Recombinant Yeast and Bacteria that Express Human P450s: Bioreactors for Drug Discovery, Development, and Biotechnology

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Summary

A wide variety of important pharmacological and toxicological properties of xenobiotics are determined by cytochrome P450-mediated metabolism. Prediction of these pathways in humans and of the P450 isoenzymes involved constitutes an essential part of drug development. Recently a variety of recombinant expression systems have been employed to predict the P450-mediated metabolism of drug candidates in humans. These include insect, bacterial, yeast, and mammalian models which all have been successfully employed recently on a small scale in drug development. However, this article focuses mainly on the comparison of bacterial and yeast systems for semi-preparative biooxidations. We demonstrated that the catalytic properties of several P450 isoforms when expressed in either Saccharomyces cerevisiae or Escherichia coli were rather similar and faithfully mimicked metabolic pathways of xeno- and endobiotics in human liver. However, taking technical considerations into account, the high level of functional expression of P450s achieved in bacteria make this system, which is patented, ideally suited for large-scale pharmaceutical biooxidations. These include the semi-preparative generation of metabolites for structural determinations and, importantly, also for drug safety studies.

This suitability is evidenced by the industrial applications presented in this review. These were conducted by two pharmaceutical companies, involving structurally diverse compounds, and utilized different bioreactor techniques. The strategies employed resulted in highly efficient, semi-preparative biooxidations of drugs and drug candidates, showing the great potential of these systems to improve and facilitate drug discovery and development.
10 Recombinant Yeast and Bacteria that Express Human P450s

10.1 Background

10.1.1 Importance of Recombinant P450s for Drug Development

The drug discovery and development process is scientifically complex, incorporating many research areas, and is inherently full of challenges and risk. It is therefore expensive and time consuming, taking typically up to 15 years and consuming up to 500 million euro per approved drug. The entire process can be divided into drug discovery and preclinical drug development, followed by clinical development and approval. Typically, new chemical entities (NCEs) are promoted from discovery into preclinical development and if they succeed to pass all hurdles, they are then submitted for investigational drug (IND) applications and eventually enter the various phases of clinical development. The attrition (failure) rate is very high in the preclinical stages, which are designed to predict the therapeutic properties (desired and adverse) in humans.

An important part of preclinical development is the prediction of drug metabolism and its implications for drug safety and pharmacokinetics in humans. The metabolism of a drug proceeds in two phases with the first phase being of greatest importance for the metabolism of pharmaceuticals. This step is mainly catalyzed by a large family of highly versatile cytochrome P450 monooxygenases (CYPs) which differ in their substrate-, regio-, and stereoselectivity. To ensure the maximum therapeutic value and safety of pharmaceuticals in humans, the metabolism of a NCE is first tested in animals or \textit{in vitro} systems that mainly rely on animal tissues \[1, 2\]. Besides being often considered ethically and economically problematic, animal-based systems have limitations partially due to large species differences in the P450s involved in drug metabolism \[3, 4\]. It is therefore essential to develop systems that are based on material derived from human tissues. These should significantly shorten the period between the discovery of drugs and their introduction into the market and simultaneously allow more accurate extrapolations to humans.

Models for human drug metabolism can roughly be classified into those that try to mimic the entire cascade of drug-metabolizing events, and those that imitate only a limited set of reactions. The former models (complex systems) include human hepatocyte and hepatoma cell cultures. The latter (simple systems) include heterologous \textit{in vivo} and \textit{in vitro} expression systems as well as enzymes purified from tissues. A disadvantage of the complex models is that drug metabolism is in most cases altered as compared to liver, either due to culture conditions, or in the case of hepatoma cells, due to phenotypic transformation \[5, 6\]. In addition, these models are only poorly suited for large-scale biooxidation reactions.

Simple models, employing microorganisms such as bacteria or yeast, are useful for characterizing specific steps in the metabolism of drugs and also the enzymes involved in them. These systems also yield recombinant enzymes for antibody production \[7\], which allows quantitation of drug-metabolizing enzymes in
human tissues, including antibody inhibition studies. Combined with the knowledge of the role of a particular enzyme in drug metabolism, as derived from the use of bacterial or yeast expression systems, this information can be used to predict the metabolic pathways of drugs in vivo. *E. coli* expression systems for P450s have been employed with great success to predict polymorphic drug oxidations [8–11]. Furthermore, these bacterial models provide a powerful means to predict drug/drug interactions which are frequently observed in vivo [12] and sometimes result in life-threatening adverse drug reactions. Bacterial expression systems for P450s are also highly suitable to express P450s mutated by site-directed mutagenesis to delineate amino acid residues in the catalytic center of these enzymes that are important for their catalytic activity [13, 14]. This information, combined with the recently available crystal structure of P450s, which was derived from bacterially expressed human P450s [15], is necessary to establish reliable *in silico* models of these proteins that will ultimately revolutionize *in silico* predictions of drug metabolism [16].

Last but not least, microbial expression systems for mammalian P450s are ideally suited to the generation of drug metabolites on a semi-preparative scale as evidenced in this review and from the literature [17, 18]. This strategy will greatly facilitate the structural elucidation of the metabolites and drug safety testing [19] employing the resulting metabolites.

