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

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Proton pump inhibitors (PPIs), metabolized by cytochrome P450 (P450) enzymes, are widely used to inhibit gastric acid secretion. This study investigated CYP116B46, a self-sufficient monooxygenase with a reductase domain, to elucidate its interaction with ilaprazole, a PPI. Binding assays and docking simulations indicate that CYP116B46 serves as a suitable model for studying PPI metabolism.

Proton pump inhibitors (PPIs) are the most widely prescribed medicines for inhibiting gastric acid release.[1–3] The pharmacological and biochemical properties of PPIs have been intensively studied to ensure their efficacy and safety.[4–6] Omeprazole was the first PPI on the market, along with lansoprazole and esomeprazole, which were developed to suppress acid secretion (Fig. 1a).[8–10] These PPIs comprise benzimidazole, pyridine, pyrrole, and sulfoxide moieties, which act as weak bases at pH <4.0 in acidic environments.[3,14] Under extremely low pH conditions in parietal cells, the sulfoxide is converted into cationic sulfenic acid and sulfonamide, and it generates covalent disulfide bonds (S–S) with H^+ and K^+-ATPase in the gastric system.[2,3] Each inhibitor forms common disulfide bonds with the thiol (–SH) group in Cys813 of phosphorylated enzymes, although other cysteines induce specific S–S bonds.[15,16] Despite the steady increase in PPI prescriptions over the past three decades, long-term administration has been associated with complications, particularly in patients with renal or hepatic issues.[17,18] The metabolism of PPIs is primarily regulated by cytochrome P450 (P450 or CYP) enzymes, and various unexpected metabolites have been identified based on PPI moieties.[6,19,20] The recently approved ilaprazole is a benzimidazole derivative that has a significantly extended half-life in plasma compared to other approved medicines and

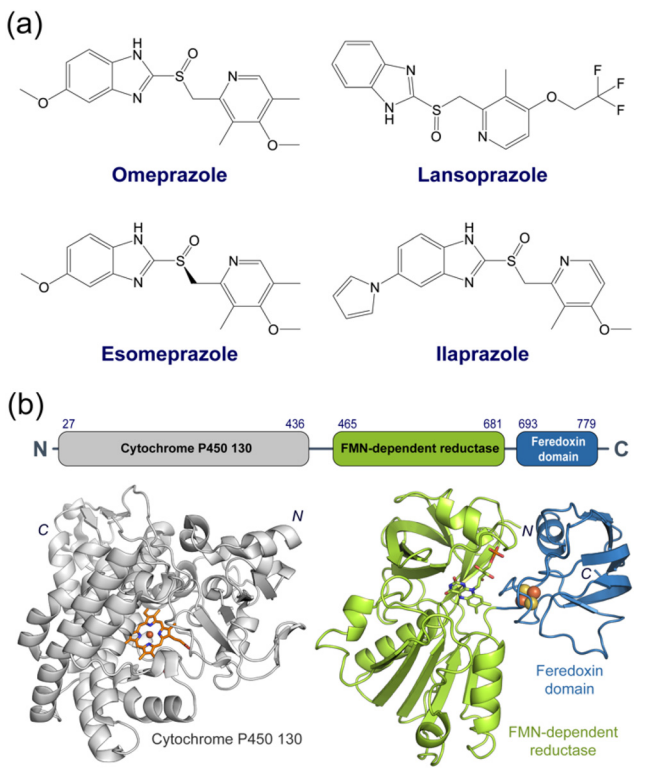

Fig. 1 Structure of drugs and metabolic enzymes. (a) Chemical structures of proton pump inhibitors. (b) Domain organization and enzyme structures of CYP116B46 from *Tepidiphilus thermophilus*. The heme domain of CYP116B46 (PDB: 6GII, gray); phthalate dioxygenase reductase (PDB: 2PIA, green and blue).[12,13] The orange ligand and sphere represent porphyrin and an Fe ion. The yellow sphere depicts a sulfur ion.

