Synthesis and biological evaluation of new pyrazolebenzene-sulphonamides as potential anticancer agents and hCA I and II inhibitors

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Abstract: Cancer is a disease characterized by the continuous growth of cells without adherence to the rules that healthy normal cells obey. Carbonic anhydrase I and II (CA I and CA II) inhibitors are used for the treatment of some diseases. The available drugs in the market have limitations or side effects, which bring about the need to develop new drug candidate compound(s) to overcome the problems at issue. In this study, new pyrazole-sulphonamide hybrid compounds 4-[5-(1,3-benzodioxol-5-yl)-3-aryl-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamides (4a - 4j) were designed to discover new drug candidate compounds. The compounds 4a - 4j were synthesized and their chemical structures were confirmed using spectral techniques. The hypothesis tested was whether an introduction of methoxy and polymethoxy group(s) lead to an increased potency selectivity expression (PSE) value of the compound, which reflects cytotoxicity and selectivity of the compounds. The cytotoxicity of the compounds towards tumor cell lines were in the range of 6.7 – 400 µM. The compounds 4i (PSE₂ = 461.5) and 4g (PSE₁ = 193.2) had the highest PSE values in cytotoxicity assays. Ki values of the compounds were in the range of 59.8 ± 3.0 - 12.7 ± 1.7 nM towards hCA I and in the range of 24.1 ± 7.1 - 6.9 ± 1.5 nM towards hCA II. While the compounds 4b, 4f, 4g, and 4i showed promising cytotoxic effects, the compounds 4c and 4g had the inhibitory potency towards hCA I and hCA II, respectively. These compounds can be considered as lead compounds for further research.

Key words: Sulphonamide, pyrazoline, chalcone, cytotoxicity, OSCC, carbonic anhydrase

1. Introduction
Cancer is a disease characterized by the continuous growth of cells without obeying the rules that normal healthy cells do. It is second amongst the reasons for death after cardiovascular diseases [1 – 4]. Based on the World Health Organization (WHO) report in 2018, 18.1 million people around the world had cancer, and 9.6 million died from the disease. It will reach 29.4 million in 2040. Although several therapeutic approaches are available, such as chemotherapy, which includes drug therapy and has great importance. The development of new chemotherapeutics is needed since the available drugs in the market have numerous side effects, resistance development to itself, or selectivity problems [1, 3, 5].

Oral cancer is ranked as the sixth most common malignancy worldwide [6 – 8]. The main carcinogens for oral squamous cell carcinoma (OSCC) are cigarette and alcohol products [9, 10]. Understanding the molecular mechanisms of tumorigenesis and metastasis process for OSCC can lead researchers to discover new chemotherapeutic strategies and improve the treatment of oral cancer.

Although there are several types of anticancer therapeutic products, novel aryl sulphonamides have recently been reported to have anticancer properties and can be used to treat different types of cancers. Among them, pazopanib, a tyrosine kinase inhibitor for renal cell carcinoma and soft tissue sarcoma, belinostat, a histone deacetylase inhibitor for peripheral T-cell lymphoma, and dabrafenib, a BRAF inhibitor for metastatic melanoma, have been approved for in the clinic treatment of patients [11] (Figure 1).

As another well-known pharmacophore in drugs or bioactive compounds are pyrazoline and its analogs. Their numerous bioactivities have been reported including their anticancer/cytotoxic activities [12 – 14].

Our research group reported anticancer activities of many pyrazoline-based sulphonamides against OSCC lines [12 – 14]. For instance, 4-[5-(2,3,4-trimethoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide

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compound (34) showed remarkable cytotoxicity with potency-selectivity expression (PSE) and tumor specificity (TS) (10.5 and 9.5) values towards OSCC lines [15]. Other studies also supported that pyrazoline-sulphonamides hybrid compounds are good candidates to develop new anticancer drugs [13 – 15]. Besides this, we reported a large library of methoxy substituted pyrazoline derivatives since this group attracted our attention with their cytotoxic properties against OSCC lines in our previous study [16]. Many other studies reporting on the valuable anticancer properties of several mono- or poly-methoxylated chemicals towards OSCC cell lines compared to substituents other than methoxy are available [16 – 18].

The hybrid approach is one of the strategies to obtain a compound or a drug with increased activity in medicinal chemistry for new drug development [19].

Of the pharmacophores used, sulphonamide has a very well-known carbonic anhydrase (CA) inhibitory effect. CA is an enzyme that catalyzes the reversible hydration/dehydration of CO₂/HCO₃⁻ [20, 21] and has various roles in physiological events such as carbon dioxide and bicarbonate transport processes, respiration, pH balancing, and CO₂ homeostasis [22, 23]. There are 16 isoforms (hCA I–XVI) that have different localizations [24, 25], of which CAs, CA I and CA II are the abundant forms. The hCA I isofrom is associated with retinal and cerebral edema, and the inhibition of CA I may help cure such conditions [22, 26 – 38]. The physiologically dominant isoform is hCA II, which is another enzyme that is associated with several diseases such as epilepsy, edema, glaucoma, and altitude sickness [22, 26 – 37]. Furthermore, it has also emerged in the past few years that these enzymes can be used as potential targets for designing antifibrotic drugs with a novel mechanism of action [39 – 41]. Of α-class carbonic anhydrases, CA IX and CA XII are the ones that are related to tumors. In cancer cases, CA IX levels especially increase.

In this study, the first aim was to synthesize pyrazolebenzene-sulphonamide hybrid compounds bearing mono- or polymethoxy (di/tri) group(s). The chemical structure of the compounds is 4-[5-(1,3-benzodioxol-5-yl)-3-aryl-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4a - j, Figure 2). Secondly, the compounds (4a - j) were tested on oral squamous cell carcinoma (OSCC) and normal oral cells to find new anticancer drug candidate compounds. As a final step, it was planned to investigate the CA inhibitory effect of the compounds on hCA I and hCA II. Since CA I and II are the widely available forms of CAs, we had the opportunity facility to study them as sulphonamides are very well-known inhibitors of CAs. If impressive results are obtained on hCA I/II, inhibition tests towards cancer-related CA IX and CA XII isoenzymes can be considered in future studies.

