Inhibition of the intrinsic NAD$^+$ glycohydrolase activity of CD38 by carbocyclic NAD analogues

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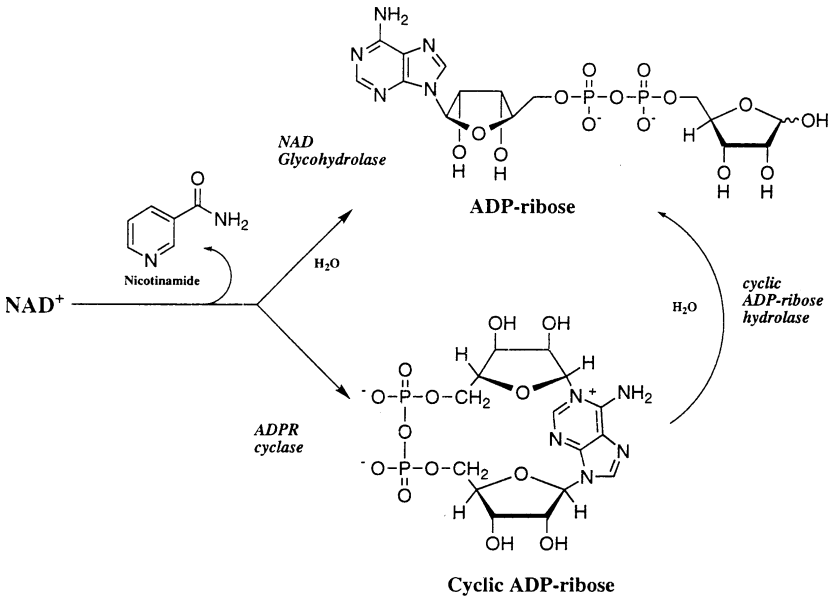
Carba-NAD and pseudocarba-NAD are carbocyclic analogues of NAD$^+$ in which a 2,3-dihydroxycyclopentane methanol replaces the β-D-ribonucleotide ring of the nicotinamide riboside moiety of NAD$^+$ [Slama and Simmons (1988) Biochemistry **27**, 183–193]. These carbocyclic NAD$^+$ analogues, related to each other as diastereomers, have been tested as inhibitors of the intrinsic NAD$^+$ glycohydrolase activity of human CD38, dog spleen NAD$^+$ glycohydrolase, mouse CD38 and *Aplysia californica* cADP-ribose synthetase. Pseudocarba-NAD, the carbocyclic dinucleotide in which L-2,3-dihydroxycyclopentane methanol replaces the D-ribose of the nicotinamide riboside moiety of NAD$^+$, was found to be the more potent inhibitor. Pseudocarba-NAD was shown to inhibit the intrinsic NAD$^+$ glycohydrolase activity of human CD38 competitively, with $K_i = 148\ \mu$M determined for the recombinant extracellular protein domain and $K_i = 180\ \mu$M determined for the native protein expressed as a cell-surface enzyme on cultured Jurkat cells. Pseudocarba-NAD was shown to be a non-competitive inhibitor of the purified dog spleen NAD$^+$ glycohydrolase, with $K_{is} = 47\ \mu$M and $K_{ii} = 198\ \mu$M. Neither pseudocarba-NAD nor carba-NAD inhibited mouse CD38 or *Aplysia californica* cADP-ribose synthetase significantly at concentrations up to 1 mM. The results underscore significant species differences in the sensitivity of these enzymes to inhibition, and indicate that pseudocarba-NAD will be useful as an inhibitor of the enzymic activity of human but not mouse CD38 in studies using cultured cells.

INTRODUCTION

CD38 is a leucocyte cell-surface protein whose expression varies with development and activation [1–3]. It is found on the plasma membranes of thymocytes, resting macrophages, activated B- and T-cells, and on many tumours. CD38 was first proposed to catalyse the synthesis of cADP-ribose based on sequence homology with ADP-ribosyl cyclase from *Aplysia californica* [4]. Indeed, CD38 contains ADP-ribosyl cyclase activity, and it also contains cADP-ribose hydrolase and NAD$^+$ glycohydrolase (NADase) activities (Scheme 1) [5–10], activities shown to be present in a family of proteins collectively termed NADases (EC



Scheme 1 Enzymic synthesis of ADP-ribose and cADP-ribose

Abbreviations used: rCD38, recombinant CD38 protein from which the membrane-spanning domain was deleted; NADase, NAD$^+$ glycohydrolase; isoniazid, isonicotinic acid hydrazide.

