## Complications of Using Fluorescent labeled peptide substrate

Back to 2005, both Denu and Kennedy labs (Borra, Smith et al, 2005; Kaeberlein, McDonagh et al, 2005) reported that it is necessary to use a peptide substrate with fluorephore to observe SIRT1 activation by resveratrol. Different assays were performed which were the Fluor de Lys assay, coumarin and rhodamine- based fluorescence assays, charcoal binding assay, and a HPLC-based assay.

- It was found that SIRT1 activation was independent of the peptide sequence investigated (three p53 peptide substrates), but was dependent on the presence of a fluorescent label, thereby showing that resveratrol failed to activate the deacetylase activity of SIRT1 by using an unlabeled substrate. Substrate competition studies demonstrated that the attachment of a fluorophore decreased the binding affinity of the corresponding peptide, but in the presence of resveratrol, the tagged substrate bound more tightly (Borra, Smith et al. 2005).
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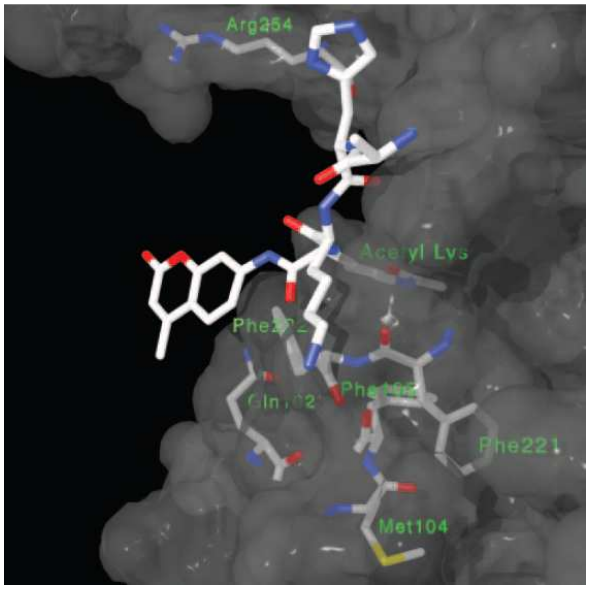
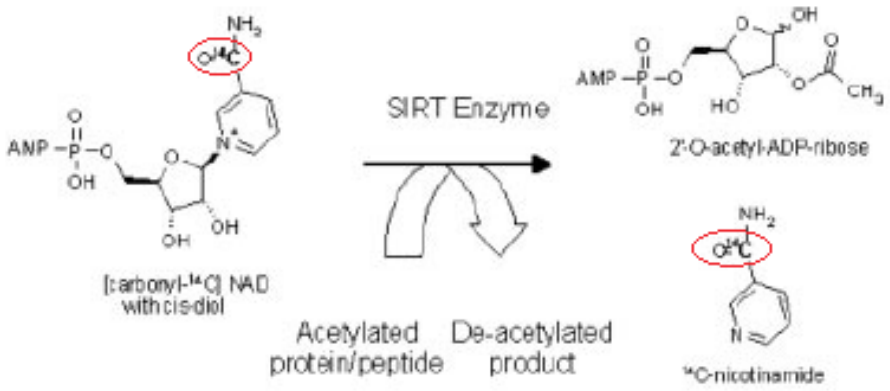


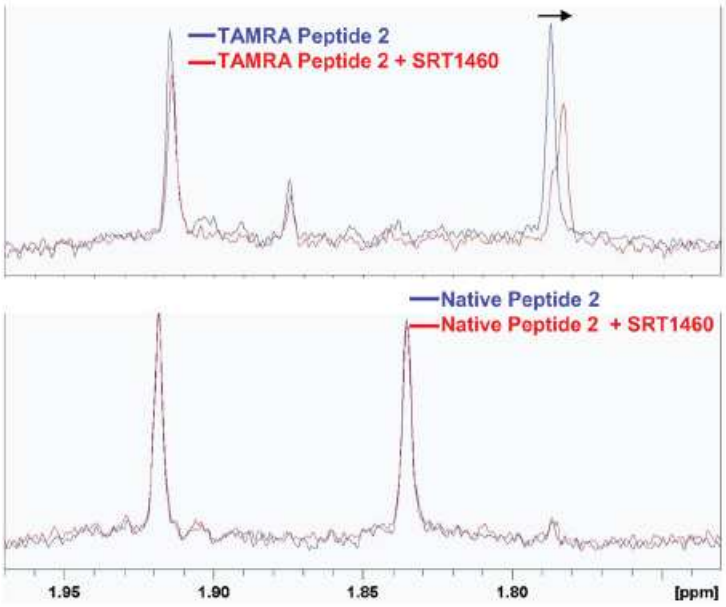

**Putative binding site of the p53-AMC peptide.** The p53-AMC peptide substrate modeled from the crystal structure of an 18-mer p53 peptide bound to Sir2-Af2 is shown bound to SIRT1 built from homology modeling. The SIRT1 residues Gln102, Met104, Phe105, Phe221, Phe222, and Arg254 are shown as possible new contacts to the coumarin of p53-AMC upon loop rearrangement resulting from resveratrol binding. These residues were chosen from the built model because of their close proximity to the coumarin ring (<10 Å). This figure was generated using Sybyl version 6.8, Swiss PDB viewer version 3.7, and POVray version 3.6.

