Comparison of P450-Glo™ Substrates with Testosterone in Determining Induction of CYP3A4 in Fresh and Cryopreserved Human Hepatocytes.

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ABSTRACT

Induction of P450 enzymes is an important determinant for a potential drug-drug interaction of new chemical entities (NCEs). It is critical data for informed drug development and decision of lead compound selection. Subsequently, induction data is being generated in early drug discovery as a screening tool versus for solely IND submission. This necessitates a need for simple diagnostic tools to determine the induction potential of cytochrome P450s (CYP) in a relevant biological system. 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. For induction associated with PXR nuclear receptor, CYP3A4 activity is the preferred model with use of testosterone or midazolam as substrate and LC/MS quantitation of the metabolite formation. An alternative to LC/MS methods is to use fluorescent or luminescent substrates on a plate reader, reducing time and cost. P450-Glo™ substrates utilize the luciferin-luciferase reaction for the production of luminescence, and through new generations, have provided specific and more active substrates for CYPs. We have tested two P450-Glo™ CYP3A4 substrates, luciferin-6'-pentafluorobenzyl ether (Luciferin-PFBE), and the newest generation, luciferin-isopropyl acetal (Luciferin-IPA), along with the non-specific luciferin-methyl 2-(6-methoxybenzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylate (Luciferin-MultiCYP) to measure the induction of CYP3A4 with rifampin in freshly isolated and plateable cryopreserved human hepatocytes and compared them to testosterone metabolism. The results demonstrate that Luciferin-MultiCYP was not sufficiently sensitive to discern CYP3A4 induction due to the fact that the majority of the activity is from CYP1A2. Luciferin-PFBE did measure induction but was below the activity and induction measured by Luciferin-IPA. Luciferin-IPA provided the greatest amount of activity and was most aligned to the induction measured by testosterone. With improved specificity and sensitivity for CYP3A4, P450-Glo™ Luciferin-IPA offers a reduction in traditional analysis time, expense and expertise, while maintaining relevant data for the measurement of induction in human hepatocytes.

INTRODUCTION

The pressures of profiling NCEs have demanded efficient screening assays that provide meaningful data. One critical assessment is for quantifying induction potential, the ability of NCEs to increase metabolically important protein levels by activating transcription and translation machinery. For example, rifampin binds to pregnane X receptor (PXR), translocates to the nucleus where it forms a heterodimer with 9-cis retinoic acid receptor (RXR)[1]. This complex binds to promoter regions, activating transcription of target genes for proteins like P450 3A4[2]. This can be perpetrated by drugs, food products or herbal supplements. The implication is the increased metabolism and transport potential for a patient which will adversely affect drug bioavailablility and efficacy leading to potential safety issues know 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[3]. 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. There are both pros and cons to each of the different platforms and methods. Cryopreserved hepatocytes that retain the ability to plate (cryoplateable) offer benefits over freshly isolated hepatocytes like scheduling of experiments, pre-characterized activities and genotypes, and ability to retest the same donor. These benefits allow for the efficient vehicle to determine induction potential. One potential bottleneck in the assessment is the quantitation of the activity using prototypical substrates that require highly technical and time-consuming bioanalytical methods like LC/MS/MS. The ideal substrate would be a highly specific and potent substrate for P450 enzyme that can be measured without traditional bioanalytical methods. A series of substrates from P450-Glo™ family of products have been developed by Promega that utilizes luciferin-luciferase reaction for the production of luminescence, and through new generations, have provided specific and more active substrates for CYPs. This system requires a luminometer and can be performed with minimal special training, unlike traditional bioanalytical methods. We have set out to compare the induction of P450 3A4 using P450-Glo™ substrates with a preferred P450 3A4 substrate testosterone in freshly isolated and 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 *InVitro*Gro™ CP medium and the culturing and dosing in *InVitro*Gro HI followed instructions for use as prescribed by BioreclamationIVTThe 48-well plates seeded with 140,000 viable hepatocytes per well were dosed with 0.2 mL of 25 µM rifampin (PC) or 1% acetonitrile (VC) in *InVitro*Gro HI at 48 and 72 hours after initial plating. Activity of P450 3A4 was measured to assess induction of P450 3A4 enzyme levels.

Determining Testosterone Metabolism.
Dosing solution of 125 µM testosterone 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 6β-hydroxytestosterone metabolite was performed using UPLC/MS/MS.

Determining P450-Glo Substrate Metabolism.
P450-Glo kits with luciferin-IPA, luciferin-PFBE and luciferin-MultiCYP substrates were obtained from Promega. Procedures were followed as described by Promega in instructions for use of lytic P450-Glo Assays 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. Luciferin-IPA and luciferin-MultiCYP substrates were incubated for 30 minutes and Luciferin-PFBE substrate was incubated for 180 minutes. Luciferin Detection Reagent with and without esterase was reconstituted as directed by instructions for use. An equal volume (0.2 mL) of Luciferin Detection Reagent was added at the end of the incubation period. An aliquot was transferred to a white 96-well microtiter plate and luminescence determined on a Wallac Victor^2 1420 Multilabel Counter.

