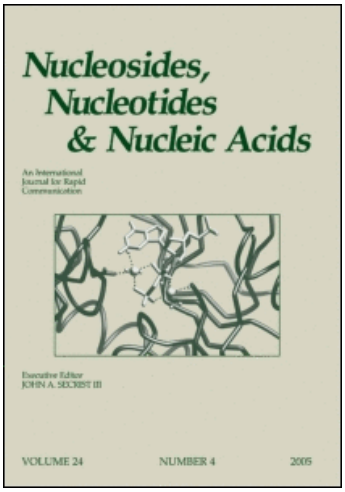

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[a] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas, USA [b] Department of Molecular Biosciences, The University of Kansas, Lawrence, Kansas, USA [c] Applied Bioinformatics Laboratory, The University of Kansas, Lawrence, Kansas, USA [d] Department of Chemistry, The University of Kansas, Lawrence, Kansas, USA

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EVALUATION OF NAD(H) ANALOGUES AS SELECTIVE INHIBITORS FOR *TRYPANOSOMA CRUZI* S-ADENOSYLHOMOCYSTEINE HYDROLASE

Qing-Shan Li,[1] Sumin Cai,[2] Jianwen Fang,[3] Ronald T. Borchardt,[1] Krzysztof Kuczera,[2,4] C. Russell Middaugh,[1] and Richard L. Schowen[1,2,4]

[1]*Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas, USA*
[2]*Department of Molecular Biosciences, The University of Kansas, Lawrence, Kansas, USA*
[3]*Applied Bioinformatics Laboratory, The University of Kansas, Lawrence, Kansas, USA*
[4]*Department of Chemistry, The University of Kansas, Lawrence, Kansas, USA*

□ *S-Adenosylhomocysteine (AdoHcy) hydrolases (SAHHs) from human sources (Hs-SAHHs) bind the cofactor NAD^+ more tightly than several parasitic SAHHs by around 1000-fold. This property suggests the cofactor binding site of this essential enzyme as a potential anti-parasitic drug target, e.g., against SAHH from* Trypansoma cruzi *(Tc-SAHH). The on-rate and off-rate constants and the equilibrium dissociation constants were determined for NAD^+/NADH analogues and suggested that NADH analogues were the most promising for selective inhibition of Tc-SAHH. None significantly inhibited Hs-SAHH while S-NADH and H-NADH (see Figure 1) reduced the catalytic activity of Tc-SAHH to $< 10\%$ in six minutes of exposure.*

Keywords S-adenosylhomocysteine hydrolase; enzyme inhibitors; anti-parasitic drugs; cofactor-site targeting

INTRODUCTION

During his long and productive career in chemistry, biochemistry, and medicinal chemistry, Professor Morris J. Robins has created novel nucleosides and other structures, often with fascinating properties, particularly in

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This paper is dedicated to Professor Morris J. Robins, in honor of and in celebration of his 70th birthday.
Address correspondence to R. L. Schowen, Department of Pharmaceutical Chemistry, 2095 Constant Avenue, The University of Kansas, Lawrence, KS, 66047, USA. E-mail: rschowen@ku.edu

their interactions with enzymes. It is our ambition in this paper to report brief studies in which we attempt to follow in his footsteps.

The parasites *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*, the agents of Chagas disease, Leishmaniasis, and malaria, respectively, infect tens of millions in the less-developed world. Effective, safe, and cheap anti-parasitic medicines are acutely required. S-adenosyl-L-homocysteine hydrolase[1] SAHH, EC 3.3.1.1), which catalyzes the reversible conversion of AdoHcy to adenosine (Ado) and homocysteine (Hcy), has emerged as a potential molecular target for design of anti-parasitic drugs.[2–4] In general, SAHH inhibition raises the cellular concentration of AdoHcy, a potent product inhibitor of all S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases. The resulting inhibition of AdoMet-dependent methylation reactions[1] produces toxic effects. Parasites express their own SAHHs[2–4] and anti-parasitic effects of known SAHH inhibitors are observed in vitro and in vivo for a variety of parasites including *L. donovani,*[5] *P. falciparum,*[6,7] and *Trypanosoma* species.[8,9] Obviously, only inhibitors specific for the parasitic enzymes have the potential for medical use.

