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Original article

Carbonic anhydrase inhibitors: Synthesis and inhibition of the human cytosolic isozymes I and II and transmembrane isozymes IX, XII (cancer-associated) and XIV with 4-substituted 3-pyridinesulfonamides

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ABSTRACT

A series of novel 4-substituted-3-pyridinesulfonamides (**2**–**27** and **31**–**33**) have been synthesized and investigated as inhibitors of five isoforms of zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), that is, the cytosolic, ubiquitous isozymes CA I and II, and transmembrane isozymes CA IX, XII (cancer-associated) and XIV. Against the human isozymes hCA I, the new compounds showed inhibition constants in the range of 0.078–11.7 μM, against hCA II in the range of 9.9–140 nM, against hCA IX in the range of 4.6–313 nM, against hCA XII in the range of 3.4–21.6 nM, and against hCA XIV in the range of 50.9–160 nM, respectively. Compounds **4**, **6**, **7**, **9**, **11**–**14**, **19**, **20**, **22**–**24**, **26**, **27**, **31** and **32** showed excellent hCA IX inhibitory efficacy, with inhibition constants of 4.6–12.0 nM, being much more effective as compared to the clinically used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** and **IND**. 4-[*N*′-(6-Chloro-7-cyano-1,1-dioxo-1,4,2-benzodithiazin-3-yl)hydrazino]-3-pyridinesulfonamide (**31**) is the prominent of the compounds due to its remarkable inhibitory activity toward hCA I (K_Is = 0.078 μM), hCA IX (K_Is = 7.2 nM) and hCA XII (K_Is = 3.4 nM).

1. Introduction

There are many connections between carbonic anhydrase (CA, EC 4.2.1.1) and cancer [1–5]. It is well known, that some CA isozymes are predominantly found in cancer cells and are lacking from their normal counterparts [3–5], these are two transmembrane isozymes CA IX and CA XII. Isozyme CA XIV was the last one to be discovered among the 15 CA isoforms of this widespread metalloprotein known up to now in human [6]. Parkkila's and Sly's groups [7–9] revealed CA XIV distribution in the human body as well as potential physiological/pathological roles. It has been observed that hCA XIV is highly abundant in the brain, kidney, colon, small intestine, urinary bladder, liver and spinal cord [7–11]. Similar to isozymes CA IX and CA XII, CA XIV is a transmembrane protein with the active site oriented extracellularly, but unlike the first two proteins, isozyme XIV is not associated with tumor cells [3,7–9,11].

Membrane-associated human carbonic anhydrase (hCAs) isozymes IX, XII and XIV [10,12] like other hCAs regulate pH and carbon dioxide (CO_2): bicarbonate anion (HCO_3^-) homeostasis, through catalysis of the reversible hydration of CO_2 to give HCO_3^- and proton (H^+). The expression level of isozymes hCA IX and XII is elevated in response to hypoxia and research on the involvement of these isozymes in cancer has progressed considerably in recent years, particularly for hCA IX [12–16]. It has been confirmed that hCA IX is a high activity CA isozyme responsible for the extracellular acidification (pH_e) of the tumor microenvironment. Multiple downstream effects of this reduced pH_e are associated with tumor progression and poor prognosis [14,15]. Aromatic sulfonamide compounds have been shown to reverse the effect of tumor acidification, to inhibit the growth of cancer cells and to suppress tumor invasion mediated by these CAs [12–16]. Thus, the data from these many physiological studies appear to have identified a CA mediated, hypoxic tumor-specific pathway. This provides firm grounds for exploring the effects of this class of compounds as a novel approach to discriminate between healthy cells and cancerous cells, specifically targeting hypoxic-tissues – an attractive attribute that is lacking in many existing cancer therapies [17,18].

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Recently, we have reported on the strong inhibition of human cytosolic isozymes I and II and tumor-associated isozymes IX and XII with some *S*-substituted 4-chloro-5 or 6-methyl-benzenesulfonamides of type **I** and **II** (Chart 1) [19–21]. Some of those compounds also showed a certain degree of selectivity for the inhibition of the tumor-associated over the cytosolic CA isoforms [19–21]. These findings prompted us to synthesis and investigate the inhibitory activity of the related 4-substituted 3-pyridinesulfonamides of type **III** (Chart 1).

2. Results and discussion

2.1. Chemistry

Syntheses of the target pyridinesulfonamides **4**–**14** were achieved in 76–94% yields by the convenient two-step procedure starting from commercially available 4-chloro-3-pyridinesulfomide **1** and the corresponding hydroxyl or thiol compounds, as shown in Schemes 1 and 2.

We have found in our studies, that the reaction of **1** with thiouera led to the formation of the expected thiouronium chloride **A** which in the presence of methanol undergoes a further transformation. The proposed mechanism leading to the formation of the products **15** and **16** is outlined in Scheme 3. The initial step is believed to be formation of unstable $1H^{+}$-pyridinium chloride **B**, which after spontaneous elimination of urea or 2-methyluronium chloride gives two products **15** and intermediate **C**, respectively. In the final stage of the reaction the non-isolable intermediate **C** reacts with starting compound **1** to afford the corresponding sulfide **16** in 53% yield (Scheme 3).

In the different conditions however, the expected thiouronium chloride **A** (**17**) was successfully obtained in excellent yield by the reaction of **1** with thiourea in boiling acetonitrile (Scheme 4). Then, upon treatment of thiouronium chloride **17** with NaOH and corresponding alkyl halides in tetrahydrofuran–water solution at temperature 0–20 °C, the desired 4-(*R*-methylthio)-3-pyridinesulfonamide **18**–**22** were obtained in 81–88% yields. The desired 4-(amino-or hydrazine)-3-pyridinesulfonamide derivatives **22**–**27** were obtained in 79–85% yields by reacting the 4-chloro-3-pyridinesulfomide **1** with an excess of corresponding amines or hydrazines in methanol at reflux (Scheme 5). Then, the reaction of **26** (Scheme 6) with appropriate methylthio compounds **28**–**30** under reflux in methanol furnished the expected 4-(*N*′-heteroarylhydrazino)-3-pyridinesulfonamides **31**–**33** in 89–92% yields.

The structures of the newly obtained compounds **2**–**27** and **31**–**33** were confirmed by elemental analysis (C, H, N) and spectroscopic data presented in the experimental section.

