Biochemical Pharmacology, Vol. 41, No. 11, pp. 1615–1624, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

STRUCTURE–ACTIVITY RELATIONSHIP OF LIGANDS OF HUMAN PLASMA ADENOSINE DEAMINASE$_2$

JOHN G. NIEDZWICKI* and DARRELL R. ABERNETHY
Division of Clinical Pharmacology, Brown University, Providence, Rhode Island, U.S.A.

(*Received* 27 *December* 1989; *accepted* 10 *December* 1990)

Abstract—Diethylaminoethyl-cellulose chromatography was used to separate the two isoenzymes of adenosine deaminase (EC 3.5.4.4), adenosine deaminase$_1$ (ADA$_1$) and adenosine deaminase$_2$ (ADA$_2$), in human plasma. One hundred and fifteen purine base, nucleoside, and nucleotide analogs were tested as inhibitors of this partially purified preparation of ADA$_2$. Coformycin and 2′-deoxycoformycin were by far the most potent inhibitors of this isoenzyme (apparent K_i values 20 and 19 nM, respectively). ADA$_2$ was also inhibited by nebularine (apparent K_i 1.5 mM) but was resistant to the potent ADA$_1$ inhibitor (+)-*erythro*-9(2-*S*-hydroxy-3-*R*-nonyl)adenine. α-D-Adenosine also inhibited ADA$_2$, as did several halogenated purine and adenine base analogs. Structural requirements for the binding of purine analogs to ADA$_2$ are presented which provide a general basis for the design of specific inhibitors of ADA$_2$. Such inhibitors may be useful in studies designed to provide an understanding of the physiological role of ADA$_2$ both in the normal state and in diseases such as human immunodeficiency virus-1 infection where levels in plasma are increased markedly.

In humans and other mammals, adenosine deaminase (ADA†, adenosine aminohydrolase, EC 3.5.4.4) exists as two distinct isoenzymes which have been termed ADA$_1$ and ADA$_2$ [1]. ADA$_1$ typically constitutes 90–100% of the total intracellular ADA activity [1, 2] and has been studied extensively and characterized. The physiological significance of ADA$_1$ is exemplified by the fact that the congenital absence of this isoenzyme is associated with a form of severe combined immunodeficiency disease [3, 4]. By contrast, relatively little is known about ADA$_2$ and it is intriguing that this isoenzyme constitutes the majority of ADA activity in normal human serum or plasma [5–7] and that these levels are increased in patients infected with HIV [6, 7].

Evidence accumulated to date indicates that ADA$_1$ and ADA$_2$ are distinct proteins which differ significantly in a number of biochemical characteristics. ADA$_1$ exists as two major forms: a monomer of molecular weight 33,000 ("small form") and a dimer/combining protein complex with a total molecular weight of 280,000 ("large form") [1, 8]. These small and large forms of ADA$_1$ do not differ substantially with regard to biochemical parameters such as K_m for adenosine, inhibitor affinity, and substrate specificity [1, 9, 10]. ADA$_2$, on the other hand, exists only as a monomer with a molecular weight of 100,000 [1, 5, 8]. Mammalian ADA$_1$ and ADA$_2$ isoenzymes differ greatly with regard to K_m for adenosine (25 μM and 2–3 mM for ADA$_1$ and ADA$_2$, respectively [1, 5, 11]), substrate specificity (e.g. 2′-deoxyadenosine is a much better substrate, relative to adenosine, for ADA$_1$ than ADA$_2$ [5, 12]), and in inhibitor specificity (i.e. ADA$_2$ is relatively insensitive to the ADA$_1$ inhibitor (±)-EHNA [5, 11, 12]).

In the present study, we describe a method whereby plasma ADA$_2$ can be isolated from ADA$_1$ using DEAE-cellulose chromatography. One hundred and fifteen purine analogs have been evaluated as inhibitors of partially purified ADA$_2$, and a structure–activity relationship for the binding of such ligands has been formulated. These studies provide a basis for the design of isoenzyme-specific ADA$_2$ inhibitors which have potential utility as biochemical tools for elucidating the physiological role of ADA$_2$. A preliminary report has been presented [13].

MATERIALS AND METHODS

Chemicals. The sources of the purine analogs and other compounds screened as ADA$_2$ inhibitors are indicated in Table 1 by the following abbreviations: ALD, Aldrich Chemical Co., Milwaukee, WI; BB, Dr. Balkrishen Bhat, University of Illinois at Urbana-Champaign, Urbana, IL; BW, Burroughs-Wellcome Co., Research Triangle Park, NC; CAL, Calbiochem-Behring Corp., La Jolla, CA; CDC, Chemical Dynamics Corp., South Plainfield, NJ; EA, Dr. Elie Abushanab, The University of Rhode Island, Kingston, RI; GW, Dr. George Wright,

* Author to whom all correspondence should be addressed at the Division of Hematology/Oncology (111), Veterans Administration Medical Center, 150 South Huntington Ave., Boston, MA 02130.

† Abbreviations: ADA, adenosine deaminase; ADA$_1$, adenosine deaminase$_1$, ADA$_2$, adenosine deaminase$_2$; DEAE, diethylaminoethyl; (+)-EHNA, (+)-*erythro*-9-(2-*S*-hydroxy-3-*R*-nonyl)adenine; (±)-EHNA, (±)-*erythro*-9-(2-hydroxy-3-nonyl)adenine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HIV, human immunodeficiency virus-1; and PNP, purine nucleoside phosphorylase. Except where otherwise specified, all nucleosides and nucleotides described in this study were of the β-D-ribofuranosyl configuration.

The University of Massachusetts Medical Center, Worcester, MA; JAM, Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL; JPS, Dr. Jean-Pierre Sommadossi, The University of Alabama, Birmingham, AL; NCI, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD; REP, Dr. Robert E. Parks, Jr., Brown University, Providence, RI; RPP, Dr. Raymond P. Panzica, The University of Rhode Island, Kingston, RI; SHC, Dr. Shih Hsi Chu, Brown University, Providence, RI; and SIGMA, Sigma Chemical Co., St. Louis, MO. [8-^{14}C]Adenosine (approx. 50 mCi/mmol) was obtained from either NEN Research Products, Wilmington, DE, or Moravek Biochemicals, Brea, CA; silica gel G UV-254 TLC plates and DEAE-cellulose (DE-23) from Whatman, Hillsboro, OR; and Opti-fluor O from Packard, Downers Grove, IL. All other chemicals were obtained from either the Fisher Scientific Co., Pittsburgh, PA, or the Sigma Chemical Co., St. Louis, MO.

Human plasma. Frozen recovered human plasma was purchased from the Rhode Island Blood Center, Providence, RI.

Partial purification of human plasma ADA_2 by DEAE-cellulose chromatography. Recovered human plasma was dialyzed against 20 vol. of Buffer A (20 mM potassium phosphate, pH 6.5) for 24 hr at 4° to remove salts (which interfere with DEAE-cellulose chromatography) and adenine (which is added routinely to donated blood as a preservative). This plasma (100 mL) was then applied to a column of DEAE-cellulose (90 × 2.5 cm). The column was eluted with 700 mL of Buffer A followed by a linear gradient of NaCl to 1 M over 700 mL. Fractions (20 mL) were collected, and every other fraction was assayed for ADA_1 and ADA_2 activity as described below.

