

Introduction

The deacetylase SIRT1 has been the subject of intensive study since the discovery of its purported role in lifespan extension in both invertebrate and vertebrate organisms. More importantly, overexpression of SIRT1 in a mouse model of Huntington's Disease (HD) was shown to be neuroprotective, with deletion of the SIRT1 gene demonstrating worsening of the HD-like phenotype. Numerous putative SIRT1 activator compounds, of which the natural product resveratrol and the synthetic molecule SRT1720 are the most well studied, have been identified as potential therapeutics for obesity or oncology indications. However, significant controversy over the true pharmacology of these compounds has been generated with the finding that the presence of a fluorescent tag on the substrate molecule is essential for their activity. Our objective has been to develop biochemical and cellular assays capable of identifying true activators of the SIRT1 enzyme. Here we describe the profiling of SRT1720 and resveratrol in a panel of biochemical assay formats, demonstrating a lack of activity of these compounds in assays conducted in the absence of a fluorescently tagged substrate. Future work will involve 1) using these assays to profile compounds selected by various virtual screening hypotheses; 2) identifying lead compounds capable of truly activating SIRT1; and 3) optimizing such compounds with the goal of testing them in an *in vivo* HD disease model.

Results

> SRT1720 and resveratrol generate conflicting results in two SIRT1 biochemical assay formats

