Activatable Fluorescence Probe via Self-immolative Intramolecular cyclization for Histone Deacetylase Imaging in Live Cells and Tissues

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Activatable Fluorescence Probe via Self-immolative Intramolecular cyclization for Histone Deacetylase Imaging in Live Cells and Tissues

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ABSTRACT: Histone deacetylases (HDACs) play essential roles in transcription regulation and are valuable theranostic targets. However, there are no activatable fluorescent probes for imaging of HDAC activity in live cells. Here, we develop for the first time a novel activatable two-photon fluorescence probe that enables *in situ* imaging of HDAC activity in living cells and tissues. The probe is designed by conjugating an acetyl-lysine mimic substrate to a masked aldehyde-containing fluorophore via a cyanoester linker. Upon deacetylation by HDAC, the probe undergoes a rapid self-immolative intramolecular cyclization reaction, producing a cyanohydrin intermediate that is spontaneously rapidly decomposed into the highly fluorescent aldehyde-containing two-photon fluorophore. The probe is shown to exhibit high sensitivity, high specificity and fast response for HDAC detection *in vitro*. Imaging studies reveal that the probe is able to directly visualize and monitor HDAC activity in living cells. Moreover, the probe is demonstrated to have the capability of two-photon imaging of HDAC activity in deep tissue slices up to 130 μm. This activatable fluorescent probe affords a useful tool for evaluating HDAC activity and screening HDAC-targeting drugs in both live cell and tissue assays.

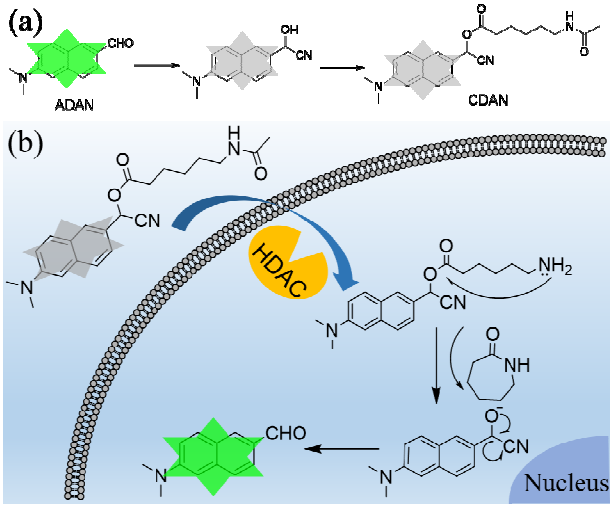
Intracellular histone deacetylases (HDACs) play crucial roles in chromatin remodeling, signal transduction and cell homeostasis.[1] HDACs are a class of hydrolytic enzymes which catalyze the removal of acetyl group from the ε-amine of lysine in histones. Upon deacetylation, the ionic interaction between histones and DNA increases, leading to more compact chromatin structures and repressed gene transcription due to limited access of transcriptional machinery to DNA. HDACs also deacetylate non-histone proteins and interact with other proteins, regulating various biological processes.[2] Aberrant levels of HDACs are recognized to be associated with various diseases including cancer, metabolic syndromes and neurodegenerative diseases.[3,4] Due to the important roles of HDACs in biomedical studies and clinical theranotics, sensitive and real-time tools enabling to monitor HDACs in live cells and even tissues are highly demanding.

