



## A study on the inhibition of adenosine deaminase

Sergio Alunni, Mara Orrù & Laura Ottavi

**To cite this article:** Sergio Alunni, Mara Orrù & Laura Ottavi (2008) A study on the inhibition of adenosine deaminase, Journal of Enzyme Inhibition and Medicinal Chemistry, 23:2, 182-189, DOI: [10.1080/14756360701475233](https://doi.org/10.1080/14756360701475233)

**To link to this article:** <https://doi.org/10.1080/14756360701475233>



Published online: 04 Oct 2008.



Submit your article to this journal [↗](#)



Article views: 2157



View related articles [↗](#)



Citing articles: 4 View citing articles [↗](#)

## A study on the inhibition of adenosine deaminase

SERGIO ALUNNI, MARA ORRÙ & LAURA OTTAVI

*Dipartimento di Chimica, Università degli Studi di Perugia, Perugia, Italy*

*(Received 5 February 2007; accepted 8 May 2007)*

### Abstract

Adenosine deaminase (ADA, EC 3.5.4.4) catalyses the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. In this study the inhibition of ADA from bovine spleen by several molecules with structure related to that of the substrate or product has been quantified. The inhibitors adenine, purine, inosine, 2-aminopurine, 4-aminopyrimidine, 4-aminopyridine, 4-hydroxypyridine and phenylhydrazine are shown to be competitive inhibitors with  $K_I$  (mM) values of 0.17, 1.1, 0.35, 0.33, 1.3, 1.8, 1.4 and 0.25, respectively. Synergistic inhibition by various combinations of molecules that imitate the structure of the substrate has never been observed. Some general conclusions are: i) the enzyme ADA from bovine spleen we have used is appropriate for kinetic studies of inhibition and mechanistic studies; it can be a reference catalytic system for the homogeneous comparison of various inhibitors; ii) this enzyme presents very rigid requirements for binding the substrate: variations in the structure of adenosine imply the loss of important interactions.

**Keywords:** *Adenosine deaminase, kinetics, mechanism, inhibition*

### Introduction

The enzyme adenosine deaminase (ADA, EC 3.5.4.4) is an important enzyme [1] of the purine metabolism which catalyses the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively (scheme 1).

This enzyme has been found in plants, bacteria [2], invertebrates [3], vertebrates [4] and mammals [5, 6], including humans [7–9]. ADA is present in many human tissues, but the highest levels have been found in lymph nodes, spleen and thymus [1]. Bovine ADA is more closely related to human ADA [10, 11].

Regarding the mechanism of the ADA reaction the evidences so far accumulated are in agreement with an aromatic nucleophilic substitution [12]. The nucleophile is formed by interaction of a water molecule with  $Zn^{++}$ , present in the active site; deprotonation of this activated  $H_2O$  molecule by the imidazole ring of His 238 allows the formation of a strong nucleophile that will substitute the  $NH_2$  group of the substrate by OH

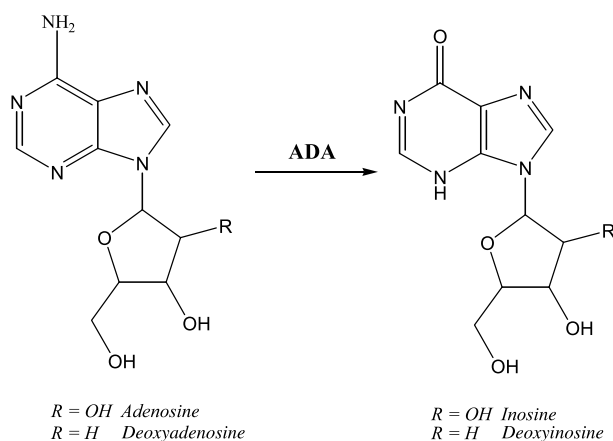
group. Glu 217 plays a key role; in fact, protonation of the N atom of the heteroaromatic substrate provides a strong stabilization of the transition state of the process.

The importance of inhibition studies of ADA is well recognized; in fact, various potential therapeutic use of ADA inhibitors have been suggested. [13–18].

Several studies on the effect of ADA inhibitors on the release of adenosine in the central nervous system [19–21] indicate that pharmacological potentiation of the formation of endogenous extra cellular adenosine may have interesting therapeutic application [22–25]. Also, ADA inhibitors attenuate myocardial ischemic injury and it has been suggested that ADA inhibition can be useful in cardiovascular protection in hypertension [26].

