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RESEARCH ARTICLE

Synthesis of 5-amino-1,3,4-thiadiazole-2-sulphonamide derivatives and their inhibition effects on human carbonic anhydrase isozymes

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Abstract

In this study, some novel inhibitors were synthesised from the further stage reactions of 4-benzoyl-1-(4-nitrophenyl)-5-phenyl-1H-pyrazole-3-carbonyl chloride with 5-amino-1,3,4-thiadiazole-2-sulphonamide **1** (inhibitor 1). They were characterised by elemental and spectral (^{1}H NMR, ^{13}C NMR, IR) analyses. Human carbonic anhydrase isoenzymes (hCA-I and hCA-II) were purified from erythrocyte cells by affinity chromatography. The inhibitory effects of inhibitor **1**, acetazolamide (**2**) and the 11 newly synthesised amides (**8–18**) on the hydratase and esterase activities of these isoenzymes (hCA-I and hCA-II) were studied *in vitro*. In relation to these activities, the inhibition equilibrium constants (K_i) were determined. The K_i values for the new compounds (**8–18**) were observed to be well below that of the parent compound inhibitor **1** and were also compared to **2** under the same experimental conditions. The comparison of the newly synthesised amides to inhibitor **1** and to **2** indicated that the new derivatives preferentially inhibited hCA-II and were more potent inhibitors of hCA-II than the parent inhibitor **1** and **2**.

Keywords: Pyrazole, pyrazole-3-carboxylic acid, inhibition effect, 1,3,4-thiadiazole-2-sulphonamide, antiglaucoma

Introduction

The sulphonamides constitute an important class of drugs which show many pharmacologic activities such as antibacterial, antihypertensive, antiviral, anti-thyroid, diuretic, hypoglycaemic and anti-tumour activity. Furthermore the sulphonamides are the best-known inhibitors of the carbonic anhydrase enzyme, currently used for the treatment of glaucoma in clinical medicine [1–20].

Glaucoma is a group of diseases characterised by a gradual loss of the visual field due to an elevation in intraocular pressure (IOP) and is the second leading cause of blindness worldwide [14,15]. Since carbonic anhydrase inhibitors have been shown to reduce intraocular pressure exclusively by lowering the aqueous humour flow, these compounds have been used for the treatment of glaucoma for years [16,17].

5-amino-1,3,4-thiadiazole-2-sulphonamide (**1**) has several biological activities and antibacterial properties [21]. Several derivatives of this drug have been synthesised and used in the treatment of glaucoma and one of them is acetazolamide (**2**). Compound **2** is used systematically (i.e. an oral medication) and reduces intraocular pressure by lowering fluid formation in the eye. A number of side effects of this drug are however experienced such

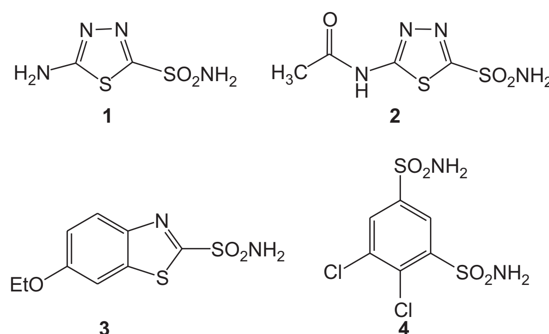

Figure 1. Structures of several CA inhibitors used in glaucoma treatment.

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as numbness and tingling in the fingers and toes, taste alterations, blurred vision, kidney stones and an increase in urination. Ethoxzolamide (**3**) and dichlorophenamide (**4**) are two other carbonic anydrase inhibitors, formulated for topical ophthalmic use (see Figure 1 for the structures) [22,23]. Nevertheless, people taking these two drugs also experience side effects as stinging, burning, blurred vision, upset stomach, dry eye, headache or dizziness [22,24–26]. The purpose of the present study was to synthesise and investigate some new inhibitors of carbonic anydrase isoenzymes with a potential use in the treatment of glaucoma.

Materials and methods

General

The chemical compounds used in this research were of analytical purity and the solvents were purified by using the appropriate purifying agents and distillation. All melting points were recorded on a Barnstead Electrothermal 9200 apparatus and were uncorrected. The IR spectrum data of the compounds were determined by a Mattson 1000 FT-IR using KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were evaluated by Bruker DPX-400, (400 MHz) and High Performance Digital FT-NMR (100 MHz) spectrometers. At the end of the each experiment TLC was performed using DC Alufolien Kiesegel 60F/254 Merck and Camag TLC devices. Elemental analyses were carried out on a Leco CHNS-932 instrument.