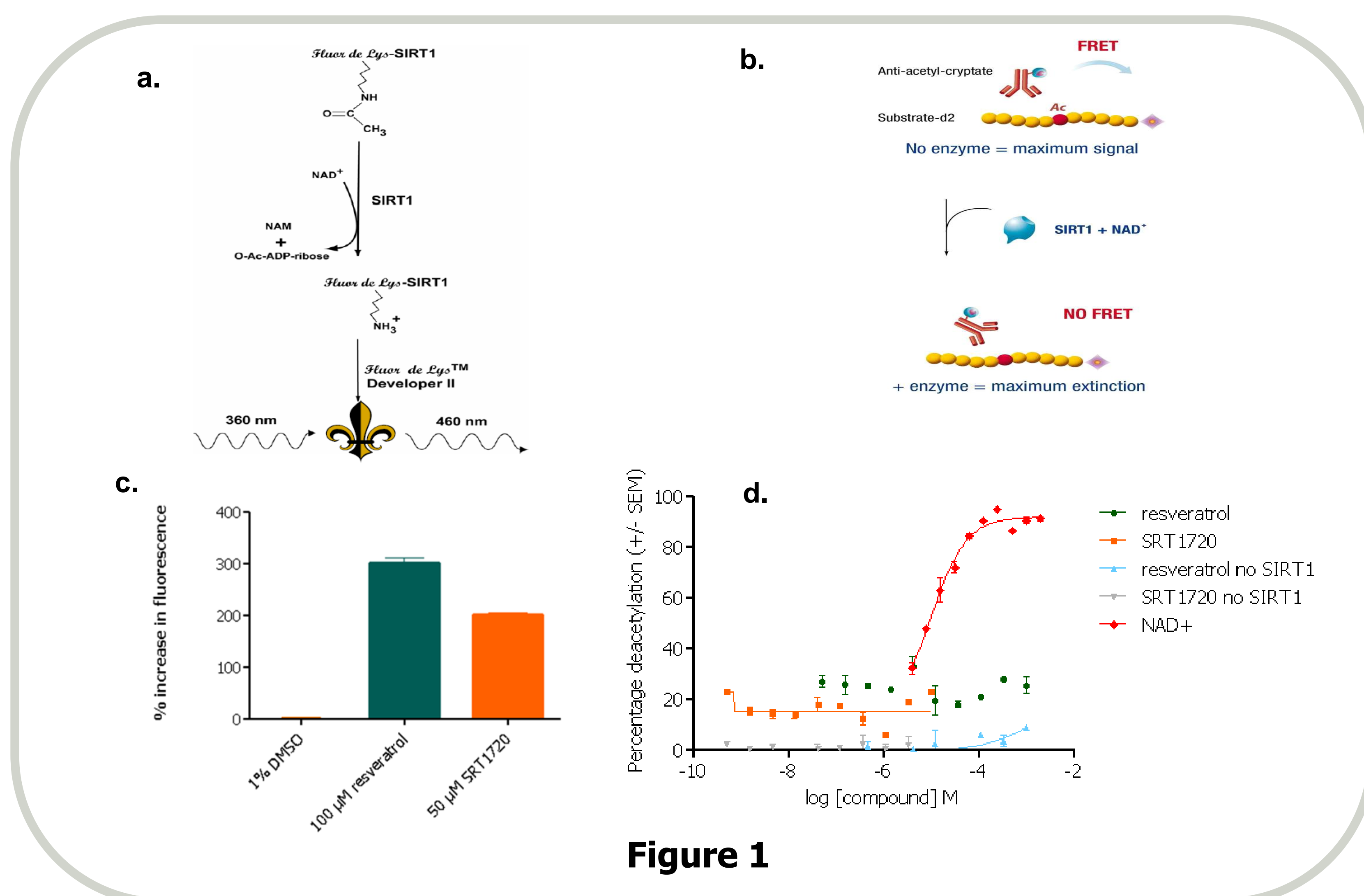


Figure 1

- SRT1720 and resveratrol have both been reported to demonstrate significant SIRT1 activation in the Fluor-de-lys assay (**Figure 1a**)¹
- These observations were confirmed in our studies (**Figure 1c**)
- The true activity of SRT1720 and resveratrol has been debated since the suggestion that the activity is due to an interaction with the fluorescent tag in the Fluor-de-lys substrate^{2,3}
- To further investigate true activation of SIRT1, we also used an alternative HTRF based assay system, based on antibody detection of an acetylated p53 peptide fragment (**Figure 1b**)
- In this assay format, neither SRT1720 nor resveratrol showed any significant activation (**Figure 1d**); increasing [NAD⁺] was used as a surrogate control for activation
- These conflicting results may be due to differences within the assay formats or an indication that the "activation" seen in the Fluor-de-lys assay is truly a false-positive result

Methods and Materials

> Fluor-de-lys assay (Enzo)

SIRT1 (0.2 U/well, produced in house by BioFocus Structural Biology) was added to SRT1720 or resveratrol (both supplied by CHDI Inc.) in a white/white low volume 384-well plate and pre-incubated for 10 min at 37 °C before the addition of 25 μM Fluor-de-lys substrate and 200 μM NAD⁺ per well. Compounds were incubated for 30 min at 37 °C before addition of kit detection reagent and detection as manufacturers' protocol at 390 nm excitation/490 nm emission on an EnVision plate reader (Perkin Elmer).

> HTRF assay (CisBio)

SIRT1 (4.2 nM/well) was added to titrations of SRT1720 or resveratrol in a black/black low volume 384-well plate in the presence of the recommended concentration of acetylated substrate before the addition of 10 μM NAD⁺ to each well. The reaction was allowed to progress for 30 min before addition of stop solution containing the detection antibody, an overnight incubation to allow signal development and detection on an EnVision plate reader on HTRF settings (665 nm, 615 nm dual read).

> LC-MS label-free detection (BioFocus)

SIRT1 (5 nM/well) was added to titrations of SRT1720 or resveratrol in a V-bottom 384-well plate together with 10 μM acetylated peptide (residues 372-389 of human p53 containing an acetylated lysine in position 382) and 30 μM NAD⁺. The reaction was progressed for 45 min, and stopped with the addition of 10% formic acid, and 4 μM heavy-labelled internal standard (as the deacetylated peptide but containing two heavy carbon and nitrogen atoms on the first two positions).

