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Benchmarking of laboratory evolved unspecific peroxygenases for the synthesis of human drug metabolites

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A B S T R A C T

By mimicking the role of human liver P450 monooxygenases, fungal unspecific peroxygenases (UPOs) can perform a range of highly selective oxyfunctionalization reactions on pharmacological compounds, including O-dealkylations and hydroxylations, thereby simulating drug metabolism. Here we have benchmarked human drug metabolite (HDM) synthesis by several evolved UPO mutants, focusing on dextromethorphan, naproxen and tolbutamide. The HDM from dextromethorphan was prepared at the semi-preparative scale as a proof of production. The structural analysis of mutations involved in the synthesis of HDMs highlights the heme access channel as the main feature on which to focus when designing evolved UPOs. These variants are becoming emergent tools for the cost-effective synthesis of HDMs from next-generation drugs.

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1. Introduction

Synthetic chemistry is increasingly being incorporated into pipelines to discover new bioactive compounds, with new drugs being designed on the basis of better understanding the appropriate biological targets [1]. The US Food and Drug Administration (FDA) guidelines for metabolites in safety testing (MIST) declare that any metabolite that is generated above 10% of the parent drug should be subject to safety testing [2]. Therefore, it is fundamental for the pharma industry to produce large amounts of pure metabolites (human drug metabolites, HDMs) in order to be able to perform pharmacokinetic and pharmacodynamics studies. However, low yields and the cumbersome processes associated with their chemical synthesis are important drawbacks to the production of HDMs, with enzymes representing the most feasible option to overcome such barriers.

Unspecific peroxygenases (UPOs; EC 1.11.2.1) are extracellular heme-thiolate enzymes considered by many as the “generational successors” to P450s for selective C–H oxyfunctionalization and they are of particular interest in organic synthesis. Using H2O2 as the final electron acceptor and exclusive oxygen donor, UPOs perform dozens of two-electron oxidation (mono(per)oxygenations) reactions [3]. Indeed, these enzymes have proven to be a valuable departure point for the synthesis of HDMs [4–6]. We previously performed directed evolution on the UPO from the basidiomycete Agrocybe aegerita (AaeUPO) to enhance its functional heterologous expression and pharmacodynamics studies. However, low yields and the cumbersome processes associated with their chemical synthesis are important drawbacks to the production of HDMs, with enzymes representing the most feasible option to overcome such barriers.

Unspecific peroxygenases (UPOs; EC 1.11.2.1) are extracellular
Consequently, we believe that the extensive portfolio of UPO variants we have generated in recent years is likely to open new avenues for the more efficient synthesis of HDMs.

In the current study, we have tested several evolved UPO variants for their capacity to synthesize authentic HDMs from three consolidated pharmaceutical agents: dextromethorphan, naproxen and tolbutamide. For each synthetic reaction, the evolved UPOs were systematically benchmarked and dextrorphan synthesis was taken to a semi-preparative scale. The differences in substrate preferences of the evolved UPOs were analyzed within a mutational context through site-directed mutagenesis and they were further discussed.

2. Results and discussion

The evolved PaDa-I, JaWa and SoLo UPO mutants were produced and purified to homogeneity (Reinheitszahl – Rz- $A_{418}/A_{280}$ ~2.2), and their activity was tested on dextromethorphan (1), an antitussive drug with sedative and dissociative properties. Native AaeUPO converts this pharmaceutical agent into dextrorphan (2) by $O$-dealkylation, its authentic HDM (Fig. 2) [5].

Previous engineered P450 BM3 variants were tested towards (1) but the product obtained was not the authentic HDM (2) [14,15]. The reactions of the selected UPO mutants were analyzed by HPLC/PDA (Fig. 3) and the products determined by HPLC/MS (see Experimental Section). In all cases, the substrate conversion indicated that each of the mutants outperformed the native AaeUPO (16%): PaDa-I, 57%; SoLo 75%, and JaWa, 82% (Table 1).

