Inhibition Effects of Some Sulfonamides on α-Carbonic Anhydrase from European seabass (*Dicentrachus labrax*)

Levrek (*Dicentrachus labrax*) Alfa-Karbonik Anhidraz üzerine Bazı Sülfonamidlerin İnhibisyon Etkisi

Research Article / Araştırma Makalesi

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ABSTRACT

Alpha-carbonic anhydrase (EC: 4.2.1.1; CA) was purified from European seabass gill and liver. The purification procedure was composed of preparation of homogenate (or hemolysate) and affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide. Some sulfonamides exhibited *in vitro* inhibitory effects on the enzyme activity. K_i constants and IC_{50} values for these drugs were determined by Lineweaver-Burk graphs and plotting activity % vs. [I], respectively. IC_{50} values of sulfanilamide, mafenide, acetazolamide, 2-amino-1,3,5-tiyadiazol-5 sulfonamide were 980 μM, 142 μM, 20 μM and 34 μM for gill carbonic anhydrase (GCA), 126 μM, 23 μM, 14 μM and 2.58 μM for liver carbonic anhydrase (LCA), respectively. Some sulfonamides exhibited much stronger inhibitory activity against GCA and LCA at low micromolar concentrations with the K_i values ranging from 0.21 to 76.0 μM as compared with other CAs.

Key Words
Carbonic anhydrase (CA), European seabass, liver carbonic anhydrase (LCA), Gill carbonic anhydrase (GCA), inhibition, sulfonamide.

ÖZET

Alfa-karbonik anhidraz (EC: 4.2.1.1; CA) levrek solungaç ve karaciğerden saflaştırıldı. Saflaştırma prosedürü homojenat hazırlama ve Sepharose 4B-tirozin-sülfanilamid afinite kramatografisinden oluştu. Bazı sülfonamidlerin enzim aktivitesi üzerine in vitro inhibisyon etkisi incelendi. Bu maddeler için K_i sabitleri ve IC_{50} değerleri Lineweaver-Burk grafikleri ve % aktivite-[I] grafikleri kullanılarak belirlendi. Sülfanilamid, mafenid, asetazolamid, 2-amino-1,3,5-tiyadiazol-5 sülfonamid için IC_{50} değerleri sırası ile solungaç karbonik anhidraz (GCA) için 980, 142, 20 ve 34 μM, karaciğer karbonik anhidraz (LCA) için 126, 23, 14 ve 2.58 μM, olarak belirlendi. Bazı sülfonamidlerin diğer CA izoenzimleri ile K_i sabitleri karşılaştırıldığında düşük mikromolar derişimlerde 0.21'den 76.0 μM'a kadar GCA ve LCA'ya karşı çok güçlü inhibitör aktiviteleri olduğu belirlendi.

Anahtar Kelimeler
Karbonik anhidraz (CA), European seabass, karaciğer karbonik anhidraz (LCA), solungaç karbonik anhidraz (GCA), inhibitör, sülfonamid.

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INTRODUCTION

Carbonic anhydrase (CA; Carbonate hydrolyase, EC 4.2.1.1) is a metalloenzyme family that catalyzes the rapid conversion of carbon dioxide to bicarbonate and protons, and involved in the biomineralization process [1]. The physiological function of the CA is to facilitate the interconversion of CO_2 and HCO_3^-, therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis. In addition, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system and inner ear [2]. The CAs are ubiquitous zinc enzymes, present in Archaea, prokaryotes and eukaryotes, being encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, eubacteria, algae and cytoplasm of green plants), the α-CAs (predominantly in eubacteria, algae and chloroplasts of both mono- as well as dicotyledons) and the α-CAs (mainly in Archaea and some eubacteria), respectively [3,4]. The α-CA family (mainly present in but not exclusive to mammals) has been thoroughly investigated from the drug design viewpoint [5].

Up to now, CA has been purified from many different tissues including human erythrocytes [6], fish erythrocytes [7]. The interactions between specific enzyme systems and different drugs, metal ions and chemicals have been extensively studied in the recent years [8-12]. These studies are very important in terms of drug design and elicit of mechanisms of the enzymes.