After it had been established that ADA_1 binds to DEAE-cellulose under the aforementioned conditions whereas ADA_2 does not (discussed below), routine preparation of ADA_2 for inhibitor screening was accomplished by applying plasma to a column as above and eluting off ADA_2 with 700 mL of Buffer A. The eluant was then brought to 80% saturation with $(NH_4)_2SO_4$, centrifuged to precipitate protein, and resuspended in 10 mL of Buffer B (100 mM sodium phosphate, pH 6.5). This preparation, subsequently referred to as partially purified ADA_2, could be stored for several months at −70° without appreciable loss of activity. When ADA_2 activity was quantitatively assayed using methodology described elsewhere [7], the yield of DEAE-cellulose purification was typically 25–50% and our partially purified ADA_2 had a specific activity of *ca.* 500 I.U./mg protein (1 I.U. = 1 μmol/min).

It should be mentioned that our preparation of partially purified ADA_2 was apparently free from PNP activity since all of the product in the reaction mixtures was in the form of inosine and none in hypoxanthine (described below). These findings are consistent with the report of Stoeckler *et al.* [14] and indicate that PNP binds to DEAE-cellulose and can thus be effectively separated (along with ADA_1) from ADA_2 by DEAE-cellulose chromatography. PNP catalyzes the phosphorolysis of many purine nucleosides, and the absence of this enzyme from our ADA_2 inhibition assay system eliminated the potentially confounding factor of base formation from nucleosides being tested.

ADA assay and screening of compounds as ADA_2 inhibitors. ADA isoenzyme activity was measured using a radioisotopic assay method with separation of substrate and products by TLC. Reaction velocities were calculated according to the percent conversion of [8-^{14}C]adenosine to [8-^{14}C]inosine. Since [8-^{14}C]hypoxanthine may be formed from [8-^{14}C]inosine via the catalysis of PNP (EC 2.4.2.1) [15], this compound was also routinely calculated as a product of the ADA reaction. No significant quantities of [8-^{14}C]hypoxanthine were formed in any of the reaction mixtures in which our partially purified ADA_2 was used.

For the assay of ADA_1 and ADA_2 in DEAE-cellulose column eluant, reaction mixtures consisted of 70 μL of eluant, 50 μL of substrate (adenosine, 0.5 mCi/mmol prepared in Buffer B, to give a final concentration of 0.1 mM) and 20 μL of Buffer B. Reaction mixtures were prepared both with and without the inclusion of 0.1 mM (+)-EHNA to distinguish ADA_1 from ADA_2 since the latter is insensitive to this concentration fo inhibitor [1, 5, 8]. (+)-EHNA is the active isomer of (±)-EHNA and was thus used in these studies [16].

Reaction mixtures prepared for the screening of compounds as ADA_2 inhibitors were 140 μL in volume and consisted of 1–2 mg of partially purified ADA_2, 0.1 mM [8-^{14}C]adenosine (0.5 mCi/mmol), 0.1 mM (+)-EHNA, and putative inhibitor, all prepared in Buffer B. Compounds to be screened were dissolved immediately prior to assay and were typically included at concentrations of 0.05, 0.1, 0.25, 0.5, and 1.0 mM although lower concentrations had to be used with compounds that were poorly soluble in water or which were potent inhibitors. The substrate concentration for these studies (0.1 mM) is far below the K_m of 2 mM for adenosine for ADA_2 [1, 8] and was chosen so as to allow for the testing of most compounds at a maximal concentration of 1 mM (i.e. 10-fold greater than substrate). Many purine analogs are not water soluble at concentrations above 1 mM, and thus it would not have been possible to assess the relative affinity of many poorly soluble, weakly binding compounds had a higher substrate concentration been used. The large amount of enzyme used in each assay was necessitated by the fact that the adenosine concentration used (0.1 mM) is far below the K_m of ADA_2 for this substrate (2 mM).

Reactions were carried out at 37° for 2 hr in the case of DEAE-cellulose column eluant and for 1 hr for inhibitor studies. Reactions were typically started by the addition of the enzyme source except when compounds were to be tested as potential "tight-binding" inhibitors [17] in which case the enzyme and inhibitor were preincubated for 20 min at 37° prior to starting the reaction with substrate. At time 0 and 1 or 2 hr, 50 μL aliquots of each reaction mixture were added to 5 μL of 40% (v/v) $HClO_4$ to stop the reaction. The samples were then centrifuged to precipitate protein, and 25 μL aliquots of the

supernates added to 55 μL of 0.2 N KOH which neutralized the samples. This KOH solution also contained 1–5 mM concentrations of adenine, adenosine, inosine, and hypoxanthine which served as carriers to facilitate visualization on TLC. The neutralized samples were then chilled and centrifuged to precipitate $KClO_4$, and 20 μL aliquots of the supernates were spotted on silica gel G TLC plates. These plates were then developed using 2-propanol:ethyl acetate:8 M ammonium hydroxide (9:4:3) for separation of nucleosides and bases as reported previously by others [18]. UV-quenching spots corresponding to hypoxanthine, inosine, adenosine, and adenine were then cut out and placed in vials containing 20 mL of Opti-fluor O, and radioactivity was quantitated.

Screening of nucleotide analogs as ADA_2 inhibitors. Nucleotide analogs (compounds **LXXIX–XC**, Table 1) were screened as ADA_2 inhibitors using a phosphate-free system since it was possible that enzyme-bound phosphate might prevent the binding of nucleotides but not nucleosides or bases. It is worthwhile to mention that such a situation exists for PNP, where nucleotide analogs of 2′-deoxyguanosine have 5- to 34-fold less affinity for the enzyme in high phosphate buffer [19]. Thus, the testing of nucleotide analogs (**LXXIX–XC**) as ADA_2 inhibitors was carried out with a preparation of partially purified human plasma ADA_2 that had been exhaustively dialyzed against 20 vol. of 20 mM HEPES, pH 6.5 (Buffer C). All components of these reaction mixtures were prepared in Buffer C. There was no appreciable difference in ADA_2 activity between control reactions carried out in Buffers B or C. Several nucleotide analogs (**LXXXII, LXXXIII, LXXXIV**) were also treated as inhibitors in reaction mixtures prepared in phosphate containing Buffer B and were non-inhibitory as was the case when Buffer C was used.

