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High-Throughput Screening Assays for CYP2B6 Metabolism and Inhibition Using Fluorogenic Vivid Substrates

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ABSTRACT

CYP2B6 is a highly polymorphic P450 isozyme involved in the metabolism of endo- and xenobiotics with known implications for the activation of many procarcinogens resulting in carcinogenesis. However, lack of validated high-throughput screening (HTS) CYP2B6 assays has limited the current understanding and full characterization of this isozyme's involvement in human drug metabolism. Here, we have developed and characterized a fluorescence-based HTS assay employing recombinant human CYP2B6 and 2 novel fluorogenic substrates (the Vivid CYP2B6 Blue and Cyan Substrates). Assay validation included testing the inhibitory potency of a panel of drugs and compounds known to be metabolized by this isozyme, including CYP2B6 substrates, inhibitors, and known inducers. Compound rankings based on inhibitory potency in the Vivid CYP2B6 Blue and Cyan Assays matched compound rankings based on relative affinity measurements from previously published data (K_i, K_d, or K_m values) for the CYP2B6 isozyme. In conclusion, these assays are proven to be robust and sensitive, with broad dynamic ranges and kinetic parameters allowing screening in HTS mode of a large panel of compounds for CYP2B6 metabolism and inhibition, and are a valuable new tool for CYP2B6 studies.

KEYWORDS: Cytochrome P450, CYP2B6, fluorescent substrate, drug metabolism, high-throughput screening (HTS)

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INTRODUCTION

Cytochrome P450 enzymes (P450s) participate in a wide array of metabolic reactions, including oxidative biotransformations of many endobiotics, xenobiotics, drugs, toxins, and carcinogens. Several well-characterized members of the human P450 superfamily, such as CYP3A4, CYP2D6, and CYP2C9, play major roles in human drug metabolism.[1,2] High-throughput screening (HTS) assays for these isoforms have enabled researchers to determine substrate specificity, inhibition, and, in some cases, metabolic rates. Possible adverse drug reactions stemming from mechanism-based enzyme inhibition and potential drug-drug interactions can be predicted from this information. As similar HTS assays for other P450 isoforms are developed, it will become possible to assess the overall contributions of these additional P450 enzymes to human drug metabolism.

CYP2B6 participates in the metabolism of a wide variety of endogenous substrates, such as steroids, fatty acids, and retinoids,[3,4] and several clinically important drugs, such as cyclophosphamide, bupropion, and midazolam.[3] In addition, CYP2B6 is linked to the metabolism of several novel chemical entities (NCEs).[5] Nonetheless, an understanding of the extent of CYP2B6's involvement in drug metabolism, especially with drug candidates in development, remains incomplete. Although this isozyme represents only a small percentage of the total human hepatic complement of cytochrome P450 enzymes,[6] CYP2B6 may play a significantly more important role in overall oxidative drug metabolism than has previously been estimated.[7-9]

Two recent developments have contributed to CYP2B6's reevaluated significance in drug metabolism. First, improved isozyme-selective, quantitative immunodetection methods have detected a considerably higher rate of CYP2B6 expression in the population than was previously estimated.[10] Second, recent studies indicate that CYP2B6 may metabolize a substantially larger percentage of drugs in humans than was previously reported.[5,9,11,12] A quick review of recent literature on CYP2B6's involvement in xenobiotic metabolism demonstrates this isozyme's growing importance.[13,14] For example, a 3-dimensional quantita-

tive structure activity relationship (3D-QSAR) based on a set of 16 substrates has been generated for CYP2B6,[15,16] and pharmacophore models for the enzyme have been developed.[16,17] Even with this progress, characterization of CYP2B6 substrate specificity remains limited, primarily because of the lack of a systematic HTS approach; HTS has been successfully applied to determine the substrate specificity of other cytochrome P450 isozymes, such as CYP3A4 and CYP2D6.[18-21] With HTS technology, researchers can assess drug metabolism and inhibition for large panels of drugs and NCEs, thus supplying additional information on a wide variety of substrates and inhibitors for modeling studies. Though several coumarin derivatives, such as 7-ethoxy-4-trifluoromethylcoumarin (7-EFC),[7,22] 7-ethoxycoumarin,[23] and 3-cyano-7-ethoxycoumarin,[9] have been used as fluorescent substrates for CYP2B6, researchers have commented on the dearth of highly sensitive CYP2B6 substrates.[15]

The fluorogenic Vivid P450 Substrates have been designed to meet this critical need for HTS assay substrates[24] and have been applied successfully to study the metabolism and inhibition of various P450 isozymes.[25] Several distinctive properties of the Vivid P450 Substrates make them strong candidates for CYP2B6 HTS assay development. Vivid P450 Substrates are referred to as fluorogenic substrates because they were developed as "blocked" fluorophores with minimal background fluorescence signal. The fluorogenic Vivid P450 Substrates generate an intense fluorescence signal only after oxidative cleavage (metabolism) by a P450 enzyme. Furthermore, each Vivid P450 Substrate contains 2 potential cleavage sites[24,25]; oxidation at either site releases the highly fluorescent product. This characteristic of the Vivid P450 Substrates may contribute to the overall high rate of the reporter metabolic reaction. The relative fluorescence signal intensity generated in the Vivid P450 reporter reaction will decrease in the presence of an isozyme-specific substrate or inhibitor.

