The recovered supernatants were analyzed on a liquid chromatograph (Perkin-Elmer, Norwalk, CT) fitted with a C18 (5-μm) reverse-phase column (150 × 4.6 mm) and a C18 precolumn (Perkin-Elmer, Milan, Italy). The mobile phase consisted of CH3CN:H2O:CH3COOH (75:23:2, vol/vol/vol) delivered at a flow rate of 2 ml/min. Detection was carried out with a Perkin-Elmer LC95 absorbance detector at 370 nm. The reference standards 4-HPR and 4-MPR were supplied by the R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). The reference standard 4-oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR), the identified metabolite, was synthesized as described in the following section. For the quantitative evaluation, reference standard curves were set up with different known amounts of each of the 3 reference standards. The limit of detectability of 4-oxo-4-HPR was 24 ng/ml, and the recovery was 95 ± 7%. The limit of detectability and the recovery of 4-HPR and 4-MPR have been reported previously (17).

Identification of the polar metabolite was made by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) analysis in positive mode. APCI mass spectra (ms) were
acquired with a ThermoQuest LCQDeca mass spectrometer (Finnigan Mat, San Jose, CA) equipped with an atmospheric pressure chemical ion source and an Xcalibur data system and connected to an HPLC system. The conditions of HPLC separation were the same as described above with the following modifications: the flow rate of the mobile phase was 1 ml/min and the size of the C18 column was 125 × 3 mm. Operating settings of the APCI-MS interface were optimized with flow injection of 4-HPR in the mobile phase used for HPLC separation at a flow rate of 10 μl/min. Optimal conditions included sheath gas flow of 80 arbitrary units, vaporized temperature of 30°C, discharge current of 6 μA, capillary voltage of 3 V, capillary temperature of 250°C, and fragmentor voltage (used for collision-induced dissociation) of 30%. Mass spectra were acquired over a range m/z 200–600.

**Synthesis of 4-oxo-4-HPR.** Synthesis of 4-oxo-4-HPR, to be used as reference standard in HPLC assays, was accomplished as follows. Methyl 4-oxoretinoate was prepared as described previously (23) with slight modifications. That is, the unstable methyl 4-bromoretinoate was prepared as described and then treated with 9:1 acetone/water containing 1.5 equivalents of potassium carbonate, instead of the previously used potassium acetate, to generate methyl 4-hydroxyretinoate directly. The latter compound was then converted to 4-oxo-retinoic acid as described previously. The 4-oxo-retinoic acid (13 mg) was activated as its acid chloride and treated with 4-aminophenol according to the method of Villeneuve and Chan (24). Purification of the resultant 4-oxo-4-HPR by silica gel preparative thin-layer chromatography (1:1 ethyl acetate/hexane) gave a 28% yield (4.6 mg) of product as a yellow oil with the following properties: UV (methanol) λmax 371 nm (ε 63,210); 1H NMR (acetone-d6) δ 1.26 [s, 6, (CH3)2], 1.82 (s, 3, 5-CH3), 1.84 (t, 2, 2-CH3), 6.01 (s, 1, 11-H), 6.35–6.42 (m, 4, vinyls), 6.74 (d, 2, Ar, J = 8.9 Hz), 7.06 (dd, 1, 13-H), 7.52 (d, 2, Ar, J = 8.9 Hz), 8.12 (s, 1, O/N-H), 9.03 (s, 1, O/N-H); HPLC on a 4.6 mm Ultrasphere ODS column with 85% methanol/water at 1 ml/min, tR 1.84 (t, 2, 2-CH2), 6.01 (s, 1, 13-H), 6.35 (d, 2, Ar, J = 8.9 Hz), 7.06 (dd, 1, 13-H), 7.52 (d, 2, Ar, J = 8.9 Hz), 8.12 (s, 1, O/N-H), 9.03 (s, 1, O/N-H); HPLC on a Beckman Instruments (San Ramon, CA) model 127 pump with model 166 detector, and a 250 × 4.6 mm Ultrashare ODS column with 85% methanol/water at 1 ml/min, tR 4.8 min (95% purity); MS (electrospray) for C20H21NO3±Na+, calculated 428.2198, observed 428.2198. The UV spectrum was recorded on a Beckman Instruments DU-40 whereas 1H NMR spectra were recorded at 400 MHz on a Bruker Instruments (Billerica, MA) spectrometer. The MS on the synthetic material was recorded on a Micromass (Milford, MA) QTOF Electrospray mass spectrometer.