Determination and significance of apparent K_i values. Apparent K_i values were determined from Dixon plots ($1/v$ vs $[I]$) of the data by a computer program written by Dr. Sungman Cha using the general principles of Cleland [20]. Apparent K_i values are related to true K_i values by the following equation:

$$\text{apparent } K_i = \frac{K_i\,(1 - [S]/K_m)}{1 - ([S]/K_m)(K_{is}/K_{ii})},$$

where K_{is} and K_{ii} are the inhibition constants that would have been estimated from the replots of slope and intercept, respectively, of a Lineweaver–Burk plot versus $[I]$. If a compound is a competitive inhibitor with regard to a substrate, $K_{ii} = \infty$ and $K_{is} = K_i$. Therefore,

$$\text{apparent } K_i = K_i\,(1 - [S]/K_m).$$

Thus, for ADA_2, which has a K_m value of 2 mM for adenosine [1, 5], the apparent K_i value of a competitive inhibitor is approximately equal to K_i. Active compounds were not characterized with regard to the type of inhibition produced (i.e. competitive, non-competitive, or uncompetitive).

Protein estimation. Protein concentrations were estimated by the Bio-Rad Laboratories (Richmond, CA) assay procedure using bovine serum albumin as a standard.

RESULTS

Apparent K_i values for compounds that were active as ADA_2 inhibitors are presented in Table 1 along with non-inhibitory compounds. Roman numerals cited in the following text refer to compounds listed in Table 1.

The results of DEAE-cellulose chromatography of ADA activity in human plasma are shown in Fig. 1. (+)-EHNA-insensitive ADA_2 eluted from the column with the wash, whereas (+)-EHNA-sensitive ADA_1 was retained until a linear gradient of NaCl was applied.

Preincubation of coformycin (**LI**) or 2′-deoxycoformycin (**LVII**) did not appreciably change the apparent K_i values for these compounds. This finding

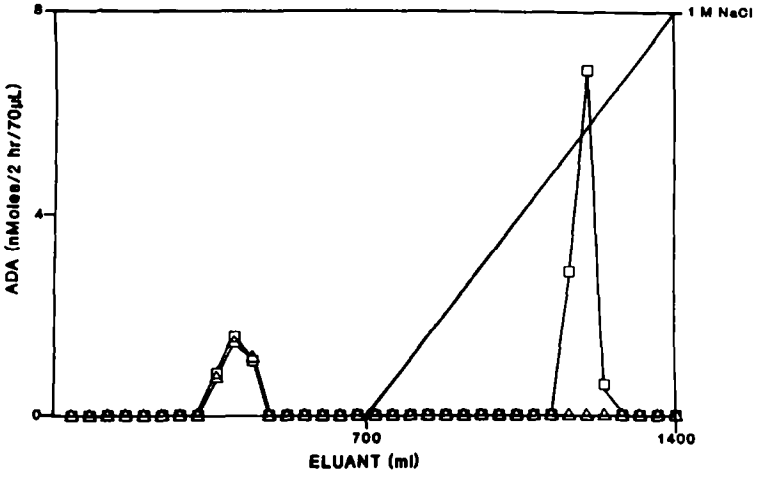

Fig. 1. DEAE-cellulose chromatography of ADA activities in human plasma. Fractions were assayed for ADA activity in the absence (□) and presence (△) of 0.1 mM (+)-EHNA. ADA_2 eluted with the solvent front, while (+)-EHNA-sensitive ADA_1 eluted after a linear gradient of NaCl was applied.

Table 1. Apparent K_i values for inhibition of human plasma ADA_2

	Compound	Source	Apparent K_i (mM)
I	Adenine	SIGMA	0.617 ± 0.089
II	Purine	CDC	N.I.*
1-Position substitution			
III	1-Methyladenine	CDC	N.I.
2-Position substitution			
IV	2-Chloroadenine	CDC	0.558 ± 0.089
V	2-Fluoroadenine	NCI	0.154 ± 0.021
VI	2-Hydroxyadenine	SIGMA	2.954 ± 0.974
VII	2-Methyladenine	SIGMA	0.370 ± 0.080
3-Position substitution			
VIII	3-Methyladenine	CDC	5.820 ± 1.708
6-Position substitution			
IX	6-Chloropurine	CDC	0.483 ± 0.074
X	*N*-6-Dimethyladenine	CDC	3.515 ± 0.892
XI	6-Dodecyladenine	CDC	N.I. (0.1†)
XII	Hypoxanthine	SIGMA	N.I.
XIII	6-Iodopurine	CDC	1.681 ± 0.989
XIV	6-Mercaptopurine	SIGMA	N.I.
XV	6-Methoxypurine	CDC	2.996 ± 0.529
XVI	*N*-6-Methyladenine	SIGMA	0.660 ± 0.080
XVII	6-Methylpurine	CDC	1.823 ± 0.529
8-Position substitution			
XVIII	8-Azaadenine	SIGMA	0.219 ± 0.015
XIX	8-Bromoadenine	CDC	0.184 ± 0.024
9-Position substitution			
XX	(+)-EHNA	EA	3.548 ± 1.196
XXI	9-Ethyladenine	CDC	1.377 ± 0.152
Purine bases substituted at more than one position			
XXII	Allopurinol	SIGMA	5.699 ± 1.131
XXIII	2-Amino-6-chloropurine	CDC	0.585 ± 0.073
XXIV	2-Amino-6,8-dithiopurine	NCI	N.I. (0.2†)
XXV	2-Aminopurine	CDC	N.I. (0.5†)
XXVI	8-Azaguanine	CDC	1.582 ± 0.319
XXVII	8-Azahypoxanthine	CDC	N.I.
XXVIII	8-Azaxanthine	CDC	N.I.
XXIX	8-Bromoguanosine	CDC	N.I.
XXX	2,6-Dichloropurine	CDC	0.110 ± 0.010
XXXI	2,6-Dimercaptopurine	CDC	N.I. (0.1†)
XXXII	2-Fluoro-8-azaadenine	JAM	0.269 ± 0.079
XXXIII	Guanine	SIGMA	N.I. 0.2†)
XXXIV	Xanthine	SIGMA	N.I. (0.5†)
Purine nucleosides and nucleoside analogs			
XXXV	Adenosine	SIGMA	2.180 ± 0.512
XXXVI	α-D-Adenosine	SIGMA	0.523 ± 0.092
XXXVII	Adenosine-*N*-1-oxide	CDC	N.I.
XXXVIII	2-Amino-6-chloropurine riboside	CDC	N.I.
XXXIX	4-Aminoimidazo[4,5-*d*]pyridazine-β-D-ribofuranoside	RPP	N.I.
XL	8-Aminopurine riboside	JAM	2.141 ± 0.569
XLI	Arabinosyladenine	SIGMA	0.580 ± 0.108
XLII	Arabinosylhypoxanthine	SIGMA	3.933 ± 0.871
XLIII	8-Azaadenosine	REP	0.285 ± 0.033
XLIV	8-Bromoadenosine	CDC	2.624 ± 0.802
XLV	8-Bromopurine riboside	JAM	N.I.
XLVI	Carbocyclic adenosine	JAM	5.949 ± 3.084
XLVII	2-Chloroadenosine	SIGMA	2.923 ± 1.036
XLVIII	6-Chloropurine riboside	CDC	N.I.
XLIX	8-Chloroadenosine	JAM	1.130 ± 0.173
L	8-Chloropurine riboside	JAM	N.I.
LI	Coformycin	CAL	0.000020 ± 0.000003
LII	Cordycepin (3'-deoxyadenosine)	SIGMA	3.514 ± 0.557
LIII	9-Deazaadenosine	REP	0.613 ± 0.085
LIV	2'-Deoxyadenosine	SIGMA	1.357 ± 0.233
LV	α-D-2'-Deoxyadenosine	GW	0.746 ± 0.126
LVI	α-D-2'-Deoxy-2-bromoadenosine	GW	1.112 ± 0.216
LVII	2'-Deoxycoformycin	EA	0.000019 ± 0.000002
LVIII	2'-Deoxyinosine	CDC	N.I.
LIX	5'-Deoxy-5'-methylthioadenosine	SIGMA	N.I.
LX	2',3'-Dideoxyadenosine	NCI	4.281 ± 0.815
LXI	2',3'-Dideoxyinosine	CAL	N.I.

