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Surface display of human cytochrome P450 enzymes 3A4, 1A2, 2C9, 2C19 and 2D6 with cytochrome P450 reductase for drug metabolism studies

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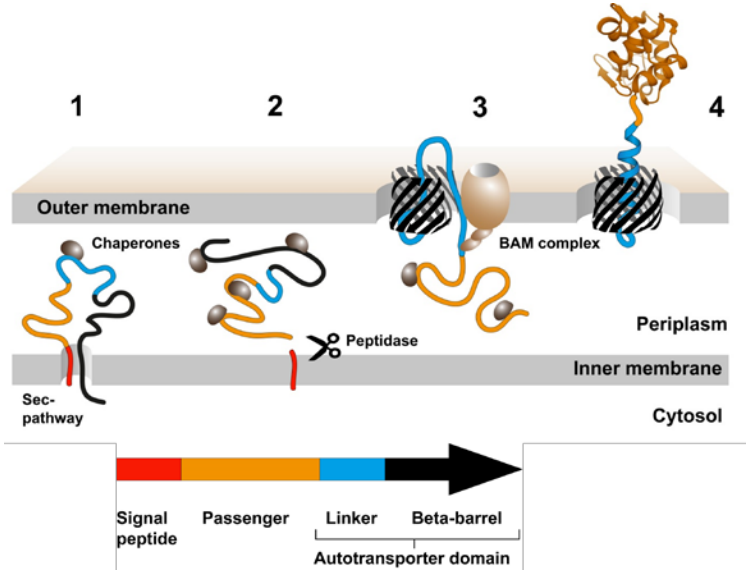
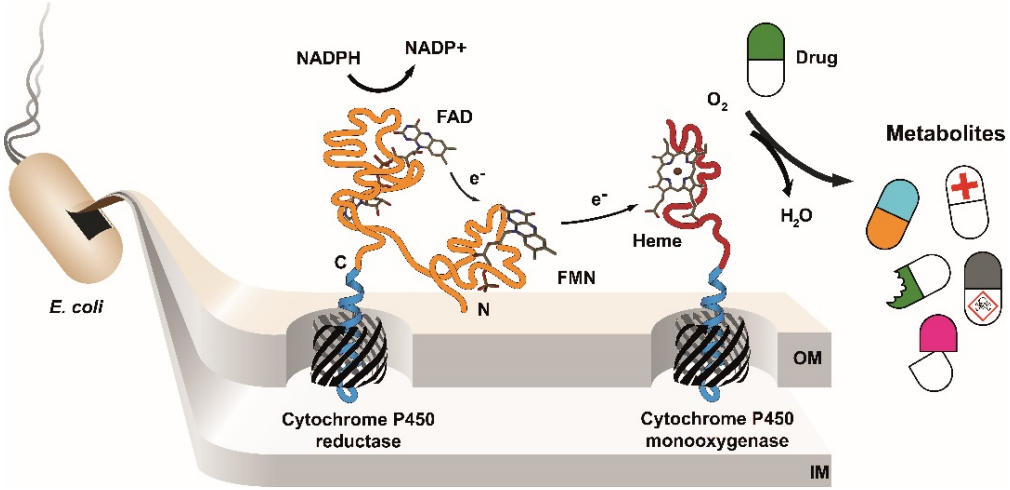
Graphical Abstract

Surface display of human cytochrome P450 enzymes 3A4, 1A2, 2C9, 2C19 and 2D6 with cytochrome P450 reductase for drug metabolism studies





A: Mechanism of passenger translocation on the surface with an autotransporter. **1**: Transport over inner membrane through Sec-Translocon; **2**: Signal peptide is cleaved off. Protein is kept in an unfolded state by chaperones; **3**: Insertion of the β-barrel into the outer membrane; **4**: Passenger is translocated onto the surface. Schematic view of the autotransporter fusion precursor protein.

B: Co-expression of cytochrome P450 monooxygenase and cytrochrome P450 reductase toward drug metabolites studies using autodisplay. With both enzymes displayed on the surface substrate accessbility and electron supply are given.

Abstract

Cytochrome P450 monooxygenases (CYPs) are responsible for the biotransformation of most known drugs and xenobiotics in human body [1]. As part of the phase-I-metabolism they catalyze a broad diversity of oxidation reactions in an extensive spectrum of substrates. The utilization of CYPs as biocatalysts is limited due to their low stability and their requirement of a membrane surrounding to fold into an active form [2]. Autodisplay of CYPs on the surface of *E. coli* has been shown an appropriate tool to overcome these limitations [3, 4].

