# Comparison of Novel Luciferin-1A2 Substrate and Phenacetin for Measuring CYP1A2 induction in Human Hepatocytes

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## ABSTRACT

Induction of drug metabolizing enzymes is one critical component in assessing drug-drug interactions. The process is controlled through drugs binding to receptors which regulate gene transcription. One mechanism entails the aryl hydrocarbon receptor (AhR), a transcription factor responsible for the up-regulation of phase I and phase II enzymes such as CYP1A1/2 and UGT1A1. The most common method to determine a drug's induction potential through AhR-dependent pathway is to measure the activity of CYP1A2 in hepatocytes using phenacetin as a substrate after exposure to the drug and compare it to the basal rate. The quantification of the metabolism of phenacetin requires bioanalytical methods like UPLC/MS/MS which demands special equipment and expertise. In contrast, the P450-Glo™ assay with the luminescent CYP1A2 substrate Luciferin-1A2 (6-methoxybenzo[d]thiazole-2-carbonitrile) is a simple and effective method to measure CYP1A2 activity requiring a plate reader and minimal expertise which will expedite data acquisition and simplify workflow. Herein, we describe the comparison of phenacetin and Luciferin-1A2 substrates for CYP specific and to determine induction of CYP1A2 in human hepatocytes using omeprazole as the inducer. The induction ratios were similar for most of the 16 donors tested for both lytic and non-lytic P450-Glo™ Luciferin-1A2 substrate methods as compared to phenacetin with significant correlation found (P value < 0.0001, R2 of 0.7). This new CYP1A2 substrate is an alternative for measuring CYP1A2 activity to traditional probe substrates that require HPLC or MS methods.

## INTRODUCTION

The pressures of profiling new chemical entities (NCE) have demanded efficient screening assays that provide meaningful data. One critical assessment is for quantifying induction potential, the ability of NCE to increase metabolically important protein levels by activating transcription and translation machinery. There are several key receptors responsible for upregulating drug metabolizing enzymes. One is example aryl hydrocarbon receptor (AhR), a cytosolic transcription factor that binds to a ligand, translocates into the nucleus which leads to gene transcription. Exogenous ligands such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons are able to activate AhR and increase transcription of drug metabolizing enzymes such as CYP1A1, CYP1A2, CYP1B1, NQ01, ALDH3A1, UGT 1A2 and GSTA1[1]. The implication is the increased metabolic capacitity for a patient, which may adversely affect drug bioavailablility and efficacy of concomitant drugs leading to potential safety issues known as drug-drug interactions (DDIs). The FDA has outlined the reasons for and methods by which to assess induction potential in a draft guidance to industry[2]. The gold standard for determining induction of CYPs is the use of hepatocytes, fresh or cryopreserved, with a positive control inducer and by measuring activity with specific substrates[3]. One preferred inducer is omeprazole while the preferred substrate is phenacetin which is metabolized to acetaminophen. The measurement of acetaminophen requires an LC/MS/MS method which is sensitive and accurate, as well as expensive in instrumentation. Expert users are also required increasing the number of scientist required to produce the necessary data.

Alternatives are necessary in order to streamline the process of screening for DDIs while maintaining relevance. One scenario is to have a substrate that does not require bioanalytical methods in order to quantify the induction potential of a test article. The ideal substrate would be a highly specific and potent substrate for P450 enzyme. Recently, a bioluminescent substrate was shown to be a specific substrate for CYP3A4 and able to be used as a marker for induction of CYP3A4[4,5]. This system required a luminometer and can be performed with minimal special training, unlike traditional bioanalytical methods. The novel prosubstrate luc-1A2[6] has been developed that is specific for CYP1A family and this new substrate must be validated in specific applications to ensure its utility as performed with prior luminescent substrates. To do this, we have set out to compare the induction of CYP1A using P450-Glo™ substrate with a preferred CYP1A substrate phenacetin in cryoplateable human hepatocytes.

## MATERIALS & METHODS

**Hepatocyte Cultures.** Human hepatocytes lots, freshly isolated or cryoplateable, were obtained from BioreclamationIVT. Procedure for the thawing and plating of cryoplateable hepatocytes in

**Hepatocyte Cultures.**
Human hepatocytes lots, freshly isolated or cryoplateable, were obtained from BioreclamationIVT. Procedure for the thawing and plating of cryoplateable hepatocytes in *InVitro*Gro™ CP medium and the culturing and dosing in *InVitro*Gro HI followed instructions for use as prescribed by BioreclamationIVT. The 48-well plates seeded with 140,000 viable hepatocytes per well were dosed with 0.2 mL of 50 µM omeprazole (PC) or 1% acetonitrile (VC) in *InVitro*Gro HI at 48 and 72 hours after initial plating. Activity of CYP1A was measured to assess induction of CYP1A enzyme levels.

