



## New luciferin-based probe substrates for human CYP26A1

Shishir Sharma<sup>a</sup>, Jingyao Liu<sup>a</sup>, Xue Zhang<sup>a</sup>, Sangeeta Shrestha Sharma<sup>a</sup>, Erik J. Sorensen<sup>a,b</sup>, Matthias Bureik<sup>a,\*</sup>

<sup>a</sup> School of Pharmaceutical Science and Technology, Health Sciences Platform, Tianjin University, Tianjin, 300072, China

<sup>b</sup> Department of Chemistry, Princeton University, Princeton, NJ, 08544, USA

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### ABSTRACT

Activity of human CYP26A1 towards six proluciferin probe substrates and their ester derivatives was monitored. These included three monofluorobenzyl ether isomers and three five-membered heterocycles. Overall, luciferin substrates with a free acid group gave higher activities than the ester compounds. Also, luciferin derivatives with six-ring structures were better metabolized than those with five-rings. The best substrates identified in this study are Luciferin 6' 3-fluorobenzyl ether (Luciferin-3FBE) and its methyl ester (Luciferin-3FBEME). Taken together, we describe eleven new probe substrates for CYP26A1 and demonstrate for the first time that CYP26A1 does not only accept acid substrates but can also metabolize esters.

### 1. Introduction

In humans, the cytochrome P450 (CYP) family 26 consists of three members: CYP26A1, CYP26B1, and CYP26C1. They all contribute to homeostasis of the vitamin A-derived morphogen retinoic acid (RA), which plays a critical role in many biological processes such as reproduction, fetal development, maintenance of skin and epithelia, and regulation of the immune system. CYP26 enzymes metabolize all-*trans*-retinoic acid (atRA) as well as its isomers 9-*cis*RA and 13-*cis*RA, and mainly produce 4-hydroxy and 4-oxo metabolites [1]. This CYP family is present in all chordates and appears to be evolutionarily highly conserved due to the enzymes' critical role in vertebrate development [2]. Amino acid sequence similarities of CYP26A1 with CYP26B1 and CYP26C1 are 42 and 43%, respectively, while CYP26B1 and CYP26C1 share a similarity of 51%. This comparatively low sequence similarity is surprising as all three enzymes are thought to carry out essentially the same metabolic function. Moreover, this apparent functional redundancy is rare for P450s that catalyze the metabolism of a single endogenous substrate. By comparison, CYP19A1, CYP17A1 and CYP24A1 are the only human members of their respective families. In addition, even small differences in human CYP protein sequence typically result in distinct differences in substrate specificity and catalytic activity; a good example are the four human CYP3A enzymes. Thus, it is well possible that some of the CYP26 enzymes might also act on other (as yet unidentified) endogenous compounds. Interestingly, strong

expression of CYP26A1 is correlated with poorer survival in breast cancer patients [3].

In the human adult liver, CYP26A1 is clearly the most strongly expressed CYP26 enzyme and also the predominant atRA hydroxylase [4]. Therefore, selective inhibitors of CYP26A1 are of pharmacologic interest as they could decrease the systemic clearance of retinoic acid (see below). In the past, we have successfully employed luminogenic probe substrates for the convenient monitoring of human CYP activities and for the identification and characterization of CYP inhibitors [5–11]. Such proluciferins are converted by CYPs to beetle luciferin, which can then be detected in a subsequent reaction catalyzed by luciferase [12]. The amount of light produced in the second reaction is proportional to CYP activity. We have also previously demonstrated that one of the commercially available proluciferins, Luciferin 6' methyl ether (Luciferin-ME), is a CYP26A1 substrate [7]. Luciferin-ME is a nonselective CYP probe substrate, and hydroxylation of its methyl group yields the hemiacetal Luciferin 6' hydroxymethyl ether, which dissociates into formaldehyde and *D*-luciferin. However, bioconversion of Luciferin-ME by CYP26A1 proceeded at a comparatively low rate. For further studies of the properties of this enzyme the identification of better proluciferin substrates is desirable. In another study, we recently reported the synthesis of new proluciferin derivatives and their metabolism by human CYP4 family enzymes [13]. It was the aim of this study to test CYP26A1 for conversion of these new compounds. Moreover, we synthesized a set of complementary esters of these proluciferins

\* Corresponding author.

E-mail address: [matthias@tju.edu.cn](mailto:matthias@tju.edu.cn) (M. Bureik).