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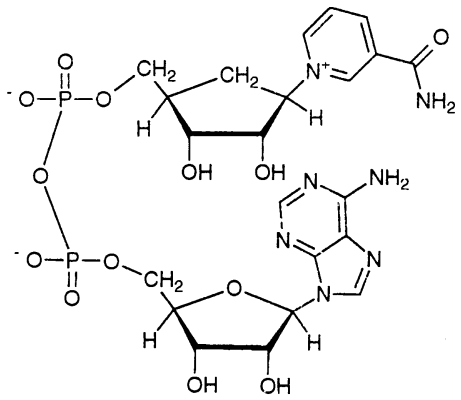
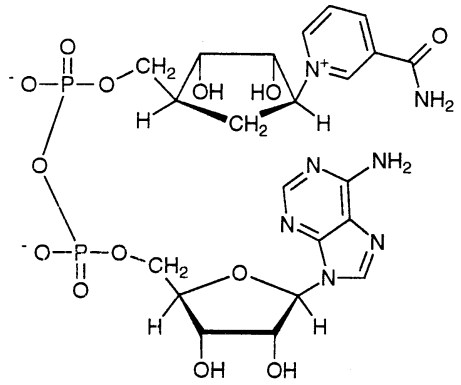


Carba-NAD (1)



Pseudocarba-NAD (2)

Figure 1 Structures of carba-NAD (1) and pseudocarba-NAD (2)

3.2.2.5) [11,12]. These results suggest that CD38 and other NADases may function by regulating the synthesis and/or degradation of cADP-ribose.

It would be useful to possess potent and specific inhibitors of the enzymic activity of CD38 to determine how cADP-ribose metabolizing activity is related to the biological function of the protein. We have previously synthesized carbocyclic analogues of NAD^+, carba-NAD (Figure 1, structure 1) and pseudocarba-NAD (Figure 1, structure 2), which are resistant to enzymic hydrolysis of the pyridinium-carbon bond [13]. One of these, pseudocarba-NAD, was shown to be a potent and specific inhibitor of the NADase from bovine spleen, bovine brain and *Bungarus fasciatus* venom [14]. Pseudocarba-NAD, however, was poorly active against closely related enzymes from *Neurospora* and rabbit. In order to employ these carbocyclic dinucleotides in studies of the function of CD38 using human or murine cells, it was necessary to characterize the inhibitory activity of carba-NAD and pseudocarba-NAD against the intrinsic NADase activity of human and murine CD38.

EXPERIMENTAL

Materials

Isonicotinic acid hydrazide (isoniazid) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BSA, fraction V, was purchased from Calbiochem (La Jolla, CA, U.S.A.). [4-^{3}H-nicotinamide]NAD^+ was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Carba-NAD and pseudocarba-NAD were synthesized as described elsewhere [13].

Enzymes

Recombinant human CD38 (rCD38) was prepared using a clone, kindly provided by Dr. Toshiaki Katada (University of Tokyo, Tokyo, Japan), which codes for a 29.7 kDa domain that contains the active site of CD38 and has the membrane-spanning domain deleted [10]. The cDNA clone was placed into a pGEX-1lT expression vector inserted in-frame into a glutathione S-transferase gene, resulting in the expression of a fusion protein of 56.7 kDa. This vector was transfected into *Escherichia coli* NM522 and expression was induced by addition of isopropylthioglucoside. The fusion protein was isolated from bacterial extracts by binding to glutathione–Sepharose 4B, treatment with thrombin, and elution of the approx. 30 kDa cleaved fragment of soluble CD38 from the column. Following purification, the preparation was denatured by dialysis in 5.5 M guanidine hydrochloride and refolded by progressive removal of the guanidine hydrochloride. From a 1 litre culture, 1.2 mg of pure enzyme was obtained. The K_m for the recombinant enzyme for hydrolysis of NAD, measured at pH 7.4 in Dulbecco's PBS (8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/149 mM NaCl/2.7 mM KCl, pH 7.4), was 28 ± 2 μM. The specific activity of this enzyme after storage at -20 °C for 1–2 years was 90×10^{-6} units/μg of protein.