**Determining Phenacetin Metabolism.**
Dosing solution of 100 µM phenacetin was prepared in *InVitro*Gro KHB. Medium was removed and 0.2 mL of dosing solution was dispensed in PC and VC wells. The culture was returned to a 37°C, 5% CO2, humidified incubator for four hours. The reaction was stopped with 0.2 mL methanol. The samples were transferred to cryovials and stored at -70° C until measurements were made. The quantification of the acetaminophen metabolite was performed using UPLC/MS/MS.

**Determining P450-Glo Substrate Metabolism.**
P450-Glo kit with Luc-1A2 was obtained from Promega. Procedures were followed as described by Promega in instructions for use of P450-Glo Assays[6] with minor changes. Briefly, all substrates were diluted in *InVitro*Gro KHB and applied to cells or control wells without cells to determine background signal of the substrates. Cultured hepatocytes were washed twice with 0.2 mL of InVitroGro KHB. Medium was removed and 0.1 mL Luc-1A2 was incubated for 30 minutes at 3 µM final in InVitroGro KHB containing 3 mM salicylamide. Luciferin Detection Reagent with D-cysteine (LDRc) was reconstituted as directed by instructions for use. An equal volume (0.1 mL) of LDRc was added at the end of the incubation period to the cells to measure activity for lytic method. A 0.1 mL aliquot was transferred to a white 96-well microtiter plate. For non-lytic method, 50 µL of cultured medium from each well was transferred to a white 96-well microtiter plate containing 50 µL of LDRc. Luminescence (RLU) was determined on a Wallac Victor[2] 1420 Multilabel Counter.

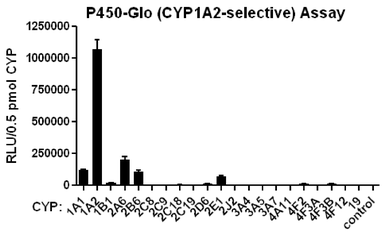
**Fold Induction.**
Raw concentrations of acetaminophen from PC and VC incubations were used to determine fold increase by the equation PC/VC. The corrected luminescent units (RLU) of the samples was determined by subtracting the raw background RLU from the raw RLU from the samples. The corrected RLU from PC and VC incubations were used to determine fold increase by the equation PC/VC.

**Data Analysis**
Statistical analysis was performed using Prism 5.0

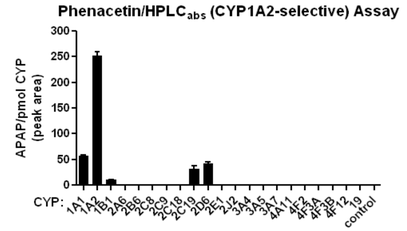
**Specificity of P450-Glo luc-1A2 and Phenacetin**
Specificity of Luc-1A2 and phenacetin was determined as previously described[6]. Briefly, the P450 enzymes used were recombinant human forms in microsomes from insect cells that coexpressed a human CYP cDNA with P450 reductase (Gentest™ Supersomes™, BD Biosciences). In the P450-Glo Assay with Luc-1A2, reaction of 5nM enzyme, 6µM Luc-1A2, NADPH Regeneration System in phosphate buffer was incubated for 10 minutes. Luminescence was read after 20 minutes with the GloMax® 96 Microplate Luminometer and reported in relative light units (RLU). In the P450 assay with phenacetin, reaction of 20nM enzyme, 0.2mM phenacetin, NADPH Regeneration System in phosphate buffer was incubated for 20 minutes. The reactions were stopped by adding equal volume of 50% methanol and the reaction product was quantitated by HPLC.

## Specificity of P450-Glo Luc-1A2 and Phenacetin

**Substrate Specificity with Recombinant P450 Enzymes.**
A panel of 21 recombinant cytochrome P450s were used to test the specificity of the Luc-1A2 and phenacetin as substrates for CYP enzymes (Graph 1). The substrate Luc-1A2 was primarily metabolized by the CYP1 family. Notable metabolism was also observed with CYP2A6, 2B6 and 2E1, along with minor contributions from CYP2D6, 4F2 and 4F3B. CYP1A2 was the major enzyme contributing approximately 70% of activity if CYP enzymes are equally distributed. Phenacetin metabolism as measured by acetaminophen formation was observed mainly by CYP1 family with the majority by CYP1A2. CYP2C19 and 2D6 also contributed to the metabolism, but as seen with luc-1A2, CYP1A2 is responsible for approximately 70% of phenacetin's metabolism.




A.




B.

Graph 1. Specificity of Luc-1A2 (Panel A) and phenacetin (Panel B) as measured by activity of recombinant cytochrome P450 enzymes.

