Structures of human cytochrome P450 1A1 with bergamottin and erlotinib reveal active-site modifications for binding of diverse ligands

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Human cytochrome P450 1A1 (CYP1A1) is an extrahepatic enzyme involved in the monooxygenation of structurally diverse compounds ranging from natural products to drugs and protoxins. Because CYP1A1 has a role in human carcinogenesis, inhibiting its activity may potentially aid in cancer chemoprevention, whereas utilizing CYP1A1’s oxidative activity could help selectively activate anticancer prodrugs. Such potential therapeutic purposes require detailed knowledge of CYP1A1’s interactions with potential ligands. Known CYP1A1 ligands also vary substantially in size, and it has not been apparent from a single existing CYP1A1 structure how larger, structurally diverse ligands are accommodated within the enclosed active site. Here, two new X-ray structures with the natural product furanocoumarin bergamottin (at 2.85 Å resolution) and the lung cancer drug erlotinib (3.0 Å) revealed binding orientations consistent with the formation of innocuous metabolites and of toxic metabolites, respectively. They also disclosed local changes in the roof of the active site that enlarge the active site and ultimately form a channel to the protein exterior. Although further structural modifications would be required to accommodate the largest CYP1A1 ligands, knowing which components of the active site are malleable provides powerful information for those attempting to use computational approaches to predict compound binding and substrate metabolism by this clinically relevant monooxygenase.

The superfamily of enzymes known as cytochrome P450 monooxygenases has vital functions in the metabolism and detoxification of a large variety of drugs and environmental toxins. However, in other instances, P450 enzymes have been known to initiate deleterious effects, such as the production of reactive intermediates leading to toxic and carcinogenic effects. One human CYP2 enzyme with a particular propensity toward activation of protoxins and procarcinogens is CYP1A1, a highly inducible, extrahepatic P450. CYP1A1 has a preference for substrates that are polycyclic aromatic hydrocarbons, such as the environmental pollutant benzo[a]pyrene. Due to the roles of CYP1A1 in human carcinogenesis, inhibition of CYP1A1 activity has been considered as a potential target for cancer chemoprevention. In contrast, other efforts have been explored for utilizing CYP1A1 oxidative metabolism to activate anticancer prodrugs in a tissue-selective manner.

To employ either CYP1A1 inhibition or leverage its oxidative power for therapeutic purposes requires detailed knowledge of the active-site interactions with potential ligands. A single crystal structure of CYP1A1 was previously determined, in complex with the small-molecule inhibitor α-naphthoflavone (ANF). This structure revealed a narrow and closed active site not much larger than the ANF ligand itself. The other family 1 human P450 enzymes, CYP1A2 and CYP1B1, also have a single structure each, also containing ANF as the bound ligand, and similarly reveal relatively small, planar active sites. However, xenobiotic-metabolizing P450 enzymes often have rather flexible structures to accommodate the variety of substrates an individual enzyme interacts with, so it can be difficult to extrapolate a range of potential P450/ligand interactions from a single structure. In particular, a number of known 1A1 substrates and inhibitors reported in the literature reveal substrate and inhibitor sizes and geometries that are not compatible with the dimensions of the currently defined CYP1A1/ANF active site.

The purpose of the current study was to probe CYP1A1 active-site flexibility and potentially capture CYP1A1 in conformationally distinct states that would be useful in trying to employ CYP1A1 as a potential therapeutic target. To accomplish this, ligands with high CYP1A1 affinity and larger sizes were co-crystallized with the CYP1A1 enzyme. This study describes two novel CYP1A1 structures, one with the epidermal growth factor receptor (EGFR) inhibitor erlotinib used in anticancer therapy and another with the furanocoumarin P450 inhibitor bergamottin. These new structures of CYP1A1 provide insight into some of the structural rearrangements.

