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Halogen- π Interactions in the Cytochrome P450 Active Site: Structural Insights into Human CYP2B6 Substrate Selectivity

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Abstract

Numerous cytochrome P450 (CYP)2B6 substrates including drugs and environmental chemicals are halogenated. To assess the role of halogen- π bonds in substrate selectivity and orientation in the active site, structures of four CYP2B6 monoterpeneoid complexes were solved by X-ray crystallography. Bornyl bromide exhibited dual orientations in the active site with the predominant orientation revealing a bromine- π bond with the Phe108 side chain. Bornane demonstrated two orientations with equal occupancy; in both the C2-atom that bears the bromine in bornyl bromide was displaced by more than 2.5 Å compared with the latter complex. The bromine in myrtenyl bromide π -bonded with Phe297 in CYP2B6, whereas the two major orientations in the active site mutant I114V exhibited bromine- π interactions with two additional residues, Phe108 and Phe115. Analysis of existing structures suggests that halogen- π interactions may be unique to the CYP2B enzymes within CYP family 2 but are also important for CYP3A enzymes.

Keywords

Cytochrome P450; X-ray crystallography; halogen- π interactions; CYP2B6

The halogen- π interaction or halogen bond is a directional and electrostatically driven non-covalent interaction formed between a region of positive electrostatic potential on the outer lobe of the covalently bonded halogen atom C-X (X = Cl, Br, I) and a nucleophile or the π electrons of an unsaturated system such as an aromatic ring of an amino acid residue.¹⁻³ The halogen bond plays an important role in drug discovery⁴⁻⁶, and addition of halogen atoms is a critical tool in drug design with approximately 1/3 of drugs in clinical trials bearing halogens.⁷⁻¹⁰ The role of halogen atoms in altering physicochemical properties of the ligand by conferring affinity and/or selectivity to the drug target often creates unique advantages over other substitutions.¹¹ Interestingly, the ratio of heavy organohalogens (i.e.,

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Supporting Information: Materials and methods for protein expression, purification, crystallization and structure determination, and Supporting Figures 1-4 and Supporting Table 1-2. This material is available at <http://pubs.acs.org>.

organochlorine¹⁰ and organobromines) to organofluorine increases in the later stages of the drug development process, which is consistent with the weaker halogen bonds from fluorine.^{9,12,13} Despite the ubiquity of halogen atoms in potent and selective drugs, the structural basis of the interaction with biological macromolecules or drug targets has remained poorly understood until recently. Numerous crystal structures in the Protein Data Bank (PDB) reveal halogen- π interactions between protein and ligand that may enhance substrate affinity and selectivity, leading to more potent or selective drugs.¹⁴⁻¹⁸

Multiple studies have demonstrated the effect of -Cl containing functional groups on π bond coordination with aromatic side chains in two geometries at approximate distance ranging between ~ 3.7 Å to ~ 4.1 Å: i) the edge-on, where the halogens in the ligand approach aromatic atoms on the periphery of the ring, and ii) the face-on, where such functional groups interact with the electron density at the center of an aromatic ring.^{19,20} In addition, the angle ($\sim 120^\circ$ or more) from the carbon atom bearing the halogen in the ligand to the centroid of the aromatic ring is crucial for considering such π -bonds.⁶ Most of the emphasis has been on understanding and optimizing the interaction of drug candidates with their targets, and the contribution of halogen interactions with drug metabolizing enzymes has not been explored systematically. Enzymes from cytochrome P450 (CYP) families 1, 2 and 3 carry out metabolism of drugs and several endogenous substrates, and many of these enzymes are involved in disposition of more than one drug.²¹ Characterization of protein-ligand interactions, in particular the interaction of halogenated ligands in the active site of various drug-metabolizing CYPs, may yield better understanding of molecular recognition or substrate preference.

