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# Application of drug metabolising mutants of cytochrome P450 BM3 (CYP102A1) as biocatalysts for the generation of reactive metabolites

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**Abstract**

Recently, several mutants of cytochrome P450 BM3 (CYP102A1) with high activity toward drugs have been obtained by a combination of site-directed and random mutagenesis. In the present study, the applicability of these mutants as biocatalysts in the production of reactive metabolites from the drugs clozapine, diclofenac and acetaminophen was investigated. We showed that the four CYP102A1 mutants used in this study formed the same metabolites as human and rat liver microsomes, with an activity up to 70-fold higher compared to human enzymes. Using these CYP102A1 mutants, three novels GSH adducts of diclofenac were discovered which were also formed in incubations with human liver microsomes. This work shows that CYP102A1 mutants are very useful tools for the generation of high levels of reference metabolites and reactive intermediates of drugs. Producing high levels of those reactive metabolites, that might play a role in adverse drug reactions (ADRs) in humans, will facilitate their isolation, structural elucidation, and could be very useful for the toxicological characterization of novel drugs and/or drug candidates.


*Keywords:* Reactive metabolites; Adverse drug reactions; Biocatalysis; Directed evolution; Biotechnology; Drug metabolism

## 1. Introduction

Cytochromes P450 (P450s) are involved in the metabolism of approximately 80% of the drugs currently on the market [1,2]. Most often, metabolism by P450s increases solubility of the compound, facilitating urinary and biliary excretion. However, in some cases, metabolism of drugs by P450s leads to the formation of highly reactive electrophilic metabolites that can subsequently react with macromolecules, leading to covalent adducts to proteins. These events are thought to be related to serious adverse drug reactions (ADRs) and to rare

*Abbreviations:* P450, cytochrome P450; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; $t_r$, retention time; ADRs, adverse drug reactions; IDRs, idiosyncratic drug reactions; GSH, glutathione; RLM, rat liver microsomes; HLM, human liver microsomes.

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idiosyncratic drug reactions (IDRs). Consequently, it is important to assess the potential of novel drug candidates to form reactive electrophilic metabolites early in the drug discovery process [3].

The most common way to determine the formation of electrophilic metabolites is to screen for the formation of glutathione (GSH) adducts by LC–MS/MS analysis. Currently, several methods exist for the sensitive and selective detection of GSH conjugates [4–6]. Reactive intermediates (RIs) are usually generated in *in vitro* incubations using rat or human liver microsomes in the presence of GSH. However, due to the relatively low activity of human P450s and the occurrence of suicide inhibition of the enzymes by the RIs, generally only low concentrations of reactive metabolites are formed by liver microsomes [7]. Consequently, only major GSH adducts can be identified easily while minor adducts might stay undetected.

The soluble cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* is considered a good candidate for use as a biocatalyst in biotechnology, because this very stable enzyme has the highest catalytic activity ever recorded for a P450 [8]. In a previous study we described a site-directed mutant of CYP102A1, R47L/F87V/L188Q that is able to metabolise various drug-like molecules, including acetaminophen. Acetaminophen is metabolised by this triple mutant into the reactive *N*-acetyl-*p*-benzoquinoneimine (NAPQI) intermediate, albeit with an activity still 15-fold lower than human CYP3A4 [9]. In a subsequent study, this mutant was subjected to several rounds of random mutagenesis, which resulted in four mutants with an up to 90-fold increased activity towards drug substrates compared to human CYP2D6 [10].

In the present study we evaluated whether these novel drug metabolising CYP102A1 mutants can be used as biocatalysts for the biosynthesis of high amounts of reactive metabolites of drugs. We chose clozapine, diclofenac and acetaminophen as model compounds since these drugs have been involved in serious ADRs in humans [11–13]. These compounds are known to be bioactivated to several reactive electrophilic intermediates that can be trapped by GSH [14–18]. We therefore investigated whether the CYP102A1 mutants (Table 1) were able to metabolise these drugs and to produce GSH adducts. The metabolites formed were subsequently compared to those formed by rat liver microsomes (RLM) and human liver microsomes (HLM). It was determined whether the same metabolites were formed and it was studied whether the CYP102A1 mutants produce higher levels of GSH adducts when compared to human and/or rat enzymes. In previous studies, *E. coli* cytosolic fractions were used for metabolism studies with these CYP102A1 mutants. However, to prevent conjugation of the reactive metabolites to cytosolic components instead of GSH, for this study we used His-tagged CYP102A1 mutants, that were based on the mutants described in [10], which were purified by nickel column chromatography.

## 2. Experimental procedures

### 2.1. Enzymes and plasmids

The CYP102A1 mutants M01, M02, M05 and M11 were prepared in the pT1-P450BM3 plasmid as described previously [10]. These mutants were cloned into a pET28a+ vector system, which contains an N-terminal His-tag to allow purification by nickel column chromatography. The pET28a+ vector containing wild-type CYP102A1 as described in [19] was kindly provided by dr. V. Urlacher (Institut für Technische Biochemie, Universität Stuttgart, Germany). The genes of the mutants M01, M02, M05 and M11 were cloned into this pET28a+ vector as described in [19]. The resulting His-tagged CYP102A1 mutants $M01_{his}$, $M02_{his}$, $M05_{his}$ and $M11_{his}$ were used in this study. Sequencing showed that one or two extra mutations were introduced in the genes of M01, M02 and M11 during the cloning process (Table 1). Enzyme kinetic analysis of these mutants using benzoxyresorufin and dextromethorphan as substrates did not show significant differences when compared to the corresponding non-His-tagged mutants M01, M02, M05 and M11 (data not shown), suggesting that the extra mutations are without effect.

Table 1
Mutations present in the CYP102A1 mutants used in this study

| Mutant $M01_{his}$ | Mutant $M02_{his}$ | Mutant $M05_{his}$ | Mutant $M11_{his}$ |
|---|---|---|---|
| R47L | R47L | R47L | R47L |
| | | | E64G |
| | | F81I | F81I |
| | L86I | | |
| F87V | F87V | F87V | F87V |
| | | | E143G |
| L188Q | L188Q | L188Q | L188Q |
| | | | Y198C* |
| E267V | | E267V | E267V |
| | | | H285Y* |
| | N319T | | |
| G415S | | G415S | G415S |
| | A964V* | | |
| G1049E* | | | |

Mutations indicated by asterisk are additional in comparison to the mutations present in the non-His-tagged CYP102A1 mutants M01, M02, M11, as previously described in [10].