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Thiophene-derivatized Fluorescent Benzamides as Possible Probes for Histone Deacetylases

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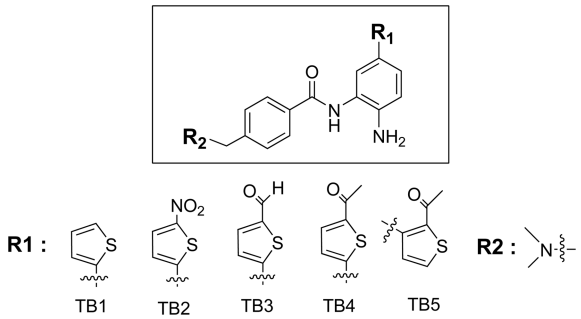
Histone deacetylases (HDACs) are crucial gene regulating enzymes that control the expression of histones-epigenetic targets in research related to developing new therapies for cancer, central nervous system disorders, and heart disease.[1] The deacetylation of histones is a vital repression process in transcriptional gene expression; it also affects apoptosis, cell-cycle arrest, and angiogenesis.[2] Therefore, a number of HDAC inhibitors have been developed; some are already used as antitumor drug candidates and many others are in phase I-III clinical trials.[3] In October 2006, the HDAC inhibitor Vorinostat (SAHA) was the first FDA-approved HDAC-targeting drug for the treatment of cutaneous T cell lymphoma (CTCL).[4] Several benzamide inhibitors, advanced second-generation inhibitors of HDACs, are currently in clinical development and clinical trials for the treatment of various cancers, including follicular lymphoma and acute myeloid leukemia.[5]

Accordingly, the use of HDACs as epigenetic biomarkers may be general across many diseases, with HDAC tracers possibly having widespread translational impact because the development of such a new diagnostic technology will allow researchers to directly probe HDACs.

Fluorescence probes are powerful molecular recognition systems with many merits.[6] These attractive imaging tools have been applied in many biological and molecular imaging studies.[7] They typically provide high sensitivity, fully three-dimensional imaging capabilities over large fields of view, and highly quantitative results; in addition, when used as tracers, they minimize biological disturbance during the imaging process, thereby enabling repeated studies. Thus, fluorescence-based HDAC probing systems would be useful and powerful diagnostic tools for imaging these enzymes. The development of fluorescent tracers for HDACs has been difficult because such tracers must have two intrinsic properties -strong fluorescence and strong binding to an HDAC enzyme (much like an inhibitor)- if they are to confer high resolution, high sensitivity, and high specificity of detection to expand the potential impact of imaging. Thus, for this study we chose to modify a benzamide drug, having a hydrophobic planar structure of two aromatic rings, with a fluorescent signaling unit, with the aim of not interfering with its binding affinity toward HDAC enzymes. Attaching a preformed fluorophore to a benzamide drug would inevitably interfere with its binding affinity; therefore, we wished to impart the fluorescence properties to the benzamide derivatives themselves.



Scheme 1. Designed thienyl-benzamide derivatives.

In this paper, we report the development of several fluorescent benzamide derivatives as selective HDAC inhibitors. Here, we chose thiophene derivatives to confer fluorescence to the aromatic groups of the benzamides.[8] To ensure that they would not interfere with the binding properties of the benzamides, we synthesized several derivatives substituted at the R_1 and R_2 positions, which are more flexible for modification; we avoided substitutions at the benzamide and free amino positions because they are essential structures for zinc binding in HDAC enzymes, as confirmed computationally (Scheme 1).[9]

