Hit-optimization using target-directed dynamic combinatorial chemistry: Development of inhibitors of the anti-infective target 1-deoxy-D-xylulose-5-phosphate synthase

Ravindra P. Jumde,1 Melissa Guardigni,4 Robin M. Gierse,1,2,3 Alaa Alhayek,1,2 Di Zhu,1,3 Zhoor Hamed,1,2 Sandra Johannsen,1,2 Walid A. M. Elgaher,1 Jörg Haupenthal,1 Anna K. H. Hirsch1,2,*

1 Department of Drug Design and Optimization, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Campus Building E8.1, 66123 Saarbrücken, Germany
2 Department of Pharmacy, Saarland University, Campus Building E8.1, 66123 Saarbrücken, Germany
3 Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
4 D3-PharmaChemistry, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genoa, Italy
* Corresponding author: Anna.Hirsch@helmholtz-hips.de

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Abstract
Target-directed dynamic combinatorial chemistry (tdDCC) enables the identification, as well as optimization of ligands for un(der)explored targets such as the anti-infective target 1-deoxy-D-xylulose-5-phosphate synthase (DXS). We report the unprecedented use of tdDCC to first identify and subsequently optimize inhibitors of the anti-infective target DXS. Using tdDCC, we were able to generate acylhydrazone-based inhibitors for DXS. The tailored tdDCC runs also provided insights into the structure–activity relationship of this novel class of DXS inhibitors. This approach holds the potential to expedite the drug discovery process and could be generally applied to a range of biological targets.

Introduction:
In dynamic combinatorial chemistry (DCC), compound libraries can be generated under thermodynamic control from a range of appropriate building blocks. The addition of external stimuli or targets can alter this thermodynamic equilibrium and change the composition of these libraries. In target-directed dynamic combinatorial chemistry (tdDCC), the target protein can select its binders and favors their formation over non-binders, resulting in their amplification in the library pool. Since the first report of tdDCC by Lehn and coworkers,1,2 it has emerged as a powerful tool to identify new ligands of biological targets.3-7 This self-screening approach reduces the synthetic efforts given that only the amplified hits need to be synthesized. Ultimately, it results in acceleration of the discovery of hit compounds in the early stages of drug discovery.

Discovered in 1993, the 2C-methyl-D-erythritol 4-phosphate pathway (MEP) pathway is an alternative biosynthetic route to generate the universal isoprenoid precursors and corresponding essential metabolites for cell survival.8 The enzyme DXS catalyzes the first, rate-limiting step for the production of isoprenoid precursors in the MEP pathway. It is also involved in the biosynthesis of thiamine (vitamin B1) and pyridoxal (vitamin B6) in bacteria.9,10 The absence of the MEP pathway in humans makes it a promising new target for the development of novel medicines against many life-threatening diseases such as tuberculosis and malaria. Despite substantial efforts dedicated to the discovery of inhibitors for DXS, to date, very few active compounds are known, which fulfills the requirements as an ideal candidate for further development.11-15 The scarcity of inhibitors and X-ray crystal structures of the anti-infective target DXS makes tdDCC a particularly attractive approach for the discovery of new inhibitors.
To date, the use of tdDCC in drug discovery has been limited to the initial hit-identification process. We decided to explore the possibility of using tdDCC for hit-optimisation along with hit-identification for DXS. In tdDCC, the target selects and amplifies its best binders/inhibitors from the library. Thus, it can be used for further optimization of the initial hits in subsequently tailored libraries inspired by the initial hits.

**Results:**

For this study, we used acylhydrazone formation as a reversible reaction of choice and the DXS enzyme from *Deinococcus radiodurans* (drDXS) as a target protein. Considering the stability issues of drDXS at lower pH and the slow acylhydrazone formation at neutral pH, the use of aniline as a nucleophilic catalyst was required. The template effect of drDXS on the dynamic combinatorial library (DCL) was evident by comparing it with the blank DCC experiment (without protein) using LC-MS/MS analysis.