Given its excellent behavior under operational conditions during the synthesis of the HDM 5′-hydroxypropranolol [10], the SoLo variant was further evaluated on a semi-preparative scale. By applying a gradual supply of $H_2O_2$ to avoid oxidative damage, we produced up to 102.1 mg of (2) from (1), with a yield of 75.2% (see Experimental Section for details). We then tested the selective demethylation of naproxen (3), a non-steroidal anti-inflammatory drug, achieving the highest substrate conversion with the PaDa-I
when considering the large-scale production and purification of enzymes. In the crystal structure of the PaDa-I variant recently made available, we noted that the F311L mutation is implicated in broadening the access channel, and it is also responsible for the dual conformational state of Phe191, conferring unheralded plasticity to the heme entrance in some of the evolved UPO variants [18]. Such flexibility at the heme access channel is not a feature of the native AaeUPO and accordingly, it is reasonable to think that this modifications might explain the improved yields with dextromethorphan (1) given its bulk. It is also worth noting that JaWa and SoLo produced higher yields with (1) than PaDa-I, differences that could be attributed to the G241D mutation situated at the entrance of the heme channel that is carried by both JaWa and SoLo but that is absent in PaDa-I.

In our previous evolution experiments with JaWa and SoLo, computational analysis revealed that G241D favored substrate anchoring (for naphthalene and propranolol, respectively), better orientating these substrates for oxygenation. To confirm that this beneficial effect also applies to (1), we reverted the G241D mutation in SoLo by site-directed mutagenesis. The SoLo-D241G mutant produced much lower conversion from (1) (from 75% to 37%), albeit still above those of native AaeUPO (16%, Table 1). The SoLo-D241G mutant also showed lower product formation than PaDa-I (37% vs. 57%), indicating that the F191S mutation in SoLo must be responsible for this effect, which is again consistent with the small differences between the JaWa (lacking F191S mutation) and SoLo mutants (Fig. 4). In terms of naproxen (3) conversion, the effect of reverting the G241D mutation was the opposite of that observed for (1), increasing roughly two-fold in the case of SoLo-D241G (Table 1). As indicated previously, positions 191 and 241 seem to be crucial for this phenomenon, with Phe191 and Gly241 of PaDa-I representing the best combination for this substrate among the evolved variants. Finally, when tolbutamide (5) was tested as the substrate, the three mutants rendered similar amounts of hydroxyltolbutamide (6), which may be an important property when considering the large-scale production and purification of (6). The total turnover numbers (TTNs, reported as μmol product/μmol enzyme) for each evolved mutant and reaction were within the same range, from 1200 (for SoLo in the production of 0-desmethylnaproxen) to 8200 (for JaWa in the production of dextromethorphan) (Table 1).

The striking differences in substrate conversion between the distinct evolved variants, generated over 8 rounds of directed evolution, led us to analyze the role of the mutations located around the catalytic cavity (Figs. 1 and 4). The heme channel of AaeUPO is furnished with 9 aromatic residues, of which Phe76 and Phe191 define its access, while Phe69, Phe121 and Phe199 are involved in positioning the substrate for catalysis [17]. In the crystal

 secretion variant (36%), followed by JaWa (25%) and SoLo (12%), yet in all the cases less than that of the native AaeUPO (57%) (Table 1) but higher than previous reported P450 variants [16].

We also assayed tolbutamide (5), a Na+-channel blocker, the hydroxylation of which was mediated by AaeUPO through the attack of the benzylic carbon, giving rise to the HDM 4-hydroxymethyl-tolbutamide (6) (Fig. 2). In this case the substrate conversion were 20%, 14%, 19% and 15% for PaDa-I, JaWa, SoLo and native AaeUPO, respectively (Table 1). Although JaWa was associated with the lowest conversion, it did display a notable lack of overoxidation (i.e.: a further two-electron oxidation reaction of (6) to 4-formyl-tolbutamide (7)), which may be an important property when considering the large-scale production and purification of (6). The total turnover numbers (TTNs, reported as μmol product/μmol enzyme) for each evolved mutant and reaction were within the same range, from 1200 (for SoLo in the production of O-desmethylnaproxen) to 8200 (for JaWa in the production of dextromethorphan) (Table 1).