In order to establish an *in vitro* system to study drug metabolism, the five most important CYPs, CYP 3A4, CYP 1A2, CYP 2C9, CYP 2C19 and CYP 2D6 were displayed on the surface of *E. coli*. The catalytic activity of CYP 3A4 was shown by testosterone as a substrate using a HPLC assay with external addition of the cytochrome P450 reductase (CPR) [5]. A co-expression of CYP 1A2 and CPR was established with both enzymes being displayed on the surface of *E. coli*. Surface display was confirmed by a protease accessibility test and by flow cytometry. Surface displayed CYP 1A2 with co-expressed CPR was able to convert phenacetin to paracetamol, as well as 7-ethoxyresorufin and 3-cyano-7-ethoxycoumarin to the fluorescent products resorufin [6] and 3-cyano-7-hydroxycoumarin. CYP 2C9, CYP 2C19 and CYP 2D6 were co-expressed with CPR on the surface of *E. coli* as well. Combining cells with these five CYP enzymes in an active form on the bacterial cell surface is supposed to provide a suitable approach for the *in vitro* simulation of drug metabolism.

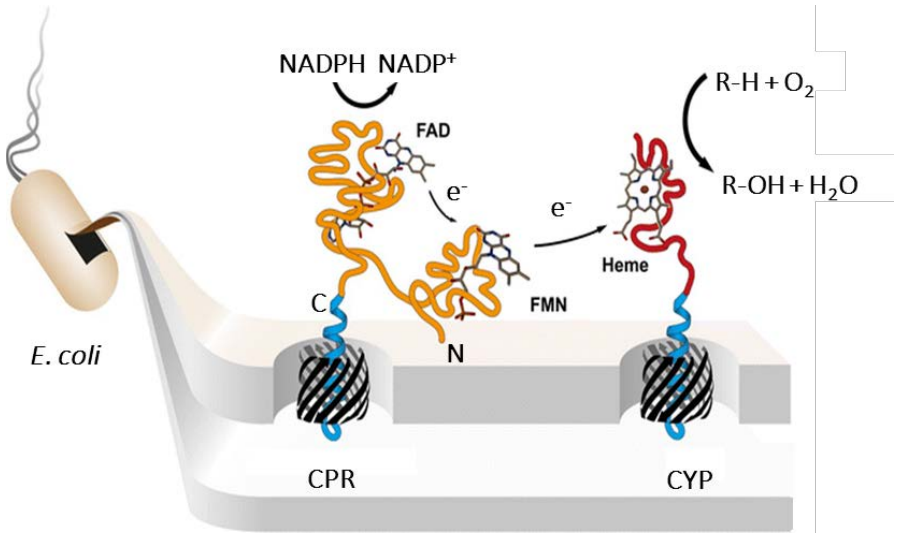
Keywords: cytochrome P450 monooxygenase; surface display; drug metabolism; autodisplay

References:

[1] Guengerich, FP.: *Chem Res Toxicol*. **2008**, 21:70–83.
[2] Nagy, P. *et al*.: *J Biol Chem*. **2011**, 286: 18048-18055.
[3] Schüürmann, J. *et al*.: *Appl Microbiol Biotechnol*. **2014**, 98(19): 8031-8046.
[4] Schumacher, S. *et al*.: *J Biotechnol*, **2012**,161:104-112.
[5] Schumacher, S., Jose, J.: *J Biotechnol*. **2012**, 161:113-120.
[6] Quehl, P. *et al*.: *Microb Cell Fact.* **2016**, 15:26-41.

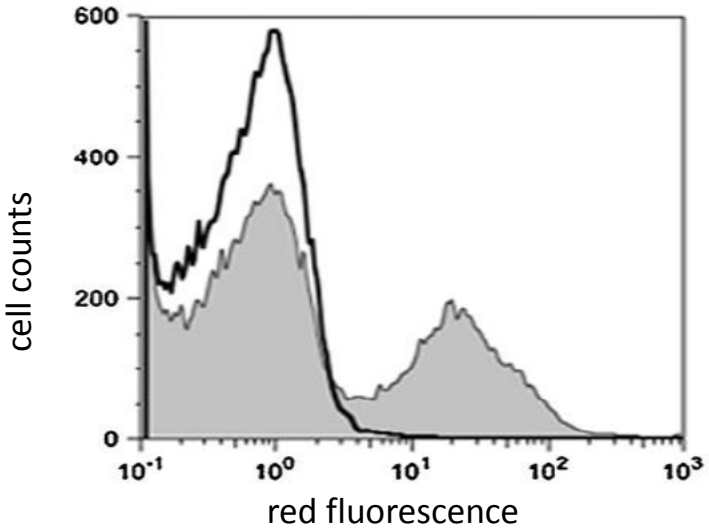
Introduction
Surface display of CYP with cytochrome P450 reductase