**Semi-quantitative Reverse Transcription-PCR Analysis.** For all semi-quantitative reverse transcription-PCR (RT-PCR) analyses, single-stranded cDNA was synthesized from 2 μg of total cellular RNA. The synthesis was accomplished by using 0.5 μg of oligo(dT) (Life Technologies, Inc., Paisley, United Kingdom), 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.), 4 μL of 5× first-strand buffer (Life Technologies, Inc.), 0.1 mol/L DTT, and a 10 mmol/L concentration of each dNTP (Amersham, Arlington Heights, IL) in a total volume of 20 μL. Reverse transcription was done for 1 hour at 37°C, then the samples were heated for 5 minutes at 95°C to terminate the reverse transcription reaction. Five μl of the retrotranscription reaction (diluted to 100 μL) was used in a final volume of 50 μL for the PCR reaction, which contained 1× PCR buffer, 1.5 mmol/L MgCl2, 200-μmol/L concentration of each dNTP, 10-pmol concentration of each primer, and 0.5 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). On the basis of the cDNA sequence of the hCYP26A1 (Genbank accession no. GI 16933529), hCRBP-I (Genbank accession no. GI 8400726), hCRBP-II (Genbank accession no. GI 18314499), and hCRABP-II (Genbank accession no. GI 6382069) genes, PCR primers were designed to amplify nucleotide sequences of 790 bp, 338 bp, 371 bp, and 413 bp, respectively. The forward and reverse primers were, respectively, for CYP26A1, 5'-GGGAAACCTTGGCATGTTGA-3' and 5'-GCCCGAGTGTAACGATCGA-3'; for CRBP-I, 5'-GGTACTGGAAGATGTTG-3' and 5'-CTCTAGGTGCAAGCTCATC-3'; for CRABP-I, 5'-CGGCACCTGGAGATGCGCA-3' and 5'-CCACGTCTAGCCGGCAACTAC-3'; and for CRABP-II, 5'-CCCAACTTCTCGGGCAACTGG-3' and 5'-CTCTCGGACGTAGACCCCTGTG-3'. To account for quantitative differences in the RNA preparation, the β-actin gene was amplified in each experiment with the following forward and reverse primers: 5'-GAAATCTGCGTACATTAAG-3' and 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3', respectively. The amplification reaction was initiated by incubation of PCR samples at 94°C for 3 minutes, followed by the cyclic program, at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds (35 cycles) for CYP26A1 amplification; at 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 60 seconds (35 cycles) for CRBP-I amplification; at 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds (35 cycles) for CRABP-I and CRABP-II amplifications. For each primer set, an increasing number of PCR cycles was performed with otherwise fixed conditions to determine the optimal number of cycles to be used. The PCR products (15 μL) were subjected to electrophoresis in 1% (w/v) agarose gel and were visualized by UV after ethidium bromide staining.

**Immunoblot Analysis.** Cells were lysed in Laemmli sample buffer containing 5% β-mercaptoethanol and were boiled for 3 minutes. Aliquots containing 80 μg of total cell proteins were fractionated on 12% SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Membranes were blocked in 5% nonfat milk in Tris-buffered saline for 1 hour at room temperature and then were incubated overnight at 4°C with rabbit polyclonal antibodies raised against human CYP26A1 (Alpha Diagnostic International, San Antonio, TX). After washing in Tris-buffered saline containing 0.1% Tween 20, the filters were incubated with peroxidase antirabbit IgG, and specific complexes were revealed by chemiluminescence according to the enhanced chemiluminescence Western blotting detection system kit (Amersham).
Fenretinide-Autoinduced Metabolism to 4-Oxo-Fenretinide and 4-Oxo-4-HPR in 4-HPR-treated Patients.

