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Effect of chirality in *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) on adenosine deaminase inhibition

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Inhibitors of adenosine deaminase (ADA*; adenosine aminohydrolase, EC 3.5.4.4) act as immunosuppressive agents and potentiate the cytotoxic effects of a number of adenosine analogs. Two classes of adenosine deaminase inhibitors have attracted the greatest attention: the antibiotics coformycin and deoxycoformycin which have been proposed to act as transition state analogs [1] and 9-(hydroxyalkyl)adenines, such as *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), which presumably bind to a hydrophobic region adjacent to the catalytic site [2]. The development of EHNA by Schaeffer *et al.* [3, 4] offers a classic example of the rational design of enzyme inhibitors.

Coformycin and deoxycoformycin are tight binding inhibitors of ADA with K_i values in the range of $2.5–15 \times 10^{-12}$ M, whereas EHNA is categorized as a semi-tight binding inhibitor with K_i values on the order of $2–4 \times 10^{-9}$ M [5, 6]. With the former inhibitors, spontaneous reactivation of the enzyme is exceedingly slow, i.e. $T_{1/2}$ = 8–29 hr, and in intact cells, such as erythrocytes, reactivation of inhibited ADA has not been demonstrated. With EHNA, however, the inhibition of ADA is more readily reversible, i.e. $T_{1/2} < 5$ min. In addition, EHNA has biochemical effects that are not readily explained solely on the basis of ADA inhibition [7]. Commercially available EHNA is, in fact, a racemic mixture of *erythro*-(+)-9-(2S-hydroxy-3R-nonyl)adenine and *erythro*-(−)-9-(2R-hydroxy-3S-nonyl)adenine and will be designated here as (±)-EHNA. Questions have arisen as to which chiral isomer is responsible for the inhibition of ADA and the additional biochemical effects on cellular metabolism. Therefore, chiral syntheses of these two *erythro* as well as the two *threo* isomers (THNA) have been developed, the testing of which should provide answers to these questions. This report describes the effects of the four isomers on human erythrocytic and calf intestinal ADA.

Materials and methods

Synthesis of chiral isomers of EHNA and THNA. The synthetic methods and characterizations described briefly here will be detailed elsewhere.† Starting with the sugar, L-rhamnose, the chiral *erythro* and *threo* isomers of 9-(2-hydroxy-3-nonyl)adenine have been prepared. A common known intermediate (I) was used to synthesize the chiral amines (IV, V, VI, and VII) [8]. As shown in Fig. 1, the 2,3-dideoxyrhamnose derivative (I) was converted via two isomeric tosylates at C-4 by treatment with lithium azide to compounds II and III. These were reduced catalytically to their respective amines and were then acetylated with acetic anhydride to the corresponding acetamide derivatives. Acid hydrolysis of the acetal linkage followed by chain extension with the proper Wittig reagent gave mixtures of olefins from which the saturated "tails" (C_6H_{13}) were secured by hydrogenation. Hydrolysis of the acetamide group with either hydrazine or 1 N hydrochloric acid furnished amines IV and V. The remaining amines VI and VII were obtained by inversion of the stereochemistry at C-2 when the acetamides were first treated with thionyl chloride followed by acid hydrolysis according to known procedures [9, 10]. Incorporation of the chiral amines into the adenine moiety at N-9 followed established routes [11]. All new compounds described here have been fully characterized (^{1}H NMR, ^{13}C NMR, mass spec., u.v., and $[\alpha]_D$).

Enzymatic procedures. The partial purification of human erythrocytic ADA and the spectrophotometric assay of adenosine deamination have been reported elsewhere [12, 13]. Calf intestinal ADA (sp. act. *ca.* 200 units/mg protein) was purchased from Boehringer Mannheim, Indianapolis, IN, and the Sigma Chemical Co., St. Louis, MO (Type III). The inhibition constants were determined from replots of the slopes of a double-reciprocal plot at five fixed inhibitor concentrations. A weighted linear regression analysis program was adapted from Cleland [14] to a Wang computer and extended by Dr. Sungman Cha to compute both K_m and K_i values from plots of $1/v$ vs $1/S$ at multiple inhibitor concentrations. Samples of (±)-EHNA were obtained from the Drug Development Branch of the

* Abbreviations: ADA, adenosine deaminase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; (±)-EHNA, racemic mixture of EHNA; THNA, *threo*-9-(2-hydroxy-3-nonyl)adenine; (+)-(2S,3R)EHNA, *erythro*-(+)-9-(2S-hydroxy-3R-nonyl)adenine; (−)-(2R,3S)EHNA, *erythro*-(−)-(2R-hydroxy-3S-nonyl)adenine; (+)-(2R,3R)THNA, *threo*-(+)-(2R-hydroxy-3R-nonyl)adenine; and (−)-(2S,3S)THNA, *threo*-(−)-(2S-hydroxy-3S-nonyl)adenine.

