**Abstract**

Probe substrates are an important tool for activity monitoring of human drug metabolizing enzymes such as cytochromes P450 (CYPs). In the present study we have tested human CYPs towards five proluciferin ester substrates which had previously only been known to be hydroxylated by CYP26A1. It was found that these substrates were converted by another 21 human CYPs, which belong to the CYP families 1 to 4, 7, and 26. Thus, 64 new pairs of enzyme and substrate were identified. Correlation analysis indicated the presence of three distinct sets of enzymes with high similarity in their activity profiles that encompass a total of 16 individual enzymes. Some of these newly identified correlations may serve as a starting point for further study of those human CYPs whose activities are not yet satisfactorily understood.

**Keywords:** cytochrome P450; drug metabolism; fission yeast; luminescence; proluciferin ester.

**1. Introduction**

Members of the superfamily of cytochrome P450 enzymes (CYPs or P450s) are present in all kingdoms of life and are extensively involved in many anabolic and catabolic processes [1, 2]. In humans, P450s are involved in Phase I drug metabolism, but they also fulfill important functions in endogenous pathways such as steroid biosynthesis [3]. The 57 human CYPs are all membrane bound proteins that are either located on the cytoplasmic side of the endoplasmic reticulum or on the matrix side of the inner mitochondrial membrane. In order to achieve the reductive activation of inert oxygen for substrate monooxygenation, human CYPs depend on electron transfer proteins such as cytochrome P450 reductase (CPR or POR), adrenodoxin (Adx), and adrenodoxin reductase (AdR) [4]. A CYP enzyme together with its redox partners therefore constitutes a CYP system. Such systems are capable of catalyzing aliphatic or aromatic hydroxylation reactions of a vast variety of substrates, and for many human family members their function and substrate specificity are well characterized.

However, a number of human CYPs are still considered to be ‘orphans’ in the sense that their main substrates and functions are still not well understood; and deorphanization of some of these enzymes might also contribute to the development of new treatment options for malignant diseases such as breast cancer [5, 6]. Identification of the substrates of orphan P450s is a difficult task, as a comprehensive screening of all possible target molecules is virtually impossible due to the high numbers involved. One approach to this problem is to first try to obtain informations about what a given enzyme *can* do in order to make better informed guesses at what the enzyme actually *does* do within the human body. An advantage of such an approach is that in the initial phase of data collecting one is not limited to working with potentially physiological substrates; instead, use of convenient probe substrates can be considered and is actually indicated, if there are chances for such probes being metabolized by CYPs in general. One group of such probe compounds are proluciferins that are converted by CYPs to luciferin, which in turn produces light upon oxidation by luciferase [7]. In many instances, the main value of this class of compounds is not that they are highly specific for a single human CYP enzyme and would thus allow to monitor its activity in a mixture of enzymes such as those present in human liver microsomes (HLMs). Rather, they are well suited to compile activity profiles for a range of enzymes for two purposes: A better understanding of their substrate preferences and the elucidation of activity correlations between them. Such correlations can help to understand the distinct enzyme properties of the individual members within a CYP family or subfamily; but of equal interest are correlations between CYPs belonging to different families, as these might indicate new and previously unsuspected perspectives for future investigations. For example, identifying strong activity correlations between an orphan and a non-orphan CYP would be expected to facilitate the deorphanization process of the former.

We have recently introduced the use of permeabilized fission yeast cells (enzyme bags) that recombinantly express full-length CYPs together with their respective redox partners for the convenient monitoring of the enzymatic activities of all human P450s [8, 9]. Such enzyme bags are permeabilized cells that can easily be prepared from many microbes and function as enzymes *in situ* [10]. Many of the proluciferin substrates that are currently commercially available contain a free acid group while a few others are hydroxyethyl esters; in addition, Luciferin 6' methyl ether methyl ester (Luciferin-MultiCYP) is a general CYP substrate that shows activity with a range of human P450 enzymes [11]. We have recently described the synthesis of several new proluciferin methyl esters and analyzed their conversion by HLMs and by CYP26A1 [12]. It was the aim of the present study to expand these data by screening other human CYPs for activity towards such probe substrates.

