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Non-isotopic dual parameter competition assay suitable for high-throughput screening of histone deacetylases

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ABSTRACT

Histone deacetylases reside among the most important and novel target classes in oncology. Selective lead structures are intensively developed to improve efficacy and reduce adverse effects. The common assays used so far to identify new lead structures suffer from many false positive hits due to auto-fluorescence of compounds or triggering undesired signal transduction pathways. These drawbacks are eliminated by the dual parameter competition assay reported in this study. The assay involves a new fluorescent inhibitor probe that shows an increase in both, fluorescence anisotropy and fluorescence lifetime upon binding to the enzyme. The assay is well suited for high-throughput screening.

The regulation of gene expression is largely regulated by epigenetic mechanisms involving the modification of histones. Histones can be methylated, phosphorylated, ubiquitinylated, SUMOylated and acetylated,[1–3] thus, forming the so-called histone code which can be recognized by effector proteins that interact with nucleosomes and transcription factors.

In contrast to methylation, acetylation is reversible with much smaller half lifes in the range of minutes. Discovered nearly 40 years ago,[4] it has only recently become clear that acetylation of the ε-amino group of specific lysine residues within the positively charged N-terminal tail of core histones H2A, H2B, H3 and H4 results in localized chromatin relaxation and a change in both histone–DNA and histone–nonhistone protein interaction. In general, acetylation by histone acetyltransferases (HAT) is correlated with transcriptional activation, whereas deacetylation activity executed by histone deacetylases (HDACs) is accompanied by transcriptional repression.[5] Mostly in this way, HDACs affect angiogenesis, cell-cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease. Not surprisingly, many HDAC inhibitors were shown to inhibit cell proliferation, induce differentiation and/or apoptosis, induce cell cycle arrest, mitotic catastrophe and reversal of transformed cell morphology. Consequently, a number of HDAC inhibitors show a potency as promising antitumor agents with several drug candidates currently being in phase I–III clinical trials.[6–8] The first drug in class, vorinostat (Zolinza), has been approved in 2006 by the FDA for the treatment of cutaneous T-cell lymphoma. The discovery of novel more specific HDAC inhibitors requires particular assay systems.[9] So far, in high-throughput screening campaigns fluorogenic enzyme activity assays or cell-based reporter gene assays have been used.[10,11] Both assay formats suffer from many false positive hits due to auto-fluorescence of the compounds or inhibition of the signal generating trypsin in case of the fluorogenic assay and unspecific regulation of the tumor suppressive pathway used as a reporter system in the cell based assay. Very recently, a fluorescence polarization based assay, which also uses a fluorescent ligand, has been described.[12] This assay uses a ligand conjugated to fluorescein and measures ligand binding to HDACs under equilibrium conditions. The assay already significantly reduced the number of false positives. However, there still are problems with strongly auto-fluorescent compound at the relatively small wavelengths where fluorescein is measured. In this Letter, we present the first non-isotopic and dual parameter competition assay which is suitable for high-throughput screening of HDAH and HDACs. The assay principle is demonstrated using the well established HDAC homolog HDAH from Bordetella/Alcaligenes species FB188. Proof-of-concept experiments with the well-known HDAC inhibitor CypX and in the presence of escalating concentrations of 7-amino-4-methyl-coumarin as a strongly auto-fluorescent model compound

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prove that the new assay is well suited for high-throughput screening applications to identify novel HDAC inhibitors.

