Supplementary Information

for

Biophysical interactions between self-sufficient cytochrome P450 from *Tepidiphilus thermophilus* and ilaprazole

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Table of Contents

Experimental

Materials and chemicals

All chemicals and solvents used in protein expression were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris; Daejung, Siheung, Republic of Korea), sodium chloride (NaCl; Daejung), dithiothreitol (DTT; GoldBio, St. Louis, MO, USA), glycerol (Samchun Chemical, Seoul, Republic of Korea), DNase I (Takara Bio, Shiga, Japan), phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific, Waltham, MA, USA), and EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were used in protein purification. Buffer solutions were prepared using 3-(N-morpholino) propanesulfonic acid (MOPS; Thermo Fisher Scientific), 2-(N-morpholino) ethanesulfonic acid (MES; Sigma-Aldrich), and sodium acetate (Sigma-Aldrich). Fast protein liquid chromatography (FPLC; ÄKTA Pure 25 L, Cytiva, Marlborough, MA, USA) and an Econo-column (Bio-Rad Laboratories, Hercules, CA, USA) along with Ni-Sepharose (Cytiva) and Superdex 200 (Cytiva) were used in protein purification. An ultracentrifuge (Hanil Science, Gimpo, Republic of Korea) and bench-top centrifuge (Beckman Coulter, Brea, CA, USA) were used for separating cell debris and supernatant.

Expression of *Tepidiphilus thermophilus* CYP116B46

The DNA sequence of CYP116B46 from *Tepidiphilus thermophilus* (*T. thermophilus*) was ligated into the pET-28a(+) vector. This vector was transformed into *Escherichia coli* C2566 (New England BioLabs, Hitchin, UK) cultured in kanamycin (50 μg mL^{-1})-containing Luria Bertani broth (250 mL) at 37 °C with shaking at 200 rpm for 16 h. The culture was scaled-up using kanamycin (50 μg mL^{-1})-containing terrific broth media (500 mL) supplemented with 1.0 mM thiamine, 0.025% (*v/v*) trace element solution, 50 μM $FeCl_3 \cdot 6H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, and 2.5 mM $(NH_4)_2SO_4$.[1] Incubation was performed at 37 °C and 200-rpm shaking until reaching an optical density at 600 nm (OD_{600}) of 0.8–1.0. The whole culture was cooled to 26 °C and treated with 0.5 mM isopropyl-β-D-thiogalactopyranoside and 1.5 mM δ-aminolevulinic acid (δ-ALA) to induce CYP116B46 expression with shaking at 150 rpm for 20 h. CYP116B46 was overexpressed until the concentration of CtCYP116B reached 0.20 μM, as determined by the CO-bound difference spectrum at an extinction coefficient ($\varepsilon_{450–490}$) of 91,000 M^{-1} cm^{-1}, and harvested via centrifugation at 13,000 rpm and 4 °C for 30 min.[2]

Purification of *T. thermophilus* CYP116B46

The cell pellet obtained from overexpressed CYP116B46 (1 L) was dissolved in buffer A (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 0.01 μL/mL DNase I, 0.002 mg/mL PMSF, and EDTA-free Protease Inhibitor Cocktail at pH 8.0), sonicated for 60 min (15s on and 45 s off), and the supernatant was filtered through a 0.22-μm membrane syringe filter (Sartorius). The Ni-Sepharose in the gravity column was equilibrated with buffer B (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole at pH 8.0) before applying the supernatant at 4 °C and 200 rpm for 12–16 h. The flow-through was collected under argon gas with imidazole (50, 100, 150, 200, 250, and 300 mM)-containing buffer B. The eluates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the target eluates were concentrated via filtration through a 30-kDa membrane filter (Merck Millipore) with centrifugation at 4 °C and 3,000 rpm. The concentrated eluates were applied in the second purification using the Superdex-200 column on the FPLC system, which was activated using buffer C (25 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, and 5% glycerol at pH 8.0). The target eluates were concentrated via filtration through a 30-kDa membrane filter with centrifugation at 4 °C and 3,000 rpm. The concentration of CYP116B46 was calculated at ε_{418} = 121 mM^{-1} cm^{-1} for the soret peak of the oxidized enzyme using a UV-Vis spectrophotometer (Cary 3500, Agilent Technologies, Santa Clara, CA, USA) and stored at –88 °C for subsequent experiments.[3]