We have previously demonstrated that 4-HPR was metabolized to 4-oxo-4-HPR, also in the plasma of patients and cell extracts. The results of MS analysis of plasma from 4-HPR-treated patients and cell extracts and medium from A2780/HPR cells were, therefore, characterized to be the 4-oxo-4-HPR metabolite. By using A2780/HPR cells and culture media, we showed that the compound, by oxidation in the retinoic ring, was 4-oxo-4-HPR. The compound, by fragmentation, yielded, by loss of the amino-phenyl-hydroxy group, the fragment ion [M + H]⁺ at m/z 406. Fragmentation of the positive molecular ion with m/z 406 yielded, by loss of the amino-phenyl-hydroxy group, the fragment ion [M + H]⁺-(NH₂C₆H₄OH) at m/z 297. By APCI-MS analysis. Mass spectra (ms) of 4-HPR polar metabolite (top panel) and 4-HPR (bottom panel) extracted from plasma of 4-HPR treated subjects. Spectra were acquired over a range m/z 200–600.

RESULTS

Identification of 4-HPR Polar Metabolite in Plasma of 4-HPR-treated Patients and in A2780/HPR Cells. We have previously reported (17) that in plasma of patients treated with 4-HPR, besides the parent drug 4-HPR and the metabolite 4-MPR, another metabolite, more polar than the parent drug, was present. A polar metabolite was also detected in cell extracts and in media of A2780/HPR cells obtained from A2780 cells after continuous exposure to 4-HPR, whereas the same metabolite was not present in A2780 cells treated only once with the drug (19). To identify the nature of the polar metabolite, we used HPLC-MS to analyze plasma samples from 4-HPR-treated patients and cell extracts and medium from A2780/HPR cells. The results of MS analysis of plasma from 4-HPR-treated patients are reported in Fig. 1. The polar metabolite was a compound with a positive molecular ion [M + H]⁺ at m/z 406. Fragmentation of the positive molecular ion with m/z 406 yielded, by loss of the amino-phenyl-hydroxy group, the fragment ion (NH₂C₆H₄OH) at m/z 297. The ensuing fragmentation of this ion made it possible to record a ms2 spectrum containing a fragmentation pattern in which the main fragments differed again by 14 units more than the fragment ion recorded in ms2 of 4-HPR (bottom panel, ms2). The ensuing fragmentation of this ion made it possible to record a ms3 spectrum containing a fragmentation pattern in which the main fragments differed again by 14 units more than the fragment present in the pattern recorded in the ms3 spectrum of 4-HPR (bottom panel, ms3), suggesting that 4-HPR was oxidized in the retinoic ring. The compound, by fragmentation, was, therefore, characterized to be the 4-oxo-4-HPR. MS analysis of polar metabolite from A2780/HPR cells and culture media gave similar results (data not shown), which indicated that 4-HPR was metabolized to 4-oxo-4-HPR, also in A2780/HPR cells.

