Targeting lysine acetylation in inflammatory lung diseases

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Chapter 6

Summary and General Discussion

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Lysine acetylation is a post-translational modification of proteins which regulates protein activity. Lysine acetylation plays a crucial role in the regulation of signaling pathways such as the NF-κB pathway. NF-κB signaling is involved in processes such as cell survival and inflammation. The NF-κB transcription factor is regulated by dynamic lysine acetylation which determines its cellular localization and DNA binding capability, among others. Lysine acetylation is installed by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Small molecule inhibitors of these enzymes can modulate NF-κB activity, and have potential in attenuating NF-κB-mediated inflammatory responses. NF-κB activity has been reported to be increased in the inflammatory airway diseases asthma and COPD. Furthermore, in macrophages, NF-κB is involved in pro-inflammatory gene expression, which plays an important role in the orchestration of asthma and COPD. Based on this, small molecule HAT inhibitors (HATi) and small molecule HDAC inhibitors (HDACi) have potential in the treatment of these diseases. Such alternative therapeutic strategies are needed, since not all patients are responsive to the current therapy which is based on glucocorticoids. In **chapter 1** an introduction to the thesis is given, addressing these topics in detail. In this thesis we explore the potential of small molecule HATi and HDACi in attenuating NF-κB-mediated inflammatory responses in model systems for inflammatory airway diseases such as asthma and COPD. In total, 3 HATi and 1 HDACi were studied, and the findings are summarized and discussed below.

In **chapter 2** we investigate C646; an inhibitor for the HAT p300 (K_i of 0.4 μM). The small molecule inhibitor C646 for the histone acetyltransferase p300 is one of the few potent molecules for histone acetyltransferases described to date. From this perspective, numerous studies have focused on applications of C646 in oncology. In view of recent literature on the role of p300 in inflammation we focused our studies on model systems for airway inflammation with the aim to resolve the question if C646 is a valuable lead compound for further development in this disease area. We demonstrate that C646 attenuates LPS and IFNγ-induced NF-κB activity and pro-inflammatory gene expression in RAW264.7 murine macrophages and pro-inflammatory gene expression in murine precision-cut lung slices. Considering the role of p300 in acetylation in the NF-κB pathway, this was in line with our hypothesis. Next to this, we found C646 to increase histone acetylation in RAW264.7 macrophages, and to inhibit recombinant histone deacetylases (by either covalent or allosteric binding) from 7 μM and higher concentrations. These finding were unexpected. Considering the importance of C646, which is being used in an increasing amount of studies, we believe that this finding has a considerable impact on the field. Its lack of selectivity at higher concentrations needs to be taken into account, since in biochemical experiments, C646 is sometimes used at concentrations where HDAC inhibition also takes place. Clearly, this can hamper the elucidation of effects connected to presumed inhibition of p300 in the investigated model systems. Notably, the highest potency for HDAC inhibition by C646 was observed for HDAC6. We investigated the acetylation of α-tubulin, an acetylation target

of HDAC6, to confirm the inhibition of HDAC6 by C646 in cells. However, no increased α-tubulin acetylation upon C646 treatment was found. Instead, C646 decreased α-tubulin acetylation. This could be explained by inhibition of p300, which could reduce α-tubulin acetylation. However, p300 has not been described to acetylate α-tubulin directly. p300 has been described to mediate acetylation of HDAC6, which renders HDAC6 less active. Therefore, inhibition of p300 by C646 could decrease HDAC6 acetylation, thereby increasing HDAC6 activity, and thereby resulting in less α-tubulin acetylation. Altogether, despite its potential to suppress pro-inflammatory gene expression, these results indicate that further optimization of C646 is necessary with respect to its HDAC inhibitory potency, and to its HAT inhibitory selectivity over HDACs. It remains unclear if the suppression of pro-inflammatory gene expression is due to its HAT inhibitory activity, due to its HDAC inhibitory activity, or inhibition of other enzymes (or a combination of these factors). We anticipate that the balance between HAT and HDAC inhibition depends on the precise target lysine(s) for acetylation or deacetylation, the enzymes that regulate them, and the extent of inhibition of these enzymes by C646. Finally, in support of our findings, another recent study where C646 was modified into a probe (C646-yne; an alkyne allowing for click chemistry) and its covalent targets in HEK293 cell lysates were identified using mass spectrometry, demonstrated that C646 binds cysteine rich proteins (1), which include the HDACs.

