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Short Communication

1-Aminobenzotriazole Coincubated with (*S*)-Warfarin Results in Potent Inactivation of CYP2C9[S]

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ABSTRACT

1-Aminobenzotriazole (ABT) is a nonselective, mechanism-based inactivator of cytochrome P450 (P450) and a useful tool compound to discern P450- from non-P450-mediated metabolism. ABT effectively inactivates major human P450 isoforms, with the notable exception of CYP2C9. Here we propose that ABT preferentially binds to the warfarin-binding pocket in the CYP2C9 active-site cavity; thus, ABT bioactivation and subsequent inactivation is not favored. Therefore, coincubation with (*S*)-warfarin would result in displacement of ABT from the warfarin-binding pocket and subsequent binding to the active site, converting ABT into a potent inactivator of CYP2C9. To test this hypothesis, in vitro studies were conducted using various coincubation combinations of ABT and (*S*)-warfarin or diclofenac to modulate the effectiveness of CYP2C9 inactivation by ABT. Coincubation of ABT with (*S*)-warfarin (diclofenac probe substrate) resulted in potent inactivation, whereas weak inactivation was observed following coincubation of ABT with diclofenac [(*S*)-warfarin probe substrate]. The kinetic parameters of time-dependent inhibition of ABT for CYP2C9 in the absence and presence of (*S*)-warfarin (20 μM) were 0.0826 and 0.273 min^{-1} for k_{inact} and 3.49 and 0.157 mM for K_I, respectively. In addition, a 73.4-fold shift was observed in the in vitro potency (k_{inact}/K_I ratio), with an increase from 23.7 ml/min/mmol (ABT alone) to 1740 ml/min/mmol [ABT with (*S*)-warfarin (20 μM)]. These findings were supported by in silico structural modeling, which showed ABT preferentially binding to the warfarin-binding pocket and the displacement of ABT to the active site in the presence of (*S*)-warfarin.

Introduction

1-Aminobenzotriazole (ABT; Fig. 1A), a useful tool compound that nonselectively inhibits and inactivates human and nonhuman cytochrome P450 (P450) enzymes, is used in in vitro reaction phenotyping and enzyme identification studies to assess P450- versus non-P450-mediated metabolism (Emoto et al., 2005; Strelevitz et al., 2006). Inactivation of P450 by ABT is known to require bioactivation via oxidation of the aminotriazole to form the reactive intermediate, benzyne, which subsequently reacts with the heme to form a covalent adduct (Ortiz de Montellano and Mathews, 1981). The effects of ABT pretreatment on major human P450 enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A) in human liver microsomes (HLM) have been reported (Emoto et al., 2003; Linder et al., 2009) and resulted in almost complete loss of activity for most P450 isoforms; however, ABT was not an effective inactivator for CYP2C9. ABT was shown to be a weak inactivator, with 58% of diclofenac 4′-hydroxylase activity remaining after a 30-minute preincubation with 1 mM ABT (Linder et al., 2009).

Many therapeutic drugs are metabolized by CYP2C9, which exhibits selectivity for the oxidation of small, lipophilic anions such as the anticoagulant (*S*)-warfarin (Fig. 1B) (Rettie et al., 1992; Kaminsky and Zhang, 1997; Miners and Birkett, 1998; Takahashi and Echizen, 2001) and the nonsteroidal anti-inflammatory drug diclofenac (Fig. 1C) (Leemann et al., 1993). CYP2C9 has a relatively large active-site cavity (approximately 470 Å^3) containing two major binding sites (the distant binding pocket is referred to here as the "warfarin-binding pocket" and the binding region near the heme is referred to as the "active site") that can simultaneously accommodate multiple small molecules (Williams et al., 2003).

Here we investigated the lack of inactivation of CYP2C9 by ABT in HLM. ABT was coincubated with either (*S*)-warfarin or diclofenac, two preferred probe substrates of CYP2C9 known to bind to different regions within the active-site cavity and show differential inhibition potency toward competitive inhibitors of CYP2C9 (Kumar et al., 2006; U.S. Department of Health and Human Services, Food and Drug Administration, 2006). The in vitro results revealed preferential binding of ABT to the warfarin-binding pocket in the CYP2C9 active-site cavity, and these findings were further rationalized using in silico structural modeling tools.

