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RESEARCH ARTICLE

Synthesis of novel sulfonamides under mild conditions with effective inhibitory activity against the carbonic anhydrase isoforms I and II

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Abstract

Novel sulfonamide derivatives **6a–i**, as new carbonic anhydrase inhibitors which candidate for glaucoma treatment, were synthesized from the reactions of 4-amino-*N*-(4-sulfamoylphenyl) benzamide **4** and sulfonyl chloride derivatives **5a–i** with high yield (71–90%). The structures of these compounds were confirmed by using spectral analysis (FT-IR, ^{1}H NMR, ^{13}C NMR, LC/MS and HRMS). The inhibition effects of **6a–i** on the hydratase and esterase activities of human carbonic anhydrase isoenzymes, hCA I and II, which were purified from human erythrocytes with Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide affinity chromatography, were studied as *in vitro*, and IC_{50} and K_i values were determined. The results show that newly synthesized compounds have quite powerful inhibitory properties.

Keywords

Amidation reaction, carbonic anhydrase, enzyme inhibition, glaucoma, sulfonamide

History

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Introduction

Sulfonamide compounds are an important class of drugs for the medicine industry and they possess various types of biological activities such as antibacterial[1], anticancer[2], anticarbonic anhydrase for glaucoma treatment[3–6], acetylcholinesterase inhibitor agents for Alzheimer's disease[7,9], antiobesity[10] and high-ceiling diuretic[11]. They are commonly used in human and veterinary medicine for therapeutic and prophylactic purposes to fight many dangerous illnesses[12].

Carbonic anhydrase (CA, EC 4.2.1.1) is a metalloenzyme that contains Zn^{2+} ion in its active site, which catalyzes the interconversion between carbon dioxide and bicarbonate and proton[13]. Although this reaction is simple, it plays an important role in many physiological and pathological processes such as transport of carbon dioxide and bicarbonate between metabolizing tissues and lungs, electrolyte secretion, pH and carbon dioxide homeostasis, some biosynthetic reactions, calcification and tumorigenicity[14]. These enzymes were encoded by six different unrelated gene families (α, β, γ, δ, ζ, η) in living organisms[15,16]. Nowadays, it is known that there are 16 different isozymes of mammalian CAs that belongs to α gene family[17]. Among these isozymes CA I, CA II, CA III, CA VII and CA XIII are cytosolic; CA IV, CA IX, CA XII, CA XIV and CA XV are membrane bound; CA VA and CA VB are mitochondrial and CA VI is secreted into saliva and milk[15–17]. CA I is a major CA isozyme in most of the vertebrates and CA II is also present in human eye[15–18]. hCA II is involved in ciliary processes for aqueous humor secretion. Therefore, the inhibition of hCA II is therapeutic for glaucoma, characterized by the elevation of intraocular pressure as a result of excessive secretion of aqueous humor[19,20]. Some sulfonamide class CA inhibitors (acetazolamide (AAZ), dorzolamide (DZA) and brinzolamide (BRZ)) have been developed for the treatment of glaucoma so far[3]. But they have some side effects including metallic taste, depression, weight loss, blurred vision, burning of the eye, etc.[20] This situation reveals that there is a need for the development of new inhibitory agents.

This paper reports the synthesis, characterization and investigation of inhibitory properties of some new sulfonamide derivatives on hCA I and hCA II (ciliary process isozyme). Inhibition effects of the compounds have been investigated under *in vitro* conditions and structure–activity relationships of them have been explained.

Methods

Chemistry

The chemicals used in the synthesis of sulfonamide derivatives were provided by Merck and Aldrich Chemical Company and CNBr activated Sepharose®4B for affinity column and electrophoresis reagents were obtained from Sigma Chem. Co. All chemicals and solvents used for the synthesis were spectroscopic reagent grade. Melting points were measured on a Bibby Scientific Stuart Digital, Advanced, SMP30 apparatus. Fourier Transform Infrared (FT-IR) spectra were recorded on a Bruker Optics, ALPHA FT-IR spectrometer. The ^{1}H NMR, and ^{13}C NMR spectra were obtained with a Bruker AV 400 Ultra Shield instrument in DMSO-d_6 as solvent with trimethylsilane as the internal reference, at 400 and 100 MHz, respectively. HRMS spectra were detected by an Agilent Technologies 6530

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Accurate-Mass Q-TOF LC/MS at the Advanced Technology Research Center of Dumlupinar University (ILTEM).

