Highly Miniaturized Formats for In Vitro Drug Metabolism Assays Using Vivid® Fluorescent Substrates and Recombinant Human Cytochrome P450 Enzymes

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Highly miniaturized P450 screening assays designed to enable facile analysis of P450 drug interactions in a 1536-well plate format with the principal human cytochrome P450 enzymes (CYP3A4, 2D6, 2C9, 2C19, and 1A2) and Vivid® fluorogenic substrates were developed. The detailed characterization of the assays included stability, homogeneity, and reproducibility of the recombinant P450 enzymes and the kinetic parameters of their reactions with Vivid® fluorogenic substrates, with a focus on the specific characteristics of each component that enable screening in a low-volume 1536-well plate assay format. The screening assays were applied for the assessment of individual cytochrome P450 inhibition profiles with a panel of selected assay modifiers, including isozyme-specific substrates and inhibitors. IC_{50} values obtained for the modifiers in 96- and 1536-well plate formats were similar and comparable with values obtained in assays with conventional substrates. An overall examination of the 1536-well assay statistics, such as signal-to-background ratio and Z′ factor, demonstrated that these assays are a robust, successful, and reliable tool to screen for cytochrome P450 metabolism and inhibition in an ultra-high-throughput screening format. (*Journal of Biomolecular Screening* 2005:56-66)

Key words: cytochrome P450, CYP1A2, CYP2C9, CYP2D6, CYP3A4, CYP2C19, fluorescent substrate, drug metabolism, P450 assay, P450 inhibition, high-throughput screening (HTS), ultra-high-throughput screening (uHTS)

INTRODUCTION

A LARGE NUMBER OF POTENTIAL DRUG CANDIDATES fail in the later stages of drug development due to lack of efficacy and unpredicted effects of human metabolism, such as toxicity and unfavorable pharmacokinetic properties.[1,2] At the present time, development of high-throughput screening (HTS) and automation technologies, together with substantial advances in combinatorial chemistry, predictive modeling, and bioinformatics, has significantly increased the number of new chemical entities entering drug discovery programs. In addition, the recent completion of the Human Genome Project is expected to raise the number of molecular targets from 500 to as many as 3000 to 4000,[3] further increasing the flow of compounds into pharmaceutical screens. The current situation is changing the role of in vitro drug metabolism and toxicology studies, which have traditionally been performed with a limited set of compounds in the latest stages of drug development, and is shifting it toward integrating absorption, disposition, metabolism, and excretion (ADME), and toxicology tests at the earliest possible stages in the drug discovery process.[4] Cytochrome P450s, a superfamily of critical enzymes involved in human drug metabolism, are implicated in many clinical cases of adverse drug reactions and toxicity stemming from mechanism-based enzyme inhibition and drug-drug interactions.[5] Performing HTS for P450 inhibition or induction earlier in the process helps to eliminate molecules with unwanted metabolic properties and guides medicinal chemists to produce better clinical candidates based on predictive cytochrome P450 structure-activity relationship models.[6] The need to screen for ADME properties at the stage of compound libraries is driven by the necessity not just to accelerate the development of the new chemical entities but to decrease rates of drug failure later in the drug development process,[2,7] creating a demand for a new generation of assays with higher throughput capability alongside higher sensitivity and reproducibility.[8] A number of approaches to screen for metabolism and inhibition of the major human cytochrome P450 enzymes have been developed. These techniques include radiometric assays based on the isolation of radiolabeled metabolites,[9-12] the use of colorimetric or fluorescent reagents for quantitation of formaldehyde formation during P450-dependent demethylation reactions,[13,14] and high-performance liquid chromatography (HPLC) methods, including rapid liquid

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chromatography/mass spectrometry (LC/MS) approaches for metabolite analysis.[15,16] However, most of these assays include relatively cumbersome postreaction separation steps that limit their usefulness for HTS. The approach that shows the most promise for high-throughput inhibitor screens is the use of fluorescent or luminescent[17] assays, which can be done in a homogeneous format (i.e., they require no postreaction separation steps). Fluorescent HTS methods employ fluorescent P450 substrates that are efficiently metabolized by specific P450 isozymes to yield a product with altered fluorescent properties, usually increased fluorescent intensity.[18,19] Metabolism assessment with fluorogenic substrates is based on the ability of an unknown drug to inhibit or compete with the fluorogenic compound in the P450 reaction and is dependent on the kinetic parameters of such an interaction.[20,21] The hits from these competitive inhibition screens must be further evaluated to determine whether they are inhibitors or substrates for the indicated isozyme. Several of the generic fluorescent cytochrome P450 substrates have been applied for the development of 96-well compatible fluorometric assays employing different cytochrome P450 isozymes.[20-22] In addition, fluorometric assays in a 384-well format for the CYP3A4 and CYP2D6 isozymes have also been described.[23] However, many undesirable features of the generic (unmodified) fluorescent molecules, such as poor aqueous solubility, high background fluorescence (e.g., the fluorescence of unmetabolized substrate), and low signal-to-background ratios, limit their applications for low-volume ultra-high-throughput formats. Recently, highly miniaturized formats for primary screening assays involving several important pharmaceutical targets were developed.[24,25] The ability to perform primary screening assays in microwell plates at 1- to 3-μL volumes has made a significant impact on the early stages of drug discovery by accelerating and reducing the cost of the drug discovery process.[8] The ultra-high-throughput screening (uHTS) assays currently available for many drug discovery targets demand similar speed, precision, and high-throughput capabilities from the ADME assays used to prescreen compound libraries, particularly for the cytochrome P450 metabolism and inhibition assays.[1,2,6,7] Here we describe the development and characterization of fluorescent assays for the detection of substrates and inhibitors of major human cytochrome P450 isozymes, enabling rapid and sensitive evaluation of P450 activity in a 1536-well plate format. The assays use the Vivid® fluorogenic substrates that were originally developed to address many issues existing with fluorescent substrates of the previous generations, such as poor solubility, high background fluorescence of the substrate, undesirable excitation and emission wavelengths, and insufficient rates of metabolism.[26] Previous applications of Vivid® substrates in the development of several cytochrome P450 screening assays in a 96-well plate format have resulted in highly robust and reliable HTS assays,[27-29] indicating that these substrates are excellent candidates for further miniaturization and development of low-volume reactions.[30] To estimate the performance of Vivid® fluorogenic substrates in a 1536-well plate format, we combined these substrates with baculovirus-expressed human P450 isozymes CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2[31] and applied them for the study of isozyme-specific drug interactions and inhibition. The IC_{50} values obtained in the 1536-well assays demonstrated a high degree of correlation with data obtained in 96-well assays and conventional HPLC-based assays.[32] In addition, the 1536-well assay characteristics, such as stability and reproducibility of the enzyme component, signal-to-background ratio, Z′ factor in the range of 0.66 to 0.82, and tolerance to organic solvent, were indicative of highly robust and reliable assays. An overall extension of the current assays for the principal human cytochrome P450 enzymes to the uHTS format is viewed as an advanced and cost-effective approach for ADME screening and presents a valuable new addition to the modern drug discovery process. The advantages and limitations of the assays for various applications are further discussed.