Among the human CYP enzymes, those of the CYP2B subfamily are involved in metabolizing numerous halogenated compounds including drugs such as efavirenz, bupropion, sertraline, and ticlopidine.²²⁻²⁵ Over 30 crystal structures of CYP2B enzymes from various species have been solved to date and deposited to the PDB, more than any other subfamily of enzymes involved in drug metabolism. This includes structures of rabbit CYP2B4, human CYP2B6, and woodrat CYP2B35 and 2B37 in the presence or absence of ligands of various size and shapes. The structures have provided essential information on the role of enzyme plasticity in the remarkable ability of a single CYP2B enzyme to bind a wide array of compounds of very different size and shape.²⁶⁻²⁹ However, in the absence of ionic or hydrogen bonds in the active site, the structural basis of CYP2B selectivity has remained obscure. Recent retrospective analysis of the active site of all the CYP2B structures solved in the presence of halogenated ligands, and comparison with the structures solved from other drug metabolizing CYP enzymes, revealed that many CYP2B substrates form halogen- π interactions with multiple phenylalanine side chains in the predominantly hydrophobic active site. For example, of the two orientations of the drug ticlopidine in the active site of a CYP2B4 structure, halogen bonding with Phe297 was only observed in the predominant conformer that is conducive to metabolism (Figure 1A).³⁰ Furthermore, the -Cl atom in the second molecule of amlodipine in the CYP2B4 structure forms a stable π bond via face-on geometry with the Phe365 side chain in the access channel as shown in Figure 1B. Such a π -bond is not observed in CYP2B6 where the residue at position 365 is methionine, and the orientation of second amlodipine is significantly altered (Figure 1C).²⁷

The central hypothesis of our ongoing work is that: 1) the selectivity of CYP2B6 for certain halogenated compounds results from a sufficiently malleable active site to accommodate the compound and form halogen- π interactions with conserved phenylalanine residues in the active site, and 2) species differences among CYP2B enzymes result from differences in variable hydrophobic active site residues that modulate halogen bonding to phenylalanine residues. As an initial test of this hypothesis we first crystallized and solved X-ray crystal structures of CYP2B6 with the halogenated monoterpene bornyl bromide and its non-halogenated analog bornane. We next crystallized CYP2B6 and its I114V mutant with (-)-myrtenyl bromide, a monoterpene structurally similar to (+) α -pinene with a bromine atom at the C10 position (Supporting Figure 1). The halogen interactions with aromatic residue side chains in the current structures were considered based on two previously analyzed criteria (Figure 2): i) C-X-- π with an approximate angle (θ) of about 120° or higher ($\alpha < 60^\circ$) from the carbon atom bearing the halogen (bromine) to the centroid of the aromatic side chains,³¹ and ii) the edge-on geometry with a distance range from ~ 3.7 Å to ~ 4.3 Å from the bromine atom to the nearest atom of the phenylalanine side chains.³¹ This criteria is inclusive of the analysis by Imai et al. that used over 38,000 PDB sets and 367,000 ligand data, representing a Cl- π interaction as any contact where the interatomic distance between the centroid of the aromatic side chain and the Cl of a ligand is shorter than 4.5 Å.^{15,20} It should be noted that the mean intermolecular distances for halogen- π interactions increase in the order Cl < Br < I, while the mean angles (θ and α) remain similar.³¹ The interatomic distances based on edge-on geometry calculated in this study included Br to the edge of the Phe side chain with distance of up to 4.35 Å at appropriate θ and α angles.