Two main classes of CA inhibitors (CAIs) are known: the metal complexing anions, and the unsubstituted sulfonamides. Sulfonamides have found extensive use as both carbonic anhydrase and matrix metalloproteinase inhibitors, which are a family of zinc containing enzymes implicated in a number of diseases such as arthritis and cancer [1, 2,13]. Studies performed on sulfonamides revealed that inhibition of these enzymes is brought about by their ability to mimic the tetrahedral transition state when binding to catalytic zinc ions located at the active site of the enzymes (Figure 1) [1,12,13]. Sulfonamides have also shown antibacterial, diuretic, hypoglycemic, antithyroid, and most recently, antitumor activity. Sulfonamide/sulfamate CAIs targeting various mammalian α-CAs have been in clinical use for more than 50 years [1].

Taking into account above information, the current study aims in purification of carbonic anhydrase enzyme from European seabass (*Dicentrachus labrax*) gill, liver, and investigation of impacts of some sulfonamides on the enzyme activity (Figure 2).

METHODS AND MATERIALS

Chemicals

Sulfanilamide, mafenide, acetazolamide, 2-amino-1,3,5-tiyadiazol-5 sulfonamide, Sepharose 4B, protein assay reagents, 4-nitrophenylacetate

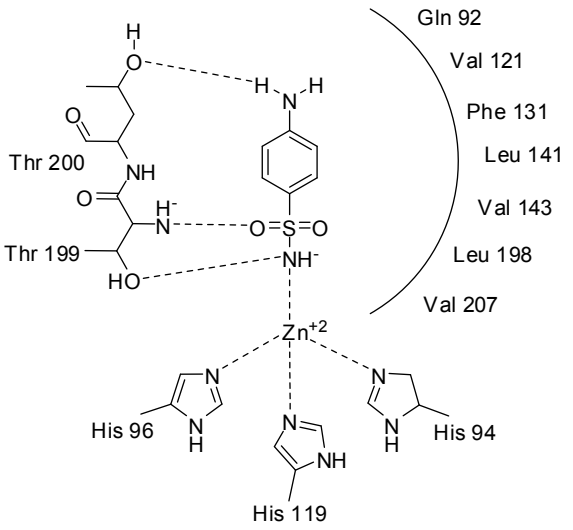

Figure 1. Active site region in the hCA II-Sulfanilamide complex X-ray structure, showing residues participating in recognition of the inhibitor molecule [13].

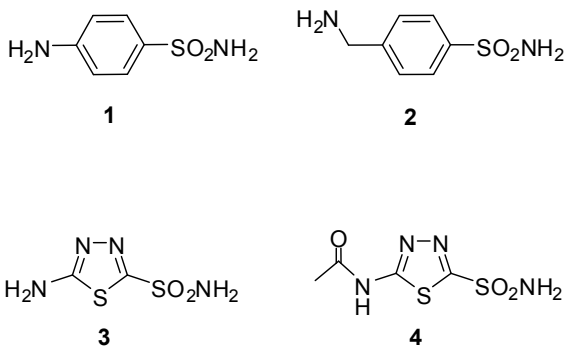

Figure 2. The structures of sulfonamides used in this study.

were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

Purification of carbonic anhydrase from fish liver by affinity chromatography

Fish gills and livers were purified from frozen fish tissues obtained from a commercial fish farm in Black Sea region in Turkey. The tissue samples were centrifuged at 10000 rpm for 30 min and the plasma and precipitate were removed. The pH of the homogenate was adjusted to 7.5 with solid Tris. The homogenate was applied to the prepared Sepharose 4BL-tyrosine-sulfanilamide affinity column equilibrated with 10mM Tris-HCl/0.1 M Na_2SO_4 (pH 7.5). The affinity gel was washed with 10 mM Tris-HCl/22 mM Na_2SO_4 (pH 7.5). The fish liver carbonic anhydrase enzyme was eluted with 1.2 M NaCl/25 mM Na_2HPO_4 (pH 6.3). All procedures were performed at 4ºC [7].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [14]. CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (*to*-*tc*/*tc*) where t_0 and *t*c are the times for pH change of the non-enzymatic 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 (NPA) 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. [15]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05M 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. The inhibitory effects of sulfonamides were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. European seabass GCA enzyme activities were measured for sulfanilamide (0.295-1.475 mM), mafenide (0.01022-0.0511 mM), acetazolamide (0.00625-0.0312 mM), 2-amino-1,3,5-tiyadiazol-5 sulfonamide (0.0135-0.0675 mM) at cuvette concentrations. European seabass LCA enzyme activities were measured for sulfanilamide (0.0029-0.435 mM), mafenide (0.0113-0.0565 mM), acetazolamide (0.00625-0.0317 mM), 2-amino-1,3,5-tiyadiazol-5 sulfonamide (0.000046-0.00046 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity (%)-[Inhibitor] graphs were drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.10–0.60 mM). The Lineweaver-Burk curves were drawn [16].

