CYP3A4 P450-Glo™ Assays with Luciferin-IPA: The Most Sensitive and Selective Bioluminescent CYP3A4 Assay

James J. Cali[1], Mary Sobol[1], Dongping Ma[1], H. Tetsuo Uyeda[2], Poncho Meisenheimer[2],
[1]Promega Corporation, [2]Promega Biosciences, Inc.

Introduction

The cytochromes P450 (CYPs) are the main enzyme family for metabolism of therapeutic drugs (1). CYP3A4 is the most active drug metabolizing CYP, and it plays a prominent role in adverse drug-drug interactions (DDIs). A drug that inhibits CYP3A4 enzyme activity or induces its expression respectively either slows or increases clearance of a co-administered CYP3A4-metabolized drug, leading in the first case to toxicity and in the second to reduced efficacy. To predict the potential for these outcomes early in drug discovery, compounds are tested in vitro for their capacity to induce or inhibit the conversion of a probe substrate by CYP3A4 to a specific product. CYP3A4 inhibition is typically measured with a cell-free enzyme assay. Inductions that are initiated at the transcription level require a cell-based assay, but the induction can be monitored at a post transcriptional level by measuring changes in CYP3A4 enzyme activity.

There are various CYP assay methods including mass spectrometry, absorbance, radioactivity, fluorescence and bioluminescence. P450-Glo™ bioluminescent CYP assays[(a)] give highly predictive results and typically rank highest for sensitivity and ease of use (2–4). The P450-Glo™ assay technology is a rapid high-throughput approach that monitors the conversion by CYPs of inactive D-luciferin derivatives to an active form that makes light when firefly luciferase is added to the reaction mixture. Light intensity is proportional to CYP activity (3,4).

CYP3A4 Substrates

Four distinct P450-Glo™ CYP3A4 substrates are now available. The original CYP3A4 substrate, Luciferin-BE, was followed by Luciferin PPXE and Luciferin-PFBE; the latest substrate is the luciferin isopropyl acetal or Luciferin-IPA. With the development of each new substrate a general trend was followed toward increased sensitivity, improved CYP3A4 selectivity and improved cell-based assay performance. An increased capacity to detect a wider range of CYP3A4 inhibitors was also achieved along with decreased sensitivity to inhibition by the common solvent DMSO. A detailed comparison of Luciferin-BE, Luciferin-PPXE and Luciferin-PFBE was published previously (5), so we will focus here on the newest bioluminescent CYP3A4 substrate, Luciferin-IPA.

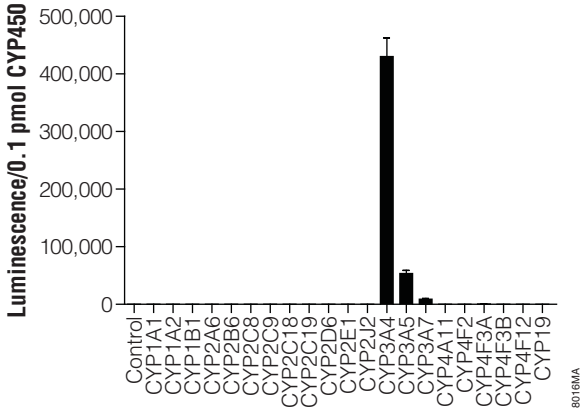

Figure 1. Selectivity of Luciferin-IPA for human CYP enzymes. Recombinant CYP enzymes in microsomes were assayed with 3µM Luciferin-IPA at 37 °C for 10 minutes, then combined with a luciferase reaction mixture (P450-Glo™ Luciferin Detection Reagent with esterase) that stops the CYP reactions and initiates luminescence. CYPs were co-expressed in insect cells with CYP450 reductase only or with reductase plus cytochrome b5 (CYP2A6, 2C8, 2C9, 2C19, 2E1, 2J2, 3A4, 3A5, 3A7 and all 4F enzymes). Control reactions used insect cell microsomes devoid of CYP activity. Values are means ± SD, n = 3. Luminescence was read on a Promega GloMax® luminometer. Full assay details are described in Technical Bulletin #TB325.

Recombinant CYP enzymes were used initially to characterize Luciferin-IPA as a CYP substrate. The activity profile of Luciferin-IPA shows high selectivity for CYP3A4 with minimal cross-reactivity with the closely related enzymes CYP3A5 and 3A7 (Figure 1).

The Luciferin-IPA reaction with recombinant CYP3A4 was linear with increasing enzyme concentrations up to at least 0.25 pmol CYP3A4/50 µl reaction (5 nM CYP3A4; $r^2 = 0.99$) (Figure 2). The enzyme concentration curve demonstrates that Luciferin-IPA is a highly sensitive probe for detecting CYP3A4 activity with greater sensitivity than earlier generation luminogenic substrate and much greater sensitivity than fluorescent substrates. A large signal-to-noise ratio (mean value = 2580) was observed at 0.16 pmol CYP3A4 per 50 µl reaction (0.3 nM CYP3A4), the lowest enzyme concentration tested. This indicates that the CYP3A4/Luciferin-IPA reaction can be employed using substantially less than the 20–30 nM CYP3A4 enzyme concentrations that are often used in CYP3A4 assays (6,7).

