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SIĞIR KARACİĞERİ KARBONİK ANHİDRAZ ENZİMİNİN BAZI İLAÇLAR İLE *İN VİTRO* İNHİBİSYONU

IN VITRO INHIBITION OF BOVINE LIVER CARBONIC ANHYDRASE II BY SOME DRUGS

İmdat AYGÜL[1*], Nuri GÜLEŞCİ[2]

[1]Gümüşhane Üniversitesi,SağlıkBilimlerFakültesi,BeslenmeveDiyetetikBölümü, Gümüşhane, Türkiye

[2]Gümüşhane Üniversitesi, SağlıkBilimlerFakültesi, İşSağlığıveGüvenliğiBölümü, Gümüşhane

ÖZET

Karbonik anhidraz enzimi (EC: 4.2.1.1; CA) sığır karaciğerinden saflaştırılarak bazı antibiyotiklerin inhibisyon etkileri incelendi. Saflaştırma prosedürü, homojenatın hazırlanması ve Sepharoz 4B-Tirozin-Sulfanamid Kromatografisinden oluştu. Birbirini takip eden bu iki yöntem ile enzim 65,24 kat, % 50,86 verimle ve spesifik aktivitesi 1117.24 U/mg protein olarak saflaştırıldı. Enzim üzerine bazı antibiyotik ilaçların *in vitro* inhibisyon etkileri belirlendi. Bu inhibitörler için IC_{50} değerleri, % aktiviteye karşı inhibitor konsantrasyon [I] grafiği çizilmek suretiyle belirlendi. Siprofloksazin ve seftazidim inhibitörlerinin IC_{50} değerleri sırasıyla 1.27 ve 5.29 mM olarak tespit edildi.

Anahtar Kelimeler*: Karbonik anhidraz(CA), Sığır, Karaciğer, İnhibitör, Antibiyotik*

ABSTRACT

Carbonic anhydrase (EC: 4.2.1.1; CA) was purified from bovine liver and some antibiotic inhibition properties were investigated. The purification procedure was composed of preparation of homogenate and affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide. By means of two consecutive procedures, the enzyme was purified 65.24-fold with a yield of 50.86%, and a specific activity of 1117.24 U/mg proteins. Some antibiotic drugs exhibited in vitro inhibitory effects on the enzyme activity. IC50 values for these inhibitos were determined by plotting activity % vs. [I], respectively. IC50 values of ciprofloxacin and ceftazidime were 1.27 and 5.29 mM.

Keywords**:** *Carbonic anhydrase (CA), Bovine, Liver, Inhibition, Antibiotic*

***SorumluYazar/Coresponding Author**: İmdat AYGÜL, GümüşhaneÜniversitesi, SağlıkBilimlerFakültesi, BeslenmeveDiyetetikBölümü, 29000-Gümüşhane, E-mail: imdat_aygul25@hotmail.com

1. INTRODUCTION

Carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1) isozyme, an ubiquitous enzyme family present in almost all living organisms, is a zinc metal ion containing metalloenzyme family. The molecular characteristics of the CAs across plants, animals, and other organism are similar [1]. The enzyme has high specific activity in many tissues such as muscle, heart, and liver of vertebrates [2,3].

Sixteen CA isoforms were determined in mammals (humans have only 15 isoforms) that differ in their subcellular localization and catalytic activity [4-6]. Some of the CAs are cytosolic isozymes (CA I, CA II, CA III, CAVII, and CA XIII), others are membrane-bound isozymes (CA IV, CA IX, CA XII, and CA XIV), two are mitochondrial isozymes (CA VA and CA VB), and one isozymes is especially secreted saliva (CA VI) [5]. However, many physiological functions, such as respiration, acid-base regulation and calcification are connected with these isozymes activity [2]. Also, some nonphysiological reactions are catalyzed by CAs in in vitro conditions [7], for instance, the enzyme has been observed to display 4-nitrophenylacetate (4-NPA) esterase activity in in vitro studies [8]. The enzyme catalyzes reversible hydration of carbon dioxide to bicarbonate ion and proton. CA therefore plays key roles in diverse processes, such as it indicates the above mentioned physiological functions [5]. The CA enzyme has high specific activity in many different tissues such as liver, kidney, heart, etc. [9,10].

