Chemistry-based enzyme detection and inhibition in epigenetics

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CHAPTER 8

Summary and future perspectives

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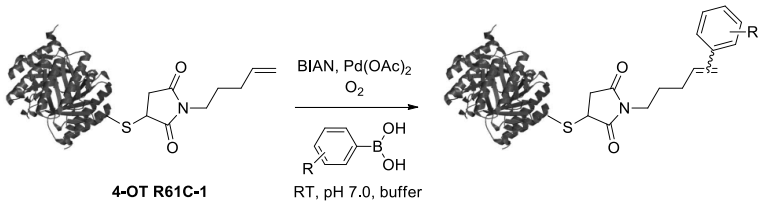
The aim of this dissertation is the development of novel diagnostic tools to trace endogenous post-translational protein modifications and study the activity of chromatin-remodeling enzymes in an attempt to unravel their role in pathological conditions.

Oxidative Heck reaction as a bioorthogonal protein-labeling strategy

The currently used methods for detection of enzyme activity have serious limitations that hamper their applications. For instance, antibody-based techniques suffer from high costs and lack of specificity, while analytical strategies (i.e. NMR spectroscopy, mass spectrometry) are either ineffective in complex protein mixtures or require long sample treatment. The expanding field of chemical biology has led to the discovery of a plethora of bioorthogonal chemical reactions employed to monitor enzyme activity *in vitro*.

Chemical labeling involves the coupling of biomolecules to reactive functionalities and subsequent visualization through a detection handle attached *via* bioorthogonal chemistries. In combination with metabolic labeling, where functionalized reporters, analogues of the natural substrates, are enzymatically incorporated to the target protein, chemistry-based methods have been proven to be a powerful tool in the study of numerous endogenous protein modifications. The most popular bioorthogonal coupling reaction is the copper-catalyzed Huisgen cycloaddition, known as the 'click' reaction, that links azides to alkynes. Despite the possibilities that they offer as chemical handles, the polarity of azides and the potential of intracellular cross-reactivity of terminal alkynes due to the acidity of the alkynic proton are serious drawbacks. Terminal alkenes are advantageous due to their higher similarity to alkanes and lower reactivity and thus represent attractive functional groups.

In **chapter 2**, we describe the development of the oxidative Heck reaction as a novel bioorthogonal reaction to detect alkene-labeled proteins *in vitro*. Before moving to real applications, in order to optimize the reaction conditions, we started by exploring the scope of the reaction on a model protein. We selected the enzyme 4-oxalocrotonate tautomerase mutated at the pre-last amino acid position with a cysteine that allows for coupling to a maleimide-bound terminal alkene *via* a Michael addition. The catalyst included $Pd(OAc)_2$ and BIAN (bis-imine of acenaphthenequinone and mesitylamine) as a ligand and was prepared in dimethylformamide (DMF). Different substituted arylboronic acids were used as coupling partners (Scheme 1). Reactions were performed for 24h at room temperature, under oxygen atmosphere and in aqueous environment with a final ratio of buffer:DMF 6:1 to keep the reagents in solution. Nevertheless, subsequent chelation of Pd with EDTA (ethylenediaminetetraacetic acid) appeared to be necessary due to the non-specific binding of the metal to amino acid residues in the protein. Mass spectrometric analysis revealed the conversion to the desired reacted protein. Varying the amount of the reagents, we found that the optimal



Scheme 1. Oxidative Heck reactions on protein-bound alkene 4-OT R61C-1.

conditions for full bioconjugation were 20 equiv. of the catalyst and 100 equiv. of the arylboronic acid with respect to the protein. Interestingly, when the same reaction conditions were applied to *cis* and *trans* internal alkenes high conversion was observed in case of the *cis* while only 5% of the protein-bound *trans* alkene reacted. This indicates that great potential lies on the use of the oxidative Heck reaction for selective detection of *cis*, in presence of *trans*, unsaturated fatty acids.

The oxidative Heck reaction was then used to detect 4-OT R61C-1 by gel electrophoresis (SDS-PAGE) after linkage to the fluorescent 3-(dansylamino) phenylboronic acid resulting in full conversion. The same coupling was performed in presence of a cell lysate from RAW267.4 macrophages mixed with 4-OT R61C-1 in ratios 1:1 and 10:1. Pleasingly, in both cases only the protein-bound alkene was visible by fluorescence. These findings introduce the oxidative Heck reaction as a useful detection tool to efficiently and selectively monitor alkene-labeled proteins *in vitro*.

