Structure-Guided Optimization of Small-Molecule Folate Uptake Inhibitors Targeting the Energy-Coupling Factor Transporters

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KEYWORDS

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ABSTRACT

Here, we report on a potent class of substituted ureidothiophenes targeting energy-coupling factor (ECF) transporters, an unexplored target, which is not addressed by any antibiotic on the market. Since the ECF module is crucial for the vitamin transport mechanism, prevention of substrate uptake should ultimately lead to cell death. By utilizing a combination of virtual and functional

whole-cell screening of our in-house library, the membrane-bound protein mediated uptake of folate could be effectively inhibited. Structure-based optimization of our hit compound yielded low-micromolar inhibitors, whereby the most active compounds showed in addition potent antimicrobial activities against a panel of clinically relevant Gram-positive pathogens without significant cytotoxic effects.

Introduction

Due to the increasing emergence of antimicrobial resistance (AMR) and the accompanying rise in morbidity and mortality rates, society is under immense pressure. In particular, multiple drug resistant (MDR) pathogens, such as *Enterococcus faecalis* or *Streptococcus pneumoniae* were prioritized by the World Health Organization due to their difficult-to-treat behavior.¹ Such pathogens need to be tackled by drugs, which address new biological targets resulting in a novel mode of action.² The energy-coupling factor (ECF) transporters are transmembrane proteins widely distributed among Gram-positive bacteria. They are considered as highly attractive antimicrobial targets, since they are required for the uptake of essential micronutrients, making them indispensable for both growth and survival of bacteria.³ These micronutrients are involved in cell metabolism and include water-soluble B-type vitamins such as folate⁴, pantothenate⁵, riboflavin⁶ and metal ions^{7,8}. In general, ECF transporters belong to the family of the adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporters, consisting of four domains.⁹ Three of them form a complex that is called ECF module, containing two nucleotide-binding domains (NBDs)—ECFA and ECFA'—which hydrolyze ATP¹⁰, as well as one transmembrane domain ECF-T (or T-component). The fourth domain ECF-S — the S-component — is a substrate-specific binding protein.⁹ Different vitamins, for example folate, are able to bind to dedicated S-components.¹¹ A common feature of ECF transporters is that the same ECF module can interact

with various S-components, facilitating the uptake of a range of vitamins in the same cell. We have recently reported a druggability assessment of the ECF-FolT as a novel and attractive antimicrobial target¹² alongside with a set of inhibitors as first tool compounds to explore the ECF transporters from a medicinal chemistry perspective.¹³

Since previous studies were limited to tool compounds, we herein present the discovery of another chemical class that inhibits the folate uptake mediated by the ECF-FolT transporter and its further biological evaluation. By combining structure-based virtual screening of our in-house small molecule library and further examination of selected molecules *via* a *Lactobacillus casei* whole-cell assay our endeavors revealed in a highly promising hit compound bearing an ureidothiophene carboxylate core motif.¹⁴ This compound served as an excellent starting point for further structure-based optimization. Synthesized derivatives and small-molecules from our in-house library enabled a straightforward structure-activity relationship (SAR) study. Ultimately, the most active compounds were evaluated for their solubility, cytotoxicity and against several clinically relevant Gram-positive pathogens.

Results and Discussion

To discover new inhibitors of the ECF transporters, we screened our in-house library consisting of more than 2,000 small molecules by structure-based virtual screening. The first step was an *in silico* screening using docking calculations to select molecules with a predicted target affinity. For this purpose, we selected the P2 pocket — located inside the ECF-T module —, which exhibits a good druggability score of 0.79 (Figure 1).¹² Since the ECF module is crucial for the vitamin transport mechanism, potential inhibitors should inhibit uptake of all substrates *via* ECF-T into the cell, ultimately leading to cell death.

Figure 1: Cartoon representation of the group-II ECF-FolT transporter crystal structure responsible for folate uptake (PDB code 5JSZ).¹¹ ECFA, ECFA', ECF-T and ECF-S colored in orange, blue, green and pink, respectively. P2 pocket depicted as yellow colored surface and ureidothiophene **1** in gray. Image generated with CHIMERA software.¹⁵

The identified 111 virtual hits were then clustered according to their structural similarity, and we selected the 22 top-ranked representatives and evaluated in a *L. casei* cell-based assay.¹⁴ Hit compound **1** displayed an inhibition of 54.3% at 50 μ M, qualifying this class of substituted ureidothiophenes as new inhibitor ECF transporter (Figure 2).

