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In Vitro Effects of Some Purine Analogue Drugs on Enzyme Activities of Carbonic Anhydrase Isozymes I and II from Human Erythrocytes

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Abstract

In vitro effects of some purine analogues on human carbonic anhydrase (CA, EC 4.2.1.1) were investigated in this study. Human erythrocyte CA-I and CA-II isozymes were purified using Sepharose-4B-aniline-sulfanilamide affinity gel chromatography. Specific activity and yields were determined as 904.08 EU mg^{-1}, 59.86%; 6469.23 EU mg^{-1}, 56.82%, respectively. The overall purification was approximately 105.74-fold for hCA-I and 756.64-fold for hCA-II. The inhibitory effects of different nucleoside analogues on CA activity were determined at low concentrations using the esterase method under *in vitro* conditions. I_{50} values for diprophylline, acyclovir, proxyphylline, aminophylline and caffeine were calculated from activity % -[I] as 4.12 μM, 5.58 μM, 9.96 μM, 19.17 mM, and 23.21 mM for hCA-I and 1.19 μM, 2.57 μM, 4.29 μM, 4.71 mM and 4.94 mM for hCA-II respectively. Additonally, the Lineweaver-Burk curves obtained were used for the determination of K_i and the inhibitor type for diprophylline, acyclovir and poxyphylline.

Abbreviations: CA, carbonic anhydrase; hCA-I, human carbonic anhydrase I; hCA-II, human carbonic anhydrase II.

INTRODUCTION

The carbonic anhydrases (EC. 4.2.1.1) are an expanding family of zinc-containing enzymes, which classically participate in the maintenance of pH homeostasis in human body, catalyzing the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and proton [1,2]. Sixteen isozymes of the zinc binding enzyme have been described that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Basically, there are five cytosolic forms (CA I-III, CA VII and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial ones (CA VA and CA VB), a secreted CA isozyme, CA VI as well as three acatalytic ones, CA VIII, X and XI, denominated also CA-related proteins (CARPs) [3]. CA XV is not present in primates but is found in other vertebrates, such as rodents, birds, and fish [4]. CAs are produced in a variety of tissues where they participate in several important biological

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processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation [3-5]. The two major CA isozymes (CAI and CAII) are present at high concentrations in the cytosol in erythrocytes, and CAII has the highest turnover rate of all the CAs [5]. Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis [6].

Many chemical susbstances and synthesized compounds affect metabolisms by changing enzyme activities. Chemicals are generally known to activate or inhibit several body enzymes *in vitro* [7-10] and affect the metabolic pathways. Purine analogues are widely used for treatment of various diseases. For example, caffeine takes part in most food, is commonly used in medical industry. Acyclovir is used in antiviral theraphy. It differs from other purine analogues by its partial purine structure. Diprophylline and proxyphylline are derivatives of xanthine and commonly used as vasodilator and bronchodilator. Another bronchodilator aminophylline is used for treatment of asthema, bronchitis and emphysema. In the present study, we have purified carbonic anhydrase I and II from human erythrocytes and examined the *in vitro* inhibition effects of purine analogue drugs on these important enzymes.

MATERIALS AND METHODS

Chemicals

Sepharose 4B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich). All other chemicals were analytical grade and obtained from Merck.

Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20 000 rpm for 30 min at 4ºC. The pH of the hemolysate was adjusted to 8.7 with solid Tris [11]. The hemolysate was applied to Sepharose 4B-aniline-sulfanylamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA-I and hCA-II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4ºC [12].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [13]. CO_2–hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_o\text{-}t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenylate ion over a period of 3 min at 25ºC using a spectrophotometer (CHEBIOS UV-VIS) according to the method described by Verpoorte et al. [14]. The enzymatic

reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [15].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli. A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye [16]. The electrophoretic pattern was photographed (see Figure 1).

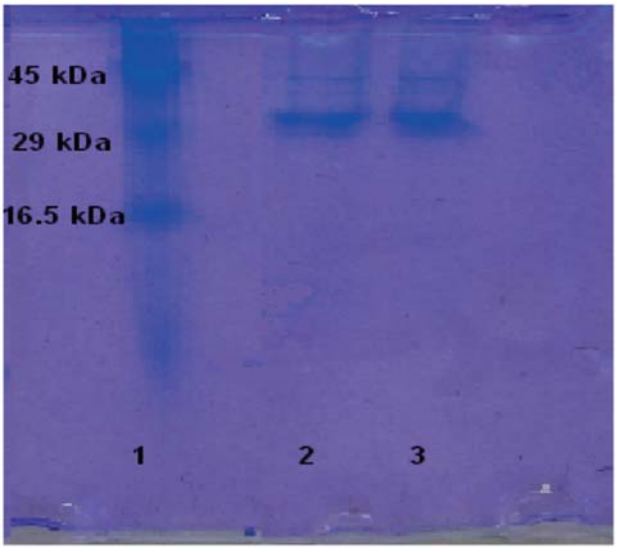

Figure 1. SDS-PAGE analysis of purified hCA-I and hCA-II. Lane 1: Standards: Egg albumin (45 kDa), bovine carbonic anhydrase (29 kDa) and chicken egg white lysozyme (16.5 kDa), Lane 2: purified hCA-I, Lane 3: purified hCA-II.

