A Novel Human Cytochrome P450, CYP26C1, Involved in Metabolism of 9-cis and All-trans Isomers of Retinoic Acid*

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Retinoids are potent regulators of cell proliferation, cell differentiation, and morphogenesis and are important therapeutic agents in oncology and dermatology. The gene regulatory activity of endogenous retinoids is effected primarily by retinoic acid isomers (all-trans and 9-cis) that are synthesized from retinaldehyde precursors in a broad range of tissues and act as ligands for nuclear retinoid acid receptors. The catabolism of all-trans-retinoic acid (atRA) is an important mechanism of controlling RA levels in cell and tissues. We have previously identified two cytochrome P450s, P450RAI-1 and P450RAI-2 (herein named CYP26A1 and CYP26B1), which were shown to be responsible for catabolism of atRA both in the embryo and the adult. In this report, we describe the identification, molecular cloning, and characterization of a third member of the CYP26 family, named CYP26C1. Transiently transfected cells expressing CYP26C1 convert atRA to polar water-soluble metabolites similar to those generated by CYP26A1 and -B1. Competition studies with all-trans, 13-cis, and 9-cis isomers of retinoic acid demonstrated that atRA was the preferred substrate for CYP26C1. Although CYP26C1 shares extensive sequence similarity with CYP26A1 and CYP26B1, its catalytic activity appears distinct from those of other CYP26 family members. Specifically, CYP26C1 can also recognize and metabolize 9-cis-RA and is much less sensitive than the other CYP26 family members to the inhibitory effects of ketocazole. CYP26C1 is not widely expressed in the adult but is inducible by RA in HPK1a, transformed human keratinocyte cell lines. This third CYP26 member may play a specific role in catabolizing both all-trans and 9-cis isomers of RA.

Retinoic acid (RA)1 regulates the expression of genes involved in cell proliferation, cell differentiation, and apoptosis and is essential for normal embryonic development and for health in the adult (1). The effects of RA are mediated by two classes of nuclear receptors, retinoic acid receptors (α, β, γ), which are activated by both all-trans- (atRA) and 9-cis- (9-cis-RA) isomers of RA and RXRs (α, β, γ), which are selectively activated only by 9-cis-RA (2). These nuclear receptors regulate transcription by binding to specific response elements in promoters of RA target genes (2). Although essentially all normal cell types have the capacity to respond to RA, the tissue distribution of RA is uneven due to tightly controlled, localized synthesis and catabolism (3–7). This tight regulation of RA distribution is critical for normal embryonic development and tissue maintenance in the adult. Most of the specific enzymes involved in this process have recently been cloned and characterized.

The biosynthesis of RA in vertebrates involves oxidation steps converting retinol to retinaldehyde and subsequently to RA (8). This first step is effected by a family of alcohol dehydrogenases, primarily ADHs 1, -2, -3, and -4, which are involved in converting all-trans retinol and 9-cis retinol to the corresponding all-trans and 9-cis retinoldehydes (9, 10). Next, the dispersal of RA is tightly controlled by enzymes involved in localized RA synthesis from retinaldehyde (retinaldehyde dehydrogenases; ALDHs). Several class I aldehyde dehydrogenases (ALDH1A1, ALDH1A2, and ALDH1A3) have been cloned from several species, including human, and are characterized by their ability to form atRA and 9-cis-RA from their aldehyde precursors (11–15). These enzymes are differentially expressed in tissues demonstrated to have higher levels of RA and correspondingly higher levels of transcription of RA-regulated genes (16).

The diffusion of RA emanating from regions of localized synthesis is confined by the expression of cytochrome P450s, belonging to the CYP26 family, which specifically convert RA to less active and more readily excreteable polar metabolites (3). Two distinct members of this family, CYP26A1 and CYP26B1, have been characterized in several species (3, 17–20). The catalytic activities of CYP26 enzymes A1 and B1 have been studied both in cultured cells and in microsomal extracts. Both enzymes similarly convert all-trans retinoic acid to 4-hydroxy-, 4-oxo-, and 18-hydroxy-RA intermediates (20). These intermediate metabolites may then be further hydroxylated to form more polar metabolites (8) or conjugated prior to elimination (21). Although in culture, some of these metabolites have activity when present at high concentration, recent genetic studies indicate that these metabolites do not have an essential role in regulating embryonic development and that this role is carried out by RA (22). Both of these enzymes exhibit a high degree of specificity for atRA (atRA → 9-cis-RA → 13-cis RA → retinaldehyde → retinol) and, as with most cytochrome P450s, can be inhibited by imidazole-based compounds such as ketoconazole (20).