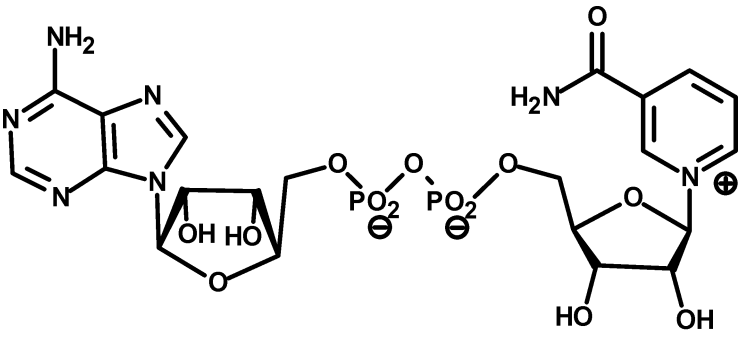
X-ray crystallographic structures for Hs-SAHH,[10,11] Pf-SAHH (from *P. falciparum,*[12]) and Tc-SAHH (from *T. cruzi,*[13]) are available. All these enzymes are highly conserved homotetrameric proteins[12,14,15] with one NAD^+ molecule bound into the active site of each subunit. All residues that interact directly with substrate or cofactor are conserved among these three enzymes. Such observations and other data[16] suggest that selective inhibition of parasitic enzymes poses a difficult challenge.

However, it has been reported that Ld-SAHH (from *L. donovani*) and Tc-SAHH bind NAD^+ much less tightly than Hs-SAHH,[17] although these parasitic SAHHs bind the reduced form NADH very tightly and persist in the inactive form containing reduced cofactor.[17] Therefore, the possibility exists to develop highly selective inhibitors for parasitic SAHHs through designing NAD^+ analogues that bind to the cofactor binding site. In this study, we have investigated NAD^+ analogues obtained by modification of the nicotinamide group and analogues obtained by modification of adenine part (Figure 1) and compared their inhibitory effects on Hs- and Tc-SAHHs, to provide information for eventual development of anti-parasitic drugs.

MATERIALS AND METHODS

NAD(H) Analogues (Figure 1)

Thionicotinamide adenine dinucleotide (S-NAD), 3-pyridinealdehyde adenine dinucleotide (H-NAD), 3-acetylpyridine adenine dinucleotide (C-NAD) and its reduced form (C-NADH), nicotinic acid adenine dinucleotide (O-NAD), nicotinamide hypoxanthine dinucleotide (NHD) and its reduced



NAD^+

Name	Structure
Left: Thionicotinamide adenine dinucleotide (S-NAD) Right: 3-Pyridinealdehyde adenine dinucleotide (H-NAD)	
Left: Nicotinic acid adenine dinucleotide (O-NAD) Right: 3-Acetylpyridine adenine dinucleotide (C-NAD)	
Left: Nicotinamide hypoxanthine dinucleotide (NHD) Right: Nicotinamide guanine dinucleotide (NGD)	
Nicotinamide 1, N^6-ethenoadenine dinucleotide (etheno-NAD)	

FIGURE 1 Structures of NAD^+ and its analogues.

