Drug Discovery

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In-Bead Screening of Hydroxamic Acids for the Identification of HDAC Inhibitors

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Dedicated to Professor Stuart L. Schreiber on the occasion of his 60th birthday[a]

Abstract: *A one bead–one compound screening format is presented. Following solid-phase synthesis on a photolabile linker, library compounds were readily released and screened inside polymer beads. The release of screening compounds was readily controlled by varying photolysis time and light intensity. Dose-response experiments were carried out to effectively distinguish high- and low-affinity ligands. A library containing 55 800 compounds was synthesized and screened in a fluorometric assay, thereby identifying potent HDAC inhibitors with IC_{50} values in the nanomolar range.*

The development of split-and-mix combinatorial synthesis has enabled the generation of huge chemical libraries of immobilized compounds.[1] Although technologies have been developed for the on-bead screening of solid-supported compounds,[2] these methods may not be predictive of the activity of the compound in solution.[3] The nature of the immobilization and non-specific interactions between the target molecule and the bead polymer pose an interfering set of thermodynamic, kinetic, and electrostatic factors that affect bioactivity. This situation may lead to false screening data. Compounds may be physically removed from the support prior to screening, but this approach is highly resource-demanding with respect to instrumentation. Off-bead screening strategies may rely on gels to reduce diffusion of library members released from beads,[4] but the assay environment is critically influenced by the nature of the gel, and post-screen hit identification is not straightforward. The use of microarrays to keep beads spatially separated is hampered by the issue of sequential, homogeneous filling of micro-wells with beads, substrates and other assay components.[5] To address these challenges, we now report a highly efficient approach for bead-based screening, where library compounds are released and subsequently screened inside respective polymeric beads. The approach is referred to as "in-bead" screening, and its unique utility is demonstrated by the identification of potent HDAC inhibitors.

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It was envisioned that organic compounds will preferentially localize or accumulate in the organic environment inside a polymeric bead whenever the surrounding media is aqueous. To confirm this hypothesis, the bead/media distribution of various organic compounds, including hydroxamic acids, was examined for PEG-based polymeric beads (Supporting Information). In general, when an aqueous solution of an organic compound was added to PEGA beads swelled in aqueous media, the organic compound was extracted into the organic environment of the bead.[6] On the other hand, the compound readily left the beads upon exposure to an organic solvent. Therefore, compounds released from beads through methods compatible with aqueous media, such as pH adjustment, enzymatic activity, or photolysis, should remain inside the bead (Figure 1 A).

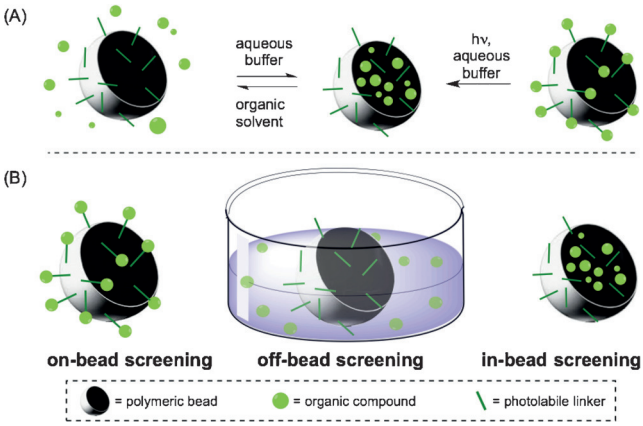


Figure 1. A) Illustration of the partition of organic compounds between polymeric beads and solvents. B) Illustration of the on-bead, off-bead, and in-bead screening techniques.

The distribution studies led us to suggest that beads may confine a spatially separated compartment useful for high-throughput biological screening. Following library synthesis on a photolabile resin, and infusion of suitable assay reagents in aqueous buffers, compounds may then be released under biocompatible conditions and screened inside each bead. Ideally, compounds would only be active upon release and not escape the parent bead swelled in appropriate assay buffer. Depending on the assay readout, beads containing hits may then be isolated through various methods, including automated approaches.