2. Materials and methods

2.1. Chemistry

The chemical structures of the final compounds 4a – j were confirmed by nuclear magnetic resonance (NMR) spectra; ¹H NMR (400MHz), ¹³C NMR (100 MHz) (Varian Mercury Plus spectrometer, Varian Inc., Palo Alto, CA, U.S.) and mass spectra (HRMS) (Shimadzu Corporation, Kyoto, Japan). Chemical shifts (δ) are reported in ppm and coupling constants (J) are expressed in hertz (Hz). Mass spectra (HMRS) for the compounds were taken using a liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu Corporation) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization modes. Shimadzu's LCMS Solution software was used for data analysis. Melting points were determined using an Electrothermal 9100/IA9100 instrument (Bibby Scientific Limited, Staffordshire, UK), which is uncorrected. Reactions were monitored by thin layer chromatography (TLC) using silica gel 60 HF254 (Merck KGaA). A solvent mixture of chloroform: methanol (4:8:0.2) was used as a thin-layer chromatography (TLC) solvent system. DMSO-d₆ (Merck) was used as a NMR solvents.

**Synthesis of chalcone compounds 3a - j, 3-(Benzo[d][1,3]dioxol-5-yl)-1-arylprop-2-en-1-one (Figure 2)**

The title compounds were synthesized by Claisen – Schmidt condensation following to the procedure reported [25, 42] in the literature. All of the intermediate compounds (3a – j) were recorded in the literature [43 – 49]. Briefly, a mixture of suitable acetophenone (1 mmol) and benzo[d][1,3]dioxole-5-carbaldehyde (1 mmol) was dissolved in ethanol (5 mL). An aqueous sodium hydroxide solution (30%, 10 mL) was added into the mixture under cold conditions (0 – 5 °C). After stirring overnight at room temperature, the reaction mixture was poured into an ice-water mixture and acidified with an HCl solution (10%) to pH = 6 – 7 (Figure 2). The compounds were used as a starting materials without further purification for the synthesis of pyrazoline derivatives.

**2.1.1. General procedure for the synthesis of pyrazolines (Figure 2, 4a - j)**

A suitable chalcone (1.00 mmol) and 4-hydrazinobenzenesulphonamide hydrochloride (1.10 mmol) were dissolved in ethanol (50 mL) and then a catalytic amount of glacial acetic acid was added. The mixture was refluxed for 18 – 24 h [12, 50, 51]. Reactions were followed by thin layer chromatography (TLC). After the reaction was stopped, some of the solvent was removed under a vacuum. The obtained solid was filtered, dried at room temperature, and crystallized from methanol-diisopropylether. The compounds, 4-[5-(1,3-benzodioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-
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1-yl]benzenesulphonamide (4a), 4-[5-(1,3-benzodioxol-5-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4b) and 4-[5-(1,3-benzodioxol-5-yl)-3-(3-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4c) were reported previously [45, 52, 53]. The compound’s chemical structures were confirmed with $^1$H NMR, $^{13}$C NMR, and HRMS in this study.

4-[5-(1,3-Benzodioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4a)

Isolated as a dark yellow solid, (yield: 30%) : mp : 196 – 198 °C (212 – 214 ºC, [45]). $^1$H NMR (400 MHz, DMSO - $d_6$) δ, ppm (J, Hz) : 7.66 (d, 1H, J = 5.0 Hz, Ar-), 7.60 (d, 2H, J = 8.8 Hz, Ar-), 7.33 (s, 1H, Ar-), 7.13 (d, 1H, J = 8.6 Hz, Ar-), 7.05 – 7.01 (m, 6H, Ar-), 6.87 (d, 1H, J = 7.9 Hz, Ar-), 6.76 – 6.72 (m, 2H, Ar-), 5.98 (s, 2H, methylene, piperonal ring), 5.56 (dd, 1H, J = 5.0, 11.9 Hz, pyrazoline ring) 3.93 (dd, 1H, J = 11.9, 17.5 Hz, pyrazoline ring), 3.19 (dd, 1H, J = 5.1, 17.4 Hz, pyrazoline ring); $^{13}$C NMR (100 MHz, DMSO - $d_6$) δ, ppm : 147.8, 146.6, 145.9, 145.6, 135.1, 134.9, 133.0, 128.5, 128.3, 127.9, 127.1, 118.9, 111.9, 108.6, 105.9, 101.1, 62.1, 43.6. HRMS, found, m / z: 421.1096 [M+4H]$^+$; C$_{22}$H$_{19}$N$_3$O$_4$S. Calculated, m / z: 425.9793.
4-[5-(1,3-Benzodioxol-5-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4b)

Isolated as a light yellow solid (yield: 38%): mp: 171 – 173 °C; 1H NMR (400 MHz, DMSO - d6) δ ppm (J, Hz): 7.73 (d, 2H, J = 8.7 Hz, Ar-), 7.59 (d, 2H, J = 8.7 Hz, Ar-), 7.06 (d, 2H, J = 8.8 Hz, Ar-), 7.01 (d, 2H, J = 8.7 Hz, Ar-), 6.86 (d, 2H, J = 7.9 Hz, Ar-), 6.75 – 6.72 (m, 2H, Ar-), 5.97 (s, 2H, methylene, piperonal ring), 5.51 (dd, 1H, J = 4.9, 11.8 Hz, pyrazoline ring), 3.88 (dd, 1H, J = 11.9, 17.6 Hz, pyrazoline ring), 3.80 (s, 3H, OCH3), 3.15 (dd, 1H, J = 5.0, 17.6 Hz, pyrazoline ring); 13C NMR (100 MHz, DMSO - d6) δ ppm: 160.7, 150.2, 148.2, 147.1, 146.5, 136.1, 133.1, 128.2, 127.6, 124.8, 119.5, 114.7, 112.3, 109.1, 106.5, 101.6, 62.4, 55.8, 43.6. HRMS, found, m / z: 452.1260 [M+H]+; C23H22N2O5S. Calculated, m / z: 452.1275.