Plasma Levels of 4-HPR and Its Metabolites 4-MPR and 4-Oxo-4-HPR in 4-HPR-treated Patients. We have previously found that the plasma levels of 4-HPR and 4-MPR in women treated with 200 mg/day 4-HPR were constant during 5-year treatment (18, 25). The plasma levels of the new identified metabolite, 4-oxo-4-HPR, were measured after 5 years of 4-HPR treatment by HPLC assay. All of the chromatograms of plasma taken from 4-HPR-treated patients showed a peak, identified as 4-oxo-4-HPR, that was absent in the chromatograms of plasma taken from untreated patients (data not shown). The concentrations of 4-HPR and its metabolites, 4-MPR and 4-oxo-4-HPR, in the plasma of treated women, collected at a median interval of 16 hours (range, 6–24 hours) after the last drug intake, are reported in Table 1. The plasma levels of 4-HPR were, on average, 0.84 μmol/L, i.e., ~1 μmol/L, as previously reported (18, 25). The concentrations of 4-MPR were, in most patients, slightly higher than those of the parent drug (on average, 1.3-fold), whereas the concentrations of 4-oxo-4-HPR were lower (on average, 0.7-fold) than those of 4-HPR.
Expression of CYP26A1 in A2780 and A2780/HPR Cells. CYP26A1, a RA-inducible enzyme that plays an important role in the metabolism of RA, is expressed in several human tumor cell lines (20, 21). To investigate the possible involvement of CYP26A1 in the 4-HPR metabolic pathway, we analyzed its expression in A2780 cells and in A2780/HPR cells continuously grown in the presence of 4-HPR. The results of the evaluation of CYP26A1 mRNA and protein expression by semi-quantitative RT-PCR and Western blot analysis, respectively, are reported in Fig. 2. CYP26A1 mRNA was slightly detectable in A2780 cells, whereas very high levels (25 times higher) of CYP26A1 transcript were detected in A2780/HPR cells. One single treatment of A2780 cells with 5 μmol/L 4-HPR caused only a slight induction at 24 hours, and the same treatment for 6 hours had no effect. (Fig. 2A) The results were substantiated by the measurement of protein levels. An 18-fold up-regulation of CYP26A1 protein was observed in A2780/HPR as a consequence of continuous treatment with the retinoid, whereas one single treatment of A2780 cells with 5 μmol/L 4-HPR caused only a slight induction at 24 hours (Fig. 2B). The results indicate that, in A2780 cells, 4-HPR induces CYP26A1 expression after 24 hours, and that this effect is amplified after chronic treatment.

Effect of RA on CYP26A1 Expression in A2780 Cells. Modulation of CYP26A1 expression by the natural retinoid RA has been demonstrated in a variety of human and mouse tumor cell lines and tissues (20, 21). We analyzed whether RA induced CYP26A1 expression in A2780 human ovarian cancer cells. A2780 cells were exposed to 1, 3, and 5 μmol/L RA for 24 hours, and CYP26A1 mRNA expression was analyzed by RT-PCR (Fig. 3A). CYP26A1 expression was markedly induced by RA in a dose-dependent manner. Time course analysis of CYP26A1 induction by 1 μmol/L RA (Fig. 3B) showed that CYP26A1 expression was already increased at 6 hours, and its expression further increased up to 72 hours (19-fold), indicating an early and time-dependent effect. Induction of CYP26A1 required continuous exposure to RA, because when cells, treated for 24 hours with 1 μmol/L RA, were analyzed 24 hours after RA removal from the culture medium, CYP26A1 levels were already reduced to baseline levels (Fig. 3C).

Effect of CYP26A1 Overexpression on 4-HPR Metabolism and Sensitivity. To establish a causal relationship between the increase in CYP26A1 expression and the metabolism of 4-HPR to 4-oxo-4-HPR, metabolism studies were made in cells overexpressing CYP26A1. An expression vector containing the full-length CYP26A1 cDNA was transiently transfected into A2780 cells (A2780-CYP26A1). CYP26 mRNA levels, analyzed by semiquantitative RT-PCR, confirmed the expected overexpression in A2780-CYP26A1 cells relative to cells trans-

<table>
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<th>Patient no.</th>
<th>Interval from last treatment (h)</th>
<th>Age (y)</th>
<th>4-HPR μmol/L</th>
<th>4-MPR μmol/L</th>
<th>4-oxo-4-HPR μmol/L</th>
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Mean ± SD 0.84 ± 0.53 1.12 ± 0.85 (1.33 ± 0.33) 0.52 ± 0.17 (0.73 ± 0.29)