form (NHDH), nicotinamide guanine dinucleotide (NGD), and nicotinamide 1, N^6-ethenoadenine dinucleotide (etheno-NAD) were bought from Sigma (St. Louis, MO, USA). The reduced forms of thionicotinamide adenine dinucleotide (S-NADH) and 3-pyridinealdehyde adenine dinucleotide (H-NADH) were prepared from the oxidized forms and ethanol with catalysis by alcohol dehydrogenase (Sigma, A-3263) as follows. A solution (usually 4 ml) containing 80 U/mL alcohol dehydrogenase, 40 mM ethanol, 4 mM of the oxidized analogue and 1 mM EDTA in 50 mM phosphate buffer, pH 8.4, was incubated at 25°C for 30 minutes and then filtered on a Centricon column (30k, Millipore, Billerica, MA, USA) to remove alcohol dehydrogenase. Reduction of the oxidized forms of H-NAD and S-NAD was analyzed by HPLC by use of the procedure for enzyme-activity assay described below. H-NAD was completely reduced, and S-NAD was 90% reduced. Purity was in general determined by HPLC.

Expression and Purification of Hs-SAHH and Tc-SAHH

The expression and purification of Hs-SAHH and Tc-SAHH were the same as previously described.[16–18]

Preparation of apo Forms of Hs-SAHH and Tc-SAHH

The apo forms of Hs-SAHH and Tc-SAHHs were prepared by the same method as previously described.[17,18]

Enzyme Activity Assay

SAHH activity was assayed in the synthetic direction by measuring the rate of formation of AdoHcy from Ado and Hcy using HPLC as previously described.[19,20] The enzyme activity in the hydrolytic direction was determined by coupling the AdoHcy hydrolysis reaction to Ado deamination catalyzed by Ado deaminase as previously described.[20]

Determination of the Degree of Occupancy of Enzymes Reconstituted with NAD(H) Analogues

A solution (1 mL) containing 50 μM apo enzyme (all enzyme concentrations used are subunit concentrations), 500 μM of an NAD(H) analogue, 0.4 M $(NH_4)_2SO_4$ and 1 mM EDTA in 50 mM phosphate buffer, pH 7.4, was incubated for 5 hours at 22°C. The free analogue that remained was then removed by passage through a PD10 column (GE Healthcare, Uppsala, Sweden) which had been equilibrated with 50 mM phosphate buffer at 4°C. The enzyme-analogue complex was further concentrated by Centricon treatment (30K, Millipore) at 4°C and the filtrate was collected

to determine the amount of analogue bound. The concentrated solution of enzyme-analogue complex was mixed with 2 volumes of ethanol, followed by centrifugation. The precipitated enzyme was re-dissolved and treated with 2 volumes of ethanol again as above. The supernatants were combined and dried in vacuum. The residue was then dissolved in water and applied to the HPLC column using the same procedure as for the enzyme-activity assay. The concentration of each compound was determined by comparison with a calibration curve obtained from authentic samples.

Determination of the Rate Constant k_{on} for Binding to apo Enzyme of NAD^+, NADH and NAD Analogues

Typical procedures for determination of the rate constant for binding of NAD^+, NGD, NHD, and etheno-NAD to Hs-SAHH and Tc-SAHH are as follows.[17,18] One μM of Hs-SAHH apo enzyme was mixed with 5 μM NAD^+ or 100 μM NAD^+ analogue and the resulting solution was pre-incubated at 37°C for 5 minutes. For binding to the Tc-SAHH apo enzyme of NAD^+ and its analogues, the procedure was the same except that the concentrations of NAD^+ or its analogues were 25 μM and 500 μM, respectively. Samples were taken for activity assay after selected time periods. The measured activities A were either converted to the amount of enzyme:NAD^+ complex or enzyme:analogue complex or used directly in Equation (1).

$$A = A_f + (A_o - A_f)[\exp(-k_{app}t)] \quad (1)$$

Here, A is the activity measured at time t, A_o is the activity at the apparent time zero (reflecting reaction during the dead-time of the experiment), A_f is the activity at the end of the experiment, and k_{app} is a first-order rate constant for association that generally will be a function of [NAD^+] or [NAD^+ analogue]. Under the above measurement conditions, A_o is quite small and k_{app} is approximately proportional to [NAD^+] or [NAD^+ analogue]. An apparent second-order rate constant k_{on} for the association reaction was therefore calculated as $k_{on} = k_{app}/[NAD^+]$ or $k_{on} = k_{app}/[NAD^+$ analogue] for the analogue and k_{on} was used in the calculation of the equilibrium dissociation constant.