Figure 2: Hit compound **1** obtained from a combination of structure-based virtual and whole-cell screening.

For the library generation of ureidothiophenyl derivatives **1–48**, we used the combination of available in-house compounds (**1–21**, **24**, **33–41**) and synthesized derivatives (**22–23**, **25–32**, **42–48**), which can differ in the Western and Eastern part.^{16–18} We commenced our synthesis from commercially available acetophenones **I**. These were converted *via* a Vilsmaier–Haack-type

reaction to the corresponding β -chloro-cinnamionitrile followed by cyclization utilizing methylthioglycolate obtaining the thiophene anthranilic acids **II** (Scheme 1).¹⁹ Subsequent saponification and treatment of the resulting acid **III** with phosgene afforded the thiaisatoic anhydrides **IV**.^{20,21} Final conversion with a variety of amines gave rise to the 5-aryl-3-ureidothiophene-2-carboxylic acids **22–23**, **25–32** in good yield.^{17,18,22}

Scheme 1: Synthesis of 5-aryl-3-ureidothiophene-2-carboxylic acids **22–23**, **25–32**. Reagents and conditions: a) i) POCl₃, DMF, 50 °C to rt, ii) NH₂OH·HCl, up to 150 °C, 75–90%. iii) methylthioglycolate, NaOMe, MeOH, reflux, 65–85%. b) KOH, MeOH, THF, H₂O, reflux, 40–80%. c) COCl₂, THF, 50–70%. d) amine, H₂O, 100 °C, 40–80%.¹⁸

To get access to 4-aryl-3-ureidothiophene-2-carboxylic acids and their bioisosteric benzene analogue, we developed a robust and variable synthetic route that would allow us to substitute the urea moiety present in hit compound **1** with specific functional groups such as the corresponding amide and amine (Scheme 2). We began our synthesis with the commercially available methyl 3-aminothiophene-2-carboxylate **V**, which was transformed into the corresponding urea derivative **43** with a yield of 51% using triphosgene (BTC) and hexylamine after saponification. To install the aryl moiety, **V** was first regioselectively brominated under acidic conditions.²³ Subsequent Suzuki cross coupling yielded **VII**, **XIII** and **XV**.^{24,25} These intermediates depicted extremely valuable precursors for further modifications and allowed us to firstly isolate urea derivatives **47**,

48 in a two-step procedure. Installation of the appropriately truncated amine linker was achieved by sodium hydride-mediated deprotonation of amine **VII**, followed by alkylation with hexyl bromide. Final treatment with lithium hydroxide gave the desired acid **45** in 91% yield. The truncated amide linker was incorporated by reacting valeroyl chloride with precursor **VII** at low temperature. At this point, final saponification of **VIII** with lithium hydroxide led to the desired product **44** in 89%. We accessed the bioisosteric product **46** in the previously described manner by combining Suzuki cross coupling between brominated methyl anthranilate **X** and (3,4-dichlorophenyl)boronic acid, subsequent saponification and final BTC-mediated urea formation. Purification by preparative HPLC gave biphenyl urea derivative **46** in 38% yield.

Scheme 2: Synthesis of ureidothiophene-carboxylic acid derivatives **44–48**. Reagents and conditions: a) i) KOH, H₂O, 90 °C, 2 h ii) BTC, THF, 0 °C, 4 h, iii) amine, THF, rt, 24 h, 51%. b) Br₂, AcOH, rt, 24 h, 32%. c) boronic acid, cat. [Pd(Ph)₄], Na₂CO₃, dioxane/H₂O (4:1), 80 °C, 65–83%. d) LiOH·H₂O, THF/H₂O (4:1), 0 °C – rt, 89–97%. e) i) BTC, THF, rt, 2 h ii) amine, THF/H₂O (1:1), rt, 24 h, 14–38%. f) DIPEA, valeroyl chloride, –20 °C – rt, 12 h, 64%. g) NaH, hexylbromide, DMF, 12 h, 67%.