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 [17].

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 procedure. A 20 μg 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 [18].

In Vitro Effects of Drugs

In order to determine the effects of some sulfonamides on fish GCA and LCA, different concentrations of inhibitors were added to the reaction medium. The enzyme activity was measured, and an experiment in the absence of inhibitor was used as control (100% activity). The IC_{50} values were obtained from activity (%) vs. inhibitor concentration plots.

In order to determine K_i constants in the media with inhibitor, the substrate (NPA) concentrations were 0.01, 0.02, 0.035, 0.05, and

0.07 mM. Inhibitor solutions (sulfonamides) were added to the reaction medium, resulting in 3 different fixed concentrations of inhibitors in 1 ml of total reaction volume. Lineweaver-Burk graphs [16] were drawn by using 1/V vs. 1/[S] values and K_i constant were calculated from these graphs. Regression analysis graphs were drawn for IC_{50} using inhibition % values by a statistical package (SPSS-for windows; version 10.0) on a computer (student *t*-test; n= 3).

RESULTS AND DISCUSSION

Carbon dioxide, produced in fish tissues, is hydrated rapidly by carbonic anhydrase enzyme, converted into bicarbonate, and transported in the blood. Approximately 98% of the transported and stored carbon dioxide is in bicarbonate form. At the respiratory epithelium, erythrocytic CA catalyses the rapid dehydration of HCO_3^- to molecular CO_2, which then diffuses passively into the ventilatory water stream. Moreover, the CO_2/HCO_3^- system constitutes one of the most important physiological buffers for acid–base regulation [1,3,19].

Studies regarding influences of various substances on fish CA enzymes have gained a great attention recently. For instance, in vitro effect of some inhibitors on enzymes, such as intestinal and branchial carbonic anhydrase and Na^+/K^+–ATPase, which play a key role in salt and osmoregulation and acid–base balance in the teleost fish, was studied. Carbonic anhydrase activities in gill and intestinal homogenates were significantly inhibited by heavy metals [20]. In another study, freshwater rainbow trout exposed to 10 μg l^{-1} Ag for 48 h had significantly lower activities of the branchial enzymes Na^+/K^+ ATPase (85% inhibition) and carbonic anhydrase (28% inhibition). The results suggested that a disturbance of branchial ionoregulation, as a result of inhibition of branchial enzymes involved in ion transport, is the principal mechanism of the physiological toxicity of silver nitrate to freshwater fish [21]. Another study demonstrated that Cl^- uptake in P. *promelas* acclimated to soft water occurs through both a Na^+:K^+:2Cl^- co-transporter and a Cl^-/HCO_3^- exchanger, but is not dependent on carbonic anhydrase [19-23].

Table 1. IC_{50} values for some sulfonamide.

Inhibitor	GCA (μM)	LCA (μM)
Sulfanilamide (1)	980	126
Mafenide (2)	142	23
Acetazolamide (3)	20	14
2-Amino-1,3,5-tiyadiazol-5 sulfonamide (4)	34	2.58

Table 2. K_i values obtained from regression analysis graphs for GCA and LCA in the presence of some sulfonamides concentrations.

Inhibitor	GCA (μM)	LCA (μM)
Sulfanilamide (1)	76.0	17.2
Mafenide (2)	33.0	1.24
Acetazolamide (AZA) (3)	0.27	0.21
2-Amino-1,3,5-tiyadiazol-5 sulfonamide (4)	0.32	5.47

In this study, European Sebass (*Dicentrarchus labrax*) gill and liver CA enzymes were first isolated. Purification procedure was carried out by the preparation of the homogenate and affinity chromatography on Sepharose 4B tyrosine-sulfanilamide. As a result of the two consecutive steps, the enzymes were purified up to 69.7 and 73.4-fold with a recovery ratios of 43% and 46% compared to homogenate, respectively. After the sample had completely passed through, the column was washed with 10mM Tris-HCl/0.1M Na_2SO_4 buffer whose pH was 7.5. During washing, absorbences of fractions were spectrophotometrically measured at 280 nm and 348 nm. These values of the absorbance showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, GCA enzyme was eluted with 1 M NaCl/25mM Na_2HPO_4 pH 7.0. LCA enzyme was eluted with 1.2 M NaCl/25 mM Na_2HPO_4 pH 6.3. At the end of the last step, highly purified enzymes were obtained exhibiting a single band on SDS-PAGE. We used only two chromatographic techniques, Sepharose 4B tyrosine-sulfanilamide affinity chromatography by modification of washing and elution conditions. These results mean that the procedure used in the purification is good enough to be used in further studies, and also has an advantage of an experimental period as short as a day.