Our interest for this enzyme is related to the particular mechanism of its reaction, where a key activation of the process is provided by protonation of N1 atom of the adenosine substrate, with strong stabilization of the transition state related to the intermediate carbanion. Other examples of biological

Correspondence: S. Alunni, Dipartimento di Chimica, Università degli Studi di Perugia, Via Elce di Sotto, 8, 06100, Perugia, Italy.  
Fax: +39 075 5855560. Email: E-mail: alunnis@unipg.it



Scheme 1.

systems, where a similar effect of stabilization of an intermediate carbanion by a protonated N atom part of an heteroaromatic system is operating, are the mechanism of reaction with the enzyme Histidine Ammonia Lyase [27] and the chemistry of pyridoxal-phosphate [28, 29]. In previous studies we have quantified the effect of protonation of the N atom of a pyridine ring on the rate and mechanism of related elimination reactions [30–35].

In literature there are several reports [1, 36–43] of studies of ADA inhibition but various types of ADA or different enzyme purification procedures have been used. With this work we decided to explore the characteristics of the inhibition of ADA from bovine spleen (SIGMA, type V) so that the inhibition of various molecules is compared with respect to the same enzyme, which could be then a reference system for studies of ADA inhibition. In this paper the inhibition study has focused mainly on the inhibition potentiality of various parts of the substrate or parts of the product. The synergistic inhibition of mixture of molecules that imitate the substrate structure has been also tested.

## Materials and methods

Adenosine deaminase from bovine spleen, type V, was from Sigma. 8  $\mu$ l of commercial ADA (1.6 ml, 1.3 mg prot/ml, 60–130 units/mg protein) were diluted with 6 ml of a solution of BSA. The solution of BSA was prepared with  $\approx$  10 mg of protein in 10 ml of phosphate buffer (pH = 7.5). The solution of the diluted enzyme was kept at 0°C and it was found to be stable for  $\approx$  2–3 weeks. The substrate adenosine and all the inhibitors tested were commercial, recrystallised materials. The water used was redistilled and freshly boiled.

Initial rate studies were performed with 20  $\mu$ l of the diluted solution of ADA in 2 ml of 50 mM solution of phosphate buffer, pH = 7.5, in a cuvette at 25°C.

The determination of  $V_0$  was made following the formation of the product inosine at  $\lambda = 235$  nm (the

difference in the extinction coefficients of inosine and adenosine is  $\Delta\epsilon_{235\text{ nm}} = 3500 \text{ M}^{-1} \text{ cm}^{-1}$ ), or at  $\lambda = 265$  nm, following the disappearance of adenosine ( $\Delta\epsilon_{265\text{ nm}} = 8400 \text{ M}^{-1} \text{ cm}^{-1}$ ). It was useful to have two possibilities for the kinetic studies at the two  $\lambda$ , because some inhibitors showed strong absorption, so it was chosen for the study the more appropriate wavelength. The  $K_M$  determined at the two  $\lambda$  were in good agreement and the average value is  $K_M = 40 \mu\text{M}$ . These studies were performed by using the standard linearization method of Lineweaver-Burk. The adenosine concentration was 0.014–0.3 mM.

The values of  $K_I$  were determined by the standard linearization method of Lineweaver-Burk (DRP) or by the Dixon Plot (see below). The choice between the two procedures was determined by the absorbance of the starting systems.

## Results and discussion

As a first approach to the study of potential inhibitors, we have made a screening of several molecules with various structures.

A comparison of the activity in the presence or absence of inhibitor,  $V_I/V_0$ , can give an indication of the strength of inhibition:  $V_I$  is the initial rate in the presence of inhibitor I and  $V_0$  is the initial rate in the absence of inhibitor. In Table I are reported the values of  $V_I/V_0$  for the molecules tested. The concentration of adenosine was 0.034 mM; 20  $\mu$ l of the diluted solution of ADA were injected in 2 ml of phosphate buffer, pH = 7.5, 25°C. The concentration of the inhibitor was  $\approx$  0.7 mM or higher or eventually compatible with the absorbance of the solution. For some compounds giving modest inhibition, shown in Table I, it has not been possible the determination of the  $K_I$  values for the high absorbance of their solutions.