**2. Materials and methods**

*2.1 Chemicals and reagents*

Ammonium chloride, Na2HPO4, glucose, KH2PO4, and potassium hydrogen phthalate were from Chemart Chemical (Tianjin, China). Agar, Biotin, CaCl2 • H2O, Citric acid, CuSO4 • 5 H2O, FeCl3 • 6 H2O, H3BO3, inositol, KCl, KI, MnSO4, MgCl2 • 6 H2O, MoO4 • 2 H2O, Na2SO4, nicotinic acid, sodium pantothenate, HPLC grade methanol, thiamine, and ZnSO4 • 7 H2O were from Kermel Chemical (Tianjin, China). The NADPH regeneration system was from Promega (Madison, USA); Triton-X100 was from Leagene (Beijing, China); Tris-HCl was from AKZ-Biotech (Tianjin, China); glycerol was from Dingguo (Tianjin, China); acetonitrile and ethyl acetate of analytical grade were from Yuanli Chemical (Tianjin, China); DMSO for molecular biology was from Sigma (St. Louis, MO), white 96-well microtiter plates were from Nunc (Thermofisher scientific, Lagenselbold, Germany). All other chemicals and reagents used were of the highest grade available.

*2.2 Fission yeast strains, media and general techniques*

All strains used in this study have been described previously [8]. In these strains, expression of human CPR and all human CYPs is regulated by the strong thiamine-repressible *nmt1* promoter of fission yeast [13]. Preparation of media and basic manipulation methods of *S. pombe* were carried out as described [14]. Briefly, strains were generally cultivated at 30°C in Edinburgh Minimal Medium (EMM) with supplements of 0.1 g/L final concentration as required. Liquid cultures were kept shaking at 150 rpm. Thiamine was used at a concentration of 5 μM throughout.

*2.3 Preparation of enzyme bags and enzyme bag cocktails*

Fission yeast strains were cultured on EMM plates with 5 µM thiamine at 30°C for 3 days and then precultured in 10 mL EMM broth at 230 rpm and 30°C for 24 h. Precultures were then used to inoculate 200 mL EMM broth in 500 mL Erlenmeyer flasks, which were then incubated under the same conditions for 24 h. For each assay 5 × 107 cells were transferred to 1.5 mL Eppendorf tubes, pelleted and incubated in 1 mL of 0.3 % Triton-X100 in Tris-KCl buffer (200 mM KCl, 100 mM Tris-HCl pH 7.8) at room temperature for 60 min at 230 rpm for permeabilization. The different sets of cocktails were prepared by mixing cells of different strains prior to permeabilization with a final cell density of 5 × 107 cells/cocktail/reaction, so that regardless of the number of CYPs included, each cocktail contained the same number of cells. After three washing steps with cold 50 mM NH4HCO3 buffer, enzyme bags were gently resuspended in 100 µL PBS with 50 % glycerol, flash frozen in liquid nitrogen, and stored at -80°C until use.

*2.5. Biotransformation of proluciferins and bioluminescence detection*

Enzyme bag preparations were thawed on ice, once washed with 100 µL 100 mM potassium phosphate buffer, pH 7.4, and then used for biotransformations as described previously [15]. Briefly, a concentrated CYP reaction mixture (containing fourfold concentrated substrate and potassium phosphate buffer) was added to the cell pellets in 1.5 mL Eppendorf tubes after permeabilization and washing. Substrates were used at a final concentration of 150 µM. CYP reactions were started by adding the twofold concentrated NADPH regeneration system. Samples were incubated for 3 h at 37°C and 1,000 rpm. After centrifugation at 16,000 *g* for 1 min the supernatants were transferred to white microtiter plates and an equal amount of reconstituted luciferin detection reagent was added to each well. Plates were then incubated at room temperature for 20 min and luminescence recorded on a Magellan infinite 200 Pro microplate reader (Tecan; Männedorf, Switzerland). In all cases, reaction parameters (reaction times and enzyme concentrations) were within the linear range. Enzyme bag cocktails were always tested three times in triplicates, with one or more positive outcomes being considered as a positive result and three negative ones counting as negative.