4-[5-(1,3-Benzodioxol-5-yl)-3-(3-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4c)

Isolated as a cream color solid. (yield: 46%): mp: 184 – 186 °C (219 – 221 °C, [45]). 1H NMR (400 MHz, DMSO - d6) δ ppm (J, Hz): 7.61 (d, 2H, J = 8.8 Hz, Ar-), 7.36 (d, 2H, J = 4.9 Hz, Ar-), 7.31 (s, 1H, Ar-), 7.10 (d, 2H, J = 8.9 Hz, Ar-), 7.05 (s, 2H, SO2N), 7.00 – 6.97 (m, 1H, Ar-), 6.87 (d, 1H, J = 7.8 Hz, Ar-), 6.76 – 6.73 (m, 2H, Ar-), 5.97 (s, 2H, methylene, piperonal ring), 5.56 (dd, 1H, J = 5.0, 11.9 Hz, pyrazoline ring), 3.88 (dd, 1H, J = 11.9, 17.7 Hz, pyrazoline ring), 3.82 (s, 3H, OCH3), 3.15 (dd, 1H, J = 5.1, 17.7 Hz, pyrazoline ring); 13C NMR (100 MHz, DMSO - d6) δ ppm: 159.9, 150.1, 148.2, 147.1, 146.3, 135.9, 133.6, 130.3, 127.6, 119.5, 119.1, 115.7, 113.9, 112.6, 111.4, 109.1, 106.4, 101.6, 62.7, 55.7, 43.4; HRMS, found, m / z: 452.1262 [M+H]+. C23H22N2O5S. Calculated, m / z: 452.1275.

4-[5-(1,3-Benzodioxol-5-yl)-3-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4d)

Isolated as a cream color solid. (yield: 35%): mp: 183 – 185 °C; 1H NMR (400 MHz, DMSO - d6) δ ppm (J, Hz): 7.93 (d, 2H, J = 7.7 Hz, Ar-), 7.60 (d, 2H, J = 8.8 Hz, Ar-), 7.42 – 7.38 (m, 1H, Ar-), 7.1 – 7.01 (m, 5H, Ar-, SO2N), 6.86 (d, 1H, J = 7.8 Hz, Ar-), 6.76 – 6.73 (m, 2H, Ar-), 5.97 (s, 2H, methylene, piperonal ring), 5.56 (dd, 1H, J = 4.8, 11.8 Hz, pyrazoline ring), 3.98 (dd, 1H, J = 12.0, 18.3 Hz, pyrazoline ring), 3.80 (s, 3H, OCH3), 3.22 (dd, 1H, J = 5.1, 18.2 Hz, pyrazoline ring); 13C NMR (100 MHz, DMSO - d6) δ ppm: 157.9, 149.4, 148.2, 147.0, 147.6, 145.3, 133.1, 133.3, 131.3, 128.8, 127.6, 121.2, 119.4, 113.9, 112.8, 112.4, 109.1, 106.4, 101.6, 62.5, 56.1, 46.8. HRMS, found, m / z: 452.1267 [M+H]+. C23H22N2O5S. Calculated, m / z: 452.1275.

4-[5-(1,3-Benzodioxol-5-yl)-3-(3,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4e)

Isolated as a cream color solid (yield: 72%): mp: 243 – 245 °C; 1H NMR (400 MHz, DMSO - d6) δ ppm (J, Hz): 7.59 (d, 2H, J = 8.8 Hz, Ar-), 7.42 (s, 1H, Ar-), 7.25 (d, 1H, J = 8.4 Hz, Ar-), 7.08 (d, 2H, J = 8.8 Hz, Ar-), 7.03 (s, 2H, SO2N), 7.00 (d, 1H, J = 8.5 Hz, Ar-), 6.87 (d, 1H, J = 7.9 Hz, Ar-), 6.76 – 6.73 (m, 2H, Ar-), 5.97 (s, 2H, methylene, piperonal ring), 5.52 (dd, 1H, J = 4.8, 11.8 Hz, pyrazoline ring), 3.88 (dd, 1H, J = 11.9, 17.6 Hz, pyrazoline ring), 3.85 (s, 3H, OCH3), 3.80 (s, 3H, OCH3), 3.17 (dd, 1H, J = 5.0, 17.6 Hz, pyrazoline ring); 13C NMR (100 MHz, DMSO - d6) δ ppm: 150.1, 149.9, 148.8, 147.7, 146.6, 145.9, 135.6, 132.6, 127.1, 124.4, 119.6, 118.9, 111.8, 111, 5, 108.7, 108.6, 105.9, 109.1, 101.6, 61.9, 55.3, 55.5, 43.1. HRMS, found, m / z: 482.1358 [M+H]+. C24H24N2O6S. Calculated, m / z: 482.1380.
ring was under DMSO solvent peak); $^{13}$C NMR (100 MHz, DMSO - $d_6$) δ ppm: 153.3, 151.8, 149.3, 148.2, 147.0, 146.5, 143.4, 136.3, 132.9, 127.5, 119.4, 112.3, 112.2, 111.5, 109.1, 106.4, 101.6, 99.0, 62.4, 57.0, 56.7, 56.2, 46.8. HRMS, found, $m/z$: 512.1486 [M + H]$^+$. C$_{27}$H$_{25}$N$_5$O$_6$S. Calculated, m/z: 512.1478.

