Molecular Vision 2021; 27:601-607 <http://www.molvis.org/molvis/v27/601>
Received 5 March 2021 | Accepted 4 October 2021 | Published 6 October 2021

A convenient test system for the identification of CYP4V2 inhibitors

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Purpose: Polymorphisms in the gene that codes for the human cytochrome P450 enzyme CYP4V2 are a cause of Bietti crystalline dystrophy (BCD). Therefore, inhibition of CYP4V2 activity may well be a cause of visual disability. However, monitoring the fatty acid hydroxylation reactions catalyzed by this enzyme is tedious and not well suited for inhibitor screening.
Methods: We investigated the use of proluciferin compounds as probe substrates for efficient and convenient determination of CYP4V2 activity.
Results: Ten proluciferins were tested for conversion by CYP4V2, and eight were found to be substrates of this enzyme. One point inhibitor assays were performed using luciferin 6' 3-furfuryl ether methyl ester (luciferin-3FEME) as the probe substrate and 12 test compounds. As expected, HET0016 had by far the strongest effect, while two other compounds (including osilodrostat) also displayed statistically significant inhibitory potency. The half maximal inhibitory concentration (IC_{50}) for HET0016 was determined to be 179 nM. A recently identified potent inhibitor of human CYP4Z1 was found not to inhibit CYP4V2. To explore the selectivity of this compound between CYP4Z1 and CYP4V2, we developed a homology model of CYP4V2 and conducted docking experiments.
Conclusions: We provide the first protocol for a robust and convenient CYP4V2 inhibitor assay that does not depend on fatty acid analysis but can be simply monitored with luminescence. Moreover, we demonstrate additional evidence for the concern that compounds with CYP-inhibitory properties may inhibit CYP4V2 activity and thus, possibly cause visual disability.

Bietti crystalline dystrophy (BCD) is an autosomal recessive inherited disease strongly associated with genetic polymorphisms of the *CYP4V2* gene (Gene ID 285440, OMIM 210370) [1,2]. This gene codes for a cytochrome P450 (CYP or P450) enzyme that according to the Human Protein Atlas is expressed in many tissues, including the eye [3]. The *CYP4V2* enzyme exhibits catalytic activity toward a range of saturated and unsaturated fatty acids, and alteration of this activity is thought to induce BCD [4,5]. Therefore, it is conceivable that inhibition of CYP4V2 activity may be a cause of visual disability. Among other possibilities, such inhibition could be an off-target effect of drugs. In this context, it is important to remember that many active pharmaceutical ingredients are, to some extent, inhibitors of human CYPs. Several CYPs are even drug targets themselves [6]; well-known examples are CYP11B2 (aldosterone synthase) [7], CYP17A1 (steroid 17-hydroxylase/17,20-lyase), and CYP19A1 (aromatase) [8]. Thus, convenient test systems for identifying compounds with inhibitory potency against CYP4V2 are desirable. However, fatty acid hydroxylation assays are not well suited for this task as analytics are comparatively tedious.

Proluciferins are a group of probe substrates that can be converted by CYPs to beetle luciferin, which, in turn, produces light upon oxidation by luciferase [9]. The amount of light is proportional to P450 activity and can conveniently be determined using a luminometer. Moreover, for the majority of human CYPs such proluciferin substrates are known. We recently reported the functional expression of all human CYPs in fission yeast, and in the course of that study, it was found that CYP4V2 has weak but statistically significant activity toward the two probe substrates, luciferin-H and luciferin-ME [10]. However, for performing robust inhibitor assays it is desirable to start with more efficient substrates. Therefore, the aim of the present study was to test ten other proluciferin compounds for bioconversion by CYP4V2 and to establish a protocol for an inhibitor test using a positive control and several additional compounds that are known to inhibit other human P450s.