Measurement of binding affinities between CYP116B46 and ilaprazole

The binding affinities (K_ds) between CYP116B46 and ilaprazole were measured using a spectrofluorometer (FP-8300, JASCO, Tokyo, Japan) through tryptophan quenching at 282 nm (λ_{ex}) and 336 nm (λ_{em}).[4, 5] All experiments were performed at a bandwidth of 2.5 nm, response of 1 s, PMT voltage of 800 V, and 25 °C. One equivalent of CYP116B46 (2.56×10^{-10} mol) in buffer D (25 mM buffer; *vide infra*, 50 mM NaCl, 1 mM DTT) was titrated in a cuvette (J/3 type material Q, JASCO) with ilaprazole to saturation (~60 equivalents). The experiments were conducted in buffer D adjusted to various pH levels using 25 mM buffers: Tris-HCl (pH 8.0 or 7.0), MOPS (pH 6.0), MES (pH 5.0), and sodium acetate (pH 4.0 or 3.0). The changes in fluorescence intensity were curve-fitted using the 1:n binding model (Hill equation) to estimate K_ds using Origin 2018 (OriginLab, Northampton, MA, USA), as represented by the following equation:

$$F_{332} = Start + (End - Start) \times \frac{{I_{total}}^n}{({K_d}^n + {I_{total}}^n)}$$

F_{332}: Fluorescence intensity at 332 nm

Start: Initial fluorescence intensity at 332 nm

End: Saturated fluorescence intensity at 332 nm

I: Concentration of ilaprazole

K_d: Dissociation constant

n: Hill coefficient

Molecular docking simulation between CYP116B46 and ilaprazole

The docking of ilaprazole with CYP116B46 was simulated using AutoDock Vina.[6] The structure of Ilaprazole was optimized through MMFF94 energy minimization in ChemBio3D Ultra 11.0.[7] Structural files for both ilaprazole and CYP116B46 were generated using AutoDock Tools and then imported into PyRx for simulation.[8] The search space covered the entire CYP116B46 protein (PDB ID: 6GII), and the exhaustiveness parameter was set to 1024 to ensure a comprehensive search.[9] The docked structures of CYP116B46 and ilaprazole were analyzed using PyMol v3.0.3.[10] Binding energies were converted into dissociation constant (K_d) values using the following equation[11]:

$$\Delta G_{binding} = RT\ln K_d$$

$\Delta G_{binding}$: Free binding energy

R: Gas constant; 8.314 J/(mol·K)

T: Temperature; 298 K (at 25 °C)

K_d: Dissociation constant

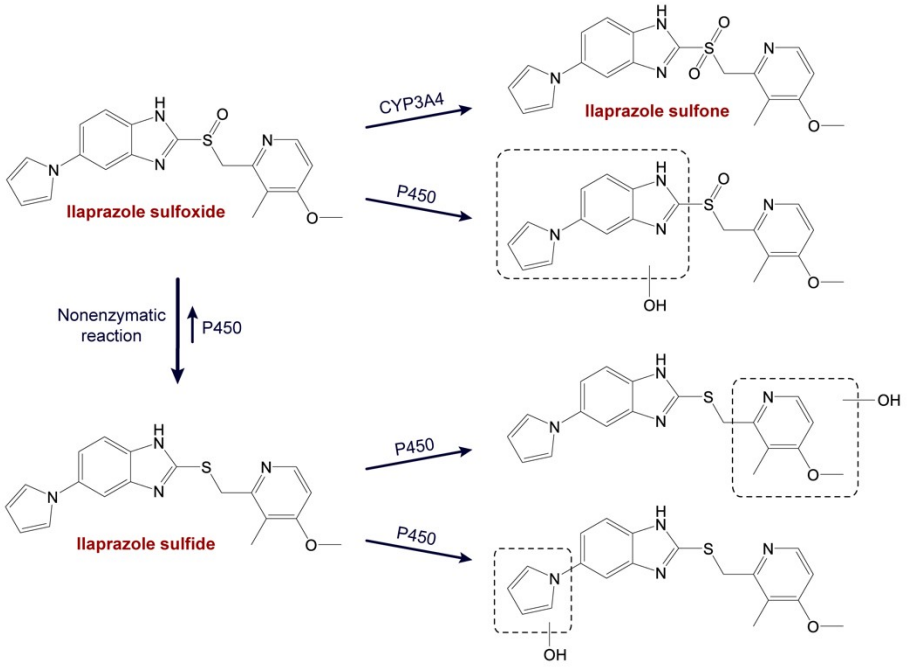

Figure S1. Metabolism of ilaprazole in human liver microsomes.[12]