Fold Induction.
Raw concentrations of 6β-hydroxytestosterone 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.

Determination of P450-Glo substrate Specificity.
Specific of P450-Glo substrate was determined as previously described[4,5,6]. 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). Reaction of 20 nM enzyme, P450-Glo substrate, NADPH Regeneration System in phosphate buffer was incubated for 10 minutes (luciferin-IPA) or 30 minutes (luciferin-MultiCYP and luciferin-PFBE). Luminescence was read after 20 minutes with the GloMax® 96 Microplate Luminometer and reported in relative light units (RLU).

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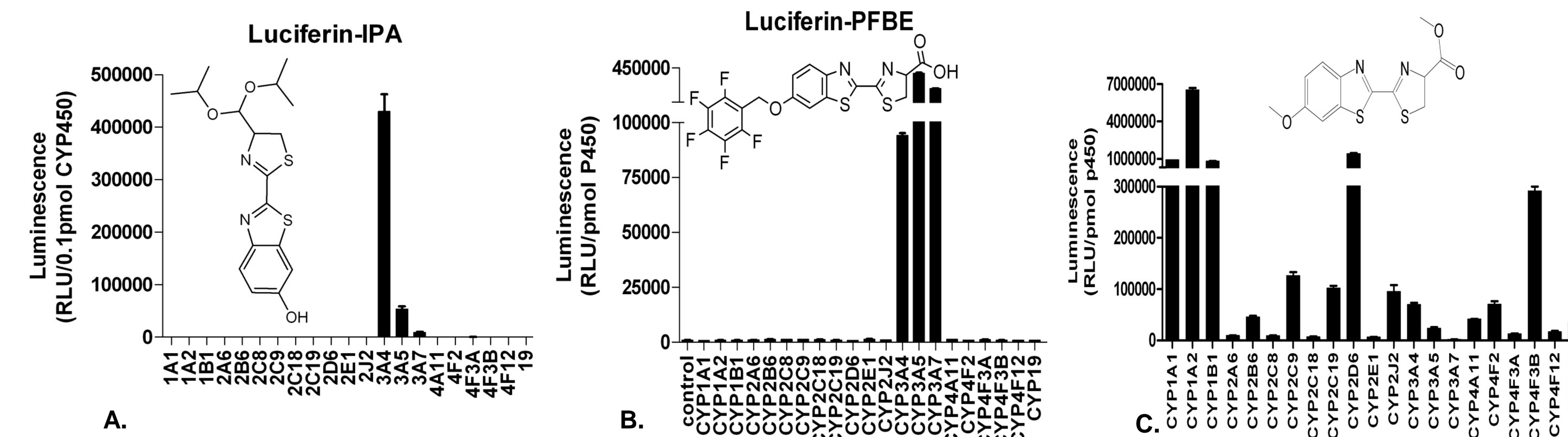
Specificity and Comparative Function of P450-Glo Substrates

Substrate Specificity with Recombinant P450 Enzymes.

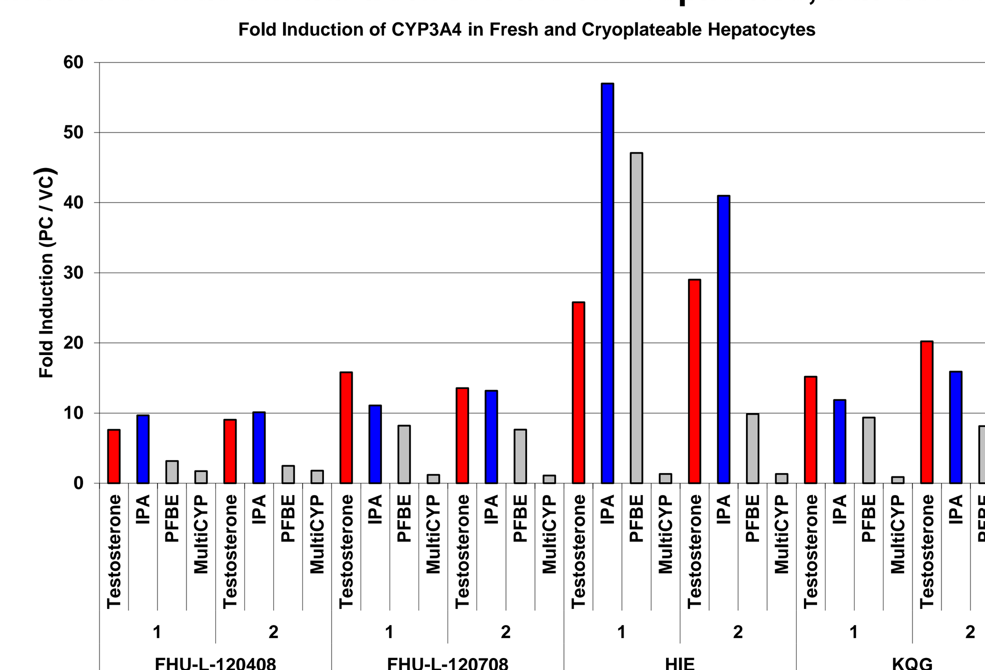
Recombinant cytochrome P450s were used to test the specificity of the luciferin-linked substrates. Luciferin-IPA has a high selectivity for P450 3A family with majority of activity from P450 3A4 (Graph 1 Panel A). Luciferin-PFBE has high selectivity for the P450 3A family with significant contributions from P450 3A5 and 3A7 (Graph 1 Panel B). However, luciferin-IPA demonstrates higher activity per pmol of enzyme as compared to luciferin-PFBE. Luciferin-MultiCYP is a non-selective, non-proportionate substrate for P450s with high contribution of P450 1A2 (Graph 1 Panel C).



