Ruprecht-Karls-Universität Heidelberg
Medizinische Fakultät Mannheim
Dissertations-Kurzfassung

Quantitative proteomic investigation of enzyme inhibitors for targeted therapy

Autor:	Isabelle Becher
Institut / Klinik:	Institut für Medizintechnologie
Doktorvater:	Prof. Dr. C. Hopf

The majority of small molecule and biopharmaceutical drugs target proteins. These proteins do not act in isolation but are embedded in cellular pathways and networks and are thus functionally tightly interconnected with many other proteins and cellular components. During development of small molecule drugs it is of high interest to identify targets as well as potential off-targets to be able to determine potency, selectivity, mode of action as well as reducing the risk of adverse events. Recent progress in mass spectrometry-based proteomic approaches enables the quantitative analysis of proteins in unprecedented depth.
Both, kinases and histone deacetylases (HDACs) are attractive drug targets, for which inhibitors have been approved for targeted cancer therapy. Protein kinases represent the single largest mammalian enzyme family with more than 500 members in the human proteome. Most kinase drugs target the ATP binding site and compete with cellular nucleotides. To what extent intracellular co-factors influence binding of kinase inhibitors is, however, unknown. Besides kinases the group of histone deacetylases has spurred a lot of medical interest in the last years. HDAC inhibition results in apoptosis and cell cycle arrest of tumor cells, as well as changes in gene expression levels. To date, two HDAC inhibitors (HDACi) have been approved for treatment of cutaneous T-cell lymphoma (Vorinostat and Romidepsin) and furthermore several clinical trials are ongoing for other inhibitors that could be used in single or combination therapy. From large-scale proteomic studies it is known that besides histones thousands of proteins are acetylated, however, only few are affected by HDAC inhibition using the broadly selective inhibitor Vorinostat.

The aim of this thesis was:

- To apply quantitative chemoproteomic-based approaches for characterizing the effect of intracellular cofactor concentration on drug affinity and for characterizing inhibitors regarding selectivity as well as binding kinetics.
- To reveal the molecular mechanism of action by identifying HDAC substrates using current proteomic methods.

The assays were conducted directly in cell extracts, ensuring close-to-physiological conditions preserving protein folding, post-translational modifications and protein-protein interactions. With respect to the effect of intracellular cofactor concentration and its effect on inhibitor affinity, protein kinases and their cosubstrate ATP were analyzed using ‘kinobeads’ technology. Furthermore, to investigate binding selectivity and binding kinetics of HDACi to HDACs as well as HDAC complexes chemoproteomic approaches were applied. In this thesis the cellular targets of HDAC enzymes using seven different HDAC inhibitors were analyzed in a large-scale acetylation study, which was combined with the quantitative analysis of protein expression levels.

The key new insights on enzyme inhibitors for targeted therapy were:

- The affinity of dasatinib for the efficacy target kinase ABL1 decreased more than 50-fold in the presence of 2 mM ATP. Additional target kinases with very high ATP affinities also displayed a clear drop in dasatinib potency: especially MAP3K4, MA3K2, ZAP70 and BRAF showed more than 100-fold decrease in dasatinib affinities in presence of 2 mM ATP
    - In summary, more than 200 kinases were identified and their affinities for ATP as well as the inhibitor dasatinib (Sprycel®) were determined

    - Combined information about cosubstrate and kinase inhibitor affinities can be used for the determination of the intracellular potency of the inhibitor
- Using immobilized Vorinostat it was possible to identify differences binding kinetics of two inhibitor classes against single enzymes and also against protein complexes. The benzamides turned out to be slow binding inhibitors, and also exhibited a high residence times
    - The slow binding of the benzamide inhibitor was reflected in a slow increase in substrate acetylation level, but also the signal amplitude after several hours was similar for fast and slow binding inhibitors
    - An enzyme activity assay revealed the benzamide-selective inhibition of the HDAC complexes CoREST and NuRD but not Sin3, emphasizing the importance of a comprehensive characterization of potential drugs
- The analysis of the ‘acetylome’, and its modulation upon HDAC inhibition with seven different HDACi led to the identification of more than 3,600 acetylated peptides on >1,700 proteins
    - Identification and verification of new HDAC6/10 target sites, such as acetylation of HN1, PIN4 and DDC3X, which were for the most part located on cytoplasmic proteins
    - Identification of new, potential HDAC8 target sites and verification of SMC3 as a HDAC8 substrate
    - Elucidation of the short-chain fatty acid Sodium butyrate as potential HAT activator
    - Only 10% of the identified acetylome were found to be significantly upregulated upon HDAC inhibition suggesting a high selectivity

In summary, this study underscored how chemoproteomic and other proteomic approaches contribute to the characterization of intracellular affinities and selectivities of enzyme inhibitors for targeted therapy. Furthermore, the use of unbiased mass spectrometry combined with a quantitative strategy identified new targets and could contribute to the identification of molecular mechanism of action of small molecule inhibitors.