> Development of an LC-MS label free SIRT1 deacetylation assay

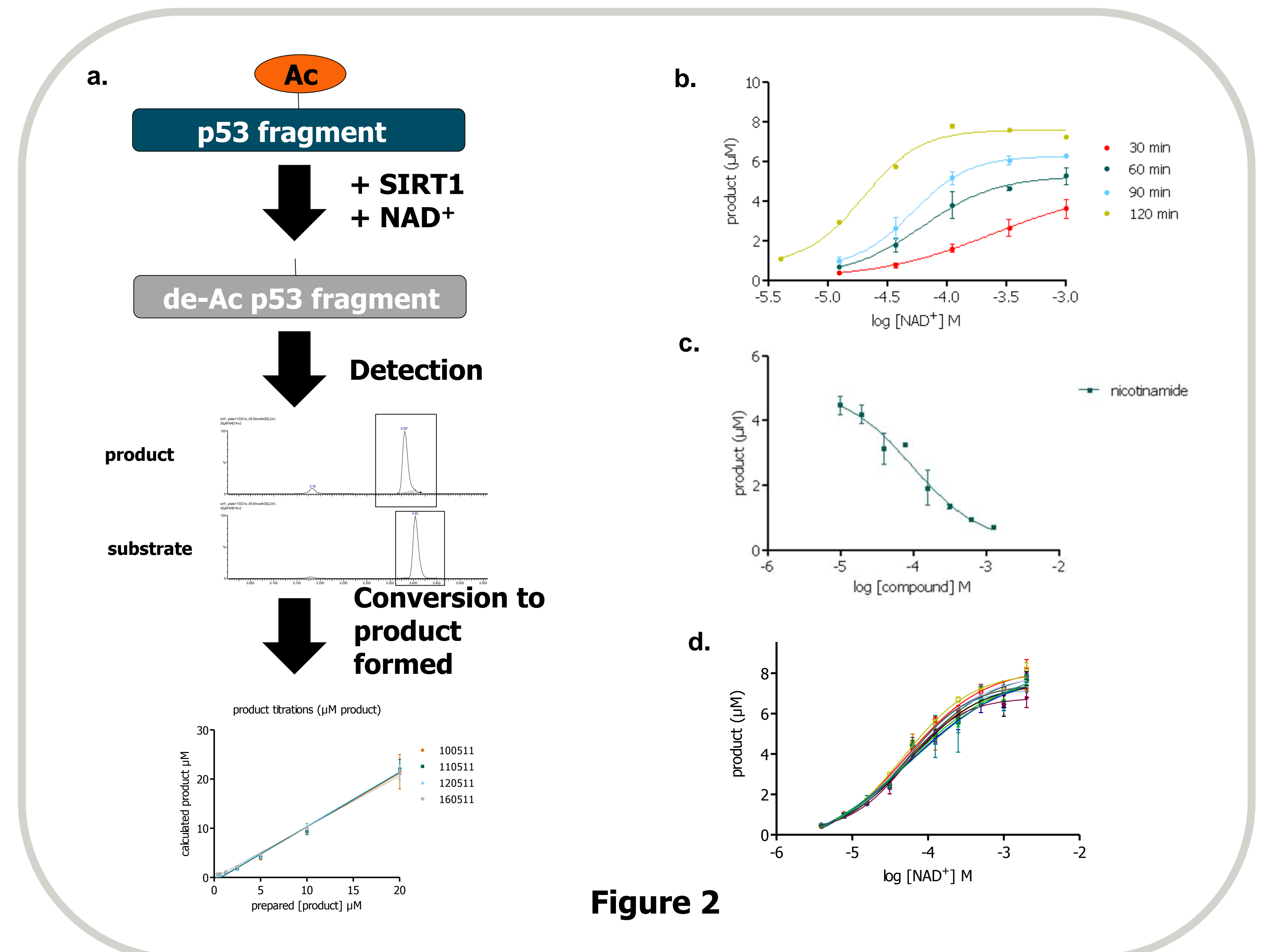


Figure 2

- To avoid any fluorescent tag or reagent interference complications completely, a LC-MS assay was developed to assess SIRT1 deacetylase activity using an unlabelled 18 amino acetylated peptide centered around a known SIRT1 substrate [p53^{lys382}]
- Deacetylation of the acetylated peptide causes a mass-shift, which is able to be detected by mass-spectroscopy (Waters Xevo TQ; **Figure 2a**); conversion to the peptide product is quantified by comparing product peak height to a standard curve; inclusion of a heavy-labelled internal standard peptide corrects for any interference
- Initial experiments demonstrated good substrate conversion over time with recombinant SIRT1; from this data, a 45 min timepoint was chosen to assess activation (**Figure 2b**)
- The pharmacology of SIRT1 activity in this assay was further characterized using inhibition by nicotinamide (**Figure 2c**) and "pseudo-activation" by NAD⁺ (**Figure 2d**)
- A good correlation between the LC-MS and HTRF assay format has been observed

