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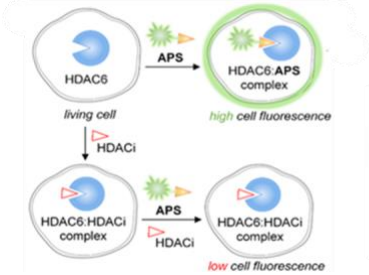

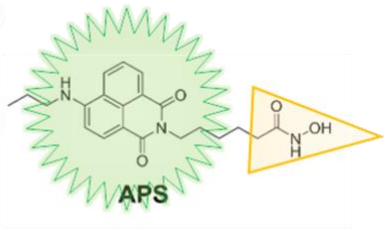

Intracellular fluorescence competition assay for inhibitor engagement of histone deacetylase.

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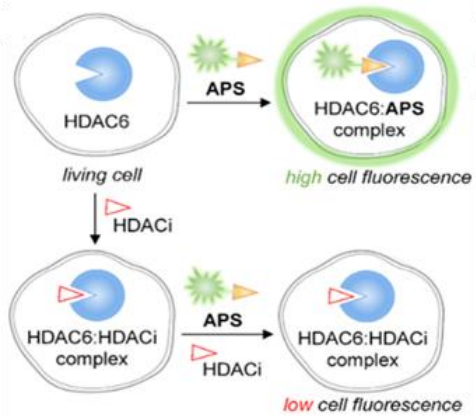
KEYWORDS: Naphthalimide, histone deacetylase, inhibitor, scriptaid, fluorescence cell microscopy, fluorescent molecular probe

ABSTRACT: An intracellular fluorescence competition assay was developed to assess the capability of inhibitor candidates to engage histone deacetylase (HDAC) inside living cells and thus diminish cell uptake and staining by the HDAC-targeted fluorescent probe **APS**. Fluorescence cell microscopy and flow cytometry showed that pre-incubation of living cells with candidate inhibitors led to diminished cell uptake of the fluorescent probe. The assay was effective because the fluorescent probe (**APS**) possessed the required performance properties, including bright fluorescence, ready membrane diffusion, selective intracellular HDAC affinity, and negligible acute cytotoxicity. The concept of an intracellular fluorescence competition assay is generalizable and has broad applicability since it obviates the requirement to use the isolated biomacromolecule target for screening of molecular candidates with target affinity.

Small molecule medicinal chemistry typically aims to find lead candidate molecules that are selective and have high affinity for biomacromolecule targets, and often these targets are intracellular. A classic discovery algorithm is to screen libraries of candidate molecules for binding to the isolated and purified biomacromolecule in a cuvette.[1 2] There are many ways to detect binding and one possibility is a fluorescence competition assay, which employs a fluorescent probe molecule that has affinity for a biomacromolecule target such as a protein or oligonucleotide.[3 4 5 6 7 8] The assay looks for displacement of the fluorescent probe from the target due to competitive binding by a candidate molecule. If the natural location of the biomacromolecule target is inside cells, then the typical next step in the discovery pathway is to determine if a candidate molecule can penetrate cells and engage the intracellular target.

There are several potential pitfalls with this traditional discovery pathway. For a start, the intracellular biomacromolecule target may be unstable when it is removed from the cellular environment or it may be technically difficult to produce the isolated biomacromolecule in pure form on a large scale. Even if a candidate molecule is found to exhibit high affinity for the isolated biomacromolecule target in a cuvette, there are several factors that could prevent engagement of the target when it is intracellular. For example, the candidate molecule may be too polar to permeate the cell plasma membrane, or there may non-selective association of the candidate with other off-target biomacromolecules (or organelles) within the cell cytoplasm.

