1. Introduction

Vitamins are essential organic components of the human diet. According to their solubility, they can be classified into two categories: water-soluble (B1, B2, B3, B5, B6, B9, B12, C, and H) and fat-soluble (A, D, E, and K). Vitamin deficiency leads to a wide variety of diseases. For example, folic acid (B9) deficiency can result in macrocytic anemia, cardiovascular disease, birth defects, carcinogenesis, muscle weakness, and Alzheimer’s disease.[1] Most human vitamins are also essential for bacteria. Whereas many bacterial species can synthesize them de novo, it is not uncommon for prokaryotes to be auxotrophic for one or more vitamins, which makes them dependent on uptake from the environment.

For instance, organisms such as *Lactobacillus casei*,[2] *Pediococcus cerevisiae*, and *Enterococcus faecium*,[3] which do not encode key enzymes for the de novo biosynthesis of folic acid, must acquire folate from external sources. Specifically, in this review, we will address B-type vitamins, which mostly function as precursors for enzymatic cofactors. In addition, selected pathogens will be evaluated in silico for their ability to obtain vitamins either by de novo biosynthesis or by utilizing dedicated membrane transporters.

To tackle infectious diseases, especially in light of the problem of antimicrobial resistance (AMR), it is urgent to find new antibiotics against unexplored drug targets with a novel mode of action.[4] Several antibiotics on the market or in development target enzymes involved in the de novo biosynthesis of cofactors: Pantothenate kinase, for instance, which is involved in the biosynthesis of coenzyme A, lumazine synthase, which regulates the biosynthesis of riboflavin, adenylytranferase, nicotinamide adenine dinucleotide synthetase, and nicotinamide adenine dinucleotide kinase, which are involved in the biosynthesis of NAD(P).[5] Additionally, dihydrofolate reductase (DHFR) and dihydropterate synthetase (DHP) are well-known and studied targets in folic acid biosynthesis and a plethora of currently used antibiotics target them. For instance, sulfamethoxazole, sulfanilamide, and dapsone are DHFS inhibitors,[6] while methotrexate and trimethoprim are selective inhibitors of DHFR.[7]

Besides the inhibition of vitamin biosynthesis, interfering with their import may also reduce their availability for pathogens, but this field still remains unexplored from a medicinal point of view. Here, we will evaluate the pharmacological importance of the vitamin transporters of the energy-coupling factor (ECF) family.
2. Energy-Coupling Factor and Adenosine 5′-Triphosphate-Binding Cassette Transporters

Adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters constitute the largest superfamily of membrane transport proteins and are found in all prokaryotic and eukaryotic species.\textsuperscript{[8–10]} ABC transporters mediate uptake or extrusion of compounds from cells and organelles. Recently, a subclass has emerged, which is present only in prokaryotes and is used for the salvage of B-type vitamins as well as nickel and cobalt ions and a few other micronutrients. This subclass of transporters was named as ECF transporters in 1979, in a study of folate, thiamine, and biotin transport by \textit{L. casei}.\textsuperscript{[11]} The term “ECF” refers to the presence of a shared protein component that is required for coupling of ATP hydrolysis to the uptake of different vitamins (folate, thiamine, and biotin). At the time, the identity and the function of the postulated common component were unknown. Only in 2009, Rodionov et al. performed a genomic analysis in which the identity of the ECF transporters was revealed.\textsuperscript{[12]} ECF transporters are expressed in a great number of bacterial species, in particular among Gram-positive bacteria, many of which are pathogens. This finding opened a new avenue for the exploration of vitamin transporters as novel drug targets.

All ABC transporters have a characteristic architecture with two subunits or domains that are embedded in the lipid bilayer, which together form a pore, and two peripheral ATPase subunits or nucleotide-binding domains (NBDs).\textsuperscript{[9]} Prokaryotic ABC transporters that mediate the import of nutrients often employ a specific substrate-binding protein (SBP) displaying high substrate specificity, which either resides in the periplasmic space (in Gram-negative bacteria) or is tethered to the membrane via a lipid or protein anchor. ECF transporters do not make use of an extracytoplasmic SBP but instead use an integral membrane protein, called S-component.\textsuperscript{[12]} The S-component interacts with a ternary complex (the ECF module), which consists of two ATPase subunits (EcfA and EcfA′), or A-components) and an integral membrane protein (EcfT or T-component), which is characteristic for ECF transporters.

ECF transporters are classified into two groups.\textsuperscript{[12]} Group-I consists of a “dedicated” ECF module that interacts only with a single S-component. The genes encoding the subunits of group-I ECF transporters are encoded by the same operon. The biotin transporter (BioMNY)\textsuperscript{[13]} and cobalt transporter (CbiMNQO)\textsuperscript{[14]} from \textit{Rhodobacter capsulatus} are examples of ECF transporters that belong to group-I.

In group-II, the genes that encode multiple S-components are not located in the same operon as those encoding the ECF modules but are usually scattered across the chromosomes. The S-components from group-II ECF transporters compete with each other for binding to the ECF module (Figure 1).\textsuperscript{[11,13]} The thiamine and folate transporters ECF-ThiT and ECF-FolT found in \textit{Lactococcus lactis}\textsuperscript{[12]} as well as the first ECF transporters for which crystal structures were solved (ECF-FoIT and ECF-HmpT in \textit{Lactobacillus brevis}) belong to group-II.\textsuperscript{[16,17]}

2.1. Structural Features of ECF Transporters

While the NBDs are highly conserved in structure and sequence between transporters that belong to the ABC transporter superfamily, including those of the ECF transporters, the membrane subunits display substantial differences (Figure 2).\textsuperscript{[17–19]}

The two NBDs transform the free energy provided by the hydrolysis of ATP into work, enabling the transport of substrates into cells. They display twofold (pseudo) symmetry and two ATP-binding sites are located at the interface of the monomers.\textsuperscript{[20,21]}

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The trans-membrane domain of ECF transporters, which connects the two NBDs with the S-component, is called EcfT. The interactions between the cytoplasmic NBDs and EcfT are mediated by two long α-helices of EcfT protrude from the membrane on the cytoplasmic side and form an X-shape (Figure 2). The function of these two coupling helices is likely to transfer conformational changes triggered by ATP hydrolysis in the ATPases to the S-component. Amino acid sequences of EcfT proteins are highly diverse; they can, however, be identified by two short conserved Ala–Arg–Gly motifs. These motifs are located in the two X-shaped helices of EcfT at the C-terminal ends. One of the motifs interacts with EcfA, and the other with EcfA′. Hence, these conserved Ala–Arg–Gly residues act as anchors. Although, the T-component usually contains five membrane-spanning α-helices, this number ranges from four to eight membrane-spanning α-helices in some homologues.

2.1.2. S-Component

The second transmembrane subunit of the ECF transporters is the S-component (EcfS). S-components are small integral membrane proteins, usually \( \approx 20 \) kDa. Although S-components for different substrates share the same 3D conformation, their sequence is not well-conserved. Most of them consist of six hydrophobic α-helices. Loop L1 connects helices 1 and 2, and loop L3 bridges helices 3 and 4. These loops cover the substrate-binding pocket, shielding it from surrounding solvent upon substrate binding.