METHODS

Chemicals

The Vivid® substrates, their corresponding fluorescent standards, and $NADP^+$ were obtained from Invitrogen Drug Discovery Solutions (Madison, WI). Ellipticine was obtained from Calbiochem (San Diego, CA), and (±)-bufuralol hydrochloride was purchased from BD Biosciences (San Jose, CA). Amitriptyline, clomipramine, diclofenac, erythromycin, furafylline, imipramine, ketoconazole, α-naphthoflavone, omeprazole, (±)-propranolol, quinidine, sulfaphenazole, tamoxifen, ticlopidine, tranylcypromine, troleandomycin, (±)-verapamil, and warfarin were obtained from Sigma-Aldrich Corp. (St. Louis, MO). All other reagents used were of the highest commercial grade available.

Enzymes

Microsomes from baculovirus-infected cells coexpressing human CYP1A2, CYP2D6, CYP2C9, CYP2C19, or CYP3A4 and NADPH-cytochrome P450 reductase (P450 BACULOSOMES®), as well as the NADPH regeneration system containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase, were obtained from Invitrogen Drug Discovery Solutions (Madison, WI). Cytochrome P450 content was determined spectrally by the method of Omura and Sato.[33] Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL).

Particle sizing

The particle-sizing experiment was performed using a Zeta Plus Particle Analyzer (Brookhaven Instruments Corp., Holtsville, NY) equipped with a solid-state laser operating at the wavelength of 532 nm. BACULOSOMES® Reagents were diluted in 100 mM potassium phosphate buffer, pH 8.0, at a concentration of 0.1 mg/

Table 1. General Reaction Conditions Used in 1536-Well Plate Assays

P450 BACULOSOMES®	*Vivid® Substrate*	*CYP450 (nM)*	*Vivid® Substrate (μM)*	*Acetonitrile from Vivid® Substrate (%)*	*Buffer (mM)*	*Reaction Time (min)*
1A2	1A2 Blue	5	3	0.15	100	20
2C9	2C9 Green	10	2	0.1	50	60
2C9	2C9 Red	10	2	0.1	50	60
2C19	2C19 Blue	5	10	0.5	50	30
2D6	2D6 Blue	10	10	0.5	100	60
3A4	3A4 Green	5	2	0.1	100	10
3A4	3A4 Red	5	3	0.15	100	20

All assays were performed at room temperature with 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 100 μM $NADP^+$ in varying amounts of potassium phosphate at pH 8.0.

mL of protein. The scattering signal was acquired for 2 min at 25 °C. The particle size was assessed using Multimodal Size Distribution (MSD) software.

96-Well assay incubation conditions

The 100-μM stock solutions of the Vivid® assay standards were prepared in DMSO, and 2-mM stock solutions of Vivid® substrates were prepared in acetonitrile according to the manufacturer's instructions. Costar 96-well black, nontreated, flat-bottom assay plates (#3915, Corning Costar, Acton, MA) were used for the fluorescent assays with a volume of 100 μL per well. All incubations were performed at room temperature using buffer conditions and enzyme concentrations as described in assay protocol (www.invitrogen.com/content/sfs/panvera/L0504.pdf). Fluorescent readings were obtained for 96-well plates kinetically over the course of the reaction using a Gemini XS instrument (Molecular Devices Corp., Sunnyvale, CA). The excitation and emission wavelengths used for each Vivid® substrate were as follows: Vivid® Red substrates (excitation 530 nm, emission 585 nm, and cutoff 570 nm), Vivid® Green substrates (excitation 485 nm, emission 530 nm, and cutoff 515 nm), and Vivid® Blue substrates (excitation 409 nm, emission 460 nm, and cutoff 455 nm). The same excitation and emission wavelength sets were used for both the Vivid® substrates and corresponding standards. Product formation was determined using a calibration curve of the corresponding fluorescent standard prepared by dilution into the appropriate buffer.

