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A fluorescent histone deacetylase (HDAC) inhibitor for cellular imaging

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Fluorescence microscopy studies using 4-morpholinoscriptaid (4MS) demonstrated rapid cellular uptake of this scriptaid analogue into the cytoplasm but no nuclear penetration. As 4MS and Scriptaid have the same in vitro activity against HDACs and KASUMI-1 cells; 4MS exemplifies a rational approach to subtly modify ‘profluorogenic’ substrates for intracellular studies.

Fluorescent tagging of bioactives for cell based microscopy studies is conventionally achieved by conjugating, by means of a spacer, a bulky fluorophore to the drug of interest. While fluorescent, the extraneous conjugation to a fluorophore invariably leads to an increase in molecular weight and has an impact on cLogP—parameters that medicinal chemists spend considerable effort optimising. Indeed, the behaviour of the ‘tagged’ compound in cells might not accurately reflect that of the parent.

Ideally, only subtle structural modifications would be performed on a bioactive to render it fluorescent. Despite the seemingly obvious benefits of such an approach, no examples could be found. While this strategy is limited somewhat in that only aromatic compounds can be considered, the dominance of highly aromatic scaffolds in drug discovery makes it complementary to the conventional ‘tagging’ approach. A bioactive that is identified as being structurally related to an existing fluorophore might be termed ‘profluorogenic’; for example naphthalene is a common component of biologically active compounds and is the core of several highly fluorescent entities including the 4-aminonaphthalimides.

Scriptaid (1) is a well-studied histone deacetylase (HDAC) inhibitor with established anticancer activity. More recently, Scriptaid (1) has been shown to have beneficial effects in the treatment of HIV and neurodegenerative disorders. Scriptaid (1) possesses the typical pharmacophore of HDAC inhibitors which includes (i) zinc binding group (chelates the Zn²⁺ ion of the active site, see Figure 1) (ii) linker (occupies a hydrophobic tunnel) which connects to the (iii) capping group (solvent exposed).

Recognising the structural similarities of the 4-aminonaphthalimide fluorophore and the known HDAC inhibitor Scriptaid (1), the fluorescent Scriptaid analogue 4MS was developed (Figure 1); the key feature being the introduction of the relatively small 4-morpholino substituent (MW = 87).

![Figure 1. Structure of Scriptaid (1) and the fluorescent 4MS. Regions of the HDAC pharmacophore are also indicated.](image)

While SAR studies of Scriptaid do not extend to modifications of the ‘capping’ group, we reasoned that, given the non-critical role of the capping group with respect to HDAC inhibition, introduction of a small amino moiety would not have deleterious effects on HDAC activity. Indeed, calculated properties (cLogP and PSA) of Scriptaid (1) and 4MS showed only subtle differences (see ESI for full details) and thus it was anticipated that other than fluorescence, 4MS would behave in a near identical fashion to the parent.

![Scheme 1. Synthesis of 4MS](image)
Table 1. Inhibition of individual HDAC isoforms, IC_{50} (µM)

| Compound | HDAC Isoform (SF), SF = Selectivity Factor vs HDAC6 | 1 | 3 | 6 | 8 | 11 | KASUMI-1 |
|----------|--------------------------------------------------|---|---|---|---|----|---------|
| Scriptaid (1) |                                    | 1.74 ± 0.06* (145) | 0.37 ± 0.06 (31) | 0.012 ± 0.0026 (27) | 1.52 ± 0.01 (127) | 0.36 ± 0.03 (30) | 0.49*     |
| 4MS      |                                    | 1.43 ± 0.13 (119) | 0.32 ± 0.04 (27) | 0.012 ± 0.0019 (27) | 1.81 ± 0.17 (151) | 0.29 ± 0.02 (24) | 0.29      |

* Values represent the average of two measurements (n = 2). * Error < 1% (n = 3).

To construct 4MS, 4-bromonaphthalimide 2 (see ESI for synthesis) was converted to the corresponding 4-morpholino derivative 3 using a palladium mediated Buchwald–Hartwig approach (Scheme 1). Treatment of 4-bromonaphthalimide 3 with morpholine (3 equiv), Pd_2(dba)_3·CHC1_3 (4 mol%) and the commercially available ligand, Xantphos (4 mol%) in the presence of Cs_2CO_3 (3 equiv) at 40 °C for 24 h gave the desired aryl amine 3 in excellent yield (90%). Carboxylic acid 4 was obtained in high yield (91%) using excess LiOH·H_2O. The free acid was converted to the mixed anhydride (using CI_2O·Et and Et_3N) then in situ treated with hydroxylamine (in methanol) to give the desired hydroxamic acid 4MS in good yield (67%, 50% over 4 steps).