Graph 1. Specificity of luciferin-IPA (Panel A), luciferin-PFBE (Panel B) and luciferin-MultiCYP as measured by activity of recombinant cytochrome P450 enzymes.

Comparative Function of Substrates to Measure Induction of P450 3A4.

Human hepatocytes, freshly isolated and cryoplateable, were used to measure the induction of P450 3A4 with 25 µM of rifampin (Graph 2). The activity of P450 3A4 was determined using luciferin-IPA, luciferin-PFBE, luciferin-MultiCYP and testosterone. The fold induction (PC/VC) was determined from triplicate samples from two replicate tissue culture plates per donor. The induction was similar between the two replicates, but donor-to-donor variability was, as expected, observed. Testosterone (red), one of the preferred substrates for measuring P450 3A4 activity, was used to represent the gold standard for determining induction of P450 3A4. Luciferin-IPA (blue) closely matched the induction as measured by testosterone, while luciferin-PFBE was not as correlative. The difference may be due to the affinity of the substrates to P450 3A4 as indicated by the fact that luciferin-IPA was incubated for 30 minutes at 3 µM versus three hours at 50 µM with luciferin-PFBE. In the case of lot HIE, the basal level (VC) was very low and approached background, especially in replicate 1 which may have skewed the apparent fold induction. Luciferin-MultiCYP does not correlate with induction of P450 3A4 due to the lack of P450 specificity. This illustrates the necessity for high specificity and high affinity substrates.



Graph 2. Comparison of fold induction as measured by P450 3A4 substrates testosterone (red), luciferin-IPA (blue), luciferin-PFBE (gray) and luciferin-MultiCYP (grey) from two freshly isolated hepatocytes and two cryoplateable hepatocyte lots.

Comparison of Testosterone and Luciferin-IPA

Induction.

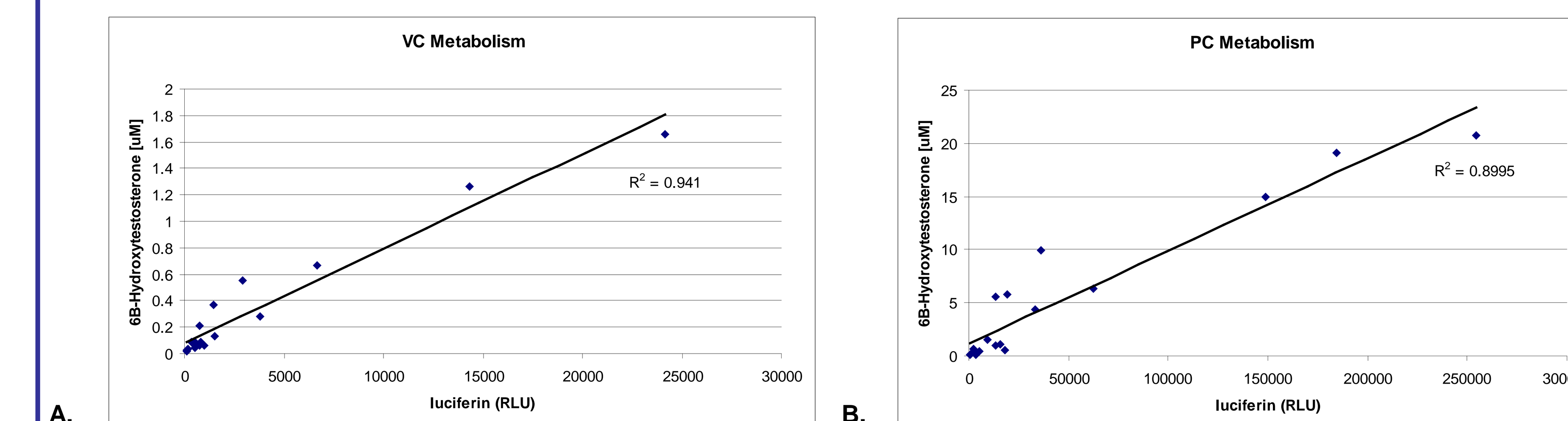
Cryoplateable human hepatocytes were used to compare testosterone and luciferin-IPA as substrates to assess induction of P450 3A4 by rifampin. Samples with luciferin-IPA as substrate were measured by the metabolic formation of luciferin and subsequent relative light units (RLU). Samples with testosterone as substrate were measured by the metabolic formation of 6β-hydroxytestosterone. Cryoplateable human hepatocytes were induced with 25 µM rifampin (PC) or 1% acetonitrile (VC) for 48 hours prior to metabolism with P450 3A4 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 6β-hydroxytestosterone. This may be due to the fact that these samples were not sufficiently seperated from the limit of detection (LOD).

