JOURNAL OF ENZYME INHIBITION AND MEDICINAL CHEMISTRY, 2017
VOL. 32, NO. 1, 169–175
http://dx.doi.org/10.1080/14756366.2016.1243536

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ORIGINAL ARTICLE

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Designing, synthesis and bioactivities of 4-[3-(4-hydroxyphenyl)-5-aryl-4,5-dihydro-pyrazol-1-yl]benzenesulfonamides

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ABSTRACT

In this study, 4-[3-(4-hydroxyphenyl)-5-aryl-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (**1**–**9**) types compounds were synthesized and their chemical structures were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectra. Cytotoxic and carbonic anhydrase (CA) inhibitory effects of the compounds were investigated. Cytotoxicity experiments pointed out that compound **4,** (4-[5-(4-chlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide), exerting the highest tumor selectivity (TS) and potency selectivity expression (PSE) values, can be considered as a lead compound of this study in terms of development of novel anticancer agents. All synthesized sulfonamides showed a good inhibition profile on hCA IX and XII in the range of 53.5–923 nM and 6.2–95 nM, respectively. These compounds were 2.5–13.4 times more selective for the inhibition of hCA XII versus hCA IX, except compound **2** which had similar inhibitory action towards both isoenzymes.

ARTICLE HISTORY
Received 4 August 2016
Revised 28 September 2016
Accepted 28 September 2016

KEYWORDS
Benzenesulfonamide; carbonic anhydrase; cytotoxicity; phenol; pyrazoline

Introduction

Cancer is a disease characterized by uncontrolled cell division which spread throughout the body and cause damage to essential organs. Although there are several strategies for the treatment of cancer, chemotheraphy is the most preferable method for inoperable cancers and medicinal chemists interest chemotheraphy parts. Despite several drugs are available in market, they have several problems such as side effects, stability, selectivity or gained resistance problems. So there is an urgent need to find new drug candidate compounds with high selectivity to the cancer cells[1,2].

Carbonic anhydrases (CAs, EC 4.2.1.1) belong to the family of zinc metalloenzymes found in a diversity of organisms and primarily responsible for catalyzing simple fundamental reaction, i.e. CO_2 hydration to bicarbonate (HCO_3^-) and proton (H^+)[3,4]. Sixteen CA isoenzymes have been identified till now. These enzymes differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of them are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI)[5,6]. Different CAs play vital roles in various physiological processes, including respiration, calcification, acid-base balance, bone resorption, etc.[7–9]. They are also involved in a number of biosynthetic pathways such as gluconeogenesis, ureagenesis, and lipogenesis as well as in pathological disorders including edema, glaucoma, obesity and epilepsy[7]. Targeting/inhibiting a particular CA is often associated with treatment of a particular disease/syndrome, e.g. CA II for antiglaucoma drug, CA VA/VB for antiobesity drug, CA VII/XIV for anticonvulsant, CA IX/XII for antitumor drug etc.[10].

In the past few years, several new tumor cell targets have been identified which led to the emergence of CA isozymes as promising target[11]. Since hCA IX and XII have been established to contribute to pH regulation of tumor cells, cell proliferation, cell adhesion and malignant cell invasion, they have been considered as valuable markers for cancer and are being targeted for designing anticancer drugs. In addition, CA IX and XII isoenzymes play a critical role in cell survival of hypoxic tumors[12–14].

The selective inhibition of CA IX and XII provide significant antitumor/antimetastatic effects[6,15,16]. Unfortunately, classical CA inhibitors do not selectively target CA IX and XII. They also inhibit other types of CA isoenzymes which have physiological relevance such as CA I and II[17,18].

The sulfonamides are an important drug class more than 70 years for their antibacterial, antiCA, diuretic, hypoglycemic, and anticancer activities[19–27]. In addition, sulfonamide derivatives E7010, ER-34410 and E7070 have recently been reported as potent antitumor agents and are in advanced clinical trials[28]. Aromatic or heterocyclic compounds containing primary sulfonamide group have been extensively studied as important scaffolds for the development of new carbonic anhydrase inhibitors (CAIs). Sulfonamide derivatives such as acetazolamide (AZA), methazolamide (MZA), ethoxzolamide (EZA), pazopanib etc. (Figure 1) are widely used as CAIs in clinical trials[29–31].