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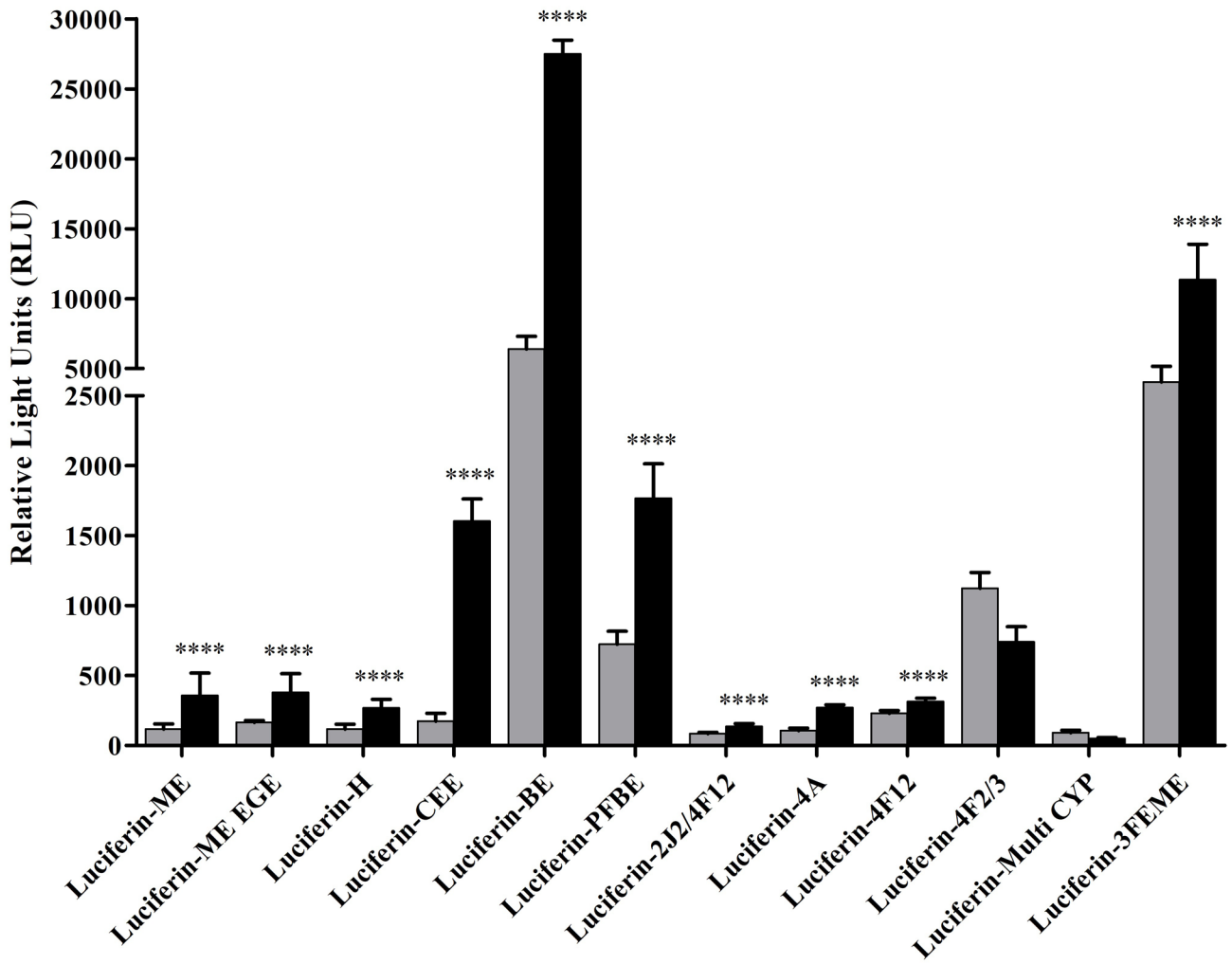


Figure 1. Screening of luminogenic substrates for conversion by CYP4V2. Permeabilized fission yeast cells (enzyme bags) were used for the biotransformation of different luminogenic substrates as indicated. The activities of strains *RAJ232* (coexpressing human CYP4V2 and CPR; black columns) and *CAD62* (control strain expressing only CPR; gray columns) are shown in relative light units (RLUs). Data shown were calculated from three independent experiments performed in triplicate. For comparison, data for luciferin-ME and luciferin-H are also shown [10]. ****p<0.001.