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2 The abbreviations used are: CYP, cytochrome P450; ANF, α-naphthoflavone; EGFR, epidermal growth factor receptor; NSCLC, nonsmall-cell lung cancer; CV, column volume(s); NCS, non-crystallographic symmetry.
Inhibition of CYP1A1 by structurally diverse ligands

The previous crystal structure of CYP1A1 was with the planar, fused multicyclic ligand α-naphthoflavone enclosed within a narrow, planar active site (7). Many CYP1A1 substrates and inhibitors do include features of various fused aromatic ring systems, often elongated in one dimension. The active-site cavity for the CYP1A1/ANF structure conforms to these aspects, but it is not particularly helpful in discerning how other CYP1A1 ligands with larger sizes and/or varying dimensions are accommodated by the active site. Whereas some human cytochrome P450 enzymes have multiple structures demonstrating significant flexibility in active-site conformation to accommodate different ligand classes, the CYP1 family enzymes have often been assumed to be comparatively static, in part because of the absence of structural evidence otherwise. To probe the malleability of the CYP1A1 active site, reported CYP1A1 ligands (substrates and/or inhibitors) that possessed a range of different chemical features and shapes were identified as potential candidates for crystallography. The ligands were broadly segmented into the following classes: azole inhibitors, tyrosine kinase inhibitors, anticancer prodrugs, and natural products from the furanocoumarin and stilbenoid classes.

To provide a ranking according to the potency of their interactions with CYP1A1, IC_{50} values were determined (e.g., see Fig. 1) for each ligand. About half of the compounds tested had reasonably potent inhibition of CYP1A1-mediated activity (Table 1), defined herein as IC_{50} < 1 μM. This series included a number of azoles, which are often potent, broad spectrum P450 inhibitors. Not surprisingly, then, the azoles clotrimazole, miconazole, and tioconazole were three of the four most potent inhibitors. These are all smaller azoles (344–417 g/mol) with a compact overall structure. Larger azoles with extended structures like ketoconazole and posaconazole (531 and 700 g/mol) were 4–7-fold less potent but still had IC_{50} values in the nanomolar range. However, fluconazole, which is smaller and has an overall structure more similar to miconazole and tioconazole, did not significantly inhibit CYP1A1, so size is certainly not the only consideration.

One of the interests of CYP1A1 in human health is its role in cancer, both as a potential generator of carcinogenic species and in the metabolism of certain anti-cancer drugs, such as tyrosine kinase inhibitors. Some of the tyrosine kinase inhibitor drugs CYP1A1 has been demonstrated to interact with include erlotinib (10), imatinib (11), and ponatinib (12), which were also tested for their inhibitory potency. These drugs displayed nanomolar to low micromolar IC_{50} values, with erlotinib being the most potent (IC_{50} = 180 ± 10 nM). Again, it was the more extended imatinib and ponatinib that were less potent inhibitors (IC_{50} > 1 μM), perhaps indicating that they might not fit the CYP1A1 active site as well. Metabolism of erlotinib and ponatinib by CYP1A1 has been reported to generate reactive electrophilic metabolites that may contribute to some of the observed life-threatening toxicities associated with these drugs (12, 13).

CYP1A1 has yet another connection to cancer therapeutics in that the tissue location and metabolic capability of CYP1A1 have suggested its utility in the activation of several different prodrugs to reactive electrophilic species leading to DNA damage and cell death (6, 14, 15). This included the benzothiazole GW610 (16), which current results suggested had a fairly potent IC_{50} of 300 ± 80 nM. In contrast, the compound AQ4N is acti-
vated by CYP1A1 to the active topoisomerase inhibitor AQ4 under hypoxic conditions (17) but under normoxic conditions evaluated herein showed essentially no inhibition of CYP1A1.

Two natural products were suggested as CYP1A1 ligands in the literature and were tested. The first was the furanocoumarin bergamottin found in grapefruit and other citrus and well-known to inhibit CYP3A4 and cause variations on CYP3A4-mediated metabolism of common drugs (18). Bergamottin also displayed potent inhibition of CYP1A1, with an IC50 of 140 ± 20 nM. Bergamottin also inhibits CYP1A2, but with attenuated potency (19, 20). This could potentially be due to size and shape complementarity of the respective active sites. At least in the ANF-bound structures, CYP1A1 possesses a slightly larger active-site volume compared with CYP1A2 (21), which may allow for greater accommodation of the larger furanocoumarin compounds. Additionally, comparison of selective ligand features between CYP1A1 and CYP1A2 suggested a preference of CYP1A1 for ligands with longer strip-like geometry, whereas CYP1A2-selective compounds were more triangular in shape (22). The other natural product suggested in the literature to interact with CYP1A1 was the trans-stilbene resveratrol found in red wine. Herein, resveratrol was an order of magnitude less potent than bergamottin at inhibiting CYP1A1 activity. The resveratrol IC50 value of −1.4 μM was more similar to the tyrosine kinase drugs imatinib and ponatinib.

