

# Bioluminescent Approaches for *In Vitro* ADMET

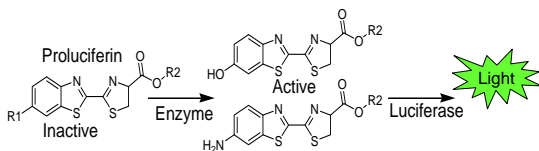
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## Abstract

ADMET targets were coupled to firefly luciferase in assays that used the convenience of light output as a readout. In a cell-based approach test compounds were tested for glutathione depletion, P450 transcription induction and induction of P450 enzyme activity. Inductions of P450 enzyme activities with selective luminogenic substrates were measured and observed as markers for identifying ligands for PXR, aryl hydrocarbon or PPAR- $\alpha$  nuclear receptors. Cell-free membrane assays with luminogenic substrates were used to measure P450 or monoamine oxidase enzyme activities and their inhibition by test compounds. IC50s from these bioluminescent assays correlated well with conventional methods in terms of rank order and absolute potency. Each bioluminescent assay relied on the light generating reaction of firefly luciferase, coupling light output with target activity. The assays were insensitive to interference from fluorescent analytes and had low intrinsic background signals giving them high levels of sensitivity and large dynamic ranges 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 <sub>3</sub> C-O	-H	CYP1A2, 2C8, 2C9, 2J2, 4A11, 4F3B, 19	Dealkylation
Cl-CH <sub>2</sub> -CH <sub>2</sub> -O	-H	CYP1A1, 1B1, 3A7	Dealkylation
H-	-H	CYP2C9	Hydroxylation
	-H	CYP3A4, 3A5, 3A7, 4F12	Dealkylation
	-H	CYP3A4, 3A5, 3A7	Dealkylation
	-H	CYP3A4, 3A5, 3A7	Dealkylation
H <sub>3</sub> C-O	-CH <sub>2</sub> CH <sub>2</sub> OH	CYP1A1, 1A2, 2D6	Dealkylation
H-	-CH <sub>2</sub> CH <sub>2</sub> OH	CYP1A1, 1A2, 2C19	Hydroxylation
H <sub>2</sub> N-CH <sub>2</sub> -CH <sub>2</sub> -O	-CH <sub>3</sub>	MAO-A, MAO-B	Oxidation
	-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™ CYP Inhibition & Induction Assays

Comparing Luminogenic to published Non-luminogenic Assays

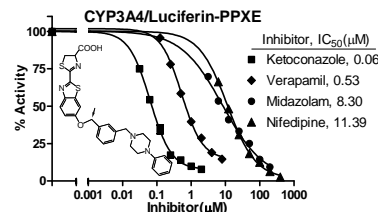
Inhibition	Enzyme/Substrate	Inhibitor/IC <sub>50</sub> (μM)	Assay [ref]
	<b>CYP1A2/Luciferin-ME</b>	<b>α-Naphthoflavone/0.08</b>	<b>Luminogenic [5]</b>
	CYP1A2/Phenacetin	α-Naphthoflavone/0.04	LC/MS [33]
	CYP1A2/CEC	α-Naphthoflavone/0.02	Fluorogenic [34]
	<b>CYP2C9/Luciferin-H</b>	<b>Sulfaphenazole/0.2</b>	<b>Luminogenic [5]</b>
	CYP2C9/Diclofenac	Sulfaphenazole/0.26	LC/MS [33]
	CYP2C9/CEC	Sulfaphenazole/0.18	Fluorogenic [35]
	<b>CYP2C19/Luciferin-H-EGE</b>	<b>Omeprazole/2.4</b>	<b>Luminogenic [5,47]</b>
	CYP2C19/Diazepam, mephenytoin	Omeprazole/6, 6.1	Radiometric [36, 37]
	CYP2C19/DBF	Omeprazole/4.0	Fluorogenic [104]
	<b>CYP2D6/Luciferin-ME-EGE</b>	<b>Quinidine/0.008</b>	<b>Luminogenic [5,35]</b>
	CYP2D6/Bufuralol	Quinidine/0.02	LC/MS [33]
	CYP2D6/CEC	Quinidine/0.009	Fluorogenic [35]
	<b>CYP3A4/Luciferin-PPXE</b>	<b>Ketoconazole/0.06</b>	<b>Luminogenic [5,6]</b>
	CYP3A4/midazolam	Ketoconazole/0.008	LC/MS [33]
	CYP3A4/testosterone	Ketoconazole/0.05	LC/UV [38]
	CYP3A4/BzRes	Ketoconazole/0.083	Fluorogenic [35]

**Inhibition Assays:** All enzyme inhibition assays, either luminogenic or non-luminogenic, were performed with recombinant enzymes (microsomes) expressed in the baculovirus/insect cell system using substrates at their K<sub>m</sub> concentrations. References are from Cali et al (2008) *Exp. Op. Drug Metab. Tox.* vol4(1):103-120