We decided to apply the in-bead screening technology to a common fluorometric assay for HDAC inhibition

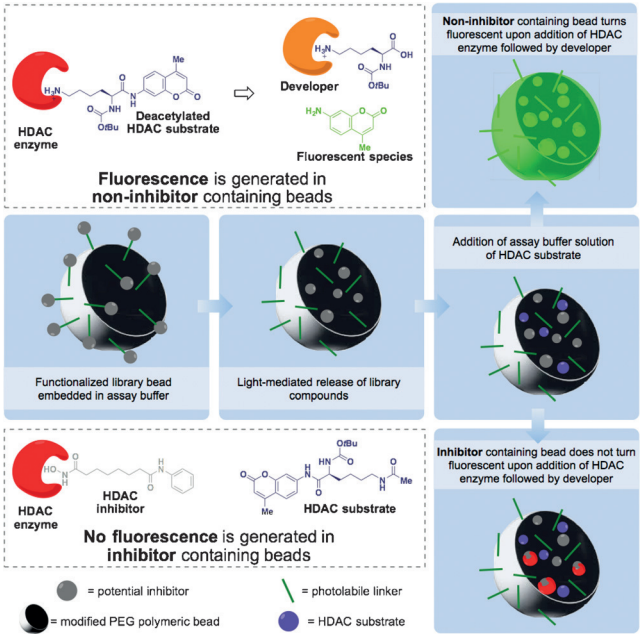
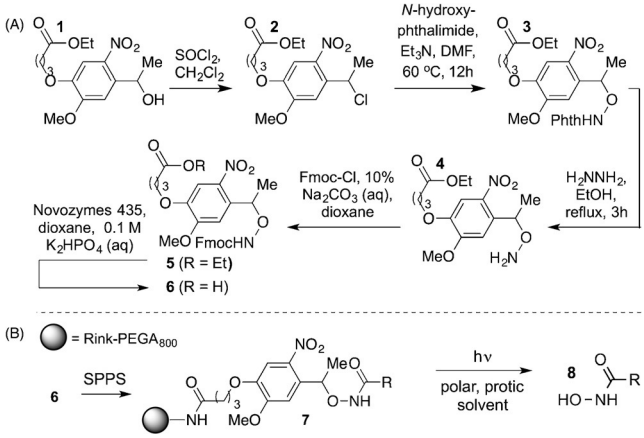


Figure 2. In-bead HDAC inhibition screening assay.

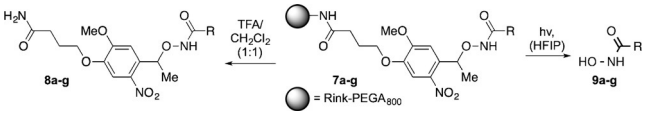


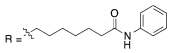
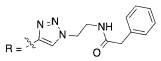
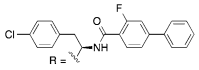
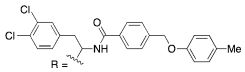
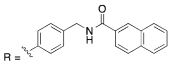
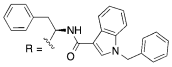
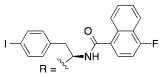
Scheme 1. A) Synthesis of Fmoc-protected hydroxylamine-functionalized carboxylic acid linker **6**. B) Application of linker **6** in SPPS for the photolytic release of hydroxamic acids.

(Figure 2), which has been widely used in academic and industrial settings in the hunt for new drugs against cancer and other diseases.[7] In this context, huge libraries of hydroxamic acid compounds have been generated through massive synthesis efforts, then screened and biologically evaluated, ultimately resulting in advanced clinical candidates and approved drugs.[8] To support our efforts, a photolabile linker **6** capable of releasing hydroxamic acids was developed (Scheme 1).

The linker was synthesized in few, high-yielding steps starting from the known alcohol **1**.[9] Using standard reagents for solid-phase peptide synthesis, the linker was immobilized and synthetically elaborated on an amino-functionalized PEGA resin. Gratifyingly, hydroxamic acids were cleanly

Table 1: Synthesis and photolytic release of hydroxamic acids **9a–g**.



Entry	Substrate	Purity[a]
A		**8a**: >95%; **9a**: >95%
B		**8b**: >95%; **9b**: >95%
C		**8c**: >95%; **9c**: >95%
D		**8d**: >95%; **9d**: >95%
E		**8e**: >95%; **9e**: >95%
F		**8f**: >95%; **9f**: >95%
G		**8g**: >95%; **9g**: >95%

[a] Photolytic cleavage was carried out for 2 h with an LED UV-lamp (365 nm). Cleavage of the Rink linker was carried out with TFA/CH_2Cl_2 (1:1) for 2 h. Purities were determined by RP-HPLC (254 nm). HFIP: hexafluoroisopropanol.

released when photolysis was performed in aqueous media (Table 1).