Dog spleen NADase was purified as described previously [11]. ADP-ribose cyclase from *A. californica* was obtained from Sigma.

Cell culture

Jurkat cells were cultured in RPMI-1640 (JRH Biosciences, Lenexa, KS, U.S.A.) supplemented with media additions to give final concentrations of 10 mg/l folic acid, 36 mg/l asparagine, 200 mg/l arginine, 800 mg/l glutamine, 100 mg/l sodium pyruvate and 10 % fetal bovine serum (HyClone Laboratories, Logan, UT). All assays were performed on cells in logarithmic growth with 100 % viability as determined by exclusion of Trypan Blue (Gibco BRL, Gaithersburg, MD, U.S.A.). EL-4 cells (ATCC TIB 39) were cultured in Dulbecco's modified Eagle's medium (JRH Biosciences) supplemented with media additions as described above and 10 % fetal bovine serum. Viable cells were isolated by centrifugation over Ficoll-Hypaque (Sigma). All cell types were washed at least twice with PBS containing 0.1 or 1 mg/ml BSA prior to assay of enzyme activity.

Enzyme assay

Dog spleen NADase was assayed as described elsewhere [11]. The assays for human and murine CD38 were performed essentially the same way, with slight modifications. rCD38 (1 μg), ADP-ribose cyclase (4 ng) or washed cells ($1–4 \times 10^6$/tube) were incubated together with substrate and inhibitor at 22 °C (cells) or 37 °C (enzymes) for 15 or 30 min in a total volume of 300 μl of PBS (pH 7.4) with 0.1 or 1 mg/ml BSA. Cell suspensions were then centrifuged at 650 ***g*** for 2 min and 200 μl of the supernatant was transferred to a clean tube. The reaction was stopped by addition of 0.67 volumes of 0.5 M sodium borate, pH 8.0. Released nicotinamide was extracted by addition of 1 ml of water-saturated ethyl acetate, vortexing and transfer of 0.7 ml of the organic fraction to 3 ml of EcoLite scintillation fluid (ICN, Cleveland, OH, U.S.A.). [4-^{3}H]Nicotinamide was quantified by scintillation counting and quench correction. NADase activity

(1 unit) is the quantity of enzyme that catalyses the hydrolysis of 1 μmol of NAD^+/min.

The effect of varying substrate concentration on initial rates of enzyme-catalysed hydrolysis was determined in the presence of several fixed inhibitor concentrations. The data for each fixed concentration of inhibitor were fitted by a non-linear regression procedure to the hyperbolic form of the Michaelis–Menten equation: $v = V_{max}[S]/(K_m + [S])$. The line so determined was displayed on a double-reciprocal plot. Kinetic parameters were determined using the non-linear regression analysis developed by Cleland [15] using a translation of the program for the IBM workstation and compatibles (obtained from Dr. R. E. Viola, Department of Chemistry, University of Akron, Akron, OH, U.S.A.). Each data set was fitted to equations describing linear-competitive, non-competitive and uncompetitive inhibition. The best description of the data was chosen according to the criteria set by Cleland [15] for the evaluation of a successful fit.

HPLC analysis of NAD hydrolysis

HPLC was performed on a system consisting of a Thermo-separation Products ConstaMetric 4100 Quaternary Solvent Delivery System, a Waters Associates 991 Photodiode array detector operated at 210 and 260 nm and a Vydac C-18 column (4.6 mm × 25 mm). The mobile phase consisted of a programmed gradient formed between buffer A (20 mM $NaHPO_4$/2 mM tetrabutylammonium phosphate, adjusted to pH 6.1 with NaOH) and buffer B (1:1 buffer A/acetonitrile) at a constant flow rate of 1.5 ml/min. The program began upon the injection of the sample and consisted of isocratic elution with buffer A for 2 min, followed by application of a linear gradient formed between buffers A and B over 20 min starting with 100 % A and ending with 80 % A/20 % B, immediately followed by a second linear gradient starting with 20 % B and ending with 70 % B over the next 8 min. Elution times of the metabolites of NAD were: NMN, 2.5 min; nicotinamide, 10 min; adenosine, 12 min; NAD, 14 min; AMP, 17 min; ADP-ribose, 22 min.

