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Carbonic anhydrase inhibitors. Inhibition of human tumor-associated isozymes IX and cytosolic isozymes I and II with some 1,3,4-oxadiazole-thiols

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Abstract

A series of chiral 1,3,4-oxadiazole-5-thiols incorporating 2-substituted-benzenesulfonamide moieties has been prepared from amino acids, via the ester and carbohydrazide intermediate, followed by cyclization with carbon disulfide. Some of these compounds have been investigated for the inhibition of three physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the human cytosolic hCA I and II, and the human, transmembrane, tumor-associated isozyme hCA IX. All these compounds showed weak (millimolar) affinity for the three isozymes, except two carbohydrazides and two heterocyclic thiols which selectively inhibited the tumor-associated isozyme with inhibition constants around 10 μM. Such compounds constitute interesting lead molecules for the possible design of CA IX-selective inhibitors.

Introduction

The α-carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in all life kingdoms [1–5]. In higher vertebrates, including humans, 16 isoforms have been described up to now, of which at least CA II, IV, VA, VB, VII, IX, XII, XIII and XIV constitute interesting targets for the development of novel antiglaucoma, antitumor, antiobesity or anticonvulsant drugs [6–13]. Indeed, by catalysing a simple but fundamental reaction, the reversible hydration of CO_2 to bicarbonate and a proton, these metalloenzymes are involved in a multitude of physiological and pathological processes, and their inhibition leads to responses that may be exploited therapeutically [1–5]. Three other genetically distinct classes of CAs have been described in various organisms, the β-CA - δ-CA families, proving that such a critical catalyst for life processes has been invented by nature at least in four different occasions [1–6]. Among them, very recently, representatives of the α- or β-CA class have been cloned and characterized in *Plasmodium falciparum* [14], *Mycobacterium tuberculosis* [15,16], *Cryptococcus neoformans* [17] or *Candida spp.* [18], some of them being also investigated from the inhibition aspect [14,17] as it has been proved that these enzymes are critical for the growth or virulence of the pathogens [14–18].

Tumor cells over-express at least two α-CA isoforms, the transmembrane CA IX and XII [1–5]. As tumors have a lower extracellular pH (pH_e) than normal cells, and this acidic medium contributes to tumor growth and metastasis through various mechanisms, it has been thought that cancer-associated CAs may play a role in the acidification processes [19]. Indeed, recently CO_2 (in addition to lactic acid) was demonstrated to be a significant

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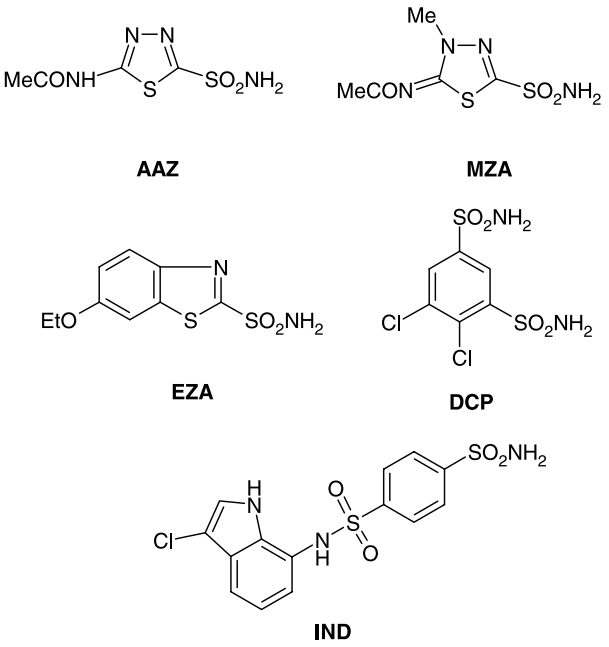
source of acidity in tumors [20], pointing to the involvement of CA IX and XII in tumor biology [19,20]. Noteworthy, CA IX-mediated acidification of tumor cells was reversed by sulfonamide CA inhibitors not only in transfected cells, but also in tumor cell lines that naturally express the CA IX protein [19,20]. This effect was observed only under hypoxia (the cellular stress triggering CA IX over-expression) [21] and not in normoxic conditions [19,20]. A potent fluorescent sulfonamide CA IX inhibitor was also shown to bind only to hypoxic cells expressing CA IX and not to their normoxic counterparts, or to control cells lacking CA IX [19,20]. Such experiments represented the proof-of-concept that inhibitors accumulate only in cells containing CA IX with an intact catalytic domain, and may thus become useful tools for reducing tumor acidosis or for imaging of hypoxic tumor cells [19,20]. CA IX inhibitors may thus prevent the decrease of pH_e observed in many tumors and might be used in combinations with other antitumor drugs to increase the efficacy or the uptake of weakly basic anticancer drugs [19,20]. Continuing our investigations in this field, the aim of the present study was the design, synthesis and *in vitro* evaluation of novel CA IX inhibitors, belonging to different classes of derivatives, other than the widely used sulfonamides and sulfamates [22–26].

Materials and methods

Chemistry

Melting points were determined on a Gallenkamp digital melting point apparatus and are uncorrected. Optical rotation data were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were recorded in KBr discs on a FT-IR model FTS 3000 MX spectrometer. Elemental analysis was performed on a Carlo Erba 1106 elemental analyzer. ^{1}H NMR (400 and 500 MHz) spectra were recorded on a Bruker NMR spectrophotometer. The chemical shifts of proton signals are in parts per million (ppm) downfield from tetramethylsilane (TMS) as an internal standard. EI-MS spectra were recorded on a MAT 312 and MAT 311A mass spectrometer. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} aluminum sheets (Merck). The purity of the synthesized compounds was established by TLC in two different solvent systems (ethyl acetate: petroleum ether 1:2 and acetone: petroleum ether 1:1). The clinically used sulfonamide CA inhibitors (CAIs) acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP** and indisulam **IND**, employed as standard inhibitors in the enzyme assays, were commercially available from Sigma-Aldrich or have been prepared as previously described [26]. Recombinant human CA isoforms hCA I, II and IX have been prepared as reported earlier by our group [28–31], and their activity measured by a stopped flow CO_2 hydration assay [32].