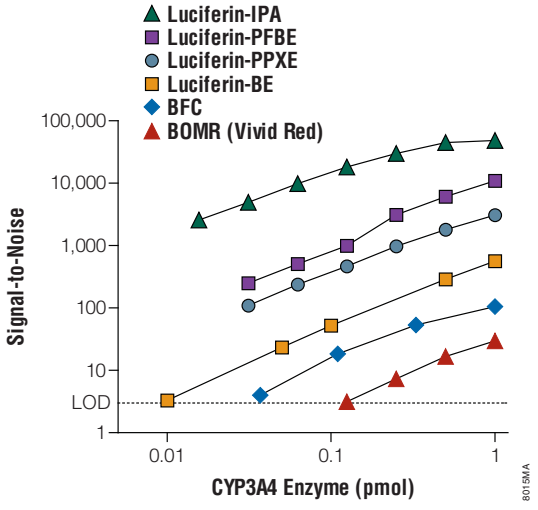

Figure 2. Titration of CYP3A4. CYP assays were performed with recombinant insect cell-expressed enzymes using the luminogenic substrates Luciferin-IPA, -PPXE, or -BE as described in Promega Technical Bulletin #TB325. Fluorescent assays with BOMR or BFC were performed as described by the substrate manufacturers (BOMR, Invitrogen; BFC, BD/Gentest) Minus-CYP control values were used to calculate signal-to-noise ratios. LOD = limit of detection.

Detecting CYP3A4 Inhibitors

The CYP3A4/Luciferin-IPA proved to be a sensitive probe reaction for detecting CYP3A4 inhibitors. Dose-dependent inhibition by midazolam, testosterone and nifedipine was observed (Figure 3). These three compounds are widely used as CYP3A4 probe substrates, so they are acting here as competitive inhibitors of the CYP3A4/Luciferin-IPA reaction (8). Because the CYP3A4/Luciferin-IPA reaction is inhibited by these three compounds it is expected in turn to detect any inhibitor also detected by each of these compounds when they are used as CYP3A4 probe substrates. Since the subsets of CYP3A4 inhibitors detected by midazolam, testosterone and nifedipine reactions do not show complete overlap, it can be expected that Luciferin-IPA will prove to be a broader spectrum probe substrate than any one of these three.

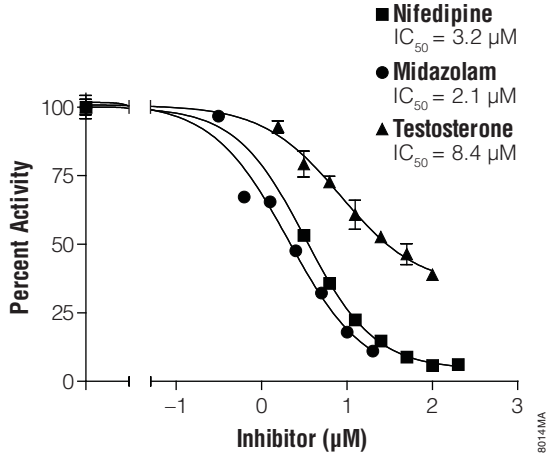

Figure 3. Using Luciferin-IPA to detect CYP3A4 inhibition. Reactions were performed using 2.0 nM recombinant CYP3A4 in 10-minute reactions at 37 °C with 2 µM Luciferin-IPA as described in Figure 1. Values are means ± SD, n = 3. Full assay details are described in Promega Technical Bulletins #TB325 and #TB340.

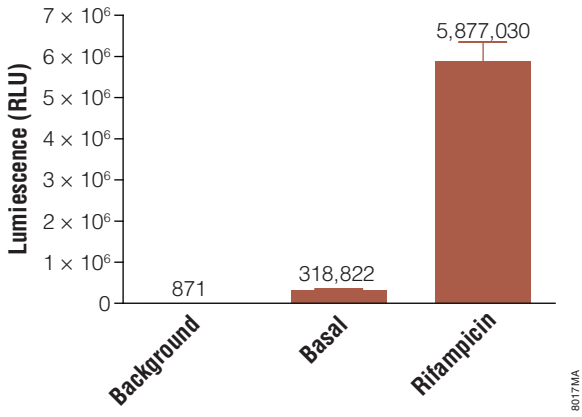

Figure 4. Detection of CYP3A4 gene induction in a cell-based assay. Fresh hepatocytes from an 84-year-old male donor (provided by Celsis In Vitro Technologies) were cultured as a monolayer in a 96-well plate and treated for 48 hours with 10 µM rifampicin or with the vehicle 0.1% DMSO (basal). Assay background was measured in medium without cells. After treatments the medium was replaced with medium containing 4 µM Luciferin-IPA, and cells were incubated for an additional 60 minutes. A sample of the medium was then combined with an equal volume of a luciferase reaction mixture (P450-Glo™ Luciferin Detection Reagent with esterase) in a separate, white luminometer plate. Luminescence was read on a Promega GloMax® luminometer. Values are means ± SD, n = 3. Full assay details are described in Promega Technical Bulletin #TB325.

