Kinetics and inhibition of enzymes in early stage drug discovery

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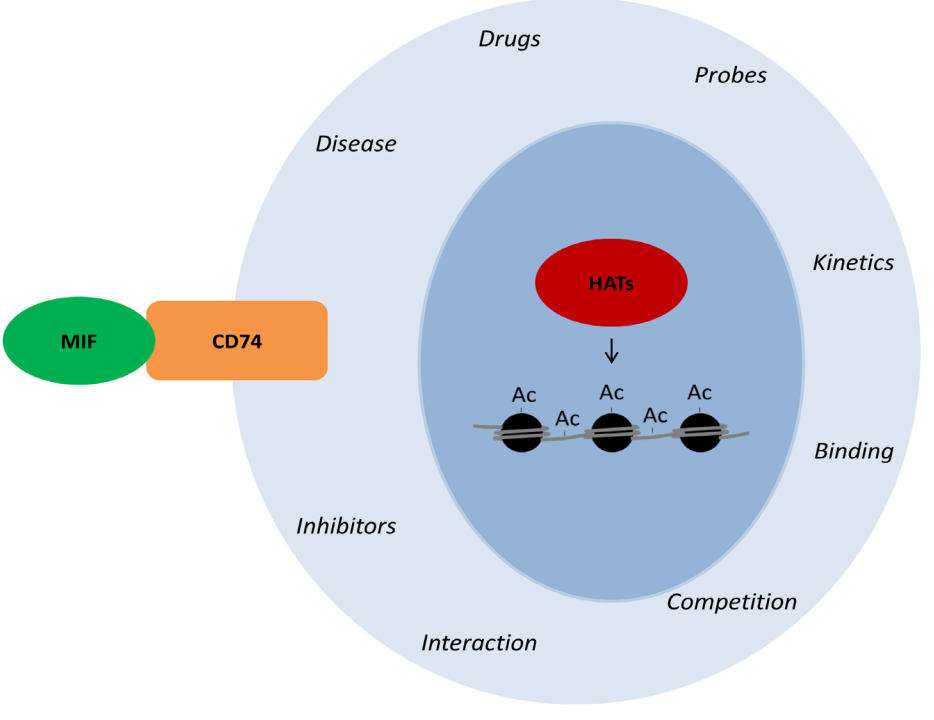
Chapter 1

Introduction and scope of the thesis



Inhibition of enzymes in early stage drug discovery

The discovery of inhibitors is an essential part of drug discovery. Not only can inhibitors be developed into therapeutic agents or diagnostics, they may also be used as tools to study biological or pathological processes. Many enzymes are drug targets and many drugs are enzyme inhibitors, for example the well-known COX1-2 inhibitors against pain and fever, kinase inhibitors in cancer and ACE inhibitors against high blood pressure. Lysine acetyltransferase 8 (KAT8) and macrophage migration inhibitory factor (MIF) are potential targets in diseases and there is a need for (novel) inhibitors for these enzymes. Therefore, this thesis describes the discovery of KAT8 and MIF inhibitors. The thesis is divided in three parts: PART 1: Inhibition of lysine acetyltransferase 8 (KAT8); PART 2: Inhibition of macrophage migration inhibitory factor (MIF); PART 3: Summary and future perspectives.

PART 1: Inhibition of lysine acetyltransferase 8 (KAT8)