The investigated 1,3,4-oxadiazole-5-thiol derivatives **5a-o** were prepared from commercially available (from Sigma-Aldrich) amino acids **1a-e** whereas the corresponding esters **3a-o** were obtained by literature procedures [33–35]. Compound **2 g** (yield 72%, m.p. 149°C, lit. 149°C) was previously reported in the literature [36].

*General procedure for the synthesis of compounds **4a-o**.* A mixture of **3a** (10 mmole) and hydrazine hydrate (80%) in absolute ethanol (50 mL) was refluxed for 9 h. The excess solvent was distilled off and the residue was filtered, washed with water and recrystallized from 60% aqueous ethanol. Other hydrazides **4b-o** were prepared in a similar fashion. The spectral data for **4a-o** are given below.

*General procedure for the synthesis of compounds **5a-o**.* Compound **4a** (5.5 mmole) was dissolved in 80 ml absolute ethanol, and 6.6 mmole of carbon disulfide was added followed by 5.5 mmole of potassium hydroxide dissolved in 10 ml of water. The reaction mixture was stirred for 15 min and then refluxed for 16.5 h. The progress of the reaction was monitored by TLC. The excess ethanol was distilled off. The reaction mixture was diluted with water and acidified with 4N HCl to pH 2-3 (Congo red). The solid obtained was filtered, washed with water and recrystallized from 60% aqueous ethanol. Other compounds **5b-o** were synthesized by the same procedure. Micro analytical, optical rotation, IR, ^{1}H-NMR and mass spectral data of compounds **5a-o** are given below.

*General procedure for the synthesis of compounds **6a-b, 6k***. An amount of 0.75 mmole (250 mg) of **5a,** 0.22 mmole **(**0.3 ml) of Et_3N and a catalytic amount (25 mg) of 4-dimethylamino-pyridine (DMAP) were stirred in 25mL of dry CH_3Cl for 15 min. 0.8 mmole **(**0.1 mL) of benzyl bromide was added and the mixture was stirred for 5 h at 30– 70°C. The reaction mixture was washed with dilute HCl, brine, water and dried over anhydrous Na_2SO_4. The excess solvent was distilled off and the product was recrystallized from aqueous ethanol. The synthesized compounds **6a-b** and **6k** were characterized by IR, ^{1}H-NMR and MS data.

2b: Micro crystals (aq. ethanol) m.p. 135–137°C, Yield = 71%; IR (KBr)(ν, cm^{-1}) 3311– 2750(NH, OH), 1707(C = O), 1375 and 1157(SO_2).

2c: Micro crystals (aq. ethanol) m.p. 140–142°C, Yield = 72%, IR (KBr)(ν, cm^{-1}) 3289– 2680 (NH, OH), 1716(C = O), 1376 and 1167(SO_2).

2e: Micro crystals (aq. ethanol) m.p. 145–147°C, Yield = 77%, IR (KBr)(ν, cm^{-1}) 3307– 2731 (NH, OH), 1725(C = O), 1365 and 1159(SO_2).

2f: Crystalline (aq. ethanol) m.p. 139–142°C, Yield = 74%, IR (KBr)(ν, cm^{-1}) 3351– 2690 (NH, OH), 1733(C = O), 1375 and 1166(SO_2).

2j: Micro crystals (aq. ethanol) m.p. 135–137°C, Yield = 75%, IR (KBr)(ν, cm^{-1}) 3315– 2695(NH, OH), 1717(C = O), 1376 and 1165(SO_2).

2 l: Micro crystals (aq. ethanol) m.p. 133–135°C, Yield = 71%, IR (KBr)(ν, cm^{-1}) 3285– 2711(NH, OH), 1714(C = O), 1375 and 1166(SO_2).

3b: Needles (aq. ethanol) m.p. 47–48°C, Yield = 71%, IR (KBr)(ν, cm^{-1}) 3237(NH), 1729(C = O), 1375 and 1165(SO_2).

3c: Needles (aq. ethanol) m.p. 51–53°C, Yield = 67%, IR (KBr)(ν, cm^{-1}) 3255 (NH), 1717(C = O), 1376 and 1166(SO_2).

3e: Needles (aq. ethanol) m.p. 87–89°C, Yield = 81%, IR (KBr)(ν, cm^{-1}) 3256(NH), 1721(C = O), 1355 and 1159(SO_2).

3f: Micro crystals (aq. ethanol) m.p. 87–89°C, Yield = 67%, IR (KBr)(ν, cm^{-1}) 3277(NH), 1726(C = O), 1377 and 1167(SO_2).

3 g: Crystalline (aq. ethanol) m.p.79°C, Yield = 78%, IR (KBr)(ν, cm^{-1}) 3256 (NH), 1717 (C = O), 1355 and 1159 (SO_2).

3 h: Crystalline (aq. ethanol) m.p. 81°C, Yield = 62%, IR (KBr)(ν, cm^{-1}) 3309(NH), 1709(C = O), 1357 and 1171(SO_2).

3i: Crystalline (aq. ethanol) m.p. 89°C, Yield = 62%, IR (KBr)(ν, cm^{-1}) 3309(NH), 1709(C = O), 1357 and 1171(SO_2).

3j: Micro crystals (aq. ethanol) m.p. 77–78°C, Yield = 80%, IR (KBr)(ν, cm^{-1}) 3287(NH), 1717(C = O), 1372 and 1165(SO_2).

3 l: Micro crystals (aq. ethanol) m.p. 76–78°C, Yield = 71%, IR (KBr)(ν, cm^{-1}) 3285(NH), 1716(C = O), 1372 and 1166(SO_2).

