CHARACTERIZATION OF NONSPECIFIC INHIBITION OF THE SIRTUINS BY SUBSTRATE NAD^+ PRECURSORS AND INHIBITORS OF OTHER NAD^+-UTILIZING ENZYMES

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Characterization of nonspecific inhibition of the sirtuins by substrate NAD^+ precursors and inhibitors of other NAD^+-utilizing enzymes

CHARACTERIZATION OF NONSPECIFIC INHIBITION OF THE SIRTUINS BY SUBSTRATE NAD^+ PRECURSORS AND INHIBITORS OF OTHER NAD^+-UTILIZING ENZYMES

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ABSTRACT

The niacin-derived biomolecule NAD+ is not only central to cell energy metabolism, but is also involved in mediating cell signaling via its use as the substrate for poly(ADP-ribose) polymerases (PARPs), ADP-ribosyl cyclases, protein-mono-ADP-ribosyl transferases, and NAD+-dependent protein deacetylases (sirtuins). These NAD+-consuming enzymes perform a number of diverse functions, including maintenance of genomic stability, regulation of senescence, transcriptional control, and other cellular regulation. Because proteins frequently share structurally and functionally similar domains, it is not unexpected that a molecule known to modulate the function of one protein also affects that of another. Such was hypothesized to be the case between the sirtuin family of protein deacetylases and i) enzymes whose activities are tied to NAD+ utilization, particularly the genotoxic stress-attenuating PARPs and PARGs, and ii) particular NAD+ metabolites and salvage pathway precursors. As such, we tested the specificity and potency of a number of established inhibitors of PARPs and PARGs, and NAD+ precursors and metabolites for their abilities to inhibit SIRT-1, -2, and -3, mammalian orthologues of Sir2. Selected PARP inhibitors had no off-target ramifications, but the PARG inhibitor ADP-HPD exhibited mild to potent inhibitory activity depending on the sirtuin tested. NAD+ precursors NAM, NR, and NMN inhibited sirtuins differentially. It was also observed that substituting various NAD+ congeners hindered catalysis, indicating an exquisite requirement for NAD+ for deacetylase activity. Finally, we showed that sirtuin activity dropped off linearly as the assay NAD+:NADH ratio was decreased, suggesting that redox state might regulate sirtuin function. Knowledge of the potential for unintended sirtuin inhibition will have clinical implications in treatment regimens employing modulators of enzymes whose ability is tied to NAD+ utilization, particularly the PARPs.

INTRODUCTION

The importance of the intracellular nucleotide nicotinamide adenine dinucleotide (NAD^+) cannot be overemphasized. Not only is it integral as a redox cofactor in energy metabolism, but it also mediates DNA damage repair via its use as the substrate by the genotoxic stress-attenuating poly(ADP-ribose) polymerases (PARPs), and is involved in other aspects of cell metabolism as the substrate for ADP-ribosyl cyclases, protein-mono-ADP-ribosyl transferases, and the sirtuin family of protein deacetylases. NAD^+ is produced biosynthetically either *de novo* from tryptophan [4], or via salvage pathways

from nicotinamide (NAM) and nicotinic acid (NA) [6,7], whereby the pyridine ring moiety is recovered and recycled via phosphoribosylation followed by adenylylation, or from nicotinamide riboside (NR) [8], which is phosphorylated and then adenylylated to regenerate NAD^+. There is mounting evidence that connects increased intracellular NAD^+ concentration with neuroprotection [9, 10], protection from cell death induced by high-energy compound deprivation [19], and the epigenetic regulation of aging [12]. As such, supplementation with precursors to increase intracellular NAD^+ concentrations represents an attractive therapeutic approach.

