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Short communication

Screening of the whole human cytochrome P450 complement (CYPome) with enzyme bag cocktails

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ABSTRACT

We have previously introduced the use of permeabilized fission yeast cells (enzyme bags) that recombinantly express full-length CYPs for drug metabolism studies. Such enzyme bags are cells with pores that function as enzymes *in situ*. They can easily be prepared without a need for ultracentrifugation and may be used in similar protocols as microsomes. In this study we report the preparation of enzyme bag cocktails that permit the testing of multiple CYPs in a single enzyme bag reaction. Moreover, we established a convenient testing scheme that permits a rapid screen of all human CYPs for activity towards any given candidate substrate. An important aspect of this approach is the reduction of individual CYP test assays. If a cocktail containing many CYPs tests negative, it follows that all CYPs included in that cocktail need not be tested individually, thus saving time and resources. The new protocol was validated using two probe substrates.

1. Introduction

The majority of drugs used in human patients are substrates of drug metabolizing enzymes [1], which are classified into the two groups of Phase I and Phase II enzymes depending on the type of reaction they catalyze: Phase I is characterized by functionalization reactions (such as redox reactions), while in Phase II conjugation reactions occur (such as glucuronidation). The most important enzymes in Phase I metabolism are the cytochrome P450 enzymes (CYPs or P450s), which belong to a large superfamily of monooxygenases present in all biological kingdoms [2]. The 57 human CYPs are all membrane bound proteins that are primarily found either on the cytoplasmic side of the endoplasmic reticulum or on the matrix side of the inner mitochondrial membrane. CYPs need to be reduced in order to catalyze redox reactions and therefore depend on electron transfer proteins. In mitochondria, there is a short electron transfer chain encompassing adrenodoxin (Adx) and adrenodoxin reductase (AdR), while in the endoplasmic reticulum, there is a single electron transfer partner, cytochrome P450 reductase (CPR or POR) [3]. Human CYPs metabolize a large variety of compounds belonging to many different chemical classes and typically catalyze aliphatic or aromatic hydroxylations; however, in principle they can also perform many other reaction types [4,5]. In analogy to the genome or the proteome, the total complement of human CYPs can be referred to as the human CYPome [6].

In the field of drug metabolism, many so-called cocktail approaches were described in the past. These include protocols that use cocktails of drug or candidate drug molecules, those that employ mixtures of recombinantly expressed and purified human CYPs, and even double cocktails where a compound mixture is given to an enzyme mixture [7]. Such techniques are used for the study of the metabolism of candidate drug compounds, for CYP inhibition assays, and for investigations of drug-drug interactions [8].

While such approaches have efficiency advantages of parallel incubation and parallel LC/MS/MS analysis of multiple probes, they suffer from the significant mutagenesis (such as removal of parts that serve as membrane anchors) needed to allow for recombinant high-level expression in bacteria; thus, the resulting proteins can hardly be considered as 'wild-type'. A solution to these issues is the recombinant expression of unmodified full-length enzymes in eukaryotic hosts such as yeast, insect, or mammalian cells, where the membrane localization of the enzymes is retained. Such recombinant eukaryotic cells have typically been used for drug metabolism studies either in whole-cell biotransformations or for the preparation of microsomes.

Whole-cell biotransformations are easy to perform and

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convenient because the cofactor NADPH is directly produced inside the living cells. But substrates and products need to pass several biological barriers (such as plasma membrane and cell wall), which is a severe problem for compounds that display very low membrane permeability. An alternative is the use of microsomes prepared from recombinant eukaryotic cells, but this method has its own issues (tedious long-term ultracentrifugation and lack of scalability). Thus, there is no perfect P450 assay as all methods have their respective advantages and disadvantages. We have recently introduced the use of permeabilized fission yeast cells (enzyme bags) that recombinantly express full-length CYPs [9] or UDP-glucuronosyltransferases (UGTs) [10] for drug metabolism studies. Such enzyme bags are cells with pores that function as enzymes *in situ*. They can easily be prepared without a need for ultracentrifugation and may be used in similar protocols as microsomes.