The aim of this study was to develop an improved assay for HDAC inhibitors. Riester et al. introduced 2-furylacryloylhydroxamate, another chromophor-hydroxamate conjugate, in a FRET-based assay to determine binding constants of non-fluorescent inhibitors of HDAH.[13] Since the intrinsic tryptophans of the enzyme had to be excited, the signal readout was highly prone to the auto-fluorescence of compounds and therefore less suitable for high throughput screening. To avoid the simultaneous excitation of compounds, we chose a dye with strongly red-shifted excitation and emission wavelengths: 1-(3-carboxypropyl)-11-ethyl-2,2,8,10,10-pentamethyl-4-(sulfomethyl)-2,10-dihydro-1*H*-13-oxa-1,6,11-triazapentacen-11-ium, called Atto700 or JA 408 (Atto-Tec, Germany).[14] This dye was conjugated to an aminohexanoyl-spacer which was functionalized with a hydroxamate moiety. All reagents for organic synthesis and biochemical experiments were obtained from Sigma (Taufkirchen, Germany). At first *N*-Fmoc-hydroxylamine-2-chlorotrityl resin (111 mg, 50 μM; Novabiochem, Bad Soden, Germany) was soaked with DCM for 1 h and subsequently Fmoc-deprotected with 30% piperidine/DMF (v/v). The resin was then washed six times with DMF and acylated with 2 × 250 μM 6-(Fmoc-amino)hexanoic acid (Aldrich, Taufkirchen, Germany) activated with TBTU (2 × 5 equiv) and NMM (2 × 15 equiv) at room temperature overnight. The resin was subsequently washed with DMF, DCM and *tert*-butylether and dried. After a second Fmoc-deprotection step the resin was reacted with 1 mg *N*-hydroxysuccinimidylester of Atto700. After cleavage with 20% TFA/DCM and lyophilization, 1 mg of a blueish powder (75% yield) was obtained. The molecular weight of the conjugate was determined using LC–MS (ESI) to be 693.5 (The molecular structure of Atto700 has not been published so far). The product Atto700-aminohexanoyl-hydroxamate (Atto-HA) was dissolved in DMSO to give a 1 mM stock solution. His-tagged HDAH was prepared as described elsewhere.[15] CypX was synthesized according to standard methods.[16–18] The assay buffer consisted of 250 mM sodium chloride, 15 mM Tris–HCl, and 20 mM potassium chloride including 0.001% Pluronic F-127 at pH 8.0.

Both, Fluorescence lifetime as well as fluorescence anisotropy were measured on a LF502 nanoscan instrument from IOM GmbH exciting at 635 nm using vertical polarized light pulses. The emitted light was split into two beams of roughly the same intensity. Both beams were simultaneously detected by two different photomultipliers each equipped with a 740/30 nm band pass and a polarization filter to collect the light polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization plane of the incident light. The polarized components were calculated by integrating the corresponding time resolved fluorescence decay curve between 4 and 20 ns (see Fig. 1). The fluorescence lifetime was calculated by fitting the fluorescence decay curves to a single exponential function using a deconvolution algorithm implemented in the instruments software to correct for the contribution of the instrument response function. The anisotropy was calculated from the linearly polarized components of the detected fluorescence emission to be $\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+2I_{\perp}}$.

This Atto700-HA conjugate (see Fig. 2A) was bound by HDAH in assay buffer resulting in the increase of fluorescence anisotropy due to the increase in mass upon complex formation. Surprisingly, a significant increase in fluorescence lifetime was observed as well. Since the binding of Atto-HA to HDAH was reversible, other non fluorescent inhibitor molecules were able to displace Atto-HA from its binding site at HDAH (see Fig. 2B). Consequently, both fluorescence signal readouts were reversed to the values of free Atto-HA. For the determination of the mean fluorescence signal readouts and their variance 48 independent samples were prepared for both free and bound Atto700-HA. The fluorescence lifetimes of free

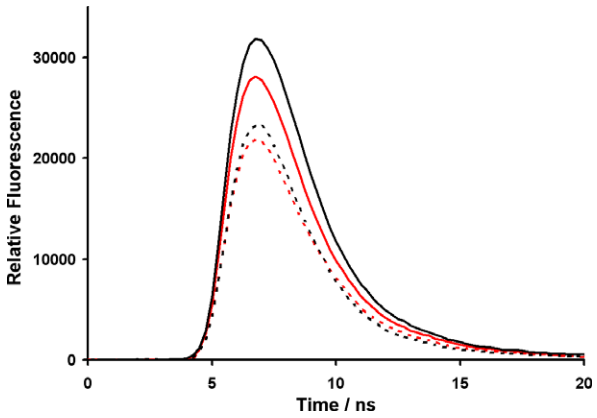


Figure 1. Time resolved decay curves of polarized fluorescence. The red solid and dotted lines denote the decay curves of emitted light, which is polarized parallel and perpendicular to the incident light, of free Atto-HA ligand at a concentration of 50 nM. The black solid and dotted curves correspond to the decay curves of emitted light, which is polarized parallel and perpendicular to the incident light, of the ligand–HDAH complex which is formed by mixing 50 nM ligand and 4 μM HDAH. All measurements were performed in assay buffer exciting at 635 nm and collecting the fluorescence emission using a band path filter at 700 nm (bandwidth 30 nm).