The expression pattern of ALDHs and CYP26 genes are often complementary, particularly in the embryo, but are also re-
required to control RA distribution in the adult (12, 23, 24).

Studies employing a transgenic retinoic-acid reporter gene (RARα-lacZ) indicated enriched regions of RA activity coincident with those of expressing ALDHs (25, 26). CYP26A1 is expressed during early embryonic development in the hindbrain and retina (17). Genetic ablation of CYP26A1 is embryonic lethal and causes many of the defects seen when embryos are exposed to a teratogenic excess of RA, including spina bifida (3). Three genetic studies have very clearly demonstrated that the role of CYP26 enzymes is to inactivate RA by converting it to more polar metabolites and by doing so, protects certain tissues from RA exposure.

**CYP26A1** is expressed in a number of adult tissues following exposure to RA. This induction of RA is regulated at the transcriptional level (27) and is a mechanism whereby spikes in vitamin A or RA intake from dietary sources can be normalized. This autoregulation feedback loop can limit the effectiveness of RA therapy in skin disorders and cancer such as acute promyelocytic leukemia (28–30). CYP26B1 is expressed in the adult brain in several regions, which include pons, cerebellum, and hippocampus, and is expressed in hair follicles and the dermal layer of skin (7). CYP26B1 expression can also be induced by RA (20), however, the expression of this enzyme in adult appears to be mainly constitutive, in particular in brain and skin (7). Differential expression of these enzymes distinguishes their roles in protecting tissues from inappropriate levels of atRA.

Although CYP26A1 and B1 can efficiently catabolize atRA, their ability to oxidize 9-cis-RA is less so. It has been speculated that another enzyme might be responsible for this latter activity (31). Presently, we describe the cloning and characterization of a third member of this family, namely **CYP26C1**. Although **CYP26C1** shares extensive sequence similarity with **CYP26A1** and **CYP26B1**, we show that it exhibits catalytic activity distinct from those of other CYP26 family members. Specifically, **CYP26C1** can metabolize atRA and can metabolize 9-cis-RA and is not sensitive to inhibition by ketoconazole.

**MATERIALS AND METHODS**

**Identification of CYP26C1 cDNA—**CYP26C1 was first identified by screening the Unfinished High Throughput Genomic Sequence (HTGS) data base at the National Center for Biotechnology Information using the amino acid sequences of human CYP26A1 (Accession number O43174) and the human CYP26B1 (Accession number AAF76003). A bacterial stab of a BAC clone containing the CYP26C1 gene (AL358613) was obtained from the Sanger Genome Center. Colonies were screened by PCR using two sets of primers to identify positives containing the known fragments of CYP26C1. One set was specific for the first predicted exon, 5′-CTCATACGTTCCCTTGGGTTCTGA and 5′-CTGC-TGAATACTACCCATGAGCCTTTC. The other set was derived from the predicted exon 4, 5′-GCAAGGACACGCTGCTGCAGCAGTCT GAAGCTCTCGACGCTGAGGARTGAGCACGTCCG and 5′-CCCCTCCCTCGCATGACCATTAGTACGCG. BAC DNA was prepared and sent to the Centre for Applied Genomics at the Hospital for Sick Children in Toronto, Ontario, and sequencing was performed to complete the genomic sequence around the identified regions containing CYP26C1.

Using sequence information from AL358613 and the sequence obtained from the Centre for Applied Genomics, a 1569-bp nucleotide sequence corresponding to a 522-amino acid protein sequence was predicted. The intron/exon boundaries were deduced based on comparison with CYP26A1 and CYP26B1.