**Selection of initial dynamic combinatorial library.** The choice of the first DCL was based on some structural similarity of building blocks with the cofactor thiamine diphosphate (ThDP) of the enzyme DXS (Figure 1a). We selected three different aldehydes containing pyrimidine and pyridine moieties and seven different hydrazides mostly featuring various heterocyclic cores. The docking study of the 21 possible acylhydrazones products from DCL-1 using LeadIT and SeeSAR showed that they are accommodated in the active site of the enzyme drDXS (Figure 1, Supplementary Figures 1–6).

![Figure 1](image1.png): a) Crystal structure of drDXS (PDB code: 2O1X); b) active site of drDXS with bound cofactor thiamine diphosphate (ThDP) and the subpockets.

**tdDCC-1.** We performed the first DCC experiment by reacting three aldehydes (A1–A3) with seven hydrazides (H1–H7) in a phosphate buffer (pH 6.25) with an excess of aniline and 5% DMSO (Figure 2a). We evaluated the composition of DCL-1 in the blank reaction after it had reached equilibrium; after 6 h, no changes in the relative peak areas (RPAs) were visible (Figure 2b). Comparison of an adaptive DCC experiment in presence of drDXS protein (40 mol%) with the blank DCL-1 revealed the amplification of five acylhydrazones (1–5) (Figure 2c and Supplementary Figure 8). Subsequently, we evaluated the effect of protein concentration on the amplification of these hits in an adaptive DCL, where 20 mol% of drDXS was used, and the effect of pre-equilibration, where drDXS (20 mol%) was added to the pre-equilibrated library (after 6 h). The systematic effect of protein concentration on the amplification is rarely studied, as the use of a large amount of protein is often considered necessary for tdDCC. Comparison of the composition of these DCLs with the blank DCLs still revealed the template effect, although the amplification of all five hits (1–5) was substantially reduced when 20 mol% of drDXS was used as compared to 40 mol% of drDXS (Figure 2e and Supplementary Figure 9). When drDXS (20 mol%) was added to the pre-equilibrated library, and the composition of the DCL was checked after an additional 6 h, the amplification of these hits was further reduced. Nonetheless, in these three experiments, all five acylhydrazones (1–5)
were amplified (Figure 2e and Supplementary Figure 10). These data suggest that even a minute amount of the protein drDXS can alter the equilibrium of the library and induce the template effect. These findings are particularly important for future DCC experiments with precious proteins.

![Diagram](image)

Figure 2. a) DCC-1, building blocks, Conditions: experiment was run in phosphate buffer (pH 6.25) and 5% DMSO, aldehydes (100 µM each, in DMSO), hydrazides (300 µM each, in DMSO), aniline (10 mM in DMSO), drDXS protein (20–40 µM in phosphate buffer); b) evaluation of the equilibrium state of DCL-1; c) UV-chromatogram of the blank and protein-templated adaptive DCL (40 mol% protein); d) amplified acylhydrazone hits (1–5) in DCL-1 and other experiments; e) effect of protein concentration and pre-equilibration on amplification of hits.

**Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-1.** We synthesized all five hits (1–5) from the tdDCC-1 and evaluated their binding affinities for drDXS using surface plasmon resonance (SPR, Figure-3). The five hits showed moderate binding affinity ($K_D$) ranging from 55–270 µM. Interestingly, the trend of binding affinities of these hits mostly correlates with their amplification in the tdDCC. Furthermore, we evaluated the enzymatic activity of these hits against drDXS and the homologous target in *M. tuberculosis* mtDXS, which showed a similar trend, the most amplified hit was the most active one (1, IC$_{50}$: drDXS = 51 ± 3 µM; mtDXS = 78 ± 3 µM). Unfortunately, none of these hits showed considerable antibacterial activity against *Escherichia coli* TolC (Inhibition = < 25% at 50 µM), which may be ascribed to permeability issues. Nonetheless, using tdDCC, we were able to find a new class of inhibitors of the anti-infective target DXS, which provides a good starting point for further optimization. Instead of traditional medicinal-chemistry approach, we decided to employ tdDCC for this purpose, letting the target protein DXS choose its best binders.
Figure 3. Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-1. Inhibition of drDXS was determined at 120 μM. Inhibition of mtDXS was determined at 200 μM. n.d. = not determined. n.i. = no inhibition. Percent (%) growth inhibition of E. coli TolC was determined at 50 μM.