In **chapter 3** we investigate MG149; an inhibitor for the MYST type histone acetyltransferases Tip60 (KAT5) and MOF (KAT8). MG149 was derived from anacardic acid (AA), which is a well-known and well-studied natural product HAT inhibitor. Interestingly, previous literature has reported that the 6-alkylsalicylate AA can attenuate inflammation in models for lung inflammation. Compared to AA, MG149 displays increased selectivity towards the MYST HATs Tip60 and MOF, which was determined in a previous study by our group (2). We determined the K_i value for inhibition of the MYST type histone acetyltransferase MOF by MG149 to be 39 ± 7.7 μM, which is an improvement compared to AA (K_i value of 64 ± 8.9 μM). In line with the inhibition of these MYST type HATs, inhibition of histone acetylation was observed in murine precision-cut lung slices in this thesis, using mass spectrometry. This correlated with inhibition of LPS and IFNγ-induced pro-inflammatory gene expression in the murine precision-cut lung slices. These results, together with literature on other 6-alkylsalycilates, highlight the potential of 6-alkylsalycilates which inhibit HATs for the treatment of inflammatory lung diseases such as asthma and COPD.

In **chapter 4** we investigate 4-amino-1-naphtol (compound **13**); a compound discovered at our lab to potently inhibit the histone acetyltransferases PCAF (KAT2B), p300 (KAT3B) and MOF (KAT8) non-selectively. The chapter focuses on the biological characterization of **13** in the context of inflammatory lung diseases. We demonstrate that **13** potently inhibits histone acetylation in RAW264.7 macrophages (using western blot and mass spectrometry), and that **13** inhibits pro-inflammatory gene expression in

murine precision-cut lung slices. **13** has anti-oxidant activity, which should be kept in mind. However, our studies indicate that the concept of HAT inhibition for the treatment of inflammatory lung diseases, is promising.

In **chapter 5** we address new therapeutic options for COPD. We approach this through a novel concept of selective histone deacetylase (HDAC) inhibition in a COPD model using MS-275; a selective inhibitor for HDAC1-3. In macrophages, MS-275 upregulated the expression of the anti-inflammatory cytokine *IL10*. Since macrophages are crucial players in COPD, and IL-10 can determine their functional role, this is particularly relevant. The increased *IL10* expression in macrophages was due to increased acetylation, nuclear translocation and binding to the *IL10* promoter of the NF-κB transcription factor. This mechanism is in line with literature which describes that LPS stimulation of macrophages activates NF-κB, which induces the expression of *IL10* along with *TNFα*, *IL1β*, and *IL12b* (3), which is further enhanced by MS-275 in our study. Importantly, when proceeding to studies in cigarette-smoke exposed mice, we observed anti-inflammatory effects based on several parameters including increased *IL10* expression in lung macrophages, reduced KC (murine IL-8) expression and reduced neutrophilic influx upon MS-275 treatment. The mechanism through which MS-275 increases *IL10* expression in connection to NF-κB signal transduction, along with the anti-inflammatory effects in mice, highlight the potential of isoform selective HDAC inhibition for the treatment of inflammatory lung diseases like COPD. HDACi are currently being used in the treatment of hematological cancer types, and MS-275 is in clinical trials for such applications. This chapter, however, points towards an important alternative potential application.

Compared to MS-275 more selective inhibitors may have more selective effects on NF-κB-mediated inflammatory responses in macrophages. For example, the HDAC3 selective inhibitor RGFP966 is an interesting candidate, based on the important role of HDAC3 in deacetylation of specific NF-κB p65 lysines (including K122 and K123, acetylation of which inhibits NF-κB activity) (4), and our previous results. RGFP966 increased anti-inflammatory IL-10 expression, and reduced pro-inflammatory gene expression in murine precision-cut lung slices. In RAW264.7 murine macrophages, pro-inflammatory gene expression was decreased, which correlated with reduced NF-κB activity (5). This is further supported by RNAi experiments for HDAC3 which demonstrated inhibition of pro-inflammatory gene expression in the macrophages (5). An interesting hypothesis is that our observations could be attributed to the capability of more selectively regulating the acetylation status of NF-κB lysines such as K122 and K123 by selective HDAC3 inhibitors such as RGFP966.

A number of important insights have been acquired in the field of HDACi over the past recent years, which will be addressed in the following sections. While it is inevitable that these sections focus on HDACi because these inhibitors have been studied much more extensively, these considerations could also be relevant for HATi.