Atto700-HA and bound Atto700-HA were (1.48 ± 0.05) and (2.03 ± 0.02) ns, respectively. Since the half width of the exciting N_2-laser pulse was about 0.5 ns, the determined lifetimes may deviate to some extent from the true value. But for screening purposes small variances in the signal readout is largely more important than absolute accuracy. The anisotropies of bound and free Atto700-HA were (0.24 ± 0.01) and (0.080 ± 0.008) ns, respectively.

The HDAC6 assay was performed as described previously.[19] In short the activity of HDAC6 (BPS Bioscience, USA) was measured in a two step fluorogenic assay using Boc-Lys(Ac)-AMC (Bachem, Germany) as substrate in the first step and a mixture of Trypsin and SAHA for the development of the fluorescence signal in the second step.

A titration of Atto700-HA with increasing amounts of HDAH resulted in a simultaneous increase of the fluorescence anisotropy and lifetime upon binding. The dissociation constant of Atto700-HA and HDAH under the standard assay conditions was calculated to be 3 μM at 21 °C (see Fig. 3).

A collection of known inhibitors was tested to validate the dual parameter competition assay. The dose–response curves of TSA and Apicidin are shown in Fig. 4 yielding K_i values of 0.7 μM and 9.2 μM, respectively. The binding constants of the whole set of inhibitors were summarized in Table 1. The binding constants for inhibitors of HDAH are in reasonable agreement with data from a FRET based binding assay[13] and a fluorogenic enzyme activity assay.[20,10] The binding constants of the dual parameter competition assay of the standard inhibitors SAHA and TSA to HDAH also agree with IC_{50} values of a fluorogenic enzyme assay using HeLa extract instead of purified enzyme. In contrast, the binding affinity of known HDAC inhibitors to purified HDAC1 or HDAC6 is usually about 10–100 times higher as compared to their binding affinity to HDAH.[21,12,22,23] But the ranking of compound potencies against HDAC6 and HDAH is similar.

The performance of a screening assay has to be assessed by measuring many replicates of positive and negative controls. If available, it is useful to measure dose–response curves of reference compounds in replicates as well. The most accepted statistical parameter to evaluate the performance of a screening assay and to compare it with other assay formats is the Z′ factor as proposed by Zhang et al.[24] In general, Z′-values greater than 0.5 are acceptable for a high throughput assay. The Z′-factors with respect to

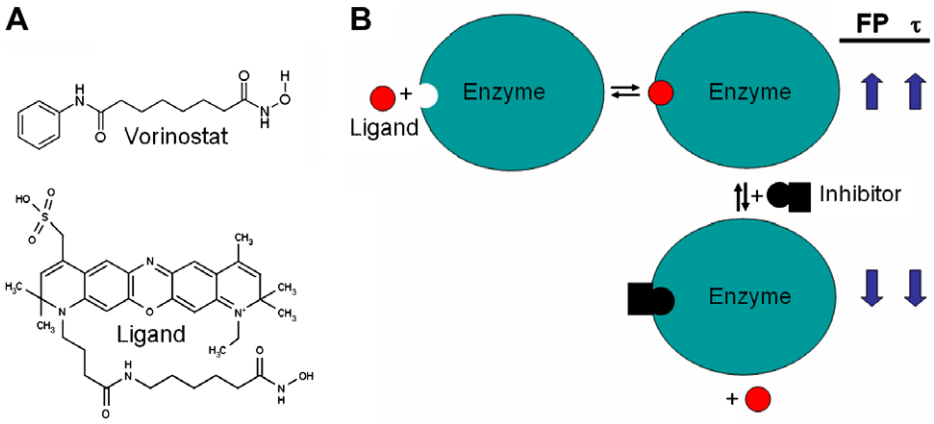


Figure 2. Principle of the homogeneous dual parameter competition assay. (A) The chemical structure of the fluorescent Atto-HA ligand is similar to the known HDAC inhibitor vorinostat. (B) The filled red circle represents the Atto-HA ligand which binds to an HDAC or HDAH enzyme, thereby increasing fluorescence anisotropy (FP) and fluorescence lifetime (τ). The filled black symbol denotes an inhibitor which binds to the active site of the enzyme. The inhibitor displaces the Atto-HA ligand which results in a decrease of fluorescence anisotropy and lifetime.

