



P450-Glo™ CYP3A4 Biochemical and Cell-Based Assays

ABSTRACT The bioavailability and clearance of most therapeutic drugs are primarily mediated by cytochrome P450 enzymes, and adverse drug-drug interactions frequently occur because of altered P450 activities. In this article, we describe cytochrome P450 assays that can predict both P450 induction-based and P450 inhibition-based drug-drug interactions at the earliest stages of drug discovery. We focus on assays for the CYP3A4 enzyme, which accounts for about 30% of the P450 in human liver and oxidizes about half of all drugs that are eliminated after prior metabolism. We have developed three luminogenic P450-Glo™ CYP3A4 substrates: Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE. Each substrate is used as a probe for in vitro biochemical assays of recombinant CYP3A4 to detect P450 inhibition by test compounds. Additionally, Luciferin-PFBE is ideal for cell-based measurements of CYP3A gene inductions by drugs. The cell-based assay is rapid and simple and can be performed in a nonlytic mode that leaves cells intact for additional analysis.

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INTRODUCTION

Cytochrome P450 enzymes (P450s) are the major catalyst for oxidative metabolism of a vast array of hydrophobic chemicals, including therapeutic drugs (1,2). Consequently, the bioavailability and clearance of many drugs are influenced by P450s, and adverse drug-drug interactions (DDI) frequently occur because of altered P450 activities (3). One type of DDI occurs when one drug inhibits a P450 that metabolizes a second co-administered drug, causing the second drug to accumulate to a toxic level. A second type of DDI occurs when the first drug induces expression of a P450 gene, leading to increased P450 activity, accelerated clearance and reduced efficacy of a second drug. Therefore, as drugs are developed and prescribed, it is important to recognize how they affect P450s.

Of the several P450s that metabolize drugs, CYP3A4 is the most important (4). CYP3A4 features prominently in DDIs because it oxidizes and is inhibited by many small organic compounds, and the CYP3A4 gene is inducible by a spectrum of compounds. As with any P450, CYP3A4 induction and inhibition are studied by measuring the effect of a test compound on enzyme activity with a probe substrate.

The P450-Glo™ Assay^(a,b) technology (Figure 1) is a rapid high-throughput approach to predict both induction- and inhibition-based DDIs at the earliest stages of drug discovery and development (5). The luminescent P450-Glo™ Assays provide several advantages over fluorescent and nonoptical assays. The assays overcome throughput limitations of HPLC-based assays and radioactive hazards associated with radiochemical assays and obviate the problem of interference by fluorescent analytes in fluorescent probe-based assays. The P450-Glo™ Assays measure the analyte D-luciferin, and this results in high P450 sensitivity because of the remarkable

sensitivity of firefly luciferase to detect D-luciferin. In this article, we focus on P450-Glo™ substrates for the key drug-metabolizing enzyme CYP3A4.

LUMINOGENIC P450-GLO™ CYP3A4 SUBSTRATES

We have developed three luminogenic CYP3A4 substrates for the luciferase-based P450-Glo™ technology (5). Each substrate is a luciferase-inactive derivative of the light-generating luciferase substrate D-luciferin. P450 enzymes convert the substrates to the luciferase-active product D-luciferin in the first reaction. The luminescent signal from the second reaction with luciferase is proportional to D-luciferin concentration and reflects P450 enzyme activity (Figure 1). The CYP3A4 substrates are Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE, which share the D-luciferin core structure but differ in the cleavable group (Figure 2). Factors that influence the choice of substrate include P450 enzyme selectivity, DMSO sensitivity and source of CYP3A4 (microsomes versus cells).

The selectivity of each luminogenic P450-Glo™ CYP3A4 substrate is shown in Figure 2, where a panel of 21 recombinant human P450s was screened against the three substrates. Luciferin-BE reacted with CYP3A4, 3A5, 3A7 and 4F12, while Luciferin-PFBE and Luciferin-PPXE showed substantial activity with only CYP3A enzymes. While CYP3A4, 3A5 and 3A7 share many substrates and inhibitors, they differ in expression patterns. CYP3A4 is the major form

CYP3A4 accounts for about 30% of the P450 in human liver, the major site for drug metabolism, and oxidizes about half of all drugs eliminated after prior metabolism (4).