In vitro inhibition studies with purine analogues

The inhibitory effects of diprophylline, acyclovir, poxyphylline, aminophylline and caffeine were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. hCA-I enzyme activities were measured for diprophylline (3.0-5.5 μM), acyclovir (3.5-7.2 μM), poxyphylline (4.8-16.2 μM), aminophylline (10-25 mM) and caffeine (15-30 mM) at cuvette concentrations and hCA-II enzyme activities were measured for diprophylline (0.7-2.5 μM), acyclovir (1.5-5.3 μM), poxyphylline (3.2-6.5 μM), aminophylline (3.2-5.7 mM) and caffeine (3.0-8.5 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity%- [Inhibitor] graph was drawn.

To determine K_i values for diprophylline, acyclovir and poxyphylline, three different inhibitor concentrations were tested; in these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver-Burk curves were drawn [17]. The K_i values and the inhibitor types obtained were determined.

RESULTS

The purification of the enzymes was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column. hCA-I enzyme was purifed, 105.74-fold with a specific activity of 904.08 $EUmg^{-1}$ and overall yield of 59.86% and the hCA-II enzyme was purifed, 756.62-fold with a specific activity of 6469.23 $EUmg^{-1}$ and overall yield of 56.82 % (Table 1). Figure 1 shows the SDS-PAGE obtained for determining the purity of the enzymes. Inhibitory effects of drugs on enzyme activities were tested under *in vitro* conditions; I_{50}

Table 1. Summary of purification procedure for hCA-I and hCA-II.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	148	40	17.3	692.00	5920	8.55	100.00	1.00
hCA-I	443	8	0.49	3.92	3544	904.08	59.86	105.74
hCA-II	827	4	0.12	0.52	3364	6469.23	56.82	756.64

values were calculated Activity%- [Inhibitor] graphs and are given in Table 2 and K_i values were calculated from Lineweaver–Burk graphs and are given in Table 3.

DISCUSSION

The isozymes of CA play important roles in different tissues [18,19]. It is known that carbonic anhydrase has been purified many times from different organisms and the effects of various chemicals, organic compound and drugs on its activity have been investigated [7-11]. In this study, CA-I and II isoenzymes were purified from human eyrtrocytes by a simple one step procedure using Sepharose 4B-aniline-sulfanilamide affinity column. The activities of the effluents were determined by the hydratase method [13] and other further kinetic studies were performed using the esterase activity method [14].

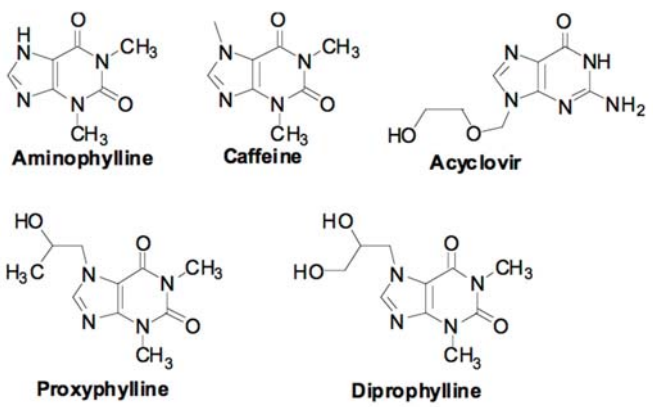

Figure 2. The structures of nucleoside analogue drugs used in this study.

Table 2. I_{50} values from in vitro study of hCA-I and hCA-II in the presence of difference nucleoside analogue drugs.

	I_{50} values	
Inhibitor	hCA-I	hCA-II
Diprophylline	4.12 μM	1.19 μM
Acyclovir	5.58 μM	2.57 μM
Proxyphylline	9.96 μM	4.29 μM
Aminophylline	19.17 mM	4.71 mM
Caffeine	23.21 mM	4.94 mM

We have not encountered any studies on the inhibitory effects of purine analogue drugs on CA esterase activity. In fact, most purine analogues inhibit CA I/II in the micromolar range as determined by the CO_2 hydratase method. However the esterase method usually gives much higher K_i values with all inhibitors. Advantage of the esterase activity determination is that it is a spectrophotometric and easy method.