10.1.2

**Fundamentals of Heterologous Expression in Bacteria**

*Escherichia coli* has been most frequently used for the bacterial expression of human drug-metabolizing enzymes. *E. coli* is an attractive system because high levels of expression as well as growth to very high cell densities can be achieved [20]. In addition, *E. coli* is easily manipulated and a wide variety of strain variants and vectors with powerful promoters are available. A limitation of the bacterial systems is that in almost all cases mammalian cDNAs have to be modified before they can be expressed [20]. Aside from trimming the 5′ and 3′ untranslated regions of the P450 cDNAs, the region around the initiation codon of protein biosynthesis should be modified to remove the rigid secondary structures that can occur in mammalian mRNAs. We have developed a strategy that circumvents changes in the coding region of the P450s by inframe fusions of a modified *ompA* leader sequence to the various human P450 cDNAs. This leader is removed during P450 synthesis, thus releasing the native P450. To obtain a functional monooxygenase complex in *E. coli*, it is also necessary to coexpress cytochrome P450 reductase, which is lacking in this organism and which supplies electrons to P450s. Several strategies have been developed to this end. Because our strategies and a detailed methodology for the bacterial expression of human P450s on a small scale has been given in various reviews and original articles [11, 17, 21–24] and is patented (US 6,566,108; WO98/02554), it will only be touched upon here. We will focus more on the utilization of these microorganisms for biooxidation on a larger scale.
10.1.3 Fundamentals of Heterologous Expression in Yeast

Bakers yeast, *Saccharomyces cerevisiae*, has been almost exclusively used for the heterologous expression of drug-metabolising enzymes in yeast. *S. cerevisiae* has proved suitable for the production of a wide variety of eukaryotic proteins in both basic research and industrial and pharmaceutical application [25]. This unicellular organism has some of the advantages of the bacterial expression systems but the additional advantage of being a eukaryotic cell with many similarities to mammalian cells in protein synthesis and processing and membrane compartmentalization.

Two types of yeast vector are available [26]: episomally replicating vectors and vectors with the potential for genomic integration. Most episomal vectors contain sequences from the 2µm endogenous yeast plasmid that confer high copy number and stable maintenance. Integrating plasmids lack a yeast origin of replication but contain regions of homology with the yeast genome flanking the cDNA cloning site. The stable integration of an ancillary protein, such as cytochrome P450 reductase, required for functional cytochrome P450 expression [27], increases the versatility of yeast for the subsequent expression of further proteins. *S. cerevisiae*-based expression biotechnology benefits from the availability of numerous selection markers for gene transfer, making it possible to cotransfect several cDNAs. Interestingly, expression of the steroidogenic CYP17A in the yeast *Yarrowia lipolytica* yielded an active P450 enzyme in the absence of coexpressed recombinant reductase [28]. This seems to be a feature of the CYP17A isoform, which also does not require exogenous reductase in *E. coli*, as another human P450 isoform (CYP1A1) expressed in *Yarrowia* required coexpression of P450 reductase to be catalytically active [29]. Similarly, the presence of mammalian P450 reductase was required for the catalytic activity of CYP2D6 when expressed in *Pichia pastoris* [30].

There are also several powerful regulatable *S. cerevisiae* promoters that can yield high levels of heterologous protein expression. However, some features of mammalian cDNAs are not optimal for the expression of proteins in *S. cerevisiae*. It has been shown that efficient expression of several proteins, including cytochrome P450s, requires the deletion of most of the 5′ untranslated region [27]. For example, a CYP1A1 cDNA under the control of the GAL10-CYC1 promoter containing either 15 bp or 5 bp of 5′ untranslated region, yielded 1µg and 6µg of functional CYP1A1 per mg of microsomal protein respectively [31]. Even though it does not appear to be necessary to change the coding region of mammalian cDNAs for their expression in *S. cerevisiae*, certain mammalian proteins are difficult to express unless the amino acid sequence is modified. An example is the rat cytochrome P450 reductase, which could not be stably expressed unaltered in *S. cerevisiae*. However, upon fusion of the N-terminal sequence of this protein with the N-terminus of the P450 reductase from *S. cerevisiae*, high levels of the rat P450 reductase were achieved [32]. Coexpression of mammalian P450 reduc-
tase and P450s in S. cerevisiae is required to achieve a catalytically highly active monooxygenase system, since the host P450 reductase couples poorly with mammalian P450s [33].

We have established a functional monooxygenase system in S. cerevisiae by stable integration of the P450 reductase linked to the 3′ phosphoglycerol kinase (PGK) promoter into the yeast genome. The resulting strain had a cytochrome c reductase activity of more than 200 nmol min⁻¹ mg⁻¹ microsomal protein which was 20-fold higher than the activity in the parental strain. The recombinant strain was subsequently transformed with an episomally replicating vector containing the various P450 cDNAs under the control of the powerful constitutive PGK promoter. In the absence of human reductase, the human P450s were catalytically inactive, however upon expression of this ancillary factor, CYP3A4 and CYP2D6 in S. cerevisiae displayed an activity towards prototypical substrates such as testosterone and bufuralol respectively. Interestingly the expression level of the various P450 isoforms varied widely, with the level of CYP2C9 being only 91 pmol mg⁻¹ microsomal protein whereas the level of CYP3A4 in microsomes was found to be 500 pmol mg⁻¹ (Table 10.1).