Following the indication of the screening of Table I we have studied the inhibition of adenine, purine, inosine, 2-aminopurine, 4-aminopyrimidine, 4-aminopyridine, and 4-hydroxypyridine, phenyl hydrazine. We have used the Dixon plot or the DRP methods, choosing the treatment (and the  $\lambda$  of work) more convenient in relation to the problems of absorbance of the solutions. With all the inhibition studied we have found competitive inhibition. In Table II are reported the calculated values of  $K_I$  (mM) and the conditions of the studies.

As an example of Dixon Plot [44, 45] is reported in Figure 1 the plot with adenine; with this treatment  $V_0$  are determined at fixed [adenosine] and varying the [I]. A plot of  $1/V_0$  vs the [I] at the various [substrate] tested gives straight lines with a common intersection on the X-axis of value =  $-K_I$ , if the inhibition is a competitive inhibition. A check of the consistency of the data can be made because the y value of the intersection must be  $1/V_m$ . An example of calculation

Table I. Values of fractional activity  $V_I/V_0$  for ADA (25°C, phosphate buffer 0.05 M, pH 7.5 and [adenosine] = 0.034  $\mu\text{M}$ ).

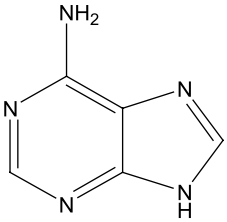
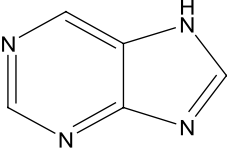
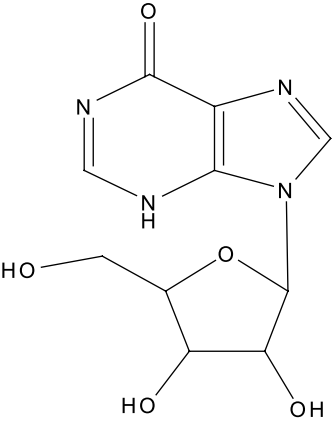
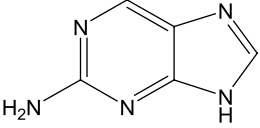
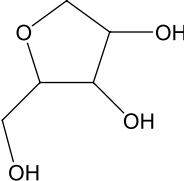
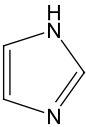
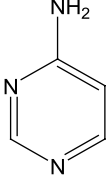
<i>Inhibitor (I)</i>	[I] (mM)	$V_0$ ( $\mu\text{M min}^{-1}$ )	$V_I$ ( $\mu\text{M min}^{-1}$ )	$V_I/V_0$
<i>Adenine</i> 	0.334	1.56	0.84	0.54
<i>Purine</i> 	0.668	1.54	1.28	0.83
<i>Inosine</i> 	0.662	1.54	0.82	0.53
<i>2 amino-purine</i> 	0.660	1.56	0.89	0.57
<i>Ribose</i> 	0.713	1.54	1.54	1
<i>Imidazole</i> 	0.796	1.54	1.54	1
<i>4 amino-pyrimidine</i> 	0.672	1.54	1.30	0.84

Table I – continued

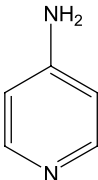
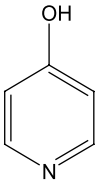
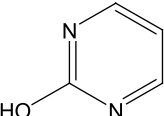
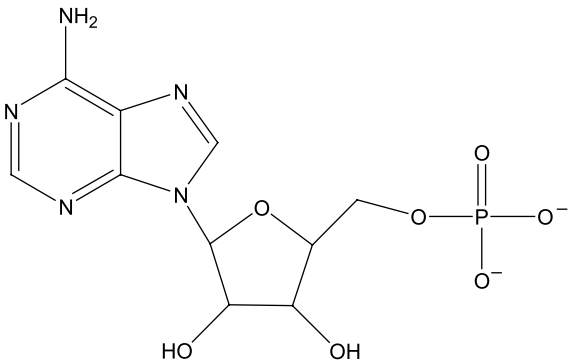
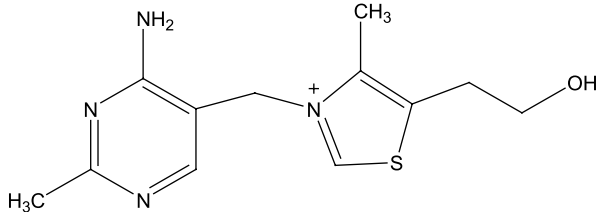
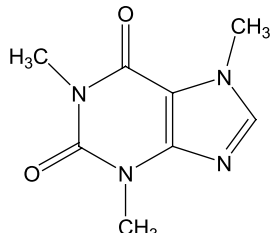
	Inhibitor ( <i>I</i> )	[ <i>I</i> ] (mM)	<i>V</i> <sub>0</sub> (μM min <sup>-1</sup> )	<i>V</i> <sub><i>I</i></sub> (μM min <sup>-1</sup> )	<i>V</i> <sub><i>I</i></sub> / <i>V</i> <sub>0</sub>
4 amino-pyridine		0.657	1.54	1.42	0.92
4 hydroxy-pyridine		0.668	1.54	1.35	0.88
2 hydroxy-pyrimidine		1.6	1.56	1.56	1
AMP		0.586	1.54	1.54	1
Thiamine		0.561	1.54	1.21	0.79
Caffeine		0.672	1.54	1.40	0.91