It was the aim of this study to create a new procedure that allows for the testing of multiple CYPs in a single enzyme bag reaction. Moreover, we wanted to establish a convenient testing scheme that permits a rapid screen of all human CYPs for activity towards any given candidate substrate.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium chloride, Na_2HPO_4, glucose, KH_2PO_4, and potassium hydrogen phthalate were from Chemart Chemical (Tianjin, China). Agar, biotin, $CaCl_2 \cdot H_2O$, citric acid, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, H_3BO_3, inositol, KCl, KI, $MnSO_4$, $MgCl_2 \cdot 6H_2O$, $MoO_4 \cdot 2H_2O$, Na_2SO_4, nicotinic acid, sodium pantothenate, thiamine, and $ZnSO_4 \cdot 7H_2O$ were from Kermel Chemical (Tianjin, China). Luciferin-BE, Luciferin-H, Luciferin-ME, and the NADPH regeneration system were 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); 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 [11]. In these strains, expression of human CPR and all human CYPs is regulated by the strong thiamine-repressible *nmt1* promoter of fission yeast [12]. Preparation of media and basic manipulation methods of *S. pombe* were carried out as described [13]. 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 230 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 5×10^7 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 5×10^7 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 NH_4HCO_3 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.

2.4. 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 [9]. 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 Luciferin-H and Luciferin-ME were both used at a final concentration of 100 μM while preliminary experiments with Luciferin-BE were done at a final substrate concentration of 50 μM. CYP reactions were started by adding the twofold concentrated NADPH regeneration system. Samples were incubated for 3 h at 37 °C and 1000 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 was recorded on a Magellan infinite 200Pro microplate reader (Tecan; Männedorf, Switzerland). In all cases, reaction parameters (reaction times and enzyme concentrations) were within the linear range.

2.5. 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. Preparation of enzyme bag cocktails and testing strategy

In order to allow for the simultaneous testing of several CYPs in the same enzyme bag assay, we developed a new methodological approach. In this procedure, fission yeast strains are cultivated as previously described but after determination of cell densities and before cell permeabilization, equal numbers of cells from different strains were mixed. In this way, roughly comparable amounts of CYPs should be contained in every cocktail. For validation of this approach, preliminary experiments were performed using the test substrate Luciferin-BE and enzyme bag cocktails that only included up to four different CYPs. In addition, we also compared the activity of freshly prepared enzyme bag cocktails with those that underwent one freeze-thaw cycle and were retested after one day, five days, or one year, respectively. These experiments demonstrated that the combination of several CYPs into a single enzyme bag cocktail is possible, and they also showed a reasonable stability of results even after one year of storage (Fig. S1).

Next, we designed a testing tree that encompasses a hierarchy of cocktails (Fig. S2). On top of the tree is the Master cocktail (M) that contains enzyme bags made from all 57 individual strains. On the second level, there are four cocktails (A to D) that contain enzyme bags made from 14 or 15 individual strains as indicated. In selecting the individual enzymes contained in each cocktail we followed the official CYP nomenclature, which means that cocktail A contains CYP1A1 to CYP2F1, cocktail B contains CYP2J2 to CYP4F3, and so on. On the third level of the hierarchy, there are twelve cocktails (1–12)