Induction	Enzyme/Substrate	Inducer/Fold induction	Nuclear Receptor	Assay
	<b>CYP3A/Luciferin-PFBE</b>	<b>Rifampicin/7</b>	<b>PXR</b>	<b>Luminogenic [5]</b>
	CYP3A/Testosterone	Rifampicin/9.6	PXR	LC/UV [40]
	<b>CYP1A/Luciferin-CEE</b>	<b>Omeprazole/12.1</b>	<b>AHR</b>	<b>Luminogenic [5]</b>
	CYP1A/Ethoxyresorufin	Omeprazole/~5-7	AHR	Fluorogenic [41]
	CYP1A/Phenacetin	Omeprazole/12.5-41.4	AHR	LC/MS [42]
	<b>CYP2C9/Luciferin-H</b>	<b>Rifampicin/3.5</b>	<b>PXR</b>	<b>Luminogenic [5]</b>
	CYP2C9/Diclofenac	Rifampicin/2.1-3.4	PXR	LCMS [42]
	<b>CYP4A/Luciferin-ME</b>	<b>Clofibrate/2.0</b>	<b>PPARα</b>	<b>Luminogenic [5]</b>
	CYP4A	Clofibrate/~1.5	PPARα	Immunoblot [43]
	<b>CYP4A/Luciferin-ME (rat)</b>	<b>Clofibrate/6.9</b>	<b>PPARα</b>	<b>Luminogenic*</b>
	CYP4A/Lauric Acid (rat)	Clofibrate/14	PPARα	Radiometric [44]

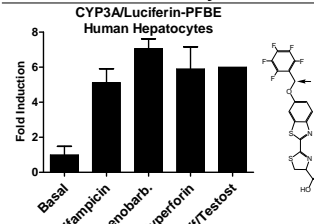
**Induction Assays:** Induction assays were performed with monolayer cultures of human hepatocytes except where rat hepatocytes are indicated. When fold induction is expressed as a range multiple hepatocytes donors were tested. References are from Cali et al (2008) *Exp. Op. Drug Metab. Tox.* vol4(1):103-120. For luminogenic assays hepatocytes were exposed to inducer for 48 hours then to a luminogenic substrate in culture medium for 4 hours. A sample of medium was then combined with luciferin detection reagent and relative luminescence was measured.

### Inhibition Assays



Insect cell-expressed CYP3A4/OR/b5

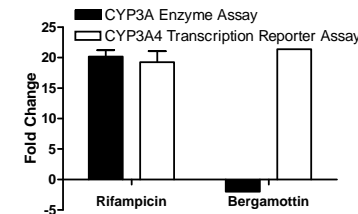
### Inhibition Assays



Cryopreserved human hepatocytes from Celsis/IVT

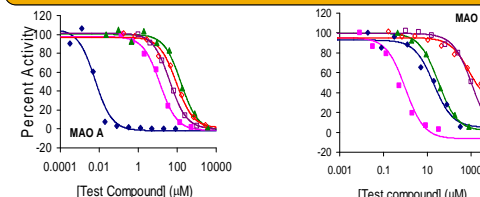
## CYP3A4 reporter assay and CYP3A enzyme Assay in DPX-2 Cells

Examining Induction of Transcription and Enzyme Activity



DPX-2 cells (Puracy Inc.) harbor 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.

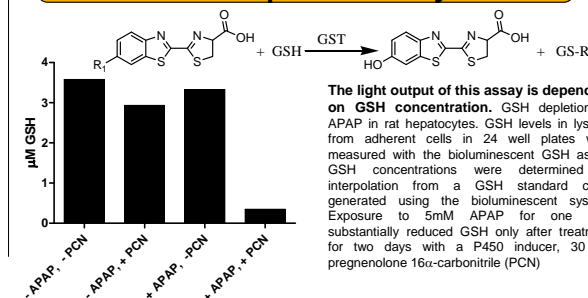
## MAO-Glo™ Monoamine Oxidase Inhibition Assays



Compound	MAO A		MAO B	
	K <sub>i</sub> or K <sub>m</sub> value (μM)	published value (μM)	K <sub>i</sub> or K <sub>m</sub> value (μM)	published value (μM)
clorgyline $\blacklozenge$	0.003 ± 0.001	0.0039	10 ± 4	4
deprenyl $\blacklozenge$	7 ± 1	5	0.5 ± 0.2	0.13
Phenylethylamine $\blacktriangle$	78 ± 16	78	16 ± 1	20
Serotonin $\blacklozenge$	45 ± 8	80	410 ± 140	2032
Dopamine $\square$	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<sub>m</sub> concentrations. Published K<sub>i</sub> & K<sub>m</sub> values referenced in: MP Valley et al. *Anal. Bioch.* (2006) 359:238-246.

## GSH-Glo™ GSH Depletion Assay



The light output of this assay is dependent on GSH concentration. GSH depletion by APAP in rat hepatocytes. GSH levels in lysates from adherent cells in 24 well plates were measured with the bioluminescent GSH assay. GSH concentrations were determined by interpolation from a GSH standard curve generated using the bioluminescent system. Exposure to 5mM APAP for one hour substantially reduced GSH only after treatment for two days with a P450 inducer, 30 μM pregnenolone 16 $\alpha$ -carbonitrile (PCN)