Epigenetics and the histone language

Epigenetics is a field of study that includes reversible changes in gene activity without changing the DNA sequence (1). Epigenetic processes include DNA methylation, histone modifications, and chromatin remodeling, which all, directly or indirectly, modify the expression of the target genes. Histone modification is an indirect mechanism controlling chromosome structure and gene expression. Histones are octameric complexes of four different histone proteins (H2A, H2B, H3 and H4) that each wrap 147 base pairs of the DNA. Protruding from these complexes are the N-termini of the histones, which can be modified trough post-translational modification such as acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. These modifications form what is sometimes called a “language”, which can be “written”, “read” and “erased” (2). The “readers” are proteins containing domains such as bromodomains (“reading” acetylated lysines), chromodomains (“reading” methylated lysines), Tudor domains (“reading” methylated lysines and arginines), BRCT and 14-3-3 (“reading” serine phosphorylation) (3). The “writers” and “erasers” of histone modification are (nuclear) enzymes including kinases, phosphatases, methyltransferases, demethylases, ubiquitinases, deubiquitinases, acetyltransferases and deacetylases. Together these “readers”, “writers” and “erasers” form a dynamic process controlling gene transcription and influencing many down-stream pathways.

Histone acetylation and KAT8

Lysine acetylation is one of the main post-translational modifications of histones and plays an important role in the regulation of gene transcription by controlling the chromatin structure of DNA (4). One of the mechanisms proposed to be responsible for the regulation of transcription is the difference in charge between acetylated and non-acetylated lysines. Lysines are positively charged residues that can interact with the negatively charged DNA, resulting in a more condensed chromatin structure and a reduced accessibility for transcription factors. When the lysines are acetylated, they lose

their positive charge, resulting in a more open chromatin structure and more accessibility to gene transcription. The “writers” and “erasers” of this post-translational modification are the histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although these enzymes are primarily known for their function in histone (de)acetylation, they do not exclusively modulate histones. Many other proteins, such as nuclear enzymes and receptors, are substrates as well (5). As such, the function of these enzymes is not limited to chromatin remodeling, but extends to modulating the function of many nuclear processes. Although the most common names for these enzymes are histone acetyltransferases and histone deacetylases, more suitable names that are becoming more in use are lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). In this thesis, the acronyms HATs and HDACs are used for the enzyme classes to limit the discussion to those enzymes that are indeed histone modifying enzymes and exclude non-histone acetyltransferases/deacetylases such as α-tubulin acetyltransferase. In case of the human HAT iso-enzymes, a preference has been given for the KAT acronym to include the broad spectrum of targets.

Here, we focus on the “writers’ of histone acetylation, the HATs. HATs can be assigned to families based on primary structure homology. Three families have been studied extensively, which are the GNAT (GCN5-related N-acetyltransferase) family, the p300/CBP (p300/CREB binding protein) family and the MYST (acronym for MOZ, Ybf2, Sas2, and Tip60) family (6). Lysine (K) acetyltransferase 8 (KAT8) is a member of the MYST HAT family. KAT8 was initially discovered as a homologue of males absent on the first (MOF) in drosophila, which is the crucial histone acetyltransferase of the male-specific-lethal (MSL) complex. This complex is responsible for dose compensation of X chromosomal gene expression in drosophila males and is largely conserved in humans. As part of this complex in humans, KAT8 specifically acetylates histone H4 lysine 16 (7). Although this human complex is not responsible for dose-compensation, H4 lysine 16 acetylation has been shown to be important for chromatin structure (8). KAT8 forms additional complexes with WD repeat domain 5 (WDR5), the KAT8 Regulatory NSL Complex Subunit (KANSL) proteins and the methyl transferase mixed lineage leukemia 1 (MLL1) (9-11). This broadens the substrate specificity to histone H4 lysines 5 and 8 and non-histone targets, such as lysine 120 on the tumor-suppressor protein p53. Recently it has also become clear that KAT8, together with the KANSL proteins, is present in mitochondria and influences mitochondrial DNA transcription (12). KAT8 has been described as a housekeeping enzyme involved in cell proliferation, DNA damage response and stem cell pluripotency (13). Therefore, investigations of its role in cancer has started to arise recently. Although KAT8 clearly does not play identical roles in different types of cancer, it has been suggested as a potential drug target in some cases (14-17). The exact functions of KAT8 are, however, still under investigation and there is a limited number of tools available to study the enzyme. To aid the investigation of KAT8 function and its use as a potential drug target, KAT8 inhibitors would be a great advantage.