Synthesis of organic compounds

1-(4-Aminophenyl)-4-benzoyl-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazole-2-yl)-1H-pyrazole-3-carboxamide (7)

The starting compound (**7**) containing aromatic primary amine function was prepared as described previously [2] and crystallised from methanol. 465 mg, 85%; mp 183°C; IR (ν, cm^{-1}): 3312–3216 (NH), 3026 (Ar CH), 1665 (C=O), 1596–1426 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d$_6$) δ (ppm): 6.51 (m, 2H, Ar NH$_2$), 8.37 (m, 2H, SO$_2$NH$_2$), 7.8–6.82 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO–d$_6$) δ (ppm): 190.58 (benzoyl C=O), 165.36 (amide C=O), 161.44 and 160.44 (thiadiazole C–2 and C–5), 152.2, 148.8, 144.57, 143.36, 137.58, 134.1, 130.48, 130.13, 129.64, 129.06, 127.65, 127.4, 127.3, 122.91, 114.4; Anal. Calcd. for $C_{25}H_{19}N_7O_4S_2$: C, 55.04; H, 3.51; N, 17.97; S, 11.75. Found: C, 55.32; H, 3.59; N, 17.75; S, 11.6.

General procedure for the synthesis of compounds 8–17

Sodium acetate (3 g) was dissolved in 10 mL H_2O and 2 mL HCl. Aromatic amine (1 mmol) and alcohol were added to the mixture until it dissolved. The temperature was adjusted to 0–5°C. A solution of $NaNO_2$ (83 mg, 1.2 mmol) in 10 mL H_2O was added to the mixture slowly and the process of diazotisation was performed. The aromatic and β-diketone (1 mmol) compounds were dissolved in ethanol and added dropwise to the diazonium salt which had been prepared previously. Finally, the coloured precipitate was filtered and purified from the ethanol.

4-Benzoyl-1-(4-((2-hydroxynaphthalen-1-yl)diazenyl)phenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (8)

This was produced according to the method in the general procedure; 567 mg, 81%; mp 284–285°C; IR (ν, cm^{-1}): 3388 (OH), 3200 (NH), 3027 (Ar CH), 1686 (C=O), 1597–1448 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d$_6$) δ (ppm): 15.7 (s, 1H, Ar–OH), 14.19 (br, s, 1H, CONH), 8.39 (s, 2H, SO$_2$NH$_2$), 8.56–6.69 (m, 20H, ArH); ^{13}C NMR (100 MHz, DMSO–d$_6$) δ (ppm): 190.55 (benzoyl C=O), 165.35 (amide C=O), 161.44 and 160.35 (thiadiazole C–2 and C–5), 144.74 (=C–OH), 144.47, 143.22, 141.61, 137.69, 137.13, 134.1, 133.09, 130.37, 130.34, 130.32, 130.21, 130.12, 129.75, 129.66, 129.48, 129.09, 128.42, 127.88, 127.47, 126.81, 125, 123.12, 122.13, 119.22; Anal. Calcd. for $C_{35}H_{24}N_8O_5S_2$: C, 59.99; H, 3.45; N, 15.99; S, 9.15. Found: C, 59.82; H, 3.49; N, 16.03; S, 9.11.

4-Benzoyl-1-(4-((4-hydroxyphenyl)diazenyl)phenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (9)

This was produced according to the method in the general procedure; 429 mg, 66%; mp 175–177°C; IR (ν, cm^{-1}): 3375 (OH), 3195 (NH), 3028 (Ar CH), 1679 (C=O), 1595–1447 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d$_6$) δ (ppm): 13.2 (s, 1H, CONH), 8.55–7.83 (br, s, 1H, Ar–OH), 7.79 (s, 2H, SO$_2$NH$_2$), 7.5–6.89 (m, 18H, ArH); ^{13}C NMR (100 MHz, DMSO–d$_6$) δ (ppm): 195.63 (benzoyl C=O), 169.18 (amide C=O), 165.56 and 165.35 (thiadiazole C–2 and C–5), 148.96 (=C–OH), 148.61, 143.49, 142.48, 138.15, 138.11, 134.57, 134.29, 134.15, 133.9, 133.54, 133.43, 133.28, 132.5, 130.25, 130.21, 127.58, 121.53, 120.89; Anal. Calcd. for $C_{31}H_{22}N_8O_5S_2$: C, 57.22; H, 3.41; N, 17.22; S, 9.86. Found: C, 57.11; H, 3.45; N, 17.21; S, 9.92.