From histones to transcription factors

It is interesting to note that the effects of HDACi on histone acetylation are oftentimes used as a read–out indicating the effects of these inhibitors in cells. However, the studies discussed above indicate that effects of HDACi on transcription factors and their acetylation status are also important to the outcome in disease models, including those for asthma and COPD. Several studies also indicate that effects on histone acetylation do not always correlate with effects on biological processes such as gene expression. For example, upon HDACi treatment, some genes have been reported to be upregulated, while others go down (roughly as many go up as down), whereas global histone acetylation is generally pronouncedly increased (6). Interestingly, the balance between effects on histone acetylation and gene expression also changes depending on the employed incubation time (7). In HeLa cells it was demonstrated that valproic acid (VPA) and suberanilohydroxamic acid (SAHA) have an early onset effect on gene expression after 12 hrs of incubation. Upon 48 hrs of incubation, these changes in gene expression had returned to baseline levels, while increases in histone acetylation had not (7). Another study found that upon HDACi treatment there was a precisely timed increase in histone H3 lysine 27 trimethylation (H3K27me3) at transcription start sites, but little or no increase in histone acetylation, whose role seemed to be to provide a stable chromatin environment that allows transcription to be modified by other factors (8). In another study, the non-selective HDACi Romidepsin and SAHA were tested in different cancer cell lines, and their effects on histone acetylation were investigated together with their effects on apoptosis (9). Treatment with Romidepsin or SAHA for 6 hours caused similar increases in histone acetylation in all cell lines, but not all of the cell lines underwent apoptosis upon Romidepsin or SAHA treatment. Therefore, the effects on histone acetylation did not correlate with effects on apoptosis in all cell lines (9). In summary, recent research indicates that HDACi-induced changes in histone acetylation cannot (fully) explain their effects on gene expression and apoptosis. Effects of HDACi on the acetylation status of specific transcription factors may therefore be particularly important in explaining their effects in disease models, such as those for asthma and COPD.

From recombinant enzymes to HDAC activity in cells

Isoenzyme selectivity of HDACi has important consequences for their effects. Hence, development of highly potent molecules selectively targeting specific HDACs or a select group of HDACs is important. This is likely important in obtaining selective effects on specific pathways such as the NF-κB pathway in the context of asthma and COPD models, which occurs through fine-regulation of its acetylation on specific lysines (4). A selectivity profile for HDACi is generally obtained by testing the inhibition on recombinant HDAC enzymes (10). However, it has become apparent that several other factors need to be taken into account. Firstly, it has been demonstrated that binding characteristics of HDACi in cells differ from the profile on recombinant enzymes due to the fact that HDACs exist in

multiprotein complexes, adding another level of complexity. For example, one study used a chemoproteomics strategy, where an affinity capture method was employed using Sepharose beads derivatized with HDACi hydroxamic acid analogues (11). This allowed for a competition binding assay, where extracts of cells treated with increasing HDACi concentration competed with the immobilized HDACi analogue probe matrix for binding protein-targets. The reduction in protein capture resulting from the competition with the increasing concentration of the 'free inhibitor' was quantified by mass spectrometry using isobaric tandem mass tags (11). Target protein complexes interacting with HDACi could be identified, and importantly, inhibitor selectivity for native drug target complexes deviated from literature values obtained using recombinant enzymes, revealing an unexpected degree of selectivity of certain HDACi. For example, benzamide-based inhibitors displayed a preference for the HDAC3-NCoR complex (11). As a side-note, interestingly, chemoproteomic approaches also enable the identification of possible off-targets of HDACi (11). Another option to investigate HDACi specificity is to analyze HDACi-induced changes at the level of all lysine acetylation sites (acetylome). A study from Scholz *et al.* elucidated the selectivity profile of HDACi at the level of the global acetylome (12). HeLa cells were treated with a panel of widely used HDACi and changes at the acetylome were monitored by quantitative bottom-up proteomics. Stable isotope labeling of amino acids in cell culture (SILAC) was combined with enrichment of acetylated peptides using anti-acetyllysine antibodies and analysis by mass spectrometry. The fraction of upregulated acetylation sites in HDACi-treated cells was greater than the fraction of downregulated sites (12). For several HDACi, the number of acetylation sites affected was not proportional to the number of HDACs they were found to inhibit when testing on recombinant HDACs, which is in line with chemoproteomics studies demonstrating unexpected selectivities of HDACi for multiprotein complexes (as for example described in (11)). In principle, differential proteomics approaches as described by Scholz *et al.* (12) enable to elucidate HDACi selectivity profiles at the level of the nuclear, cytosolic or mitochondrial acetylome. Altogether, while HDACi are currently often characterized by their selectivity profile on recombinant HDACs, they can be more accurately characterized by generating a selectivity profile on HDACs in cells, which can be complemented with acetylome analysis. We envision that such better characterizations of HDACi targets and specificities allow for a more selective targeting of HDACs and their substrates, which will drive the further development of HDACi as potential therapeutics for diseases such as asthma and COPD.

