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INHIBITION OF ADENOSINE DEAMINASE FROM SEVERAL SOURCES BY DEAZA DERIVATIVES OF ADENOSINE AND EHNA

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Deaza analogues of adenosine and EHNA were tested as inhibitors of the enzyme adenosine deaminase (ADA) obtained from several sources including human erythrocytes, calf intestine, Saccaromices cerevisiae, *Escherichia coli* and Takadiastase.

K_i values of the inhibitors suggest differences among the enzymes both at purine and *erythro*-nonyl binding site. Among the ribofuranosyl derivatives, 1-deazaadenosine is the best inhibitor, its K_i ranging between 3.5×10^{-7} and 4×10^{-5} M for ADA from erythrocytes and Takadiastase respectively. Only ADA from erythrocytes and calf intestine bind EHNA and some of deazaEHNA analogues; 3-deaza-EHNA behaves very similarly to EHNA both in affinity and slow binding mechanism, whereas 1-deaza-EHNA, though less potent, is a good inhibitor.

KEY WORDS: Adenosine deaminase inhibition, adenosine derivatives, inhibition by substrate analogues, adenosine deaza analogues, *erythro*-9-(2-hydroxy-3-nonyl)-adenine deaza analogues

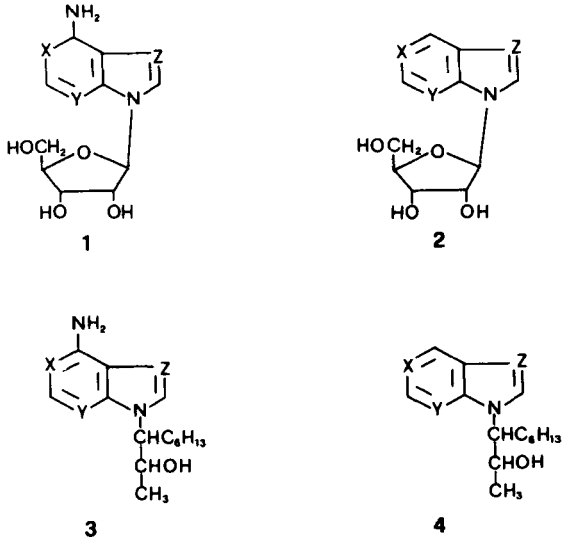
ABBREVIATIONS: EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-adenine; 1-deazaEHNA, *erythro*-9-(2-hydroxy-3-nonyl)-1-deazadenine; 3-deazaEHNA, *erythro*-9-(2-hydroxy-3-nonyl)-3-deazaadenine; 7-deaza-EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-7-deazaadenine; 1,3-dideazaEHNA, *erythro*-9-(2-hydroxy-3-nonyl)-1,3-dideazaadenine; 6-deaminoEHNA, *erythro*-9-(2-hydroxy-3-nonyl)-purine; 6-deamino-1-deaza-EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-3-deazapurine.

INTRODUCTION

Deamination of adenosine and deoxyadenosine to form inosine and deoxyinosine is catalyzed by adenosine deaminase (ADA, adenosine aminohydrolase EC 3.5.4.4.). This reaction, playing a central role in purine interconversion, allows adenosine to enter into purine salvage pathways and it is also related to nucleotide synthesis via adenosine kinase[1].

Absence of ADA, which results in severe immunodeficiency, leads to accumulation of deoxyadenosine. As a consequence the level of dATP in lymphoid tissues is greatly enhanced by a tissue-specific effect[2]. This event in turn might allosterically inhibit production of other deoxynucleotides by nucleoside diphosphate reductase and ultimately (might) effect DNA replication.

The inhibition of this enzyme is the object of many investigations with the aim of finding inhibitors which might be used as co-drugs, in combination with adenine nucleoside analogues, for treatment of both cancer and viral diseases. In fact recent experiments have demonstrated marked potentiation by ADA inhibitors of the



	X	Y	Z
a	N	N	N
b	CH	N	N
c	N	CH	N
d	N	N	CH
e	CH	CH	N
f	CH	N	CH

FIGURE 1 Structures of substrate analogues.

