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RESEARCH ARTICLE

Synthesis, cytotoxicity and carbonic anhydrase inhibitory activities of new pyrazolines

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Abstract

A series of polymethoxylated-pyrazoline benzene sulfonamides were synthesized, investigated for their cytotoxic activities on tumor and non-tumor cell lines and inhibitory effects on carbonic anhydrase isoenzymes (hCA I and hCA II). Although tumor selectivity (TS) of the compounds were less than the reference compounds 5-Fluorouracil and Melphalan, trimethoxy derivatives **4**, **5**, and **6** were more selective than dimethoxy derivatives **2** and **3** as judged by the cytotoxicity assay with the cells both types originated from the gingival tissue. The compound **6** (4-[3-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl] benzene sulfonamide) showed the highest TS values and can be considered as a lead molecule of the series for further investigations. All compounds synthesized showed superior CA inhibitory activity than the reference compound acetazolamide on hCA I, and II isoenzymes, with inhibition constants in the range of 26.5–55.5 nM against hCA I and of 18.9–28.8 nM against hCA II, respectively.

Keywords

Carbonic anhydrase, cytotoxicity, dental cells, pyrazoline, sulfonamide, tumor selectivity

History

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Introduction

Cancer is one of the most important causes of deaths in the world[1]. Various classes of drugs such as alkylating agents, antimetabolites, antibiotics, hormones and plant alkaloids are used in the traditional cancer chemotherapy[2]. Although selective toxicity is preferable in cancer treatment, the drugs used in clinics have several side effects such as resistance development to the anticancer agents in addition to low selectivities[3].

Carbonic anhydrase (CA, EC 4.2.1.1) a zinc-dependent metalloenzyme that catalyzes the reversible hydration of CO_2. Sixteen different α-CA isoforms have been isolated and characterized so far in mammals: CA I, CA II, CA III, CA VII, CA XIII are cytosolic; CA IV, CA IX, CA XII, CA XIV and CA XV are associated with the cell membrane; CA VA and CA VB are in mitochondria and CA VI is secreted into saliva and milk[4].

The CAs contribute many physiological and pathological processes such as pH homeostasis, electrolyte secretion in various tissues and organs, gluconeogenesis, lipogenesis, ureagenesis, bone resorption calcification and tumorigenicity[5–7]. Thus, the CAs have become remarkable therapeutic targets for the treatment of a wide range of disorders. Several CA inhibitors show anticancer[7], diuretics[8], antiglaucoma[9], antiinfective[10] and anti-obesity[11] activities.

Pyrazole/pyrazolines are an important class of heterocyclic compounds containing three carbon atoms and two nitrogen atoms in adjacent positions. These heterocyclic compounds can be seen in nature as alkaloids, vitamins, pigments and constituents of plant and animal cells widely[12]. Medicinal chemists have great attention of pyrazolines and substituted pyrazolines because of their various biological activities such as antimicrobial, anticonvulsant, anticancer, anti-inflammatory, antitubercular, antiviral, analgesic and antidepressive activities[13–17]. Sulfonamide is an important functional group in medicinal chemistry having a wide range of bioactivities such as cytotoxic, carbonic anhydrase inhibitory, antibacterial, antimalarial, antihypertensive, antiviral, anti-inflammatory and diuretics activities[8,13,18–25].

In this study it was aimed to synthesize new compounds bearing pyrazoline and sulfonamide pharmacophores and to test their carbonic anhydrase inhibitory effects on hCA I and II isoenzymes. It was also planned to investigate their cytotoxic activities towards four cancer cell lines (Ca9–22, HSC-2, HSC-3, and HSC-4) and three non-tumor cells (HGF, HPLF and HPC) to see whether any of compounds synthesized is/are tumor-specific cytotoxin/s.