4-[5-(1,3-Benzodioxol-5-yl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4i)

Isolated as a cream color solid. (yield: 54%): mp: 212 – 214 °C; $^1$H NMR (400 MHz, DMSO - $d_6$) δ ppm (J, Hz): 7.59 (d, 2H, $J = 8.9$ Hz, Ar-), 7.11 – 7.04 (m, 6H, Ar-), 6.87 (d, 1H, $J = 7.8$ Hz, Ar-), 6.75 – 6.72 (m, 2H, Ar-), 5.98 (s, 2H, methylene, piperonal ring), 5.57 (dd, 1H, $J = 4.7, 11.9$ Hz, pyrazoline ring), 3.90 (dd, 1H, $J = 12.0, 17.6$ Hz, pyrazoline ring), 3.85 (s, 6H, OCH$_3$), 3.70 (s, 3H, OCH$_3$), 3.26 (dd, 1H, $J = 4.9, 17.8$ Hz, pyrazoline ring); $^{13}$C NMR (100 MHz, DMSO - $d_6$) δ ppm: 153.0, 149.8, 147.7, 146.6, 145.8, 138.7, 135.5, 132.9, 127.3, 127.1, 118.9, 111.9, 108.6, 105.9, 103.5, 101.0, 62.1, 60.1, 55.9, 43.1. HRMS, found, $m/z$: 512.1486 [M+H]$^+$. C$_{27}$H$_{25}$N$_5$O$_6$S. Calculated, m/z: 512.1480.

4-[5-(1,3-Benzodioxol-5-yl)-3-(4-hydroxy-3-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide (4j)

Isolated as a dark yellow solid. (yield: 54%): mp: 161 – 163 °C; $^1$H NMR (400 MHz, DMSO - $d_6$) δ ppm (J, Hz): 9.56 (bs, 1H, OH), 7.58 (d, 2H, $J = 8.8$ Hz, Ar-), 7.37 (s, 1H, Ar-), 7.16 (d, 2H, $J = 8.1$ Hz, Ar-), 7.06 – 7.03 (m, 4H, Ar-), 6.86 (d, 1H, $J = 7.8$ Hz, Ar-), 6.75 – 6.72 (m, 2H, Ar-), 5.98 (s, 2H, methylene, piperonal ring), 5.50 (dd, 1H, $J = 4.7, 11.7$ Hz, pyrazoline ring), 3.85 (s, 6H, OCH$_3$), 3.15 (dd, 1H, $J = 4.9, 17.6$ Hz, pyrazoline ring), (one of the proton peaks of the pyrazoline ring was under DMSO solvent peak); $^{13}$C NMR (100 MHz, DMSO - $d_6$) δ ppm: 150.7, 148.9, 148.23, 148.20, 153.3, 151.8, 149.3, 148.2, 147.0, 146.5, 145.8, 143.4, 136.3, 132.9, 127.6, 123.6, 120.4, 119.4, 115.9, 112.2, 110.0, 109.1, 106.4, 101.6, 62.3, 56.1, 43.7. HRMS, found, m/z: 468.1224 [M+H]$^+$. C$_{27}$H$_{23}$N$_5$O$_6$S. Calculated, m/z: 468.1205.

3. Results and discussion

3.1. Chemistry

The compounds (4a - j), 4-[5-(1,3-benzodioxol-5-yl)-3-aryl-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide, were synthesized successfully. The aryl part was changed as phenyl (4a), 4-methoxyphenyl (4b), 3-methoxyphenyl (4c), 2-methoxyphenyl (4d), 3,4-dimethoxyphenyl (4e), 2,5-dimethoxyphenyl (4f), 2,4-dimethoxyphenyl (4g),
2,4,5-trimethoxyphenyl (4h), 3,4,5-trimethoxyphenyl (4i), 3-methoxy-4-hydroxyphenyl (4j). The chemical structure of the final pyrazoline compounds were elucidated by $^1$H NMR, $^{13}$C NMR, and HRMS. The NMR data proved that the target pyrazoline ring was successfully synthesized under the reaction conditions applied. A peak belonging to the proton on the fifth position of the pyrazoline ring was seen in the range of 5.57 – 5.42 ppm as doublet (dd). Also, one of the protons on the fourth position of pyrazoline was seen in the range of 3.98 – 3.88 ppm while another proton was observed in the range of 3.22 – 3.15 ppm as dd. Two aliphatic carbons of pyrazoline (C-4 and C-5) also appeared in the range of 62.4 – 43.1 ppm in $^{13}$C NMR spectra as expected. HRMS results were found compatible with predicted and calculated values for the compounds.

3.2. Cytotoxicity

Cytotoxicity's of the compounds 4a - 4j were evaluated towards Ca9–22, HSC-2, HSC-3, and HSC-4 human oral squamous cell carcinoma cell lines as tumor cell lines and HGF, HPLF, and HPC human normal oral cells as nontumor cells (Table 1) according to the procedure in the literature [42, 54 – 56]. 5-Fluorouracil (5-FU) was used as a reference anticancer drug.

First, the question that whether the compounds have cytotoxic/anticancer properties should be answered. The cytotoxicities of the compounds towards tumor cell lines were in the range of 6.7 – 400 µM (Table 1). This shows that the compounds had anticancer properties. The compounds having more potent cytotoxicity than 5-FU and their times of potency (shown in the parenthesis) were as follows towards cell lines: Ca9–22 cell line: 4a (1.3), 4b (1.6), 4d (1.6), 4f (2.3), 4g (2.2), 4i (2.4), and 4j (1.3), and HSC-2 cell line: 4a (10), 4b (14.5), 4c (14.3), 4d (15.3), 4f (16.6), 4g (19.3), 4i (8.4), 4j (9.8). On the other hand, compounds did not show considerable cytotoxicity towards HSC-3 and HSC-4 cell lines, except compound 4i towards HSC-4.