- Important role in biotransformation (Phase I) of xenobiotics and drugs
  - metabolism intermediate studies
- $RH + O_2 + 2e^- + 2 H^+ => ROH + H_2O$
- Broad range of regio- and stereospecific oxidations
- Crucial for the activity of CYP:
  - electron donator
  - membrane surrounding
- Co-expression of CYP and CPR on the surface using autodisplay technology



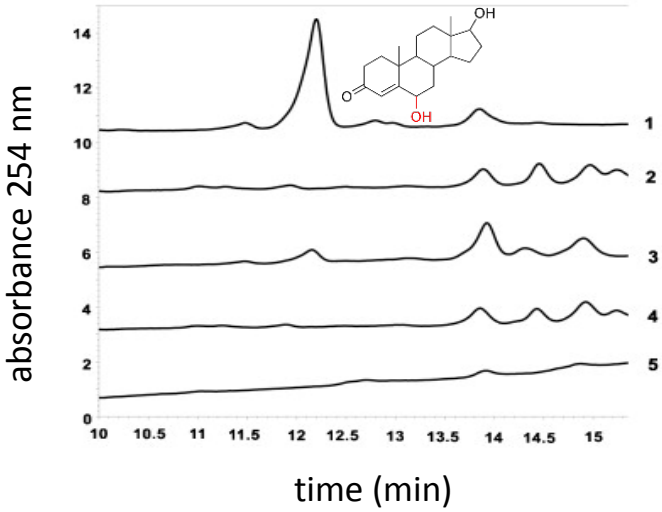
Results and discussion
Surface display of CYP3A4

Proof of surface display



Flow cytometry analysis of immunolabeled cells [7]. Cell samples were treated with a primary monoclonal anti-CYP3A4 antibody and a secondary Dylight647 conjugated anti-IgG antibody, washed and then analyzed via flow cytometry. Black: *E. coli* UT5600(DE3) control cells, Grey: cells displaying CYP 3A4.

Hydroxylation assay

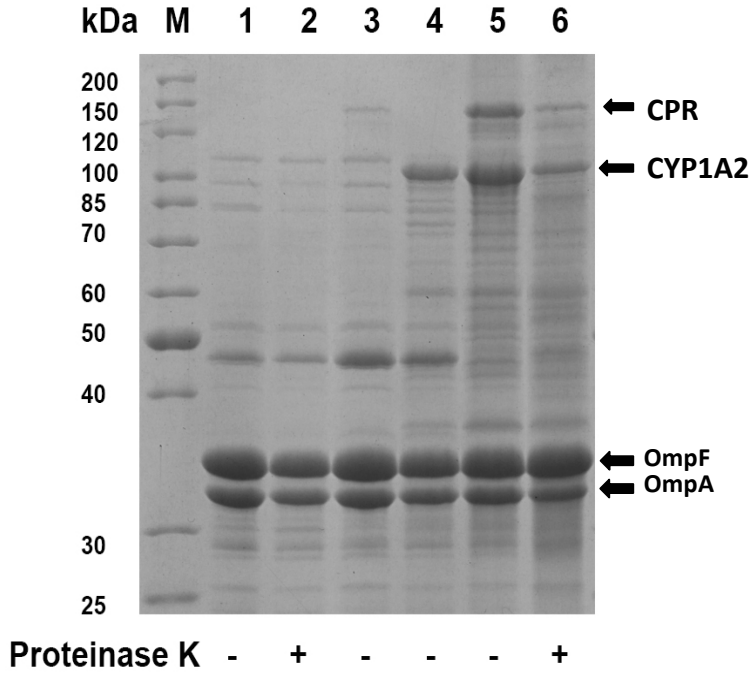


Hydroxylation assay with testerone as substrate using the whole cell biocatalyst displaying CYP 3A4 (OD_{578nm}10, 72h) with HPLC [7]. 1: purified CYP 3A4, showing only the product peak; 2: *E. coli* UT5600 (DE3) cells without protein induction; 3: cells displaying CYP 3A4; 4: *E. coli* UT5600 (DE3) cells; 5: reaction buffer.