> SRT1720 and resveratrol are inactive in a label-free LC-MS assay

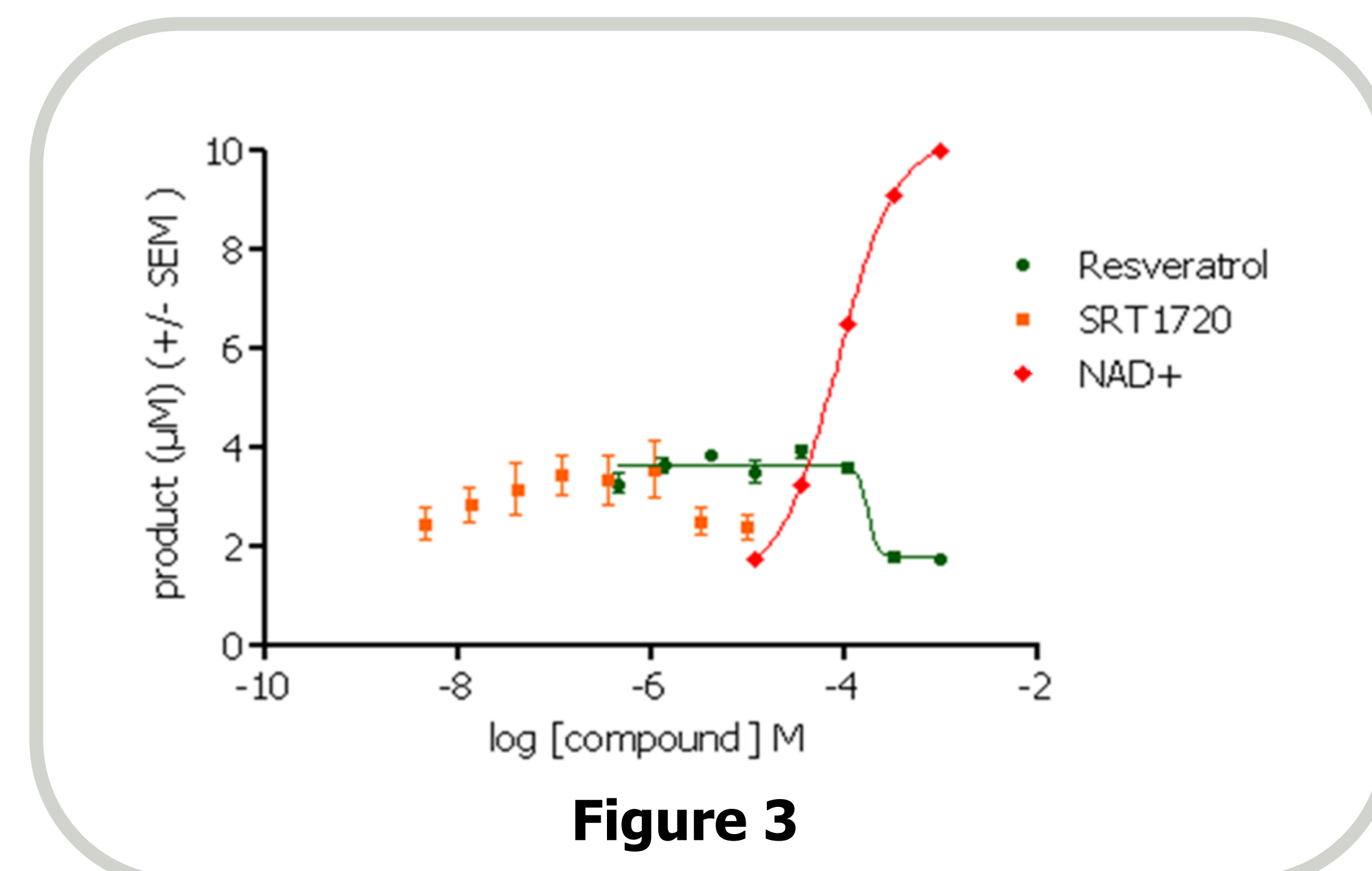


Figure 3

- In contrast to the Fluor-de-lys assay, and in agreement with the HTRF assay format, no significant activation of SIRT1 was observed with either compound (**Figure 3**)
- Higher throughput led us to choose the HTRF assay format for primary screening, with the LC-MS assay being used for confirmation of any potential hits identified

Conclusions and next steps

- Multiple assay platforms were evaluated for their ability to identify true SIRT1 activators
- Assay-specific false-positive hits with literature compounds were observed in the Fluor-de-lys assay format
- Good correlation between the HTRF assay platform and the in-house developed LC-MS assay has led to these assays being incorporated into our screening cascade
- Currently, we are profiling compounds, selected by a variety of virtual screening hypotheses, for activator activity in these assays
- We are also developing cell-based assays of SIRT1 deacetylase activity to allow testing of hits in a cellular context using full length native substrates

References

- Milne et al., (2007) *Nature* 450, 712-716;
- Pacholec et al., (2010) *J. Biol. Chem.* 285, 8340-8351;
- Huber et al., (2010), *Future Med Chem.* 2, 1751-1759