Fluorescence competition assays have been described for ligand binding to a protein target on the cell surface,[9 10] and there are intracellular target engagement assays based on resource-intensive methods such as mass spectrometry,[11] and radiolabeling.[12] While a number of approaches have been examined there is no specific report of a straightforward "small molecule" intracellular fluorescence competition assay.[13 14 15 16 17] The general concept in Scheme 1A is based on a fluorescent probe that possesses four crucial properties, (A) bright fluorescence emission that is easily observed using a fluorescence microscope or flow cytometer, (B) good plasma membrane permeability such that the fluorescent probe can spontaneously diffuse both into, *and* out of, cells, (C) selective affinity for a desired intracellular target and negligible cell toxicity, (D) negligible affinity for other intracellular biomacromolecules, such that no intracellular accumulation of the fluorescent probe occurs if the intracellular target is blocked. The assay uses cell microscopy or flow cytometry to determine if pre-treating a population of cells with a candidate molecule leads to decreased engagement of the fluorescent probe at the intracellular site.



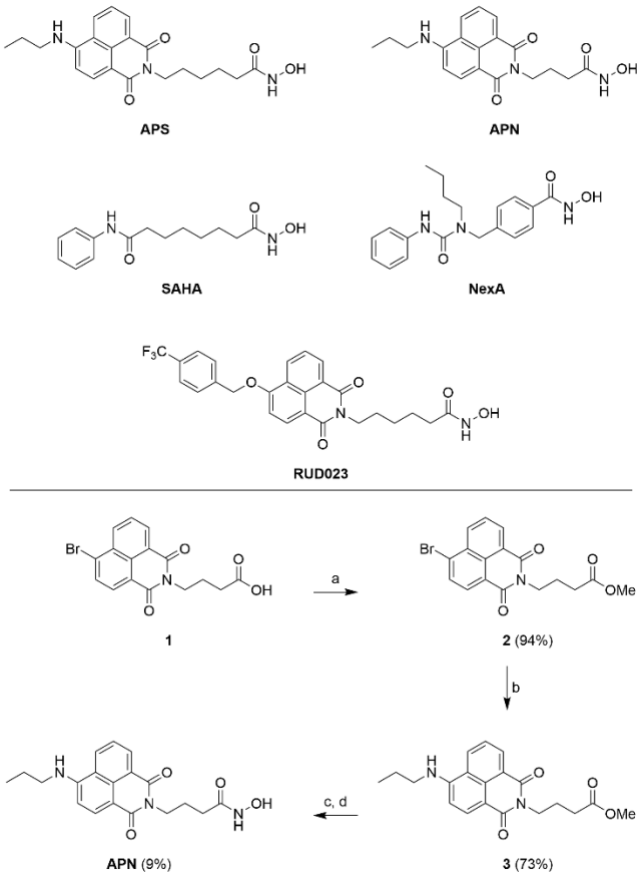
Scheme 1: Schematic summary of intracellular fluorescence competition assay and structure of **APS**.

We demonstrate proof of concept by developing an intracellular fluorescence competition assay that reports molecular targeting of intracellular histone deacetylase (HDAC). HDACs are a class of enzyme that regulate transcription by removing acetyl groups from the lysine residues present in histones.[18] After this epigenetic modification, the structure of the DNA:chromatin complex is altered and transcription is less favoured.[19] Targeting misregulated epigenetic pathways has become an established strategy in the development of new therapeutics to treat a range of diseases including cancer.[20–22] A large number of HDAC inhibitors (HDACi) have now been developed, and several have been FDA approved for the treatment of T-cell lymphoma and multiple myeloma.[23–26]

In recent years, several different fluorescent probes have been reported with HDAC targeting capability and they can be sorted into two separate groups according to their mechanism of operation. One group is a small collective of responsive fluorescent substrates that change signal once they are cleaved by HDAC enzyme.[27 28 29] Enzyme catalysis produces signal amplification which enhances imaging sensitivity, but a technical drawback with this enzymatic cleavage process is exit of the released fluorophore from the cell due to exocytosis or spontaneous membrane diffusion. This leads to a time dependent decrease in fluorescence image intensity and loss of image resolution. The other group of fluorescent HDAC probes are fluorescent HDACi that possess selective affinity for the HDAC active site. Examples of fluorescent HDACi include a near-infrared probe for imaging cancer cells[30] and tumors in mice,[31 32] and visible probes with dansyl,[33] nitrobenzoxadiazole,[34] naphthalimide,[35 36] fluorescein[31] and coumarin[37] fluorophores for cell microscopy. The fluorescence of most of these HDACi probes does not change upon enzyme engagement; thus, they can be used to report the location and abundance of intracellular HDAC.

