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Synthesis and Investigation of Inhibition Effects of New Carbonic Anhydrase Inhibitors

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Abstract—Three new derivatives of 2-substituted 1,3,4-thiadiazole-5-sulfonamide have been synthesized. These compounds are 2-(3-chloropropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**1**); 2-(2,2-dichloroacetylamino)-1,3,4-thiadiazole-5-sulfonamide (**2**); and 2-(3-phenylpropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**3**). Inhibition effects of these compounds on carbonic anhydrase I and II have been investigated. By comparing I_{50} and K_i values of the compounds, it has been found that compound **1** is a more potent inhibitor than acetazolamide (**b**) on carbonic anhydrase II. © 1997 Elsevier Science Ltd. All rights reserved.

Introduction

There are seven recognized isozymes of carbonic anhydrase (CA); they have broad structural and evolutionary features in common.[1] The M_r is about 30,000, with zinc at the center, the active site including three histidine molecules, and zinc-bound water or hydroxide.[2] The enzyme has a simple role in nature: the catalytic interconversion of CO_2 and HCO_3^- (or CO_3^{2-}) ion with discharge of a proton.[3]

Since the discovery 56 years ago that sulfonamides inhibit CA,[4] it has appeared that powerful inhibitors are restricted to the structures RSO_2NH_2, where R is an aromatic or heteroaromatic ring.[3,5,6] Several small amendments have been made recently; there can be an oxygen bridge between R and S,[7] and NH_2 may be oxidized to NHOH.[8] In general, there are two constants in comparing each of the inhibitors, inhibitor–enzyme dissociation constant K_i and inhibitor–enzyme binding constant I_{50}.

Parenteral sulfonamides (i.e., acetazolamide, methazolamide, dichlorphenamide and ethoxzolamide) have been used for 40 years to reduce intraocular pressure (IOP) in glaucoma.[9] These inhibitors, both topical and systemic, lower IOP by reducing HCO_3^- formation in the ciliary process, thus lowering Na^+ transport and flow of aqueous humor; this is the basis for their use in glaucoma.[10] The pharmacological effect of them is believed to be due to the inhibition of CA II in the ciliary epithelium.[11,12] Unfortunately, systemic therapy with parenteral sulfonamides and their derivatives leads to significant side-effects.[13,14] Many of the side-effects observed are probably due to inhibition of the isozymes in other tissues. The main problem of designing new inhibitors is therefore to make them more organ-selective and isozyme-selective by adjusting the pharmacokinetic properties.

These undesirable side-effects obligate synthesis of new derivatives of sulfonamides that are more selective against CA II to be used in glaucoma. In this paper, we present the synthesis of new CA inhibitors and an in vitro inhibition study of them on CA I and II.

Synthesis

The synthesis of inhibitors is outlined in Scheme 1. 2-Acetylamino-1,3,4-thiadiazole-5-sulfonamide (**b**) (acetazolamide) used as a parenteral sulfonamide was synthesized from 2-amino-1,3,4-thiadiazole-5-thiol (**a**) by first acetylating and secondly oxidative chlorination to the sulfonyl chloride followed by amidation with an anhydrous liquid ammonia. Compound **b** was converted to the 2-amino-1,3,4-thiadizole-5-sulfonamide (**c**) by acidic hydrolysis. The compound **c** was reacted with 3-chloropropionyl chloride in pyridine to produce 2-(3-chloropropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**1**) and with 3-phenylpropionyl chloride in pyridine to produce 2-(3-phenylpropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**3**). In addition to these reactions, thirdly, the compound (**c**) was reacted with dichloroacetic anhydride in the presence of acetic acid to give 2-(2,2-dichloroacetylamino)-1,3,4-thiadiazole-5-sulfonamide (**2**).[15]

Biochemistry

CA I and II were purified from human erythrocytes by Sepharose-4B-L-tyrosine-*p*-aminobenzenesulfonamide affinity column.[16] Carbonic anhydrase activity was determined by hydrolysis of *p*-nitrophenylacetate.[17] The in vitro inhibition study of newly synthesized compounds **1**, **2**, **3** and acetazolamide (**b**) is summarized in Table 1. Results are expressed as I_{50} and K_i values.