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Experimental

Materials and methods

Chemical structure of the compounds were determined by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopies using a Varian Mercury Plus spectrometer (Varian Inc., Palo Alto, CA). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are expressed in hertz (Hz). Mass spectra for the compound **1** were taken using a liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization mode. Shimadzu's LCMS Solution software was used for data analysis. Mass spectra for the compounds **2–6** were undertaken on a HPLC-TOF Waters Micromass LCT Premier XE (Milford, MA) mass spectrometer using an electrospray ion source (ESI). Melting points were determined using an electrothermal 9100/IA9100 instrument (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. The reactions were monitored using silica gel HF254–366 TLC plates (E. Merck, Darmstadt, Germany). IR spectra of the compounds were taken using a FT-IR spectrometer (Perkin Elmer Spektrum One FT-IR, Bridgeport Avenue Shelton, CT).

*Synthesis of chalcone compounds, **1a–6a***

4-Methoxyacetophenone and suitable aldehyde in 1:1 mol ratio were dissolved in ethanol (10 ml)[23]. Aqueous NaOH solution (10%, 20 ml) was added into the reaction flask. Reactions were monitored by TLC. When at least one of the starting compounds finished, reactions were stopped. Reaction content was poured on ice-water and neutralized by HCl (10%). The precipitates obtained were washed with cold water and ethanol, filtered, and dried. The purities were checked by TLC and used for the synthesis of pyrazoline derivatives **1–6** without further purification.

*Synthesis of pyrazoline containing benzene sulfonamides, **1–6***

A mixture of a suitable chalcone compound (**1a–6a**) and 4-hydrazinobenzenesulfonamide hydrochloride in 1:1.1 mol ratio in ethanol (25 ml for **1–3** and 30 ml for **4–6**) in the presence of glacial acetic acid (0.05 ml) was refluxed[26]. Reactions were monitored by TLC using $CHCl_3$:MeOH (4.8:0.2) as a solvent system. When the reactions were stopped, the precipitate obtained was filtered, dried and crystallized from suitable solvent system. Experimental data of the compounds are presented in Table 1. Spectral data of the compounds are presented in Table 2.

Biological activity

Carbonic anhydrase inhibition assay

The purification of cytosolic CA isoenzymes (CA I and II) were previously described with a simple one-step method by a sepharose-4B-L tyrosine-sulphanilamide affinity chromatography[27]. The protein quantity in the column effluents was determined spectrophotometrically at 280 nm. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied with a Bio-Rad Mini Gel system (Mini-PROTEAN Tetra System, China) after purification of both CA isoenzymes[28]. Briefly, it was performed in acrylamide for the running (10%) and the stacking gel (3%) contained SDS (0.1%), respectively. Activities of CA I and II isoenzymes were determined according to the method of Verpoorte et al[29]. The increasing in absorbance of reaction medium was spectrophotometrically recorded at 348 nm (Shimadzu, UV-VIS Spectrophotometer, UVmini-1240, Kyoto-Japan). Also, the quantity of protein was determined at 595 nm according to Bradford method[30]. Bovine serum albumin was used as standard protein. The IC_{50} values were obtained from activity (%) versus compounds plots. For calculation of K_i values, three different concentrations were used. The Lineweaver–Burk curves were drawn and calculations were realised[31].

Cytotoxicity assay

The cytotoxicity of the compounds were assayed towards human oral squamous cell carcinoma cell lines (Ca9–22, HSC-2, HSC-3, HSC-4) and human normal oral cells (HGF, HPLF, HPC) as described with some minor modifications[32–37]. All cells were cultured in DMEM supplemented with 10% fetalbovine serum (FBS). All test samples were dissolved in dimethylsulfoxide (DMSO). Near confluent cells were incubated in 96-microwell (Becton Dickinson, Franklin Lakes, NJ) for 48 h with 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 or 200 μM of each test compound. Any cytotoxicity induced by DMSO (0.002, 0.004, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5 or 1%) were subtraced from the corresponding treated groups. The viable cell numbers were determined by the MTT method. In brief, the treated cells were incubated for another 3 h in fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO and the absorbance at 562 nm of the cell lysate was determined using a microplate reader (Sunrise Rainbow RC-R; TECAN, Männedorf, Switzerland). CC_{50} (the concentrations of the compounds in micromoles which reduce the viable cell number by 50%) was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from triplicate assays.