2.2. CA inhibition studies

The compounds **4**–**7**, **9**–**14**, **16**, **18**–**27** and **31**–**33** as well as standard, clinical used CAIs, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND** (Chart 1), have been tested for the inhibition of two cytosolic, ubiquitous isozymes of human origin, that is, hCA I and II, and three transmembrane isoforms hCA IX, XII (cancer-associated) and hCA XIV isozyme (Table 1). The following should be noted regarding CA inhibitory data of Table 1: (i) against the slow cytosolic isoform hCA I, the sulfonamides investigated here showed high to weak inhibitory properties. Thus, derivatives **7**, **9**, **10**, **14**, **16**, **18**–**24** and **33** showed weak inhibition of this isoform, with K_Is in the range of 8.32–11.77 μM, being thus much weaker inhibitors as compared to the clinically used compounds **AAZ**–**IND** (Table 1). The compounds **4**–**6**, **11**–**13**, **25**–**26** and **33** were slightly more effective inhibitors against hCA I with K_Is in range of 5.62–7.91 μM. However, the other three compounds **27**, **31** and **32** showed hCA I inhibitory activity (K_Is = 0.078–1.52 μM) in the same range as the clinically used compounds **AAZ**–**IND** (Table 1); (ii) against the ubiquitous and dominant rapid cytosolic isozyme hCA II, compounds **4**–**7**, **9**–**14**, **16**, **18**–**20**, **22**–**27** and **31**–**33** acting as weaker inhibitors (K_Is in the range 45.8–140.0 nM) (Table 1), but the other only compound **21** acting as a stronger CA II inhibitors, with K_Is = 9.9 nM, of the some order magnitude as must of the clinically used compounds (Table 1); (iii) a quite larger variation of inhibitory activity was observed for the inhibition of the tumor-associated isoform, hCA IX (Table 1). Thus, the compounds **5** and **10** showed weak CA IX inhibitory activity, with K_Is in range of 300–313 nM. Another groups of compounds such as **16**, **18**, **21**, **25** and **33**, showed moderate CA IX inhibition, with K_Is range 15.8–47.3 nM, whereas the remaining derivatives **4**, **6**, **7**, **9**, **11**–**14**, **19**, **20**, **22**–**24**, **26**, **27**, **31** and **32** were very effective hCA IX inhibitors

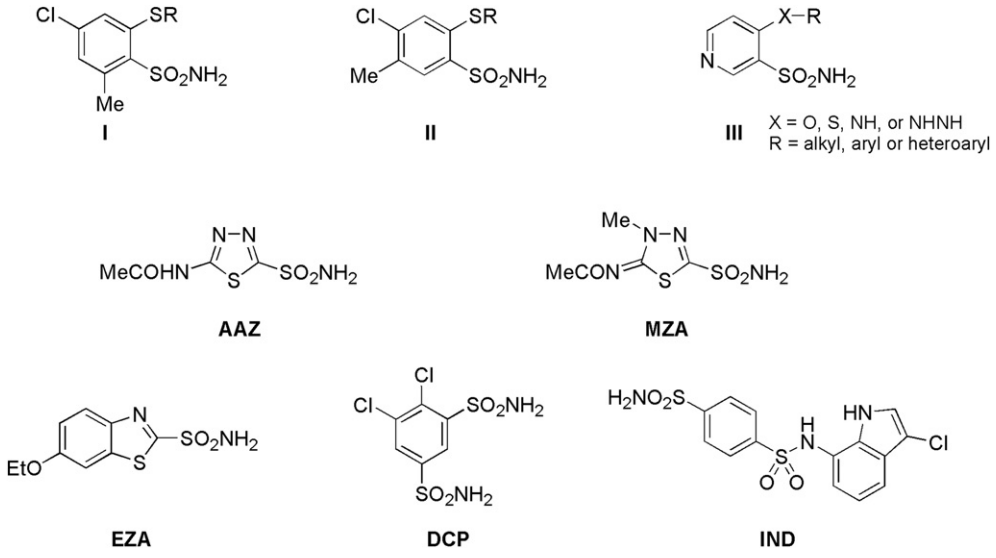
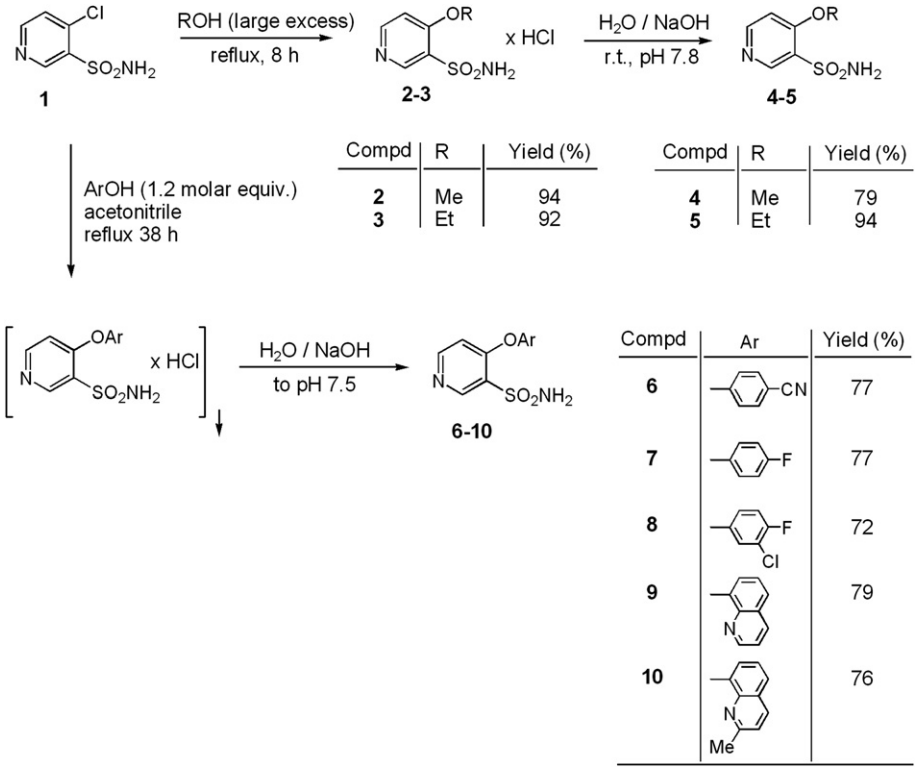


Chart 1.

