Energy-coupling factor transporters: exploration of the mechanism of vitamin uptake and inhibitory potential of novel binders
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Summary and Future Perspectives

Energy-coupling factor (ECF) transporters are a group of membrane proteins within the family of ATP-binding cassette (ABC) importers that couple the uptake of substrates across the membrane by harnessing the free energy from ATP hydrolysis\(^1\)-\(^6\). The two ATPase subunits found in ECF transporters (often heterodimers of EcfA and EcfA') are connected to a transmembrane domain, EcfT to form a three-subunit complex (ECF-module). This ECF-module activates another transmembrane domain named S-component that translocates the substrate from the environment into the cytosol. A common property of the S-components is their high affinity for transported compounds, with dissociation constants in the low to sub-nanomolar range to scavenge the substrate: water soluble vitamins (folate, riboflavin, cobalamin, biotin, niacin and thiamine), including their precursors; metal ions (for example Ni\(^{2+}\), Co\(^{2+}\) and heme); amino acid (tryptophan) and a precursor of methionine\(^2\)-\(^5\),\(^7\)-\(^14\). Those vitamins and minerals are micronutrients that are essential components of the diets, however needed in a small amount, and most of those human vitamins are also vital for the bacteria.

Like other ABC importers, the ECF transporters are found mostly in prokaryotic microorganisms. They are particularly abundant in the Firmicutes phylum of Gram-positive bacteria, where many members are human pathogens\(^15\). However, they have never been identified in humans. Because of the important functions, properties and availability of these transporters in pathogenic bacteria, the transport mechanism of ECF transporters may yield potential insights to develop specifically novel antibiotics because.

Here, we investigated B-type vitamin transporters from the energy-coupling factor (ECF) family. For my PhD project, I started with the development of the antibacterial agents that bind to the S-component of the energy-coupling factor (ECF) transporter for thiamine transport in, ThiT from *Lactococcus lactis* (Chapter 2). In this project, structure-based design was used to generate thiamine derivatives, which turned out to be not suitable to produce potential drugs\(^{16\text{-}18}\). To obtain new extended thiamine derivatives, we used a dynamic combinatorial chemistry (DCC) for fragment growing, maintaining a deazathiamine moiety. An acylhydrazone motif was used as a linker along with an aldehyde, and eight hydrazides were selected to form a small pre-equilibrated dynamic combinatorial library of eight acylhydrazones. To evaluate the quality of the binders, we determined the binding affinity by isothermal titration calorimetry (ITC). The binding was weak (\(K_D\) values in the micromolar range) compared to the previous derivatives that showed \(K_D\) values in the nanomolar range.

In an *in silico* study, we assessed the druggability of an ECF transporter for folic acid (ECF-FolT2) from *L. delbrueckii* (PDB code 5JSZ)\(^2\) (Chapter 1). This protein was selected because the crystal structure had the highest resolution at that time. By aligning sequences of the proteins from seven pathogens (*S. aureus*, *S. pneumoniae*, *E. faecium*, *E. faecalis*, *C. tetani*, *C. novyi*, and *C. difficile*), we considered them promising drug targets from the *in silico* point of view with a reasonable volume and sequence conservation. The results revealed potentially new druggable pockets which are located within EcfT and EcfA. We also analyzed the presence of *de novo* biosynthetic routes for the B-type vitamins in question.
for the above pathogens to confirm that this target class holds promise for the discovery of antimicrobial
drugs possibly on a broad-spectrum level.

The small binders described in a previous study\textsuperscript{16–19} and Chapter 2 have been developed for the target
S-component ThiT from \textit{Lactococcus lactis}. However, the compounds could not be promising candidates
for starting the development of new antibiotics, because the $K_D$ values did not always correlate very well
with the experimental values. Based on the in silico study in Chapter 1, new hits for the ECF transporters
were developed to increase the chance of being specific for a group II of the ECF transporters that was
extensively discussed in Chapter 3. In the group II, various S-components with different substrate
specificity are able to bind the same ECF module. Therefore, the small-molecule binders would not
only inhibit folate uptake by ECF FolT2, but also the uptake of other vitamins by other ECF transporters
present in \textit{L. delbrueckii}. Here, the so-called P2 pocket situated between coupling helices of EcfT was
selected. In this way, movement of the coupling helices could inhibit the FolT2 from dissociating from
the ECF module and this might abolish transport activity if a small molecule can be found to bind in
this pocket. The first class of compounds was virtually screened and tested using a transport assay with
radiolabeled folate resulting in a promising compound 1 ($IC_{50} 282 \mu$M). The potency and drug-likeness
of 1 encouraged us to identify structural features using structure–activity relationship (SAR) study.

At the first stage of SAR study, we explored the role of 2-hydroxybenzoic acid found compound 20
with a sterically bulky carbamate moiety that inhibited ECF activity by a 2-fold increase compared to 1 (20, $IC_{50} = 134 \mu$M). The next stage, we investigated the role of the naphthalene ring. However,
we were just able to synthesize two compounds (22 and 23) when an ether linker presents between
the 2-hydroxybenzoic acid and the naphthalene ring. With Br-substituted naphthalene ring (22), the
potential inhibition improved by four times ($IC_{50} 69 \mu$M) and it is the best inhibitor up to date.