Pyrazol(in)e derivatives were reported their wide range of bioactivities such as anticancer, antiinflammatory, antiinfective, carbonic anhydrase inibitory and analgesic activities[32–38]. Celecoxib, a clinically used nonsteroidal antiinflammatory drug that selectively inhibits COX-2, has sulfonamide and pyrazole scaffolds in its chemical structure[39]. On the other hand, it was reported that compounds carring phenol moiety had CA inhibitory effect[40–45].

Our group recently focused on the synthesis of the compounds having both pyrazole-sulfonamide pharmacophores in a molecule to search for several bioactivities[32,33,37]. To further

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extend these lines of studies, the present study aims to synthesize of 4-[3-(4-hydroxyphenyl)-5-aryl-4,5-dihydro-pyrazol-1-yl]benzenesulfonamides which has pyrazole, sulfonamide and phenolic pharmacophores all together to investigate their cytotoxic/anticancer activities and also their effects on hCA IX and XII which are tumor associated CA isoenymes, expecting to find out new candidate compound/s for further studies.

Materials and methods

Experimental

Melting points were determined using an Electrothermal 9100 (Bibby Scientific Limited, Staffordshire UK) instrument and are uncorrected. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained using a Varian Mercury Plus spectrometer (Palo Alto, CA). Chemical shifts (δ) are reported in ppm. Mass spectra were undertaken on an HPLC-TOF Waters Micromass LCT Premier XE (Milford, MA) mass spectrometer using an electrospray ion source (ESI).

General procedure for the synthesis of chalcones (Scheme 1, 1′–9′)

Aqueous solution of NaOH (10%,10 ml) was added into the ethanol (6 ml) solution of suitable arylaldehyde (20.0 mmol) and 4-hydroxyacetophenone (20.0 mmol). The mixture was stirred overnight at room temperature and then it was poured on ice-water (100 ml) in a beaker. The mixture was neutralized with solution of HCl (10%, 8.5 ml)[46]. The colored precipitate formed was filtered and crystallized from suitable solvent at room temperature. The crystallization solvent was ethanol-water (**1′**, **3′**, **4′**, **6′**, **9′**) or methanol-water (**2′**, **5′**, **7′**, **8′**). The yields of the chalcones were in the range of 15–38% [**1′** (38%), **2′** (29%), **3′** (18%), **4′** (15%), **5′** (37%), **6′** (17%), **7′** (34%), **8′** (29%), **9′** (27%)].

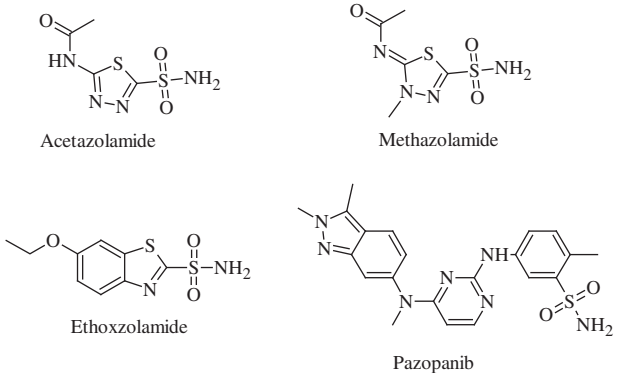


Figure 1. Chemical structures of some carbonic anhydrase inhibitors which are in clinical use.

