Synthesis, cytotoxicity and carbonic anhydrase inhibitory activities of new pyrazolines

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Synthesis, cytotoxicity and carbonic anhydrase inhibitory activities of new pyrazolines

Kaan Kucukoglu 1, Fatih Oral 1, Tefvik Aydin 1, Cem Yamali 1, Oztekin Algul 2, Hiroshi Sakagami 3, Ilhami Gulcin 4,5, Clauidi T. Supuran 6, and Halise Inci Gul 1

1Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey, 2Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mersin University, Mersin, Turkey, 3Division of Pharmacology, School of Dentistry, Meikai University School of Dentistry, Sakado, Saitama, Japan, 4Department of Chemistry, Faculty of Science, Ataturk University, Erzurum, Turkey, 5Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia, and 6Neurofarba Departemente Laboratorio di ChimicaBioinorganica, Universita degli Studi di Firenze, via U. Schiff 6, SestoFiorentino (Florence), Italy

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-Fluourouracil 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-1H-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.

Introduction
Cancer is one of the most important causes of deaths in the world 1. Various classes of drugs such as alkylating agents, antimitabolites, antibiotics, hormones and plant alkaloids are used in the traditional cancer chemotherapy 2. Although selectivity 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 CAIs 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 CAIs have become remarkable therapeutic targets for the treatment of a wide range of disorders. Several CA inhibitors show anticancer 7, diuretics 8, antiglaucoma 9, antinfective 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, antimarial, 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.
Experimental

Materials and methods

Chemical structure of the compounds were determined by 1H NMR (400 MHz) and 13C 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 Spectrum 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-hydrizinobenzensulfonamide 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 refluxed26. Reactions were monitored by TLC using CHCl3: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 chromatography27. 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 isoenzymes28. 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 al29. 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 method30. Bovine serum albumin was used as standard protein. The IC50 values were obtained from activity (%) versus compounds plots. For calculation of KI values, three different concentrations were used. The Lineweaver–Burk curves were drawn and calculations were realised31.

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 modifications32–37. 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 subtracted 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). CC50 (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 CC50 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, 1H NMR, 13C 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.

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

PHBS: 4-Hydrizinobenzensulfonamide hydrochloride.
The compounds were compared with reference drugs 5-Fluorouracil (5-FU) and Melphanal (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 Melphanal.

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>
<thead>
<tr>
<th>Compounds</th>
<th>1H NMR, 13C NMR, and HRMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-(5-(2,5-dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)benzenesulfonamide. 1H NMR (DMSO-d6, 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, OCH3), 3.82 (s, 3H, OCH3), 3.77 (s, 3H, OCH3), 3.74 (s, 3H, OCH3), 3.69 (s, 3H, OCH3). 13C NMR (DMSO-d6, δ ppm): J Enzyme Inhib Med Chem, 2016; 31(S4): 20–24.</td>
</tr>
</tbody>
</table>
First TS calculation was made by dividing the mean CC50 value of each compound against three human oral normal cells (Column D) to the mean CC50 value against four human OSCC cell lines (Column B, Table 3). Second TS calculation was also calculated by dividing the CC50 value of each compound against HGF cells (Column C) to the CC50 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 selectivity 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.

IC50 (the drug concentration causing 50% inhibition of the desired activity) of the compounds on hCA I isoenzyme were in the range of 23.8–30.1 nM while acetazolamide (AZA) has IC50 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. 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.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (nM)</th>
<th>K_i (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hCA I</td>
<td>hCA II</td>
</tr>
<tr>
<td></td>
<td>r^2</td>
<td>r^2</td>
</tr>
<tr>
<td>1</td>
<td>30.1</td>
<td>0.99</td>
</tr>
<tr>
<td>2</td>
<td>40.8</td>
<td>0.98</td>
</tr>
<tr>
<td>3</td>
<td>46.2</td>
<td>0.92</td>
</tr>
<tr>
<td>4</td>
<td>38.5</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>49.2</td>
<td>0.93</td>
</tr>
<tr>
<td>6</td>
<td>36.5</td>
<td>0.94</td>
</tr>
<tr>
<td>AZA</td>
<td>293.0</td>
<td>0.98</td>
</tr>
</tbody>
</table>

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

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-1H-pyrazol–yl]-1 benzene sulfonamide) 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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ORCID

Ilhami Gulcin http://orcid.org/0000-0001-5993-1668

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