1536-Well assay incubation conditions

For the ultra-high-throughput assays, Greiner 1536-well black plates (#782076, Greiner Bio-One, Inc., Longwood, FL) were used with a total reaction volume of 8 μL using conditions described in Table 1. Reagents were dispensed using the Flying Reagent Dispenser I (FRD I) from Aurora Biosciences (San Diego, CA). Assays were conducted in end-point mode by dispensing 1 μL of inhibitor to stop the reaction at the end of the incubation period for a final concentration of 10 μM ketoconazole (CYP3A4), 10 μM α-naphthoflavone (CYP1A2), 1 μM quinidine (CYP2D6), 10 μM sulfaphenazole (CYP2C9), or 100 μM tranylcypromine (CYP2C19). The plates were read on an Ultra plate reader (Tecan, Inc., Durham, NC) using the following filter sets: Vivid® Red substrates (excitation 535 ± 25 nm, emission 590 ± 20 nm), Vivid® Green substrates (excitation 485 ± 20 nm, emission 535 ± 25 nm), and Vivid® Blue substrates (excitation 405 ± 20 nm, emission 465 ± 35 nm).

96-Well inhibition studies

Vivid® assays conducted in 96-well plates were used to determine IC_{50} values of a selected panel of drugs known to be metabolized by specific P450 isozymes, as indicated in Table 2. Available information about each drug's solubility was used to prepare stock solutions and serial dilutions of the drugs employed in a current study. The 10-mM stock solutions of amitriptyline, (±)-bufuralol, clomipramine, diclofenac, imipramine, (±)-propranolol, ticlopidine, tranylcypromine, and (±)-verapamil were prepared and serially diluted in water to generate 20 concentrations in duplicate of each inhibitor. Ellipticine, erythromycin, furafylline, ketoconazole, omeprazole, quinidine, sulfaphenazole, tamoxifen, troleandomycin, and (R,S)-warfarin were prepared in DMSO at concentrations ranging from 5 to 100 mM and serially diluted into water containing an appropriate, uniform amount of DMSO; final DMSO content in most cases ranged from 0.1% to 0.5% and did not exceed 1%. Each experiment was conducted twice. The inhibition assays were performed as described in the section on 96-well assay incubation conditions, with final concentrations as described in Table 1. At twice the final reaction concentration, 50 μL of a mixture of the BACULOSOMES® Reagent and the NADPH regeneration system in phosphate buffer (pH 8.0) was preincubated for 15 min in the presence of varying concentrations of 40 μL inhibitor prior to dispensing 10 μL of a mixture of 1 mM $NADP^+$ and Vivid® substrate. Final concentrations of the Vivid® substrates were 2 μM Vivid® 2C9 Green, 2 μM Vivid® 2C9 Red, 2 μM Vivid® 3A4 Green, 3 μM Vivid® 1A2 Blue, 3 μM Vivid® 3A4 Red, 10 μM Vivid® 2C19 Blue, or 10 μM Vivid® 2D6 Blue. Fluorescence values were then read in end-point mode after a 10-min (3A4 Green), 20-min (1A2 Blue and 3A4 Red), 30-min (2C19 Blue), or 60-min

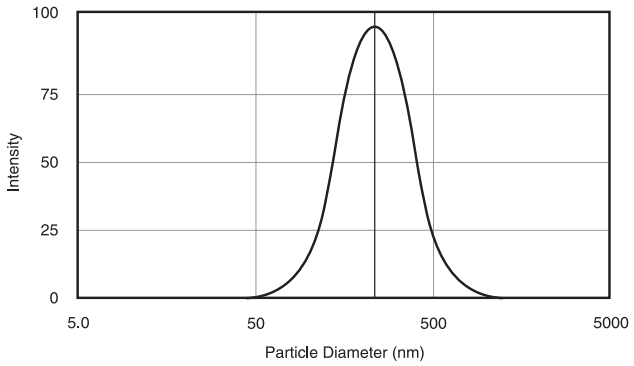

FIG. 1. Particle size distribution in CYP3A4 BACULOSOMES® suspension. Measurements were performed as described in the section on particle sizing conditions.

(2C9 Green, 2C9 Red, and 2D6 Blue) incubation period. Background fluorescence was established by wells containing all of the assay components, except BACULOSOMES® Reagent, and subtracted before further analysis. Percent inhibition was then calculated for each concentration of inhibitor by comparison of fluorescence values to those wells without inhibitor. Controls were used to account for inhibition by DMSO where appropriate. IC_{50} values were then determined by nonlinear regression using Prism software (version 3.03, GraphPad Software, San Diego, CA).

1536-Well inhibition studies

For inhibition studies in the 1536-well format, serial dilutions were made in the same manner as described for 96-well inhibition studies in quadruplicate, and final reaction concentrations were the same as described for the 1536-well assay incubation conditions. The volume of reagents was scaled down to 4 μL of a mixture of the BACULOSOMES® Reagent and the NADPH regeneration system in phosphate buffer (pH 8.0) at twice the final reaction concentration, 3.2 μL of various concentrations of the inhibitor, and 0.8 μL of a 1-mM $NADP^+$ and Vivid® substrate mixture. Final concentrations of the Vivid® substrates were 2 μM Vivid® 2C9 Green, 2 μM Vivid® 2C9 Red, 2 μM Vivid® 3A4 Green, 3 μM Vivid® 1A2 Blue, 3 μM Vivid® 3A4 Red, 10 μM Vivid® 2C19 Blue, or 10 μM Vivid® 2D6 Blue. Then, 1 μL of isozyme-specific inhibitor was added to stop the reaction, as described for the 1536-well assay incubation conditions, after a 10-min (3A4 Green), 20-min (1A2 Blue and 3A4 Red), 30-min (2C19 Blue), or 60-min (2C9 Green, 2C9 Red, and 2D6 Blue) incubation period. Fluorescence values were measured using the Ultra plate reader. Background fluorescence was established by wells containing all of the assay components, except BACULOSOMES® Reagents, and was subtracted before analysis. Percent inhibition was calculated for each concentration of the inhibitor by comparison of fluorescence values to those wells without the inhibitor. Controls were used to account for inhibition by DMSO where appropriate. IC_{50} values were determined by nonlinear regression using Prism software.