Results and discussion

All the compounds studied here are reported for the first time with their detailed synthesis, spectral analysis, and bioactivities except **3** and **6**. The synthetic pathway of the compounds are summarized in Scheme 1 and their experimental data (Table 1), spectral data (Table 2), cytotoxicities of the compounds against human oral squamous cell carcinoma cell lines and human normal oral cells (Table 3), the inhibitory potential of the compounds on hCA I and II isoenzymes (Table 4) are summarized in Tables 1–4.

Structures of the compounds synthesized were established on the basis of spectral data taken from FT-IR, ^{1}H NMR, ^{13}C NMR and HRMS. The IR spectra of the compounds showed absorption bands in the regions 1599–1571 cm^{-1} corresponding to C=N stretching bands because of ring closure. Infrared spectra revealed

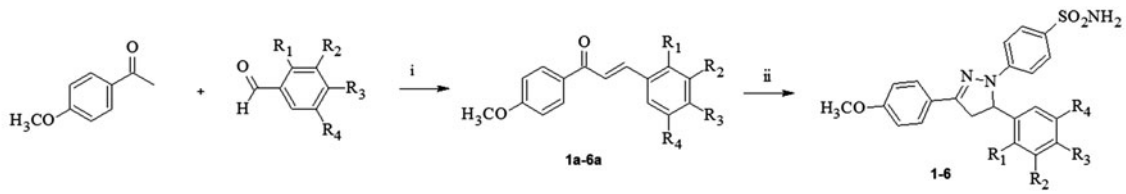
Table 1. Experimental data of the compounds **1–6**.

Compounds	Chalcone mmol	PHBS mmol	Time h	Yield %	Crystallization Solvents	Mp, °C
1	1.68	1.84	5	53	Ethanol/Chloroform	267–269
2	1.68	1.84	18	71	Ethanol/Chloroform	197–199
3	1.68	1.84	4	19	Methanol	200–202
4	1.52	1.67	6	67	Methanol	168–170
5	1.52	1.67	4	85	Methanol/Chloroform	242–244
6	1.52	1.67	1	72	Methanol	238–240

PHBS: 4-Hydrazinobenzensulfonamide hydrochloride.

Table 2. Spectral data of the compounds **1–6**.