Among the many enzyme subfamilies that utilize NAD^+ as a cofactor is the sirtuin family of type-III histone deacetylases (HDACs). NAD^+ and a protein acetylated on lysine are concomitantly bound by sirtuins, resulting in ternary complex formation [21] where the weakly nucleophilic Ɛ-N-acetyl-lysine protein residue is poised to attack the C1 ribose of ADP-ribose, thereby releasing NAM. Intramolecular attack by the 2'-hydroxyl group results in the formation of 2'-O-acetyl-ADP-ribose [5], the physiological role of which has not yet been conclusively established. Such deacetylation of histones has been implicated in a number of processes, including transcriptional silencing, cellular senescence, and DNA recombination [12]. Among varied targets of sirtuin-mediated deacetylation that have been described are p53 [3], PGC-1α [1,2], and nuclear factor-κB [11], suggesting many points of regulation by the activities of sirtuins.

As proteins frequently share similar structural and functional folds due to their divergent evolutionary histories, it should not be surprising if a small-molecule inhibitor/enhancer known to modulate the function of one protein is also observed to affect that of another, or if a metabolite known to be involved in one biochemical process, by merit of structural and/or chemical similarity, exhibits a stimulatory or inhibitory effect on an enzyme in an extraneous process. This was hypothesized to be the case between the sirtuins and i) inhibitors of those enzymes whose activities are intimately tied to intracellular NAD^+ consumption, particularly the genotoxic stress-attenuating PARPs and PARGs, and ii) specific NAD^+ nuclear salvage pathway precursors and other NAD^+ metabolites. Of particular salience are the new-age, high-potency PARP inhibitors. A number of reports have demonstrated the increased antitumor activity afforded by PARP inhibitors in conjunction with ionizing radiation [15], temozolomide [13,14], topoisomerase I inhibition [16], and in BRCA mutant systems [17,18], and so a number of therapeutic regimens are being evaluated clinically.

Accordingly, we assayed inhibitors of PARPs and PARGs as well as NAD^+ metabolites and salvage pathway precursors for their abilities to modulate the deacetylase activity of SIRT-1, -2 and -3, nuclear, cytosolic, and mitochondrial species, respectively. Also, the questions of sirtuin cosubstrate discrimination and catalytic activity as a function of redox state were addressed by employing an array of NAD^+ congeners and varying the NAD^+:NADH assay ratio, respectively.

METHODS

Deacetylase assay: type-III HDAC activity assay kits were purchased from Biomol International and Cyclex MBL

International Corporation. As per Biomol instructions, 25µM fluorogenic, lysine-acetylated peptide patterned after SIRT-1 substrate p53 composed of the sequence $_{N}$RHKK(Ɛ-N-Ac)$_{C}$ was incubated with 250µM NAD^+ and the particular test compound under question, and the reaction was initiated by the addition of recombinant hSIRT. SIRT-2 and -3 required a different peptide sequence, $_{N}$QPKK(Ɛ-N-Ac)$_{C}$, also fashioned from p53. Solutions were made up in prescribed assay buffer consisting of 50mM TrisHCl, pH 8.0, 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, and 1mg/mL BSA. The reaction was allowed to proceed for 1hr at 37ºC, and was quenched with a solution containing 2mM NAM and a proprietary reagent that potentiated fluorophore development.

The MBL/Cyclex assay was set up very similarly, with a fluoro-peptide substrate and sirtuin of proprietary sequence and assay concentration incubated with 250µM NAD^+, the test compound under question, and 0.5mAU/mL lysl endpeptidase, which cleaved the deacetylated peptide substrate at lysine residues concomitantly with the deacetylase reaction. Solutions were made up in an assay buffer consisting of 5mM TrisHCl, pH 8.8, 0.4mM $MgCl_2$, and 50µM DTT. The reaction was carried out at room temperature, and quenched with a proprietary "stop" solution an hour after enzyme was added.

In both protocols, fluorescence intensity was measured at Ex.355 and Em.460 on a Spectramax Gemini XS fluorometer, and was directly proportional to enzyme activity. Data were corrected against the enzyme-minus condition AFU magnitudes, and were expressed in percentage activity relative to the corresponding enzyme-plus control condition, in which the sirtuin was incubated only with NAD^+ and fluorogenic peptide. Per condition, two assays were performed in duplicate, and, when the data converged, the corresponding inter-assay data were normalized and fitted in accordance with a four-parameter logistic model, the equation for which is:

% activity of control = 100/(1+10 ^(logIC50 – log[test compound])*H),

where H denotes the Hill slope, assumed to equal 1.0, and IC_{50} is fixed at the midpoint of the curve.