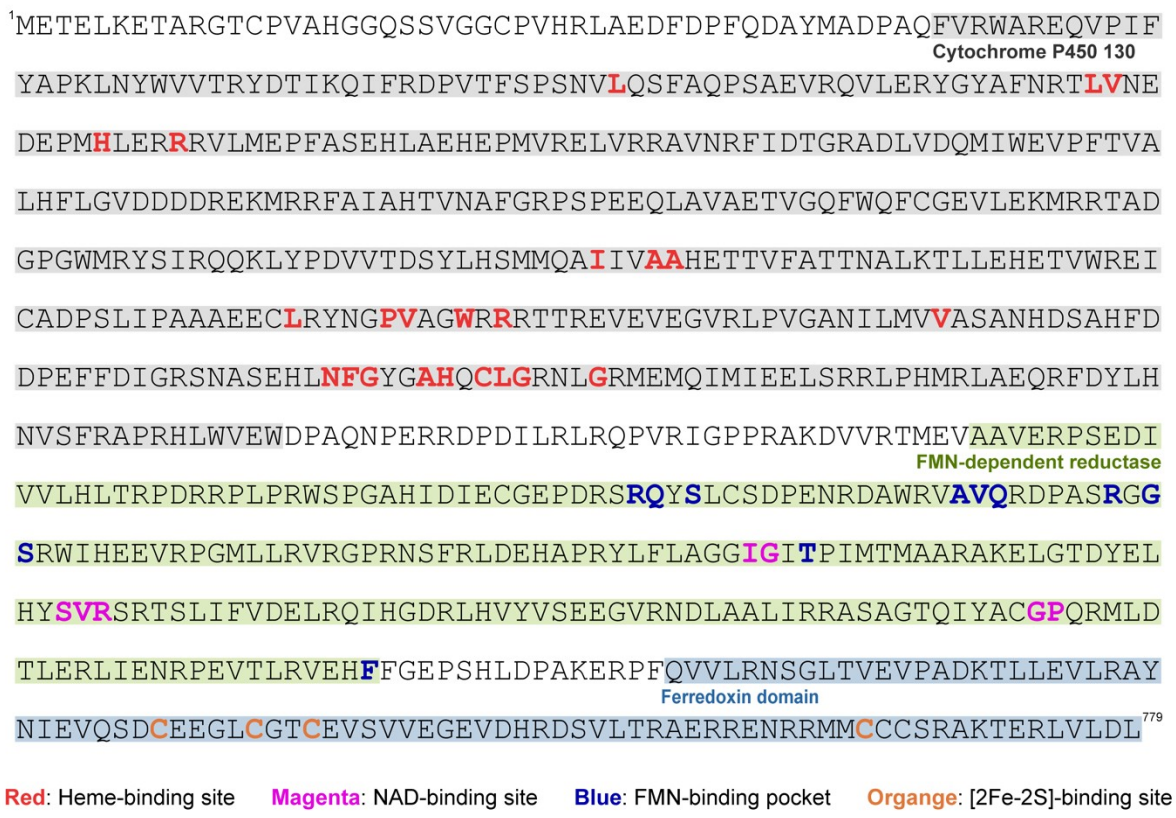

Figure S2. Amino acid sequences of *T. thermophilus* CYP116B46 (NCBI: WP_055423153).

Figure S3. Sequence alignment of heme domain in *T. thermophilus* CYP116B46 (NCBI: WP_055423153) with *Mycobacterium tuberculosis* CYP130 (NCBI: ALB18420.1), *Homo sapiens* CYP3A4 (NCBI: AAF13598.1), and *H. sapiens* CYP2C19 (NCBI: NP_000760.1).