Starting from hit compound **1**, we examined the structurally similar analogues available in our in-house library for extending the SAR and further hit optimization. Firstly, we focused on the Eastern part of the molecule, where we varied the substituent R by introducing several mono- or di-substituted aliphatic, aromatic and heterocyclic groups (Table 1). All compounds were tested for their proportional inhibition of the radiolabeled folic acid uptake into *L. casei* cells. For all compounds showing more than 70% inhibition, we additionally determined their half maximal inhibitory concentration (IC₅₀).

Table 1: Eastern part modifications with their % inhibition and IC₅₀ values against ECF-FoIT.

Compound	R	% Inhibition ECF-FoIT @50μM	IC ₅₀ (μM)
2		32.8	n.d. ^{a)}
3		33.5	n.d.
4		57.2	n.d.
5		70.6	21.4
6		10.3	n.d.
7		84.2	7.9
8		71.7	12.3
9		90.6	9.1

10		39.3	n.d.
11		53.5	n.d.
12		60.7	n.d.
13		78.4	18.5
14		83.8	10.1
15		99.1	2.1
16		34.7	n.d.
17		52.4	n.d.
18		89.6	9.7
19		87.8	9.4

20		90.8	4.7
21		82	12.2

^{a)} n.d. = not determined.

Variation of the Eastern part of the molecule revealed that increasing the size of the aliphatic side chain enhances the activity (**3**, **4**, **5**) compared to the non-substituted urea motif present in **1**. Adding a polar carboxylic group (**4** vs **6**) causes a drop in activity. Introducing a directly linked phenyl ring (**7**) significantly boosts the activity, while a spacer between the urea and the phenyl ring (**8** and **9**) slightly decreases the activity. Replacing the phenyl ring (**8**) by a pyridyl (**10**, **11**, **12**) results in generally less potent derivatives. The position of the heteroatom seems to be further influencing the activity with the *para* position (**12**) being preferred. Additionally, the activity was influenced by having two aromatic groups or one aromatic and one aliphatic in addition to the presence or absence of a spacer in the disubstituted derivatives. Accordingly, adding a methyl as a second substituent besides the phenyl (**13**) decreases the activity, while further elongation to an ethyl (**14**) or a butyl (**15**) boosts the activity. Keeping the butyl group constant and introducing a spacer to the phenyl results in a further decrease in activity (**16**, **17**). Keeping the benzyl group constant, while adding an extra methyl (**18**) or ethyl (**19**) results in slight enhancement in activity relative to the benzyl alone (**8**) with both compounds being equipotent. Replacing the alkyl groups with a phenyl ring (**20**) enhances the activity, while having another benzyl substituent (**21**) further decreasing the activity.

For the next set of modifications, we maintained the Eastern part of compounds **5** and **19** as they were among the most potent derivatives and various structurally related analogues were available

in our in-house library. This set of modifications included changes in the Western part of the molecule (Table 2) besides studying the influence of the different regioisomers on activity (Table 3).

Table 2: Western part modifications with their % inhibition and IC₅₀ values against ECF-FoIT.

Compound	Core	R	% Inhibition ECF-FoIT @50μM	IC ₅₀ (μM)
22	A		25.1	n.d. ^{a)}
23	A		36.2	n.d.
24	A		27.2	n.d.
25	A		67.9	n.d.
26	A		96.8	21.8
27	A		59	n.d.
28	A		12.1	n.d.

29	A		73.0	44.4
30	A		81.7	23.5
31	A		52.3	n.d.
32	A		27.1	n.d.
33	B		22.1	n.d.
34	B		52.3	n.d.
35	B		38.9	n.d.
36	B		67.2	n.d.
37	B		18.2	n.d.
38	B		97.1	10.2

^{a)} n.d. = not determined.