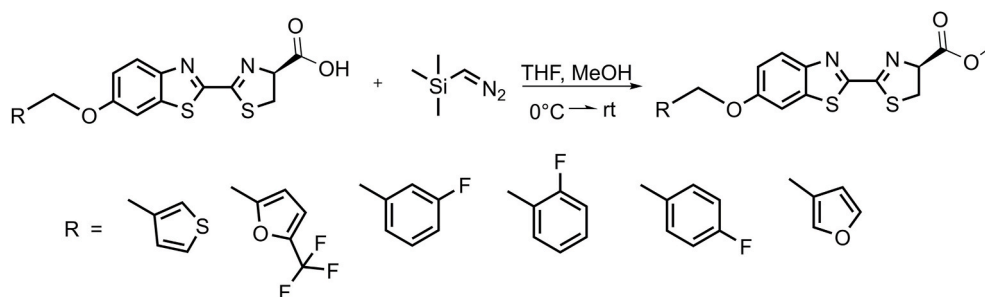


Fig. 1. Synthesis scheme for proluciferin esters.

and also tested them with human liver microsomes (HLMs) and with CYP26A1.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Triton-X100 was from Leagene (Beijing, China); Tris-HCl, from AKZ-Biotech (Tianjin, China); potassium chloride, ammonium bicarbonate, potassium dihydrogen phosphate and potassium hydrogen phosphate were from Jiangtian Chemical (Tianjin, China); glycerol was from Dingguo (Tianjin, China); 1 X PBS buffer was from Corning (Manassas, VA); human liver microsomes (HLMs) were from Sekisui XenoTech (Kansas City, KS, USA); Luciferin 6' 2-fluorobenzyl ether (Luciferin-2FBE), Luciferin 6' 3-fluorobenzyl ether (Luciferin-3FBE), Luciferin 6' 2-furfuryl ether (Luciferin-2FE), Luciferin 6' 3-furfuryl ether (Luciferin-3FE), Luciferin 6' 2-(5-trifluoromethyl)furfuryl ether (Luciferin-TFM2FE), and Luciferin 6' 3-thenyl ether (Luciferin-3TE) were synthesized as described [13]; the NADPH regeneration system (20X solution A: 26 mM  $\text{NADP}^+$ , 66 mM glucose-6-phosphate, 66 mM  $\text{MgCl}_2$ ; and 100X solution B: 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, pH 5.5), Luciferin detection reagent (Cat# V8921), and Luciferin detection reagent with esterase (Cat# V8931) were from Promega (Madison, USA); white opaque 96-well microtiter plates were from Nunc (ThermoFisher scientific, Lagensfeld, Germany); all other chemicals and reagents used were of the highest grade available.

### 2.2. General experimental details for chemical synthesis

Unless otherwise indicated, commercial reagents of high purity were purchased and used without further purification in the reactions described herein. Reactions were monitored by thin-layer chromatography (TLC) carried out on Merck silica gel plates (60 F<sub>254</sub>). Visualization was accomplished with UV light (254 nm). Crude reaction products were purified by Flash column chromatography using Merck silica gel (200–300 mesh).

### 2.3. Instrumentation

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on 400 MHz or 600 MHz spectrometers at 25 °C using a Bruker Avance III instrument with chemical shifts reported relative to tetramethylsilane (TMS).  $^1\text{H}$  NMR data are referenced to the residual solvent peak at 7.26 ppm ( $\text{CDCl}_3$ ) or 2.50 ppm ( $(\text{CD}_3)_2\text{SO}$ ).  $^{13}\text{C}$  NMR spectra, recorded at 101 and 151 MHz, are referenced to the solvent peak at 77.16 ppm ( $\text{CDCl}_3$ ) or 39.52 ppm ( $(\text{CD}_3)_2\text{SO}$ ). High resolution mass spectrometry (HRMS) data were obtained on a Thermo scientific Q Exactive HF mass spectrometer. Luminescence measurements were done on a Infinite M200 PRO (Tecan, Austria).

### 2.4. General procedures for the preparation of proluciferin methyl esters

To a solution of the appropriate acid (1.0 mmol) in THF:MeOH (4:1, 0.3 M) at 0 °C was slowly added (trimethylsilyl)diazomethane (2.0 M in hexane, 1.2 equiv). The reaction was allowed to warm to room temperature over a period of 3 h. The mixture was then concentrated *in vacuo* and the residue purified by flash chromatography on silica gel using a mobile phase consisting of a mixture of 10:1 petroleum ether/ethyl acetate to provide the desired ester compounds [14]. Product identity was confirmed by NMR and HRMS (data not shown).