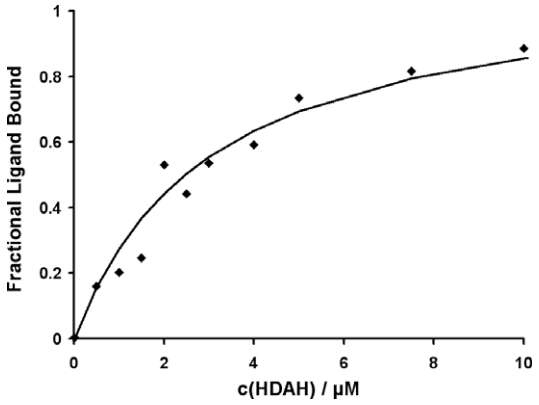


Figure 3. Binding of HDAH to Atto700-HA. Binding of HDAH to 50 nM Atto700-HA in assay buffer pH 8.0. The binding degree is calculated from the fluorescence anisotropy which increases upon binding to HDAH. The dissociation constant of HDAH and Atto700-HA has been obtained from fitting the data to a simple bimolecular binding model (smooth line) and was calculated to be 3 µM.

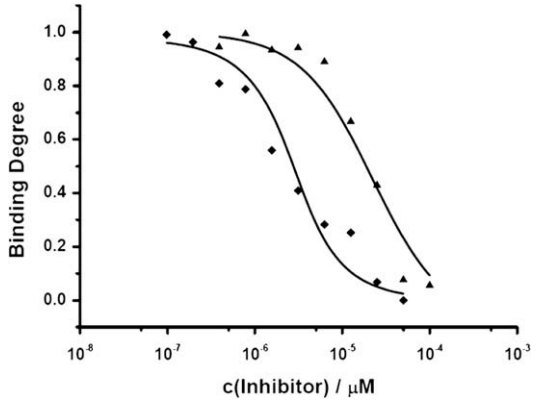
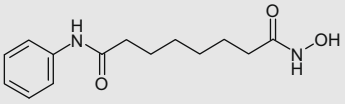
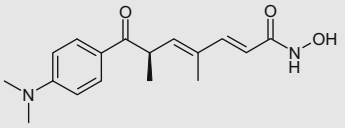
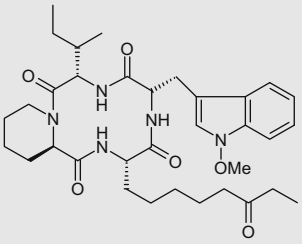
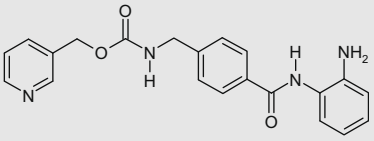
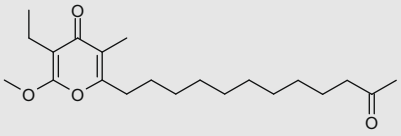
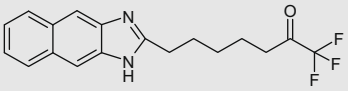
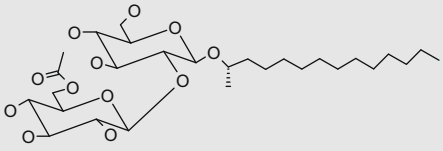
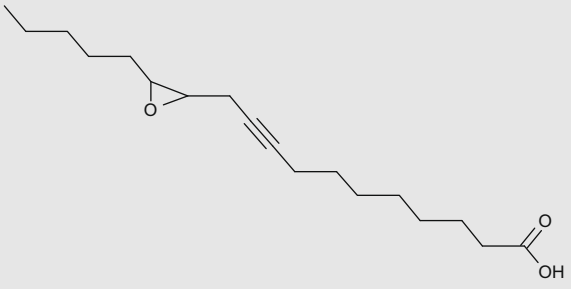


Figure 4. Dose–response curves of HDAH/HDAC inhibitors. The binding degree of 50 nM Atto700-HA to 2.5 µM HDAH is plotted versus the concentrations of added TSA (diamond) or Apicidin (triangle). The K_i-values are calculated to be 0.7 µM for TSA and 9.2 µM for Apicidin.