*2.6. Statistical analysis*

All data were calculated from experiments done at least twice in triplicates and are presented as mean ± SD. Statistical significance was determined using a two-tailed t-test. Differences were considered significant if *P* < 0.05. Statistical analysis was done using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA; USA).

**3. Results and discussion**

3.1 Testing strategy and enzyme bag cocktail results

Recently, we have described a new methodological approach for the concomitant monitoring of the activity of several CYPs within the same experiment [16]. According to this protocol, equal numbers of cells from different CYP-expressing strains are mixed before cell permeabilization, thus providing approximately similar amounts of individual CYPs within the resulting cocktails. We have also described a corresponding testing scheme, with the Master cocktail (M) that comprises all 57 human CYPs strains being on top, four cocktails (A to D) that include either 14 or 15 individual CYPs constituting the second level, and twelve cocktails (1 to 12) with either four or five individual CYPs on the third level. On the lowest level of this testing tree there are the enzyme bags made from the individual strains that express one human CYP each in the order of the CYP nomenclature. The screening of enzyme bag cocktails was mainly intended to yield qualitative information (yes/no answers), while individual enzyme testing was used to obtain quantitative activity data. The chemical structures of the proluciferin ester probe substrates used in this study are shown in Table 1. Activity monitoring of the cocktails on the highest two levels of the testing scheme (cocktails M, A, B, C, and D) always gave positive findings with at least some substrates (Fig. 1). The cocktails on the third level yielded six positive results (cocktails 1, 3, 5, 7, 8, and 11). This approach therefore significantly reduced the amount of further testing, since only the 28 enzymes contained in these six cocktails were further studied, while the remaining 29 CYPs were left out. In view of the testing being done thrice in triplicates, this means that the total number of individual CYP activity assays was thus reduced from 513 to 252.

3.2 CYP profiling of luciferin derivatives containing monofluorobenzyl ethers

The 28 individual enzymes contained in the six cocktails identified above were individually tested for activity towards the three proluciferin ester substrates containing monofluorobenzyl ethers. 14 enzymes were found to catalyze the biotransformation of Luciferin-2FBEME (Fig. 2). Highest activities were observed for CYP26A1, CYP3A4, CYP4Z1 and CYP2E1, respectively (in this order). In the case of Luciferin-3FBEME, seven CYP enzymes were able to catalyze its biotransformation, with highest activities being were observed for CYP26A1, CYP2E1, CYP4Z1, CYP4F8, and CYP3A4, respectively (Fig. 3). Finally, 14 enzymes catalyzed the biotransformation of Luciferin-4FBEME, with CYP26A1, CYP2E1, and CYP4Z1, respectively, being most efficient (Fig. 4). A graphical overview of these results is shown in Fig. 5. It shows that a total of 17 CYPs were found to metabolize at least one of the luciferin fluorobenzyl ether methyl esters, and among these, nine were acting on all three. Only two enzymes (CYP1A2 and CYP2A6) could hydroxylate two substrates, while the remaining six were active for one. Not surprisingly, in almost all cases the strongest activities were seen with the enzymes that could metabolize all three substrates; the only exception is CYP4F12, which only displayed activity towards Luciferin-4FBEME.

3.3 CYP profiling of luciferin derivatives containing five-membered heterocycles

17 enzymes were able to catalyze the biotransformation of Luciferin-3FEME (Fig. 6), and highest activities were observed for CYP4F8, CYP4V2 and CYP4F11, respectively. Interestingly, this is the first proluciferin substrate that shows much higher activity with CYP4F8 than with CYP4Z1; for all previously tested compounds, CYP4Z1 always had been the more efficient enzyme [17]. For the biotransformation of Luciferin-3TEME, again 17 enzymes were found to be positive, although these were not exactly the same ones as for the former substrate (Fig. 7). The best activities were observed for CYP4Z1, CYP2E1, and CYP4F8, respectively. Thus, 14 of the enzymes tested could metabolize both luciferin heterocycle ether methyl esters, and all of these could also act on at least on of the other three substrates (Fig. 5). Overall, the differences in actvities seen in the CYP-dependend biotransformations of the two luciferin heterocycle ethers substrates were not as large as in the case of the luciferin fluorobenzyl ether methyl esters (no enzyme gave a result that was more than tenfold higher than any other enzyme).