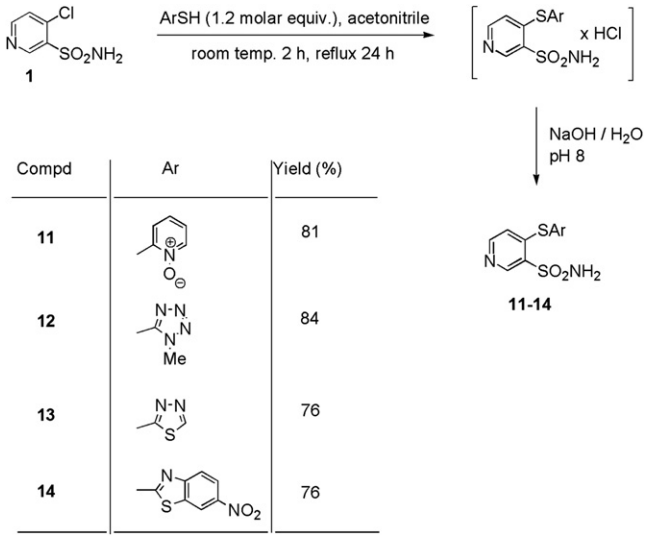


Scheme 1. Synthesis of 4-(alkoxy and aryloxy)pyridine-3-sulfonamides **2**–**10**.

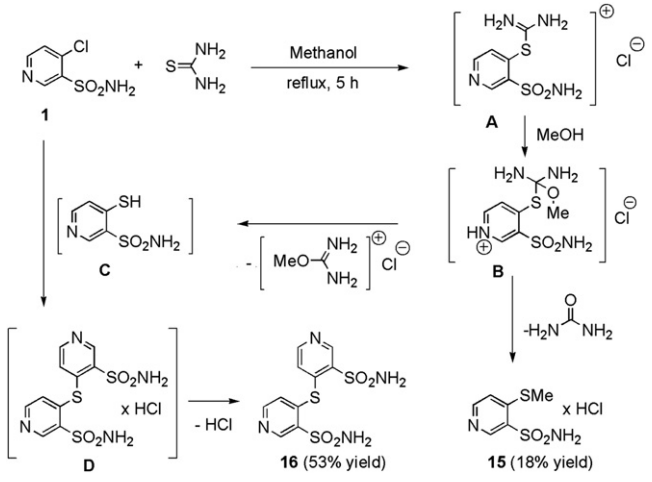
(K_Is in range of 4.6–12.0 nM, Table 1); (iv) a quite good inhibition profile of the second tumor-associated isoform, hCA XII has also been observed for all investigated sulfonamides of type **4**–**33** (Table 1). Thus, compounds **11**, **14**, **19**, **20**, **22**–**24**, **26** and **27** were the moderate inhibitors (K_Is in the range of 10.1–21.6 nM), whereas the remaining compounds were very effective hCA XII inhibitors (K_Is in the range of 3.4–8.9 nM, Table 1); (v) all of the investigated sulfonamides **5**, **9**, **10**, **16**, **18**–**27** and **31**–**33** showed moderate to weak inhibition properties towards hCA XIV isozyme with K_Is in the range of 50.9–160.0 nM (Table 1).

3. Conclusions

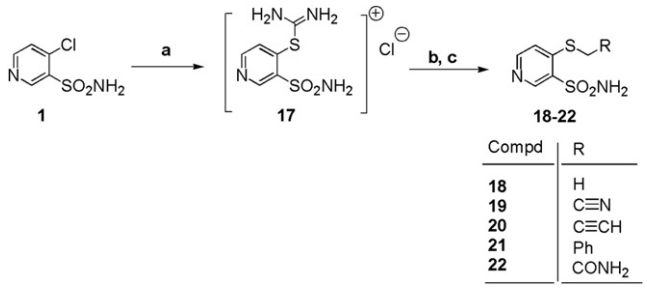
We have developed methods for the preparation of novel series of 4-substiuted 3-pyridiesulfonamides (ethers, sulfides, amines and hydrazines). The 24 new sulfonamides have been assayed for the inhibition of five physiologically relevant CA isozymes, such as CA I and II, the tumor-associated isozymes CA IX, XII as well as transmembrane CA XIV. Against the human isozyme hCA I the new compounds showed inhibition constants in the range of 0.078–11.7 μM, against hCA II in the range 9.9–140.0 nM, against hCA IX in the range 4.6–313 nM, against hCA XII in the range



Scheme 2. Synthesis of 4-(heteroarylthio)pyridine-3-sulfonamides **11**–**14**.



Scheme 3. Proposed mechanism of the formation of the 4-mercapto-3-pyridinesulfonamide derivatives **15** and **16** in the reaction of 4-chloro-3-pyridinesulfonamide with thiourea in boiling methanol.



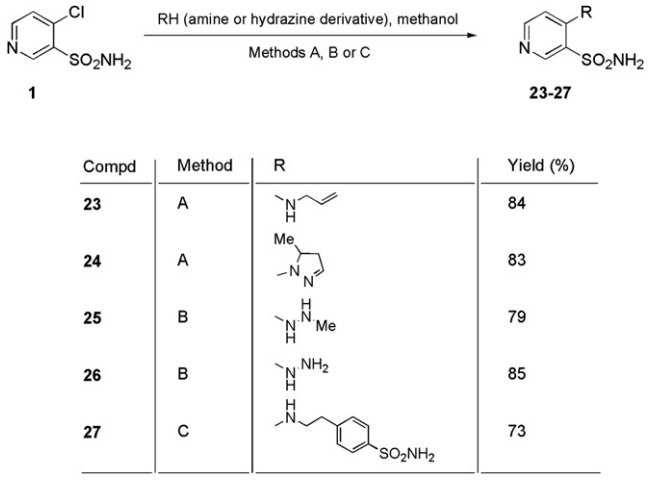
Scheme 4. Synthesis of 2-(3-sulfamoyl-4-pyridyl)thiouronium chloride **17** and its application to the synthesis of sulfides **18**–**22**. Reagents, conditions and yields: (a) thiourea (1.02 molar equiv.), acetonitrile, reflux, 3 h, 98%; (b) NaOH (2.14 molar equiv.), water, tetrahydrofuran, 0–5 °C; (c) alkyl halide R–CH_2X (1.14 molar equiv.), tetrahydrofuran, 0–7 °C, 0.5 h, room temperature, 5 h, 81 88%.

3.4–21.6 nM and against hCA XIV in the range 50.9–160 nM, respectively. Compounds **27**–**32** having an activity against hCA I (K_Is = 0.075–1.52 μM) comparable the clinical used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** or **IND** (K_Is = 0.025–1.2 μM). Several of the new compounds including **4**, **6**, **7**, **9**, **11**–**14**, **19**, **20**, **22**–**24**, **26**, **27**, **31** and **32** showed excellent hCA IX inhibitory efficacy, with inhibition constants of 4.6–12.0 nM, being much more effective as compared to the clinically used **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (K_Is = 24–50 nM).