4b: Crystalline (aq. ethanol) m.p. 91–93°C, Yield = 75%, IR (KBr)(ν, cm^{-1}) 3377, 3285(NH), 1671(C = O), 1376 and 1155(SO_2). ^{1}H NMR (acetone-d_6) δ 1.66–1.75(m, 2H, CH_2), 2.55(t, 2H, CH_2, J = 7.4 Hz), 2.81(t, 2H, CH_2, J = 6.9 Hz), 7.45(d, 2H, ArH J = 8.0 Hz**)**, 7.89(d, 2H, ArH J = 8.0 Hz), 9.61(bs, 2H, NH), 11.29(bs, 1H, NH). EI-MS (%) 291 (M^+, 19), 175(100), 111(87), 76(9), 57(11).

4c: Crystalline (aq. ethanol) m.p. 110–112°C, Yield = 62%, IR (KBr)(ν, cm^{-1}) 3332, 3260(NH), 1656(C = O), 1372 and 1161(SO_2). ^{1}H NMR (acetone-d_6) δ 1.69–1.78(m, 2H, CH_2), 2.78(t, 2H, CH_2, J = 6.9 Hz), 2.84(t, 2H, CH_2, J = 7.8 Hz), 3.89 (s, 3H, OCH_3), 7.25(d, 2H, ArH J = 8.1 Hz**)**, 7.59(d, 2H, ArH J = 8.2 Hz), 9.57(bs, 2H, NH), 10.75(bs, 1H, NH). EI-MS (%) 287 (M^+, 11), 223(10), 185(100), 107(57), 76(8), 57(23).

4e: Crystalline (aq. ethanol) m.p. 161–163°C, Yield = 85%, IR (KBr)(ν, cm^{-1}) 3319, 3292(NH), 1653(C = O), 1365 and 1149(SO_2). ^{1}H NMR (acetone-d_6) δ 0.98(dd, 3H, CH_3, J = 7.2 Hz, J = 7.2 Hz), 1.82–1.92(m, 2H, CH_2), 4.38(t, 1H, CH, J = 7.5 Hz), 7.65(d, 2H, ArH J = 8.0 Hz**)**, 8.01(d, 2H, ArH J = 8.0 Hz), 9.72(bs, 2H, NH), 11.25(bs, 1H, NH). EI-MS (%) 291 (M^+, 14), 175(25), 111(100), 76(19), 57(9).

4f: Crystalline (aq. ethanol) m.p. 165–167°C, Yield = 65%, IR (KBr)(ν, cm^{-1}) 3297, 3242(NH), 1671(C = O), 1375 and 1166(SO_2). ^{1}H NMR (Acetone-d_6) δ 0.89(t, 3H, CH_3, J = 7.4 Hz), 1.84–1.89(m, 2H, CH_2), 3.91(s, 3H, OCH_3), 4.37(t, 3H, CH_3, J = 7.4 Hz), 7.12(d, 2H, ArH J = 8.2 Hz**)**, 7.72(d, 2H, ArH J = 8.2 Hz), 9.75(bs, 2H, NH), 10.75(bs, 1H, NH). EI-MS (%) 287 (M^+, 100), 230(7), 211(11), 185(9), 107(21), 76(5), 57(9).

4 g: Powder (aq. ethanol) m.p. 171–172°C, Yield = 77%, IR (KBr)(ν, cm^{-1}) 3319, 3292 (NH), 1641 (C = O), 1323 and 1149 (SO_2). ^{1}H NMR (acetone-d_6) δ = 0.65 (d, 3H, CH_3, J = 6.7 Hz), 0.82 (d, 3H, CH_3, J = 7.0 Hz), 2.02–2.05 (m, 1H, CH), 2.10–2.15 (m, 1H, CH), 2.40 (s, 3H, CH_3), 4.25 (dd, 1H, *CH, J = 8 Hz, J = 9.0 Hz), 7.24 (d, 2H, ArH, J = 8.0 Hz), 7.60 (d, 2H, ArH, J = 8.0 Hz), 9.20 (bs, 2H, NH_2), 10.41 (bs, 1H, NH). EI-MS (%) 285 (M^+,10), 225(12), 197(8), 171 (20), 155 (66), 130 (11), 91 (100), 85 (26), 57 (15), 41 (11).

4 h: Crystalline (aq. ethanol), m.p. 172–174°C, Yield = 69%, IR (KBr)(ν, cm^{-1}) 3322, 3292(NH), 1671(C = O), 1369 and 1165(SO_2). EI-MS (%) 305(M^+, 19), 111(100).

4i: Crystalline (aq. ethanol), m.p. 169–171°C, Yield = 71%, IR (KBr)(ν, cm^{-1}) 3376, 3282(NH), 1651(C = O), 1375 and 1166(SO_2). EI-MS (%) 301(M^+, 11), 117(100).

4j: Micro crystals (aq. ethanol) m.p. 151–153°C, Yield = 81%, IR (KBr)(ν, cm^{-1}) 3366, 3251(NH), 1675(C = O), 1378 and 1165(SO_2). EI-MS (%) 257(M^+, 57), 91(100).

4l: Crystalline (aq. ethanol) m.p. 160–162°C, Yield = 64%, IR (KBr)(ν, cm^{-1}) 3388, 3282(NH), 1672(C = O), 1375 and 1166(SO_2). ^{1}H NMR (Acetone-d_6) δ 1.35(d, 3H, CH_3, J = 7.4 Hz), 3.91(s, 3H, OCH_3), 4.55–4.63 (m, 1H, CH), 7.12(d, 2H, ArH J = 8.0 Hz), 7.66(d, 2H, ArH J = 8.0 Hz), 9.45(bs, 2H, NH), 10.92 (bs, 1H, NH). EI-MS (%) 273(M^+, 6), 214(100), 185(5), 107(67), 76(5), 57(10).