Inhibitors and their potency

The development of inhibitors for HAT enzymes has been a challenging task so far and no inhibitors for KAT8 were available at the start of this project. A complicating factor is

the kinetic behavior of the HAT enzymes and the consequences of this for determination of the potency of inhibitors. The potency of enzyme inhibitors is often measured by determining the 50% inhibitory concentration (IC_{50}). This is done by measuring the enzyme activity in the presence of increasing concentrations of inhibitor relative to the enzyme activity without inhibitor. The concentration of inhibitor giving 50% inhibition of enzyme activity, the IC_{50}, is then used as a measure for the potency. However, this IC_{50} value depends on the conditions used in the assay, such as the concentration of the enzyme substrates and their affinities for the enzyme, the K_m values. A change in assay conditions will therefore give a change in IC_{50} value. For an initial screen of the potency of inhibitors relative to a reference compound for a certain enzyme, this IC_{50} can be used and is relatively straightforward to obtain. However, for the comparison of the potency between different assays and for the determination of selectivity this value is not suitable. The inhibitory potency (K_i) value is a potency value independent of the assay conditions and is therefore much more representative as measure for the potency of an inhibitor (18). In case of competitive inhibitors of enzymes converting only one substrate, this K_i can be calculated using well-known methods like the Cheng-Prusoff equation, Dixon plot or using double reciprocal plots (19-21). However, since HATs, including KAT8, use a cofactor, acetyl coenzyme A (Ac-CoA), as an acetyl donor for the acetylation of the lysine on their target proteins, they are bisubstrate enzymes: they convert two substrates into two products. In this case, it is not possible to use these methods for calculation of the K_i and more elaborate kinetic evaluations are necessary (22). It is therefore important to investigate the kinetic behavior of HAT inhibitors for the determination of reproducible inhibitory constants. PART 1 of this thesis describes novel inhibitors of KAT8 and the kinetic evaluations that enable the calculation of their K_i values.

In **chapter 2**, a thorough summary is given from the known literature on HATs and HAT inhibitors. The specificity of the histone acetylation of each HAT is described as well as their function in cells. The role of HATs in diseases such as cancer, inflammatory diseases, viral infections and neuronal diseases is discussed and it is described where and how HATs have been suggested as suitable targets in these diseases. Finally, the current HAT inhibitors with their limitations and opportunities are described.

In **chapter 3**, the aim was to find inhibitors for the HAT subtype KAT8, since no inhibitors for this enzyme were described as yet. Anacardic acid, an inhibitor of other HAT subtypes, was discovered as KAT8 inhibitor and several derivatives were synthesized to investigate the structure-activity relationship. Using biochemical and biophysical methods, the catalytic mechanism of KAT8 and the mechanism of inhibition of anacardic acid was investigated. This led to a proposed model for the catalytic activity of KAT8 and enabled calculation of the inhibitory potency (K_i) values.

In **chapter 4**, a fragment screening method was used to discover compound **13** as a novel KAT8 inhibitor. A broad scope of control experiments and kinetic evaluations was employed to investigate this inhibitor. This showed that compound **13** was non-selective over other enzymes and had anti-oxidant activity, but did not show interference with the

KAT8 assay. Therefore this inhibitor was used to investigate the kinetic behavior of the enzyme and the inhibitor. Due to the enzyme catalytic mechanism and the remarkable mechanism of action of the inhibitor, this inhibitor was shown to have two K_i values. This stresses the importance of calculating the K_i values for inhibitors of KAT8 and other HATs.

In **chapter 5**, the aim was to discover novel KAT8 inhibitors based on C646, an inhibitor for the HAT subtype p300. Two classes of inhibitors showed inhibition in the KAT8 assay and their kinetic behavior was investigated. For the first class was shown that these were irreversible inhibitors of KAT8. The second class was shown to be reactive with the enzymatic product, which made it impossible to say whether these are true KAT8 inhibitors. This chapter shows the importance of further investigating the behavior of inhibitors and describes suitable and straightforward experiments to use as control experiments.