A number of other diverse compounds ranging from moieties such as imidazole and indole to the analgesic acetaminophen were also tested for inhibitory potency toward CYP1A1. Additionally, endogenous fatty acids, such as arachidonic acid (23), have been reported to be efficiently metabolized by CYP1A1. The small chemical moieties indole, imidazole, and the drug acetaminophen resulted in exceedingly weak inhibition of CYP1A1 activity, with IC50 values >100 μM. The binding affinity of these small compounds is likely weak, and they may not make extensive interactions with the active site. The endogenous arachidonic acid substrate was only weakly inhibitory, with an IC50 value of 3.8 μM.

Overall, this screen of many CYP1A1 ligands suggested that several of the imidazole azoles, bergamottin, and erlotinib had significantly different structures from α-naphthoflavone but IC50 values less than 200 nM and might be good candidates for structural studies probing the flexibility of the CYP1A1 active site.

Characterization of bergamottin and erlotinib binding to CYP1A1

As examples of structurally diverse CYP1A1 ligands with relatively low IC50 values, the binding of bergamottin and erlotinib were further characterized by determining their affinities and binding modes via UV spectroscopy. Tandem cuvettes were employed to blank out the intrinsic absorbance of both bergamottin and erlotinib, which overlap the wavelength range of the P450 heme Soret peak monitored to detect ligand binding. Titration of CYP1A1 with either bergamottin or erlotinib resulted in a blue shift of the Soret peak, with a peak forming at ∼391 nm and a trough at 427–429 nm when observed in difference mode (Fig. 2). Such a Type I binding profile is indicative of displacement of the water from the heme iron. The Kd for bergamottin was 370 ± 40 nM (Fig. 2), which is in a similar range to its IC50 value determined herein and the dissociation constant for ANF (∼300 nM) reported previously (7). The Kd measured for erlotinib was 1.07 ± 0.04 μM (Fig. 2), indicating that erlotinib binds to the CYP1A1 resting state with lower affinity compared with bergamottin and ANF. However, both ligands have affinities and solubilities that can support saturation of CYP1A1 for structure determination.

Overall CYP1A1 X-ray structures

Structures were subsequently determined with both bergamottin and erlotinib at resolutions of 2.85 and 3.0 Å, respectively. Both structures were solved in the P212121 space group with very similar unit cell dimensions and contained four copies of the protein within the asymmetric unit. For both structures, all four copies within the asymmetric unit were very similar, although two of them tended to be more disordered with regions of weaker electron density for some protein side chains and the active-site ligand. The 1A1/erlotinib structure disorder prevented reliable modeling of the loop between E and F helices and the active-site ligand. The 1A1/bergamottin was 370 ± 40 nM (Fig. 2), which is in a similar range to its IC50 value determined herein and the dissociation constant for ANF (∼300 nM) reported previously (7). The Kd measured for erlotinib was 1.07 ± 0.04 μM (Fig. 2), indicating that erlotinib binds to the CYP1A1 resting state with lower affinity compared with bergamottin and ANF. However, both ligands have affinities and solubilities that can support saturation of CYP1A1 for structure determination.
Cytochrome P450 1A1 structures

(molecule A) will be specifically referenced in the following discussion.

The overall tertiary structures of the two new CYP1A1 ligand complexes with bergamottin and with erlotinib are highly similar to the previously determined structure of CYP1A1 with the ligand ANF (Fig. 3). Compared with the previous CYP1A1/ANF structure, the new structures with bergamottin and erlotinib both had root mean square deviations of 0.37 Å for Ca atoms. A distinctive feature of the previous CYP1A1/ANF structure, one conserved in the homologous CYP1A2 and CYP1B1 structures