RESULTS

Inhibition of rCD38

The enzymic activity of the extracellular domain of rCD38 was assayed by measurement of the release of [4-^{3}H]nicotinamide from [4-^{3}H-nicotinamide]NAD^+. This assay primarily measures the NADase activity of CD38, since the cyclase activity is approx. 1–10 % of the glycohydrolase activity [5,8,11] and both activities release nicotinamide. The assay was conducted in PBS at pH 7.4 containing 100 μg/ml BSA to compare activities of soluble rCD38 and cell-surface CD38. The rate of production of nicotinamide was linear over 60 min under our assay conditions, indicating that the enzyme is not subject to self-inactivation during assay. The concentrations at which activity was inhibited by 50 % (IC_{50} values) were determined for carba-NAD and pseudocarba-NAD (Table 1). Inhibition by isoniazid, a nicotinamide analogue that inhibits certain NADases [16], plateaued at less than 20 % maximum inhibition. Assay of rCD38 with varying concentrations of NAD^+ and using several fixed concentrations of pseudocarba-NAD gave a K_i value of 148 ± 12 μM (Figure 2). Analysis of the kinetic data indicated that pseudocarba-NAD acts as a linear-competitive inhibitor.

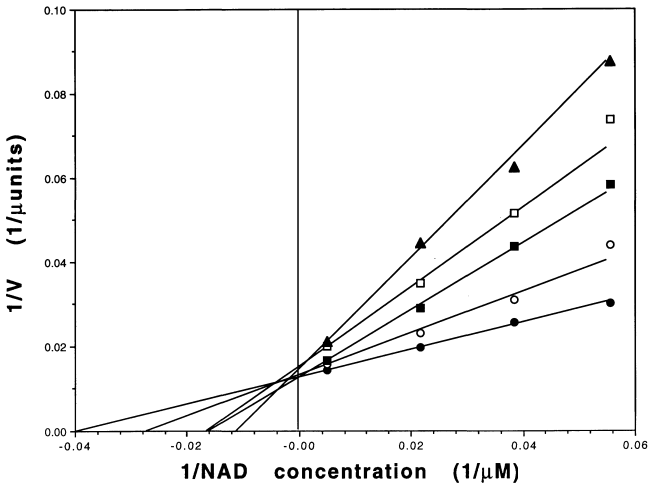


Figure 2 Lineweaver–Burk plot showing the effect of the addition of pseudocarba-NAD on the initial rates of release of nicotinamide from NAD^+ catalysed by human rCD38

The assay was conducted at 37 °C and pH 7.4 as described in the Experimental section. The concentration of substrate NAD^+ varied from 18 to 200 μM, and the initial velocity was measured as pmol of nicotinamide released/min. Inhibitor concentrations were: ●, no inhibitor; ○, 100 μM; ■, 200 μM; □, 400 μM; ▲, 600 μM. Replots of intercept ($1/V_{max\ apparent}$) versus [I] and slope (K_m/V_{max}) values versus [I] were linear (results not shown).

Inhibition of cell-surface human CD38

Since the eventual goal of this study was the inhibition of CD38 activity on cells, it was important to show that carba-NAD and pseudocarba-NAD also inhibited cell-surface CD38. Jurkat cells

Table 1 Inhibition of NADase activity from various sources by substrate analogues

IC_{50} values are expressed to two significant figures. For rCD38, Jurkat cells and *Aplysia* ADP-ribosyl cyclase, assays were in PBS with 0.1 mg/ml BSA at $[NAD^+] \cong K_m$, which was 37 μM for rCD38, 27 μM for Jurkat cells and 160 μM for *Aplysia* ADP-ribosyl cyclase. Dog spleen NADase was assayed in Hepes, and mouse EL-4 cells were assayed in PBS with 1 mg/ml BSA at 60 μM NAD^+. ND, not determined.

