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Original Article

A Kinetic Comparison on the Inhibition of Adenosine Deaminase by Purine Drugs

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Abstract

The effects of allopurinol, acyclovir and theophylline on the activity of adenosine deaminase (ADA) were studied in 50 mM sodium phosphate buffer pH 7.5 at 27°C, using a UV– Vis spectrophotometer. Adenosine deaminase is inhibited by these ligands, via different types of inhibition. Allopurinol, as a transition state analog of xanthine oxidase, and acyclovir competitively inhibit the catalytic activity of ADA. Inhibition constant values are 285 and 231 μM for allopurinol and acyclovir, respectively. Theophylline acts as a non-competitive inhibitor for ADA, which shows different affinity binding sites at various drug concentrations. There were two different types of inhibition constant, one of them due to a low concentration of the drug (K_i = 56 μM) and the other appearing at higher concentrations of theophylline (K_i = 201 μM). Thermodynamic parameters also show that ADA has two binding sites for theophylline.

The comparison of inhibition constant for inosine (K_i = 143 μM) and acyclovir (K_i = 231 μM) elucidates the critical role of the ribose ring within the inosine structure, relative to the open ring of acyclovir. Comparison of the inhibition constant of theobromine (K_i= 311 μM) with inosine (K_i= 143 μM) shows the critical binding role of N^7 position within the purine ring. Interestingly, the N^7 position in allopurinol is replaced by a CH_2 group, which demonstrates the lower inhibiting potency of allopurinol (K_i = 285 μM) relative to inosine (K_i= 143 μM). In a structural sense, a comparison made between the structure of theophylline and theobromine besides a comparison between the inhibition constant of theophylline (K_i = 56 μM at low and 201 μM at higher concentrations) and caffeine (K_i = 342 μM) indicate that substitution of a bulky group in N^1 and N^7 positions of purine has a critical role in the binding affinity of the above- mentioned inhibitors to the enzyme.

Keywords: Adenosine deaminase; Allopurinol; Acyclovir; Theophylline; Kinetic.

Introduction

Adenosine deaminase (ADA), (E.C. 3.5.4.4.) is one of the major enzymes in purine metabolism, catalyzing the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine nucleosides to their respective inosine derivatives, nucleosides and ammonia, with a rate enhancement of 2×10^{12} relative to the nonenzymatic reaction (1). K_m values for adenosine and deoxyadenosine are 45 and 34

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micromolar, respectively. The optimum pH for the enzyme activity is around the neutral range (2-4).

This enzyme has a wide phylogenetic distribution and its amino acid sequence is highly conserved from bacteria to humans (5, 6). ADA has been found in plants, bacteria, invertebrates, vertebrates, and mammals including humans (6, 7). This enzyme is present in virtually all human tissues, but the highest levels are found in the lymphoid system such as lymph nodes, spleen and thymus (8). Among these, the activity in T-cells and glia are higher than in B-cells and neurones (9).

In humans, the ADA activity occurs mainly in two distinct iso-enzymes and they are referred to as ADA_1 and ADA_2 (10). ADA_1 exists in 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), this complex has no significant effect on its' catalytic activity (11). ADA_2 exists as a monomer, with molecular weight of 100,000 (10). In humans, ADA_1 constitutes the majority of intracellular ADA activity, although the predominant isoenzyme in human plasma and serum is ADA_2 (11).

Gene sequences have been reported for human (12), mouse (13), *E. coli* (5) and bovine ADA(14). The homology of the three mammalian enzymes is very high. The sequences of human and mouse ADA 83% identical, those of bovine and mouse ADA are 85% identical, and those of human and bovine ADA being 91% identical. In particular, the amino acid residues around the active site are highly conserved. Only one active-site residue is different between human ADA and mouse ADA, and all the residues around the active site are identical in human ADA and bovine ADA (15).

The crystal structure of mouse ADA was first reported in 1991. ADA has a central parallel α/β barrel with eight β- strands and eight peripheral α - helices, a commonly observed structural motif of several other enzymes. The structure has five additional helices, three of which (H1 to H3) are located at the COOH-terminal end of the β barrel, between β1 and α1. The other two (H4 and H5) form an antiparallel loop over the opposite end of the barrel. The active site of ADA residues lies at the C- terminal end of the β barrel, in a deep oblong-shaped pocket. A penta-coordinated Zn^{2+} cofactor is embedded in the deepest part of the pocket. The zinc ion is located deep within the substrate binding cleft and coordinated in a tetrahedral geometry to His 17, His 214, and Asp 295. A water molecule, which shares the ligand coordination site with Asp 295, is polarized by the metal giving rise to a hydroxylate ion that replaces the amine at the C6 position of adenosine through a stereo-specific addition/elimination mechanism (16, 17). Mutation studies of amino acids in the proposed active site near the zinc-binding site in adenosine deaminase confirm the essential role of these residues in catalysis (18, 19).