4-Benzoyl-1-(4-iodophenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (10)

The diazonium salt solution of the aromatic amine compound was prepared according to the method in the general procedure. The KI (166 mg, 1 mmol) was dissolved in ethanol and cooled to 0–5°C and added dropwise to the diazonium salt solution which was prepared previously. The precipitated yellow product was filtered and purified from ethanol. 400 mg, 61%; mp 160–162°C; IR (ν, cm^{-1}): 3320 (NH), 3005 (Ar CH), 1672 (C=O), 1595–1447 (Ar C=C and C=N); ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 12.15 (br, s, 1H, CONH), 7.77 (s, 2H, SO$_2$NH$_2$), 7.61–6.75 (m, 14H, ArH); ^{13}C NMR (100 MHz, CDCl$_3$) δ (ppm): 190.5 (benzoyl C=O), 165.45 (amide C=O), 161.43 and 160.38 (thiadiazol C–2 and C–5), 97.09 (ArC–I), 144.48, 143.25, 143.06, 139.7, 139.61, 137.63, 134.07, 130.26, 129.98, 129.83, 129.63, 129.07, 128.92, 122.5; Anal. Calcd. for $C_{25}H_{17}IN_6O_4S_2$: C, 45.74; H, 2.61; N, 12.80; S, 9.77. Found: C, 45.63; H, 2.64; N, 12.82; S, 9.81.

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4-benzoyl-1-(4-(2-(2,4-dioxopentan-3-ylidene) hydrazinyl)phenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (11)

433 mg, 66%; mp 271–273°C; IR (ν, cm^{-1}): 3170 (NH), 3028 (Ar CH), 2971 (aliphatic CH), 1686 (C=O), 1596–1445 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 13.81 (s, 1H, CONH), 8.36 (s, 2H, SO_2NH_2), 2.48 and 2.41 (s, 6H, $2CH_3$), 7.82–7.24 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 197.49 and 196.87 (acetyl C=O), 190.56 (benzoyl C=O), 165.34 (amide C=O), 161.42 and 160.36 (thiadiazole C–2 and C–5), 31.71 and 26.87 (CH_3), 144.49, 143.06, 142.59, 137.68, 135.31, 134.79, 134.06, 130.29, 130.02, 129.63, 129.08, 129.03, 127.85, 127.54, 122.91, 116.91; Anal. Calcd. for $C_{30}H_{24}N_8O_6S_2$: C, 54.87; H, 3.68; N, 17.06; S, 9.77. Found: C, 54.80; H, 3.69; N, 17.08; S, 9.75.

4-Benzoyl-1-(4-(2-(1,3-dioxo-1-phenylbutan-2-ylidene) hydrazinyl)phenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (12)

445 mg, 62%; mp 187–190 °C; IR (ν, cm^{-1}): 3190 (NH), 3005 (Ar CH), 2971 (aliphatic CH), 1674 (C=O), 1597–1447 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 13.86 (br, s, 1H, CONH), 11.31 (s, 1H, Ar–NH–N=C), 8.35 (s, 2H, SO_2NH_2), 1.92 (s, 3H, CH_3), 7.81–7.11 (m, 19H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 196.6 (acetyl C=O), 195.43 and 190.66 (benzoyl C=O), 165.11 (amide C=O), 161.74 and 160.7 (thiadiazole C–2 and C–5), 25.42 (CH_3), 144.38, 143.85, 140.27, 137.74, 136.01, 134.91, 134.01, 133.45, 130.58, 130.26, 129.9, 129.6, 129.52, 129.25, 129.06, 128.99, 128.4, 127.96, 127.48, 122.86, 122,72, 115.15; Anal. Calcd. for $C_{35}H_{26}N_8O_6S_2$: C, 58.49; H, 3.65; N, 15.59; S, 8.92. Found: C, 58.38; H, 3.68; N, 15.63; S, 8.96.