General procedure for preparation of 4-nitro-*N*-(4-sulfamoylphenyl)benzamide compound (3)

4-Aminobenzenesulfonamide **(1)** (1.739 g, 10.1 mmol), 4-nitro benzoylchloride **(2)** (1.856 g, 10 mmol), 3 mL dry triethylamine (TEA) and dry 30 mL THF were stirred for 5 h at room temperature. Afterwards the solvent was removed *in vacuo* and the precipitated crude product was washed with 1000 mL distilled H_2O. The product was purified by recrystallization from ethanol[4,5].

Procedure for preparation of 4-amino-*N*-(4-sulfamoylphenyl)benzamide compound (4)

$Na_2S{\cdot}9H_2O$ (1 mmol) and sulfur (2 mmol) were dissolved by boiling 20 mL of water. This solution (sodium poly-sulfur) was then added dropwise to a stirred and warm solution of 4-nitro-*N*-(4-sulfamoylphenyl)benzamide (**3**) (1 mmol) in ethanol–water. The progress of the reaction was monitored by TLC. Once the reaction was completed, the mixture was cooled to room temperature and solid was filtered off and washed with H_2O. The sulfonamide product was purified and recrystallized from the ethanol (90%). The melting point of compound **(4)** was found to be 313 °C[4,5].

General procedure for preparation of sulfonamide derivatives 6a–i

A mixture of a 4-amino-*N*-(4-sulfamoylphenyl)benzamide **(4)** 0.5 mmol (0.1455 g) and methanesulfonyl chloride **(5a)** 0.55 mmol (0.06325 g) in dry 5 mL pyridine were stirred for 4 h at room temperature. The progress of the reaction was monitored by TLC. Once the reaction is completed, the solvent was removed *in vacuo* and residue was washed with 1000 mL water. The sulfonamide derivatives were obtained as pure products.

4-(Methylsulfonamido)-*N*-(4-sulfamoylphenyl)benzamide (6a)

As white solid, 132.98 mg, 72%, mp 291 °C (dec.). IR (cm^{-1}): 3377, 3306 and 3280 w (–NH and $–NH_2$), 3039 w (Ar–H), 2939 w (C–H), 1650 s (C=O), 1509 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.48 (s, 1H, –NH), 10.27 (s, 1H, –NH), 8.00–7.95 (m, 4H, Ar–H), 7.83 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.34 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.30 (s, 2H, SO_2NH_2), 3.12 (s, 3H, $–CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.22, 142.19, 141.84, 138.56, 128.93, 129.33, 126.51, 119.78, 117.82, 39.75; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{14}H_{15}N_3O_5S_2$: 369.0453; found $[M-H]^-$: 368.0377.

4-(Ethylsulfonamido)-*N*-(4-sulfamoylphenyl)benzamide (6b)

As white solid, 141.87 mg, 74%, mp 311 °C (dec.). IR (cm^{-1}): 3399, 3306 and 3280 w (–NH and $–NH_2$), 3095 w (Ar–H), 2944 w (C–H), 1650 s (C=O), 1507 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.38 (s, 1H, –NH), 10.20 (s, 1H, –NH), 7.90–7.86 (m, 4H, Ar–H), 7.73 (d, 2H, $J = 9.0$ Hz, Ar–H), 7.27 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.21 (s, 2H, SO_2NH_2), 3.13 (q, 2H, $J = 7.36$ Hz, $–CH_2$), 1.14 (t, 3H, $J = 7.3$ Hz, $–CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.22, 142.19, 141.90, 138.56, 128.88, 129.34, 126.49, 119.75, 117.65, 45.56, 8.02; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{15}H_{17}N_3O_5S_2$: 383.0610; found $[M-H]^-$: 382.0535.