### 2.5. Fission yeast media and strains

Recombinant fission yeast strain CAD62 (genotype *h-ura4-D.18 leu1::pCAD1-CPR*) expressing human cytochrome P450 reductase (CPR) and strain RAJ267 (genotype *h-ura4-D.18 leu1::pCAD1-CPR/pREP1-CYP26A1*) coexpressing human CPR and human CYP26A1 have been described before [7,15]. In both strains, expression of the human genes is regulated by thiamine-repressible *nmt1* promoter [16].

### 2.6. Fission yeast cultivation and biotransformation procedures

Fission yeast media, biotransformations with enzyme bags, bioluminescence detection, and statistical analysis have all been described before [5,17]. More details are given in the [Supplemental Informations](#).

### 2.7. Biotransformations with human liver microsomes (HLMs)

Luminescence-based CYP activity assays were essentially done according to the instructions of the commercial manufacturer of Luciferin-BE (Promega; Madison, USA). Briefly, for HLM assays (final reaction volume: 50  $\mu\text{l}$ ) 100 mM potassium phosphate buffer, pH 7.4, contained 20  $\mu\text{g}$  of total HLM protein and either 20  $\mu\text{M}$  (acids) or 150  $\mu\text{M}$  (esters) of CYP probe substrates. Reactions were started by adding NADPH to a final concentration 1 mM. Control reactions were identical except that no NADPH was added. For all experiments, the amount of substrate consumed was within the linear range with respect to time and enzyme concentration used. Incubation was done by shaking at 400 rpm at 37 °C for 30 min, then reactions were stopped by adding luciferin detection reagent.

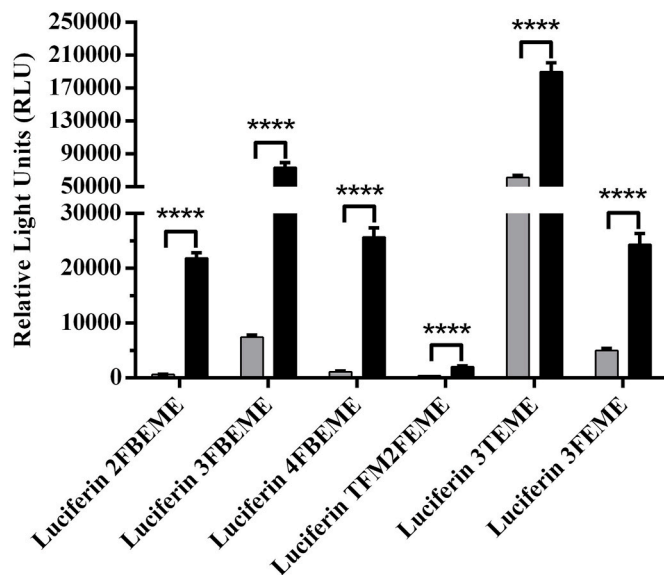
## 3. Results

In a recent study, we demonstrated that the probe substrate Luciferin-ME can be hydroxylated by human CYP26A1 [7]; however, the activities detected were rather low in comparison to those of many other human P450 enzymes. Therefore, we wanted to identify other proluciferin substrates that can be metabolized much more efficiently by this enzyme. For this purpose, six recently described new proluciferin acid compounds were employed [13]. In addition, ester derivatives of these compounds were synthesized in the present study and also tested. Chemical synthesis of the new proluciferin esters was done according to

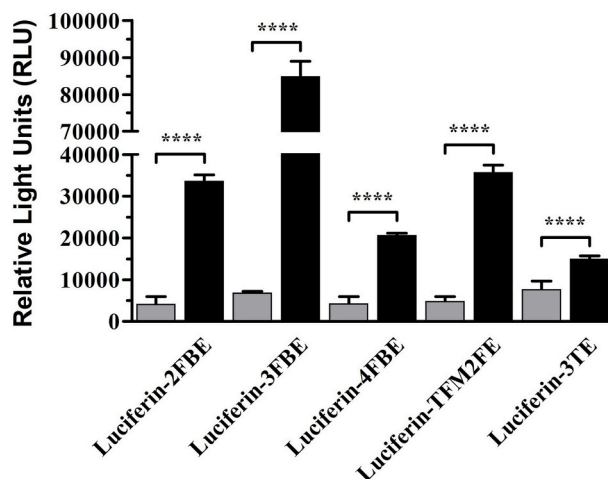
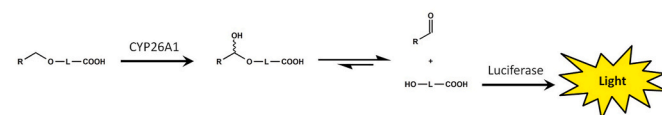
**Table 1**  
Chemical structure of luminogenic CYP substrates used in this study.