Bergamottin binding to CYP1A1

In all four of the molecules in the asymmetric unit, bergamottin is positioned similarly in the CYP1A1 active site. Its fused psoralen ring system is oriented distal from the heme, with \( \pi-\pi \) interactions to the parallel side chain of the F-helix residue Phe-224. The geranyloxy chain of bergamottin extends toward the heme, bending at the 3' position so that the rest of the chain lies over the heme, slightly off center of the iron. The terminal dimethyl portion of this ligand chain is directed toward a cluster of hydrophobic residues that are part of the \( \beta_4 \) and K/\( \beta_1-4 \) loops, including Val-382 (Fig. 4A). These residues close off the bottom portion of the CYP1A1 active-site cavity opposite from the I-helix. There is a hydrogen bond formed between the ketone oxygen of the bergamottin psoralen moiety and the side chain nitrogen of Asn-222. This combination of interactions between bergamottin and the CYP1A1 active site results in the 6' and 7' carbons of the geranyloxy chain closest to the heme iron, at distances of 4.8 and 5.1 Å, respectively, suggesting that oxidation might occur at one or more of these positions.

Reports of bergamottin metabolism by P450 enzymes in the literature are few but important in terms of the “grapefruit juice

Figure 3. CYP1A1 structures bound to bergamottin (orange), erlotinib (green), and previously determined \( \alpha \)-napthoflavone (purple), all superimposed by Ca atoms.

Figure 4. CYP1A1 active-site interactions with bergamottin (A) and erlotinib (B). Shown are polar CYP1A1 active-site residues (sticks) that make hydrogen bonds (black dashed lines) with the ligands. Electron density is shown as 2F\( _{o} \) - F\( _{c} \) simulated annealing composite omit maps (blue mesh) contoured at 1.0 \( \sigma \). Heme is shown as black sticks. Key sites of metabolism are indicated by red dashed lines to the heme iron. Distances for hydrogen bonds and ligand–heme iron distances (red dashed lines) are shown in Å.
effect” wherein metabolism of the furan ring of the psoralen moiety results in reactive intermediates causing mechanism-based inhibition of the P450 enzyme, whereas metabolism on the geranyloxoy chain produces stable metabolites (23). Metabolism by CYP1A1 has not been reported previously, but the Hollenberg group investigated bergamottin metabolism by CYP3A4 (18), CYP3A5, and CYP2B6 (23). Bergamottin is a potent mechanism-based inhibitor of CYP3A4, consistent with metabolism on the psoralen ring. For CYP3A5, the primary metabolite involved oxidation at the 1’-carbon of the geranyloxoy chain, leading to cleavage of the geranyl chain to form bergaptol. In contrast, CYP2B6 produced major metabolites from oxidations on the terminal end of the geranyl-oxoy chain. Both CYP3A5 and CYP2B6 were also capable of generating bergamottin metabolites that formed GSH conjugates following furan ring oxidation, but the dominant metabolite was different for each enzyme, suggesting different preferences for bergamottin orientation in the respective active sites.

The current crystal structure of CYP1A1 with bergamottin suggests that this enzyme prefers to bind bergamottin more like CYP2B6 than CYP3A5 and would be more likely to oxidize the terminal portion of the geranyl chain. The current CYP1A1 structure does not indicate the potential for complete reorientation of bergamottin within the active-site cavity, which would be necessary for reactive metabolites. Although this cannot be ruled out, the observed orientation would suggest that CYP1A1 would have less tendency toward bergamottin bioactivation compared with other P450 enzymes, due to the specific interactions with the CYP1A1 active-site cavity that dictate ligand orientation.

CYP1A1 bergamottin metabolism

To determine whether the binding pose for bergamottin in the CYP1A1 active site is consistent with metabolite generation, turnover assays were performed with human CYP1A1 and human NADPH-cytochrome P450 reductase. CYP1A1 produced at least four major metabolites at different substrate concentrations. At low concentrations of bergamottin, only one major metabolite was observed. At higher concentrations, a second metabolite was observed and then a third metabolite (Fig. 5). The third metabolite coelutes with authentic 6’,7’-dihydroxybergamottin, whereas the other two metabolites are slightly less polar, suggesting that they are likely to be the individual 6’-hydroxy and 7’-hydroxy products. The identity of the much more polar fourth metabolite is unknown. Overall, however, the metabolite profile is generally consistent with the binding orientation observed in the X-ray structure.

