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Kinetic method for the large-scale analysis of the binding mechanism of histone deacetylase inhibitors

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ABSTRACT

Performing kinetic studies on protein ligand interactions provides important information on complex formation and dissociation. Beside kinetic parameters such as association rates and residence times, kinetic experiments also reveal insights into reaction mechanisms. Exploiting intrinsic tryptophan fluorescence a parallelized high-throughput Förster resonance energy transfer (FRET)-based reporter displacement assay with very low protein consumption was developed to enable the large-scale kinetic characterization of the binding of ligands to recombinant human histone deacetylases (HDACs) and a bacterial histone deacetylase-like amidohydrolase (HDAH) from *Bordetella/Alcaligenes*. For the binding of trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and two other SAHA derivatives to HDAH, two different modes of action, simple one-step binding and a two-step mechanism comprising initial binding and induced fit, were verified. In contrast to HDAH, all compounds bound to human HDAC1, HDAC6, and HDAC8 through a two-step mechanism. A quantitative view on the inhibitor-HDAC systems revealed two types of interaction, fast binding and slow dissociation. We provide arguments for the thesis that the relationship between quantitative kinetic and mechanistic information and chemical structures of compounds will serve as a valuable tool for drug optimization.

The rational design of selective and highly potent drug candidates is a challenging task of medical and pharmaceutical chemistry. Traditional approaches of rational drug design are often focused only on optimizing the affinity of a compound to a target protein. This approach has been recently highlighted by Stahl et al. as the only reasonable way for structure-based drug design, since other approaches are often highly dependent on the system under investigation [1]. Classically, this gain in affinity is often achieved by adding hydrophobic moieties to the core of a lead structure, which leads to a favorable change in entropy on binding. This procedure generates disadvantages including unfavorable effects on the physiochemical properties of the ligand and a loss of target specificity, since enhanced hydrophobic effects are unspecific in nature [2].

Another approach to increase the affinity of a ligand and even more importantly its selectivity is to optimize lead structures with respect to their thermodynamic signature. Therefore, it is necessary to determine both the entropic and the enthalpic contributions to the binding process. Favorable enthalpic contributions are gained from highly system-specific effects such as hydrogen bonding and van der Waals interactions and should therefore be a major driving force in the binding process. In contrast to binding entropy, optimizing for favorable binding enthalpy is extraordinary difficult [3]. Both approaches, based exclusively on affinity or thermodynamic signature, are based on evaluation of ligand binding under equilibrium conditions in a closed system. In open systems, like the human body, ligand concentrations are fluctuating over the time- and equilibrium-based constants, such as the equilibrium dissociation constant, K_d, and are insufficient to reflect the amount of formed complexes or predict the physiological response. In order to overcome these limitations it is useful to determine the kinetic parameters of the binding reaction and add them to the drug design process [4].

One strategy focusing on optimizing kinetic parameters of ligand binding was introduced with the residence time concept by Copeland et al. in 2006 [5]. In this approach the residence time refers to the half-time of a ligand target complex and indicates how long a ligand stays in the bound state under nonequilibrium conditions. To obtain long-lasting ligand target complexes the residence time must be increased during the drug development processes. Residence times can be determined from ligand displacement

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kinetics. Several methods exist for the determination of on- and off-rates of ligand binding. One of the most commonly used methods is surface plasmon resonance (SPR)[1] which involves the attachment of proteins or ligands to a surface and enables the continuous measurement of binding of unlabeled molecules to the immobilized binding partners [6]. The major limitations of this rather expensive and maintenance-intensive method are common problems to conjugate functional proteins to the surface, unspecific surface binding artifacts, and mass transport issues. In principle, enzyme activity dilution assays, e.g., using a Caliper LabChip reader, can also be employed to investigate the dissociation kinetics of ligands from their enzyme target [7]. Since such studies are rather indirect and depend on the turnover number of the enzyme, the method is not suitable for the detailed investigation of fast binding kinetics and more complicated mechanisms. In addition, the interactions between the typically artificial substrate and the test compound could impair the measuring result. Homogeneous solution phase binding assays are a useful alternative to assays employing binding to surfaces. An experimental procedure for the thermodynamic and kinetic characterization of ligand binding was suggested by Neumann et al. [8], based on displacement of a fluorescent probe on binding of a ligand, which causes a change in the measured fluorescence intensity. A recent example to assess structure–kinetics relationships with this method was reported by Schneider et al. [9]. The particular fluorescent probe employed in these studies was not specified however. One major requirement for the setup of a reporter displacement assay is that the designed probe exhibits a faster dissociation rate than the association rate of the ligand. Beside determination of residence times a kinetic characterization can be used to analyze binding mechanisms in more detail. The simplest and most often assumed binding mechanism is a one-step association and dissociation model. In many cases binding reactions are more complex and include target isomerization, conformational selection, multiple binding sites, or modes. Compounds, whose binding mechanism includes target isomerisation, often show prolonged residence times. The determination of the corresponding binding mechanism is believed to bear great potential as an additional criterion to categorize and select compounds for further optimization in a relatively early phase of drug development [4].