Data analysis was carried out using GraphPad Prism version 4 software. Validation assays of variable enzyme and NAD^+ concentration, as well as incubation time were performed in order to determine assay parameters that reflected a constant rate of fluorophore formation. Test compounds were serially diluted to 10X the desired assay concentrations in assay buffer before being diluted 1:10 in assay wells. Cosubstrate discrimination studies were carried out by substituting various NAD^+ analogues for NAD^+ at 250µM while holding all other assay conditions constant. Redox state dependence studies were performed by varying the assay concentration of NADH (from 5 to 250µM) while holding NAD^+ concentration constant.

RESULTS

Of the NAD+ precursors and metabolites tested, NAM, NR, and NMN exhibited inhibitory activity.

An array of NAD^+ precursors and metabolites were tested in the deacetylase assay at concentrations ranging from 5 to 500µM for their abilities to inhibit SIRT-1, -2, and/or -3. Not surprisingly, NAM was the

most effective sirtuin inhibitor in its class, with an IC_{50} value for SIRT-1 of 102μM. Interestingly, SIRT-2 and -3 were much more sensitive to NAM inhibition, with IC_{50} values some ten times smaller at 10 and 13μM, respectively. Such inhibition by NAM is thought to be mediated by a transglycosidation reaction between an enzyme-ADP-ribosyl-acetyl-peptide intermediate and NAM whereby NAD^+ is reconstituted, thus driving the reaction in the reverse direction [22]. NA, the acid analogue of NAM, completely failed to inhibit the enzyme at the concentrations tested. At physiological pH, the carboxyl group of NA is expected to be deprotonated. That NA is unable to inhibit the reaction by means of transglycosidation is probably the case because the negative charge contributes to repulsion in the enzyme active site, favoring dissociation. NR differentially inhibited the tested enzymes, but less potently than NAM, with IC_{50} values for SIRT-1, -2, and -3 calculated at 389, 61, and 344μM, respectively. The addition of a ribosyl group presumably contributes to steric strain, and probably explains the reduced inhibitory efficacy relative to that of NAM. NMN inhibited the deacetylase reaction very mildly, with calculated IC_{50} values of 2365, 1128, and 1547μM, respectively. In a similar vein, any reduced inhibitory efficacy of NMN relative to NR is probably due to increased steric resistance experienced on the introduction of the phosphoryl group, and on the attendant energetically unfavorable introduction of negative charge. Finally, NAD^+ metabolites cADP-ribose and ADP-ribose were assayed, and displayed no discernible inhibitory power.

Inhibitors of PARPs and PARGs affected sirtuin activity differentially.

All PARP and PARG inhibitor test compounds were assayed at concentrations ranging from 5 to 500μM in the deacetylase assay except for the high-potency PARP inhibitor PJ-34, which was assayed at concentrations between 10 and 100nM. Classical PARP inhibitors benzamide and 3-amino benzamide are somewhat structurally similar to NAM; while in the former, benzene is substituted for pyridine, the latter contains a benzene scaffold with an exocyclic amine function at the *meta* position relative to the amide group. This structural similarity supported the conjecture that there might be some modulation of sirtuin activity by these PARP inhibitors, but no such inhibitory activity was observed. Thus, the heterocyclic aromatic ring structure might be requisite for inhibition. The tricyclic PARP inhibitor PJ-34 failed to inhibit the sirtuins at nanomolar concentrations. The PARG inhibitor ADP-HPD, a nitrogen-in-the-ring analogue of ADP-ribose, was observed to inhibit each of the sirtuins differentially, with SIRT-3 being the most sensitive to inhibition. IC_{50} values for SIRT-1, -2, and -3 were determined at 466 5485, and 546μM, respectively. Presumably, ADP-HPD was able to share many of the active-site contacts that NAD^+ does when it binds, as it is very similar structurally. Moreover, the positively charged pyrrolidine nitrogen of ADP-HPD presumably confers binding stability by making possible electrostatic interactions with the enzyme. This idea might reasonably account for the lack of similar inhibition by the almost structurally identical ADP-ribose.