Structure	Name	Reference
	Luciferin 6' benzyl ether (Luciferin-BE)	[12]
	Luciferin 6' 2-fluorobenzyl ether (Luciferin-2FBE)	[13]
	Luciferin 6' 2-fluorobenzyl ether methyl ester (Luciferin-2FBEME)	This study
	Luciferin 6' 3-fluorobenzyl ether (Luciferin-3FBE)	[13]
	Luciferin 6' 3-fluorobenzyl ether methyl ester (Luciferin-3FBEME)	This study
	Luciferin 6' 4-fluorobenzyl ether (Luciferin-4FBE)	[13]
	Luciferin 6' 4-fluorobenzyl ether methyl ester (Luciferin-4FBEME)	This study
	Luciferin 6' 3-furfuryl ether (Luciferin-3FE)	[13]
	Luciferin 6' 3-furfuryl ether methyl ester (Luciferin-3FEME)	This study
	Luciferin 6' 2-(5-trifluoromethyl)furfuryl ether (Luciferin-TFM2FE)	[13]
	Luciferin 6' 2-(5-trifluoromethyl)furfuryl ether methyl ester (Luciferin-TFM2FEME)	This study
	Luciferin 6' 3-thenyl ether (Luciferin-3TE)	[13]
	Luciferin 6' 3-thenyl ether methyl ester (Luciferin-3TEME)	This study

the scheme shown in Fig. 1 following a general procedure described previously [14]. Table 1 contains a list of all pro-luciferin substrates used in this study. Biotransformation of the new pro-luciferin ester compounds demonstrated that they are all excellent substrates for HLMs (Fig. 2). Permeabilized cells (enzyme bags) of the recombinant fission yeast strain RAJ267 that recombinantly coexpresses human CYP26A1 and CPR [7] were used for the biotransformation of the pro-luciferin

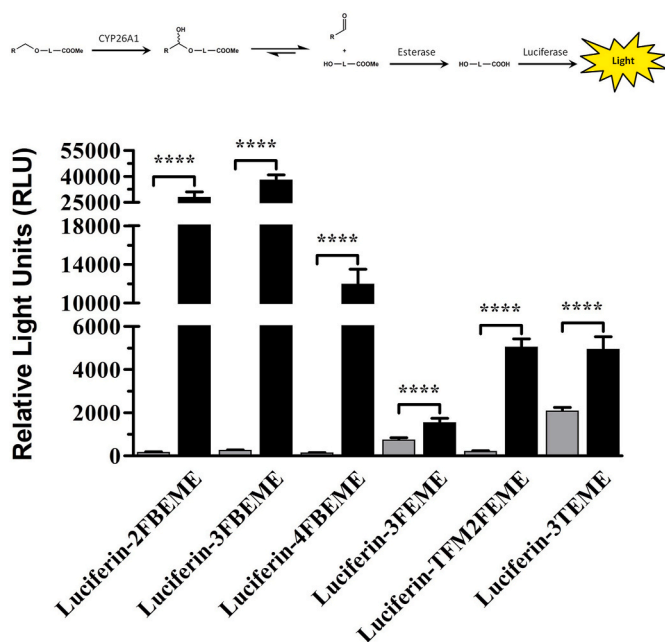


**Fig. 2.** Screening of pro-luciferin ester substrates for conversion by human liver microsomes (HLMs). Experiments were performed in the presence (black columns) or absence (grey columns) of NADPH and activities are shown in relative light units (RLU). Data shown were calculated from three experiments done in triplicates. \*\*\*\* $P < 0.001$  versus control.



**Fig. 3.** Screening of pro-luciferin substrates for conversion by human CYP26A1. Permeabilized fission yeast cells (enzyme bags) of strains RAJ267 (coexpressing human CYP26A1 and CPR; black columns) or CAD62 (control strain expressing only CPR; grey columns) were used for the biotransformation of different luminogenic substrates as indicated. The hydroxylation reaction yields the respective hemiacetals, which dissociate into an aldehyde and D-luciferin (shown as HO-L-COOH). The amount of the latter was determined in a second reaction catalyzed by luciferase. All activities are shown in relative light units (RLU). Data shown were calculated from three independent experiments done in triplicates. \*\*\*\* $P < 0.001$  versus control.