* Ratio between metabolite (4-MPR or 4-oxo-4-HPR) and parent drug concentrations.
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A2780-mock cells treated with the empty vector (Fig. 4C). The antiproliferative effect of exogenously added 4-oxo-4-HPR in A2780 cells was also assessed and compared with that of 4-HPR. Cells were treated with 0.3, 1, 3, 5, and 10 μmol/L 4-oxo-4-HPR or 4-HPR and cell number was evaluated 72 hours later (Fig. 4D). 4-oxo-4-HPR inhibited A2780 cell proliferation, and it was slightly more effective than 4-HPR (IC₅₀ = 0.6 μmol/L and 1 μmol/L for 4-oxo-4-HPR and 4-HPR, respectively).

Effect of RAR Overexpression on CYP26A1 Expression and 4-HPR Metabolism. The role of RARs on CYP26A1 induction by RA is well established (20, 21). To investigate whether RAR expression can affect 4-HPR metabolism through CYP26A1 modulation, we analyzed the expression of CYP26A1 in parallel with 4-HPR metabolism in A2780 cells overexpressing RARs. We used one clone overexpressing RARβ (indicated as α5), two clones overexpressing RARβ (β7 and β13) and one clone overexpressing RARγ (γ19; Fig. 5A), which were established by stable transfection of A2780 cells with RARα, RARβ, and RARγ, respectively, as described previously (10). No difference in the transcript (Fig. 5B) and protein (Fig. 5C) levels of CYP26A1 was observed between RARα transfected cells (α5) and cells transfected with the empty vector, whereas RARβ (β7 and β13) and RARγ (γ19) cells constitutively expressed higher levels. The results indicate that the expression of CYP26A1 in A2780 cells was affected by RARβ and RARγ expression.

In parallel, 4-HPR metabolism was analyzed in clones that overexpressed RARs. To this aim, α5, β7, β13, γ19, and empty vector- clones were cultured in the presence of 5 μmol/L 4-HPR for 72 hours. Cells and medium samples were harvested and analyzed by HPLC. Figure 5D shows the HPLC profiles of the extracts from the media. In the α5 clone, similarly to the empty vector-clones, besides peaks 2 and 3 corresponding to an isomer form of 4-HPR and to 4-HPR, respectively, no detectable peak corresponding to 4-oxo-4-HPR was found. In contrast, the chromatographic profiles of β7, β13, and γ19 clones showed peak 1, corresponding to 4-oxo-4-HPR, demonstrating the ability of RARβ and RARγ-overexpressing clones to metabolize 4-HPR to the polar metabolite 4-oxo-4-HPR. Cellular extracts from the transfected clones were also analyzed. No peak corresponding to 4-oxo-4-HPR was detected in any of the clones, including those showing the metabolite in the medium, suggesting that the possible intracellular content of 4-oxo-4-HPR was below the sensitivity limit of the assay (data not shown).

Analysis of CRABP-I, CRABP-II, and CRBP-I Expression in A2780 and A2780/HPR Cells. In addition to CYP26A1, RA transcriptionally modulates CRBP-I, CRABP-I and CRABP-II, which are thought to play a role in RA metabolism by regulating its intracellular concentrations (22). We evaluated whether A2780/HPR cells expressed different levels of these proteins compared with A2780 parental cells. The expression of CRBP-I and CRABP-II and CRBP-I mRNA was detected by semiquantitative RT-PCR analysis (Fig. 6). CRBP-I and CRABP-II mRNA were similarly expressed in the two cell lines, whereas the expression of CRBP-I mRNA was markedly increased (28-fold) in A2780/HPR cells.
DISCUSSION

4-HPR has shown promise as a therapeutic (7, 8) and preventive agent against ovarian tumors (6). Little is known about 4-HPR metabolism, and nothing is known about 4-HPR metabolism in tumor cells. In the present study, we identified a new metabolite of 4-HPR, 4-oxo-4-HPR, which is present in human plasma and which is formed in human ovarian tumor cells through the induction of CYP26A1 expression.