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exerts potent therapeutic effects in long- and short-term treatment of gastro-esophageal reflux disease.[21,22]

P450s are involved in diverse biochemical reactions that are essential for drug metabolism, owing to their critical roles in human liver microsomes (HLMs).[8,19] The catalytic activities of P450 are of major interest in the monitoring of prodrug metabolic activities.[2–4] The unexpected side reactions of benzimidazole PPIs have been widely investigated with regard to their distribution and metabolism in circulation.[19] The major metabolites of PPIs such as ilaprazole include hydroxylation metabolites in pyrrole and pyridine moieties (Fig. S1†).[19,23] In other PPIs, including omeprazole and esomeprazole, the sulfoxide is converted into sulfide metabolites or highly oxidized sulfone derivatives as well as hydroxylated metabolites, which are crucial in pharmacokinetics.[4,24] Sulfoxide oxidation is mainly influenced by CYP3A4 in HLMs, particularly *via* the activity of P450 monooxygenase.[25–27]

The recently reported structure of the self-sufficient enzyme CYP116B46 from *Tepidiphilus thermophilus* (*T. thermophilus*) suggested that it could be a good receptor for studying PPI metabolism (Fig. S2†).[28] Biophysical aspects of the P450 monooxygenase of ilaprazole remain unclear and docking simulation of the ilapraole–P450 complex and its substrate–protein binding pockets is needed to elucidate its metabolism.[29–31] CYP116B46 contains reductase and ferredoxin domains that are crucial for electron transfer and determine the appropriate electron sources, such as NADH and NADPH, in P450 engineering (Fig. 1b). In this study, we successfully over-expressed and purified CYP116B46 and characterized its biophysical interaction with ilaprazole. Docking simulations were performed based on the reported structure to elucidate the substrate reaction pathways. The complex structures suggested that the sulfoxide from ilaprazole is not favorably oxidized by CYP116B46 due to substrate interactions with different binding preferences. These self-sufficient P450 systems serve as suitable models for elucidating the biochemical interactions and biophysical properties of PPIs.

Understanding the dynamic expression of P450 is complicated by its differences from other soluble proteins. CYP116B46 from *T. thermophilus* was over-expressed in TB media and purified through a two-step process. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed a single prominent band with a molecular weight of 91.58 kDa and purity exceeding 95%. The UV-vis spectra of the purified CYP116B46 revealed the typical absorption characteristics of its heme, flavin mononucleotide (FMN), and [2Fe–2S] cofactors (Fig. 2a). The Soret band was observed at 420 nm, along with α- and β-bands at 570 and 535 nm, respectively. These findings are closely consistent with those reported for CYP116B1, which exhibits the Soret band at 418 nm and α- and β-bands at 566 and 532 nm, confirming the presence of the heme cofactor (Fig. S3†).[32] The UV spectra revealed additional characteristic peaks at 355 and 490 nm, which were difficult to discern due to the high extinction coefficient of the Soret band. The oxidized form of flavin typically shows peaks at 360 nm and 450–475 nm, characteristic of