Many different chemical compounds, prodrugs, and drugs synthesized in laboratory conditions or that exist naturally at relatively low concentrations affect the metabolism of different organisms by changing enzyme activity. Inhibitory or activatory effect showed compounds of this CA isozymes have several medical applications, such as among others in the treatment of glaucoma, as diuretics, in the management of several neurological disorders, including epilepsy, whereas several agents are in clinical evaluations as anticancer drugs or diagnostic tools [5].

So far inhibitory effects of different compounds, organic and inorganic anions, metal ions, phenol or poly phenolic compounds, and various species prodrugs or drugs have been investigated against many different CA isozymes [10-18]. In this study, carbonic anhydrase II, which has a vital importance in metabolism, was purified from bovine liver via a fast and simple technique and investigated some kinetic properties. Moreover, its in vitro inhibition by some antibiotic drugs was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

CNBr-activated Sepharose 4B, sulfanilamide, protein standards, 4-nitrophenylacetate, ciprofloxacin, and ceftazidime were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were obtained from Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt Germany).

2.2. Bovined Liver Homogenate Preparation

Bovine carbonic anhydrase II was purified from bovine liver tissue obtained from a local municipal slaughterhouse. Fresh bovine liver samples were taken and washed three times with 0.9 % NaCl, an isotonic saline solution. The liver cells were lysed by immersion in liquid nitrogen. The lysed sample was transferred to the buffer solution (25 mM Tris-HCl / 0.1 M Na_2SO_4, pH 8.7) and centrifuged at 4°C, 10000 x g for 30 min. The supernatant was centrifuged again and the second supernatant was used in subsequent studies [9].

2.3. Affinity Chromatography

The pH of bovine liver homogenate was adjusted to 8.7 using solid Tris and 50 ml of supernatant was applied to the prepared Sepharose 4B-L-tyrosine-sulfanilamide affinity column (1.36x30 cm). The CA enzyme was purified by using the affinity gel according to the published method [3].

The affinity gel was washed with 10 mM Tris-HCl / 22 mM Na_2SO_4 (pH 7.5). The bovine liver CA II enzyme was eluted with 1.2 M NaCl / 25mM Na_2HPO_4 (pH 6.3) [9]. Purified CA enzyme was dialyzed for 20 minute against 0.05 M Tris-SO_4 / 1 mM 2-mercaptoethanol (pH 7.4). All procedures were performed at 4°C. Protein concentrations in the column effluents were determined at 280 nm spectrophotometrically.

2.4. Hydratase Activity Assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [20]. CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (to-tc/tc) where t_0 and tc are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

2.5. Esterase Activity Assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25ºC using a spectrophotometer according to the method described by Verpoorte et al. [8]. The inhibitory effects of ciprofloxacin and ceftazidime were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity (%)-[Inhibitor] graphs were drawn.

2.6. Protein Determination

Protein during the purification steps was determined spectrophotometrically at 595nm according to the Bradford method, using bovine serum albumin as the standard [21].

3. RESULTS AND DISCUSSION

Ciprofloxacin and ceftazidime are generally mammalian used an antibiotic that can treat lots of bacterial infections. Each drugs are spectrum of activity includes most strains of bacterial pathogens responsible for including Gram-negative and Gram-positive bacterial pathogens [22]. Ciprofloxacin and other fluoroquinolones are valued for this broad spectrum of activity, excellent tissue penetration, and for their availability in both oral and intravenous formulations [23]. Ceftazidime is a third-generation cephalosporin. The balance of activity tips toward Gram-positive organisms for earlier generations; later generations of cephalosporins have more Gram-negative coverage. Ceftazidime is one of the few in this class with activity against Pseudomonas [24].