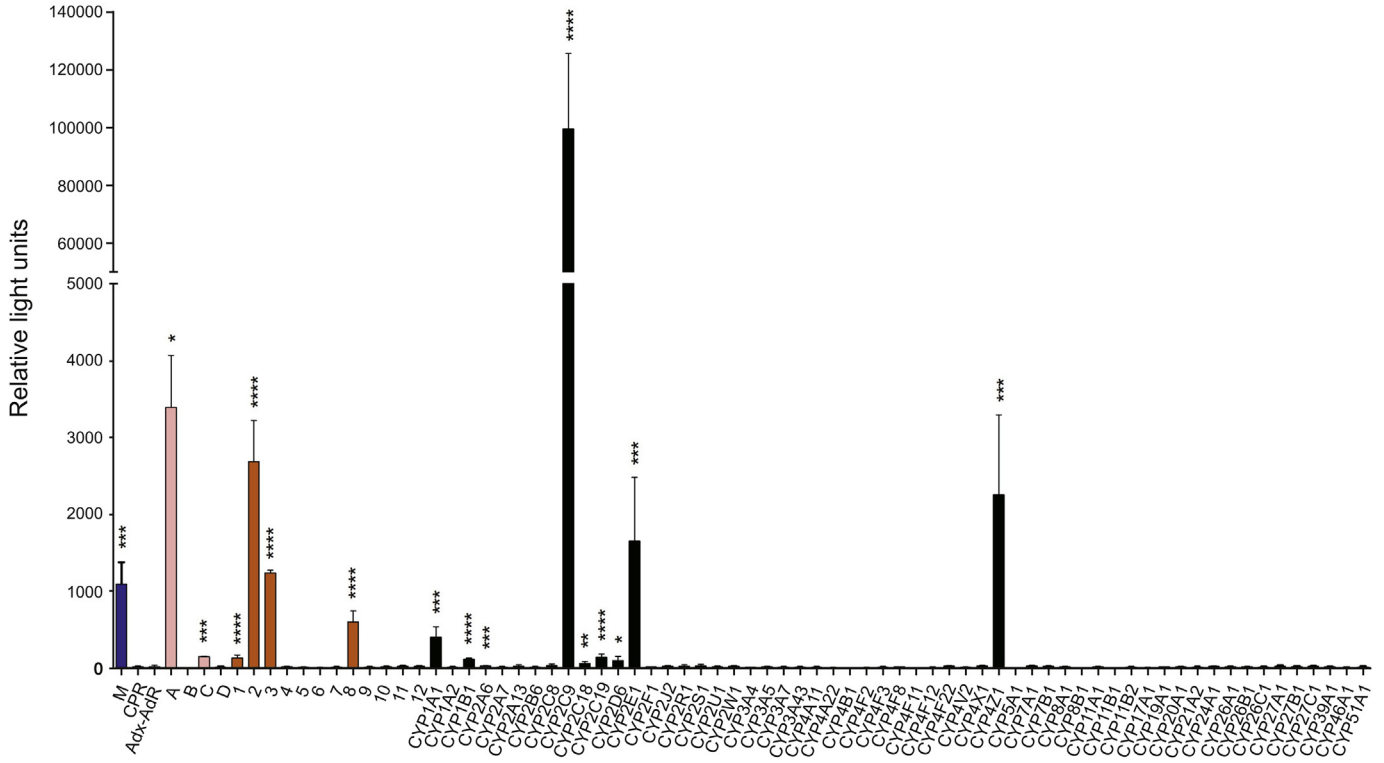

Fig. 1. Enzymatic activity of enzyme bag cocktails and enzyme bags containing single CYPs towards the substrate Luciferin-H. Cocktail M (containing all 57 CYPs) is shown in dark blue, cocktails A-D (containing 14 or 15 CYPs) are shown in pink, cocktails 1–12 (containing 4 or 5 CYPs) are shown in orange, and individual CYPs are shown in black. CPR: Control with enzyme bags containing only CPR. Adx-AdR: Control with enzyme bags containing only Adx and AdR. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$.