We fixed the R_2 position with a tertiary amino group to ensure a polarity balance, while modifying the R_1 position with several thiophene derivatives to provide a diverse set of electronic structures (with H, NO_2, CHO, and CH_3CO as electron withdrawing groups) to produce spectra variety of fluorescence properties. We used Boc-protected 2-nitro-4-bromophenyl amine as our starting material, which we coupled with pinacol diborane to provide the boronic ester compound (See Scheme S1). Only when 1,4-dioxane was the solvent did we produce the cross-coupling product; DMF and DMSO provided the homocoupled side product, while toluene gave a low conversion. Next, we followed Suzuki-coupling to attach thiophene derivative to R1 position. Interestingly, prior to reduction of the electron-withdrawing nitro group, none of these derivatives exhibited fluorescence. In contrast, after reduction to form the electron-donating amino group, each derivative displayed a strong fluorescence signal. Thus, the fluorescence properties presumably involv-

Table 1. Absorption, fluorescence, and quantum yields of the benzamide thiophene derivatives

Compound	Φ^a in MeOH	λ_{ab} in MeOH (nm)	λ_{em} in MeOH (nm)
TB1	0.53	317	403
TB2	none	419	none
TB3	0.13	316, 379	558
TB4	0.31	366	475, 547
TB5	0.17	297	495

[a]Determined using 2,5-diphenyloxazole[10] as a standard (1×10^{-5} M in MeOH); λ_{ex} = 316 nm.

ed the electron-donating amino groups interacting with the electron-withdrawing thiophene units. When we coupled these thienyl amines with chloromethyl benzoyl chloride, we could not obtain the benzamide products as a major compound because of instability of fluorophore product. Therefore, we reversed our synthetic strategy; first we reacted chloromethyl-benzoyl chloride with the boronic ester compound **3** to produce the benzamide scaffold **4** and then we attached the thiophene derivatives to provide the fluorescent benzamides (See Scheme S1). Finally, we attached a dimethylamine unit to the R_2 position and then deprotected the Boc group to produce final compounds **TB1-TB5** as free amines.

Table 1 lists the absorption, fluorescence, and quantum yields of the benzamide thiophene derivatives **TB1-TB5**. The range of absorption maxima extended from 317 to 419 nm. Interestingly, **TB1** emits a band at a value of λ_{max} of 403 nm, with **TB3** at 558 nm, **TB4** at 475 and 547 nm, and **TB5** at 495 nm, thereby presenting colors ranging from blue to green (Figure S1). Interestingly, the presence of the strongly electron withdrawing NO_2 group in **TB2** resulted in no fluorescence emission, whereas is the other tested substituents provided fluorescence.

Next, we checked the quantum yields of these compounds to determine their fluorescence efficiencies, using 2,5-diphenyloxazole as a reference compound. **TB1** exhibited the highest quantum yield with a blue signal; **TB3**, presenting an aldehydic substituent, provided a relatively low quantum yield; **TB4** and **TB5**, which differ only by the position of their attached methylketone units on the thiophene ring, exhibited medium quantum efficiencies and green colors. When we tested the solubility of these benzamide derivatives, we found that only two of them, **TB1** and **TB4**, were soluble in water. Accordingly, we suspect that **TB1** and **TB4** would be good candidates for use as diagnostic materials for imaging HDAC enzymes. Finally to determine the potency and selectivity as a HDAC inhibitor we examined IC_{50} of our thiophene benzamide derivatives using fluorometric assay method.[11] The binding affinity of **TB1** showed high binding affinity (IC_{50} = 2.9 nM and 5.5 nM) to the HDAC 1 and HDAC 2 (Figure S2).

In summary, we have synthesized a series of novel fluorescent benzamides inhibitors possessing intrinsic fluorescence properties. Most of these benzamide fluorophores exhibit high quantum yields, making them suitable for use in imaging studies, with colors ranging from blue to green; a couple of them were also water-soluble. Notably, **TB1** and **TB2** display a high quantum yield and **TB1** exhibits high binding affinity to HDAC enzymes. We believe that these new fluorescent benzamide inhibitors might be useful diagnostic tools for *in vitro* studies of HDACs.

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Supporting Information. Experimental details; Syntheses and characterization data, fluorescence spectra, and binding affinity of thiophene-derivatized fluorescent benzamide.

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