† G. Bastian, M. Bessodes, R. P. Panzica, E. Abushanab, S-F. Chen, J. D. Stoeckler and R. E. Parks, Jr. *J. med. Chem.*, in press.

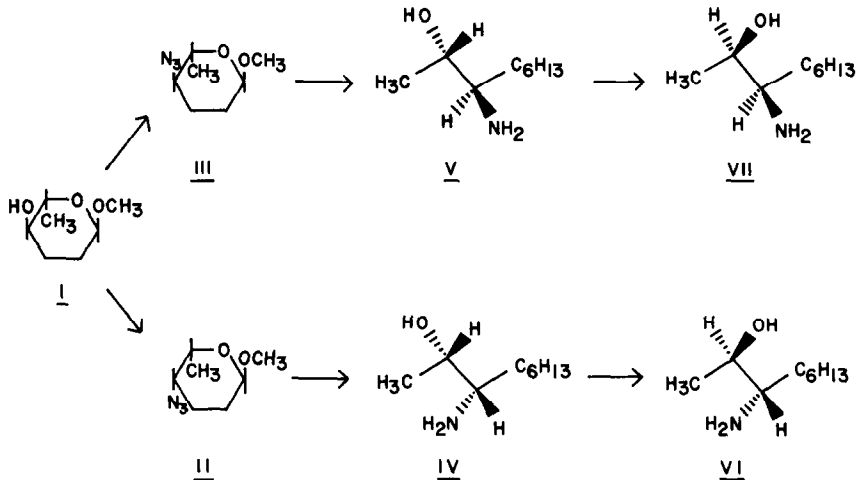


Fig. 1. Reaction pathway for the synthesis of the EHNA and THNA "tails".

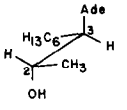
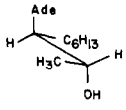
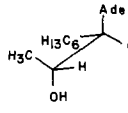
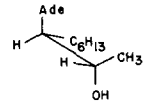
National Cancer Institute. Preincubation of inhibitors with ADA for 15 min prior to the addition of substrate affected the extent of inhibition only with (±)-EHNA and (+)-(2S,3R)EHNA. Increases in this preincubation time to as long as 2 hr did not affect further the nature of the inhibition. Since a time lag may occur before attainment of steady-state with semi-tight binding inhibitors [5], the reaction rates were determined 2 min after initiating the reaction by addition of adenosine. No correction was made for depletion of substrate during this time lag. With the less active isomers, a time lag does not occur and preincubation with enzyme was not required. The K_i values for each isomer of Table 1 are the averages of two to four individual experiments which were in agreement with S.D. values of <15%.

Results and discussion

Figure 2A presents a double-reciprocal plot of the inhibition of human erythrocytic ADA by (+)-(2S,3R)EHNA and is representative of data obtained with the other three isomers with human and calf intestinal ADA. The inset is a replot of the slopes vs inhibitor concentrations. Panels B, C and D are replots of the slopes vs inhibitor concentrations derived from similar data with human erythrocytic ADA and the other EHNA and THNA isomers. All of these replots are linear.

The inhibition constants (K_i) of the chiral isomers shown in Table 1 identify (+)-(2S,3R)EHNA as the most active inhibitor of both human and calf ADA. The K_i of (±)-EHNA with human ADA was determined at this time to be 4 nM which is about twice the value for (+)-(2S,3R)EHNA. The lower K_i value reported earlier for (±)-EHNA might be attributable to differences in the enzyme preparations. Thus, the difference in potency of the two enantiomers that comprise (±)-EHNA with both enzymes is in the order of 200 to 250-fold. Of interest is that the least potent isomer results from inversions in stereochemistry at both chiral centers. On the other hand, inversion at one of the chiral carbons yields decreases in inhibitory potency in the range of 40 to 75-fold. In agreement with earlier observations [5], the EHNA compounds are slightly less potent inhibitors of the calf enzyme. The relative differences between human and calf ADA in inhibition by the THNA isomers may reflect structural differences in enzymatic protein. A recent preliminary

Table 1. Inhibition constants (K_i) of the chiral isomers of EHNA and THNA with human erythrocytic and calf intestinal ADA

	EHNA		THNA	
				
ISOMER	(+)-2S,3R	(−)-2R,3S	(+)-2R,3R	(−)-2S,3S
$[\alpha]_D^*$	+37.7	−38.7	+38.5	−41.0
Ki (nM), Human ADA	2	500	122	80
Ki (nM), Calf ADA	32	625	120	240

The K_i values are the means of determinations made in two to four separate experiments with S.D. < 15%. Calf intestinal ADA from two commercial sources gave essentially identical results.

