

Hydroxylation reactions with P450 enzymes

Hydroxylation reactions catalysed using P450 enzymes have a range of applications in drug metabolite production and lead diversification

Facilitating the identification of toxic metabolites early in the drug development process and enabling the diversification of lead compounds through the generation of a broad range of hydroxylated derivatives may lead to the discovery of safer, more potent drugs with a reduced risk of failure in clinical trials.

In humans and animals most xenobiotic compounds including drugs are metabolised and eliminated via a reaction sequence carried out predominantly in the liver.¹ Commonly in drug metabolism the first step is catalysed by cytochrome P450 monooxygenases, forming primary metabolites that are hydroxylated, de-alkylated, or oxidised in other ways.^{2,3}

During preclinical and clinical testing, drugs frequently fail not due to problems with the drug candidate itself but due to problems arising from the metabolites.⁴ For this reason, the FDA has published guidance that strongly recommends that all significant metabolites be characterised early in the drug development process.⁵ It is highly desirable that such characterisation be done prior to the initiation of human testing. Often, once the site of hydroxylation or other modification is identified, a drug can be redesigned to improve its activity and/or half-life. A common strategy is to replace a reactive hydrogen atom with fluorine, blocking P450-catalysed hydroxylation at that site and thereby eliminating the formation of a toxic or problematic metabolite and/or extending the serum half-life for a drug.

Metabolites are not always the source of negative data for a drug. In a number of cases metabolites have been shown to display biological activities superior to or different from that of the drug from which they are derived. The identification of these so-called 'active metabolites' can lead to safer, more active drug substances. A well-known example is the antihistamine fexofenadine (Allegra™), which is a metabolite of the older drug terfenadine (Seldane™). Fexofenadine was found to have all the biological activity of terfenadine but cause fewer adverse reactions in patients, leading to its approval in 1996 by the FDA. As a result, the metabolite fexofenadine is now marketed as an improved drug in place of the original drug substance terfenadine.⁶

Producing human metabolites in vitro in amounts sufficient for structural identification and preliminary toxicity and activity testing poses significant challenges. Chemical synthesis has been traditionally employed to produce metabolites, but often the synthetic route requires many steps and months of time to develop and carry out, resulting in a low yield and a high cost in both time and materials. An alternative to chemical synthesis is to use cytochrome P450 enzymes to generate the desired metabolites. The most common source is pooled human liver microsomes (HLM), but these microsomal preparations contain a mixture of many different enzymes, and their cost, batch-to-batch variability in activity, and restriction on availability limit the usefulness of human liver microsomes for preparative synthetic work. Liver microsomes from alternative animal sources have been used as an in vitro alternative to HLM.^{7,8} Although lower in cost than the human analogues, animal liver microsomes suffer from most of the same caveats as HLM and, in addition, have the disadvantage of sometimes producing different metabolites compared to their human counterparts. For the laboratory preparation of drug metabolites, better systems for production were needed.

Human Cytochrome Biocatalysts

Many of the problems encountered in the use of microsomes or in vivo systems can be solved through the use of recombinant human P450 enzymes. The biggest advantage of the use of recombinant human P450 enzymes is the ability to generate a desired metabolite in a one-step reaction. The complete, functionally-active P450 system is complex, requiring at a minimum the P450 enzyme component and a reductase component to be functional. The reductase requires a reduced nicotinamide cofactor, typically NADPH, and this cofactor must be regenerated to provide a steady supply of reducing equivalents for the reductase. The overall complexity of the reaction mixtures and the cost have been barriers to the widespread use of recombinant human P450s for metabolite synthesis in the past.

A product developed and offered by Codexis, Inc dramatically simplifies the production of metabolites using

recombinant human P450 enzymes. The Human Cytochrome Biocatalyst (HCB) is a recombinant human cytochrome P450 enzyme preparation produced from a synthetic, redesigned gene and formulated with all the components necessary for a fully functional catalytic P450 system. Unlike other preparations of recombinant human cytochromes which are only offered as microsomes, the HCB is available as a lyophilised powder containing the P450 enzyme, the reductase, NADPH, an enzymatic cofactor regeneration system, and an optimised reaction buffer. As a lyophilised preparation, the HCB is easier to ship, store, and handle, and because it is fully formulated with all the necessary components for activity, the user need only add water and the drug to be metabolised.

All the major human liver cytochromes are available as a Human Cytochrome Biocatalyst, allowing each human cytochrome enzyme to be screened in parallel to determine which is the active enzyme for metabolism of a given drug. Once identified, the most active cytochrome system can be scaled up and used to produce larger quantities of the desired metabolite.

Some examples illustrate the advantages in using HCBs for the synthesis of drug metabolites. Table 1 shows the reaction of various Human Cytochrome Biocatalysts used in the production of key metabolites. Yields range from 70-100%, and the reactions are all complete within 6 hours. In the case of HCB 2C9 used for the production of the 4-hydroxy derivative of diclofenac, the reaction is essentially complete within 1 hour, with a quantitative conversion of drug to the desired metabolite.