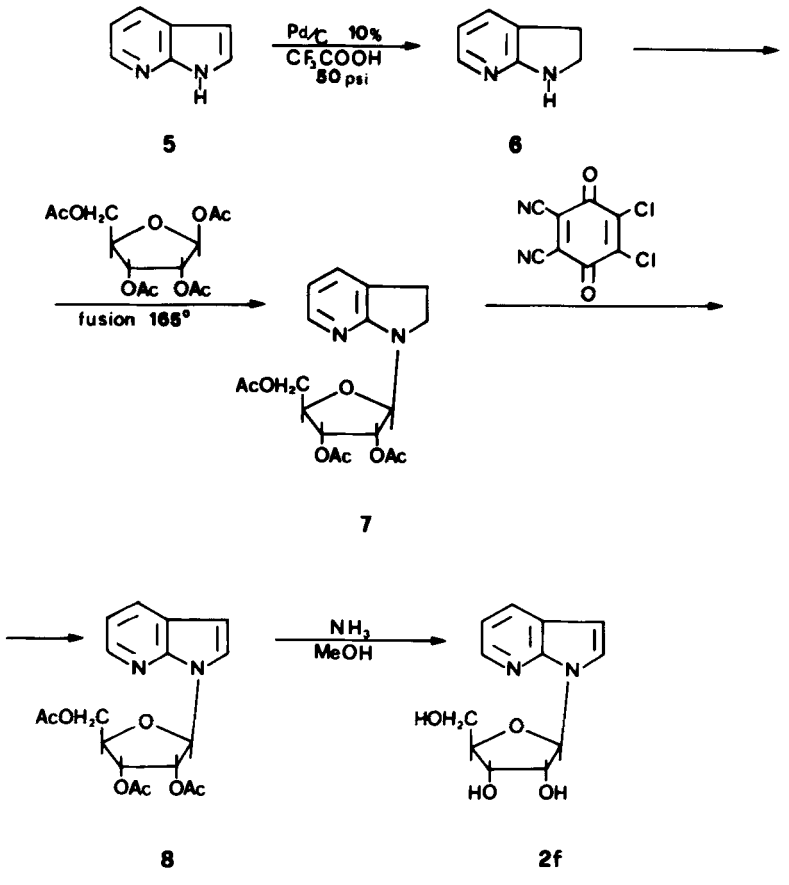
cytotoxic effects of cordycepin, xylosyladenine, adenine esalinoside and other adenosine analogues in a number of tumor systems[3].

ADA inhibitors might also have application in the chemotherapy of lymphoproliferative disorders, in the immunosuppressive therapy and in the study of the genetic deficiency of the enzyme[4,5].

To this purpose several adenosine analogs have been synthesized and some of them bind to the enzyme with high affinity[6]. One of the most interesting is EHNA, which is postulated to bind by its aliphatic chain to a hydrophobic site of ADA, different from the site normally occupied by the ribosidic moiety of the substrate.

As little was known on the effect of modifications of the heterocyclic moiety of EHNA, we have recently investigated the effect of isosteric substitution of nitrogen atoms 1, 3 or 7 in the purine moiety by carbon, either in EHNA or adenosine[7,8].

During these preliminary experiments we observed that ADA from calf intestine and yeast showed a different sensitivity to deaza derivatives. Since ADA structure is poorly elucidated and little comparative information is available on ADA from various species, analysis by means of inhibitors may stress differences that could be exploited for selective inhibition and drugs design.



Scheme 1

To evaluate whether the deaza analogues of EHNA or of adenosine may be sensitive probes at the active site of different molecular forms of ADA, we purified ADA from various sources including human red cells, yeast, *Escherichia coli* and Takadiastase. This paper reports the comparative effectiveness of such analogues on these enzymes and on the commercial ADA from calf intestine. The structure of the analogues is shown in Figure 1.