Table I – *continued*

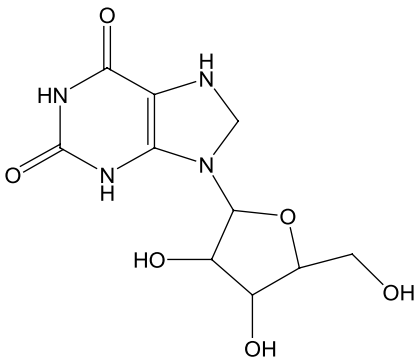
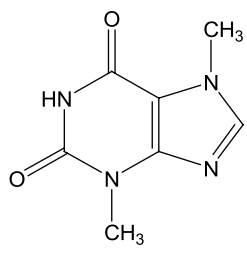
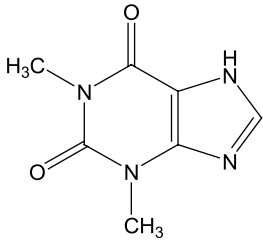
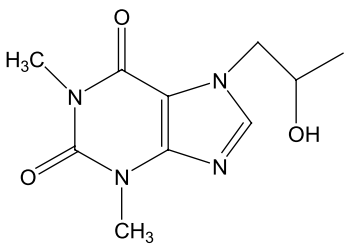
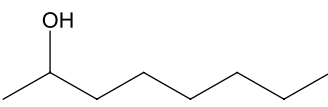
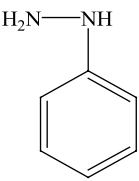
<i>Inhibitor (I)</i>	[I] (mM)	$V_0$ ( $\mu\text{M min}^{-1}$ )	$V_I$ ( $\mu\text{M min}^{-1}$ )	$V_I/V_0$
<i>Xanthosine</i> 	0.664	1.54	1.36	0.88
<i>Theobromine</i> 	0.532	1.54	1.25	0.81
<i>Theophylline</i> 	0.665	1.54	1.33	0.86
<i>7-(β-hydroxy-propyl)-theophylline</i> 	0.658	1.54	1.19	0.77
<i>2 Octanol</i> 	1.47	1.56	1.56	1
<i>Hydrazine</i> $\text{H}_2\text{N}-\text{NH}_2$	0.684	1.56	1.55	1
<i>Phenyl-hydrazine</i> 	0.510	1.56	0.44	0.28

Table II. Calculated values of  $K_I$  (mM) for inhibition of ADA (25°C, phosphate buffer 0.05 mM, pH 7.5) by various inhibitors. The error on  $K_I$  values is  $\pm 10\%$ .

Inhibitor (I)	$\lambda$ (nm)	Method	$K_I$ (mM)
Adenine	235	Dixon Plot	0.17
Purine	235	Dixon Plot	1.1
Inosine	265	DRP	0.35
2-aminopurine	265	Dixon Plot	0.33
4-aminopyrimidine	265	Dixon Plot	1.3
4-aminopyridine	235	Dixon Plot	1.8
4-hydroxypyridine	235	Dixon Plot	1.4
Phenylhydrazine	265	DRP	0.25

of  $K_I$  by DRP for competitive inhibition is shown in Figure 2 for phenylhydrazine.

