Bioluminescent Approaches for *In Vitro* ADMET

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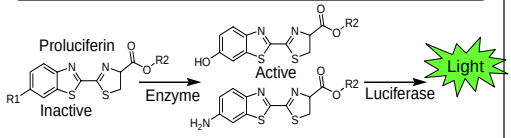
Abstract

Bioluminescent ADMET assays couple targets of interest to the photon emitting reaction of firefly luciferase. Multiplexed cell-based assays examine test compounds for cytotoxicity and P450 induction at the transcriptional level and at the level of P450 enzyme activity. Cell-free membrane assays measure P450 or monoamine oxidase enzyme activities and their inhibition by test compounds. Each bioluminescent assay relies on the light generating reaction of firefly luciferase, correlating light output with target activity. The assays are insensitive to interference from fluorescent analytes and also have low intrinsic background signals making them highly sensitive and giving them a large dynamic range for robust, high throughput applications.

Proluciferin Assays



Assays rely on release of free luciferin from inactive luciferin precursors by enzymes of interest. Luciferase then uses the free luciferin to generate light in proportion to the amount of target enzyme activity. Available Assays:

P450, MAO, GST/GSH, caspase 3/7, cytotoxicity.

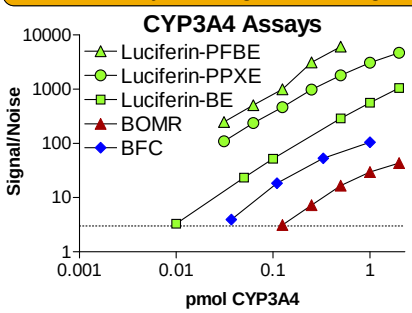
Proluciferin Luminogenic Substrates

Enzyme Selectivity

R1	R2	Enzyme	Reaction
H_3C O	-H	CYP1A2, 2C8, 2C9, 2J2, 4A11, 4F3B, 19	Dealkylation *(Luciferin-ME)*
Cl O	-H	CYP1A1, 1B1, 3A7	Dealkylation *(Luciferin-CEE)*
H-	-H	CYP2C9	Hydroxylation *(Luciferin-H)*
O	-H	CYP3A4, 3A5, 3A7, 4F12	Dealkylation *(Luciferin-BE)*
F F F O	-H	CYP3A4, 3A5, 3A7	Dealkylation *(Luciferin-PFBE)*
N O	-H	CYP3A4, 3A5, 3A7	Dealkylation *(Luciferin-PPXE)*
H_3C O	$-CH_2CH_2OH$	CYP1A1, 1A2, 2D6	Dealkylation *(Luciferin-ME-EGE)*
H-	$-CH_2CH_2OH$	CYP1A1, 1A2, 2C19	Hydroxylation *(Luciferin-H-EGE)*
H_2N O	$-CH_3$	MAO-A, MAO-B	Oxidation *(luciferin-APE)*
O_2N O O	-H	GST	Luciferin displacement
Z-DEVD-NH	-H	Caspase 3/7	Peptide bond cleavage
AAF-NH	-H	Marker protease for cell death	Peptide bond cleavage

P450-Glo™ Luminescent CYP Assay

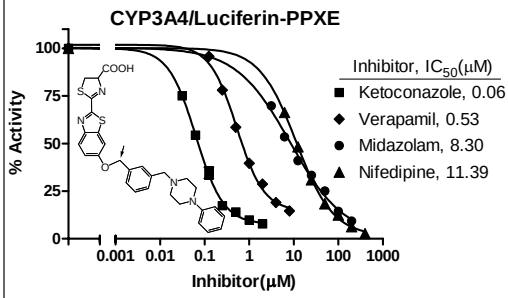
Sensitivity: luminogenic >fluorogenic



Recombinant CYP3A4/OR/b5 was assayed against three luminogenic substrates and two fluorescent substrates, BOMR (vivid red) and BFC.

P450-Glo™ Luminescent CYP Assay

Measuring P450 Inhibition



Inhibition of recombinant CYP3A4 reaction with luminogenic substrate Luciferin-PPXE.

CYP3A4 IC_{50} (μM) Comparison Luminogenic Assay vs. Literature

Compound	P450-Glo™ Luciferin-BE	P450-Glo™ Luciferin-PPXE	Literature*
Clotrimazole	0.01	0.02	0.002 - 0.06
Ketoconazole	0.1	0.1	0.01 - 0.2
Troleandomycin	0.1	0.3	0.3 - 6.1
Verapamil	0.4	0.5	0.4 - 8.4
Erythromycin	1.2	6.5	1.8 - 74
Fluvoxamine	15.2	nd	10.7 - *24*
Midazolam	17.4	8.3	1.3 - 59.8
Disopyramide	29.0	nd	~30
Omeprazole	61	nd	78
α-Naphthoflavone	+	+	+
Nifedipine	+	19.9	+
Testosterone	+	+	+

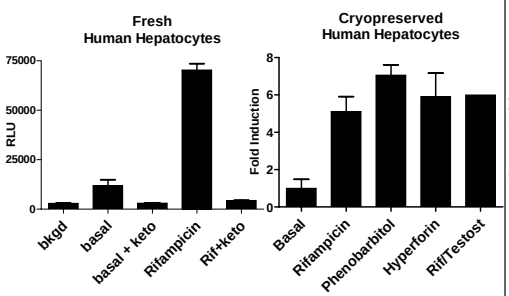
IC_{50}s were measured against recombinant CYP3A4 with P450-Glo™ luminogenic assays at 37ºC using substrates at their K_m conc. Incubation times were within the linear range of the reactions.