ADA is present in virtually all mammalian cells and has a central role in maintaining competence. Aberrations in the expression and function of ADA have been implicated in several disease states such as severe combined immuno deficiency (SCID), which is characterized by impaired B- and T-cell-based immunity resulting from an inherited deficiency in ADA (20, 21). ADA is also specifically involved or its levels are changed in a variety of other diseases including acquired immunodeficiency syndrome (AIDS), anemia, various lymphomas, and leukemias. Higher levels of ADA in the alimentary tract and decidual cells of the developing fetal-maternal interface put ADA among those enzymes performing unique roles related to the growth rate of cells, embryo implantation, and other undetermined functions (22-24). The ADA activity can be found at variable amounts in all cell types present in the nervous system.

Understanding the interaction of ADA with its inhibitors and substrates at the molecular level is important in the development of the next generation of pharmaceutical agents acting as inhibitors or substrates. Following our previous studies on modified histidine residues (25) and inhibition of ADA with inosine (26), caffeine (27), acetaminophen (28), theophylline (29) and theobromine (30), in this study the kinetic of inhibitory effects of allopurinol, acyclovir, theophylline and other purine ligands on the enzymatic reaction of ADA have been investigated.

Experimental

Materials

Adenosine deaminase (type IV, from calf intestinal mucosa), adenosine, inosine, caffeine, allopurinol, acyclovir, theophylline and theobromine were all obtained from Sigma. The other related chemicals, of the highest grade, were obtained from different industrial sources. The solutions were prepared in double distilled water.

Methods

Enzyme Assay

Enzymatic activities were assayed by UV-Vis spectrophotometry with a Shimadzu-3100 instrument, based on the Kaplan Method, in an attempt to follow the decrease in absorbance at 265 nm resulting from the conversion of adenosine to inosine (31). This method uses the change in extinction coefficient of adenosine (8400 M^{-1} cm^{-1}), on conversion to inosine by the catalytic activity of enzyme. The concentration of enzyme in the assay mixture (50 mM of a pH 7.5 sodium phosphate buffer), was 0.94 nM, with a final volume of 1 ml. Activities were measured using at least seven different concentrations of adenosine and the assays repeated at least three times. The range of adenosine concentration used was between 0.25–2.5 K_m. Care was taken to use experimental conditions where the enzyme reaction was linear during the first minute of the reaction.

A plot of 1/V versus 1/S, called the Lineweaver– Burk plot, yields a straight line with an intercept of $1/V_{max}$ and a slope of K_m / V_{max}. Measurement of the rate of catalysis at different concentrations of substrate and inhibitor serves to distinguish between competitive and noncompetitive inhibition (39).

In a competitive inhibition; V_{max} is unaltered, whereas K_m is increased. The slope of competitive plot is equal to:

$$\text{Slope} = K_m / V_{max} (1 + [I] / K_1) \text{ or}$$

$$\text{Slope} = K_m.[I] / V_{max}.K_1 + K_m / V_{max}$$

A plot of K_m values versus concentration of inhibitors [I] (named as the secondary plot) yields a straight line. The intercept on the horizontal axis would be K_i and is used in order to obtain the inhibition constant.

In a non-competitive inhibition; K_m is unaltered, whereas V_{max} is decreased.

In a non-competitive inhibition (39),

$$1/ V_{max\ app} = 1/ V_{max} (1 + [I] /K_1) .$$

Inhibition constant in a non-competitive inhibition is obtained from a plot of $1/ V_{max\ app}$ values versus the concentration of inhibitor [I]. The intercept on the horizontal would be equal to K_i.