EXPERIMENTAL

Materials

Adenosine (**1a**), 7-deazaadenosine (**1d**), and purine riboside (**2a**) were purchased from Sigma Chemical Co. The other purine and nucleoside derivatives were synthesized by the published method: 1-deazaadenosine (**1b**)[9]; 3-deazaadenosine (**1c**)[10]; 1,3-dideazaadenosine (**1e**)[9]; 1,7-dideazaadenosine (**1f**)[11]; 1-deazapurine riboside (**2b**)[9]; 3-deazapurine riboside (**2c**)[12]; 7-deazapurine riboside (**2d**)[13]; 1,3-dideazapurine riboside (**2e**)[14]; EHNA (**3a**)[6]; 1-deazaEHNA (**3b**)[8]; 3-deazaEHNA (**3c**)[8]; 7-deazaEHNA (**3d**)[15]; 1,3-dideazaEHNA (**3e**)[15]; 6-deaminoEHNA (**4a**)[16]; 6-deamino-1-deazaEHNA (**4b**)[8]; 6-deamino-3-deazaEHNA (**4c**).[8] Calf intestine adenosine deaminase (200 U/mg at 25°C) was purchased from Boehringer Mannheim. Takadiastase crude powder was obtained from Serva.

Chemistry

Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H n.m.r. spectra were obtained with a Varian EM-390 90-MHz spectrometer, using tetramethylsilane as internal standard. TLC were carried out on precoated TLC plates with silica gel 60F-254 (Merck). For column chromatography, silica gel (Merck) was used. 1,7-Dideazapurine riboside (**2f**) is a new nucleoside which was synthesized following Scheme 1.

Several attempts to synthesize **2f** by direct glycosilation of 1H-pyrrolo[2,3-*b*]pyridine (**5**) led exclusively to the 1-*N*-acetyl derivative of **5**. For this reason compound **5** was hydrogenated with 10% Pd/C in trifluoroacetic acid at 50 psi to give the 2,3-dihydro derivative **6**. Fusion of **6** with tetra-*O*-acetyl-β-D-ribofuranose in the presence of a catalytic amount of *p*-toluenesulfonic acid afforded the protected nucleoside **7**. Oxidation of **7** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) followed by deacetylation with methanolic ammonia gave the desired compound **2f**.

2,3-Dihydro-1H-pyrrolo[2,3-b]pyridine (**6**). To a solution of 5 g (0.042 mol) of 1H-pyrrolo[2,3-b]pyridine (**5**) in 100 ml of trifluoroacetic acid was added 1.5 g of 10% Pd/C, and the mixture was shaken with hydrogen at 50 psi for 0.5 h. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was made basic (pH 9) with 12 N NaOH. The solution was extracted several time with $CHCl_3$, and the combined extracts were dried (Na_2SO_4) and evaporated. The residue was chromatographed on a silica gel column eluting with EtOAc–MeOH (90 : 10) to give 3 g (60%) of **6** as a pure white solide: mp 81–82°C; ^{1}H n.m.r. ($CDCl_3$) δ 3.03 (*t*, 2, H-3), 3.58 (*t*, 2, H-2), 4.57 (*s*, 1, NH), 6.47 (*m*, 1, H-5), 7.23 (*d*, 1, $J_{4,5}$ = 7 Hz, H-4), 7.82 (*d*, 1, $J_{5,6}$ = 5 Hz, H-6). (Found C, 69.62; H, 6.82; N, 23.07; requires C, 69.97; H, 6.71; N, 23.32%.)

1-(2′,3′,5′-Tri-O-acetyl-β-D-ribofuranosyl)-2,3-dihydro-1H-pyrrolo[2,3-b]pyridine (**7**). An intimate mixture of **6** (2.5 g, 20.8 mmol), 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (12 g, 37.3 mmol), and *p*-toluensulfonic acid (150 mg) was heated at 165°C with stirring *in vacuo* (25 mm) for 50 min. The resulting solid was neutralized with saturated Na_2CO_3 solution and extracted several times with EtOAc. The organic phase was dried (Na_2SO_4) and evaporated to give a residue, which was chromatographed on a silica gel column. Elution with EtOAc–C_6H_6 (90 : 10) yielded 2.4 g (30%) of **7** as a chromatographically pure oil and 1.2 g of 1-*N*-acetyl derivative of **6**. ^{1}H n.m.r. ($CDCl_3$) δ 3.02 (*t*, 2, H-3), 3.68 (*t*, 2, H-2), 4.23 (*s*, 3, CH_2-5′ and H-4′), 540 (*m*, 2, H-2′ and H-3′), 6.18 (*d*, 1, *J* = 7 Hz, H-1′), 6.59 (*m*, 1, H-5), 7.27 (*d*, 1, $J_{4,5}$ = 6 Hz, H-4), 7.95 (*d*, 1, $j_{5,6}$ = 5 Hz, H-6). (Found C, 57.06; H, 5.93; N, 7.25; requires C, 57.13; H, 5.86; N, 7.40%.)

