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The *In Vitro* and *In Vivo* Inhibitory Effects of Some Sulfonamide Derivatives on Rainbow Trout (*Oncorhynchus Mykiss*) Erythrocyte Carbonic Anhydrase Activity

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The *in vitro* and *in vivo* inhibitory effects of 5-(3α, 12α-dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (1), 5-(3α, 7α, 12α-trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (2), 5-(3α, 7α, 12α-triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (3) and acetazolamide on rainbow trout (*Oncorhynchus mykiss*) (RT) erythrocyte carbonic anhydrase (CA) were investigated. The RT erythrocyte CA was obtained by affinity chromatography with a yield of 20.9%, a specific activity of 422.5 EU/mg protein and a purification of 222.4-fold. The purity of the enzyme was confirmed by SDS-PAGE. Inhibitory effects of the sulfonamides and acetazolamide on the RT erythrocyte CA were determined using the CO_2-Hydratase method *in vitro* and *in vivo* studies. From *in vitro* studies, it was found that all the compounds inhibited CA. The obtained I_{50} value for the sulfonamides (1), (2) and (3) and acetazolamide were 0.83, 0.049, 0.82 and 0.052 μM, respectively. From *in vivo* studies, it was observed that CA was inhibited by the sulfonamides (1), (2) and (3) and acetazolamide.

Keywords: Sulfonamides; Carbonic anhydrase; Rainbow trout; Erythrocyte

Abbreviations: CA, carbonic anhydrase; RT, rainbow trout

INTRODUCTION

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is member of the zinc metalloenzymes.[1,2] This enzyme is a well characterized pH regulatory enzyme in most tissues including erythrocytes; it catalyses the reversible hydration of CO_2 to HCO_3^- and H^+.[3–5]

Fourteen different CA isozymes have been described up to the present in higher vertebrates.[6,7] Among the CA isozymes are cytosolic (such as CA I, CA II, CA III, CA VII), membrane-bound (CA IV, CA IX, CA XII and CA XIV), mitochondrial (CA V), secretory forms (CA VI) and several acatalytic forms (CA VIII, CA X and CA XI).[8,9] Some of them have also been identified in tumor cells.[10]

Sulfonamides were the effective chemotherapeutic agents used systematically in the cure and prevention of bacterial infections. They were most important and popular medicines against bacterial infections before the advent of antibiotics and the development of bacterial resistance to sulfonamides in the course of time. However, they are still used successfully against various infections today. They are reliable medicines and ensure a cheap cure.[11,12]

Some sulfonamides are also the strongest inhibitors of CA isozymes, for example acetazolamide, dorzolamide and brinzolamide are three important CA inhibitors.[13] The sulfonamides exhibit their action by interacting with the zinc ion and the hydroxyl and amino groups of the amino acid Thr-199.[14] They have a diuretic effect by inhibiting CA isozymes in human kidney tubule cells.[13] In addition, those CA inhibitors which reduce intraocular pressure are widely used pharmacological agents for the treatment of glaucoma.[15–18]

In this study, we investigated the inhibition effects of some sulfonamide derivatives on the activity of CA from rainbow trout (*Oncorhynchus mykiss*) (RT) erythrocytes, *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Materials

RT were used in all experiments (*in vitro* and *in vivo* studies). Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie, Taufkirchen, Germany). *p*-Aminobenzene sulfonamide and L-tyrosine were from Merck (Merck KGaA, Darmstadt, Germany). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck. The sulfonamides (**1**), (**2**), and (**3**) were synthesized in our laboratory.[19]

Purification of RT Erythrocyte CA by Affinity Chromatography

CA was purified from RT blood, which was obtained from the Department of Fishery Sciences at Atatürk University. After the animals were anesthetized (MS-222 was used), blood was collected via heart puncture with a syringe flushed with EDTA and then placed into EDTA-containing tubes.[20,21] These samples were centrifuged (15 min, 3000 × g) (MSE, MISTRAL 2000) and plasma, and leucocytes were removed. The pack of red cells was washed twice with 0.9% w/v NaCl, and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation (48745 × g for 30 min.) (Heraeus Sepatech, Suprafuge 22) at 4°C and the pH of the haemolysate adjusted to pH 8.7 with solid Tris. The pH-adjusted haemolysate was then subjected to affinity chromatography [*Chromatography system*: chromatography column: 1.36 × 30 cm (Sigma Chemical Company); bed volume: 25 ml; peristaltic pump (Pharmacia Chemical Company); fraction collector (AO Instrument Company, U.S.A.)] at 4°C for the purification of CA.[22]