The 2.0 Å resolution crystal structure of CYP2B6 in complex with bornyl bromide demonstrated multiple orientations of the ligand in the active site (Figure 3, A and B). The F_o-F_c omit map clearly illustrated that in the predominant ligand orientation, the bromine formed π bond with the aromatic side chain of Phe 108 in an edge-on fashion. The minor orientation with the bromine atom toward the heme was fit without generating negative peaks in F_o-F_c maps. Moreover, the high-resolution (1.7 Å) crystal structure of CYP2B6 with bornane exhibited an identical closed conformation and active site to the bornyl bromide complex (root-mean-square deviation (RMSD) of 0.15 Å in a C α overlay). The electron density from the F_o-F_c omit map was suggestive of two bornane orientations of equal occupancy in the active site, as shown in Figure 3C. However, the C2 atom that bears the bromine in bornyl bromide, was offset by > 2.5 Å in bornane compared with bornyl bromide (Figure 3D). In this orientation, a bromine atom introduced at C2 of bornane would be too far to form π bonds with any of the phenylalanine residues, suggesting that such bonds in bornyl bromide indeed lead to re-orientation of the ligand.

A crystal structure of CYP2B6 (2.27 Å) in complex with (-)-myrtenyl bromide also revealed halogen bonds. The overall conformation and the active site residues were identical to the previously determined (+) α -pinene structure and to the current bornyl bromide and bornane complexes. The RMSD between each of these structures in a C α overlay was < 0.2 Å. Data collection and refinement statistics for all the CYP2B6 complexes represented in this work are shown in Supporting Table 1. The F_o-F_c omit map demonstrated equal occupancy of the ligand in two orientations, as shown in Figure 4A, in contrast to the bornyl bromide complex, where the bromine up orientation was preferred. For comparison, in the available

CYP2B6 complex with (+) α -pinene there is only a single ligand orientation in the active site with the C10 atom facing the heme (Supporting Figure 2).³² The bromine up orientation of (-)-myrtenyl bromide showed a π interaction between the bromine and the aromatic side chain of Phe297 in an edge-on fashion, whereas the bromine down orientation was modeled near the heme iron (Figure 4B).

To test how active site residues that vary among CYP2B enzymes might alter halogen bonding to the conserved phenylalanine residues, we crystallized CYP2B6 I114V in the presence of (-)-myrtenyl bromide. The Ile114 in CYP2B6 is valine in dog CYP2B11, which has been shown previously to be critical for the hydroxylation of polychlorobiphenyls by that enzyme.^{33,34} Despite the remarkable similarity in the overall protein conformation and the active site residues to the wild-type complex, the crystal structure of CYP2B6-I114V revealed three orientations of (-)-myrtenyl bromide in the active site as illustrated in the omit map (Figure 4C). Two predominant bromine up orientations of the ligand were found in which π bonds with multiple phenylalanine residues were observed, whereas a minor conformer was present with bromine down near the heme iron. As shown in Figure 4D, one of the predominant conformers exhibited bromine- π interactions with Phe297 in the I114V active site, as in the wild-type complex. However, the new and additional predominant conformer filled the extra space near Val114 and exhibited two new π interactions with Phe108 and Phe115. It should be recognized that the halogen- π distances in this and the previous analysis¹⁵ based on crystal structures are greater than the sum of van der Waals radii. In a crystallographic snapshot, the electron density of the ligand is an average of conformations packed in the crystal lattice. It is also important to note that the sigma hole of the bromine in these alkyl bromide type molecules is typically less pronounced than in arylhalides, leading to weaker interactions.³⁵ Overall, apart from the described angle and distance criteria based on the crystallographic structures, future studies involving multiple methodologies could help to further characterize halogen- π interactions. Possible approaches include measurement of melting thermodynamics using differential scanning calorimetry and computational methods that generate scoring functions for halogen- π contacts and predict associated energies via quantum mechanical analysis.³⁶