METHODS

Chemicals and reagents: Proluciferin compounds (except luciferin-3FEME) and the NADPH regeneration system (20X solution A: 26 mM $NADP^+$, 66 mM glucose-6-phosphate, 66 mM $MgCl_2$; and 100X solution B: 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, pH 5.5), the luciferin detection reagent (Cat# V8921), and the luciferin detection reagent with esterase (Cat# V8931) were from Promega (Madison, WI). Luciferin 6' 3-furfuryl ether methyl ester (luciferin-3FEME) was synthesized as described [11]. Triton X-100 was from Leagene (Beijing, China), and Tris-HCl was from AKZ-Biotech (Tianjin, China). Potassium chloride, ammonium bicarbonate, potassium dihydrogen phosphate, and potassium hydrogen phosphate were from Jiangtian Chemical (Tianjin, China), and 1X TBS buffer, 1X PBS (0.1 mM $CaCl_2$ (anhydrous), 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) buffer were from Corning (Manassas, VA). White opaque 96-well microtiter plates were from Nunc (Thermo-Fisher Scientific, Lagenselbold, Germany). All other chemicals and reagents used were of the highest grade available.

Fission yeast media and strains: Recombinant fission yeast strain *CAD62* (genotype h-ura4-D.18 leu1::pCAD1-CPR) expressing human cytochrome P450 reductase (CPR) and

strain *RAJ232* (genotype h-ura4-D.18 leu1:: pCAD1-CPR/pREP1-CYP4V2) coexpressing human CPR and human CYP4V2 have been described previously [10,12]. In both strains, expression of the human genes is regulated by the thiamine-repressible *nmt1* promoter [13].

Fission yeast cultivation and biotransformation procedures: Fission yeast media, biotransformations with enzyme bags, bioluminescence detection, and statistical analysis have all been described previously [14,15]. Details are given in Appendix 1.

Statistical analysis: All data are presented as mean ± standard deviation (SD). Statistical significance was determined using a two-tailed *t* test. P values of less than 0.05 were considered statistically significant. Statistical analysis was conducted

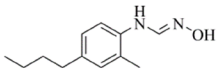
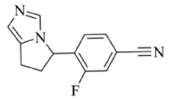
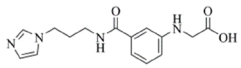
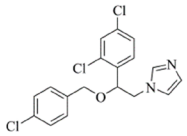
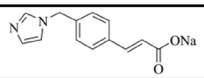
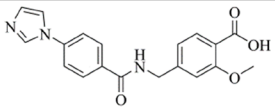
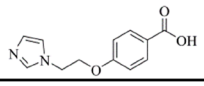
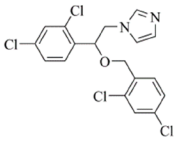
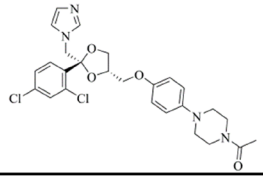
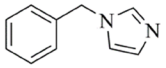
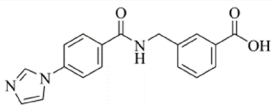
Compound	Structure	Percentage Inhibition	*P* value
HET0016		98 ± 1	$8.4 * 10^{-8}$
Osilodrostat		36 ± 19	0.0038
Inhibitor 2		21 ± 11	0.0025
Econazole		29 ± 35	0.0555
Ozagrel sodium		8 ±13	0.2272
1-Amino-benzotriazole		5 ± 52	0.8138
Inhibitor 6		-2 ± 16	0.7722
Dazoxiben		-4 ± 10	0.4667
Miconazole		-10 ± 16	0.1342
Ketoconazole		-10 ± 75	0.6883
1-Benzyl-imidazole		-13 ± 20	0.0870
Inhibitor 9		-29 ± 19	0.0022

Figure 2. Percentage inhibition of human CYP4V2 by twelve test compounds. Inhibitor concentration: 1 µM. Probe substrate: Luciferin-3FEME (150 µM).

using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA).