80 ml of pH-adjusted RT erythrocyte haemolysate was applied to the Sepharose 4B-L-tyrosine-sulfanylamide affinity column pre-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 RT erythrocyte CA was eluted with 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6) (flow rate: 20 ml h^{-1}; fraction volume: 4 ml). Purified CA was dialyzed for 72 h against 0.01 M K_2HPO_4/0.1 M KCl/5 mM 2-mercaptoethanol (pH 7.4). All procedures were performed at 4°C.[23]

The absorbance at 280 nm of the protein in the column effluents was determined spectrophotometrically. CO_2-hydratase activities in the eluants were determined and the active fractions were collected.[24,25]

In Vitro Inhibition Studies

CA activities were measured with increasing concentration of the sulfonamides (**1**) (0.43, 0.64, 1.07, 1.50 and 1.93 μM), (**2**) (0.02, 0.04, 0.06, 0.08 and 0.10 μM), (**3**) (0.17, 0.86, 1.03, 1.37 and 1.71 μM) and acetazolamide (0.01, 0.02, 0.05, 0.08 and 0.10 μM), respectively. CA activity was determined colorimetrically using the CO_2-hydration method of Wilbur and Anderson as modified by Rickli *et al.*[25,26] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_o - t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

The mathematical relationship among the sulfonamides (**1**) (**2**) and (**3**) and acetazolamide concentrations and CA activities (expressed as a percentage of that in the absence of the sulfonamides (1), (2), (3) and acetazolamide) were determined using conventional polynomial regression software (Microsoft Office 97, Excel).

Fish Husbandry and Maintenance

RT (n = 40) used in this study were mature with an average weight of 250 ± 24 g. The average water temperature was 9 ± 2°C (mean ± SD) during the tests. RT were fed twice daily with a commercial pelleted trout feed (at 1% body weight).

In Vivo Inhibition Studies

For these studies, ten RT (250 ± 24 g) were selected for intramuscular administration of each sulfonamide and acetazolamide respectively. Before the administration of compound, blood samples (0.5 ml) were taken from each RT and then 200 mg kg^{-1} body weight of sulfonamide and acetazolamide respectively was injected into each RT. A blood sample was also taken from each RT at 2, 4 and 6 h after injection. All blood samples were added to test tubes containing EDTA (2 mM) and subjected to centrifugation at 2500 × g for 15 min at 4°C (HERMLE Z383K). The erythrocyte pellet was then washed with cold 0.16 M KCl three times and the supernatant was discarded. One volume taken from the erythrocyte pellet obtained was hemolysed in five volumes of ice-water to give the hemolysate. Activity was determined according to the method of Wilbur and Anderson as described above.[24,25] The obtained data were subjected to statistical analysis applying student-t-test and presented as means ± SD.

Protein Determination

During the purification steps, protein levels were determined spectrophotometrically (595 nm)