To date, the only identified metabolite of 4-HPR is 4-MPR, which is less polar than the parent drug and which has been found in all of the in vivo studies in mice, rats, and humans (15–18). 4-MPR does not seem to play a role in 4-HPR tumor growth-inhibitory effect because it was ineffective in inhibiting tumor cell proliferation in vitro (19, 26) and in vivo (8). In addition to 4-MPR, all of the aforementioned studies, and a study in organ cultures of mammary gland (27), also reported the presence of an unidentified metabolite more polar than the parent drug. Moreover, we recently reported that A2780/HPR cells, which were obtained by continuous exposure of A2780 cells to 4-HPR, differed from parental cells for the ability to metabolize 4-HPR into a polar metabolite, whereas 4-MPR was not detected in either cell line, i.e., A2780 or A2780/HPR (19). The polar metabolite was herein identified by MS as 4-oxo-4-HPR, which is an oxidized form of 4-HPR bearing modification in position 4 of the cyclohexene ring. We had previously shown that in women participating in a breast tumor prevention trial, treated for 5 years with 200 mg/day 4-HPR, the plasma concentrations of 4-HPR were stable during the 5-year treatment and that the concentrations were, on average, 1 mol/L (18, 25). The results of the present study, carried out in a small group of women participating in the same trial, indicate that, after 5 years of treatment, the plasma levels of 4-oxo-4-HPR, the identified polar metabolite, were, on average, lower than that of the parent drug (i.e., 0.5 mol/L). In the same patients, the levels of 4-MPR were, as reported previously, slightly higher than those of 4-HPR (25). However, it is worthwhile pointing out that, despite the higher levels of 4-MPR in plasma and in breast tissues of women participating in the same breast cancer prevention trial, 4-MPR was mainly localized in the fatty tissue, whereas 4-HPR and an unidentified polar metabolite, presumably 4-oxo-4-HPR, were concentrated in epithelial cells, that is, the location in which the drug is supposed to exert its effect (16).

The finding of 4-oxo-4-HPR in human plasma and in A2780/HPR cells prompted us to investigate the enzyme involved in its formation. It is known that cytochrome P450 enzymes belonging to the CYP26 family are involved in retinoid metabolism. However, no data are available about their potential involvement in retinoid metabolism in tumor cells. Therefore, we investigated the expression and the activity of CYP26A1, which is known to be involved in retinoid metabolism in non-tumor cells (28). The results of this study indicate that CYP26A1 is expressed in A2780/4-HPR cells, and that the expression is induced by 5 μmol/L 4-HPR. The induction of CYP26A1 expression in A2780/4-HPR cells was confirmed by RT-PCR analysis. The results of the present study suggest that the expression and activity of CYP26A1 are involved in the metabolism of 4-HPR and 4-oxo-4-HPR in A2780/HPR cells, and that the expression and activity of CYP26A1 are involved in the formation of 4-oxo-4-HPR in A2780/HPR cells.
metabolism (20, 21, 28, 29). CYP26A1, the first of the three members of the CYP26 family to be identified, is a RA-inducible enzyme that metabolizes RA and retinol into oxidized products (20, 21, 30). This enzyme plays an important role in the regulation of RA levels during embryogenesis and in adult tissues (20, 21). In tumor cells, CYP26A1 can be constitutively expressed, induced by RA, or not expressed at all (20, 21). CYP26A1, which was only slightly expressed in A2780 cells, was induced by one single treatment with 4-HPR and resulted markedly up-regulated in A2780/HPR cells. The results indicate that 4-HPR, after prolonged treatment, may substantially increase the expression of CYP26A1 in tumor cells and suggest an association between high levels of CYP26A1 enzyme and production of 4-oxo-4-HPR. We do not know whether the ability of 4-HPR to up-modulate CYP26A1 expression might be of relevance for its preventive effects, because nothing is known about CYP26A1 expression during carcinogenesis (31, 32).

