**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-[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.

**Keywords**

Benzenesulfonamide; carbonic anhydrase; cytotoxicity; phenol; pyrazoline

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**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, chemotherapy is the most preferable method for inoperable cancers and medicinal chemists interested in chemotherapy 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.

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 XI 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, lipogenesis as well as in pathological disorders including edema, glaucoma, obesity and epilepsy.10 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 target11. 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 effects5,15,16. 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 II17,18.

The sulfonamides are an important drug class more than 70 years for their antibacterial, antiCA, diuretic, hypoglycemic, and anticancer activities19–27. In addition, sulfonamide derivatives E7010, ER-34410 and E7070 have recently been reported as potent antitumor agents and are in advanced clinical trials28. 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), ethozolamide (EZA), pazopanib etc. (Figure 1) are widely used as CAIs in clinical trials29–31.

Pyrazol(ine) derivatives were reported their wide range of bioactivities such as anticancer, antiinflammatory, antinfecive, carbonic anhydrase inibitory and analgesic activities32–38. Celecoxib, a clinically used nonsteroidal antiinflammatory drug that selectively inhibits COX-2, has sulfonamide and pyrazole scaffolds in its chemical structure39. On the other hand, it was reported that compounds containing phenol moiety had CA inhibitory effect40–45.

Our group recently focused on the synthesis of the compounds having both pyrazole-sulfonamide pharmacophores in a molecule to search for several bioactivities32,33,37. To further...
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 pyrazolines (Scheme 1, 1–9)**

A suitable chalcone (1.00 mmol) and 4-hydrazinobenzenesulfonylhydrochloride (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$^{32,33,37}$ for 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 chromatography (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-water (3:2) for 1–5, 7, 9; methanol-chloroform (1:3) for 6; methanol (3:2) for 8. Since hydrogens of SO$_2$NH$_2$ exchanged with deuterium of CD$_3$OD, sulfonamide hydrogens were not observed on $^1$H NMR spectra.

**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)$^{46}$ and then it was extracted with ether. 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%)].

**Scheme 1.** Synthesis of compounds 1–9.

**Reagents and conditions.** (i) 10% aq NaOH, EtOH, 0–5 °C, 12 h; (ii) 4-Hydrazinobenzenesulfonylhydrochloride, EtOH, glacial acetic acid, reflux 6–12 h. Ar: C$_6$H$_5$ for 1’, 1; 4-CH$_3$C$_6$H$_4$ for 2’, 2; 4-CH$_3$OC$_6$H$_4$ for 3’, 3; 4-CIC$_6$H$_4$ for 4’, 4; 2,4-(Cl)$_2$C$_6$H$_4$ for 5’, 5; 4-FC$_6$H$_4$ for 6’, 6; 4-BrC$_6$H$_4$ for 7’, 7; 4-NO$_2$C$_6$H$_4$ for 8’, 8; C$_6$H$_5$S(2-yl) for 9’, 9.

**Figure 1.** Chemical structures of some carbonic anhydrase inhibitors which are in clinical use.
(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, –CH3); 13C NMR (100 MHz, CD3OD, ppm) δ = 159.4, 150.3, 147.5, 139.6, 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 C23H22N3O3S[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: 92%. 1H NMR (400 MHz, CD3OD, ppm) δ = 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, –OCH3), 3.09 (dd, 1H, J = 12.1, 5.5 Hz); 13C NMR (100 MHz, CD3OD, ppm) δ = 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 C22H22N3O3S[M + H]+ 424.1313; found 424.1312.

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

M.p. 152–154 °C. Yield: 62%. 1H NMR (400 MHz, CD3OD, ppm) δ = 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 (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); 13C NMR (100 MHz, CD3OD, ppm) δ = 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 C22H19ClN3O3S[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%. 1H NMR (400 MHz, CD3OD, ppm) δ = 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 (d, 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); 13C NMR (100 MHz, CD3OD, ppm) δ = 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 C21H18Cl2N3O3S[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%. 1H NMR (400 MHz, CD3OD, ppm) δ = 7.63 (d, 4H, J = 8.8 Hz), 7.30–7.26 (m, 2H), 7.07–7.03 (m, 4H), 6.82 (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); 13C NMR (100 MHz, CD3OD, ppm) δ = 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 C21H16F3N3O3S[M + H]+ 412.1113; found 412.1115.

Carboxyl anhydrase enzyme assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO2 hydration reaction51. 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 Heps (pH
Table 1. Cytotoxic activity of compounds 1–9 against human oral malignant and nonmalignant cells.

| Compounds | HGF (C) | HPLF (D) | HPC (D) | SI | SD |
|-----------|---------|----------|---------|----|----|
| 1         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 2         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 3         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 4         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 5         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 6         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 7         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 8         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |
| 9         | 57.0    | 1.4      | 94.7    | 1.2 | 81.0 |

The results were shown in Table 1. The compounds which have SI values of 1 can be considered as tumor-specific antineoplastic agents. 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. The first calculation was made by dividing the average CC50 value towards normal cells into the average CC 50 value towards OSCC (TS = Column D/Column C, Table 1). The second calculation is the comparison of malignant towards 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. The first calculation was made by dividing the average CC50 value towards normal cells into the average CC 50 value 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. 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 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 CC50 value of the nonmalignant cells and the CC50 value of a compound towards a specific tumor derived from gingiva (Ca9–22) and nonmalignant (HGF) cells by MTT method. The results were shown in Table 1.

Results and discussion

The compounds were successfully synthesized and their chemical structures were elucidated by 1H NMR, 13C 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. The results were shown in Table 1. The first question to be answered is whether the compounds have antineoplastic property or not. CC50 (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-Fluorouracil (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 CC50 value of the nonmalignant cells and the CC50 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. 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. The first calculation was made by dividing the average CC50 value towards normal cells into the average CC 50 value towards a total of four cancer cell lines (TS = Column D/Column C, 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 CC50 value towards HGF cells by the CC50 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 CC50 values towards cancer cell lines and the average SI values towards these cell lines and expressed as a percentage.
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 = 76.6 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 oxygen 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 oxygen 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 inhibition profile on hCA IX and XII, with Ks 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.

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**Disclosure statement**

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

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