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Dynamic Combinatorial Chemistry to Identify Binders of ThiT, an S-Component of the Energy-Coupling Factor Transporter for Thiamine

Leticia Monjas[+],[a] Lotteke J. Y. M. Swier[+],[b] Inda Setyawati,[b, c] Dirk J. Slotboom,*[b] and Anna K. H. Hirsch*[a, d, e]

We applied dynamic combinatorial chemistry (DCC) to identify ligands of ThiT, the S-component of the energy-coupling factor (ECF) transporter for thiamine in *Lactococcus lactis*. We used a pre-equilibrated dynamic combinatorial library (DCL) and saturation-transfer difference (STD) NMR spectroscopy to identify ligands of ThiT. This is the first report in which DCC is used for fragment growing to an ill-defined pocket, and one of the first reports for its application with an integral membrane protein as target.

Dynamic combinatorial chemistry (DCC) is a powerful tool for hit identification and optimization. Over the past 20 years, DCC has been successfully applied in medicinal chemistry and chemical biology for the discovery of binders to DNA, RNA and protein targets.[1–3] The DCC technique involves the generation of a library of compounds by reversible reaction of different building blocks. The main advantage of DCC is that several potential ligands for a protein can be screened simultaneously, avoiding the individual synthesis, purification and biochemical evaluation of every member of the dynamic combinatorial library (DCL). Up to date, there are only a few reports in which DCC has been applied to identify binders of transmembrane proteins.[4–6] Here, we have applied DCC to identify possible binders for ThiT, the S-component of the energy-coupling factor (ECF) transporter for thiamine (Figure 1 A) in *Lactococcus lactis*.[7,8] ECF transporters represent an interesting target for the development of antibacterial agents with a novel mode of action by blocking vitamin transport.[9]

In our previous work, we studied binding of thiamine derivatives to ThiT, including compounds that occupy a subpocket within the substrate binding pocket of ThiT at the hydroxy end of thiamine (Figure 1 B).[10–12] Although we described strong binders (K_D values in the nano- and micromolar range), our predicted K_D values did not always correlate very well with the experimental values. A possible explanation is that when the unliganded substrate binding pocket is "open" to the surrounding solvent (the loop L1 is in a different conformation), this subpocket adopts a different conformation than the one in the available crystal structure, in which the loop L1 closes the substrate binding pocket as a lid (PDB ID: 3RLB).[8] This could then be the reason why structure-based design was unsuccessful in this case.

To obtain new extended thiamine derivatives, we here use DCC for fragment growing, maintaining the deazathiamine moiety (Figure 1 A), and screen various substituents to occupy the possibly flexible part of the thiamine binding pocket. Fragment growing by DCC has been done but not into flexible pockets.[13] Identification of ligands for ill-defined pockets is challenging and DCC is an ideal method to address this challenge. We used an acylhydrazone motif as a linker, which has been extensively used in DCC,[1–3,14,15] and selected aldehyde **A** and eight hydrazides (**H1**–**H8**) to form a small pre-equilibrated dynamic combinatorial library of eight acylhydrazones (**AH1**–**AH8**) (Scheme 1).

We synthesized aldehyde **A** as previously described.[10] Hydrazides **H1**–**H8** were obtained from their corresponding methyl esters (ethyl ester in the case of **H6**), which were commercially available or synthesized by esterification of the corresponding carboxylic acid, using HCl in methanol at reflux, in 91–93 % yield. Next, the reaction of the corresponding ester with hydrazine monohydrate at reflux afforded the hydrazides in 24–97 % yield (details of the synthesis are available in the Supporting Information).

We used saturation-transfer difference (STD) NMR spectroscopy to identify which of the eight compounds bound to ThiT. STD-NMR spectroscopy is a powerful technique to study protein-ligand interactions in solution. Usually, the concentration of ligand(s) is 10- to 100-fold the concentration of protein,

[a] Dr. L. Monjas,[+] Prof. Dr. A. K. H. Hirsch
Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen (The Netherlands)

[b] Dr. L. J. Y. M. Swier,[+] I. Setyawati, Prof. Dr. D. J. Slotboom
Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen (The Netherlands)
E-mail: d.j.slotboom@rug.nl

[c] I. Setyawati
Biochemistry Department, Bogor Agricultural University, Bogor (Indonesia)

[d] Prof. Dr. A. K. H. Hirsch
Current address: Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Department of Drug Design and Optimization, 66123 Saarbrücken (Germany)

[e] Prof. Dr. A. K. H. Hirsch
Department of Pharmacy, Medicinal Chemistry, Saarland University, Campus Building E8.1, 66123 Saarbrücken (Germany)
E-mail: Anna.Hirsch@helmholtz-hzi.de

[+] These authors contributed equally to this work.