Materials and Methods

Pooled HLM (both male and female; 150 donors; protein content of 20 mg/ml) were purchased from BD Biosciences (San Jose, CA) and stored at −80°C. Potassium phosphate buffer (KPi; pH 7.4; 100 mM) was provided by the Media Preparation facility at Genentech, Inc. Dimethylsulfoxide, formic acid, NADPH, and (*S*)-warfarin were purchased from Sigma-Aldrich (St. Louis, MO). Diclofenac and tienilic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). ABT was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Phenyl-d_5-7-hydroxywarfarin and 4′-hydroxydiclofenac-[$^{13}C_6$] (stable labeled metabolite internal standards) were purchased from BD Biosciences. High-performance liquid chromatography–grade acetonitrile was purchased from Honeywell Burdick & Jackson (Muskegon, MI).

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ABBREVIATIONS: ABT, 1-aminobenzotriazole; HLM, human liver microsomes, K_I, inactivator concentration that supports half the maximal inactivation rate, k_{inact}, maximal rate of enzyme inactivation; LC-MS/MS, liquid chromatography–tandem mass spectrometry; P450, cytochrome P450; TDI, time-dependent inhibition.

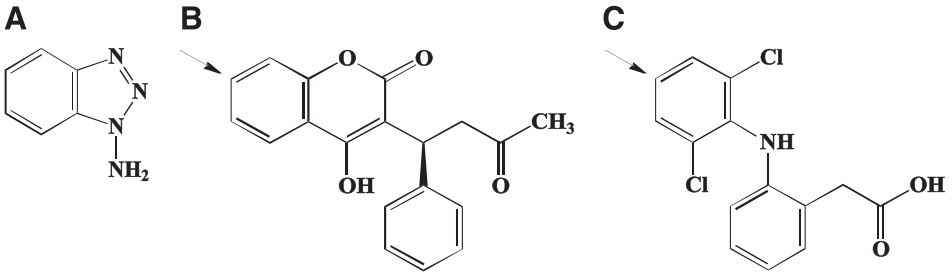

Fig. 1. Chemical structures of ABT (A), (*S*)-warfarin (B), and diclofenac (C). The CYP2C9-mediated sites of oxidation leading to 7-hydroxywarfarin (B) and 4′-hydroxydiclofenac (C) are indicated with arrows.

Inhibition of CYP2C9 Activity. ABT (0–10 mM), ABT (0–10 mM) with (*S*)-warfarin (2 μM), or (*S*)-warfarin (0–100 μM) was coincubated with the CYP2C9 probe substrate diclofenac (5 μM) and ABT (0–10 mM), ABT (0–10 mM) with diclofenac (5 μM), or diclofenac (0–100 μM) was coincubated with the CYP2C9 probe substrate (*S*)-warfarin (2 μM) at 37°C with HLM (final protein concentrations of 0.05 mg/ml [(*S*)-warfarin probe] and 0.2 mg/ml [diclofenac probe]). The reactions (n = 3) were initiated by the addition of NADPH (1 mM final concentration), incubated for 30 minutes [(*S*)-warfarin probe] or 10 minutes (diclofenac probe), and terminated by the addition of 0.1% formic acid in acetonitrile containing a stable labeled metabolite internal standard. The optimal incubation conditions (K_m values, HLM protein concentrations, and incubation times) for the probe substrates (*S*)-warfarin and diclofenac were determined in our laboratory and the formations of 7-hydroxywarfarin and 4′-hydroxydiclofenac were linear up to 60 minutes (data not shown). The samples were centrifuged (2000*g* for 5 minutes); the supernatants (50 μl) were removed, diluted with 0.1% formic acid in water (100 μl), and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Time-Dependent Inhibition of CYP2C9 Activity. The maximal rate of enzyme inactivation (k_{inact}) and the inactivator concentration that supports half the k_{inact} (K_I) for CYP2C9 were determined in HLM. Either ABT (0.014–10 mM) or tienilic acid (0.01–5.0 μM) alone, ABT (0.014–10 mM) or tienilic acid (0.01–5.0 μM) and (*S*)-warfarin (0.2, 2.0, or 20 μM), or ABT (0.014–10 mM) or tienilic acid (0.01–5.0 μM) and diclofenac (0.5, 5.0, or 50 μM) were preincubated at 37°C with HLM (1.0 mg/ml) and NADPH (1.3 mM) in KPi (100 mM; pH 7.4) for 0.5, 2.5, 9, 17, and 25 minutes for ABT (n = 3) or 0.5, 1, 2.5, 5, and 10 minutes for tienilic acid (n = 3). The final incubation volume was 100 μl, and the final solvent content in the incubation was 0.05% dimethylsulfoxide and 0.95% acetonitrile. After the initial incubation, a 20-fold dilution was performed into a secondary incubation containing diclofenac (30 μM final concentration) or (*S*)-warfarin (20 μM final concentration) and NADPH (1.3 mM) in KPi (100 mM; pH 7.4). The secondary incubation (200 μl final volume) proceeded for 15 minutes and was quenched with formic acid (3%) in acetonitrile (50 μl) containing a stable labeled metabolite internal standard. The samples were centrifuged (2000*g* for 5 minutes) and analyzed by LC-MS/MS.