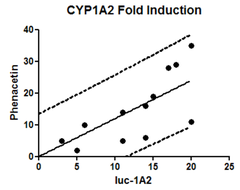
## Comparison of luc-1A2 and Phenacetin

**Induction.**
Cryoplateable human hepatocytes were used to compare Luc-1A2 and phenacetin as substrates to assess induction of CYP1A2 by omeprazole. Samples with Luc-1A2 as substrate were measured by the metabolic formation of luciferin and subsequent relative light units (RLU). This was performed by a lytic and non-lytic methods. Samples with phenacetin as substrate were measured by the metabolic formation of acetaminophen. Cryoplateable human hepatocytes were induced with 50 µM omeprazole (PC) or 1% acetonitrile (VC) for 48 hours prior to metabolism with CYP1A2 substrates. The fold induction (PC/VC) was determined and the two substrates were compared. The fold induction were similar for the two substrates except when the VC level was very low and approached background for luciferin or lower limit of quantitation (LLOQ) for acetaminophen as observed in lot AWG. This may be due to the fact that these samples were not sufficiently separated from the limit of detection (LOD).

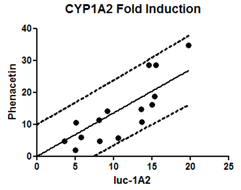
| | luc-1A2 | | | | | | Phenacetin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Non-lytic (RLU) | | | Lytic (RLU) | | | Acetaminophen (µM) | | |
| Lot | VC | PC | Fold | VC | PC | Fold | VC | PC | Fold |
| AAA | 415 | 759 | 3 | 401 | 770 | 4 | 0.031 | 0.151 | 5 |
| AAS | NA | NA | NA | 1509 | 18564 | 6 | 0.031 | 0.185 | 6 |
| AWG | 296 | 3574 | 52 | 292 | 4155 | 61 | 0.025 | 0.475 | 19 |
| ETA | NA | NA | NA | 1790 | 13429 | 8 | 0.110 | 1.263 | 11 |
| HIE | 6916 | 42085 | 6 | 10006 | 49519 | 5 | 0.071 | 0.747 | 10 |
| IPH | 2791 | 52078 | 20 | 3168 | 58479 | 20 | 0.157 | 5.454 | 35 |
| LHO | 829 | 9038 | 14 | 1302 | 11578 | 11 | 0.056 | 0.321 | 6 |
| LOF | 2294 | 34408 | 17 | 3622 | 49232 | 15 | 0.020 | 0.564 | 28 |
| NLX | 2073 | 27747 | 15 | 2661 | 37194 | 15 | 0.018 | 0.336 | 19 |
| QSI | NA | NA | NA | 502 | 2155 | 14 | 0.119 | 1.748 | 15 |
| SCT | 2384 | 24229 | 11 | 3527 | 26801 | 8 | 0.197 | 0.940 | 5 |
| SKD | 2541 | 42221 | 18 | 3199 | 45802 | 15 | 0.139 | 3.960 | 29 |
| SMK | 731 | 5856 | 11 | 872 | 6142 | 9 | 0.037 | 0.523 | 14 |
| VEP | 1827 | 23555 | 14 | 2555 | 34185 | 15 | 0.178 | 2.881 | 16 |
| VNL | 666 | 2341 | 5 | 809 | 3101 | 5 | 0.072 | 0.146 | 2 |
| YRT | 2426 | 42993 | 20 | 3344 | 42117 | 14 | 0.181 | 1.944 | 11 |

Table 1. Correlation of the formation of luciferin and acetaminophen from uninduced (VC) and induced (PC) human hepatocytes.

**Correlation of Signals.**
The fold inductions were compared between the substrates Luc-1A2 and phenacetin. Panel A. represents the data derived by non-lytic method and panel B. by lytic method for the luminescence reaction. While both lytic and non-lytic showed significant correlation to phenacetin (P values < 0.01), the lytic method resulted in a more statistically significant correlation with a P value < 0.0001. As well, the $R^2$ value was better for lytic than non-lytic, 0.7016 and 0.5260, respectively.




A.




B.

Graph 2. Correlation of the fold induction as measured by phenacetin and Luc-1A2 human hepatocytes. Panel A. represents non-lytic method and panel B. represents lytic method. Values from lot AWG were excluded for correlation calculations.

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## CONCLUSIONS

Luc-1A2 was a specific substrate for P450 1A family and compares well to phenacetin. Some differences were observed and should be taken into account depending on the application. Lytic method compared more favorably than the non-lytic method for the application of determining induction of CYP1A2.

Luc-1A2 data was determined less than one hour after incubation with a simple plate reader luminometer. The phenacetin data was determined by traditional bioanalytical methods which took hours to run requiring highly technical UPLC/MS/MS equipment and personnel.

Luc-1A2 in conjunction with cryoplateable human hepatocytes offers a fast, reliable and low-cost alternative to the current gold standard of assessing induction potential of new chemical entities with substrates requiring traditional bioanalytical methods.