Compounds	^{1}H NMR, ^{13}C NMR, and HRMS
1	4-(5-(2,5-dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide. ^{1}H NMR (DMSO-d_6, δ ppm): 7.69 (d, $J = 8.8$ Hz, 2H), 7.57(d, $J = 9.2$ Hz, 2H), 7.02-6.94 (m, 6H), 6.79 (dd, $J = 2.9$, 9.0 Hz, 2H), 6.35 (d, $J = 2.9$ Hz, 1H), 5.62 (dd, $J = 11.9$, 5.0 Hz, 1H), 3.84 (s, 3H, -OCH_3), 3.82 (s, 3H,OCH_3), 3.77 (s, 3H, -OCH_3), 3.04 (dd, $J = 17.6$, 5.0 Hz, 1H)(One of the proton peak of the pyrazole ring under the solvent peak). ^{13}C NMR (DMSO-d_6, δ ppm):160.9, 153.8, 150.9, 150.8, 146.6, 133.1, 130.2, 128.4, 127.9, 125.0, 114.8, 113.3, 112.8, 112.1, 57.8, 56.8, 55.9, 55.8, 42.6. HRMS (ESI-MS) Calc. for$C_{24}H_{26}N_3O_5S$ $[M + H]^+$, 468.1588; found 468.1582
2	4-(5-(2,4-dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1- yl)benzenesulfonamide. ^{1}H NMR (DMSO-d_6, δ ppm): 7.69 (d, $J = 8.8$ Hz, 2H), 7.56 (d, $J = 8.8$ Hz, 2H), 6.99-6.93 (m, 6H), 6.73 (d, $J = 8.8$ Hz, 1H), 6.62 (d, $J = 2.2$ Hz, 1H), 6.38 (dd, $J = 8.4$, 2.6 Hz, 1H), 5.58 (dd, $J = 11.7$, 4.8 Hz, 1H), 3.87 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3), 3.69 (s, 3H, -OCH_3), 3.01 (dd, $J = 17.4$, 4.8 Hz, 1H)(One of the proton peak of the pyrazole ring under the solvent peak). ^{13}C NMR (DMSO-d_6, δ ppm): 160.9, 160.7, 157.8, 150.8, 146.6, 132.9, 128.3, 127.9, 127.2, 125.2, 121.2, 114.8, 112.1, 105.7, 99.7, 57.5, 56.5, 55.9, 55.8, 42.8. HRMS (ESI-MS) Calc. for $C_{24}H_{26}N_3O_5S$ $[M + H]^+$ 468.1593; found 468.1590
3	4-(5-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide. ^{1}H NMR (DMSO-d_6) δ (ppm): 7.69 (d, $J = 8.8$ Hz, 2H), 7.53 (d, $J = 9.0$ Hz, 2H), 7.04-6.97 (m, 6H), 6.90 (d, $J = 2.2$ Hz, 1H), 6.84 (d, $J = 8.4$ Hz, 1H, Ar-H), 6.63 (dd, $J = 8.2$, 2.0 Hz, 1H), 5.45 (dd, $J = 11.6$, 5.3 Hz, 1H), 3.11 (dd, $J = 17.2$, 2.9 Hz, 1H) (One of the proton of the pyrazole ring and methoxy peaks under the solvent peak).^{13}C NMR (DMSO-d6) δ (ppm): 160.9, 150.4, 149.7, 148.7, 146.9, 134.7, 133.1, 128.4, 127.7, 125.0, 118.0, 114.9, 112.7, 112.5, 110.3, 62.8, 56.1, 56.0, 55.9, 43.9. HRMS (ESI-MS) Calc. for $C_{24}H_{26}N_3O_5S$ $[M + H]^+$ 468.1593; found 468.1599
4	4-(3-(4-methoxyphenyl)-5-(2,3,4-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide. ^{1}H NMR (DMSO-d_6, δ ppm): 7.71 (d, $J = 8.8$ Hz, 2H), 7.56 (d, $J = 9.1$ Hz, 2H), 6.99-6.97 (m, 6H), 6.69-6.65 (m, 2H), 5.57 (dd, $J = 12.1$, 5.1 Hz, 1H), 3.86 (s, 3H, -OCH3), 3.78 (s, 3H, -OCH3), 3.75 (s, 3H, -OCH3), 3.71 (s, 3H, -OCH3), 3.09 (dd, $J = 17.6$, 5.1 Hz, 1H) (One of the proton peak of the pyrazole ring under the methoxy peak). ^{13}C NMR (DMSO-d_6, δ ppm): 160.9, 153.7, 151.2, 150.7, 146.6, 142.6, 133.1, 128.3, 127.9, 127.1, 125.2, 121.4, 114.8, 112.2, 108.6, 61.5, 61.0, 58.3, 56.4, 55.9, 43.1. HRMS (ESI-MS) Calc. for $C_{25}H_{28}N_3O_6S$ $[M + H]^+$ 498.1699; found 498.1691
5	4-(3-(4-methoxyphenyl)-5-(2,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide. ^{1}H-NMR (DMSO-d_6, δ ppm): 8.24 (s, 1H), 7.69 (d, $J = 8.4$ Hz, 2H), 7.54 (d, $J = 8.8$ Hz, 2H), 6.98-6.95 (m, 4H), 6.72 (s, 1H), 6.49 (brs, 2H), 5.55 (dd, $J = 12.1$, 5.6 Hz, 1H), 3.81 (s, 3H, -OCH3), 3.76 (s, 3H, -OCH3), 3.74 (s, 3H, -OCH3), 3.63 (s, 3H, -OCH3), 3.05 (dd, $J = 17.4$, 5.6 Hz, 1H) (One of the proton peak of the pyrazole ring). ^{13}C NMR (DMSO-d_6, δ ppm): 160.9, 151.4, 150.8, 149.9, 146.9, 143.4, 132.9, 128.3, 127.8, 125.1, 120.4, 114.9, 112.2, 111.9, 99.3, 79.7, 57.2, 57.1, 56.4, 55.9, 42.7. HRMS (ESI-MS) Calc. for $C_{25}H_{28}N_3O_6S$ $[M + H]^+$ 498.1699; found 498.1701
6	4-(3-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide. ^{1}H NMR (DMSO-d_6, δ ppm): 8.2 (s, 1H), 7.70 (d, $J = 8.8$ Hz, 2H), 7.56 (d, $J = 9.1$ Hz, 2H), 7.04 (d, $J = 8.8$ Hz, 2H), 7.01 (brs, 2H), 6.98 (d, $J = 8.8$ Hz, 2H), 6.54 (s, 1H), 5.40 (dd, $J = 12.0$, 5.0 Hz, 1H), 3.77 (s, 3H, -OCH3), 3.65 (s, 3H, -OCH3), 3.10 (dd, $J = 17.6$, 5.0 Hz, 1H)(Other methoxy peaks under the solvent peak). ^{13}C NMR (DMSO-d_6, δ ppm): 160.9, 153.9, 150.6, 147.2, 138.3, 137.1, 133.3, 128.4, 127.8, 124.9, 114.9, 112.5, 103.4, 79.2, 63.5, 60.6, 56.5, 55.9, 43.9. HRMS (ESI-MS) Calc. for $C_{25}H_{28}N_3O_6S$ $[M + H]^+$ 498.1699; found 498.1698