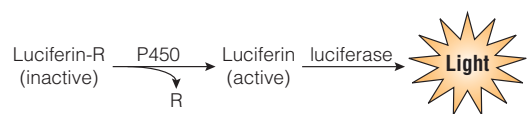


Figure 1. Conversion of P450-Glo™ substrate by cytochrome P450. Cytochrome P450 enzymes act on a luminogenic substrate to produce a luciferin product that generates light with the Luciferin Detection Reagent, which is added after the P450 reaction is complete.

The shared “3A” designation of CYP3A enzymes reflects P450 nomenclature conventions. The “3” gene family encodes P450s with >40% sequence identity at the amino acid level, and “A” is a subfamily with >55% identity (6).

expressed in adult liver and intestine, and CYP3A5 is typically expressed at much lower levels than CYP3A4 and then in only a subset of the adult population (6). CYP3A7 is the predominant CYP3A in fetal liver and disappears gradually from birth through early childhood and is replaced by CYP3A4 and 3A5 (7). The patterns of cross-reactivity for the luminogenic CYP3A substrates are not at issue when assaying recombinant or purified P450 preparations that only contain a single P450. In contrast, activity toward any of the substrates from a native preparation such as human liver microsomes or in a cell-based hepatocyte assay may reflect contributions from any or all cross-reacting enzymes.

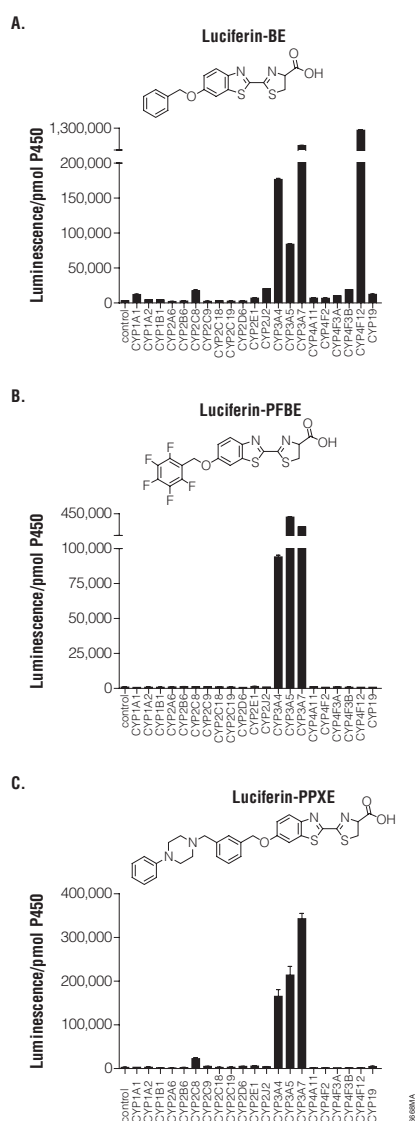


Figure 2. Substrate selectivity for P450 enzymes. Twenty one recombinant human P450 isoforms, expressed in microsomes from an insect cell expression system, were assayed for activity against Luciferin-BE, Luciferin-PFBE, and Luciferin-PPXE in 96-well white luminometer plates. One picomole of each enzyme was incubated in KPO_4 buffer (pH 7.4) with $50\mu M$ substrate and an NADPH regenerating system in a $50\mu l$ reaction for 30 minutes at $37^\circ C$. Luciferin Detection Reagent was added, and luminescence was read using a GloMax™ 96 Microplate Luminometer (Cat.# E6521). Results are shown as the mean \pm SD, $n = 3$.