In this study, we employed the novel fluorogenic Vivid CYP2B6 Blue and Cyan Substrates to develop a fluorescence-based HTS assay with recombinant human CYP2B6 enzyme. CYP2B6 is a highly polymorphic enzyme with several identified allelic variants.[26] Recombinant human CYP2B6*1 (wild type), the most common variant of CYP2B6 in Caucasian and Japanese populations,[26,27] has been employed for assay development. We used this assay to evaluate the inhibitory potency of a selected panel of drugs and compounds, including known CYP2B6 substrates, mechanism-based inhibitors, and inducers. In addition, we compared the performance of the Blue and Cyan Substrates in HTS reactions with recombinant human CYP2B6 by evaluating each Vivid CYP2B6 Substrate's fluorescence and kinetic properties. Subsequently, we used both Vivid CYP2B6 Substrates to investigate the inhibitory potential of drugs and compounds having either a presumed or an unknown association with the CYP2B6 pathway. We obtained K_i values for these compounds in assays with the Blue and Cyan Substrates and compared them with published values, where available. As part of testing the reliability and quality of Blue and Cyan screening assays in HTS format, we assessed the assay linearity range, kinetic parameters (apparent V_{max} and K_m), solvent sensitivity, and inhibitory potential. Furthermore, we discuss the relative benefits of each fluorogenic Vivid CYP2B6 Substrate in specific applications.

MATERIALS AND METHODS

Chemicals

All reagents used were of the highest commercial grade available. The Vivid CYP2B6 Blue Substrate, Vivid CYP2B6 Cyan Substrate, corresponding fluorescent dye standards for the Blue and Cyan Substrates, and $NADP^+$ were obtained from PanVera (Madison, WI). The 7-EFC was obtained from Molecular Probes (Eugene, OR). Propofol (2,6-diisopropylphenol) was obtained from Acros Organics (Morris Plains, NJ). All other chemicals were obtained from Sigma-Aldrich Corp (St Louis, MO).

Enzymes

Microsomes from baculovirus-infected cells coexpressing CYP2B6 and NADPH-cytochrome P450 reductase (CYP2B6 BACULOSOMES Reagent) and an NADPH regeneration system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were obtained from PanVera.

Generation of Standard Curves With Fluorescent Dye Standards

All measurements were acquired at room temperature in Costar black, flat-bottom, 96-well plates (Corning Incorporated Life Sciences, Acton, MA) with a total volume of 0.1 mL/well. A stock solution of each fluorescent dye standard was prepared, serial dilutions were made, and fluorescence values were collected, following PanVera's Vivid Assay Protocol (http://www.panvera.com/tech/protocols/L0504.pdf). Fluorescence values were determined with a GEMINI XS fluorescence plate reader (Molecular Devices Corporation, Sunnyvale, CA) using the following settings: excitation wavelength 409 nm, emission wavelength 460 nm, and wavelength cutoff 455 nm

for the Blue Substrate; and excitation wavelength 400 nm, emission wavelength 502 nm, and wavelength cut-off 475 nm for the Cyan Substrate. The fluorescence values obtained in the reaction of each Vivid CYP2B6 Substrate with CYP2B6 enzyme were plotted to a calibration curve of a corresponding Vivid Standard and used to calculate the concentration of the fluorescent metabolite formed in the reaction. In some cases, the observed changes in fluorescence intensity were measured directly as relative fluorescence units (RFUs).

Assay Incubation Conditions

The reactions were performed in Costar black, flat-bottom, 96-well plates with a total reaction volume of 0.1 mL/well. The plates were incubated at room temperature, and fluorescence values were measured on a GEMINI XS fluorescence plate reader using the settings described above. Briefly, the CYP2B6 BACULOSOMES Reagent and the NADPH regeneration system (in the absence of $NADP^+$ and a fluorogenic Vivid CYP2B6 Substrate, but in the presence of one of the test compounds) were preincubated for 20 minutes at room temperature. The reaction was initiated with the addition of the combined $NADP^+$ and either the Blue or Cyan Substrate solution. The final assay components and conditions for the Blue Substrate were as follows: 5 µM Blue Substrate, CYP2B6 BACULOSOMES Reagent (5 nM recombinant human CYP2B6, coexpressed with NADPH-P450 reductase), the NADPH regeneration system (3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase), and 100 µM $NADP^+$ in a 100 mM potassium phosphate buffer (pH 8.0). The final assay components and conditions for Cyan Substrate were as follows: 2 µM Cyan Substrate, CYP2B6 BACULOSOMES Reagent (1 nM recombinant human CYP2B6, coexpressed with NADPH-P450 reductase), the NADPH regeneration system, and 30 µM $NADP^+$ in a 100 mM potassium phosphate buffer (pH 8.0).

Apparent V_{max} and K_m Determination

The assays for determining kinetic parameters for the CYP2B6 reaction with the Blue and Cyan Substrates were performed essentially as described in Assay Incubation Conditions above, except that the Blue or Cyan Substrate concentrations were varied for these determinations. The reaction rates were measured by following the increase in RFUs over the course of the reaction and using the calibration curve generated from the corresponding fluorescent dye standard. The kinetic parameters for the reactions were obtained by curve fitting and analysis using Prism software (GraphPad Software, Inc, San Diego, CA).

Comparison of Reaction Rate With Blue and Cyan Substrates and Another Commercially Available Fluorescent CYP2B6 Substrate

The assays for comparing the relative signal intensity generated in reactions of the CYP2B6 BACULOSOMES Reagent with the fluorogenic Blue and Cyan Substrates and with another generally available fluorescent substrate and reporter probe, 7-EFC,[28] were performed essentially as described in Assay Incubation Conditions above, except that each substrate concentration was 5 µM and recombinant human CYP2B6 concentration was 5 nM. The reaction rates were measured by following the increase in RFUs over the course of each reaction.