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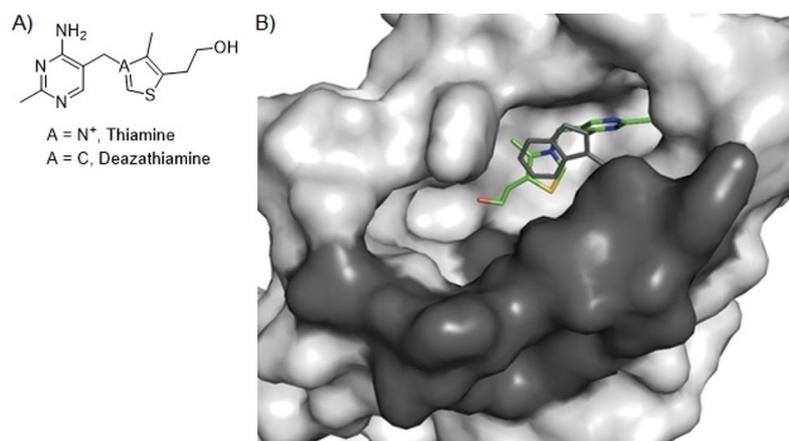https://doi.org/10.1002/cmdc.201700440.

Figure 1. A) Structures of thiamine and deazathiamine. B) Crystal structure of ThiT in complex with thiamine (PDB ID: 3RLB):[8] close-up of the thiamine binding pocket, with ThiT shown in surface representation, except for the Trp34 residue and thiamine that are shown in stick representation to visualize the substrate binding pocket. Color code: ThiT in gray, with the residues of the loop L1 (residues Leu26–Ile39) highlighted in darker gray, and thiamine with the C atoms in green, O in red, N in blue, and S in yellow.
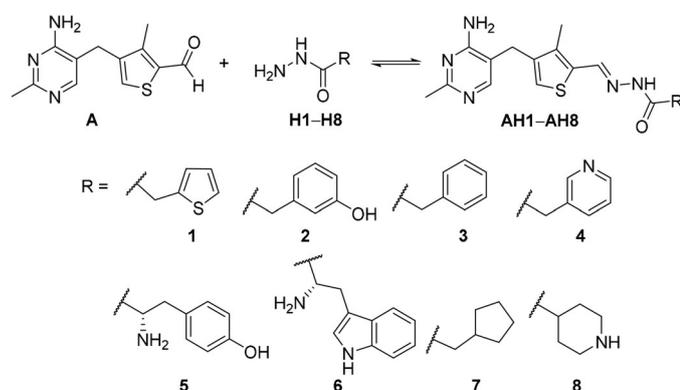
Scheme 1. Dynamic combinatorial library (DCL) to afford acylhydrazones as binders of ThiT.

which allows to work with low protein concentrations (in the micromolar range).[16] ^{1}H-STD-NMR spectroscopy has been successfully applied in combination with DCC in some studies,[17,18] and in a few cases to study ligand binding to transmembrane proteins.[19,20] In previous reports in which STD-NMR spectroscopy was applied to transmembrane proteins, these proteins were embedded in the lipid bilayer of a liposome or in a membrane preparation derived from cells, and not in detergent solution as in our case. Hence, we wanted to show that DCC can be conveniently analyzed by STD-NMR spectroscopy also for a transmembrane protein in detergent solution. Using ThiT in a detergent-solubilized state, allowed us to work at higher protein concentrations than if we would have performed the additional reconstitution step into liposomes. In addition, having the protein solubilized in a detergent micelle eased the buffer-exchange procedure that we performed to obtain a sample of ThiT in deuterated buffer. For our target, we first ran a control experiment with ThiT and a known binder (**B1**) (Figure S1). This control experiment served two purposes: first, the amounts of ThiT that we can obtain are not enough to record a ^{1}H-NMR spectrum to identify a suitable irradiation frequency (i.e., a fre-