### 10.2 Comparison of P450 Levels and Enzymic Activities in Various Models

Table 10.1 lists the P450 isoforms that have been expressed by us in the different microorganisms, their expression levels, and their enzymatic characterization. P450 isoforms that have been expressed in at least two systems (bacteria and yeast) are listed in bold. The levels of recombinant P450s (pmol min⁻¹ mg⁻¹ protein) in the different cellular systems were surprisingly similar. However, we found that the level of some P450s in E. coli can be increased to about 1000 pmol mg⁻¹ protein when growing the cells in the controlled environment of a biofermenter. This level represents approximately 5% of the bacterial membrane protein, which translates into a yield of 1000 nmol P450 L⁻¹ of culture. Similarly it has been shown by others that the yield of P450s in S. cerevisiae can be improved to about 500 nmol L⁻¹ of culture when using vectors that are stably maintained in rich medium, which allows the cells to grow to very high cell densities [33]. Therefore E. coli and S. cerevisiae are ideally suited for the production of sufficient quantities of enzymes for structural studies. The testosterone 6β-hydroxylase and the bufuralol 1′-hydroxylase activity (pmol min⁻¹ mg⁻¹) found in membranes isolated from bacteria which expressed CYP3A4 or CYP2D6 respectively were 2- and 30-fold respectively higher than in human liver microsomes (see Table 10.1 footnote). Importantly, these activities in bacterial membranes were 120- and 6-fold higher than the activities found in yeast membranes (Table 10.1). This also strongly suggests that, apart from the ease of handling, the bacterial systems are far superior to the yeast-based systems for large-scale biooxidations.
For pharmacokinetic investigations it is essential that the catalytic properties of the recombinant P450 isozymes are similar across the various recombinant models. The data presented in Table 10.1 show that the $K_m$ values of the CYP3A4-mediated 6β-hydroxylation of testosterone and the CYP2D6-mediated 1′-hydroxylation of bufuralol were closely similar in the two models developed in this work. These values are also in good agreement with the $K_m$ reported for CYP2D6 expressed either in the baculovirus system or in human lymphoblastoid

<table>
<thead>
<tr>
<th>Expression system</th>
<th>Expressed P450 isoform</th>
<th>P450 expression level$^a$ (pmol mg$^{-1}$)</th>
<th>Yield (nmol L$^{-1}$)</th>
<th>Catalytic activity$^b$ (pmol min$^{-1}$ mg$^{-1}$)</th>
<th>Turnover number (min$^{-1}$)</th>
<th>$K_m$ (μmol L$^{-1}$)</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>1A1</td>
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<td>725</td>
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<td>287</td>
<td>180</td>
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<td></td>
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<td>150</td>
<td>550</td>
<td>2.9</td>
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<tr>
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<td>30</td>
<td>37</td>
<td>1.2</td>
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</tr>
<tr>
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<td>n.d.</td>
<td>n.a.</td>
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<td>498</td>
<td>2400</td>
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<td>n.a.</td>
<td>1.43</td>
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<td>370</td>
<td>1200</td>
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<td></td>
<td>2E1</td>
<td>70</td>
<td>190</td>
<td>1360</td>
<td>30</td>
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<tr>
<td></td>
<td>3A4</td>
<td>300</td>
<td>210</td>
<td>12 000</td>
<td>40</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>3A5</td>
<td>150</td>
<td>250</td>
<td>700</td>
<td>4</td>
<td>n.a.</td>
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<tr>
<td></td>
<td>4A11</td>
<td>80</td>
<td>70</td>
<td>1600</td>
<td>20</td>
<td>n.a.</td>
</tr>
<tr>
<td>S. cerevisiae</td>
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<td>9</td>
<td>0.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>2D6</td>
<td>100</td>
<td>2.6</td>
<td>209</td>
<td>8.6</td>
<td>11.1</td>
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<tr>
<td></td>
<td>3A4</td>
<td>500</td>
<td>4.4</td>
<td>104</td>
<td>1.5</td>
<td>99</td>
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</tbody>
</table>

Heterologous expression levels for P450s were determined in whole bacteria or yeast cells (yield) or in bacterial membranes or yeast microsomes (pmol mg$^{-1}$ protein) and represent typical values. Enzyme activities were determined in bacterial membranes or yeast microsomes. P450 isoforms that were expressed in E. coli as well as in yeast are in bold.

The various P450 isozymes were coexpressed with human P450 reductase. The cytochrome c reductase activities found in E. coli strains coexpressing P450s and P450 reductase (hOR) were typically 400–1200 nmol cytochrome c reduced min$^{-1}$ mg$^{-1}$ membrane protein with the exception of strains expressing CYP1A2 for which this value was 70–120. In S. cerevisiae coexpressing P450s and hOR the levels of reductase were 200–250 nmol cytochrome c reduced min$^{-1}$ mg$^{-1}$ membrane protein.

n.a., not available.