Drug and Test Compound Screening and Determining Apparent K_i Values

The Vivid CYP2B6 Assay with either the Blue or the Cyan Substrate can be used to rank compounds by their relative inhibitory potency by comparison of the compound's inhibition potency in the assay, for example, by apparent K_i values. The apparent K_i values for the drugs and test compounds listed in **Table 1** were determined. Each drug and test compound was initially prepared as a stock solution in a solvent as indicated in **Table 1**. The final concentration of the solvent did not exceed 0.5% (vol/vol), and proper solvent controls were used to rule out solvent inhibition at this concentration. Dose-response curves were generated as described previously.[25] The apparent K_i values were calculated for the test compounds using Prism software.

All assay components were prepared and combined, and all incubations were performed under conditions shown to be linear with respect to protein and substrate concentration and time following PanVera's Vivid Assay Protocol. Following this method, we screened commercially available drugs and test compounds (see **Table 1**) that could be classified into 3 groups:

1. compounds known to be CYP2B6 substrates, inducers, or inhibitors with published K_m, K_i, or IC_{50} (Inhibitory Concentration 50) values;
2. compounds that are associated with the CYP2B6 pathway but for which no published K_m, K_i, or IC_{50} values exist; and

Table 1. Comparison of Apparent K_i Values Determined in the Blue and Cyan Assay With Published Values for the CYP2B6 Enzyme*

Drug or Compound	Apparent K_i Values in Vivid CYP2B6 Assay (μM)		Solvent	Published Relative Affinity Values (μM)
	Blue	Cyan		
Acetaminophen	ND	ND	Water	—
Amitriptyline	29	43	Water	144 [16]
Bupropion	42	51	Water	85-156 [15, 29]
Caffeine	ND	ND	DMSO	—
Clotrimazole	0.52	0.99	DMSO	12 [30]
Cyclophosphamide	>1000	>1000	Water	—
Dextromethorphan	46	50	Water	350 [16]
Diallyl sulfide	ND	ND	Water	—
Diclofenac	200	260	Water	—
Diethyldithiocarbamate	410	545	Water	—
Diltiazem	290	460	Water	—
Ibuprofen	ND	ND	Methanol	—
Imipramine	47	85	Water	383 [16]
Ketoconazole	4.1	5.7	Methanol	—
Lidocaine	170	220	Water	—
S-Mephenytoin†	780	850	Water	564 [16]
Methimazole	>1000	>1000	Water	—
Metoprolol	ND	ND	DMSO	—
Miconazole	0.32	0.26	DMSO	0.05 [30]
Midazolam	65	80	DMSO	46 [9]
Nifedipine	8.3	7.1	DMSO	—
N-Nitrosodimethylamine	ND	ND	Water	—
Orphenadrine	82	180	Water	750 [31]
Phenethyl isocyanate	0.34	0.22	DMSO	1.5 [32]
Progesterone†	25	55	DMSO	—
Propofol	2.8	3.6	DMSO	10 [33]
Retinol	9.2	4.3	DMSO	—
Rifampicin	47	48	DMSO	—
Tamoxifen	4.2	1.6	Methanol	—
Thio-TEPA	6.2	5.7	Water	6.2 [34]
Tolbutamide	ND	ND	DMSO	—
Tranylcypromine	3.1	6	Water	—
Troleandomycin	ND	ND	DMSO	—
Verapamil	110	130	Water	137 [15]

*DMSO indicates dimethyl sulfoxide; ND, no inhibition detected when screened at 100 μM and 1 mM concentrations. The assays were performed essentially as described in Assay Incubation Conditions, but the concentrations of the different test compounds varied and the plates were read continuously for 10 minutes for assays with the Cyan Substrate and 20 minutes for assays with the Blue Substrate. Three groups of test compounds were assayed: drugs or compounds known to be CYP2B6 substrates or inhibitors (bold font); drugs or compounds that are associated with the CYP2B6 pathway but for which no published relative affinity values exist (standard font); and drugs or compounds that have not been previously associated with CYP2B6 metabolism (italic font). The solvent concentration in the reactions was 0.5% or less (vol/vol).

†Indicates data limited by solubility.

3. compounds that have not been implicated in CYP2B6 metabolism (a control group).

Solvent Effects

The effects of varying the concentrations of several organic solvents—ethanol (EtOH), methanol (MeOH), dimethyl sulfoxide (DMSO), and acetonitrile (ACN)—on the Blue and Cyan Assay were explored. The assays were performed essentially as described in Assay Incubation Conditions above, but each reaction contained varying amounts of one of the solvents (0%-1% levels [vol/vol]), and each assay was run for 10 minutes.

Dynamic Range and Z'-Factor

The assays for these experiments were performed essentially as described in Assay Incubation Conditions above but were run for 60 minutes. The dynamic range was calculated as a net fold increase in fluorescence signal intensity over the background signal. The Z'-factor was determined as described by Zhang and coworkers.[35]

RESULTS

Assay Linearity and Apparent V_{max} and K_m Determinations With the Fluorogenic Blue and Cyan Substrates

The linearity of metabolite formation in the reaction of the CYP2B6 isozyme with the fluorogenic Blue and Cyan Substrates was determined using varying concentrations of the CYP2B6 enzyme, as indicated in **Figure 1**. The readings were obtained in the kinetic mode using the instrument settings described in Generation of Standard Curves With Fluorescent Dye Standards above. With the Blue Substrate, the upper limit of the CYP2B6 linearity range was 2.5 nM protein and 5 µM Blue Substrate (**Figure 1A**), while with the Cyan Substrate, the upper limit of the CYP2B6 linearity range was 0.5 nM enzyme and 2 µM Cyan Substrate (**Figure 1B**), over 60 minutes, in room-temperature reaction. Protein concentrations of 5 nM and 1 nM were used in 15-minute assays for the apparent V_{max} and K_m determinations with the Blue and Cyan Substrates, respectively (**Figure 2**).