4-Benzoyl-1-(4-(2-(1,3-dioxo-1,3-diphenylpropan-2-ylidene) hydrazinyl)phenyl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (13)

656 mg, 84%; mp 181–182°C; IR (ν, cm^{-1}): 3180 (NH), 3027 (Ar CH), 1656 (C=O), 1596–1448 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 13.7 (br, s, 1H, CONH), 11.7 (s, 1H, Ar–NH–N=C), 8.35 (s, 2H, SO_2NH_2), 8.2–7.2 (m, 24H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 194.17, 191.01 and 190.59 (benzoyl C=O), 165.31 (amide C=O), 161.44 and 160.38 (thiadiazole C–2 and C–5), 144.39, 143.69, 142.89, 138.5, 137.68, 137.31, 136.44, 135.04, 134.62, 134.06, 133.78, 133.52, 133.02, 130.57, 130.27, 129.95, 129.61, 129.53, 129.33, 129.12, 128.99, 128.67, 127.9, 127.46, 122.79, 115,51; Anal. Calcd. for $C_{40}H_{28}N_8O_6S_2$: C, 61.53; H, 3.61; N, 14.35; S, 8.21. Found: C, 61.45; H, 3.64; N, 14.35; S, 8.22.

Ethyl 2-(2-(4-(4-benzoyl-5-phenyl-3-(5-sulphamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1H-pyrazol-1-yl)phenyl) hydrazono)-3-oxo-3-phenylpropanoate (14)

434 mg, 58%; mp 133–135°C; IR (ν, cm^{-1}): 3197 (NH), 3027 (Ar CH), 2971 (aliphatic CH), 1664 (C=O), 1597–1448 (Ar C=C and C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.62 (s, 1H, CONH), 8.14 (s, 2H, SO_2NH_2), 4.36 (q, 2H, OCH_2), 1.32(t, 3H, CH_3), 7.78–7.08 (m, 19H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 191.39 and 189.46 (benzoyl C=O), 164.63 (ester C=O), 163.59 (amide C=O), 161.67 and 158.64 (thiadiazole C–2 and C–5), 61.72 (CH_2), 13.99 (CH_3), 144.65, 142.09, 141.85, 137.17, 137.08, 134.73, 134.04, 133.71, 132.87, 130.33, 129.8, 129.68, 128.81, 128.69, 128.53, 128.45, 128.17, 127.91, 127.27, 126.47, 122.59, 115,51; Anal. Calcd. for $C_{36}H_{28}N_8O_7S_2$: C, 57.74; H, 3.77; N, 14.96; S, 8.56. Found: C, 57.61; H, 3.79; N, 14.99; S, 8.54.

Ethyl 2-(2-(4-(4-benzoyl-5-phenyl-3-(5-sulphamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1H-pyrazol-1-yl)phenyl) hydrazono)-3-oxobutanoate (15)

439 mg, 64%; mp 193–195°C; IR (ν, cm^{-1}): 3212 (NH), 3018 (Ar CH), 2971 (aliphatic CH), 1664 (C=O), 1600–1427 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 14.02 (br, s, 1H, CONH), 11.73 (s, 1H, Ar–NH–N=C), 8.33 (s, 2H, SO_2NH_2), 4.32 (q, 2H, OCH_2), 2.51 (s, 3H, $CO\underline{CH_3}$) 1.28(t, 3H, CH_3), 7.79–7.08 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 194.29 (acetyl C=O), 190.72 (benzoyl C=O), 164.86 (ester C=O), 162.86 (amide C=O), 162.15 and 160.23 (thiadiazole C–2 and C–5), 61.79 (CH_2), 25.79 ($CO\underline{C}H_3$), 14.32 (CH_3), 154.77, 144.29, 143.31, 137.81, 137.62, 133.96, 133.19, 130.25, 129.88, 129.59, 129.25, 129.04, 128, 127.51, 122.72, 115,67; Anal. Calcd. for $C_{31}H_{26}N_8O_7S_2$: C, 54.22; H, 3.82; N, 16.32; S, 9.34. Found: C, 54.15; H, 3.82; N, 16.30; S, 9.37.