Table 1. *continued.*

	Compound	Source	Apparent K_i (mM)
LXII	2,8-Dichloroadenosine	CDC	4.573 ± 1.314
LXIII	2',3'-Diisopropylidene adenosine	SIGMA	1.718 ± 0.262
LXIV	2-Fluoro-8-azaadenosine	JAM	0.425 ± 0.112
LXV	Formycin A	SHC	0.188 ± 0.016
LXVI	Formycin B	SIGMA	N.I.
LXVII	Guanosine	SIGMA	N.I. (0.5†)
LXIII	8-Hydroxymethyladenosine	JAM	N.I.
LXIX	8-Hydroxymethylpurine riboside	JAM	N.I.
LXX	Inosine	SIGMA	N.I.
LXXI	6-Mercaptopurine riboside	CDC	N.I.
LXXII	2'-*O*-Methyladenosine	SIGMA	N.I.
LXXIII	3'-*O*-Methyladenosine	SIGMA	N.I.
LXXIV	*N*-6-Methyladenosine	SIGMA	4.971 ± 1.646
LXXV	*N*-6-Methylmercaptopurine riboside	CDC	N.I.
LXXVI	Nebularine (purine riboside)	CDC	1.481 ± 0.283
LXXVII	*N*-6-Phenyladenosine	JAM	N.I.
LXXVIII	Tubercidin	SIGMA	N.I.
Nucleotides and nucleotide analogs			
LXXIX	Adenosine-3':5'-cyclic monophosphate	SIGMA	N.I.
LXXX	Adenosine-5'-diphosphate	SIGMA	N.I.
LXXXI	Adenosine-3'-monophosphate	SIGMA	N.I.
LXXXII	Adenosine-5'-monophosphate	SIGMA	N.I.
LXXXIII	Adenosine-5'-triphosphate	SIGMA	N.I.
LXXXIV	Cytidine-5'-triphosphate	SIGMA	N.I.
LXXXV	2'-Deoxyadenosine-5'-diphosphate	SIGMA	N.I.
LXXXVI	2'-Deoxyadenosine 5'-monophosphate	SIGMA	N.I.
LXXXVII	2'-Deoxyadenosine-5'-triphosphate	SIGMA	N.I.
LXXXVIII	2'-Deoxycytidine-5'-triphosphate	SIGMA	N.I.
LXXXIX	Guanosine-5'-monophosphate	SIGMA	N.I.
XC	Inosine-5'-monophosphate	SIGMA	N.I.
Other compounds			
XCI	7-Acetyl-7,8-dihydro-8-ethoxyimidazo-[1,5-*a*]pyridazine	EA	N.I.
XCII	7-Acetyl-7,8-dihydro-8-hydroxy-3-methylimidazo[1,5-*a*]pyridazine	EA	4.286 ± 1.227
XCIII	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside	SIGMA	2.616 ± 1.271
XCIV	4-Aminoimidazo[4,5-*d*]pyridazine	RPP	1.352 ± 0.318
XCV	4-Aminopyrazolo[3,4-*d*]pyrimidine	CDC	1.559 ± 0.261
XCVI	4-Amino-1,2,3-triazolo[4,5-*d*] pyridazine	RPP	0.983 ± 0.218
XCVII	Arabinosylcytosine	SIGMA	N.I.
XCVIII	3'-Azidothymidine (Zidovudine, AZT)	BW	N.I.
XCIX	*lin*-Benzoadenine	BB	N.I. (0.125†)
C	Benzotriazole	CDC	6.740 ± 2.460
CI	6-Chloro-2,4-diaminopyrimidine	CDC	N.I.
CII	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	ALD	5.277 ± 1.106
CIII	4-Chloro-7-sulfobenzofurazan	CDC	N.I.
CIV	Cytidine	SIGMA	N.I.
CV	Cytosine	SIGMA	N.I.
CVI	2'-Deoxycytidine	SIGMA	N.I.
CVII	2',3'-Didehydro-2',3'-dideoxythymidine (D4T)	JPS	N.I.
CVIII	5,7-Dimethyl-*s*-triazolo-[1,5-*a*]-pyrimidine	ALD	N.I.
CIX	5-Fluorocytosine	CDC	N.I.
CX	4-Fluoro-7-nitrobenzofurazan	ALD	0.167 ± 0.017
CXI	4-Hydroazinoimidazo[4,5-*d*]pyridazine	RPP	2.437 ± 0.553
CXII	4-Hydrazino-1,2,3-triazolo[4,5-*d*] pyridazine	RPP	N.I.
CXIII	5-Nitrobenzotriazole	ALD	1.176 ± 0.137
CXIV	Ribavirin	SIGMA	N.I.
CXV	1,2,4-Triazolo[1,5-*a*]pyrimidine	ALD	N.I.

Apparent K_i values ± SE were determined from triplicate assays using five concentrations of inhibitor and a control. Statistical estimations were calculated according to the general method of Cleland [20].

* Less than 10% inhibition of ADA_2 at an inhibitor concentration of 1.0 mM (i.e. 10-fold in excess of substrate).

† The maximum millimolar concentration that could be tested because of poor water solubility.

suggests that these compounds are not "tight-binding" inhibitors [17] of ADA_2.

DISCUSSION

The purpose of this work was to systematically define the structural requirements for the binding of purine analogs to human ADA_2. Starting with the simple purine adenine (**I**), our strategy was to quantitate the effects of substituents on binding with the aim of ultimately providing a basis for the design of inhibitors of this isoenzyme.

It must be emphasized that we examined compounds only as potential inhibitors of ADA_2 and it is possible that many of the adenosine analogs which bind to this isoenzyme are also substrates. Indeed, it has been shown that chicken ADA_2 will catalyze the deamination of 2′-*O*-methyladenosine, 3′-*O*-methyladenosine and various other adenosine analogs, albeit at velocities much slower than observed with adenosine [8]. This property has led some investigators to refer to ADA_2 as a "nonspecific aminohydrolase" [11, 12, 21] although evidence to date indicates that adenosine is the single best substrate. With regard to the present study, we feel that it is reasonable to assume that any compound which was a substrate for ADA_2 also would by definition compete with adenosine and thus be identified as a ligand.