1-(2′,3′-5′-Tri-O-acetyl β-D-ribofuranosyl) pyrrolo [2,3-b]pyridine (**8**). A mixture of 2.1 g (5.55 mmol) of **7**, dissolved in 120 ml of dry xylene, and 1.35 g (5.94 mmol) of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was heated under reflux for 2 h. The solid was removed by filtration and the filtrate was evaporated to dryness in vacuo. The residue was chromatographed on a silica gel column eluting with C_6H_6–EtOAc (60 : 40) to give 800 mg (40%) of **8** as a chromatographically homogeneous oil; ^{1}H n.m.r. ($CDCl_3$) δ 4.41 (*s*, 3, CH_2–5′ and H-4′), 5.66–5.88 (*m*, 2, H-3′ and H-2′), 6.63 (*m*, 2, H-3 and H-1′), 7.15 (*m*, 1, H-5), 7.41 (*d*, 1, *J* = 4 Hz, H-2), 7.95 (*d*, 1, $J_{4,5}$ = 7.5 Hz, H-4), 8.35 (*d*, 1, $J_{5,6}$ = 5 Hz, H-6). (Found C, 57.28; H, 5.52; N, 7.49; requires C, 57.44; H, 5.35; N, 7.44%.)

1-β-D-Rybofuranosyl-1H-pyrrolo[2,3-b]pyridine (**2f**). A solution of 750 mg (1.99 mmol) of **8** in 40 ml of methanol saturated with ammonia was set aside at room temperature for 24 h. The solvent was removed in vacuo and the residue was chromatographed on a silica gel column eluting with EtOAc–MeOH (90 : 10) to give 350 mg (71%) of **2f** as a pure solid: mp 151–153°C: ^{1}H n.m.r. (DMSO) δ 3.64 (*m*, 2, CH_2-5′), 3.95 (*m*, 1, H-4′), 4.14 (*m*, 1, H-3′), 4.52 (*m*, 1, H-2′), 6.28 (*d*, 1, *J* = 6 Hz, H-1′), 6.75 (*d*, 1, *J* = 4 Hz, H-3), 7.16 (*m*, 1, H-5), 7.78 (*d*, 1, *J* = 4 Hz, H-2), 8.02 (*d*, 1, $J_{4,5}$ = 7 Hz, H-4), 8.28 (*d*, 1, $J_{5,6}$ = 5 Hz, H-6). (Found C, 57.38; H, 5.81; N, 11.03; requires C, 57.59; H, 5.64; N, 11.20%.)

Purification of the Enzymes

Purification of ADA from human erythrocytes. The hemolyzate prepared as described by Brownson and Spencer[17], equilibrated in 50 mM imidazole buffer, pH 5.8, was treated with dry CH-Sephadex C 50 and stirred for 30 min at 4°C; the filtrate, essentially free of hemoglobin, was fractionated with ammonium sulfate at 4°C. The 30–60% fraction was collected and dialyzed against 0.1 M potassium phosphate buffer pH 6.9, 100 μM dithiothreitol overnight. Further purification was achieved by affinity chromatography on 6-amino-9-(*p*-aminobenzyl)adenine-Sepharose 4B according to Rossi *et al.*[18] The specific activity of the enzyme was 10.5 U/mg with K_m for adenosine 3.2 × 10^{-5} M.