Key words: carbonic anhydrase isozymes, carbonic anhydrase inhibitors.
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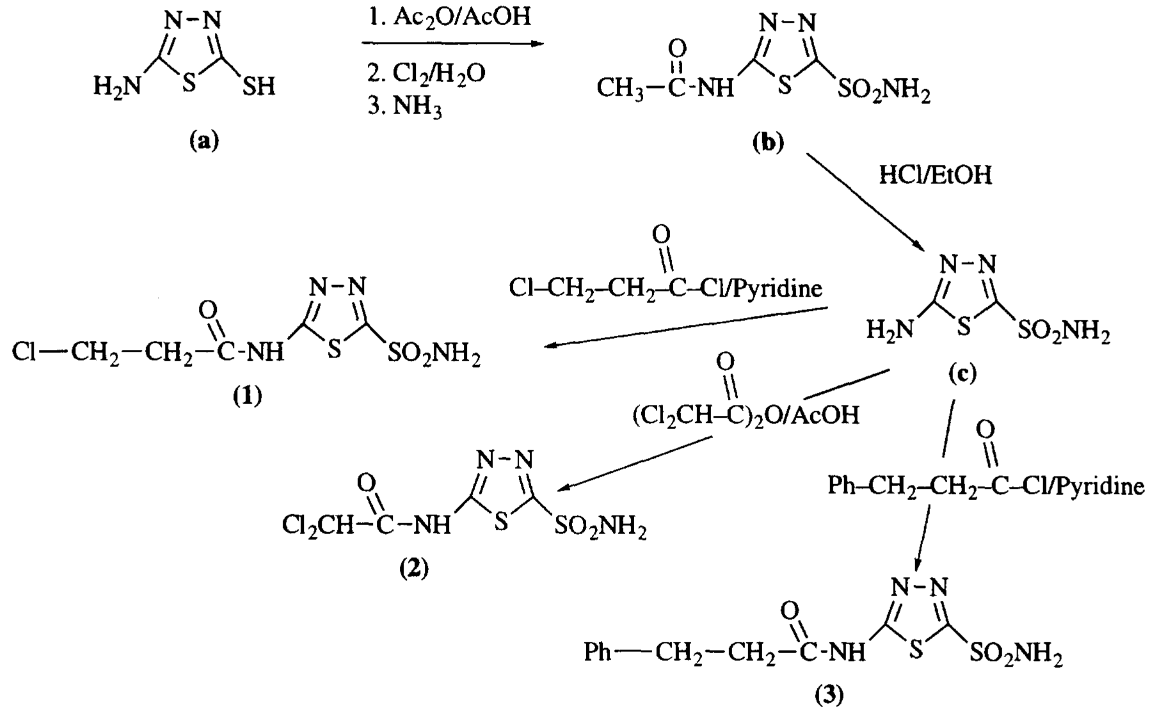


Scheme 1.

Results and Discussion

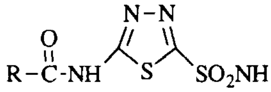
Carbonic anhydrase inhibitors lower intraocular pressure by reducing HCO_3^- formation in the ciliary process, thus lowering Na^+ transport and flow of aqueous humor; this is the basis for their use in glaucoma.[10] However none of the CA isozyme selective inhibitors can be used therapeutically. It remains, therefore, to use the presently available inhibitors as effectively as possible. Acetazolamide is the most widely used inhibitor and has advantages over the others because it is 20 times less active against CA I than CA II in erythrocytes.[18] A new CA inhibitor called MK-417 is also extremely weaker against CA I than CA II.[14] This 'protects' the red cell and avoids the development of respiratory acidosis. The respiratory acidosis is probably responsible for some of the side-reactions observed during acetazolamide therapy: i.e., fatigue, headache, taste sensations and for the distress in the 'chronic lunger'.[19–21] Unfortunately, since systemic therapy with parenteral sulfonamides and their derivatives leads to significant side-effects, these undesirable side-effects obligate synthesis of new derivatives of sulfonamides.