Chapter 6 gives an overview of HAT inhibitors and discusses the challenges that are met in the discovery of inhibitors for these enzymes. Challenges such as the substrate specificity of HATs, the molecular properties of the inhibitors, the catalytic mechanism of HATs and the lack of kinetic investigation of HATs and their inhibitors complicate the development of HAT inhibitors. Suggestions are given for strategies to improve the discovery of HAT inhibitors.

PART 2: Inhibition of macrophage migration inhibitory factor (MIF)

Inflammation and MIF

Inflammatory processes have been found to play a key role in diseases like asthma (23), rheumatoid arthritis (24) and inflammatory bowel disease (25). Additionally, inflammation seems to be involved in cancer and neurodegenerative diseases such as Alzheimer's (26) and Parkinson's (27) disease. The inflammatory response involves several different cell types that work together to fight a potential threat to the body. Communication between these immune cells is therefore essential for a fast and efficient mobilization of the immune response. Cytokines are the signaling molecules of the cells. They are expressed and excreted by immune cells upon recognition of a danger signal and interact with membrane receptors on target cells. This can lead to activation or inhibition of the inflammatory response depending on the type of cytokine and interacting target. Therefore, targeting pro-inflammatory cytokines and their interaction with receptors can be an efficient approach to inhibit the inflammatory response in disease. That this may be a feasible method, has been shown by studies with recombinant cytokine analogs, antibodies, decoy receptors and several small-molecules (28-31) in inflammatory diseases.

Macrophage migration inhibitory factor (MIF), is one of the pro-inflammatory cytokines involved in signaling between immune cells. Unlike other cytokines, MIF is constitutively expressed and stored in cytoplasmic pools (32). Therefore, it is rapidly released in response

to a stimulus. MIF is expressed by several immune cells, such as T-cells, macrophages, basophiles, eosinophils and B-cells (33). MIF interacts with surface receptors on B-cells, T-cells, macrophages and some epithelial cells, such as type II cluster of differentiation 74 receptor (CD74) and mediates signaling through complex formation of CD74 with CD44 and chemokine receptors CXCR2 and CXCR4 (34-36). This results in a stimulation of inflammation through for example leukocyte recruitment, upregulation of toll-like receptor 4 (TLR4) and activation of the NF-κB pathway. It was shown that neutralization of MIF with antibodies or genetic deletion provides benefits in several disease models, such as sepsis, inflammatory bowel disease and rheumatoid arthritis (37-40). MIF has also been shown to play a role in cancer through the downregulation of p53, which enhances the proliferation and survival of malignant cells. MIF has been shown to be overexpressed in several cancer cell types such as colon cancer, lung cancer, breast cancer, glioblastoma and melanoma (41-45). In a tumor mice model, the small molecule MIF inhibitor, ISO-66, was able to reduce the volume growth of mice inoculated with melanoma or colon cancer cells (46). Therefore, MIF is a potential target in both immune diseases and cancer and inhibitors of MIF may be ultimately used as therapeutic agents.

In **chapter 7**, the aim was to discover novel inhibitors of macrophage migration inhibitory factor (MIF). Making use of the tautomerase activity of MIF, novel derivatives based on known triazole and isoxazole MIF inhibitors, were synthesized. Additionally, a library of compounds of the chromene scaffold were screened for inhibition of MIF and several hits were found. The active compounds from these two groups will be further investigated for their inhibition of the interaction between MIF and CD74 and may ultimately lead to novel therapeutics against inflammatory diseases or cancer.

PART 3: Summary and future perspectives

Finally, both parts of the thesis are summarized and discussed in chapter 8 and future perspectives are described. Hoofdstuk 8 bevat ook een samenvatting in het Nederlands.

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