Homology modeling: As no X-ray structure of CYP4V2 has been reported thus far, a homology model of CYP4V2 was constructed to serve as input for docking experiments of inhibitor 9. The homology model of CYP4V2 was conducted on the I-TASSER server [16-18] with the CYP4V2.1 sequence as input (UniProt, Identifier: Q6ZWL3–1). The best of the five obtained structures according to the highest C-score (0.23, calculated by I-TASSER to suggest model confidence) was selected after visual inspection in Molecular Operating Environment (MOE)2019.0102 software (Chemical Computing Group ULC, Montreal, Canada). The X-ray structure of rabbit CYP4B1 (PDB code: 5TQ6) [19] was recommended by I-TASSER as the top structural analog, from which the heme cofactor coordinates were inserted into the model for CYP4V2. A covalent bond between the 5-methyl of the heme moiety and Glu329 was manually built in the model followed by local energy minimization in the optimized potentials for liquid simulations - all atoms (OPLS-AA) force field [20]. Glu329 is at a similar spatial position as the covalent linker Glu310 in CYP4B1, as well as in the primary sequence in the multisequence alignment of the CYP4 family [19]. Trp143, Thr333, and Phe398 were rotated using the Rotamer generator in MOE to allow accommodation of the heme. Protein geometry was checked, and atom clashes were solved by minimizing local energy.

Inhibitor docking: Docking experiments for inhibitor 9 with the homology model of CYP4V2 were performed using Genetic Optimization for Ligand Docking (GOLD) suite version 5.2 (CCDC Software, Cambridge, UK) [21] using the standard settings. Search efficiency was set to 200%, and the genetic algorithm (GA) was set to run ten times. The GoldScore P450 scoring function was used to evaluate docking poses [22]. The heme iron and space within a radius of 18 Å were defined as the docking site. Poses obtained were energy minimized in MOE in the presence of the CYP4V2 homology model, and the most plausible poses were selected. The OPLS-AA force field [20] was used consistently for all minimization calculations.

RESULTS AND DISCUSSION

We recently observed that CYP4V2 has weak but statistically significant activity toward the two probe substrates luciferin-H and luciferin-ME [10]. These results prompted us to search for additional proluciferin substrates for this enzyme. Therefore, we tested ten such probe substrates using permeabilized cells (enzyme bags) of the recombinant fission yeast strain *RAJ232* that recombinantly coexpresses human CYP4V2 and CPR. It was found that eight of these compounds could be metabolized by CYP4V2, with luciferin-BE and luciferin-3FEME the most efficient substrates (Figure 1). Interestingly, luciferin-4F2/3 and the promiscuous compound luciferin-Multi Cyp were not found to be CYP4V2 substrates, although they can be converted by other human CYP4 enzymes [9].

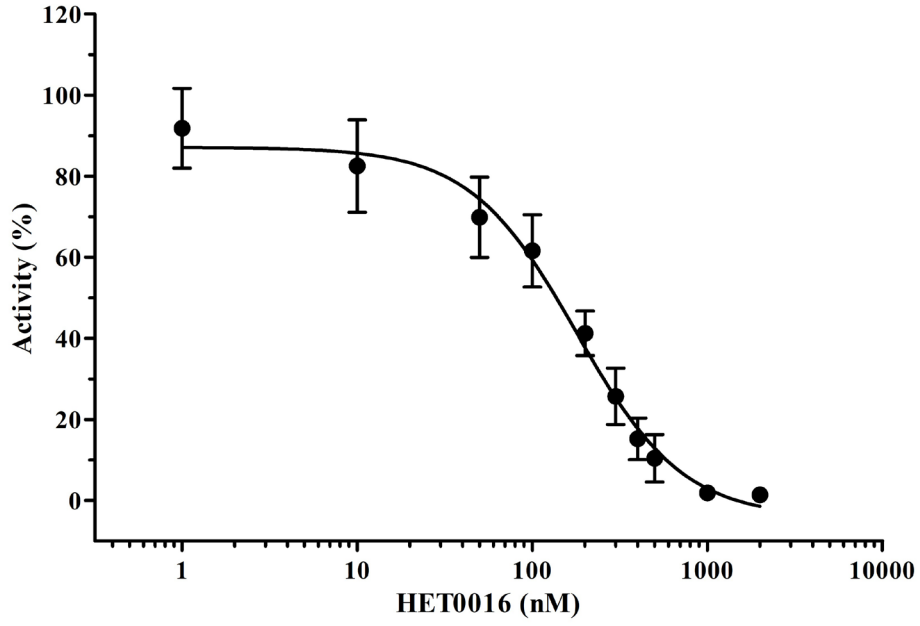


Figure 3. IC_{50} determination for inhibition of CYP4V2 by HET0016. Activity of CYP4V2 toward luciferin-3FEME was determined either without an inhibitor (with dimethyl sulfoxide (DMSO) as a vehicle control) or in the presence of HET0016 at final concentrations between 1 nM and 2 µM as indicated. Data shown were calculated from three independent experiments performed in triplicate.