2.2. Mechanism of Transport

The transport mechanism used by ECF transporters is proposed to be different from any other transport protein, but a complete picture is still lacking. We will discuss the model presented in Figure 3 but other models have been proposed. The S-component binds the transported substrate with high affinity from the extracellular environment. Subsequently, the substrate-bound protein is proposed to “topple” in the membrane as a compact body. During the toppling movement, the substrate-binding site reorients from
Figure 3. Possible mechanism of transport of group-II ECF transporters. 1) ATP binding releases the empty S-component, leading to 2) separation of the ECF module and the S-component oriented in an outward-facing state. 3) The S-component binds its transported substrate. 4) The substrate-bound S-component topples over in the membrane. 5) The toppled S-component interacts with the ECF module, releasing the substrate into the cytoplasm.

the extracellular side of the membrane to the cytoplasmic side. Several membrane-spanning helices end up parallel to the plane of the membrane.

Possibly, substrate-loaded S-components can topple over in the lipid bilayer without support from the ECF module or ATP hydrolysis (step 4, Figure 3), but this issue is debated. This spontaneous toppling may be possible because the substrate-loaded S-components form compact structures without exposing charged residues to the outward face of the membrane. In contrast, the apo S-component cannot topple over spontaneously because its open loops L1 and L3 expose charged and hydrophilic residues.

The toppled S-component interacts with a hydrophobic binding platform on the T-unit, which breaks the high-affinity binding of the substrate and promotes release of the substrate into the cytoplasm (step 5, Figure 3). ATP binding is necessary to reorient the S-component and to dissociate the protein from the complex (at least in group-II transporters) so that it can start a new round of transport (steps 1 and 2, Figure 3).

The ATPases of the ECF transporters can be in a closed (tightly packed) conformation and an open one (partially dissociated). Closing is promoted by binding of ATP molecules, which interact with both NBDs. The closed conformation of the ATPases presumably pushes the C-terminal ends of the X-shaped helices in the EcfT subunit toward each other. After hydrolysis, the dimer is destabilized by releasing phosphate and ADP, which allows the NBDs to partially dissociate again. The chemical energy released from the hydrolysis of ATP can be transmitted via the EcfT subunit to the S-component to promote alternating access of the substrate-binding site. The S-component does not interact directly with the two ATPase units.

The proposed mechanism is based on the evaluation of crystal structures of ECF FolT2 (the group-II ECF transporter for folate from Lactobacillus delbrueckii) in the apo-state, a complex of the ECF FolT2 with AMP-PNP, a complex of FolT1 (an S-component from the same organism) with folate, and functional data from several publications. However, crystal structures of ECF transporters with the ATPases in a closed, tightly packed state have not yet been reported. An important question that still needs to be addressed is in which conformational state the ECF module interacts with the S-component. It needs to be demonstrated whether the toppling of the S-component might depend on the hydrolysis of ATP as it has been postulated by alternative models.

3. Decrease of Vitamin Uptake: a New Antimicrobial Approach?

Vitamins are essential for the survival and growth of many bacteria. Decreasing the intracellular concentration could be an attractive strategy to attenuate growth of infectious bacteria. Vitamins can be obtained through de novo biosynthesis and/or via uptake from the environment by membrane transporters. In bacteria that use only one of these processes, it is expected that
inhibition of the relevant process will prevent growth. However, in the case that bacteria have more than one way to obtain these micronutrients, the expected physiological outcome of inhibition of one of the routes is difficult to predict.

Several research groups demonstrated the vital role of vitamins in different species by adopting a number of approaches:

Zachary et al. investigated how the growth phenotype of Bacteroides thetaiotaomicron is influenced in thiamine-acquisition mutants.\[31\] *B. thetaiotaomicron* is a Gram-negative bacterium that obtains thiamine both through biosynthesis and by using uptake mechanisms. Mutants with disturbed biosynthesis failed to grow in a thiamine-deficient environment. In order to examine the effect of the vitamin transporters on the growth of the bacteria, single and double mutants were generated. Since *B. thetaiotaomicron* utilizes an inner (pnuT) and outer (OMthi) transporter to take up thiamine, individual transporter deletions were made. Double mutants containing deletions of both pnuT and OMthi transporters are highly attenuated at thiamine concentrations of below 1000 nM, but no effect on the growth was observed when only one of the transporters was lacking. Mutants lacking both the biosynthesis and one transporter (pnuT or OMthi) exhibit severe defects in growth at thiamine concentrations below 100 nM.

In contrast, the growth of Vibrio cholerae was unaffected when the transport of riboflavin was disrupted.\[32\] *V. cholerae* is a highly pathogenic Gram-negative bacterium that is known to utilize both transporters and biosynthesis to maintain its riboflavin homeostasis. Flores et al. demonstrated that upon deletion of ribN, a gene encoding a riboflavin transporter, no growth defects were observed compared to the WT. However, upon deletion of ribD, an essential gene for the biosynthesis of riboflavin, the strains were dependent on the presence of external riboflavin. Bacteria bearing a deletion of ribD or ribN faced a difficulty to compete against the WT when growing in river water. On the other hand, ribN mutants could compete against the ΔribD strain; this effect was reverted when an excess of riboflavin is added to the water.

Fuller et al. demonstrated for the first time that riboflavin-auxotrophic mutants can lead to the attenuation of a bacterial pathogen in its natural host. Specifically, a mutation was introduced in *Actinobacillus pleuropneumoniae* in a bifunctional enzyme containing guanosine triphosphate cyclohydrolase (GTPCH) and 3,4-dihydroxy-2-butanoate-4-phosphate synthase suppressing de novo biosynthesis of riboflavin.\[33\] *A. pleuropneumoniae* is a Gram-negative bacterium, which belongs to the family of Pasteurellaceae, and is the causative agent for acute necrotizing hemorrhagic bronchopneumonia in pork. This strain was unable to grow in the absence of exogenous riboflavin. Experimental infection studies in pigs demonstrated that the riboflavin-requiring mutant was unable to cause the disease on the basis of mortality, lung pathology, and clinical signs, at dosages as high as 500 times the LD_{50} for the wild-type.

Following a similar approach, Ahmed et al. immunized adult rabbits and monkeys from lethal shigellosis by oral administration of a thiamine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y.\[34\] A mutant strain was created in which thyA, the gene responsible for the first step in the de novo biosynthesis of thiamine was deleted. The stable thiamine-auxotrophic cells were administered to rabbits and monkeys, none of which developed the regular symptoms attributed to *Shigella* infection.

A more comprehensive study that targets the flavin mononucleotide (FMN) riboswitch that controls riboflavin homeostasis in Gram-positive bacteria was published recently.\[35\] *Staphylococcus aureus* among other Gram-positive bacteria obtains riboflavin either from external sources or by de novo biosynthesis.\[36\] Wang et al. demonstrated that in an in vivo model, the bacterial burden within spleens of infected mice between MRSA COL ΔribD and MRSA COL strains showed no significant difference, indicating that inhibiting only the biosynthesis of riboflavin in MRSA is not enough to influence its growth in vivo. However, use of a small-molecule inhibitor of FMN riboswitches enabled the simultaneous inhibition of both riboflavin de novo biosynthesis (rib operon) and riboflavin uptake (ribU) in the same infectious setting. This dual inhibition led to a decrease of the bacterial burden within spleens of infected mice exhibiting an antibacterial ability, suggesting that in the case of *S. aureus*, an antibiotic effect can be achieved by disrupting the riboflavin homeostasis through simultaneous inhibition of both de novo biosynthesis and uptake of riboflavin.