*a* The following enzyme activities have been determined for the different P450 isoenzymes (the typical specific activities reported in literature for human hepatic microsomes are given in brackets): CYP1A1 and CYP1A2, 7-ethoxyresorufin O-dealkylase (95 pmol min$^{-1}$ mg$^{-1}$); CYP2B6, diazepam-demethylase; CYP2C9, diclofenac 4′-hydroxylase; CYP2D6, bufuralol 1′-hydroxylase (70 pmol min$^{-1}$ mg$^{-1}$); CYP2E1, chlorozoxazone 6-hydroxylase (1500–4000 pmol min$^{-1}$ mg$^{-1}$); CYP3A4 and CYP3A5, testosterone 6β-hydroxylase (5000 pmol min$^{-1}$ mg$^{-1}$); CYP4A11, lauric acid omega-hydroxylation; P450 reductase, cytochrome c (230 nmol min$^{-1}$ mg$^{-1}$).

*b* The P450 content was determined spectrophotometrically and is expressed as pmol mg$^{-1}$ membrane protein.
cells which were $4.7 \mu\text{mol L}^{-1}$ and $18 \mu\text{mol L}^{-1}$ respectively [34, 35]. Similarly the $K_m$ of CYP3A4 expressed in the two models presented here agrees with the $K_m$ reported for CYP3A4 expressed in a baculovirus system, which was $56 \mu\text{mol L}^{-1}$ [36]. In many cases, however, the catalytic properties of recombinant P450s cannot be directly compared with those of P450s in human liver microsomes, since here several P450s can contribute to the metabolism of a given substrate. For example, it is known that the bufuralol 1′-hydroxylation is catalyzed by CYP2D6 but also with a lower affinity by CYP1A2 [35]. Similarly, the testosterone 6β-hydroxylation is catalyzed not only by CYP3A4 but also by CYP3A5 [36].

It can be said that except for CYP1A2, the substrate turnover numbers (min$^{-1}$) of the P450 isozymes expressed in E. coli differed by a factor of less than four from those found in liver (Table 10.1 footnote). The relatively low turnover number of bacterially expressed CYP1A2 for the O-dealkylation of 7-ethoxyresorufin is most likely due to the low level of P450 reductase in this particular strain of E. coli, which was at least 4-fold lower than in the other E. coli lines. Lower levels of reductase may have been also partially responsible for the low substrate turnover number of CYP3A4 expressed in S. cerevisiae. Another reason for the low enzyme activity of CYP3A4 in yeast membranes could be the absence of cytochrome $b_5$, which has been shown to stimulate the activity of CYP3A4 towards several substrates. It should be noted that another group has coexpressed CYP3A4 together with cytochrome $b_5$ and P450 reductase in S. cerevisiae [37], however the turnover number given in that report is similar to our value for the testosterone 6β-hydroxylase activity of CYP3A4 in the absence of cytochrome $b_5$. Interestingly this ancillary factor is absent in E. coli in which CYP3A4 displayed a surprisingly high catalytic activity. One may speculate that an E. coli protein substituted for cytochrome $b_5$ in stimulating the activity of CYP3A4 [21] and indeed simultaneous expression of human cytochrome $b_5$ together with CYP3A4 and P450 reductase stimulated the testosterone 6β-hydroxylase activity of this P450 isoform only 1.6-fold compared with the activity in the absence of cytochrome $b_5$ [38]. The substrate turnover numbers of the recombinant CYP3A4 and of the recombinant CYP2D6 expressed in E. coli appears to be rather similar to the substrate turnover number estimated for these enzymes in human liver microsomes, which are $55 \text{ min}^{-1}$ [36] and $10 \text{ min}^{-1}$ [39], respectively. However, one caveat in this comparison is that more than one P450 may metabolize a given substrate and that P450 isoforms are difficult to quantitate exactly in human liver microsomes.

Since the catalytic properties ($K_m$, turnover number) of the P450 isoforms are rather independent of the cellular system used for their expression, recombinant models could be very valuable in predicting P450-mediated metabolism in humans. However it remains to be seen if the substrate specificities of all the P450 isoforms are preserved in the expression systems. In order to address this our 14 industrial partners have assayed various recombinant P450s using a panel of isoform-specific substrates [40, 41] and their and our data indicate that the catalytic properties (substrate-, regioselectivity and $K_m$ values) of the recombinant P450s are the same as their microsomal counterparts.
10.3
Use of *E. coli* P450 Expression Systems in Bioreactors

10.3.1
General Considerations

Drug development requires the detailed characterization of metabolic pathways and their relevance for drug safety. This type of analysis necessitates that milligram quantities of metabolites need to become available, a task which until now had to be performed by elaborate chemical syntheses which become even more demanding when the metabolites result from stereoselective oxidations. It is obvious that recombinant P450s are ideally suited for the production of sufficient quantities of P450 metabolites, provided that the cellular system expresses these enzymes at high levels in a catalytically highly active form. In addition, it is important that the catalysis proceeds for sufficient time and that the fermentation system is suitable for scale-up. Our results show that the activity (expressed as pmol min$^{-1}$ mg$^{-1}$ membrane protein) of either CYP2D6 or of CYP3A4 in membranes isolated from recombinant bacteria were by a factor of 6- and 110-fold respectively higher than that of membranes isolated from yeast. In addition the yield of P450 in *E. coli* was between 50- and 100-fold higher than that in *S. cerevisiae*. Thus at least in our hands, *E. coli* appears to be more suitable as a bioreactor system than *S. cerevisiae*, even though it may be likely that under growth conditions which give higher yields of P450 [33] the yeast system could also be employed as biofermenter.