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### 3. Conclusions

Evolved UPO variants with different substrate scopes are suitable biocatalysts to synthesize known and novel HDMs. The heme access channel of these evolved variants is malleable and it can be adapted through additional evolutionary campaigns to achieve cost-effective production of HDMs. Accordingly, future structure-guided evolution experiments focusing on this region may

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**Table 1**

<table>
<thead>
<tr>
<th>Substrate (m/z)</th>
<th>Product (m/z)</th>
<th>PaDa-I (% product)</th>
<th>JaWa (% product)</th>
<th>SoLo (% product)</th>
<th>AaeUPO* (% product)</th>
<th>SoLo-D241G (% product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextromethorphan [M+H]+ 272</td>
<td>0-desmethylnaproxen [M+H]+ 215</td>
<td>36</td>
<td>12</td>
<td>57</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Naproxen [M-H]- 229</td>
<td>4-hydroxymethyl-tolbutamide [M+H]+ 287</td>
<td>14</td>
<td>19</td>
<td>15</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Tolbutamide [M+H]+ 271</td>
<td>4-formyl-tolbutamide [M+H]+ 285</td>
<td>15</td>
<td>–</td>
<td>4</td>
<td>n.q.</td>
<td>16</td>
</tr>
</tbody>
</table>

Reactions were performed at room temperature for one hour (after enzyme activity deceased). Each reaction mixture contained 0.1 μM of purified enzyme, 1 mM substrate (dissolved in 10% acetonitrile), 4 mM ascorbic acid, and 1 mM H2O2 in 100 mM potassium phosphate buffer pH 7.0 (1 mL final volume).

* Data obtained from Ref. [5]. (-): not detected n.q.: not quantified. TTNs for mutants can be calculated from the ratio (in μM) between product conversion and enzyme concentration after 1 h reaction (i.e. multiplying each value of product conversion by 100). Standard errors were in all cases lower than 3%.
expand the substrate range of UPOs towards next-generation drugs with different chemical structures.

4. Experimental

Tolbutamide, ascorbic acid and Yeast Transformation kit were purchased from Sigma-Aldrich/Merck (Darmstadt, Germany). Naproxen, dextromethorphan, dextrorphan were purchased from Santa Cruz Biotechnology (CA, USA). The high-fidelity DNA polymerase iProof was acquired from BioRad (CA, USA). The BamHI and XhoI restriction enzymes were purchased from New England Biolabs (MA, USA) and the protease-deficient *S. cerevisiae* strain BJ5465 from LGCPromochem (Barcelona, Spain). The Zymoprep Yeast Plasmid Miniprep kit and Zymoclean Gel DNA Recovery kit were from Zymo Research (CA, USA). The NucleoSpin Plasmid kit was purchased from Macherey-Nagel (Düren, Germany) and the oligonucleotides were synthesized by IDT (IA, USA). All chemicals were reagent-grade purity or analytical standards.

4.1. Expression and purification of UPO variants

UPO variants were produced and purified as described before [8,10].

4.2. Reactions and product characterization

Reaction mixtures (1 mL) contained purified peroxynogenases (PaDa-I, JaWa, SoLo and SoLo-D241G mutants, 0.1 μM), substrate (1 mM, dissolved in 10% acetonitrile), potassium phosphate buffer (100 mM, pH 7.0), ascorbic acid (4 mM) and a single dosage of H₂O₂ (1 mM). All reactions were stirred at 30 °C for one hour until reaction stopped. The reaction mixtures were analyzed by reversed-phase chromatography (HPLC) using a quaternary pump (Agilent Technologies, model 1100) coupled to a Phenomenex Zorbax Eclipse plus C18 column (4.6 mm diameter by 100 mm length, 3.5 μm particle size), with an autosampler (Hitachi, model L-2200) and a photodiode array detector (PDA, Varian Prostar). Column temperature was kept at 30 °C and flow rate at 1 mL/min. Each injection had a volume of 10 μL and the analytes were eluted with a gradient from 100% of CH₃CN to 100% of H₂O in 5 min (with 0.1 vol/vol of formic acid in both solvents), followed by 10 min linear gradient from 100% of H₂O to 100% of CH₃CN. UV detection wavelengths were 238 nm for tolbutamide, 280 nm for dextromethorphan and 235 nm for naproxen. Integration of peaks was carried out using the Varian Star LC workstation 6.41.