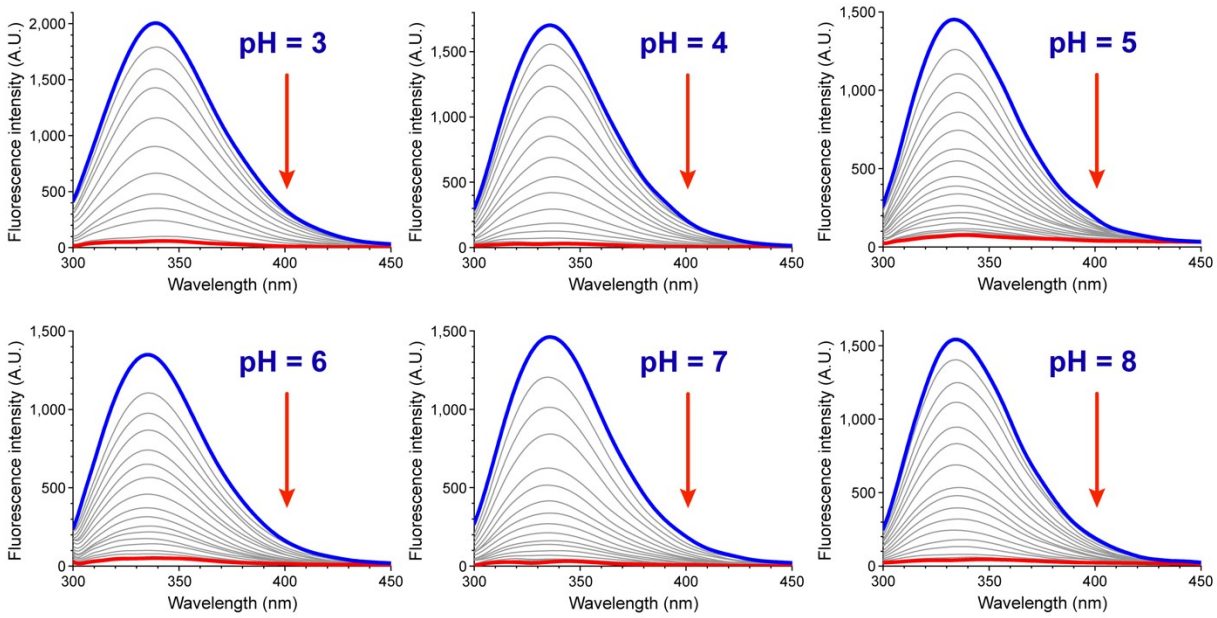

Figure S4. Interaction of CYP116B46 with ilaprazole based on tryptophan quenching. Conditions: [CYP116B46] = 0.32 μM; λ_{ex} = 282 nm; λ_{em} = 332 nm; 25 mM buffer (varying pH from 3 to 8), 50 mM NaCl, 1 mM DTT; 25 °C.