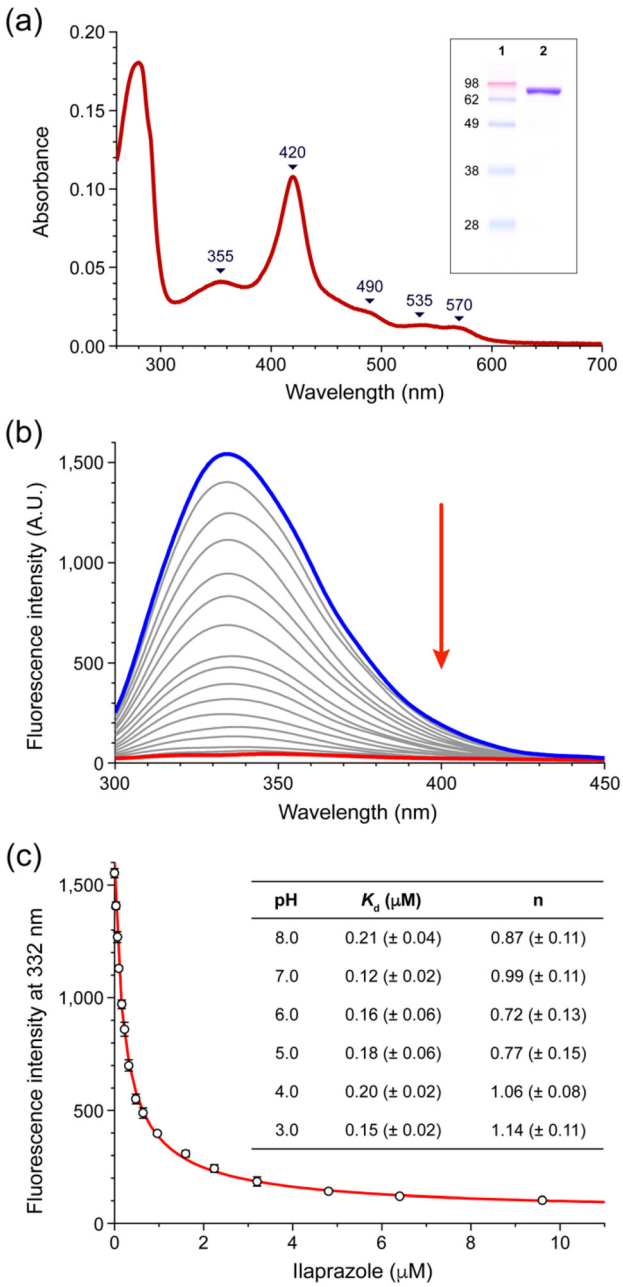

Fig. 2 Biophysical studies on CYP116B46 with ilaprazole. (a) UV-visible spectrum of purified CYP116B46 from *T. thermophilus*. The inlet represents purified CYP116B46 (91.58 kDa) in sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Lanes 1 and 2 indicate the molecular marker and purified CYP116B46, respectively. (b) Interaction of CYP116B46 with ilaprazole analyzed through tryptophan quenching at pH 7.0. (c) The dissociation constant (K_d) was determined by curve fitting using a 1 : *n* binding equation based on fluorescence intensities at 332 nm between CYP116B46 and ilaprazole at pH 7.0. The inset table presents K_d values analyzed through tryptophan quenching under varying pH conditions.

oxidized FMN.[33,34] The [2Fe–2S] clusters exhibit peaks at 332, 418, and 467 nm, with the highest absorbance at 332 nm.[35,36] Therefore, the peaks observed at around 355 and 490 nm in CYP116B46 may be attributed to the presence of FMN and

[2Fe–2S] clusters. These findings demonstrate the structural integrity and functional versatility of the CYP116B family, which catalyzes the hydroxylation of various substrates, including medium-chain alkanes, alkylbenzenes, and aromatic compounds.[32,37–39]

Previous studies did not clearly characterize the binding affinity between ilaprazole and P450s. Accordingly, we investigated the interaction between CYP116B46 and ilaprazole in tryptophan quenching experiments (Fig. 2b and Fig. S4†). The fluorescence intensities of the 12 tryptophan residues near the heme prosthetic group of CYP116B46 decreased in a concentration-dependent manner with the titration of ilaprazole, indicating a direct interaction between CYP116B46 and ilaprazole. These findings provide clearer evidence of the binding process, which is crucial for understanding the metabolism of ilaprazole and its potential drug interactions.[19] Through curve fitting of the $1:n$ binding model, we observed reasonable binding affinity between CYP116B46 and ilaprazole, as evidenced by the dissociation constants (K_d) within the micromolar range. The binding affinity remained consistent under diverse acidic conditions (pH 3.0–8.0, Fig. 2c and Fig. S5†). This pH independence of the K_d values is supported by the structural flexibility of CYP116B46, as demonstrated by molecular dynamics (MD) simulations, which enables pH-induced conformational changes that prevent excessive unfolding.[39,40] Recent cryo-EM studies revealed that CYP102A1 assembles into homodimers, potentially enhancing structural stability by shielding active sites or key regions under acidic conditions.[41,42] Our finding reflected a slightly stronger interaction between CYP116B46 and ilaprazole compared to previously reported values for other PPIs, with K_d values for omeprazole with CYP3A4 and CYP2C19 at 18.0 ± 2.0 and 8.6 ± 0.3 μM, respectively.[10,43] The Hill coefficient at pH 7.0 was 0.99 ± 0.11, which is very close to 1, suggesting that the interaction is most favorable under neutral conditions (Fig. 2c). At a pH below 4.0, the Hill coefficient exceeded 1. This suggests that, in a protonation environment, there is an increase in non-specific binding between CYP116B46 and ilaprazole.

Heme containing P450 monooxygenases catalyzes diverse oxidation in the biosynthesis and metabolism of xenobiotics, thus these enzymes are widely applied in regio- and stereoselective reactions.[44–47] The C–H activation, which requires high energy, can be achieved using P450 monooxygenase under mild conditions through O_2 activated intermediates, although it requires electron transfer cofactors including the [2Fe–2S] cluster and FMN for the catalytic cycle.[48] The structure of CYP116B46 (PDB: 6GII) provides crucial information on its binding behavior.[28] We performed docking simulations to further elucidate the interaction between CYP116B46 and ilaprazole, which are pivotal for understanding the metabolic pathways and potential efficacy of PPIs. The structure of CYP116B46 was used to identify the cavity serving as the binding pathway for ilaprazole (Fig. 3a). This cavity, extending from the surface to the heme, clearly delineates the substrate ingress and is well suited for accommodating ilaprazole, which measures 3–5 Å, allowing easy access and entry. Turner *et al.*