Reagents and conditions. i: EtOH, aq. NaOH (10%), rt. ii: 4-Hydrazinobenzensulfonamide.HCl, EtOH, glacial CH_3COOH, reflux. **1**: ($R_1 = R_4 = OCH_3$, $R_2 = R_3 = H$), **2**: ($R_1 = R_3 = OCH_3$, $R_2 = R_4 = H$), **3**: ($R_2 = R_3 = OCH_3$, $R_1 = R_4 = H$), **4**: ($R_1 = R_2 = R_3 = OCH_3$, $R_4 = H$), **5**: ($R_1 = R_3 = R_4 = OCH_3$, $R_2 = H$), **6**: ($R_2 = R_3 = R_4 = OCH_3$, $R_1 = H$)

Scheme 1. Synthetic pathway of the pyrazoline bearing benzenesulfonamides **1–6**. Reagents and conditions. i: EtOH, aq. NaOH (10%), rt. ii: 4-Hydrazinobenzensulfonamide.HCl, EtOH, glacial CH_3COOH, reflux. **1**: ($R_1 = R_4 = OCH_3$, $R_2 = R_3 = H$), **2**: ($R_1 = R_3 = OCH_3$, $R_2 = R_4 = H$), **3**: ($R_2 = R_3 = OCH_3$, $R_1 = R_4 = H$), **4**: ($R_1 = R_2 = R_3 = OCH_3$, $R_4 = H$), **5**: ($R_1 = R_3 = R_4 = OCH_3$, $R_2 = H$), **6**: ($R_2 = R_3 = R_4 = OCH_3$, $R_1 = H$).