Diethyl 2-(2-(4-(4-benzoyl-5-phenyl-3-(5-sulphamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1H-pyrazol-1-yl)phenyl) hydrazono)malonate (16)

358 mg, 50%; mp 170–172°C; IR (ν, cm^{-1}): 3184 (NH), 3019 (Ar CH), 2970 (aliphatic CH), 1740 (C=O), 1610–1429 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 12 (br, s, 1H, CONH), 8.3 (s, 2H, SO_2NH_2), 4.34–4.12 (q, 4H, $2OCH_2$), 1.28(t, 6H, $2CH_3$), 7.79–6.55 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 190.79 (benzoyl C=O), 172.50 (ester C=O), 164.59 (amide C=O), 162.7 and 161.25 (thiadiazole C–2 and C–5), 61.89 and 61.31 (CH_2), 14.56 and 14.32 (CH_3), 149.85, 144.17, 138.93, 137.86, 133.92, 130.24, 129.89, 129.59, 129.37, 129.03, 128.96, 128.81, 128.04, 127.26, 126.22, 122.86, 122.27, 113,67; Anal. Calcd. for $C_{32}H_{28}N_8O_8S_2$: C, 53.62; H, 3.94; N, 15.63; S, 8.95. Found: C, 53.49; H, 3.97; N, 15.65; S, 8.92.

tert-Butyl 2-(2-(4-(4-benzoyl-5-phenyl-3-(5-sulphamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)-1H-pyrazol-1-yl)phenyl) hydrazono)-3-oxobutanoate (17)

366 mg, 51%; mp 206–207°C; IR (ν, cm^{-1}): 3190 (NH), 3025 (Ar CH), 2974 (aliphatic CH), 1673 (C=O), 1600–1426 (Ar C=C and C=N); ^{1}H NMR (400 MHz, DMSO–d_6) δ (ppm): 14.18 (s, 1H, CONH), 11.92 (s, 1H, Ar–NH–N=C), 8.2 (s, 2H, SO_2NH_2), 2.37 (s, 3H, CH_3), 1.53 (s, 9H, $C(CH_3)_3$), 7.76–6.55 (m, 14H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 193.88 (acetyl C=O), 190.46 (benzoyl C=O), 165.26 (ester C=O), 162.03 (amide C=O), 161.38 and 160.06 (thiadiazole C–2 and C–5), 83.29 ($O\underline{C}(CH_3)_3$), 28.32 ($OC(\underline{C}H_3)_3$),

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26.66 (CH_3), 144.11, 142.95, 142.81, 137.64, 133.74, 130.02, 129.47, 128.81, 128.78, 128.73, 127.74, 126.93, 126.87, 122.83, 115.79, 113.8; Anal. Calcd. for $C_{33}H_{30}N_8O_7S_2$: C, 55.45; H, 4.23; N, 15.68; S, 8.97. Found: C, 55.32; H, 4.23; N, 15.71; S, 9.02.

4-Benzoyl-1-(2-methyl-1-(2-oxopropyl)-1H-indol-5-yl)-5-phenyl-N-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)-1H-pyrazole-3-carboxamide (18)

The aromatic amine compound (1 mmol, 545 mg) was dissolved in 15 mL DMF and chloroacetone (0.182 mL) was added to the mixture. The reaction was maintained at 60°C for three days in a condenser with a $CaCl_2$ headpiece. After the evaporation of the solvent of the dark coloured mixture in a rotary evaporator, the remainder composed of a dense liquid was added to water and mixed. The granulated product was filtered and purified from ethanol. 537 mg, 84%; mp 158–160°C; IR (ν, cm^{-1}): 3240 (NH), 3059 (Ar CH), 2922 (aliphatic CH), 1689 (C=O), 1598–1448 (Ar C=C and C=N); ^1H NMR (400 MHz, DMSO–d_6) δ (ppm): 13.78 (br, s, 1H, CONH), 8.37 (s, 2H, SO_2NH_2), 6.64 (s, 1H, indole CH), 2.89 (s, 2H, CH_2), 2.51 and 2.24 (s, 6H, $2CH_3$), 8.08–7.28 (m, 13H, ArH); ^{13}C NMR (100 MHz, DMSO–d_6) δ (ppm): 203.14 (acetyl C=O), 190.52 (benzoyl C=O), 165.33 (amide C=O), 162.79 and 161.41 (thiadiazole C–2 and C–5), 48.81 (CH_2), 36.27 and 31.25 (CH_3), 146.62, 144.48, 143.15, 137.75, 137.64, 134.1, 130.29, 130.21, 130.05, 129.92, 129.71, 129.63, 129.07, 128.93, 128.04, 127.33, 126.58, 123.76, 123.07; Anal. Calcd. for $C_{31}H_{25}N_7O_5S_2$: C, 58.20; H, 3.94; N, 15.33; S, 10.02. Found: C, 58.07; H, 3.98; N, 15.35; S, 10.03.