3.4 Correlation analysis

Out of the 28 CYPs tested in this study, nine could metabolize all five substrates, while six did not act on any of them. In order to group enzymes that show pairwise similar behavior towards these proluciferin ester substrates, a correlation matrix was calculated (Fig. S11). The correlations quantify the similar or dissimilar nature of the behavior of two given enzymes with respect to all five substrates, with a value close to 1 corresponding to similar behavior, -1 to opposite behavior and 0 to uncorrelated behavior. Fig. S12 shows a histogram of the correlation data extracted from the correlation matrix. The distribution of occurrences at the high end of the spectrum (red) suggests a possible grouping criterion for enzymes with similar activity of having pairwise correlations of at least 0.85. Retaining only the enzymes that satisfy this criterion, there remain 21 overlapping pairs, each with a correlation of at least 0.85. Grouping different variables together based on only pairwise correlations is a subtle task, since correlations are not à priori a transitive property. It has been shown, however, that in the limit of strong positive correlations the transitive property holds [18], which allows us to group correlating enzymes together in the strong correlation limit defined by our criterion. Applying transitivity, one obtains four mutually disjunct sets of correlating enzymes (one of which is trivial, as it contains only a single correlation relation between the two enzymes CYP2A7 and CYP1B1). The remaining three sets include a total of 16 enzymes (see below). From this quantitative analysis, one would expect each member of a set to have a comparable activity profile towards the five ester substrates as any other member of the same set, with the sets as a whole showing different behavior from one another. In order to provide a visualization of this correlation analysis, we show radar charts that show the substrate preferences of all 22 enzymes based on the signal-to-background ratio (Fig. 8). In addition, the three sets of enzymes mentioned above are indicated by different colors.

The first set encompasses CYP2D6, CYP2C19, CYP4F11, and CYP4V2, with the latter two displaying much higher activity towards Luciferin-3FEME. Interestingly, these two CYP4 family enzymes do not group together with the other four family members that showed activity in these assays. Instead, CYP4F8, CYP4F12, and CYP4Z1 belong to the second set, while CYP4X1 did not have high similarity in its activity profile with any of these three sets. These data are in good agreement with previous observations on the CYP4 family which suggested that they may be divided into two subgroups depending on the presence or absence of a covalent link to the heme [19]. In this classification CYP4F8, CYP4F12, CYP4X1 and CYP4Z1 form the group of enzymes that lack a heme covalent link, while the other CYP4 enzymes all have one. The second set includes CYP3A4, CYP1A2, CYP3A5, CYP3A7, CYP7A1, and CYP26A1, with the latter being the most active enzyme overall. Here it is noteworthy that CYP26B1 and CYP26C1 are not in this set, even though they belong to the CYP26 family. In addition, three of the four members of the human CYP3A family group together in this set; the exception being CYP3A43. In the third set we find CYP3A43, CYP2A6, CYP4F8, CYP2E1, CYP4F12, and CYP4Z1; among these, CYP2E1 displayed the highest activity towards the proluciferin ester substrates. Despite its high sequence homology with CYP2A6, CYP2A7 was not found to be in this group of enzymes. However, CYP3A43 is a member of this set, and it is thus tempting to speculate that in order to identify additional substrates for this understudied CYP, one might want not only to consider activities of its CYP3A subfamily group members, but also try compounds that are well metabolized by CYP2A6 or CYP2E1, for instance.