Going beyond human drug metabolites

The recognition that many drugs can be converted into 'active metabolites' has led medicinal chemists to consider ways to incorporate cytochrome P450-like reactions into lead diversification strategies. In order to cast a wide net in the search for compounds having improved biological activity, fewer drug-drug interactions and reduced toxicity, the ability to diversify a lead compound using reactions that produce many different hydroxylated derivatives is highly desirable. These

reactions would include not only human and animal metabolites but also other hydroxylated derivatives not produced by mammalian enzyme systems.

Codexis, Inc has recently launched a new product that simplifies the production of a wide range of hydroxylated derivatives. Based on the pioneering work of Frances Arnold at Caltech, the Codex™ MicroCyp Plate contains 96 variants of the bacterial cytochrome 102A1, commonly designated as P450-BM3. The distinctive feature of the P450-BM3 class of cytochromes is that such enzymes are self-sufficient P450s, meaning that both the P450 component and the reductase component are contained on a single polypeptide chain. Since the P450-BM3 is entirely self-sufficient, no separate reductase enzyme is required. By creating a platform of variants based on the BM3 enzyme offered in a microtitre-plate format, Codexis has simplified the process of lead diversification. A set of enzyme variants having broad hydroxylation capability can be screened quickly on a target compound, and the useful reactions can be scaled up to produce useful quantities of hydroxylated derivatives.

The robustness of the BM3 class of enzymes provides important benefits in both the screening and the production of hydroxylated derivatives of lead compounds. The BM3 mutants are more thermostable than human cytochrome enzymes and more tolerant of higher concentrations of substrates, leading to better scalability. Table 1 illustrates this advantage, comparing HCB 2C9 to a BM3 MicroCyp variant for the hydroxylation of diclofenac. The BM3 variant was able to tolerate 50-fold higher concentration of diclofenac

and sustain a longer reaction time, resulting in a much higher final titre of hydroxylated derivative, albeit with a lower overall conversion. In addition, a wide range of different hydroxylated derivatives can be produced with the MicroCyp BM3 variants, going beyond hydroxylated derivatives produced by human P450 enzymes. The breadth of scope in the MicroCyp enzyme platform offers medicinal chemists the opportunity to rapidly diversify lead compounds through hydroxylation chemistry.

The Codex™ MicroCyp Plates and Human Cytochrome Biocatalysts are innovative products that are helping to improve the rate of success in drug discovery, while reducing the cost and expense of the drug development process. Facilitating the identification of toxic metabolites early in the drug development process and enabling the diversification of lead compounds through the generation of a broad range of hydroxylated derivatives may lead to the discovery of safer, more potent drugs with a reduced risk of failure in clinical trials. ^{sp2}

REFERENCES

1. Guengerich F.P. Cytochromes P450, drugs, and diseases. *Mol Interv.* 2003; 3(4):194–204.
2. Guengerich F.P. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* 2001;14(6): 611–650.
3. Masubuchi Y., Hosokawa S., Horie T., Suzuki T., Ohmori S., Kitada M., Narimatsu S. Cytochrome P450 isozymes involved in propranolol

metabolism in human liver microsomes.

The role of CYP2D6 as ring hydroxylase and CYP1A2 as N-desisopropylase. *Drug Metab Dispos* 1994; 22(6):909–915.

4. Bajpai, M. and Adkison, K.K. High-throughput screening for lead optimisation: a rational approach. *Current Opinion in Drug Discovery and Development* 2000; 3, 63-71.

5. Guidance for Industry, Safety Testing of Drug Metabolites, Center for Drug Evaluation and Research, Food and Drug Administration, June 2005.

6. Markham A. and Wagstaff A.J. Fexofenadine. *Drugs* 1998; 55(2): 269-274.

7. Ueng Y.F., Yu H.J., Lee C.H., Peng C., Jan W.C., Ho L.K., Chen C.F., Don M.J. *Journal of Chromatography A* 2005; 1076, 103-109.

8. Hubl U., Stevenson D.E. *Enzyme and Microbial Technology* 2001; 29, 306-311.

This article is by Martin Mayhew, Spiros Kambourakis, and David Rozzell of Codexis.

Further information

David Rozzell
Codexis, Inc
129 N. Hill Avenue
Pasadena, CA 91106
USA

Tel: +1 626 685 9494
Fax: +1 626 356 39999

Codexis, Inc
200 Penobscot Drive
Redwood City, CA 94063
USA

Tel: +1 650 421 8100
Fax: +1 650 421 8102

Internet Links:

Email:
david.rozzell@codexis.com
Web: www.codexis.com

	Biocatalyst	mg/l drug	% conversion	mg/l metabolite(s)	Reaction time
Testosterone	HCB 3A4	100	70%	74	4 hours
Nifedipine	HCB 3A4	69	70%	48	3 hours
Dextromethorpan	HCB 2D6	54	89%	48	4 hours
Diclofenac	HCB 2C9	30	100%	32	1 hour
Diclofenac	MicroCyp	1500	30%	530	6 hours

Table 1. Metabolite production of selected drugs using Human Cytochrome Biocatalysts and a BM3 mutant from the Codexis MicroCyp plate. Each reaction was performed with a P450 biocatalyst concentration of 1 μM (1000 nmol / litre), in the presence of the corresponding P450 reaction mix, at 30°C with agitation to promote oxygen transfer to the reaction solution. The final column also represents biocatalyst productivity as milligrams of metabolite(s) produced per μmol of P450 biocatalyst.