4m: Crystalline (aq. ethanol) m.p. 160–162°C, Yield = 64%, IR (KBr)(ν, cm^{-1}) 3266(NH), 1651(C = O), 1322 and 1151(SO_2). ^{1}H NMR (acetone-d_6) δ 1.71–1.82 (m, 2H, CH_2), 2.38(s, 3H, CH_3), 4.37(dd, H, CH, J = 7.6 Hz, J = 7.6 Hz), 7.32(d, 2H, ArH J = 8.0 Hz), 7.66(d, 2H, ArH J = 8.0 Hz), 9.45 (bs, NH), 10.34 (bs, NH). EI-MS (%) 267 (M^+, 17), 155(65), 91(100), 75(15). Anal. Calcd for $C_{13}H_{21}O_3N_3$: (267.33) C, 58.41; H, 7.92; N, 15.72. Found: C, 58.23; H, 7.81; N, 16.03%.

4n: Crystalline (aq. ethanol), m.p. 165–167°C, Yield = 71%, IR (KBr)(ν, cm^{-1}) 3331, 3210(NH), 1665(C = O), 1364 and 1155(SO_2). EI-MS (%) 319(M^+, 7), 111(100).

4o: Crystalline (aq. ethanol), m.p. 172–174°C, Yield = 67%, IR (KBr)(ν, cm^{-1}) 3369, 3241(NH), 1659(C = O), 1375 and 1165(SO_2). EI-MS (%) 315 (M^+, 15), 171(100).

5b: Powder (aq. ethanol), m.p 171–172°C, Yield 85%, IR (KBr)(ν, cm^{-1}) 3287(-NH), 2545(- C = N), 1375 and 1165(-SO_2). ^{1}H NMR (acetone-d_6) δ1.92–1.98 (m, 2H, CH_2), 2.80 (t, 2H, CH_2, J = 7.5 Hz), 3.09 (t, 2H, CH_2, J = 6.7 Hz), 7.63(d, 2H, ArH J = 8.1 Hz), 7.87 (d, 2H, ArH J = 8.2 Hz), 12.80 (bs, NH). EI-MS (%) 335(M^+ + 2), 333 (M^+, 7), 260(14), 177(25), 175(69), 158(10), 141(13), 129 (91), 113(32), 112(10), 111(100), 98(24), 76(11), 75(48), 69(29), 56(22), 55(16). Anal. Calcd for $C_{11}H_{12}O_3N_3S_2Cl$ (333.7921): C, 39.58; H, 3.62; N, 12.59; S, 19.21. Found: C, 39.68; H, 3.74; N, 12.77; S, 19.20%.

5c: Powder (aq. ethanol), m.p 159–161, Yield 87%, IR (KBr)(ν, cm^{-1}) 3216(-NH), 2548(-SH), 1589(- C = N), 1369 and 1179(-SO_2). ^{1}H NMR (acetone-d_6) δ 1.89–1.96 (m, 2H, CH_2), 2.79(t, 2H, CH_2 J = 6.7 Hz), 3.00(q, 2H, CH_2, J = 6.8 Hz), 3.88(s, 3H, OCH_3), 7.07 (d, 2H, ArH J = 8.1 Hz), 7.76 (d, 2H, ArH J = 8.2 Hz), 12.92(bs, NH). EI-MS (%) 330 (M^+, 1), 329(M^+, 67), 300(10), 256(14), 191(27), 173(12), 172(25), 171(100), 158(33), 155(27), 141(21), 136(23), 129(69), 123(35), 108(15), 107(27), 92(31), 77(43), 34(11), 56(6). Anal. Calcd for $C_{12}H_{15}O_4N_3S_2$ (329.3897): C, 43.76; H, 4.60; N, 12.76; S, 19.47. Found: C, 43.44; H, 4.56; N, 12.87; S, 19.71%.

5e: Crystalline (ethyl. acetate/ pet. ether), $[\alpha]_D^{20}$ = +41.64 (C = 1.00 g/100 cm^3, acetone); m.p 191–192°C, Yield 81%. IR (KBr)(ν, cm^{-1}) 3233(-NH), 2545(-SH), 1589(- C = N), 1365 and 1149(-SO_2). ^{1}NMR (acetone-d_6) δ 0.94 (t, 3H, CH_3 J = 7.4 Hz), 1.84–1.90 (m, 2H, CH_2), 4.39 (dd, H, CH J = 7.8 Hz, J = 7.5 Hz,), 7.61 (d, 2H, ArH J = 8.0 Hz), 7.85 (d, 2H, ArH J = 8.0 Hz) 12.89(bs, NH). EI-MS (%) 313 (M^+, 12), 260(14), 177(25), 175(68), 129(91), 111(100), 76(11), 69(29). Anal. Calcd for $C_{11}H_{12}O_3N_3S_2Cl$ (333.7921): C, 39.58; H, 3.62; N, 12.59; S, 19.21. Found: C, 39.59; H, 3.65; N, 12.20; S, 19.48.

5f: Crystalline (ethyl. acetate/ pet. ether), $[\alpha]_D^{20}$ = +44.32 (C = 1.05 g/100 cm^3, acetone); m.p 211–213°C, Yield 91%. IR (KBr)(ν, cm^{-1}) 3266(-NH), 2565(-SH), 1582(- C = N), 1356 and 1145(-SO_2). ^{1}H NMR (acetone-d_6) δ 0.92 (t, 3H, CH_3 J = 7.5 Hz), 1.82–1.89 (m, 2H, CH_2), 3.86(s, 3H, OCH_3), 4.36 (dd, H, CH J = 7.6 Hz, J = 7.7 Hz,), 7.01 (d, 2H, ArH J = 8.0 Hz), 7.71 (d, 2H, ArH J = 8.2 Hz). EI-MS (%) 330(M^+, 12), 260(14), 191(26), 172(25), 171(100), 158(32), 155(28), 129(70), 107(86), 92(30), 77(42), 64(10). Anal. Calcd for $C_{12}H_{15}O_4N_3S_2$ (329.3897): C, 43.76; H, 4.60; N, 12.76; S, 19.47. Found: C, 43.56; H, 4.37; N, 12.78; S, 19.57%.