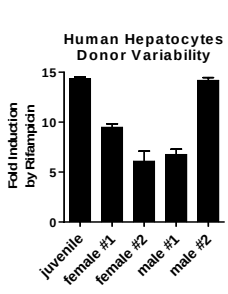
*Literature values from LC/MS, fluorogenic and radiometric assays (JJ Cali *et al. Expert Opin. Drug Metab. Toxicol*. (2006) **2**(4):629-645.

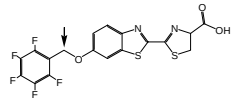
nd=not determined; "+"=positive cooperativity

Rapid Cell-based P450 Assay

CYP3A Induction in Human Hepatocytes



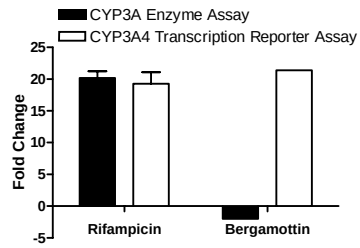




CYP3A enzyme activity in hepatocyte monolayers on 96 well plates. 48 hr inducer treatments were followed by 4 hr incubations with luciferin-PFBE and ketoconazole where indicated (keto) in medium. A sample of this medium was then combined with P450-Glo™ luciferin detection reagent and luminescence (RLU) was measured. After treatments cell viabiltiy was measured using the CellTiter-Glo™ viability assay and P450 activities were nomalized to cell number before calculating fold induction: (Induced P450 RLU/cell#)/(basal P450/cell#). Rifampicin induction measured in this way was comparable to the conventional CYP3A testosterone hydroxylation assay (Rif/testost, data provided by Celsis/IVT).

CYP3A4 reporter assay and CYP3A enzyme Assay in DPX-2 Cells

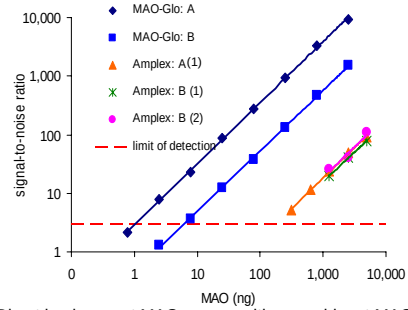
The Importance of Examining Induction at Levels of Both Transcription and Enzyme Activity



DPX-2 cells (Puracyp Inc.) are a HepG2 derivative harboring a constitutively expressed human PXR cDNA and a luciferase pGL3 reporter vector with the human CYP3A4 promoter. Endogenous CYP3A enzyme activity was induced through PXR and measured with the P450-Glo™ Luciferin-PFBE Assay. Induction of the CYP3A4 reporter construct was measured with the Bright-Glo™ Luciferase Reporter Assay System as a reflection of CYP3A transcriptional activation. The PXR ligands rifampicin and bergamottin induced the CYP3A4 transcriptional reporter and rifampicin also caused an increase in CYP3A enzyme activity. In contrast bergamottin inhibited CYP3A enzyme activity because it is a mechanism-based inhibitor of CYP3A4.

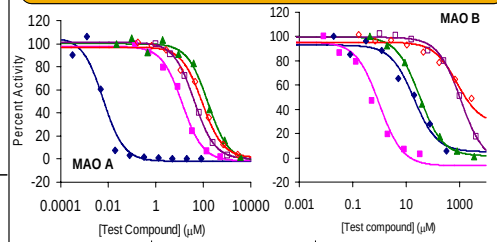
MAO-Glo™ Monoamine Oxidase Assay

Sensitivity: luminogenic >fluorogenic



Direct luminescent MAO assays with recombinant MAOs and the luminogenic substrate luciferin-APE were compared to indirect fluorogenic MAO assays that use the H_2O_2 sensing dye, Amplex Red™ and MAO substrates p-tyramine (1) or benzylamine (2).

Measuring MAO Inhibition with MAO-Glo™



Compound	MAO A K_i or K_m value (μM)	MAO A published value (μM)	MAO B K_i or K_m value (μM)	MAO B published value (μM)
clorgyline ◆	0.003 ± 0.001	0.0039	10 ± 4	4
deprenyl ■	7 ± 1	5	0.5 ± 0.2	0.13
Phenylethylamine▲	78 ± 16	78	16 ± 1	20
Serotonin ◇	45 ± 8	80	410 ± 140	2032
Dopamine □	21 ± 1	120	570 ± 120	301

Dose-dependent inhibition of recombinant MAO-A and -B was measured using the luminogenic MAO substrate luciferin-APE at the respective K_m concentrations. Published K_i & K_m values referenced in: MP Valley *et al*. *Anal. Bioch*. (2006) **359**:238-246.

Summary

Luminogenic substrates for drug metabolizing enzymes make it possible to harness the key advantages of bioluminescent assay technology for ADMET applications. The advantages include high sensitivity, simplicity, scalability, low false hit rate, HTS applications, safety and cost effectiveness.