Z′ factor and signal-to-background determination

A full 96- or 1536-well plate was used for each signal-to-background and Z′ factor determination. Volumes of reagents and incubation times were identical to those used for the inhibition studies. In 1536-well plates, the experiments were performed in the absence and presence of 1% DMSO; 3.2 μL of 2.5% DMSO in water was substituted in place of the inhibitor described in the 1536-well inhibition studies. The Z′ factor was determined as described previously by Zhang and coworkers.[34]

RESULTS

Stability and homogeneity assessment of the P450 enzyme component

P450 BACULOSOMES® Reagents were used as a source of recombinant cytochrome P450 enzymes to perform metabolic reactions with Vivid® substrates in HTS and uHTS formats. Enzyme stability is a feature of high importance for uHTS technology, in which preservation of the enzyme activity for several hours may be required. To address these issues, we performed a characterization of BACULOSOMES® Reagents by particle sizing to assess the homogeneity of the preparation and to check for possible aggregate formation. Such aggregates could cause variability in P450 activity levels and could increase light scattering, affecting fluorescent assay performance. Particle size was measured in BACULOSOMES® suspension and followed a peak distribution in a range from 50 to 500 nm, with the majority of BACULOSOMES® present as 250-nm diameter particles (Fig. 1). Relatively narrow variation in particle size in membrane preparations is an indicator of system homogeneity, which is an important parameter contributing to the stability and reproducibility of the enzyme component of the assay. To follow up the changes in particle size after multiple freeze/thaw cycles, we subjected BACULOSOMES® to 5 freeze/thaw cycles and then assessed homogeneity. No significant shift in particle size distribution was observed following the freeze/thaw cycles, indicating the absence of aggregates due to the procedure (data not shown). In addition, we tested the stability of BACULOSOMES® Reagents (CYP3A4, 2D6, 1A2, 2C9, and 2C19) by measuring their activity with Vivid® fluorogenic substrates before and after a 1- to 7-h incubation on ice or at room temperature (Fig. 2). The tube containing P450 BACULOSOMES® was stored on ice or at room temperature, and at the indicated time points, aliquots were taken out and assayed for activity. No significant loss of P450 activity was observed for the nondiluted stock of CYP3A4, 2D6, 2C9, or 2C19 BACULOSOMES® within the indicated time frame and conditions, as shown in Figure 2. At the same time, an increase in CYP1A2 activity during the first 60 min of storage on ice or at room temperature with Vivid® CYP1A2 Blue substrate was observed (Fig. 2). The latter effect is probably related to the conformational rearrangements previously reported for this isozyme.[35] After the initial 1-h equilibration stage, CYP1A2 BACULOSOMES® activity remained stable for up to 7 h when

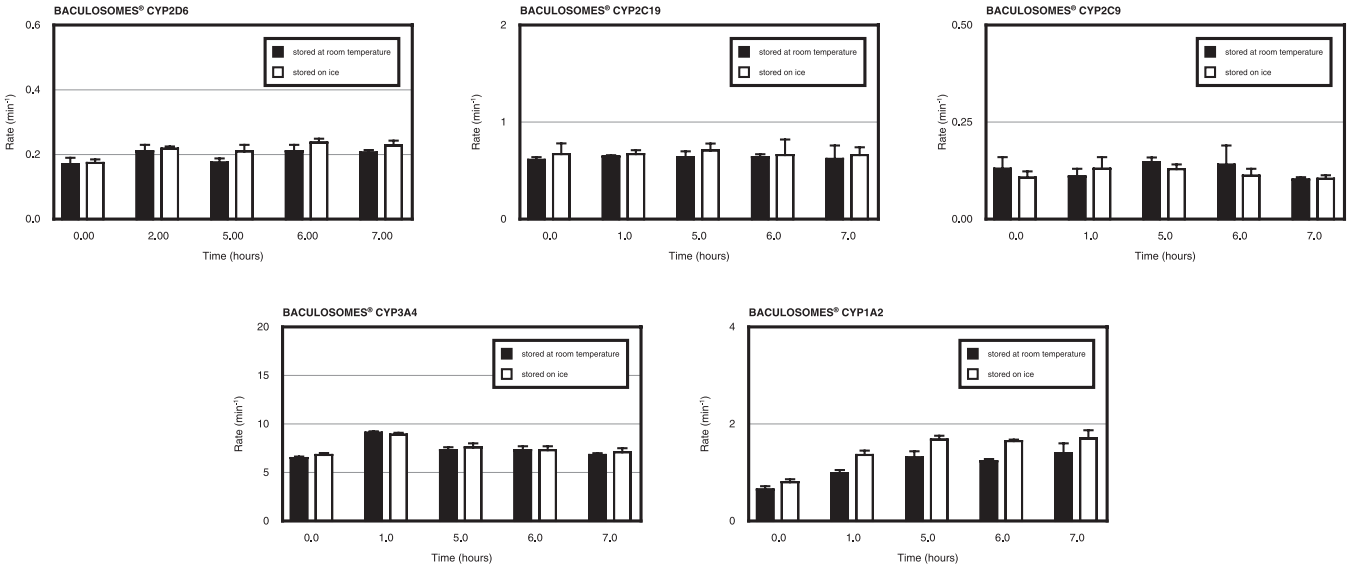

FIG. 2. Nondiluted P450 BACULOSOMES® stability at different temperatures. Reactions were performed in triplicate in a 96-well plate, essentially as described in the section on 96-well assay incubation conditions.

stored on ice or at room temperature (Fig. 2). The overall 1- to 7-h stability studies performed for all BACULOSOMES® Reagents indicated that 1 thawed tube of P450 enzyme could be stored in nondiluted format and used in uHTS applications within the several hours of experimental procedures without significant precipitation or loss of enzymatic activity. At the same time, stability of the diluted P450 BACULOSOMES® can be affected by the presence of the components of the NADPH regeneration system and does not allow multiple reactions with the 1-step diluted enzyme preparation. The optimal conditions for the storage of the diluted P450 enzymes without significant loss of their activity are subjects of further investigations.