General procedure for the synthesis of pyrazolines (Scheme 1, 1–9)

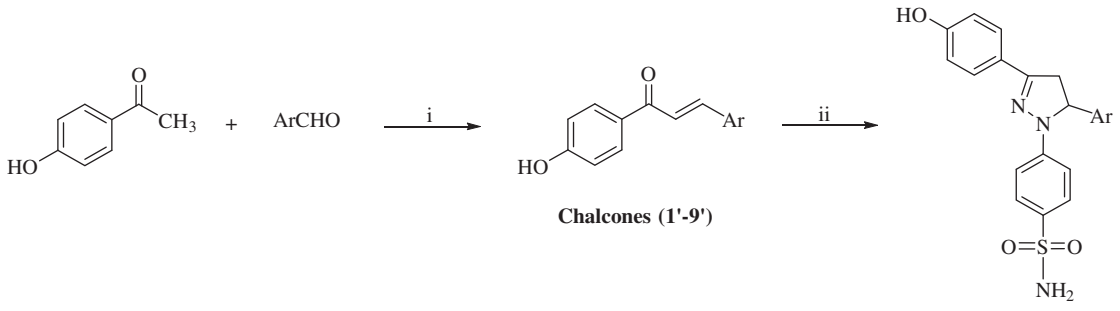
A suitable chalcone (1.00 mmol) and 4-hydrazinobenzenesulfonamide hydrochloride (1.10 mmol) were solved in ethanol [25 ml (**7**), 30 ml (**2**, **3**, **6**, **8**), 50 ml (**1**), 60 ml (**4**), 70 ml (**5**, **9**)] and then catalytic amount of glacial acetic acid was added and the mixture was refluxed[32,33,37] [6 h (**9**), 9 h (**2**, **7**), 10 h (**1**), 11 h (**4**, **6**), 12 h (**3**, **5**, **8**)]. Reactions were followed by thin layer chromotography (TLC). After the reaction was stopped, some of the solvent was removed under vacuum and the mixture was stirred for 12 h. The obtained solid was filtered, dried at room temperature and crystallized from suitable solvent. It was methanol-chloroform (**1**), methanol (**2**, **3**), methanol-ether (**4**, **5**, **6**, **7**, **8**, **9**). Since hydrogens of SO_2NH_2 exchanged with deuterium of CD_3OD, sulfonamide hydrogens were not observed on ^{1}H NMR spectra.

4-[3-(4-Hydroxyphenyl)-5-phenyl-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (1)

M.p. 208–210 °C. Yield: 47%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.65$ (d, 2H, $J = 8.8$ Hz), 7.64 (d, 2H, $J = 9.0$ Hz), 7.36–7.24 (m, 5H), 7.08 (d, 2H, $J = 9.0$ Hz), 6.84 (d, 2H, $J = 8.8$ Hz), 5.43 (dd, 1H, $J = 12.0$, 5.7 Hz), 3.92 (dd, 1H, $J = 17.4$, 12.0 Hz), 3.13 (dd, 1H, $J = 17.4$, 5.7 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 160.2$, 151.5, 148.7, 143.5, 132.8, 130.3, 128.9, 128.8, 128.5, 126.9, 125.0, 116.5, 113.3, 64.5, 44.7; HRMS (ESI-MS): calcd. for $C_{21}H_{20}N_3O_3S$ $[M + H]^+$ 394.1225; found 394.1217.

4-[3-(4-Hydroxyphenyl)-5-p-tolyl-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (2)

M.p. 163–164 °C. Yield: 75%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.62$ (d, 2H, $J = 8.8$ Hz), 7.61 (d, 2H, $J = 9.1$ Hz), 7.20–7.10



Reagents and conditions. (i) 10% aq NaOH, EtOH, 0-5 °C, 12 h; (ii) 4-Hydrazinobenzenesulfonamide hydrochloride, EtOH, glacial acetic acid, reflux 6-12 h. **Ar:** C_6H_5 for **1′, 1;** 4-$CH_3C_6H_4$ for **2′, 2;** 4-$CH_3OC_6H_4$ for **3′, 3;** 4-ClC_6H_4 for **4′, 4;** 2,4-$(Cl)_2C_6H_3$ for **5′, 5;** 4-FC_6H_4 for **6′, 6;** 4-BrC_6H_4 for **7′, 7**; 4-$NO_2C_6H_4$ for **8′, 8;** C_4H_3S(2-yl) for **9′, 9**.