Since 2-(propionylamino)-1,3,4-thiadiazole-5-sulfonamide and 2-(2-chloroacetylamino)-1,3,4-thiadiazole-5-sulfonamide are known compounds and their inhibitions on CA are stronger than acetazolamide,[22,23] in this study, 2-(3-chloropropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**1**); 2-(2,2-dichloroacetylamino)-1,3,4-thiadiazole-5-sulfonamide (**2**); and 2-(3-phenylpropionylamino)-1,3,4-thiadiazole-5-sulfonamide (**3**) derivatives of 2-substituted 1,3,4-thiadiazole-5-sulfonamide which are not in the literature have been synthesized as shown in Scheme 1. Since the difference in interaction between the enzyme and inhibitor can be rationalized from the varied shape of the inhibitors, compounds **1**, **2**, and **3** having different groups were synthesized. In order to obtain maximal inhibition activity, the C-linked hydrogen atom(s) must be substituted by halogen.[14] Therefore, the compounds **1** and **2** having chlorine were synthesized.

In vitro inhibition effects of **1**, **2**, **3**, and acetazolamide (**b**) on CA I and II purified by affinity chromatography were studied to obtain I_{50} and K_i values given in Table 1. While the compounds **1** and **3** have higher I_{50} and K_i values than **b** on CA I, **2** has a lower K_i than **b** on CA I. Although compounds **2** and **3** have higher I_{50} and K_i values than **b** on CA II, **1** has lower I_{50} and K_i than **b** on CA II. By comparing I_{50} and K_i values of the compounds, it has been found that compound **1** is a more potent inhibitor than **b** on CA II and is a weaker inhibitor than **b** on CA I. Compound **1** was also approximately 20 times less active against CA I than CA II. Therefore, compound **1** can have fewer side-effects on body as discussed above.

2-(2-Chloroacetylamino)-1,3,4-thiadizole-5-sulfonamide having $ClCH_2-$ as R group gave 0.6 activity ratio on CA when it was compared with acetazolamide as 1.0.[23] When the compound **2** having Cl_2CH- as R group is compared with **b**, the I_{50} ratio of them on CA II is 1.17. 2-(2-Chloroacetylamino)-1,3,4-thiadizole-5-sulfonamide has an approximately twofold higher inhibition on CA II than compound **2**. In order to obtain high inhibition, it appears that one chlorine is better than two chlorines for substitution in acetazolamide (**b**).

Table 1. The results of inhibition studies for the synthesized compounds on carbonic anhydrase isozymes



		CA I		CA II	
Compound	**R**	I_{50} (nM)	K_i (nM)	I_{50} (nM)	K_i (nM)
b	H_3C–	445	718	75	70
1	$ClCH_2$–CH_2	644	875	40	46
2	Cl_2CH–	433	454	88	100
3	Ph–CH_2–CH_2–	990	1270	127	120

When 2-propionylamino-1,3,4-thiadizole-5-sulfonamide having CH_3–CH_2– as R group was compared with acetazolamide, the I_{50} ratio of them was found to be 0.87 on CA II.[22] In this study, when the compound **1** having $ClCH_2$–CH_2– as R group is compared with **b**, the I_{50} ratio of them on CA II is 0.53. As can be seen compound **1** shows better inhibition on CA II than 2-propionylamino-1,3,4-thiadizole-5-sulfonamide as a parent compound **1**, because of chlorine. Compound **1** also has a lower ratio (0.53) than 2-(2-chloroacetylamino)-1,3,4-thiadizole-5-sulfonamide (0.6), when they are compared to acetazolamide (**b**).