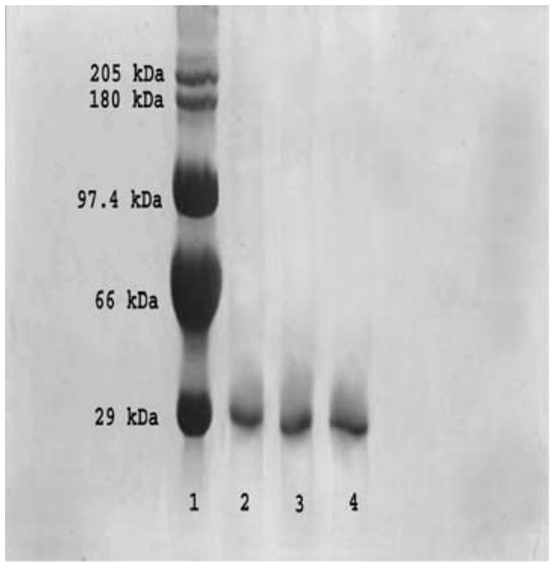

FIGURE 1 SDS-PAGE bands of CA. Lane 1: Standard proteins; Rabbit muscle myosin (205,000 Da), α_2-macroglobulin (180,000 Da), rabbit phosphorylase B (97,400 Da), bovine serum albumin (66,000 Da) and bovine carbonic anhydrase (29,000 Da). Lane 2: rainbow trout erythrocyte CA, Lane 3: rainbow trout gill CA, Lane 4: rainbow trout lens CA.

according to the Bradford's method, using bovine serum albumin as standard.[27] The amount of protein in column fractions was determined from absorbance measurements at 280 nm.

SDS-polyacrylamide Gel Electrophoresis

Enzyme purity was controlled by SDS-polyacrylamide gel electrophoresis. This technique was performed according to the Laemmli method using a vertical slab gel apparatus.[28] It was carried out in 10% and 4% acrylamide concentrations for running and stacking gel, respectively, containing 10% SDS. The gel easily stained with Coomassie Brillant Blue R-250 dye reagent overnight. The electrophoretic pattern obtained is shown in Figure 1.

RESULTS

The RT erythrocyte CA was purified by Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography (Table 1) and purity confirmed by SDS-PAGE (Figure 1). The overall purification gave CA in a yield of 20.9% with a specific activity of 422.5 EU/mg protein, which is a 222.4-fold purification (Table 1). Figures 2–5 show the *in vitro* effects of the sulfonamides and acetazolamide on the enzyme activity. I_{50} values were estimated at 0.83, 0.049, 0.82 and 0.052 μM for the sulfonamides (**1**), (**2**), (**3**) and acetazolamide, respectively (Table 2). The results of the *in vivo* effects of the sulfonamides and acetazolamide are presented in Table 3. In the sulfonamide (**1**) treated group, the control enzyme activity was 1638.9 ± 143.5 EU $(g\,Hb)^{-1}$, while the respective values determined 2, 4 and 6 h after sulfonamide (**1**) administration were 495.4 ± 103.1 ($p < 0.05$), 455 ± 114.4 ($p < 0.05$), and 485 ± 91.6 EU $(g\,Hb)^{-1}$ ($p < 0.001$), respectively. The greatest inhibition was found 4 h after injection. For sulfonamide (**2**) the control activity was determined as 1289.2 ± 46 EU $(g\,Hb)^{-1}$. The activities of the groups after sulfonamide (**2**) injection were measured at 2 h ((318 ± 67.9 EU $(g\,Hb)^{-1}$ ($p < 0.001$)), 4 h ((30.4 ± 6.3 EU $(g\,Hb)^{-1}$ ($p < 0.001$)) and 6 h ((304.4 ± 36.2 EU $(g\,Hb)^{-1}$ ($p < 0.001$)). The greatest inhibition was found 4 h after injection. For sulfonamide (**3**), the control activity was determined as 1792.5 ± 311 EU $(g\,Hb)^{-1}$. The activities of the groups after sulfonamide (**3**) injection were measured at 2 h ((762.2 ± 62.7 EU $(g\,Hb)^{-1}$ ($p < 0.05$)), 4 h ((99.5 ± 55.3 EU $(g\,Hb)^{-1}$ ($p < 0.05$)) and 6 h ((282.6 ± 75.8 EU $(g\,Hb)^{-1}$ ($p < 0.05$)). The greatest inhibition was found 4 h after injection. In the acetazolamide-treated group the control CA activity was determined as 1662.5 ± 67 EU $(g\,Hb)^{-1}$. The activities of the groups after the acetazolamide injection were measured at 2, 4 and 6 h, and the corresponding activities were 105.7 ± 27.9 ($p < 0.001$), 38.9 ± 11.8 ($p < 0.001$) and 339.2 ± 137.9 EU $(g\,Hb)^{-1}$ ($p < 0.05$), respectively (Table 3). The greatest inhibition was found 4 h after injection.

