SUPPLEMENTAL MATERIAL

Evaluation of luminogenic substrates as probe substrates for bacterial Cytochrome P450 enzymes: Application to *Mycobacterium tuberculosis*

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Figure S1: Structures of luminogenic substrates used in this study.  

Figure S2: UV-Vis spectra of recombinant FNR and Flda from *Escherichia coli*.  

Figure S3: Time course of D-luciferin production in incubation of Luciferin-BE with CYP130A1-BM3 fusion protein  

Figure S4: Luminescence production after addition of luciferin detection reagent to incubations of luminogenic substrates in presence and absence of Mtb CYPs: A) CYP121A1, B) CYP124A1, C) CYP125A1, D) CYP130A1-BM3R and E) CYP142A1.  

Figure S5: Comparison of D-luciferin production in incubation of Luciferin-BE with CYP130A1-BM3R fusion protein and in incubations of Luciferin-BE with CYP130A1 supported by FNR and Flda from *Escherichia coli*.  

Figure S6: Comparison of D-luciferin production by CYP130A1-BM3R and CYP130A1 supported by FNR and Flda from *Escherichia coli*.  

Figure S7: Concentration dependence of the specific activity of D-luciferin production in incubations of CYP142A1 with Luciferin-ME EGE as substrate.
**Figure S1:** Structures of luminogenic substrates used in this study. Arrows indicate the site of hydroxylation leading to D-luciferin formation. Arrows indicate the position of hydroxylation required for luminescence production after addition of luciferin detection reagent. Human CYPs (hCYPs) capable to perform oxidation are indicated below the substrates.

**a:** After oxidation by CYP, hydrolysis of ester-group by esterases is required for D-luciferin formation.\(^1\)

**b:** After O-dealkylation by CYP, coupling of 2-cyano-6-hydroxybenzothiazole to D-cysteine is required for D-luciferin formation.

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1. Technical bulletin P450-GloTM Assays, https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/101/p450-glo-assays-protocol.pdf
Figure S2: UV-Vis spectra of 40 µM FNR (A) and 190 µM oxidized Flda from *Escherichia coli* in 100 mM potassium phosphate buffer, pH 7.4. The reference cuvette contained 100 mM potassium phosphate buffer, pH 7.4. Protein concentrations were quantified using the extinction coefficients: $\varepsilon_{456\text{ nm}} = 7.1 \text{ mM}^{-1}$ for FNR and $\varepsilon_{466\text{ nm}} = 8.2 \text{ mM}^{-1}$ for oxidized Flda.1,2

1. Jenkins, C. M.; Waterman, M. R. Flavodoxin and NADPH-Flavodoxin Reductase from *Escherichia coli* Support Bovine Cytochrome P450c17 Hydroxylase Activities. *Biochemistry* **1994**, 269, 27401–27408.

2. McIver, L.; Leadbeater, C.; Campopiano, R. L.; et al. Characterisation of Flavodoxin NADP$^+$ Oxidoreductase and Flavodoxin; Key Components of Electron Transfer in *Escherichia coli*. *Eur. J. Biochem.* **1998**, 257, 577–585.
Figure S3. Time course of D-luciferin formation in incubation with 0.5 µM CYP130A1-BM3R fusion protein with 37.5 µM Luciferin-BE as substrate. Reactions without CYP130A1-BM3R were taken along as negative control. All conditions were incubated in triplicate. D-Luciferin formation is indicated by luminescence formation after addition of P450-Glo luciferin detection reagent. Points represent mean ± standard deviation of triplicate experiments.

The solid line represents the curve fit obtained by linear regression.
Figure S4. Luminescence production after addition of luciferin detection reagent to incubations of luminogenic substrates in presence and absence of Mtb CYPs: A) CYP121A1, B) CYP124A1, C) CYP125A1, D) CYP130A1-BM3R and E) CYP142A1.

Blue bars indicate luminescence formation after 60 minutes incubation of luminogenic substrates in presence of Mtb CYPs. Orange bars indicate luminescence formation after 60 minutes incubation of luminogenic substrates in absence of Mtb CYPs. Error bars represent standard deviation of triplicate experiments. Background reflects the presence of trace amounts of free D-Luciferin. Values on top of the bars represent the signal-to-background (S/B) ratio: luminescence in presence of Mtb CYP / luminescence in absence of Mtb CYP.

RLU, relative light units.
Figure S5. Concentration of D-luciferin formed after 60 minutes of incubation of seventeen luminogenic substrates at 100 µM in presence of 0.5 µM Mtb CYPs and NADPH-regenerating system.
Figure S6: Comparison of D-luciferin production in incubations of Luciferin-BE with 0.5 μM CYP130A1-BM3R and in incubations of 0.5 μM CYP130A1 supported by 2.5 μM FNR and 5 μM Flda from *Escherichia coli*. Luciferin-BE (50 μM) was incubated for 1 h at 37 °C in presence of NADPH-regenerating system, after which the D-luciferin formed was quantified using the P450-Glo LDR-reagent, as described in Materials and Methods. Activity of CYP130-BM3R was set as 100% and relative activities represent means of triplicate measurements. Error bars represent standard deviations.
Figure S7. Concentration dependence of the specific activity of D-luciferin production in incubations of CYP142A1 with Luciferin-ME EGE as substrate.

All conditions were incubated in triplicate. Points represent mean ± standard deviation of triplicate experiments.

The solid line represents the curve fit obtained by nonlinear regression according to the Michaelis-Menten equation. Incubations were analyzed as described in Materials and Methods. Enzyme kinetic parameters obtained were: \( K_m \), 270 ± 22 µM; \( V_{max} \), \((61 \pm 6) \times 10^6\) (nmol D-luciferin/min/nmol CYP).