Modifications of the Western part showed that the di-chlorinated derivatives are more potent than the mono-chlorinated ones. Among the created derivatives, the 2,5-dichlorophenyl (**26**) and the 3,4-dichlorophenyl (**5**) were the most potent ones. Having in mind the relative potencies of compounds **5** and **19**, we compared the different substituents in *para* position. The results revealed

that the NO₂ group (**30**) showed the highest increase in activity followed by OCF₃ (**36**) > Br (**34**) > Me (**35**) > CF₃ (**32**), while the CN (**37**) was the least potent. Among the 3,4 di-substituted derivatives, the di-chloro was always more potent than the CF₃ congener (**5** vs **27**). Interestingly, further extension with a phenoxy (**38**) is tolerated and results in enhanced activity.

As a next step, we wanted to compare the impact of the different regioisomers on activity (Table 3). This revealed that switching the positions of the carboxylic and the urea groups results in a complete loss in activity (**5** vs **39**). Interestingly, the 2,3,4-substitution pattern is more favorable than 2,3,5 and enhanced the activity (**5** vs **40**). Flipping the carboxylic with the urea in the 2,3,4 pattern turned out again not to be beneficial (**40** vs **41**).

Table 3: The different regioisomers with their % inhibition and IC₅₀ values against ECF-FoIT.

Compound	Structure	% Inhibition ECF-FoIT @50μM	IC ₅₀ (μM)
5		70.6	21.4
39		n.i. ^{a)}	n.d. ^{b)}

40		98.6	9
41		89.6	17.2

a) n.i. = < 10% inhibition; b) n.d. = not determined.

A final set of modifications was performed based on the more potent regioisomer **40** where we evaluated the importance of the urea and the main thiophenyl core in addition to the effect of truncation (Table 4). It was clearly seen that any truncation of the Eastern or the Western part leads to significant loss of activity (*cf.* **42** and **43**). To explore the importance of urea linker, we replaced it with an amide (**44**) and an amine (**45**). Although the urea remained the most potent, the amine (**45**) was just slightly less potent, while the amide (**44**) showed a more pronounced decrease in activity. Finally, scaffold hopping was done by replacing the main thiophenyl core with a phenyl ring (**46**). Interestingly, this was acceptable with only a slight decrease in activity.

Based on the results of all the modifications tested, we finally combined the best features. We used the most potent Eastern part in compound **15**, which incorporates a phenyl and a butyl residue. For the Western part, we chose phenoxy-phenyl and 2-5, dichlorophenyl residues as incorporated in the most potent compounds **38** and **26**, respectively. Combination of the Eastern and the Western parts using the more potent 2,3,4-substituted core resulted in compounds **47** and **48**. While merged 2-5, dichlorophenyl compound **47** did not show the desired enhancement in activity, this came true for phenoxy-phenyl derivative **48**, which revealed an excellent folate uptake inhibition with an

IC₅₀ value of 2.5 μ M. This could in part be due to the 2,3,4-substituted core has a different SAR requirement than the 2,3,5- substituted core from which we adopted the SAR.

Table 4: Variable modifications and merged derivatives on the more potent regioisomer with their % inhibition and IC₅₀ values against ECF-FolT.

Compound	Structure	% Inhibition ECF-FolT @50 μ M	IC ₅₀ (μ M)
42		n.i. ^{a)}	n.d. ^{b)}
43		n.i.	n.d.
44		31.8	n.d.
45		95.9	15.7
46		65.5	n.d.

47		82.4	17.8
48		99.4	2.5

a) n.i. = < 10% inhibition; b) n.d. = not determined.

The superb inhibition of **15** and **48** encouraged us to further evaluate their minimal inhibitory concentrations (MIC) against a broad panel of clinically relevant Gram-positive pathogens, alongside with their solubility and cytotoxic effects on human liver cancer cells (HepG2) (Table 5). Both **15** and **48** showed good kinetic solubility²⁶ indicating adequate access the envisaged P2 pocket of the transmembrane protein since further cell uptake is not necessary. Unfortunately, we observed only a slight antimicrobial effect of **15** and **48** for *Staphylococcus aureus*, suggesting that *S. aureus* not necessary relies on the folate uptake and predominantly obtains it directly via the biosynthesis.^{12,27} Beyond that, compound **48** exhibited a good activity of 4-8 μM and 8 μM for both, wild-type *E. faecalis* and the vancomycin-resistant *E. faecalis*, whereas **15** showed an even stronger inhibition of the wild-type and an equipotent effect on the antibiotic-resistant strain revealing a MIC of 2 μM . Also the non-resistant *S. pneumoniae* strain could be effectively treated by our optimized compounds showing an MIC of 2 μM (**15**) and 4 μM (**48**), whereas compound **48** lost its effect on the penicillin-resistant strain with 16 μM . The latter displayed only mild