As previously mentioned in the introduction, novel anticancer drugs that show less side effects and higher selectivity towards cancer cells urgently need to be developed [1, 3, 5]. Normal cells surround tumor cells in humans. Consequently, candidate compounds that are planned to be used in future clinical applications should show higher cytotoxicity against tumor cells rather than normal cells. The selectivity index (SI) value reflects this property. The SI values of the compounds are as follows towards cell lines: Ca9–22 cell line: 4a (1.3), 4b (1.6), 4d (1.6), 4f (2.3), 4g (2.2), 4i (2.4), and 4j (1.3), and HSC-2 cell line: 4a (10), 4b (14.5), 4c (14.3), 4d (15.3), 4f (16.6), 4g (19.3), 4i (8.4), 4j (9.8).

Table 1. Cytotoxicity results of pyrazoline derivatives (4a - 4j).

| Tumor cell lines CC$_{50}$ (µM) | Non-tumor cell lines CC$_{50}$ (µM) |
|---------------------------------|----------------------------------|
| Ca9–22                          | HSC-2                            |
| SI                              | SI                               |
| SI                              | SI                               |
| SI                              | SI                               |
| mean                            | SD                               |
| HGF                             | HPLF                             |
| HPC                             | mean                             |
| SD                              | D                                |
| D/B                             | C/A                              |
| (D/B)$^1$ ×100                  | (C/A)$^2$ ×100                   |
| 4a                              | 17.1                             | 3.1                            | 26.1                           | 2.0   | 20.8                           | 2.5   | 26.1                           | 2.2   | 22.5                           | 4.4   | 56.3                           | 37.6                           | 63.2                           | 52.4                           | 13.3                           | 2.3                             | 3.3                             | 10.3                           | 19.3                           |
| 4b                              | 14.0                             | 21.4                           | 18.0                           | 16.7                           | 16.2                           | 18.5                           | 18.2                           | 16.5                           | 16.6                           | 2.0                             | 400                            | 400                            | 98.7                           | 299.6                          | 174.0                          | 18                             | 28.6                           | 108.6                          | 205.1                          |
| 4c                              | 19.5                             | 6.1                            | 18.3                           | 6.5                            | 13.9                           | 8.6                            | 18.0                           | 6.6                            | 17.4                           | 2.4                             | 38.2                           | 178                            | 140                            | 118.7                          | 72.3                           | 6.8                             | 2.0                            | 39.1                           | 10.1                           |
| 4d                              | 13.8                             | 2.7                            | 17.1                           | 2.2                            | 12.4                           | 3.0                            | 14.9                           | 2.5                            | 14.6                           | 2.0                             | 36.9                           | 39.7                           | 37                             | 37.8                           | 1.6                             | 2.6                             | 2.7                            | 17.9                           | 19.5                           |
| 4e                              | 47.2                             | 8.5                            | 400                            | 1.0                            | 61.0                           | 6.6                            | 400                            | 1.0                            | 227                           | 199.8                          | 400                            | 400                            | 400                            | 400                            | 0.0                             | 1.8                             | 8.5                            | 0.8                            | 17.9                           |
| 4f                              | 9.7                              | 28.2                           | 15.7                           | 17.4                           | 11.5                           | 23.8                           | 12.0                           | 22.8                           | 22.2                           | 2.5                             | 400                            | 276.3                          | 147                            | 274.4                          | 126.5                          | 22.4                           | 41.2                           | 183.6                          | 425.1                          |
| 4g                              | 9.9                              | 25.8                           | 13.5                           | 18.9                           | 9.8                            | 26.0                           | 12.7                           | 20.1                           | 11.5                           | 1.9                             | 400                            | 234.3                          | 131                            | 255.1                          | 135.7                          | 22.2                           | 40.3                           | 193.2                          | 405.4                          |
| 4h                              | 400                              | 1.0                            | 400                            | 1.0                            | 21.3                           | 18.8                           | 21.8                           | 18.3                           | 211                           | 218.5                          | 400                            | 400                            | 400                            | 400                            | 0.0                             | 1.9                             | 1.0                            | 0.9                            | 0.3                            |
| 4i                              | 9.3                              | 30.6                           | 30.9                           | 9.2                            | 11.3                           | 25.2                           | 6.7                            | 42.5                           | 14.6                           | 11.0                           | 400                            | 83.7                           | 371.3                          | 285                            | 174.9                          | 19.6                           | 43                             | 134.6                          | 461.5                          |
| 4j                              | 17                               | 3.8                            | 26.7                           | 2.4                            | 26.3                           | 2.5                            | 16.8                           | 3.9                            | 21.7                           | 5.6                            | 76.7                           | 64.7                           | 54.3                           | 65.2                           | 11.2                           | 3.0                             | 4.5                            | 13.8                           | 26.4                           |
| 5-FU                            | 22                               | 44.8                           | 261                            | 3.8                            | 7.8                            | 126.4                          | 12.5                           | 78.9                           | 75.8                           | 123.6                          | 1000                           | 1000                           | 958.3                          | 986.1                          | 24.1                           | 13.0                           | 45.4                           | 17.1                           | 206                           |