For measurement of k_{on} for NADH, the bound NADH was monitored by fluorescence as previously described.[19]

Determination of the Rate Constants k_{off} for Dissociation of NAD^+ and NAD(H) Analogues from Their Complexes with Hs-SAHH and Tc-SAHH

Determination of k_{off} for the dissociation of NAD^+, NGD, NHD, and etheno-NAD from complexes with either Hs-SAHH or Tc-SAHH was carried out as follows. A solution (usually 4 ml) containing 4 μM SAHH, 80 U/ml

alcohol dehydrogenase, 30 mM 2-propanol, 2 mM NADH, and 1 mM EDTA in 50 mM phosphate buffer, pH7.4, was incubated at a chosen temperature. Samples were taken after selected time periods for determination of enzyme activity and NADH content. NADH concentration did not change during all these experimental periods. As a control, the stability of SAHH was also determined under the same conditions as above except in the absence of NADH and alcohol dehydrogenase. The enzyme activities relative to initial activity were plotted against time and fitted to $A/Ao = \exp(-kt)$, where A/Ao is the relative activity and k is the enzyme inactivation rate constant. Enzyme thermal inactivation rate constants were obtained by the same method. The dissociation rate constants for NAD^+ and NAD(H) analogues were obtained by subtracting the enzyme thermal inactivation rate constants from the value of k. For determination of k_{off} for NADH analogues, the enzyme:(NADH analogue) complex was mixed with 1 mM of NAD^+ and the enzyme-activity recovery rate indicated the rate of the analogue dissociation.

Dissociation Constant (K_d)

Equilibrium dissociation constants of NAD^+, NGD, NHD, and etheno-NAD were calculated from the relationship $K_d = k_{off}/k_{on}$. Dissociation constants of NADH, NADH analogues, S-NAD, H-NAD, O-NAD and C-NAD were calculated by fitting (Microcal Origin 7.0 software) the enzyme activity as a function of ligand concentration in the presence of NAD^+ to the following equation, previously derived.[17]

$$\text{Activity}_0/\text{Activity} = 1 + ([\text{Ligand}]/K_d^{\text{Ligand}})(K_d^{\text{NAD}}/(K_d^{\text{NAD}} + [NAD^+]))$$

"Activity_0" refers to the enzyme activity in the absence of ligands other than NAD^+ and $K_d{}^{NAD}$ is the known dissociation constant of NAD^+.

Inhibition of Tc-SAHH and Hs-SAHH by NAD(H) Analogues

The inhibition of Hs-SAHH and Tc-SAHH activity by the NAD(H) analogues was investigated by incubation of 20 μM of each analogue with Tc-SAHH and Hs-SAHH in 50 mM phosphate buffer, pH 7.4, containing 50 μM NAD^+ at 37°C for 6 minutes (refer to Table 3). 50 μM NAD^+ was included because the concentration of NAD^+ inside cells was estimated to be in this general range.[21]

RESULTS

Table 1 reports that NAD^+ analogues can be reconstituted into the active sites of both Hs-SAHH and Tc-SAHH with 82–98% of the expected unit

TABLE 1 Fraction of occupancy and cofactor activities of NAD^+ analogues as ligands of Hs-SAHH and Tc-SAHH[a]