Below we describe in detail several examples of the use of the bacterial expression systems that express human P450s in the production of drug metabolites in industrial settings. Note that the bioreactor conditions employed by the two companies were rather different and yielded the desired biooxidations of structurally highly diverse compounds at the desired efficiencies.

10.3.2
The Roche Experience

10.3.2.1 Background and Utility of P450 Systems in Pharma Research

Roche obtained 14 recombinant human CYP450 isozymes coexpressed with NADPH-P450 reductase (CPR) in *E. coli* as part of a collaboration with the University of Dundee, Scotland (LINK Program). During the last 10 years these enzymes have been used extensively within Roche both as biocatalysts for the preparation of metabolites of drug candidates and for DMPK applications such as high-throughput CYP450 inhibition screening. The availability of state of the art fermentation facilities at our site has allowed the production of sufficient biomass for metabolite preparation on the milligram to gram scale for applications such as structure identification, analytical reference material, elucidation of toxicological properties, and so on. Equally important is the in-house expertise in downstream processing, as purification of the metabolites from biobroths,
although often neglected as a factor in biocatalysis, is often the most time-consuming and difficult step in the process. The procedure for the preparation of one such metabolite in 100 mg amounts is described below.

10.3.2.2 Fermentation of Recombinant *E. coli*
A generic fermentation protocol was developed which can be applied with minor modifications to all 14 recombinant CYP450s received from the LINK program. Fermentations were carried out at 30°C using modified Terrific Broth medium in 150L Braun fermenters with a working volume of 100L. Stirring speed and aeration was set such that dissolved oxygen (DO) decreased to below detectable levels after 4–6 h cultivation. Such low DO levels were also found to be necessary for formation of active CYP450 by other authors [42]. The inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) was added when OD₆₀₀nm reached 0.6–0.8, generally after 4 h growth. After 24–48 h fermentation time, depending on the CYP450 isofrom, cells were harvested by continuous flow centrifugation, resuspended in a high sucrose-containing buffer and after shock freezing in dry ice were stored at −80°C.

10.3.2.3 Biotransformations Catalyzed by Recombinant CYP450
In an early protocol, the frozen *E. coli* biomass was thawed and the cells disrupted by treatment with lysozyme/EDTA followed by resuspension in hypotonic buffer. Biotransformations using this crude membrane preparation were carried out with an NADPH-regenerating system based on glucose-6-phosphate dehydrogenase. This system is rather costly in terms of reagents, and so we looked for a method in which the lysozyme treatment could be omitted and the cells used intact (i.e. still with capacity to regenerate NADPH). It was found that frozen cells which were thawed and resuspended in phosphate buffer pH 7.4 supplemented with a small amount of NADP had the capability to generate metabolites albeit with lower yields than when crude membranes were used. It was decided to optimize this process using CYP3A4 as the model enzyme as this isoform makes the largest contribution to drug metabolism in the liver [43] and is therefore the most important for metabolite synthesis. It was found that supplementation of the reaction buffer with a carbon source such as citrate or isocitrate greatly stimulated both hydroxylation (M1) and N-demethylation (M2) of an endothelin receptor antagonist (Table 10.2). Although the effect of addition of these C-sources is substrate dependent, in most cases citrate and isocitrate are superior to glucose.

A second example is the hydroxylation of chlozoxazone also by recombinant CYP3A4 shown in Fig. 10.1. In this case the yield of 6-OH chlozoxazone was 8-fold higher with isocitrate than glucose and it can be postulated that NADPH regeneration is more efficient, probably due to the requirement for only one functioning enzyme, isocitrate dehydrogenase. It is possible that the freeze–thaw undergone by these cells adversely affects glycolytic/TCA cycle enzyme(s) and this may result in less efficient NADPH regeneration when glucose is used as cosubstrate.
Table 10.2 Effect of carbon source addition on biotransformation of an endothelin receptor antagonist by frozen and thawed *E. coli* JM109 containing CYP3A4, P450 reductase and cytochrome b₅.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Metabolite M1 (mg L⁻¹)</th>
<th>Metabolite M2 (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>1.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Reactions were carried out for 24 h at 27°C with shaking at 220 rpm in 24 deep-well plates containing in a total volume of 1.0 mL: 0.1 mol L⁻¹ phosphate buffer pH 7, 1.2 nmol CYP3A4, 50 nmol NADP, and 50 nmol substrate.

Fig. 10.1 Hydroxylation of chlorzoxazone by “frozen and thawed” *E. coli* cells containing CYP3A4, NPR, and cytochrome b₅, with addition of 20 mmol L⁻¹ glucose or isocitrate. Reactions were carried out at 27°C with shaking at 220 rpm in 100 mL baffled flasks containing 10 mL 0.1 mol L⁻¹ phosphate buffer pH 7.4: 8 nmol CYP3A4, 2.0 mmol chlorzoxazone, 0.5 mmol of either disodium citrate or glucose and 0.5 mmol NADP.