We next performed CYP4V2 inhibitor tests using luciferin-3FEME as the probe substrate, HET0016 as a positive control [4], and 11 test compounds with known inhibitory effects on other human CYPs. This set encompassed two other known CYP4 inhibitors (1-aminobenzotriazole and 1-benzylimidazole [23-25]), four recently described CYP4Z1 inhibitors (ozagrel and inhibitors 2, 6, and 9 [26]), the antifungals econazole, miconazole, and ketoconazole [27], the CYP5A1 inhibitor dazoxiben [28], and the CYP11B inhibitor osilodrostat [29]. For these test compounds, we determined the percentage inhibition of CYP4V2 at an inhibitor concentration of 1 µM. HET0016 had by far the strongest inhibitory effect (percentage inhibition 98 ± 1.0, P = 8.4 * 10-8), while osilodrostat (36 ± 19, P = 0.0038) and inhibitor 2 (21 ± 11, P = 0.0025) also displayed statistically significant potency (Figure 2). Inhibitor 9 showed a small but significant activating effect (−29±19***), and none of the other compounds influenced the enzyme's activity. The half maximal inhibitory concentration (IC_{50}) for the inhibition of CYP4V2 by HET0016 was determined to be 179 nM (Figure 3). In a previous study using reconstituted His-tagged CYP4V2 expressed in Sf9 cells, an IC_{50} of 38 nM was reported