Assay linearity

In addition to the enzymatic component, the chemical and fluorescent characteristics of Vivid® fluorogenic substrates contributed to the successful development of uHTS applications. The suggested reaction time for each assay was established by exploring the linearity of metabolite formation in the reactions of CYP3A4, 2D6, 1A2, 2C9, and 2C19 BACULOSOMES® with their corresponding Vivid® substrates (Fig. 3). The readings were obtained in kinetic mode using the assay and instrument settings described in Methods and were compared to control reactions performed in the absence of the enzymatic component. Metabolic rates for the CYP2D6 Blue and CYP2C9 Red and Green reactions were linear for at least 60 min at room temperature (Fig. 3). The time linearity of the other Vivid® assays was restricted to 10- to 30-min reactions, depending on the P450 isozyme and fluorescent substrate used (Fig. 3). These reaction times were also used as guidelines to conduct all inhibition assays, as described in the Methods section.

P450 inhibition assays in 1536-well format

The characterization and validation studies in the 1536-well plate format were carried out to establish the usefulness of the assay in predicting isozyme-specific metabolism and inhibition. This type of analysis included a titration of test compounds and the generation of dose-dependent curves used for calculating apparent IC_{50} values as described previously.[27-29] Therefore, 20 concentration point curves for 19 different compounds with 7 different P450/Vivid® fluorogenic substrate combinations using 3 known substrates and 1 inhibitor per each pair were generated. The inhibition studies were conducted at room temperature under optimized buffer conditions (Table 1) at Vivid® substrate concentrations approaching the apparent K_m, enabling the detection of weak inhibitors.[27,36] The inhibition studies in the 1536-well plate format were performed in end-point mode, after the fluorescent reaction was stopped by the addition of an isozyme-specific inhibitor. Apparent IC_{50} values obtained in the 1536-well reactions were similar to IC_{50} values obtained for the same drugs in the 96-well plate format (Table 2), therefore allowing an easy cross-format transition of data. In all of the cases studied, ranking of a drug's inhibition potency was similar between fluorescent and conventional assays, though apparent IC_{50} values obtained with either fluorescent or conventional assays were not identical (Table 2). For a majority of the drugs, apparent IC_{50} values in fluorescent assays were similar to IC_{50} values obtained by screening with conventional substrates.[33]