5 g: Powder (aq. ethanol), $[\alpha]_D^{20}$ = +28.64 (C = 0.69 g/100 cm^3, acetone); m.p. 190–191°C, Yield = 93%, IR (KBr)(ν, cm^{-1}) 3287 (NH), 2555 (SH), 1597 (C = N), 1351 and 1161 (SO_2), 669 (C(S). ^{1}NMR (acetone-d_6) δ = 0.87 (d, 3H, CH_3, J = 6.7 Hz), 1.03 (d, 3H, CH_3, J = 6.7 Hz), 2.02–2.05 (m, 1H, CH), 2.1–2.12 (m, H, CH), 2.37 (s, 3H, CH_3), 4.17 (dd, 1H, *CH, J = 8.4 Hz, J = 9.2 Hz), 7.24 (d, 1H, NH, J = 9.0 Hz), 7.30 (d, 2H, ArH, J = 8.0 Hz), 7.63 (d, 2H, ArH, J = 8.2 Hz), 12.80 (bs, NH + SH). EI-MS(%) 327(M^+,9), 284 (16), 225 (4), 172 (10), 155 (74), 130 (19), 91 (100), 65 (29). Anal. Calcd for $C_{13}H_{17}O_3N_3S_2$ (327.414)): C, 47.69; H, 5.23; N, 12.83; S, 19.58. Found: C, 47.36; H, 4.95; N, 13.12; S, 19.95%.

5 h: Micro crystals (aq. ethanol), $[\alpha]_D^{20}$ = +29.32 (C = 0.70 g/100 cm^3, acetone); m.p. 195–199°C, Yield = 91%, IR (KBr)(ν, cm^{-1}) 3287(NH), 2553(-SH), 1595(C = N), 1351and 1161(SO_2), 669 (C-S). ^{1}NMR (acetone-d_6) δ 0.91(d, 3H, CH_3 J = 7.1 Hz), 1.07(d, 3H, CH_3 J = 7.1 Hz), 2.21–2.25(m, H, CH), 4.25(dd, 1H, CH, J = 8.4 Hz, J = 8.4 Hz), 7.65(d, 2H, ArH J = 8.0 Hz), 7.89(d, 2H, ArH J = 8.0 Hz), 12.89 (bs, NH). EI-MS (%) 347(M^+, 9), 175(100), 155(11), 111(74), 76(7). $C_{12}H_{14}O_3N_3S_2Cl$ (347.8463): HRMS: (347.7452).

5i: Micro crystals (aq. ethanol), $[\alpha]_D^{20}$ = +44.45 (C = 1.07 g/100 cm^3, acetone); m.p. 201–203°C, Yield = 65%, IR (KBr)(ν, cm^{-1}) 3262(NH), 1582(C = N), 1376and 1155(SO_2), 702 (C-S). ^{1}H NMR (acetone-d_6) δ 0.87(d, 3H, CH_3 J = 6.7 Hz), 1.03(d, 3H, CH_3 J = 6.7 Hz), 2.10– 2.13(m, H, CH), 3.89(s, 3H, OCH_3), 4.20 (dd, 1H, CH, J = 9.2 Hz, J = 9.2 Hz), 7.28(d, 2H, ArH J = 8.0 Hz), 7.64(d, 2H, ArH J = 8.0 Hz), 12.80 (bs, NH). EI-MS (%)

343(M^+, 11), 234(21), 171(100), 107(57), 76(5). $C_{13}H_{17}O_4N_3S_2$ (343.4278): HRMS: (343.4365).

5j: Crystalline (aq. ethanol), $[\alpha]_D^{20} = +43.41$ (C = 1.06 g/100 cm^3, acetone); m.p.205–207°C, Yield 83%, IR (KBr)(ν, cm^{-1}) 3282(-NH), 2551(-SH), 1589(-C = N), 1375 and 1116(-SO_2). ^{1}H NMR (acetone-d_6) δ 1.46 (d, 3H, CH_3), 2.4 (s, 3H, CH_3), 4.57–4.65 (m, H, CH), 7.34 (d, 2H, ArH J = 8.2 Hz), 7.68 (d, 2H, ArH J = 8.2 Hz), 12.85(bs, NH). EI-MS (%) 299(M^+, 45), 235(12), 198(21), 155(66), 91(100), 65(18). Anal. Calcd for $C_{11}H_{13}O_3N_3S_2$ (299.3633): C, 44.13; H, 4.38; N, 14.04; S, 21.42. Found: C, 44.07; H, 4.43; N, 14.31; S, 21.13%.

5l: Powder (aq. ethanol), $[\alpha]_D^{20} = +22.67$(C = 0.54 g/100 cm^3, acetone); m.p. 177–179°C, Yield 79%. IR (KBr)(ν, cm^{-1}) 3266(-NH), 2550(- C = N), 1586(-C = N), 1371 and 1162(-SO_2). ^{1}H NMR (acetone-d_6) δ1.53 (d, 3H, CH_3, J = 7.2 Hz), 3.89 (s, 3H, OCH_3), 4.74– 4.65(m, H, CH), 7.52(d, 2H, ArH J = 8.1 Hz), 7.76 (d, 2H, ArH J = 8.1 Hz), 12.85(bs, NH). EI-MS (%) 315(M^+, 9), 241(11), 180(9), 175(69), 135(100), 133(21), 107(57), 76(11). Anal. Calcd for $C_{11}H_{13}O_4N_3S_2$ (315.3627): C, 41.90; H, 4.21; N, 13.32; S, 20.33. Found: C, 42.13; H, 4.20; N, 13.22; S, 20.46%.

5m: Crystalline (aq. ethanol), $[\alpha]_D^{20} = +41.27$ (C = 1.00 g/100 cm^3, acetone); m.p 171–173°C, Yield 85%, IR (KBr)(ν, cm^{-1}) 3283(NH), 1595(C = N), 1375 and 1156(SO_2). ^{1}H NMR (acetone-d_6) δ 0.83–0.88 (m, 6H, $2CH_3$), 1.62–1.65 (m, 2H, CH_2), 2.39 (s, 3H, CH_3), 4.35 (dd, H, CH, J = 7.5 Hz, J = 7.5 Hz), 7.31(d, 2H, ArH J = 8.0 Hz), 7.64(d, 2H, ArH J = 8.0 Hz), 10.70 (bs, NH). EI-MS (%) 341(M^+, 48), 284(63), 186(33), 155(100), 91(82), 112(45), 111(23), 65(11), 57 (5). $C_{14}H_{19}O_3N_3S_2$ (341.4553): HRMS: (341.4678).