Scheme 1. Synthesis of compounds 1–9.

(m, 4H), 7.05 (d, 2H, $J = 8.8$ Hz), 6.82 (d, 2H, $J = 8.8$ Hz), 5.37 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.87 (dd, 1H, $J = 17.4$, 12.1 Hz), 3.08 (dd, 1H, $J = 17.4$, 5.5 Hz), 2.28 (s, 3H, $-CH_3$); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.0$, 150.3, 147.5, 139.2, 137.4, 131.5, 129.6, 127.7, 127.3, 125.6, 123.8, 115.3, 112.1, 63.1, 43.5, 19.9; HRMS (ESI-MS): calcd. for $C_{22}H_{22}N_3O_3S$ $[M + H]^+$ 408.1382; found 408.1367.

4-[3-(4-Hydroxyphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (3)

M.p. 176–178 °C. Yield: %23. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.62$ (d, 2H, $J = 8.8$ Hz), 7.61 (d, 2H, $J = 8.8$ Hz), 7.16 (d, 2H, $J = 8.8$ Hz), 7.06 (d, 2H, $J = 8.8$ Hz), 6.86 (d, 2H, $J = 8.8$ Hz), 6.82 (d, 2H, $J = 8.8$ Hz), 5.36 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.86 (dd, 1H, $J = 17.2$, 12.1 Hz), 3.74 (s, 3H, -OCH_3), 3.09 (dd, 1H, $J = 17.2$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.5$, 159.0, 150.3, 147.6, 134.1, 131.4, 127.7, 127.2, 126.9, 123.9, 115.3, 114.4, 112.1, 62.9, 54.5, 43.5; HRMS (ESI-MS): calcd. for $C_{22}H_{22}N_3O_4S$ $[M + H]^+$ 424.1331; found 424.1312.

4-[5-(4-Chlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (4)

M.p. 152–154 °C. Yield: 62%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.64$ (d, 2H, $J = 8.8$ Hz), 7.63 (d, 2H, $J = 8.8$ Hz), 7.32 (d, 2H, $J = 8.4$ Hz), 7.26 (d, 2H, $J = 8.4$ Hz), 7.05 (d, 2H, $J = 9.2$ Hz), 6.82 (d, 2H, $J = 8.8$ Hz), 5.45 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.91 (dd, 1H, $J = 17.2$, 12.1 Hz), 3.12 (dd, 1H, $J = 17.2$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.1$, 150.3, 147.3, 141.0, 133.3, 131.9, 129.1, 127.8, 127.5, 127.4, 123.6, 115.3, 112.1, 62.6, 43.3; HRMS (ESI-MS): calcd. for $C_{21}H_{19}ClN_3O_3S$ $[M + H]^+$ 428.0836; found 428.0824.

4-[5-(2,4-Dichlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (5)

M.p. 246–248 °C. Yield: 74%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.66$ (d, 2H, $J = 8.8$ Hz), 7.62 (d, 2H, $J = 8.8$ Hz), 7.56 (d, 1H, $J = 2.2$ Hz), 7.20 (dd, 1H, $J = 8.4$, 2.2 Hz), 7.02 (d, 1H, $J = 8.4$ Hz), 6.97 (d, 2H, $J = 8.8$ Hz), 6.81 (d, 2H, $J = 8.8$ Hz), 5.69 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.97 (dd, 1H, $J = 17.4$, 12.1 Hz), 3.06 (dd, 1H, $J = 17.4$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.2$, 150.6, 146.9, 137.6, 134.1, 132.8, 132.2, 129.7, 128.2, 127.9, 127.8, 127.6, 123.4, 115.4, 111.9, 60.0, 41.9; HRMS (ESI-MS): calcd. for $C_{21}H_{17}Cl_2N_3O_3S$ $[M-H]^-$ 460.0289; found 460.0282.