HGF (human gingival fibroblast), HPLF (human periodontal ligament fibroblast), HPC (human pulp cell), Ca9-22, HSC-2, HSC-3, and HSC-4 oral squamous cell carcinoma cell lines. CC$_{50}$ values refer to the concentrations of the compounds in micromolar (µM) which reduce the viable cell number by 50%. Tumor selectivity (TS) was calculated for the compounds by dividing the average CC$_{50}$ value towards normal cells into the average CC$_{50}$ value towards cancer cell lines (D/B), tumor selectivity (TS) was calculated for the compound by dividing the average CC$_{50}$ value towards HGF cells into the CC$_{50}$ value towards Ca9-22 cells (C/A). Selectivity index (SI) figures were generated which are quotients of the average CC$_{50}$ values of nonmalignant cells and CC$_{50}$ figure of a compound towards a specific cell line. A potency selectivity expression (PSE) values were calculated according to equation i.e. (D/B)$^1$ × 100 and (C/A)$^2$ × 100. CC$_{50}$ value was determined from the growth curves plotted at different concentrations of each compounds in triplicate wells. SFU: 5-fluorouracil (reference compound), SD: standard deviation.
were calculated towards a specific cell line as described before [55] and presented in Table 1. The SI figure being higher than 1 reflects the selectivity of the tested compound toward tumor cells, rather than a normal cell. In terms of SI figures, all compounds presented SI values of 2.6–30.6 towards Ca9–22 cell line (except 4h) while all compounds showed SI values of 2.0–18.9 towards HSC-2 cell line (except 4e, and 4h). On the other hand, all compounds showed SI values of 2.5–26.0 towards HSC-3 cell line while all compounds had SI values of 2.0–42.5 toward HSC-4 cell line (except 4e).

The tumor selectivity (TS₁ and TS₂) of each compound was calculated as described in previously reported literature procedures [55] and these figures are presented in Table 1. Based on the TS₁ values, 4f and 4g which have 2,5 and 2,4-dimethoxy substituents had the highest TS₁ values in a series with 22.4 and 22.2, respectively. On the other hand, the 3,4,5-trimethoxy substituted compound 4i (TS₁ = 19.6) had the second highest TS₁ value. Among mono-methoxy compounds, the para-methoxy compound 4b had a TS₁ value of 18 and was the third highest. As expected, they were in accordance with the literature findings [16 – 18]. The second calculation (TS₂) considered the differences of sensitivity between the malignant (Ca9–22) and non-malignant (HGF) cells derived from the same tissue (gingiva). According to TS₂ values obtained, 4i (3,4,5-trimethoxy) had the highest TS₂ value of 43. This result was followed by 4f (2,5-dimethoxy), 4g (2,4-dimethoxy), and 4b (4-methoxy).

As seen in both calculations poly-methoxylated compounds had higher TS value than mono derivatives, The selectivity order changed as tri > di > mono or di > tri > mono. These findings also supported in our previous reports [16 – 18], thus the methoxylated compounds can be considered for new anticancer drug designs.

The desired properties for a lead compound are being both markedly cytotoxic and selectively toxic for tumors. To identify lead compounds of the study PSE (PSE₁ and PSE₂) values were calculated as shown in Table 1 [55]. PSE₁ reflects general cytotoxicity and selectivity potency of the compound towards all cells used, whereas PSE₂ seems more specified since it was considered for the same origin cells. When the compounds tested towards OSCC lines were considered in terms of PSE₁ values of the compounds. The best poly-methoxylated compounds were 4i (with 3,4,5-trimethoxy, PSE₂ = 461.5) > 4f (with 2,5-dimethoxy, PSE₂ = 425.1) > 4g (with 2,4-dimethoxy, PSE₂ = 405.4) while the best mono-methoxylated derivative was 4b (with 4-methoxy, PSE₂ = 205). On the other hand, the non-substituted or non-methoxylated derivative 4a had a PSE₂ value of 19.3. The reference anticancer drug 5-FU had a PSE₂ value of 206, which is a similar value to 4-methoxy derivative 4b’s.

PSE₂ values of 4i, 4f, 4g were 2.2 (4i), 2.1 (4f) and 2.0 (4g) times more potent than the reference drug 5-FU. Mono- or poly-methoxylated increased the PSE₂ values in 4b (4-methoxy), 4f (2,5-dimethoxy), 4g (2,4-dimethoxy), 4i (3,4,5-trimethoxy) derivatives for 10.6, 22.0, 21.0 and 24.0 times, respectively, compared to the methoxylated compound 4a. Methoxylating did not change the PSE₂ value very much in 4d (2-methoxy), 4e (3,4-dimethoxy), 4j (4-hydroxy-3-methoxy) derivatives compared to the methylated compound 4a. Interestingly decreased PSE₂ values were observed in 4c (3-methoxy, at half the value) and 4h (2,4,5-trimethoxy, with a dramatic decrease which is 64.3 times.

Therefore, it can be said that except for 4h and 4c, mono- or poly-methoxylated mostly increased or did not change PSE₂ values of the compounds compared to non-methoxylated 4a. Even if small increases or decreases occurred with the nine methoxylated compounds, only three of them (33.3%) decreased PSE₂ value, while 6 of them (66.6%) increased the PSE₂ value. This suggests that methoxylation can be considered to be a useful modification to increase cytotoxicity and selectivity of compounds (PSE₂) in general. Among mono-methoxylated ones, the (4b, 4c, 4d), 4-methoxy derivative (4b) had the best PSE₂ value, of the dimethoxylated; 2,5-dimethoxylated (4f) and 2,4-dimethoxylated (4g) had the highest and best PSE₂ values. When PSE₂ value was considered, 2-methoxy (4d) compound had a similar PSE₂ value to non-methoxylated 4a. Adding a methoxy group to 4-position in addition to 2-position in 4g compound caused 20.8 fold increase in cytotoxicity and selectivity (PSE₂) compared to 4d (2-methoxy) and 2.0 fold increase compared to 4b (4-methoxy) derivatives. This means that the synergic effect was obtained in compound 4g when compared to 4d and 4b. Similarly, in compound 4f which is a 2,5-dimethoxy derivative, PSE₂ value increased by 21.8 times compared to 4d (2-methoxy) and 42.1 times compared to 4c (3-methoxy) derivatives. The data confirmed that, this is a synergic effect of polymethoxy derivatives when compared to its corresponding mono analogs.

The introduction of the third methoxy group into the structure in compound 4i (3,4,5-trimethoxy) increased the PSE₂ value, which reflects cytotoxicity and selectivity. Increases in PSE₂ were 2.3, 45.7, and 23.7 times in 4i compared to mono- methoxylated compounds 4b, 4c, and 4d. Increases were 1.1 and 1.12 times in 4i compared to dimethoxylated compounds 4f and 4g, respectively.