Given by the inhibitory improvement by these naphthalene ring modifications, we sought to find more
reliable linkers to be synthesized. Three different linkers of analogues were obtained: amine, amide and
sulfonamide, respectively compounds 24, 25 and 26. However, only compound 24 had an inhibitory
effect albeit less potent than hit 1. Despite compound 24 being a weak inhibitor, we made use of its
anallogues to explore the role of naphthalene ring in the hit 1. Unfortunately, up to date, we did not get
any better potential inhibitor than compound 22.

Not only small binders that were potentially used to develop novel antibiotics against the ECF transporters,
but also small-protein binders were generated \textit{in vivo} (nanobody) against ECF-PanT (Chapter 4). In
this subproject, I aimed to catch novel conformations of the ECF transporters potentially showing new
insight of mechanistic transport. By immunizing a llama with the antigen of ECF-PanT, in a membrane-
reconstituted state, we obtained 40 unique Nbs that were classified into 21 families based on the
sequence in the so-called CDR3 region. Using co-elution in gel filtration chromatography experiments,
we characterized the binding of all nine Nbs, not only to ECF-PanT, but also to ECF-FolT2 or ECF-
Pdx, which have identical ECF modules as ECF-PanT but different S-components. Furthermore, the
inhibitory effect of the Nbs on the transport activity of the ECF transporters (ECF-PanT and ECF-FolT2)
was also investigated and resulted in a complete inhibition performed by Nb 86. Three of nine Nbs (69, 81 and 86) were assessed further for their affinities of binding, showing excellent affinity ($K_D$ in the
low nanomolar range) for all of them. In addition, one of the nanobodies (Nb 81) was successfully co-crystallized with ECF-PanT yielding a structure with 2.8 Å resolution as described further in Chapter 5.

Diving into the translocation mechanism of ECF transporters may potentially help to develop the novel antibiotics. Therefore, during my PhD, an effort was also made to show the transport mechanism which was discussed extensively in chapter 5. Here, the unusual transport mechanism of group II transporters with a shared ECF module was reproduced in an in vitro system instead of in vivo that was revealed first by Henderson et al.¹. Using the proteoliposomes system, we show that the S-components FolT2 and PanT associating with, and dissociating from the identical ECF module showing a dynamic composition of the ECF transporter subunits. The exchange is part of the mechanism of translocation and the transport kinetics behavior suggests that the substrate association with FolT2 is much slower than with PanT. The differences of those kinetics could be be explained from a structural viewpoint. The crystal structures of the full complex ECF-FolT2 with the apo S-component in an inward-open (post-release state) showed a substantially different structure of the S-component compared with folate-bound FolT1, which may explain the slow binding of folate. In contrast, in the crystal structure of nanobody-bound ECF-PanT, which was also is interpreted as a post-release state, smaller conformational changes are expected upon pantothenate binding to PanT than folate binding to FolT2. It could be explained from the structural information. First the binding pocket in the PanT is more occluded (loops L1 and L3 not being splayed out as far as in the ECF-FolT2 structure). Second, the binding site geometry of the apo state suggests that only minor rearrangements are needed for pantothenate binding.

Remaining Questions

This thesis provides small molecule binders of ECF transporters (chapter 2 and 3). As discussed above, the binders in chapter 2 designed to bind the S-component were not promising to develop as antibiotics candidates, and in chapter 3 we moved to the new strategy to find new binders that potentially interact with the ECF module instead of the S-component. Some candidate compounds were obtained by showing inhibitory effect and the best one had an IC₅₀ value of ~69 µM. However, we never have biophysically proved using ITC (isothermal calorimeter), spectroscopy, crystallography or single molecule cryo-EM (electron microscopy) that the compound really binds to the ECF module.

In chapter 4, an effort was made to raise small protein binders (nanobodies). However, not all nanobodies were yet characterized to find the unique ones that help fundamental experiments to show mechanistic action of the ECF transporters. One of the nanobodies (Nb81) seems potentially promising for the exploration of ECF transporters properties because it fully inhibits the substrate transport to the level of background. One of the powerful strategies to use is structural study (crystallography or single-molecule cryo-EM) aiming to trap the ECF transporters in a novel conformation.

In chapter 5, further experimental work is still needed to test if the excess of FolT2 molecules in the co-reconstituted system (Compare Figure 1 with Figure 3a and Figure 4) might cause bilayer imperfections,
which facilitate toppling\textsuperscript{20} thus leading to increased transport rates. In addition, we also speculated there would be smaller conformational changes upon pantothenate binding to PanT than folate binding to FolT2 based on the comparison crystal structures among ECF-FolT2 (5JSZ)\textsuperscript{2}, FolT1 (5DOY)\textsuperscript{3} and ECF-PanT. To test this hypothesis, we need to show a substrate-bound structure of solitary PanT which might best be done using a crystallography approach.

Finally, another big question still remaining is the uncertain complete mechanism of the ECF transporters. More experimental work is needed to elucidate the steps of transport. Here, structures of new conformations are expected to provide insight, such as an outward-open state, the ECF module alone without attached S-component, or the complex with the closed conformation of ATPase subunits.