5n: Micro crystals (aq. ethanol), $[\alpha]_D^{20} = +23.47$ (C = 0.51 g/100 cm^3, acetone); m.p. 185–187°C, Yield = 91%, IR (KBr)(ν, cm^{-1}) 3291(NH), 1592(C = N), 1356and 1142(SO_2), 667 (C-S). ^{1}H NMR (acetone-d_6) δ 0.89–0.95(m, 6H, $2CH_3$), 1.82–1.89(m, 2H, CH_2), 4.39 (dd, 1H, CH, J = 7.6 Hz, J = 7.6 Hz), 7.65(d, 2H, ArH J = 8.1 Hz), 7.81(d, 2H, ArH J = 8.1 Hz). EI-MS (%) 361(M^+, 7), 304(5), 186(6), 175(100), 111(66), 76(5), 57 (12). $C_{13}H_{16}O_3N_3S_2Cl$ (361.8731): HRMS: (361.8620).

5o: Micro crystals (aq. ethanol), $[\alpha]_D^{20} = +39.37$ (C = 0.91 g/100 cm^3, acetone); m.p. 177–178°C, Yield = 73%, IR (KBr)(ν, cm^{-1}) 3282(NH), 1583(C = N), 1377and 1166(SO_2), 703 (C-S). ^{1}NMR (acetone-d_6) δ 0.82–0.87(m, 6H, $2CH_3$), 1.61–1.64(m, 2H, CH_2), 1.75–1.82(m, 1H, CH), 4.33(dd, 1H, CH, J = 7.5 Hz, J = 7.5 Hz), 7.31(d, 2H, ArH J = 8.0 Hz), 7.65(d, 2H, ArH J = 8.0 Hz), 12.80(bs, NH).EI-MS (%) 357(M^+, 12), 300(4), 281(3), 251(11), 186(10), 71(100), 107(55), 77(6), 57 (19). $C_{14}H_{19}O_3N_3S_2$ (357.4547): HRMS: (357.4588).

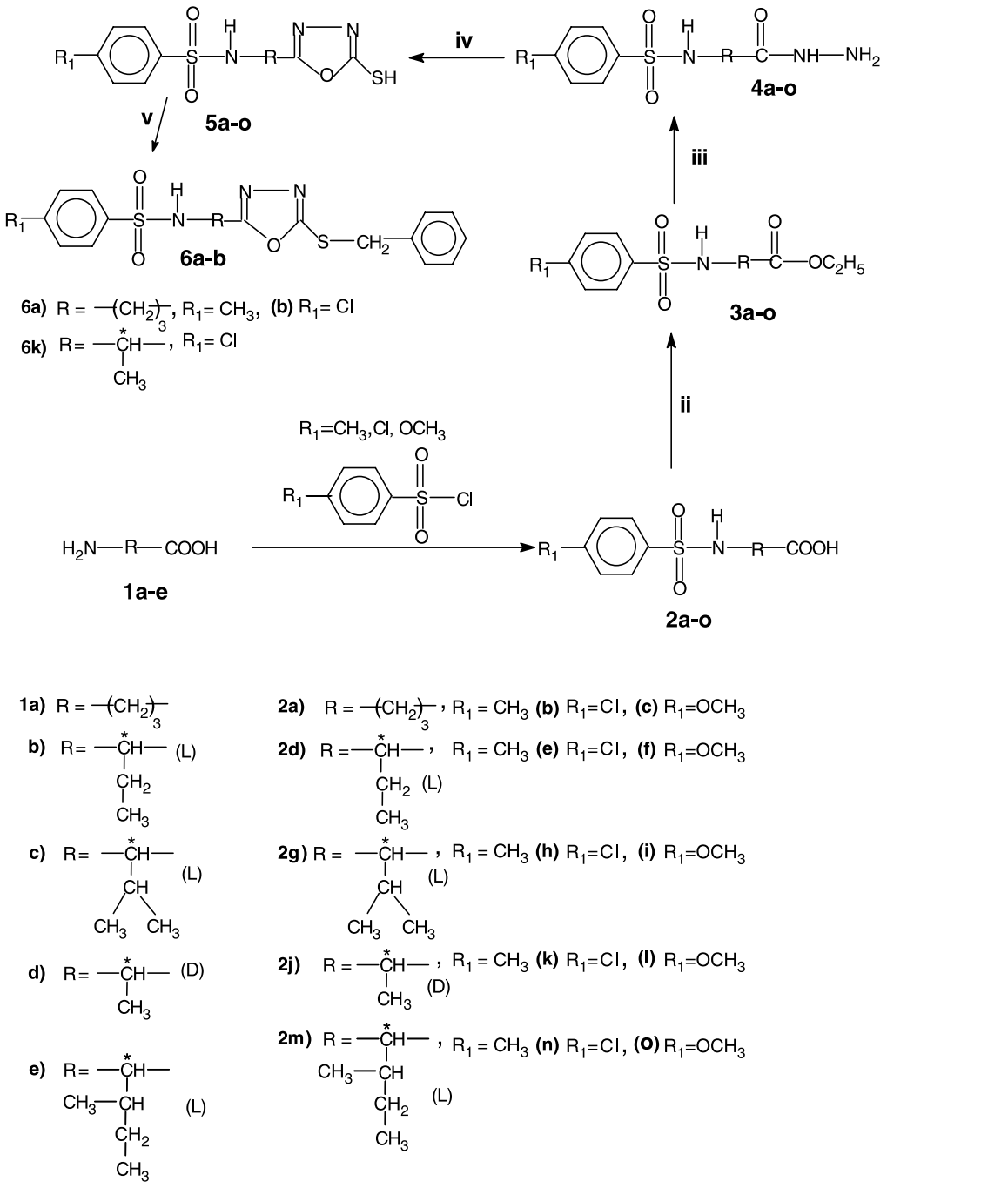
6a: Micro crystals (aq. ethanol), m.p. 117–119°C, Yield = 74%, IR (KBr)(ν, cm^{-1}) 3262 (NH), 1582(C = N), 1355 and 1165(SO_2), 672(C-S). ^{1}H NMR (acetone-d_6) δ 1.88–1.96 (m, 2H, CH_2), 2.38 (s, 3H, CH_3), 2.87 (t, 2H, CH_2, J = 6.61 Hz), 2.99 (t, 2H, CH_2, J = 6.7 Hz), 4.40 (s, 2H, CH_2-Ph), 7.21(bs, NH, replaceable with D_2O), 7.23–7.27(m, 5H, ArH), 7.40 (d, 2H, ArH J = 8.4 Hz), 7.75 (d, 2H, ArH J = 8.4 Hz). EI-MS (%) 403(M^+, 7), 312 (3), 186(2), 155(47), 129(42), 91(100), 77(13) 76(3).