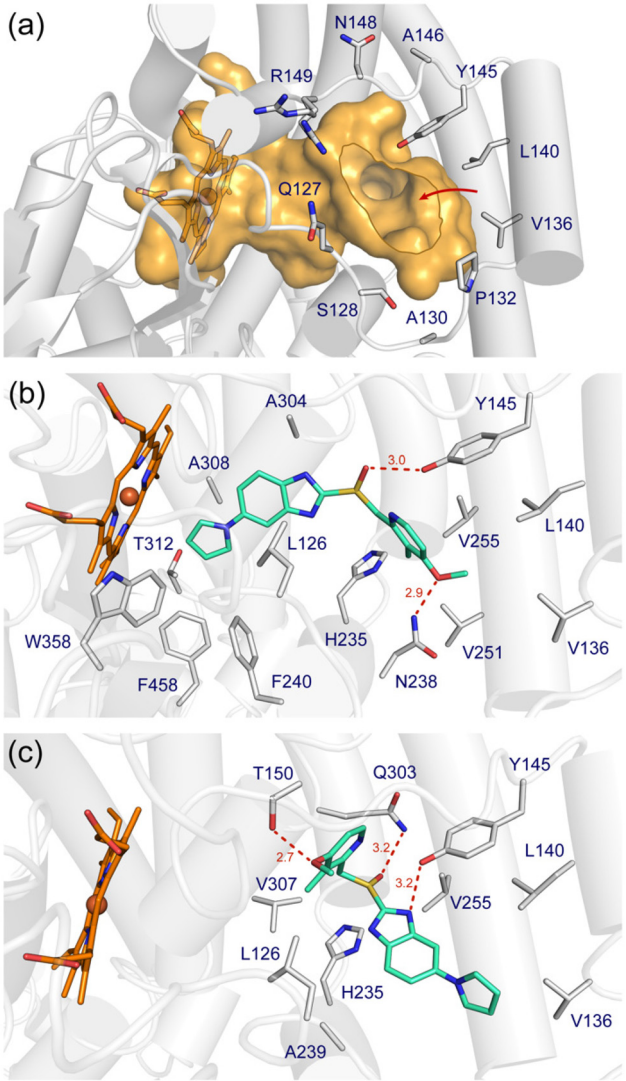

Fig. 3 Predicted interaction between ilaprazole and CYP116B46. (a) The proposed substrate binding pathway of CYP116B46 from *T. thermophilus* (PDB: 6GII). Cavities are shown as translucent van der Waals surfaces in the interior of CYP116B46.[7] The red arrow indicates the ilaprazole ingress pathway. (b and c) Docking models have been derived from molecular simulation using AutoDock Vina.[11] Red dashed lines indicate hydrogen bonds.

demonstrated that the substrate channel of CYP116B46 is so narrow that it limits the access of organic molecules, which was confirmed by its smaller cavity size compared to human P450s such as CYP3A4 and CYP2C19 (Fig. S6†).[28] Despite this structural limitation, CYP116B46 has been shown to catalyze the hydroxylation of medium-length alkyl chains, including decanoic acid.[38] Recent MD simulations have shown that the substrate channel can exhibit opening motions, suggesting that ilaprazole could bind to its active site.[39] Various hydrophobic and hydrophilic residues were observed surrounding the cavity ingress, underscoring the complexity of the immediate environment that facilitates the binding and interaction of ilaprazole.