We have also studied the synergistic inhibition of various combinations of molecules that, used in equimolar concentration, imitate the structure of the substrate or part of it. We have tried the combinations adenine + ribose, adenine + octanol, 2-aminopurine + ribose, 2-aminopurine + octanol, 4-aminopyrimidine + imidazole, 4-aminopyrimidine + imidazole + ribose, 4-hydroxypyridine + imidazole + ribose. In all the cases the inhibition observed was the one expected for the single inhibitors and we have never found synergistic inhibition.

From Table II we observe that adenine presents competitive inhibition with  $K_I = 0.17$  mM, (the value reported in literature<sup>34</sup> with ADA from *Mithilus Edulis*, 17°C, pH = 6 is 0.02 mM); with respect to  $K_M = 0.04$  mM with adenosine substrate, this value is significantly larger, then adenosine is bonded by ADA strongly than adenine. It is then important for binding the presence of the ribose structure, but we note that ribose is not inhibitor neither alone neither in synergy with adenine. We conclude that ADA has quite rigid requirements for the optimization of the interactions in the active site for binding the substrate adenosine.

Purine has  $K_I = 1.1$  mM, being weaker inhibitor with respect to adenine; this result shows the importance of the  $\text{NH}_2$  group for binding, in agreement with the suggested interactions [12] of this group with Glu 217. The inhibitor 4-aminopyrimidine has a structure that is related to adenosine missing the ribose and imidazole rings. We found  $K_I = 1.3$  mM similar to that of purine. 2-aminopurine presents  $K_I = 0.33$  mM, being then a good inhibitor, close to adenine; this result shows the importance of the presence of the  $\text{NH}_2$  group in the inhibitor relatively independent on its position. 4-aminopyridine presents  $K_I = 1.8$  mM, being weaker inhibitor than 4-aminopyrimidine. Inosine presents  $K_I = 0.35$  mM; (the value reported in literature<sup>33</sup> with ADA from calf intestinal mucosa, 27°C, pH = 7.5, is  $K_I = 0.143$  mM). 4-hydroxypyridine with a structure that is one part of inosine, has  $K_I = 1.4$  mM.

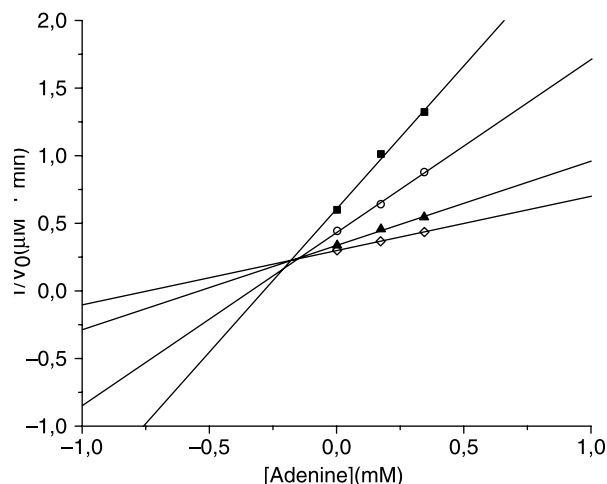


Figure 1. Dixon plot  $1/V_0$  vs [adenine] for the ADA reaction with adenosine (25°C, phosphate buffer 0.05 mM, pH 7.5) in the presence of adenine (0, 0.17, 0.34 mM) (■) [adenosine] = 0.0199 mM; (○) [adenosine] = 0.033 mM; (▲) [adenosine] = 0.066 mM; (◇) [adenosine] = 0.099 mM.

We have studied AMP and found that this molecule is not a substrate for ADA and a modest inhibitor at high concentration. In fact at 1.31 mM the ratio  $V_I/V_0$  is 0.63 ([adenosine] = 0.034 mM): clearly the presence of the phosphate group decreases strongly the possibility of the binding interactions in the active site.