A potential drawback with an assay based on fluorescent HDACi probe is low image intensity because the amount of bound fluorophore is restricted by the abundance of HDAC enzyme and the stoichiometry of the probe:HDAC enzyme complex. On the favorable side, image intensity and image resolution is unlikely to change much over time because the probe is immobilized as a complex with the intraceullar HDAC enzyme.[†] Here, the fluorescent probe is scriptaid analogue **APS** (Scheme 1), a fluorescent naphthalimide derivative with an appended hydroxamic acid group that exhibits good HDAC6 selectivity. [38 39] It targets intracellular HDAC (localizing in the cytoplasm along with HDAC6) and cells emit a bright, green fluorescence with high photostability and pH insensitivity. Its structure lacks charged groups and so it was expected to readily permeate cell membranes. We show that it can be used within an intracellular fluorescence displacement assay for detecting molecules that can target HDAC inside cells.

The first experimental unknown to address was the cell imaging outcome if intracellular HDAC was blocked by a potent small molecule candidate Would the fluorescent **APS** probe readily diffuse out of the cell and thus not be observed? We were concerned that **APS** would be retained inside the cells because its zinc-binding hydroxamic acid group might associate with off-target zinc-containing proteins inside the cell, or alternatively associate with intracellular lipophilic sites such as organelle membranes. To gain additional insight we synthesized **APN** (Scheme 2), a close structural analogue of **APS** with a shorter linker. **APN** is a fluorescent analogue of a literature compound called nullscript that is known to have negligible HDAC affinity because the shorter chain does not allow the hydroxymate group to reach the zinc cation buried within the HDAC active site.[40] We prepared **APN** using a similar process to that previously described for the synthesis **APS** (Scheme 2).[39] First, condensation of 4-bromonaphthalic anhydride with 4-aminobutyric acid (both commercially available) gave the corresponding imide **1** in 88% yield. This imide was then esterified to afford **2** in high yield (94%) which was coupled with propylamine using the Pd-XantPhos method to give aminonaphthalimide **3** (73%).[39] The methyl ester was then hydrolysed, the resultant carboxylic acid coupled with THP protected hydroxylamine, and finally deprotected using *p*-toluenesulfonic acid to afford **APN**.



Scheme 2: *top*: Structure of compounds used in this study and *bottom*: synthesis of fluorescent aminopropylnullscript (**APN**). Reagents and conditions: (a) H_2SO_4, CH_3OH, 60 °C, 4 h; (b) propylamine, $Pd_2(dba_3)_2{\cdot}CHCl_3$, XantPhos, toluene, 21 °C, 48 h; (c) $LiOH{\cdot}H_2O$, $THF:H_2O$, 21 °C, 24 h; 67% (d) i: NH_2OTHP, EDCI·HCl, HOBt, DMF, 12 °C, 48 h; ii: $TsOH{\cdot}H_2O$, *i*-PrOH, 21°C, 20 h.

As expected, **APN** exhibited the same fluorescence properties as **APS** (Table 1). The log P values were determined to be 2.2 for **APN** and 1.0 for **APS** (Figure S3.1), values that implied good cell membrane permeability in both cases.[41] We also measured bilayer membrane partition abilities using a standard liposome centrifugation assay and found that at micromolar concentrations both compounds do not accumulate to any measurable amount within the bilayer membranes of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) liposomes (Figure 1). Based on these results, we expected **APN** and **APS**

to have similar capability to permeate cells but not localize within intracellular membranes. We also measured IC_{50} values for inhibition of HDAC1 and HDAC6 enzyme activities and as expected **APN** was found to be a much weaker HDACi than **APS** (Table 2).

Table 1: Photophysical data for APN and APS.

Compound	λ_{ex}	λ_{em}	θ_F
APN	445 nm	547 nm	0.78[a]
APS	450 nm	544 nm	0.81

[a] Quantum yield measurement calculated in reference to absolute quantum yield of **APS**,[39] average of three measurements in DMSO.