4-[5-(4-Fluorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (6)

M.p. 243–244 °C. Yield: 72%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.63$ (d, 4H, $J = 8.8$ Hz), 7.30–7.26 (m, 2H), 7.07–7.03 (m, 4H), 6.82 (d, 2H, $J = 8.8$ Hz), 5.45 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.90 (dd, 1H, $J = 17.6$, 12.1 Hz), 3.11 (dd, 1H, $J = 17.6$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.1$, 150.3, 147.4, 138.2, 131.8, 127.8, 127.7, 127.3, 123.7, 115.8, 115.6, 115.3, 112.1, 62.6, 43.5; HRMS (ESI-MS): calcd. for $C_{21}H_{19}FN_3O_3S$ $[M + H]^+$ 412.1131; found 412.1115.

4-[5-(4-Bromophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (7)

M.p. 174–175 °C. Yield: 38%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.64$ (d, 2H, J = 8.8 Hz), 7.63 (d, 2H, J = 8.8 Hz), 7.48 (d, 2H, $J = 8.4$ Hz), 7.19 (d, 2H, $J = 8.4$ Hz), 7.05 (d, 2H, $J = 9.1$ Hz), 6.82 (d, 2H, $J = 8.8$ Hz), 5.42 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.91 (dd, 1H, $J = 17.6$, 12.1 Hz), 3.12 (dd, 1H, $J = 17.6$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.1$, 150.3, 147.3, 141.5, 132.1, 131.9, 127.8, 127.7, 127.4, 123.6, 121.2, 115.3, 112.1, 62.7, 43.3; HRMS (ESI-MS): calcd. for $C_{21}H_{19}BrN_3O_3S$ $[M + H]^+$ 472.0330; found 472.0317.

4-[3-(4-Hydroxyphenyl)-5-(4-nitrophenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (8)

M.p. 173–176 °C. Yield: 61%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 8.20$ (d, 2H, $J = 8.8$ Hz), 7.65 (d, 2H, $J = 9.2$ Hz), 7.64 (d, 2H, $J = 8.8$ Hz), 7.51 (d, 2H, $J = 8.8$ Hz), 7.05 (d, 2H, $J = 9.2$ Hz), 6.82 (d, 2H, $J = 8.8$ Hz), 5.60 (dd, 1H, $J = 12.1$, 5.5 Hz), 3.97 (dd, 1H, $J = 17.6$, 12.1 Hz), 3.17 (dd, 1H, $J = 17.6$, 5.5 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.2$, 150.3, 149.5, 147.7, 147.2, 132.3, 127.9, 127.5, 127.1, 124.2, 123.4, 115.4, 112.1, 62.6, 43.1; HRMS (ESI-MS): calcd. for $C_{21}H_{18}N_4O_5S$ $[M-H]^-$ 437.0920; found 437.0931.

4-[3-(4-Hydroxyphenyl)-5-(thiophen-2-yl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide (9)

M.p. 220–221 °C. Yield: 21%. ^{1}H NMR (400 MHz, CD_3OD, ppm) $\delta = 7.66$ (d, 2H, $J = 9.1$ Hz), 7.64 (d, 2H, $J = 8.8$ Hz), 7.26 (d, 1H, $J = 5.0$ Hz), 7.17 (d, 2H, $J = 8.8$ Hz), 7.06 (d, 1H, $J = 3.2$ Hz), 6.93 (dd, 1H, $J = 5.0$, 3.2 Hz), 6.83 (d, 2H, $J = 8.8$ Hz), 5.77 (dd, 1H, $J = 11.5$, 5.1 Hz), 3.88 (dd, 1H, $J = 17.2$, 11.5 Hz), 3.26 (dd, 1H, $J = 17.2$, 5.1 Hz); ^{13}C NMR (100 MHz, CD_3OD, ppm) $\delta = 159.1$, 150.5, 147.7, 145.4, 132.1, 127.8, 127.2, 126.7, 124.9, 124.8, 123.7, 115.3, 112.5, 59.3, 43.7; HRMS (ESI-MS): calcd. for $C_{19}H_{18}N_3O_3S_2$ $[M + H]^+$ 400.0790; found 400.0789.