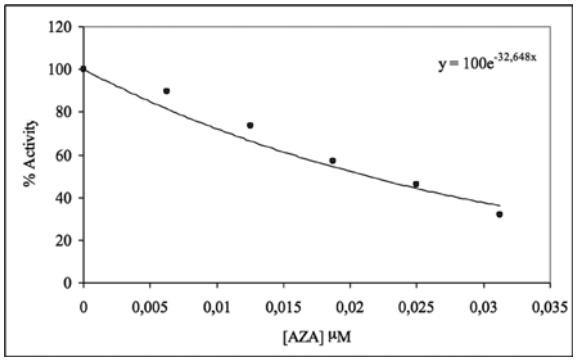

Figure 3. Activity %-[Acetazolamide] regression analysis graphs for fish GCA in the presence of 5 different acetazolamide (AZA) concentrations.

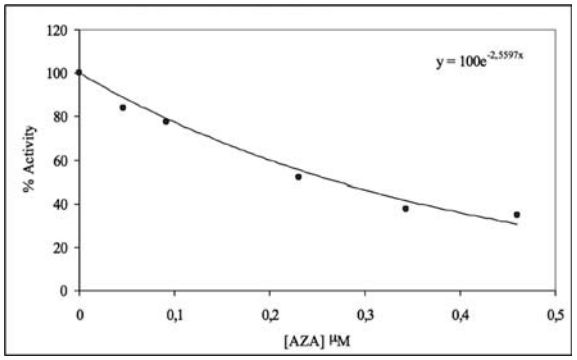

Figure 4. Activity %-[Acetazolamide] regression analysis graphs for fish LCA in the presence of 5 different acetazolamide (AZA) concentrations.

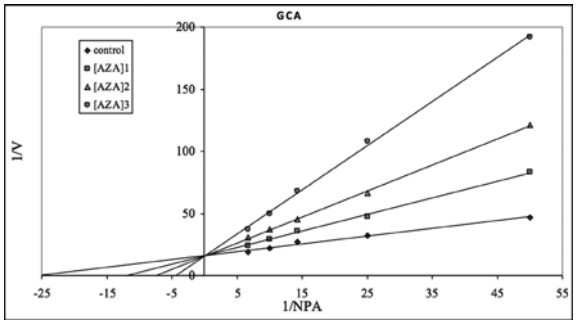

Figure 5. Lineweaver-Burk graph in 5 different substrate 4-nitrophenylacetate (NPA) concentrations and in 3 different acetazolamide (AZA) concentrations for determination of K_i for GCA.

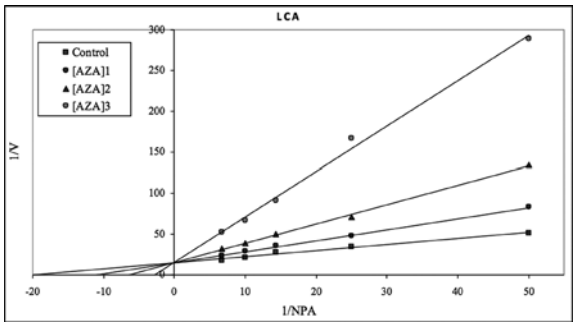

Figure 6. Lineweaver-Burk graph in 5 different substrate 4-nitrophenylacetate (NPA) concentrations and in 3 different acetazolamide (AZA) concentrations for determination of K_i for LCA.

In this study, sulfanilamide, mafenide, acetazolamide and 2-amino-1,3,5-tiyadiazol-5 sulfonamide were chosen to investigate their inhibitory effects on fish gill and liver CAs. K_i and IC_{50} parameters of these inhibitors were determined for GCA and LCA. Sulfonamides inhibited the enzyme activity at low concentrations. It is clear that sulfonamides are potent inhibitors for fish CA enzyme similar to human isoforms. K_i and IC_{50} graphs show that acetazolamide inhibits CAs in competitive manner (Figures 3-6 and Table 2). According to K_i values, the best inhibitor for fish GCA and LCA is acetazolamide.

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