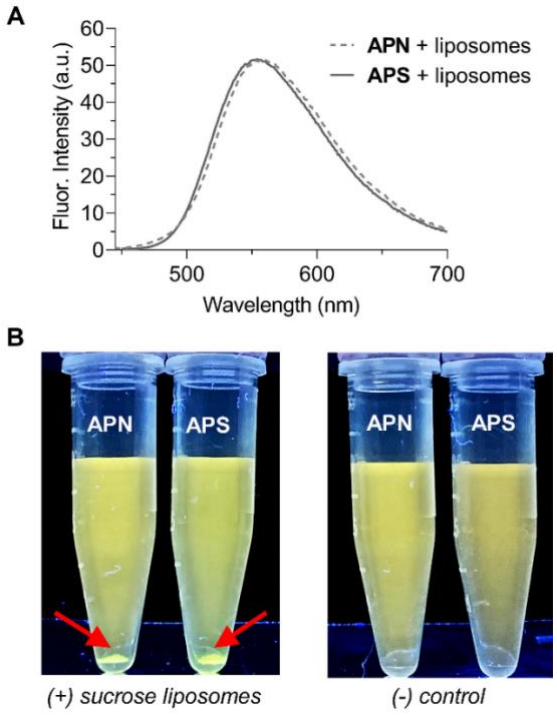


Figure 1: (A) Fluorescence emission spectra (λ_{ex} = 445 nm) of collected supernatant from **APN** or **APS** after contacting liposomes followed by centrifugation. (B) Color photographs showing that fluorescent **APN** or **APS** do not associate with pelleted, dense sucrose-encapsulated POPC liposomes. Samples illuminated by hand-held long-wave UV lamp and red arrows denote liposome pellet after centrifugation.

The two fluorescent compounds (**APN** and **APS**) were evaluated in a cytotoxicity assay, and a >90% cell survival rate after 24 h incubation was observed for both probes at 1 μM concentration (Figure S8.1). The fact that **APN** and **APS** both exhibited little short-term cytotoxicity at 1 μM concentration, encouraged us to conduct short term cell microscopy imaging experiments with the premise that observed differences in cell uptake were not due to changes in cell viability or membrane partitioning. Rather any difference in intracellular fluorescence would be due to HDAC6 binding. Human lung carcinoma A549 cells were incubated with 1 μM of either **APN** or **APS** for two hours, washed with PBS and then co-stained with the nuclear dye Hoescht 33342. The cells were again washed with PBS and fixed using paraformaldehyde before cell fluorescence imaging was performed. The fluorescent micrographs revealed a very low level of cellular uptake for **APN**, compared with **APS** which localized within the cytoplasm as previously reported (Figure 2).[39] We infer from these results that **APS** retention inside the cells is due to its HDAC6 affinity and if the intracellular HDAC enzyme was blocked by a potent HDACi, the **APS** would diffuse out of the cells. Consistent with this scenario is a recent observation that a fluorescent HDAC probe comprised of near-infrared dye with appended hydroxamic acid group was retained inside cells whereas the near-infrared dye alone slowly diffused out of the cells. [30]

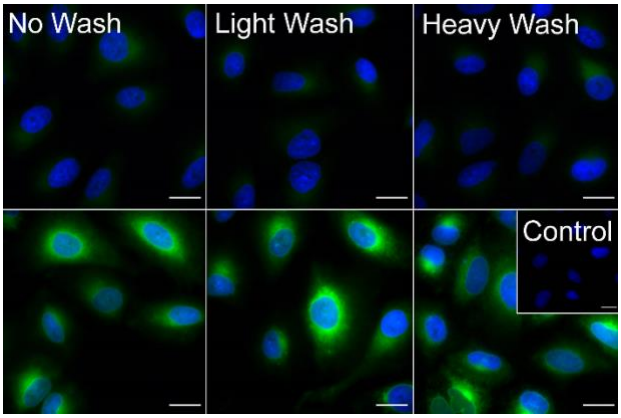


Figure 2: Representative epifluorescence micrographs of A549 cells incubated with 1 μM of either **APN** (top panel), or **APS** (bottom panel) for 30 minutes followed by cell washing (none, light or heavy). Green shows probe; blue indicates Hoechst 33342 nuclear stain; insert depicts untreated cells stained only with Hoechst 33342; length scale bar = 20 μm.