To demonstrate the in-bead technology in HDAC inhibitory screens, library beads functionalized with inhibitors and non-inhibitors were examined. Beads functionalized with the known HDAC-inhibitor SAHA[10] (**9a**) and the HDAC non-inhibitor (**10**), respectively, were subjected to irradiation with LED UV-light (0.5 min, 360 nm) in a Tris-HCl buffer (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg mL^{-1} BSA), before adding the HDAC substrate and incubating for 30 min. Upon isolation of the beads by simple filtration, HDAC enzymes were added. After an appropriate assay time, the HDAC activity in the beads was quenched by addition of the ultra-potent inhibitor trichostatin A (TSA). The degree of inhibition was then quantified by addition of trypsin, which liberates fluorescent aminocoumarin from residual deacetylated substrate. After washing with excessive amounts of buffer, beads were examined using fluorescence microscopy (excitation at 360–460 nm), allowing clear visual differentiation of non-fluorescent (inhibitor-containing) and fluorescent (non-inhibitor-containing) beads. Experiments showed complete inhibition of HDAC activity in SAHA-functionalized beads (Figure 3A), and no inhibition upon releasing non-ligands (Figure 3B). In addition, no HDAC inhibition was observed in SAHA-functionalized beads when the photolysis step was omitted (Figure 3C).

As fluorescence development requires the action of two enzymes, both HDAC and trypsin, negative control beads are at the same time indicative of a functional assay. Along these lines, both the absolute and relative concentrations of substrate, enzyme and inhibitor inside the beads could be

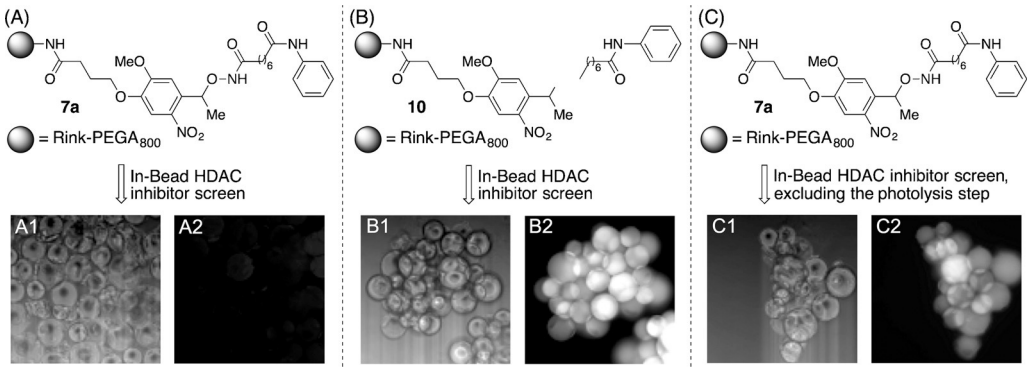


Figure 3. Representative bright-field (A1, B1, C1) and corresponding fluorescence (A2, B2, C2) microscopy images of beads after screening: A) SAHA-containing beads; B) HDAC non-inhibitor-containing beads; C) SAHA-functionalized beads excluded from the photolysis step.

varied independently. The amount of putative inhibitor correlates with the photolysis time, and the uptake of substrate depends merely on the concentration hereof in the surrounding aqueous media, with a natural upper limit (Supporting Information).

On the basis of these results, we envisioned the possibility of performing an in-bead dose-response assay by simply varying the light exposure time. Quantifiable batches of two solid-supported inhibitors (**7a** and **7b**, respectively) were separately illuminated for 5 s, 15 s, 1 min, and 5 min before carrying out the in-bead HDAC assay. The fluorescence intensity was found to decrease with increasing photolysis time as more inhibitor was being released (Figure 4). These

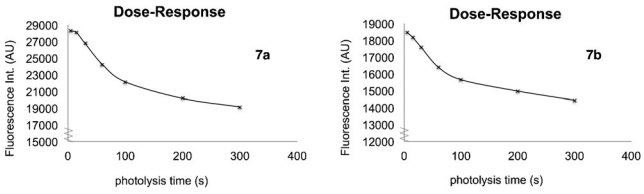


Figure 4. Dose-response measurements using library beads **7a** and **7b**, respectively.