Results

Kinetic studies

Allopurinol

Figure 1 shows the Lineweaver – Burk plot for the ADA – adenosine system, in which three different concentration of allopurinol (37.5, 56.25 and 75 μM) are incubated with the enzyme-substrate complex at 27°C. The value of V_{max} is unchanged by the allopurinol concentration, but the apparent Michaelis constant (K_m') value is increased. This confirms the competitive inhibition of adenosine deaminase by allopurinol due to the penetration of the inhibitor molecules (allopurinol) into the active site of the enzyme. The values of K_m' at any fixed concentration of allopurinol were obtained from Figure 1. Inset of Figure 1 depicts a secondary plot of the apparent Michaelis constant, (K'_m), values against the concentration of allopurinol, in an attempt to obtain the inhibition constant. The K_m value was found to be equal to 38 μM and the inhibition constant (K_i) = 285 μM.

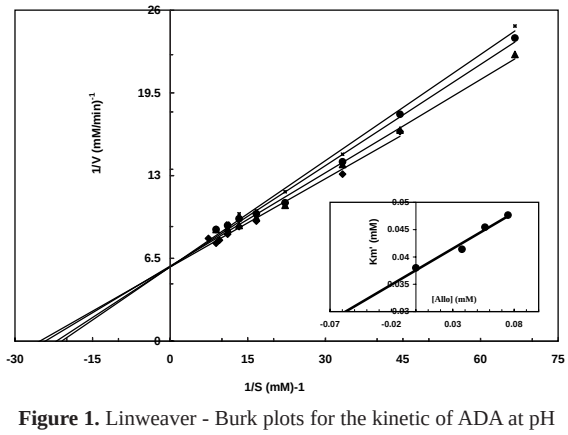


Figure 1. Linweaver - Burk plots for the kinetic of ADA at pH = 7.5 and T = 300 K in the presence of different concentrations of allopurinol (0 - 0.075 mM). Inset: secondary plot, K'_m versus [I] is shown. S and I are donated as substrate and inhibitor, respectively. (0 mM: ◆, 0.0375, mM: ▲, 0.056 mM: ●, 0.075 mM: ■).

Table 1. The values of V_{max} and K_m in the presence and absence of theophylline at 27°C

Theophylline concentration (μM)	V_{max} (μM/min)	K_m (μM)
0	23	40
8	21	40
15	18	40
30	15	40
45	19	40
60	18	40
75	17	40

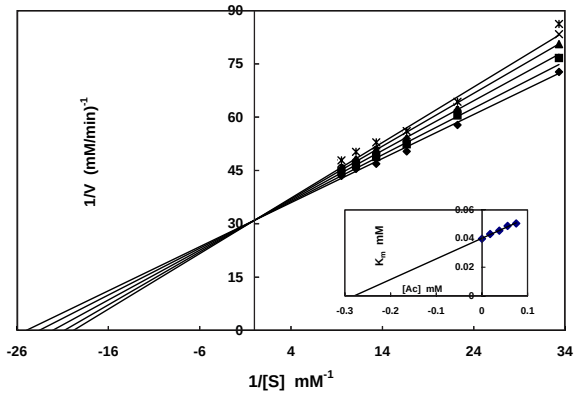


Figure 2. Linweaver - Burk plots for the kinetic of ADA at pH = 7.5 and T = 300 K in the presence of different concentrations of acyclovir (0 - 0.075 mM). Inset: secondary plot, K'_m versus [I] is shown. S and I are donated as substrate and inhibitor, respectively. (0 mM: ◆, 0.019, mM: ■, 0.038, mM: ▲, 0.056 mM: ×, 0.075 mM: ★).

Acyclovir

Figure 2 shows a Lineweaver– Burk plot for the ADA– adenosine system, where four different concentration of acyclovir (19, 38, 56 and 75 μM) are incubated with the enzyme-substrate complex (pH = 7.5 and T = 27°C). The value of V_{max} is unchanged by increasing the concentration of acyclovir, but the apparent Michaelis constant (K_m') value is increased. This confirms the competitive inhibition of acyclovir on ADA. Inset of Figure 2 depicts a plot of the K'_m values against the concentration of acyclovir, named as the secondary plot, in order to obtain the inhibition constant (K_i). The Ki value obtained was equal to 231 μM.