Purification of ADA from Saccaromices cerevisiae. Commercial baker's yeast (1 kg) was stirred overnight in 0.1 M $NaHCO_3$, and 0.6 mM phenylmethylsulfonylfluoride. The slurry was homogenized with a Waring Blender and centrifuged and the supernatant was fractionated by ammonium sulphate. The 30–80% fraction was solubilized and dialyzed against 2.5 mM phosphate buffer pH 7.4 and then applied to a DEAE 52 cellulose column (cm 2.5 × 38) equilibrated with the same buffer. The elution was carried out by 2.5 mM phosphate buffer pH 7.4 with a linear gradient from 0 to 0.7 M NaCl. The active fractions were applied to a affinity chromatography column of 6-amino-9-(*p*-aminobenzyl)adenine-Sepharose 4B. The calculated K_i for this form of enzyme with 6-amino-9-(*p*-aminobenzyl)adenine was, 2.68×10^{-4}, higher value than that reported for other forms of ADA[18]. The original elution method was therefore modified by substituting guanyl urea with 0.7 mM adenosine, 0.2 M NaCl. The specific activity of this preparation was 9.66 U/mg with K_m for adenosine 4×10^{-5} M.

Purification of ADA from Escherichia coli. The stages of the purification were performed essentially according to the procedure of Nygeard[19]. After the hydroxylapatite step, the active fractions were pooled and dialyzed against 0.1 M potassium buffer pH 7.0, and applied to the affinity column 6-amino-9-(*p*-aminobenzyl)purine-Sepharose 4B[18]. Although the ADA is only retarded and not retained by this affinity column, it was separated from the bulk of the other proteins[20]. The active fractions were pooled and concentrated by ultrafiltration. ADA obtained by this procedure was essentially free of purine nucleoside phosphorylase activity. The specific activity was 55 U/mg with K_m for adenosine 5.2×10^{-5} M.

Purification of ADA from Takadiastase. ADA from Takadiastase was prepared according to the procedure of Wolfender *et al.*[21] The specific activity was 253 U/mg, with K_m for adenosine 1.23×10^{-4} M. The enzyme was stable for several weeks at 4°C in 50 mM potassium phosphate buffer pH 7.1 and several months after lyophylization.

Enzyme assay. The initial rate of hydrolysis of adenosine or the substrate analogues was measured according to the method of Kalkar[22] by following the change in absorbance at 265 nm, after addition of the enzyme to the substrate in 50 mM potassium phosphate buffer pH 7.2 and 0.1 M KCl. The assay was carried out at 25°C, in a Cary 219 spectrophotometer. One enzymatic unit is the amount of enzyme which converts 1 μmole of adenosine to inosine/min under the reported conditions. Ultraviolet absorption spectra for all substrates were checked before and after incubation. K_i values for EHNA (**3a**), 3-deaza EHNA (**3c**) were calculated from the rates obtained after 3 min preincubation of inhibitors and ADA to establish equilibrium[23]. K_i values were determined by the Lineweaver-Burk method. The protein content of the enzyme preparations was determined according to the method of Lowry[24].

RESULTS

Tables I and II show the obtained K_i values towards ADA of adenosine and deaza-EHNA analogues respectively.

1-Deazaadenosine binds to ADA from different sources with different affinity. In fact, this compound shows high affinity towards ADA from mammalian tissues, the reaction kinetic (not reported) indicating a competitive type of inhibition without lag time in the formation of the enzyme-inhibitor complex. An affinity, lower by one and two order of magnitude, is shown by 1-deazaadenosine towards ADA from yeast and from *Escherichia coli* and Takadiastase respectively.

3-Deazaadenosine is a very weak inhibitor of mammalian enzymes although it shows some affinity towards the yeast enzyme. 1,3-Dideazaadenosine similarly binds better to yeast enzyme than to mammalian ones. 7-Deaza and 1,7-dideazaadenosine are substantially inactive as inhibitors of all types of ADA.

The affinity of these adenosine deazaanalogues towards all the adenosine deaminases largely decreases when there is no amino group in 6-position.

Table II indicates that EHNA and its deaza and dideaza analogues bind almost selectively to ADA from mammalian sources; only EHNA shows a very low affinity towards ADA from yeast.