**Cloning of the Full-length CYP26C1 cDNA—**The full-length CYP26C1 was amplified by PCR using an upstream primer encompassing the translation initiation codon, 5′-CTCATACGTTCCCTTGGGTTCTGA, and a second primer 5′-ATGTCAGGAGGCATTAGGCATTCC TTGCG. This primer sequence corresponds to the sequence downstream of the assigned stop codon. Human Adrenal Poly(A) RNA (Ambion, TX) was used as the template to make cDNA using the Thermoscript RT-PCR System and oligo(dT) primer, as per the manufacturer's directions (Invitrogen, Carlsbad, CA). A fragment of ~1600 bp in length was PCR-amplified by the following reaction conditions: 1 cycle at 94 °C for 2 min, followed by 30 cycles of 99 °C for 30 s, 60 °C for 30 s, and 68 °C for 105 s. The PCR-generated product was sequenced at Corbett DNA Service Laboratories (Kingston, Ontario, Canada) and subcloned into pcDNA3.1 (Invitrogen) to generate the CYP26C1 expression construct, pcDNA3.1-CYP26C1.

**Isolation and Reverse Transcription-PCR—**CYP26C1 expression was assayed in HPK1a and HPK1α-ras cell lines (provided by Dr. Glenville Jones, Queen's University) was determined by RT-PCR. Briefly, cells were plated in the 6-well plates at 500,000 cells per well in 2 ml of suitable growth medium (final 50–70% confluence). At different time intervals, growth medium was removed, and 1 ml of TRIzol (Invitrogen) was added per well. After 5 min at room temperature, total RNAs were prepared according to Invitrogen's instructions. For induction studies, cells were plated in 6-well plate 1 day prior treatment were treated with all-trans retinoic acid or 9-cis-RA at a concentration of 1 μM for 24 h prior to harvesting. A control experiment was conducted by adding the vehicle only (Me2SO). Total RNA (10 μg) was reverse-transcribed using Invitrogen's Thermoscript RT-PCR system.

Two microfilters of cDNA were used for RT-PCR amplification using the Qiagen's Hotstart Tag master mix. The CYP26C1-selective primers used in this study comprised a 268-bp fragment between nucleotides 541 and 809 starting from the translation initiation codon (forward primer: 5′-GTC TAC GAC GCC TCC AAA GGG CTC AC-3′ and reverse primer: 5′-ATT AGG TCG AGG GCA TCA CCC GGC T-3′). PCR was conducted using a Mastercycler Gradient PCR Machine (Eppendorf) using the following reaction conditions as follow: 95 °C for 15 min followed by 95 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, and finally 1 cycle at 72 °C for 5 min. PCR products were analyzed on a 1.8% agarose gel.

**Real-time PCR—**CYP26C1 TaqMan (FAM) probe was synthesized by Applied Biosystems and used in combination with the Universal TaqMan master mix. A 71-bp CYP26C1 fragment, corresponding to the cDNA region between nucleotides 674 and 744 from ATG initiation codon, was amplified. An ABI Prism 7000 Sequence Detection System was used for real-time PCR amplification. As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was measured simultaneously. PCR amplification procedures followed manufacturer instructions. Briefly, the thermocycling program consisted of one cycle at 50 °C for 2 min followed by 95 °C for 10 min and 50 cycles at 95 °C (15 s) and 60 °C (1 min). The reactions were then chilled to 4 °C. The data were analyzed using the ABI Prism 7000 Sequence Detection System software.

**Transient Transfection of COS-1 Cells—**Exponentially growing cells were plated in triplicate into six-well tissue culture plates at 150,000 cells/well in DMEM containing 10% FBS 1 day before transfection. Cells were transfected with 1 μg of each pcDNA3.1-CYP26C1 or pcDNA3.1-CYP26B1 or their empty vector pcDNA3.1 by using FuGENE 6 transfection reagent as described by the manufacturer (Roche Applied Science).