Most CYP2B structures to date represent complexes with inhibitors rather than substrates. This includes the first ligand complexes of CYP2B4, CYP2B6, CYP2B35 and 2B37 with the small inhibitor 4-(4-chlorophenyl) imidazole and the complexes of CYP2B4 and 2B6 with the larger amlodipine.²⁶⁻²⁸ The coordinate bond between a nitrogen in the inhibitor and heme iron likely yielded higher affinity and stable complex formation that aided in successful crystallization. More recently, monoterpenes have proven to be ideal active site probes to study human CYP2B6 interaction with environmentally important natural products.^{29,32} Unlike inhibitors that ligate to the heme iron, these molecules are substrates with relatively free rotation in the active site and yet bind very tightly with CYP2B6. Combined with their high binding affinity and freedom of rotation in the active site, the small size of these monoterpenes enabled us to determine structurally the role of halogen- π interactions in ligand binding to CYP2B6 selectivity. This would be considerably more difficult with larger and more complex ligands. The K_s values for each of these monoterpenes ranged from 0.1 to 0.3 μ M, except for the CYP2B6 (I114V) complex, which demonstrated a 2-fold decrease (0.6 μ M) in binding affinity compared with the wild-type

enzyme (Supporting Table 2). The lack of effect of the bromine on binding affinity likely reflects the enthalpy-entropy compensation discussed previously in regards to the halogen bond.³⁷ Thus, a structural and thermodynamic study conducted using the DNA-Holiday junction as the model system revealed the improvement of halogen bonding ability going from F to Cl to Br to I and the direct relation toward a more ideal geometry.³⁷ The results further indicated that the I-bond is more enthalpically favorable than that of bromine.³⁸ However, this favorable enthalpy was at an entropic cost, with the Br-bond being associated with a slight increase in entropy and the I-bond with negative ΔS , suggesting that the free energy of stabilization is less favorable for the I-bond than for the Br-bond.

To explore possible halogen- π interactions between other CYPs and their ligands besides CYP2B6, crystal structures of drug metabolizing CYP2 and CYP3 subfamily of enzymes in the PDB were analyzed (August 2016 release). Despite the presence of more phenylalanine residues in the active site of CYP2A6 than CYP2B6, none of the structures of CYP2A6 in the PDB contained a halogenated ligand. Interestingly, Phe297 in CYP2B6, which plays an important role in halogen- π interactions, corresponds to Iso300 in CYP2A6 (Supporting Figure 3A).²⁹ In contrast to the Phe-rich CYP2A6 and 2B6, the active site of CYP2C8 and CYP2C9 contains only one phenylalanine residue (Phe205 and Phe114, respectively), which does not interact with the halogenated ligand (Supporting Figure 3B). Like the CYP2C enzymes, CYP2D6 lacks the phenylalanine residues needed for halogen bonding, whereas similar to CYP2A6, CYP2E1 contains appropriate phenylalanine residues but shows no evidence of halogen bonding in known structures. However, CYP2B6 is not alone in forming halogen- π bonds. The ketoconazole complex of CYP3A4 demonstrates that the -Cl of each of the multiple drug molecules in the active site interacts with either Phe213 or Phe241, and the bromoergocriptine complex shows a Br- π bond with the aromatic Tyr53 (Supporting Figure 3, C and D).^{39,40} Thus, analysis of existing structures suggests that halogen- π interactions may be unique to the 2B enzymes within CYP family 2 but are also important for CYP3A4.

Our structural studies suggest that the mechanism by which halogenated ligands preferentially interact with CYP2B enzymes involves the presence of multiple phenylalanine side chains in the active site that form π bonds with halogens. The results provide important insights into the role of such halogen interactions with aromatic side chains that facilitate the orientation of ligands near heme that are conducive to metabolism by CYP2B enzymes. The current observation is not just limited to halogenated drugs on market that are preferred substrates of CYP2B6, but also extends to environmental contaminants. These include the most abundant polybrominated diphenyl ether congeners in humans (BDE-47),⁴¹ the polychlorinated biphenyls (PCB-153),⁴² and the commonly used halogenated pesticide, Chlorpyrifos,⁴³ each of which is metabolized with high affinity and selectivity by CYP2B6 (Supporting figure 4). A fundamental question has been how halogenated environmental contaminants make specific contacts with the active site of CYP2B6 that lead to tight binding and selective oxidation by this enzyme as opposed to other human hepatic P450 enzymes with binding pockets of comparable size and shape. The results of the present study of brominated monoterpenes provide strong suggestive evidence that halogen- π bonds are important determinants of CYP2B6 action on drugs and environmental compounds. Efforts