Compound	IC_{50} (μM)				
	rCD38	Jurkat cells	Dog spleen NADase	Mouse EL-4 cells	*Aplysia* ADP-ribosyl cyclase
Isoniazid	> 1000	> 1000	ND	> 1000	> 2000
Carba-NAD	400	900	510	> 1000	> 1000
Pseudocarba-NAD	200	360	200	> 1000	> 1000

were chosen for assay since CD38 is known to be expressed on the Jurkat cell surface [7] and the cells exhibit several well-defined functions. Expression of CD38 by the Jurkat subline was confirmed by flow cytometry using the anti-CD38 monoclonal antibodies HIT2 (Pharmigen, San Diego, CA, U.S.A.), OKT10 and THB-7 with FITC-conjugated rabbit anti-mouse IgG (Organon Teknika Corp, Durham, NC, U.S.A.). The latter two antibodies were purified from the supernatants of the OKT10 and THB-7 hybridomas obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Staining with each antibody gave a symmetrical single peak during analysis by flow cytometry, indicative of an homogeneous population of cells, all of which expressed CD38 constitutively. Once we verified that our Jurkat subline expressed CD38, we determined that CD38 activity of Jurkat cells had a K_m of 27 μM and V_{max} of 125 μunits/10^6 cells.

We were concerned that the kinetics and inhibition of the Jurkat cell-surface NADase might be influenced by a cell-surface-expressed nucleotide pyrophosphatase, which could catalyse the degradation of pyridine dinucleotides by hydrolysis of the pyrophosphoryl bond to mononucleotides AMP and NMN [17]. We first examined the effect of inhibiting the pyrophosphatase activity with 2 mM EDTA [17]. The addition of 2 mM EDTA to the NADase assay catalysed by either soluble rCD38 or Jurkat cells did not change the rate of release of nicotinamide, indicating that hydrolysis of NAD by a pyrophosphatase was not influencing the NADase activity that we measured.

In order to better assess the possible contribution of pyrophosphatase activity to the hydrolysis of NAD by cells, we analysed the products formed from the hydrolysis of [4-^{3}H]NAD catalysed by Jurkat cells. The reaction was allowed to proceed under standard conditions of assay (PBS, pH 7.4) with an NAD concentration of 200 μM using 1×10^6 cells for 30 min. At the end of the assay, cells were removed by centrifugation, and the supernatant filtered and analysed by reversed-phase HPLC with UV detection at 210 and 260 nm. Fractions of effluent were collected at 30 s intervals and radioactivity determined by liquid-scintillation counting. The HPLC system employed for analysis was capable of separating all the expected products from cleavage of either the pyridinium ribotide bond of NAD or the pyrophosphate of NAD (see the Experimental section). When we examined the purine nucleotides formed after treatment of NAD by Jurkat cells using reversed-phase HPLC, we observed that substrate NAD decreased concomitantly with an increase in ADP-ribose. We did not observe any other products, particularly AMP, formed during the reaction. Since nicotinamide has a low extinction coefficient, we examined the formation of nicotinamide and nicotinamide mononucleotide radiometrically. We observed that $> 99\,\%$ of the isotope released from [^{3}H-nicotinamide]NAD was isographic with nicotinamide. No other radiolabelled products were observed. In particular, activity isographic with NMN accounted for $\leqslant 0.1\,\%$ of the product formed by Jurkat cells. This result is in accord with a previous study which reported that Jurkat cells expressed very low levels of nucleotide pyrophosphatase activity constitutively [18].

The IC_{50} values for carba-NAD and pseudocarba-NAD (Table 1) were similar to those observed for rCD38. Again, pseudocarba-NAD was the superior inhibitor and isoniazid inhibition plateaued at less than 20 % inhibition. The K_i determined for pseudocarba-NAD for inhibition of the enzymic activity on Jurkat cells was also similar to that of rCD38; 180 ± 15 μM (Figure 3). Analysis of the data showed competitive inhibition. With the substrate NAD^+ present at a concentration equal to its K_m, it was possible to inhibit $> 99\,\%$ of the NADase activity of Jurkat cells at 1 mM pseudocarba-NAD.