6b: Crystalline (aq. ethanol), m.p. 133 −135°C, Yield = 73%, IR (KBr)(ν, cm^{-1}) 3266 (NH), 1582 (C = N), 1328 and 1156 (SO_2), 662 (C-S). ^{1}H NMR (acetone-d_6) δ 1.91–1.97 (m, 2H, CH_2), 2.81 (t, 2H, CH_2, J = 7.5 Hz), 3.10 (t, 2H, CH_2, J = 6.7 Hz), 4.35(s, $2CH_2$, S-CH_2-Ar), 7.28 (bd, NH, replaceable with D_2O, J = 8.0 Hz), 7.33–7.38 (m, 5H, ArH), 7.62 (d, 2H, ArH, J = 8.0 Hz), 7.88 (d, 2H, ArH, J = 8.2 Hz). EI-MS (%) 423 (M^+, 2), 359 (4), 346(2), 332(12), 260(16), 177(25), 175 (66), 112(9), 111(69), 91 (100), 77(47).

6k: Needles (aq. ethanol), $[\alpha]_D^{20} = +43.32$ (C = 1.05 g/100 cm^3, acetone); m.p 116–117°C, Yield 75%. IR (KBr)(ν, cm^{-1}) 3289(-NH), 1592(-C = N), 1371 and 1166(-SO_2), 679(C-S). ^{1}NMR (acetone-d_6) δ 1.51 (d, 3H, CH_3 J = 7.0 Hz), 4.35 (s, 2H, CH_2), 4.64–4.71(m, 1H, CH), 7.22 (bs, NH, replaceable with D_2O), 7.27–7.36 (m, 5H, ArH) 7.66 (d, 2H, ArH J = 8.4 Hz), 7.89(d, 2H, ArH J = 8.0 Hz). EI-MS (%) 409 (M^+, 7), 218 (20), 208 (3), 177 (37), 175(100), 139 (28), 111(39), 91(88), 75 (20).

CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument was used for assaying the CA-catalysed CO_2 hydration activity [32]. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7–17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition



Scheme 1. **Reagents and conditions:** i) Aq. 5% NaOH, ether, stirring at RT for 6 h; ii) H_2SO_4 / ethanol, refluxing for 11 h; iii) NH_2-NH_2.H_2O / ethanol (absolute), refluxing for 9 h; iv) CS_2 / KOH, ethanol, refluxing for 16.5 h. (v) Et_3N, DMAP, CH_3Cl(dry), benzyl bromide, stirring at 30–70°C for 5 h.

constants were obtained by non-linear least-squares methods from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations [28–31].

Results and discussion

A number of methods have been reported in the literature for the synthesis of heterocyclic compounds incorporating free mercapto and secondary sulfonamide moieties in their molecules [33,34]. Most of such compounds were prepared by using carboxylic acid hydrazides and sulfonic acids/sulfonylchlorides as starting materials, followed by cyclization of the sulfonylated amino acid carbohydrazide [33,34]. We report here a novel approach for preparing such heterocycles, using different chiral and achiral amino acids as starting material (Scheme 1). The advantages

of this method include easy availability of the starting materials, high yields of the final products (sulfonamides), a free mercapto group at position 5 of the oxadiazole ring, and most importantly the incorporation of chiral centres for possible stereo-selectivity in various applications of such compounds (e.g., as enzyme inhibitors). Benzyl derivatives **6a-b** and **6k** have also been prepared from the corresponding free thiols, by reaction with benzyl bromide (Scheme 1).