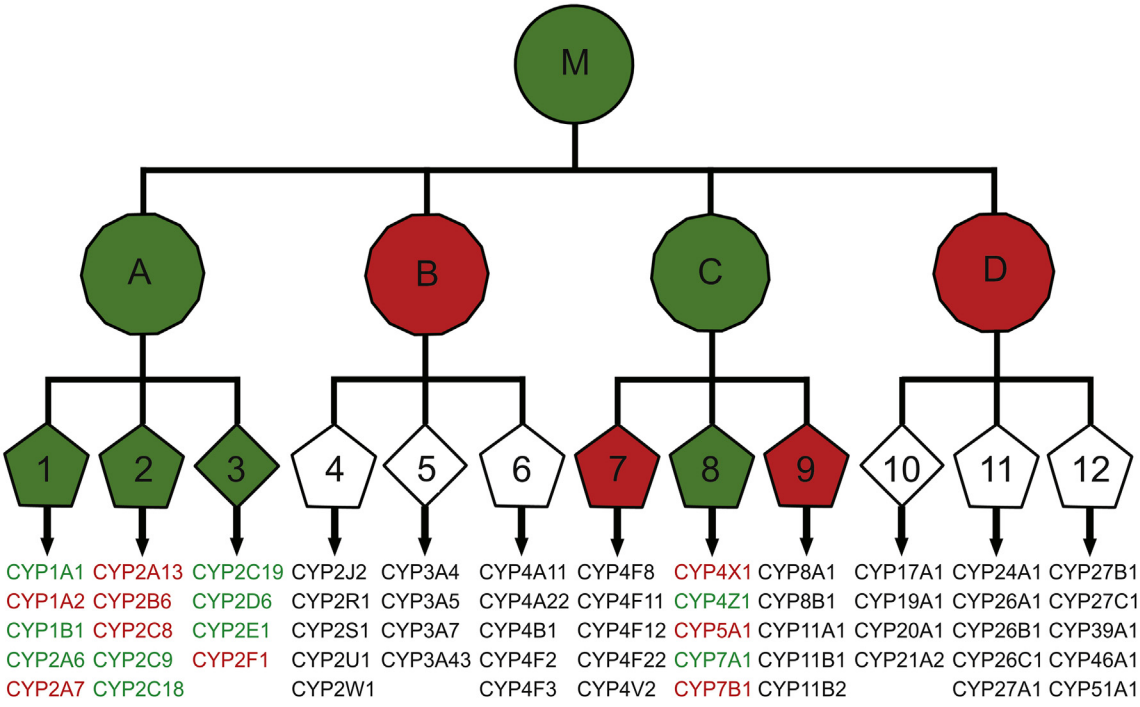

Fig. 2. Scheme of the cocktails testing tree showing an overview of the results obtained in the activity assays of enzyme bag cocktails and enzyme bags containing single CYPs towards the substrate Luciferin-H. Green: Cocktails or individual enzymes testing positive. Red: Cocktails or individual enzymes testing negative. Black: Cocktails or individual enzymes not tested.

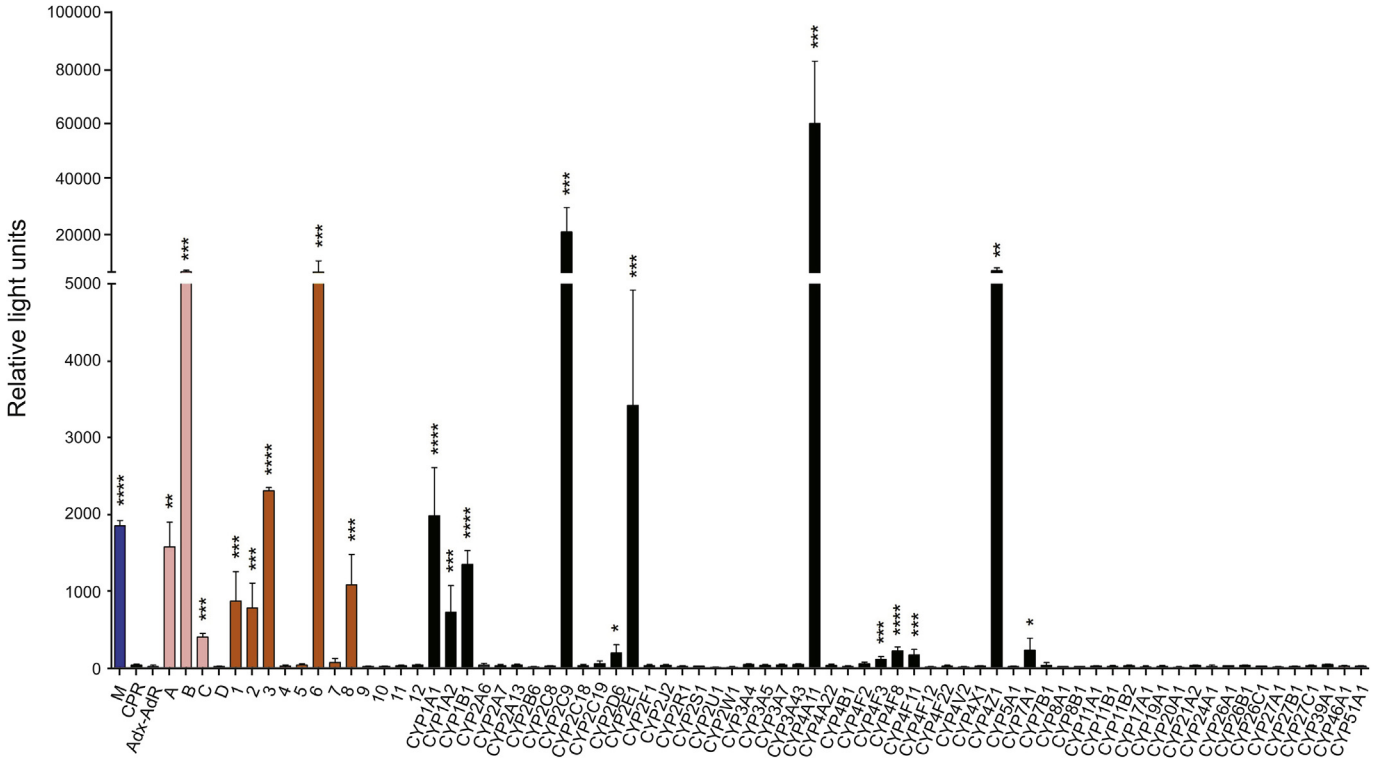

Fig. 3. Enzymatic activity of enzyme bag cocktails and enzyme bags containing single CYPs towards the substrate Luciferin-ME. Cocktail M (containing all 57 CYPs) is shown in dark blue, cocktails A-D (containing 14 or 15 CYPs) are shown in pink, cocktails 1–12 (containing 4 or 5 CYPs) are shown in orange, and individual CYPs are shown in black. CPR: Control with enzyme bags containing only CPR. Adx-AdR: Control with enzyme bags containing only Adx and AdR. *$P < 0.05$, **$P < 0.01$. ***$P < 0.005$, ****$P < 0.001$.