Sirtuins could not utilize NAD+ congeners as substrates in the deacetylation reaction.

Independently, NAD^+ congeners NADH, and NADPH, NAAD, and $NADP^+$ each were substituted for NAD^+ at an assay concentration of 250μM. Each of the tested sirtuins was unable to acetylate the ADP-ribose portions of any of the tested NAD^+ analogues in the deacetylation reaction. The

reduced congeners lose both aromaticity and the favorable planar geometry associated with oxidized pyridine groups. These qualities are presumably essential for maintaining stabilizing pi-pi interactions experienced between the oxidized pyridine ring of NAD^+ and active-site residue F35, and for productive van der Waals contacts between the oxidized pyridine ring and N101 and A24 [20]. With respect to the $NADP^+$ and NADPH analogues, the addition of a 2' phosphoryl group, as discussed earlier, probably contributes to both steric bulk and charge repulsion in the active site that favor dissociation. Indeed, crystallographic data indicate that substitution of the 2'-OH hydrogen-bond donor with a phosphoryl group would likely disrupt the active-site hydrogen bonding stabilization observed between NAD^+ and E219 [20]. Finally, it is probable that NaAD, the acid analogue of NAD^+, cannot be utilized because of unfavorable charge introduction that affects active-site coordination. The amide nitrogen of NAD^+ has van der Waals contacts with I32, and also serves as a hydrogen bond-donor to D103 [20], whereas the acid oxygen of NAAD, negatively-charged at this moiety, cannot contribute to either of these stabilizing interactions.

Sirtuin activity is affected by the assay NAD+:NADH ratio.

To determine whether sirtuin activity depends on redox conditions, NADH was added to the assay at concentrations ranging from 5 to 250μM while NAD^+ concentration was kept constant at 250μM. Deacetylase activity for the sirtuins was observed to decrease almost linearly as the assay NAD^+:NADH ratio was decreased. Though NADH cannot be utilized as a substrate, it is probable that it competes with NAD^+ for hSIRT binding given their structural similarity, and given that NADH has previously been shown to competitively inhibit Sir2 [23]. Interestingly, this inhibition was different depending on the sirtuin in question, with SIRT-2 being the most susceptible to changes in the assay NAD^+:NADH ratio. Linear regression analysis yielded Pearson's r-squared values for SIRT-1, -2, and -3, of 0.8316, 0.9146, 0.9455, respectively.

DISCUSSION

Here, we performed a number of experiments designed to investigate the potential modulation of mammalian sirtuin deacetylase activity as a function of incubation with particular NAD^+ salvage pathway precursors and metabolites, established small-molecule inhibitors of members of the PARP and PARG enzyme families, NAD^+ congeners, and the assay NAD^+:NADH ratio.

Of the NAD^+ salvage pathway precursors and metabolites tested, NAM, NR, and NMN inhibited the sirtuins, but in a non-uniform fashion. SIRT-2 and -3 (IC_{50} = 10 and 13μM, respectively) were some ten times more sensitive to NAM inhibition than SIRT-1 (102μM), while SIRT-2 (IC_{50} = 61μM) was some six times more sensitive to NR inhibition than were SIRT-1 and SIRT-3 (IC_{50} = 389 and 344μM, respectively). NMN inhibited the sirtuins at concentrations well outside physiological relevance (IC_{50} = ~1.1 and ~2.4mM). Thus, inhibition by tested amide-containing precursors is a function of molecular weight and charge. NA and potential regulatory NAD^+ feedback effectors cADPr and ADPr displayed no inhibitory activity at the concentrations tested. Such observations imply that efforts to increase intracellular NAD^+ concentrations without adversely affecting integral sirtuin activity by supplementation with NR, NMN, NA or some combination thereof might prove therapeutically useful.