P450-GLO™ CYP3A4 ASSAY PERFORMANCE

Dose dependence and linearity with respect to time and enzyme concentration for CYP3A4 reactions are shown in Figure 3. Time courses for accumulation of D-luciferin, the P450-Glo™ reaction product, showed differences for the three substrates. All reactions showed linear product accumulation for 10 minutes or longer at $37^\circ C$ (Figure 3, Panel A). Linearity persisted longer with each substrate when reactions were performed at $24^\circ C$ (data not shown). Each reaction was very sensitive, with limits of CYP3A4 detection of $<0.01 pmol$ for Luciferin-BE and Luciferin-PPXE and $<0.001 pmol$ for Luciferin PFBE (Figure 3, Panel B). Substrate dose dependence was measured under conditions of linearity with respect to time and temperature (Figure 3, Panel C). Each reaction showed substrate saturation. Substrate inhibition was observed at the highest concentrations of Luciferin-PFBE and Luciferin-PPXE and at concentrations $>200\mu M$ for Luciferin-BE (data

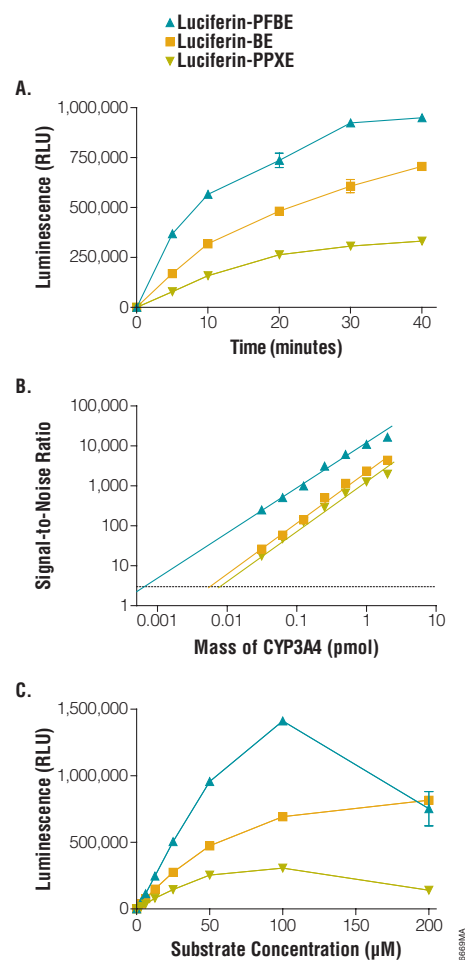


Figure 3. CYP3A4 assay characterization. CYP3A4 activities are shown with Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE substrates. **Panel A.** Time-dependent signal increases at $37^\circ C$ in $50\mu l$ reactions with $1 pmol$ CYP3A4 and $50\mu M$ Luciferin-BE, $50\mu M$ Luciferin-PFBE or $30\mu M$ Luciferin-PPXE. **Panel B.** CYP3A4 enzyme concentration dependence of 10-minute reactions at $37^\circ C$ with $50\mu M$ Luciferin-BE, $50\mu M$ Luciferin-PFBE or $30\mu M$ Luciferin-PPXE. **Panel C.** Substrate dose dependence of 10-minute reactions at $37^\circ C$ with $1 pmol$ recombinant CYP3A4.

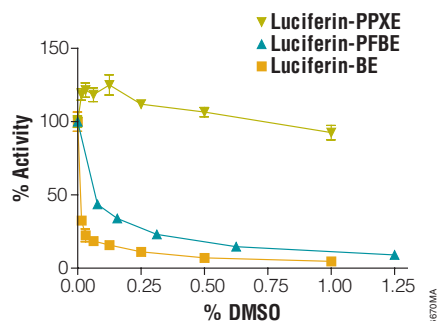


Figure 4. DMSO sensitivity of CYP3A4 reactions. CYP3A4 activity was measured in the presence of increasing amounts of DMSO in 30-minute assays at 37°C with 1 pmol of CYP3A4 and 50 μM substrate.

not shown). Substrate inhibition is a commonly observed property of CYP3A4 reactions, so it is not unexpected with luminogenic substrates. Indeed, the atypical kinetics of CYP3A4 have been extensively studied (8,9). Since CYP3A4 substrate dose dependence curves are a poor fit to a conventional hyperbolic Michaelis-Menton model, the substrate concentrations where half maximal activity is observed is sometimes expressed as S_{50} rather than K_m (10). For Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE, $S_{50} \approx 50 \mu\text{M}$, $50 \mu\text{M}$ and $25 \mu\text{M}$, respectively.