Diprophylline, acyclovir, poxyphylline, aminophylline and caffeine were chosen for investigation of their inhibitory effects on CA in this study and it was important that purine analogues inhibited the enzyme activity at low concentrations. K_i parameters of these inhibitors for hCA-I and hCA-II were determined and it was found that most purine analogue drugs were potent inhibitors of CA.

In this study, we have concluded that purine structure doesn't have significant effect on enzyme activitiy but functional grups connected to imidazole ring increase inhibition strength. These inhibitors, like diprophylline and acyclovir, can be supposed as bonding model for active side of CA-II enzyme.

Table 3. K_i values for some nucleotide analogue drugs of hCA-I and hCA-II.

Inhibitor	K_i values for hCA-I	Inhibition type	K_i values for hCA-II	Inhibition type
Diprophylline	8.53 µM	Competitive	2.62 µM	Competitive
Acyclovir	9.95 µM	Uncompetitive	3.86 µM	Competitive
Proxyphylline	24.05 µM	Uncompetitive	9.17 µM	Competitive

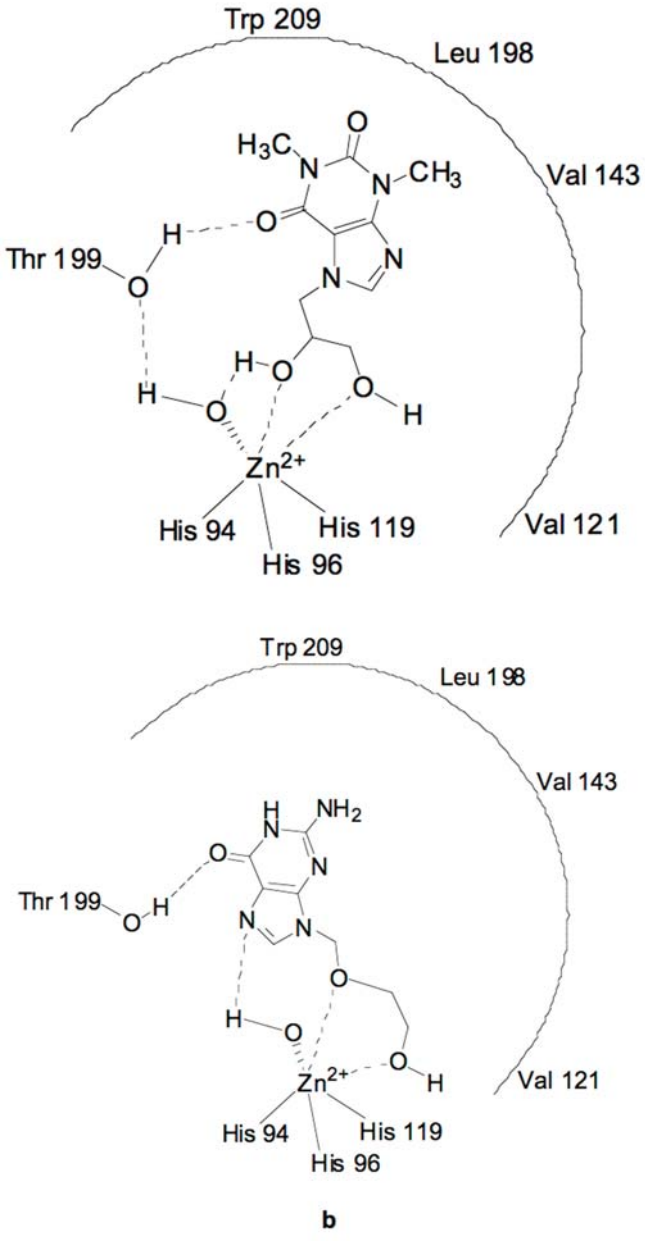

Figure 3. Putative binding model of diprophylline (a) and acyclovir (b) to the CA active side, considering the X-ray crytal structure of the hCA II-phenol adduct repoted earlier [21].

Those purine analogue drugs used in this study affect CA isoenzymes may due to the presence of different functional groups in addition to the -NH_2, -CH_3 and -OH groups bound to purine ring. It was reported in a study that thioxolone and salicylic acid derivatives lacks the sulfonamide, sulfamate, or related functional groups that are typically found in known CA inhibitors and could represent the starting point for a new class of CA inhibitors that may have advantages for patients with sulfonamide allergies [7,20]. Similarly our findings may indicate another class of CA inhibitors. If results of this study are checked, it can be easily seen that purine analogue drugs are more effective than many sulphonamides that are synthetized as spesific inhibitors of CA. These points out those purine analogue drugs may be used for the treatment of glucoma disease like sulphonamides.

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