[7]: Schumacher, S., Jose, J.: *J Biotechnol*. **2012**, 161:113-120.

Results and discussion
CYP1A2 and CPR

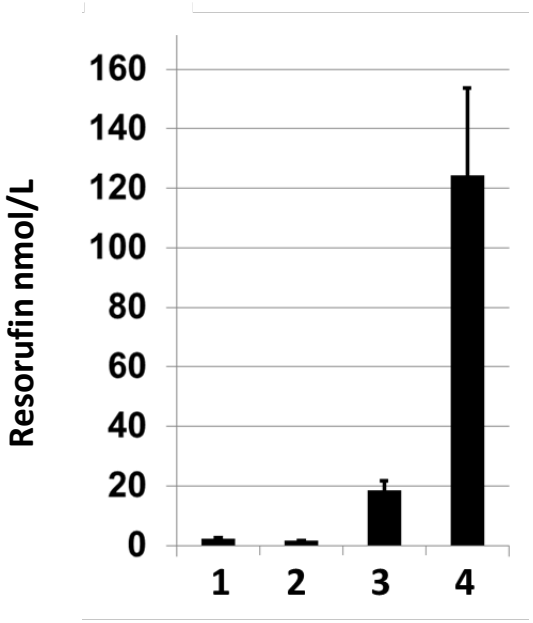
Proof of surface display



SDS-PAGE of outer membrane protein preparations. 1,2: *E. coli* BL21 (DE3); 3: expression of CPR; 4: expression of CYP1A2; 5,6: co-expression of CYP 1A2 and CPR; 2 and 6: proteinase K digest as proof of surface accessibility.

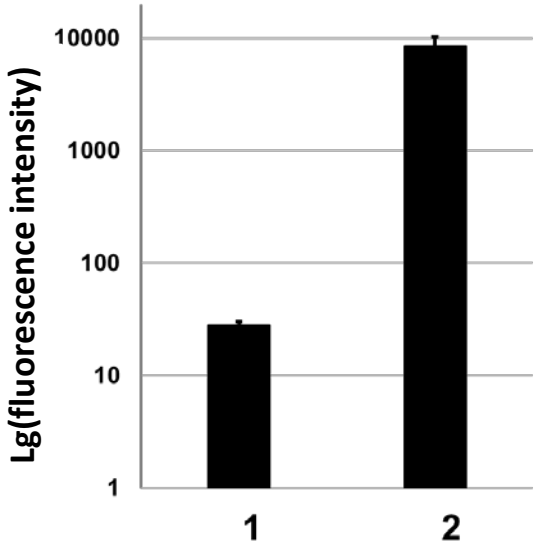
Results and discussion
CYP1A2 and CPR

7-Ethoxyresorufin-O-deethylation



7-Ethoxyresorufin-o-deethylation activity in a whole cell assay (OD_{578nm}40, 40h). 1: *E. coli* BL21 (DE3); 2: cells displaying CPR; 3: cells displaying CYP1A2; 4: cells displaying CYP1A2 and CPR. Resorufin concentrations were determined by HPLC in triplicates. The activity could be increased with an additional protein sequence ($G_4SGGS(G_4S)_3$) between passenger and linker[8].