Biological activity

Cytotoxicity assay

The cytotoxicity of the compounds **1–9** were assayed towards human oral squamous cell carcinoma cell lines derived from gingiva tissue (CA9–22) and tongue (HSC-2, HSC-3, HSC-4), and human normal oral cells (gingival fibroblasts, HGF; periodontal ligament fibroblasts, HPLF; pulp cells, HPC) with some minor modifications[33,43,47–50]. In brief, cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells (2.5×10^3 cells/well) were inoculated and incubated for 48 h to achieve complete adherence. Near confluent cells were incubated for a further 48 h in the fresh culture medium containing each test compound (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 μM) or 5-FU (positive control) (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 μM). The viable cell numbers were determined by the MTT method. Cytotoxicity induced by DMSO (0.0078, 0.156, 0.03125, 0.0625, 0.125, 0.25, 0.5 or 1%) was subtracted from each well. The CC_{50} values were determined from dose-response curves. The tumor selectivity (TS) was calculated by the following equation: TS = mean CC_{50} against normal cells/mean CC_{50} against cancer cells [shown as (D/B) or (C/A) in Table 1]. A potency selectivity expression (PSE) was devised which is the product of the reciprocal of average CC_{50} values towards cancer cell lines and the average SI values towards these cell lines and expressed as a percentage[49].

Carbonic anhydrase enzyme assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration reaction[51]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH

Table 1. Cytotoxic activity of compounds 1–9 against human oral malignant and nonmalignant cells.

	CC_{50} (μM)																	
	Human oral squamous cell carcinoma cell lines							Human oral normal cells						TS				
Compounds	Ca9–22 (A)	SI	HSC-2	SI	HSC-3	SI	HSC-4	SI	Mean (B)	SD	Mean SI	HGF (C)	HPLF	HPC	Mean (D)	SD	(D/B)	(C/A)	PSE
1	82.0	1.4	94.7	1.2	81.0	1.5	83.7	1.4	85.3	6.3	1.4	133.3	59.7	161.0	118.0	52.4	1.4	1.6	1.6
2	57.0	1.7	95.3	1.0	67.3	1.4	70.3	1.4	72.5	16.3	1.4	99.3	42.3	150.0	97.2	53.9	1.3	1.7	1.9
3	53.3	2.0	95.3	1.1	71.0	1.5	88.3	1.2	77.0	18.8	1.4	107.7	39.3	167.3	104.8	64.0	1.4	2.0	1.9
4	39.7	2.2	81.7	1.1	57.0	1.5	61.0	1.4	59.8	17.3	1.6	89.0	29.3	144.3	87.6	57.5	1.5	2.2	2.6
5	44.3	1.7	82.3	0.9	76.0	1.0	54.0	1.4	64.2	18.0	1.3	63.0	34.7	131.0	76.2	49.5	1.2	1.4	2.0
6	61.7	1.4	84.7	1.0	78.0	1.1	62.3	1.4	71.7	11.5	1.2	64.7	33.0	155.0	84.2	63.3	1.2	1.0	1.7
7	55.7	1.4	85.0	0.9	70.3	1.1	61.7	1.3	68.2	12.7	1.2	52.0	34.3	149.3	78.6	61.9	1.2	0.9	1.7
8	39.3	1.7	76.0	0.9	63.3	1.0	63.7	1.0	60.6	15.3	1.2	42.0	30.0	126.7	66.2	52.7	1.1	1.1	1.9
9	99.3	1.2	67.0	1.8	87.3	1.4	93.7	1.3	86.8	14.1	1.4	165.7	26.7	173.3	121.9	82.6	1.4	1.7	1.7
5-FU	29.0	>34.5	13.0	>76.9	16.0	>62.5	13.0	>76.9	17.8	7.6	>62.7	>1000	>1000	>1000	>1000	>1000	>56.2	>34.5	>352.2

CC_{50} values refer to the concentrations of the compounds in micromoles which reduce the viable cell number by 50%. Oral squamous cell carcinoma (OSCC) cell lines used are Ca9–22 (derived from gingiva), HSC-2, HSC-3, HSC-4 (derived from tongue). Normal oral cells used are human gingival fibroblasts (HGF), human periodontal ligament fibroblasts (HPLF), and human pulp cells (HPC). Tumor selectivity (TS) value is calculated by dividing the mean CC_{50} value of each compound against normal cells to mean CC_{50} value against OSCC. CC_{50} value was determined from the growth curves plotted at different concentrations of each compounds in triplicate wells. SI: Selectivity Index; SD: Standart Deviation; 5-FU: 5-Fluorouracil; μM: Micromolar.