As a result, in order to see inhibition of CA II in the ciliary epithelium,[11,12] compound **1** should be tested in vivo as a candidate for treatment of glaucoma. This work is of particular interest because it involves a variance of the structure–action relationship with respect to CA inhibition.

Experimental

The structures of synthesized compounds have been identified by 200 MHz ^1H NMR (Gemini Varian A 0690), 60 MHz ^{13}C NMR (Varian EM 3 60L), IR (Perkin Elmer M.377) and Elemental Analysis (EAGER 200) methods. Melting points were measured on a Buchi Rotavapor R110.

2-Acetylamino-1,3,4-thiadiazole-5-sulfonamide (acetazolamide)(b). A mixture of 3 g (0.022 mol) **a** and 10 ml (0.11 mol) of acetic anhydride containing 0.5 mL of concentrated sulfuric acid was heated in a steam-bath for 0.5 h. The resultant mixture was allowed to cool slowly. The obtained solid compound was dissolved in 40 mL of 50% acetic acid. Chlorine gas was introduced in a fine stream for 0.5 h at 5 °C during which time the character of the precipitate changed. The solid was filtered and added to anhydrous liquid ammonia. The excess ammonia was removed in vacuo and the colorless solid residue was redissolved in 250 mL of cold water. The solid obtained by acidification of this solution was crystallized. (82%); mp 259–260 °C; IR (KBr) 3290, 2915, 1685, 1540, 1360, 1170 cm^{-1}; ^1H NMR (CD_3OD) δ 2.3 (s, 3 H, CH_3); ^{13}C NMR (CD_3OD) 171.3 (C=O), 166 (HNC=N), 163 (CSO_2NH_2), 23 (CH_3) ppm. Anal. ($C_4H_6N_4O_3S_2$).

2-Amino-1,3,4-thiadiazole-5-sulfonamide (c). A mixture of 3 g (0.016 mol) of **b**, 6 mL of concentrated HCl and 40 mL of ethanol was heated in an oil bath for 3 h. After a part of ethanol had evaporated, the obtained suspension was allowed to cool slowly. The solid was filtered and crystallized from water. (86%); mp 216–217 °C; IR (KBr) 3300, 1600, 1340, 1170 cm^{-1}; ^{13}C NMR (D_2O) 170.2 (H_2NC=N), 160.2 (CSO_2NH_2) ppm. Anal. ($C_2H_4N_4O_2S_2$).

2-(3-Chloropropionylamino)-1,3,4-thiadiazole-5-sulfonamide (1). 0.2 g (1.11 mmol) of **c**, 0.143 g (1.13 mmol) of 3-chloropropionyl chloride and 0.5 mL pyridine were mixed at room temperature for 6 h. After most of the pyridine had evaporated, the obtained solid was filtered and crystallized from water. (70%); mp 174–175 °C; IR (KBr) 3250, 2915, 1685, 1530, 1395, 1165 cm^{-1}; ^1H NMR (CD_3OD) δ 3.0 (t, 2H, CH_2), 3.9 (t, 2H, CH_2); ^{13}C NMR (CD_3OD) 171 (C=O), 166 (HNC=N), 163.5 (CSO_2NH_2), 40.8 ($ClCH_2$), 39.7 (CH_2) ppm. Anal. ($C_5H_7N_4O_3S_2Cl$).

2-(2,2-dichloroacetylamino)-1,3,4-thiadiazole-5-sulfonamide (2). 0.25 g (1.38 mmol) of **c**, 0.333 g (1.38 mmol) of 2,2-dichloroacetic anhydride and 20 mL of acetic acid were refluxed in the oil bath for 2 h. The resultant mixture was allowed to cool slowly and then dissolved in 10 mL of concentrated ammonium hydroxide. The solution was acidified with concentrated hydrochloric acid. The obtained solid was filtered and then crystallized from hot water. (74%); mp 221–222 °C; IR (KBr) 3395, 3280, 3020, 2950, 1710, 1560, 1350, 1170 cm^{-1}; ^1H NMR (CD_3OD) δ 6.7 (s, H, $CHCl_2$); ^{13}C NMR (CD_3OD) 165.5 (C=O), 168 (HNC=N), 163 (CSO_2NH_2), 66.5 (Cl_2CH) ppm. Anal. ($C_4H_4N_4O_3S_2Cl_2$).