Assay kinetic parameters, such as apparent V_{max} and K_m values, provide important information about the interactions between a substrate and a metabolizing enzyme. CYP2B6 reactions with either the Blue or the Cyan Substrates followed classic Michaelis-Menten kinetics. The apparent V_{max} value calculated for the Cyan Substrate in the reaction with the CYP2B6 BACULOSOMES Reagent was approximately 3-fold higher than the apparent V_{max} value obtained in the reaction of the Blue Substrate with the CYP2B6 Baculosomes Reagent. In contrast, the apparent K_m value determined for the Cyan Substrate was almost 6-fold lower than the apparent K_m value determined for the Blue Substrate. The experimentally determined apparent K_m values (**Figure 2**) measured in the Blue and Cyan Substrates reactions with the recombinant CYP2B6 isozyme guided the concentration of each Blue or Cyan Substrate used in the competitive competition assays with many compounds.

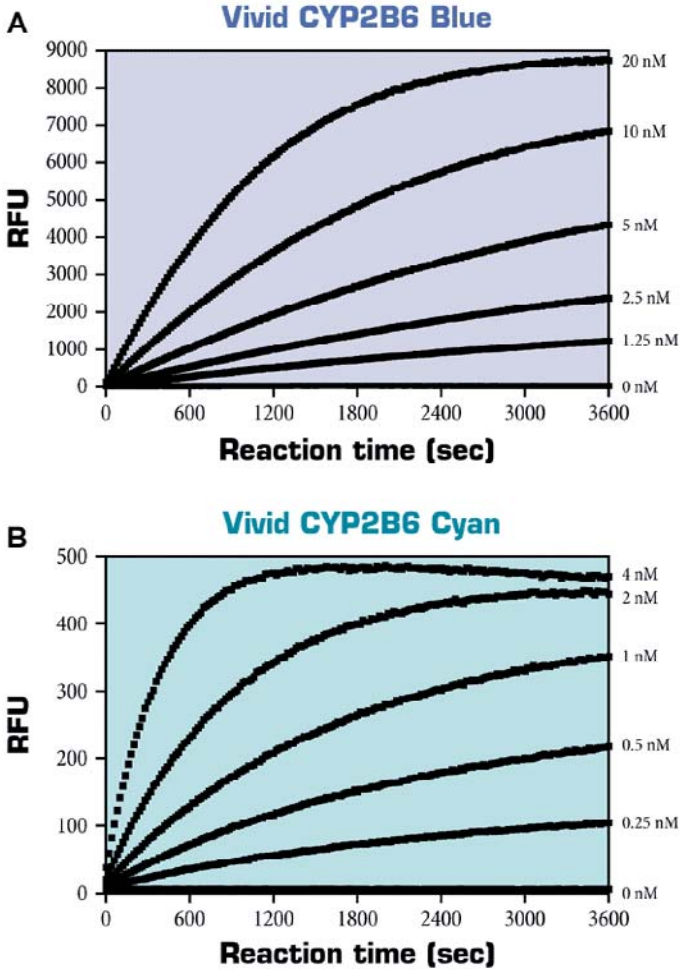

Figure 1. Linearity of the reaction over time with varying concentrations of recombinant human CYP2B6 enzyme: (A) with the Blue Substrate, and (B) with the Cyan Substrate. The assays were performed essentially as described in Assay Incubation Conditions. The plates were read continuously for 60 minutes.