**Analysis of Retinoic Acid Metabolic Activity—**Forty-eight hours post-transfection with pcDNA3.1-CYP26C1, COS-1 cells were washed twice with DMEM and then incubated in 0.5 ml of DMEM containing 5% FBS and either radiolabeled atRA (0.1 μCi/ml, PerkinElmer Life Sciences at 1 mCi/ml, 50 Ci/mmol) or radiolabeled 9-cis-RA (0.15 μCi/ml at 2 nm, PerkinElmer Life Sciences at 0.2 μCi/ml, 75 Ci/mmol) at 24 h concentration. All procedures involving retinoids were conducted in an environment protected from light. After incubation for 3 h at 37 °C, reactions were stopped by adding 10 μl of 10% acetic acid, and total retinoids were extracted using the Bligh-Dyer procedure as described previously (20). The water-soluble retinoid metabolites separated in the upper phase were quantified in a β-scintillation counter (Wallac, PerkinElmer Life Sciences). The organic-soluble metabolites were dried by speed-vacuum, and residues were resuspended in 100 μl of HPLC mobile solvent consisting of acetonitrile/water/acetic acid (50:50:0.5, v/v), and analyzed on HPLC (Alliance separation module 2695, Waters, Millford, MA). Separation of the acid metabolites by HPLC was achieved by using a reverse-phase column (150 × 4.6 mm C18 Zorbax-SB, Hewlett Packard) with a linear gradient of acetonitrile at flow rate 1 ml/min. The HPLC conditions were initially isocratic for 2 min with solvent A (acetonitrile/water/acetic acid in the ratio 50:50:0.5, v/v containing 10 mM ammonium acetate), followed by the application of a linear gradient from 0% to 100% solvent B (acetonitrile/water/acetic acid at the ratio 95:5:0.1 containing 10 mM ammonium acetate), solvent B was then held isocratically for 5 min before returning to the initial condition with solvent A for 5 min. Effluent from HPLC column flowed directly to a radioflow detector LB (EG & G, Bethesda, Bad Wildbad, Germany).
CYP26C1 Metabolizes All-trans and 9-cis RA Isomers

**Competition Assays**

**Cell-based Assay**—In cell based-assay COS-1 cells were transfected with either pcDNA3.1-CYP26C1 or pcDNA-CYP26B1 in six-well tissue culture plate as described above. 48 h after transfection, cells were harvested, pooled, washed with DMEM, and plated into 48-well tissue culture plate with 5 × 10^5 cells per well in 0.2 ml of DMEM media containing 5% FBS. Cells were incubated with radiolabeled atRA (0.05 μCi/ml [3H]RA, at 2 nM concentration), in the presence or absence of increasing concentrations of each unlabeled retinoid (atRA, 9-cis-RA, 13-cis-RA) or ketoconazole. Following a 10-min preincubation period at 37°C, the reaction was initiated by addition of 10 mM NADPH (Sigma). After incubation for 1 h at 37°C, the reaction was stopped by acidification with 5 μl of 10% acetic acid. Extraction of the samples was conducted by Bligh-Dyer procedure, and water-soluble metabolites were counted in a scintillation counter as described above.

**LCMS Analysis**—LC/MS was used to obtain more precise information on the molecular mass of the atRA metabolites. HeLa cells were transfected with pcEBV-CYP26C1, comprising CYP26C1 subcloned into an Epstein-Barr virus promoter based vector containing hygromycin B resistance gene. HeLa cells stably expressing CYP26C1 were isolated by selecting and cloning atRA-metabolism-positive cells in minimal essential medium containing 10% FBS and 100 μg/ml hygromycin B. Tissue culture plates of exponentially growing cells (70 to 80% confluency) were incubated in 5 ml of minimal essential medium containing 5% FBS and 1 μM of either atRA or 9-cis-RA. After incubation for 5 h at 37°C, total retinoids were extracted twice with an equal volume of ethyl acetate containing 1% acetic acid. The upper organic phase was removed, dried using speed vacuum, and the total residue was resuspended in solvent for LC/MS. The LC mobile phase consisted of water (solvent A), acetonitrile (solvent B), and 0.5% acetic acid (solvent C). The LC separation of retinoid metabolites was achieved by a reverse-phase column (150 × 2.1 mm Zorbax C18 Eclipse XDB 5 μm, Agilent Technologies, CA) at a flow rate of 0.2 ml/min with a linear gradient of acetonitrile. The mobile phase started with mixture of solvents A:B:C in the ratio 64:35:1 for 2 min, and a linear gradient for 28 min up to 95% acetonitrile.