	Luciferin- IPA						Testosterone						Induction	
	VC (RLU)			PC (RLU)			VC [µM]			PC [µM]			IPA	Test
Lot	Average	±	SD	Average	±	SD	Average	±	SD	Average	±	SD	Fold	Fold
AAA	251	±	20	3305	±	220	0.018	±	0.005	0.459	±	0.038	28	26
AAS	1170	±	207	18048	±	852	0.065	±	0.007	0.597	±	0.121	18	9
ASL	3041	±	476	19655	±	2141	0.550	±	0.100	5.751	±	0.812	7	10
BPB	3973	±	1201	62868	±	5373	0.278	±	0.029	6.313	±	1.209	17	23
ETA	670	±	57	9507	±	302	0.088	±	0.013	1.523	±	0.139	18	17
GCP	520	±	69	1249	±	358	0.078	±	0.009	0.181	±	0.040	3	2
HIE	6818	±	814	184498	±	22098	0.663	±	0.107	19.130	±	1.410	28	29
LHO	686	±	87	15685	±	2505	0.048	±	0.007	1.076	±	0.081	31	22
LMP	24353	±	2168	254837	±	12687	1.661	±	0.210	20.756	±	0.650	11	12
ONQ	462	±	31	2388	±	114	0.088	±	0.005	0.638	±	0.138	7	7
OSI	294	±	32	768	±	128	0.024	±	0.005	0.064	±	0.015	5	3
SCT	14484	±	639	148735	±	25200	1.265	±	0.115	14.934	±	1.239	10	12
SMK	1009	±	84	13626	±	1947	0.085	±	0.002	0.949	±	0.296	16	11
SOM	966	±	58	3536	±	1338	0.061	±	0.010	0.121	±	0.021	4	2
VEP	1578	±	266	13621	±	101	0.368	±	0.081	5.605	±	1.375	9	15
VNL	364	±	29	5440	±	2448	0.035	±	0.005	0.440	±	0.142	30	13
VUA	1681	±	265	33478	±	1144	0.127	±	0.019	4.343	±	0.743	22	34
YRT	887	±	101	36215	±	1509	0.210	±	0.017	9.885	±	0.805	48	47

Table 1. Correlation of the formation of 6β-hydroxytesosterone and luciferin from uninduced (VC) and induced (PC) human hepatocytes.

Correlation of Signals.

The signals were compared by plotting a trend line of 6β-hydroxytestosterone and corrected RLUs (raw minus background) for both the un-induced (VC) (Graph 3 Panel A) and induced (PC) (Graph 3 Panel B) hepatocytes. A good correlation was made as indicated by R^2 values of 0.941 and 0.8995 for VC and PC, respectively. Thus, luciferin-IPA gave comparable relative activity of P450 3A4 to testosterone, an FDA preferred substrate for P450 3A4.



Graph 3. Correlation of the formation of 6β-hydroxytesosterone and luciferin from uninduced (VC) and induced (PC) human hepatocytes.

CONCLUSIONS

1. Luciferin-IPA was a highly specific and potent substrate for P450 3A family, as compared to the specific but less potent substrate luciferin-PFBE. The luciferin-MultiCYP was a non-specific and non-proportionate P450 substrate.
2. Luciferin-IPA provided similar metabolic activity assessment of P450 3A to testosterone. As such, the fold induction of P450 3A was similar between the two substrates. Luciferin-IPA data was determined less than one hour after incubation with a simple plate reader luminometer. The testosterone data was determined by traditional bioanalytical methods which took hours to run with highly technical UPLC/MS/MS.
3. Luciferin-IPA 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.