tdDCC-2. We assumed that using the common structural motifs from the first hits as building blocks in the second tdDCC, along with new aldehyde and/or hydrazide counterparts would provide better chances of identifying improved hits. From the initial hits (1–5), tetrahydrobenzothiophene and indole emerged as two privileged structural motifs, which are present in hits 1–3 and hits 4 and 5, respectively. We wanted to investigate the effect of inverting the position of these two groups in the acylhydrazone. To do so, we included their corresponding aldehydes A4 and A5 in DCL-2 instead of hydrazides H1 and H7. In DCL-2, along with A4 and A5, we included A6 as an additional aldehyde and four new hydrazides H8–H11 along with the three old ones (H2, H5, and H6, Figure 4a). Analysis of DCL-2 revealed the amplification of five new hits (6–10, Figure 4b and Supplementary Figure 12).

Figure 4. a) DCC-2, building blocks, Conditions: experiment was run in phosphate buffer (pH 7.04) and 5% DMSO, aldehyde (100 μM each, in DMSO), hydrazides (300 μM each, in DMSO), aniline (10 mM in DMSO), drDXS protein (40 μM in phosphate buffer); b) amplified acylhydrazone hits in DCL-2.

Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-2. We synthesized all five new hits (6–10) and evaluated their binding affinities for drDXS by SPR (Figure 5). The $K_D$ values of these five hits were in the range of 7–150 μM, a significant increase compared to the first five hits from tdDCC-1. However, when tested against drDXS and mtDXS, these new hits (6–10) showed no significant improvement in their enzymatic activity. The most amplified hit 6 was most active against drDXS (IC$_{50}$ = 101 ± 15 μM) and hit 7 was the most active against mtDXS (Inhibition = 65 ± 4% at 200 μM). Interestingly, hits (7–10) from DCC-2 showed an improvement in antibacterial activity against E. coli TolC (Inhibition = 21–100% at 50 μM) compared to the hits from DCC-1. The most active compound 9 showed a MIC value of 19 ± 7 μM. These improvements in binding affinity and antibacterial activity confirmed our hypothesis that tdDCC can be used for the optimization of inhibitors/hits.
Inhibition of mtDXS was determined for the products from these heterocycles (thiophene, furan, imidazole, and pyrrole) are a hit. Several hits containing different phenyl rings favored. As hypothesized, the use of 1H-indole-6-carbaldehyde instead of 1H-indole-3-carbaldehyde was well-tolerated given that five hits containing this structural motif were amplified in these DCLs (26, 31, 32, 35, and 37). Moreover, several hits containing different phenyl rings (20, 21, 23, 25, and 30) were also amplified.

Figure 5. Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-2. Inhibition of drDXS was determined at 120 μM. Inhibition of mtDXS was determined at 200 μM. * = Inhibition of mtDXS was determined at 120 μM. n.d. = not determined. Percent (%) growth inhibition of E. coli TolC was determined at 50 μM. MIC values were determined only for the best compounds. ** = growth inhibition of E. coli TolC was determined at 25 μM.

tdDCC-3 & -4. To further support our hypothesis, we performed another two tailored tdDCC runs using the common structural motifs from the potent hits from DCL-2. We selected thiophene from hits 5, 6, and 7 and 2,4-dichlorophenyl from hit 9, and included the corresponding hydrazides H11 and H12 in the next tdDCC-3 (Figure 6). We also sought to unravel the influence of flexible linkers on the activity of the hits and thus included H12. In the tdDCC-3 along with hydrazides H11 and H12, we selected 22 commercially available aldehydes (A1, A2, A7–A26, see Figures 6a and 6b). For operational simplicity, we divided tdDCC-3 into two groups tdDCC-3a and tdDCC-3b, where two hydrazides H11 and H12 were reacted with two separate groups of eleven aldehydes each (Figures 6a and 6b).