	Hs-SAHH		Tc-SAHH	
Compound	Amount bound (mol/mol subunit)	Relative activity (%)	Amount bound (mol/mol subunit)	Relative activity (%)
NAD^+	0.98 ± 0.04	100	0.96 ± 0.05	100
Modifications in the carboxamide side-chain of the nicotinamide ring				
S-NAD	0.91 ± 0.06	1.4 ± 1.1	0.93 ± 0.06	1.3 ± 0.7
H-NAD	0.94 ± 0.08	0	0.98 ± 0.04	0
O-NAD	0.87 ± 0.09	0	0.83 ± 0.08	0
C-NAD	0.88 ± 0.09	21.4 ± 5.2	0.85 ± 0.08	20.5 ± 3.7
Modifications in the adenine ring				
NGD	0.84 ± 0.08	46.5 ± 6.8	0.73 ± 0.09	58.5 ± 7.2
Etheno-NAD	0.89 ± 0.07	69.0 ± 5.3	0.88 ± 0.09	80.6 ± 6.4
NHD^2	0.84 ± 0.07	38.6 ± 6.1	0.82 ± 0.08	35.2 ± 4.2

[a]Errors calculated from three independent measurements.

occupation of one active site in each of the four monomeric subunits of the homotetramer. Modifications of the adenine ring lead to occupancies in the lower range and generate catalytic activities at about 40–70% of that for NAD^+. Modifications of the nicotinamide lead to occupancies in the higher range and catalytic activities at 0–20% of that for NAD^+.

Table 2 reports values of k_{on}, the second-order rate constant for association of a ligand with Hs-SAHH or Tc-SAHH, k_{off}, the first-order rate constant for dissociation of a ligand from its complex with the enzyme, and K_d, the equilibrium thermodynamic dissociation constant of a ligand from its complex with the enzyme. The values of k_{on} range from less than 10^2 to over 10^4 $M^{-1}s^{-1}$, the values of k_{off} from about 10^{-5} s^{-1} to nearly $10^{-1}s^{-1}$, and the K_d values from near-nanomolar to millimolar.

Table 3 identifies five compounds that exhibit some selectivity in trapping the cofactor sites of Tc-SAHH in preference to those of Hs-SAHH during a 6-minutes incubation period in the face of a 2.5-fold excess of NAD^+.

DISCUSSION

Occupancy and Catalytic Activity of NAD^+ Analogues with Hs-SAHH and Tc-SAHH

The data in Table 1 show that NAD^+ analogs complex extensively with Hs-SAHH or Tc-SAHH, the numerical values of occupancy being 4 equivalents of ligand per equivalent of homotetramer, thus being equal to the expectation for cofactor-site binding. The fact that five of the seven analogs produce measurable catalytic activity also is consistent with occupancy of

TABLE 2 Rate and equilibrium constants of the association (k_{on}) with apo-Hs-SAHH and apo-Tc-SAHH and the dissociation (k_{off}, K_d) from the holo-enzymes of NAD(H) analogues at 37°C