Prior to a discussion of the binding of purine analogs to ADA_2, some general concepts regarding the chemistry of substituted purines are appropriate. As a general rule, amino-substituted purine bases and nucleosides exist in solution exclusively as the amino, rather than imino, tautomers [22, 23]. Likewise, hydroxy- and mercapto-substituted purines assume keto and thione rather than enol or thiol tautomers [22]. Furthermore, the majority of purine bases and nucleosides exist as uncharged species under the conditions of our assay (pH 6.5) as their pK_a values are generally >9 [24, 25]. Exceptions to these rules do occur and will be discussed where appropriate.

Purine bases substituted at the 1-position. Substitution of a methyl group at the 1-position of adenine (i.e. 1-methyladenine, **III**) abolished binding to ADA_2. It is difficult to draw any specific conclusions from this observation, since this chemical modification drastically changes the structure of the adenine molecule. In addition to potential steric effects of the *N*-1 methyl group, **III** is one of the few adenine analogs in which the exocyclic 6-position nitrogen exists primarily as an imine rather than an amine [23] and furthermore this nitrogen may be significantly protonated and thus positively charged at physiologic pH. The pK_a for protonation of the imino group of **III** is 7.2 [26] and this is quite different when compared to the pK_a values of adenine (9.87 and >12 for the imidazole [24] and amino [27] protons, respectively).

Adenosine-*N*-1-oxide (**XXXVII**) did not bind to ADA_2. The presence of an exocyclic negatively charged oxo group [23], steric factors, and localization of a positive charge around *N*-1 on **XXXVII** [23] are all possible contributing factors to this observation.

Purine bases substituted at the 2-position. 2-Methyladenine (**VII**) bound slightly better to ADA_2 than adenine (**I**), indicating some tolerance for substitution at this position. The 2-oxo group of 2-hydroxyadenine (**VI**) decreased binding nearly 5-fold. 2-Hydroxyadenine (**VI**) exists primarily (i.e. 95%) as the keto tautomer in solution [28] and the relative proportions of the *N*-1 and *N*-3 protonated forms are unknown, thus making it difficult to draw definitive conclusions from this finding.

2-Fluoradenine (**V**) bound 3-fold better than adenine (**I**) to ADA_2. This finding is certainly a reflection of the electron withdrawing effects of the fluorine atom [29], since it otherwise very closely resembles hydrogen in terms of Van der Waals radius and hydrophobic character [30]. It is interesting to note that the pK_a for 2-fluoradenine (**V**) does not differ greatly from that of adenine (**I**) (9.6 [31] and 9.8 [24], respectively) despite the electron withdrawing effects of fluorine. Thus, the increased proportion of an ionic species is not responsible for the tighter binding of **V** relative to **I**. It is also noteworthy that a *C*-2 fluorine group renders the 6-position of adenosine analogs resistant to enzymatic deamination as evidenced by the binding but lack of substrate activity of 2-fluoroadenosine with ADA_1 [32].

Purine bases substituted at the 3-position. 3-Methyladenine (**VIII**) bound to ADA_2 nearly 3-fold less tightly than adenine (**I**). Both steric effects of the *N*-3 methyl group and increased basicity (pK_a 6.1) [33] of the imidazole ring [34] relative to adenine (pK_a 4.12) [35] may be involved in this finding. Unlike 1-methyladenine (**III**), 3-methyladenine (**VIII**) retains the amino configuration at *C*-6 [36].

Purine bases substituted at the 6-position. Hypoxanthine (**XII**) and 6-mercaptopurine (**XIV**) did not bind to ADA_2 and neither did their corresponding ribonucleosides, inosine (**LXX**) and 6-mercaptopurine riboside (**LXXI**). Steric or electrostatic factors involving these 6-position substituents do *not* appear to be responsible for this finding as evidenced by the large variety of 6-position substitutent purines that did bind to ADA_2 [specifically, adenine itself (**I**), 6-chloropurine (**IX**), *N*-6-dimethyladenine (**X**), 6-iodopurine (**XIII**), 6-methyoxypurine (**XV**), *N*-6-methyladenine (**XVI**), and 6-methylpurine (**XVII**)]. The unique feature of hypoxanthine (**XII**), 6-mercaptopurine (**XIV**), and their ribonucleosides **LXX** and **LXXI** in comparison with the aforementioned compounds is that they exist almost exclusively as keto or thione tautomers in which *N*-1 is protonated [22]. We thus conclude that *N*-1 in purines must not be protonated in order to permit binding to ADA_2. It is conceivable that *N*-1 of adenine participates in hydrogen bonding in the active site of ADA_2, as has been proposed for ADA_1 [37].

Although 6-mercaptopurine (**XIV**) is considerably more acidic than adenine (**I**) (pK_a 7.8 vs 9.8 [25]), this fact does not seem to be playing a role in the failure of **XIV** to bind to ADA_2. In fact, one might expect that the increased acidity of **XIV** would *enhance* binding to ADA_2, as evidenced by the relatively tight binding of 6-chloropurine (**IX**) (pK_a 7.7 [25]) to this enzyme. The monoanion forms of both **IX** and **XIV** appear to be formed via loss of a

proton from the imidazole ring [25]. We concluded that the presence of a proton on *N*-1 of **XIV** is the major factor which precludes binding to ADA_2.

Purine bases substituted at the 7-position. Unfortunately, no 7-position substituted purine bases were available for study; however, some conclusions can be drawn from our data on nucleoside analogs. *N*-7 appears to be necessary for the binding of purine analogs to ADA_2, as tubercidin ("7-deazaadenosine," **LXXVIII**) was non-inhibitory. **LXXVIII** has a basic pK_a of 5.3 [38] which is higher than that of most purines (i.e. <4.5 [25]). However, ionized forms of **LXXVIII** are in such low proportion (6%) that the foregoing conclusions regarding the necessity of *N*-7 for binding to ADA_2 seem justified.

Purine bases substituted at the 8-position. 8-Azaadenine (**XVIII**) bound to ADA_2 nearly 3-fold better than adenine. While this compound retains the amino configuration at *C*-6 [39], substitution of an endocyclic nitrogen for *C*-8 drastically changes the physical properties of **XVIII** relative to adenine (**I**). In particular, **XVIII** exists to a large extent in solution as an anion (pK_a 6.27), probably via loss of a proton from *N*-9 of the triazole ring rather than the *C*-6 amino group [39]. The ribonucleoside of 8-azaadenine (i.e. 8-azaadenosine, **XLIII**) also bound well to ADA_2.

Purine bases substituted at the 9-position. Although the ethyl group of 9-ethyladenine (**XXI**) was tolerated, the long hydrophobic 9-position substituent of (+)-EHNA (**XX**) decreased binding nearly 6-fold relative to adenine (**I**). The poor binding of (+)-EHNA to ADA_2 (apparent K_i 3.5 mM) reflects a significant difference between the active site of this isoenzyme and that of ADA_1 for which (+)-EHNA is an extremely potent inhibitor (K_i 10^{-9} M) [16].