Fluorescent probes afford a powerful tool for detection of various enzyme activities *in vitro* and in living cells.[5-7] Currently, fluorescent probes for HDAC could be classified into two groups based on their design strategies, "always-on" probes and "turn-on" probes. The "always-on" probes were designed by tagging a fluorophore to HDACs targeting molecules such as inhibitors or subtrates.[8-10] These probes require tedious washing steps to avoid background fluorescence from nonspecifically bound or excessive probes, precluding real-time intracellular HDAC activity detection. Activatable or "turn-on" strategies elegantly solve this issue by designing probes that are initially non-emissive or weakly fluorescent but display enhanced fluorescence upon interacting with HDACs. Because aliphatic amide structure in the HDAC substrate does not allow direct π-conjugation to a fluorophore to modulate its electronic state directly, development of activatable fluorescence probes has been a long pursuit. A turn-on fluorescence probe has been developed and commercially available for detecting HDAC activity by conjugating a fluorophore to the peptide substrate that is protected from protease-mediated digestion due to acetylation.[11] It does not allow direct HDAC assays and an additional protease digestion step is required for fluorescence development. Activatable fluorescence probes for single-step detection of HDAC activity have been designed based on deacetylation induced changes in electrostatic interaction for fluorescence modulation.[12-14] However, relatively low reaction efficiency and specificity have limited these probes for evaluating enzymatic activity and inhibitor potency in complex biological matrices. Useful designs of activatable fluorescence probes have recently been proposed by use of the nucleophilicity of the amine group generated by deacetylation, which intramolecularly attacks to an electrophilic group and triggers enhanced fluorescence.[15-17] Nevertheless, these intramolecular reactions generally involve the formation of a very large ring with a slow kinetics and require the use of non-physiological, slightly basic buffers to increase the reaction rates. To our knowledge, no probes have been realized for monitoring or imaging of HDACs in living cells. Development of activatable fluorescence probes enabling *in situ* imaging of HDACs and evaluation of their inhibitors in live cells still remains a great challenge.

Herein, we design a novel activatable fluorescence probe (CDAN) for highly sensitive and rapid detection and imaging of HDAC activity in living cells and tissues based on a new self-immolative intramolecular cyclization reaction mechanism, as illustrated in Scheme 1. The probe is designed by conjugating an acetamidohexanoate moiety, a well-established mimic to HDAC substate,[18] to a two-photon fluorophore, 6-(dimethylamino)-2-naphthaldehyde (ADAN) via a cyanoester linker (Scheme 1a). The key design of the probe is to use the cyanoester linker with a highly electrophilic carbonyl moiety, which can facilitate nucleophilic addition with amine. Moreover, this design mitigates intramolecular charge transfer (ICT) effect responsible for fluorescence enhancement in the fluorophore by converting the strong electron-withdrawing aldehyde group into weak electron-withdrawing cyanoester group. According to this design, the probe merely delivers very low fluorescence in the absence of

HDAC. Upon deacetylation by HDAC, the probe generates an amine group able to attack the highly electrophilic carbonyl moiety in the cyanoester linker, triggering a self-immolative intramolecular cyclization. The self-immolative reaction liberates a cyanohydrin intermediate, which is spontaneously rapidly decomposed into the highly fluorescent two-photon fluorophore ADAN in aqueous solution.[19] This probe is demonstrated to have high sensitivity, high specificity and fast response towards HDAC *in vitro* and in living cells. Moreover, the activated probe also possesses a high two-photon absorption cross section, allowing fluorescence imaging of HDAC activity in deep tissue slices. To our knowledge, this is the first "turn-on" fluorescence probe that enables monitoring and imaging of HDAC activity in living cells and tissues with high sensitivity and rapid response. This activatable fluorescent probe may afford a useful tool for evaluating HDAC activity and screening HDAC-targeting drugs in live cell and tissue assays.

Scheme 1. (a) Design for activatable HDAC probe. (b) Illustration of fluorescence turn-on mechanism for HDAC detection.



The HDAC probe was synthesized by conjugating ADAN with a mimic HDAC substrate via a cyanoester linker (Scheme S1 in the Supporting Information). Briefly, ADAN was prepared in a two-step procedure: First, 6-bromo-2-naphthol was reacted with dimethylamine in the presence of $Na_2S_2O_5$ to synthesize 6-bromo-N,N-dimethylnaphthalen-2-amine, which was then reacted with n-BuLi in anhydrous THF at -78 ^{o}C to obtain ADAN.[20] Subsequently, the probe CDAN was prepared in another two steps. ADAN was reacted with trimethylsilyl cyanide in the presence of zinc iodide to obtain 2-(6-(dimethylamino) naphthalen-2-yl)-2-hydroxyacetonitrile, which was further coupled with 6-acetamidohexanoic acid to generate CDAN. In addition, a control probe (CP) without the cyano group was also prepared for comparison. CDAN and CP were characterized with mass spectroscopy (MS), ^{1}H NMR, and ^{13}C NMR (Figures S1-S6 in the Supporting Information).

