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 were 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-
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cinogenesis, is up-regulated by 4-HPR chronic treatment. (22).

Port (22).

through regulation of intracellular RA concentration and trans-

modulates the expression of cellular retinoic acid-binding pro-

sues and in some human tumor cell lines (20, 21). Moreover, RA

constitutively expressed in numerous embryonic and adult tis-

s responsible for its oxidation to polar metabolites and which is

reduced intracellular 4-HPR levels, and showed changes in

marker expression, suggestive of a more differentiated pheno-

type, 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 re-

sponsible for its oxidation to polar metabolites and which is

constitutively expressed in numerous embryonic and adult tis-

s and in some human tumor cell lines (20, 21). Moreover, RA

modulates the expression of cellular retinoic acid-binding pro-

teins (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 trans-

port (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. More-

over, we show that CRBP-I, which is lost during ovarian car-

cinogenesis, 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 iden-
tification of the polar metabolite and for evaluation of its con-
centrations, 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 con-
sent form. The design and all study features of the prevention
trial have been previously described (5). Briefly, the trial en-
rolled women who met the following criteria: age between 30
and 70 years, breast cancer (T1, T2, 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 \( \pm 24 \) 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

\( -80^\circ \text{C} \) 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% CO\(_2\) 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 \( \mu \text{mol/L} \) 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 \( \mu \text{mol/L} \), and were stored at

\( -80^\circ \text{C} \) 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 \( \mu \text{mol/L} \) 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 \( -80^\circ \text{C} \) 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 \( \mu \text{g/ml} \) butylated hydroxytoluene
(BHT; Sigma) as antioxidant and were then sonicated. An
aliquot of 200 \( \mu \text{L} \) of each cell, medium, and plasma sample was
added to 400 \( \mu \text{L} \) of \( \text{CH}_3\text{CN} \) containing 125 \( \mu \text{g/ml} \) BHT, and the
mixture was vortexed and centrifuged to pellet the precipitated
proteins. The recovered supernatants were analyzed on a liquid
chromatograph (Perkin-Elmer, Norwall, CT) fitted with a C18
(5-\( \mu \text{m} \)) reverse-phase column (150 \( \times \) 4.6 mm) and a C18
precolumn (Perkin-Elmer, Milan, Italy). The mobile phase con-
sisted of \( \text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{CH}_3\text{COOH} \) (75:23:2, vol/vol/vol) deliv-
ered at a flow rate of 2 \( \text{ml/min} \). Detection was carried out with a
Perkin-Elmer LC95 absorbance detector at 370 nm. The re-
ference 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 evalua-
tion, 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 atmo-
spheric 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 300°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-oxoretinoic acid as described previously. The 4-oxoretinoic 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-CH2), 6.01 (s, 1, 13-H), 6.35–6.42 (m, 4, vinyls), 6.74 (d, 2, Ar, J = 8.9 Hz), 7.06 (dd, 1, 11-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 121 pump with otherwise fixed conditions to determine the optimal number of cycles to be used. The HPLC 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).

Construction of Expression Vectors. The human CYP26A1 full-length cDNA was cloned into the eukaryotic expression vector pREP4 (Invitrogen, Milan, Italy) containing the Rous Sarcoma Virus long terminal repeat enhancer/promoter. To this aim, total RNA was extracted from cells that expressed high levels of CYP26A1 (A2780/HPR) and was used for retrotranscription reaction, as described in the previous section. The obtained cDNAs were amplified by PCR with the CYP26A1-specific forward primer, 5′-ATCCCAAGCTTC-CACCATGGGGCTCCCGGCGCTG-3′, containing HindIII restriction site and a Kozak sequence (CCACC) and the reverse

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Plasma Levels of 4-HPR and Its Metabolites 4-MPR and 4-oxo-4-HPR in 4-HPR-treated Patients. 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 \([M+H]^+-([\text{NH}_2\text{C}_6\text{H}_4\text{OH}])\) at m/z 297 (top panel, ms1), having 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 from those 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 ms2 and ms3, 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.

Fig. 1 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 (for details, see Materials and Methods).
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 only slight induced CYP26A1 mRNA expression after 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 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). CYP26A1 mRNA levels, analyzed by semiquantitative RT-PCR, confirmed the expected overexpression in A2780-CYP26A1 cells relative to cells trans-

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**Table 1** Plasma concentrations of 4HPR, 4-MPR, and 4-oxo-4-HPR in patients treated for 5 years with 200 mg/d 4-HPR

<table>
<thead>
<tr>
<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 (R)*</th>
<th>4-oxo-4-HPR μmol/L (R)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>57</td>
<td>0.98</td>
<td>0.86 (0.88)</td>
<td>0.49 (0.50)</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>51</td>
<td>0.85</td>
<td>0.95 (1.12)</td>
<td>0.93 (1.09)</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>61</td>
<td>0.81</td>
<td>1.17 (1.44)</td>
<td>0.43 (0.53)</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>57</td>
<td>0.92</td>
<td>1.21 (1.32)</td>
<td>0.57 (0.62)</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>59</td>
<td>0.28</td>
<td>0.44 (1.57)</td>
<td>0.23 (0.82)</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>52</td>
<td>0.57</td>
<td>0.99 (1.74)</td>
<td>0.64 (1.12)</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>49</td>
<td>0.43</td>
<td>0.60 (1.40)</td>
<td>0.47 (1.09)</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>55</td>
<td>0.52</td>
<td>0.53 (1.02)</td>
<td>0.46 (0.88)</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>47</td>
<td>0.71</td>
<td>0.59 (0.83)</td>
<td>0.39 (0.55)</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>60</td>
<td>2.28</td>
<td>3.46 (1.32)</td>
<td>0.53 (0.23)</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>61</td>
<td>0.90</td>
<td>1.62 (1.80)</td>
<td>0.57 (0.63)</td>
</tr>
</tbody>
</table>