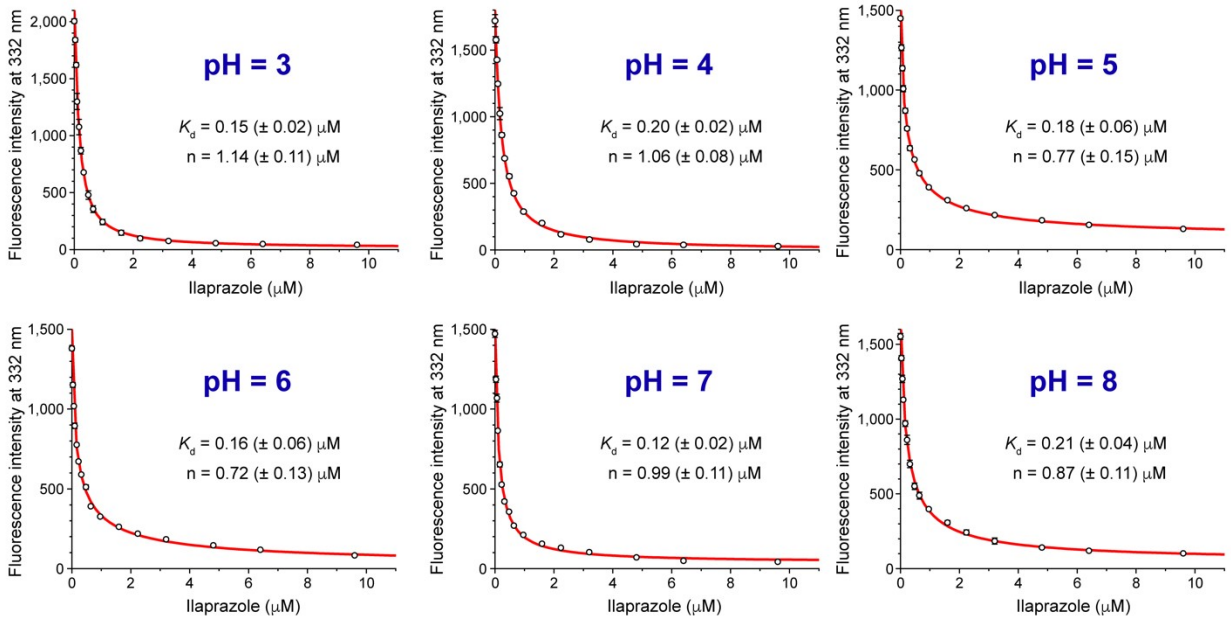

Figure S5. Binding affinities between CYP116B46 and ilaprazole. Dissociation constants (K_ds) were analyzed through tryptophan quenching under varying pH conditions and determined by curve fitting using the 1:n binding (Hill) equation based on fluorescence intensities at 332 nm.

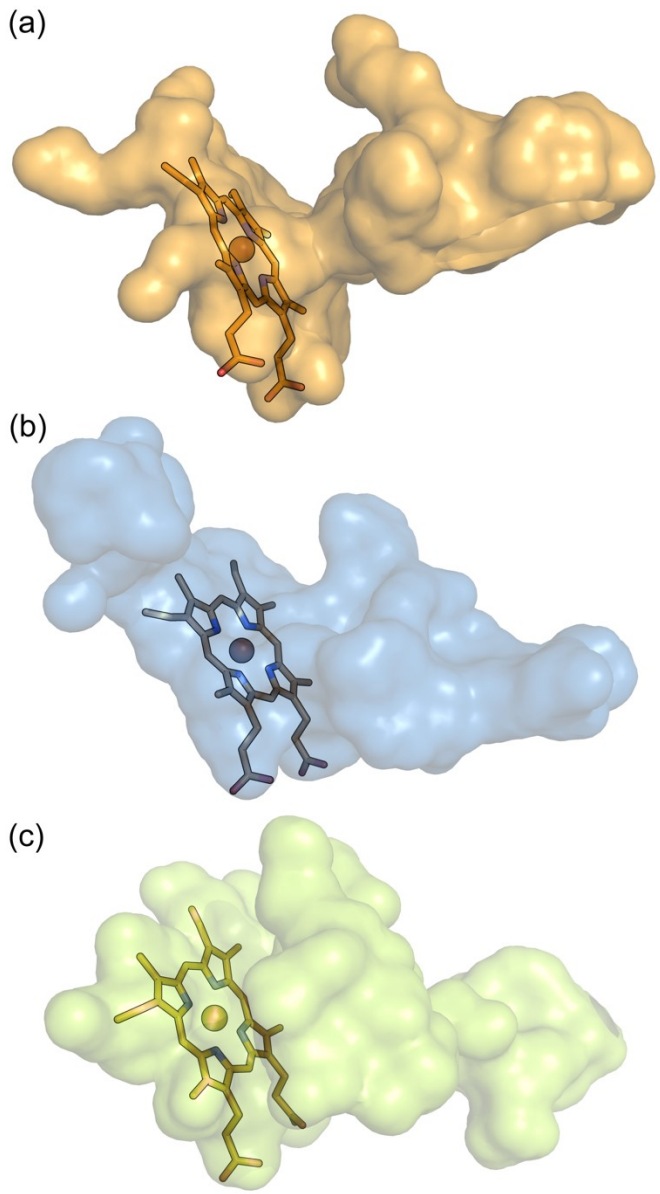

Figure S6. Analysis of cavities in CYP450s. The cavities were visualized using PyMOL 2.5.2 as van der Waals surfaces in the interior of (a) CYP116B46 (PDB: 6GII, orange), (b) CYP3A4 (PDB: 1TQN, skyblue), and (c) CYP2C19 (PDB: 4GQS, limon).