LC-MS/MS Analysis. The prepared samples were analyzed by LC-MS/MS for 7-hydroxywarfarin and phenyl-d_5-7-hydroxywarfarin as described previously (Halladay et al., 2011). The equipment and LC conditions were the same for 4′-hydroxydiclofenac and 4′-hydroxydiclofenac-[$^{13}C_6$] analysis, and the multiple reaction monitoring (MRM) transitions in positive ion mode were m/z 313.4 → m/z 232.1 (4′-hydroxydiclofenac) and m/z 319.1 → m/z 238.0 (4′-hydroxydiclofenac-[$^{13}C_6$]).

Data Analysis. The IC_{50} values were determined using the GraphPad Prism version 5.0 by GraphPad Software (La Jolla, CA). For k_{inact} and K_I calculations, the amount of metabolite formed at each concentration relative to a vehicle control was calculated, and k_{obs} (the observed first-order inactivation rate constant) was determined by linear fit of the natural logarithm of percent remaining activity versus time for each concentration of test compound ([*I*]). Apparent K_I and k_{inact} were estimated using nonlinear regression in GraphPad Prism (Wang et al., 2004).

In Silico Modeling. ABT alone and ABT simultaneously docked with another molecule of ABT, (*S*)-warfarin, or diclofenac were simulated within the active-site cavity of human CYP2C9 (PDB code 1OG5) (Williams et al., 2003). Each ligand was positioned into the active site of CYP2C9 using PyMOL Molecular Graphics System, Version 1.6 (Schrödinger, LLC, Portland, OR). Force field parameters and electrostatics for each resulting ligand-receptor complex were obtained from the CHARMM 27 biomolecular force field (MacKerell et al., 2000-2001). Severe interatomic clashes were alleviated in NAMD (Humphrey et al., 1996) via 100 steps of molecular mechanics optimization. Further structural relaxation was effected in NAMD via 1000 steps of molecular dynamics simulation at 298 K via a constant temperature and pressure ensemble. The electrostatic energies of ABT and each of the codocked ligands were calculated using Gaussian 09 (Gaussian, Inc., Wallingford, CT). The fold differences of the binding free energies of the simulations were determined by comparing the free energies with ABT and another molecule of ABT, (*S*)-warfarin, or diclofenac to the free energy with ABT alone, where a difference of 1.36 kcal/mol equates to a 10-fold difference (Freire, 2008).