compounds. These experiments showed that five out of the six acid compounds tested are indeed CYP26A1 substrates, with Luciferin-3FBE giving the highest activity (Fig. 3). Statistically significant activities were also observed for its isomers Luciferin-2FBE and Luciferin-4FBE as



**Fig. 4.** Screening of pro-luciferin ester substrates for conversion by human CYP26A1. Permeabilized fission yeast cells (enzyme bags) of strains RAJ267 (coexpressing human CYP26A1 and CPR; black columns) or CAD62 (control strain expressing only CPR; grey columns) were used for the biotransformation of different ester substrates as indicated. The hydroxylation reaction yields the respective hemiacetals, which dissociate into an aldehyde and the methyl ester of *D*-luciferin (shown as HO-L-COOMe). This ester was transformed to *D*-luciferin (shown as HO-L-COOH) by treatment with esterase, and the amount of *D*-luciferin was then determined in a subsequent reaction catalyzed by luciferase. All activities are shown in relative light units (RLU). Data shown were calculated from three independent experiments done in triplicates. \*\*\*\**P* < 0.001 versus control.

well as for Luciferin-TFM2FE, and Luciferin-3TE, respectively. Luciferin-3FE is no CYP26A1 substrate. Interestingly, all of the pro-luciferin methyl esters were also metabolized by CYP26A1 (Fig. 4). Highest activities were detected for the three isomers Luciferin-3FBEME, Luciferin-2FBEME, and Luciferin-4FBEME, while Luciferin-TFM2FEME, Luciferin-3TEME, and Luciferin-3FEME are weaker substrates. Taken together, we have thus identified eleven new probe substrates for CYP26A1. Moreover, our data demonstrate for the first time that CYP26A1 does not only accept acid substrates but can also metabolize esters.

#### 4. Discussion

In this study, activity of CYP26A1 towards six pro-luciferin probe substrates and their ester derivatives was monitored. Chemical synthesis of the new pro-luciferin esters (Fig. 1) was performed according to a procedure described previously [14]. All of the ester compounds were strongly metabolized by HLMs (Fig. 2). With the exception of the 3-furfuryl ether compounds (where no statistically significant activity for the acid analogue was found), luciferin acid substrates gave on average twice as high RLU values as the ester substrates when metabolized with CYP26A1 (Figs. 3 and 4). This could be expected as the enzyme's best characterized activity is that toward RA. The effect was less strong for the monofluorobenzylether derivatives but more pronounced for the two five-membered heterocycles where the comparison could be made. Similar to our previous observations with human CYP4 enzymes [13], luciferin ether derivatives with six-ring structures typically were better metabolized than those with five-rings. More specifically, for the three monofluorobenzylether derivatives, activity of the 3-fluoro compounds was highest for both acid and ester, followed by the 2-fluoro and the

4-fluoro isomers in that order. In terms of activity, the 3-thenyl ether compounds (Luciferin-3TE and Luciferin-3TEME) range in the middle as both of them were metabolized well, with the acid substrate showing threefold higher activity than the ester. The 3-furfuryl ether derivatives were the poorest substrates overall, as the acid compound was not metabolized and the remaining ester (3FEME) showed the lowest activity of all esters. The 2-(5-trifluoromethyl)furfuryl compounds were both well metabolized, but interestingly, with a factor of seven the difference between the acid (Luciferin-TFM2FE) and the ester (Luciferin-TFM2FEME) was the highest observed in the whole study.

Our findings naturally raise the interesting question whether retinyl esters might also be CYP26A1 substrates. Moreover, retinoic acid metabolism blocking agents (RAMBAs) hold promise for improving the clinical benefit of retinoid treatments, for instance against acute promyelocytic leukemia or neuroblastoma tumors; and interestingly, some of these compounds are esters [18,19]. We expect that the new and convenient pro-luciferin-based activity assays presented in this study will be useful for the identification of new substrates and inhibitors of CYP26A1.

#### Credit author statement

**Shishir Sharma:** Investigation, Methodology, Validation, Formal analysis, Writing - Original Draft, Visualization. **Jingyao Liu:** Investigation, Methodology. **Xue Zhang:** Investigation, Methodology. **Sangeeta Shrestha Sharma:** Investigation, Methodology. **Erik J. Sorensen:** Conceptualization, Funding acquisition. **Matthias Bureik:** Conceptualization, Project administration, Funding acquisition, Writing - Review & Editing.

#### Declaration of competing interest

The authors declare no conflict of interest.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2020.100861>.

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