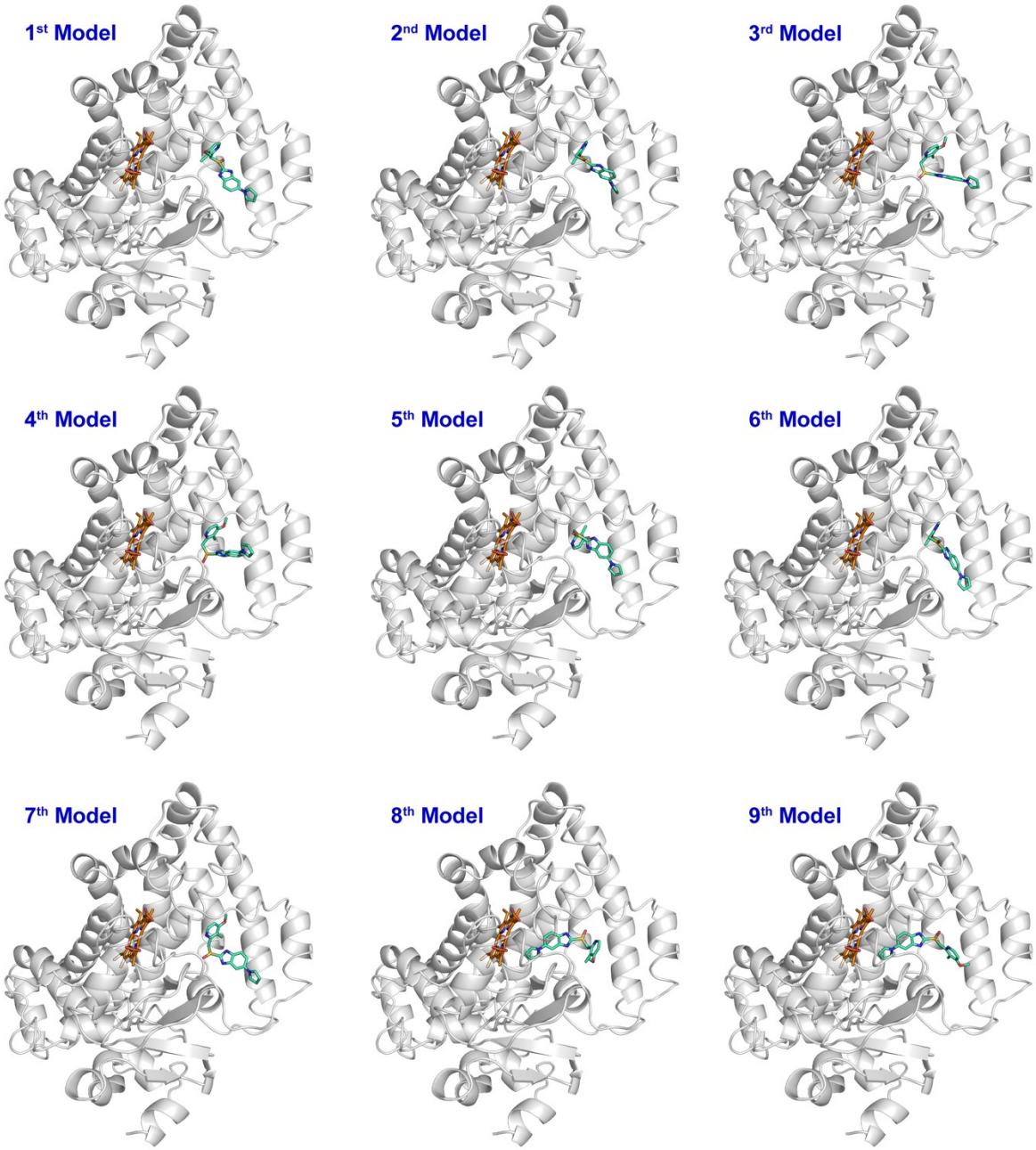

Figure S7. Overall docking models of CYP116B46 (PDB: 6GII) and ilaprazole.[9] Docking models were derived from molecular docking simulations using AutoDock Vina. The gray ribbons represent CYP116B46, orange indicates heme, and greencyan depicts ilaprazole.

Table S1. Binding affinities and RMSD for docking models of CYP116B46 and ilaprazole.

Docking model	Binding energy (ΔG, kcal/mol)	Dissociation constant (K_d, μM)	RMSD/ub	RMSD/lb
1st Model	–9.2	0.17	0.0	0.0
2nd Model	–9.1	0.21	4.044	2.617
3rd Model	–9.0	0.25	2.989	2.018
4th Model	–9.0	0.25	4.061	3.171
5th Model	–8.9	0.30	4.388	2.629
6th Model	–8.8	0.35	3.604	2.258
7th Model	–8.8	0.35	2.987	2.005
8th Model	–8.8	0.35	8.512	4.396
9th Model	–8.8	0.35	8.87	4.006

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