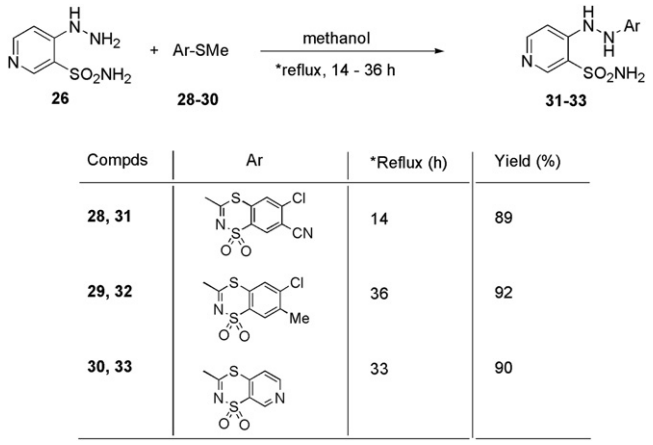
4. Experimental protocols

4.1. Synthesis

The following instruments and parameters were used: melting points Bűchi 535 apparatus; IR spectra: KBr pellets, 400–4000 cm^{-1} Perkin Elmer 1600 FTIR spectrometer; ^{1}H and ^{13}C NMR: Varian Gemini 200 apparatus at 200 and 50 MHz, respectively; chemical shifts are expressed at δ values relative to Me_4Si as standard. The results of elemental analyses for C, H and N were within ±0.3% of theoretical values. According to the methods described previously the following substrates were obtained: 6-chloro-7-(cyano- and methyl)-3-methylthio-1,4,2-benzodithiazine 1,1-dioxides **28** [22,23], **29** [24], and 3-methylthiopyrido[4,3-*e*]-1,4,2-dithiazine 1,1-dioxide **30** [25].



Scheme 5. Synthesis of 4-(amino and hydrazino)-3-pyridosulfonamide derivatives **23**–**27**.



Scheme 6. Synthesis of 4-(*N*′-heteroarylhydrazino)-3-pyridinesulfonamides **31**–**33**.

4.1.1. Procedure for the preparation of 4-alkoxy-3-pyridinesulfonamide hydrochlorides (**2**, **3**) and their transformation to free sulfonamides (**4** and **5**)

*4.1.1.1. A. Hydrochlorides **2** and **3**.* A solution of 4-chloro-3-pyridinesulfonamide **1** (3.85 g, 0.02 mol) in the corresponding alcohol (30–35 ml) was refluxed with stirring for 8 h. After cooling to room temperature the suspension was left overnight. The precipitate was collected by filtration, washed with the corresponding alcohol (3 × 5 ml) and dried at temperatures gradually increasing to 90 °C.

*4.1.1.1.1. 4-Methoxy-3-pyridinesulfonamide hydrochloride (**2**).* Starting from anhydrous methanol (30 ml), the title compound **2** was obtained (4.25 g, 94%); m.p. 168–169 °C; IR (KBr) 3305, 3140 (SO_2NH_2), 2715, 2615, 2135, 2060, 1910 (NH^+), 1630, 1590, 1505 (C=N and C=C), 1340, 1175 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.20 (s, 3H, CH_3O), 7.78 (d, J = 6.8 Hz, 1H, H-5), 7.97 (s, 2H, SO_2NH_2), 8.90 (s, 1H, H-2), 8.98 (d, J = 6.8 Hz, 1H, H-6) ppm; ^{13}C NMR (DMSO-d_6) δ 58.96, 111.38, 129.94, 141.95, 148.20, 167.23 ppm. Anal. ($C_6H_9ClN_2O_3S$) C, H, N.

*4.1.1.1.2. 4-Ethoxy-3-pyridinesulfonamide hydrochloride (**3**).* Starting from anhydrous ethanol (35 ml), the title compound **3** was obtained (4.4 g, 92%); m.p. 167–168 °C; IR (KBr) 3325, 3190 (SO_2NH_2), 2760, 2670, 2560, 2040, 1995 (NH^+), 1630, 1590, 1525 (C=N and C=C), 1340, 1165 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.44 (t, J = 7.0 Hz, CH_3), 4.57 (q, J = 7.0 Hz, 2H, CH_2), 7.78 (d, J = 6.5 Hz, 1H, H-5), 7.84 (s, 2H, SO_2NH_2), 8.90 (s, 1H, H-2), 8.94 (d, J = 6.5 Hz, 1H, H-6) ppm. Anal. ($C_7H_{11}ClN_2O_3S$) C, H, N.

*4.1.1.2. B. Free sulfonamides **4** and **5**.* To a stirred solution of the appropriate hydrochlorides **2** or **3** (0.01 mol) in water (8 ml) was added dropwise a solution of 1% NaOH in water to pH 7.8. The suspension obtained was stirred at room temperature for an additional 4 h. The precipitate was filtered off, washed with water (5 × 2 ml), and acetonitrile (3 × 1 ml), and dried at temperatures gradually increasing to 100 °C.

*4.1.1.2.1. 4-Methoxy-3-pyridinesulfonamide (**4**).* Starting from hydrochloride **2** (2.25 g), the title compound **4** was obtained (1.5 g, 79%); m.p. 163–165 °C; IR (KBr) 3315, 3275, 3225 (SO_2NH_2), 1590, 1565(C=N and C=C), 1340, 1160 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.98 (s, 3H, CH_3O) 3.98 (s, 3H, CH_3O), 7.27 (d, J = 5.8 Hz, 1H, H-5), 7.38 (s, 2H, SO_2NH_2), 8.63 (d, J = 5.8 Hz, 1H, H-6), 8.71 (s, 1H, H-2) ppm; ^{13}C NMR (DMSO-d_6) δ 56.72, 108.65, 128.08, 148.07, 155.14, 162.39 ppm. Anal. ($C_6H_8N_2O_3S$) C, H, N.

