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Expression in yeast, new substrates, and construction of a first 3D model of human orphan cytochrome P450 2U1: Interpretation of substrate hydroxylation regioselectivity from docking studies

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ABSTRACT

Background: Cytochrome P450 2U1 (CYP2U1) has been identified from the human genome and is highly conserved in the living kingdom. In humans, it has been found to be predominantly expressed in the thymus and in the brain. CYP2U1 is considered as an "orphan" enzyme as few data are available on its physiological function(s) and active site topology. Its only substrates reported so far were unsaturated fatty acids such as arachidonic acid, and, much more recently, *N*-arachidonoylserotonin.
Methods: We have expressed CYP2U1 in yeast *Saccharomyces cerevisiae*, built a 3D homology model of CYP2U1, screened a library of compounds known to be substrates of CYP2 family with metabolite detection by HPLC–MS, and performed docking experiments to explain the observed regioselectivity of the reactions.
Results: We show that some drug-related compounds, debrisoquine and terfenadine derivatives, that are subtrates of CYP2D6 and CYP2J2, two enzymes phylogenetically close to CYP2U1, are hydroxylated by recombinant CYP2U1 with regioselectivities quite different from those previously reported in the case of CYP2D6 and 2J2. Docking experiments of those compounds and of the previously described substrate arachidonic acid allow us to explain the regioselectivity of the observed hydroxylations on the basis of the interactions of these substrates with key residues of CYP2U1 active site.
Major conclusion and general significance: Our results show for the first time that human orphan CYP2U1 can oxidize several exogenous molecules including drugs. This could have consequences for the metabolism of drugs particularly in the brain.

1. Introduction

Cytochromes P450 (CYPs) constitute a superfamily of hemoproteins that play key roles in the metabolism of a large variety of endogenous compounds and xenobiotics [1]. In the human genome, 57 genes have been found to code for CYPs. Among them, about fifteen are known to be involved in the metabolism of xenobiotics and participate in the elimination of exogenous compounds such as drugs, toxins or pollutants, and about thirty human CYPs are involved in the biosynthesis of endogenous compounds such as sterols, vitamins and eicosanoids [2]. Very few data are presently available for the other human CYPs that have been recently discovered from an analysis of the human genome, and the CYPs whose biological roles and functions remain poorly known are called "orphan CYPs" [2–4].

Among them, CYP2U1 displays high sequence homology with CYP2R1, 2D6, and 2J2, and is highly conserved among the living kingdom [5–8]. Studies of the distribution of CYP2U1 mRNA and protein have

Abbreviations: AA, arachidonic acid; a.m.u., atomic mass unit; a.u., arbitrary units; CYP, cytochrome P450; Deb, debrisoquine; 1- 3-, 4-, 5-, 6-, 7- and 8-OH-Deb, 1-, 3-, 4-, 5-, 6-, 7-, and 8-hydroxy-debrisoquine, respectively; *N*-OH-Deb, *N*-hydroxy-debrisoquine; Deb-urea, urea derivative of debrisoquine; Et-Terf, ethyl-terfenadone; ESI, electrospray ionization; HETE, hydroxy-eicosatetraenoic acid; HMQC, heteronuclear multiple-quantum correlation; MD, molecular dynamics; MS/MS, tandem mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; RMSD, root mean square deviation; Rt, retention time; SAMS, solvent accessible molecular surface; Terf, terfenadone; TOCSY, total correlation spectroscopy

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shown that it is preferentially expressed in the thymus and cerebellum; its presence was also detected in the kidneys, lungs, heart, white adipose tissue, platelets, and the blood–brain barrier [5,6,9–15]. CYP2U1 was found to be up-regulated in a variety of cancer tissues such as breast or colorectal cancer tissues [16,17]. Very recently, mutations in the CYP2U1 gene were found to be related to the appearance of hereditary spastic paraplegia, a neurological disorder [18]. Other data have shown that CYP2U1 catalyzes the hydroxylation of some polyunsaturated fatty acids [6]. In the case of arachidonic acid (AA), the CYP2U1-dependent hydroxylation was found to occur in ω- and ω-1 positions [6]. Moreover, during the preparation of this manuscript, it was reported that CYP2U1 also catalyzed the oxidation of N-arachidonoylserotonin at position 2 of its indole ring [19]. However, the precise biological roles of CYP2U1, its ability to oxidize xenobiotics, and its structure remain so far unknown.

In order to find other possible CYP2U1 substrates, including drugs, and to get a first idea of the 3D structure of this protein, we have expressed CYP2U1 in yeast *Saccharomyces cerevisiae* expressing human cytochrome P450 reductase W(hR), and constructed a 3D homology model of CYP2U1. Docking of AA in the active site of this model allowed us to explain the unusual hydroxylation regioselectivity previously reported for this substrate [6], which gave a first validation of the model. Then, in order to find new CYP2U1 substrates and to know if CYP2U1 could be involved in drug metabolism, the ability of CYP2U1-expressing yeast microsomes to oxidize a library of molecules was tested, with a special focus on compounds known to be substrates of CYP2 family members. This led us to find first xenobiotic CYP2U1 substrates: debrisoquine (Deb) (Scheme 1), an anti-hypertensive drug [20] which is a well-known substrate of CYP2D6 [21,22], and some analogues of terfenadine (Terf) (Scheme 2), an antihistaminic drug known to be a substrate of CYP3A4 and CYP2J2 [23–26]. The regioselectivity of these CYP2U1-dependent oxidations greatly differed from those catalyzed by CYP2D6 and CYP2J2. Noticeably, this regioselectivity could be explained by docking experiments of Deb and Terf in our rebuilt structural 3D model of CYP2U1.