Results and Discussion

ABT is a nonselective inhibitor and time-dependent inactivator of major human P450 enzymes (Ortiz de Montellano and Mathews, 1981; Emoto et al., 2005). However, ABT ineffectively inhibits and inactivates CYP2C9, in contrast to almost complete activity loss of other P450 isoforms (Emoto et al., 2003; Linder et al., 2009). Results shown here suggest that the inefficiency of ABT to inactivate CYP2C9 may be attributed to multiple binding sites within the large active-site cavity (approximately 470 Å^3) (Williams et al., 2003; Seifert et al., 2006). For example, a crystal structure of CYP2C9 provides evidence for preferential binding of (*S*)-warfarin to a binding pocket near the active site, termed the warfarin-binding pocket (Williams et al., 2003). When bound in this predominantly hydrophobic pocket, (*S*)-warfarin is ~10 Å from the heme, representing a nonproductive binding orientation too distant to interact with the heme and consequently be hydroxylated. When bound in the warfarin-binding pocket, there is ample space for a second small molecule, such as flurbiprofen or even another molecule of (*S*)-warfarin, to bind at the active site and interact with the heme (Williams et al., 2003; Seifert et al., 2006). Similar to flurbiprofen, diclofenac shows evidence of preferential binding in a region that is much closer to the active site, with a distance of 4.7 Å between C-4′ and the heme (Melet et al., 2003; Yan et al., 2005). In vitro and in silico data reported here support this two-site model of CYP2C9 binding as well as the preferential binding of ABT to the warfarin-binding pocket. To test this hypothesis, experiments were designed to displace ABT using the preferred probe substrates of CYP2C9, (*S*)-warfarin and diclofenac, which bind to different binding sites in the active-site cavity (Kumar et al., 2006; U.S. Department of Health and Human Services, Food and Drug Administration, 2006). The potency of ABT inactivation of CYP2C9 was enhanced when copreincubated with (*S*)-warfarin but not with diclofenac. The in silico modeling results further support these findings.

In the CYP2C9 inhibition studies, ABT inhibited the formation of 4′-hydroxydiclofenac with an IC_{50} of 5320 μM (Table 1). However, when coincubated with (*S*)-warfarin (2 μM), the IC_{50} of ABT decreased 4.4-fold to 1210 μM. In contrast, ABT effectively inhibited CYP2C9 activity when (*S*)-warfarin was the probe substrate by inhibiting 7-hydroxywarfarin formation with an IC_{50} of 370 μM.

TABLE 1

Inhibition of CYP2C9: IC_{50} values (± S.D.) for ABT, (*S*)-warfarin, and diclofenac incubated discretely and ABT incubated with either (*S*)-warfarin or diclofenac

Probe Substrate (K_m; Incubation Concentration)	Inhibitor + Coincubated Analyte (Concentration)	Concentration Range of Inhibitor	IC_{50}
		μM	
Diclofenac (5 μM)	ABT	0–10,000	5320 ± 1080
	ABT + (*S*)-Warfarin (2 μM)	0–10,000	1210 ± 259
	(*S*)-Warfarin	0–100	6.28 ± 2.39
(*S*)-Warfarin (2 μM)	ABT	0–10,000	370 ± 12
	ABT + Diclofenac (5 μM)	0–10,000	394 ± 61
	Diclofenac	0–100	16.9 ± 5.0

Addition of diclofenac (5 μM) in the incubation with ABT did not modulate 7-hydroxylase activity, as the IC_{50} value was comparable (IC_{50} of 394 μM) to that of ABT alone. In summary, inhibition of CYP2C9 by ABT was probe-dependent: only (*S*)-warfarin (2 μM) modulated the inhibitory activity of ABT when coincubated, suggesting competitive binding between ABT and (*S*)-warfarin to the same binding site within the active-site cavity.

A concentration-dependent change in the time-dependent inhibition (TDI) kinetic parameters was observed when increasing concentrations of (*S*)-warfarin were copreincubated with ABT (Table 2). The k_{inact} and K_I were 0.0826 min^{-1} and 3.49 mM, respectively, when ABT (0.014–10 mM) was preincubated for 30 minutes. The k_{inact}/K_I ratio was 23.7 ml/min/mmol. When TDI was assessed with increasing concentrations of (*S*)-warfarin (0.2, 2.0, and 20 μM) and ABT (0.014–10 mM) in the preincubation, an increase in magnitude of TDI was observed. Increasing concentrations of (*S*)-warfarin up to 20 μM and ABT (0.014–10 mM) increased the k_{inact} 3.31-fold, decreased the K_I of ABT 22.2-fold, and increased the k_{inact}/K_I ratio 73.4-fold compared with ABT (0.014–10 mM) alone. We attribute this modulation of CYP2C9 inactivation to the displacement of ABT binding from the warfarin-binding pocket by (*S*)-warfarin, resulting in the binding of ABT at the active site where bioactivation occurs. When TDI was assessed with (*S*)-warfarin as the probe substrate and ABT (0.014–10 mM) with increasing concentrations of diclofenac (0.5, 5.0, and 50 μM) in the preincubation, direct inhibition dominated and TDI kinetic parameters could not be calculated. In contrast to ABT, when TDI was assessed for tienilic acid (0.01–5 μM), a CYP2C9-specific mechanism-based inactivator (Melet et al., 2003), with increasing concentrations of (*S*)-warfarin (0.2, 2.0, and 20 μM) in the preincubation, an increase in the magnitude of TDI was not observed. This observation was attributed to the preferential binding of tienilic acid at the active site and not at the warfarin-binding pocket (Melet et al., 2003). The inactivation plots of CYP2C9 for ABT or tienilic acid alone and copreincubated with various concentrations of (*S*)-warfarin using diclofenac as a probe substrate are provided (Supplemental Figs. 1 and 2).

