

Proluciferin Acetals as Bioluminogenic Substrates for Cytochrome P450 Activity and Probes for CYP3A Inhibition^[S]

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ABSTRACT:

Cytochrome P450 (P450) assays use probe substrates to interrogate the influence of new chemical entities toward P450 enzymes. We report the synthesis and study of a family of bioluminogenic luciferin acetal substrates that are oxidized by P450 enzymes to form luciferase substrates. The luciferin acetals were screened against a panel of purified P450 enzymes. In particular, one proluciferin acetal has demonstrated sensitive and selective CYP3A4-catalyzed oxidation to a luciferin ester— K_m and k_{cat} are 2.88 μM and 5.87 pmol metabolite $\cdot min^{-1} \cdot pmol enzyme^{-1}$, respectively.

The proluciferin acetal was used as a probe substrate to measure IC_{50} values of known inhibitors against recombinant CYP3A4 or human liver microsomes. IC_{50} values for the known inhibitors correlate strongly with IC_{50} values calculated from the traditional high-performance liquid chromatography-based probe substrate testosterone. Luciferin acetals are rapidly oxidized to unstable hemi-orthoesters by CYP3A resulting in luciferin esters and, therefore, are conducive to simple rapid CYP3A bioluminescent assays.

Introduction

Cytochrome P450 (P450) enzymes oxidize endogenous compounds, drugs, new chemical entities (NCEs), and other xenobiotics (Guengerich, 2007). P450 enzyme inhibition or CYP gene induction by drugs can affect the elimination of concomitantly administered drugs thereby resulting in adverse drug-drug interactions (Wienkers and Heath, 2005). Fatalities due to profound drug-drug interactions are not uncommon (Lazarou et al., 1998; Köhler et al., 2000), and efforts to identify P450 active NCEs early in the drug discovery process continue (Zientek et al., 2008; Grime et al., 2009).

In general, P450 assays use probe substrates to interrogate the influence of NCE's toward P450 enzymes. Probes based on known drugs are traditionally detected by radiometric assays (Stresser et al., 1996; Moody et al., 1999) and mass spectrometry, yet these assays are disadvantaged by concerns over the safety and disposal of radioactive isotopes or by low throughput, respectively. Chromogenic, fluorogenic (Friden et al., 2006), and bioluminogenic probes (Cali et al., 2006) are rapid and sensitive, yet concerns regarding their analogy to traditional drug probes (Bell et al., 2008) must be addressed by empirical correlation to traditional probes. A complexity in interpreting correlative studies is associated in particular with CYP3A4, which

demonstrates substrate-dependent responses to certain analytes (Wang et al., 2000). Even traditional probes such as nifedipine, midazolam, and testosterone interrogate NCEs in a substrate-dependent manner.

Bioluminescent luciferase substrates produce photons in a luciferase-catalyzed reaction and have been used extensively as tools in structural biology and drug development (Fan and Wood, 2007). Numerous structure/activity articles (White et al., 1965; White and Woerther, 1966; Branchini et al., 1989; Woodroffe et al., 2008) have outlined the structural requirements of bioluminescent luciferase substrates. In contrast to a bioluminescent substrate, a bioluminogenic substrate is a luciferase prosubstrate that does not produce light with luciferase but can be converted to a light-generating bioluminescent substrate by an enzyme or system of interest. The bioluminescent product of the reaction of interest can then be detected as a luminescent signal in a second reaction with firefly luciferase. Assays based on bioluminogenic substrates have been reported for proteases (O'Brien et al., 2005), oxidases (Zhou et al., 2006b), phosphatases (Zhou et al., 2008), glutathione transferases (Zhou et al., 2006a), and glycosidases (Cali et al., 2008). In the majority of these cases, the bioluminogenic substrates have been modified at the 6'-carbon atom of luciferin with a recognition moiety for the enzyme of interest (see Scheme 1 for luciferin numbering).

The electronic characteristics of substrate molecules play a decisive role in the site of oxidation in P450-catalyzed reactions. A strong correlation has been demonstrated between the oxidation site in a P450 reaction and the most stable radical site in the organic substrates (Jones et al., 2002). The relative stabilities of the possible radicals formed on an organic molecule relate to the homolytic bond dissociation enthalpy of the carbon-hydrogen bond. Oxygens impart radical

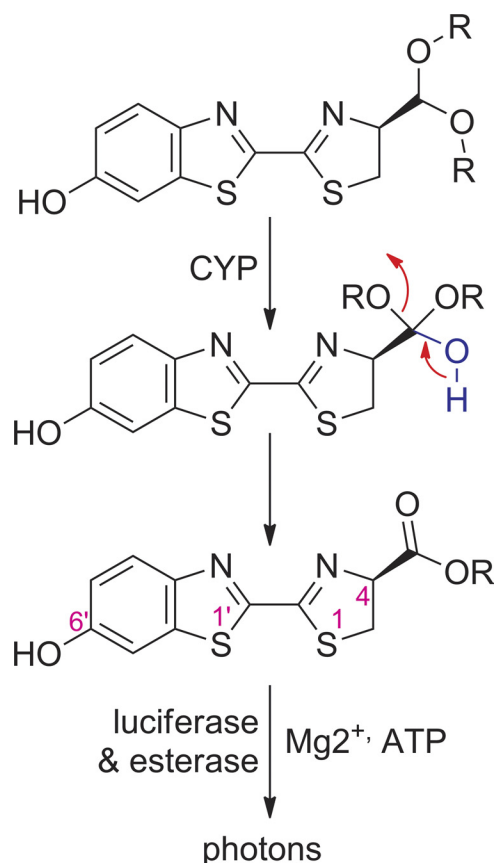
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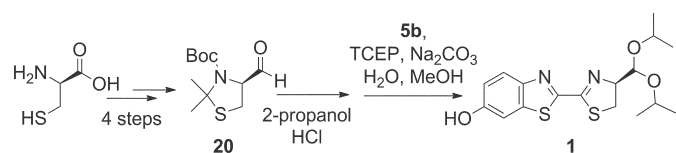
[S] The online version of this article (available at <http://dmd.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: P450, cytochrome P450; NCE, new chemical entity; HPLC, high-performance liquid chromatography; RLU, relative light units; HLMs, human liver microsomes; KPO₄, potassium phosphate buffered to the stated pH; LOD, limit of detection.