**Microsome-based Assay**—The microsome-based assay utilized microsomes prepared from the COS-1 cells transiently expressing either CYP26B1 or CYP26C1. Cells were harvested, 48 h after transfection, washed with ice-cold phosphate-buffered saline, counted, then homogenized and sonicated in lysis buffer (100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 M sucrose) containing protease inhibitor mixture (Roche Applied Science). The microsomes were isolated by differential centrifugations for 10 min at 800 g. Finally, the postmitochondrial supernatant was centrifuged at 100,000 g for 60 min using ultracentrifugation (Beckman). The microsome pellets were isolated and homogenized gently in the storage buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl2, 150 mM KCl, and 10% glycerol) containing protease inhibitor mixture. Protein concentration in the microsomal preparation was determined using the Bradford reaction assay kit using bovine serum albumin as standard (Pierce). Microsomes were aliquoted at 1 mg/ml in storage buffer, checked for RA-metabolic activity, and stored in liquid nitrogen. The microsome-based assay was conducted in the storage buffer containing 0.5% bovine serum albumin using a mixture (total volume of 200 μl) composed of an appropriate amount (10 μg) of micromolar suspension incubated with radiolabeled atRA (0.05 μCi/ml [3H]RA, at 2 nM concentration) in the presence or absence of increasing concentrations of each unlabeled retinoid (atRA, 9-cis-RA, 13-cis-RA) or ketoconazole. Following a 10-min preincubation period at 37°C, the reaction was initiated by addition of 10 mM NADPH (Sigma). After incubation for 1 h at 37°C, the reaction was stopped by acidification with 5 μl of 10% acetic acid. Extraction of the samples was conducted by Bligh-Dyer procedure, and water-soluble metabolites were counted in a scintillation counter as described above.

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of solvent B with a constant flow rate of 1% solvent C. At 30 min the mobile phase was held isocratically for 10 min before returning to the initial conditions over 5 min, and equilibrating for 5 min.

LC/MS was performed using Micromass Quattro Ultima Triple-stage quadrupole mass spectrometer (Manchester, UK) connected to a LC Alliance 2969 separation module (Waters, Milford, MA). The MS was operated in the negative electrospray mode (ES-MS) with a (-3.15 KV) capillary voltage and (-37 V) cone voltage. Nitrogen was used as a desolvation gas at a flow of 871 liter/h. For MS/MS experiments, argon was used as a collision gas at a pressure of $2.3 \times 10^{-3}$ torr with collision energy of 25 V. The mass spectrometer was operated in a full mass scan ($m/z$ 200–500), and product ion scan. The major retinoid metabolites were further characterized using tandem mass spectroscopy (MS/MS) in the product ion scan mode, and daughter ions were detected from $m/z$ 50 to $m/z$ 400.

**RESULTS**

**Identification and Cloning of CYP26C1**—A genomic region corresponding to a putative third member of the CYP26 family was identified by searching the HTGS data base for sequences with similarity to the human CYP26 family members (CYP26A1 and CYP26B1). The identified sequence (accession no. AL358613) corresponded to a 160,532-bp clone from human...
chromosome 10, which showed similarity to the nucleotide sequence encoding CYP26A1 and CYP26B1 (20). N-terminal exon 1, as well as exons 4 and 5 and part of the C-terminal exon 6 were predicted from this sequence by comparison with the other known CYP26 family members. Exon 6 contained the heme thiolate benchmark motif characteristic of all cytochrome P450s (FXXGXRXCXG, where X can be any amino acid), FGG-GA-RRSC-LG.

A BAC clone (AL358613) was obtained and sequenced to confirm that the clone contained an open-reading frame that conceptually could be translated into a novel CYP26-like protein (CYP26C1). The predicted 6 exons and intron/exon boundaries of CYP26C1 are shown in Fig. 1A. We compared the amino acid sequence of CYP26C1 with those of CYP26A1 and CYP26B1 (Fig. 1B). Overall CYP26A1 and CYP26C1 show 43% identity at the amino acid level and 52% at the nucleotide level over the region of the predicted open reading frame. The overall similarity of the two putative open reading frames is somewhat higher when conservatively substituted amino acids are considered.

Cloning of the Full-length CYP26C1 cDNA—To clone the CYP26C1 cDNA, we first analyzed the pattern of CYP26C1 expression using PCR amplification of multtissue rapid amplification of cDNA ends panels representing different human tissues (muscle, stomach, testis, placenta, pituitary gland, thyroid gland, adrenal gland, and pancreas) that showed primarily that CYP26C1 could be detected in most tissues at low levels (data not shown). We subsequently used human adrenal mRNA (Ambion, TX) to clone the full-length CYP26C1 cDNA by RT-PCR.

Expression and Inducibility of CYP26C1 in Human Cell Lines—We used RT-PCR to screen cell lines derived from a range of different tissues for expression and inducibility of CYP26C1. CYP26C1 mRNA transcripts could be detected in HPK1a (from keratinocytes) and HPK1a-ras (HPK1a cells transformed with ras). Glyceraldehyde-3-phosphate dehydrogenase transcripts were also analyzed to normalize for differences in total RNA content of the sample (Fig. 2A).