The question of the bioavailability of these niacin derivatives should be addressed at this point. While NA and NR are taken up in the diet, it is not entirely clear whether such a densely charged molecule as NMN can traverse the plasma membrane, or whether it is or can be transported from the extracellular milieu in a type of receptor-mediated manner. In any case, that NMN fails to inhibit the sirtuins in any biologically relevant sense should increase confidence in NR as a therapeutic supplement, because NR is phosphorylated intracellularly to NMN before being adenylylated to produce NAD^+.

The PARG inhibitor ADP-HPD inhibited the sirtuins, but PARP inhibitors benzamide, 3-aminobenzamide, and PJ-34 displayed no inhibitory activity. Thus, given these results, affecting poly(ADP-ribose) pools by modulating PARP activity rather than PARG activity is preferable. On the one hand, these results bode well for PARP inhibitors therapeutically, as these data suggest that there are no off-target effects on the sirtuins by these particular inhibitors of the PARPs. But on the other hand, these results also indicate that off-target inhibition is, in fact, a possibility, and so there is an obvious need to screen more inhibitors of enzymes whose activities are intimately tied to NAD^+ and its consumption so to obviate any nonspecific, deleterious interactions that such dispensation might cause. This is especially true for PARP inhibitors, as they are becoming increasingly clinically relevant.

NAD^+ congeners NaAD, $NADP^+$, NADPH, and NADH were substituted for NAD^+ in the assay and could not be used as cosubstrates by any of the sirtuins tested, presumably because of factors thwarting active-site coordination, likely caused by changes in the molecule related to introduction of charge, steric bulk, and a loss of planar geometry and associated aromaticity on reduction of the pyridine group, or some combination thereof.

To determine whether sirtuin activity might be affected by redox conditions, we varied the assay NAD^+:NADH ratio and measured deacetylase activity. For each of the sirtuins, decreasing the NAD^+:NADH ratio had the effect of linearly decreasing the extent of deacetylation, though the rate of decrease was different depending on the species tested. Specifically, the cytoplasmic SIRT-2 was most sensitive to inhibition by NADH, with activity decreased by ~70% percent when the NAD^+:NADH ratio equaled 1. The activities of SIRT-1 and -3 were maximally decreased at this ratio by ~40 and ~30%, respectively. Thus, NADH acts as an inhibitor of the sirtuins tested. The NAD^+:NADH ratio reflects cellular energy status and presumably signals whether conditions are such that the energy-expensive processes of transcription and translation of genes can be adequately subsidized. Thus, the biological justification for sirtuin inhibition as a function of increased NADH (the decreased NAD^+:NADH ratio) is presumably that the relative abundance of NADH, signaling adequate energy stores, inhibits sirtuin-mediated deacetylation, ultimately increasing gene expression. Similarly, the relative abundance of NAD^+, indicating energy deficiency, favors the deacetylation reaction, and results ultimately in gene silencing. In accordance with this model, previous reports [23,24] have implicated the NAD^+:NADH ratio as the primary factor regulating the deacetylase activity of the hSIRT yeast orthologue Sir2 under conditions of stress such as calorie restriction and exercise, in which the NAD^+ and NADH concentrations vary most markedly from steady-state concentrations. That NAD^+ alone among the nucleotide congeners tested can support the sirtuin-mediated reaction further reflects the

likelihood that intracellular NAD^+ concentration, and by logical extension, intracellular energy state, has a major part in the regulation of sirtuin activity.