Purification of isoenzymes hCA-I and II from human erythrocytes

In order to purify the hCA-I and II isoenzymes, first human blood was centrifuged at 1500 rpm for 20 min and after the removal of the plasma, the erythrocytes were washed with an isotonic solution (0.9% NaCl). After that, the erythrocytes were lysed with 1.5 volume of ice-cold water. The lysate was centrifuged at 20 000 rpm for 30 min to remove the cell membranes and non-lysed cells. The pH of the supernatant was adjusted to 8.7 with tris and loaded onto an affinity column containing Sepharose-4B-L-tyrosine-*p*-aminobenzene sulphonamide as the binding group. After extensive washing with 25 mM tris-HCl/22 mM Na_2SO_4 (pH 8.7), the hCA-I and II isoenzymes were eluted with 1 M NaCl/ 25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6) [27,28]. The amount of purified protein was estimated by the Bradford method [29] and SDS-PAGE was carried out to determine whether the elute contained the enzyme [30].

Hydratase activity assay

Carbonic anhydrase (CA) activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [31]. The CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $((t_0 - t_c)/t_c)$ where t_0 and t_c were the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. The IC_{50} values (the concentration of inhibitor producing a 50% inhibition of CA activity) were obtained *in vitro* for the synthesised compounds (**8**–**18**), with **1** and **2** as the control compounds.

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 25°C using a spectrophotometer (CHEBIOS UV-vis) according to the method described in the literature [32,33]. The enzymatic reaction, in a total volume of 3 mL, contained 1.4 mL of 0.05 M tris–SO_4 buffer (pH 7.4), 1 mL of 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 any enzyme solution. The IC_{50} values were obtained *in vitro* for the synthesised compounds (**8**–**18**), with **1** and **2** as the control compounds.

Determination of K_i values

The K_i values were determined as described in the literature [34–38]. According to this study, in its first part, the IC_{50} values were obtained as *in vitro*. IC_{50} values of the inhibitors (for the synthesised compounds **8**–**18**, with **1** and **2** as the control compounds) were assayed by the hydrolysis of 4-nitrophenylacetate on the esterase activities of the CA isoenzymes in the presence of various inhibitor concentrations. The absorbance was determined at 348 nm after 3 min [34]. Regression analysis graphs were drawn by the plotting inhibitor concentrations versus the enzyme activity by using the Microsoft Excel Program. In the second part of the study, the concentrations of the inhibitors which gave results of 30%, 50% and 70% inhibition on the isoenzymes were determined at five different substrate concentrations. At each of these inhibitor concentrations (30%, 50% and 70%), the enzyme activity was then measured in the presence of the various substrate concentrations given above and the data were linearised with a Lineweaver–Burk plot in order to obtain the K_i value.

Results and Discussion

The treatment of glaucoma primarily aims at decreasing the intraocular pressure, which may lead to optic nerve damage and subsequently to vision loss. Several of the carbonic anhydrase inhibitors currently in use act by lowering the intraocular pressure by decreasing the aqueous humor production [22]. However, all medications, whether topical or oral, have some benefits as well as some side effects. Because of the ocular side effects after administration of the sulphonamide derivatives, the development of new carbonic anhydrase inhibitors as candidate drugs with fewer side effects will increasingly become valuable for the treatment of glaucoma. In this study, 11 derivatives of sulphonamides were synthesised and their effects on purified human carbonic anhydrase isoenzymes (hCA-I and hCA-II) were also investigated.