SCHEME 1. Proposed mechanism for bioluminescent P450 activity of proluciferin acetals.

stability to neighboring carbons, and P450-catalyzed O-dealkylations result from the oxidation of these carbons. However, the oxygens of an acetal group impart significant radical stability on the carbon atom sandwiched between them. Bond enthalpies for the C-H bond of a carbon between the oxygens in an acetal are some 10 kcal/mol lower than the corresponding C-H bonds of an ether (Blanksby and Ellison, 2003). Correspondingly, P450 enzymes should oxidize acetals at the carbon between the two oxygens thereby forming an unstable hemi-orthoester, which rapidly decomposes to an ester. This expectation suggested that acetal derivatives of D-luciferin could serve as bioluminescent P450 substrates according to the hypothetical scheme shown (Scheme 1). The scheme recognizes that luciferin esters do not react with luciferase to produce light but are rapidly and quantitatively de-esterified to the luminogenic carboxylic acid form in a luciferase



SCHEME 3. Synthesis of D-luciferin isopropyl acetal (compound 1) from D-cysteine.

formulation supplemented with a carboxyl esterase (Cali et al., 2006). In such a formulation, any luciferin acetal oxidized by a P450 according to the proposed scheme would convert to the luminogenic form and produce light with luciferase.

We report the synthesis and study of a family of bioluminescent luciferin acetals that are converted to luciferase substrates selectively by the CYP3A enzymes. This study examines the activity of the acetals toward a panel of P450 enzymes, and we examine a subset of acetals as bioluminescent probes for CYP3A4 inhibition measurements.

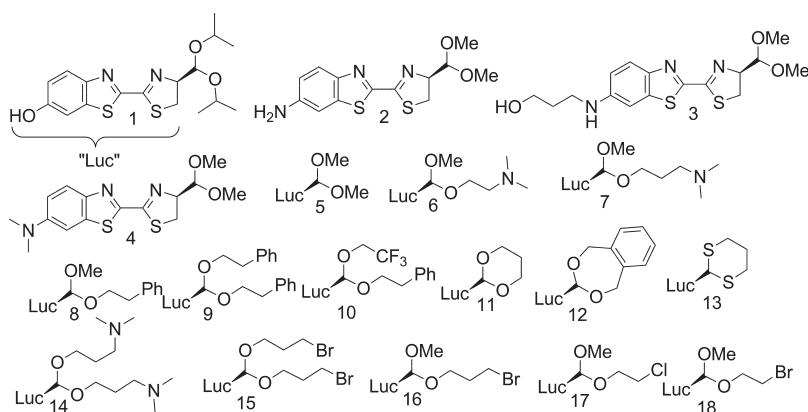
Materials and Methods

Synthesis. A series of acetals were synthesized to examine the structure/bioluminescent profile with P450 enzymes (Scheme 2). D-cysteine was converted to compound 1 in six steps (Scheme 3). D-cysteine was allowed to react with acetone to form the thiazolidine, which was subsequently protected with di-*t*-butyldicarbonate. The carboxylic acid was converted to the Weinreb amide and subsequently reduced to the aldehyde with lithium aluminum hydride to yield 20. The aldehyde was refluxed in 2-propanol and HCl. The intermediate was allowed to react with the nitrile of 5b to yield 1. Tris(2-carboxyethyl)phosphine was added to prevent any disulfide formation during the reaction. As a control, DL-cysteine was used in a parallel synthesis to generate the racemized version of 1. Chiral high-performance liquid chromatography (HPLC) was used to show that the reaction starting with D-cysteine results in enantiomerically pure 1 (data not shown).

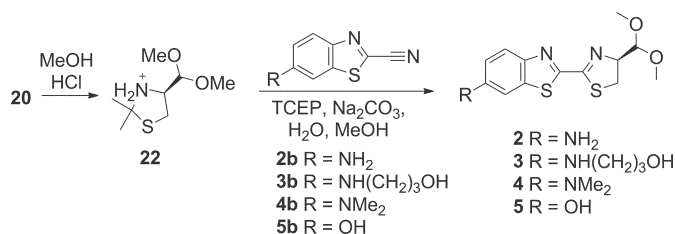
A series of dimethyl acetals based on alternative luciferase substrates were synthesized from the thiazolidine aldehyde 20 (Scheme 4). The dimethyl acetal of thiazolidine aldehyde 22 was condensed with various benzothiazole nitriles (2b–5b). Under aqueous conditions, compound 22 was proposed to establish equilibrium between the thiazolidine ring and a ring-opened β -mercaptoamine; the β -mercaptoamine rapidly reacts with the nitriles 2b to 5b to form the thiazoline products (2–5).

Aryl containing acetals (8–10), dioxanes, and dithianes (11–13) were constructed in a similar manner (Scheme 5). The acetals containing basic amines (6, 7, 14) were synthesized from a trans-acetalization of 5 with the appropriate halogenated alcohol followed by substitution with dimethylamine (Scheme 6). Detailed experimental protocols are included in supplemental materials.

Screening of P450s. Recombinant P450 enzymes in microsomal fractions (Supersomes) were purchased from BD Biosciences Discovery Labware (Bedford, MA). Each P450 enzyme is coexpressed with cytochrome P450 reductase; cytochrome b_5 is also coexpressed with some of the P450s (CYP2A6, -2B6, -2C8, -2C9, -2C19, -2E1, -2J2, -3A4, -3A5, -3A7, -4F2, -4F3A, -4F3B,



SCHEME 2. Series of proluciferin acetals tested against P450 activity.