In addition, an introduction of an electron-donating hydroxy group which is a hydrogen bonding donor group to para positions of phenyl with a meta methoxy substituent in compound 4j, increased the PSE₂ value 2.6 times in 4j comparing to 4c (3-methoxy). This was also found to be a useful modification for the increase in PSE₂ value.

When the order of PSE values was considered, it was as follows 4g (2,4-dimethoxy) > 4f (2,5-dimethoxy) > 4i (3,4,5-trimethoxy) > 4b (4-methoxy), while it was 4i > 4f > 4g > 4b in PSE₂ calculation. Mono- or poly-methoxylation (di or
tri) increased the PSE\textsubscript{2} value of the compounds by 22.2%, while it increased the PSE\textsubscript{1} value by 77.7%.

In a previous study, 4-(5-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1\textit{H}-pyrazol-1-yl) benzenesulphonamide compounds' having free methoxy groups on its chemical structure were reported as cytotoxic against OSCC [13]. The compounds' CC\textsubscript{50} values were in the range of 22 – 200 \textmu M, while their TS values were 0.7 and 3.4. When we compared our compound 4b that has piperonal moiety, which is a cyclic form of 3,4-dimethoxy groups in the previous compound [13], it can be expressed here using a piperonal structure, therefore making it a useful modification since it increased the tumor specificity of compound 4b (TS: 18 and 28.6) by 8 – 25 times towards OSCC. These significant outcomes indicate that a piperonal moiety may be used as a favorable group for designing new bioactive compounds in future studies.

The methoxy group is an electron-donating group and can form hydrogen bonds with enzymes. This is an important in the bioactivity of many compounds and drugs. Increases in the bioactivity of the compounds may be attributed to; proper interaction of the compound with the active site of the enzyme, the stability of this complex formed, and the adequate concentration of the biomacromolecules of the compound at the active site of the compound, which depends on the pharmacokinetic properties of that compound. The other factor affecting the cytotoxic potency can be the type of cell line used and the mechanism of action of the compound for the activity at issue. Furthermore, decreases in cytotoxicity may be attributed to the low stability of the compounds, which affect the concentration of the compound at the active side, or improper position of the compound at the active side which limits proper interaction. Unchanged bioactivity can bring the mind ineffectiveness of some groups to realize the activity in question partition coefficient of the compounds can also direct compound travel and its effect. Additionally, the size of molecules can be considered as an affecting factor since the behavior of the molecule in cells can be affected differently.

3.3. Carbonic anhydrase I and II inhibition

hCA I and hCA II inhibition results of the compounds are presented in Table 2 as IC\textsubscript{50} (\textmu M) and K\textsubscript{i} (\textmu M). When CA inhibitory profiles of the compounds were investigated based on the IC\textsubscript{50} values, the compounds were effective at 6.6 - 30.1 \textmu M toward hCA I while they were effective at 9.2 - 20.0 \textmu M toward hCA II isoenzyme. The 3-methoxy-bearing compound 4c was the most effective inhibitor on hCA I and hCA II while phenyl-bearing compound 4a had the least effective toward hCA I in terms of IC\textsubscript{50} values. The reference compound, acetazolamide (AZA), had IC\textsubscript{50} values of 16.6 \textmu M and 8.4 \textmu M towards hCA I and II, respectively. The compounds 4b (1.5), 4c (2.5), 4d (1.3), 4e (1.6), 4f (1.2), 4g (2.5), 4h (1.2) times were more potent than AZA towards hCA I while all compounds had less inhibition potential than AZA towards hCA II in terms of IC\textsubscript{50}.

When the inhibition constants (K\textsubscript{i}) were considered, K\textsubscript{i} values of the compounds were in the range of 12.7 ± 1.7 \textmu M – 59.8 ± 3.0 \textmu M towards hCA I and in the range of 6.9 ± 1.5 \textmu M – 24.1 ± 7.1 \textmu M towards hCA II. The K\textsubscript{i} values of AZA towards hCA I and II isoenzymes.

| Compounds | IC\textsubscript{50} (\textmu M) | K\textsubscript{i} (\textmu M) |
|-----------|-----------------------------|-----------------------------|
|           | hCA I | r\textsuperscript{2} | hCA II | r\textsuperscript{2} | hCA I | hCA II |
| 4a        | 30.1  | 0.9863 | 10.7  | 0.9846 | 59.8 ± 3.0 | 8.9 ± 2.3 |
| 4b        | 11.3  | 0.9881 | 16.3  | 0.9733 | 31.9 ± 1.9 | 11.5 ± 4.6 |
| 4c        | 6.6   | 0.9483 | 9.2   | 0.9595 | 21.5 ± 1.5 | 6.9 ± 1.5 |
| 4d        | 12.8  | 0.9487 | 10.7  | 0.9509 | 28.5 ± 1.6 | 8.9 ± 1.9 |
| 4e        | 10.2  | 0.9576 | 15.2  | 0.9679 | 25.5 ± 1.6 | 9.3 ± 0.6 |
| 4f        | 14.0  | 0.9607 | 16.3  | 0.9732 | 27.8 ± 10.1 | 12.3 ± 2.8 |
| 4g        | 6.7   | 0.9734 | 11.3  | 0.9623 | 12.7 ± 1.7 | 7.8 ± 2.2 |
| 4h        | 13.8  | 0.9875 | 14.3  | 0.9506 | 49.6 ± 1.9 | 9.3 ± 1.6 |
| 4i        | 29.7  | 0.9743 | 12.5  | 0.9412 | 52.5 ± 1.8 | 24.1 ± 7.1 |
| 4j        | 17.9  | 0.9459 | 20.0  | 0.9386 | 29.8 ± 9.4 | 9.3 ± 1.9 |
| AZA*      | 16.58 | 0.9887 | 8.4   | 0.9825 | 30.2 ± 7.8 | 4.4 ± 0.6 |

*Acetazolamide (AZA) was used as a standard inhibitor for both hCA I and II isoenzymes.
hCA I and hCA II were 30.2 ± 7.8 µM and 4.4 ± 0.6 µM, respectively. When \( K_i \) values were considered, the compound 4g, which has a 2,4-dimethoxy substituent, towards hCA I and compound 4c, which has a 3-methoxy substituent, towards hCA II had the best inhibition potential. Differences in inhibition potentials of the compounds may result from differences in their chemical structures and also differences in their interactions with the active site(s) of enzymes.