Still, consistent with this notion of sirtuin regulation by the NAD^+:NADH ratio are the findings of recent reports suggesting that glutamate dehydrogenase (GDH) and isocitrate dehydrogenase 2 (ICDH2) are *in vitro* targets activated by SIRT-3-mediated deacetylation [25], and also that GDH is an *in vivo* SIRT-3 target [26]. GDH, which deaminates glutamate to α-ketoglutarate, replenishes tricarboxylic acid cycle intermediates, thereby increasing ATP and NADH production. In the third step of the TCA cycle, ICDH2 decarboxylates isocitrate, generating α-ketoglutarate, and also increases ATP and NAD(P)H production. Thus, it is probably the case that deacetylation of these targets represents redox signaling by high NAD^+ content, characteristic of depleted energy stores, ultimately stimulating anapleurotic reactions that increase TCA cycle flux and produce energy-rich compounds.

It is becoming increasingly evident that sirtuin activity is not only integral to cellular function, but also that the sirtuins might potentially represent attractive therapeutic targets. These factors motivated inquiry into consequences on deacetylase activity possibly effected by i) NAD^+ salvage pathway precursor supplementation and ii) PARG and PARP inhibitor administration. Here, we have shown that certain NAD^+ salvage pathway precursors, the PARG inhibitor ADP-HPD, and NADH concentration differentially obstruct hSIRT activity, while other precursors and certain PARP inhibitors exert no effect. This and further characterization of nonspecific/regulatory inhibition of sirtuin activity could prove instrumental therapeutically inasmuch as potent and specific inhibitors of the case-specific NAD^+-utilizing enzymes can be opted for to affect biochemical function more finely.

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FIGURE LEGENDS

Fig. 1. Deacetylase IC_{50} plots generated on incubation with various NAD^+ precursors and metabolites. *a)* NAM *; b)* NA*; c) NR; d)* NMN*; e)*ADPr*; f)* cADPr.
Fig. 2. Deacetylase IC_{50} plots generated on incubation with PARP and PARG inhibitors. *a)* benzamide *; b)* 3-aminobenzamide*; c)* PJ-34*; d)* ADP-HPD.
Fig. 3. Relative deacetylase activies given on co-incubation with NAD^+ congeners. *a)* NAD^+*; b)* $NADP^+$*; c)* NADPH*; d)* NADH*; e)* NaAD.
Fig. 4. Deacetylase activities given on modulation of the assay NAD^+/NADH ratio.

Figure 1: Sirtuin inhibition as a function of precursor/metabolite test compound concentration