*4.1.1.2.2. 4-Ethoxy-3-pyridinesulfonamide (**5**).* Starting from hydrochloride **3** (2.38 g), the title compound **5** was obtained (1.9 g, 94%); m.p. 179–180 °C; IR (KBr) 3310, 3145 (SO_2NH_2), 1590, 1550 (C=N and C=C), 1335, 1165 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.38

(t, $J = 7.0$ Hz, 3H, CH_3), 4.30 (q, $J = 7.0$ Hz, 2H, OCH_2), 7.20 (d, $J = 5.8$ Hz, 1H, H-5), 7.32 (s, 2H, SO_2NH_2), 8.58 (d, $J = 5.8$ Hz, 1H, H-6) ppm. Anal. ($C_7H_{10}N_2O_3S$) C, H, N.

4.1.2. General procedure for the preparation of 4-aryloxy-3-pyridinesulfonamies (**5**–**10**)

A mixture of 4-chloropyridine-3-sulfonamide **1** (2.89 g, 0.015 mol) and the corresponding phenols or hydroxy derivatives of heterocyclic compounds (0.018 mol) in acetonitrile (20 ml) was refluxed with stirring for 38 h. After cooling to room temperature and standing overnight the precipitate was collected by filtration, washed with acetonitrile (2×1.5 ml) and dried. The adequate crude hydrochlorides obtained was dissolved in water (15–25 ml) and adjusted to pH 7.5 with 1% solution of NaOH in water. After 6 h of stirring, the precipitate of the adequate title products was filtered off, washed successively with water (4×3 ml) and acetonitrile (2×1.5 ml), and dried at temperatures gradually increasing to 100 °C. In this manner the following sulfonamide were obtained.

*4.1.2.1. 4-(4-Cyanophenoxy)-3-pyridinesulfonamide (**6**).* Starting from 4-cyanophenol (2.14 g), the title compound **6** was obtained (3.2 g, 77%); m.p. 208–210 °C; IR (KBr) 3360, 3255 (SO_2NH_2), 2235 (C≡N), 1640, 1575 (C=N and C=C), 1340, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.03 (d, $J = 5.5$ Hz, H-5, pyrid.), 7.39 (d, $J = 8.1$ Hz, 2H, PhCN), 7.73 (s, 2H, SO_2NH_2), 7.87 (d, $J = 8.1$ Hz, 2H, PhCN), 8.66 (d, $J = 5.5$ Hz, 1H, H-6, pyrid.) 8.93 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{12}H_9N_3O_3S$) C, H, N.

*4.1.2.2. 4-(4-Fluorophenoxy)-3-pyridinesulfonamide (**7**).* Starting from 4-fluorophenol (2.02 g), the title compound **7** was obtained (3.1 g, 77%); m.p. 182–184 °C; IR (KBr) 3330, 3172 (SO_2NH_2), 1635, 1600, 1575 (C=N and C=C), 1325, 1175, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.80 (d, $J = 5.4$ Hz, 1H, H-5, pyrid.), 7.27–7.32 (m, 2H, PhF), 7.37 (t, $J = 8.8$ Hz, 2H, PhF) 7.68 (s, 2H, SO_2NH_2), 8.58 (d, $J = 5.4$ Hz, 1H, H-6, pyrid.), 8.87 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{11}H_9FN_2O_3S$) C, H, N.

*4.1.2.3. 4-(3-Chloro-4-fluorophenoxy)-3-pyridinesulfonamide (**8**).* Starting from 3-chloro-4-fluorophenol (2.64 g), the title compound **8** was obtained (3.3 g, 72%); m.p. 210–211 °C; IR (KBr) 3315, 3160 (SO_2NH_2), 1580, 1500 (C=N and C=C), 1340, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.94 (d, $J = 5.7$ Hz, 1H, H-5, pyrid.), 7.24–7.31 (m, 1H, 3-Cl-4-FPh), 7.53–7.61 (m, 2H, 3-Cl-4-FPh), 7.67 (s, 2H, SO_2NH_2), 8.60 (d, $J = 5.7$ Hz, 1H, H-6, pyrid.), 8.72 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{11}H_8ClFN_2O_3S$) C, H, N.

*4.1.2.4. 4-(Quinolinoxy)-3-pyridinesulfonamide (**9**).* Starting from 8-quinolinol (2.61 g), the title compound **9** was obtained (3.6 g, 79%); m.p. 218–219 °C; IR (KBr) 3315, 3150 (SO_2NH_2), 1590, 1570, 1500 (C=N and C=C), 1340, 1330, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.60 (d, $J = 5.6$ Hz, 1H, H-5, pyrid.) 7.60–7.66 (m, 1H, quinolyl), 7.70–7.75 (m, 2H, quinolyl), 7.77 (s, 2H, SO_2NH_2), 8.01–8.06 (m, 1H, quinolyl), 8.43 (d, $J = 5.6$ Hz, 1H, H-6, pyrid.), 8.53 (d, $J = 8.4$ Hz, 1H, quinolyl), 8.83 (d, $J = 4.0$ Hz, 1H, quinolyl), 8.88 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{14}H_{11}N_3O_3S$) C, H, N.

*4.1.2.5. 4-(2-Methyl-8-quinolinoxy)-3-pyridinesulfonamide (**10**).* Starting from 2-methyl-8-quinolinol (2.87 g), the title compound **10** was obtained (3.6 g, 76%); m.p. 243–245 °C dec.; IR (KBr) 3300, 3180 (SO_2NH_2), 1625, 1600 1580, 1560 (C=N and C=C), 1350, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.56 (s, 3H, CH_3), 6.89 (d, $J = 4.9$ Hz, 1H, H-5, pyrid.), 7.53 (d, $J = 8.3$ Hz, 1H, H-3, quinoline), 7.68 (t, $J = 7.0$ Hz, 1H, H-6, quinoline), 7.82 (d, $J = 7.0$ Hz, 1H, H-5 quinoline), 7.97 (br s, 3H, SO_2NH_2 and H-7, quinoline), 8.41 (d, $J = 8.3$ Hz, 1H, H-4, quinoline), 8.53 (d, $J = 4.9$ Hz, 1H, H-6, pyrid.), 8.90 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{15}H_{13}N_3O_3S$) C, H, N.

4.1.3. Procedure for the preparation of 4-heteroarylthiopyridine-3-sulfonamides (**11**–**14**)

A mixture of 4-chloropyridine-3-sulfonamide **1** (1.93 g, 10 mmol) and the corresponding heteroarylthiol (12 mmol) in acetonitrile (25 ml) was stirred at room temperature for 2 h, followed by reflux for 24 h. After cooling to room temperature the resulting suspension was left overnight. The precipitate of the adequate crude hydrochloride of **11**–**14** was collected by filtration, washed with acetonitrile (3×2 ml) and dried, then treated with water (10 ml). The resulting suspension (pH 1) was slowly adjusted to pH 8 with 1% solution of NaOH in water. After stirring at room temperature for 3 h, the precipitate of the adequate sulfonamides **11**–**14** was filtered off, washed successively with water (4×1.5 ml) and acetonitrile (3×1.5 ml), and dried at temperatures gradually increasing 100 °C. In this manner the following sulfonamides were obtained.