Identification of reaction products was determined by liquid chromatography-mass spectrometry (HPLC/MS) with a Waters Instrument equipped with a chromatographic module Alliance 2695, diode array detector (PDA 2996) and a quadrupole mass spectrometer (Micromass ZQ). Reversed phase chromatography was performed on a SunFire C18 (2.1 mm diameter, 50 nm length, 3.5 μm particle size, Waters); which was eluted at 1 mL/min with aqueous/acetonitrile (0.1% vol/vol formic acid in both solvents), with 20 min linear gradient from 95% of acetonitrile to 95% of H₂O.
Samples were ionized by electrospray ionization (ESI, with nitrogen to desolvate the mobile phase) and analyzed in positive reflector mode. Naproxen and O-desmethylnaproxen were analyzed employing a mass spectrometer coupled to a hybrid QTOF analyzer (model QSTAR, Pulsari, AB Sciex). The compounds were analyzed by direct infusion and ionized by ESI in negative reflector mode. The ionizing phase was methanol basified with 1% NH₄OH.

4.3. Semi-preparative production of dextrophor and NMR analysis

Dextrmethorphan (135.6 mg, 0.5 mM) was dissolved in acetonitrile (10 mL). The solution was added to potassium phosphate buffer (90 mL, 100 mM pH 7.0) containing ascorbic acid (4 mM) and SoLo mutant (0.05 μM). Reactions (2 × 50 mL) were performed at 30 °C and 600 rpm using a thermo shaker device (Eppendorf). H₂O₂ was added with a syringe pump (0.06 mmol/h) over 16.5 h. Afterwards, the solution was heated to 70 °C for 3 min and the precipitated enzyme removed. The conversion (76.2%) was determined by HPLC (water with 0.1% TFA/acetonitrile, 5/95 to 95/5). Afterwards, the solution was concentrated to 25 mL and freeze-dried. The obtained powder was dissolved in acetonitrile (10 mL). The solution was added to potassium phosphate buffer at pH 7.08 (d, 7.8 Hz, 1H), 6.80 (d, J = 2.5 Hz, 1H), 6.71 (dd, J = 8.3, 2.5 Hz, 1H), 3.65–3.59 (m, 1H), 7.22–3.08 (m, 3H), 2.92 (s, 3H), 2.73 (td, J = 13.2, 3.6 Hz, 1H), 2.52–2.39 (m, 1H), 1.97 (dt, J = 12.4, 3.1 Hz, 1H), 1.87 (td, J = 13.8, 4.6 Hz, 1H), 1.79–1.27 (m, 7H), 1.26–1.11 (m, 1H). The obtained powder was dissolved in deuterated DMSO (3%), DMSO (0.5 μM), D214G F (0.5 μM), dNTPs (1 mM, 0.25 mM each), high-fidelity DNA polymerase ipProof (0.02 U/mL), and the template Solo (10 ng), and 2) 50 μL final volume, DMSO (3%), RMLN (0.5 μM), D214G R (0.5 μM), dNTPs (1 mM, 0.25 mM each), high-fidelity DNA polymerase ipProof (0.02 U/mL), and the template Solo (10 ng). PCR reactions were carried out on a gradient thermocycler using the following parameters: 98 °C for 30 s (1 cycle); 98 °C for 10 s, 48 °C for 30 s, and 72 °C for 30 s (28 cycles); and 72 °C for 10 min (1 cycle). PCR products were loaded onto a preparative agarose gel and purified with the Zymoclean Gel DNA Recovery kit. The recovered DNA fragments were cloned under the control of the GAL1 promoter of the pRoCo30 expression vector, with use of BamH1 and XhoI to linearize the plasmid and to remove the parent gene. The linearized vector was loaded onto a preparative agarose gel and purified with the Zymoclean Gel DNA Recovery kit. The PCR products (200 ng each) were mixed with the linearized plasmid (100 ng) and transformed into S. cerevisiae for in vivo gene reassembly and cloning by IOVE [19].

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