The cell microscopy results indicated that **APS** exhibits the required characteristics of a displacement probe, and prompted us to investigate its use in an intracellular fluorescence competition assay that reports engagement of intracellular HDAC by candidate HDACi. In Figure 3 are representative cell micrographs showing fixed A549 cells pre-blocked with 10 μM HDACi for 2 hours followed by co-incubation with 1 μM **APS** and 10 μM HDACi. Three different HDACi were tested, the pan-inhibitor (**SAHA**), the known HDAC6 selective HDACi (Nexturastat, **NexA**), and a newly reported fluorescent HDACi (**RUD023**).[42] Each HDACi was found to reduce the amount of intracellular **APS** with an observed affinity order for intracellular HDAC, **RUD023** > **SAHA** ~ **NexA**. The trend revealed by these intracellular fluorescence microscopy results was confirmed by independent flow cytometry experiments. Flow cytometry is more time consuming than cell microscopy but has value as a complementary detection method that surveys a higher number of cells and thus has higher statistical significance. The cytometry data in Figure 4 shows the same trend as the microscopy data in Figure 3.

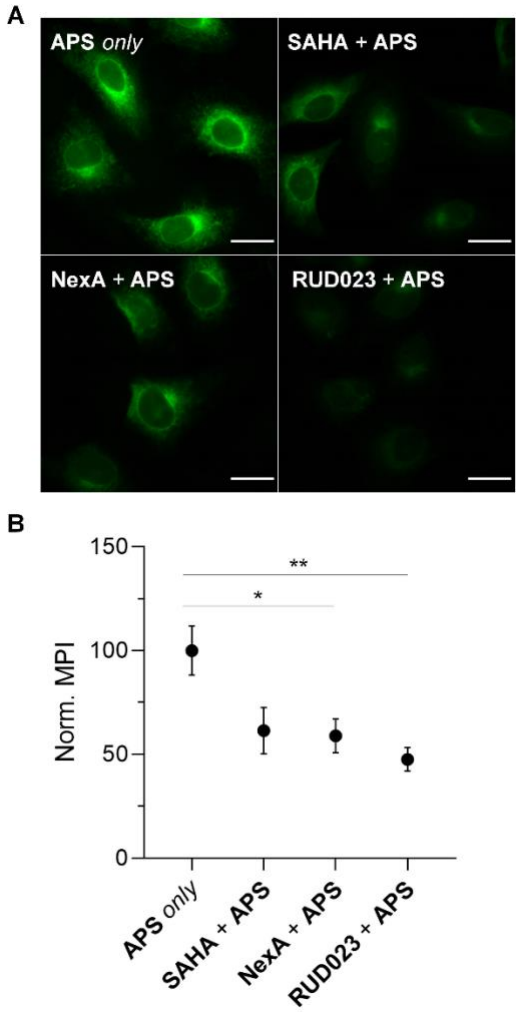


Figure 3: (A) Representative epifluorescence cell micrographs showing fixed A549 cells pre-incubated with 10 µM HDACi for 2 hours followed by co-incubation with a binary mixture of 1 µM **APS** and 10 µM HDACi. (B) Quantification of the intracellular **APS** fluorescence as mean pixel intensities (MPI) for three independent experiments. The threshold p-values are * $p < 0.05$, ** $p < 0.005$. Green shows probe; length scale bar = 20 µm.