From IC_{50} to binding kinetics

Next to these studies into selectivity profiles, there has been a study into the binding kinetics of HDACi with recombinant HDACs (13). A reporter displacement binding assay was used to quantify the association (k_{on}) and dissociation (k_{off}) kinetic rate constants as well as the binding constants (K_d) for a set of hydroxamic acid-based and benzamide-based inhibitors

against HDAC1 and HDAC2. While K_d values were similar, the k_{on} and k_{off} were slow for the benzamides. Therefore, benzamides displayed slow, whereas hydroxamic acids displayed fast binding kinetics (13). The effects upon washout of the inhibitors were then tested in the neuroblastoma cell line SH-SY5Y; after a 'pulse' treatment with the inhibitors for 6 hrs and replacement of the medium with a drug-free medium. In line with slow binding kinetics for benzamides, a sustained state of histone hyperacetylation was observed for the benzamide-based inhibitor MS-275 after washout (which was still observed after 96 hrs), while upon treatment with the hydroxamic acid-based inhibitors, acetylation returned to baseline levels much faster (within 18 hrs) (13). Becher *et al* reported similar findings using a chemoproteomic approach which allowed for a comparison of time-dependent binding of hydroxamic acid-based and benzamide-based HDACi (14). Also in line with this, another study using MS-275 found this compound to have a long lasting effect on histone acetylation of histone H4 lysine 12 (H4 K12) after washout (15). These studies indicate that the binding kinetics of HDACi have biological consequences, which is a point that needs to be taken into account next to the selectivity profile of HDACi, since it is important in evaluating their therapeutic utility.

Concluding remarks

This thesis addresses the targeting of lysine acetylation in inflammatory airway diseases. We base our strategy on the fact that lysine acetylation regulates important signaling pathways such as the NF-κB pathway. Enhanced NF-κB activity has been reported in the inflammatory airway diseases asthma and COPD. Using small molecule inhibitors to selectively regulate NF-κB acetylation status, and thereby modulate NF-κB activity, could be an alternative therapeutic strategy for these diseases.

While this thesis demonstrates the potential of HATi in attenuation of inflammatory responses in asthma and COPD models, there is still a need for more potent and selective HATi. The development of potent and cell permeable small molecule HATi is at an early stage, and a challenging issue that has been met with limited success so far. This is an area of future research in need of urgent attention.

The development of HDACi has been much more successful. Currently, HDACi are being used in the treatment of hematological cancers, however, these compounds have anti-inflammatory properties at lower concentrations than those which are being used to treat cancer. Therefore, there has been interest in these compounds as therapeutic agents in other areas, including in inflammatory airway diseases such as asthma and COPD. HDACi can modulate the NF-κB pathway, which could explain their anti-inflammatory properties. In line with this, our results indicate that the HDAC1-3 inhibitor MS-275 can modulate the NF-κB transcription factors in macrophages, which increases anti-inflammatory *IL10*

expression. In this thesis, we also demonstrate for the first time that the HDAC1-3 inhibitor MS-275 can attenuate inflammation in a COPD mouse model.

Also for the HDACi, generating more selective inhibitors is still an area of future research where important steps can be made. A number of important insights have been acquired over the past recent years. For example, it is important to study the selectivity profile of HDACi in cells, since the inhibition at the level of recombinant HDACs has been shown to vary from the inhibition on HDACs in cells which are present in multiprotein complexes. A selectivity profile can also be generated at the level of the acetylome. Mass spectrometry has been particularly useful in allowing these kinds of analyses, which allow for a better characterization of HDACi targets and selectivity at different levels complementing each other. Off-targets of inhibitors can also be investigated using mass spectrometry based techniques. Next to studying IC_{50} values, it is important to take thermodynamic and kinetic parameters into account, such as the residence time of HDACi in recombinant HDACs, which has been shown to vary between hydroxamic acid and benzamide type HDACi.

Altogether, both HATi and HDACi have great potential for the treatment of inflammatory airway diseases. However, a clinical application in this field is still far from within reach. In the field of HATi, there is a need for more potent and selective inhibitors. For the HDACi, the recent insights as discussed above are crucial in the future development of more selective HDACi, and will drive the development of such HDACi. This is essential in broadening their application towards inflammatory lung diseases such as asthma and COPD. Future steps must also include further elucidation of the effects of HDACi in more advanced disease models. Finally, a challenge lies ahead in determining which specific HATs or HDACs need to be targeted by the inhibitors.

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