In **chapter 3**, we continued our studies by employing the reaction for detection of histone acylation *in vitro* in combination with metabolic labeling techniques. First, fully aqueous conditions and high conversions of 4-OT R61C-1 were achieved with the use of EDTA as a ligand for the catalyst and the water soluble 3-(biotinylamino)phenylboronic acid and 3-(dansyl-PEG-amino)phenylboronic acid as detection handles used at the amounts optimized before. In comparison to BIAN and 2-amino-4,6-dihydroxypyrimidine, developed by Davis *et al.*, EDTA proved to be a more efficient ligand for Pd(II)-catalyzed bioorthogonal protein couplings while chelation of Pd out of the protein was no longer needed. However, opposite results were obtained when EDTA was used on a small-molecule terminal alkene in presence of 1 equiv. of the catalyst and 10 equiv. of phenylboronic acid, indicating the superiority of this ligand only in protein-bound alkenes as supported from the water solubility and stability of EDTA in presence of proteins. Moreover, conduction of the reaction at various pHs supported neutral or slightly basic conditions as the optimal for completion while full conversion was achieved at concentrations of 4-OT R61C-1 down to 5 μM. However, the efficiency of the oxidative Heck as well as the 'click' reaction dropped at lower concentrations of the corresponding functionalized proteins.

Aiming at monitoring histone acylation *in vitro*, we employed 3 olefinic carboxylates, sodium 4-pentenoate **3.4a**, its thio- and methyl ester, as chemical reporters for enzymatic transfer by histone acetyltransferases (HATs) and alkene-

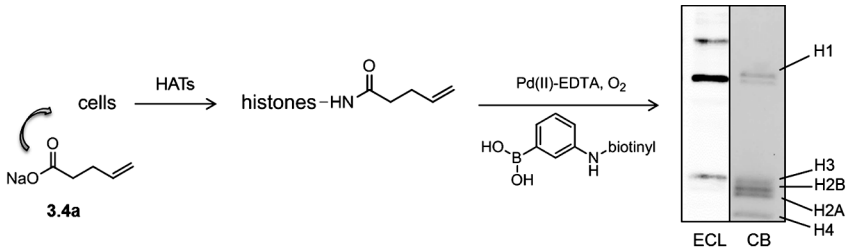


Figure 1. Metabolic alkene labeling, biotinylation and *in vitro* detection of histone acylation *via* the aqueous oxidative Heck reaction. ECL; enhanced chemiluminescence assay for on blot detection, CB; coomassie blue staining.

labeling of histone proteins. Treatment of RAW267.4 cells with 10 mM of each compound for 6h followed by histone extraction and biotinylation *via* the oxidative Heck reaction resulted in efficient labeling of histone H3 in case of **3.4a** (Figure 1) while its esters were poorly incorporated. Similar luminescent intensity was obtained after incubation with sodium-4-pentynoate and subsequent 'click' reaction but in this case histone H4 was also detected implying a better incorporation of 4-pentynoyl moiety into this histone compared to the 4-pentenoic one. Finally, inhibition studies with known HATi and histone deacetylase (HDACi) and subsequent cell treatment with reporter **3.4a** resulted in reduction of H3 labeling due to lower activity of the endogenous enzymes controlling histone acylation (HAT) and removal of the naturally introduced acetyl groups (HDACs). However, the effect of HATi on lysine acetylation levels was not visible when detection occurred *via* the anti-acetyl lysine antibody. These data support the advantageous employment of chemistry-based approaches to test the *in vivo* effectiveness of developed inhibitors against enzymes implicated in diseases.

In **chapter 4**, we review the applications of metabolic labeling techniques and bioorthogonal chemistries on the study of protein acylation *in vitro*. Specifically we emphasize on the reactions that employ terminal alkene- or alkyne-functionalized chemical reporters as they represent valuable reactive functionalities due to their small size and facile cellular uptake. Next to their advantages, we also report the downsides of each reaction seeking for the most appropriate for bioconjugation. We also point out that in order to investigate post-translational protein modifications the first step of incorporation of the reporter into the protein of interest has to occur *in vivo* using cell's metabolic machinery, while the respective bioorthogonal coupling may take place *in vitro* after cell lysis and protein extraction. Focusing on protein acylation, we then describe the metabolic pathways of acyl-CoA to outline the types of acyl-CoA alkene- or alkyne-functionalized reporters that have been used to monitor this protein modification *in vitro*. Moreover, we demonstrate our own efforts on probing acylated proteins by using olefinic α-keto carboxylates but subsequent biotinylation *via* the oxidative Heck reaction revealed no labeling of either cell lysates or extracted core of histones presumably due to the competition of the reporter with natural substrates and its less favorable uptake by endogenous