Up-modulation of CYP26A1 expression in A2780 cells also occurred after RA treatment, and the effect was much more evident than that observed with 4-HPR. However, also for RA, the up-regulation of CYP26A1 expression required the continuous presence of the retinoid because CYP26A1 transcripts returned to baseline levels within 24 hours after RA removal. Other natural retinoids like 9-cis-retinoic acid (9-cis-RA) and 13-cis-retinoic acid (13-cis-RA) may induce CYP26A1 expression (33), but studies on substrate specificity have demonstrated that CYP26A1 does not appreciably metabolize the 9-cis-RA or 13-cis-RA isomers (20, 34). CYP26A1 has been shown to be induced also by synthetic retinoids other than 4-HPR (34, 35); however, the ability of such retinoids to be metabolized by the CYP26A1 enzyme has never been investigated. Our results demonstrate that the synthetic retinoid 4-HPR can affect the expression of CYP26A1 and that 4-HPR itself can be a substrate of this enzyme. However, at variance with RA, the autoinduction of 4-HPR metabolism to 4-oxo-4-HPR does not result in a decline of 4-HPR plasma levels during chronic treatment (18, 25). The lower effect of 4-HPR on CYP26A1 induction and its lower specificity for the enzyme, compared with RA, might account for the difference in pharmacokinetics behavior. Another important consequence of the autoinduction of 4-HPR metabolism might be the inhibition of RA catabolism, due to 4-HPR competition with RA for CYP26A1. Indeed, 4-HPR has been shown to increase RA-induced differentiation by inhibiting RA catabolism into polar metabolites (36).

A direct involvement of the CYP26A1 enzyme in 4-HPR metabolism (20, 21, 28, 29). CYP26A1, the first of the three members of the CYP26 family to be identified, is a RA-inducible enzyme that metabolizes RA and retinol into oxidized products (20, 21, 30). This enzyme plays an important role in the regulation of RA levels during embryogenesis and in adult tissues (20, 21). In tumor cells, CYP26A1 can be constitutively expressed, induced by RA, or not expressed at all (20, 21).

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A direct involvement of the CYP26A1 enzyme in 4-HPR

Fig. 5 CYP26A1 mRNA expression and 4-HPR metabolism in clones that overexpressed RARs. In A, RAR expression was determined by Western blot analysis in A2780 cells stably transfected with RARα (α5), RARβ (β7, β13) RARγ (γ19) and in A2780 control cells as described previously (10). In B, CYP26A1 mRNA expression was measured by RT-PCR in α5, β7, β13, and γ19 cells and in empty vector clones (A2780). Two µg of total RNA were retrotranscribed, and the gene sequences for CYP26A1 and β-actin, used as internal standard, were coamplified in the same reaction. In C, CYP26A1 protein expression was evaluated by Western blot analysis in α5, β7, β13, and γ19 and empty vector clone (Empty vector). Eighty µg of protein were loaded onto a SDS-PAGE gel, and were immunoblotted with antibody against CYP26A1. As a control for loading, the blot was incubated with actin antibody. D, 4-HPR metabolism in the media of α5, β7, β13, γ19, and empty vector clones. Cells were treated, 24 hours after seeding, with 5 µmol/L 4-HPR. After 72 hours of treatment, retinoids were extracted from media and were analyzed by reverse-phase HPLC. The experiments were repeated two times, with similar results. The results of one experiment are reported.
metabolism to 4-oxo-4-HPR was demonstrated by transfecting A2780 cells with CYP26A1 cDNA. CYP26A1-overexpressing cells produced 4-oxo-4-HPR, and the levels of the oxidized form of 4-HPR were approximately 10-fold lower than those of parent drug. The cell sensitivity to 4-HPR was not reduced after CYP26A1 overexpression, which indicated that such conversion of 4-HPR into 4-oxo-4-HPR does not affect 4-HPR growth-inhibitory effects. Similarly to our findings, in a low-metabolizing head and neck squamous cell line, introduction of CYP26A1 cDNA resulted in an increased amount of RA polar metabolites but in no change in RA sensitivity (37). Formation of the oxidized forms of RA and retinol has generally been considered as an inactivating pathway. However, several studies have demonstrated that these metabolites have a role as mediators of retinoid effects (38, 39, 40). Consistent with these observations, evaluation of the in vitro growth-inhibitory effects of 4-oxo-4-HPR in A2780 cells revealed that this oxidized metabolite was active and even slightly more potent than the parent drug.