Although, a direct target validation for ECF transporters has not been conducted yet, there are several reports suggesting they may indeed be promising antimicrobial targets. Following a target-identification strategy, it has been shown that a gene, which encodes an ECF transporter in *Streptococcus pneumoniae*,\[37\] is one of the essential genes for the survival of this bacterium. Furthermore, a targeted gene-disruption strategy was applied in which over 300 conserved genes were selectively disrupted using a selectable marker for drug resistance. These genes were previously chosen from a list based on common availability in at least two bacterial species (*Escherichia coli*, *Bacillus subtilis*, *E. faecalis*, and *S. aureus*) while, proteins with sequence identity of more than 30% compared to proteins form a yeast were excluded. As a result, 113 conserved essential genes were discovered, including “SP_2220” that belongs to the family ABC transporters and encodes a part of an ECF transporter.

Adopting a different approach, Schauer et al. demonstrated in knock-out studies that upon deletion of thiT (Imo1429), a gene which encodes the S-component for thiamine, the proliferation of *Listeria monocytogenes* is reduced.\[38\] *L. monocytogenes* is a foodborne pathogen with a high mortality rate that has emerged as a model for intracellular parasitism. The ability to replicate within host cells during the infection process is due to disruption of the phagosomal membrane leading to its escape from the vacuole into the cytoplasm. Since ThiC, a crucial enzyme for the biosynthesis of thiamine, has not been identified in the genome of *L. monocytogenes*,\[39\] de novo biosynthesis is not an option for obtaining this vitamin. Thus, the requirement for thiamine diphosphate (ThDP) can be satisfied by two alternative pathways: thiamine uptake or hydroxymethylpyrimidine (HMP) salvage coupled to hydroxethylthiazole (HET) de novo biosynthesis. The cytosolic replication of *L. monocytogenes* was reduced 3.3-fold and 2.2-fold upon deletion of thiD and thiT, which are responsible for HMP salvage and thiamine transport, respectively. In addition, it was reported that essential genes of the de novo biosynthesis of thiamine were upregulated in case of a lower concentration of and/or a higher demand for these substrates during replication within epithelial cells. As a result, ThDP appears to play a major role for the metabolism of *L. monocytogenes* under nutrient-inefficient conditions. Therefore, the availability of thiamine, its
precursors, and diphosphates is expected to have a significant effect on the in vivo replication of \textit{L. monocytogenes}.

In summary, these examples show that availability of vitamins plays a crucial role in the growth and survival of pathogens. As a result, decreasing the intracellular concentration of these micronutrients seems to be a viable approach to reduce their proliferation or abolish their pathogenicity in the infected host. The examples for \textit{S. pneumoniae} and \textit{L. monocytogenes} show that ECF transporters may be considered as important antimicrobial targets.

4. ECF Transporters and Pathogens

Different microorganisms seem to control homeostasis of vitamins in various ways. For example, \textit{S. aureus} relies on de novo biosynthesis of folic acid,\cite{40} whereas \textit{Clostridium tetani} has abolished or partially lost this ability.\cite{41,42} As a result it solely scavenges folates from an exogenous source. On the other hand, a large number of pathogens such as \textit{E. faecalis}\cite{43} and \textit{C. botulinum}\cite{44} are capable of both de novo biosynthesis and uptake of thiamine and pantothenate, from an enriched nutrient environment, respectively.

The uptake of nutrients usually requires much less energy than the use of de novo biosynthesis. For instance, the complete synthesis of 1 mole of riboflavin requires 25 moles of ATP, whereas two moles of ATP, or even fewer depending on the transport system, are consumed for its transportation.\cite{45} As a result, microorganisms capable of expressing vitamin transporters presumably preferentially rely on the uptake of vitamins in a vitamin-enriched environment.

Among the \textit{firmicutes} species, a phylum of Gram-positive bacteria, the transport of vitamins across their membranes is often mediated by ECF transporters. This family of proteins is essential for pathogens such as \textit{L. monocytogenes}, \textit{Mycoplasma genitalium}, \textit{S. pneumoniae}, and \textit{S. aureus}. With the aim to highlight the importance of ECF transporters, we selected representative pathogenic organisms (\textit{S. aureus}, \textit{S. pneumoniae}, \textit{E. faecium}, \textit{E. faecalis}, \textit{C. tetani}, \textit{Clostridium novyi}, and \textit{Clostridium difficile}) and evaluated them in terms of their encoded ECF transporters for six B-type vitamins (folic acid, pantothenate, niacin, thiamine, riboflavin, and biotin) as their major transmembrane transport system for uptake, as well as their capability to obtain the vitamins through de novo biosynthesis. The selected bacteria are responsible for serious infectious diseases and some appear on the WHO priority list as the most problematic antibiotic-resistant organisms.\cite{46} In addition, several of these organisms are referred as “ESKAPE pathogens” (\textit{E. faecium}, \textit{S. aureus}, \textit{Klebsiella pneumoniae}, \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa}, and \textit{Enterobacter species}).\cite{47} These pathogens are known to cause serious nosocomial infections and are known for their mechanisms of drug resistance, leading to a reduced intracellular drug concentration (biofilm formation, efflux pumps, and porin loss).\cite{48,49}

4.1. \textit{Staphylococcus aureus}

\textit{S. aureus} is a Gram-positive coccus bacterium which forms characteristic grape-like clusters. Although \textit{S. aureus} is part of the normal skin microbiota in humans and animals, it is responsible for numerous infections.\cite{50} In the past, infections caused by \textit{Staphylococcus} species responded well to penicillin treatment. However, excessive use of these antibiotics led to the emergence of penicillin-resistant strains, followed by methicillin-resistant strains.\cite{48,49} The methicillin-resistant \textit{S. aureus} (MRSA) is responsible for the death of approximately 19 000 people in the USA every year.\cite{51}

4.2. \textit{Streptococcus pneumoniae}

\textit{S. pneumoniae} is a ubiquitous human respiratory bacterial pathogen associated with pneumonia and meningitis.\cite{52} It is considered to be the most important bacterial cause of pneumonia and meningitis globally, responsible for an estimated 1 million deaths annually in children alone.\cite{53} Invasive pneumococcal disease (IPD) caused by \textit{S. pneumoniae}, represents a major clinical and economic burden. Despite the availability of the pneumococcal polysaccharide vaccine (PPV), the fatality rate for patients hospitalized with IPD has remained relatively stable at about 12% since 1952. It has been estimated that only in Europe the treatment of pneumonia costs $\approx$ 10 billion euro per year.\cite{54}

4.3. \textit{Enterococcus} Species

Another class of pathogens that are frequently resistant to a number of important antibiotics is the \textit{Enterococci}, which are Gram-positive facultative anaerobes. Although these organisms are constituents of the gut microbiota in humans, they are involved in serious nosocomial infections,\cite{55} especially in immunosuppressed patients.\cite{56} \textit{Enterococci} were formerly classified as part of the genus \textit{Streptococcus}. There are more than 20 \textit{Enterococcus} species, but \textit{E. faecium} and \textit{E. faecalis} are clinically the most relevant. Specifically, these two are reported to cause a variety of infections including urinary, soft-tissue, and bloodstream infections.\cite{57} In addition, as a part of the National Antimicrobial Resistance Monitoring System, a large number of clinical isolates of the mentioned pathogens (\textit{E. faecalis} [67.5%] and \textit{E. faecium} [53.7%]) are resistant to tetracycline, a broad-spectrum antibiotic.\cite{58}