	Hs-SAHH			Tc-SAHH		
	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (M)
NAD^+	1200 ± 200	$(40 \pm 5) \times 10^{-6}$	$(31 \pm 3) \times 10^{-9}$	250 ± 10	$(47 \pm 1) \times 10^{-4}$	$(19 \pm 2) \times 10^{-6}$
NAD^+: Modifications in the carboxamide side-chain of the nicotinamide ring						
S-NAD	7900 ± 200	$(310 \pm 30) \times 10^{-6}$	$(39 \pm 1) \times 10^{-9}$	405 ± 34	$(62 \pm 3) \times 10^{-4}$	$(15 \pm 1) \times 10^{-6}$
H-NAD	11400 ± 700	$(480 \pm 20) \times 10^{-6}$	$(42 \pm 2) \times 10^{-9}$	153 ± 17	$(23 \pm 2) \times 10^{-4}$	$(15 \pm 1) \times 10^{-6}$
C-NAD	3000 ± 200	$(510 \pm 10) \times 10^{-6}$	$(170 \pm 10) \times 10^{-9}$	33 ± 4	$(200 \pm 20) \times 10^{-4}$	$(610 \pm 50) \times 10^{-6}$
O-NAD	580 ± 50	$(760 \pm 40) \times 10^{-6}$	$(1300 \pm 100) \times 10^{-9}$	36 ± 5	$(260 \pm 30) \times 10^{-4}$	$(720 \pm 60) \times 10^{-6}$
NAD^+: Modifications in the adenine ring						
NGD	34 ± 3	$(25 \pm 2) \times 10^{-3}$	$(73 \pm 9) \times 10^{-5}$	24 ± 1	$(78 \pm 1) \times 10^{-3}$	$(3.2 \pm 0.1) \times 10^{-3}$
NHD	29 ± 2	$(15 \pm 1) \times 10^{-3}$	$(50 \pm 5) \times 10^{-5}$	30 ± 1	$(63 \pm 5) \times 10^{-3}$	$(2.1 \pm 0.2) \times 10^{-3}$
Etheno-NAD	41 ± 4	$(17 \pm 1) \times 10^{-3}$	$(40 \pm 5) \times 10^{-5}$	20 ± 1	$(72 \pm 4) \times 10^{-3}$	$(3.6 \pm 0.3) \times 10^{-3}$
NADH analogues						
NADH	2000 ± 100	$(10 \pm 1) \times 10^{-6}$	$(5 \pm 1) \times 10^{-9}$	1430 ± 90	$(2.5 \pm 0.3) \times 10^{-4}$	$(0.17 \pm 0.1) \times 10^{-6}$
S-NADH	7200 ± 1300	$(28 \pm 5) \times 10^{-5}$	$(37 \pm 1) \times 10^{-9}$	470 ± 96	$(3 \pm 0.3) \times 10^{-4}$	$(0.6 \pm 0.1) \times 10^{-6}$
H-NADH	11000 ± 1700	$(44 \pm 6) \times 10^{-5}$	$(40 \pm 2) \times 10^{-9}$	191 ± 28	$2.1 \pm 0.2 \times 10^{-4}$	$(1.1 \pm 0.1) \times 10^{-6}$
C-NADH	3200 ± 500	$(32 \pm 4) \times 10^{-5}$	$(10 \pm 1) \times 10^{-8}$	46 ± 8	$4.1 \pm 0.3 \times 10^{-4}$	$(8.8 \pm 0.3) \times 10^{-6}$
NHDH	26 ± 8	$(62 \pm 1) \times 10^{-4}$	$(24 \pm 7) \times 10^{-5}$	29 ± 2	$12 \pm 1 \times 10^{-3}$	$(42 \pm 1) \times 10^{-5}$

[a]See the Materials and Methods section for the techniques of determination of k_{on}, k_{off}, and $K_d = k_{on}/k_{off}$, and for variations with individual compounds.

TABLE 3 Inhibition of Hs-SAHH and Tc-SAHH by NAD(H) analogues[a,b]

Analogue	Remaining Activity (%)	
	Hs-SAHH	Tc-SAHH
S-NAD	97 ± 1	74 ± 3
H-NAD	98 ± 1	77 ± 4
C-NAD	>99	94 ± 4
O-NAD	>99	96 ± 3
NGD	>99	96 ± 4
NHD	>99	96 ± 3
Etheno-NAD	>99	95 ± 3
S-NADH	96 ± 1	8 ± 2
H-NADH	96 ± 1	9 ± 2
C-NADH	>99	52 ± 3
NHDH	>99	98 ± 2

[a]Errors calculated from three independent measurements.

[b]After incubation with 20 μM analogue, 50 μM NAD^+ to simulate intracellular conditions, at pH 7.4 (phosphate buffer) for a period of 6 minutes.

the cofactor site. Further evidence favoring this view is provided by the fact that modifications of the adenine ring give higher activities and lower occupancies than modifications of the nicotinamide ring. This combination is consistent with the long-held view[22] that the adenine "anchor" is more important for binding than for catalysis. The fact that H-NAD (with an aldehyde side chain) and O-NAD (with a carboxylate side chain) are the only NAD^+ analogues that do not support measurable catalytic activity with Tc-SAHH renders them the only compounds in this part of the set worthy of further consideration for anti-parasitic agents.