4-(4-Methoxyphenylsulfonamido)-*N*-(4-sulfamoylphenyl)-benzamide (6c)

As white solid, 200.75 mg, 87%, mp 259 °C (dec.). IR (cm^{-1}): 3370, 3283 and 3260 w (–NH and $–NH_2$), 3105 w (Ar–H), 2976 w (C–H), 1651 s (C=O), 1503 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.60 (s, 1H, NH), 10.33 (s, 1H, NH), 7.83 (d, 2H, $J = 8.78$ Hz, Ar–H), 7.78 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.73–7.70 (m, 4H, Ar–H), 7.21 (s, 2H, SO_2NH_2), 7.16 (d, 2H, $J = 8.5$ Hz, Ar–H), 7.02 (d, 2H, $J = 8.8$ Hz, Ar–H), 3.72 (s, 3H, $–CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.18, 162.61, 142.16, 142.32, 138.55, 130.81, 129.17, 128.98, 126.49, 119.68, 118.04, 114.52, 55.65; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{20}H_{19}N_3O_6S_2$: 461.0715; found $[M-H]^-$: 460.0638.

4-(4-Methylphenylsulfonamido)-*N*-(4-sulfamoylphenyl)-benzamide (6d)

As white solid, 182.65 mg, 82%, mp 289 °C (dec.). IR (cm^{-1}): 3375, 3299 and 3276 w (–NH and $–NH_2$), 3088 w (Ar–H), 1648 s (C=O), 1506 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.66 (s, 1H, NH), 10.33 (s, 1H, NH), 7.84–7.65 (m, 8H, Ar–H), 7.30 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.20 (s, 2H, SO_2NH_2), 7.16 (d, 2H, $J = 8.5$ Hz, Ar–H), 2.27 (s, 3H, $–CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.17, 143.63, 142.14, 141.19, 138.56, 136.38, 129.38, 129.83, 129.18, 126.76, 126.48, 119.67, 118.10, 20.94; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{20}H_{19}N_3O_5S_2$: 445.0766; found $[M-H]^-$: 444.0689.

4-(Naphthalene-2-sulfonamido)-*N*-(4-sulfamoylphenyl)-benzamide (6e)

As white solid, 204.65 mg, 85%, mp 254 °C (dec.). IR (cm^{-1}): 3356 and 3324, 3262 and 3230 w (–NH and $–NH_2$), 1653 s (C=O), 1506 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.96 (s, 1H, –NH), 10.39 (s, 1H, –NH), 8.60 (s, 1H, Ar–H), 8.19 (d, 1H, $J = 7.8$ Hz, Ar–H), 8.13 (d, 1H, $J = 8.8$ Hz, Ar–H), 8.02 (d, 1H, $J = 7.8$ Hz, Ar–H), 7.91–7.79 (m, 7H, Ar–H), 7.73–7.65 (m, 2H, Ar–H), 7.32 (d, 2H, $J = 8.8$, Ar–H) 7.30 (s, 2H, SO_2NH_2); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.14, 142.13, 141.04, 138.54, 136.17, 134.33, 131. 50, 129.69, 129.39, 129.29, 129.20, 129.16, 128.29, 127.83, 127.80, 126.48, 121.85, 119.66, 118.19; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{23}H_{19}N_3O_5S_2$: 481.0766; found $[M-H]^-$: 480.0690.