DISCUSSION

There are many chemicals, which are known to have adverse or beneficial effects on enzymes of all life forms including human beings and fish. Sulfonamides have been used for the treatment of some diseases in humans. Dorzolamide and

TABLE I Scheme for purification of carbonic anhydrase from rainbow trout erythrocytes

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	53.8	80	28	2240	4304	1.9	100	1
Sepharose-4B-L tyrosine-sulfanilamide affinity chromatography	60	15	0.142	2.13	900	422.5	20.9	222.4

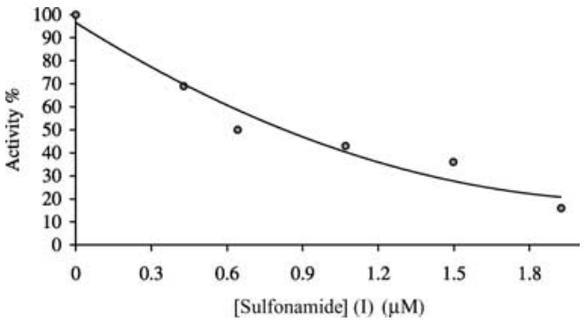

FIGURE 2 Effect sulfonamide (**1**) concentration on rainbow trout erythrocyte CA activity.

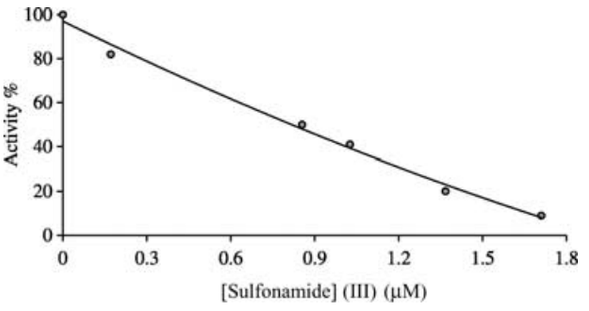

FIGURE 4 Effect of sulfonamide (**3**) concentration on rainbow trout erythrocyte CA activity.

brinzolamide are two of the topical CA inhibitors[13] and are used for the treatment of glaucoma.[15–18] Recently, it has been shown that the derivatives of simple aromatic sulfonamide carbonic anhydrase inhibitors acted as very powerful inhibitors of growth for many types of tumor cells, *in vitro* and *in vivo*.[10] Sulfonamides are also used against various diseases in fish especially furunculosis, vibriosis and coccidiosis.[29,30] It is known that some sulfonamide derivatives perform several biological activities and have antibacterial properties through the 1, 3, 4-thiadiazole ring.[31] The sulfonamide derivatives used in this study include this ring structure.

The action of these sulfonamides on CA activity has previously been reported as being due to inhibition of human CA-I and CA-II.[19] Given the importance of CA in pH regulation in most tissues, the effects of administering increasing concentrations of the sulfonamide derivatives (**1**), (**2**), and (**3**) and acetazolamide on rainbow trout CA was undertaken in this study.

Although, CA and the inhibitory effects of many chemicals on CA have been studied in most tissues and red blood cells, no study has yet been done on RT erythrocyte CA.

The sulfonamides (**1**), (**2**), and (**3**) and acetazolamide were chosen as inhibitors for investigation of inhibition effects. To show inhibition effects, remaining activity % of RT erythrocyte CA at five different concentrations of each sulfonamide and acetazolamide were determined. CA activity in the absence of the sulfonamides was taken as 100% activity (Figures 2–5).

For each inhibitor, the inhibitor concentrations causing up to 50% inhibition (I_{50} values) were determined from the regression analysis graphs. The obtained I_{50} values of the sulfonamides (**1**), (**2**), and (**3**) and acetazolamide were 0.83, 0.049, 0.82 and 0.052 μM on RT erythrocyte CA, respectively (Table 2). These results implied that sulfonamides and acetazolamide have an inhibitory effect on CA at very low concentrations and it was observed that sulfonamide (**2**) had the greatest inhibitory potency. Inhibitory effects of these sulfonamides in rainbow trout CA and due to being bound to the active center of the enzyme or another part of enzyme.

