Supporting Information

Dihydronicotinamide riboside is a potent NAD^+ concentration enhancer in vitro and in vivo.

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Running title: *NRH increases NAD^+ concentrations in cells and tissues*

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SI Material and Methods

Stability test of NRH and NR

To assess the stability of NRH and NR in different pH environments, 1 mM NRH or NR was incubated in 150 mM phosphate buffer at pH 6, 7, 8, and 9 for up to 10 hr. At different time points, aliquots were injected onto a C-18 column and HPLC system with 1 mL/min elution using 20 mM ammonium acetate as an eluent and multiwavelength detection. The 340 nm chromatogram peaks for NRH and 260 nm chromatogram peaks for NR were integrated and used to obtain quantification of the respective compounds. Plots of fraction compound area versus the initial time area as a function of time were used to assess compound stability. To test the stability of NRH and NR in cell media, 1 mM NRH or NR in media were incubated with or without confluent HEK293 cells for up to 24 hr. 50 µl of media was collected every 2 hr and injected onto a C-18 column and HPLC system for quantification as above. Plots of fraction compound area versus the initial time area as a function of time were used to assess compound stability.

NRH effect on NAD^+ cycling assay

To determine if the presence of NRH can generate a non-specific effect on the NAD^+ cycling assay used to quantitate NAD^+ concentrations, 1 µM NRH was added to all concentrations of NAD^+ standards in the assay performed as reported (1). To further control for the processing conditions, NRH was treated with 7% perchloric acid then neutralized with NaOH and K_2HPO_4. 1 µM of acid-treated NRH was also added to all NAD^+ standards for comparison.

NMN effects on NAD concentrations in cells.

F98, HEK293, Neuro2a and C2C12 cells in 6 well plates were treated in media containing 1 mM NMN or vehicle only for 6 hr and the cellular NAD^+ levels determined as reported (1).

Apoptosis test with flow cytometry

To test if NRH has cytotoxicity, 1 mM NRH was incubated with HEK293 and Neuro2a cells for 24 hr, and then cells were treated with trypsin to detach for cell counting. Cells were stained with trypan blue and counted with a hemocytometer. To examine for apoptosis in HEK293 cells, PBS, 1 mM NRH or NR was added to cells in 6 well plates treated for 24 hr. We used Annexin V/PI staining kit and Caspases3/7/SYTOX dead cell staining kit (Life Technologies) and counted cells

using a BD FACSCelesta™ flow cytometer. Quadrants for apoptotic cells, necrotic cells and live cells were identified and quantified by the manufacturer instructions.

Lactate level determination

Intracellular lactate levels were measured in the cells treated with 1 mM NRH after 6 hr using the L-Lactate Assay Kit from Cayman Chemical according to manufacturer's protocol. Cell number was counted with hemocytometer to normalize lactate amount to cell number. The used media were also collected to determine extracellular lactate concentrations.

^{1}H NMR and ^{13}C NMR data for NRH

In agreement with prior report for ^{1}H NMR(2).

^{1}H NMR (CD_3OD, 500 MHz): δ 7.08 (s, 1H, **H-2**), 6.02 (d, 1H, J = 7.1 Hz, **H-6**), 4.94-4.89 (m, 1H, **H-1'**), 4.80 (d, 1H, *J* = 7.1 Hz, **H-5**), 4.12 (t, 1H, J = 6.3 Hz, **H-3'**), 4.06 (m, 1H, **H-4'**), 3.90-3.87 (m, 1H, **H-2'**), 3.69-3.58 (m, 2H, **H-5'**), 2.99 (s, 2H, **H-4a**, **H-4b**). ^{13}C NMR (CD_3OD, 125 MHz): 137.8, 125.2, 105.2, 101.1, 94.9, 83.5, 70.9, 70.1, 61.5, 22.0

References:

1. Li, W., and Sauve, A. A. (2015) NAD(+) content and its role in mitochondria. *Methods in molecular biology* **1241**, 39-48
2. Oppenheimer, N. J., and Kaplan, N. O. (1976) Proton magnetic resonance study of the intramolecular association and conformation of the alpha and beta pyridine mononucleotides and nucleosides. *Biochemistry* **15**, 3981-3989

SI Figure Legends

Figure S1: Characterization of NRH. (a) Spectrum of NRH absorbance. (b) Linear curve of NRH concentration versus absorbance at 340 nm.