In another study conducted by our research group, a series of poly-methoxylated pyrazoline benzene sulphonamides were synthesized and their inhibitory effects on CAs were investigated. All compounds presented superior CA inhibitory activity compared to the reference compound, acetazolamide, on CAs with inhibition constants in the range of 30.1 – 49.2 nM against hCA I and of 23.8 – 30.1 nM against hCA II in terms of IC_{50} values, respectively [13]. Based on the literature findings, to obtain more potent hCA I, II inhibitors, and cytotoxic compounds, pyrazoline type compounds can be derived with poly-methoxylated phenyl rings such as 2,3,4-trimethoxy, 2,4,6-trimethoxy, and 2,4-dimethoxy groups. Additionally, bioisosteric heterocyclic rings such as furan and thiophen can be used instead of phenyl rings. Furthermore, molecular docking studies can be carried out to identify molecular interactions in future research.

4. Conclusion
A new series of pyrazole-sulphonamides, [4-[5-(1,3-benzodioxol-5-yl)-3-aryl-4,5-dihydro-1H-pyrazol-1-yl]benzenesulphonamide] were synthesized and evaluated their cytotoxicities and carbonic anhydrase inhibitory potencies. The cytotoxicities of the compounds towards tumor cell lines were in the range of 6.7 – 400 µM. The compounds 4i (PSE\(_2\) = 461.5) and 4g (PSE\(_1\) = 193.2) had the highest PSE values in cytotoxicity assays. The use of methoxy substituents in different parts of the ring severely affected bioactivity. All compounds presented hCA I and hCA II inhibition potency. The compounds 4c (\( K_i = 6.9 \pm 1.5 \) µM, hCA II) and 4g (\( K_i = 12.7 \pm 1.7 \) µM, hCA I) had the lowest \( K_i \) values as the best CA inhibitors. The compounds that show impressive bioactivities on the targets can be considered as lead compounds for further studies.

Acknowledgments
The authors are thankful to Dr. C. Kazaz and Dr. B. Anıl (Atatürk University) for their contribution of NMR spectra.

Conflict of interest
The authors declare no conflict of interest.

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hCA için;
hCA II için;
Compound 4a

Chemical Formula: C_{22}H_{19}N_2O_4S
Exact Mass: 421,1096

$^1$H NMR of compound 4a

$^{13}$C NMR of compound 4a
HRMS of compound 4a

\[ \text{SI (+)} \]

\[ \text{SI (-)} \]
Compound 4b

Chemical Formula: C_{23}H_{23}N_{3}O_{5}S
Exact Mass: 451,1202

$^1$H NMR of compound 4b

$^{13}$C NMR of compound 4b
HRMS of compound 4b
Compound 4c

Chemical Formula: C$_{23}$H$_{21}$N$_3$O$_5$S
Exact Mass: 451.1202

$^1$H NMR of compound 4c

$^{13}$C NMR of compound 4c
HRMS of compound 4c
Compound 4d

Chemical Formula: C_{23}H_{21}N_{3}O_{5}S
Exact Mass: 451.1202

$^1$H NMR of compound 4d

$^{13}$C NMR of compound 4d
HRMS of compound 4d

![HRMS graph]

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C$_{23}$ H$_{21}$ N$_3$ O$_5$ S $[\text{M+H}]^+$ Predicted region for 452.1275 m/z

| Rank | Score | Formula (M) | Ion   | Mass m/z | Prec. m/z | DX (eV) | DX (ppm) | Int. | DDE |
|------|-------|-------------|-------|----------|-----------|---------|----------|------|-----|
| 1    | 58.93 | C$_{23}$ H$_{21}$ N$_3$ O$_5$ S | [M+H]$^+$ | 452.1265 | 452.1275 | 0.0     | -1.77    | 88.84| 15.0|
Compound 4e

Chemical Formula: C_{24}H_{23}N_{3}O_{6}S
Exact Mass: 481.1308

$^1$H NMR of compound 4e

$^{13}$C NMR of compound 4e
HRMS of compound 4e
Compound 4f

Chemical Formula: C_{24}H_{23}N_{3}O_{6}S
Exact Mass: 481.1308

$^1$H NMR of compound 4f

$^{13}$C NMR of compound 4f
HRMS of compound 4f
Compound 4g

Chemical Formula: C_{24}H_{32}N_{2}O_{5}S
Exact Mass: 481.1308

$^1$H NMR of compound 4g

$^{13}$C NMR of compound 4g
HRMS of compound 4g
Compound 4h

Chemical Formula: C_{29}H_{29}N_{3}O_{7}S
Exact Mass: 511.1413

$^1$H NMR of compound 4h

$^{13}$C NMR of compound 4h
HRMS of compound 4h
Compound 4i

Chemical Formula: C_{25}H_{25}N_{5}O_{7}S
Exact Mass: 511.1413

$^1$H NMR of compound 4i

$^{13}$C NMR of compound 4i
HRMS of compound 4i
**Compound 4j**

Chemical Formula: C$_{22}$H$_{17}$N$_3$O$_3$S  
Exact Mass: 467.1151

$^1$H NMR of compound 4j

$^{13}$C NMR of compound 4j
HRMS of compound 4j