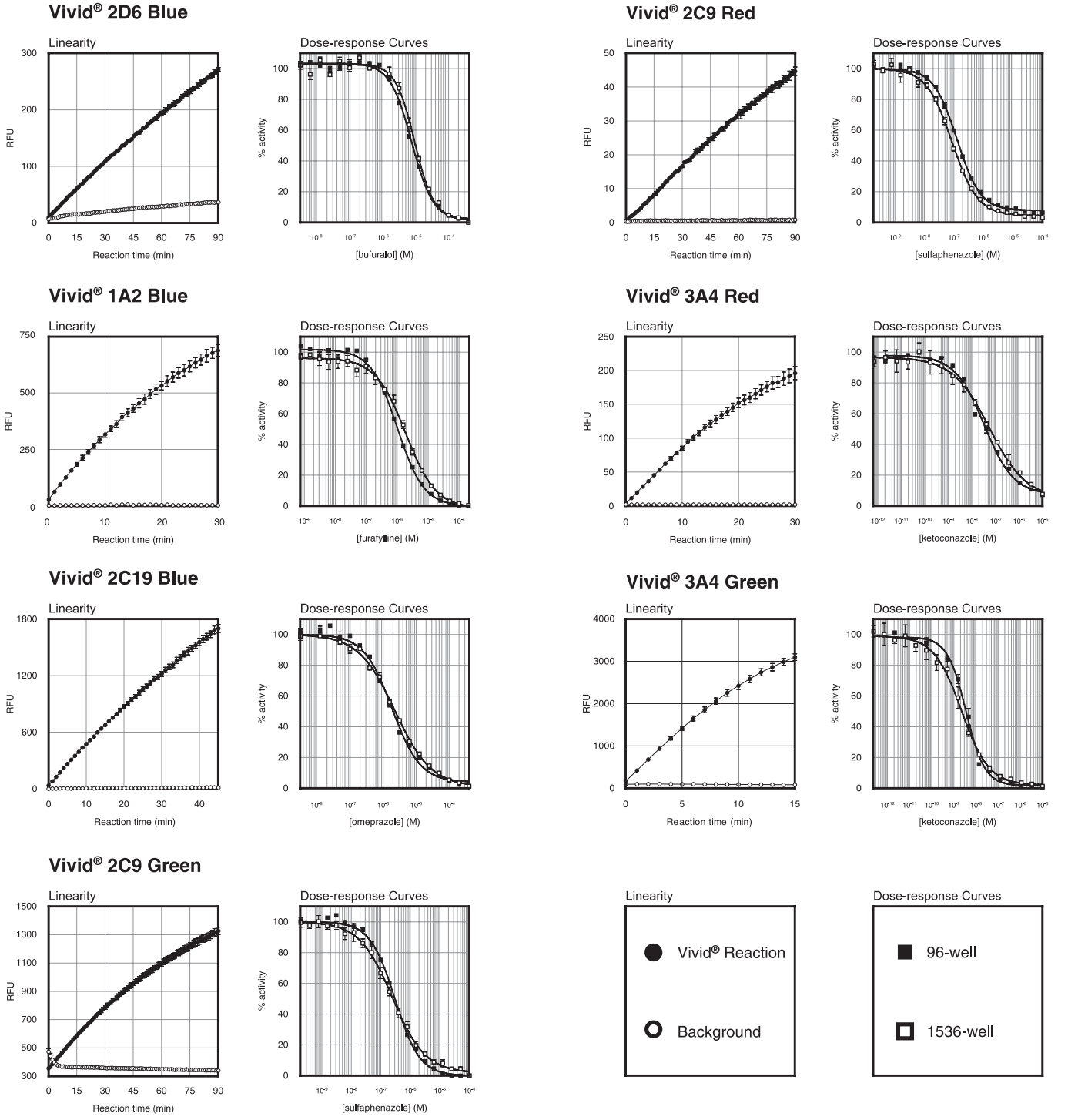

FIG. 3. Time linearity and representative dose-response inhibition curves for P450 BACULOSOMES® and Vivid® substrates reactions. Assays for linearity of the reaction over time were performed in quadruplicate in 96-well plates, as described in the section on 96-well assay incubation conditions. The plates were read continuously at room temperature for the indicated periods of time. Dose-response curves were obtained in quadruplicate in 96- and 1536-well plate formats, as described in the section on 96- and 1536-well inhibition studies.

Table 2. IC_{50} Values (μM) Obtained with Vivid® Substrates in 96- and 1536-Well Formats and Conventional Assays

Vivid® Assay	*Drug*	*96-Well IC_{50}*	*1536-Well IC_{50}*	*Conventional Assay IC_{50}*
1A2 Blue	Propranolol	0.3 ± 0.1	0.6 ± 0.04	3.84 ± 0.63
	Furafylline	1 ± 0.1	1.9 ± 0.1	1.2-2.9
	Ellipticine	0.02 ± 0.01	0.02 ± 0.001	0.11 ± 0.01
	Ticlopidine	1.2 ± 0.4	1.4 ± 0.2	6.1 ± 0.45
2C9 Green	Sulfaphenazole	0.3 ± 0.02	0.3 ± 0.02	2.3-2.5
	Diclofenac	3.3 ± 1	3.5 ± 0.8	>30
	Tamoxifen	8.6 ± 0.5	12 ± 1.0	10 ± 2
	Warfarin	18 ± 3	18 ± 0.5	22 ± 2
2C9 Red	Sulfaphenazole	0.1 ± 0.02	0.1 ± 0.001	2.3-2.5
	Diclofenac	2.1 ± 0.1	1.8 ± 0.1	>30
	Tamoxifen	5.1 ± 0.4	5 ± 2	10 ± 2
	Warfarin	5.4 ± 0.5	4.5 ± 0.3	22 ± 2
2C19 Blue	Amitriptyline	2.0 ± 0.2	2.9 ± 0.1	3.3 ± 1.2
	Tranylcypromine	2.9 ± 0.1	2.9 ± 0.1	8.2 ± 0.67
	Omeprazole	2.2 ± 0.5	2.4 ± 0.1	2.1 ± 0.36
	Ticlopidine	0.2 ± 0.02	0.3 ± 0.02	0.40 ± 0.04
2D6 Blue	Quinidine	0.004 ± 0.002	0.002 ± 0.0001	0.005 ± 0.001
	Bufuralol	7 ± 1	9.3 ± 0.8	6.1 ± 1.6
	Imipramine	1 ± 0.13	1.4 ± 0.2	3.8 ± 0.99
	Clomipramine	0.3 ± 0.03	0.4 ± 0.03	1.6 ± 0.17
3A4 Green	Ketoconazole	0.004 ± 0.0004	0.009 ± 0.003	0.005-0.046
	Erythromycin	2.4 ± 0.1	2.0 ± 0.1	2.4 ± 0.35
	Verapamil	2.7 ± 0.1	3.9 ± 0.3	2.0 ± 0.28
	Troleandomycin	0.21 ± 0.01	0.22 ± 0.03	1.0 ± 0.95
3A4 Red	Ketoconazole	0.1 ± 0.02	0.06 ± 0.01	0.005-0.046
	Erythromycin	0.4 ± 0.04	0.6 ± 0.1	2.4 ± 0.35
	Verapamil	0.3 ± 0.1	0.5 ± 0.02	2.0 ± 0.28
	Troleandomycin	0.1 ± 0.03	0.2 ± 0.02	1.0 ± 0.95

As reported by Cohen et al.[32] All assays were performed in quadruplicate, $n = 4$.

However, for some of the assay modifiers, such as propranolol (CYP1A2) or sulfaphenazole (CYP2C9), an overprediction of the inhibition potency (lower IC_{50} values) in fluorescence-based competitive assays versus the conventional assays was observed. Although similar issues have been reported previously by attempts at direct correlations between conventional and fluorescent assays,[33] they do not invalidate the application of fluorescent assays for the assessment of P450 metabolism and inhibition. Although a direct correlation between the IC_{50} values obtained in both types of assays is not always advisable, the fluorescent assays are rapid screening tools for the ranking of compounds' inhibitory potency and may be followed by detailed characterization of the identified inhibitors by lower throughput applications or in vivo assessment.

1536-Well plate assay characteristics

To assess the robustness of the uHTS format for cytochrome P450 metabolism and inhibition, several characteristics of the assay, such as signal-to-background ratio and Z′ factor, were calculated and compared to those obtained in the 96-well plate format (Table 3). All assays employing the fluorogenic Vivid® substrates in the 1536-well format demonstrated broad dynamic range (Table 3) and high Z′ factors in a range of 0.66 to 0.82, therefore exhibiting characteristics of highly reliable and reproducible assays.