7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition percantage were obtained by using PRISM 3, as reported earlier[52], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier[53].

Results and discussion

The compounds were succesfully synthesized and their chemical structures were elucidated by ^{1}H NMR, ^{13}C NMR, and HRMS spectra as shown in experimental section. The cytotoxic effects of the compounds were assayed towards human oral malignant (Ca9–22, HSC-2, HSC-3 and HSC-4) and nonmalignant (HGF, HPLF and HPC) cells by MTT method[33,43,47–50]. The results were shown in Table 1.

The first question to be answered is whether the compounds have antineoplastic property or not. CC_{50} (the concentration of the compound that kills 50% of the cells as mol/L) values of the compounds were in the range of 39.3–99.3 μM while reference compound 5-Fluorouracile (5-FU)'s changed in the range of 13–29 μM. It can be said that the compounds studied here have antineoplastic properties since they are effective at micromolar level. However, they are less cytotoxic than 5-FU towards cancer cell lines (Table 1).

The second aspect of the compounds to be considered is whether they are tumor-specific cytotoxins since tumors are surrounded by different types of normal cells in oral cavity. Selectivity index (SI) value, which is the quotient of the average CC_{50} value of the nonmalignant cells and the CC_{50} value of a compound towards a specific malignant cell line, was generated in Table 1. The compounds which have SI values of >1 can be considered as tumor-specific antineoplastic agents[54]. So, it can be said that the most of the compounds have shown tumor specificity against all cancer cell lines, except the compounds **5**, **7** and **8** towards HSC-2 cell (Table 1). The highest SI value of 2.2 was calculated for the compound **4** towards Ca9–22 cancer cell line.

The TS of the compounds were calculated by two types of calculations[49]. The first calculation was made by dividing the average CC_{50} value towards normal cells into the average CC_{50} value towards a total of four cancer cell lines (TS = Column D/Column B, Table 1). The second calculation is the comparison of malignant (Ca9–22) and nonmalignant (HGF) cells which has the same tissue origine (gingiva). TS values were calculated by dividing the CC_{50} value towards HGF cells by the CC_{50} value towards Ca9–22 cells (TS = Column C/Column A, Table 1). Both types of TS calculations demonstrated that compound **4** showed the highest tumor-specificity (TS = 1.5 and 2.2, respectively).

Lead compound should possess both marked cytotoxic potency and also selective toxicity for tumors. In order to identify such molecule, a PSE was devised which is the product of the reciprocal of average CC_{50} values towards cancer cell ines and the average SI values towards these cell lines and expressed as a percentage[49]

Table 2. Effects of compounds 1–9 on hCA IX and XII isoenzymes activity.

	Ki (nM)		
Compounds	hCA IX	hCA XII	hCA XII/hCA IX
1	923	68.9	13.4
2	100	95	1.1
3	93.1	33.6	2.8
4	97.7	38.8	2.5
5	85.7	9.2	9.3
6	84.1	8.6	9.8
7	66.2	7.6	8.7
8	63.7	7.5	8.5
9	53.5	6.2	8.6
AAZ*	25	5.7	4.4

*Acetazolamide (AAZ) was used as a standard inhibitor for all CAs investigated here.

(Table 1). When PSE values were considered, all compounds had lower PSE values than the reference compound 5-FU. PSE values of the compounds studied were in the range of 1.6–2.6. The chlorine substituted compound **4** had the highest PSE value of 2.6 among the series (Table 1).