We also examined whether CYP26C1 transcripts could be induced in HPK1a cell line following treatment with either all-trans, or 9-cis-RA. Treatment of HPK1a cells with all-trans-RA or 9-cis-RA resulted in increased expression of CYP26C1 approximately 15- and 50-fold, respectively (see Fig. 2B).

Retinoic Acid Metabolism by CYP26C1—Structural similarities between CYP26A1, B1, and C1 prompted us to establish
whether this novel enzyme could also metabolize atRA. CYP26C1 was introduced into the expression vector pCDNA3.1 to generate pCDNA3.1-CYP26C1 for transient transfection into COS-1 cells (a cell line that has been previously used to characterize CYP26A1 and CYP26B1). COS-1 cells 48 h after transfection with pCDNA3.1-CYP26C1 were incubated with radiolabeled [3H]atRA for 3 h, and cells were extracted for both aqueous and organic soluble metabolites. COS-1 cells transfected with pCDNA3.1-CYP26C1 exhibited a strong ability to convert the radiolabeled substrate into an aqueous soluble form, in contrast to COS-1 cells alone or COS-1 cells transfected with the parent vector (Fig. 3A). Organic soluble metabolites were evaluated by HPLC of the organic phase as presented in Fig. 3B. The HPLC chromatogram indicated that COS-1 cells transfected with pCDNA3.1-CYP26C1 convert the substrate peak to more polar metabolite peaks. These results clearly indicate that expression of CYP26C1 causes an extensive metabolism of atRA to more polar metabolites.

Characterization of CYP26C1 Substrate Specificity—In the previous study we have used competition analysis to establish CYP26A1 and CYP26B1 substrate preference for naturally occurring retinoids, including atRA, 9-cis-RA, 13-cis-RA, retinol, and retinal (20). Surprisingly, similar studies performed with CYP26C1 revealed that both atRA as well as 9-cis-RA could compete very effectively with labeled [3H]atRA substrate in contrast to what was found for the other CYP26 family members, neither of which exhibit preference for 9-cis-RA (Fig. 4). Moreover, CYP26C1 also exhibited a markedly reduced sensitivity to inhibition by ketoconazole (IC50 50 μM) compared with both CYP26A1 and CYP26B1 (IC50 8–10 μM) (20). Thus, although CYP26A1 and CYP26B1 share very similar substrate specificities, CYP26C1 is distinguished by its apparent higher affinity for 9-cis-RA and lower affinity for ketoconazole. As we have previously determined for CYP26A1 and CYP26B1, atRA precursors, retinol and retinal do not compete at concentration up to 100 μM. Conversely, downstream metabolites of RA such as 4-hydroxy-, 4-oxo-, and 18-hydroxy-RA do exhibit the ability to compete for CYP26C1 (data not shown).

To confirm the substrate specificity profile of CYP26C1, we prepared microsomes from COS-1 cells transiently expressing CYP26C1 or CYP26B1 (Fig. 5). Again we noted the ability of 9-cis-RA to strongly compete with [3H]atRA for CYP26C1 activity in microsomal preparations. The apparent IC50 values determined in whole cell and microsomal preparations are summarized in Table I. We also noted that when CYP26C1 enzyme activity is analyzed in cell culture based assays, the average apparent IC50 values for atRA is 0.3 μM, which is 10-fold lower than that observed for either CYP26A1 or CYP26B1. However, in microsome-based assays, the affinity for atRA is similar for all the three enzymes. We do not presently know the cause of this discrepancy.

Metabolism of 9-cis-RA by CYP26C1 and Identification of Metabolites—The physiological importance of 9-cis-RA is not clear. Nevertheless, several studies have identified components of retinoid signaling with specificity toward 9-cis-RA, including RXR nuclear receptors and retinaldehyde dehydrogenase (32). The possible importance of 9-cis-RA prompted us to more thoroughly characterize the capability of CYP26C1 to metabolize 9-cis-RA. Transient transfection experiments were carried out in a manner similar to those conducted for atRA metabolism (see above). COS-1 cells transfected with pCDNA3.1-CYP26C1 generated aqueous soluble metabolites of radiolabeled 9-cis-RA (Fig. 6A). HPLC analysis of the organic phase extracted from these cells, indicated that almost total conversion of 9-cis-RA to more polar metabolites over the 3-h incubation period (Fig. 6B).