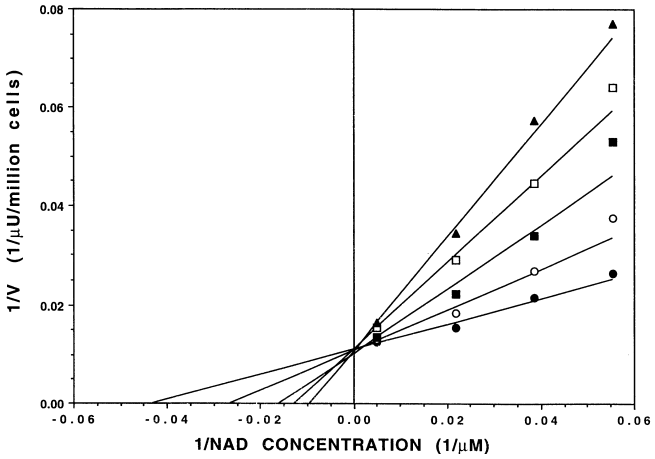


Figure 3 Lineweaver–Burk plot showing the effect of the addition of pseudocarba-NAD on the initial rates of release of nicotinamide from NAD^+ catalysed by human CD38 present on the surface of Jurkat cells

The assay was conducted in PBS at 22 °C as described in the Experimental section. The concentration of substrate NAD^+ varied from 18 to 200 μM, and the initial velocity was measured as pmol of nicotinamide released/min per million cells. Inhibitor concentrations were: ●, no inhibitor; ○, 75 μM; ■, 200 μM; □, 400 μM; ▲, 600 μM. Replots of intercept ($1/V_{max\ apparent}$) values versus [I] and slope (K_m/V_{max}) values versus [I] were linear (results not shown).

To verify that pseudocarba-NAD was stable in the presence of Jurkat cells under the conditions of assay, we incubated 330 μM pseudocarba-NAD with Jurkat cells for 15 and 30 min under the conditions of assay. Using HPLC analysis, we observed no change in the concentration of pseudocarba-NAD within the limits of experimental error. We observed further that no new peaks were formed during the incubation, which could account for more than 0.5 % of pseudocarba-NAD. In particular, AMP, a metabolite diagnostic of pyrophosphate cleavage, was not formed during the incubation.

Inhibition of NADase from dog spleen

IC_{50} values were determined for carba-NAD and pseudocarba-NAD for purified dog spleen NADase [11] (Table 1). Pseudocarba-NAD was again the superior inhibitor. In this instance, the inhibition was non-competitive (Figure 4). Analysis of the kinetics of inhibition by pseudocarba-NAD gave K_{is} 47 ± 9 μM and K_{ii} 198 ± 47 μM.

Inhibition of murine CD38 activity

We examined the effect of several inhibitors on cell-surface NADase activity present on a murine cell line that expresses CD38 [19]. Intact EL-4 cells, a murine lymphoma cell line, gave saturation kinetics with a K_m for NAD^+ of 57 μM and a V_{max} of 280 μunits/10^6 cells. In comparison, Jurkat cells under the conditions of 1 mg/ml BSA in PBS had a K_m of 63 μM and V_{max} of 80 μunits/10^6 cells. None of the inhibitors tested was effective against the NADase expressed on the surface of EL-4 cells (Table 1). We verified that pyrophosphatase activity was absent from EL-4 cells by observing no effect from the addition of the pyrophosphatase inhibitor EDTA upon our NADase assay, determining that nicotinamide and ADP-ribose were the only products of NAD hydrolysis observed, and verifying that pseudocarba-NAD was stable to the conditions of assay.