*4.1.3.1. 4-(N-Oxide-2-pyridylthio)pyridine-3-sulfonamide (**11**).* Starting from 2-mercapto- pyridine *N*-oxide (1.53 g), the title compound **11** was obtained (2.3 g, 81%); m.p. 183–185 °C dec.; IR (KBr) 3190, 3100 (SO_2NH_2), 1635, 1570 (C=N and C=C), 1345, 1170 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.28 (d, $J = 5.2$ Hz, 1H, H-5, pyridinesulfonamide), 7.34–7.49 (m, 3H, H-3, H-4 and H-5, pyridine *N*-oxide), 7.84 (s, 2H, SO_2NH_2), 8.43 (d, $J = 6.2$ Hz, 1H, H-6, pyridine *N*-oxide), 8.64 (d, $J = 5.2$ Hz, 1H, pyridinesulfonamide), 9.04 (s, 1H, H-2, pyridinesulfonamide) ppm; ^{13}C NMR (DMSO-d_6) δ 125.66, 125.96, 127.12, 129.03, 139,17, 139.42, 141.41, 145.23, 148.45, 152.77 ppm. Anal. ($C_{10}H_9N_3O_3S_2$) C, H, N.

*4.1.3.2. 4-(1-Methyl-5-tetrazolylthio)pyridine-3-sulfonamide (**12**).* Starting from 5-mercapto-1-methyltetrazole (1.4 g), the title compound **12** was obtained (2.3 g, 84%); m.p. 189–191 °C dec.; IR (KBr) 3360, 3135 (SO_2NH_2), 1570, 1540 (C=N and C=C), 1340, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.07 (s, 3H, CH_3), 6.84 (d, $J = 5,5$ Hz, 1H, H-5), 8.07 (s, 2H, SO_2NH_2), 8.54 (d, $J = 5,5$ Hz, 1H, H-6), 8.97 (s, 1H, H-2) ppm. Anal. ($C_7H_8N_6O_2S_2$) C, H, N.

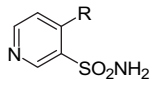
*4.1.3.3. 4-(1,3,4-Thiadiozol-2-ylthio)pyridine-3-sulfonamide (**13**).* Starting from 2-mercapto-1,3,4-thiadiazole (1.42 g), the title compound **13** was obtained (2.1 g, 76%); m.p. 163–164 °C dec.; IR (KBr) 3265, 3165 (SO_2NH_2), 1570, 1530 (C=N and C=C), 1335, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.11 (d, $J = 5,2$ Hz, 1H, H-5 pyrid.), 8.01 (s, 2H, SO_2NH_2), 8.57 (dd, $J_{ortho} = 5.2$ Hz, $J_{meta} = 1.7$ Hz, 1H, H-6, pyrid.), 8.96 (d, $J_{meta} = 1.7$ Hz, 1H, H-2, pyrid.), 9.92 (s, 1H, H-5, thiadiazole) ppm. Anal. ($C_7H_6N_4O_2S_3$) C, H, N.

*4.1.3.4. 4-(6-Nitrobenzothiazol-2-ylthio)pyridine-3-sulfonamide (**14**).* Starting from 2-mercapto-6-nitrobenzothiazole (2.55 g), the title compound **14** was obtained (2.8 g, 76%); m.p. 263–265 °C dec.; IR (KBr) 3235, 3135 (SO_2NH_2), 1570, 1515 (C=N and C=C), 1340, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.81 (d, $J = 3.9$ Hz, 1H, H-5, pyrid.), 8.04 (s, 2H, SO_2NH_2), 8.20 (d, $J = 8.3$ Hz, 1H, H-4, 6-nitrobenzothiazole), 8.37 (d, $J = 8.3$ Hz, 1H, 6-nitrobenzothiazole), 8.74 (d, $J = 3.9$ Hz, 1H, H-6, pyrid.), 9.18 (s, 1H, H-2, pyrid.), 9.23 (s, 1H, H-7, 6-nitrobenzothiazole) ppm. Anal. ($C_{12}H_8N_4O_4S_3$) C, H, N.

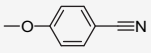
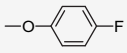
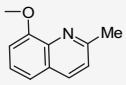
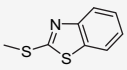
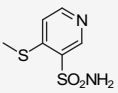
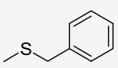
4.1.4. Synthesis of 4-methylthio-3-pyridinesulfonamide hydrochloride (**15**) and 4,4′-thiodipyridine-3-sulfonamide (**16**) by the reaction of 4-chloro-3-pyridinesulfonamide with thiouera in boiling methanol

A mixture of 4-chloro-3-pyridinesulfonamide **1** (4.81 g, 0.025 mol) and thiourea (1.92 g, 0.0252 mol) in methanol (15 ml) was stirred at reflux for 5 h. After cooling to room temperature the

Table 1
Carbonic anhydrase inhibition data for compounds **4**–**7**, **9**–**14**, **16**, **18**–**27** and **31**–**33** and standard inhibitors against human isozymes hCA I, II, IX, XII and XIV, by a stopped-flow, CO_2 hydration assay [26].

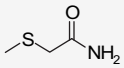
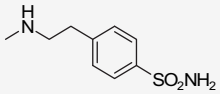
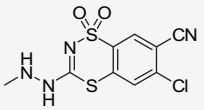
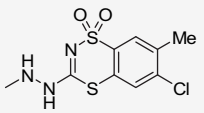
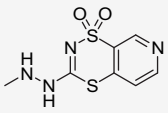