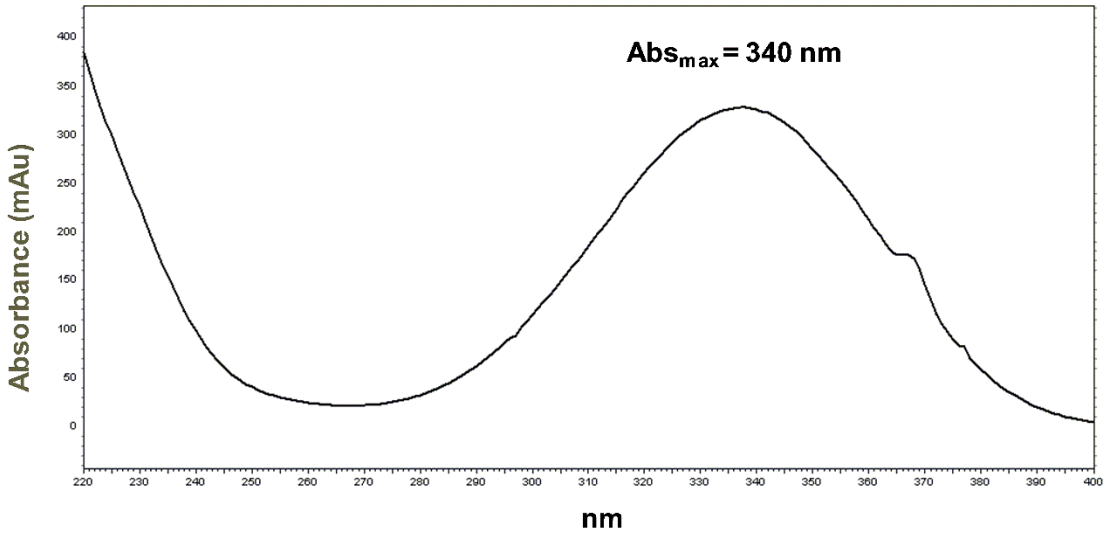
Figure S2. Stability of NRH and NR in different pH buffers and in cell media. NRH and NR were incubated in 150 mM phosphate buffers at pH 6, pH 7, pH 8 and pH 9 for up to 10 hr at room temperature. The amount of (a) NRH and (b) NR left was measured by HPLC and presented as the amount relative to amount at time 0. Also, (c) 1 mM NRH or (d) 1 mM NR were incubated with or without HEK293 cells in cell culture media at 37^{o}C for up to 24 hr. The media was collected at different times and injected onto HPLC to determine the amount of NRH and NR remaining.

Figure S3. NMN treatments mildly increase NAD^+ levels. F98, HEK293, Neuro2a and C2C12 were treated with 1 mM NMN for 6 hr and the cellular NAD^+ levels were checked. Data are expressed as mean ± SEM, n=4-6, *, $p<0.05$.

Figure S4. NRH treatment does not induce an apoptotic response in cells. (a) HEK293 and Neuro2a cells were incubated with 1 mM NRH for 24 hr, then the total cell numbers were determined. Data are expressed as mean ± SEM, n=6. *, $p<0.05$. For assay of apoptotic markers, we stained treated HEK293 cells for (b) Annexin V or (c) Caspase 3/7 and counted cells using a flow cytometer. Data are expressed as mean ± SEM, n=3. Quadrants with live cells, necrotic cells and apoptotic cells were quantified as described in methods.

Figure S5. Lactate concentrations with and without NRH treatment. (a) Intracellular and (b) extracellular lactate concentrations were measured in HEK293, Neuro2a, INS1 and C2C12 cells that were treated with or without 1 mM NRH for 6 hr as described in methods. Data are expressed as mean ± SEM, n=4, ***, $p<0.001$.

a.



