Bioluminescent Cytochrome P450 Assays.

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Abstract

P450-Glo™ Luminescent P450 assays utilize derivatives of beetle luciferin as luminogenic probe P450 substrates. Luciferin is a substrate for the light generating reaction of firefly luciferase. The derivatives are not substrates for luciferase but are metabolized by P450s to luciferin, which in turn reacts with luciferase to make light. Assays are easily configured in multi-well plates where a homogenous luciferase mixture is added directly to P450 reactions that use luciferin derivatives as substrates. The amount of luciferin produced by a CYP450 is directly proportional to light output so light is used to measure P450 activity. P450-Glo™ assays were used here to measure CYP1A2, 2C9, 2C19, 2D6 and 3A4 activities and their inhibition by chemical inhibitors. The P450-Glo™ assays provided a simple, homogenous and robust luminescent assay format for rapid screening of compounds against P450 activities.

Introduction

Cytochrome P450 enzymes (P450s) are the major catalyst for the oxidative metabolism of a vast array of hydrophobic chemicals including therapeutic drugs (1). The metabolism of drugs by P450s and the inhibition of P450s by drugs are important considerations in drug discovery because P450 inhibition can alter drug disposition and cause adverse drug-drug interactions (2). For example, if a first drug inhibits the metabolism of a second co-administered drug the second may accumulate to a toxic level. There are 57 P450 genes in humans (3) and enzymes encoded by only five of them, CYP1A2, 2C9, 2C19, 2D6 and 3A4, account for 80-90% of P450-dependent drug metabolism (4).

In small molecule drug discovery *in vitro* test systems are commonly employed to detect P450 inhibition by candidate compounds. For this purpose we developed the P450-Glo™ assays in which luminogenic P450 substrates are used as probes for P450 activity in recombinant P450 preparations or liver microsomes. Drugs that inhibit these marker activities are identified as P450 inhibitors or P450 substrates that compete with the probe for the P450 active site. The probes are derivatives of the firefly luciferase substrate D-luciferin; however, they are inactive in the light generating reaction with luciferase. For P450-Glo™ assays a first reaction is performed in which a P450 converts a luminogenic substrate to a luciferin product (Figure, part A). At the end of the P450 reaction a luciferin detection reagent is added that contains luciferase and ATP. This reagent simultaneously stops the P450 reaction and initiates a luciferase reaction that generates an amount of light that is directly proportional to the amount of luciferin product produced by P450 in the first step. P450 enzyme selectivity for a P450-Glo™ luminogenic substrate depends on the nature of the derivitizations on D-luciferin (R_1 and R_2 in the Figure). Several unique

luciferin derived substrates are available. Assays for CYP1A1, 1A2, 1B1, 2C8, 2C9, 2C19, 2D6, 2J2, 3A4, 3A7, 4A11, 4F12 and 19 have been developed (5,6,7) and here we measured inhibition of several of these P450s using the P450-Glo^{TM} assays.

Materials and Methods

P450 enzyme inhibition was measured using P450-Glo™ Screening Systems (Promega Corp.), which provided complete sets of reagents for performing luminescent P450 assays. Each system included a membrane preparation containing a recombinant human cytochrome P450 enzyme, control membranes devoid of P450 activity, a luminogenic cytochrome P450 substrate, an NADPH regeneration system, KPO_4 buffer (pH 7.4), luciferin-free water and a luciferin detection reagent that contained a stabilized recombinant firefly luciferase (UltraGlo™ Luciferase) and ATP. The luminogenic P450-Glo™ substrates, luciferin-ME, luciferin-H, luciferin-H EGE, luciferin-ME EGE and luciferin-BE are derivatives of beetle luciferin ((4S)-4,5-dihydro-2-(6-hydroxybenzothiazolyl)-4-thiazolecarboxylic acid or D-luciferin). The P450 membranes were microsomes from baculovirus-infected insect cells with cDNA-expressed human CYP1A2, 2C9, 2C19, 2D6 or 3A4 and P450 reductase (and cytochrome b5 for CYP2C9, CYP2C19 and CYP3A4). 50µl P450 reactions were performed in white opaque 96 well plates according to the manufacturer's protocol (8,9). Briefly, a luminogenic P450 substrate was incubated at 37°C with a P450 enzyme and a P450 inhibitor or its vehicle for ten minutes (0.5pmol CYP1A2 plus 100µM luciferin-ME, 0.5pmol CYP2C9 plus 100µM luciferin-H, 0.25pmol CYP2C19 plus 10µM luciferin-H EGE, 0.25pmol CYP2D6 plus 30µM luciferin-ME EGE, 1.0pmol CYP3A4 plus 50µM luciferin-BE). Reactions were then initiated by adding the NADPH regeneration system and incubated for 10 (CYP1A2), 20 (CYP2C19) or 30 minutes (CYP2C9, 2D6 and 3A4). At the end of the incubations an equal volume of the luciferin detection reagent (50µl) was added at room temperature and luminescence was read 20