Off-Rates, On-Rates, and Affinities for NAD^+ and NADH Analogues

Table 2 presents these data for all compounds studied. A useful baseline for comparison is provided by the data for the oxidized cofactor NAD^+ and the reduced cofactor NADH with Hs-SAHH and Tc-SAHH. As previously noted,[17,18] the affinity of both forms of the cofactor is in the nanomolar range for Hs-SAHH, the result of an off rate constant in the range of 10^{-6} s^{-1} and an on-rate constant in the range of 10^3 $M^{-1}s^{-1}$; in contrast, the affinity of both forms of the cofactor is in the micromolar range for Tc-SAHH, the result of an off-rate constant in the range of 10^{-3} to 10^{-4} s^{-1} and an on-rate constant in the range of 10^3 to 10^2 $M^{-1}s^{-1}$. The contrast in cofactor affinities between Hs-SAHH and Tc-SAHH thus arises mainly from a much faster off-rate constant for the latter, and it is therein one may seek the opportunity for selective inhibition of Tc-SAHH over Hs-SAHH.

Among the NAD^+ analogues, the most promising seemed to be H-NAD, with an aldehydic side-chain, and O-NAD, with a carboxylate side-chain, which gave no detectable activity when reconstituted into the active sites of either Hs-SAHH or Tc-SAHH (Table 1). H-NAD shows an on-rate constant 10-fold larger and an off-rate constant 12-fold larger than NAD^+ with Hs-SAHH so that the affinity for Hs-SAHH of H-NAD is not much different from its affinity for NAD^+. With Tc-SAHH, more or less the same thing is observed. The close similarity to the kinetics for NAD^+ does not bode well for successful inhibition in the presence of NAD^+. O-NAD presents no better picture. Its affinity for both Hs-SAHH and Tc-SAHH is smaller than their affinity for NAD^+ by factors of 35–40 and their on-rate constants are smaller than those for NAD^+.

The NADH analogs show generally poorer affinity than NADH with Hs-SAHH (factors of 3–600) but affinities with Tc-SAHH that, except for NHDH, are weaker than that for NADH by smaller factors (3–40) and are greater in all cases except NHDH than the affinity of Tc-SAHH for NAD^+, the probable competitor for inhibitor binding in vivo. We conclude that NADH analogues may offer the best opportunities for selective inhibition of Tc-SAHH.

Inhibition of Hs-SAHH and Tc-SAHH by NAD^+ and NADH Analogues

Table 3 shows for each analogue with Hs-SAHH and Tc-SAHH the remaining levels of catalytic activity after a 6-minutes exposure of the enzyme to 20 μM analogue in the presence of 50 μM NAD^+ to simulate competition by intracellular cofactor. Under these conditions, no significant inhibition of Hs-SAHH occurred with any analogue. Tc-SAHH was reasonably strongly inhibited only by the NADH analogues S-NADH and H-NADH, with a bit less than 10% activity remaining. These are the only NADH analogues for which the on-rate constants for Tc-SAHH exceed (S-NADH) or nearly match (H-NADH) the on-rate constant. This feature may in part enable the effective competition they display.

CONCLUSION

We would recommend NADH analogues as a possible starting point for the design of anti-trypanosomal drugs. Although the set of compounds studied herein does not in any sense approach a thorough exploration of structures in any part of the cofactor, we hope it serves to support the further study of cofactor analogs. Clearly the modification of the carboxamide side chain of the nicotinamide ring was more successful than modification of

the adenine ring, in agreement with previous indications of a more intimate binding relationship at the latter center.[1,22]

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