The binding of two nucleoside analogs to ADA_2 is appropriate to mention here. 9-Deazaadenosine (**LIII**) bound 4-fold more tightly than adenosine (**XXXV**) and likewise formycin A ("8-aza-9-deazaadenosine," **LXV**) bound slightly better than 8-azaadenosine (**XLIII**). Little is known of the chemical properties and tautomerism of **LIII** and experimental [40] versus theoretical [41] methodologies provide conflicting data on the preferred tautomeric form of **LXV**. We thus cannot draw any clear conclusions about replacement of *N*-9 of adenosine analogs with a carbon beyond stating that the two compounds tested (**LIII** and **LXVI**) having such a substitution bound well to ADA_2.

Nucleoside analogs. The fact that 8-bromoadenosine (**XLIV**) (a *syn* conformer [42, 43]) bound to ADA_2 only slightly less well than adenosine (**XXXV**) would appear to indicate that *syn* and *anti* conformational differences are not important in the binding of nucleoside analogs to this enzyme. Furthermore, it seems reasonable to assume that the bulky *C*-8 substituent of 8-chloroadenosine (**XLIX**) also causes a preference for the *syn* conformation and the fact that **XLIX** binds to ADA_2 with an apparent K_i value similar to that of adenosine (**XXXV**) also supports this hypothesis.

In general, purine bases had greater affinity for ADA_2 than their corresponding ribonucleosides or deoxyribonucleosides. This relationship is most clearly exemplified by the 5-fold lower apparent K_i of 2-chloroadenine (**IV**) as compared to 2-chloroadenosine (**XLVII**). The *C*-2 chloro group of **XLVII** renders the 6-position resistant to deamination [44, 45] and the molecule very likely retains the *anti* conformation of adenosine [46]; thus a direct comparison of the binding of **IV** and **XLVII** seems reasonable. Other purine bases also bound more tightly than their corresponding nucleoside analogs [e.g. adenine (**I**) vs adenosine (**XXXV**) or 2′-deoxyadenosine (**LIV**); 6-chloropurine (**IX**) vs 6-chloropurine riboside (**XLVIII**)]; however, in these cases apparent K_i values for nucleosides may be overestimated because they could be ADA_2 substrates and thus be metabolized *in situ* to poorly binding inosine analogs.

An unexpected observation was the 4-fold tighter binding of α-D-adenosine (**XXXVI**) to ADA_2 as compared to the naturally occurring anomer (β)-adenosine (**XXXV**). Similarly, α-D-2′-deoxyadenosine (**LV**) was 2-fold more potent than (β)-2′-deoxyadenosine (**LIV**) and α-D-2′-deoxy-2-bromoadenosine (**LVI**) also bound well to ADA_2. α-D-Adenosine analogs bind very poorly to ADA_1 [44, 47, 48], and this finding implies significant differences in the structural requirements for the binding of ribose modified analogs of adenosine between ADA_1 and ADA_2.

Transition state inhibitors. Coformycin (**LI**) and 2′-deoxycoformycin (**LVII**), which resemble the transition state of the ADA catalyzed reaction [49], were the most potent ADA_2 inhibitors identified in this study. These findings are consistent with the report of Daddona and Kelley who found 99% inhibition of human spleen ADA_2 with 20 μM 2′-deoxycoformycin [11]. [The apparent K_i value for 2′-deoxycoformycin (**LVII**) given in Table 1 is more accurate than the value from our preliminary studies [13] which were limited by the small quantities of **LVII** that were available to us at that time.]

The fact that nebularine (purine riboside, **LXXVI**) bound to ADA_2 slightly better than adenosine (**XXXV**) whereas the analogous base purine (**II**) was non-inhibitory may be relevant to the present discussion of transition state inhibitors. Elegant studies by Kurz and Frieden [50] and Jones *et al.* [51] have shown that purine riboside is hydrated at the 6-position in the active site of ADA_1 to yield *in situ* a tetrahedral transition state inhibitor. The sugar moiety of nebularine is evidently important in the formation of this transition state analog since the base purine does not form such an inhibitor of ADA_1. Our findings with **LXXVI** and **II** suggest that a similar mechanism may occur in the inhibition of ADA_2 by **LXXVI**.

Nucleotide analogs. A variety of nucleotide analogs were examined as ADA_2 inhibitors (**LXXIX–XC**) including adenosine-3′:5′-cyclic monophosphate (**LXXIX**) and adenosine-3′-monophosphate (**LXXXI**). Similar findings with many of these nucleotide analogs have been reported by Daddona and Kelley [11]. The failure of nucleotide 5′-monophosphate analogs to bind to ADA_2 may indicate intolerance for the 5′-monophosphate group by virtue of steric or electrostatic repulsion.

Miscellaneous compounds. It was of interest to

examine the binding of *lin*-benzoadenine (**XCIX**) to ADA_2, since this compound is the only purine base analog that has significant activity as a substrate for ADA_1 [52]. As discussed above, the ribose moiety of adenosine evidently causes a change in enzyme conformation which permits deamination and this argument has been proposed to account for the reactivity of ADA_1 with nucleosides but not bases [53]. The fact that *lin*-benzoadenine (**XCIX**) did not inhibit ADA_2 further exemplifies the difference in the active sites between ADA_1 and ADA_2.

4-Fluoro-7-nitrobenzofurazan (**CX**) was a relatively potent inhibitor of ADA_2 (apparent K_i 0.2 mM). While **CX** does bear some resemblance to a purine base, we are hesitant to draw any firm conclusions regarding the active site of ADA_2 since compounds of this class (e.g. 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, NBD chloride, **CII**) are known to be extremely reactive with amino groups [54] and the observed inhibition may be due to non-specific protein denaturation.

A number of analogs in which the atoms of the purine ring were rearranged or replaced were tested as ADA_2 inhibitors (allopurinol, **XXII**; 8-azaguanine, **XXVI**; 8-azahypoxanthine, **XXVII**; 8-azaxanthine, **XXVIII**; **XCI**: **XCII**; 4-aminoimidazolo[4,5-*d*]-pyridazine or "2-aza-3-deazaadenine," **XCIV**; 4-aminopyrazolo[3,4-*d*]pyrimidine or "7-deaza-8-aza-adenine," **XCV**; 4-amino-1,2,3-triazolo[4,5-*d*]pyridazine or "2-aza-3-deaza-8-azaadenine," **XCVI**; benzotriazole or "1,3-dideaza-8-azapurine," **C**; **CVIII**; **CXI**; **CXII**; and 1,2,4-triazolo[1,5-*a*]pyrimidine or "1-deaza-5-azapurine," **CXV**). The binding of these compounds will not be discussed in detail since no potent inhibitors emerged from this series, the binding of most were generally consistent with what might be expected from our findings with "true" purines, and the physiochemical properties of these compounds are largely unknown and difficult to predict.