Erlotinib binding to CYP1A1

Erlotinib, marketed as Tarceva, is a tyrosine kinase inhibitor that inhibits the EGFR and was approved for treatment of nonsmall-cell lung cancer (NSCLC). The use of erlotinib has been related to a number of adverse drug effects, including rash (24), skin toxicities (25), and diarrhea (26), that vary in severity, with more life-threatening effects, such as pulmonary toxicity (27) and toxic epidermal necrosis (Tarceva package insert). Metabolism of erlotinib and other kinase inhibitors by P450 enzymes can generate reactive metabolites and could explain some of the associated toxicities of these drugs (12, 13). In humans, erlotinib is metabolized primarily by P450 enzymes CYP3A4, CYP3A5, and CYP1A1, with the latter extrahepatic enzyme playing an important role in the lung and in pharmacokinetic variability (10). Of particular clinical significance is the inducibility of CYP1A1. Tobacco smoke can increase CYP1A1 expression levels in the lung (28, 29), which in turn can have large effects on erlotinib clearance (30, 31). As a result, higher concentrations of erlotinib may be required to treat NSCLC in smokers (32).

Cocrystallization of CYP1A1 with the EGFR inhibitor erlotinib revealed this ligand binding in a slot-like, planar active site, with its planar quinazoline core distal to the heme making π−π interactions with the aromatic side chain of Phe-224 (Fig. 6). The terminal alkyne moiety is clearly positioned above the heme and extends into a pocket formed by hydrophobic residues of the β4 and K/β1_4 loops, similar to positioning of the geranyloxoy chain of bergamottin. The two 2-methoxyethoxy side chains of erlotinib extend away from the heme toward the roof of the CYP1A1 active site, one positioned between the F and G helices and the other side chain protruding between the F and I helices underneath the F helix break. Erlotinib appears to potentially form a number of hydrogen-bonding contacts with the CYP1A1 active site. Two of these involve Asp-320. If protonated, Asp-320 is positioned to be a hydrogen bond donor to one of the side chain oxygens. Asp-320 also appears to make a water-mediated interaction with the nitrogen connecting the quinazoline and phenyl-acetylene moieties, but this active-site water could only be reliably modeled into molecule A and B, which had the best electron density. Additionally, Asp-222 makes a potential hydrogen-bonding interaction with an oxygen on the methoxyethoxy side chain. The substituent oxygen of the other side chain hydrogen-bonds with the side-chain Asn-255. Finally, a nitrogen of the quinazoline core forms a water-mediated hydrogen bond with the backbone of Ser-116.

The primary metabolic reactions in humans are O-demethylation of the methoxyethoxy side chain, oxidation of the terminal alkyne, and hydroxylation of the para position on the phenyl-acetylene moiety (33). The position of erlotinib observed in the CYP1A1 crystal structure is consistent with

![Figure 5. Bergamottin metabolism by CYP1A1 yields one major metabolite that coelutes with authentic 6’,7’-dihydroxybergamottin (6’,7’DHB; 26.6 min) and two slightly less polar metabolites (27.6 and 27.7 min) that occur at lower substrate concentrations and are likely the individual 6’-monohydroxy and 7’-monohydroxy metabolites. Thus, at least three major metabolites are consistent with the bergamottin orientation observed in the X-ray structure.](image)
metabolism of either the terminal alkyne carbon or the para carbon of the aromatic aniline ring. The distances to the iron are 4.6 and 4.9 Å for the terminal alkyne and para carbons, respectively. Oxidation at these two positions of erlotinib is significant in terms of its potential to produce reactive species that might underlie erlotinib-associated toxicities. The 4-hydroxylation of the phenyl-acetylene carbon results in formation of a para-hydroxyanalyne, which can be oxidized by P450 to form the reactive quinoneimine. Conversely, oxidation of the terminal alkyne can result in reactive oxirene or ketene intermediates (13).

Li et al. (13) detected GSH conjugates of erlotinib metabolism by CYP1A1. These conjugates were formed on the aniline ring, consistent with CYP1A1 hydroxylation at the para position and consistent with the orientation observed herein. Mechanism-based inactivation of CYP1A1 was not observed, suggesting that the initial hydroxylated product may be released before formation of the reactive quinoneimine. Indeed, p-hydroxyerlotinib is the major metabolite circulating in human plasma (13). In contrast, CYP3A4 and CYP3A5 oxidize the terminal alkyne (34), resulting in reactive intermediates that do result in addition of these P450 enzymes and mechanism-based inactivation. The fact that CYP1A1 is not inactivated suggests that CYP1A1 does not modify the terminal alkyne. Reports indicate that both CYP3A4/5 and CYP1A1 (10) can also generate active, nontoxic des-methyl metabolites of the side chains, suggesting that both enzymes can bind erlotinib in multiple orientations.