are currently underway to further test this hypothesis using halogenated drug substrates of CYP2B6 and their non-halogenated analogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CYP	Cytochrome P450
CYP2B6	an N-terminally truncated and modified and C-terminally His-tagged form of the cytochrome P450 2B6 genetic variant K262R with an internal mutation Y226H
RMSD	root-mean-square deviation
SSRL	Stanford Synchrotron Radiation Lightsource
PDB	Protein Data Bank

Figure 1.

Halogen- π interactions in CYP2B structures. Ligands are shown in yellow, and heme is depicted in red sticks: A, the Cl of the chlorophenyl moiety of ticlopidine coordinating Cl- π bond with the aromatic ring of F297 shown in stick representation in the rabbit CYP2B4 structure (green). B and C, demonstration of Cl- π interaction between the second amlodipine and the aromatic ring of F365(sticks) in the CYP2B4 complex (green). C, the altered orientation of amlodipine and substitution of methionine at position 365 in human CYP2B6 structure (pink) is shown in sticks.

Figure 2.

Representative geometric models of halogen- π interactions: A, the C-X- π systems considered in this study extends from bromine (X) to the centroid of the aromatic residue side chain (Phe, Tyr, Trp and His) in the protein, with θ approximately 120° or more and α less than 60° . B, the edge-on geometry where the halogen atom (X=bromine in this study) approach the periphery of the ring with a distance range of from $3.7 \text{ \AA}$ to around $4.3 \text{ \AA}$. The geometry is further defined by the difference between the halogen atom and the centroid or the nearest atom of the aromatic ring ($r-r' > 0.3 \text{ \AA}$).

Figure 3. Structures of CYP2B6-bornyl bromide complex (green) and CYP2B6-bornane complex (pink). Heme is shown as red sticks. Bornyl bromide and bornane are shown as blue sticks. A, an unbiased $F_o - F_c$ omit map in orange mesh at 3σ contour level calculated prior to inclusion of ligand demonstrates the well-defined electron density of bornyl bromide that corresponds to one large lobe with a bromine up orientation, and another minor lobe with bromine down orientation located near the heme iron. B, the bromine of bornyl bromide forming π bonds with the aromatic side chain of F108 is shown at appropriate distances and angles. C, two orientations were modeled with bornane in equal occupancy that were distinct from bornyl bromide. D, the location of C2 atom that bears the bromine atom in the major conformer of bornyl bromide is shown in the respective conformer of bornane. For clarity, the representative sketches of bornyl bromide and bornane have been incorporated in the bottom-right corner of panels A and C.

Figure 4.

Structures of CYP2B6 and CYP2B6 I114V in complex with (-)-myrtenyl bromide. A, an unbiased F_o-F_c omit map (orange mesh) at 3σ before modeling (-)-myrtenyl bromide in the CYP2B6 active site (cyan) in two orientations. The bromine up and bromine down orientations were modeled with equal occupancies in the active site. B, the bromine up orientation of (-)-myrtenyl bromide facilitates the π -bond with F297 side chain in the active site. C, the figure illustrates the two major electron density lobes in orange mesh with bromine up orientations and a minor bromine down orientation facing the heme iron in the CYP2B6 I114V structure (yellow). Compared with the CYP2B6 (-)-myrtenyl bromide complex, an additional bromine up orientation was evident in the vicinity of I114V mutation in the active site. D, the two predominant orientations of (-)-myrtenyl bromide coordinating π interactions with three crucial aromatic side chains of F108, F115 and F297 in the active site. The new orientation allows additional π -bond formation compared to the wild-type enzyme. Heme is shown in red and (-)-myrtenyl bromide in light blue sticks. The (-)-myrtenyl bromide sketch is provided in the bottom-right corner of panels A and C.