Many CYP3A4 reactions are inhibited by the common organic solvent dimethylsulfoxide (DMSO; 11). This is a concern when concentrated stock solutions of test compounds are dissolved in DMSO, as is frequently true for drug libraries. CYP3A4 reactions with Luciferin-BE and Luciferin-PFBE do show a marked sensitivity to DMSO, but reactions with Luciferin-PPXE do not (Figure 4). Therefore, Luciferin-PPXE is the substrate of choice when DMSO cannot be avoided or minimized. For CYP3A4 reactions with Luciferin-BE or Luciferin-PFBE, it is preferable to use acetonitrile as the solvent for concentrated stock solutions of test compounds. The CYP3A4 reactions with all three luminogenic substrates are insensitive to acetonitrile up to about 2% (data not shown).

When screening compounds with any CYP3A4 substrate, it is important to recognize that CYP3A4 binds substrates and inhibitors in multiple, potentially nonoverlapping orientations (12,13). Furthermore, some compounds exhibit positive cooperativity with CYP3A4 when presented in concert; the presence of one compound stimulates activity with the second (14,15). Though the exact configuration of CYP3A4 substrate/inhibitor binding is controversial, it is frequently described in terms of sites where testosterone, midazolam and nifedipine bind (12,16). If binding of a test compound overlaps with the substrate, it will score as an inhibitor; if it binds in a nonoverlapping configuration, it may activate the reaction or have no effect. Activity toward Luciferin-PPXE is inhibited by midazolam and nifedipine and activated by testosterone, so use of this substrate allows detection of compounds that bind like all three of these, usually by inhibition but also

by activation (Table 1). Luciferin-BE and Luciferin-PFBE reactions are inhibited by midazolam but activated by nifedipine and testosterone. Still, Luciferin-BE and Luciferin-PFBE can detect compounds from all three classes but with a greater frequency of activators than Luciferin-PPXE. IC_{50} values measured in CYP3A4 reactions with the three luminogenic substrates correlate well with published values using conventional assays (Table 1; 5,17–23).

CELL-BASED CYP450 ASSAYS

Luminogenic P450-Glo™ CYP3A4 substrates are cell-permeant and therefore useful for detecting CYP3A gene inductions in cell-based assays. Induction is measured as an increase in CYP3A enzyme activity. In human cells such as hepatocytes this increase can be a combination of CYP3A4, 3A5 and 3A7 activities (Figure 2). To perform cell-based assays, cells are cultured as monolayers and treated with test compounds. After a sufficient exposure time, typically 48 hours, the luminogenic substrate is added to cell culture medium and incubated with the cells for a predetermined time, typically 3–4 hours. The substrate diffuses into cells and is metabolized by P450 enzymes, and the reaction product diffuses into the culture medium. A sample of medium is combined with an equal volume of Luciferin Detection Reagent, and luminescence is read and correlated with P450 activity. Induction is observed when luminescence from test compound-treated cells is greater than untreated controls. Figure 5 shows an example using fresh hepatocytes from a human donor to examine the effect of the antibiotic rifampicin, a well known CYP3A4 gene inducer (24). Of the three substrates, Luciferin-PFBE is most effective at detecting induction (Figure 5, Panel A). The fold induction observed with Luciferin-PFBE correlates well with a conventional assay that measures testosterone hydroxy-

Table 1. Inhibition of Recombinant CYP3A4. IC_{50} Values from CYP3A4 Reactions are Compared to Values Published in the Literature and Determined Using Various Nonluminogenic Methods.

Test Compound	IC_{50} Values (μM) with Luciferin-BE ^(1,2)	IC_{50} Values (μM) with Luciferin-PFBE ^(1,2)	IC_{50} Values (μM) with Luciferin-PPXE ^(1,2)	Published IC_{50} Values ^(1,2)
Midazolam	17.4	26.0	8.3	1.3–59.8 (17)
Nifedipine	+	+	19.9	+, 0.2–17.2 (18)
Testosterone	+	+	+	+
Ketoconazole	0.1	0.2	0.1	0.1–0.2 (17)
Erythromycin	1.2	0.7	6.5	1.8–74 (17)
Troleandomycin	0.2	0.1	0.3	0.3–6.1 (17)
Verapamil	0.04	0.5	0.5	0.12–4.0 (17,21)
Clotrimazole	0.01	n.d.	0.02	0.002–0.06 (17,22)
Azamulin	0.1	0.1	0.5	0.03, 0.12 (23)

¹Luminogenic substrates were used at or near S_{50} concentrations, and reactions were performed with a range of test compound concentrations. When multiple values or a range of values is given, it typically reflects the probe substrate dependence of inhibition (16).