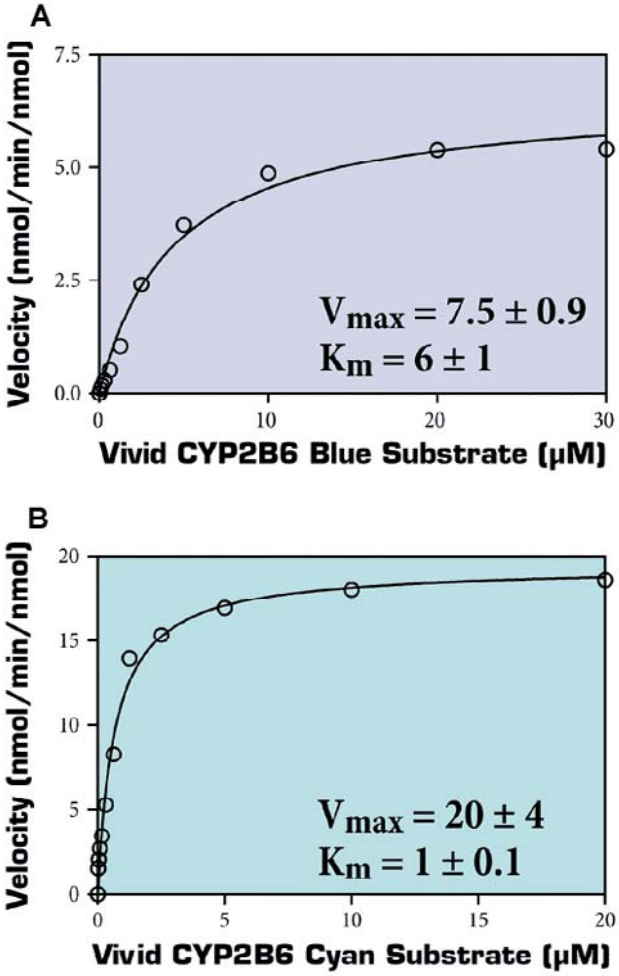

Figure 2. Kinetic parameters (apparent K_m and V_{max}) in the reaction of recombinant human CYP2B6 enzyme with (A) the Blue Substrate, and (B) the Cyan Substrate. The assays were performed essentially as described in Assay Incubation Conditions, but the Blue and Cyan Substrate concentrations ranged from 0 to 20 μM for the Cyan Substrate and from 0 to 30 μM for the Blue Substrate. Metabolite production and the reaction rate were quantified with the Vivid Blue and Cyan Standards.

Comparison of Reaction Rate With Another Fluorescent Substrate Previously Implicated in CYP2B6 Metabolism

We compared the relative signal intensity generated in the reaction of the CYP2B6 BACULOSOMES Reagent with the fluorogenic Blue and Cyan Substrates and with another generally available fluorescent substrate and reporter probe, 7-EFC, previously implicated in CYP2B6 metabolism and inhibition.[28] As **Figure 3** indicates, after CYP2B6 cleavage, Blue and Cyan Substrates produce a fluorescence signal 10- to 30-fold more intense than that obtained with 7-EFC in the reaction; the reaction with Blue Substrate yields the most intense fluorescence signal. At the substrate and enzyme concentrations used, the signal obtained with the Cyan Substrate is linear for only 10 minutes, while the signal obtained with the Blue Substrate remains linear for up to 40 minutes.

Solvent Effects

The choice of organic solvent used to solubilize drugs and test compounds can greatly affect CYP2B6 activity. Our results demonstrate that the various organic solvents commonly used to solubilize test compounds affect CYP2B6 activity with the Blue and Cyan Substrates differently (**Figure 4**). In the case of the metabolic reaction of the CYP2B6 Baculosomes Reagent with the Blue Substrate, the reaction rate was strongly affected by the solvent concentration in the reaction. For example, the presence of 1% EtOH in the assay mixture inhibited the initial reaction rate by 30% (**Figure 4A**, Panel 2) and the presence of 1% MeOH, DMSO, or ACN in the assay mixture inhibited the initial reaction rate by no more than 20% (**Figure 4A**, Panels 1, 3, and 4). In contrast, in the metabolic reaction of the CYP2B6 BACULOSOMES Reagent with the Cyan Substrate, the reaction rate was almost unaffected when the solvent concentration in the reaction was no greater than 1%. Under these conditions, only 1% EtOH inhibited the initial reaction rate, but by no more than 3% (**Figure 4B**, Panel 2). We used DMSO as the primary organic solvent for the drugs and compounds used in the Blue and Cyan Assays, with some exceptions described in **Table 1**.

Screening for Inhibitory Potency and Determining Apparent K_i Values

In an HTS screen, targets can be assessed by determining their apparent K_i values in the corresponding Vivid reporter assay. In this study, we used the Vivid CYP2B6 Assays to evaluate compounds that might be implicated in CYP2B6 metabolism—for instance, to identify CYP2B6 assay modifiers, including potential substrates, inhibitors, or inducers. Separate reporter reactions were run in parallel in HTS format with each fluorogenic Blue or Cyan Substrate, essentially as described in Assay Incubation Conditions above. As part of this quantitative assessment, we determined the apparent K_i values for various drugs and test compounds in the Vivid CYP2B6 Assay (presented in **Table 1**). The Blue and Cyan Assays detected all drugs and test compounds that were previously implicated

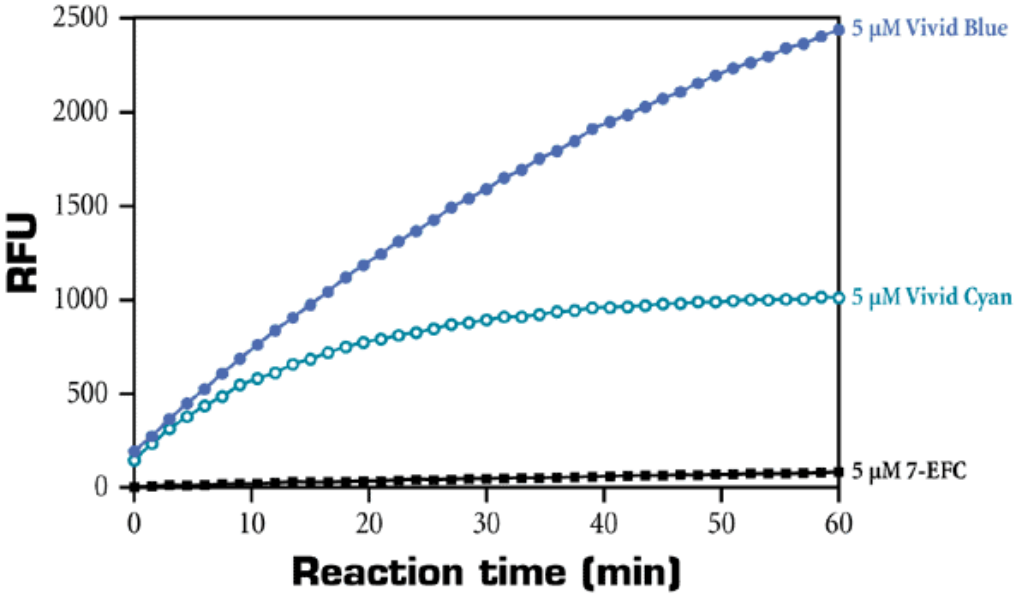

Figure 3. Comparison of the CYP2B6 reaction rate with Blue and Cyan Substrates with the CYP2B6 reaction rate with 7-EFC. The assays were performed essentially as described in Assay Incubation Conditions, but the Blue and Cyan Substrate concentrations were 5 µM for the Blue and Cyan Substrates as well as for 7-EFC. The plates were read at various time points during the 60-minute course of the reaction.