NH_2 peaks in the regions 3371–3226 cm^{-1}. FT-IR spectra of the compounds are provided in the Supplementary file. The FT-IR values of the compounds were in accordance with literature values[23]. In the ^{1}H NMR and ^{13}C NMR spectra of pyrazolines, chemical shift values of the three hydrogen atoms attached to the C-4 and C-5 carbon atoms of the heterocyclic ring and C-3, C-4 and C-5 carbon atoms of the pyrazolines were observed at expected ppms (See Table 2 and Supplementary File).

Cytotoxic activities of the compounds were tested against four cancer cell lines (Ca9–22, HSC-2, HSC-3, HSC-4) and three non-tumor normal cells (HGF, HPLF, HPC). The cytotoxicities of the compounds were compared with reference drugs 5-Fluorouracil (5-FU) and Melphalan (Table 3). The compounds **2**, **4** (2.50 times), **5** (7.16 times) and **6** (5.51 times) towards Ca9–22 cell line; the compound **6** (2.22 times) towards HSC-2 cell line, the compounds **5** (2.35 times) and **6** (5.19 times) towards HSC-3 cell line, the compounds **5** (1.95 times) and **6** (1.77 times) towards HSC-4 cell line were more cytotoxic than 5-FU. On the other hand, the compounds **2** (1.83 times), **4** (1.83 times), **5** (5.23 times), and **6** (4.03 times) towards Ca9–22 cell line, the compound **6** (1.24 times) towards HSC-2 cell line were more cytotoxic than reference compound Melphalan.

Tumor specificity (TS) of the compounds was considered by TS values. TS values were calculated by two types of calculation. Tumor-specificity (TS) value reflects the selectivity of the compounds against cancer tissues rather than normal ones.

Table 3. Cytotoxicities of the synthesized compounds towards human oral squamous carcinoma cell lines and human normal oral cells.

	CC_{50} (μM)												
	Human oral squamous carcinoma cell lines						Human normal oral cells						
Compounds	Ca9-22 (A)	HSC-2	HSC-3	HSC-4	Mean (B)	SD	HGF (C)	HPLF	HPC	Mean (D)	SD	TS	
1	*Not Tested*											D/B	C/A
2	22	200	200	200	155.5	89.0	22	119	46	62.3	50.5	0.4	1.0
3	200	200	200	200	200.0	0.0	63	120	181	121.3	59.0	0.6	0.3
4	22	200	200	200	155.5	89.0	75	135	115	108.3	30.6	0.7	3.4
5	7.7	41	31	20	24.9	14.3	11	12	12	11.7	0.6	0.5	1.4
6	10	11	14	22	14.3	5.4	20	21	23	21.3	1.5	1.5	2.0
5-FU	55.1	24.4	72.7	38.9	47.8	–	>200	>200	>200	>200	–	>4.2	>3.6
Melphalan	40.3	13.6	11	10.3	18.80	–	>200	151.0	>175.5	175.2	–	>9.3	>4.9

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-specificity (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. SD: standard deviation, 5-FU: 5-fluorouracil, μM: micromolar.

Table 4. Carbonic anhydrase inhibitory activities of the compounds on hCA I and II isoenzymes.

	IC_{50} (nM)				K_I (nM)	
Compounds	hCA I	r^2	hCA II	r^2	hCA I	hCA II
1	30.1	0.99	24.8	0.96	26.5 ± 4.6	18.9 ± 9.0
2	40.8	0.98	30.1	0.98	39.7 ± 8.3	20.5 ± 3.7
3	46.2	0.92	26.7	0.97	55.5 ± 19.4	28.8 ± 6.5
4	38.5	0.98	23.8	0.98	31.4 ± 7.3	19.9 ± 2.7
5	49.2	0.93	25.7	0.98	29.9 ± 5.5	25.9 ± 7.0
6	36.5	0.94	23.9	0.98	34.1 ± 7.3	20.2 ± 3.0
AZA	293.0	0.98	146.5	0.98	276.3 ± 98.1	117.8 ± 14.1

Acetazolamide (AZA) was used as a standard inhibitor for all hCA I and II isoenzymes.