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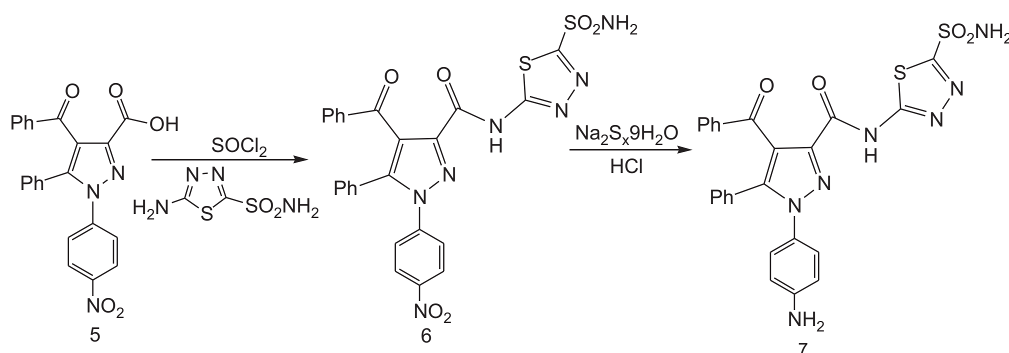
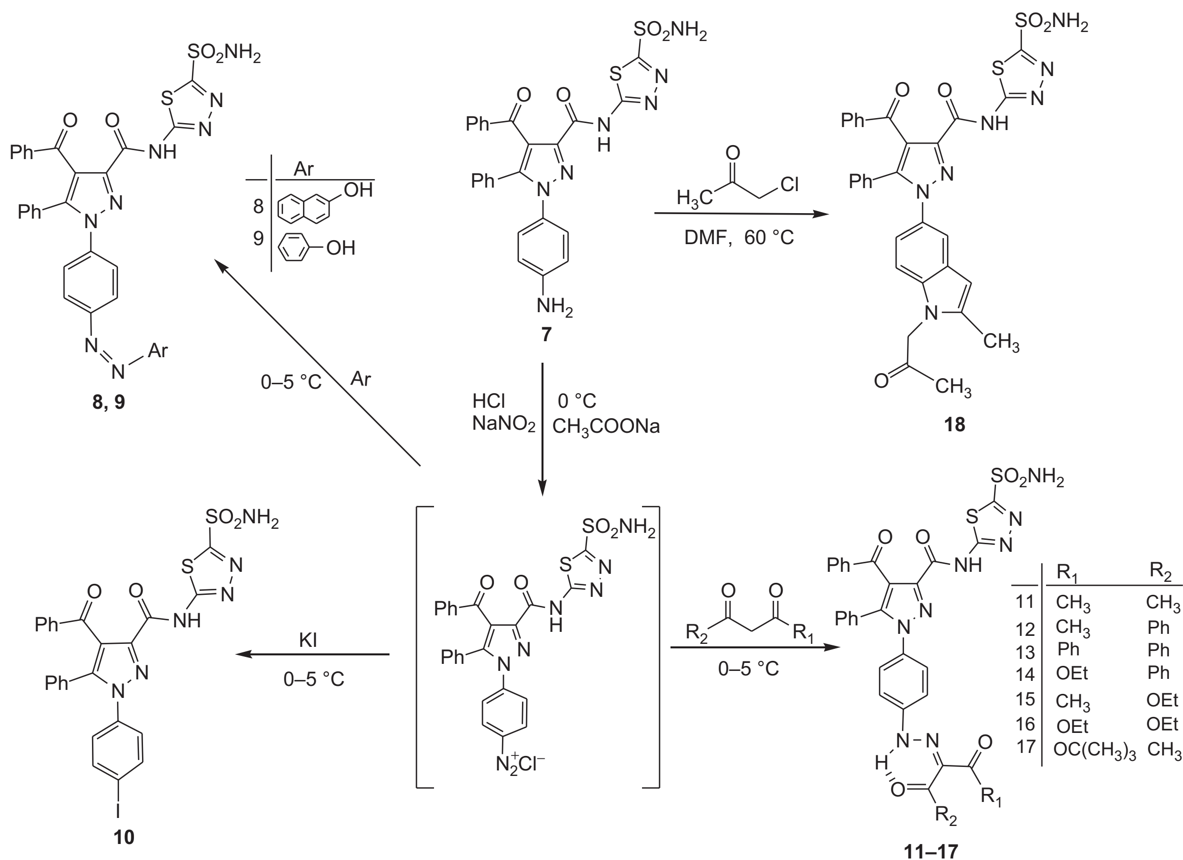
Chemistry

In order to synthesise the new compounds, firstly the activated pyrazole carboxylic acid was reacted with inhibitor **1** and a new amide derivative (**6**) was obtained (Scheme 1). After that, using this newly synthesised amide derivative, our target inhibitor was prepared by reducing the NO_2 group to the NH_2 group (**7**) [39,40].

In our work, the diazotisation method was generally used to synthesise some new derivatives of compound **7** which was an aromatic primer amine. As described previously, the diazonium salt of the compound was prepared [2] and then the coupling and replacement reactions were performed. During the reactions, the temperature was controlled (0–5°C). In our experiments, different pH ranges were tested and the best yield was observed in the range of pH = 3.5–4. The products were mostly coloured. The purity of each product was checked by TLC which was performed for each of the reactions. The structures of the compounds were characterised by spectral data (see experimental). Compound **7** gave azo compounds by the diazo coupling reaction with aromatic compounds such as phenol or β–naphthol. Compound **8** was produced in 81% yield and the derivatives of compound **9** were produced in a 66% yield. Compound **10** was produced in 61% yield by interference of the diazonium compound with KI. For the transfer of ion of I^-, known as a strong nucleophile, KI was sufficient without any need for a catalyser.