Theophylline

Figure 3a shows the Lineweaver-Burk plot for ADA in different concentrations of theophylline (0, 7.5, 15, 30, 45, 60 and 75 μM), when incubated with enzyme – substrate complex at a pH of 7.5 and at 27°C. The value of Michaelis constant (K'_m) is unchanged by increasing the theophylline concentrations, but the V_{max} values are decreased (Table 1). This confirms the non-competitive inhibition of adenosine deaminase by theophylline, indicating that the inhibitor does not interfere with the binding of substrate to the active site. The decrement of V_{max}, which is obtained from each concentration of theophylline, did not show a normal process, as expected, and showed two sets of binding sites. Hence, there seems to be two different types of inhibition constant, one of them due to low drug concentrations (7.5, 15 and 30 μM) and the other showing at higher concentrations of theophylline (45, 60 and 75 μM). Figures 3a,b show Lineweaver-Burk plots for ADA at

Table 2. Thermodynamic and kinetic parameters (enthalpy, dissociation binding and inhibition constant) of adenosine deaminase (ADA), when interacting with inosine, caffeine, theophylline, theobromine, allopurinol and acyclovir.
(1) Obtained by calorimetry. (2) Obtained by spectroscopic method.

Inhibitors	Dissociation binding constant (1)	Enthalpy (ΔH) kJ/mol (1)	Inhibition constant (2)
Inosine (a)	140 μM	- 32 kJ/mol	143 μM
Caffeine (b)	350 μM	+ 75.0 kJ/mol	342 μM
Theophylline	K_d = 167 μM (c) K_d = 19 μM (c)	ΔH_1 = -12.2 kJ/mol ΔH_2 = - 14.9 kJ/mol	201 μM 56 μM
Theobromine (d)	318 μM	- 15.80 kJ/mol	311 μM
Allopurinol	—	—	285 μM
Acyclovir	—	—	231 μM

(a) Taken from ref, (26). (b) Taken from ref, (27).
(c) Taken from ref, (29). (d) Taken from ref, (30).

low and higher concentrations of theophylline, respectively. In the inset of Figures 3b and c the values of V_{max} at varying concentrations of theophylline were obtained from Figures 3b and c plotted versus low and high concentrations of theophylline (secondary plot), in order to obtain the inhibition constants (K_i). The K_i values obtained at low and high concentrations of theophylline were found to be equal to 56 μM and 201 μM, respectively.

Thermodynamic studies

The raw data obtained from the isothermal titration microcalorimetry of ADA interaction with these inhibitors in two different concentrations of proteins shows the heat of binding of inhibitor molecules per mole of ADA. Our previous method was used for determination of binding constant and enthalpy of inhibitors binding, using the ITC data (32, 33). Table 2 shows the dissociation binding constants and the molar enthalpy of inhibitors binding, using the ITC data, as well as the inhibition constants obtained from the spectroscopic method. The results show that ADA has two binding sites for theophylline binding.

Discussion

Carbon-13 nuclear magnetic resonance and ultraviolet spectra measurements suggested that purine ribonucleoside is bound in a form that is tetrahedral (sp^3) at the C-6 position (34). The protonated Glu 217 facilitates the reaction by donating a hydrogen bond to N^1 position of purine, thus enabling the formation of tetrahedral C-6 (18). The bound form could either be the extremely rare species of purine ribonucleoside that is hydrated at the N^1 and C-6 position (35) or a covalently bonded complex formed by the additional of an active site sulfur or oxygen nucleophile at the active site (16). When adenine derivatives are compared, electron-withdrawing groups are found to increase the limiting rate of enzymatic deamination (35).

Adenosine deaminase is inhibited by theobromine and inosine, as a product of the enzymatic reaction. Competitive inhibitions were observed for inosine and theobromine (26, 30). A fitting method was used for the determination

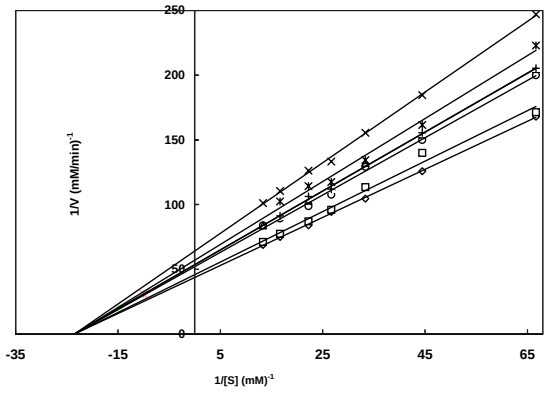


Figure 3a. Linweaver - Burk plots for the kinetic of ADA at pH = 7.5 and T = 300 K in the presence of different concentrations of theophylline
a: (0 - 0.075 mM). (0 mM: ◊, 0.008 mM: ○, 0.015 mM: Δ, 0.030 mM: ×, 0.045 mM: ○, 0.060 mM: +, 0.075 mM: ⋆).