N-(4-sulfamoylphenyl)-4-(2,4,6-trimethylphenylsulfonamido)benzamide (6f)

As white solid, 179.95 mg, 76%, mp 262 °C (dec.). IR (cm^{-1}): 3378 and 3359, 3314 and 3285 w (–NH and $–NH_2$), 3188 w (Ar–H), 2979 w (C–H), 1658 s (C=O), 1508 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.78 (s, 1H, NH), 10.37 (s, 1H, NH), 7.91 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.85 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.79 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.29 (s, 2H, SO_2NH_2), 7.10 (d, 2H, $J = 8.8$ Hz, Ar–H), 7.05 (s, 2H, Ar–H), 2.63 (s, 6H, 2x–CH_3), 2.23 (s, 3H, $–CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.14, 142.43, 142.19, 141.18, 138.73, 136.51, 133.42, 128.62, 131.92, 129.22, 126.48, 119.60, 116.82, 22.38, 20.94; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{22}H_{23}N_3O_5S_2$: 473.1079; found $[M-H]^-$: 472.1001.

4-(3,5-Dichloro-2-hydroxyphenylsulfonamido)-*N*-(4-sulfamoylphenyl)benzamide (6g)

As white solid, 209.13 mg, 81%, mp 266 °C (dec.). IR (cm^{-1}): 3390 br (OH), 3347, 3282 and 3250 w (–NH and $–NH_2$), 3090 w (Ar–H), 1653 s (C=O), 1505 s (C=C); ^{1}H NMR (DMSO–d_6, 400 MHz) δ (ppm): 11.27 (s, 1H, OH), 10.85 (s, 1H, NH), 10.41

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(s, 1H, NH), 7.91 (d, 2H, $J = 9.0$ Hz, Ar–H), 7.87–7.85 (m, 3H, Ar–H), 7.80–7.78 (m, 3H, Ar–H), 7.28 (s, 2H, SO_2NH_2), 7.25 (d, 2H, $J = 8.8$ Hz, Ar–H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.15, 150.26, 142.16, 140.66, 138.53, 129.25, 128.83, 123.88, 122.86, 134.21, 129.15, 128.41, 126.48, 119.64, 117.83; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{19}H_{15}C_{l2}N_3O_6S_2$: 514.9779; found $[M-H]^-$: 513.9723.

4-(Phenylsulfonamido)-*N*-(4-sulfamoylphenyl)benzamide (6h)

As white solid, 153.17 mg, 71%, mp 279 °C (dec.). IR (cm^{-1}): 3386 and 3274 w (–NH and $–NH_2$), 3090 and 3066 w (Ar–H), 1649 s (C=O), 1505 s (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.84 (s, 1H, NH), 10.43 (s, 1H, NH), 7.93–7.80 (m, 8H, Ar–H), 7.67–7.58 (m, 3H, Ar–H), 7.30 (s, 2H, SO_2NH_2), 7.27 (d, 2H, $J = 8.5$ Hz, Ar–H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.18, 142.15, 141.06, 139.25, 138.56, 133.21, 129.42, 129.21, 126.70, 126.50, 119.70, 118.25; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{19}H_{17}N_3O_5S_2$: 431.0610; found $[M-H]^-$: 430.0535.

4-(4-Bromophenylsulfonamido)-*N*-(4-sulfamoylphenyl)-benzamide (6i)

As white solid, 229.67 mg, 90%, mp 287 °C (dec.). IR (cm^{-1}): 3417 and 3387, 3291 and 3273 w (–NH and $–NH_2$), 3089 w (Ar–H), 1649 s (C=O), 1507 s (C=C); H^1 NMR (DMSO-d_6, 400 MHz) δ (ppm): 10.91 (s, 1H, NH), 10.45 (s, 1H, NH), 7.95–7.89 (m, 4H, Ar–H), 7.84–7.77 (m, 6H, Ar–H), 7.31 (s, 2H, SO_2NH_2), 7.27 (d, 2H, $J = 8.5$ Hz, Ar–H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 165.15, 142.14, 140.72, 138.58, 138.47, 129.74, 127.16, 132.53, 129.29, 128.72, 126.51, 119.73, 118.58; HRMS (QTOF-ESI): *m/z* [M] calcd. for $C_{19}H_{16}BrN_3O_5S_2$: 508.9715; found $[M-H]^-$: 507.9619.