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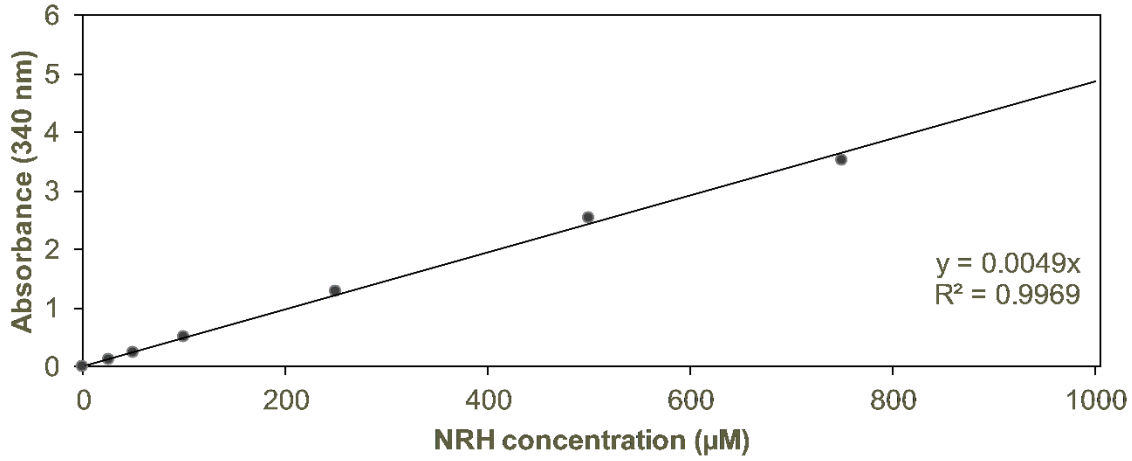
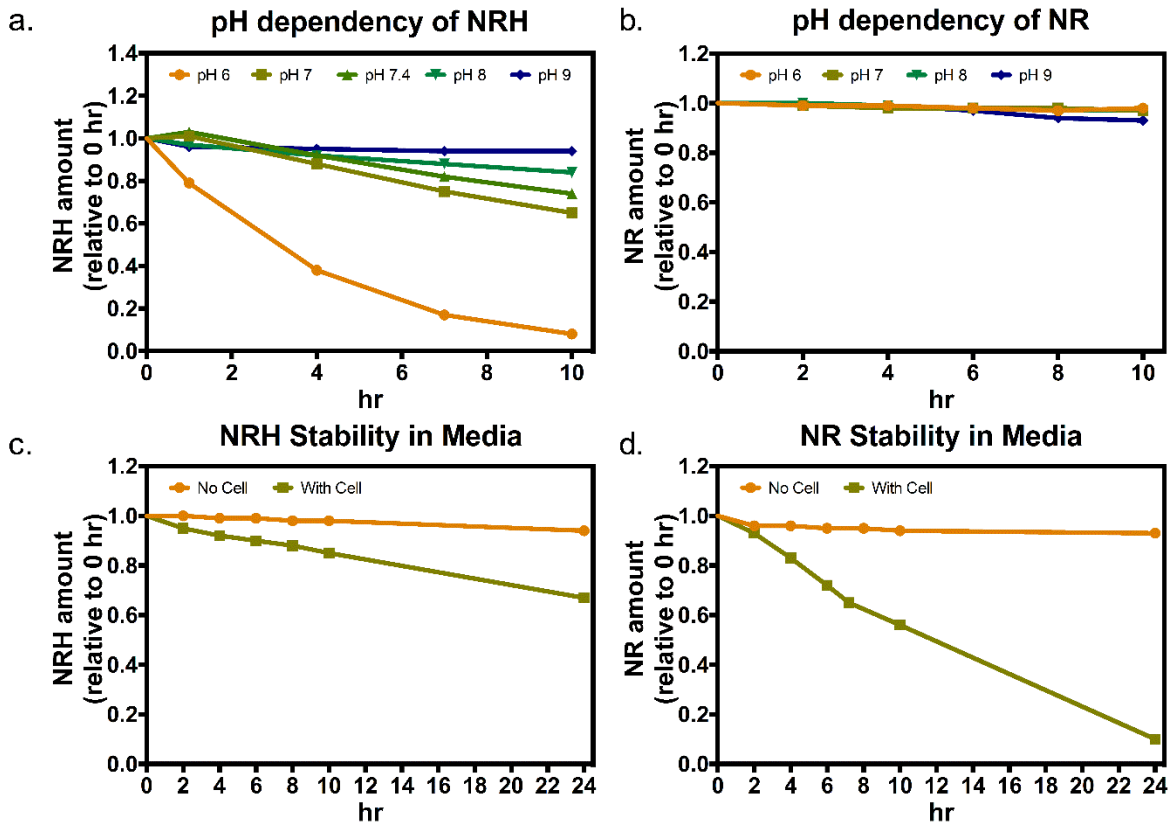