4-7, 9-14, 16, 18-27 and **31-33**

Compound	R	K_I[a]				
		hCA I[b] (μM)	hCA II[b] (nM)	hCA IX[c] (nM)	hCA XII[c] (nM)	hCA XIV[d] (nM)
AAZ		0.31	12	25	5.7	41
MZA		0.78	14	27	3.4	43
EZA		0.025	8	34	22	25
DCP		1.20	38	50	50	345
IND		0.031	15	24	3.4	104
4	–OMe	7.34	93.8	5.7	5.8	NT
5	–OEt	6.10	76.7	300.0	5.9	52.2
6		6.64	94.5	7.2	6.3	NT
7		9.16	94.4	7.9	6.9	NT
9		10.32	105.0	9.1	6.8	59.7
10		10.32	80.4	313.0	6.7	70.0
11		7.62	84.5	7.5	13.8	NT
12		6.53	45.8	4.6	4.2	NT
13		6.67	56.9	7.7	5.1	NT
14		8.84	94.8	9.6	21.6	NT
16		11.17	65.0	47.3	4.7	160.0
18	–S–Me	9.03	40.1	47.0	6.7	98.3
19		11.71	91.1	11.7	15.5	140.0
20		8.32	108.0	6.3	15.6	56.4
21		9.09	9.9	30.4	6.5	68.2

(*continued on next page*)

Table 1 (*continued*)

Compound	R	K_I[a]				
		hCA I[b] (μM)	hCA II[b] (nM)	hCA IX[c] (nM)	hCA XII[c] (nM)	hCA XIV[d] (nM)
22		9.51	131.0	11.8	15.9	142.0
23		8.86	133.0	12.0	16.9	143.0
24		10.30	140.0	7.8	15.7	152.0
25		5.62	87.2	15.8	8.9	95.7
26		7.91	85.6	4.9	10.1	93.4
27		1.52	52.0	7.5	15.1	149.0
31		0.078	88.3	7.2	3.4	92.3
32		0.101	46.1	5.8	8.1	50.9
33		8.90	117.0	16.1	4.6	83.7

[a] Errors in the range of ±5–10% of the reported value (from 3 different assays).
[b] Human (cloned) isozymes, by CO_2 hydration method.
[c] Catalytic domain of human, cloned isozymes [16,27], by the CO_2 hydration method.
[d] Full-length human (cloned) isozyme [30].

suspension was left overnight. The precipitate of title compound **16** was filtered off, washed with methanol (3 × 1 ml) and dried. Yield 2.3 g (53%), m.p. 216–218 °C dec.; IR (KBr) 3300, 3205 (SO_2NH_2), 1635, 1560 (C=N and C=C), 1335, 1160 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.25 (d, J = 5,5 Hz, 2H, H-5 and H-5′), 7.87 (s, 4H, 2× SO_2NH_2), 8.65 (d, J = 5,5 Hz, 2H, H-6 and H-6′), 9.16 (s, 2H, H-2 and H-2′) ppm. Anal. ($C_{10}H_{10}N_4O_4S_3$) C, H, N.

The methanol–filtrate mixture was evaporated to 1/3 volume at diminished pressure. After cooling to room temperature the suspension was left overnight. The precipitate of compound **15** was filtered off, washed with methanol (2 × 1 ml) and dried. Yield 1.1 g (18%), m.p. 238–239 °C dec.; IR (KBr) 3285, 3170 (SO_2NH_2), 2715, 2520, 2325, 2030 (NH^+), 1620, 1585, 1530 (pyridine ring), 1335, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.69 (s, 3H, CH_3), 7.80 (d, J = 6.2 Hz, 2H, H-5), 8.03 (s, 2H, SO_2NH_2), 8.73 (d, J = 6.2 Hz, 1H, H-6), 8.86 (s, 1H, H-2), 11.75 (br s, 1H, NH^+) ppm; ^{13}C NMR (DMSO-d_6) δ 15.06, 121.87, 136.81, 141.29, 144.68, 158.41 ppm. Anal.

4.1.5. 2-(3-Sulfamoyl-4-pyridyl)thiouronium chloride (**17**)

A suspension of **1** (9.63 g, 0.05 mol) and thiourea (3.88 g, 0.051 mol) in acetonitrile (85 ml) was stirred at reflux for 3 h and left to stand at room temperature overnight. The precipitate was filtered off, washed with acetonitrile (4 × 4 ml) and dried. Yield 13.2 g (98%), m.p. 149–151 °C dec.; IR (KBr) 3540, 3355, 3200, 3160 (SO_2NH_2 and C–NH_2), 2990, 2720, 2630 (NH^+), 1650, 1565, 1535 (NH_2 and pyridine ring), 1335, 1165 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.80 (d, J = 5,4 Hz, 1H, H-5), 8.02 (s, 2H,

SO_2NH_2), 8.81 (d, $J = 5{,}4$ Hz, 1H, H-6), 9.08 (s, 1H, H-2), 9.63 and 9.71 [2s, 2× 2H, S–C(NH_2)$_2$] ppm. Anal. ($C_6H_9ClN_4O_2S_2$) C, H, N.

*4.1.6. Procedure for the preparation of 4-(R-methylthio)pyridine-3-sulfonamides (**18**–**22**)*

To an ice-cold solution of NaOH (0.6 g, 15 mmol) in water (3 ml) and tetrahydrofuran (15 ml) was added with stirring thiouronium chloride **17** (1.88 g, 7 mmol) and solution of the appropriate alkyl halide (8 mmol) in tetrahydrofuran (8 ml). After 0.5 h the ice bath was removed and the reaction mixture was stirred at room temperature for 5 h. The precipitate of the adequate sulfides obtained was filtered off, washed successively with water (6 × 2 ml) and methanol (3 × 1.5 ml), and dried. In this manner the following sulfides were obtained.

*4.1.6.1. 4-Methylthiopyridine-3-sulfonamide (**18**).* Starting from methyl iodide (1.14 g), the title compound **18** was obtained (1.26 g, 88%); m.p. 222–223 °C; IR (KBr) 3295, 3160 (SO_2NH_2), 1570$_{vs}$ (C=N and C=C), 1345, 1165 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.55 (s, 3H, CH_3S), 7.45 (d, $J = 5{,}5$ Hz, 1H, H-6), 7.60 (s, 2H, SO_2NH_2), 8.53 (d, $J = 5{,}5$ Hz, 1H, H-6), 8.79 (s, 1H, H-2) ppm. Anal. ($C_6H_8N_2O_2S_2$) C, H, N.

*4.1.6.2. 4-(Cyanomethylthio)pyridine-3-sulfonamide (**19**).* Starting from bromoacetonitrile (0.96 g), the title compound **19** was obtained (1.3 g, 81%); m.p. 208–209 °C dec.; IR (KBr) 3340, 3170 (SO_2NH_2), 2250 (C≡N), 1570$_{vs}$ (C=N and C=C), 1340, 1170 (SO_2NH_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.53 (s, 2H, CH_2), 7.61 (d, $J = 5{,}4$ Hz, 1H, H-5), 7.84 (s, 2H, SO_2NH_2), 8.71 (d, $J = 5{,}4$ Hz, 1H, H-6), 8.91 (s, 1H, H-2) ppm. Anal. ($C_7H_7N_3O_2S_2$) C, H, N.