The fluorescence response of CDAN toward HDAC was investigated using HDAC6 as a model enzyme, because it could shuttle between the nucleus and cytoplasm, allowing direct detection of its activity in the cytoplasm.[21] The probe only displayed a negligible fluorescence signal, which was ascribed to the very weak electron withdrawing ability of the cyanoester group (Figure 1a). Incubation of CDAN with HDAC6 showed an intense fluorescence peak at ~525 nm with 70-fold enhancement in the fluorescence intensity (Figure 1a). Concurrently, there was a red shift from 300 nm to 380 nm in the absorption spectrum (Figure S7 in the Supporting Information). The enhancement of fluorescence signal and red shift in absorption peak were due to the HDAC6 induced deacetylation of CDAN. It was clear that the fluorescence spectrum of CDAN after incubation with HDAC6 was almost identical to that of ADAN, indicating the formation of ADAN after deacetylation of CDAN. Moreover, there was a remarkable decrease in fluorescence signal for HDAC pretreated with a specific HDAC inhibitor vorinostat,[22] implying that the fluorescence enhancement was correlated to the HDAC activity. A further control experiment was performed using HeLa cell lysates and the HDAC inhibitor. The probe displayed an intense fluorescence peak after incubation with the cell lysates, indicating substantial HDAC activity in HeLa cells. In contrast, the cell lysate pretreated with an excessive amount of the HDAC inhibitor showed almost complete attenuation of the fluorescence. This result suggested that cell lysate with inhibited HDAC activity did not activate the fluorescence of the probe, confirming that other species present in the cell lysate such as non-specific esterases did not degrade the probe and the probe was highly specific to HDAC (Figure S8 in the Supporting Information). In addtion, we found that reaction of CP with HDAC6 merely exhibited negligible fluorescence enhancement (Figure 1a). These results indicated that the design of a cyano group in the CDAN probe was essential for its responsiveness toward HDAC6.

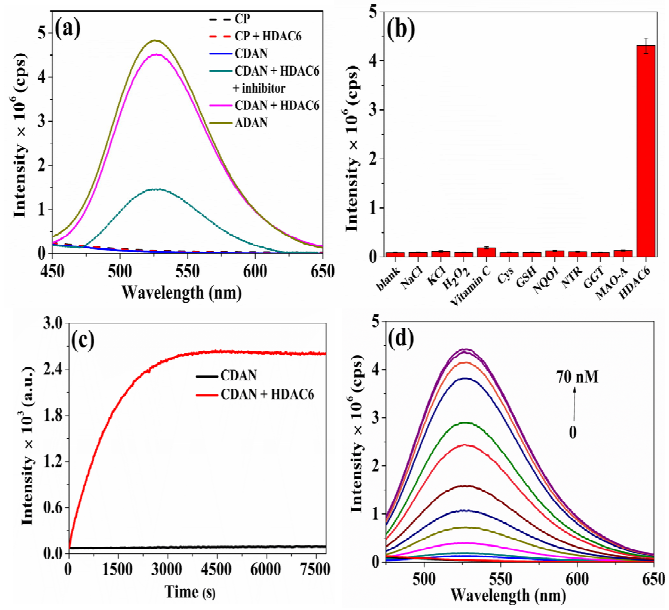


Figure 1. (a) Fluorescence spectra of ADAN (5 μM), CDAN (5 μM), CDAN (5 μM) + HDAC6 (60 nM), HDAC6 (60 nM) + vorinostat (1 μM) + CDAN (5 μM), CP (5 μM) and CP (5 μM) + HDAC6 (60 nM). (b) Fluorescence response of CDAN (5 μM) to various substances. (c) Real-time response of CDAN (5 μM) and CDAN (5 μM) towards HDAC6 (60 nM). (d) Fluorescence response of CDAN (5 μM) in PBS buffer (pH 7.4, 10 mM) to HDAC6 at varied concentrations (0-70 nM).