The in vitro data were supported by in silico molecular dynamics docking studies with CYP2C9 (PDB code 1OG5), which were performed with ABT alone, as well as with another molecule of either ABT, (*S*)-warfarin, or diclofenac (Fig. 2, A–D). Identical docking parameters and settings were used in these simulations, and the free energies of binding (kcal/mol) and distances from the molecules to the heme are recorded in Table 3. The simulation with ABT alone showed preferential binding of ABT at the warfarin-binding pocket, the same hydrophobic pocket that (*S*)-warfarin favorably binds to in CYP2C9 (Fig. 2A). In this model, residues Gly98, Ile99, Phe114, Leu366, and Pro367 surrounded ABT. More specifically, the triazole moiety of ABT interacted with the hydrophobic residue Gly98 and the aminotriazole of ABT faced away from the heme, providing further evidence for the ineffectiveness of ABT to be bioactivated and consequently inactivating the enzyme. The free energy calculation was favorable at −7.1 kcal/mol (Table 3). The distance from the heme to the nearest carbon of ABT was 13 Å, which would be too distant from the heme for oxidation to occur. Two molecules of ABT could be docked simultaneously, with one ABT molecule bound at the warfarin-binding pocket (as described above) and the other ABT molecule bound at the active site in proximity to the heme (Fig. 2B). Val113, Phe114, Ala297, and Thr301 surrounded ABT at the active site, and the aminotriazole was oriented 1.9 Å from the heme to allow oxidation (Table 3). The binding energy of two ABT molecules was less favorable, with a 4.4-fold decrease in the free energy (−6.5 kcal/mol) compared with only one ABT molecule docked, which may explain the observed weak inactivation of CYP2C9 by ABT. ABT and (*S*)-warfarin codocked favorably with ABT bound at the active site and (*S*)-warfarin bound at the warfarin-binding pocket (Fig. 2C). In this simulation, residues Gly98, Ile99, Phe100, Phe114, Leu366, Pro367, Leu388, and Phe476 surrounded (*S*)-warfarin in the warfarin-binding pocket. In particular, the phenyl group of (*S*)-warfarin interacted with the residues Phe100, Pro367, and Phe476. The position of (*S*)-warfarin was 9.7 Å from the heme (Table 3). ABT bound at the active site (as described above) oriented in close proximity to the heme for oxidation of the aminotriazole. The free

TABLE 2

Kinetic parameters (± S.D.) of time-dependent inhibition for ABT (0.014–10 mM) in the absence and presence of (*S*)-warfarin with diclofenac probe substrate

30-Minute Preincubation Conditions		k_{inact}	K_I	k_{inact}/K_I
ABT	(*S*)-Warfarin			
mM	*μM*	*min^{-1}*	*mM*	*ml/min/mmol*
0.014–10	0	0.0826 ± 0.0157	3.49 ± 1.72	23.7
	0.2	0.0856 ± 0.0092	1.01 ± 0.21	84.7
	2.0	0.188 ± 0.009	0.396 ± 0.047	475
	20	0.273 ± 0.032	0.157 ± 0.044	1740