2. Materials and methods

2.1. Chemicals

Most chemicals and biochemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), Alfa-Aesar (Schiltigheim, France) or Difco Laboratories (COGER, Paris, France). All organic solvents were purchased from SDS (Peypin, France) and were of the highest purity available. [1-^{14}C]Arachidonic acid (sp. act, 50 mCi/mmol) was provided by Perkin-Elmer (Courtaboeuf, France). Ebastine came from Pharmafarm (Paris, France), and 4-hydroxy-debrisoquine (4-OH-Deb) and 8-hydroxy-debrisoquine (8-OH-Deb) from Toronto Research Chemicals (Toronto, Canada). 4-[4-(Hydroxydiphenylmethyl)piperidin-1-yl]-1-(4-*tert*-butylphenyl)butan-1-one (terfenadone), 4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-(4-methylphenyl)butan-1-one (methyl-terfenadone), **7**, 4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-(4-ethylphenyl)butan-1-one (ethyl-terfenadone, Et-Terf), **8**, 4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-(4-propylphenyl)butan-1-one (propyl-terfenadone), **9**, 4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-(4-butylphenyl) butan-1-one (butyl-terfenadone), **10**, 4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-[4-(3-hydroxypropyl)phenyl]butan-1-one, **11**, and, 3-({4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-oxobutyl}phenyl)propyl acetate, **12**, were synthesized as described previously [24–26]. Benzylguanidine, **4**, 2-phenyl-ethylguanidine, **5**, and 6,7-dimethoxy-1,2,3,4-tetrahydro-*iso*-quinoline carboxamidine, **6**, were prepared by reaction of the corresponding amines with di-Boc-*1H*-pyrazole-1-carboxamidine followed by HCl deprotection of the guanidine [27]. Synthesis and characterization of authentic Deb metabolites and Deb analogues **4–6** are detailed in the Supporting information. All authentic Deb metabolites were fully characterized by UV–vis, ^{1}H NMR and mass spectroscopy (see Supporting information). Their ^{1}H NMR and MS spectra were in accordance with previously described data [28].

2.2. Origins of recombinant CYPs

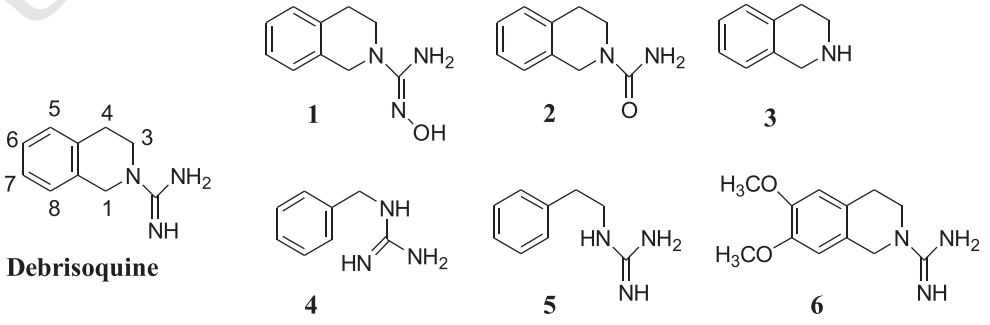
Microsomes from baculovirus-infected *Spodoptera frugiperda* insect cells coexpressing one of the following CYPs (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D1, CYP2D6, CYP2E1, CYP2R1 or CYP3A4) with CYP reductase (Supersomes®) were purchased from BD Biosciences (Le Pont de Claix, France). CYP2J2 with the human CYP reductase and cytochrome *b5* over-expressed in *Escherichia coli* (Bactosomes®) was provided by Cypex (Dundee, UK).

Preparation of full-length cDNA of *cyp2u1*, expression of CYP2U1 in several strains of yeast *S. cerevisiae*, electrophoresis and western blot analysis for CYP2U1 were performed following usual procedures [29–31] and are detailed in the Supporting information. The protein concentrations were measured by the Bradford method using bovine serum albumin as a standard [32]. The CYP concentrations were measured on a Cary300 spectrophotometer (Varian, Les Ulis, France) by UV–visible difference spectroscopy of the Fe^{II}–CO complexes using an ε value of 91,000 $M^{-1} \cdot cm^{-1}$ [33]. The cytochrome *c* reductase activity of yeast microsomes was measured by monitoring the absorbance at 550 nm at 25 °C and using a Δε value of 21,000 $M^{-1} \cdot cm^{-1}$ [33].

2.3. Search for substrates and identification of metabolites

2.3.1. Oxidation of arachidonic acid by yeast microsomes expressing CYP2U1

The ability of microsomes from W(hR) yeast expressing CYP2U1 to hydroxylate AA was studied after incubation of microsomes containing 0.1 nmol CYP2U1 in 0.1 M phosphate buffer pH 7.4, in the presence of 1 mM EDTA, 5 mM $MgCl_2$, and 30 μM [1-^{14}C]arachidonic acid in a final volume of 50 μL. Incubation mixtures containing all of the reagents except NADPH were incubated for 5 min at room temperature, then 2 min at 28 °C before initiation of the reactions with 1 mM NADPH.



Scheme 1. Structure of Deb with numbering of its C-atoms, and structure of the Deb analogues **1–6** evaluated as substrates of yeast microsomes expressing CYP2U1.