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

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Energy-coupling factor transporters:
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potential of novel binders

Inda Setyawati

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university of
 groningen

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

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 18 June 2021 at 9.00 hours

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*Dedicated to my beloved parents,
My lovely husband,
My dearest son, and
My sisters*

"When I travelled the world over in search of what I
needed, and returned home to find it."
-George Moore-

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Scope of This Thesis

This thesis presents investigations of small molecule ligands that inhibit vitamin uptake by Energy Coupling Factor (ECF) transporters, which form a subclass of ATP-binding cassette (ABC) transporters. The long-term vision is that these molecules may lead to development of novel antibiotics with an unrivalled mechanism of action. In addition, I explored the mechanism how vitamins are translocated across a membrane bilayer via these transporters. For these goals, the ECF transporters from *Lactococcus lactis* and *Lactobacillus delbrueckii* specific for thiamin, folate and pantothenate were studied using biochemical, biophysical and structural biological techniques.

Chapter 1 introduces the family of ECF transporters. It provides information on their abundance among microorganisms, substrate specificity, three-dimensional structure and mechanism of action. Furthermore, the potential of ECF transporters as targets for new antibiotics is discussed.

In Chapter 2 the identification of small molecule ligand for the substrate binding subunit (S-component) ThiT from *L. lactis* using a dynamic combinatorial library (DCL) is presented. ThiT was chosen for this work for a continuity of the previous studies that led to the discovery of binders based on structure-based design approaches. This is the first application of dynamic combinatorial chemistry (DCC) used for fragment growing to an ill-defined pocket and also the first report in which an integral membrane protein as the target. Six acylhydrazone binders were synthesized and we determined the binding affinity by isothermal titration calorimetry (ITC). These binders have K_D values in the micromolar range and are considered as weaker binders compared to the similar compounds that showed K_D values in the nanomolar range.

Chapter 3 describes virtual screening, design, synthesis and structure–activity relationship (SAR) of a first class of small inhibitors based on the X-ray crystal structure of the folate-specific transporter ECF-FolT2 from *L. delbrueckii*. As discussed in chapter 2, small molecule agents have been developed for the S-component ThiT from *L. lactis*. Albeit, no binder has been a promising candidate for developing new antibiotics. In this chapter active agents were designed against the ECF module instead of the S-component. Since the ECF FolT2 is the group II ECF transporter in which distinct S-components with different substrate specificity interact with the same ECF module, the binders would not only inhibit folate transport by ECF FolT2, but also the transport of other vitamins that make use of different S-components, but the same ECF module in *L. delbrueckii*. In this way, the inhibitors would form an interesting scaffold for the development of novel antibiotics. As the result, the optimized compound opens up the possibility to use this chemical class to investigate the role of the ECF transporters in health and disease since it has been tested against *Streptococcus pneumoniae* with an MIC value of 2 µg/mL.

In Chapter 4, small-protein (nanobody) binders were generated *in vivo*, in contrast to the *in vitro* approaches used in chapter 2 and 3. Different from chapter 2 and 3, the binders described in this chapter would not be used for antibiotic activity, yet to catch novel conformations of the ECF transporters potentially showing the mechanistic action of ECF transporters. Here, an effort was made to raise and

characterize nanobodies against the pantothenate-specific ECF-PanT from *Lactobacillus delbrueckii*. The nanobodies (Nbs) were generated by immunizing a llama with the ECF-PanT reconstituted in liposomes. We obtained 40 unique Nbs, however currently only nine were assayed that showed their association 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. Three of them were investigated further for their binding affinities showing excellent affinity (K_D in the low nanomolar range). One of the nanobodies (Nb 81) has been extensively characterized and co-crystallized with ECF-PanT which is further discussed extensively in **chapter 5**.

In **chapter 5** an effort was made to show intricate kinetics behavior of group II ECF transporters (ECF-Fol2 and ECF-PanT) observed *in vivo* can be reproduced in an *in vitro* system. Specifically, the both ECF transporters were co-reconstituted into proteoliposomes, and assessed for kinetics of transport for the respective substrates. The results show exchange of S-components is part of the mechanism of substrate translocation and suggest much slower substrate association with FolT2 than with PanT. Using one of the Nbs (Nb 81) discovered in **chapter 4**, we successfully crystalized ECF-PanT and solved a structure. Comparison with ECF-FolT2 revealed more substantial conformational changes upon binding of folate than pantothenate, which could explain the kinetic differences.