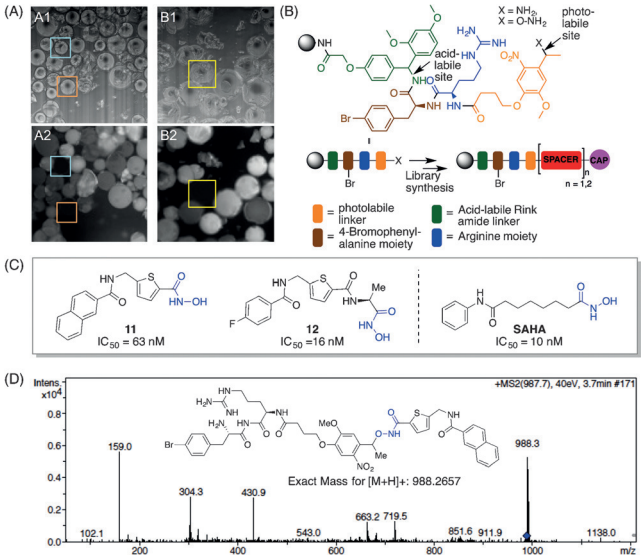


Figure 5. In-bead HDAC inhibitor screen. A) Fluorescence and corresponding bright-field microscopic images of selected beads after screening (10x magnification; D360-460 filter). The boxes indicate beads that contain a HDAC inhibitor. B) Synthetic strategy and structure of the library employed in the screen. C) Selected hit-compounds identified by the in-bead HDAC inhibitor screen. D) A representative QTOF MS-MS spectrum derived from selected hit-bead.

results clearly show the possibility of performing dose-response-type measurements, which is a unique advantage offered by the in-bead technology compared with other bead-based techniques.

The screening and analysis of combinatorial libraries demand effective methods for the sorting and identification of bioactive compounds from single beads. To facilitate QTOF-MS analysis, a bromine-containing dipeptide sequence, Phe-(4-Br)-Arg, was positioned between the photolabile linker and a Rink-linker functionalized support. Cleavage of the acid-labile Rink linker then provides a product with sufficient mass to be out of range of low-mass noise and matrix ions. The bromine tag generates mass peaks with a characteristic isotope pattern, and the arginine moiety facilitates appropriate ionization, altogether rendering relevant peaks from library products readily identifiable.

The in-bead HDAC inhibitor screen was applied to a library of 55800 compounds (Figure 5B) using Boc-Lys-(Ac)-AMC as substrate and HELA nuclear extract as a source of HDAC activity. All of the library compounds share the common structural features of known amidohydrolase inhibitors, that is, a metal-binding moiety (hydroxamic acid or amide moiety), a hydrophobic spacer unit (one or more proteinogenic or non-proteinogenic amino acids), and an aromatic cap group[11] (Supporting Information, Figure S2). Rewardingly, when trypsin was added towards the end of the assay, the majority of the beads turned fluorescent. Hit beads that remained dark were transferred to analysis vials using a needle, then individually treated with TFA, before elucidating structures using MS-MS. Selected hits (**11** and **12**) and a representative QTOF MS-MS spectrum used to identify **11** are shown in Figure 5C,D. Fluorescent beads were also routinely isolated and decoded by QTOF MS-MS spectrometry, and these beads were shown not to contain inhibitors, that is, typically non-hydroxamic acid compounds (Figure 5B, typically $X = NH_2$). All hits were resynthesized, purified, and screened in solution. Compounds **11** and **12** displayed IC_{50} values of 63 and 16 nM,

respectively, which are similar to that of the known HDAC-inhibitor SAHA.

In summary, the developed in-bead technology constitutes a viable and exceptionally resource-effective approach to the integrated synthesis and screening of large combinatorial libraries. By adapting a common fluorescence-based screening assay and a photolabile linker to the format, a series of highly potent hydroxamic acid inhibitors of HDACs were identified. In addition to the identification of enzyme inhibitors, we foresee that the in-bead technology can be applicable to a range of other biological targets and amenable to colorimetric binding assays.

Keywords: combinatorial libraries · HDAC inhibitors · in-bead screening · photolabile linker

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