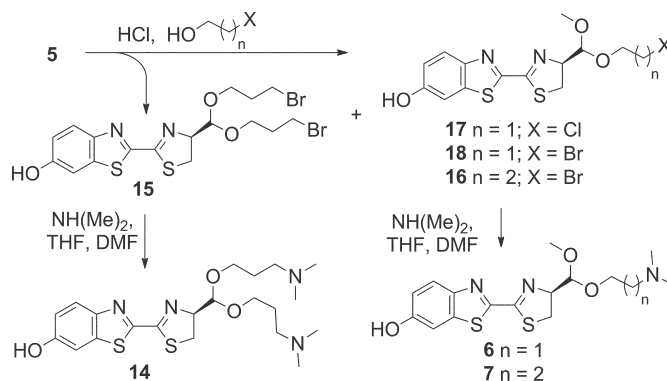


SCHEME 4. Dimethyl acetal derivatives of *N*-alkyl aminoluciferins were synthesized by converting **20** (Scheme 3) to the isolated intermediate **22** followed by the condensation with previously reported benzothiazole nitriles **2b** to **5b**.

and -4F12). The P450-negative controls are Supersomes devoid of P450 enzyme expression. To screen the proluciferin acetal compounds as P450 substrates, they were first prepared as 50 mM stock solutions in dimethyl sulfoxide and then combined at 50 μ M with P450 enzymes at 20 nM recombinant P450 enzyme and an NADPH-generating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 U/ml glucose-6-phosphate dehydrogenase) in an appropriate buffer as recommended by the P450 enzyme manufacturer (200 mM KPO₄, pH 7.4 for CYP3A4, 100 mM KPO₄, pH 7.4 for CYP1A1, -1A2, -1B1, -2D6, -2E1, -3A5, -3A7, -2J2, -4F12, and -19; 50 mM KPO₄, pH 7.4 for CYP2B5, -2C8, -2C19, -4F2, -4F3A, and -4F3B; 25 mM KPO₄, pH 7.4 for CYP2C9, 100 mM Tris-HCl, pH 7.5 for CYP2A6, -2C18, and -4A11). The 50 mM luciferin acetal stock solutions contributed 0.1% dimethyl sulfoxide to each reaction mixture. Reactions were performed in a volume of 50 μ l in opaque white 96-well plates that were incubated in a 37°C H₂O bath for 30 min. Reactions were initiated by addition of the NADPH-generating system. Reactions were stopped, and luminescence was initiated by adding 50 μ l of P450-Glo Luciferin Detection Reagent (Promega Corporation, Madison, WI) supplemented with 20 units/ml porcine esterase (Sigma-Aldrich, St. Louis, MO). After 20 min at room temperature, luminescence was read as relative light units (RLU) on a plate reading luminometer [Polarstar Optima (BMG Labtech, Durham, NC) or Veritas (Promega Biosystems, Sunnyvale, CA)].

HPLC Analysis of CYP3A4-Catalyzed Oxidation of 1. Fluorescence chromatograms (Supplemental Fig. 1) were used for the analysis of compound **1** with a control Sf9 membrane preparation, with CYP3A4 membranes, and with CYP3A4 membrane preparation with esterase treatment. Luciferin at 2.2 min was identified with an authentic standard. A peak at 7.5 min disappeared on treatment with esterase and was therefore assigned as the putative luciferin ester intermediate. Separation was obtained on an Adsorbosphere HS C18 column (5 μ , 150 \times 4.6 mm; Grace Davison Discovery Sciences, Deerfield, IL) with a 50 mM ammonium acetate buffer (pH6) and linearly modified acetonitrile gradient. Component elution was monitored with fluorescence by means of 330 nm excitation and 520 nm emission.

Measuring P450 Inhibition. Several known CYP3A4 inhibitors were applied in a dose-response mode to reactions of recombinant CYP3A4 or human liver microsomes (HLMs) with compound **1** or with the CYP3A4 substrate testosterone. The HLMs were a mixed gender pool from nine male and six female donors (BD Biosciences Discovery Labware). Reaction conditions for interrogation of inhibitors were established as described under *Results*, and GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to

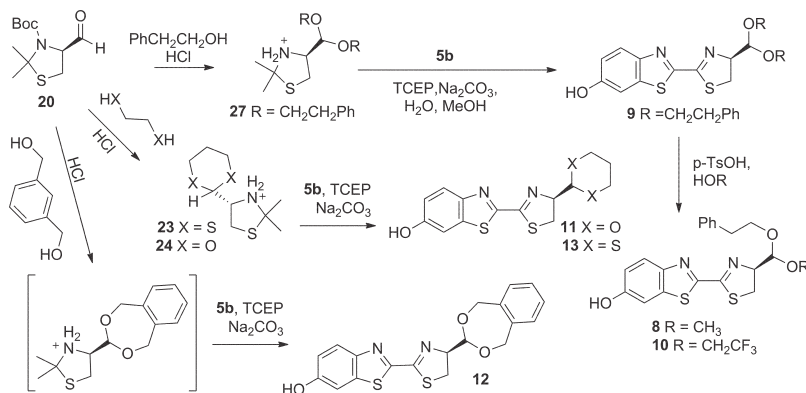


SCHEME 6. Mixed acetals and amine containing acetals were synthesized from **5**.

calculate IC₅₀ values. Inhibition reactions were performed in 100 mM KPO₄ buffer, pH 7.4 with 20 nM recombinant CYP3A4 and 10 μ g/ml HLMs using 50 μ l and 100 μ l volumes, respectively, for the compound **1** and testosterone assays. Testosterone reactions were performed as described previously (Kerwin et al., 1999). Compound **1** concentrations of 3 μ M for recombinant P450 or 8 μ M for HLMs were used. Testosterone concentrations of 50 μ M for recombinant P450 or 10 μ M for HLMs were used. Substrates and inhibitors were allowed to incubate for 10 min at 37°C with CYP3A4 or HLMs before initiating reactions by adding the NADPH-generating system. The reactions with compound **1** were stopped, and luciferin product was detected by luminescence in the same manner as described for P450 screening. The reactions with testosterone were stopped by addition of 50 μ l of acetonitrile and then centrifuged at 10,000g for 3 min. The supernatant (50 μ l) was injected into a 3 \times 100 mm, 3 μ C18 HPLC column and separated with a mobile phase initially of 15% acetonitrile increasing to 100% acetonitrile over 12 min, at a flow rate of 0.75 ml/min. The CYP3A4 reaction product 6 β -hydroxytestosterone was detected by its absorbance at 240 nm (HPLC retention time = 5 min) and quantitated by comparison to absorbance of authentic 6 β -hydroxytestosterone standards.