Purification of carbonic anhydrase I and II isoenzymes from human erythrocytes

Erythrocytes were purified from human blood. The blood samples were centrifuged at 1500 rpm for 20 min and plasma was removed. Later, red cells were washed with isotonic solution (0.9% NaCl), and the erythrocytes were hemolyzed with 1.5 volumes of ice-cold water. Cell membranes were removed by centrifugation at 4 °C, 20 000 rpm for 30 min. The pH of hemolysate was adjusted to 8.7 with solid TRIS (tris(hydroxymethyl)aminomethane). The hemolysate was applied to affinity column (Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide) pre-equilibrated with 25.0 mM TRIS–HCl/0.1 M Na_2SO_4 (pH 8.7). After the extensive washing with a solution of 25.0 mM TRIS–HCl/22.0 mM Na_2SO_4 (pH 8.7), the hCA I and hCA II isoenzymes were eluted with the solution of 1.0 M NaCl/25.0 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively[21]. For quantitative protein determination, the Bradford method was used with bovine serum albumin as a standard[22]. Also purity control of the isoenzymes was performed with SDS-PAGE after the purification[23].

Determination of hydratase and esterase activities of hCA I and hCA II

The CO_2 hydratase activity of the enzyme was determined at 0 °C in a veronal buffer (pH 8.15) with the pH-stat method as the indicator and saturated carbon dioxide solution as the substrate in a final volume of 4.2 mL. The time (in seconds) taken for the solution to change from pH 8.15 to pH 6.50 was measured. The enzyme unit (EU) is the enzyme amount that reduces the non-enzymatic reaction time by 50%. The activity of an enzyme unit was calculated by using the equation $((t_0-t_c)/t_c)$, where t_0 and t_c are times for pH change of the non-enzymatic and enzymatic reactions, respectively[24].

Esterase activity was assayed by following the change in the absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer according to the method described in the literature[25]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M TRIS–SO_4 buffer (pH 7.4), 1.0 mL of 3.0 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.

Determination of IC_{50} and K_i values of the compounds

To determine the IC_{50} values of the inhibitors, hydratase and esterase activities of CA isoenzymes were assayed in the presence of various inhibitor concentrations as mentioned above. Regression analysis graphs were drawn by plotting the percent enzyme activity versus inhibitor concentration and IC_{50} values were calculated[3,6].

To determine K_i values as well as the inhibition type, three different inhibitor concentrations giving 30%, 50% and 70% inhibition were selected. At each of these inhibitor concentrations, enzyme activity was measured in the presence of various substrate concentrations (0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM and 0.7 mM) and the data were linearized with Lineweaver–Burk plot for V_{max} and the K_i determination. Enzyme activity was also measured in the presence of the same substrate concentrations but in the absence of any inhibitor to determine the V_{max}[3,6].

Statistical analysis

All the presented data were confirmed in at least three independent experiments and are expressed as the mean ± standard deviation (SD). Data were analyzed by using a one-way analysis of variance for multiple comparisons (SPSS 13.0, SPSS Inc., Chicago, IL). $p < 0.00001$ was considered to be statistically significant.

Result and discussion

Chemistry

The general synthetic procedure to obtain sulfonamide derivatives in three step reaction is described in Scheme 1. The 4-nitro-*N*-(4-sulfamoylphenyl)benzamide **(3)** was prepared in the presence of TEA in THF at room temperature[3–5]. The 4-amino-*N*-(4-sulfamoylphenyl)benzamide **(4)** was obtained in polysulfide solution by reduction of the nitro compound **(3)**. Novel sulfonamide derivatives **6a–i** were synthesized from the reactions of 4-amino-*N*-(4-sulfamoylphenyl)benzamide **4** and sulfonyl chloride derivatives **5a–i** in pyridine.

The novel sulfonamide derivatives were obtained under mild conditions, in short times and in high yields.

Spectral data are consistent with the chemical structures of the compounds. When the IR spectrums were examined; the C=O groups stretching vibrations, which belong to the structure of sulfonamide derivatives **6a–i,** were observed between 1658 and 1648 cm^{-1} (Supplemental Figures).