Amino acids **1a-e** were converted to the corresponding sulfonamides **2a-o** by reaction with 4-methylbenzenesulfonyl chloride, 4-chlorobenzenesulfonyl chloride or 4-methoxybenzenesulfonyl chloride, respectively, in alkaline medium using the standard literature procedure [37]. The carboxylic acid group of these sulfonamides **2a-o** was esterified with ethanol in acidic medium [35], and esters **3a-o** thus obtained were reacted with hydrazine hydrate (80%) to furnish the corresponding hydrazides **4a-o** in good yields [36]. The sulfonamides bearing the 2,5-disubstituted-1,3,4-oxadiazole moiety **5a-o** were then prepared by the reaction of hydrazides **4a-o** with carbon disulfide and potassium hydroxide [38,39]. Mass spectroscopy (see Materials and Methods for details) showed that the disubstituted 1,3,4-oxadiazoles **5** were formed by elimination of H_2S from carbohydrazides **4** and CS_2, and not the corresponding 1,3,4-thiadiazoles (which might be formed from the same reagents by elimination of water). The synthesis of compounds **2a, 2d, 2k, 3a, 3d, 3k, 4a, 4d, 4k, 5a, 5d** and **5k** has been reported earlier by our group [40]**.** The structures of synthesized compounds was supported by physical, optical rotation data (for the chiral derivatives), microanalytical data, IR, ^{1}H NMR and mass spectral data. The IR spectrum of the representative sulfonamide **5 g,** revealed the presence of characteristic bands for -NH at 3286 cm^{-1}, and 2551 cm^{-1} for -SH in addition to the -SO2 functional group. In the mass spectrum of **5 g,** the molecular ion peak was observed at m/z 327(M^+, 9). In the ^{1}H NMR spectrum of **5 g,** the following important signals were observed: $\delta = 0.87$ (d, 3H, CH_3, J = 6.7 Hz), 1.03 (d, 3H, CH_3, J = 6.7 Hz) two doublets of two methyl groups attached to pro-chiral centre, 2.02–2.05 (m, 1H, CH), 2.1–2.12 (m, 1H, CH), pro-chiral centre, 4.17 (dd, H, CH, J = 8.4 Hz, J = 9.2 Hz) chiral centre, 7.24 (d, 1H, NH, J = 9.0 Hz), 7.30 (d, 2H, ArH, J = 8.0 Hz), 7.63 (d, 2H, ArH, J = 8.2 Hz), 12.80 (bs, NH + SH, replaceable with D_2O) with [?] $^{20}_{D=} + 28.64^{o}$(0.69 g / 100 cm^3 of acetone). Sulfonamides **5b-c, 5e-j, 5l-o, 6a-b** and **6k** are new compounds not reported previously in the literature. None of these compounds has been investigated previously for their interaction with CA, although inhibition studies of these enzymes with heterocyclic thiols are available in the literature [41–43].

Some of the compounds **4–6** reported in this study and clinically used CA inhibitors of the sulfonamide type (as standard drugs), such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP** and indisulam **IND**, have been tested for the inhibition of two cytosolic, ubiquitous isozymes of human origin, i.e., hCA I and hCA II [1–6], as well as the human tumor-associated isoform hCA IX (Table I). The main goal of this investigation was that of detecting CA IX inhibitors (possibly with selectivity towards this isozyme over the ubiquitous cytosolic isoforms CA I and II [1–6]), belonging to different classes of compounds than the widely investigated sulfonamides and sulfamates [21–30]. Data in Table I show the following: (i) against the slow cytosolic isozyme hCA I, thiols **5a–5 g** behave as weak inhibitors, with K_Is in the range 129–678 μM, whereas carbohydrazides **4k** and **4m**, thiols **5j** and **5k**, as well as the S-benzylated derivative **6b** showed very weak inhibitory properties (K_Is in the range 1059–1675 μM); (ii) against the rapid cytosolic isoform hCA II; a weak inhibitory activity was shown only by thiols **5a, 5b** and **5d** (K_Is in the range 552–720 μM), whereas all other investigated compounds showed very weak inhibition (K_Is > 1200 μM); (iii) against the tumor-associated isozyme hCA IX, four derivatives showed good inhibition, with K_Is in the range 9.9–10.1 μM. Unexpectedly, two of these compounds (**4k** and **4m**) are carbohydrazides, a class of derivatives not previously investigated as possible CA inhibitors, whereas the remaining two are thiols (**5f** and **5 g**). Such compounds, mainly 1,3,4-thiadiazole-5-thiol derivatives, were in fact shown to behave as micromolar CA IX inhibitors in an earlier

Table I. Inhibition data for compounds **4-6** reported in the present paper and standard sulfonamide CA inhibitors, against isozymes I, II, and IX, by a stopped-flow, CO_2 hydration assay [32].

Inhibitor	K_I^*		
	hCA I[a] (μM)	hCA II[a] (μM)	hCA IX[b] (μM)
AAZ	0.31	0.012	0.025
MZA	0.78	0.014	0.027
EZA	0.025	0.008	0.034
DCP	1.20	0.038	0.050
IND	0.031	0.015	0.024
4k	1059	9092	10.0
4m	1440	1266	9.9
5a	501	552	1381
5b	390	566	1203
5d	170	720	820
5e	129	1783	560
5f	552	1486	10.1
5 g	678	1314	9.9
5j	1088	1215	1467
5k	1675	1364	1509
5m	1110	1665	1187
6b	1250	3960	2830

*Errors in the range of 5–10% of the reported value (from 3 different assays). [a] Human (cloned) isozymes, by the CO_2 hydration method; [b] Catalytic domain of human, cloned isozyme [30,31], by the CO_2 hydration method [32].

work of our group [42]. Surprisingly, the remaining thiols **5a–5e** and **5j–5m**, as well as the S-benzylated derivative **6b**, showed quite weak CA IX inhibitory activity (K_Is in the range 560–2830 μM. It should also be mentioned that although the four efficient CA IX inhibitors detected here (**4k**, **4m**, **5f** and **5 g**) possess only micromolar affinity for the enzyme, these compounds on the other hand may be considered as CA IX selective inhibitors, since they have very low affinity for the ubiquitous isoforms hCA I and II. With the sulfonamide CA inhibitors, this is generally not observed, as most of such compounds usually have very high affinity both for CA I and II [1–7]. Thus, derivatives **4k**, **4m**, **5f** and **5 g** reported here may be considered as very interesting lead molecules for the possible design of CA IX-selective inhibitors. It would also be important to understand why carbohydrazide **4k**, **4m** bind to the CA IX active site, whereas their binding to CA I and II is very ineffective.

Conclusions

A series of chiral 1,3,4-oxadiazole-5-thiols incorporating 2-substitutedbenzenesulfonamide moieties has been prepared from amino acids, via the ester and carbohydrazide intermediate, followed by cyclization with carbon disulfide. Some of these compounds have been investigated for the inhibition of three physiologically relevant CA isoforms, the human cytosolic hCA I and II, and the human, transmembrane, tumor-associated isozyme hCA IX. All these compounds showed weak (millimolar) affinity for the three isozymes, except two carbohydrazides and two heterocyclic thiols which selectively inhibited the tumor-associated isozyme with inhibition constants around 10 μM. Such compounds constitute interesting lead molecules for the possible design of CA IX-selective inhibitors.

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