

Novel Bioluminescent Substrates to Measure CYP3A4 Activity in Cell-based and Microsome Assays



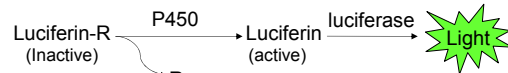
Promega

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Abstract

We have developed three distinct luminogenic substrates for measuring the enzyme activity of CYP3A4, the principal drug-metabolizing cytochrome P450 (CYP) in the liver and small intestine. The substrates are luciferin derivatives used with the luciferase based P450-Glo™ technology. Factors that influence the choice of substrate include CYP enzyme selectivity, DMSO sensitivity and source of CYP3A4 (microsomes vs. cells). The selectivity of each substrate for CYP enzymes is demonstrated in assays with recombinant CYPs. These microsome assays can utilize any of the three substrates for detecting inhibition and measuring IC₅₀s. Assays of CYP3A4 from cultured cells use the substrates in a non-lytic approach that detects basal and induced CYP3A enzyme activity in freshly isolated and cryopreserved hepatocytes and DPX-2 cells (a stable cell-line over-expressing the PXR nuclear receptor). The cell-based assays can be multiplexed with a cell viability assay to measure cytotoxicity of test compounds and for normalization of CYP activity measurements to cell number. These cell and microsome based assays are homogeneous and easily configured in high throughput multi-well formats to measure CYP gene induction or enzyme inhibition by new chemical entities.

P450-Glo™ Assay Scheme

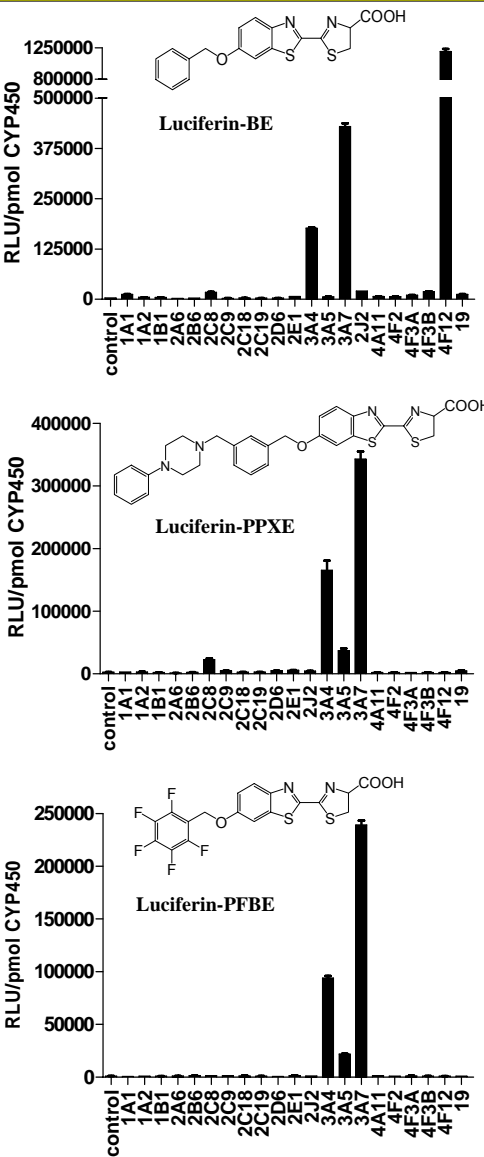


Luminogenic substrate assays are based on the enzymatic release of free luciferin by a CYP450 from an inactive luciferin precursor. The luciferase enzyme uses the CYP450 generated luciferin as a substrate with ATP, and oxygen to create light. The light production is directly proportional to CYP450 activity. Test compounds that inhibit CYP450 activity result in less light production and those that induce activity result in greater light production.