4.4. \textit{Clostridium} Species

Bacteria of the genus \textit{Clostridium} appear to cause serious infections with a tremendous impact on the health-care system. Clostridial infections are characterized by a disease produced by toxins. Species of \textit{Clostridia} produce very large clostridial cytotoxins (LCCs).\cite{59} Tetanus and botulism are two examples of this type of infection, both caused by LCC. \textit{C. tetani} is a Gram-positive non-encapsulated obligate anaerobe that produces round terminal end spores in broth culture. Tetanus inhibits \gamma\textsubscript{-}aminobutyric acid (GABA) and glycine neurotransmitter release, causing spastic paralysis followed by respiratory failure and severe impairment of the autonomous nervous system.\cite{60}

\textit{C. novyi} is another example of an organism that produces LCC, also causing severe infections in injection-drug users.\cite{61} Furthermore, \textit{C. difficile} is considered to be responsible for...
a larger number of cases of nosocomial acquired antibiotic-associated diarrhea, causing an estimated 435,000 cases with 29,000 deaths yearly in the USA.\textsuperscript{61} From the economical point of view, \textit{C. difficile} causes an annual economic burden ranging from $436 million to $3 billion in the USA. Incidence and severity are increasing, which is in part associated with the emergence and prevalence of fluoroquinolone-resistant \textit{C. difficile} clones.\textsuperscript{62}

5. B-Type of Vitamins

In this section, we focus on six B-type vitamins: folate, pantothenate, niacin, thiamine, riboflavin, and biotin. The de novo biosynthesis of B-type vitamins in prokaryotes will be briefly introduced, limited only to the main routes that pathogens utilize to obtain these micronutrients.

5.1. Folic Acid

Folic acid (or vitamin B9, Figure 4) plays a major role in numerous biochemical reactions. Tetrahydrofolic acid (THF), a reduced analogue of folic acid is involved in a broad set of transformations ranging from the synthesis of purines, building blocks of RNA and DNA, and synthesis of thymidylate, to the remethylation of homocysteine to methionine. These biochemical transformations are classified as one-carbon (1C) metabolism, because of the transfer of one-carbon groups into biological molecules during anabolism. In addition, 1C metabolism is required for amino-acid homeostasis. Specifically, it functions as a regulator and sensor of the cell’s nutrient status through cycling of 1C-groups.\textsuperscript{63} In particular, the levels of three amino acids (methionine, serine, and glycine) are directly controlled by it.

De novo synthesis of folate was described by Bermingham and Derrick.\textsuperscript{64} The first step in folate synthesis is the formation a pterin ring from guanosine-5′-triphosphate (GTP) by GTP-cyclohydrolase I, followed by the production of 6-hydroxymethyl-7,8-dihydropterin diphosphate (DHPDP). A kinase, an aldolase and a pyrophosphokinase are the enzymes catalyzing these steps. Subsequently, the pterin moiety is linked with the paraaminobenzoic acid (PABA), by the formation of a C–N bond, catalyzed by the dihydropteroate synthase (DHPS) to produce dihydropteroate. Then, dihydrofolate (DFH) is synthesized by the coupling of a glutamate moiety to dihydropteroate accomplished by folylpoly-γ-glutamate synthetase (FGPS). Finally, DHFR transforms DHF into tetrahydrofolate.
5.2. Thiamine

Thiamine (or vitamin B1, Figure 4) plays a crucial role in various cell functions and it is a precursor for cofactors of enzymes involved in several metabolic steps,[65] including energy metabolism and degradation of sugars and other carbon-based molecules. ThDP, the phosphorylated form of thiamin, participates in the conversion of pyruvate to acetyl-CoA, where NAD+ is the oxidizing agent.[66] In addition, ThDP is crucial in several other functions, for instance, activating an anion channel of large-unit conductance in neuroblastoma cells and functioning in the intestinal lumen as phosphate ester forms.[67]

In E. coli, thiamine triphosphate (ThTP) is produced from thiamine monophosphate (ThMP) in a diphosphorylation by thiamine phosphokinase (ThiL).[68] Alternatively, thiamin phosphate synthase (ThiE) assembles ThMP de novo by coupling hydroxymethylpyrimidinediphosphate (HMP-DP) and hydroxyethylthiazole phosphate (HET-P). The two heterocyclic precursors, HMP-DP and hydroxypyruvate synthase (ThiE) assembles ThMP de novo by coupling hydroxymethylpyrimidinediphosphate (HMP-DP) and hydroxyethylthiazole phosphate (HET-P). The two heterocyclic precursors of ThDP, HMP-DP, and Thy-P, are biosynthesized through yethylthiazole phosphate (HET-P). The two heterocyclic precursors of ThDP, HMP-DP, and Thy-P, are biosynthesized through independent pathways. In E. coli, ThiMP-PP is produced from a precursor common to thiamine and de novo purine biosynthesis by ThiC,[69] whereas, Thy-P is synthesized in a series of steps catalyzed by ThiFSHG, ThiL, Dsx, and ThiW.

5.3. Pantothenate

Pantothenate (or vitamin B5, Figure 4) is the precursor of coenzyme A. Its name is derived from the Greek “παντοθενός” meaning “from everywhere” and small quantities of pantothenic acid are found in nearly every food. Due to the fact that it is a precursor of coenzyme A (CoA), it is considered indispensable for all living organisms.[69] The biosynthesis of CoA from pantothenic acid is an essential and universal pathway in prokaryotes and eukaryotes. CoA is assembled in five steps from pantothentic acid.[70] It plays a leading role in cellular metabolism and fatty-acid biosynthesis, and is involved in over 100 different reactions in intermediary metabolism.[71]

An intermediate from the biosynthesis of valine and one molecule of aspartate are combined to produce pantothenate de novo. This typical pathway is found in most bacteria, such as in E. coli, S. typhimurium, and C. glutamicum. In a first step, the aceotolactate isomeroreductase (IlvC) and the ketopantoate reductase (PanE) transform -acetolactate to -ketoisovalerate.[72] Next, the enzyme ketopantoate hydroxymethyltransferase (PanB) transforms -ketoisovalerate to -ketopantoate. The enzymes, PanE and IlvC reduce the -ketopantoate to pantoate. In addition, an aspartate is decarboxylated to yield -alanyl. In the last step, -alanyl and pantoate are condensed to afford pantothenate.[73]

5.4. Niacin

Niacin (or vitamin B3, Figure 4) is the precursor of the coenzymes NAD+ and NADP, which play an important role in oxidative respiration as electron carriers.[74] NAD+ is also necessary in the catabolism of fat, carbohydrates, proteins, and alcohol, as well as in cell signaling and in DNA repair. On the other hand, NADP mostly participates in anabolic reactions such as fatty-acid and cholesterol synthesis.[75]

Nicotinic acid and nicotinamide are collectively termed niacin. Nicotinamide is converted to nicotinic acid in E. coli by nicotinamide homologue (PncA).[76] In prokaryotes, quinoline, a precursor of nicotinamide, is synthesized from aspartate and dihydroxycetone phosphate. However, recent findings suggest that some bacteria such as P. fluorescens, Cytophaga hutchinsonii, and Ralstonia metallidurans synthesize quinolines from tryptophan.[77] Specifically, they use an alternative pathway, which exists in eukaryotes.