We have also studied the inhibition by molecules such as hydrazine ( $\text{NH}_2\text{-NH}_2$ ), hydroxylamine ( $\text{NH}_2\text{OH}$ ) and phenyl hydrazine. The reason for the choice of these potential inhibitors was related to the fact that a key aspect of ADA catalysis in the active site is the protonation by Glu 217 of the  $\text{N}_1$  nitrogen heteroaromatic atom of the substrate. These molecules are known to give the “ $\alpha$  effect” [46], in fact they are able to interact with a proton in amplified way

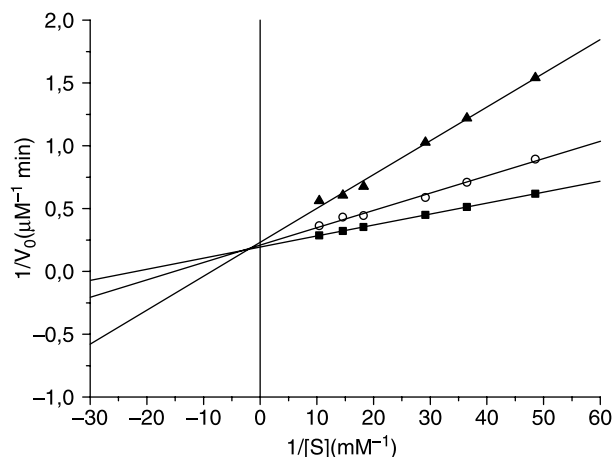


Figure 2.  $1/V_0$  vs  $1/[S]$  plot for the ADA reaction with adenosine (25°C, phosphate buffer 0.05 mM, pH 7.5) in the presence of phenylhydrazine at various concentrations. (■) [I] = 0; (○) [I] = 0.17 mM; (▲) [I] = 0.425 mM.



with respect to their basicity. We have found that hydrazine and hydroxylamine are not inhibitors, while there is a significant inhibition by phenyl hydrazine, with  $K_I = 0.25$  mM. As long as the  $pK_a$  of the molecules are 8.2, 6.17, 5.27 respectively [47], we propose that the inhibition is related to the interaction of the  $NH_2$  group with the proton of Glu 217 in the active site, but that the presence of the hydrophobic phenyl ring is important for binding; then probably close to Glu 217 in the active site there could be an hydrophobic task.

Finally, we tried iodoacetamide as "affinity label" inhibitor [44] to see if it was possible the covalent binding by  $S_N2$  reaction with a nucleophile (ex. imidazole ring) in the active site and then have irreversible inhibition, but no inhibition was observed.

Some general conclusions that can be made from this study are:

The ADA enzyme has very rigid requirements for binding adenosine substrate: variations in the structure of adenosine imply the loss of important interactions; in agreement with this conclusion is also the finding that synergistic inhibition has not been observed in any case studied;

The quantification of the inhibition by molecules that are part of the substrate or of product is useful for the identification of structures potentially inhibitors, then for the project of new inhibitors; The ADA enzyme from bovine spleen we have used in our study is a quite stable enzyme, appropriate for kinetic studies of inhibition and mechanistic studies and can be a reference catalytic system for the homogeneous comparison of various inhibitors.

## Acknowledgements

Thanks are due to the Ministero dell'Istruzione dell'Università e della Ricerca (MIUR) for the financial support.