in CYP2B6 metabolism and inhibition (**Table 1**, standard font). None of the drugs and test compounds in the control group exhibited any inhibitory effect (**Table 1**, italic font), indicating the high specificity of the Blue and Cyan Assays. The apparent K_i values we obtained enabled us to rank the test compounds by their inhibitory potency. Our ranking matches that based on previously published relative affinity measurements with the CYP2B6 isozyme (K_i, K_d, or K_m values) determined in more traditional assays (**Table 1**). We also performed a cross-comparison of the apparent K_i values obtained with the Blue and Cyan Substrates with the test compounds listed in **Table 1** (**Figure 5**). The data show a very close correlation; the R^2 value is 0.97.

DISCUSSION

We compared the performance of the Vivid CYP2B6 assays with the fluorogenic Blue and Cyan Substrates based on several criteria, including assay linearity, apparent kinetic parameters (V_{max} and K_m), solvent sensitivity, preferential excitation, and emission wavelengths, and by the inhibitory effect on the reaction of CYP2B6 assay modifiers (eg, CYP2B6 substrates, inducers, and inhibitors). The rate of the CYP2B6 oxidative reaction was significantly higher with the Blue or Cyan Substrate than with another conventional coumarin derivative, 7-EFC, the most commonly used fluorescent CYP2B6 substrate and reporter probe,[7,36] as illustrated in **Figure 3**. Under similar reaction conditions, the signal intensity generated by the Blue or Cyan Substrate far exceeds that of 7-EFC. Even under conditions in which the 7-EFC concentration was 8 times higher than that of either the Blue or the Cyan Substrate, the signal intensity generated in the 60-minute, room-temperature CYP2B6 reactions with the Blue or Cyan Substrate exceeded that generated with 7-EFC by nearly 30-fold for the Blue Substrate and 10-fold for the Cyan Substrate (data not shown).

The fluorescence signal intensity generated with the fluorogenic Blue Substrate is greater than that generated with the Cyan Substrate, as illustrated in **Figures 1** and **3**. This feature of the Blue Substrate offers the advantage of developing assays with a broader dynamic range. On the other hand, the emission spectrum from the oxidized product (metabolite) of the Cyan Substrate (emission detected at 502 nm) will presumably interfere less with the fluorescence emission of NADPH (340-440 nm) than will the oxidized Blue Substrate (metabolite emission detected at 460 nm). Interference with NADPH fluorescence has presented significant problems in the past, especially in screening some compound libraries that absorb or fluoresce very close to UV range. Some methods to resolve