Compound **6g** aromatic O–H stretching band was observed at 3390 cm^{-1}. Besides, sulfonamide derivatives **6a–i** aromatic C–H stretching bands and aliphatic C–H stretching bands were observed between 3188–3039 cm^{-1} and 2979–2939 cm^{-1}, respectively. –NH and $–NH_2$ groups stretching vibration bands that belong to these compounds **6a–i** were observed in the region between 3399–3317 cm^{-1} and 3314–3230 cm^{-1}, respectively[26,27]. Stretching vibration of the C=C bonds in the aromatic structures was observed at 1509–1503 cm^{-1}. Symmetric and

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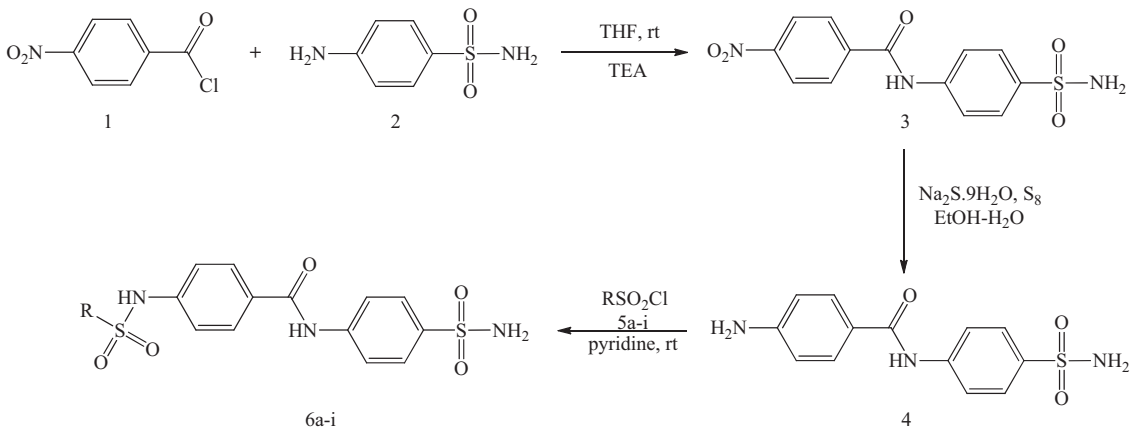

	6a	6b	6c	6d	6e	6f	6g	6h	6i
R	CH_3-	C_2H_5-	4-$OCH_3C_6H_4$-	4-$CH_3C_6H_4$-	2-Naphtyl	2,4,6$(CH_3)C_6H_2$-	3,5-diCl, 2-OHC_6H_2-	C_6H_5-	4-BrC_6H_4-

Scheme 1. Synthesis of novel sulfonamide derivatives.

asymmetric vibration bands of SO_2, existing in the structure of the novel sulfonamide compounds were determined to give severe vibrations in the range of 1159–1135 cm^{-1} (Supplemental Figures).

The ^1H NMR spectra of sulfonamide compounds **(6a**, **6c**, **6d**, and **6f)**, which belong to protons of the methyl groups, showed singlet peaks in between 2.27 and 3.72 ppm. The compound **6b** ethyl group protons were observed in triplet peak at 1.14 ppm (3H) and quartet peak at 3.12 ppm (2H) (Supplemental Figures).

Aromatic protons of all the sulfonamide derivatives **6a–i** showed signals in the region between 7.02 and 8.60 ppm. The compound **6g** hydroxyl group proton was observed as a broad peak at 11.27 ppm. SO_2NH_2 group protons of sulfonamide compounds **6a–i** were observed as singlet peaks between 7.20 and 7.31 ppm. The carboxamide group protons (–CONH–) of all compounds **6a–i** showed broad peaks between 10.20 and 10.96 ppm (Supplemental Figures).

Carbonic anhydrase inhibition

In this part of the study we aimed to:

- investigate the inhibition potentials of synthesized compounds on hCA I and II,
- compare the contribution of different functional groups on the inhibition potentials of the compounds.