5.5. Riboflavin

Riboflavin (or vitamin B2, Figure 4) participates as cofactor in redox metabolic reactions involving energy production from carbohydrates, fatty acids, ketone bodies, and proteins, as well as in amino acid and fat metabolism. The importance of riboflavin derivatives for mitochondrial function was demonstrated in the 1970s and 1980s in studies conducted in riboflavin-deficient animals. Additionally, riboflavin is necessary for the production of glutathione, which is a free-radical scavenger.[78]

Riboflavin is synthesized de novo from one molecule of GTP and two molecules of ribulose 5-phosphate. In B. subtilis, the biosynthesis is regulated by a rib operon encoding all the essential enzymes, namely pyrimidine deaminase/reductase (RibG), the - and -subunits of a riboflavin synthase, and GTP cyclohydrolase (RibA)/3,4-dihydroxy 2-butanone 4-phosphate (3,4-DHBP) synthase.[78]

5.6. Biotin

Biotin (or vitamin B7, Figure 4) plays an essential role in metabolism. It is a physiologically active cofactor for carbon dioxide carriers in numerous metabolic carboxylation, decarboxylation, and transcarboxylation reactions. The biotin biosynthetic pathway is widespread among microbes; B. subtilis and E. coli served as model system to elucidate the biosynthesis.

The pathway consists of two stages, the synthesis of the pemeloy-CoA precursor and an assembly of the bicyclic ring structure of biotin. In B. subtilis, the biosynthesis involves biol and bioW genes to produce pemeloy-CoA from pimelic acid (a seven-carbon β,ω-dicarboxylate), whereas in E. coli, pemeloy-CoA is synthesized from malonyl-CoA instead of pimelic acid. The pathway in E. coli uses products of bioF and bioW genes. The pathway of biotin assembly is similar in both organisms, involving products of the bioF, bioA, bioD, and bioB genes.[79]

6. Vitamins and Bacteria

As mentioned before, many bacteria and plants have the ability to utilize de novo biosynthesis to obtain vitamins. In contrast, humans merely depend on their uptake through food. This fact explains why the biosynthesis of vitamins has emerged as an effective and attractive target in drug discovery. The bacteria described in chapter four are considered the cause of several diseases with
an enormous economic burden. In addition, the development of AMR against last-line-defense antibiotics poses an increasing demand on discovering novel strategies to combat these infections.

In this context, we will evaluate how these bacteria (S. aureus, S. pneumoniae, E. faecium, E. faecalis, C. tetani, C. novyi, and C. difficile) are able to obtain their essential vitamins (folic acid, pantothenate, niacin, thiamine, riboflavin, and biotin) through biosynthesis.

We searched the genome of each bacterium for the existence of genes that encode proteins which are involved in key steps in the biosynthesis of vitamins by searching the KEGG database for signature enzymes that catalyze biosynthetic reactions for each of the mentioned vitamins. Furthermore, we summarize any experimental evidence whether or not each of these organisms relies on de novo biosynthesis to obtain the vitamins mentioned above.

Since not all the selected pathogens encode the same S-components, we focused our search only on the biosynthetic pathways to the vitamins for which S-components of group-II of ECF transporters are expressed in the respective bacteria (Table 1). For example, in S. aureus, only RibU, ThiW, the S-components for riboflavin and a thiamine precursor, respectively, and BioY, the S-component for biotin, are expressed. In E. faecalis all six S-components for vitamins and for a thiamine precursor are encoded (PanT, RibU, FoT, NiaX, ThiT, ThiW, BioY for pantothenate, riboflavin, folic acid, niacin, thiamine, a precursor of thiamine and biotin, respectively).

**Table 1.** Collection of pathogenic bacteria that have genes for expressing group-I and group-II ECF transporters; the ability to biosynthesize specific vitamins (folic acid, pantothenate, niacin, thiamine, riboflavin, and biotin) is indicated. S-components that have been experimentally verified to bind the indicated substrate are depicted in bold. Organisms with the ability to biosynthesize the respective vitamins are represented with (+), while those that cannot synthesize de novo are represented with (−). The experimental evidence is depicted in blue color, while the in silico evaluation is shown in red.

| No | Organism | ECF group-I | ECF group-II S-components | Biosynthesis |
|----|----------|-------------|----------------------------|--------------|
|    |          |             |                            | Folic acid  |
|    |          |             |                            | Panthenate  |
|    |          |             |                            | Niacin      |
|    |          |             |                            | Thiamine    |
|    |          |             |                            | Riboflavin  |
|    |          |             |                            | Biotin      |
| 1  | S. aureus| YkoEDC      | RibU, ThiW, BioY           | N/A         |
| 2  | S. pneumoniae| HisTUV YkoEDC | PanT, RibU, NiaX, QueT, PdxU, ThiW, BioY | N/A |
| 3  | E. faecium| QrtTUVW/HisTUV | PanT, QueT, PdxU, RibU, FoT, NiaX, ThiT, BioY | +/−[84] |
| 4  | E. faecalis| QrtTUVW/HisTUV | PanT, RibU, FoT, NiaX, ThiT, QueT, PdxU, ThiW, BioY | +/−[89] |
| 5  | C. tetani| CbiMNQOChrTUV | PanT, QueT, CbiT, PdxU, ThiW, TrpP, RibU, ThiT, NiaX, FoT, BioY | −/−[41,42] |
| 6  | C. novyi | CbiMNQO     | PanT, QueT, PdxU, TrpP, RibU, NiaX, ThiT, FoT, BioY | −/−[41,42] |
| 7  | C. difficile| CbiMNQOChrTUV | PanT, QueT, PdxU, TrpP, RibU, BioY | N/A |

6.2. *Streptococcus pneumoniae*

*S. pneumoniae* only encodes the S-components PanT, NiaX, RibU, and BioY for panthenate, niacin, riboflavin, and biotin, respectively (Table 1). According to the KEGG database, it encodes all the essential genes responsible for the de novo biosynthesis of niacin, thiamine, and riboflavin, but lacks more than one gene involved in the biosynthesis of pantothenate and biotin. Regarding the biosynthesis of thiamine, it has several essential genes: tenA1 and 2, thiM1 and 2, thiE, thiW, thiE2, and thiD. In detail, among the missing genes are panB and panC encoding 3-methyl-2-oxobutanoate hydroxymethyltransferase and pantotheta-alanine ligase, respectively. In an attempt to correlate these results with the experimental evidence, we found out that *S. pneumoniae* is able to grow in a chemically defined medium lacking niacin by upregulating putative genes from the biosynthetic route to niacin such as nadC. No experimental evidence concerning panthenate, riboflavin, and biotin biosynthesis is available.
6.3. Enterococcus faecium

*E. faecium* encodes all six S-components (FolT, PanT, NiaX, ThiT, RibU, and BioY) for the selected vitamins (Table 1). According to our search in KEGG database, this bacterium does not appear to have access to de novo biosynthesis of any of the six vitamins, because in each case it is missing one or more crucial genes for each biosynthetic pathway. Several genes are missing, which are involved in folic acid biosynthesis, namely: folE, phoZ, folB, folk, and folP. It is considered that *E. faecium* can only grow in presence of a folic acid derivative such as folic acid, pteroic acid, DHF, and leucovorin, enriched with serine, methionine, purines, and pyrimidines or grow slowly in a relatively large amount of thymidine.\(^{[86–88]}\)

According to the KEGG database, *E. faecium* does not encode PanB and PanC: two crucial enzymes for pantothenate biosynthesis. In addition, it does not grow in CDM without the presence of pantothenate indicating the essentiality of this vitamin.\(^{[88]}\)

As far as niacin biosynthesis is concerned, nadC is the missing gene. In the case of riboflavin, the whole biosynthetic arsenal is absent. Experimental evidence suggests that *E. faecium* can still grow in the absence of these two vitamins but only at a very low rate; the presence of both of them in the medium produces a stimulatory effect to the growth.\(^{[86]}\) Biosynthesis of thiamine is not possible according to the KEGG database, since at least three genes are missing, including thiC, thiE, and thiM. The essential genes (bioF, bioA, bioD, and bioB) involved in biotin biosynthesis are also absent in *E. faecium*. Nevertheless, this pathogen grows well and produces enterocin in CDM lacking biotin.\(^{[75]}\)

In other words, this pathogen may not depend on biotin for its growth.