minutes later on a Glomax™ 96 Microplate Luminometer (Promega Corp.). The luciferin detection reagent stops the P450 reaction and simultaneously initiates a glow-style luminescent reaction with a half-life of greater than two hours. The magnitude of the light signal is dependent on and directly proportional to the amount of luciferin product generated by the P450 reaction.

Results

The P450-Glo™ assays were designed primarily as a tool for measuring P450 inhibition by compounds of interest. Libraries of compounds can be screened at a single concentration to identify P450 inhibitors or compounds can be screened at a range of concentrations to measure inhibitory potency. To demonstrate the value of these assays for detecting inhibitory potency dilution series of known P450 inhibitors were applied to P450-Glo™ reactions (Figure 1, part B). Assays were configured with luminogenic substrates at K_m concentrations for the respective P450s. IC_{50} values were calculated after measuring the dose-dependent effects of inhibitors. The IC_{50}s measured with P450-Glo™ were similar to published values from other assays that used fluorescent or non-optical methods (11,12).

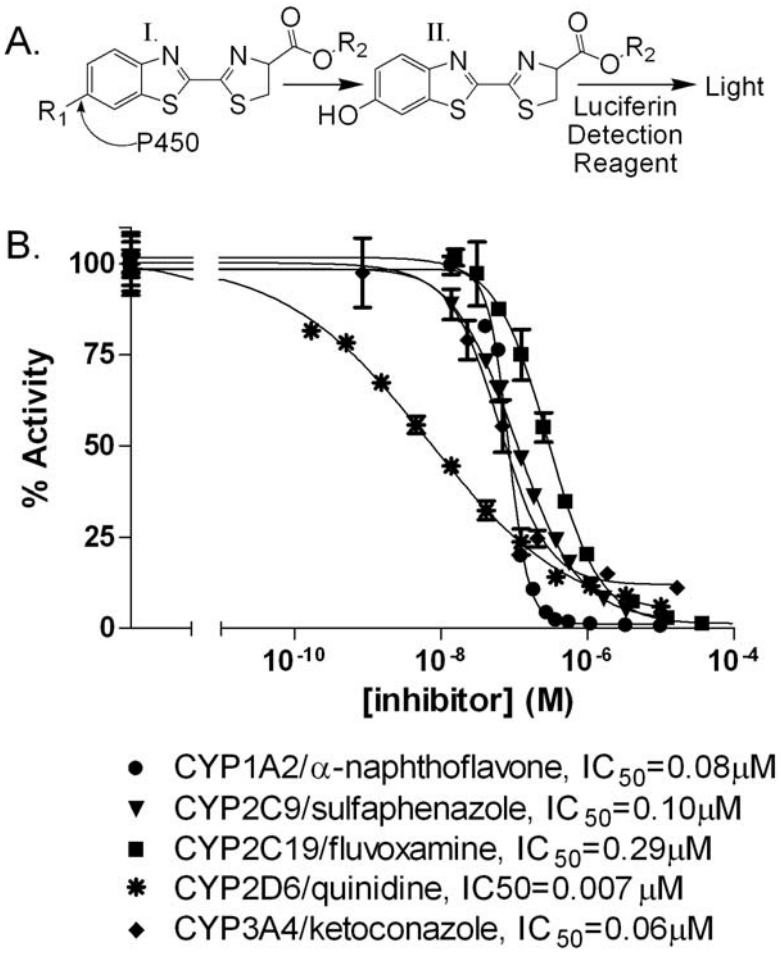

Figure 1. ***Measuring P450 Inhibition with P450-Glo™***. **A**. Compound I represents the P450-Glo™ substrates and compound II, the luminogenic reaction products that generate light with the P450-Glo™ luciferin detection reagent. P450 enzyme selectivity is determined by the nature of R_1 and R_2 , which represent derivitizations on the core D-luciferin structure. **B**. Recombinant P450s were assayed with their respective luminogenic substrates and the indicated concentrations of inhibitors. Curve fits and IC_{50} calculations were performed with the GraphPad Prism® program.

Discussion

The bioluminescent approach of P450-Glo™ assays has certain fundamental advantages over fluorescent assays. For example, the excitation light required for fluorescent methods can create high-background signals that limit assay sensitivity. Bioluminescence does not require an excitation light, so background signals are lower and assays more sensitive (6). Also, misleading results due to overlap between fluorescent spectra of the probe substrates and fluorescent analytes or other assay components (e.g., NADPH) are eliminated with the bioluminescent approach. An additional advantage is found in the improved water solubility of the P450-Glo™ luminogenic substrates compared to typical fluorescent substrates, so little or no organic solvent is introduced with P450-Glo™ substrates. Luminescent assays also show improved linearity with respect to enzyme concentration, a feature that is likely influenced by the water solubility of the luminogenic substrates.