DISCUSSION

During the past decade of drug discovery, the application of HTS approaches has succeeded in accelerating the generation of new lead compounds in the pharmaceutical industry. One of the new challenges for HTS now lies in the area of early ADME screening, demanding the development of technologies amenable to highly miniaturized formats. Here we have described the development of fluorescence-based uHTS assays for inhibitor screening, employing Vivid® fluorogenic substrates and recombinantly expressed principal human cytochrome P450 isozymes in the 1536-well plate format. Drug metabolism assays using human liver microsomes (HLMs), hepatocytes, and tissue slices are widely used in preclinical drug metabolism studies and ADME screening in vitro. Many in vitro studies are done with HLMs, but high interindividual variability in the expression levels of particular P450 isoforms, population-dependent polymorphism, and heterogeneity due to the presence of multiple cytochrome P450 isoforms limit their use in HTS applications. Recently, progress in

Table 3. Vivid® Assay Signal-to-Background (S/B) and Z′ Factor

	96-Well, No DMSO		*1536-Well, No DMSO*		*1536-Well, 1% DMSO*	
Vivid® Assay	*S/B*	*Z′*	*S/B*	*Z′*	*S/B*	*Z′*
1A2 Blue	57 ± 7	0.89	12 ± 2	0.71	10.8 ± 0.9	0.73
2C9 Green	3.0 ± 0.1	0.87	10.3 ± 1.0	0.77	9.6 ± 0.9	0.79
2C9 Red	52 ± 10	0.90	66 ± 8	0.75	55 ± 4	0.82
2C19 Blue	53 ± 4	0.80	25 ± 2	0.80	19.6 ± 1.4	0.81
2D6 Blue	5.5 ± 0.4	0.86	3.5 ± 0.2	0.76	2.10 ± 0.12	0.66
3A4 Green	29 ± 2	0.92	40 ± 6	0.72	22 ± 3	0.72
3A4 Red	80 ± 8	0.88	90 ± 11	0.76	41 ± 4	0.80

recombinant human protein production has allowed a variety of recombinant human P450 enzymes to be expressed in *Escherichia coli*, insect, and mammalian cell expression systems.[31,37,38] A typical cytochrome P450 system of this type consists of a microsomal fraction of the baculovirus- or lymphoblast-expressed P450, coexpressed with P450 oxidoreductase and, in some cases, cytochrome b_5.[39] One of the main advantages of these heterologously expressed individual P450 isozymes is that they serve as a more reliable and consistent source of P450 enzyme and show significantly increased rates of metabolism that are suitable for detection in the uHTS format. Although recombinant P450s do not have exactly the same properties as HLMs, they have an advantage in that only 1 cytochrome P450 isozyme is present, allowing one to exclude the contributions from other P450 isozymes and other drug-metabolizing enzymes, such as flavin monooxygenases and UDP-glycosyltransferases. This simplifies the assessment of isozyme involvement in the metabolism of a particular drug, the conclusions about inhibitor potency, and determining relative affinities and contributions of the involved enzymes without requiring the use of isozyme-specific inhibitors or inhibitory antibodies. To be used as a tool in HTS or uHTS assays, the recombinant P450 proteins must be reliable in terms of homogeneity, enzyme stability, and reproducibility. In many cases, recombinant enzymes produced as a microsomal fraction from overexpressing cell lines have limited stability due to the heterogeneity of the preparation and the precipitates form following a limited storage at room temperature. Exposing the enzyme to an extended storage at room temperature is often a necessary step in an HTS assay in which more than 1 multiwell plate at a given time is used. The precipitated fractions may reduce enzyme activity and affect the accuracy and predictability of the assay. This is especially important for membrane and lipid-containing fractions, such as microsomes, in low-volume fluorescent assays in which precipitation may significantly increase the background fluorescence due to a light-scattering effect. In the current study, extended stability (7 h at room temperature) and homogeneity of the nondiluted BACULOSOMES® stock were demonstrated. In addition to the stability, the high specific content and activity of these recombinant enzymes made them appropriate for the development of low-volume uHTS fluorescent assays with Vivid® substrates, which have been previously employed in studies of metabolism and inhibition with several P450 isozymes in the 96-well plate format.[27,28]

Several distinctive features of the Vivid® substrates employed in the current study made the development of uHTS screening assays possible. Vivid® substrates (http://gcms.invitrogen.com/content. cfm?pageid=10058) were originally developed as benzyloxymethyl and alkoxymethyl ethers of available phenolic dyes, such as resorufin, fluorescein, 3-cyano-7-hydroxycoumarin, and the structurally related 7-hydroxy-4-trifluoromethylcoumarin.[26,30] The nonmodified phenolic dyes and their commercially available benzyl derivatives often suffer from high initial fluorescent background, interference from NADPH fluorescence, and low turnover rates that prevent or complicate their use in uHTS assays, necessitating several additional steps or coupled reactions. For example, an application of DFB, a fluorescent CYP3A4 substrate, requires an additional step for the removal of excess NADPH at the end of incubation to prevent NADPH interference with the fluorescent metabolite detection. This is accomplished by adding oxidized glutathione and glutathione reductase to convert NADPH to $NADP^+$, which is not fluorescent.[21] In other cases, a derivatization step with alkali to enhance the signal-to-background ratio may be required.[40]