For tdDCC-4, another common structural motif (indole) from the active hits of DCC-1 and DCC-2 was included as a building block (Figure 6c). Here we used 1H-indole-6-carbaldehyde (A23) instead of 1H-indole-3-carbaldehyde (A5) to understand the effect of substitution patterns of ligands on the binding to the protein and eventually in the activity of the potential acylhydrazones. Similarly, to understand the effect of inverting the position of the thiophene in the acylhydrazone, we included the corresponding aldehyde A7. Along with these two aldehydes we also included 1H-pyrrole-2-carbaldehyde (A20) and eight hydrazides, including five new hydrazides (H13–H17). Here, using DCC approach, and including a range of heterocyclic building blocks to cover a wide chemical space with good structural diversity, we aimed at studying to some extent the traditional medicinal chemistry approach driven by structure–activity relationships. On the contrary to the traditional medicinal chemistry approach, where all possible 68 acylhydrazone products from these three DCLs should be synthesized and tested for their biological activity, we let the protein select its best binders and synthesized only the amplified derivatives.

The analysis of these target-directed DCLs revealed the amplification of ten hits each for DCL 3a and 3b and nine hits for DCL4 (Figure 7, and Supplementary Figures 14, 16, and 18). Carefully inspecting the hits from all three DCLs revealed that the 2,4-dichlorophenyl motif and five-membered heterocycles (thiophene, furan, imidazole, and pyrrole) are favored. As hypothesized, the use of 1H-indole-6-carbaldehyde instead of 1H-indole-3-carbaldehyde was well-tolerated given that five hits containing this structural motif were amplified in these DCLs (26, 31, 32, 35, and 37). Moreover, several hits containing different phenyl rings (20, 21, 23, 25, and 30) were also amplified.
Figure 6. a) DCC-3a, building blocks; b) DCC-3b, building blocks, conditions: experiment was run in phosphate buffer (pH 7.04) and 5% DMSO, aldehyde (100 μM each, in DMSO), hydrazides (2000 μM each, in DMSO), aniline (10 mM in DMSO), drDXS protein (40 μM in phosphate buffer); c) DCC-4, building blocks, conditions: experiment was run in phosphate buffer (pH 7.04) and 5% DMSO, aldehyde (100 μM each, in DMSO), hydrazides (300 μM each, in DMSO), aniline (10 mM in DMSO), drDXS protein (40 μM in phosphate buffer).

Figure 7. a) Amplified acylhydrazone hits in DCL-3a. b) in DCL-3b; c) in DCL-4.

Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-3a, -3b and -4. We decided to synthesize a total of eleven out of 29 hits, based on their representative class
(five-membered and bicyclic heterocycles, functionalized phenyl rings, and chiral compounds) and/or their amplification in the DCL and evaluated their binding affinities for drDXS by SPR (Figure 8). Most of the hits show substantial improvements in binding affinity compared to the hits from the first and second round of tdDCC (Figure 8). Hits 11, 12, 21, 26, and 37 showed single-digit micromolar affinities (2–8 μM) and hits 22, and 35 showed binding affinity of 15 ± 3 μM and 32 ± 3 μM, respectively. When we evaluated these eleven hits for their inhibitory activities, six compounds (22, 23, 25, 26, 35 and 37) emerged as moderate inhibitors of drDXS (activity inhibition = 40–62% at 120 μM) similar to the hits from DCL-1 and -2, out of which compound 23 showed the best IC$_{50}$ value of 34 ± 4 μM. However, five hits (21, 23, 25, 26, and 35) showed improved antibacterial activities against E. coli (TolC), out of which compound 26 and 35 featured the best MIC values (14 ± 4 μM and 16 ± 5 μM, respectively). Overall, the hits from the last three rationally designed DCLs showed significant improvements in the binding affinity and antibacterial activity compared to the hits from tdDCC-1 and tdDCC-2. Moreover, the compound 23 emerged as the most potent inhibitor of the drDXS.

![Figure 8](image)

**Figure 8.** Binding affinity, enzymatic- and antibacterial-activity of hits from tdDCC-3, and 4. Inhibition of drDXS was determined at 120 μM. Percent (%) growth inhibition of E. coli TolC was determined at 50 μM. MIC values were determined only for the best compounds. n.d. = not determined. n.i. = no inhibition.

**Mode of inhibition studies.**

To obtain information about the binding site of the inhibitors, we selected representative compound 1 for the mode of inhibition studies. We performed competition enzyme activity assays, where, inhibition of drDXS was measured with varying substrate concentrations (curves are shown in Supplementary Figures 139–141).