10.3.2.4 Preparation of N-Desethyl Amodiaquine

N-Desethyl amodiaquine was required in multi-milligram amounts as an analytical reference for a CYP2C8 assay. CYP2C8, containing a modification at the N-terminus in which the first eight residues are replaced by those from bovine 17α-hydroxylase [44], was coexpressed with P450 reductase in *E. coli* JM109 using...
a two plasmid system identical to that described for CYP2D6 [23]. Using the fermentation conditions described earlier, expression levels of CYP2C8 were typically 700–1000 nmol L\(^{-1}\) of culture after 24 h growth as determined by CO + red – red difference spectra. Cytoplasmic membranes prepared from such cells contained 1000–1200 pmol mg\(^{-1}\) CYP2C8 and CPR activity of 300–400 nmol cyt c red min\(^{-1}\) mg\(^{-1}\) protein. Optimization of amodiaquine biotransformation was carried out on the 10 mL scale in 100 mL shake flasks. Frozen *E. coli* cells were thawed slowly at room temperature and diluted in phosphate buffer pH 7.4 to a CYP2C8 concentration of 0.8 nmol mL\(^{-1}\). The concentrations of biocatalyst, substrate, citrate, and NADP were optimized using GALOP experimental design software (Forschungszentrum Jülich GmbH, Germany). Two rounds of optimization resulted in a doubling of substrate concentration from 0.2 to 0.4 mmol L\(^{-1}\) and a 4-fold reduction in the amount of biocatalyst (800 to 200 nmol L\(^{-1}\)) required for complete conversion. Using the optimized conditions a 2 L reaction with 372 mg of amodiaquine dihydrochloride was carried out in 5 × 2 L flasks each containing 80 nmol CYP2C8. The biotransformation yield reached 93% after 32 h incubation as determined by HPLC (Fig. 10.2). The metabolite was purified by extraction followed by flash chromatography on silica gel. After concentration of the pure

![Fig. 10.2 Biotransformation of amodiaquine by “frozen and thawed” *E. coli* cells containing CYP2C8 and NPR. Reactions were carried out at 27 ºC with shaking at 220rpm in 2000 mL baffled flasks containing 400 mL 0.1 mol L\(^{-1}\) phosphate buffer pH 7.4: 80 nmol CYP3A4, 4.0 mmol amodiaquine, 20 mmol disodium citrate and 0.5 mmol NADP.](image-url)
fractions and drying of the residue, 172 mg of desethyl amodiaquine dihydrochlo-
ride was obtained with >99% purity in 55% overall yield.

10.3.3
The Novartis Experience

10.3.3.1 **Introduction**
Already in the discovery phase, an understanding of the metabolic pathways of
the lead drug candidates can be highly useful for improving the overall pharma-
cokinetic profiles of compounds in a lead structural series [45]. Although HPLC-
MS provides a powerful tool for the identification of low concentrated metabolites
in solution, it is often still necessary to synthesize and isolate the metabolites on
milligram to hundred milligram scale for final structure confirmation by NMR
and biological testing. For biocatalytic synthesis of phase I metabolites, in the
Expertise Bioreactions of Novartis Pharma AG, Basel (Switzerland), 14 different
recombinant human (rh) cytochrome P450 isoenzymes functionally coexpressed
with human P450 reductase in *E. coli* are established as whole cell biotransfor-
mation systems. The genetic constructs were established at the Biomedical Research
Centre of the University of Dundee, Scotland, UK in the course of an academia/industry
collaboration between the said institute and 14 pharmaceutical compa-
nies. In a first step, often only the six major human CYP systems (CYP 1A2, 2C9,
2C19, 2D6, 3A4, 2E1) or otherwise, all 14 CYP-expressing *E. coli* cell lines available
to us are screened to identify the relevant isoenzyme. This is then used as the
biocatalyst for metabolite production via whole cell biotransformation. If the de-
sired compound is not formed by human CYPs or the conversion is too low, a
suitable microorganism can often be found producing the desired structure in
high yield. For this purpose, a screening of 150 microbial strains with known
oxidative capabilities is carried out in a 96-well format within 8 days (System
Duetz, Kühner AG, Basel, Switzerland). The technology for preparative biotrans-
formation involving CYPs is demonstrated below, showing the production of four
metabolites from AAG561 (see Table 10.3), a new drug compound in development
at Novartis Pharma AG against anxiety and depression. For completion, the latest
developments of the methodology are given.

10.3.3.2 **Production of *E. coli* Cells with CYP Activity**
From frozen glycerol stocks (−80°C, see below) of *E. coli* strains coexpressing
CYPs and P450 reductase, some material was streaked onto a Luria Bertani (LB)
agar plate containing 100 μg mL⁻¹ ampicillin or 100 μg mL⁻¹ of ampicillin plus
50 μg mL⁻¹ of chloramphenicol, depending on the construction system used (both
antibiotics for the systems with CYPs 1A2, 2C9, 2D6, and 3A5, where the two
human enzymes are cloned into two plasmids, just ampicillin for the one plasmid
systems with CYPs 1A1, 2C18, 2C19, 3A4, and 2E1). After incubation at 37°C for
16 h, a single colony was used to inoculate the preculture consisting of 25 mL solu-
tion of LB broth (Fluka, Switzerland) with pH 6.8 in a 100 mL shake flask contain-
ing antibiotics as mentioned above. The preculture was placed in an orbital shaker set at 37 ºC and a rate of 220 rpm until it reached an optical density at 600 nm (OD600) between 0.7 and 1. Then it was stored in a refrigerator until use (4 ºC, 1–2 days maximum).