The presence of RA-responsive elements in the promoter region of CYP26A1 has been identified (41), and the involvement of different RARs in the induction of CYP26A1 by RA has been demonstrated in different cells (20, 33, 42). Our results provide evidence that, in A2780 cells, RARβ and RARγ are the main RAR subtypes involved. A2780 clones overexpressing RARβ and, to a lesser extent, those overexpressing RARγ showed higher constitutive CYP26A1 levels than did mock-transfected cells and the ability to metabolize 4-HPR to 4-oxo-4-HPR. The enhanced constitutive expression of CYP26A1 in clones that overexpress RARβ and RARγ is probably due to CYP26A1 induction by the natural retinoids present in the serum added to the culture medium.

The metabolism of RA involves, besides enzymes that regulate the synthesis and the catabolism of the retinoid, specific proteins such as CRBPs and CRABPs, which sequester and transport intracellularly retinol and RA, respectively (22), and whose expression, similarly to CYP26A1, is modulated by RA (43). Our results on CRABPs and CRBP-I mRNA expression in A2780 and A2780/HPR cells indicated that the continuous presence of 4-HPR in the medium did not affect CRABP-I and CRABP-II mRNA expression but was able to increase CRBP-I transcript. The role of increased levels of CRBP-I on 4-HPR metabolism is difficult to understand, because 4-HPR has little or no binding affinity for this protein (44). However, by up-regulating CRBP-I, 4-HPR might increase the intracellular concentration of retinol. Interestingly, human breast cancer cells pretreated with 4-HPR and then treated with [3H]retinol, exhibited much higher [3H]retinol concentration than did cells that were not pretreated (45). It is reasonable to hypothesize that, in these cells, 4-HPR pretreatment caused retinol accumulation through CRBP-I up-regulation. We do not known whether this effect may contribute to the decrease of retinol plasma levels that occurs after 4-HPR treatment and that has been shown to be caused by the binding of 4-HPR to RBP (46, 47). Another interesting consequence of the ability of 4-HPR to up-modulate CRBP-I expression is that this effect might in part account for the observed protective effect of 4-HPR against ovarian cancer (6), because the loss of CRBP-I expression has been recently described as an early event in ovarian carcinogenesis (48).

Moreover, the increased expression of CRBP-I in A2780/HPR cells that are continuously treated with 4-HPR substantiates our previous observations of a more differentiated phenotype compared with A2780 cells (19).

In summary, we have identified a new metabolite of 4-HPR, 4-oxo-4-HPR, that is present in humans and that has tumor growth-inhibitory effects. We have also presented evidence that, after repeated treatments, 4-oxo-4-HPR is formed in tumor cells through the induction of CYP26A1 expression. These findings might have important clinical implications because they indicate that the same autoinduction of 4-HPR metabolism might occur in vivo. 4-HPR might be differently metabolized in organs and/or tumors according to different expression of CYP26A1, which is, in turn, associated with RAR expression. Another important consequence of CYP26A1 induction by 4-HPR is that 4-HPR might affect not only its own metabolism, but it might also interfere with the metabolism of endogenous retinoids. Finally, we have shown that 4-HPR up-regulates the expression of CRBP-I transcript that is lost during ovarian carcinogenesis.

REFERENCES


Identification of the Fenretinide Metabolite 4-Oxo-Fenretinide Present in Human Plasma and Formed in Human Ovarian Carcinoma Cells through Induction of Cytochrome P450 26A1

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