both signal readouts have been calculated on the basis of 48 independent samples of free and bound Atto700-HA under standard assay conditions to be 0.66 for fluorescence anisotropy and 0.62 for fluorescence lifetime. Both independent signal readouts showed a high correlation which was also reflected by the dose dependent change of the signals in the presence of increasing concentrations of the inhibitor CypX (see Fig. 5).[13] The assay performance was so high, that the signal readouts in the presence of different inhibitor concentrations could be well distinguished. It may be advantageous, that the assay is measured in chemical equilibrium. It has been shown, that microtiter plates measured 2 h later again showed essentially the same results than before (data not shown). Beyond assay performance the necessity to differentiate between hits and non active compounds puts some emphasis on the robustness of a good high throughput screening assay. The assay signals can be disturbed by assay interferences originating from assay reagents and screening compounds like auto-fluorescence, inner-filter-effect or variable tracer concentrations. A major concern in many fluorescence based assays is the auto-fluorescence of compounds which are frequently found as false positive hits. We have designed the dual parameter competition assay to minimize the impact of auto-fluorescence on the signal readouts. The robustness of the assay was demonstrated using 7-amino-4-methyl-coumarin (AMC) as a highly fluorescent model compound. Up to 50 µM AMC caused no significant impact on both fluorescence anisotropy and fluorescence lifetime (see Fig. 6). Even at such a high concentration as 100 µM AMC there is only a minor change in both signal readouts which is comparable to the effect of a weak inhibitor causing about 20% replacement of Atto700-HA.

Drugs which interfere with the activity of histone deacetylases represent a new strategy in the treatment of neoplastic diseases. Although vorinostat is an effective inhibitor, it shows little selectivity for the Class I and II enzymes. More selective HDAC inhibitors may improve the clinical efficacy while minimizing side effects. This study was motivated by the high interest in more selective inhibitors and the necessity of better performing and robust screening assays for HDACs. The frequently used fluorogenic assay principle is prone to auto-fluorescent compounds which strongly interfere with the fluorescence readout in the UV to near UV range of light. In addition, the signal generating enzyme trypsin could be inhibited by a compound pretending to be an inhibitor of HDAC. Therefore, many false positive hits would be found which means extra testing and ultimately more effort and time to be spent for validating hits using a different assay principle. On the other hand, fluorescent compounds which actually are good inhibitors could be overlooked, if strongly fluorescent compounds would be excluded from the hits. Improved assay setups suited to high throughput screening should fulfill several criteria. One of the most important

Table 1
Binding constants and IC_{50} values of HDAH / HDAC inhibitors

K_i and IC_{50} values in μM		HDAH			HDAC6	RT extract	HDAC1	HeLa-extract
Inhibitor	Structure	K_i[a]	IC_{50}	K_i[d]	IC_{50}	IC_{50}	IC_{50}	IC_{50}
SAHA		1.3 ± 1.0	1.0[b]	1.0	0.022[f]	0.01[i]	0.027[g]	0.7[e]
Trichostatin A		0.7 ± 0.3	1.2[b]	–	0.017[f]	0.001[i]	0.006[h]	0.3[e]
Apicidin		9.2 ± 2.5	–	–	0.67[f]	–	0.044[g]	–
MS-275		>100	118[i]	–	>10[j]	8.8[i]	0.12[j]	–
45E09		1.1 ± 0.9	0.6[c]	–	>50[a]	>50[k]	–	–
46F08		1.4 ± 0.6	0.6[c]	–	0.07[a]	0.6[k]	–	–
40H03		10 ± 4.0	6.2[c]	2.0	>50[a]	>50[k]	–	–
22G03		20 ± 4.4	9.2[c]	4.3	>20[a]	–	–	–

Binding constants and IC_{50} values of inhibitors blocking HDAH and HDACs using different assays.
[a] This study.
[b] Fluorimetric assay.[20]
[c] Fluorogenic enzyme activity assay.[10]
[d] FRET assay.[13]
[e] Fluorogenic enzyme activity assay.[21]
[f] Fluorescence polarization assay.[12]
[g] Fluorogenic enzyme activity assay.[22]
[h] Radiometric histone deacetylation assay.[23]
[i] Fluorogenic enzyme activity assay.[20]
[j] Fluorogenic enzyme activity assay.[25]
[k] Fluorogenic enzyme activity assay.[10]