²A “+” indicates that positive cooperativity was observed; the test compound stimulated CYP3A4 activity with the luminogenic substrate.
n.d. = not determined

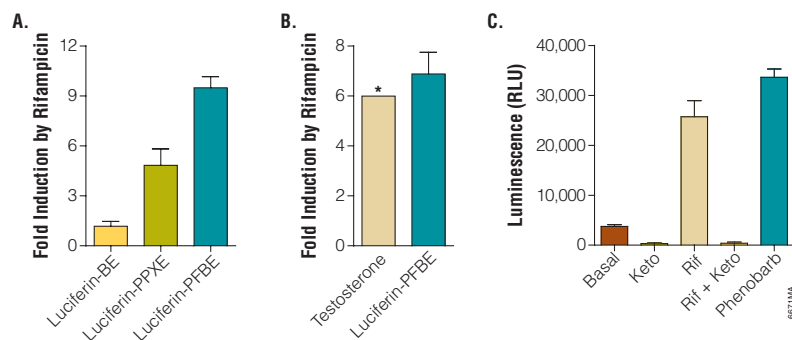


Figure 5. Cell-based CYP3A4 induction assays. Human hepatocytes in monolayers in 96-well plates were treated for 48 hours with 10 μ M rifampicin or 0.5mM phenobarbital. Luminogenic substrates (50 μ M final concentration) were added to culture medium and incubated for 4 hours before removing a sample of medium to combine with Luciferin Detection Reagent in a separate plate. Luminescence was read using a GloMax™ 96 Microplate Luminometer (Cat.# E6521). **Panel A.** Fresh human hepatocytes with Luciferin-BE, Luciferin-PPXE or Luciferin-PFBE. Fold induction is the activity with rifampicin treatment (induced activity) divided by activity from untreated cells (basal activity). **Panel B.** Cryopreserved human hepatocytes with Luciferin-PFBE (The asterisk denotes the fold induction from testosterone assay reported by the supplier of the cells, In Vitro Technologies). **Panel C.** Ketoconazole (keto, 10 μ M) was co-incubated with Luciferin-PFBE in control and rifampicin (rif)-treated cells.

lation as a marker of CYP3A enzyme activity (Figure 5, Panel B). Luciferin-PFBE-directed activity is also induced by other known CYP3A gene inducers, such as phenobarbital (25). Basal and rifampicin-induced activities with Luciferin-PFBE were confirmed to be CYP3A by showing sensitivity to inhibition by the CYP3A inhibitor ketoconazole (Figure 5, Panel C).

CONCLUSION

We show that Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE are ideal probe substrates for CYP3A4, the predominant P450 enzyme in adult human hepatocytes, to monitor P450 enzyme inhibition and gene induction. Results obtained with these substrates correlate well with published results from conventional CYP3A4 assays. The Luciferin-PFBE is suitable for nonlytic cell-based assays, which allow intact cells to be used in additional analyses. Thus, the P450-Glo™ Assays provide a rapid, safe and convenient way to predict the two most common causes of CYP3A4-mediated adverse drug-drug interactions using cell-based or biochemical assays.

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ORDERING INFORMATION

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay*	10ml	V8751
P450-Glo™ CYP1B1 Assay*	10ml	V8761
P450-Glo™ CYP1A2 Assay*	10ml	V8771
P450-Glo™ CYP2C8 Assay*	10ml	V8781
P450-Glo™ CYP2C9 Assay*	10ml	V8791
P450-Glo™ CYP3A4 Assay*	10ml	V8801
P450-Glo™ CYP3A7 Assay*	10ml	V8811
P450-Glo™ CYP2C19 Assay*	10ml	V8881
P450-Glo™ CYP2D6 Assay*	10ml	V8891
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890

* Other sizes are available.

(i) U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

(ii) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

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