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Metabolism of retinoids by microsomal fractions of CYP26C1- and CYP26B1-transfected cells. Microsomes were isolated from the transiently transfected COS-1 cells expressing either CYP26B1 or CYP26C1. An appropriate amount of microsomal suspension was incubated with radiolabeled atRA (2 nM) in the presence or absence of increasing concentrations of each unlabeled retinoid (atRA, 9-cis-RA, and 13-cis-RA), and ketoconazole. Following a 10-min preincubation period at 37 °C, the reaction was initiated by addition of NADPH followed by 1 h incubation at 37 °C. The reaction was stopped, retinoid metabolites were extracted by Bligh-Dyer procedure, and the aqueous upper phase was counted in a scintillation counter.

**Table I**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Cell-based assay</th>
<th>Microsome-based assay</th>
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<tr>
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<td>CYP26C1</td>
<td>CYP26B1</td>
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<tr>
<td>atRA</td>
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<td>3.5</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>1.2</td>
<td>&gt;10</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Retinol</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Retinal</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>50</td>
<td>7</td>
</tr>
</tbody>
</table>

* ND, not determined.
Fig. 6. CYP26C1 metabolism of 9-cis-RA. A, conversion of 9-cis-RA to aqueous soluble metabolites. COS-1 cells transiently transfected with CYP26C1 were incubated with 2 nM of radiolabeled 9-cis-RA for 3 h at 37 °C, reactions were stopped, and retinoid metabolites were extracted by Bligh-Dyer procedure. The upper phase containing the water-soluble polar RA metabolites was counted in a scintillation counter. B, HPLC analysis of CYP26C1 metabolism of 9-cis-RA. The organic soluble metabolites were dried by speed vacuuming, resuspended in HPLC mobile solvent, and analyzed on HPLC. Separation of retinoid metabolites by HPLC was achieved by using a reverse-phase column (150 × 4.6 mm C18 Zorbax-SB, Hewlett Packard) with a linear gradient of acetonitrile at flow rate 1 ml/min. Effluent from HPLC column flowed directly to a radioflow detector, which resolves radioactive 9-cis-RA metabolites peaks.

Fig. 7. LC/MS analysis of 9-cis-RA and all-trans-RA metabolism in stably transfected HeLa cells expressing CYP26C1. HeLa-CYP26C1 cells were incubated with 1 μM of atRA or 9-cis-RA for 5 h at 37 °C, and total retinoids were extracted twice with an equal volume of ethyl acetate containing 1% acetic acid. The upper organic phase was removed, dried in speed vacuum, and the total residue was resuspended in solvent for HPLC and analyzed in LC/MS. The mass spectrometry was operated in the negative electrospray mode (ES−), with a full scan mode scanning from m/z 200 to m/z 500.
CYP26C1 Metabolizes All-trans and 9-cis RA Isomers

9-cis RA metabolites. The 9-cis RA major metabolites, peak 1 and peak 2, were further characterized using tandem mass spectroscopy (MS/MS). The mass spectrometry was operated in the negative electrospray mode (ES−) using product ion scan mode, and daughter ions were detected from m/z 50 to m/z 400.

**Table II**

<table>
<thead>
<tr>
<th>Retinoids</th>
<th>LC-MS Retention time (min)</th>
<th>MS/MS, major fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>atRA</td>
<td>33.2</td>
<td>299, 255, 239, 119</td>
</tr>
<tr>
<td>4-hydroxy-RA</td>
<td>15.8</td>
<td>315, 271, 253, 151, 119</td>
</tr>
<tr>
<td>4-oxo-RA</td>
<td>16.9</td>
<td>313, 269, 254, 191, 163, 119</td>
</tr>
<tr>
<td>18-hydroxy-RA</td>
<td>19</td>
<td>315, 271, 253, 151, 109</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>32.6</td>
<td>299, 255, 239, 119</td>
</tr>
<tr>
<td>Peak 1</td>
<td>20.7</td>
<td>313, 269, 254, 191, 163, 119</td>
</tr>
<tr>
<td>Peak 2</td>
<td>19.6</td>
<td>315, 271, 253, 151, 119</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Our present work describes the cDNA cloning and characterization of enzymatic activity of CYP26C1, the third member of the CYP26 family of cytochrome P450s specifically involved in the metabolism of RA. CYP26C1 shares 43 and 51% amino acid identity with CYP26A1 and CYP26B1, respectively. Although the overall similarity is moderate, the high degree of conservation of functional domains first described for CYP26A1 (18) clearly identifies CYP26C1 as a CYP26 family member. Interestingly, CYP26C1 gene is located on chromosome 10 within 13 kb from CYP26A1 (33) suggesting that a recent gene duplication event may have given rise to these two CYPs. Each of these three CYP26 genes may have distinct roles in normal and disease tissue providing unique targets for retinoid-based therapies.