Comparison of all three CYP1A1 structures yields insights into residues involved in accommodating diverse ligand structures

The new co-crystal structures of CYP1A1 in this study have many overall structural elements consistent with the previous CYP1A1/ANF structure. Thus, the CYP1A1 structure accommodates bergamottin and erlotinib with minimal perturbations of the tertiary and secondary structure. Both bergamottin and erlotinib are positioned in the active site with their respective planar components in the same plane that ANF adopted and other known CYP1A1 ligands that were previously docked into the experimental CYP1A1/ANF structure (7). This common binding plane is likely driven by the conserved orientation of Phe-224, which has favorable π–π interactions and sandwiches the planar ligand core against the I helix.

Within this overall context, however, important differences arise in a key localized portion of the structure. The unusual break in the F helix for the CYP1A1 structure appears to assist in accommodation of different CYP1A1 ligands. Whereas the active sites with ANF and bergamottin are enclosed (Fig. 6, A and B), in the erlotinib structure, Asn-222 in the F helix break adjusts its positioning, resulting in an opening that extends the active-site cavity to the surface (Fig. 6C). One of the methoxye- 
thoxy chains of erlotinib extends into this channel between the F-helix break and portions of the β-loop. Because the open channel in the CYP1A1/erlotinib structure extends to bulk solvent, the end of the channel was artificially closed to permit comparison of active-site volumes. It appears that concerted torsioning of the Asn-222 and Leu-254 side chains approximately doubles the volume of the CYP1A1/erlotinib active site (806 Å³) compared with those for the CYP1A1/ANF (408 Å³) and CYP1A1/bergamottin (456 Å³) structures (Fig. 6). In the CYP1A1/ANF structure, this channel is closed by hydrogen bonding between Asn-222 and I helix residue Asp-320 (Fig. 6A). In the CYP1A1/bergamottin structure, Asn-222 is torsioned in yet a different way, breaking the interaction with Asp-320 but not opening the channel (Fig. 6B). In each case, the various rotamers of Asn-222 (Fig. 7) are accommodated by differential positioning of the spatially adjacent G helix residue Leu-254. Larger CYP1A1 substrates and inhibitors might utilize an expanded version of this channel to access the active site. Certainly, the channel as currently observed would have to widen further to accommodate entry/exit of just the current substrates or their metabolites.

Conclusions

In summary, two new experimental structures with erlotinib and bergamottin are consistent with known metabolism. The erlotinib structure suggests that CYP1A1 participates in the first step in the formation of chemically reactive metabolites that may contribute to lung and other toxicities. On the other hand, bergamottin interacts with CYP1A1 in an orientation suggestive of innocuous metabolite generation. In combination, the known structures suggest that, whereas many of the structural elements of the CYP1A1 active site are rigid, structurally diverse, somewhat larger ligands can be accommodated by local modifications. The structures of CYP1A1 with bergamottin and erlotinib in this study establish one approach by which CYP1A1 accommodates somewhat larger ligands. This consists of side-chain repositioning in the roof of the CYP1A1 active site, even generating a channel to the surface in the erlo-
tinib structure. It is possible that even larger CYP1A1 ligands

Cytochrome P450 1A1 structures

Figure 6. Active-site cavities for CYP1A1 structures with α-naphthoflaveone (A), bergamottin (B), and erlotinib (C). The side chains of Asn-222 and Leu-254 (sticks) display altered positioning between the structures, contributing to expansion of the CYP1A1 active-site cavity.
like ketoconazole and posaconazole might be accommodated by further expanding the channel observed in the erlotinib structure. Knowing which components of the active site are malleable and which are not provides powerful information for those attempting to use computational approaches to predict compound binding and substrate metabolism for such enzymes.