enzymes. Despite the undeniable success of metabolic labeling strategies, we conclude that the need for high concentrations of chemical reporters or/and the use of inhibitors against the enzymes that remove the added label question the true representation of endogenous protein modifications in their physiological context. Therefore, scientific efforts should be oriented towards the discovery of improved diagnostic tools that will allow not only for identification but also for quantification of these modifications and will offer a closer look on the biological mechanisms underlying pathological responses.

Our work justifies the use of the oxidative Heck reaction as an attractive alternative to the widely-used 'click' reaction as it presents comparable efficiency. Moreover, considering its successful application in *in vitro* monitoring protein acylation and, hence, endogenous HAT activity, future work should be focused on employing the reaction for detection of the activity of other enzymes involved in serious diseases. However, despite its efficiency and chemoselectivity to protein-bound alkenes, the long reaction time needed for the carbon-carbon bond formation hampers its use in cases where bioconjugation has to be performed rapidly. In addition to limitations of other bioorthogonal chemical reactions developed so far, novel chemistry-based biocompatible methods are required to explore enzyme function and validate small-molecule inhibitors as candidates for therapeutic approaches.

Phoswitchable HDACi as promising anticancer agents

Extensive literature supports the important role of HDACs in epigenetic regulation of gene expression and the link between aberrant HDAC activity and tumorigenesis. A wide variety of HDACi has been identified over the years but only few of them have been approved for clinical use or are under investigation in clinical trials for the treatment of several malignancies. A common side effect of classic chemotherapy is cytotoxicity due to non-selectivity of clinically-used agents. This problem can be addressed by the use of photopharmacological approaches where light is employed for local, external activation of a drug containing a photoswitchable part limiting its cytotoxic effects and allowing for the use of higher concentrations.

In **chapter 5** of this dissertation we present the development of photoswitchable analogues of the clinically-used hydroxamic acid-type HDACi vorinostat, panobinostat and belinostat as potential chemotherapeutic agents. As a 'photoswitch' we selected an azobenzene moiety introduced either in the cap (first design) or the linker region (second design) of the parental agent allowing for two isomeric forms, the *trans* and the *cis*. Ideally, the thermodynamically stable *trans* form is less potent than the *cis* and application of UV irradiation will alter the binding properties providing the photoactivatable, active *cis* form. Towards this aim, we designed and synthesized 12 compounds with various chain lengths, substitutions and positions of the azobenzene group in respect to the cap of the parental agent. Photochemical studies revealed a satisfactory photostationary state (PSS>76%) while almost all the compounds exhibited a half-life of their *cis* form of more than 2h in aqueous environment.

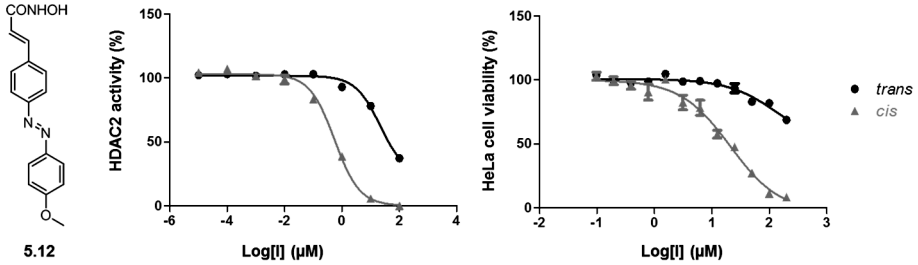


Figure 2. Studies on the performance of the photoswitchable HDACi **5.12**.