TABLE I
Inhibition constants for deazaadenosine analogues towards adenosine deaminase from different sources.

Inhibitors	Human erythrocytes K_i	Calf intestine K_iM	*Saccaromices cerevisiae* K_iM	Takadiastase K_iM	*Escherichia coli* K_iM
1b 1-deazaadenosine	3.55×10^{-7}	6.6×10^{-7}	4.67×10^{-6}	5.52×10^{-5}	4.02×10^{-5}
1c 3-deazaadenosine	2.42×10^{-4}	3.6×10^{-4}	3.81×10^{-5}	–	–
1d 7-deazaadenosine	–	–	–	–	–
1e 1,3-dideazaadenosine	3.86×10^{-5}	1.1×10^{-4}	3.66×10^{-6}	–	–
1f 1,7 dideazaadenosine	–	–	–	–	–
2a purin riboside	8.7×10^{-6}	8.8×10^{-6}	8.46×10^{-6}	1.83×10^{-5}	6.07×10^{-5}
2b 1-deazapurine riboside	3.02×10^{-5}	4.2×10^{-5}	4.97×10^{-5}	1.81×10^{-5}	1.27×10^{-4}
2c 3-deazapurine riboside	–	–	2.55×10^{-5}	–	–
2d 7-deazapurine riboside	–	–	–	–	–
2e 1,3-dideazapurine riboside	3.08×10^{-4}	7.6×10^{-4}	1.63×10^{-4}	–	–
2f 1,7-dideazapurine riboside	–	–	–	–	–

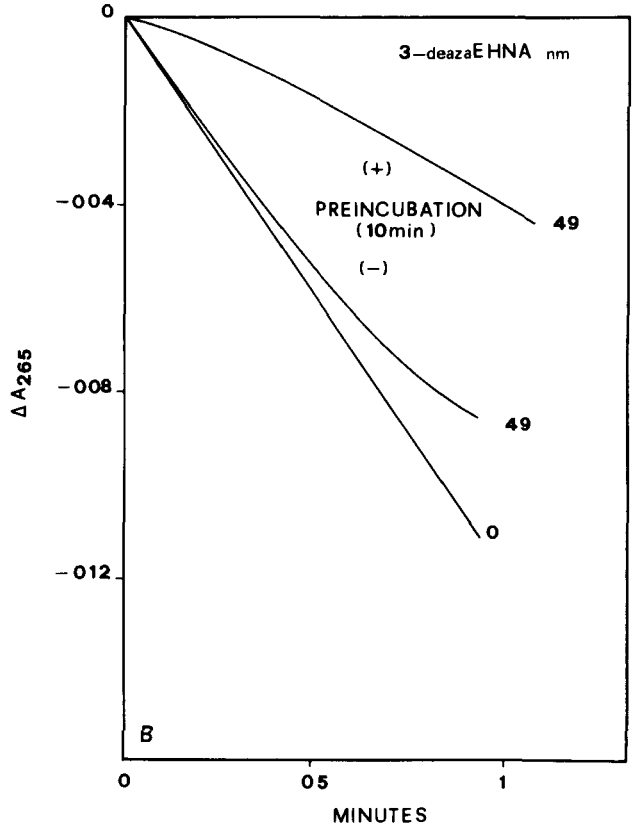


FIGURE 2 Spectrophotometric tracing of the reaction of human erythrocyte adenosine deaminase in the presence of 3-deazaEHNA with (+) and without (–) preincubation.

TABLE II
Inhibition constants for deazaEHNA analogues towards adenosine deaminase from different sources.