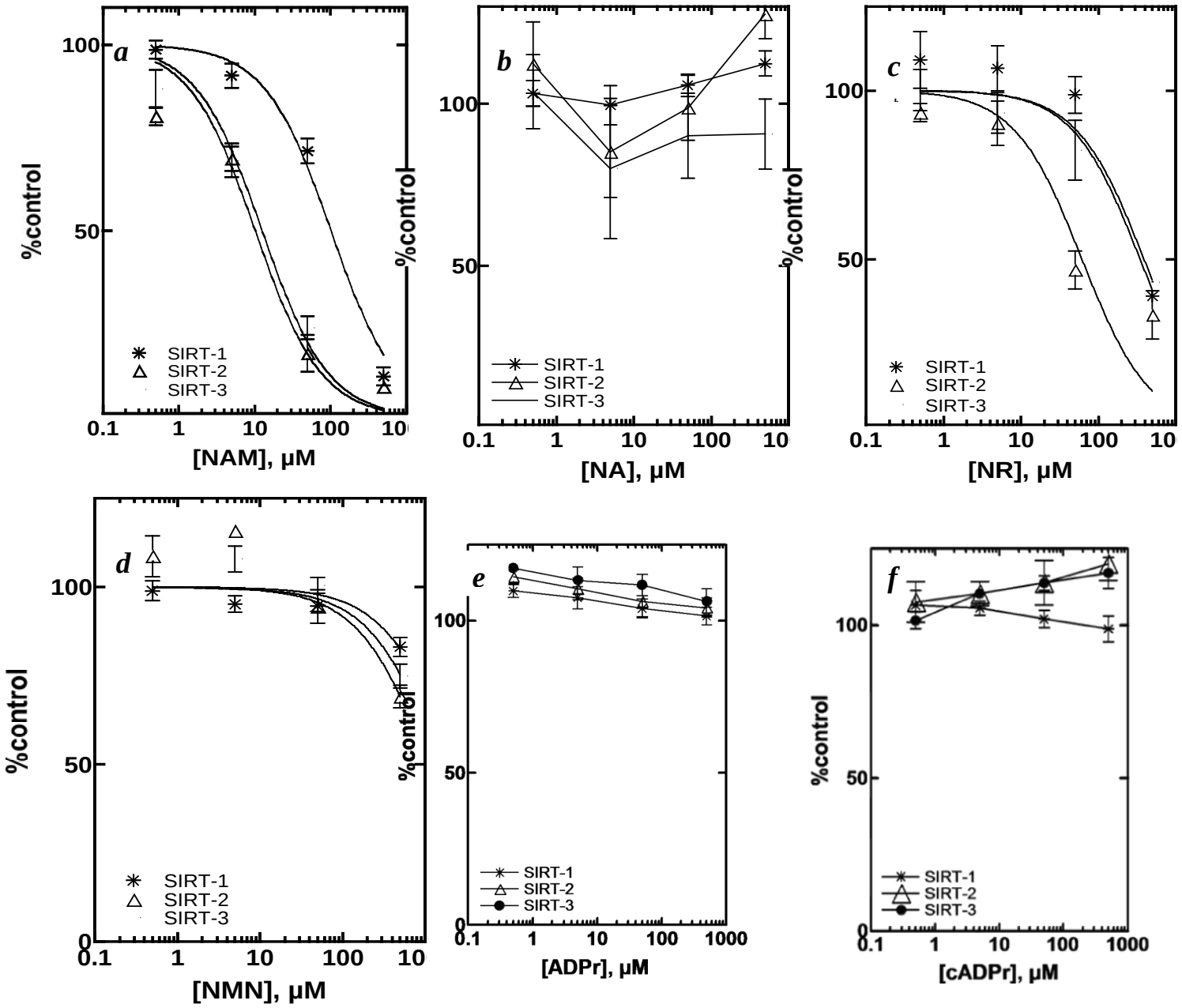

Fig. 2: Sirtuin inhibition as a function of inhibitor test compound concentration