cytotoxic effects at an IC₅₀ of 57 μ M revealed. Most interestingly, ureidothiophene **15** inhibits the penicillin-resistant *S. pneumonia* strain with an MIC of 0.5 μ M, while only showing insignificant cytotoxic effect. These findings make ureidothiophene **15** an excellent candidate for further development as an antibiotic, showing a superb therapeutic window while presenting a novel mode of action.

Table 5: Antibacterial profiles, cytotoxicity and kinetic solubility of compounds **15** & **48**

Strain	15	48
	MIC [μ M]	
<i>Enterococcus faecium</i> DSM-20477	2	16
VR- <i>Enterococcus faecium</i> DSM-17050 ^[a]	8	8
<i>Streptococcus pneumoniae</i> DSM-20566	2	4
PR- <i>Streptococcus pneumoniae</i> DSM-11865 ^[b]	0.5	16
<i>Staphylococcus aureus</i> Newman	23	16
	IC ₅₀ [μ M]	
HepG2	95.6 $\pm$ 9.4	57.0 $\pm$ 0.9
	Kinetic solubility S_{kin} [μ M]	
	106.6 $\pm$ 1.9	>200

^[a]VR: Vancomycin-resistant; ^[b] PR: Penicillin-resistant

Conclusions

We discovered a potent class of substituted ureidothiophenes targeting ECF transporters, an unexplored target, which is not addressed by any antibiotic on the market. Starting from an in-house library screening hit, an extensive SAR study was done by exploring the Eastern and Western parts of the molecule as well as different possible regioisomers. This finally led to two highly potent ECF inhibitors **15** and **48**, revealing submicromolar inhibition. Beyond the superb on-target activity of those two molecules, potent antibacterial activities against a panel of Gram-positive clinically relevant strains were observed, first and foremost the penicillin-resistant *S. pneumoniae* inhibition at 0.5 μ M. Both frontrunners only showed mild cytotoxic effects on human cell lines, paving the way for the development of further ureidothiophene-based antibacterial drugs targeting ECF-transporters.

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Author Contributions

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‡Spyridon Bousis contributed to the conception of this study, designed and performed the virtual screening, evaluated and interpreted resulting data as well as writing and editing of the manuscript.

‡Mostafa M. Hamed contributed to the design and conception of this study, evaluated and interpreted resulting data as well as the writing and editing of the manuscript. Additionally, the author coordinated the ECF bioassays.

Eleonora Diamanti contributed to the conception of this study, designed and performed the virtual screening, evaluated and interpreted resulting data, edited and proofread of the manuscript.

Jörg Haupenthal contributed to editing and proofreading of the manuscript and coordination of the solubility, antimicrobial and cytotoxicity assays.

Anna K. H. Hirsch contributed to conception and supervised this study, as well as editing and proofreading of the manuscript.

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ABBREVIATIONS

ATP-binding cassette (ABC), antimicrobial resistance (AMR), adenosine 5'-triphosphate (ATP), energy-coupling factor (ECF), *Enterococcus faecalis* (*E. faecalis*), *Enterococcus faecium* (*E. faecium*), half maximal inhibitory concentration (IC₅₀), high-performance liquid chromatography (HPLC), *Lactobacillus casei* (*L. casei*), minimum inhibitory concentration (MIC), multiple drug resistant (MDR), nucleotide-binding domain (NBD), penicillin-resistant (PR), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*), structure-activity relationship (SAR), triphosgene (BTC), vancomycin-resistant (VR)

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By utilizing a combination of virtual and functional whole-cell screening of our in-house library and structure-based optimization later on, the membrane-bound energy-coupling factor (ECF) transporter mediated uptake of folate could be effectively inhibited by a potent class of substituted ureidothiophenes.