Main cultures were prepared with 100–200 mL of Modified Terrific Broth (MTB, Sigma-Aldrich, Buchs, Switzerland) in 500 mL flasks with 1–4 baffles (all combinations serve). The inoculation volume was 1% v/v. The flasks were incubated in an orbital shaker at 37 ºC and 180 rpm. Before induction of enzyme expression, the temperature was reduced to 30 ºC. Induction was performed at an OD600 of 0.7–1 by addition of 1 mmol L⁻¹ of IPTG and 0.5 mmol L⁻¹ of δ-aminolevulinic acid. The total cultivation time was around 24 h, in which all cell lines reached an OD600 of 14–16. The cells were centrifuged at 5000 rpm and 4 ºC for 15 min. The pellet was resuspended in one tenth of the original main

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**Table 10.3** Proposed structures and relative abundance of metabolites from AAG561 after whole cell biotransformation with E. coli JM109 coexpressing CYP3A4 and P450 reductase.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Structure</th>
<th>MH⁺ m/z</th>
<th>rel. peak area</th>
<th>tR (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG561</td>
<td><img src="image" alt="Structure AAG561" /></td>
<td>411, 357</td>
<td>62.9%, 9.2%</td>
<td>15.4, 13.0</td>
</tr>
<tr>
<td>M4</td>
<td><img src="image" alt="Structure M4" /></td>
<td>357</td>
<td>9.2%</td>
<td>13.0</td>
</tr>
<tr>
<td>M2</td>
<td><img src="image" alt="Structure M2" /></td>
<td>373</td>
<td>2.1%</td>
<td>11.24</td>
</tr>
<tr>
<td>M3</td>
<td><img src="image" alt="Structure M3" /></td>
<td>385</td>
<td>2.9%</td>
<td>11.6</td>
</tr>
<tr>
<td>M4</td>
<td><img src="image" alt="Structure M4" /></td>
<td>357</td>
<td>9.2%</td>
<td>13.0</td>
</tr>
<tr>
<td>M5</td>
<td><img src="image" alt="Structure M5" /></td>
<td>369</td>
<td>15.7%</td>
<td>13.24</td>
</tr>
<tr>
<td>M6</td>
<td><img src="image" alt="Structure M6" /></td>
<td>385</td>
<td>1.1%</td>
<td>13.8</td>
</tr>
<tr>
<td>M7</td>
<td><img src="image" alt="Structure M7" /></td>
<td>427</td>
<td>1.1%</td>
<td>13.8</td>
</tr>
<tr>
<td>M8</td>
<td><img src="image" alt="Structure M8" /></td>
<td>425</td>
<td>2.8%</td>
<td>15.2</td>
</tr>
</tbody>
</table>

No structure proposal, molecule contains chloro atom.
culture volume using PSE buffer (KH$_2$PO$_4$/NaOH pH 7.2 50 mmol L$^{-1}$, sucrose 250 mmol L$^{-1}$, EDTA 0.25 mmol L$^{-1}$) and, if not immediately applied, stored at $-80^\circ$C until use as biocatalyst. For long-term preservation, precultures with an OD$_{600}$ of 0.7–1 were frozen in the presence of 15% sterile glycerol at $-80^\circ$C. On preparative scale, 14 $\times$ 500 mL Erlenmeyer flasks each filled with 200 mL of MTB medium plus 100 mg L$^{-1}$ ampicillin were inoculated with *E. coli* JM109 coexpressing CYP3A4 and P450 reductase and further processed as described above.

### 10.3.3.3 Whole Cell Biotransformation

In order to permeabilize the cells for biotransformation, 5% v/v of a Tween 80 solution (20 g L$^{-1}$) was mixed into fresh or defrosted 10$\times$ cell suspension. After incubation for 4 min at room temperature, the cells were harvested by centrifugation (see above) resuspended in PSE buffer, centrifuged again and finally resuspended in this buffer (cells 10-fold concentrated compared to the original culture volume).

Biotransformations on an analytical scale serve as activity assay for freshly grown cells and for optimizing the reaction conditions before preparative application. Furthermore, they are used for the identification of the CYP isoforms relevant for a specific compound and for the elaboration of its metabolite profile. 0.5 mL of cell suspension were mixed with 2.5 $\mu$L of a methanolic solution of AAG561 (20 mg mL$^{-1}$) in an Eppendorf cap closed with a membrane lid (Eppendorf Lid Bac, Eppendorf, Hamburg, Germany) and incubated at 30$^\circ$C and 1100 rpm in a temperature-controlled Eppendorf mixer for 4 and 20 h. The reactions were stopped by mixing with 0.5 mL of acetonitrile for 15 min. After centrifugation in a Heraeus Biofuge at 14 000 rpm, the supernatant was subjected to RP$_{18}$-HPLC-DAD and RP$_{18}$-HPLC-MS analysis.