There are a few studies existing in the literature on the interactions of drugs and CA isozymes. The interactions of ceftriaxone, morphine, imipenem, diprophylline, acyclovir, proxyphylline, aminophylline, caffeine, midazolam, and diazepam with two human isozymes, CA I-II, has only recently been investigated [16-19], evidencing some low milimolar inhibitors as well as the possibility to design isozyme selective CA inhibitors. Indeed, the inhibition profile of various CA isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the sub-micromolar level for many simple functional group containing molecules [17]. Thus, it seemed reasonable to us to extend the previous studies [9-19], including this study on antibiotic drugs with common clinical applications.

We report here the first study on the inhibitory effects of ciprofloxacin and ceftazidime of type on the esterase activity of bovin CA II (bCA II). The previous studies by Coban et al.[16], investigated morphine by using a Verpoorte method, 4-nitrophenyl acetate esterase assay for monitoring hCA inhibition [8]. The data in Table 2 show the following regarding inhibition of bCA II with ciprofloxacin and ceftazidime, by an esterase assay [8], with 4-nitrophenyl acetate (4-NPA) as the substrate: Inhibitory activity has been observed with ciprofloxacin and ceftazidime for the inhibition of the rapid cytosolic isozyme bCA II (Table 2). Ciprofloxacin and ceftazidime showed moderate bCA II inhibitory activity with a IC_{50} values of 1.27 and 5.29 mM (Table 2).

The CAIs belong to two classes: (i) phenols (such as the simple phenol C_6H_5OH) [25], which bind to the zinc-coordinated water molecule/ hydroxide ion from the active site, through a network of two hydrogen bonds (Fig. 3A) [25], (ii) the ciprofloxacin which bind rather similar but not identical to phenols, that is, by anchoring to the water molecule/hydroxide ion coordinated to Zn(II) (Fig.3B).

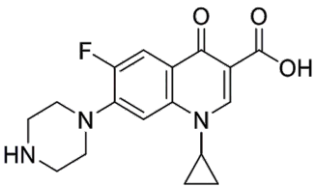
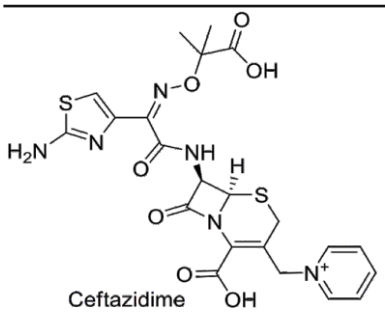
3. CONCLUSIONS

Ciprofloxacin and ceftazidime drugs affected the activity of bCA II isozyme due to the presence of the different functional groups (cyclopropane, piperazine, pyridinium, -COOH, $-CH_3$, -F and $-NH_2$) present in their aromatic scaffold. Our findings here a novel class of efficient CAIs, interacting with the CA II (main cytosolic) in a different manner compared to sulfonamides, sulfamates, and other classes of inhibitors, is reported in this paper. Kinetic measurements allowed us to identify different functional group substituted two antibiotics as well as ciprofloxacin as micromolar inhibitors of the bCA II isozyme. This new class of inhibitors binds differently from all other CAIs known to date, being found between the phenol-binding site within the enzyme cavity. They exploit different interactions with amino acid residues and water molecules from the CA active site compared to other classes of inhibitors, offering the possibility to design compounds with a better inhibition profile compared to the clinically used sulfonamides/sulfamates.

Table 1. Summary of purification procedure of CA from bovine liver.

Purification step	Activity (EU/ml)	Total volume (ml)	Protein mg/ml	Total protein (mg)	Total activity (U)	Spesific activity (U/mg)	Yield (%)	Purification factor
Homogenate	112	20	6.54	130,8	2240	17,125	100	1
Affinity Chromatography	324	4	0.29	1.16	1296	1117.24	50.86	65.24

Table 2. IC_{50} values for bovine liver CA.