In this study a previously reported FRET-based reporter displacement assay, which is suitable for thermodynamic characterization of histone deacetylase inhibitors (HDACi), is adapted to allow for the kinetic characterization of HDACi [10]. HDACi have been developed to target different types of cancer [11]. As first-in-class drugs suberoylanilide hydroxamic acid (SAHA) and romidepsin have been approved by U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [12]. Particularly SAHA is rather unselective and inhibits most isoforms of Zn^{2+}-dependent histone deacetylases (HDACs). Zn^{2+}-dependent HDACs are divided into class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), and class IV (HDAC11). Class III consists of 7 isoforms, the sirtuins, which require NAD^+ as a cofactor for their catalytic activity instead of Zn^{2+} [13]. Together with their opponents, the histone acetyl transferases (HATs), HDACs are mostly recognized for their roles in cellular differentiation, proliferation, and apoptosis by altering genetic expression through determining the acetylation status of lysine residues of histones [14]. Beside histones a number of proteins, including cancer-relevant proteins like p53 and other cytosolic proteins such as HSP90 and α-tubulin, have been identified as substrates of HDACs, which exemplifies the involvement of HDACs in the regulation of many cellular processes [15,16]. The role of HDACs in different cancer types has been intensively investigated and reviewed over the last decade [11,17]. Consequently, efforts in the development of HDACi have been increased in order to generate more specific drugs, which are hypothesized to cause fewer side effects such as fatigue, nausea, dehydration, diarrhea, thrombocytopenia, or electrocardiogram changes [12,18].

Beside eukaryotic HDACs a number of bacterial homologues, such as the histone deacetylase-like protein (HDLP) form *Aquifex aeolicus* and the histone deacetylase-like amido-hydrolase (HDAH) from *Bordetella*/*Alcaligenes*, have been described [19,20]. HDAH is structurally closely related to HDACs and shares an identity of 35% with the second catalytical domain of human HDAC6 [21]. Several experimental results, including similar substrate and inhibitor selectivities, prove that HDAH serves as a good model for HDAC6 [22,23]. Beyond the use as model enzymes for human HDACs several bacterial and protozoan parasite deacetylases are currently investigated as protein targets for the treatment of bacterial infections or parasitic diseases [24–27].

The results of this study provide arguments and evidence that kinetic data are important for an understanding of the mode of action between ligands and proteins and for differentiating between compounds having different association rates and residence times on target proteins with occasionally undistinguishable equilibrium binding affinities.

Materials and methods

All chemicals if not stated otherwise were obtained from Sigma/Aldrich (USA), AppliChem (Germany), Roth (Germany), and Merck (Germany). Boc-Lys(Ac)-AMC and Boc-Lys(trifluoracetyl)-AMC were obtained from Bachem (Switzerland). SAHA and TSA were purchased from Cayman Chemical (USA). LU210 and SATFMK were prepared as described elsewhere [28,29]. Dansyl-conjugate probes with varying spacer lengths were synthesized as described previously [10]. HDAC1 and 6 were purchased from BPS Bioscience (USA). His-tagged FB188 HDAH and His-tagged recombinant HDAC8 were prepared as described elsewhere [20,30]. The assay buffer consisted of 250 mM sodium chloride, 15 mM Tris–HCl, 0.001% Pluronic F-167, and 50 mM potassium phosphate at pH 8.0. For experiments with FB188 HDAH the assay buffer was supplemented with 250 μM EDTA. All reactions were performed in black half-area 96-well microplates (Greiner Bio-One, Germany). The obtained data were fitted using Prism 5 (GraphPad Software, USA) or Gepasi 3.3 [31].

Enzyme activity assay

The determination of the inhibitory effect of all SAHA derivatives on the catalytic activity of HDAC1, HDAC6, and HDAC8 was carried out by an enzyme activity assay, which was reported earlier by Wegener et al. [32], In these experiments a serial dilution of the respective compound was incubated with 1 nM of the respective HDAC for 30 min at 25 °C in assay buffer. The catalytic reaction was initiated by the addition of 40 μM Boc-Lys(Ac)-AMC for HDAC1 and 6 or 20 μM Boc-Lys(trifluoracetyl)-AMC for HDAC8. After an incubation for 60 min at 25 °C the reaction was stopped by the addition of 40 μM SAHA and the deacetylated substrate was converted into a fluorescent product by the addition of 0.5 mg/ml trypsin. The release of AMC was correlated to the enzyme activity. From the obtained data points the K_d values of

[1] *Abbreviations used:* AMC, 7-amino-4-methylcoumarin; Dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; FRET, fluorescence resonance energy transfer; HDAH, histone deacetylase-like amidohydrolase from Bordetella/Alcaligenes; HDAC, histone deacetylase; K_d value, dissociation constant; LU210, 2,2,3,3,4,4,5,5,6,6,7,7-dodecafluorooctanedioic acid hydroxyamide phenyl-amide; SAHA, suberoylanilide hydroxamic acid; SATFMK, suberoylanilide trifluoromethylketone; SPR, surface plasmon resonance; TSA, trichostatin A.