Materials and methods

Protein expression and purification

Expression of human CYP1A1 was performed using a construct encoding an N-terminal truncation spanning residues 2–34 that was replaced with the sequence AKTSS and a C-terminal 6-histidine tag (7). Expression was based on the method described previously (7) with a number of modifications. For clarity, a detailed protocol is included herein. CYP1A1 purification was initiated by resuspending cells in resuspension buffer consisting of 20 mM potassium phosphate, 20% (v/v) glycerol, pH 7.4, and 1 mM PMSF. The resulting resuspension was lysed by a French press using one pass at 16,000 p.s.i. internal cell pressure. Detergent extraction of CYP1A1 was performed on this lysate by adding 1% (w/v) CHAPS and stirring for 1 h. Cell lysate was clarified by ultracentrifugation at 142,000 \( \times g \) for 15 min to isolate spheroplasts, which were gently washed with resuspension buffer twice and then resuspended with the aid of a Dounce homogenizer in lysis buffer consisting of 3 mM histidine. CYP1A1 was then eluted with a linear gradient from 3 mM histidine-containing loading buffer to elution buffer (10 mM potassium phosphate, 100 mM NaCl, 20% (v/v) glycerol, 0.5% (w/v) CHAPS, 80 mM histidine, 2 mM EDTA) over 6 CV. Eluted fractions possessing a UV absorbance of \( A_{419}/A_{280} > 0.8 \) were pooled, diluted 2-fold with CM wash buffer (10 mM potassium phosphate, 100 mM NaCl, 20% (v/v) glycerol, 0.5% (w/v) CHAPS, 1 mM EDTA, pH 7.4) and loaded onto three 5-ml pre-equilibrated Hi-Trap carboxymethyl-Sepharose fast-flow columns (GE Healthcare) connected in series (total 15-ml column volume). The column was washed with 10 CV of CM wash buffer, and then CYP1A1 was eluted using a 6-CV linear gradient from CM wash buffer to SEC buffer (50 mM potassium phosphate, 500 mM NaCl, 20% (v/v) glycerol, 0.5% (w/v) CHAPS, 1 mM EDTA, pH 7.4) followed by 100% SEC buffer for an additional 4 CV. Fractions with \( A_{419}/A_{280} > 1 \) were pooled and concentrated to 4 ml, centrifuged at 10,000 \( \times g \) to remove any precipitated protein, and then loaded onto a pre-equilibrated Superdex 200 gel filtration column (GE Healthcare) run with the same SEC buffer. Major peaks exhibiting an \( A_{419}/A_{280} > 1.1 \) were pooled and evaluated by SDS-PAGE, UV-visible spectroscopy, and the reduced-carbon monoxide difference assay (36). Protein was quantitated using the Soret in the absolute spectrum in the presence of saturating imidazole and an extinction coefficient of 100 mM\(^{-1}\) cm\(^{-1}\). Purified CYP1A1 was flash-frozen in aliquots in a dry ice/ethanol slurry, and stored at \(-80^\circ C\) until use for experiments.
A final concentration of 100 nM CYP1A1 and 20 nM reductase in reaction) were performed starting with pre-incubation of CYP1A1 and full-length human reductase at a 1:2 ratio (to give a final concentration of 10 nM CYP1A1 and 20 nM reductase in reaction). The other chamber contained only SEC buffer. Stock solutions of ligands were created in DMSO (0.2–10 mM). Ligands were titrated into the CYP1A1-containing chamber of the sample cuvette and the buffer-only chamber of the reference cuvette. An equal volume of DMSO was titrated into the sample cuvette and the buffer-only chamber of the reference cuvette. Ligands were evaluated according to their ability to inhibit CYP1A1-mediated metabolism of the P450 Glo™ substrate luciferin-ME EGE (Promega). Inhibition assays for CYP1A1 were performed starting with pre-incubation of CYP1A1 and full-length human reductase at a 1:2 ratio (to give a final concentration of 10 nM CYP1A1 and 20 nM reductase in reaction) in 400 mM potassium phosphate, pH 7.4, buffer at room temperature for 20 min. The substrate luciferin-ME EGE was added to the pre-incubated protein to give a final concentration during the assay of 4 μM, which is equal to the previously determined Km. Protein with substrate was then dispensed into a white 96-well microplate (Corning), and the respective inhibitor concentration was evaluated in duplicate.