Results

Screening Cytochrome P450s for Activity with Proluciferin Acetals. A series of proluciferin acetals (Scheme 2) were screened for bioluminogenic activity against a panel of recombinant human P450 enzymes in microsomal fractions (Fig. 1). A moderate to high substrate concentration of 50 μ M was used with 20 nM P450 enzymes and a liberal reaction time of 30 min at 37°C. A control microsome fraction with no recombinant P450 expression was used to monitor intrinsic instability or non-P450-catalyzed conversion of the proluciferin acetals to luciferin. The bioluminogenic reactions are performed by incubating the P450 enzymes with proluciferin P450 substrates and NADPH in a first reaction where an active enzyme/substrate combination generates luciferin ester. In a second reaction,



SCHEME 5. Aryl containing acetal derivatives, dioxanes, and dithianes were synthesized from intermediate **20**.

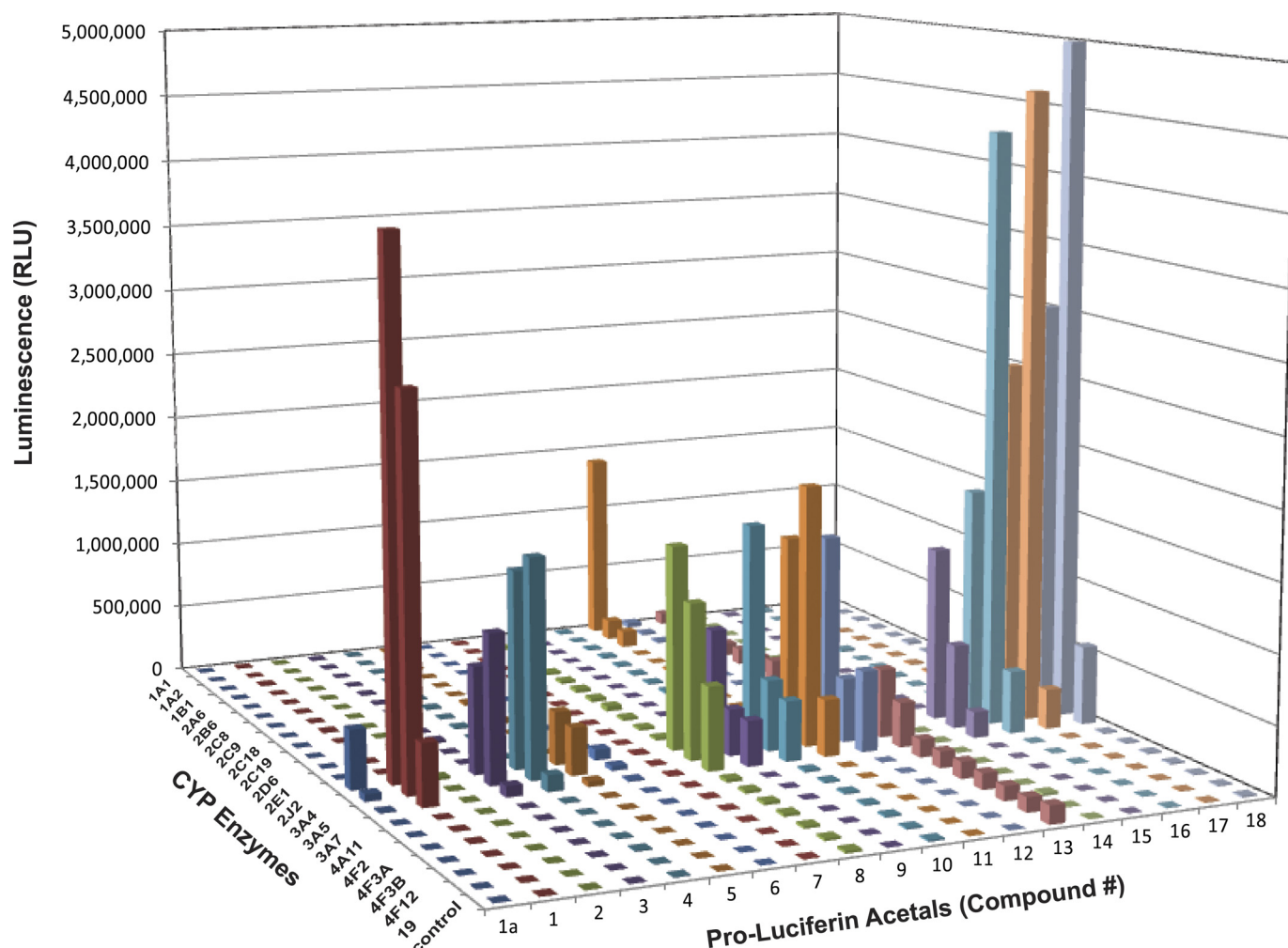


FIG. 1. Recombinant P450 (CYP) enzyme screen against proluciferin acetals. Compounds **1** to **18** are the respective acetals illustrated in Scheme 2 exposed at 50 μM to 20 nM recombinant P450 enzymes for 30 min at 37°C. **1a** is compound **1** exposed at 3 μM to 2 nM recombinant P450 enzymes for 10 min at 37°C.

carboxyl esterase and luciferase convert any luciferin ester to a proportional number of photons. P450 activity is detected on a luminometer as RLU. Other than **13**, compounds **1** to **18** showed only a minor luminescence signal in the control, indicating that non-P450 enzymes and/or uncatalyzed conversions of the proluciferin do not contribute to the signals. The diisopropyl acetal **1** displayed the largest CYP3A4 signal. CYP3A5 activity was highest for **16**, **17**, and **18**. CYP3A7 activity was highest for **8**. Compounds **2**, **7**, and **14**, gave no light in excess of the P450-negative control. The 1,3 dioxane **11** showed some CYP3A activity along with detectable CYP1A and -1B activity. The dithiane **13** displayed CYP3A5 and CYP3A7 activity only with substantial light in the control, indicating some level of luciferin contamination in the sample. Fourteen of 18 proluciferin acetals were bioluminescent primarily against CYP3A. Detailed CYP3A net luminescent signals were extracted from Fig. 1 and displayed with error bars in 2 dimensions for better visualization (Fig. 2).