* The specific rotations were determined in ethanol.

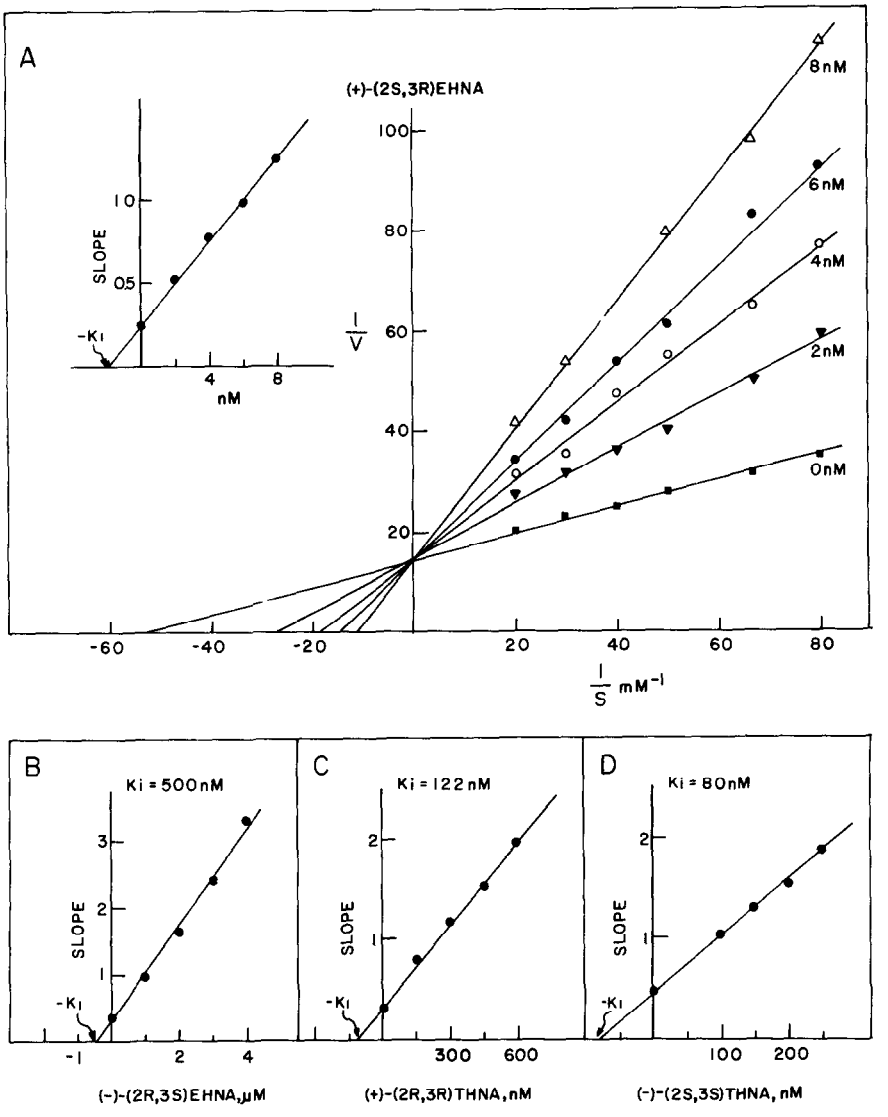


Fig. 2. (A) Double-reciprocal plot of the reaction of human erythrocytic ADA with adenosine in the presence of different concentrations of (+)-(2S,3R)EHNA under conditions described in Materials and Methods. From a replot of slopes vs (+)-(2S,3R)EHNA concentrations, the K_i was estimated to be 2×10^{-9} M. Panels B, C and D are replots of the slopes vs inhibitor concentrations derived from similar data with human erythrocytic ADA and the other EHNA and THNA isomers.

communication [15] that appeared after submission of this manuscript examined the L and D isomers of EHNA [corresponding to the (+)-(2S,3R)EHNA and (−)-(2R,3S)EHNA respectively] and calf intestinal ADA and reported that the L isomer has a K_i value of 0.764 nM and is about 80-fold more potent than the D isomer. These compounds were prepared by a synthetic route different from that employed here. At this point we cannot account for the differences between these values and those reported in Table 1. It is unlikely that differences in the enzyme preparations are responsible because the calf intestinal ADA from two sources, Sigma and Boehringer Mannheim, gave essentially the same results. It should be noted that Baker *et al.* [15] did not examine human ADA or the THNA isomers. Significantly, the findings uncovered independently in both laboratories are in excellent agreement with predictions made by Schaeffer and Schwender [11].