Yang et al.
S. Figure 1



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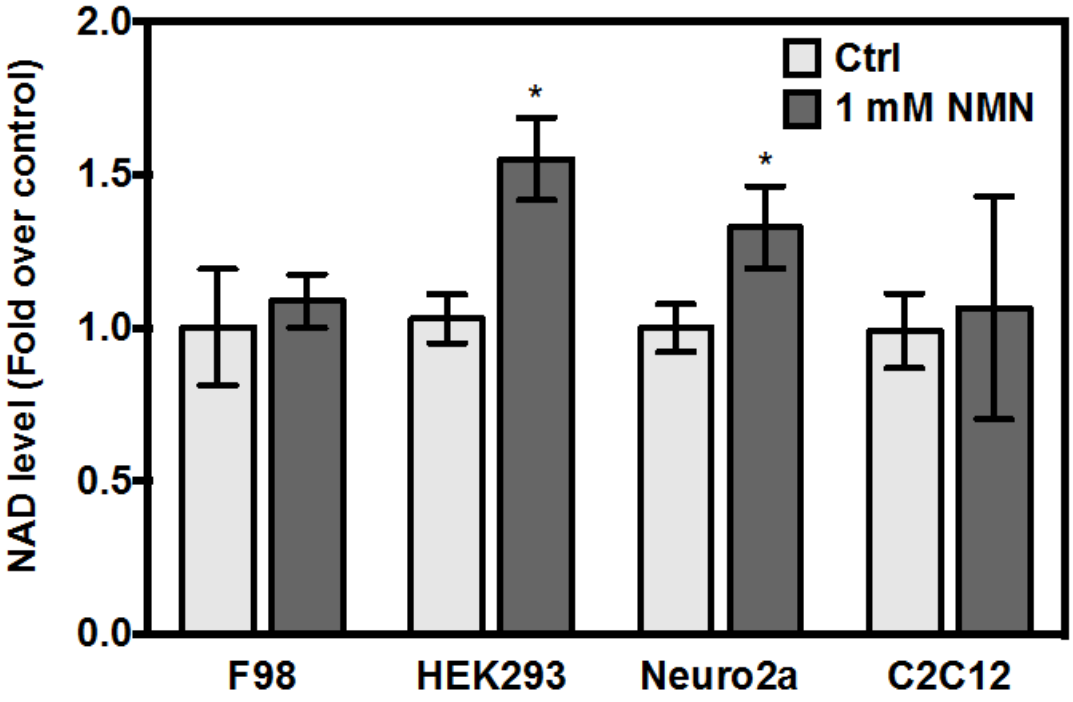
S. Figure 2