Characterization of P450 Activity with Compound 1. The diisopropyl acetal **1** was studied in further detail because it displayed the largest signal with CYP3A4, an enzyme of particular interest because of its prominent role in drug metabolism. Preferential activity with CYP3A4, -3A5, and -3A7 suggested this compound could be used as a selective CYP3A probe. The recombinant CYP3A4 reaction with **1** was performed using fluorescence HPLC to detect the proluciferin

acetal and its CYP3A4 reaction products with and without addition of carboxyl esterase. Incubation of **1** with CYP3A4 gave rise to two apparent reaction product peaks (supplemental data). The smaller of the two peaks comigrated with an authentic D-luciferin standard. Esterase treatment shifted the second intermediate peak to the position of D-luciferin. This analysis is consistent with the model of Scheme 1 where the P450 enzyme initiates a reaction sequence that yields a luciferin ester that is processed to luciferin by added esterase. The appearance of a small D-luciferin peak in the absence of added esterase is likely due to esterase activity already present in the CYP3A4 microsome preparation, and this was confirmed by observing elimination of the peak by pretreatment of the microsomes with the carboxylesterase inhibitor phenylmethylsulfonyl fluoride (data not shown). The absence of luciferin and luciferin ester peaks after incubation with control membranes lacking P450 enzyme confirms that conversion of **1** to these products is P450-dependent. Nuncatalytic or non-P450 enzymatic conversions of **1** do not occur to a significant extent in the Supersome system.

Kinetic Characterization of CYP3A4-Catalyzed Oxidation of Compound 1. An initial test that varied the concentration of the common P450 reaction buffer KPO_4 (pH 7.4) showed the CYP3A4 reaction with compound **1** was optimal at 100 mM KPO_4 (data not shown), and this concentration was used for subsequent characterizations. The reaction was characterized by varying CYP3A4 enzyme

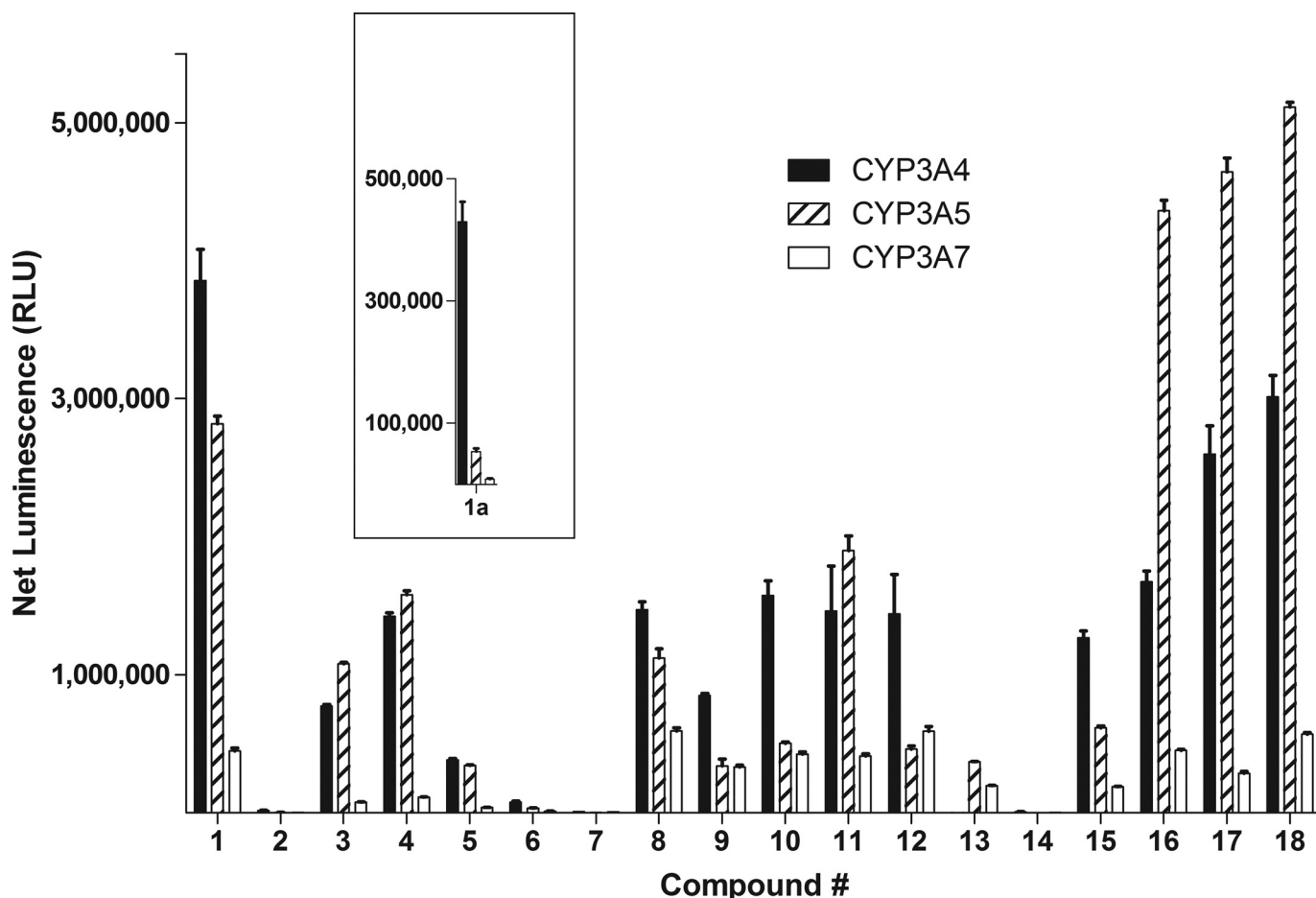


Fig. 2. Recombinant CYP3A enzyme screen against proluciferin acetals. Net luminescence values are signals from the P450 reactions shown in Fig. 1, minus the respective P450-independent controls ($n = 3$, \pm S.D.).