For all the sulfonamides, the greatest inhibition was found 4 h after injection. These results indicated that sulfonamides (**1**), (**2**) and (**3**) and acetazolamide entered into the erythrocytes. Our data showed that *in vivo* studies relating to these compounds

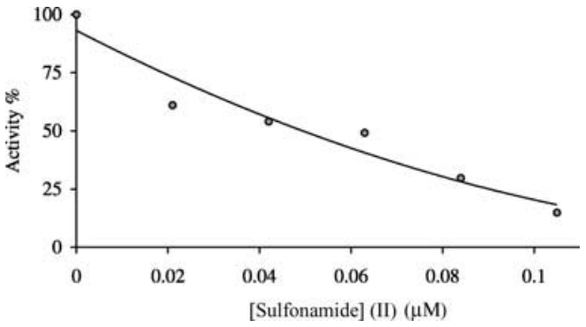

FIGURE 3 Effect of sulfonamide (**2**) concentration on rainbow trout erythrocyte CA activity.

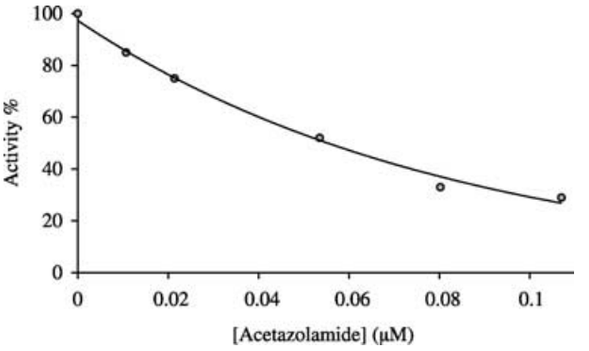

FIGURE 5 Effect of acetazolamide concentration on rainbow trout erythrocyte CA activity.

TABLE II I_{50} values from *in vitro* study of CA inhibition

Sulfonamides	I_{50} value (μM)
5-(3α,12α-dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**1**)	0.83
5-(3α,7α,12α-trihydroxy-5-β-cholanamido)-1,3,4- thiadiazole-2-sulfonamide (**2**)	0.049
5-(3α,7α,12α-triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**)	0.82
Acetazolamide	0.052

supported the results of the *in vitro* studies. Since, the blood concentration of administered sulfonamide derivatives is maximal at four hours, inhibition may probably then be at its maximum level.

In conclusion, the physiological effects of these sulfonamides on rainbow trout erythrocyte CA activity were observed. From the findings of the present study, sulfonamide (**2**) and acetazolamide had the greatest potency than the others in *in vitro* and *in vivo* experiments. In addition, due to the absence of any feasible curative measures for tumors, it might be that both sulfonamide (**2**) and acetazolamide could be useful as chemotherapeutic agents for the treatment of trout tumors. Future work needs to investigate the effect of the inhibitors on the growth of tumors cells by *in vitro* and *in vivo* studies and also determine at which stage these two inhibitors inhibited CA activity.

TABLE III Statistical values obtained from *in vivo* studies for the following compounds

The Sulfonamides	Time	X+SD EU(g Hb)$^{-1}$	P
5-(3α,12α-dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**1**)	Control	1638.9 ± 143.5	–
	2 h	495.4 ± 103.1	<0.05
	4 h	455 ± 114.4	<0.05
	6 h	485 ± 91.6	<0.001
5-(3α,7α,12α-trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**2**)	Control	1289.2 ± 46	–
	2 h	318 ± 67.9	<0.001
	4 h	30.4 ± 6.3	<0.001
	6 h	304.4 ± 36.2	<0.001
5-(3α,7α,12α-triacetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (**3**)	Control	1792.5 ± 311	–
	2 h	762.2 ± 62.7	<0.05
	4 h	99.5 ± 55.3	<0.05
	6 h	282.6 ± 75.8	<0.05
Acetazolamide	Control	1662.5 ± 67	–
	2 h	105.7 ± 27.9	<0.001
	4 h	38.9 ± 11.8	<0.001
	6 h	339.2 ± 137.9	<0.05

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