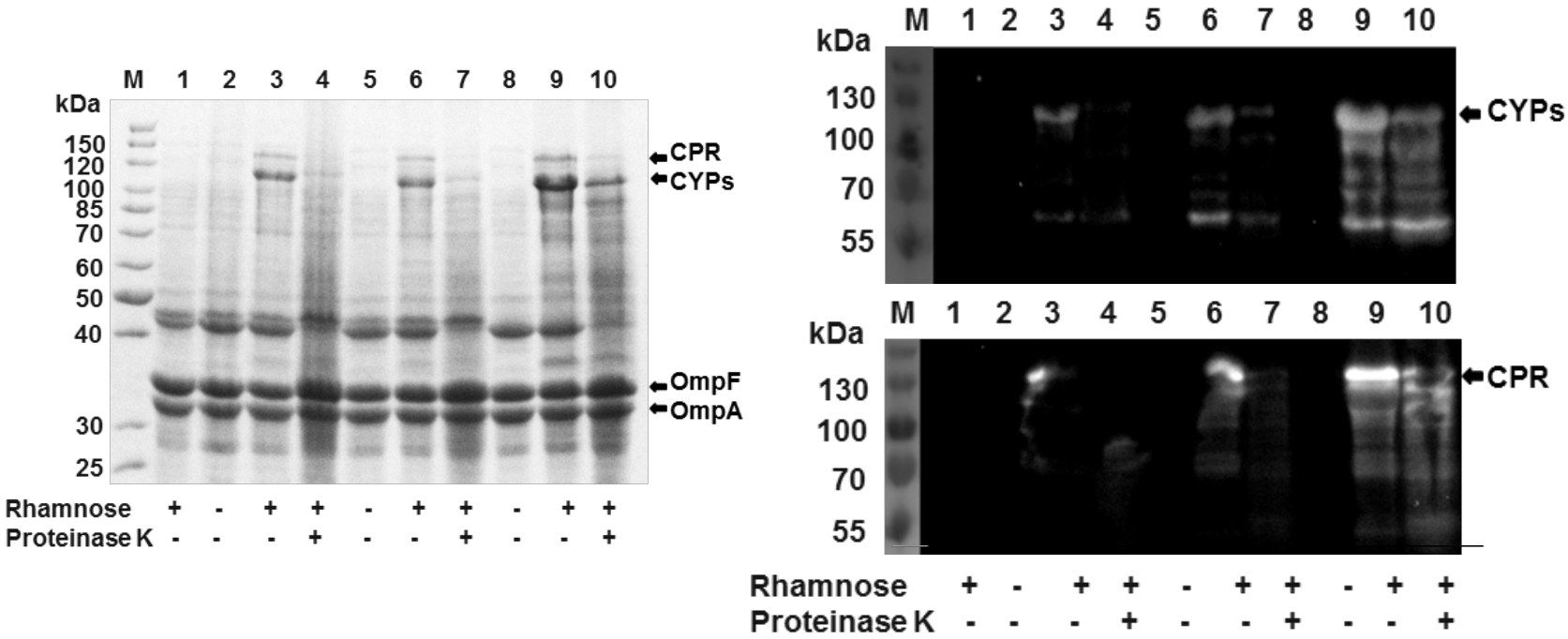
3-Cyano-7-ethoxycoumarin-O-deethylation



3-Cyano-7-ethoxycoumarin-O-deethylation activity in a whole cell assay (OD_{578nm}40, 1h). 1: *E. coli* BL21 (DE3); 2: cells displaying CYP1A2 and CPR. Fluorescence was measured with a microtiter plate reader at an emission wavelength of 460 nm.

[8]: Quehl, P. *et al*.: *BBB Biomembranes.* **2017**, 1859(1):104-116.

Results and discussion
Co-expression of CPR with CYP2C9; CYP2C19; CYP2D6



SDS-PAGE and Western Blot analysis of outer membrane protein preparations. 1: *E. coli* BL21 (DE3); 3: co-expression of CYP2C9 and CPR; 6: co-expression of CYP2C19 and CPR; 9: co-expression of CYP2D6 and CPR, lane 2,5 and 8: outer membrane protein preparations of non-induced co-expression; 4, 7 and 10: proteinase K digest as proof of surface accessibility. For the upper Western Blot the outer membrane protein preparations were treated with a primary monoclonal anti-myc antibody and a secondary HRP conjugated anti-IgG antibody. For the lower Western Blot the samples were treated with a primary monoclonal anti-CPR antibody and a secondary HRP conjugated anti-IgG antibody.

Conclusions

- Surface display of CYP3A4, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 on the surface of *E. coli.* is proven via flow cytometry analysis and/or protease K accessibility assay
- For CYP1A2, CYP2C9, CYP2C19 and CYP2D6, the co-expression with CPR was successful, so no external electron supplying enzyme is necessary.
- Activity of surface displayed CYP3A4 was proven using testosterone as a substrate in an HPLC-based assay.
- The functional interaction between surface displayed CPR and CYP1A2 was shown with 7-ethoxyresorufin and 3-cyano-7-ethoxycoumarin as substrates.
- These five important human CYPs with catalytic activities on the surface of bacterial cell surface could provide a convenient platform for the in *vitro* simulation of drug metabolism.

Acknowledgments

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