Inhibitors	Ic_{50} (mM)
 Ciprofloxacin	1.27
 Ceftazidime	5.29

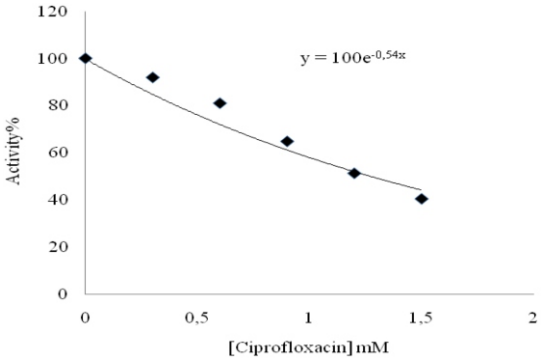


Figure 1. Activity %- [Ciprofloxacin] regression analysis graphs for bovine liver CA in the presence of 5 different drug concentrations.

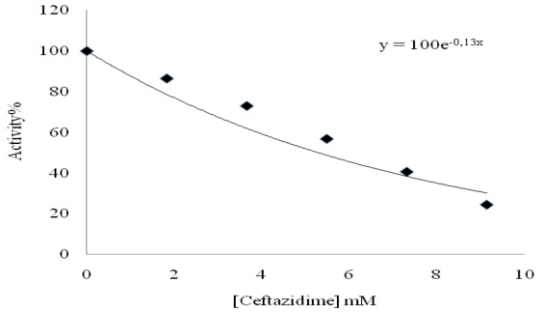
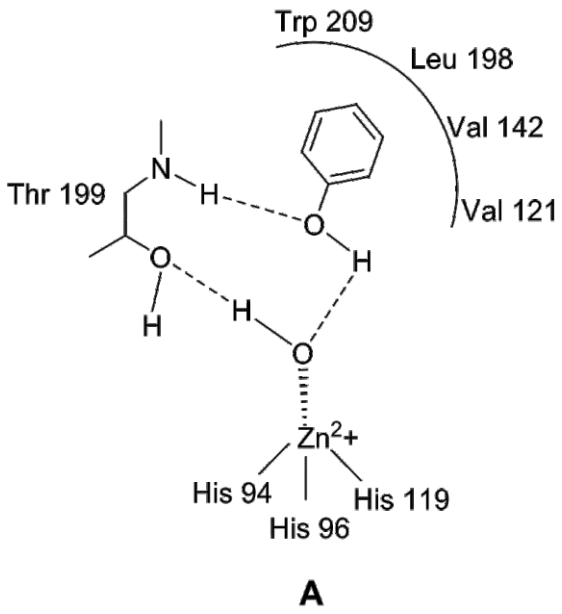
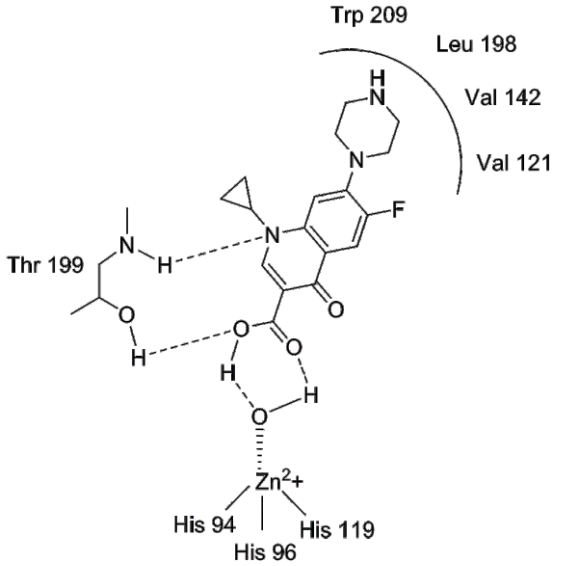


Figure 2. Activity %-[Ceftazidime] regression analysis graphs for bovine liver CA in the presence of 5 different drug concentrations.



A



B

Figure 3. CA inhibition with: such as compounds anchoring to the zinc-bound water/hydroxide ion, such as phenol (A) and ciprofloxacin (b). Figures represent distances (in Å), as determined by X-ray crystallographic techniques [25]. All these binding modes have been proven by means of X-ray crystallography on enzyme-inhibitor adducts [25]

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