P450-Glo™ Assay Methods

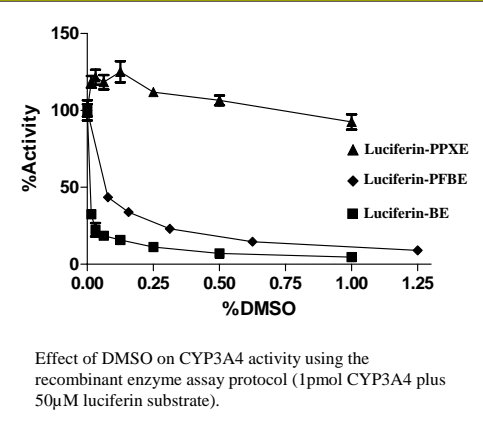
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| <p>Recombinant enzyme assay</p> <ol style="list-style-type: none"> 1. Prepare P450 reactions by combining a P450 sample, NADPH regenerating system and P450 substrate in a multi-well plate format. 2. Incubate at 37°C 30 minutes. 3. Terminate P450 reactions and start luminescent detection by adding one volume of a luciferase/ATP mixture (Luciferin Detection Reagent or LDR) to each P450 reaction well. 4. Read luminescence. | <p>Cell-based assay</p> <ol style="list-style-type: none"> 1. Culture hepatocytes in monolayers and treat with test compounds. 2. After treatment replace culture medium with medium containing the P450 substrate. Inhibitors can also be added at this point. 3. Incubate for 4 hours. 4. Transfer a volume of medium to a white multi-well plate and combine with an equal volume of LDR. 5. Read luminescence. <p>Optional: perform cell viability assay on cells left in culture wells (e.g. CellTiter-Glo™).</p> |
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Substrate Structures and Enzyme Selectivity



21 Recombinant human P450s were assayed for activity against Luciferin-BE, Luciferin-PPXE, and Luciferin-PFBE using the recombinant enzyme assay protocol (mean ± SD, n=3).

DMSO Tolerance

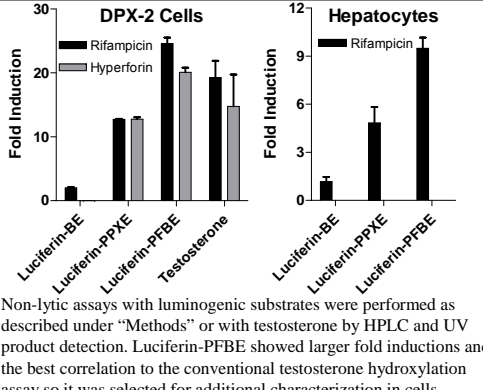


Measuring CYP3A4 Inhibition: IC₅₀ (μM)

	Luciferin-BE	Luciferin-PFBE	Luciferin-PPXE	Literature*
Midazolam	17.4	26.0	8.3	1.3-59.8
Nifedipine	+	+	19.9	+, 0.2-17.2
Testosterone	+	+	+	+
Ketoconazole	0.1	0.2	0.1	0.1-0.2
Erythromycin	1.2	0.7	6.5	1.8-74
Troleandomycin	0.2	0.1	0.3	0.3-6.1
Verapamil	0.4	0.5	0.5	0.12-4.0
Clotrimazole	0.01	nd	0.02	0.002-0.06
Azamulin	0.1	0.1	0.5	0.03, 0.12

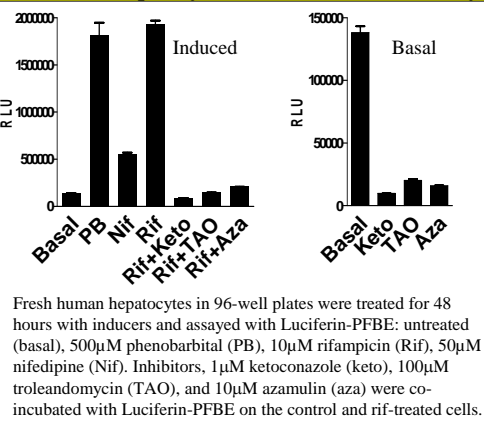
(nd) not determined; (+) stimulated
*Inconsistency of literature values reflects substrate-dependence of CYP3A4 inhibition.