According to TS and PSE values; it seems that the compound **4**, 4-[5-(4-chloro-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide, can be considered as a leader compound of this study in terms of cytotoxicity and can be used for further developments.

The compounds **1–9** were also tested in terms of CA inhibition profile on hCA IX and XII which are important isoenzymes taking part important roles in cancer biology, especially at the regulation of extracellular pH of cancer cells. The inhibitory constant (Ki) values of the compounds synthesized were in the range of 53.5–923 nM towards hCA IX while they were in the range of 6.2–68.9 nM towards hCA XII (Table 2). The compounds tested were 2.5–13.4 times more selective towards hCA XII isoenzyme than hCA IX isoenzyme, except compound **2**.

When the effects of the substituents on CA inhibition were considered the compound **8** which has electron attracting nitro substituent on phenyl ring had the lowest Ki values towards both isoenzymes among the compounds studied **1**–**8**. Compound **8** having nitro substituent had 14.5 and 9.2 times more powerful inhibition potential than the compound **1**, which is nonsubstituted phenyl derivative. Replacement of benzene ring by thiophene ring is often used in medicinal chemistry to modify bioactivity of a compound since benzene and thiophene are bioisosteric rings. In this study, replacement of benzene ring by thiophene increased CA inhibitory potential by decreasing the Ki value. When the compounds **1** with benzene and **9** with thiophene were compared, **9** was more potent inhibitor than **1**. Inhibitory potential of **9** was 17.3 and 11.1 times more potent than **1** towards hCA IX and XII, respectively.

Any type of substitution on phenyl ring increased the inhibition potential of the compounds by decreasing the Ki values towards both isoenzymes, except the compound **2** towards hCA XII isoenzyme. When halogen bearing compounds were compared, the order of inhibition potency of the compounds was as follows: compound **7** with bromine (Ki = 66.2 nM) > compound **6** with fluorine (Ki = 84.1 nM) > compound **4** with chlorine (Ki = 97.7 nM) towards hCA IX isoenzyme. It was as follows towards hCA XII isoenzyme: compound **7** (Ki = 7.6 nM) > compound **6** (Ki = 8.6 nM) > compound **4** (Ki = 38.8 nM). The potency order of the compounds towards hCA IX an XII the same as **7** > **6** > **4**. There was no relation between the electronegativity of halogen and Ki values. Dichlorine substitution was found useful to increase the inhibition potency of the compound towards both isoenzymes in compound **5** comparing to compound **4**, which has mono chlorine atom. Inhibition potential increased 7.5 times in compound **5** comparing to compound **1** towards hCA XII isoenzyme while **5** was 10.8 times more potent towards hCA IX isoenzyme than **1**. When compounds **2** with methyl substituent and **3** with methoxy substituent were compared, introduction of oxygene into molecule **3** increased the inhibition potential 2.8 times towards hCA XII while there is a slight increase towards hCA IX (1.1 times) by the introduction of oxygene in **3** comparing with **2**. The increased inhibition potential may be attributed to the possibility of hydrogen bonding with **3** comparing to **2**.

Conclusion

Cytotoxicity results of the synthesized compounds revealed that compound **4**, 4-[5-(4-chloro-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]benzenesulfonamide, may be considered as a leader compound in terms of cytotoxic/anticancer activity. All studied compounds showed an impressive inhibiton profile on hCA IX and XII, with K_Is in the range of 53.5–923 nM and 6.2–95 nM, respectively. Except **2**, all compounds were 2.5–13.4 times more selective inhibitor towards hCA XII than hCA IX while compound **2** had similar selectivity towards both isoenzymes. All compounds reported here can be considered as leader compounds to develop new selective hCA XII inhibitors for further detailed studies.

Acknowledgements

The authors Halise Inci Gul and Ebru Mete thank to Ataturk University for financial support. This work was also supported by Grant-in-Aid for Challenging Exploratory Research from The Ministry of Education, Culture, Sports, Science and Technology (Sakagami H. 25670897).

Disclosure statement

The authors report no conflict of interest and are responsible for the contents and writing of the paper.

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