concentration, the initial concentration of **1**, and incubation time. Plots of time-dependent (Fig. 3, A–C) and enzyme concentration-dependent (Fig. 3, E–H) signal increases showed initial linear phases with decreases in linearity at longer time points, increased enzyme concentration, and decreased initial concentrations of **1**, indicating that **1** might become limiting in the nonlinear phases. The dose-dependent activity curves of compound **1** were fit to a hyperbola for each of the various reaction times and enzyme concentrations (Fig. 3, I–K). The average of the K_m values derived from the curve fits for 12 sets of conditions was $2.78 \pm 0.80 \mu\text{M}$. The substrate concentration curve was repeated under linear conditions of reaction time and enzyme concentration (10 min, 2 nM CYP3A4) (Fig. 3L). In this case, CYP3A4 reaction rates were calculated after converting RLU values to luciferin product concentrations by interpolation from a luciferin standard curve (data not shown). Under these conditions the curve fit found K_m and k_{cat} values of $2.88 \pm 0.18 \mu\text{M}$ and $5.87 \pm 0.14 \text{ pmol metabolite} \cdot \text{min}^{-1} \cdot \text{pmol enzyme}^{-1}$, respectively, for CYP3A4 (Table 1). A contribution from substrate depletion to the loss of linearity observed at lower substrate concentration, higher enzyme concentration, and greater incubation times is consistent with the reaction rates calculated for Fig. 3L. In contrast, steady-state reaction conditions can be assumed in 10 min reactions with 2 nM CYP3A4 at K_m substrate concentration where we estimate $<2\%$ of the substrate is consumed, and these conditions are reasonable for interrogation against CYP3A4 inhibitors. Figure 3F includes a CYP3A4 enzyme titration curve performed under nearly identical conditions: 10 min with $2.5 \mu\text{M}$ compound **1**. From a linear regression analysis, the limit

of detection (LOD) for the CYP3A4 enzyme was estimated at 0.04 nM. LOD is defined as the concentration in which the luminescent value falls within three S.D.s of the background.

These steady-state CYP3A4 conditions were also used in a repeat screen of compound **1** against the recombinant human P450 enzyme panel, where a substantial increase in selectivity for CYP3A4 over CYP3A5 and -3A7 was observed compared with the conditions of the initial screen with 20 nM enzyme, $50 \mu\text{M}$ substrate, and 30-min incubations (Fig. 1). This preference of compound **1** for CYP3A4 over CYP3A5 and -3A7 is explained in terms of the K_m , k_{cat} , and enzymatic efficiencies (k_{cat}/K_m) of the three enzymes (Fig. 3, D and L; Table 1). Ratios of the enzymatic efficiencies, CYP3A4/CYP3A5 or CYP3A4/CYP3A7, indicate a 9-fold and 102-fold selective preference for CYP3A4 over the other two enzymes.

Inhibition Assays. Compound **1** (Luciferin-IPA; Promega) was used as a probe substrate to measure IC_{50} values of known inhibitors against recombinant CYP3A4 or HLMs. The linear reaction conditions for recombinant CYP3A4 at substrate K_m were used ($3 \mu\text{M}$ compound **1**, 2 nM CYP3A4, 10-min reactions). Recombinant CYP3A4 inhibition was also measured against the commonly used testosterone 6β -hydroxylation reaction (Kenworthy et al., 1999; Wang et al., 2000), and IC_{50} values from this study were compared with those determined with compound **1**. Conditions for the testosterone assays were first established so that reactions were performed at substrate K_m ($50 \mu\text{M}$) under conditions of linearity with respect to time and CYP3A4 concentration (10 min, 20 nM). The IC_{50} values for known CYP3A4 inhibitors correlated closely between the compound