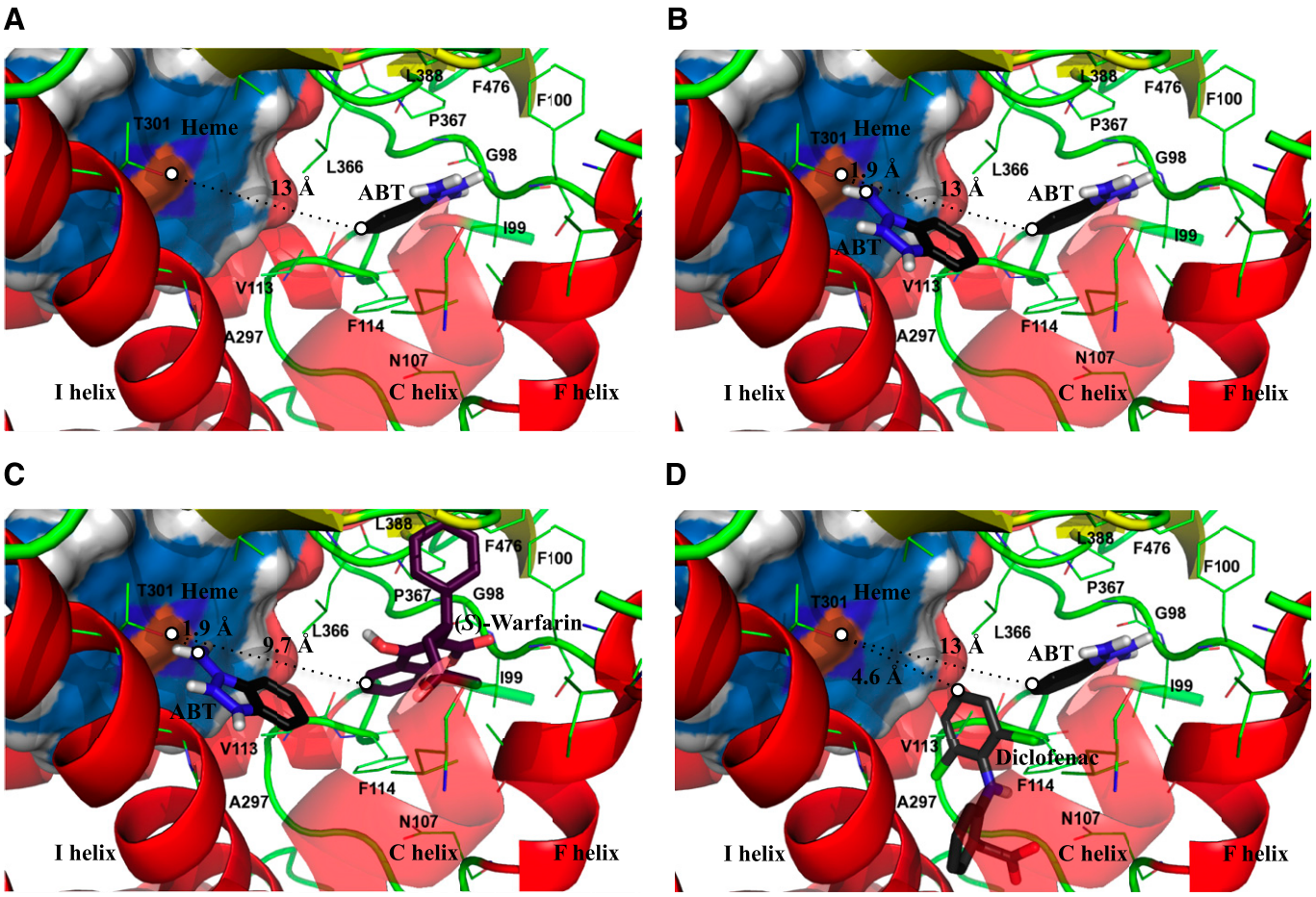

Fig. 2. Molecular docking of CYP2C9 (PDB code 1OG5) with ABT docked individually (A) and ABT simultaneously docked with another molecule of ABT (B), (*S*)-warfarin (C), or diclofenac (D). The figure shows the CYP2C9-drug complexes deduced from molecular docking simulations using PyMOL Molecular Graphics System, Version 1.6. Identical docking parameters and settings were used in these simulations, and the distances (Å) from the molecules to the heme are represented.

energy calculation for this orientation was highly favorable at −8.1 kcal/mol, which was 7.4-fold and 11.8-fold more favorable than docking either ABT alone or docking two ABT molecules, respectively. This simulation demonstrates how the coincubation of ABT and (*S*)-warfarin results in effective inhibition and inactivation of CYP2C9 in the in vitro studies. Codocking simulations of ABT and diclofenac predict favorable and preferential binding of ABT at the warfarin-binding pocket and binding of diclofenac at the active site (Fig. 2D). In this model, ABT was bound at the warfarin-binding pocket as described above and diclofenac was bound at the active site with a free energy calculation of −7.0 kcal/mol (Table 3). Diclofenac was surrounded by Asn107, Val113, Phe114, and Ala297 and bound at the active site. In addition, the dichlorophenyl moiety (C-4′) of diclofenac was positioned 4.6 Å away from the heme, which is comparable to the reported distance by Yan et al. (2005), and 4′-hydroxylation is favored.

