Discovery of Inhibitors by Combinatorial-Chemistry Approaches

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Chapter 6

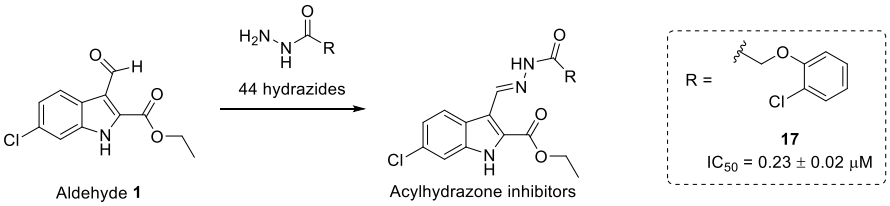
Summary and Future Perspectives

6.1 Summary

Initial drug discovery usually consists of testing chemically synthesized small molecules or isolated natural products against a protein target. After finding a hit, numerous iterative cycles of synthesis and biochemical evaluation are performed. Besides the discovery of a hit being uncertain, the process costs lots of time, money, energy and chemical resources. The work in this thesis focusses on finding and optimizing methods for initial drug discovery. Herein we describe the development of inhibitors using combinatorial screening with acylhydrazones, an attempted kinetic-target guided synthesis approach using reductive amination and a dynamic combinatorial chemistry (DCC) approach. We worked with three different enzyme targets: 15-lipoxygenase-1 (15-LOX-1), murine double minute 2 (MDM2) oncoprotein and macrophage migration inhibitory factor (MIF). Despite the large differences between the proteins, they can be linked to each other: MIF is a cytokine that interacts with numerous receptors and proteins. Among them is p53, a tumor-suppressor protein that is activated by cellular stress or damage and leads to cell-cycle arrest, apoptosis and DNA repair. MDM2 is the negative regulator of the p53 protein and its overexpression leads to loss of p53 function. By stabilizing the p53-MDM2 complex, MIF is a potent modulator of the p53 signaling pathway. Human 15-LOX-1 is a mammalian lipoxygenase, which plays an important regulatory role in several CNS and inflammatory lung diseases. It converts arachidonic and linoleic acid into hydroperoxyl fatty acids, which cause oxidative stress and can lead to ferroptosis. The hydroperoxyl fatty acids can also be further metabolized into lipid-signaling molecules, playing important regulatory roles in many diseases. MIF also has several pro-inflammatory functions and both MIF and 15-LOX-1 are involved in, for example, asthma. This thesis contains the following parts:

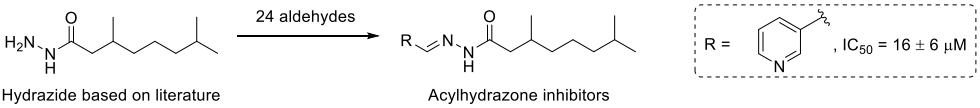
In **Chapter 1**, recent developments in DCC are discussed with a special focus on the analytical methods. First the concept of DCC and the general requirements and features are introduced. Then all the common analytical techniques, such as liquid- and size-exclusion chromatography, different nuclear magnetic resonance (NMR) spectroscopy methods, mass spectrometry, fluorescence spectroscopy and X-ray crystallography are discussed using literature examples. Last, a modification of DCC, tethering, is reviewed.

Chapter 2 describes our development of a combinatorial screening approach based on acylhydrazone chemistry to discover new inhibitors of 15-LOX-1. We focus mainly on the improvement of physicochemical properties, rather than on potency alone. An indole aldehyde hit, which was previously found in a fragment screening, is coupled to 44 hydrazides (Scheme 1). The diverse library is subsequently tested, without prior purification, in an activity-based assay against 15-LOX-1. The four most promising hits, as well as selected other compounds, are then synthesized, purified and re-tested against the enzyme. Three new inhibitors are found with IC_{50} values below 0.5 μM. One compound, acylhydrazone **17**, has an IC_{50} value of 0.23 ± 0.02 μM, falls into an interesting SAR and has an improved lipophilic ligand efficiency compared to a previously reported reference compound.



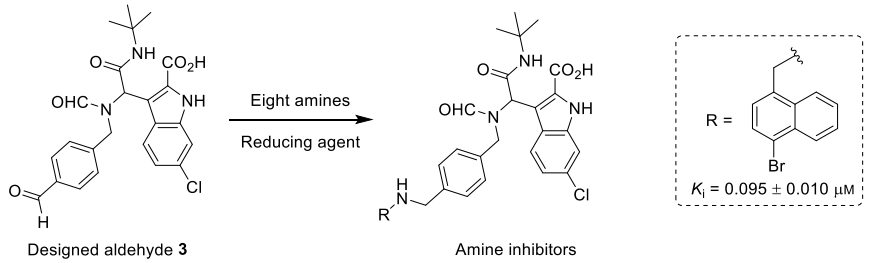
Scheme 1. Left, generation of *in situ* formed acylhydrazones that are tested against 15-LOX-1 and; right, the structure of most the promising inhibitor (**17**).