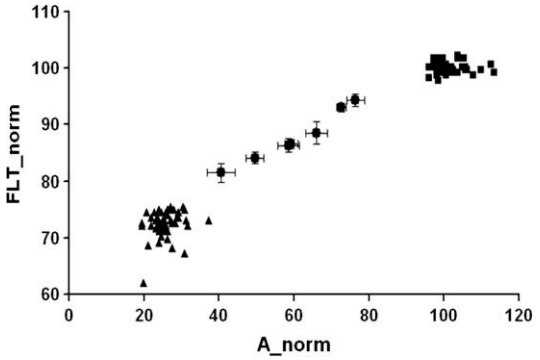


Figure 5. Dual parameter competition assay performance. 96 independent samples, 48 containing free (triangles) and 48 containing bound Atto-HA (squares), were measured under standard assay conditions. The circles denote equilibrated mixtures of HDAH-/Atto-HA complexes and 100, 50, 25, 12.5, 6.25 and 3.125 μM CypX, respectively. Increasing concentrations of CypX result in decreasing anisotropy and lifetime values. Each data point of the CypX concentration series represents five independent measurements with corresponding standard deviations of both signal readouts. The fluorescence anisotropy (A_norm) and lifetime (FLT_norm) were normalized by the corresponding mean signal of bound Atto-HA and plotted versus each other. The performance of the assay was determined by calculating the Z′-factors with respect to fluorescence anisotropy (0.66) and fluorescence lifetime (0.62).

criteria is the Z′-factor by which assay quality of the control signals are assessed. But of equal importance is the ability of the assay to avoid perturbations by numerous unspecific effects that can originate from the assay components or the screening compounds themselves. These undesired interferences impact directly on the screening campaign as false positive or false negative hits. Key interferences are auto-fluorescence and inner-filter effect of compounds and variable tracer concentrations. A high throughput assay should also have a simple protocol to minimize accumulating errors from liquid handling.

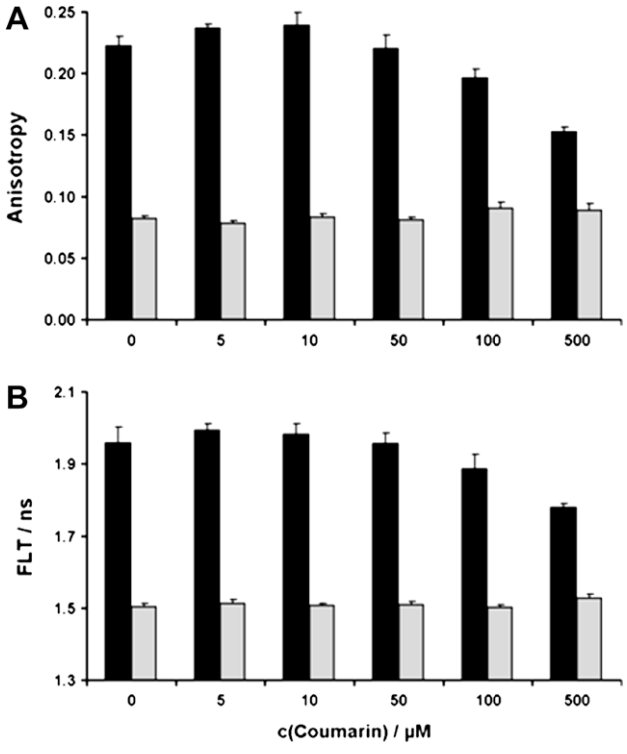


Figure 6. Impact of auto-fluorescent coumarin on signal readouts. The effect of increasing amounts of 7-amino-4-methyl-coumarin on the fluorescence readouts fluorescence anisotropy (A) and lifetime (B) of complexed (black bars) and free Atto700-HA (grey bars) was measured under standard assay conditions. Each data point represents five independent experiments each measured in a total volume of 40 μl.