Scheme 1. Synthesis of title compound.



Scheme 2. Synthesis of compounds 8-18.

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Table 1. IC_{50} and K_i values of hCA-I and hCA-II isoenzyme hydratase and esterase activities obtained after the inhibition experiments.

İnhibitor	Hydratase IC_{50} (μM)		Esterase IC_{50} (μM)		K_i values (μM)	
	hCA-I	hCA-II	hCA-I	hCA-II	hCA-I	hCA-II
1	3.35	2.75	2.8	7.4	2.7	3.5
2	4.2	4.02	3.5	7.9	3.3	7.75
8	2.86	2.4	4.75	2.1	5.83	0.848
9	0.2	0.8	0.23	0.03	0.095	0.018
10	0.27	0.18	0.35	0.05	0.36	0.056
11	0.33	0.08	0.57	0.13	0.572	0.103
12	0.164	0.16	1	0.37	1.532	0.244
13	0.7	1	4.8	1.42	1.018	3.184
14	1.32	1.3	0.8	0.85	1.532	0.498
15	1.21	0.8	0.91	0.14	1.19	0.125
16	0.85	1	0.38	0.07	0.3	0.065
17	0.78	0.2	0.33	0.08	0.31	0.108
18	0.75	0.6	2.4	1.74	6.671	3.916

Some of the hydrazo derivatives (**11**–**17**) were produced in high yields by reaction of compound **7** with aliphatic 1,3–dicarbonile compounds which included active C–H protons [2,41]. When the results of the ^{1}H NMR spectra were examined, it was obvious that the signals of compounds **11**–**17** at δ= 11.92–11.31 ppm intervals resulted from the hydrogen atoms in covalent linkage with nitrogene. Thus, these compounds including aliphatic active C–H groups prefer keto-hydrazo (–NH–N=C) tautomeric structures after coupling.

Compound **18** was produced in 84% yield by reaction of compound **7** with chloroacetone in DMF at 60°C for 72 h [42]. Syntheses of compounds **8**–**18** from compound **7** are shown in Scheme 2.

In vitro inhibition studies

The effects of these new inhibitors on human carbonic anhydrase I (hCA-I) and II (hCA-II) purified from red blood cells were investigated. The CA isozymes were purified to homogeneity by affinity chromatography.

The inhibition effects of the parent compounds (**1** and **2**) and the newly synthesised compounds (**8**–**18**) as the control compounds on the hCA-I and hCA-II enzymes were studied by the hydratase and esterase activity methods, the IC_{50} values were determined and then the K_i values were determined for each compound. According to the *in vitro* studies, the average IC_{50} values for the hydratase activity of the newly synthesised compounds (**9**–**18**) ranged from 0.164 to 1.32 μM for hCA-I and from 0.08 to 1.3 μM for hCA-II lower than the IC_{50} values of **1** (3.35 and 2.75 μM for hCA-I and hCA-II respectively) and of **2** (4.2 and 4.02 μM for hCA-I and hCA-II respectively). The IC_{50} values obtained from the esterase activities of the synthesised compounds (**9**–**12, 14**–**17**) ranged from 0.23 to 1 μM for hCA–I. The IC_{50} values of the studied compounds for esterase activity (**8**–**18**) ranged from 0.03 to 2.1 μM for hCA–II. The IC_{50} values of these compounds were lower than **1** and **2** (2.8 and 3.5 μM for hCA–I and 7.4 and 7.9 μM for hCA–II respectively). Consequently the novel compounds have significant K_i values. The K_i values of some of the compounds (**9**–**17**) ranged from 0.095 to 1.532 for hCA-I. The K_i values of some of the compounds (**8**–**12, 14**–**17**) were also ranged from 0.018 to 0.848 for hCA-II (Table 1).

Generally our synthesised compounds showed remarkable inhibition effects on hCA-I and hCA-II and showing higher inhibition than **1** and **2**. Hence these compounds may be candidates for the treatment of glaucoma.

Declaration of interest

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