- Borra et al. proposed a model for the effect of resveratrol on a coumarin labeled peptide. Without resveratrol, the coumarin attached to a p53 peptide would be solvent-exposed, and would exist in an energetically unfavorable conformation. Resveratrol may induce a conformational change that creates a binding pocket, which better accommodates coumarin, thereby resulting in enhanced substrate binding. Therefore, it is possible that a SIRT1 substrate containing the appropriate hydrophobic or aromatic amino acids might in fact be a target for resveratrol and other small-molecule-based activation (Borra, Smith et al, 2005).

- By using [3H]acetate and [14C]nicotinamide release assays, Kaeberlein et al. also demonstrated fluorescent tag-dependent activation of SIRT1 by resveratrol, and determined that the Km for the Fluor de Lys p53 substrate was approximately 8.5-fold higher than a p53 peptide lacking the fluorophore (Kaeberlein, McDonagh et al, 2005).




- More recently, Pacholec et al. published an independent study of the Sirtris compounds. By using an HPLC method to separate acetylated and deacetylated products, Pacholec et al. reported that like resveratrol, several Sirtris compounds led to SIRT1 activation in the presence of a peptide substrate covalently linked to a fluorophore, but not with an unlabeled peptide. NMR chemical shift studies (CH3) investigated the perturbation of the acetylated lysine group in peptide substrates with and without a covalently linked fluorophore. A resonance shift was detected when SRT1460 was incubated with a TAMRA-p53 peptide, but not with an unlabeled p53 peptide, thus providing evidence that SRT1460 interacted with the fluorophore.




(*Up*) 1H NMR spectrum of 10 µM of TAMRA Peptide 2 in the presence (*red*) or absence (*blue*) of 50µM SRT1460. *(Down)* 1H NMR spectrum of 10 µM of the Native Peptide 2 in the presence (*red*) or absence (*blue*) of 50 µM SRT1460. *Arrows* indicate the upfield shift of the acetyl (CH3) signal at 1.78 ppm in *A* and *B* (*blue*) upon the addition of 50µM SRT1460 (*red*), whereas the acetyl (CH3) signal at 1.83ppmin *C* showed no shift (*blue* and *red*) upon the addition of 50µM SRT1460. The amino acid sequence of the TAMRA Peptide 2 and the Native Peptide 2 are identical and differ only in the TAMRA group.

Surface plasmon resonance was used to demonstrate an interaction with the fluorophore, as concentration-dependent binding was observed with a TAMRA-containing peptide and not with a native peptide. ITC studies demonstrated that SRT1460 bound to SIRT1 in the presence of the fluorescently labeled peptide. However, SRT1460 did not bind to a SIRT1-unlabeled native p53 peptide complex; this indicates that SRT1460 bound only in the presence of the fluorophore (Pacholec, Bleasdale et al, 2010).

- Dai et al. confirmed the binding of SRT1460 and SRT1720 to TAMRA labeled peptides. However, the authors provide evidence that some compounds identified by high-

throughput screens similar to Milne et al. (Milne, Lambert et al 2007) bind SIRT1 independent of the TAMRA tag, while others appear to increase SIRT1 activity in a substrate- dependent manner (Dai, Kustigian et al, 2010).

- Dai et al. also reported the substrate-dependent activation of SIRT1 with a few small-molecule compounds. These results suggest that SIRT1 activation could be dependent upon the hydrophobicity of residues located at the C-terminal of the acetylated lysine residue (Dai, Kustigian et al, 2010).

The fluorescence-based assays have been shown to be very efficient approaches for screening a large number of compounds. However, as with all highthroughput screening techniques, the results are best interpreted within the limiting context of the physical assay used. Numerous sirtuin assays have been reported, with each measuring different components of the reaction. To directly monitor deacetylation, MS, HPLC(Pacholec, Bleasdale et al. 2010; Borra and Denu, 2003) and microfluidic mobility shift assays (Liu, Gerber et al, 2008) have been described. A continuous microplate assay (Smith, Hallows 2009), [14C]nicotinamide release assay (Kaeberlein, McDonagh et al, 2005; McDonagh, Hixon et al. 2005), nicotinamide exchange assay (Landry, slama et al. 2000; Landry, Sutton et al. 2000), and TLC methods (Borra and Denu 2003; Landry, Sutton et al. 2000; Tanny and Moazed 2001) measure nicotinamide formation. To monitor the production of OAADPr, charcoal binding (Borra and Denu 2003), TLC (Landry, Sutton et al. 2000; Tanny and Moazed 2001), and HPLC assays can be used. These published assays afford a range of techniques capable of validating small-molecule effects.

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