The docking simulation revealed nine distinct binding models for ilaprazole within the active site of CYP116B46, along with their corresponding binding energies (Fig. S7 and Table S1†). These binding energies were converted into K_d values, which closely aligned with those obtained from tryptophan quenching experiments (Fig. 2c and Table S1†). This consistency between experimental and calculated values supports the reliability of our binding models. The simulations consistently showed ilaprazole positioned within the cavity. The complex formation simulation indicated that, while ilaprazole interacts strongly with CYP116B46, it has limited access to the heme active site (Fig. S7†). Despite the generally poor association with the heme, the 8th and 9th models showed the pyrrole ring of ilaprazole positioned 4.4 Å from the heme. This proximity suggested that hydroxylation reaction may occur through electron transfer, aligning with previous studies that identified the hydroxylation of the pyrrole ring as an important metabolic pathway (Fig. S1†).[19] A detailed analysis of the 9th model revealed that ilaprazole primarily interacts with hydrophobic residues in CYP116B46 (Fig. 3b), minimizing the influence of pH on the binding affinity. Here, ilaprazole stabilizes its binding through hydrogen bonds—the sulfoxide group bonded with Tyr145 of CYP116B46 and the methoxy group bonded with Asn238. The interaction with asparagine was unaffected by protonation or deprotonation, and the hydroxyl group of tyrosine remained protonated across the pH range of 3–8, indicating that the binding is pH-independent. Ilaprazole engages in hydrophobic interactions with Trp358, which may have a direct influence on its quenching effect against CYP116B46 fluorescence. The 1st model, which exhibited the highest binding energy (−9.2 kcal mol^{-1}), demonstrated that ilaprazole was suitably bound at the entrance of the cavity; the 2nd to 6th models displayed similar binding patterns (Fig. 3c). Both the 1st and 9th models revealed that the sulfoxide group of ilaprazole forms hydrogen bonds with CYP116B46, suggesting that these interactions may inhibit the metabolism of ilaprazole by restricting direct interactions or transformations through its limited access to the active site of CYP116B46, potentially increasing its stability and prolonging its bioactivity.

Zhu and co-workers demonstrated that ilaprazole is converted into reduced sulfide and its derivatives.[19] These reduced metabolites do not depend on NADPH-based electron transfers in HLMs, and no further oxidation of sulfone derivatives has been observed for ilaprazole.[19] This suggests that CYP3A4 plays a minor role in ilaprazole metabolism. Consequently, the pharmacological effects of ilaprazole are largely unaffected by the concurrent administration of CYP3A4 inhibitors or inducers, indicating its potential for use in multidrug therapy environments. Our biophysical assay and docking simulations revealed that the binding energies closely aligned with the K_d values obtained from tryptophan quenching. This consistency reinforces the accuracy of the binding models, offering valuable insights into its metabolism. CYP116B46, a naturally fused enzyme with a phthalate family oxygenase reductase, provides a promising model for studying the hydroxylation of PPIs. The well-conserved structure featuring FMN and [2Fe–2S] clusters is suitable for exploring P450 binding abilities for potential pharmaceutical applications (Fig. 1b) and is useful for understanding PPI metabolism and P450 complex dynamics.

This study provides significant insights into the biophysical interactions between PPIs and a self-sufficient P450 and presents an appropriate model for characterizing PPI metabolism. The higher binding affinity of ilaprazole-CYP116B46 compared to other PPI complexes confirms its suitability for detailed metabolic studies. The substrate-binding simulation indicated that ilaprazole interacted with CYP116B46 despite the limited size of the ingress pathway to the heme active site, and that the hydroxylation of the pyrrole ring of ilaprazole could influence its metabolism. This interaction pattern may contribute to the increased and prolonged action of ilaprazole, distinguishing it from other PPIs that primarily undergo metabolism *via* CYP3A4.[44,45,49] Our findings further suggest those of previous studies regarding the minimal role of CYP3A4 in ilaprazole metabolism, which proposed reduction to sulfide derivatives rather than oxidation.[19] Understanding PPI metabolism is crucial, because it directly influences pharmacokinetics including absorption, distribution, metabolism, and excretion.[19,22] The role of hepatic electron transport systems in redox reactions and metabolite formation is quite complicated and P450 induces unexpected metabolic pathways.[19,49] CYP116B46 proved to be useful in elucidating P450 complex generation and the pharmacokinetics of prodrugs including PPIs. This research not only enhances our understanding of P450 and PPI metabolism but also provides a useful framework for optimizing the efficacy and safety of PPI in *in vitro* assays.

Author contributions

C.H.Y. prepared plasmid DNA; Y.J. and S.-J.Y. expressed protein; J.Y., Y.H., H.G.K and S.J.L. performed protein purification; J.Y., Y.H., and S.J.L. performed tryptophan quenching; Y.H. H.G.K., and S.J.L analyzed the docking simulation; Y.H., S.-J.Y., C.H.Y., H.G.K., and S.J.L. wrote the manuscript; H.G.K. and S.J.L. designed the experiments, compiled the manuscript, and edited and reviewed the experiments and manuscript with input from all the authors.

Data availability

The data supporting this article have been included as part of the ESI.† The data were plotted with Origin software.

Conflicts of interest

There are no conflicts to declare.

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