6.4. Enterococcus faecalis

The pathogen *E. faecalis*, encodes all six S-components: FolT, PanT, NiaX, ThiT, RibU, and BioY (Table 1). Based on our in silico study using the KEGG database, the biosynthesis of folic acid and pantothenate is feasible for this pathogen, while de novo biosynthesis of niacin, thiamine, riboflavin, and biotin is not. *E. faecalis* lacks most of the essential genes for enzymes involved in riboflavin synthesis, among them ribA, ribD, ribH, and ribE, and at least two genes, nadC and nadA, from the niacin pathway. In addition, it lacks ThiC, the first enzyme involved in thiamine biosynthesis. As far as the first three vitamins (folic acid, pantothenate, and niacin) are concerned, the experimental results are in line with our in silico study, proving that *E. faecalis* grows in the absence of folic acid\(^{[89]}\) and has putative operons encoding enzymes for pantothenate biosynthesis\(^{[89]}\) while its growth in a CDM depends on the presence of niacin.\(^{[89]}\) Although it lacks the first enzyme in the biosynthesis of thiamine, it is still able to grow in a CDM without thiamine.\(^{[89]}\) Since the missing gene is at the very beginning of the pathway, this effect may be explained by the existence of a transporter for a precursor of thiamine. It was shown that in *E. faecalis*, riboflavin is biosynthesized in the presence of folic acid\(^{[92]}\) and no experimental result showed that biotin is essential for the growth of *E. faecalis*.

6.5. Clostridium tetani

In *C. tetani*, all six S-components (FolT, PanT, NiaX, ThiT, RibU, and BioY) are encoded (Table 1). According to the KEGG database, de novo biosynthesis of both thiamine and riboflavin are feasible for *C. tetani*, while folic acid, pantothenate, niacin, and biotin demands must be completely satisfied from an exogenous source. In more detail, this pathogen lacks the majority of the genes required for the biosynthesis of folic acid including folB, folK, folP, and folE, three genes from the biosynthesis of niacin, and at least three essential genes (panB, panC, and panE) for the biosynthesis of pantothenate. This bacterium does not contain bioF, bioA, and the genes to produce the pimeloyl moiety, all of which are involved in the biosynthesis of biotin. In this case, the experimental data are in excellent agreement with the results obtained from our in silico analysis. *C. tetani* is unable to grow in CDM lacking folic acid, pantothenate, niacin or biotin, respectively, while the absence of thiamine and riboflavin in the CDM does not arrest the cell cycle of the bacterium, still allowing a slow growth.\(^{[91,42]}\)

6.6. Clostridium novyi

*C. novyi* like *E. faecium*, *E. faecalis*, and *C. tetani* encodes all six S-components (FolT, PanT, NiaX, ThiT, RibU, and BioY) (Table 1). According to the KEGG database, it encodes all the essential enzymes for the de novo biosynthesis of pantothenate, niacin, thiamine, and riboflavin. In particular, it has access to all of the required enzymes for folic acid biosynthesis except PhoZ. Due to a lack of experimental evidence, it cannot be clarified if this pathogen is able to grow in the absence of folic acid. Since *C. novyi* misses only one enzyme for the de novo biosynthesis of folic acid, it is possible that this specific enzyme may have been overlooked in the genome sequence of this organism. In addition, this pathogen misses some essential genes for biotin synthesis just like *C. tetani*.

6.7. Clostridium difficile

*C. difficile* encodes only the S-components PanT, RibU, and BioY for pantothenate, riboflavin, and biotin, respectively (Table 1). The biosynthetic pathways of these micronutrients are available according to the KEGG database. This pathogen has all the essential genes to obtain these vitamins through de novo biosynthesis. In addition, experimental evidence seems to confirm our finding, given that the lack of riboflavin does not arrest the cell cycle of *C. difficile* in a CDM, while Miller et al. demonstrated that this pathogen has putative operons encoding the enzymes for pantothenate biosynthesis.\(^{[89]}\) However, the biosynthetic route to biotin is not available. Some essential genes are missing. In parallel, the experimental evidence seems to confirm this statement, as biotin is an essential vitamin for *C. difficile* growth.\(^{[90]}\)

Due to the fact that not all bacteria have access to de novo biosynthesis, we investigated the way in which these pathogens obtain vitamins, through either synthesis or by taking them up from the environment. Considering that all of the organisms mentioned utilize ECF transporters for the uptake of specific
vitamins, by inhibiting this transport mechanism, the uptake of various vitamins will be affected. For example, since \textit{C. tetani} expresses all six S-components, the inhibition of the ECF transporter may lead to a reduction of the concentration of all six vitamins inside the cell, of which only two can be obtained via biosynthesis.

7. Druggability Assessment

In order to evaluate the potential of ECF transporters as valuable antimicrobial drug targets, we performed in silico studies. Although, a direct target validation has not been reported so far, our findings indicate that these transporters may be broad-spectrum targets against several pathogenic bacteria.

The first step in this approach is the discovery of potentially druggable pockets. Due to the fact that only a handful of crystal structures of the whole ECF transporter is reported in the literature (Table S1, Supporting Information), we chose the X-ray crystal structure of ECF-FolT2 from \textit{L. delbrueckii} (PDB code 5JSZ) given that it has the highest resolution. In particular, this protein belongs to the group-II of ECF transporters and its S-component is dedicated to the uptake of folic acid. Second, we conducted a sequence-conservation study in specific organisms.

7.1. Druggable Pockets

DoGSiteScorer, a fully automated algorithm, enables identification of pockets and prediction of their druggability. Druggability is the likelihood of finding a selective, low-molecular weight molecule that binds with high affinity to the target.\textsuperscript{[93]} The program provides a druggability score from 0 (undruggable) to 1 (druggable). Druggable pockets tend to be larger, deeper, more hydrophobic, and complex in shape. The geometric and physicochemical properties, such as volume, surface, lipophilic surface, and depth are calculated for the predicted pockets by DoGSiteScorer. For the ECF-FolT2 X-ray crystal, DoGSiteScorer identified 36 pockets of which twelve are considered druggable (Table S2, Supporting Information; \textbf{Figure 5}). In addition, we used CHIMERA software to illustrate the figure.\textsuperscript{[94]}

We filtered the identified pockets by applying a threshold of \textgreek{z}0.5 based on their “Drug Score” feature (Table S2, Supporting Information). Nine of the twelve pockets identified are found at the interface of two or three out of the four subunits that constitute the whole complex of the ECF-FolT2 transporter: P3 (orange), P4 (magenta), and P9 (firebrick) located between EcFT and the S-component; P2 (yellow) and P8 (purple) between EcFT, EcFA’, and the S-component; P5 (magenta) between EcFT and EcFA’; and P0 (medium blue), P6 (green) and P7 (gray) between

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\textbf{Figure 5.} Druggability assessment of the ECF-FolT2 transporter. The ECF-FolT2 transporter (PDB code 5JSZ) is represented in cartoon and colored in cyan. a) Side view (from the membrane plane) of ECF-FolT2 along with the 12 identified pockets: P0, P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, and P11 colored in medium blue, red, yellow, forest green, orange, magenta, green, gray, purple, firebrick, pink, and aquamarine, respectively. b) Top view (from the extracellular side) of ECF-FolT2. Images were generated with CHIMERA software. The color coding of the pockets and the ECF-FolT2 transporter is maintained throughout the manuscript for figures showing the ECF transporter in ribbon.
EcfA and EcfA’. The remaining three pockets: P1 (red), P10 (pink) and P11 (aquamarine) are located within EcfT, EcfA, and the S-component, respectively (Figure 5).