Despite the good potency and LLE values, the indolyl compounds suffer from their low aqueous solubility. Therefore, in **Chapter 3** we investigate the possibility of scaffold hopping using the combinatorial screening approach developed in Chapter 2. In this case, the tail of a previously reported potent molecule is used as hydrazide and various aromatic aldehydes are used for indole replacement (Scheme 2). This results in the identification of the 3-pyridyl ring as a suitable replacement for the indolyl core. However, the compound potency dramatically decreases to an IC_{50} value of 16 ± 6 μM. Attempts in optimizing the 3-pyridyl by installing various electron-donating and electron-withdrawing groups do not improve the potency. Additionally, replacing the hydrazide tail with hydrazides of the four most potent acylhydrazones from Chapter 2 also shows no improvement in inhibitory activity.



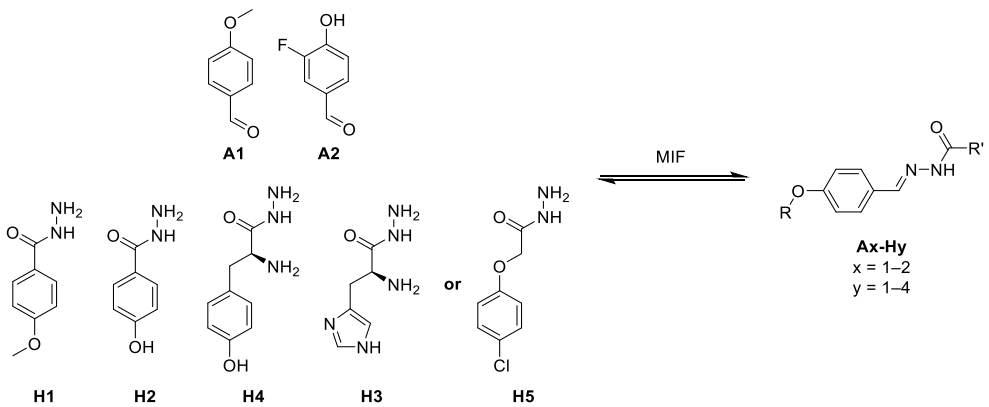
Scheme 2. Left, generation of *in situ* formed acylhydrazones that are tested against 15-LOX-1 to find a replacement for the indolyl core reported in Chapter 1 and; right, the structure of the most potent inhibitor.

In **Chapter 4**, we try to perform a protein-templated reductive amination reaction with MDM2. Based on the finding that the Leu26 pocket is rather flexible and is enlarged upon ligand binding, we envisioned this would be a particularly interesting target for a kinetic-target guided synthesis approach, given that flexible pockets are notoriously difficult to target using structure-based drug design (SBDD). Based on modeling with an X-ray crystal structure of MDM2 in complex with a reported inhibitor, we design and optimize a new scaffold, which can be assembled from an aldehyde and an amine using a reductive amination reaction. After synthesizing the aldehyde building block, it is combined with MDM2 and several amines in the presence of $NaCNBH_3$. After estimating the detection limit of our analytical method, we conclude that the reaction does not take place in the presence of the protein. After synthesis of all library members and testing against MDM2 and MDMX, we find that one of the products is a very potent and also rather selective inhibitor of MDM2 (Scheme 3).



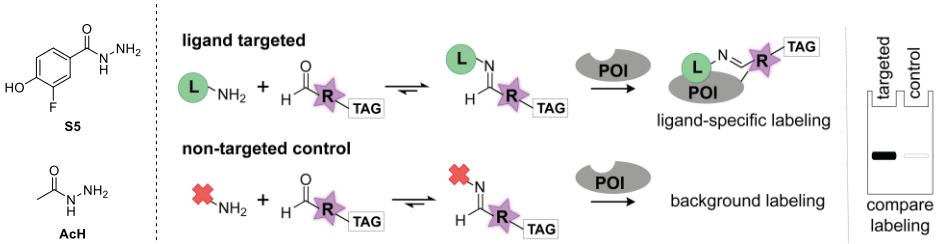
Scheme 3. Left, synthesis of reductive amination products as inhibitors of MDM2, and; right, the structure of the most potent inhibitor.