## References

- [1] Cristalli G, Costanzi S, Lambertucci C, Lupidi G, Vittori S, Volpini R, Campioni E. Adenosine deaminase: Functional implications and different classes inhibitors. *Med Res Rev* 2001;21:105–128.
- [2] Lupidi G, Marmocchi F, Falasca M, Venardi G, Cristalli G, Grifantini M, Withehead E, Riva F. Adenosine deaminase from *Saccharomyces cerevisiae*: Kinetics and interaction with transition and ground state inhibitors. *Biochim Biophys Acta* 1992;1122:311–316.
- [3] Aikawa T, Umemori-Aikawa Y, Fisher JR. Purification and properties of the adenosine deaminase from the midgut gland of a marine bivalved mollusc. *Atrina spp Comp Biochem Physiol* 1977;58B:357–364.
- [4] Ma PF, Fisher JR. Two different hepatic adenosine deaminase in the chicken. *Biochim Biophys Acta* 1968;159:153–159.
- [5] Brady TG, O'Donovan CI. Tissue distribution of adenosine deaminase in six mammal species. *Comp Biochem Physiol* 1965;14:101–119.
- [6] Lupidi G, Marmocchi F, Falasca M, Venardi G, Cristalli G, Riva F. Adenosine deaminase from bovine brain: Purification and partial characterization. *Biochem Int* 1992;26:1053–1063.
- [7] Argwal RP, Sagar SM, Parks RE, Jr. Adenosine deaminase from human erythrocytes: Purification and effects of adenosine analogs. *Biochem Pharmacol* 1975;24:693–701.
- [8] Daddona PE, Kelly WN. Analysis of normal and mutants forms of human adenosine deaminase. *J Biol Chem* 1980;252:110–115.
- [9] Daddona PE. Human adenosine deaminase: properties and turnover in cultured T and B lymphoblast. *J Biol Chem* 1981;256:12496–12501.
- [10] Kelly MA, Vestling MM, Murphy CM, Hua S, Sumpter T, Fenselau C. Primary structure of bovine adenosine deaminase. *J Pharm Biomed Anal* 1996;14:1513–1519.
- [11] Kinoshita T, Nishio N, Sato A, Murata M. Crystallization and preliminary analysis of bovine adenosine deaminase. *Acta Crystallogr* 1999;D55:2031–2032.
- [12] Gleeson MP, Burton NA, Hillier IH. The mechanism of adenosine deaminase catalysis studied by QM/MM calculations: The role of histidine 238 and the activity of the alanine 238. *Phys Chem Chem Phys* 2003;5:4272–4278.
- [13] Cass CE, Au-Yeung TH. Enhancement of 9-beta-d-arabino-furanosyladenine cytotoxicity to mouse leukemia L1210 in vitro by 2'-deoxycoformycin. *Cancer Res* 1976;36:1486–1491.
- [14] Johns DG, Adamson RH. Enhancement of the biological activity of cordycepin(3'-deoxyadenosine) by the adenosine inhibitor 2'-deoxycoformycin. *Biochem Pharmacol* 1976;25:1441–1444.
- [15] LePage GA, Worth IS, Kimball AP. Enhancement of the antitumor activity of arabinofuranosyladenine by 2'-deoxycoformycin. *Cancer Res* 1976;36:1481–1485.
- [16] Adamson RH, Zaharevitz DW, Johns DG. Enhancement of the biological activity of adenosine analogs by the adenosine deaminase inhibitor 2'-deoxycoformycin. *Pharmacology* 1977;15:84–89.
- [17] Glazer RI, Lott TJ, Peale AL. Potentiation by 2'-deoxycoformycin of the inhibitory effect by 3'-deoxyadenosine(cordycepin) on nuclear RNA synthesis in L210 cells in vitro. *Cancer Res* 1978;38:2233–2238.
- [18] Iliakis G, Ngo FQH. Effects of adenosine deaminase inhibitor 2'-deoxycoformycin on the repair and expression of potentially lethal damage sensitive to  $\beta$ -araA. *Radiat Environ Biophys* 1985;24:81–88.
- [19] Sciotti VM, Van Wylen DGL. Increases in interstitial adenosine and cerebral blood flow with inhibition of adenosine kinase and adenosine deaminase. *J Cerebral Blood Flow Metab* 1993;13:201–207.
- [20] Llyod HGE, Fredholm BB. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int* 1995;26:387–395.
- [21] Golembiowska K, White TD, Sawynok J. Modulation of adenosine release from rat spinal cord by adenosine deaminase and adenosine kinase inhibitors. *Brain Res* 1995;699:315–320.
- [22] White TD. Pharmacological augmentation of excitatory amino acid-evoked adenosine release from rat cortical slices. *Drug Dev Res* 1994;31:333.
- [23] White TD. Potentiation of excitatory amino acid-evoked adenosine release from rat cortex by inhibitors of adenosine kinase and adenosine deaminase and by acadesine. *Eur J Pharmacol* 1996;303:27–38.
- [24] Hebb MO, White TD. Co-administration of adenosine kinase and deaminase inhibitors produces supra-additive potentiation