First TS calculation was made by dividing the mean CC_{50} value of each compound against three human oral normal cells (Column D) to the mean CC_{50} value against four human OSCC cell lines (Column B, Table 3). Second TS calculation was also calculated by dividing the CC_{50} value of each compound against HGF cells (Column C) to the CC_{50} value of each compound against Ca9–22 cell line (Column A, Table 3) since these cells are originated from the same tissue (gingiva). When TS values were considered, all the compounds had lower TS values than two reference compounds (5-FU and Melphalan) in both the calculations.

When the first TS calculation of values was considered the compound **6** had the highest TS value (1.5) while the second calculation of TS pointed out compounds **4** (TS = 3.4), **6** (TS = 2.0) and **5** (TS = 1.4) with higher TS values greater than 1. At the second TS calculation, compound **4** was the most tumor selective one. Tri-methoxy derivative compounds **4**, **5**, and **6** had superior tumor specificity than di-methoxy derivatives **2** and **3.** It seems that addition of one methoxy group made a valuable contribution to the cytotoxic activities of the compounds in compounds **4**, **5**, and **6**. This may result from increased possibility to form hydrogen bonding in trimethoxy derivatives.

IC_{50} (the drug concentration causing 50% inhibition of the desired activity) of the compounds on hCA I isoenzyme were in the range of 30.1–49.2 nM while acetazolamide (AZA) has IC_{50} value of 293.0 nM. It means that the compounds **1–6** [**1** (30.1), **2** (40.8), **3** (46.2), **4** (38.5), **5** (49.2), **6** (36.5)] were 5.9–9.7 times more potent inhibitor than AZA on hCA I. On the other hand, IC_{50} values of the compounds on hCA II were in the range of 23.8–30.1 nM while AZA had IC_{50} value of 146.5 nM. This means that the compounds **1–6** [**1** (24.7), **2** (30.1), **3** (26.7), **4** (23.8), **5** (25.7), **6** (23.9)] were 4.8–6.3 times more potent inhibitor than AZA on hCA II. When K_i values of the compounds were considered they were in the range of 26.5 ± 4.6–55.5 ± 19.4 nM towards hCA I, while they were in the range of 18.9 ± 9.0–28.8 ± 6.5 nM towards hCA II. K_i values of the reference compound AZA were 276.3 ± 98.1 nM and 117.8 ± 14.1 nM towards hCA I and hCA II, respectively.

Introduction of a methoxy group to 4 positions of 2,5-dimethoxy derivative **1** decreased both hCA I and II inhibition in compound **5**, while introduction of a methoxy group to 3 positions of 2,4-dimethoxy derivative **2** decreased or had similar inhibitory effect on hCA I and II in compound **4** according to K_i values. On the other hand, introduction to a methoxy group to 5 positions of compounds **2** and **3** increased hCA I inhibitory effect in both cases in compounds **5** and **6**, while it decreased the hCA II inhibitory effects in compounds **5** and **6** compared with compounds **2** and **3**.

Conclusion

In conclusion; although tumor selectivity of the compounds were less than reference compounds 5-FU and Melphalan, trimethoxy derivatives compounds **4**, **5**, and **6** were more selective than dimethoxy derivatives **2** and **3** as judged by the cytotoxicity assay with the cells both cell types originated from the gingival tissue. Compound **6** (4-[3–(4-methoxyphenyl)-5–(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl] benzenesulfonamide) having the highest TS values in both calculations can be considered as the leading molecule of the series for further investigations in terms of cytotoxicity. Since all the compounds had shown superior inhibitory activity than reference compound AZA on hCA I and II isoenzymes, the compounds synthesized can be

candidate compounds for further testing on other CA isoenzymes and new synthetic designs.

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Declaration of interest

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Supplementary material available online