TABLE 3

Free energy calculations and binding distances from the heme after molecular docking of ABT alone and codocking of ABT and (*S*)-warfarin or diclofenac to CYP2C9

Docking Combinations	Free Energy of Binding	Fold Difference of Free Energy[a]	R(X-Fe)[b]	
			ABT	Coanalyte[c]
	kcal/mol		*Å*	
ABT	−7.1	N.A.	13	N.A.
ABT + ABT	−6.5	−4.4	1.9	13
ABT + (*S*)-warfarin	−8.1	7.4	1.9	9.7
ABT + diclofenac	−7.0	−0.74	13	4.6

N.A., not applicable.

[a]Fold differences of free energy of binding for ABT with either another molecule of ABT, (*S*)-warfarin, or diclofenac were based on ABT alone. A free energy of binding difference of 1.36 kcal/mol is equal to a 10-fold difference. A negative fold difference indicates a decrease in the fold difference.

[b]R(X-Fe) = closest atom to the heme.

[c]Coanalyte includes ABT, (*S*)-warfarin, or diclofenac, depending on the docking combination.

These results may have broader implications, as careful consideration must be used when choosing appropriate probe substrates for assessing CYP2C9-mediated interactions, as well as other P450 isoforms with similar dual-binding active sites, such as CYP3A4 (Korzekwa et al., 1998; Shou et al., 1999; Schrag and Wienkers, 2001). As noted here and previously, CYP2C9 will be inhibited or inactivated to various degrees depending on how and where the drug of interest binds in the CYP2C9 active-site cavity (i.e., warfarin-binding pocket versus active site), and this will impact which probe is more appropriate to assess the activity change of CYP2C9 [i.e., (*S*)-warfarin versus diclofenac] (Kumar et al., 2006; Hutzler et al., 2009). When the drug of interest is suspected or known to bind in the warfarin-binding pocket, (*S*)-warfarin would be a more sensitive CYP2C9 probe substrate than other probe substrates, such as diclofenac, tolbutamide, and phenytoin (Kumar et al., 2006), because these substrates will be able to enter and exit the CYP2C9 active site freely, resulting in an underprediction of potential inhibition. Similarly, when the drug of interest is known to bind at the active site, diclofenac would be an appropriate probe substrate to assess inhibition. To minimize the risk of probe-dependent differences in drug discovery, in vitro inhibition and inactivation studies should include either two probes assessed separately, such as (*S*)-warfarin and diclofenac, or a single probe, (*S*)-warfarin, as this compound appears to be a more sensitive probe to determine changes of CYP2C9 activity (Kumar et al., 2006; Hutzler et al., 2009). This screening paradigm is routinely used for assessing the inhibition and inactivation of CYP3A using probe substrates such as testosterone and midazolam (Kenworthy et al., 1999).

Department of Drug Metabolism and Pharmacokinetics (J.K.S., S.M., S.W., C.E.C.A.H., S.C.K., J.S.H.) and Department of Safety Assessment (K.A.F.), Genentech, Inc., South San Francisco, CA

Jasleen K. Sodhi
Kevin A. Ford
Sophie Mukadam
Susan Wong
Cornelis E. C. A. Hop
S. Cyrus Khojasteh
Jason S. Halladay

Authorship Contributions

Participated in research design: Sodhi, Halladay.

Conducted experiments: Sodhi, Ford, Mukadam, Wong, Halladay.

Contributed new reagents or analytic tools: Sodhi, Ford, Halladay.

Performed data analysis: Sodhi, Mukadam, Halladay.

Wrote or contributed to the writing of the manuscript: Sodhi, Hop, Khojasteh, Halladay.

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Address correspondence to: Jason S. Halladay, Genentech, Inc., 1 DNA Way, MS 412a, South San Francisco, CA 94080. E-mail: halladay@gene.com