For this purpose, firstly, the carbonic anhydrase isozymes (hCA I and II) were purified separately from human erythrocyte cells with a single-step method, Sepharose®4B-*p*-aminobenzene sulfonamide affinity chromatography. Purity control of the isozymes was performed by SDS-PAGE and single bands were observed and then the inhibition potentials of newly synthesized compounds on hydratase and esterase activities of hCA I and II were investigated as *in vitro*[24,25].

Kinetic studies showed that the compounds have inhibitory properties on hydratase and esterase activities of hCA I and II isoforms. The inhibition effects of synthesized sulfonamide derivatives on the hydratase activities of the isozymes demonstrate that these compounds are attached to zinc ion in the active site[16].

As shown in Table 1, synthesized derivatives have more potential inhibition effects on hCA II than hCA I. Because hCA II is a target enzyme for glaucoma treatment, more powerful inhibition of this isozyme is clinically important. Also the compounds showed remarkable inhibition effects on the esterase activities of hCA I and hCA II when compared with **AAZ**, which is intraocular pressure reducing agent.

The compounds have inhibitory effects, with micromolar range, on the hydratase activities of the isozymes. The IC_{50} values were in the range of 0.518–1.654 μM for hCA I and 0.138–1.060 μM for hCA II. Compound **6c** has the most powerful inhibition effect on the hydratase activity of hCA I (IC_{50} value 0.518 μM), whereas **6d** is the strongest inhibitor for the hydratase activity of hCA II (IC_{50} value 0.138 μM). Also **6i** has the weakest inhibition effect on the hydratase activities of the isoforms, hCA I and II (IC_{50} values 1.654 μM and 1.060 μM, respectively). Synthesized compounds are the more potent inhibitors for hydratase activity of hCA II than the other isoform's hydratase activity. According to hydratase IC_{50} values, the inhibition potentials were in the order of **6c > 6d > 6e > 3 > 4>6f > 6a > 6g > 6b > 6h > 6i** for hCA I, and **6d > 6a > 6c > 3 > 6e > 4 > 6f > 6b > 6g > 6h > 6i** for hCA II.

When the inhibition effects of the compounds on the esterase activities of the isozymes, hCA I and II were examined, a similar situation to the inhibition of the hydratase activity could be observed clearly. But the compounds showed more effective inhibition, nearly in nanomolar range, on the esterase activities of the isozymes. The IC_{50} values were in the range of 0.049–0.276 μM for hCA I and 0.027–0.237 μM for hCA II. Unlike the inhibition of the hydratase activity, newly synthesized compounds **6a–i** have more powerful inhibition effects on the esterase activities of hCA I and hCA II than the starting compounds **3** and **4**. **6f**, containing 2,4,6–trimethylphenyl, has the strongest inhibition effect on the esterase activities of hCA I and II (IC_{50} values 0.049 and 0.027 μM, respectively). Also **6h** and **6d** have nearly the same inhibition potentials on the isozymes (see Table 1). These compounds contain phenyl and tolyl moieties, respectively. Esterase IC_{50} values of **6d**, **6f** and **6h** are close to each other. Furthermore, their substituents are similar to each other as structurally. Therefore it can be thought that these compounds make similar interactions with the active site. However, contrary to our expectations, the increase of the polarity in the substituents, reduced the inhibitory potentials of the compounds. This situation is clearly observed on **6c**, **6g** and **6i**. Also the compounds containing non-aromatic substituents, **6a** and **6b**, have weaker inhibition potentials on the esterase activities of hCA I and hCA II. The reason why compounds **6d**, **6f** and **6h**, which have apolar

Table 1. The effects of synthesized compounds on hCA I and II isozymes under *in vitro* conditions.