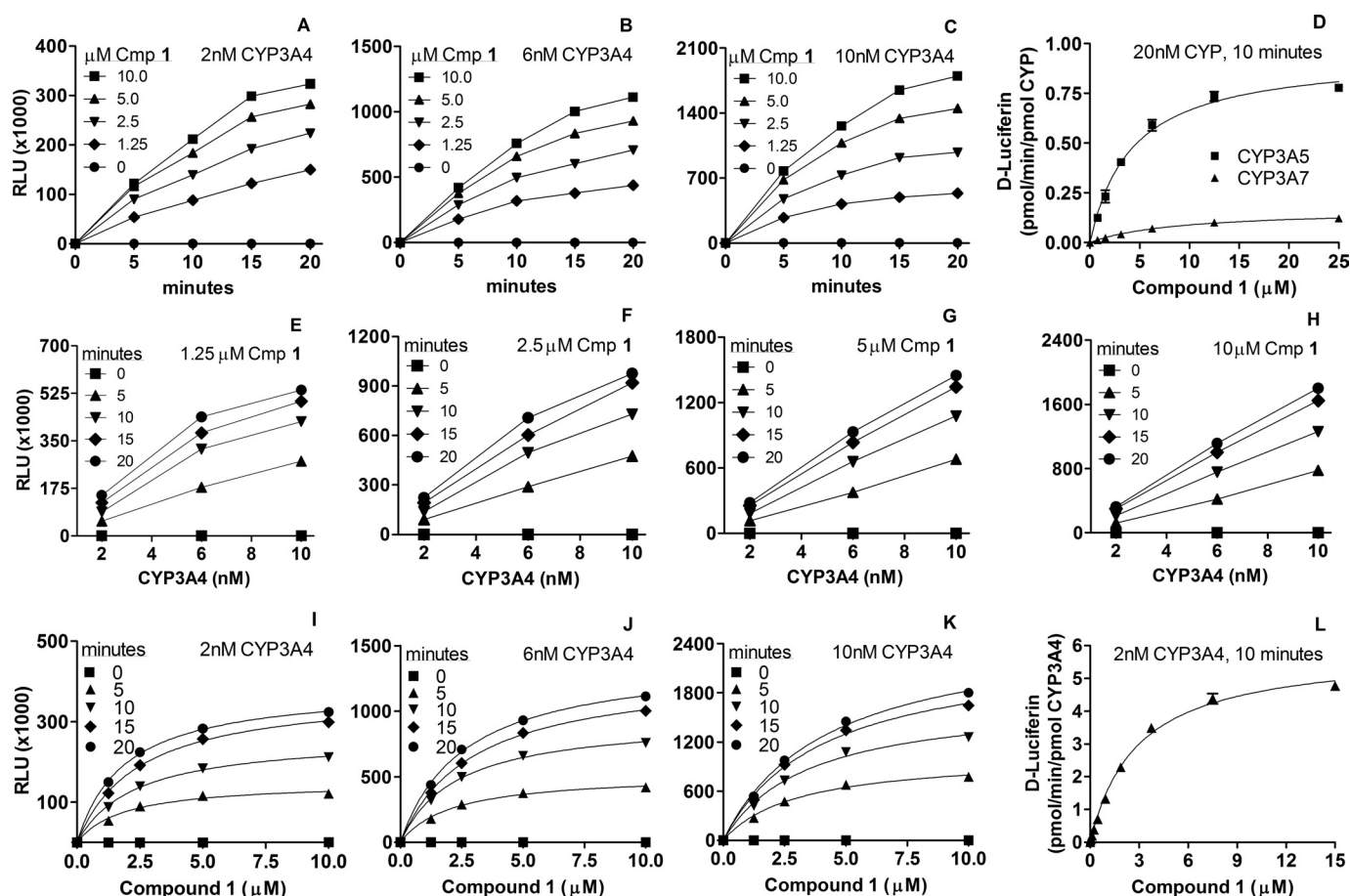


FIG. 3. Characterization of the CYP3A4 reaction with compound 1. Fifty-microliter reactions in duplicate or triplicate were performed at 37°C. Recombinant CYP3A4 activity was scored as RLU after adding a luciferase reaction mixture for detection of the compound 1/CYP3A4 reaction product. A–C, time-dependent luminescence; E–H, CYP3A4 concentration-dependent luminescence; I–K, compound 1 dose-dependent luminescence. For D, recombinant CYP3A5 or -3A7 were substituted for CYP3A4. For D and L, rates of D-luciferin production were calculated after conversion of RLU to D-luciferin concentration by comparison to a D-luciferin standard generated with the luciferase reaction mixture.

1 assay and testosterone assays (Fig. 4A). A similar comparison was performed using HLMs in place of recombinant CYP3A4 enzyme. Reaction conditions for interrogation of inhibitors were first established and found to be somewhat different from those observed with recombinant CYP3A4. For compound 1, an apparent K_m concentration of 8 μM was observed and used in 10-min reactions with 20 $\mu\text{g}/\text{ml}$ HLMs. It was also noted that significant luminescent signals were not observed in HLMs when NADPH was withheld from the reactions (data not shown). This was consistent with a P450-dependent mechanism and the absence of noncatalytic or non-P450 enzyme-dependent substrate conversion. Using testosterone as a substrate, an apparent K_m of 50 μM was observed and used in 10-min reactions with microsomes at 20 $\mu\text{g}/\text{ml}$. As with recombinant CYP3A4, a close correlation of IC_{50} values between the testosterone and compound 1 assay was observed for the eight inhibitors tested (Fig. 4B).

TABLE 1

Kinetic data for CYP3A-catalyzed oxidation of proluciferin acetal 1 based on analysis of Fig. 3, D and L

Reaction	K_m μM	k_{cat}	k_{cat}/K_m
CYP3A4	2.88 ± 0.18	5.87 ± 0.14	2.04
CYP3A5	4.21 ± 0.31	0.94 ± 0.02	0.22
CYP3A7	8.85 ± 0.53	0.17 ± 0.004	0.02

Discussion

The P450-catalyzed oxidation of proluciferin acetals results in bioluminescent luciferase substrates. Scheme 1 describes the proposed mechanism by which light is produced from P450 activity. The oxidation of the acetal yields an unstable hemi-orthoester, which is proposed to decompose to the luciferin ester. The resulting luciferin ester is converted to D-luciferin by esterase, and its concentration is measured using the bioluminescent output from a luciferase reaction. Light output is proportional to P450 activity.

The acetals were designed to explore the structural effects of hydrophobicity (1, 5, 9, 11, 12, 15) and charge (6, 7, 14) on the P450 activity. Mixed acetals (10, 16–18) and a dithiane (13) were also produced. Furthermore, alkyl aminoluciferin luciferase substrates (Woodroffe et al., 2008) have been reported, and a series of related acetals (2, 3, 4) were generated. The screen of P450 enzymes was designed to push the system to detect any reasonable activity.

CYP3A converts 1 to a compound that is easily measured in a bioluminescent reaction. A direct relationship between the P450 activity and light output exists because the concentration of luciferin produced by P450 is within the linear range of a standard curve of luciferin bioluminescence. A high degree of selectivity by this compound 1 for the CYP3A subfamily was expected based on screening a panel of recombinant P450 enzymes (Fig. 1), and greater selectivity for CYP3A4 could be dialed in with reaction conditions that favor

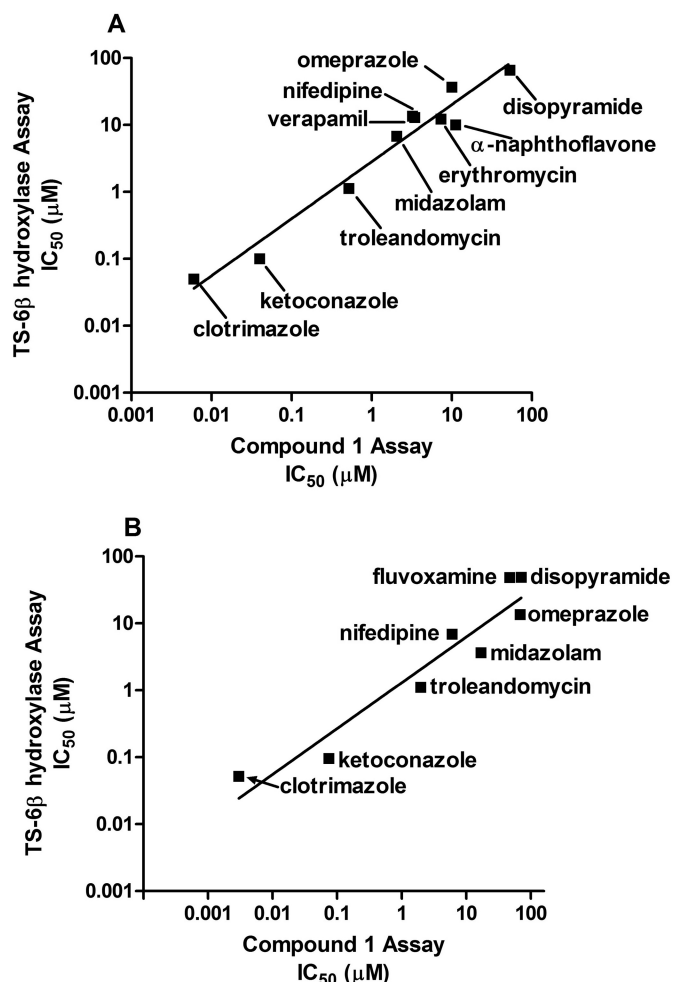


Fig. 4. Correlation of testosterone and compound **1** as probe substrates for P450 inhibition assays. IC₅₀ comparisons of recombinant CYP3A4 assays ($r^2 = 0.96$, $P < 0.0001$) (A) and human liver microsome assays ($r^2 = 0.91$, $P = 0.0003$) (B).