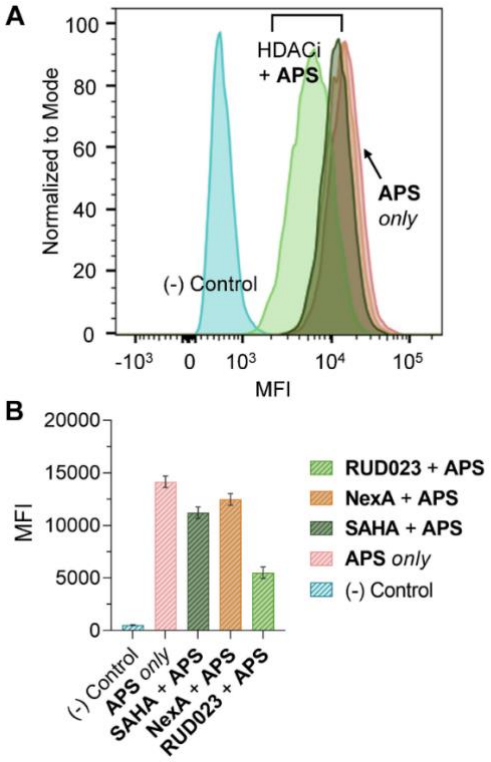


Figure 4: (A) Representative flow cytometry data for cells that were treated with 10 µM HDACi for 2 hours at 37 °C followed by co-incubation with a binary mixture of 10 µM HDACi and 500 nM **APS** for 1 hour at 37 °C followed by wash, trypsinization, and fixation. (B) Quantification of the **APS** mean fluorescence intensity (MFI) as geometric mean intensity for three independent experiments.

Shown in Table 2 are the IC_{50} values for inhibition of HDAC1 and HDAC6 by the compounds examined in this study. The data shows that while **APS** has high affinity and selectivity for HDAC6 the cell studies employed the probe at 1 and 10 µM to ensure sufficient fluorescence for microscopy and counting, respectively. In both cases, the added **APS** likely saturated *all* available isoforms of intracellular HDAC which in turn means the assay conditions were biased to identify HDACi with HDAC affinities beyond HDAC6. Although **RUD023** has weaker affinity than **NexA** for HDAC6, it has higher affinity for the other isoforms which likely explains why **RUD023** was more effective than **NexA** at reducing the total amount of fluorescent intracellular **APS**.

Fine-tuning this intracellular competition assay as a method to identify HDAC6-selective inhibitors is an attractive future goal but likely requires a version of **APS** that has exceptional affinity for HDAC6 and very low affinity for the other isoforms. This high level of selectivity would ensure that the fluorescent probe would only diffuse out the cells when HDAC6 was blocked by a high affinity HDAC6i.

Table 2: IC_{50} Values for HDAC Inhibition and Isoform Selectivity Factor.*

	IC_{50}, µM (Selectivity Factor)	
Compound	**HDAC1**	**HDAC6**
SAHA (vorinostat)	0.033 (1)	0.033[43]
NexA (nexturastat A)	3.02 (604)	0.005[44]
APS	0.59 (123)	0.0048[39]
APN	9.50 (8)	1.12
RUD023	0.28 (24)	0.012

*Selectivity Factor describes selectivity for HDAC6 over HDAC1, calculated by dividing IC_{50} value for HDAC1 by that for HDAC6.

To summarize, an intracellular fluorescence competition assay was developed to assess the capability of HDACi candidates to engage HDAC inside living cells and thus diminish cell uptake and staining by the HDAC-targeted fluorescent probe **APS**. In general terms, a successful intracellular fluorescence competition assay requires a fluorescent probe, such as **APS**, to possess several performance criteria, including: (A) bright fluorescence emission, (B) ready diffusion in and out of cells, (C) selective affinity for a desired intracellular target and at concentrations that do not induce acute cell toxicity, (D) negligible affinity for other intracellular biomacromolecules, such that no intracellular accumulation of the fluorescent probe occurs if the intracellular target is blocked. The concept of an

intracellular fluorescence competition binding assay is generalizable and has broad applicability since it obviates the requirement to use isolated biomacromolecule target for candidate molecule screening. Moreover, it can likely be incorporated into high content screening paradigms that look for candidate-induced changes in cell phenotype. The information output of a high content screen would be improved if the assay could also report if there was target engagement in the cells that have undergone a predetermined phenotypic change. [13 45 46]

Supporting Information

Supporting information is provided containing full details for synthesis and characterization of the new compounds as well as a full description of the biological and imaging protocols used.

Author Information

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no conflict of interest.

Footnotes

[†]A fluorescent coumarin probe was shown to be quenched upon binding intracellular HDAC and switched back on by a competing HDACi.[37] While an assay based on "turn on fluorescence" is attractive, a drawback is diffusion of the displaced fluorescent probe out of the cells which counters the probe signal increase and thus produces a false negative screening outcome).

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