	Hydratase IC_{50}[a,b] (μM)		Esterase IC_{50}[a,b] (μM)		K_i[a,b] (μM)	
Compound	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II
AAZ	0.224 ± 0.0042	0.198 ± 0.0071	0.321 ± 0.0051	0.212 ± 0.0048	0.210 ± 0.0035	0.120 ± 0.0049
3	0.741 ± 0.0011	0.312 ± 0.0002	0.276 ± 0.0003	0.237 ± 0.0012	0.149 ± 0.0009	0.141 ± 0.0008
4	0.860 ± 0.0005	0.370 ± 0.0003	0.184 ± 0.0005	0.085 ± 0.0009	0.081 ± 0.0001	0.048 ± 0.0005
6a	1.125 ± 0.0003	0.203 ± 0.0004	0.136 ± 0.0005	0.070 ± 0.0009	0.052 ± 0.0002	0.029 ± 0.0005
6b	1.248 ± 0.0008	0.418 ± 0.0009	0.160 ± 0.0005	0.082 ± 0.0007	0.065 ± 0.0005	0.036 ± 0.0003
6c	0.518 ± 0.0001	0.250 ± 0.0006	0.112 ± 0.0005	0.059 ± 0.0001	0.050 ± 0.0005	0.028 ± 0.0007
6d	0.547 ± 0.0007	0.138 ± 0.0004	0.058 ± 0.0005	0.032 ± 0.0005	0.025 ± 0.0008	0.015 ± 0.0006
6e	0.738 ± 0.0004	0.362 ± 0.0007	0.105 ± 0.0005	0.051 ± 0.0005	0.043 ± 0.0003	0.023 ± 0.0005
6f	0.925 ± 0.0009	0.394 ± 0.0005	0.049 ± 0.0005	0.027 ± 0.0008	0.020 ± 0.0003	0.011 ± 0.0001
6g	1.143 ± 0.0002	0.596 ± 0.0002	0.195 ± 0.0005	0.113 ± 0.0002	0.081 ± 0.0006	0.045 ± 0.0004
6h	1.416 ± 0.0006	0.620 ± 0.0005	0.053 ± 0.0005	0.029 ± 0.0004	0.022 ± 0.0002	0.013 ± 0.0001
6i	1.654 ± 0.0004	1.060 ± 0.0001	0.106 ± 0.0005	0.065 ± 0.0006	0.044 ± 0.0004	0.025 ± 0.0008

AAZ was used as a reference compound.
[a]Mean ± standard error, from three different assays.
[b]$p < 0.00001$ for all analysis.

substituents, are potentially better inhibitors is that these compounds better interact with the hydrophobic pocket of the active site. A similar compound that has been studied previously supports this claim and PDB code of this compound (3N3J) shows how this compound interacts with this enzyme[28].

The esterase K_i values are in agreement with the esterase IC_{50} values. Therefore, the aforementioned structure–activity relationships for esterase activity can also be said for K_i values. These values were in the range of 0.020–0.149 μM for hCA I, and 0.011–0.141 μM for hCA II. hCA I and hCA II isozymes have more affinity against **6d**, **6f** and **6h** derivatives that have apolar aromatic substituents (K_i values 0.025 ± 0.008 μM, 0.020 ± 0.003 μM and 0.022 ± 0.002 μM for hCA I, and 0.015 ± 0.006 μM, 0.011 ± 0.001 μM and 0.013 ± 0.001 μM for hCA II, respectively). But the affinity of the isozymes against inhibitors decreased in the presence of non-aromatic substituents (**6a** and **6b**) and polar aromatic substituents (**6c**, **6g** and **6i**). According to K_i values, inhibition potentials were in the order of **6f** > **6h** > **6d** > **6e** > **6i** > **6c** ≈ **6a** > **6b** > **6g** ≈ **4** > **3** for hCA I and II isoforms.

Conclusion

Today, some carbonic anhydrase inhibitors for the treatment of glaucoma are present on the market, but such drugs have many side effects. Strong carbonic anhydrase inhibitors can be developed to reduce dosage, and as a result of this, reduction of side effects can be achieved. In the present study, the synthesized compounds have remarkable inhibition effects on hCA I and II. Consequently, these potential inhibitors may be sufficient to reduce the dosage. So we can say that the synthesized inhibitors can be candidates for *in vivo* studies in the treatment of glaucoma.

Declaration of interest

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Supplementary material available online

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