The selectivity of the probe was then studied by incubating CDAN with various biologically relevant species including KCl NaCl, H_2O_2, vitamin C, cysteine (cys), glutathione (GSH), human NAD(P)H: quinone oxidoreductase-1 (NQO1), nitroreductase (NTR), gamma-glutamyl transpeptidase (GGT) and monoamine oxidase A (MAO-A). All the tested substances showed negligible fluorescence signals, similar to that of the blank. However, there was a substantial increase in fluorescence intensity with the addition of HDAC6 (Figure 1b). These results indicated that CDAN was highly selective towards HDAC6, attributed to its stringent substrate specificity. Furthermore, the pH effect on the fluorescence emission of CDAN and its product after incubation with HDAC-6 were also investigated. The fluorescence intensity of CDAN was small and stable from pH 4.0 to pH 8.4, suggesting

CDAN was stable in the physiological conditions (Figure S9 in the Supporting Information). Meanwhile, large fluorescence enhancement was achieved at physiological conditions of pH 7.4 for CDAN incubated with HDAC6, which might be attributed to the optimal activity of HDAC6. These results indicated that CDAN could detect HDAC6 activity under physiological conditions. Moreover, the response time of CDAN towards HDAC6 was studied by measuring fluorescence signal at 525 nm in real time (Figure 1c). The fluorescence signal gradually increased upon addition of HDAC6 and reached a plateau in 50 min, which was much faster than the current activatable probes.[15,16] Additionally, the two-photon cross sections (δs) of CDAN and ADAN were also characterized. The δs of CDAN was only 2.7 GM at 760 nm while that of ADAN is about 56 GM (Figure S10 in the Supporting Information), demonstrating that ADAN had a large δs with excellent signal to background ratio.[23] These results suggested that our probe held great potential for deep imaging of cells and tissues with great contrast using two-photon microscopy.

The ability of CDAN to quantify HDAC6 concentrations was then investigated. With increasing concentrations of HDAC6 in the range from 0 to 70 nM, the fluorescence signals displayed a gradual increase with a saturated response at 60 nM (Figure 1d). This saturated signal might be attributed to complete depletion of the probe by 60 nM HDAC6, indicating thousands of turnovers of an enzyme in catalyzing the cleavage of the probe. Such a large turnover indicated the high sensitivity of CDAN for HDAC6 detection. The fluorescence intensities at 525 nm were linearly proportional to the concentrations of HDAC6 in the range of 3.0 to 60 nM with an estimated detection limit of 0.9 nM (Figure S11 in the Supporting Information). Furthermore, the IC_{50} value of the HDAC inhibitor vorinostat was estimated to be 91 nM (Figure S12 in the Supporting Information), which was comparable to previous reports.[24] This result indicated the ability of our probe for measuring IC_{50} for HDAC inhibitors. Additionally, HDAC concentrations in cell lysates detected with CDAN and a commercial kit were consistent with variations within 10 % (Figure S13 in the Supporting Information), implying that our probe could potentially quantify HDAC in complex biological samples. A further investigation revealed that the Michaelis constant K_m was 4.2 μM for the probe toward HDAC6 (Figure S14 in the Supporting Information), which was comparable to those reported values using commercial fluorogenic peptide substrates (4.0-11.3 μM).[25] This result indicated our probe was a desirable substrate for HDAC6, preserving its excellent enzymatic activity.

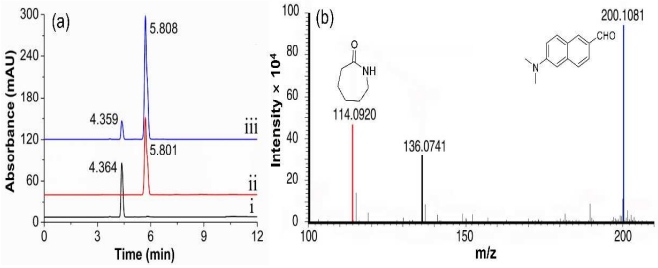


Figure 2. (a) HPLC profiles of (i) CDAN, (ii) ADAN, (iii) CDAN reacted with HDAC6 for 1 h at 37 ºC. (b) HR-ESI-MS spectrum of the reaction products of CDAN and HDAC6.