All compounds were initially tested for inhibition against HDAC activity from HeLa nuclear extracts where molecules of the first design displayed higher potency than the ones of second design with the *trans* isomeric form being more active than the *cis*. Afterwards, selected compounds were screened for potency against human recombinant HDAC1-3, 6 and 8. Regarding the first design, *trans*-compounds showed great potency in the low nanomolar range (even 10-fold or greater comparing to the original vorinostat) but small difference in activity (expressed in IC_{50} values) between *cis*/*trans* isomers. Gratifyingly, this difference was significantly high for the second design with *cis*-**5.12** being nearly 40 times more potent than *trans* against HDAC2 (Figure 2). Additional studies involved incubation of HDAC2 with *cis*-**5.12** and successful *in situ* photoisomerization (using white light) resulting in increase of enzyme activity due to the formation of the less potent *trans*-**5.12**. Furthermore, treatment of HeLa cells with both isomeric forms for 16h led to dose-dependent depletion of cell viability in case of the *cis* isomer while the *trans* had minor influence (Figure 2). Taking these data together, compound **5.12** fulfills most of the criteria required for a privileged photocontrolled chemotherapeutic agent and introduces the exciting opportunity that photopharmacology offers to the clinical applications of such molecules in cancer treatment.

Our studies provided the proof of principle for the discovery of additional light-activated inhibitors that target enzymes with central roles in oncogenesis, thus minimizing the risk of off-target effects usually observed in classic chemotherapy. Nevertheless, the need for lower toxicity and higher tissue penetration points towards the development of visible-light switchable inhibitors. Aiming at fully non-invasive photopharmacology, therapeutic applications of such molecules in combination with the recent advances in tumor imaging are very promising.

8

Anti-inflammatory effects of azobenzene analogues of Entinostat

Next to carcinogenesis, it is well established that HDACs are involved in the pathogenesis of several inflammatory diseases. Among other activities, their deacetylation function on NF-κB p65 transcriptional factor has defined them as crucial regulators of NF-κB-mediated inflammatory signaling. A considerable

number of efforts has been made to target HDACs in inflammation but the lack of specificity of the majority of the clinically-used HDACi or, more importantly, the ineffectiveness and divergent pharmacological effects of selective HDACi call for the development of novel diagnostic tools to assess the role of individual isoforms in inflammatory pathways.

In **chapter 6**, we introduce a chemical epigenetic strategy to generate closely related structural analogues of the clinically-used potent HDAC1-3 inhibitor Entinostat in an attempt to investigate the function of each isoform in NF-κB-mediated inflammatory gene expression in macrophages. Three compounds were designed with an azobenzene moiety at the *para*, *meta* and *ortho* position with respect to the amine group of the connection unit of Entinostat (compounds **6.4**, **6.7**, and **6.9** respectively). Inhibition and kinetic studies using the *trans* isomeric form of each inhibitor revealed a non-selective profile for HDAC1-3 inhibition (low micromolar range), with the exception of **6.7** which displayed a K_i of 0.63 μM over HDAC1.

Further pharmacological studies on LPS/IFNγ-stimulated RAW264.7 macrophages pre-treated for 20h with each inhibitor showed inhibition of transcriptional activity and nuclear translocation of NF-κB p65 for compounds **6.4** and **6.9** while **6.7** and the reference Entinostat exhibited the opposite effects. We then monitored the expression levels of the pro-inflammatory genes TNFα, iNOS, IL-1β, IL-6 and IL-12b where we were pleased to find that, apart from iNOS where no influence was observed, compound **6.4** resulted in dose-dependent reduction of the mRNA levels of all the other cytokines. Analogues **6.7** and **6.9** suppressed the expression of IL-6 but, as Entinostat, they caused either upregulation or had no effect on the rest target genes. Notably, the remarkable induction of iNOS expression observed with compound **6.7** and Entinostat supports the link between HDAC1 inhibition and transcriptional regulation of this gene. In addition, the phenomenal increase of the anti-inflammatory cytokine IL-10 caused by Entinostat was not observed in any of its azobenzene analogues. We conclude that minor alterations in the structure of an inhibitor can lead to significant changes in its pharmacological behavior and that an overall HDAC1-3 inhibition is favorable to obtain the desirable anti-inflammatory profile.

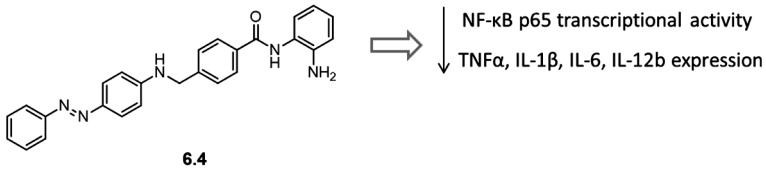


Figure 3. Structure and anti-inflammatory effects of a *para* azobenzene analogue of Entinostat.