2-(3-phenylpropionylamino)-1,3,4-thiadiazole-5-sulfonamide (3). 0.88 g (4.44 mmol) of **c**, 0.842 g (5 mmol) of 3-phenylpropionyl chloride and 0.5 mL pyridine were mixed at room temperature for 6 h. After most of the pyridine had evaporated, the obtained solid was filtered and crystallized from water. (58%); mp 240–241 °C; IR (KBr) 3290, 3020, 2950, 1680, 1520, 1370, 1175 cm^{-1}; ^1H NMR (d_6-DMSO) δ 2.9 (m, 4H, CH_2), 7.25 (m, 5H, Ar), 8.35 (s, 2H, $-SO_2NH_2$), 2.5 (s, 2H, $-NH_2$); ^{13}C NMR (d_6-DMSO) 171 (C=O), 164 (HNC=N), 162 (CSO_2NH_2), 36.5 (CH_2), 30 (CH_2) 126.5, 128.5 and 140.5 (Ar) ppm. Anal. ($C_{11}H_{12}N_4O_3S_2$).

Purification of carbonic anhydrase I and II from human erythrocytes: Erythrocytes were purified from human blood. The blood samples were centrifuged at 1500 rpm for 20 min and the plasma and buffy coat were removed. After the packed red cells were washed with NaCl (0.9%), the erythrocytes were hemolyzed with cold water. The ghost and intact cells were removed by centrifugation at 4 °C, 20,000 rpm for 30 min. The pH of hemolysate was brought to 8.5 with solid Tris. The hemolysate was applied to the affinity column having a structure of Sepharose-4B-L-tyrosine-sulfanylamide and equilibrated with 25 mM Tris-HCl:0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with the solution of 25 mM Tris-HCl:22 mM Na_2SO_4 (pH 8.5). CA I and CA II isozymes were eluted with the solution of 1 M NaCl:25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$:0.5 M $NaClO_4$ (pH 5.6), respectively.[16] The absorbance at 280 nm was used to monitor protein in the column effluents. For protein content estimation the method of Coomassie brilliant blue[24] was used with bovine serum albumin as a standard.

Determination of I_{50} and K_i values of compounds. Carbonic anhydrase activity was assayed by the hydrolysis of *p*-nitrophenylacetate.[17] I_{50} and K_i values of **b**, **1**, **2** and **3** were determined on CA I and CA II. In order to determine I_{50} values, 1.2 mL of 3 mM *p*-nitrophenylacetate was used as substrate. The volume of substrate was added to 1.5 mL with five different concentrations of inhibitors (50, 100, 150, 200, and 300 μL) and water. Reaction was started by adding of 1.4 mL of 0.05 M Tris-SO_4 (pH 7.4) and 0.1 mL enzyme solution for total volume of 3 mL. The absorbance was determined at 348 nm after 3 min. This study was repeated three times for each inhibitor. In order to determine I_{50} values, Regression analysis graphs were drawn by using % inhibition values by a statistical packing program on a computer. The inhibitor concentrations causing up to 50% inhibition were determined from the graphs. This way was followed to determine K_i values. In the media with or without inhibitor, the substrate concentrations were 0.6, 0.8, 1.0, and 1.2 mM. For this purpose, the substrate was used between 0.6 and 1.2 mL. Inhibitor solutions were added to the reaction medium as 0.1, 0.2 and 0.3 mL resulting in three different fixed concentrations of inhibitor. The Linewear–Burk graphs were obtained and K_i values were calculated.

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