The concentration of cofactor ThDP had a strong influence on the inhibitory activity, shifting the IC$_{50}$ value from 35 ± 10 μM at 0.3 μM ThDP to only 20% inhibition at 5 μM ThDP. Pyruvate concentrations showed a similar effect, increasing the IC$_{50}$ value from 19 ± 5 μM at 0.1 mM pyruvate to 86 ± 15 μM at 0.4 mM and just 32% inhibition at the highest concentration 0.6 mM. Concerning D/L-GAP, no influence of the substrate concentration on inhibition could be observed, all measured IC$_{50}$ values are...
in the range of 50 µM. These first findings show that compound 1 is ThDP- and pyruvate-competitive and non-competitive to D/L-GAP.

In the first step of the reaction catalyzed by DXS, ThDP and pyruvate are forming a covalent intermediate in the active site, while the binding of D/L-GAP to its charged pocket is accelerated 600 fold after the formation of an intermediate.\(^{22}\) A compound binding to the ThDP binding site is likely to also occupy parts of the pyruvate pocket, as both are located adjacent to each other. To bind to the cationic binding site of D/L-GAP, compounds with negatively charged functional groups, enabling ionic interactions, would be beneficial. The type of inhibition characteristics observed for compound 1 fits well to the initial docking study of acylhydrazones products from DCL-1 in the ThDP binding site (Supplementary Figures 1−6). In particular, the docked compound 1 is accommodated well in the ThDP active site and occupies a part of the substrate pocket (Figure 9).

![Figure 9](image)

**Figure 9.** a) Docked binding mode of compound 1 (cyan) compared to ThDP (pink) in the active site of drDXS (PDB code: 2O1X); b) Interaction of compound 1 with the residues in the active site of drDXS.

The reported inhibitor butylacetylphosphonate (BAP), containing an acetylphosphonate mimic of pyruvate,\(^{23}\) binds in the active site of DXS and forms a covalent complex with the ThDP and extends into the substrate pocket of the active site (for the active site of drDXS, see Figure 1b).\(^{24}\) The presence of ThDP and/or BAP in the protein-templated DCL can influence the amplification of acylhydrazones negatively if they are ThDP- and substrate-competitive inhibitors.

To provide support for this notion, two competition DCC experiments using DCL-1 were performed in the presence of ThDP (DCC-5) and ThDP + BAP (DCC-6). The composition of tdDCC-5 and -6 were analyzed (Supplementary Figure 19 and 20) and compared with the composition of tdDCC-1 (Figure 10). Addition of ThDP leads to a considerable decrease in the amplification of compounds 1, 2, 4, and 5, while compound 3 showed no change in the amplification (tdDCC-5, Figure 10). Comparing the results from tdDCC-6 (ThDP + BAP), with tdDCC-1 and tdDCC-5, we observed a further decrease in amplification of compounds 1 and 2, while compound 3 and 5 showed a minor or no decrease in amplification, and compound 4 showed a slight increase in amplification (Figure 10). These findings suggest that most of the identified hits from DCL-1 compete with ThDP and substrate(s) and supports the results from the MOI study for compound 1.
Discussion

This study demonstrated that tdDCC can be effectively used as a hit-optimization technique along with its well-established application for hit-identification. The process exhibits the potential of identifying potent hits in less time compared to the traditional medicinal chemistry approach. Importantly, using tdDCC, no prior knowledge of inhibitors is required, which allows generating new classes of hit compounds for important drug targets including underexplored ones like DXS with little or no structural information. In the first round, using tdDCC, we were able to identify a new class of hits for the anti-infective target DXS displaying good binding affinity and enzymatic activity. These initial hits were optimized in terms of binding affinity, enzymatic activity, and antibacterial activity during subsequent tdDCC runs. Compound 23 from the last round of DCC emerged as a most potent inhibitor of drDXS. A tailored tdDCC also allowed to shed light on the structure–activity relationships of this new class. Altogether, these results demonstrate a translational potential of tdDCC in the drug-discovery process. Additionally, docking study, competition-assays and -DCC experiments provided very important information about a mode of inhibition of compound 1.

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Competing interests

The authors declare no competing financial interest.
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**TOC image.** Hit-identification and -optimisation using target-directed dynamic combinatorial chemistry.