Our findings strongly support the useful prospects that chemical epigenetics can offer to the discovery and identification of HDACi with anti-inflammatory characteristics. Such studies can further confirm the connection between HDAC activities and regulation of NF-κB signaling. Furthermore, careful design of closely related structural derivatives of a clinically-used agent can pave the way for the apprehension of the role of isoforms of other enzyme classes in various disease models and, in this way, guide focused therapeutic efforts.

Activity-based probes for detection of LSD1 activity

Recent advances demonstrate a versatile but critical role of lysine-specific demethylase-1 (LSD1) in tumorigenesis. Its effects on activation or suppression of gene expression, depending on the interactions with other proteins, have attracted the interest of many research groups aiming at elucidating the biological function of this enzyme and its implications in cancer initiation and progression.

In **chapter 7**, we describe our efforts on labeling human recombinant and endogenous LSD1 by employing five tranylcypromine (TCP) analogues to ensure for covalent binding to the cofactor FAD and activity-based protein profiling. Four compounds contained a *para*-benzoylamino or alkoylamino group in respect to the phenyl ring of TCP and were functionalized either with a terminal alkyne for subsequent detection *via* the 'click' reaction or with a terminal alkene allowing for bioconjugation *via* the oxidative Heck reaction. Due to the non-covalent interactions of LSD1 with the cofactor, we also designed an alkyne-functionalized probe (compound **7.8**) including a benzophenone moiety that serves for photocrosslinking and irreversible binding to the enzyme. All compounds exhibited high potency, fast binding and a time-dependent mechanism of inhibition.

Initial failures on labeling endogenous LSD1 *via* a two-step process, urged us to continue our studies by using the biotinylated forms of alkyne-containing probes subjected to 'click' reactions with azide-PEG3-biotin. Surprisingly, for all compounds, incubation with human recombinant LSD1 and, in case of probe **7.8**, UV irradiation, resulted in a dose-dependent protein labeling demonstrating strong interactions between LSD1 and its FAD cofactor. Further control reactions included probing after protein heat-inactivation or treatment with a non-TCP-bearing biotinylated compound. In both cases no luminescence was observed indicating tagging based on active enzyme. Nevertheless, treatment with LSD1 inhibitors followed by brief incubation with pre-clicked probes resulted in no clear reduction of labeling, but further analysis is needed in order to explore the scenario of enzyme activity-dependence as well as another site of modification for the TCP-based probes. Finally, pre-clicked probe **7.8** was applied on HeLa nuclear extracts where, apart from other intracellular proteins, no apparent labeling of endogenous LSD1 was visible. Additional studies on the identification of labeled proteins and investigation of the mechanism of LSD1 inactivation of the developed probes are currently ongoing. Ultimately, the results of such experiments will set the stage for the deeper investigation of LSD1 as an emerging pharmacological target.

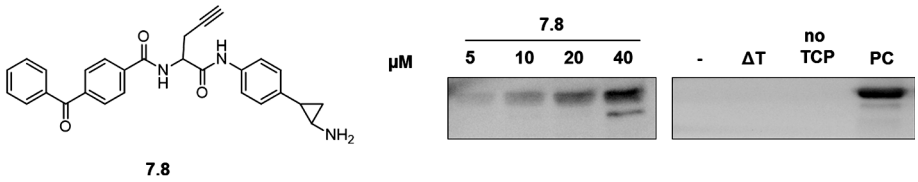


Figure 4. Structure of probe **7.8** and on blot luminescence detection of human recombinant LSD1 treated with various concentrations of the pre-clicked biotinylated probe. Control reactions are also shown. ΔT; heat-denaturation control, PC; positive control.

The results described in this dissertation enable successful application of the bioorthogonal oxidative Heck reaction to detect metabolic labeling using alkene-labeled metabolic precursors *in vitro*. Our work also aided the discovery of novel potent photoswitchable HDAC inhibitors as potential anticancer agents as well as HDAC inhibitors with promising anti-inflammatory properties. Finally, initial efforts on monitoring LSD1 activity *via* an ABPP method suggest further investigation of the mechanism of action of TCP-based inhibitors in order to explore the importance of this enzyme in carcinogenesis.