The synthetic methods described have several advantages. All four isomers are synthesized from a single synthon derived from a relatively inexpensive sugar, L-rhamnose, and no step in the syntheses requires resolution of racemic mixtures. These methods allow facile structural modifications of the most active EHNA which could lead to the development of analogs with more potent and/or more desirable biochemical or pharmacologic properties. Another interesting prospect is the introduction of functional groups, e.g. NH_2 or COOH, to develop reagents for the purification of ADA by affinity chromatography. In studies currently in progress, all four isomers are being examined for cytoxicity with various human tumor lines. Also, the effects of the four isomers on various aspects of the purine metabolism of Sarcoma 180 cells are under study. These chiral isomers of EHNA will be examined for antiviral and cytotoxic effects to determine possible relationships with inhibition of ADA.

In summary, chiral syntheses have been develped for the four *erythro* and *threo* isomers related to *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA). As starting material, the synthetic routes employed a common synthon derived from the relatively inexpensive sugar, L-rhamnose. All steps were performed without need for resolution of racemic mixtures. These methods are readily adaptable to the preparation of additional related compounds.

The four chiral isomers have been examined with adenosine deaminase (ADA) from human erythrocytes and calf intestine. With both human and calf enzymes, (+)-(2S,3R)EHNA was the most potent isomer (K_i values, human ADA = 2 nM, calf ADA = 3.2 nM), whereas its enantiomer (−)-(2R,3S)EHNA was 200 to 250-fold less active (K_i values, human ADA = 500 nM, calf = 625 nM). With both enzymes the two *threo* isomers were about 40–75 times less potent than (+)-(2S,3R)EHNA. The K_i values for THNA were: (+)-(2R,3R)THNA, human ADA = 122 nM, calf = 120 nM; (−)-(2S,3S)THNA, human ADA = 80 nM, calf = 240 nM. Current studies include examination of these four isomers for cytotoxicity and antiviral properties.

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Calcium-stimulated glutamate decarboxylase activity in brain slices

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Calcium ions are established as critically important in excitation secretion coupling in neuronal synaptic function and in numerous other secretory cells in the mammal (for a review, see Ref. 1). Typical of the classical role of Ca^{2+} in synaptic function is the inhibition of stimulus-dependent γ-aminobutyric acid (GABA) release from synaptosomes when Ca^{2+} is omitted from the incubation medium [2]. At the molecular level, however, it is less clear how calcium couples neuronal stimulation to transmitter secretion and the extent to which the cation is important in other basic cellular functions. For example, there are voltage-sensitive Ca^{2+} channels in neuronal membranes and these channels participate in the generation of the action potential [3]. Data from studies with synaptosomes have shown that Ca^{2+} is sequestered within cells [4]. Ca^{2+}-dependent protein phosphorylation has been localized to neurons in brain [5], and calmodulin, as the intracellular receptor for calcium, is of key importance in cellular regulation [6].

There is some evidence that Ca^{2+} participates in the regulation of transmitter biosynthesis in serotonergic neurons [7], noradrenergic neurons [8], and in dopaminergic neurons [9]. The present study was carried out to define a role for Ca^{2+} in the regulation of GABA biosynthesis in brain GABAergic neurons.

The animals used were male Sprague–Dawley-derived rats from the Charles River Laboratories (Wilmington, MA). Labeled compounds and Aquassure were purchased from the New England Nuclear Corp. (Boston, MA), and ionophore A23187 was obtained from CalBiochem (La Jolla, CA). Amino acids and imidazole were purchased from the Sigma Chemical Co. (St. Louis, MO), $CoCl_2 \cdot 6H_2O$ was obtained from Fisher Scientific (Fair Lawn, NJ), and $LaCl_3 \cdot 7H_2O$ ("Gold Label") was supplied by the Aldrich Chemical Co. (Milwaukee, WI). Verapamil · HCl was a gift of the Knoll Pharmaceutical Co. (Whippany, NJ). All other chemicals and reagents were obtained