In **Chapter 5**, we apply DCC to identify inhibitors of MIF. We design a small library of acylhydrazones, based on the interaction between Asn97 and a phenol (Scheme 4). Initially, building blocks **A1–2** and **H1–4** are used, but **H3** turns out to catalyze acylhydrazone formation after increasing the pH to "freeze" the equilibrium. Therefore, **H3** was substituted by **H5**. The DCC experiment is repeated and shows amplification of two compounds. **A1-H5** is also highly amplified in the BSA control experiment and was not yet further studied. The other compound, **A2-H5**, shows very little amplification in the control experiment and is synthesized separately. The compound shows indeed to be potent against MIF (K_i value of 5.5 ± 0.5 μM). All other DCC compounds do not give a significant amplification of one of the possible products, although we show that e.g. **A2-H1** is also an inhibitor of MIF (K_i value of 6.7 ± 0.6 μM). We synthesize more compounds to establish a small SAR around the acylhydrazones and find that the position of the acylhydrazone motif does not affect the potency. Consistent with literature, we find that **A1-H1** is inactive against MIF and that the fluorine substituent in **A2-H1** lowers the potency by approximately a factor four. The addition of a second *ortho*-fluorine results in a decrease in inhibitory activity. Compared to inhibitors in the literature and from ongoing work in our group, the phenol acylhydrazones are a lot less potent. And we think that drastically improving these numbers would be very difficult.



Scheme 4. Designed dynamic combinatorial library to afford acylhydrazones as binders of MIF.

We realized that we could potentially use the fluorine-substituted phenol as targeting group of MIF for labeling purposes using a combinatorial approach, recently reported by the Witte group (Scheme 5). By combining aldehydes bearing reactive groups with hydrazide **S5**, we find time-dependent labeling for one of the *in situ* formed probes. Control experiments using blocking of the hydroxyl moiety, denaturation prior to labeling and competition with a known covalent inhibitor show that it is very likely the labeling takes place in the active site of MIF.



Scheme 5. Schematic representation of the combinatorial approach to synthesize probes that modify the protein target with a tag. The tag can subsequently be visualized by reacting it with a fluorophore. By comparing this signal with a non-targeted control, specific labeling can be detected. Adapted from Van der Zouwen *et al.*; *Chem. Commun.* **2019**, *55*, 2050.

6.2 Future Perspectives

Protein-templated DCC has now been used for more than two decades. The concept of library generation in the presence of a pharmacologically relevant target that enables the selective amplification of high-affinity binders is still fascinating. However, although it is a very promising concept which can revolutionize the drug-discovery process, there are still many hurdles to overcome. First of all, bigger and more complex libraries have to be used so that the method has a real advantage over traditional synthesis of each possible product. Moreover, equilibration times have to be shortened so that less stable enzymes can be used. One example would be the development of novel reversible reactions or the use of special substrates that accelerate commonly used reactions, such as acylhydrazone chemistry. Alternatively, the development of microflow-based DCC, of which recently the first example was reported, can be a solution.[1]

Another issue is the “freezing” of the equilibrium before analysis. Every handling step between having established an equilibrium and analysis of the library, can disturb the equilibrium. This is a serious problem, since it is unknown to what extent the disturbance takes place. Ideally, a library should be instantly analyzed without addition of base, co-solvent, heating, filtration or other treatment. So far, this has only been possible using special NMR methods, but especially in this case the library size is limited. The application of ^{19}F-NMR in the analysis of protein-templated DCC might be very convenient. Though, it is a commonly used analytical method in organic chemistry and of dynamic systems, so far, it has only been performed once in protein-templated DCC.[2] Taking into account that this example was using poor inhibitors (IC_{50} ± 3 mM), only standard 1D ^{19}F-NMR and that some

of the MIF inhibitors in Chapter 5 already contain a fluorine, this would be a very interesting start of a new project.

Regarding Chapter 5, several open ends remain. In order to be able to find a suitable phenol replacement, the DCL design should be optimized using inhibitors with known binding affinities/inhibition constants. The conditions should be varied in pH, catalyst, reaction times and buffer. After having established a working DCC system, new building blocks using possible phenol replacements should be used to see if amplification can be observed.

An important issue we would like to raise is the question how the acylhydrazone motif looks like in solution. It might sound self-evident, but it is essential information for modeling and docking, as well as the design of inhibitors and possible isosteres of the acylhydrazones. A detailed analysis using 2D NMR spectroscopy of the compounds in Chapter 2 showed that these acylhydrazones in DMSO likely do not exist as *Z*-isomer, but as $E_{\text{synperiplanar}}$ and $E_{\text{antiperplanar}}$ conformers. This effect has been reported in 2013.[3] However, some literature reports the existence of *E*- and *Z*-isomers due to observed splitting in NMR, while others do not specify the conformation or 2D structure of their molecules at all. In order to interpret inhibitory data of acylhydrazone inhibitors, it is crucial to understand how the molecules look like. Furthermore, we do not know if an inhibitor can easily rotate around its N–N bond in order to engage in more interactions with the protein target. The same holds for rotation around the amide bond. If one of the rotations takes place, this would result in large structural changes. Future research focusing on the flexibility and conformations of acylhydrazones, *e.g.* using NMR spectroscopy, protein-crystallography and *in silico* methods should answer these questions.

6.3 References

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