7.2. Sequence Conservation

To find out whether the pockets hold the potential to be valuable drug targets for the development of broad-spectrum antibiotics, we performed a sequence alignment of the ECF module of the seven selected pathogens: *S. aureus*, *S. pneumoniae*, *E. faecium*, *E. faecalis*, *C. tetani*, *C. novyi*, and *C. difficile*. Since the ECF module of the group-II ECF transporters is predicted to interact with different S-components, it can be considered a particularly appealing drug target, because by inhibiting the function of the ECF module, the uptake of different vitamins is disrupted. As a result, we limited the sequence alignment only to the group-II ECF module.

In order to align the protein sequences of the selected organisms, we retrieved the FASTA files of the seven organisms from the UniProtKB database using the accession numbers indicated in Table S3, Supporting Information. The FASTA files represent the three parts of the ECF module: EcfT, EcfA, and EcfA’ of each of the pathogens indicated above. We conducted three different multiple-sequence alignments between each of the three domains (Figures S1–S3, Supporting Information).

Even though the sequence conservation of the ECF module among the selected pathogens does not exhibit a high total identity score (Table S4, Supporting Information), it is still considered a protein worth targeting. As depicted in Figure 6, there are areas in the module with very high sequence-identity conservation, which are colored in bright red. These sites are located primarily in the areas where subunits of the ECF complex interact with each other and natural ligands are bound. The EcfA and EcfA’ interface as well as the ATP-binding pocket are highly conserved (Figure 6). In addition, the locations where the EcfT-unit interacts with the EcfA and EcfA’ or where EcfT binds to the S-component are depicted in bright red color. The high conservation of specific residues in these seven pathogens indicates that these regions are essential for maintaining the transport function of the ECF complex. Thus, it can be hypothesized that molecules which interact with these residues may lead to the inhibition of the transport process.

Finally, residues depicted in deep blue do not seem to play a conserved role in the transport mechanism (Figure 6). This can be hypothesized based on the high percentage of dissimilarity between the species mentioned, which is likely to affect the binding interactions of potential inhibitors between species.
Furthermore, this region is mostly exposed to the solvent with few hydrophobic grooves to target, making it less suitable for the design of small-molecule inhibitors.

7.3. Combined Features

In an attempt to identify druggable pockets for structure-based drug design (SBDD), we combined the results of the druggability assessment (Table S2, Supporting Information) with our analysis of the sequence conservation of the ECF module from the seven different pathogens (Table 1).

Few of the identified pockets are predicted not to be good drug targets for the development of broad-spectrum antibiotics because of the low conservation: Pockets P7 (gray), P9 (firebrick), and P11 (aquamarine) in Figure 7 exhibit a very poor overall score, in particular a low drug score and a small volume (Table S2, Supporting Information).

In contrast, pockets such as P0 (medium blue), P2 (yellow), and P5 (magenta) in Figure 7 and P1 (red), P3 (forest green), and P6 (green) in Figure 8 exhibit very good drug scores, with a reasonable volume and sequence conservation. All of them can be considered good drug targets from the in silico point of view. However, one should be aware of the mechanism of the ECF transporter before addressing an identified pocket as future target for an SBDD project. For instance, although P0 and P5 seem to be good targets, these pockets contain residues that are responsible for ATP binding and hydrolysis. So, these residues should be avoided, otherwise selectivity over other human ABC transporters might be lost.

Finally, pockets such as P8 (purple) and P10 (pink) have acceptable sequence-identity scores, but do not exhibit a good drug score or volume, resulting in a lower priority.

7.4. Targeting S-Components

An alternative approach to prevent vitamin transport is either the inhibition of binding of the S-components to the ECF module or the inhibition of their ability to bind vitamins. Their unique features, namely the mechanism of transport and their structure, can enable the discovery of novel compounds, which interfere with the uptake of vitamins into the bacterial cell. These components are present only in prokaryotes, making them potential antibiotic targets. We performed chemical-biology studies with substrate analogues to unravel the intriguing mechanism of transport. In Table S5, Supporting Information, all available X-ray crystal structures of S-components are listed.

We reported the first binders of S-components in 2015.[96,97] Specifically, we designed thiamine derivatives for ThiT, a thiamine-dedicated S-component, expressed in L. lactis. Our molecules were designed to bind in a similar way as thiamine but
their bulky substituents would induce a conformational change, preventing the loop 1 from closing like a lid required to occlude the vitamin. These probes should interfere with the binding of thiamine and inhibit its uptake.

To realize these goals, in a first step, we examined how the natural substrate is bound in the S-component (PDB code 3RLB). By designing and synthesizing a series of derivatives of the vitamin, we evaluated the contribution of each of the functional groups of the substrate to the binding affinity. We determined six new crystal structures (Table S5, Supporting Information) of ThiT in complex with our derivatives. Even though the binding affinity and the structure of these derivatives differed from those of thiamine, the substrate-binding site in ThiT remained almost unchanged, providing new insights into the mode of action of S-components.

In a second step, we designed and synthesized a new series of thiamine analogues aiming to improve the selectivity over other thiamine-binding proteins in mammalian cells, such as human thiamine pyrophosphokinase 1, human transketolase, and human thiamine triphosphatase. Importantly, the methyl group of the pyrimidine ring of thiamine can be modified without a significant loss of its high binding affinity. A docking study of our thiamine derivatives and four human thiamine-dependent enzymes (human thiamine pyrophosphokinase 1, human transketolase, human thiamine triphosphatase, and human branched-chain α-ketoacid dehydrogenase) suggested that modification of the methyl group may provide selectivity over these human enzymes.

An analysis of the substrate-binding pocket of ThiT led to the discovery of a new subpocket. Apparently, the natural ligand seems to partially occupy a hydrophobic groove extending in the direction of the hydroxethyl moiety of thiamine. This time, our workflow included design (using the software KRIPO), synthesis as well as biochemical evaluation and molecular-dynamics (MD) simulations. We determined the binding affinities of the compounds designed to fill this groove of ThiT by isothermal titration colorimetry (ITC), exhibiting $K_D$ values in the nanomolar range.
8. Concluding Remarks