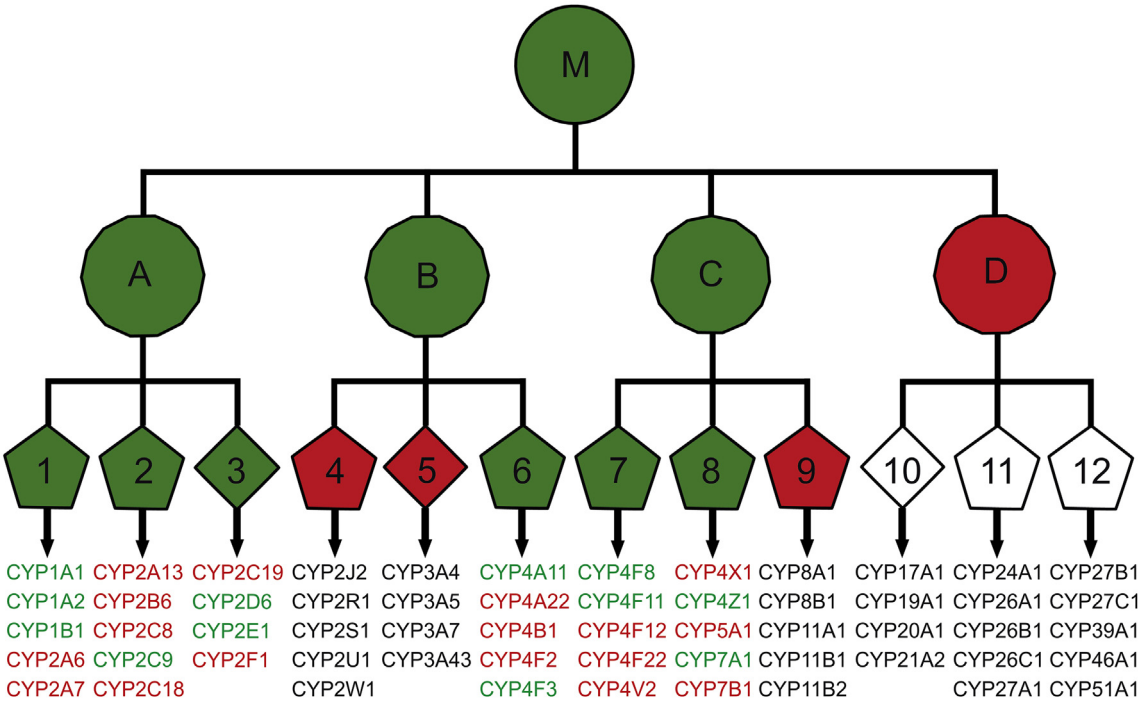

Fig. 4. Scheme of the cocktails testing tree showing an overview of the results obtained in the activity assays of enzyme bag cocktails and enzyme bags containing single CYPs towards the substrate Luciferin-ME. Green: Cocktails or individual enzymes testing positive. Red: Cocktails or individual enzymes testing negative. Black: Cocktails or individual enzymes not tested.

that contain enzyme bags made from four or five individual strains, again in the order of the nomenclature. The procedure we established makes an important distinction between qualitative and quantitative analysis: Experiments with enzyme bag cocktails are essentially only used for obtaining yes/no answers, whereas experiments with enzyme bags made from individual strains (i.e. containing only one human CYP) are intended to yield quantitative activity data (as in our previous publications). In order to account for small activities that might be observed in cocktails where only one CYP present can catalyze the reaction and/or that activity is low, we decided to consider any cocktail that gave a positive result once to be positive, which means that the corresponding cocktails on the next level of the hierarchy would be tested. By contrast, we only considered a cocktail to be negative for a certain reaction if a negative result was obtained in three individual assays. This would then result in the corresponding cocktails on the next level of the hierarchy not to be tested. The advantage of such an approach becomes apparent if we consider the situation where a candidate compound is only metabolized by a single CYP, for instance CYP2C9. Without the enzyme bag cocktail approach, all 57 human CYPs would have to be tested to verify this fact. By contrast, using our testing tree only eight cocktails and five individual enzymes need to be tested: Cocktail M would be positive, as would be cocktail A, but not B, C or D. Next, cocktails 1 to 3 (those corresponding to A) would be tested, with cocktail 2 testing positive and both 1 and 3 negative; finally, the five enzymes that make up cocktail 2 would be tested individually, revealing CYP2C9 to be positive and the other four negative. Thus, with only 13 assays the single CYP responsible for the observed activity could be identified. Having obtained that information, detailed enzymatic studies using the one CYP in question can then be performed with the aim of yielding quantifiable data.