Compound 4MS was then assessed for inhibitory activity against HDAC isoforms 1, 3 and 8 (class I), 6 (class IIb) and HDAC 11 (class IV). The introduction of the morpholine group was not deleterious towards HDAC inhibition. Indeed, when compared to Scriptaid (1) 4MS had similar activity against both HDAC6 (IC_{50} = 12 nM, Table 1) and HDAC3 (4MS, IC_{50} = 0.32 µM; cf 1, IC_{50} = 0.37 µM) and was slightly more efficacious against HDAC1 (IC_{50} = 1.43 µM) than Scriptaid (1, IC_{50} = 1.74 µM). Consequentially, 4MS still displayed similar selectivity as Scriptaid (1) for HDAC6 over HDAC1 and HDAC3. For the other class I isoform investigated (HDAC8) 4MS inhibited with an IC_{50} of 1.81 µM compared with 1.52 µM for 1 which corresponds to improved selectivity (4MS, SF = 151; cf 1, SF = 127). A moderate improvement in activity was also obtained at HDAC11 (4MS, IC_{50} = 0.29 µM; cf 1, IC_{50} = 0.36 µM).

Both Scriptaid (1) and 4MS exhibit modest selectivity towards cytosolic HDAC6, which is known to modulate a number of processes that can lead to cell stress and apoptosis including α-tubulin deacetylation and binding to the stress granule protein, G3BP1. Indeed, HDAC6 inhibitors have been proposed as therapies for acute myeloid leukemia, a hematological cancer represented by KASUMI-1 cells. As such the ability of 4MS to decrease cell growth was evaluated in KASUMI-1 cell lines (Table 1) and 4MS inhibited the growth of the leukemia cell line with an IC_{50} of 0.29 µM, which is comparable with Scriptaid (1, IC_{50} = 0.49 µM).

The photophysical properties of 4MS were typical of 4-dialkylamino-1,8-naphthalimide derivatives and proved to be well suited for fluorescence microscopy. In DMSO, the absorption maximum (Figure 2) was 399 nm with an emission maximum at 534 nm (Stokes shift = 135 nm). This compares favourably with Scriptaid (1), which presents no visible fluorescence.

To demonstrate the potential of 4MS as a tool to provide pharmacokinetic information, confocal microscopy was used to visualise the cellular penetration and subcellular location of 4MS in the human breast cancer MDA-MB-231 cell line. The cells were treated in situ with a 1.0 µM solution of 4MS and images were taken at 10 second time points (Figure 3A–F). At each time point, a significant increase in emission intensity from the treated cells was evident, clearly indicating rapid cellular uptake. Cellular uptake of biologically active molecules is currently generally monitored by radio-labeling or fluorescent tagging. While radio-labeling can be achieved through isotopic replacement it requires specialised equipment and precautions. As mentioned, fluorescent tagging often leads to a significant increase in the molecular weight of the known biologically active compound. Large increases in molecular weights may result in poor absorption and cell permeation of the fluorescent therapeutic.

By utilising the inherent fluorescent properties of 4MS, rapid cellular uptake was visualised in less than 50 seconds.

Additional time course studies were conducted at lower concentrations (0.025–0.30 µM), in which 4MS was incubated in MDA-MB-231 cells for 48 h and co-stained with propidium iodide to monitor cell death (Figure 4). No fluorescence was observed in the nucleus, suggesting that 4MS did not penetrate the nuclear envelope. It is possible that the lack of observed fluorescence in the nucleus was due to DNA mediated fluorescence quenching rather than lack of penetration therefore titrations of 4MS with calf thymus (ct)-DNA were conducted. While a slight decrease in the emission intensity of 4MS was observed upon the addition of ct-DNA in phosphate buffer (10 mM at pH 7.4), the emission band of 4MS was still clearly visible after a large excess of ct-DNA had been added (see ESI). These results indicate that 4MS was not entering the nucleus and that the lack of fluorescence emission from the nucleus was not a result of DNA binding/quenching. The observed cytoplasmic accumulation is interesting given the concentration of

**Figure 2.** Normalised excitation and emission spectra of 4MS in DMSO.
HDACs in the nucleus and may ultimately prove advantageous as the class II HDAC isoforms, such as HDAC6, are known to shuttle between the nucleus and the cytoplasm. Based on our results, it might be possible to design selective inhibitors for class II HDAC by refining the localisation profile of the therapeutic rather than focussing solely on enhancing its specific enzyme binding.

Upon incubation of MDA-MB-231 cells with the fluorescent 4MS at 0.025 and 0.1 µM no significant changes were observed (Figure 4A and B, respectively). However, the treatment of MDA-MB-231 cells with 0.3 µM 4MS, cell death was evident within 48 h (Figure 4C).

In summary, the rational design and synthesis of 4MS has been performed. Structural changes from Scriptaid (1) were minimal and were not detrimental to isoform selectivity or anticancer activity (KASUMI-1). The strategic location of the morpholino substituent at the 4-position of the naphthalimide imparted photophysical properties and enabled the study of these compounds in cells using fluorescence microscopy; demonstrated with the visualisation of rapid cellular uptake and subsequent distribution in MDA-MB-231 cells. This fluorescent analogue has very quickly contributed to our understanding of how the well-studied Scriptaid (1) acts at a cellular level and is likely to be a value to the many researchers currently investigating Scriptaid (1) as a potential neuroprotectant and anticancer agent.

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