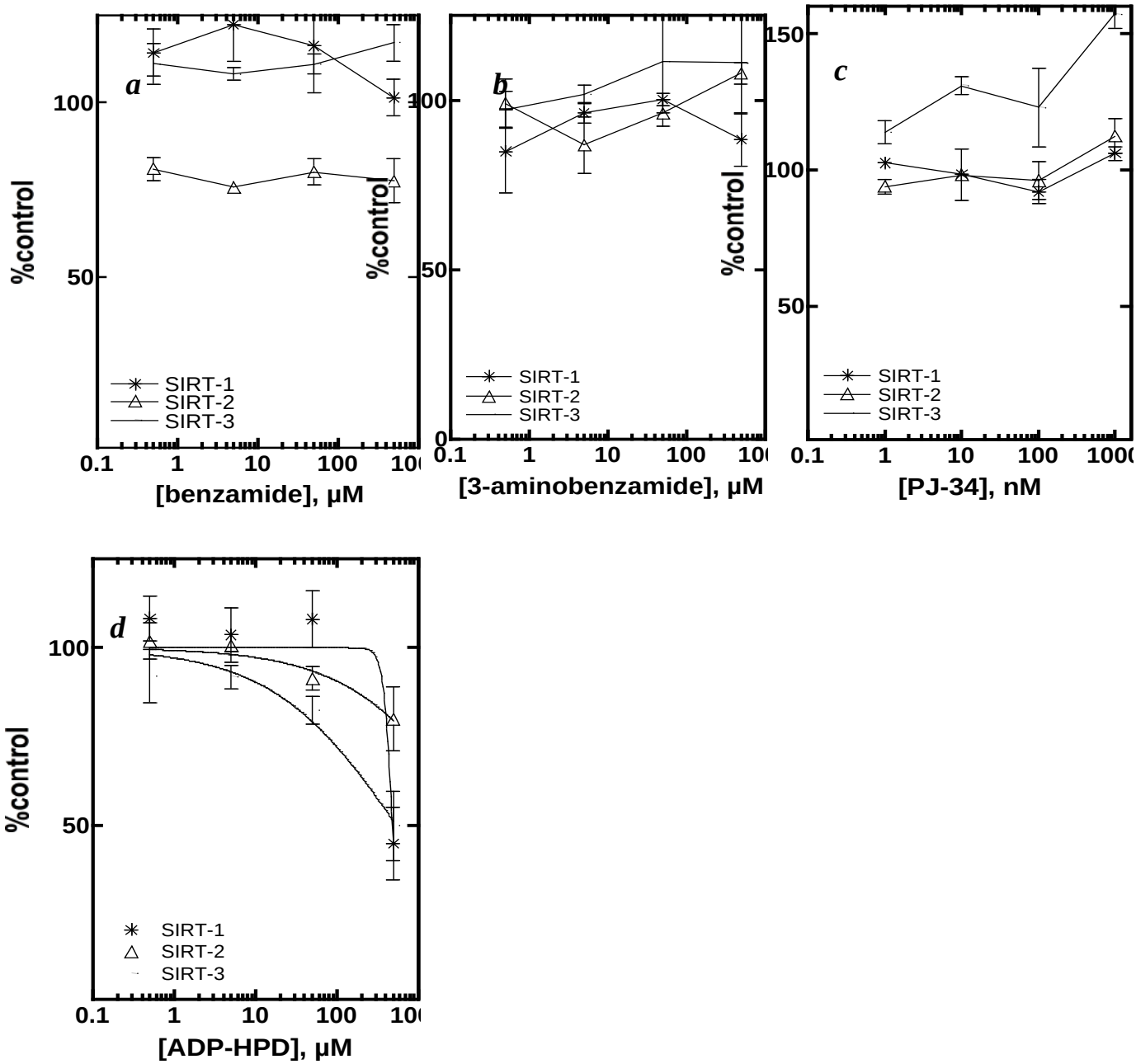

Fig 3: NAD^+ analogue cofactor discrimination.

cosubstrate, 250μM	*% NAD^+ control*		
	SIRT-1	*SIRT-2*	*SIRT-3*
NAD^+ a)	100	100	100
$NADP^+$ b)	>1	>1	>1
NADPH c)	2	1	1
NADH d)	2	2	1
NaAD e)	>1	>1	>1

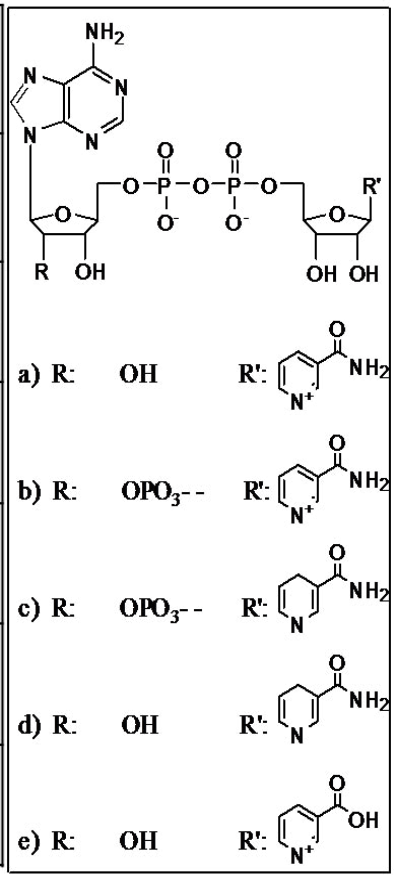

Fig. 4: Sirtuin inhibiion as a function of the NAD^+/NADH assay ratio.