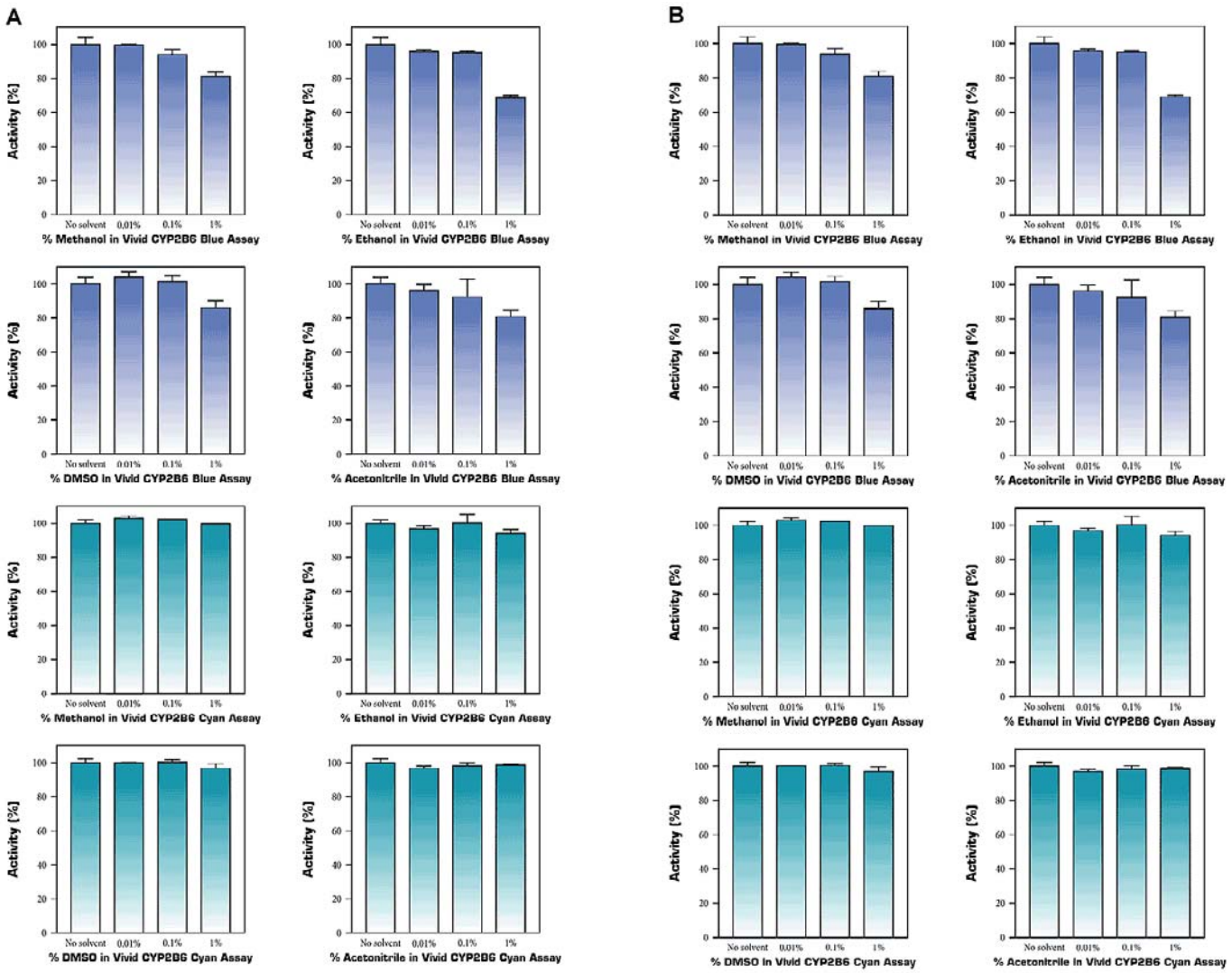

Figure 4. Effects of solvents on (A) Blue and (B) Cyan Assays. The assays were performed essentially as described in Assay Incubation Conditions, but the amounts of solvent varied (from 0% to 1% levels [vol/vol]) and the reaction time was 10 minutes. Reactions were performed in the presence of varying amounts of (1) MeOH, (2) EtOH, (3) DMSO, and (4) ACN.

this problem relied on inserting an additional step in the assay protocol either to enhance fluorescence over the background fluorescence from NADPH or to remove excess NADPH from the reaction mixture.[37] Using the Blue or Cyan Substrate does not require NADPH removal or an additional separation step; the homogeneous format of these assays allows fluorescence readings in the kinetic mode. Nonetheless, screening with the Cyan Substrate may present a valuable alternative for particularly UV-sensitive applications.

Knowing an assay's sensitivity to solvents enables investigators to choose the proper solvent for screening test compounds or compound libraries that will interfere only minimally with the reporter system. Solvent sensitivity of a reaction usually depends on the nature of the enzyme and the substrate pair used in the reaction. As our results indicate, the Cyan Substrate pairing with CYP2B6 BACULOSOMES exhibits greater tolerance for the presence of an organic solvent in the reaction than does the Blue Substrate. Therefore, the Cyan Substrate can be used without significant interference with any of the solvents tested in the Vivid CYP2B6 Assay if the final solvent concentration does not exceed 1% (**Figure 4**). The solvent concentration in a Vivid CYP2B6 Blue Assay should not exceed 0.1%; DMSO is the preferred solvent. As a general guideline, the recommendation is to apply proper controls to account for the inhibitory effect of any solvent used.

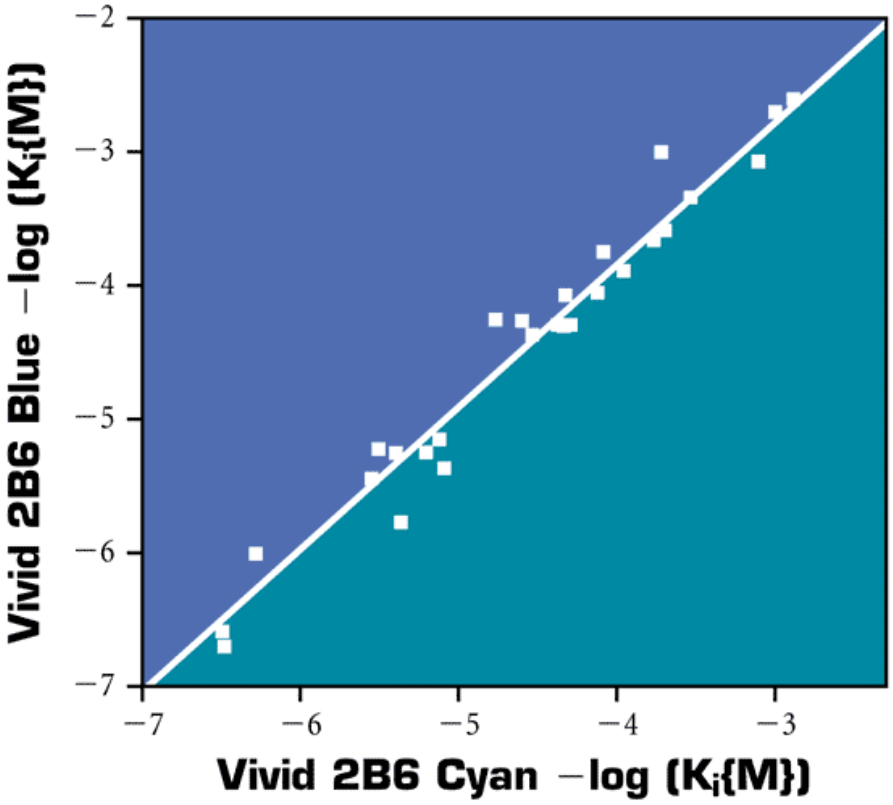

Figure 5. Correlation of apparent K_i values determined in the Blue and Cyan Assays. The assays were performed essentially as described in Assay Incubation Conditions, but the concentrations of the different test compounds varied and the reaction time was 10 minutes for assays with the Cyan Substrate and 20 minutes for assays with the Blue Substrate.