**CYP1A inhibition assays**

Ligands were evaluated according to their ability to inhibit CYP1A1-mediated metabolism of the P450 Glo™ substrate luciferin-ME EGE (Promega). Inhibition assays for CYP1A1 were performed starting with pre-incubation of CYP1A1 and full-length human reductase at a 1:2 ratio (to give a final concentration of 10 nM CYP1A1 and 20 nM reductase in reaction) in 400 mM potassium phosphate, pH 7.4, buffer at room temperature for 20 min. The substrate luciferin-ME EGE was added to the pre-incubated protein to give a final concentration during the assay of 4 μM, which is equal to the previously determined Km. Protein with substrate was then dispensed into a white 96-well microplate (Corning), and the respective inhibitor concentration was evaluated in duplicate.

**Ligand-binding assays**

Binding of ligands to CYP1A1 was monitored using a double-beam UV-visible spectrophotometer (Shimadzu Scientific Instruments) at 20 °C. CYP1A1 was diluted into SEC buffer to a concentration of 1 μM and equally divided into the two chambers of two 1-cm path length tandem cuvettes (sample and reference). The other chamber contained only SEC buffer. Stock solutions of ligands were created in DMSO (0.2–10 mM). Ligands were titrated into the CYP1A1-containing chamber of the sample cuvette and the buffer-only chamber of the reference cuvette. An equal volume of DMSO was titrated into the buffer chamber of the sample cuvette and the CYP1A1-containing chamber of the reference cuvette to correct for solvent and dilution effects to the spectra. Absorbance was monitored from 300 to 500 nm. Dissociation constants were determined by nonlinear regression fitting (GraphPad Prism) of the change in absorbance versus ligand concentration using a tight binding equation described previously (39).

**Co-crystallization, data collection, and structure determination**

Purified CYP1A1 was concentrated to 17 mg/ml (bergamottin) or 20 mg/ml (erlotinib) by three iterative centrifugal ultrafiltration steps that involved dilution with SEC buffer containing 0.4 M ammonium nitrate and either 20 μM bergamottin or 20 μM erlotinib. Crystals of CYP1A1 with the respective ligands were grown using the sitting-drop vapor diffusion method in either 24-well plates (bergamottin) or 96-well plates (erlotinib).
Plates were set up at 20 °C by mixing 1 μl of CYP1A1/bergamottin with 1 μl of a crystallization solution (0.2 M potassium phosphate dibasic, 20% (w/v) PEG 3350, 15% (v/v) glycerol) or 0.75 μl of CYP1A1/erlotinib with 0.75 μl of crystallization solution (0.2 M potassium phosphate dibasic, 20% (w/v) PEG 3350, 10% (v/v) glycerol, 0.5% (w/v) n-dodecyl-N,N-dimethylamine-N-oxide). Plates were sealed and equilibrated against 300 μl (bergamottin) or 50 μl (erlotinib) of the respective crystallization solution at 4 °C. Triangular prism crystals grew over 2–3 days and were harvested at 5–6 days. CYP1A1/bergamottin crystals were cryoprotected using the bergamottin crystallization solution containing 30% (w/v) PEG 3350, whereas CYP1A1/erlotinib crystals were cryoprotected with erlotinib crystallization solution containing 20% (v/v) glycerol. Crystals were flash-cooled in liquid nitrogen, and diffraction data were collected on beamline 9-2 at the Stanford Synchrotron Radiation Lightsource (Table 2). Integration of the data and scaling were performed using XDS (40) and AIMLESS (41). Structures were solved using molecular replacement via Phaser (42) with the CYP1A1/ANF structure (7) as a search model (Protein Data Bank code 48V, molecule A), resulting in log likelihoods of 20,276 (1A1/bergamottin) and 12,779 (1A1/erlotinib). Building and refinement of the models were iteratively performed using the programs Coot (43) and PHENIX (44), respectively (Table 2). Torsion-angle NCS restraints were used during refinement with chain A as the reference selection for the other NCS-related chains. Ligand restraints were generated using PHENIX eLBOW (45) with AM1 geometry optimization. Active-site void volumes were calculated using VOIDOO (46), with probe radius = 1.4 Å and grid spacing = 1.0. All figures were prepared using PyMOL (47).

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