- of N-methyl-D-aspartate-evoked adenosine formation in cortex. *Eur J Pharmacol* 1998;344:121–125.
- [25] Poon A, Saynok J. Antinociceptive and anti-inflammatory properties of an adenosine kinase inhibitor and an adenosine deaminase inhibitor. *Eur J Pharmacol* 1999;384:123–138.
- [26] Tofovic SP, Kusaka H, Li P, Jackson EK. Effects of adenosine deaminase inhibition on blood pressure in old spontaneously hypertensive rats. *Clin Exp Hyperten* 1998;20:329–344.
- [27] Langer M, Pauling A, Rétey J. The role of dehydroalanine in catalysis by histidine ammonia lyase. *Angew Chem Int Ed Engl* 1995;34:1464–1465.
- [28] Abeles RH, Frey PA, Jencks WP. *Biochemistry*. Jones and Bartlett Publisher; 1992.
- [29] Voet D, Voet JG. *Biochemistry*. John Wiley and sons; 1990.
- [30] Alunni S, Conti A, Palmizio ER. Mechanism and proton activating factors in base-induced  $\beta$ -elimination reactions of N-[2-(4-pyridyl)ethyl]quinuclidinium and N-[2-(2-pyridyl)ethyl]quinuclidinium salts. *J Chem Soc Perkin Trans* 2000;2:453–457.
- [31] Alunni S, Conti A, Palmizio ER. Use of solvent isotope effect to identify an intermediate carbanion in the  $\beta$ -elimination reactions from N-[2-(4-pyridyl)ethyl]quinuclidinium and N-[2-(2-pyridyl)ethyl]quinuclidinium induced by acetohydroxamate/acetohydroxamic acid buffers. *Res Chem Intermed* 2001;27:635–641.
- [32] Alunni S, Busti A. Mechanism and proton activating factors in base-induced  $\beta$ -elimination reactions of 2-(2-chloroethyl)pyridine. *J Chem Soc Perkin Trans* 2001;2:778–781.
- [33] Alunni S, Laureti V, Ottavi L, Ruzziconi R. Catalysis of the  $\beta$ -elimination of HF from isomeric 2-fluoroethylpyridines and 1-methyl-2-fluoroethylpyridinium salts. Proton-activating factors and methyl-activating factors as a mechanism test to distinguish between concerted E2 and E1cb irreversible mechanisms. *J Org Chem* 2003;68:718–725.
- [34] Alunni S, Ottavi L. Mechanism of acid-base catalysis of  $\beta$ -elimination reactions in systems activated by a pyridine ring. *AJ Org Chem* 2004;69:2272–2283.
- [35] Alunni S, De Angelis F, Ottavi L, Papavasileiou M, Tarantelli F. Evidence of a borderline region between E1cb and E2 elimination reaction mechanisms: A combined experimental and theoretical study of systems activated by the pyridine ring. *J Am Chem* 2005;127:15151–15160.
- [36] Frieden C, Kurz LC, Gilbert HR. Adenosine deaminase and adenylate deaminase: comparative kinetic studies with transition state and ground state analog inhibitors. *Biochemistry* 1980;19:5303–5309.
- [37] Lerner LM, Rossi RR. Inhibition of adenosine deaminase by alcohols derived from adenine nucleosides. *Biochemistry* 1972;11:2772–2777.
- [38] Schaeffer HJ, Johnson RN, Odin E, Hansch C. Structure-activity relationships in adenosine deaminase inhibitors. *J Med Chem* 1970;13:452–455.
- [39] Saboury AA, Divsalar A, Jafari GA, Mossavi-Movahedi AA, Housaindokht MR, Hakimelahi GH. A product inhibition study on adenosine deaminase by spectroscopy and calorimetry. *J Biochem Mol Biol* 2002;35:302–305.
- [40] Ogawa T, Aikawa Y, Aikawa T. Kinetic characteristics and binding process of substrate analogs to the adenosine deaminase in the marine mussel, *mytilus edulis*. *Comp Biochem Physiol* 1987;88B(1):91–100.
- [41] Wolfenden R. On the rate-determining step in the action of adenosine deaminase. *Biochemistry* 1969;8(6):2409–2412.
- [42] Saboury AA, Divsalar A, Ataie G, Amanlou M, Moosavi-Movahedi AA, Hakimelahi GH. Inhibition study of adenosine deaminase by caffeine using spectroscopy and isothermal titration calorimetry. *Acta Biochem Pol* 2003;50:849–855.
- [43] Aikawa T, Aikawa Y, Brady TG. The pH-dependence of the inhibitory effects of several divalent cations on the bovine intestine adenosine deaminase activity. *Int J Biochem* 1980;12:493–495.
- [44] Copeland A. *Enzymes*. VCH Publisher, Inc. 1996.
- [45] Dixon M. The effect of pH on the affinities of enzymes for substrates and inhibitors. *Biochem J* 1953;55:161–170.
- [46] Isaac NS. *Physical organic chemistry*. Harlow, Essex, England: Langman Scientific and Technical; 1987.
- [47] Bruice TC, Dixon JE.  $\alpha$ -effects IV. Additional observation on the  $\alpha$ -effect employing malachite green as substrate. *J Am Chem Soc* 1971;93:6592–6597.