The kinetic plots for CYP2B6-mediated reactions with the Blue or Cyan Substrate shown in **Figure 2** demonstrate that the enzymatic reaction of the Blue and Cyan Substrates with the CYP2B6 BACULOSOMES Reagent follows a classical Michaelis-Menten equation. In contrast, the 7-EFC substrate interaction with CYP2B6 is better fitted to a sigmoidal curve,[9] typically attributed to a more complex model of interactions. The low apparent K_m values (~0.7-1 µM) obtained in CYP2B6 reactions with the Cyan Substrate reflect the Cyan Substrate's high-affinity binding to the CYP2B6 active site. High-affinity binding of the reporter Cyan Substrate may prevent competition by reaction modifiers with a lower affinity and therefore complicate their detection; for instance, weak inhibitor interactions may be underestimated. On the other hand, the significantly higher apparent K_m value (~6 µM) of the Blue Substrate makes it a better competitive substrate for various lower-potency inhibitors and reaction modifiers (**Figure 2**). Thus, the Blue Substrate may be the preferred substrate for screening less potent CYP2B6 interactions. For example, some relatively weak binding CYP2B6 substrates (orphenadrine and diethyldithiocarbamate, **Table 1**) appeared to be less potent inhibitors in the Cyan Assay than in the Blue Assay. These results may be explained by a higher-affinity binding of the Cyan Substrate, compared to the Blue Substrate, to the CYP2B6 active site.

Nonetheless, for the majority of the compounds studied, both the Blue and Cyan Assays yield comparable compound ranking by inhibitory potency (**Figure 5**). The relative compound potency data obtained with either the Blue or Cyan Substrate, therefore, generally will be transferable to studies done with the other Vivid CYP2B6 Substrate. An ability to perform assays with Blue or Cyan Substrates at room temperature presents an additional advantage for the HTS applications by eliminating the need for the thermostatic equipment. However, either assay could also be performed at 37°C without significant changes in IC_{50} values or compound inhibitory potency (data not shown).

Obtaining information about the relative affinity of a particular test compound for the CYP2B6 enzyme can enable approximate comparisons with other relative affinity measurements, such as apparent K_i, K_m, or K_d values (see **Table 1**) obtained in in vivo or traditional in vitro assays. For most test compounds, the in vitro data obtained in assays with the Blue and Cyan Substrates were similar to the published data. In some instances, however, our in vitro data differed from the published in vivo studies. Although similar issues have been reported previously in attempts to correlate in vitro and in vivo data obtained with other P450 enzymes and substrates,[38] these issues do not negate the overall concept of ranking compounds based on their inhibitory potency.

Therefore, the Blue and Cyan Substrates were used to determine the inhibitory potential of a group of compounds that were implicated in CYP2B6 metabolism but for which no published calculated values were available (**Table 1**), by measuring their K_i values in the corresponding Blue or Cyan Assay. These assays enabled us to expand the number of compounds included in the ranking. No control compounds exhibited any inhibitory effect (**Table 1**, italic font), indicating the high specificity of the Blue and Cyan Assays. However, the design of the Blue and Cyan Assays means that all CYP2B6 assay modifiers function as competitive inhibitors in the Blue and Cyan Assays. Consequently, although the Blue and Cyan Assays can clearly detect substrates, inhibitors, or inducers that interact with the CYP2B6 enzyme, discrimination among the different classes of compounds is problematic. For example, although some compounds, such as dexamethasone[39] and rifampicin,[40] have been previously reported as CYP2B6 inducers in vivo, the Blue and Cyan Assays could not distinguish these inducers from inhibitors. Therefore, the Blue and Cyan Assays can initially identify all compounds that interfere with CYP2B6 metabolism, but for more thorough investigations of the CYP2B6 pathway, secondary cell-based or HPLC (high performance liquid chromatography)-based assays may be necessary to differentiate substrates, inducers, and inhibitors of CYP2B6.

The Blue and Cyan Assays present a new competitive and sensitive tool for predicting CYP2B6 drug metabolism and inhibition by evaluating the inhibitory potency of existing drugs and potential drug candidates in a timely and effective manner. Either fluorogenic Vivid CYP2B6 Substrate can be used in screening applications with recombinant CYP2B6. Although compound ranking by inhibitory potency is similar with either the Blue or the Cyan Substrate, the features and characteristics of each can make one preferred for particular screening needs (eg, apparent kinetic parameters, solvent sensitivity, assay dynamic range, preferential excitation and emission wavelengths). Both screening assays were reliable, confirming the interactions with the known panel of CYP2B6 substrates, inducers, and inhibitors, and were able to assess the inhibitory potency of some other, less characterized substances. One potential drawback of the Blue and Cyan Substrates, however, is that they are not isozyme-specific and therefore cannot be used as reporter substrates for CYP2B6 in human liver microsomes (HLM), in intact cell systems, or in vivo. Nonetheless, they fulfill the need for novel sensitive fluorescent substrates for in vitro applications with recombinant human CYP2B6 in an HTS format with singularly expressed CYP2B6.

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