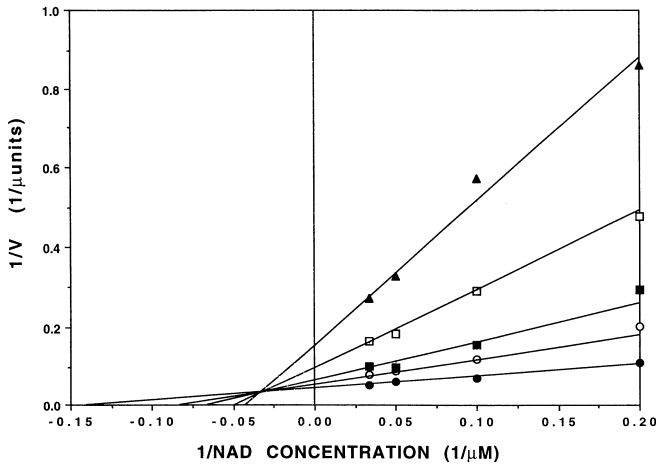


Figure 4 Lineweaver–Burk plot showing the effect of the addition of pseudocarba-NAD on the initial rates of release of nicotinamide from NAD+ catalysed by dog spleen NADase

The assay was conducted at 37 °C and pH 7.2 as described in the Experimental section. The concentration of substrate NAD+ varied from 5 to 30 μM, and the initial velocity was measured as pmol of nicotinamide released per min. Inhibitor concentrations were: ●, no inhibitor; ○, 50 μM; ■, 100 μM; □, 250 μM; ▲, 500 μM. Replots of intercept ($1/V_{max\ apparent}$) values versus [I] and slope (K_m/V_{max}) values versus [I] were linear (results not shown).

Effect of inhibitors on *Aplysia* ADP-ribosyl cyclase

The identification of enzymic activity in CD38 was prompted due to its sequence similarity with the ADP-ribosyl cyclase of *A. californica* [4]. This enzyme displays ADP-ribosyl cyclase and NADase activity, but not cADP-ribose hydrolase activity [20]. Under our assay conditions, the *Aplysia* enzyme had a K_m of 200 μM and a V_{max} of 142 units/mg. Isoniazid inhibited with an IC_{50} of greater than 2 mM (Table 1). Inhibition by carba-NAD and pseudocarba-NAD plateaued at less than 20% inhibition, indicating that these compounds are not good inhibitors of the ADP-ribosyl cyclase (Table 1).

DISCUSSION

Our objective is to discover inhibitors of the intrinsic enzymic activity of CD38 and to apply these in studies to determine the relation of this enzymic activity to proposed biological function(s) of CD38. Like NADases from dog spleen, bovine spleen, and *B. fasciatus* venom, CD38 catalyses the conversion of NAD+ to ADP-ribose and the conversion of NAD+ to cADP-ribose, albeit in low yield [11,12]. This suggests that NADases and CD38 are related. Although these proteins differ in the kinetic parameters concerning cADP-ribose hydrolysis to ADP-ribose [21], they share most fundamental attributes with respect to catalysis of transglycosidation reactions, stereochemistry and reaction mechanism, which supports the contention that CD38 is an authentic NADase [22,23].

Carba-NAD and pseudocarba-NAD are analogues of NAD+ in which 2,3-dihydroxycyclopentane methanol replaces the ribotide ring of the nicotinamide riboside of NAD+. Both carba-NAD and pseudocarba-NAD are resistant to enzymic cleavage of the pyridinium-carbon bond, and therefore inhibit NADases and related mono(ADP-ribosyl) transferases [13]. A comparison of the relative inhibitory activities revealed that pseudocarba-NAD was the superior NADase inhibitor [14]. This was surprising since pseudocarba-NAD is a carbocyclic analogue of an unnatural diastereomer of NAD+ containing β-nicotinamide-L-ribotide. Pseudocarba-NAD was found to be superior to carba-NAD as an inhibitor of the purified NADase from *B. fasciatus* venom [14], the purified calf spleen NADase [24] and unpurified NADase-containing extracts from bovine, ovine, porcine and rabbit brains [14]. This trend is now extended to include rCD38, purified dog spleen NADase and the murine CD38, all of which exhibit a preference for binding pseudocarba-NAD.

This shared preference for binding pseudocarba-NAD suggests a spatial relation among sensitive binding sites, and further suggests that pseudocarba-NAD can assume a folded conformation that mimics the folded conformation proposed for the natural cyclase substrate NAD+. We further expect that sensitivity to inhibition by pseudocarba-NAD will be relatively specific, since dehydrogenases are not expected to recognize pseudocarba-NAD as a substrate or as an inhibitor [13].