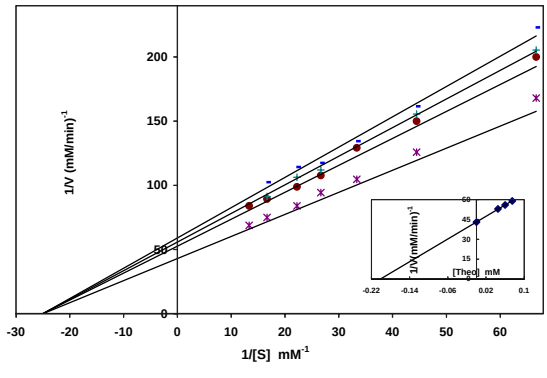


Figure 3b. (0 - 0.030 mM). Inset: secondary plot, $1/V_{max}$ versus [I] is shown. S and I are donated as substrate and inhibitor, respectively. (0 mM: ◊, 0.008 mM: ○, 0.015 mM: Δ, 0.030 mM: ×).

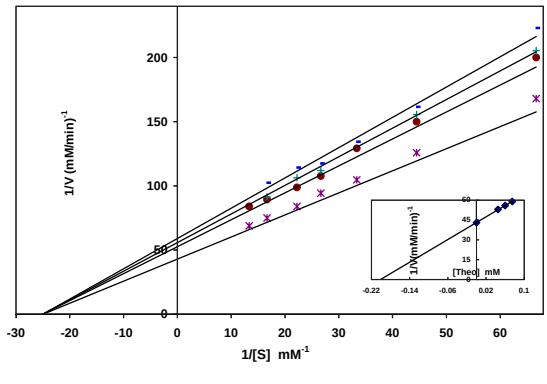


Figure 3c. (0.045 - 0.075 mM). Inset: secondary plot, $1/V_{max}$ versus [I] is shown. S and I are donated as substrate and inhibitor, respectively. (0 mM: ◊, 0.045 mM: ○, 0.060 mM: +, 0.075 mM: ⋆).

of binding constant and the enthalpy of inhibitor binding, using the isothermal titration microcalorimetry data. The dissociation binding constant values are equal to 140 μM and 318 μM for inosine and theobromine, respectively, obtained from the microcalorimetry method. These results agree well with the values of inhibition constants (143 μM and 311 μM) for inosine and theobromine, obtained from the spectroscopy method. The exothermic process of inosine and theobromine binding to ADA leads to an increase in the inhibition constant, when the temperature is increased.

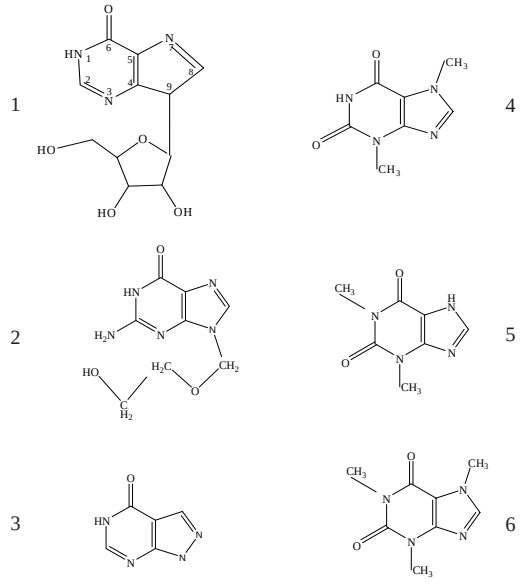
Adenosine deaminase is also inhibited by allopurinol and acyclovir. The single cross-over point indicates competitive inhibition of adenosine deaminase by allopurinol and acyclovir due to the penetration of the inhibitor molecules (allopurinol or acyclovir) into the active site of the enzyme. Inosine, allopurinol, acyclovir and theobromine inhibit ADA competitively, which agrees well with the literature in which it is reported that substitutions in the C-6 position of adenosine result in compounds that are competitive inhibitors (36).