The analytical biotransformations (4 h incubation time) showed metabolite formation with $>10\%$ conversion (rel. HPLC-MS peak area of the most abundant metabolite) by CYPs 1A1, 2C18, 3A4, and 3A5. The reactions involved were oxidative dealkylation and hydroxylation (see Table 10.3), whereby the dealkylated products M4 and M5 were the major reaction products. The additionally hydroxylated derivatives M2 and M3 as well as the di-dealkylated species M1 represented only minor metabolites. The data indicated that dealkylation preceded hydroxylation in the metabolic pathway.

Since CYP3A4 showed the broadest metabolite pattern, the preparative biotransformation was performed with 280 mL of a 10$\times$ concentrated cell suspension of *E. coli* JM109 coexpressing this CYP isoform and P450 reductase after treatment with Tween 80 as described above. AAG561 was added in form of 1.4 mL of a methanolic solution (20 mg mL$^{-1}$) and the incubation was performed in two 500 mL Erlenmeyer flasks with one baffle at 30$^\circ$C and 180 rpm in a laboratory shaker for 20 h. The biotransformation broth was extracted twice with 200 mL of CH$_2$Cl$_2$/isopropanol (9:1). Phase separation was achieved by centrifugation. The organic layers were pooled, dried with MgSO$_4$ and the solvent was removed under reduced pressure yielding ~360 mg of solid. Further purification was effected by prep. RP$_{18}$-HPLC (water–acetonitrile gradient in the presence of 0.05% trifluoro-
acetic acid) with UV and MS detection. All preparations obtained thereby still contained some Tween 80 originating from the cell treatment for permeabilization. These contaminations, however, could be removed by normal phase chromatography. The structures of the preparations of M1–M5 were elucidated by NMR spectroscopy and the identity and purity was verified by HPLC-MS (see above) and, except for M3, by HPLC-UV (220 nm).

Up to 13.6 mg of the different metabolites were obtained with purities between 74 and 98%. Metabolites M2 and M3 proved to be hydroxylated at the methyl group on the quinoxaline ring (see Fig. 10.3). The metabolite pattern in respect to the structure and even the quantity of the reaction products was identical to that from in vitro incubations of [14C]AAG561 with human postmitochondrial liver fractions (S9) measured by HPLC with radiodetection (Wirz B and Weber B, internal report, Novartis Basel, Switzerland).

Fig. 10.3 Metabolites prepared from AAG561 via whole cell biotransformation using CYP3A4/P450 reductase coexpressed in E. coli JM109.
10.3.3.4 Recent Developments

*E. coli* cells with high activity of CYPs can also be obtained on larger scale by cultivation in a stainless steel stirred tank bioreactor. The aeration should be sufficient for good growth but during CYP expression the dissolved oxygen concentration should be kept very low in order to avoid enzyme deactivation [42]. Recently we have grown the cells on a routine basis in a BioWave 50SPS bioreactor (Wave-Biotech AG, Tagelswangen, Switzerland) in order to lower the costs by use of a cheaper and simpler technology. A disposable polyethylene bag serves as the reactor, which is rocked on a temperature-controlled table. Oxygen is supplied via a stream of sterile air through the headspace of the bag. Under conditions recommended for the cultivation of *E. coli* (40 rocks/min, 10.5° rocking angle) and an airflow of 0.5 L min$^{-1}$ (maximum value of the built-in membrane pump), the CYP3A4-expressing *E. coli* cell line grew only to an OD 600 of 8 instead of 16 commonly achieved in MTB, probably because of oxygen limitation. By supplying an air flow supplemented with 10% of pure oxygen, cell density and CYP activity are identical to the ones obtained in shake flasks.

The treatment of the biocatalytically active cells with the Tween 80 implicated an additional purification step for separating off the detergent. Alternatively, substrates with low water solubility can be coated onto highly dispersed silica gel before supplying to the reaction mixture. The considerable increase in conversion is probably due to the enhanced dissolution via offering a large solid–liquid interface.

Applying these more recent technologies, a compound of undisclosed structure with a molecular weight (MW) of 533 and a calculated log$P$ of 2.29 (program CPOGP, BioByte Corp., Claremont, CA, USA) could be converted to 80% under catalysis of CYP3A4 to 100 mg of a N-demethylated metabolite, another compound (MW 241, calc. log$P$ 2.56) by CYP2D6 even in a concentration of 200 mg L$^{-1}$ to 100% to a monohydroxylated derivative (300 mg). In comparison it can be assumed that the relatively low conversion of AAG561 (see Table 10.3) was probably, at least to some extent, due to its high hydrophobicity (calc. log$P$ 6.9) and hence, low water solubility.

10.4 Conclusion

*E. coli* systems expressing human P450s have been used on a small scale in preclinical drug development (e.g. metabolic high-throughput screening). However, we have also convincingly demonstrated the effectiveness of *E. coli* expressing human CYP450 isozymes as biocatalysts for the production of drug metabolites on the 100 mg to 1 g scale. For some purposes it may be advantageous to employ broken cells, while for others intact cells may be preferable. Often such metabolites are either not accessible via chemical synthesis or only via very tedious multistep procedures. The biocatalytic route offers a convenient, relatively fast preparation of drug metabolites for a variety of applications. The availability
of 14 cloned human enzymes means that the authentic metabolites found in vivo will be accessible in the vast majority of cases. In order to fully exploit the potential of these enzymes the respective fermentation and downstream processing capacities should be available.

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