quency where only the protein resonates and not the ligands); second, we wanted to establish whether the conditions we use are optimal and enable us to detect a known binder. The final concentration of ThiT in 500 μL deuterated buffer (pD 7.0) in the NMR tube was 7.8 μм. Running the ^{1}H-STD NMR experiment using a 100-fold excess of ligand, and measuring for 11 h irradiating at −1 ppm or −2 ppm, resulted in difference spectra featuring peaks corresponding to our known binder **B1**, as well as the detergent (n-decyl-β-ᴅ-maltopyranoside) that is present in the buffer (Figure S1). As a result, any of these frequencies are suitable for the experiment with the DCL. We performed the experiment with our library of compounds using a 100-fold excess of ligand and irradiation at −1.1 ppm for 11 h. To analyze our DCL, we divided it into two sub-libraries, containing the aldehyde and four hydrazides each as building blocks, selected in a way that the characteristic NMR signals do not overlap. For the experiment with ThiT, the building blocks were left to react in a buffer at pD 5.0 with shaking for 24 h. We checked the DCL by UPLC–MS and NMR spectroscopy before adding the protein, and all the constituents were formed in about equal amounts. Then, the DCL was added to the solution of ThiT in buffer at pD 7.0, given than ThiT does not tolerate lower pD, forcing us to use a static DCL,[21] and the on-resonance and off-resonance spectra were recorded. For the first library, DCL-A, we included the aldehyde **A** and the hydrazides **H1**, **H2**, **H6** and **H7**. The second library, DCL-B, consisted of the same aldehyde **A** and the hydrazides **H3**, **H4**, **H5** and **H8** (Figure S2). In DCL-A, we observed that three of the four possible acylhydrazones bind to ThiT. In principle, after 24 h of incubation to form the DCL, all of the aldehyde **A** was consumed according to ^{1}H-NMR spectroscopy, but apparently, a small amount was still in solution, and after many scans during the experiment with protein we could see it in the ^{1}H-STD-NMR spectrum. In DCL-B, three of the other four possible acylhydrazones emerged as binders of ThiT. Taken together, the analysis of both libraries shows that all the compounds derived from aromatic hydrazides (**AH1–AH6**) bind to
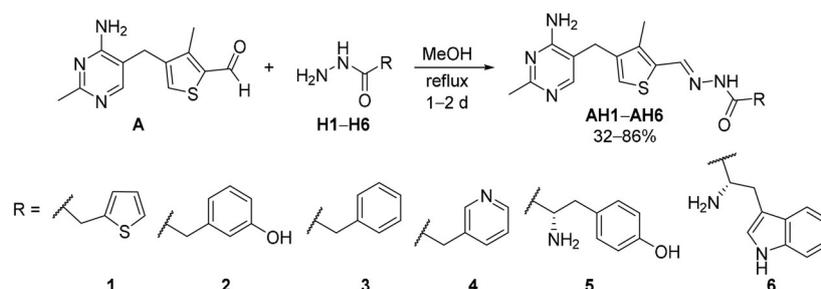
Scheme 2. Synthesis of the acylhydrazones identified as binders of ThiT.

ThiT, whereas those derived from aliphatic hydrazides (**AH7** and **AH8**) do not bind.

We synthesized the six identified binders by reaction of aldehyde **A** and the corresponding hydrazides **H1–H6** at reflux in MeOH, affording the desired products **AH1–AH6** in 32–86% yield (Scheme 2). We determined the binding affinity of the synthesized acylhydrazones by isothermal titration calorimetry (ITC), and tested the compounds as mixtures of *E/Z* isomers using ThiT stabilized with detergent as we performed the STD-NMR experiments (Table 1). We observed that ThiT has a higher binding affinity for acylhydrazones **AH1**, **AH4** and **AH5** than for acylhydrazones **AH2**, **AH3** and **AH6**.

Table 1. Binding affinities of ThiT for thiamine, deazathiamine and acylhydrazones **AH1–AH6** determined by ITC.

Compound	K_D [μM]
Thiamine[7]	$(0.122 \pm 0.013) \times 10^{-3}$
Deazathiamine[10]	$(4.23 \pm 1.69) \times 10^{-3}$
AH1	$5.30 \pm 1.19^{[a]}$
AH2	$28.8 \pm 6.89^{[b]}$
AH3	$20.9 \pm 10.5^{[a]}$
AH4	$3.02 \pm 0.172^{[a]}$
AH5	$8.41 \pm 5.54^{[c]}$
AH6	$44.5 \pm 8.06^{[a]}$

[a–c] Values are the mean ± SD obtained from [a] 4, [b] 6, or [c] 3 experiments.

In conclusion, we successfully applied DCC in combination with STD NMR spectroscopy to identify binders of ThiT. The advantage of this method is that it requires a low concentration of unlabeled protein (in the micromolar range), which is particularly advantageous for proteins that are difficult or expensive to produce, such as integral membrane proteins. The disadvantages are the limited size of the library and the requirement to determine the ¹H-NMR reference spectrum of each individual product for comparison with the ¹H-STD-NMR spectrum. This study is the first example in which DCC is applied to a challenging target for fragment growing to an ill-defined pocket. In addition, it is one of the first applications of ¹H-STD-NMR spectroscopy to transmembrane proteins, and the first one that uses detergents instead of liposomes to embed the protein. The acylhydrazones identified by ¹H-STD-NMR spectroscopy bind to ThiT with K_D values in the micromolar range. Comparing their binding affinities with similar compounds,[12] in which most of the compounds show K_D values in the nanomolar range, the acylhydrazones are weaker binders. We need to take into account, however, that the K_D values were determined for mixtures of *E/Z* isomers, and probably only one of them binds with high affinity to ThiT. Furthermore, the linker required for acylhydrazone formation makes these compounds longer and more rigid than those previously reported. As a result, the compounds may not be able to adopt a favorable conformation to interact with ThiT, explaining the decrease in binding affinity. Even if the acylhydrazones are not better binders than the compounds reported previously, it is remarkable that DCC enables fragment growing into flexible pockets that cannot be addressed by structure-based design, opening up opportunities in medicinal chemistry.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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