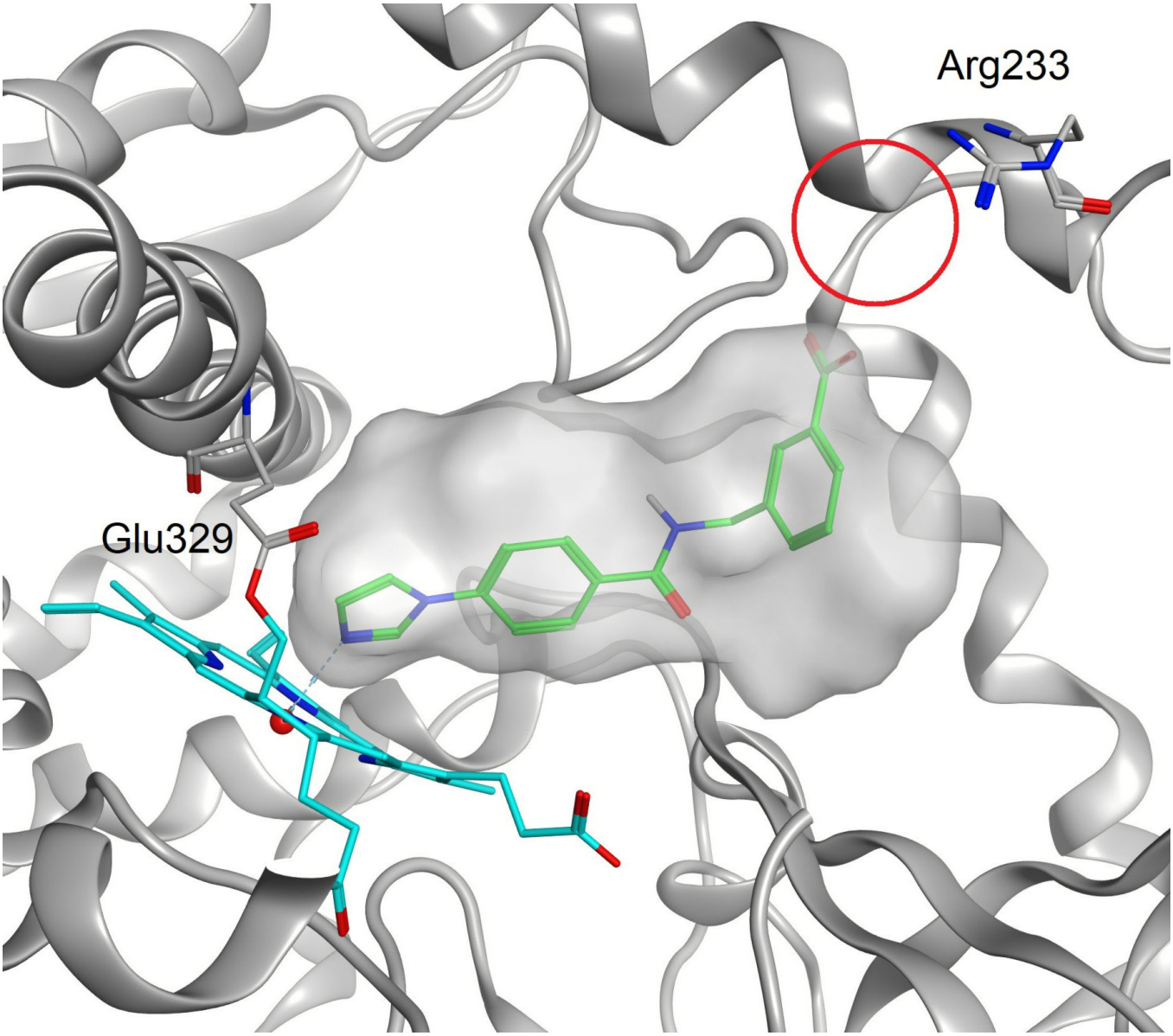


Figure 4. Docking suggests that inhibitor 9 does not bind to the active site of CYP4V2. In the CYP4V2 homology model, the heme (light blue) is connected to Glu329 by a covalent bond. Inhibitor 9 (green) might complex the heme iron (the red sphere in the center of the heme) but is too far from Arg233 to interact. The lack of interaction between inhibitor 9 and Arg 233 is illustrated by a red cycle.

[4]; considering the different experimental approaches, these findings are in reasonable agreement.

Inhibitor 9 has recently been shown to be a potent inhibitor of CYP4Z1 [26]. In the present study, inhibitor 9 was found not to inhibit CYP4V2. To explore the selectivity of inhibitor 9 between CYP4Z1 and CYP4V2, we developed a homology model of CYP4V2 and conducted docking experiments (Figure 4). In the homology model of CYP4V2, the heme iron is coordinated by Cys467, and the porphyrin ring is connected to Glu329 by a covalent bond, as suggested by Hsu and colleagues [19]. The active site close to the heme consists of hydrophobic residues, including Met123, Phe126, Leu136, Phe328, Leu397, Leu505, and their nearby residues. The docking results suggest that inhibitor 9 does not bind to the active site of CYP4V2. The imidazole moiety of inhibitor 9 might be able to coordinate the heme iron. However, the carboxylate moiety of inhibitor 9 is unlikely to form a putatively important charge-assisted hydrogen bond to Arg233, which corresponds to Arg487 in CYP4Z1 [30]. The distance between the oxygen atom of the carboxylate moiety of inhibitor 9 and the nitrogen atom of Arg233 is large with 8.0 Å, and therefore, the salt bridge is weak. Compared to Arg487 in CYP4Z1, Arg233 of CYP4V2 is modeled at a position further away from the heme. According to the present homology model, Arg233 is sandwiched between Tyr245 and Phe126 and forms cation-Π interactions. Thus, the strong salt bridge is missing, the suggested pose for inhibitor 9 is highly improbable, and therefore, affinity to CYP4V2 is decreased drastically.

In conclusion, in this publication we provide the first protocol for a robust and convenient CYP4V2 inhibitor assay that does not depend on fatty acid analysis but can be simply monitored with luminescence. The probe substrates used in this study are not specific for CYP4V2 but can also be metabolized by other human CYPs. However, when using a recombinant system that contains only CYP4V2, this lack of specificity is not a problem. If a test system based on mammalian cells is desired, use of the probe substrates luciferin-CEE or luciferin-PFBE would be indicated. Both also show good activity with CYP4V2 (Figure 1), are commercially available, and moreover, are explicitly recommended for cell-based activity assays by the manufacturer. Finally, we demonstrated additional evidence for the concern that compounds with CYP-inhibitory properties may inhibit CYP4V2 activity and therefore, possibly cause visual disability. Such off-target effects are especially worrisome in marketed drugs such as osilodrostat.

APPENDIX 1. DETAILS OF THE EXPERIMENTAL PROCEDURES.

To access the data, click or select the words “Appendix 1.”

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