**4. Conclusions**

After an initial enzyme bag cocktail screen, 28 human CYPs enzymes were tested for activity towards a set of five proluciferin ester substrates which had previously only be known to be metabolized by CYP26A1. In addition to this enzyme, 21 other CYPs could be identified which can metabolize at least one of these substrates and in total, 64 new pairs of enzyme and substrate were found. A correlation analysis indicated the presence of three distinct sets of enzymes with high similarity in their activity profiles that encompassed a total of 16 individual enzymes. Some of these newly identified correlations may serve as a starting point for further study of those human CYPs whose activities are not yet satisfactorily understood.

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None.

**Conflicts of interest**

Authors declare that there are no conflicts of interest.

**Figure legends**

**Fig. 1.** Scheme of the cocktails testing tree showing an overview of the results obtained in the activity assays of enzyme bag cocktails towards five proluciferin ester substrates. Cocktails testing positive are shown in shades of green as follows: Dark green (testing positive with all five substrates), green (4 positives), fluorescent green (3 positives), and light green (2 positives). Cocktails testing negative with all substrates are shown in red and the individual enzymes that are contained in the third level cocktails are shown in black.

**Fig. 2.** Enzymatic activity of enzyme bags containing single CYPs contained in cocktails 1 (grey), 3 (black), 5 (blue), 7 (pink), 8 (red), and 11 (orange), respectively, towards the substrate Luciferin 6' 2-fluorobenzyl ether methyl ester (**Luciferin-2FBEME**). CPR: Control with enzyme bags containing only the microsomal electron transfer protein. Adx-AdR: Control with enzyme bags containing only the mitochondrial electron transport proteins. \*\*\**P* < 0.005; \*\*\*\**P* < 0.001.

**Fig. 3.** Enzymatic activity of enzyme bags containing single CYPs towards the substrate Luciferin 6' 3-fluorobenzyl ether methyl ester (**Luciferin-3FBEME**). CPR: Control with enzyme bags containing only the microsomal electron transfer protein. Adx-AdR: Control with enzyme bags containing only the mitochondrial electron transport proteins. \*\**P* < 0.01; \*\*\*\**P* < 0.001.

**Fig. 4.** Enzymatic activity of enzyme bags containing single CYPs towards the substrate Luciferin 6' 4-fluorobenzyl ether methyl ester (**Luciferin-4FBEME**). CPR: Control with enzyme bags containing only the microsomal electron transfer protein. Adx-AdR: Control with enzyme bags containing only the mitochondrial electron transport proteins. \*\*\**P* < 0.005; \*\*\*\**P* < 0.001.

**Fig. 5.** Distribution of CYP enzyme activity across the five pro-luciferin ester substrates in enzyme bag activity assay following cocktail testing strategy. Category of CYP activity is based on signal to background ratio (S/B) calculated from average relative light units (RLU) of three independent experiments and their respective redox partner (human CPR (CAD62) for microsomal P450s and AdR:Adx (RAJ4) for mitochondrial CYPs, respectively).

**Fig. 6.** Enzymatic activity of enzyme bags containing single CYPs towards the substrate Luciferin 6' 3-furfuryl ether methyl ester (**Luciferin-3FEME**). CPR: Control with enzyme bags containing only the microsomal electron transfer protein. Adx-AdR: Control with enzyme bags containing only the mitochondrial electron transport proteins. \**P* < 0.05; \*\**P* < 0.01. \*\*\**P* < 0.005; \*\*\*\**P* < 0.001.

**Fig. 7.** Enzymatic activity of enzyme bags containing single CYPs towards the substrate Luciferin 6' 3-thenyl ether methyl ester (**Luciferin-3TEME**). CPR: Control with enzyme bags containing only the microsomal electron transfer protein. Adx-AdR: Control with enzyme bags containing only the mitochondrial electron transport proteins. \*\*\**P* < 0.005; \*\*\*\**P* < 0.001.

**Fig. 8.** Radar charts showing substrate preferences based on the ratio of signal to background (S/B). The three fluorinated benzene isomers follow a clockwise arrangement from the top of plot, followed by the two heterocycle side groups. The three sets of enzyme groups with high similarity in their activity profiles are indicated by colors (set 1: green; set 2: yellow; set 3:blue). Enzymes shown in grey do not have high similarity in their activity profiles with any of these three sets.

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