CYP3A4 over CYP3A5 and CYP3A7 (Fig. 2; Table 1). The selectivity of compound **1** was confirmed by inhibition studies in HLMs that contain a mixture of potentially cross-reacting P450 enzymes. Known CYP3A4 inhibitors inhibited the HLM activity at IC₅₀ values consistent with selective CYP3A4 inhibition, as indicated by close correlation to the IC₅₀ values determined by the well established CYP3A4-selective testosterone 6 β -hydroxylation reaction (Fig. 4A).

The 0.04 nM LOD for CYP3A4 activity with substrate **1** is very low. This allows for an assay of exquisite sensitivity. By reducing the amount of P450 containing microsomes in a reaction, the extent of nonspecific substrate or analyte binding is kept to a minimum or even nondetectable level. A very sensitive assay also enables detection of activity in samples with low P450 expression. The limit of CYP3A4 detection is potentially influenced by three scenarios: 1) contamination of **1** with D-luciferin, 2) non-P450-catalyzed conversion of **1** to D-luciferin, or 3) uncatalyzed conversion of **1** to D-luciferin. Due to the drastic difference in functional groups and intrinsic stability of the acetal functionality, contaminating luciferin was typically reduced to less than 0.0001% of proluciferin acetals using either silica or reversed-phase chromatography. Non-P450-catalyzed and uncatalyzed conversions apparently do not contribute significantly to background signals because there was no evidence of conversion in the P450-negative control of the recombinant P450 system (Fig. 1; Supplemental Fig. S1), and conversion in HLMs was NADPH-dependent, a hallmark of P450-dependent catalysis.

IC₅₀ comparisons for known CYP3A4 inhibitors between the compound **1** assay and the testosterone 6 β -hydroxylation assay showed nearly identical rank ordering for both recombinant CYP3A4 and HLM reactions, indicating that **1** can be used as a surrogate for the testosterone CYP3A4 assay (Table 2). The HLM correlation is also consistent with the high degree of selectivity of compound **1** for CYP3A4 enzymes. The IC₅₀ values were closely correlated for most of the inhibitors showing fold differences between unity and approximately 4-fold. A few compounds showed larger fold differences, which is not unexpected given the substrate-dependent nature of CYP3A4 inhibition (Wang et al., 2000). Differences between HLMs and recombinant CYP3A4 inhibition are known from the literature, although they are not explained within the scope of this study (Nomeir et al., 2001).

In addition to cell-free enzyme inhibition studies, the utility of compound **1** for nonlytic cell-based assays of CYP3A induction in cultured hepatocytes has also been described previously (Li, 2009; Doshi and Li, 2011). The CYP3A compound **1** was also applied to hepatocytes in a three-part multiplex format that provided luminescent CYP3A, a fluorescent CYP1A, and a luminescent viability measurement from a single culture well (Larson et al., 2011).

The simple nature of the luminogenic proluciferin acetal assays likely provides an advantage to P450 activity screens of large chemical libraries and other applications that benefit from a high-throughput approach. P450 assays based on HPLC or liquid chromatography mass spectrometry analysis are widely accepted, but they are often too slow for high-throughput applications and are limiting when rapid turnaround times are needed. Furthermore, the ease of implementing a bioluminescent P450 assay facilitates expansion from assays of a single concentration of test compound to more informative full-dose response screens. Data from such quantitative high-throughput P450 screens reveal structure/activity relationships and inhibition trends in large compound sets, and enable the annotation of compound libraries with P450 data (MacArthur et al., 2009; Veith et al., 2009). The strong correlation between inhibitory data from the CYP3A4 luciferin acetal and the testosterone assays supports the expectation that the bioluminescent assays will provide relevant data while meeting throughput requirements in large P450 screens.

TABLE 2

IC₅₀ comparisons

Data compare inhibition with the compound **1** assay and testosterone 6 β -hydroxylase assay using recombinant CYP3A4 or human liver microsomes.

CYP3A4 Inhibitors	rCYP3A4			HLMs		
	1 ^a	TS ^b	Δ ^c	1 ^a	TS ^b	Δ ^c
	IC ₅₀ (μM)			IC ₅₀ (μM)		
Clotrimazole	0.006	0.05	8	0.003	0.052	16.7
Ketoconazole	0.04	0.10	2.5	0.074	0.10	1.3
Troleandomycin	0.52	1.13	2.2	1.96	1.11	1.8
Midazolam	2.06	6.80	3.3	16.82	3.64	4.6
Nifedipine	3.24	13.55	4.2	5.99	6.90	1.2
Verapamil	3.45	12.95	3.8	115	14.86	7.7
Erythromycin	7.33	12.27	1.7	NC ^d	NC ^d	
Omeprazole	11.21	10.00	1.1	68.27	13.52	5.0
α-Naphthoflavone	10.00	36.64	3.7	NC ^e	NC ^e	
Fluvoxamine	ND ^f	ND ^f		46.94	48.31	1.0
Disopyramide	53.29	65.88	1.2	71.09	48.80	1.5

r, recombinant; TS, testosterone; ND, not determined; NC, not calculated.

^a IC₅₀s determined using bioluminescent assay with compound **1**.

^b IC₅₀ determined using testosterone 6 β -hydroxylase assay.

^c Δ is the fold difference in IC₅₀ values between the two assays.

^d IC₅₀ was not calculated due to only modest inhibition of both assays.

^e IC₅₀ was not calculated due to a modest stimulation.

^f Fluvoxamine was not applied to recombinant CYP3A4.

Authorship Contributions

Participated in research design: Meisenheimer, Ma, Sobol, Klaubert, and Cali.

Conducted experiments: Meisenheimer, Uyeda, Ma, Sobol, McDougall, Corona, Simpson, and Cali.

Contributed new reagents or analytic tools: Meisenheimer and Simpson.

Performed data analysis: Meisenheimer, Ma, Sobol, Simpson, and Cali.

Wrote or contributed to the writing of the manuscript: Meisenheimer, Uyeda, Ma, Sobol, McDougall, Corona, Simpson, Klaubert, and Cali.

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