CYP3A Induction in DPX-2 Cells & Human Hepatocytes

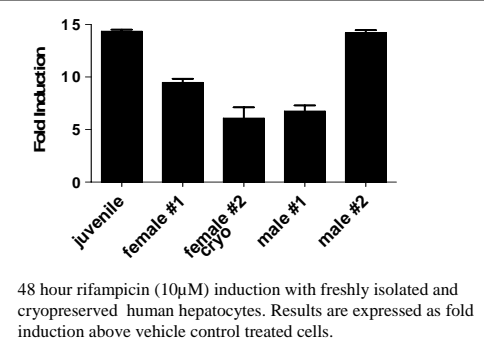


Non-lytic assays with luminogenic substrates were performed as described under "Methods" or with testosterone by HPLC and UV product detection. Luciferin-PFBE showed larger fold inductions and the best correlation to the conventional testosterone hydroxylation assay so it was selected for additional characterization in cells.

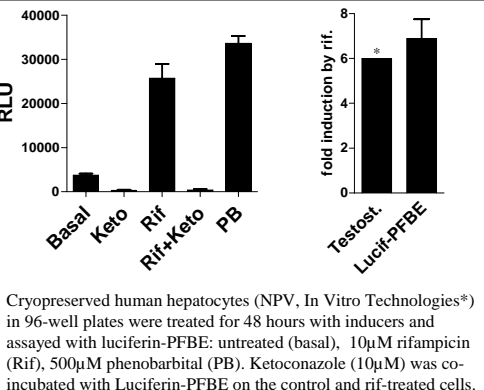
CYP3A Induction and Inhibition in Human Hepatocytes: Luciferin-PFBE Activity



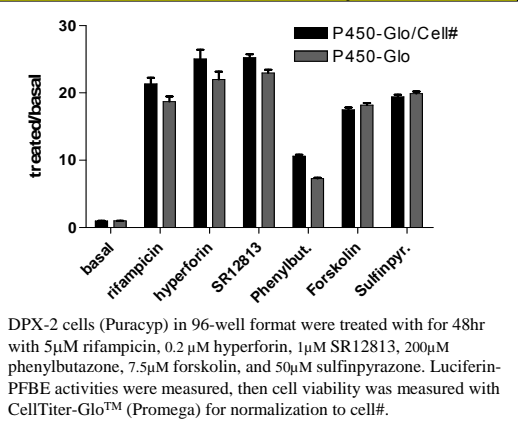
Donor Variability in Human Hepatocytes: Luciferin-PFBE Activity



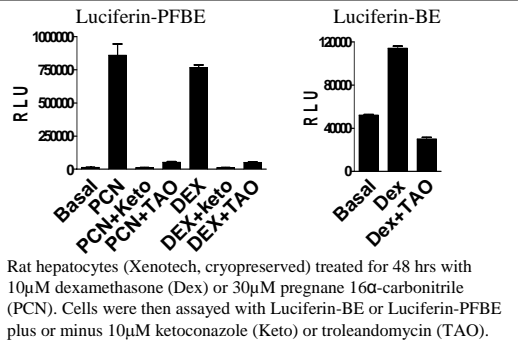
CYP3A/Luciferin-PFBE Activity in Cryopreserved Human Hepatocytes



CYP3A Induction in DPX-2 Cells: Luciferin-PFBE Activity



CYP3A Induction in Rat Hepatocytes



Summary

- Three luminogenic CYP450 substrates were examined in biochemical and cell-based P450-Glo™ assays. Luciferin-PPXE and -PFBE showed a high degree of selectivity for human CYP3A enzymes while Luciferin-BE cross-reacted with CYP4F12.
- Each substrate proved effective as a probe for measuring CYP3A4 inhibition by known inhibitors showing IC₅₀s that correlate with literature values measured against other substrates in different assay technologies.
- Luciferin-PPXE has an advantage over Luciferin-BE and -PFBE for biochemical inhibition assay in that its reaction with CYP3A4 is insensitive to the DMSO vehicle from typical test compounds. Luciferin-PPXE may also prove a more sensitive probe for inhibition in that its reaction with CYP3A4 was inhibited by nifedipine while Luciferin-BE and PFBE reactions were stimulated.
- Luciferin-PFBE has advantages over Luciferin-PPXE and -BE for cell-based assays, showing higher fold inductions by prototypical inducers and closer correlation to a conventional CYP3A4 testosterone hydroxylation assay.