Vivid® fluorogenic substrates exhibit a very low level of background fluorescence (Fig. 3) and do not require an additional step or coupled reaction to achieve high signal-to-background ratios in P450 reactions (Table 3). Metabolites of the Vivid® substrates have favorable fluorescent characteristics, such as excitation in the visible range of the light spectrum, preventing interference from NADPH and the majority of compounds with an absorbance peak in the UV range. Although recently developed alternative technology employing chemiluminescent P450 substrates presents an additional advantage for screening fluorescent compound libraries and exhibits high signal-to-background ratios,[17] these assays require a coupled reaction with luciferase and are prone to the detection of false positives due to the inhibition of luciferase by reaction modifiers. In addition, high aqueous solubility of the Vivid® substrates allows lowering the organic solvent concentration used in assays by keeping it at or below 0.5% acetonitrile in all final reac-

tion mixtures (Table 1). This latter feature is extremely important for several cytochrome P450 isozymes due to known effects of organic solvents on the course of their metabolism.[41]

Assay characterization and validation studies were carried out to establish the usefulness of the 1536-well format for the prediction of isozyme-specific metabolism and inhibition. Dose-response curves for 19 different compounds were generated with 7 different P450/Vivid® fluorogenic substrate combinations (3 isozyme specific substrates and 1 inhibitor per each pair). Here we demonstrate that Vivid® fluorescent assays with recombinant P450 isozymes performed in the 1536-well format were able to detect isozyme-specific substrates and inhibitors for CYP1A2, 2C9, 2C19, 2D6, and 3A4. The apparent IC_{50} values obtained for each drug in the 1536-well plate format were in good agreement with the values obtained in the 96-well plate format, as indicated in Table 2. Application of only 1 fluorescent probe to metabolism and inhibition studies with CYP1A2, 2D6, and 2C19 was sufficient. At the same time, more complex interactions between multiple substrates at the active site of CYP3A4[42] and CYP2C9[43] have been reported, indicating a need to apply multiple probes for these isoforms' metabolic assessment. The advantages of applying several structurally unrelated fluorescent probes have been discussed previously for CYP2C9[29] and CYP3A4.[44] In our studies, we applied both Vivid® Green and Red fluorescent substrates to CYP3A4 and CYP2C9 metabolism and inhibition. Apparent IC_{50} values obtained with Vivid® Green substrates better correlated with results obtained with conventional substrates for both isozymes (Table 2). At the same time, Vivid® Red fluorescent substrates may have the advantage of more sensitive detection and may avoid interference from library compounds with fluorescent background in the blue and green ranges of the fluorescent spectrum.

Typically, assay miniaturization will result in greater data variability and affect several assay parameters such as dynamic range and Z′ factor. It is important that Vivid® assay miniaturization did not show any significant negative effects on assay performance. Vivid® assays carried out in the 1536-well plate format demonstrated only a slight deviation from the assay parameters obtained in the 96-well format. All Vivid® assays performed in the 1536-well plate format have a very high Z′ factor in a range of 0.66 (CYP2D6 Blue) to 0.82 (CYP2C9 Red), making them highly reliable for uHTS applications. One of the most significant effects of assay miniaturization was reduction in the signal-to-background ratio observed for CYP1A2 Blue and CYP2C19 Blue Vivid® reactions performed in the 1536-well plate format, but broad dynamic range was maintained in both applications (Table 3) and did not affect the detection of assay modifiers. Some of the variations in dynamic range observed between the 96- and 1536-well formats could be due to the use of filter-based instruments (Ultra plate reader) versus monochromator-based instruments (Gemini XS plate reader) for detection. The effects of several organic solvents on the performance of assays employing Vivid® fluorogenic substrates and recombinant cytochrome P450 enzymes have been reported previously.[27-29] Here we explored assay tolerance to the presence of DMSO, an organic solvent commonly used to dissolve compound libraries in HTS applications. The presence of 1% DMSO did not significantly affect the Z′ factor in any of the 1536-well Vivid® assays but did result in the reduction of signal-to-background ratios, which was most dramatic in the CYP3A4 Vivid® Green and Red assays. However, high signal-to-background ratios in a range from 22 to 41 were still maintained under these conditions (Table 3), thus confirming the robustness and reliability of the assay performance in the presence of 1% DMSO. As a general guideline, the recommendation is to apply a proper control to account for the effect of DMSO or any organic solvent used in Vivid® assays.[27-29] Alternatively, to minimize the negative effect of DMSO on assay performance, test compounds could be delivered as redissolved dry films, according to recent data demonstrating comparable activity for compounds delivered in DMSO stocks or as dried compound films.[23] The latter approach may be preferable for further development of fully automated miniaturized assays and robotic applications.

Overall, we proved that HTS methods for in vitro cytochrome P450 metabolism and inhibition studies employing the Vivid® P450 fluorescent substrates and major human P450 isozymes described here are sensitive, homogeneous, robust, and amenable to miniaturization, allowing for ultra-high-throughput formats. The strong fluorescent signal, high signal-to-background ratio, and excellent Z′ factor value make these assays valuable tools for screening large compound libraries for cytochrome P450 metabolism and inhibition in a cost-effective, low-volume screening format. Furthermore, reduced amounts of enzyme used in uHTS present the advantage of minimizing enzyme variability by conducting multiple P450 assays with the same batch of the recombinant cytochrome P450 enzyme. This enables more accurate and consistent data generation during the extended lead optimization process.

One potential drawback of assays based on fluorescent reporter reactions is that both substrates and inhibitors act as assay modifiers by inhibiting the reporter fluorescent reaction, therefore preventing discrimination between allosteric and mechanism-based inhibition. However, this is a common issue for all of the known fluorescent reporter reactions and may be resolved in the future by application of more sophisticated kinetic models[45] that were not a subject of the current investigation. In summary, highly miniaturized formats of the Vivid® fluorescent assays for the assessment of human cytochrome P450 metabolism and inhibition present a valuable contribution to the area of early ADME toxicology studies.

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