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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Fig. 2 Effect of 4-HPR on the expression of CYP26A1 in A2780 and A2780/HPR cells. CYP26A1 gene expression was analyzed in A2780 cells after one single treatment with 5 μmol/L 4-HPR for 6 (6h) and 24 hours (24h) and in A2780/HPR constantly maintained in the presence of 4-HPR. CYP26A1 mRNA expression was measured by RT-PCR assay. 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 A, CYP26A1 protein expression was analyzed by Western blot analysis. Eight μg of protein were loaded onto an SDS-PAGE gel, and the blot was incubated with actin antibody. +, presence of 4-HPR, −, absence of 4-HPR.
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A2780-mock cells. Twenty-four hours after transfection, cells were treated with 5 μmol/L 4-HPR for 48 hours. Afterward, cells were harvested and counted. No change in sensitivity to 4-HPR was found between A2780-CYP26A1 and A2780-mock cells (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 ($IC_{50} = 0.6 \mu\text{mol/L}$ 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 RARα (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 RARα 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-clone, 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 CRABP-I, CRABP-II, and CRBP-I, 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 CRABP-I and CRABP-II and CRBP-I mRNA was detected by semiquantitative RT-PCR analysis (Fig. 6). CRABP-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, 110 ± 26 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 ± 0.2 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. Four CYP26 enzymes (CYP26A1, CYP26B1, CYP26C1, and CYP26C2) have been described, of which CYP26A1 is specifically liver-expressed. CYP26A1 has been shown to play a crucial role in the metabolism of all-trans-retinoic acid and its derivatives (20–23). In order to verify the hypothesis that CYP26A1 could be involved in the metabolism of 4-HPR, we transiently transfected A2780 cells with CYP26A1 cDNA and analyzed the expression of the enzyme by RT-PCR. As shown in Fig. 4A, cells transiently transfected with CYP26A1 cDNA expressed higher levels of CYP26A1 mRNA than mock-transfected cells. The induction of CYP26A1 expression was confirmed by Western blotting (data not shown). The level of CYP26A1 expression was then correlated with the formation of 4-oxo-4-HPR. As shown in Fig. 4B, 4-HPR metabolism in A2780-CYP26A1 cells was significantly higher than in mock-transfected cells. Furthermore, the antiproliferative activity of 4-oxo-4-HPR was significantly higher in A2780-CYP26A1 cells compared with mock-transfected cells (Fig. 4C). These results suggest that CYP26A1 is involved in the metabolism of 4-HPR and that this enzyme is responsible for the formation of 4-oxo-4-HPR.

**Fig. 4** Metabolism and growth-inhibitory effects of 4-HPR in A2780 cells transiently transfected with CYP26A1 and sensitivity of A2780 cells to 4-oxo-4-HPR. A, RT-PCR analysis for detection of CYP26A1 mRNA levels in A2780 cells transfected with CYP26A1 cDNA (A2780-CYP26A1) or with an empty vector used as control (A2780-mock). 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. B, 4-HPR metabolism in A2780-CYP26A1 and in A2780 mock-transfected cells. Transfected cells were treated with 5 μM 4-HPR for 48 hours. 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. C, 4-HPR antiproliferative activity in A2780-CYP26A1 (▲) and A2780-mock cells (▪). Cells were treated with 5 μmol/L 4-HPR 24 hours after transfection, and surviving cell number was evaluated 2 days later. Results are the means of two independent experiments. D, 4-oxo-4-HPR (○) and 4-HPR (●) antiproliferative activity in A2780 cells. Cells were treated with the indicated concentrations of the two retinoids, and surviving cell number was evaluated 3 days later. Results are the means of three independent experiments.

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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. However, it is interesting to point out that aberrant differentiation of neoplastic cells results, in part, from defects in retinoid metabolism (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). 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. However, it is interesting to point out that aberrant differentiation of neoplastic cells results, in part, from defects in retinoid metabolism (31, 32).

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clones that overexpress RARβ/H9252 4-HPR. The enhanced constitutive expression of CYP26A1 in transfected cells and the ability to metabolize 4-HPR to 4-oxo-4-HPR showed higher constitutive CYP26A1 levels than did mock-transfected cells. A2780 clones overexpressing RARβ/H9252 provide evidence that, in A2780 cells, RARβ/H9253 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 CRBP-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 the 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-modulates the expression of CRBP-I transcript that is lost during ovarian carcinogenesis.

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Fenretinide-Autoinduced Metabolism to 4-Oxo-Fenretinide


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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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