Rational design of ADA_2 inhibitors. Substituent groups on purine analogs often have complex and unpredictable effects on the chemical properties of such compounds, and thus the rational design of ADA_2 inhibitors is difficult. This problem is clearly illustrated by the fact that 2-fluoro-8-azaadenine (**XXXII**) bound less tightly to ADA_2 than either 2-fluoroadenine (**V**) or 8-azaadenine (**XVIII**), the opposite of what one would predict based on individual substituent effects.

With the foregoing caveat in mind, α-D-nucleosides with appropriately substituted aglycones (e.g. α-2-fluoroadenosine, α-coformycin, etc.) would seem to be worthwhile to synthesize and evaluate as ADA_2 inhibitors. α-Nucleosides in general bind poorly to ADA_1 [44, 47, 48], whereas we have found that α-D-adenosine (**XXXVI**) and α-D-2′-deoxyadenosine (**LV**) bind *more* tightly to ADA_2 than (β)-adenosine (**XXXV**) and (β)-2′-deoxyadenosine (**LIV**), respectively. In theory, the α-D-configuration of compounds such as α-D-coformycin will hinder or prevent binding to ADA_1 while enhancing ADA_2 inhibition.

Potential uses of ADA_2 inhibitors. Specific ADA_2 inhibitors may be useful as pharmacological tools to aid in understanding the physiological role of this isoenzyme particularly in HIV infection where plasma levels are elevated markedly [6, 7]. While ADA_2 constitutes the majority of human plasma ADA activity even in the absence of this disease [5–7], it is not clear that this isoenzyme can play a role in circulating adenosine metabolism since its K_m (2 mM) [1, 5] is far above plasma adenosine concentrations (<150 nM) [55, 56]. In addition, few mammalian cells appear to contain significant levels of this isoenzyme [11, 12, 57] and thus the cellular source of ADA_2 in plasma is not obvious. Further studies on this unusual isoenzyme appear to be warranted.

Acknowledgements—The authors would like to thank Drs. Elie Abushanab, Balkrishen Bhat, Shih Hsi Chu, Nelson J. Leonard, John A. Montgomery, Raymond P. Panzica, Robert E. Parks, Jr., Jean-Pierre Sommadossi, and George Wright for providing some of the nucleoside analogs used in this study. We are also indebted to Drs. Elie Abushanab, Clair J. Cheer, and Johanna D. Stoeckler for helpful discussions and advice regarding this work. This study was supported by Grant AG-08226 from the United States Public Health Service.

REFERENCES

1. Hirschhorn R and Ratech H, Isozymes of adenosine deaminase. In: *Isozymes: Current Topics in Biological and Medical Research* (Eds. Rattazzi MC, Scandalios JG and Whitt GS), Vol. 4, pp. 131–157. Alan R. Liss, New York, 1980.
2. Van der Weyden MB and Kelley WN, Human adenosine deaminase. Distribution and properties. *J Biol Chem* **251**: 5448–5456, 1976.
3. Giblett ER, Anderson JE, Cohen F, Pollara B and Meuwissen HJ, Adenosine-deaminase deficiency in two patients with severely impared cellular immunity. *Lancet* **ii**: 1067–1069, 1972.
4. Hirschhorn R, Beratis N and Rosen FR, Characterization of residual enzyme activity in fibroblasts from patients with adenosine deaminase deficiency and combined immunodeficiency: Evidence for a mutant enzyme. *Proc Natl Acad Sci USA* **73**: 213–217, 1976.
5. Ratech H and Hirschhorn R, Serum adenosine deaminase in normals and in a patient with adenosine deaminase deficient-severe combined immunodeficiency. *Clin Chim Acta* **115**: 341–347, 1981.
6. Yokoyama MM and Tsuboi I, Adenosine deaminase isoenzymes and HIV/HTLV-1 infections. *J Natl Cancer Inst* **80**: 698, 1988.
7. Niedzwicki JG, Kouttab NM, Mayer KH, Carpenter CCJ, Abushanab E, Parks RE Jr and Abernethy DR, Plasma adenosine deaminase$_2$: A marker for human immunodeficiency virus infection. *J Acquired Immune Defic Syndr* **4**: 178–182, 1991.
8. Ratech H, Thorbecke GJ, Meredith G and Hirschhorn R, Comparison and possible homology of isozymes of adenosine deaminase in aves and humans. *Enzyme* **26**: 74–84, 1981.
9. Akedo H, Nishihara H, Shinkai K, Komatsu K and Ishikawa S, Multiple forms of human adenosine deaminase. I. Purification and characterization of two molecular species. *Biochim Biophys Acta* **276**: 257–271, 1972.
10. Constine J, Glazer RI and Johns DG, Adenosine deaminase inhibitors: Differential effects on multiple forms of adenosine deaminase. *Biochem Biophys Res Commun* **85**: 198–202, 1978.
11. Daddona PE and Kelley WN, Characteristics of an aminohydrolase distinct from adenosine deaminase in cultured human lymphoblasts. *Biochim Biophys Acta* **658**: 280–290, 1981.