3.2. CYP profiling of the luminogenic probe substrate Luciferin-H

In our previous study we have reported the functional expression of all human CYPs in fission yeast and reported their activity towards the two probe substrates Luciferin-H and Luciferin-ME [11]. Such proluciferins can be converted by CYPs to luciferin, which in turn produces light upon oxidation by luciferase [14]. Therefore, these two substrates are perfect candidates for the validation of the enzyme bag cocktail approach as activity data for all cocktails can be predicted. Firstly, we tested Luciferin-H, which is converted to luciferin by a CYP-dependent aryl hydroxylation reaction. In our previous study we found that CYP2C9, CYP2E1, and CYP4Z1 (in this order) displayed by far the highest activities towards this substrate [11]. When subjecting this substrate to the testing tree procedure, we observed significant activity with the Master cocktail M as could be expected (Fig. 1). On the second level of the hierarchy, cocktails A and C gave positive results while B and D did not. Cocktail A showed much higher activity than C as the former contains both CYP2C9 and CYP2E1 while the latter only contains CYP4Z1. The third level of the testing scheme gave positive results for cocktails 1 to 3 and 8, again as expected. The 19 enzymes contained in these four cocktails were then tested individually, with eleven giving positive results and the remaining eight being negative (Fig. 2). These results are in good agreement with our previous data. All enzymes that showed strong activity in the earlier study also did so in these experiments. There are some borderline cases where activities are quite low in comparison to controls and where statistical significance therefore may vary.

3.3. CYP profiling of the luminogenic probe substrate Luciferin-ME

The second part of the enzyme bag cocktail validation consisted of activity measurements for Luciferin-ME, which is converted to luciferin by a CYP-dependent aliphatic hydroxylation reaction followed by dissociation of the resulting hemiacetal [14]. For this substrate, CYP4A11, CYP2E1, CYP4Z1, and CYP2C9 (in this order) had previously shown the highest activities [11]. Again, cocktail M showed good activity as expected, and cocktails A to C (but not D) were also positive (Fig. 3). Of the former, cocktail B displayed the highest activity as it contains CYP4A11, with A (containing CYP2E1 and CYP2C9) and C (CYP4Z1) showing lower but still significant activity. On the third level of the testing scheme, positive results were obtained for cocktails 1 to 3 and 6 to 8, again as expected. Individual testing of the 29 enzymes contained in these six cocktails led to 12 positive and 17 negative results (Fig. 4). Again, results are in good agreement with previous data.

4. Conclusions

Experiments using HLMs (or microsomes from other tissues) are necessarily biased towards those CYPs that display high activity in the tissue of origin. Moreover, there is no human cell type that expresses all CYPs, so some enzymes will always be missed. With the availability of all human CYPs being recombinantly produced in the same host microbe we now have demonstrated the preparation of much more unbiased cocktails as equal amounts of the cells that produce the different CYPs were combined. Moreover, additional special cocktails can also be made, such as cocktails containing all CYPs known to be expressed in any given tissue, or cocktails containing all CYPs involved in the metabolism of certain chemical classes of compounds (e.g. steroids). Therefore, in this study we present a platform technology that has a huge variety of applications in the future.

The attribution of observed CYPs activities displayed by human microsomes is often done by blocking this activity using ‘specific’ inhibitors of the CYP enzyme in question. However, so far there is no published data where any such inhibitors were tested against all human CYPs; thus, there is always a risk that these compounds inadvertently coinhibit other CYPs for whom the inhibitory effect is not yet known. The enzyme bag cocktail approach presented in this study avoids this problem, as the different CYPs contained in any of the cocktails can always be individually tested. Thus, validation of the cocktail results at the level of the individual enzymes can always be performed.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.05.003.

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