CYP26C1, although closely related in amino acid sequence to CYP26A1 and CYP26B1, exhibits some unique enzymatic properties. As with CYP26A1 and -B1, it can be demonstrated that CYP26C1-transfected cells can metabolize atRA. Competition analysis indicates that CYP26C1 is at least as efficient as CYP26A1 or -B1 in this regard. Moreover, CYP26C1 substrate preference profiles for other naturally occurring retinoids such as retinol, retinaldehyde, and 13-cis-RA do not discriminate it from the other CYP26 family members. However, our studies using transfection assays clearly demonstrate that CYP26C1 has the ability to bind 9-cis-RA with comparative affinity at least 100-fold greater than that observed for either CYP26A1 or -B1. Microsomal extracts from transfected cells also demonstrate the different substrate preferences of these enzymes for the 9-cis metabolite of RA. Metabolism studies showed that CYP26C1 could metabolize both all-trans and 9-cis isomers of RA, producing metabolites that by LC/MS analysis are consistent...
ent with mono- and di-hydroxylated derivates. The two major metabolites of 9-cis-RA generated by CYP26C1 closely correspond to 4-hydroxy-9-cis-RA and 4-oxo-9-cis-RA (32, 34). These studies suggest that the substrate binding pocket for CYP26C1 is functionally distinguishable from those of the other CYP26 enzymes. This is further substantiated by the observation that, although ketoconazole can block CYP26A1- and CYP26B1-mediated RA metabolism, it is less effective at blocking CYP26C1 activity.

We do not presently know whether the ability of CYP26C1 to metabolize 9-cis-RA has any physiological significance. Indeed, the physiological role of 9-cis-RA remains controversial. Nevertheless, some significant differences in the biosynthetic machinery giving rise to atRA and 9-cis-RA; ALDH1A1 for example, has recently been shown to have preference for 9-cis-RA over atRA (32). Cellular retinol-binding proteins (CRBPs) have been isolated that bind all-trans-, but have low affinity for 9-cis-retinol (CRBPs I and II), and a putative CRBP-II has been identified with affinity for both (35). Also, cellular retinoic acid-binding proteins (CRABPi and -II) both bind atRA but not 9-cis-RA (36). Pharmacokinetic studies also suggest that there may be differences in the way these two isomers of RA are metabolized (37). Biochemical studies and x-ray crystallography has implicated 9-cis-RA as a physiological ligand for RXRs. Because RXRs have also been shown to be activated by docosahexanoic acid, we examined the possibility that CYP26C1 might also metabolize this long-chain fatty acid, however, we did not observe any docosahexanoic acid metabolism affected by this enzyme (data not shown). Because of the promiscuous role played by RXRs in signaling mediated by other nuclear receptors, the presence of CYP26C1 in cells could potentially influence the activities of several important pathways, including those for vitamin D, thyroid hormone, and fatty acids whose corresponding receptors form heterodimers with RXRs (31).

The full-length CYP26C1 cDNA was derived from RNA isolated from human adrenal tissue. Attempts to characterize CYP26C1 expression did not convincingly show the presence of CYP26C1 transcript in any of the other RNA samples isolated from adult human tissues. In comparison, CYP26A1 transcripts could not be detected in any of the tissues analyzed, however, CYP26A1 levels are highly induced by RA in a number of human cell lines, and it has been demonstrated that several tissues exhibit induced expression of CYP26A1 transcript following exposure to RA (19, 23). CYP26C1 has so far only been detected in a small number of human cell lines.


A Novel Human Cytochrome P450, CYP26C1, Involved in Metabolism of 9-cis and All-trans Isomers of Retinoic Acid
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