These issues were addressed by choosing Atto700, a dye which absorbs in the red part of the visual spectrum, thereby largely preventing interference of auto-fluorescent compounds and designing an assay with anisotropy readout. The anisotropy readout also corrects for variable tracer concentrations and is quite robust against inner-filter effects caused by screening compounds. Upon binding of the 39.4 kDa HDAH to the small Atto700-HA ligand not only the fluorescence anisotropy but also the fluorescence lifetime increased significantly. This may be attributed to the changed micro environment of the Atto700 fluorophore which is more hydrophobic in the complex as compared with the free Atto-HA conjugate. Both signal readouts enabled the development of the dual parameter competition assay which is extremely robust against autofluorescent and inner-filter effects of screening compounds as well as variations of tracer Atto-HA concentration. The dual parameter competition assay has been validated by using a set of known HDAH or HDAC inhibitors. The calculated K_i values are in reasonable agreement with K_i values of a FRET-based binding assay[13] and IC_{50} values of an enzyme activity assay.[10] The K_i values for HDAH are 10–100 times higher than the corresponding IC_{50} values for HDAC6, but the ranking of inhibitors from the well known inhibitor classes hydroxamates (SAHA, TSA), cyclic peptides (Apicidin), benzamides (MS-275) and trifluomethylketones (46F08) is similar for HDAC6, but not for HDAC1. The hydroxamates are unselective and inhibit HDAH as well as HDAC1 and HDAC6, representatives from HDAC enzyme classes 1 and 2, respectively. The trifluormethylketone 46F08 is a potent inhibitor of HDAH and HDAC6. In contrast, Apicidin and MS-275 are potent inhibitors of HDAC1, but are clearly less active on HDAC6 and HDAH. This pronounced correlation of inhibitor selectivity is in agreement with the study of Hildmann et al.,[20] who pointed out that HDAC6 is the closest eukaryotic homologue of HDAH and exhibits very similar substrate and inhibitor specificity, based on enzyme activity assays. The only exception of inhibitor selectivity is the ketone 45E09 which only binds to and inhibits HDAH rather than HDACs, irrespective of enzyme class. It appears that substituents which increase the electron deficiency of ketones like fluorine are important to increase the potency of HDAC inhibitors whereas plain ketones are able to interact with HDAH.

The new competition assay is suited to measure dose response curves and to quantify the binding affinity of HDAH inhibitors. In the case of HDAH micro molar amounts of enzyme are required. This limits the accurate calculation of dissociation constants much smaller than the enzyme concentration. But in case of a very strong inhibitor in the nanomolar range it can be estimated that its K_i value would be less than, for example, 1 μM. However, in a screening situation strong inhibitors will not be overlooked. Increasing concentrations of inhibitors displace Atto700-HA from its binding site at HDAH in a dose dependent manner giving rise to a decrease of fluorescence anisotropy and lifetime. The sensitivity of the assay can be estimated based on the dissociation constant of Atto700-HA and HDAH and the compound concentration of, for example, 10 μM. A compound with dissociation constant of 20 μM would cause 16 percent reduction of the signal readouts. Therefore, compounds with dissociation constants of about 20 μM and smaller should be identified as hit. The performance of the assay and its robustness against variations of tracer concentration were judged by calculating the Z′-factors for both fluorescence signal readouts to be 0.66 for anisotropy and 0.62 for lifetime. Both Z′-factors on their own were excellent and demonstrated that the performance criteria of a high throughput assay were met. By using both signal

readouts for defining the hit threshold, the quality of the assay is even better and the number of statistically false positive hits would be reduced further. The robustness of the dual parameter competition assay against auto-fluorescent compounds, which usually emit in the near UV range up to about 500 nm, was demonstrated using different concentrations of the very highly fluorescent AMC. There was no assay interference up to 50 μM AMC which is 1000 times higher than Atto700-HA which is used as tracer. Most screening compounds are used at 10 μM or even lower. Only very few screening compounds are excitable at 635 nm and these compounds should be readily identified by looking at the simultaneously measured fluorescence intensity. Therefore, it is very unlikely, that the dual parameter competition assay is disturbed by auto-fluorescent compounds. In addition, the measurement of microtiter plates is highly reproducible and can be repeated even 2 h later with essentially the same result. This may be advantageous, if the screening run is stopped by an unexpected event. In this case, the screening can simply be continued after fault clearance without loosing plates.

In summary, we have reported a dual parameter competition assay with excellent performance in both signal readouts fluorescence anisotropy and fluorescence lifetime. The assay procedure is extremely simple and involves only the addition of preformed complex consisting of Atto700-HA and HDAH to screening compounds or controls, equilibrating and measuring. One of the strengths of the presented assay is its robustness against auto-fluorescent compounds. These features render the dual parameter competition highly suitable for high throughput screening campaigns to identify novel inhibitors of HDAH and HDACs.

Supplementary data

Supplementary data (the equations for data fitting and calculation of binding constants and K_i values are included in the supplementary material) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.102.

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