The reaction mechanism was further validated with reversed-phase HPLC and HR-ESI-MS. CDAN and ADAN had retention times of 4.364 min and 5.801 min, respectively (Figure 2a). After CDAN being incubated with HDAC6 for 1 h, there was a small elution peak at 4.359 min and a large elution peak at around 5.808 min, corresponding to CDAN and ADAN, respectively (Figure 2a). This result indicated that CDAN was converted to ADAN by HDAC6. In addition, the appearance of peaks at m/z= 200.1081 ($[M+H]^+$) and m/z=114.0920 ($[M+H]^+$) for the reaction products in HR-ESI-MS spectrum were consistent with the parent ion peaks of ADAN and caprolactam, directly proving that the reaction of CDAN with HDAC6 produced ADAN (Figure 2b). The products of CP after incubation with HDAC6 were also investigated with both HPLC and HR-ESI-MS. After CP incubated with HDAC6, there were three chromatographic peaks (Figure S15 in the Supporting Information). Based on the relative polarity of the compounds and the peaks in ESI-MS spectrum (Figure S16 in the Supporting Information), the elution peaks were ascribed to the deacetylated CP, (6-(dimethylamino)naphthalen-2-yl)methanol and the residual CP, respectively. The large elution peak at 6.274 min suggested that the deacetylated product was quite stable within the time scale of study. These results indicated the cyano group in CDAN is essential for the fast response and recovery of the ADAN fluorophore. Taken together, these results gave clear evidences that HDAC6 could turn on the fluorescence signal of CDAN via deacetylation cascaded generation of ADAN, which supported our proposed reaction mechanism.

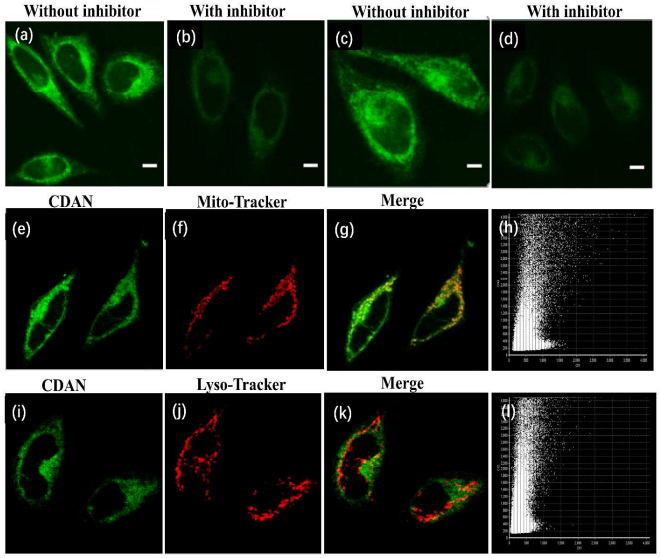


Figure 3. One-photon and two-photon images of Hela cells after being incubated with 10 μM CDAN (a, c) or pretreated with 1 μM vorinostat and then incubated with 10 μM CDAN (b, d). Scale bar= 20 μm. Intracellular localization of CDAN in HeLa cells (e-l). Images of HeLa cells pretreated with 10 μM CDAN for 2 h and subsequently 1 μM Mito-Tracker Red (or 0.05 μM Lyso-Tracker Red) for 10 min.

The ability of CDAN to visualize HDAC6 activity in live cells was then investigated. HeLa cells, known for their high expression of HDACs, were chosen as a model cell line. Before cell imaging assay, the cytotoxicity of CDAN to HeLa cells was evaluated with a standard MTT assay. The cells had over 90% cell viability for concentrations of CDAN up to 20 μM, revealing that CDAN is of low toxicity and good biocompatibility (Figure S17 in the Supporting Information). Both one-photon and two-photon fluorescence images were obtained with HeLa cells treated with or without the HDAC inhibitor. There were bright fluorescence signals for both one-photon and two-photon fluorescence images when CDAN was incubated with the cells (Figure 3a, 3c, Figure S18 in the Supporting Information). However, the fluorescence images became much dimmer for cells pretreated with the HDAC inhibitor (Figure 3b, 3d, Figure S18 in the Supporting Information). This was because endogenous HDACs could remove the acetyl group on CDAN, initiating a cascade of reactions and regenerating ADAN with strong fluorescence signal. Meanwhile, decetylation process was largely hindered in the presence of the inhibitor, thereby failing to turn on bright fluorescence signal. There was still weak fluorescence for cells pretreated with the inhibitor, which might be due to the limited concentration of the