Luciferin-IPA was also used to detect CYP3A4 gene induction by the well known CYP3A4 inducer rifampicin in a cell-based assay with fresh human hepatocytes (Figure 4; ref. 9). Because Luciferin-IPA is cell permeant, as is the luciferin product of the CYP3A4 reaction, it is possible to configure a nonlytic assay that detects the CYP reaction product in the cell culture medium. This provides the possibility of performing an assay such as a cell viability assay in multiplex. A substantial basal signal representing CYP3A4 enzyme activity endogenous to hepatocytes was detected from the vehicle-treated wells showing a signal-to-background ratio of 366. The basal signal was increased 18.5-fold by rifampicin, reflecting an increase in CYP3A4 enzyme activity due to CYP3A4 gene induction.

Conclusion

The bioluminescent CYP3A4 substrate, Luciferin-IPA, is a highly sensitive and selective probe for detecting CYP3A4 activity. This sensitivity means that the CYP3A4 enzyme can be used at substantially lower concentrations than are typically used in CYP3A4 assays. Because Luciferin-IPA is cell permeant and CYP3A4-selective, it can be used to detect CYP3A4 gene induction and be multiplexed with a cell viability assay. The CYP3A4/Luciferin-IPA assay is also a sensitive means for detecting CYP3A4 inhibitors in a dose-dependent manner. This new generation bioluminescent substrate proves to be sensitive, selective and versatile for all bioluminescent CYP3A4 assay applications.

References

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Protocol

P450-Glo™ Assay Technical Bulletin #TB325
(www.promega.com/tbs/tb325/tb325.html)

P450-Glo™ Screening SystemsTechnical Bulletin #TB325
(www.promega.com/tbs/tb325/tb325.html)

Ordering Information

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	10 ml	V9001
	50ml	V9002
P450-Glo™ CYP3A4 Screening System with Luciferin-IPA		V9920

[a]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. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

More Cytochrome P450 Substrates!

SIX BIOLUMINESCENT SUBSTRATES FOR P450 ENZYME ASSAYS

A series of articles in *eNotes* describe six new substrates available from Promega for bioluminescent CYP assays.

Luminogenic CYP assays use prosubstrates for the light-generating reaction of firefly luciferase. CYPs convert the prosubstrates to an active luciferin, which produces light in a second reaction with luciferase. The amount of light generated is proportional to the amount of luciferin produced by the CYP and, therefore, to CYP enzyme activity.

LUCIFERIN-4A

CYP4A enzymes are cytochromes P450 that catalyze the ω-hydroxylation of fatty acids and the formation of arachidonic acid metabolites. Genes encoding CYP4A enzymes are induced by agonists of the peroxisome proliferator-activated receptor alpha (PPARα) nuclear receptor. CYP4A assays, including human CYP4A11 and rat CYP4A1, CYP4A2 and CYP4A3, commonly require chromatographic steps or cell lysate preparations that limit ease-of-use and throughput.

The luciferin derivative, 2-(6-methoxyquinolin-2-yl)-4,5-dihydrothiazole-4-carboxylic acid, referred to as Luciferin-4A, is selectively converted to quinolylluciferin, an active alternative to native beetle luciferin, by the human CYP4A11 enzyme. The eNotes article demonstrates use of the substrate in a luminogenic CYP4A11 biochemical assay and also in a cell-based assay that measures CYP4A basal and induced activity in intact rat hepatocytes.

See more:
www.promega.com/enotes/applications/ap0092.htm

LUCIFERIN-4F2/3

The cytochromes P450 CYP4F2 and CYP4F3B catalyze ω-hydroxylation of fatty acids and arachidonic acid and metabolize certain drugs. Enzyme assays for CYP4F2 and CYP4F3B typically include a chromatographic separation step that limits ease-of-use and throughput.

The luciferin derivative, 2-(6-(4-(methylthio)benzyloxy) benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid, referred to as Luciferin-4F2/3, is shown to have a high degree of selectivity for CYP4F3B and CYP4F2.

See more:
www.promega.com/enotes/applications/ap0093.htm

LUCIFERIN-4F12

CYP4F12 is a cytochrome P450 enzyme that catalyzes the oxidation of leukotrienes, arachidonic acid and certain drugs. CYP4F12 is expressed predominantly in the liver with lower levels in the kidney, colon, small intestine and heart. Enzyme assays for CYP4F12 typically include a chromatographic separation step (e.g., radiometric, HPLC or LC/MS-based assays), limiting the ease-of-use and throughput.