The comparison made between the inhibition constant of inosine (K_i=143 μM) and acyclovir, (K_i=231 μM) elucidates the critical role of the ribose ring in the inosine structure, relative to the open ring of acyclovir. In other word, inosine as a product of the enzymatic reaction binds more tightly to the active site of the enzyme related to acyclovir. Comparison of the inhibition constant of theobromine (K_i=311 μM) with inosine (K_i= 143 μM) shows the critical binding role of N^7 in purine ring, which is substituted by a methyl group instead of a hydrogen atom on the N^7 position of theobromine. Asp 296 and Gly 184 residues in the active site of adenosine deaminase participate in hydrogen bonding with N^7 and N^9 of purine (18). Allopurinol as a transition state analog of xanthine oxidase inhibits competitively the catalytic activity of ADA. Interestingly, N^7 position in allopurinol is replaced by a CH_2 group, which demonstrates the lower inhibiting potency of allopurinol (K_i=285 μM) relative to inosine (K_i=143 μM).

ADA is non-competitively inhibited by theophylline under the experimental condition. This indicated that the inhibitor does not interfere with the binding of substrate to the active site. Theophylline has bulky groups in N^1 and N^3 position, thus the inhibitor molecules could not penetrate into the active site of adenosine deaminase. Non-competitive inhibition of ADA by theophylline shows different affinity binding sites at various drug concentrations. Therefore, there seems to be two different types of inhibition constant. One of them being due to the low concentration of the drug and the other occuring at higher concentrations of theophylline. Thermodynamic parameters also show that ADA has two binding sites for theophylline, which shows positive cooperativity in its sites (29). An enhancement in the order of 8.7 times that of the binding affinity of the second site due to the binding of theophylline on the first binding site may be followed by a fine conformational change in ADA, leading to the activation of the protein at low doses of the drug (29).

Caffeine with bulky groups in N^1, N^3 and N^7 positions, acts as an uncompetitive inhibitor for ADA at 27°C (27). N^1, N^3 and N^7 positions of the purine ring in caffeine are substituted by the methyl group. Purine base should not lack N^1, N^3 or N^7 positions. C^6-N^7-C^8 side is especially important for the recognition by the enzyme. Nucleosides bearing a bulky group in this area become, therefore, inactive as the substrate (38). The inhibition constant for caffeine is equal to 342 μM, obtained from the spectroscopic method, which agrees well with the value of 350 μM for the dissociation binding constant obtained from the microcalorimetric method (27). The endothermic process of caffeine binding to ADA leads to a decrease of inhibition constant when the temperature increases. Moreover, the interaction between ADA and caffeine might be a hydrophobic interaction, as increasing the temperature improves the binding.

In a structural sense, comparison between the structure of theophylline and theobromine, besides a comparison between the inhibition constant of theophylline (K_i=56 μM at low and 201 μM at higher concentrations) and caffeine (K_i=342 μM), indicates that substitution of a bulky group in N^1 and N^7 positionsof purine has a critical role in the binding affinity of the mentioned inhibitors to the enzyme.



Scheme 1. The open formula for 1 – Inosine, 2- Acyclovir, 3- Allopurinol, 4- Theobromine, 5- Theophylline, 6- Caffeine

(QSAR) analysis has revealed that binding affinity of the purine nucleosides upon interaction with ADA depends on the molecular volume, dipole moment of the molecule, and the electric charge around the N^1 atom (37). Nucleosides lacking the N^1 atom will not be a substrate, but are good inhibitors of adenosine deamination. Theophylline and caffeine, which are substituted at the N^1 position, could not behave as common competitive inhibitors.

Conclusion

ADA is competitively inhibited by inosine, allopurinol, theobromine and acyclovir at 27°C. Those drugs haven no substitution on the N^1 position of the purine ring. Therefore, inhibitor molecules competitive with substrate molecules could penetrate into the active site of the enzyme. In other way, caffeine and theophylline act as uncompetitive and non-competitive inhibitors for ADA, respectively. These types of inhibition indicate that the inhibitor molecules could not penetrate into the active site of adenosine deaminase. The above-mentioned drugs are methylated at the N^1 position of the purine rings. So, N^1 purine is a critical place for binding to the active site of ADA. Structural comparison between caffeine and theophylline shows that the N^7 purine ring in caffeine is substituted, therefore the difference in inhibition should be related to the structure of the inhibitor molecule. Thermodynamic parameters of the dissociation binding constant are comparable with the kinetic parameters of the inhibition constant, obtained from the spectroscopic method for inosine, caffeine, theophylline and theobromine.

Acknowledgements

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