Vitamins are essential micronutrients for the growth and survival of bacteria. However, not all pathogens are able to utilize de novo biosynthesis. An alternative means of obtaining vitamins is the use of membrane transporters. Given that ECF transporters are expressed in a great number of bacterial species and given that they are absent from eukaryotes, they are considered potential drug targets. To highlight the importance of ECF transporters and investigate their druggability, we selected seven pathogens (S. aureus, S. pneumoniae, E. faecium, E. faecalis, C. tetani, C. novyi, and C. difficile), which are responsible for serious diseases, leading to an enormous societal economic burden. Although they have not yet been validated as drug targets, experimental findings demonstrating their necessity for bacterial growth have been reported. We evaluated these pathogens in terms of their ability to utilize de novo biosynthesis or to take up vitamins by using ECF transporters. Since they are not well-explored, from the medicinal point of view, a sequence-alignment study shed light on their potential to be broad-spectrum antibiotic targets. Use of the algorithm DoGSiteScorer enabled the discovery of potentially druggable pockets. Initial chemical-biology studies have helped to elucidate the intriguing mechanism of transport. Combined with the recently reported structures of full ECF transporters, the data available set the stage for medicinal-chemistry campaigns aimed at the discovery of the first inhibitors of this important protein family as novel antibiotics with an unprecedented mechanism of action.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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[1] S. Y. Hwang, Y. J. Kang, B. Sung, J. Y. Jang, N. L. Hwang, H. J. Oh, Y. R. Ahn, H. J. Kim, J. H. Shin, M.-A. Yoo, C. M. Kim, H. Y. Chung, N. D. Kim, J. Cell. Physiol. 2018, 233, 736.
[2] M. Rogosa, R. F. Wiseman, J. A. Mitchell, M. N. Disraely, A. J. Beaman, J. Bacteriol. 1953, 65, 681.
[3] R. G. Crittenrend, N. R. Martinez, M. J. Playne, Int. J. Food Microbiol. 2003, 80, 217.
[4] Q. Du, H. Wang, J. Xie, Int. J. Biol. Sci. 2011, 7, 41.
[5] a) G. Magni, M. Di Stefano, G. Orsomando, N. Raffaelli, S. Ruggieri, Curr. Med. Chem. 2009, 16, 1372; b) K. Mdluli, M. Spigelman, Curr. Opin. Pharmacol. 2006, 6, 459; c) C. Spry, K. Kirk, K. J. Saliba, FEMS Microbiol. Rev. 2008, 32, 56.
[6] A. Nzila, J. Antimicrob. Chemother. 2006, 57, 1043.
[7] B. I. Schweitzer, A. P. Dicker, J. R. Bertino, FASEB J. 1990, 4, 2441.
[8] J. ter Beek, A. Guskov, D. J. Slotboom, J. Gen. Physiol. 2014, 143, 419.
[9] C. F. Higgins, Annu. Rev. Cell Biol. 1992, 8, 67.
[10] a) G. Ames, C. Mimura, V. Shyamala, FEMS Microbiol. Rev. 1990, 75, 429; b) A. L. Davidson, E. Dassa, C. Oreille, J. Chen, Microbiol. Mol. Biol. Rev. 2008, 72, 317.
[11] G. B. Henderson, E. M. Zevely, F. M. Huennekens, J. Bacteriol. 1979, 137, 1308.
[12] D. Rodionov, P. Hebbeln, A. Eudes, J. ter Beek, I. Rodionova, G. Erkens, D. Slotboom, M. Gelfand, A. Osterman, A. Hanson, T. Eitinger, J. Bacteriol. 2009, 191, 42.
[13] F. Finkenwirth, M. Sippach, H. Landmesser, F. Kirsch, A. Ogienko, M. Gronzel, C. Kiesler, H. Steinhoff, E. Schneider, T. Eitinger, J. Biol. Chem. 2015, 290, 16929.
[14] Z. Bao, X. Qi, S. Hong, K. Xu, F. He, M. Zhang, J. Chen, D. Chao, W. Zhao, D. Li, J. Wang, P. Zhang, Cell Res. 2017, 27, 675.
[15] M. Majsnerowska, J. ter Beek, W. K. Stanek, R. H. Duurkens, D. J. Slotboom, Biochemistry 2015, 54, 4763.
[16] K. Xu, M. Zhang, Q. Zhao, F. Yu, H. Guo, C. Wang, F. He, J. Ding, P. Zhang, Nature 2013, 497, 268.
[17] T. Wang, G. Fu, X. Pan, J. Wu, X. Gong, J. Wang, Y. Shi, Nature 2013, 497, 272.
[18] P. Zhang, J. Wang, Y. Shi, Nature 2010, 468, 717.
[19] G. B. Erkens, R. P.-A. Berntsson, F. Fulyani, M. Majsnerowska, A. Vujčić-Žagar, J. ter Beek, B. Poolman, D. J. Slotboom, Nat. Struct. Mol. Biol. 2011, 18, 755.
[20] N. K. Karpowich, D.-N. Wang, Proc. Natl. Acad. Sci. U. S. A. 2013, 110, 2534.
[21] P. C. Smith, N. Karpowich, L. Millen, J. E. Moody, J. Rosen, P. J. Thomas, J. F. Hunt, Mol. Cell 2002, 10, 139.
[22] O. Neubaier, A. Alfandega, J. Schoknecht, U. Sternberg, A. Pohlmann, T. Eitinger, J. Bacteriol. 2009, 191, 6482.
[23] T. Eitinger, D. A. Rodionov, M. Grote, E. Schneider, FEMS Microbiol. Rev. 2011, 35, 3.
[24] M. Majsnerowska, I. Hänelt, D. Wunnicke, L. V. Schäfer, H.-J. Steinhoff, D. J. Slotboom, Structure (London, England: 1993) 2013, 21, 861.
[25] L. J. Y. M. Swier, A. Guskov, D. J. Slotboom, Nat. Commun. 2016, 7, 11072.
[26] N. K. Karpowich, J. M. Song, N. Cocco, D.-N. Wang, Nat. Struct. Mol. Biol. 2015, 22, 565.
[27] P. Hebbeln, D. A. Rodionov, A. Alfandega, T. Eitinger, Proc. Natl. Acad. Sci. U. S. A. 2007, 104, 2909.
[28] G. B. Erkens, D. J. Slotboom, Biochemistry 2010, 49, 3203.
[29] D. J. Slotboom, Nat. Rev. Microbiol. 2014, 12, 79.
[30] F. Finkenwirth, F. Kirsch, T. Eitinger, Biochemistry 2017, 56, 4578.
[31] Z. A. Costilow, P. H. Degnan, mSystems 2017, 2, e00116.0.
[32] A. Fuentes Flores, I. Sepúlveda Cisternas, J. I. Vásquez Solís de Ovando, A. Torres, V. A. García-Angulo, Gut Pathog. 2017, 9, 64.
[93] A. Volkamer, D. Kuhn, F. Rippmann, M. Rarey, Bioinformatics (Oxford, England) 2012, 28, 2074.
[94] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, J. Comput. Chem. 2004, 25, 1605.
[95] The UniProt Consortium, Nucleic Acids Res. 2017, 45, D158.
[96] L. J. Y. M. Swier, L. Monjas, A. Guskov, A. R. de Voogd, G. B. Erkens, D. J. Slotboom, A. K. H. Hirsch, ChemBioChem 2015, 16, 819.
[97] L. Monjas, L. J. Y. M. Swier, A. R. de Voogd, R. C. Oudshoorn, A. K. H. Hirsch, D. J. Slotboom, MedChemComm 2016, 7, 966.
[98] D. J. Wood, J. de Vlieg, M. Wagener, T. Ritschel, J. Chem. Inf. Model. 2012, 52, 2031.
[99] L. J. Y. M. Swier, L. Monjas, F. Reeßing, R. C. Oudshoorn, A. Aisyah, T. Primke, M. M. Bakker, E. van Olst, T. Ritschel, I. Faustino, S. J. Marrink, A. K. H. Hirsch, D. J. Slotboom, MedChemComm 2017, 8, 1121.