HDAC inhibitor. Furthermore, colocalization assays were performed to study the intracellular distributions of HDACs using Mito-Tracker red and Lyso-Tracker red to stain the mitochondria and lysosomes, respectively. Fluorescence signal of CDAN did not colocalize with those of Mito-Tracker red or Lyso-Tracker red, with correlation coefficients of 0.65 and 053 (Figure 3e-3l), respectively. These results indicated that there were HDACs distributed in the cytoplasm, consistent with previous findings.[26] These results demonstrated that our probe could visualize HDAC activity in live cells, which held potential for live cell based quantification of HDAC activity and screening for its inhibitors.

The ability of CDAN toward monitoring HDAC activity in tissue slices with two-photon excitation was studied using cervical carcinoma tissue slices as a model. A slice of carcinoma tissue was incubated with CDAN (5 μM) for 2 h at 37 ^{o}C before two-photon imaging. Fluorescence images at different depths were collected in a Z-scan mode. The result demonstrated that CDAN could image HDAC activities in tissue slices with an imaging depth of 130 μm (Figure 4). Moreover, we found that the tissue slice pretreated with the specific HDAC inhibitor displayed dramatically decreased fluorescence contrast (Figure S19 in the Supporting Information). Taken together, these results suggested that our probe had excellent tissue penetrating ability and staining specificity for HDAC imaging, implying the potential of the probe for HDAC-targeted drug screening, disease diagnosis and image-guided cancer surgery.

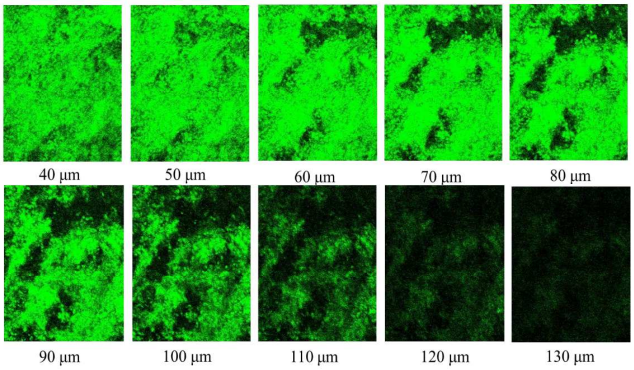


Figure 4. Z-axis scanning of cervical carcinoma tissues labeled with CDAN. Two-photon images were obtained in a depth of 40-130 μm under a 100×Oil immersion lens. Excitation wavelength: 760 nm.

In conclusion, we have developed a small-molecule turn-on fluorescence probe for studying HDAC activity, based on a novel reaction mechanism. CDAN was composed of a latent naphthaldehyde fluorophore and an acetamidohexanoate group as a mimic substrate for HDAC. The probe was essentially nonfluorescent in its free form, but exhibited intense fluorescence signal upon HDAC-catalyzed deacetylation. The probe exhibits high sensitivity, high specificity and rapid response towards HDACs *in vitro*. Its ability to visualize the activity of HDACs was demonstrated in live cells. It was also demonstrated that CDAN probe was capable of monitoring HDAC activity in tissue slices with two-photon microscopy. Therefore, we established a new strategy for designing turn-on fluorescence probes for HDAC detection, which holds a great potential for developing new tools for imaging HDAC activity and evaluating drug efficacy *in vivo*.

ASSOCIATED CONTENT

Supporting Information

Experimental methods including synthetic procedures, compounds characterizations, *in vitro* assay, cell culture and fluorescence imaging as well as additional figures. These materials are available free of charge on the ACS Publications website.

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Author Contributions

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Notes

The authors declare no competing financial interests.

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