Inhibitors	Human erythrocytes $K_i M$	Calf intestine $K_i M$	*Saccaromices cerevisiae* $K_i M$	Takadiastase $K_i M$	*Escherichia coli* $K_i M$
3a EHNA	4.3×10^{-9}	7×10^{-9}	1.17×10^{-4}	–	–
3b 1-deazaEHNA	1.22×10^{-7}	1.6×10^{-7}	–	–	–
3c 3-deazaEHNA	6.35×10^{-9}	1×10^{-8}	–	–	–
3d 7-deazaEHNA	1.84×10^{-4}	4×10^{-4}	–	–	–
3f 1,3-dideazaEHNA	1.54×10^{-5}	7.05×10^{-5}	–	–	–
4a 6-deaminoEHNA	3.08×10^{-6}	8.4×10^{-7}	–	–	–
4b 6-deamino 1-deazaEHNA	2.1×10^{-7}	5.5×10^{-7}	–	–	–
4c 6-deamino 3-deazaEHNA	7.8×10^{-7}	1.2×10^{-7}	–	–	–

The affinity of ADA from red cells and calf intestine is higher for EHNA deaza-derivatives than for the corresponding ribofuranosyl derivatives. In particular the inhibitory activity of 3-deazaEHNA is strongly increased in comparison with that for 3-deazaadenosine. 3-DeazaEHNA also shows slow binding kinetics typical of ENHA, as illustrated in Figure 2. The K_i values calculated for 7-deazaEHNA are so high that this compound is considered inactive.

The presence of the amino group in position-6 is important also for this class of inhibitors with the exception of 1-deazaEHNA.

DISCUSSION

The results obtained by using deaza adenosines as inhibitors suggest the occurrence of some differences at the purine binding sites among ADA from several sources. In particular, the different affinity for 1-deazaadenosine, which is a good inhibitor of ADA from erythrocytes and calf intestine but is a weaker inhibitor of ADA from yeast and still weaker for ADA from *Escherichia coli* and Takadiastase, suggests variations of the fine structure at the active site. These variations might be related to the evolutionary level and apparently increase the flexibility of the active site of the mammalian enzymes.

3-Deazadenosine is a poor inhibitor. Its weak affinity supports the hypothesis that the N^3 of adenosine is involved in hydrogen bonding with the ribose 5′ hydroxylic group and therefore contributes to stabilize the molecule in just the right conformation to bind ADA[7,8,25]. The smaller K_i value shown by the yeast enzyme among the enzymes investigated, may suggest a broader specificity. Further studies to characterize this protein are being carried out.

Indirect evidence for the role of N^3 in mainly stabilizing the structure of the ribofuranosyl derivatives comes from the tight binding of 3-deazaEHNA. In fact the substitution of ribose by the *erythro*-nonyl chain in 3-deazaadenosine leads to a potent inhibitor of ADA from erythrocytes and calf intestine, which is very similar to EHNA also in its slow binding mechanism[23]. The same effectiveness is not obtained in the case of 1-deazaEHNA which, instead, behaves very similarly to 1-deazaadenosine.

1-DeazaEHNA is also noteworthy for the small contribution made to binding by the amino group in the 6-position which by contrast is an important requirement for the ADA derivatives. This data suggests that modifications at the purine moiety which lead to increased affinity for ADA may not be additive in the case of the *erythro*-nonyl derivatives, probably because of an unfavorable fit at the active site.

A general requirement to bind ADA from all species is the presence of the nitrogen atom in position-7, as no or very poor binding of 7-deaza derivatives is seen.

The specificity of adenosine deaminase towards the 9-substituted adenines such as EHNA can be explained by assuming the existence at or near the active site of a hydrophobic region able to bind the *erythro*-nonyl moiety. Such a region could restrict the area of the hydropholic site normally occupied by the ribosidic moiety of the substrate by freely rotating around the N9–C1 bond.

As ADA from various sources shows unequal sensitivity to EHNA, it is possible that differences occur in the hydrophobic region among the enzymes which were investigated. Table II shows that the sensitivity is high in enzymes from mammalian sources, very low in yeast enzyme and none in enzymes from Takadiastase and

Escherichia coli. This may be explained either by a different conformation of the active site or by the absence of the hydrophobic region. It is worthy of mention that the presence of molecular species of ADA insensitive to EHNA in invertebrates[20,26–28], or with reduced sensitivity in vertebrates[20,29] has been reported. This finding might suggest that the lack of EHNA binding capacity is not a peculiar property of the enzymes from organisms, though the EHNA-sensitive enzyme seems to be the representative form in vertebrates.

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