Our initial characterization of carba-NAD and pseudocarba-NAD revealed a significant species variability in sensitivity towards inhibition using pseudocarba-NAD. NADases from cow and sheep, and from snake venom, exhibited high sensitivity to pseudocarba-NAD, whereas enzymes from rabbit or *Neurospora* were relatively resistant [14]. It has been observed previously that NADases of vertebrate species differ greatly in sensitivity to inhibition by isoniazid [16]. NADases from ruminants, birds and *B. fasciatus* venom are sensitive to inhibition by concentrations of isoniazid in the low millimolar range, whereas the NADases from human and mouse are resistant. We have observed previously that the enzymes most sensitive to inhibition by pseudocarba-NAD are those that are also most sensitive to inhibition by isoniazid. The structural basis for this difference in sensitivity to inhibition between similar enzymes from different species is not yet known.

Differences in sensitivity to inhibition by carbocyclic dinucleotides among species required us to establish the sensitivity of human CD38 to the inhibitors before applying the compounds in studies designed to illuminate function. Pseudocarba-NAD was shown to be a linear competitive inhibitor of the enzymic activity of human CD38 with a K_i of 150–190 μM. There is only a small difference between the soluble recombinant enzyme and the transmembrane form of the enzyme in sensitivity to inhibition by pseudocarba-NAD.

Dog spleen NADase catalyses the synthesis of cADP-ribose from NAD+ [11], as well as the hydrolysis of NAD+ to ADP-ribose and nicotinamide. Its enzymic activity categorizes it as a cADP-ribose synthetase and preliminary sequence data support structural similarity between the dog enzyme and vertebrate CD38. Pseudocarba-NAD inhibits the dog spleen enzyme non-competitively, with inhibitor dissociation constants of $K_{is} = 47 \pm 9$ μM and $K_{ii} = 198 \pm 47$ μM. This is the first case of non-competitive inhibition observed for pseudocarba-NAD; this mechanism requires the formation of a ternary enzyme–substrate–inhibitor complex, and suggests that for the dog enzyme an additional inhibitory nucleotide binding site exists. Substrate inhibition in certain related enzymes has been observed [25], and is supportive of the existence of an inhibitory dinucleotide binding site.

Mouse is a particularly important experimental animal for analysis of immune functions, and for which CD38 is well characterized [9]. CD38 from murine B-cells has been shown to be homologous with human CD38. Mouse EL-4 (thymoma) cells are known to transcribe CD38 mRNA [19]. We detected the characteristic enzymic activity by assay of nicotinamide release from NAD+ catalysed by the ecto-NADase activity of intact mouse EL-4 cells, and measured for it a K_m of 57 μM. Neither carba-NAD nor pseudocarba-NAD inhibited this activity sig-

nificantly at concentrations of up to 1 mM. Clearly the murine CD38 is relatively insensitive to inhibition by the carbocyclic dinucleotides, as well as to inhibition by the nicotinamide analogue isoniazid. Indeed, we are unaware of any potent inhibitor of the mouse NADase. *Aplysia* ADP-ribosyl cyclase was similarly insensitive to inhibition either by carba-NAD or by pseudocarba-NAD, with IC_{50} values > 1 mM.

Other potent NADase inhibitors are known. Arabinosyl-NAD and 2-fluoroarabinosyl-NAD have been shown to inhibit bovine NADase as slow-binding reversible inhibitors with dissociation constants in the micromolar to nanomolar range [26]. 2-Fluoroarabinosyl-NAD was further shown to possess high potency as an inhibitor of human CD38/NADase [23]. The specificity of this class of inhibitors has not yet been investigated thoroughly.

Pseudocarba-NAD was shown to possess significant activity as an inhibitor of human CD38 as well as dog spleen NADase. The unusual stereochemistry of the dinucleotide implies high specificity for the pyridine dinucleotide binding site of sensitive enzymes. Dinucleotide pseudocarba-NAD will therefore be useful for inhibiting cADP-ribose synthetase/NADase activity in cultured human cells in studies of CD38 function, and will serve as a lead structure for the development of superior cADP-ribose synthetase inhibitors.

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