12. Van Der Weyden MB, Jack I and Ziegler JB, Characterization of adenosine deaminating activity in normal and adenosine deaminase deficient human tissue. *Adv Exp Med Biol* **165B**: 87–70, 1984.
13. Niedzwicki JG and Abernethy DR, Structure–activity relationship of human adenosine deaminase$_2$ (ADA_2). *Pharmacologist* **31**: 161, 1989.
14. Stoeckler JD, Agarwal RP, Agarwal KC and Parks RE Jr, Purine nucleoside phosphorylase from human erythrocytes. *Methods Enzymol* **51**: 530–543, 1978.
15. Stoeckler JD, Purine nucleoside phosphorylase, a target for chemotherapy. In: *Developments in Cancer Chemotherapy* (Ed. Glazer RI), pp. 35–60. CRC Press, Boca Raton, FL, 1984.
16. Bessodes M, Bastian G, Abushanab E, Panzica RP, Berman SF, Marcaccio EJ Jr, Chen S-F, Stoeckler JD and Parks RE Jr, Effect of chirality in *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) on adenosine deaminase inhibition. *Biochem Pharmacol* **31**: 879–882, 1982.
17. Cha S, Tight-binding inhibitors—I. Kinetic behavior. *Biochem Pharmacol* **24**: 2177–2185, 1975.
18. Arch JRS and Newsholme EA, Activities and some properties of adenylate cyclase and phosphodiesterase in muscle, liver and nervous tissues from vertebrates and invertebrates in relation to the control of the concentration of adenosine 3′:5′-cyclic monophosphate. *Biochem J* **158**: 603–622, 1976.
19. Tuttle JV and Krenitsky TA, Effects of acyclovir and its metabolites on purine nucleoside phosphorylase. *J Biol Chem* **259**: 4065–4069, 1984.
20. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol* **29**: 1–32, 1967.
21. Schrader WP, Pollara B and Meuwissen HJ, Characterization of the residual adenosine deaminating activity in the spleen of a patient with combined immunodeficiency disease and adenosine deaminase deficiency. *Proc Natl Acad Sci USA* **75**: 446–450, 1978.
22. Chenon M-T, Pugmire RJ, Grant DM, Panzica RP and Townsend LB, Carbon-13 magnetic resonance. XXVI. A quantitative determination of the tautomeric populations of certain purines. *J Am Chem Soc* **97**: 4636–4642, 1975.
23. Lister JH, Physiocochemical aspects of the chemistry of purines. *Adv Heterocycl Chem* **6**: 1–43, 1966.
24. Izatt RM Christensen JJ and Rytting JH, Sites and thermodynamic quantities associated with proton and metal ion interaction with ribonucleic acid, deoxyribonucleic acid, and their constitutent bases, nucleosides, and nucleotides. *Chem Rev* **71**: 439–481, 1971.
25. Lawley PD, The spectra of purines. In: *Fused Pyrimidines, Part II, Purines* (Ed. Brown DJ) pp. 439–528. Wiley-Interscience, New York, 1971.
26. Brookes P and Lawley PD, The methylation of adenosine and adenylic acid. *J Chem Soc* 539–545, 1960.
27. Budo G and Tomasz J, Second protonation of adenine and guanine. *Acta Biochim Biophys Acad Sci Hung* **9**: 217–221, 1974.
28. Sepiol J, Kazimierczuk Z and Shugar D, Tautomerism of isoguanosine and solvent-induced keto-enol equilibrium. *Z Naturforsch* **31c**: 361–370, 1976.
29. Charton M, The estimation of Hammett substituent constants. *J Org Chem* **28**: 3121–3124, 1963.
30. Fujita T, Iwasa J and Hansch C, A new substituent constant, π, derived from partition coefficients. *J Am Chem Soc* **86**: 5175–5180, 1964.
31. Montgomery JA and Hewson K, Synthesis of potential anticancer agents. XX. 2-Fluoropurines. *J Am Chem Soc* **82**: 463–468, 1960.
32. Agarwal RP, Sagar SM and Parks RE Jr, Adenosine deaminase from human erythrocytes: Purification and effects of adenosine analogs. *Biochem Pharmacol* **24**: 693–701, 1975.
33. Lawley PD and Brookes P, Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem J* **89**: 127–138, 1963.
34. Leonard NJ and Deyrup JA, The chemistry of triacanthine. *J Am Chem Soc* **84**: 2148–2160, 1962.
35. Lewin S and Tann NW, Reactions of nucleic acids and their components. Part II. Thermodynamic constants of adenine. *J Chem Soc* 1466–1467, 1962.
36. Pal BC and Horton CA, The structure of 3-methyladenine and methylation of 6-dimethylaminopurine. *J Chem Soc* 400–405, 1964.
37. Weiss PM, Cook PF, Hermes JD and Cleland WW, Evidence from nitrogen-15 and solvent deuterium isotope effects on the chemical mechanism of adenosine deaminase. *Biochemistry* **26**: 7378–7384, 1987.
38. Suzuki S and Marumo S, Chemical structure of tubercidin. *J Antibiot (Tokyo)* **14A**: 34–38, 1961.
39. Albert A, Chemistry of 8-azapurines (1,2,3-triazolo-[4,5-*d*]pyrimidines). *Adv Heterocycl Chem* **39**: 117–180, 1986.
40. Krugh TR, Tautomerism of the nucleoside antibiotic formycin, as studied by carbon-13 nuclear magnetic resonance. *J Am Chem Soc* **95**: 4761–4762, 1973.
41. Orozco M, Canela EI, Lluis C, Mallol J and Franco R, Theoretical study of the tautomerism of adenosine and formycin: Functional implications. In: *Progress in Clinical and Biological Research. QSAR: Quantitative Structure Activity Relationships in Drug Design* (Ed. Fauchere JL), Vol. 291, pp. 365–368. Alan R. Liss, New York, 1989.
42. Ikehara M, Uesugi S and Yoshida K, Studies on the conformation of purine nucleosides and their 5′-phosphates. *Biochemistry* **11**: 830–836, 1972.
43. Stolarski R, Dudycz L and Shugar D, NMR studies on the *syn-anti* dynamic equilibrium in purine nucleosides and nucleotides. *Eur J Biochem* **108**: 111–121, 1980.
44. Coddington A, Some substrates and inhibitors of adenosine deaminase. *Biochim Biophys Acta* **99**: 442–451, 1965.
45. Rockwell M and Maguire MH, Studies on adenosine deaminase. I. Purification and properties of ox heart adenosine deaminase. *Mol Pharmacol* **2**: 574–584, 1966.
46. Sundaralingham M, Structure and conformation of nucleotides and nucleotides and their analogs as determined by X-ray diffraction. *Ann NY Acad Sci* **225**: 3–42, 1975.
47. LePage GA and Junga IG, Metabolism of purine nucleoside analogs. *Cancer Res* **25**: 46–52, 1965.
48. Bennett LL Jr, Allan PW, Hill DL, Thomas HJ and Carpenter JW, Metabolic studies with an α-nucleoside, 9-α-D-arabinofuranosyl-8-azaadenine. *Mol Pharmacol* **12**: 242–250, 1976.
49. Agarwal RP, Inhibitors of adenosine deaminase. *Pharmacol Ther* **17**: 399–429, 1982.
50. Kurz LC and Frieden C, Adenosine deaminase converts purine riboside into an analogue of a reactive intermediate: A ^{13}C NMR and kinetic study. *Biochemistry* **26**: 8450–8457, 1987.
51. Jones W, Kurz LC and Wolfenden R, Transition-state stabilization by adenosine deaminase: 1,6-Addition of water to purine ribonucleoside, the enzyme's affinity for 6-hydroxy-1,6-dihydropurine ribonucleoside, and the effective concentration of substrate water at the active site. *Biochemistry* **28**: 1242–1247, 1989.
52. Leonard NJ, Petrič A and Rykowski A, Defined dimensional alterations in enzyme substrates. Birch reduction of *lin*-benzopurines. A contribution to information concerning the binding sites of adenosine deaminase and xanthine oxidase. *J Org Chem* **53**: 3873–3875, 1988.

53. Wolfenden R, Wentworth DF and Mitchell GN, Influence of substituent ribose on transition state affinity in reactions catalyzed by adenosine deaminase. *Biochemistry* **16**: 5071–5077, 1977.
54. Fager RS, Kutina CB and Abrahamson EW, The use of NBD chloride (7 chloro-4-nitrobenzo-2-oxa-1,3-diazole) in detecting amino acids and as an N-terminal reagent. *Anal Biochem* **53**: 290–294, 1973.
55. German DC and Kredich MN, A radioenzymatic assay for plasma adenosine. *Anal Biochem* **142**: 536–541, 1984.
56. Möser GH, Schrader J and Deussen A, Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* **256**: C799–C806, 1989.
57. Ratech H, Martiniuk F, Borer WZ and Rappaport H, Differential expression of adenosine deaminase isozymes in acute leukemia. *Blood* **72**: 1627–1632, 1988.