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S. Figure 3

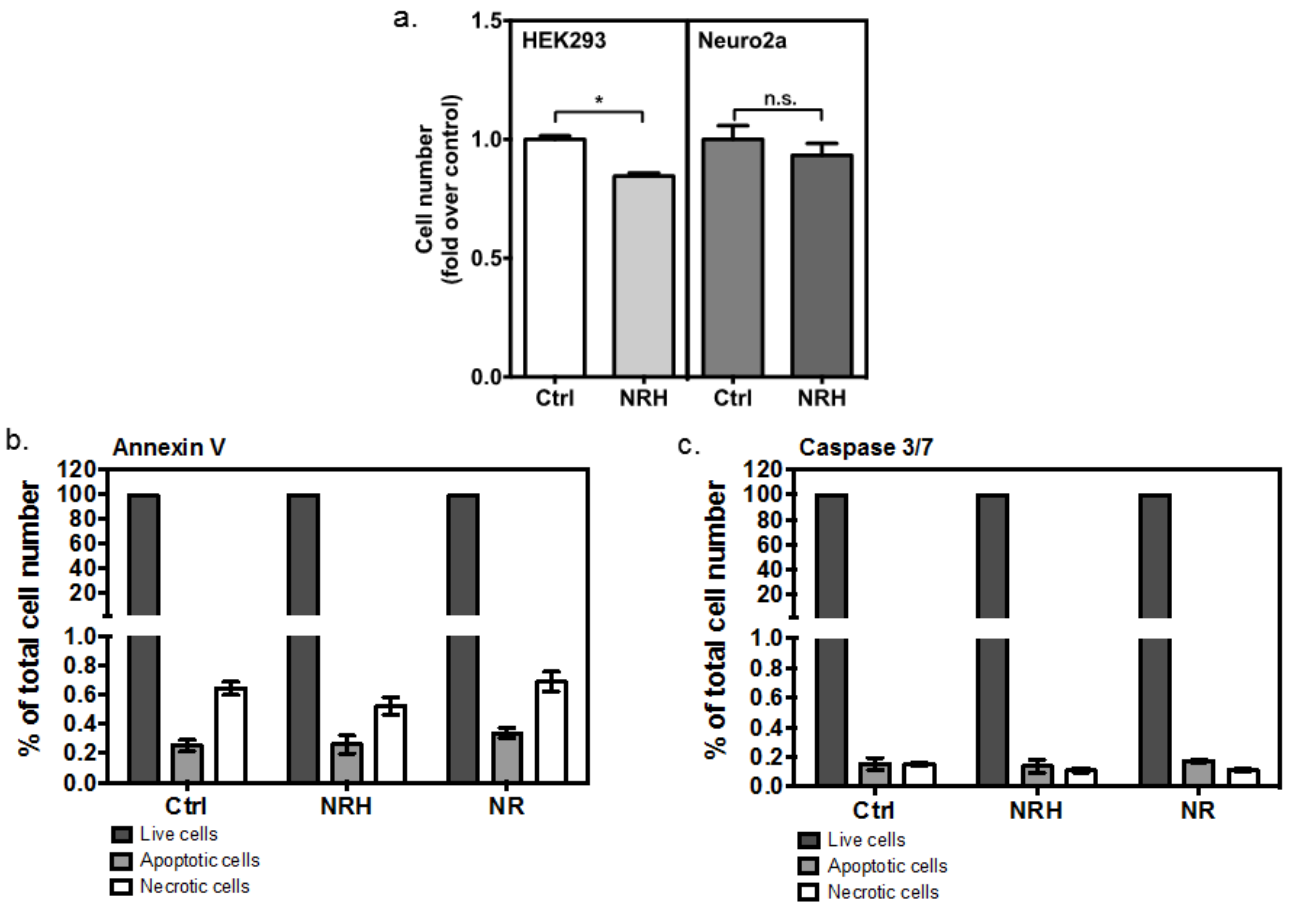

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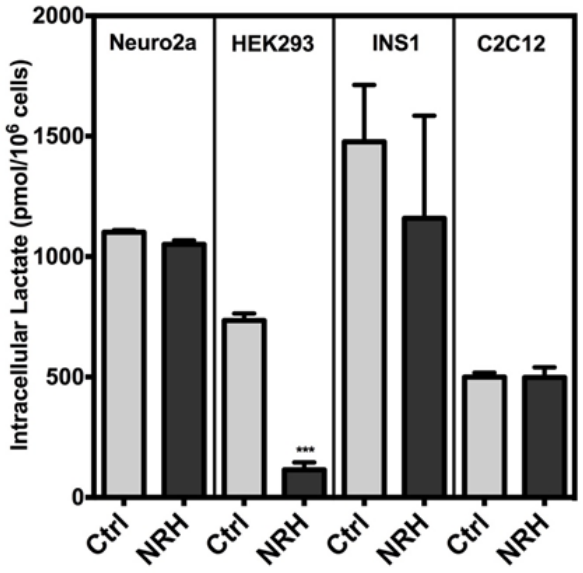
Figure S4

a.



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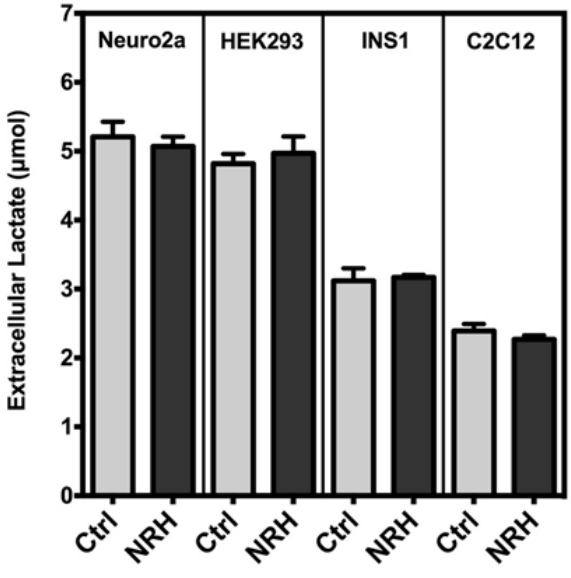

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Figure S5