A large signal-to-noise ratio from P450-Glo™ assays result in robust, high-quality assays. Z´-factor is the commonly accepted numerical assessment of assay quality. A perfect assay has a Z´-factor value equal to 1.0, and assays with a Z´-factor greater than 0.5 are usually viewed as acceptable for HTS (12). Z´-factor values for P450-Glo™ Assays are typically greater than 0.8.

By adapting the fundamental advantages of bioluminescence P450-Glo™ assay technology allows researchers to move beyond many of the limiting features of traditional P450 assay methods. These simple-to-use robust assays are highly sensitive and amenable to automation. These features, combined with the low incidence of false positives resulting from compound interference, make the P450-Glo™ assays an excellent approach for high throughput P450 Assays.

References

1. Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol 2001; 14(6): 611-50.
2. Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, et al. The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective Drug Metab Dispos 2003; 31: 815-832.
3. Nelson DR. Cytochrome P450 Homepage. http://drnelson.utmem.edu. (accessed July, 2005).
4. Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, et al. drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_I/AUC) ratios. Drug Metab Dispos 2004; 32(11):1201-1208.
5. Cali J. Screen for CYP450 inhibitors using P450-GloTM luminescent cytochrome P450 assays. Cell Notes 2003; 7:2-4 (www.promega.com).
6. Cali JJ, Sobol M, Ma D. Screen for cytochrome P450 activity using a luminescent assay. Cell Notes 2005; 13:8-10 (www.promega.com).
7. Cali JJ, Ho S, Ma D, Simpson DJ, Frackman S, Daily WJ, et al. Bioluminescent P450 assays that use D-luciferin derivatives as substrates for CYP1A1, 1A2, 1B1, 2C8, 2C9, 2J2, 3A4, 3A7, 4A11, 4F3B, 4F12 and 19. In: Proceedings of the 14th International Conference on Cytochrome P450s. Medimond S.r.l., 2005:77-84.
8. P450-Glo™ Assay Systems Technical Bulletin #TB325. www.promega.com/tbs/tb325/tb325.html (accessed July, 2005).

9. P450-Glo™ Screening Systems Technical Bulletin #TB340. www.promega.com/tbs/tb340/tb340.html (accessed July 2005).

10. Sai Y, Dai R, Yang TJ, Krausz KW, Gonzalez FJ, Gelboin HV, Shou M. Assessment of specificity of eight chemical inhibitors using cDNA expressed cytochromes P450. Xenobiotica 2000; 30:327-43.

11. Bennett MA, Prueksaritanont T, Lin JL. Drug interactions with calcium channel blockers: possible involvement of metabolite-intermediate complexation with CYP3A. Drug Metab Dispos 2000; 28:125-130.

12. Zhang J-H, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Scr 1999; 4:67-73.