*4.1.6.3. 4-(2-Propynylthio)pyridine-3-sulfonamide (**20**).* Starting from propargyl bromide (0.95 g), the title compound **20** was obtained (1.3 g, 81%); m.p. 210–211 °C dec.; IR (KBr) 3335, 3260 (SO_2NH_2), 2120 (C≡CH), 1570$_{vs}$ (C=N and C=C), 1340, 1165 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.34 (s, 1H, C≡CH), 4.08 (s, 2H, CH_2), 7.54 (d, $J = 5{,}5$ Hz, 1H, H-5), 7.70 (s, 2H, SO_2NH_2), 8.59 (d, $J = 5{,}5$ Hz, 1H, H-2), 8.84 (s, 1H, H-2) ppm; ^{13}C NMR (DMSO-d_6) δ 19.11, 74.94, 79.14, 120.56, 135.98, 146.96, 151.64 ppm. Anal. ($C_8H_8N_2O_2S_2$) C, H, N.

*4.1.6.4. 4-Benzythiopyridine-3-sulfonamide (**21**).* Starting from benzyl bromide (1.37 g), the title compound **21** was obtained (1.6 g, 81%); m.p. 229–230 °C; IR (KBr) 3380, 3170 (SO_2NH_2), 1575$_{vs}$ (C=N and C=C), 1335, 1165 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.43 (s, 2H, CH_2), 7.30 (t, $J = 7.3$ Hz, 1H, H-4, Ph), 7.37 (t, $J = 7.3$ Hz, 2H, H-3 and H-5, Ph), 7.51 (d, $J = 7.8$ Hz, 2H, H-2 and H-6, Ph), 7.58 (d, $J = 5.4$ Hz, 1H, H-5, pyrid.), 7.66 (s, 2H, SO_2NH_2), 8.53 (d, $J = 5.4$ Hz, 1H, H-6, pyrid.), 8.82 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{12}H_{12}N_2O_2S_2$) C, H, N.

*4.1.6.5. 4-(Carbamolymethylthio)pyridine-3-sulfonamide (**22**).* Starting from 2-bromoacetamide (1.1 g), the title compound **22** was obtained (1.4 g, 81%); m.p. 198–200 °C dec.; IR (KBr) 3400, 3315, 3170 (NH_2), 1690, 1660 (C=O), 1635 (C=N) 1330, 1160 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.86 (s, 2H, CH_2), 7.33 (s, 1H, $CONH_A$), 7.50 (d, $J = 4.4$ Hz, 1H, H-5), 7.65 (s, 2H, SO_2NH_2), 7.70 (s, 1H, $CONH_B$), 8.56 (d, $J = 4.4$ Hz, 1H, H-6), 8.82 (s, 1H, H-2) ppm. Anal. ($C_7H_9N_3O_3S_2$) C, H, N.

*4.1.7. Procedures for the preparation of 4-(amino and hydrazino)-3-pyridinesulfonamide derivatives (**23**–**27**), (Methods A, B and C)*

*Method A (for compounds **23** and **24**).* To a solution of allylamine (2.85 g, 50 mmol) or 5-methyl-2-pyrazoline (4.2 g, 50 mmol) in methanol (20 ml) 4-chloro-3-pyridinesulfonamide **1** (1.93 g, 10 mmol) was added. The reaction mixture was stirred at reflux for 7 h. The solvent and excess of amine was evaporated under reduced pressure. To the resulting oily residue water (25 ml) was added dropwise and stirred at room temperature for 4 h. The precipitate thus obtained was collected by filtration, washed with water (5 × 4 ml) and methanol (3 × 1 ml), and dried.

*4.1.7.1. 4-(Allylamino)-3-pyridinesulfonamide (**23**).* Starting from allylamine the title compound **23** was obtained (1.8 g, 84%); m.p. 150–151 °C; IR (KBr) 3370, 3305 (SO_2NH_2), 1600, 1560, 1520 (C=N and C=C), 1340, 1145 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.85–4.00 (m, 2H, NCH_2–CH=CH_2), 5.12–5.25 (m, 2H, NCH_2–CH=CH_2), 5.79–5.98 (m, 1H, NCH_2–CH=CH_2), 6.62 (t, $J = 5.4$ Hz, 1H, NH–CH_2), 6.68 (d, $J = 5.9$ Hz, 1H, H-5), 7.54 (s, 2H, SO_2NH_2), 8.19 (d, $J = 5.9$ Hz, 1H, H-6), 8.50 (s, 1H, H-2) ppm; ^{13}C NMR (DMSO-d_6) δ 44.36, 107.04, 116.31, 122.03, 134.26, 148.63, 149.47, 152.53 ppm. Anal. ($C_8H_{11}N_3O_2S$) C, H, N.

*4.1.7.2. 4-(5-Methyl-2-pirazolino)-3-pyridinesulfonamide (**24**).* Starting from 5-methyl-2-pirazoline the title compound **24** was obtained (2.0 g, 83%); m.p. 206–207 °C; IR (KBr) 3295, 3110 (SO_2NH_2), 1590, 1525 (C=N and C=C), 1340, 1155 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.13 (d, $J = 6.1$ Hz, 3H, CH_3), 2.60–2.69 (m, 1H, H_A-4, pyrazoline), 3.11–3.25 (m, 1H, H_B-4, pyrazoline), 4.67 (sext, 1H, H-5, pyrazoline), 7.01 (d, $J = 5.8$ Hz, 1H, H-5, pyrid.), 7.20 (s, 2H, SO_2NH_2), 7.24 (s, 1H, N=CH, pyrazoline), 8.34 (d, $J = 5.8$ Hz, 1H, H-6, pyrid.), 8.82 (s, 1H, H-2 pyrid.) ppm.; ^{13}C NMR (DMSO-d_6) δ 18.60, 41.28, 53.97, 110.60, 125.79, 146.23, 146.62, 150.59, 152.44 ppm. Anal. ($C_9H_{12}N_4O_2S$) C, H, N.

*Method B (for compounds **25** and **26**).* To a solution of methylhydrazine (2.8 g, 60 mmol) in methanol (10 ml) 4-chloro-3-pyridinesulfonamide (1.93 g, 10 mmol) was added. The reaction mixture was stirred at room temperature for 16 h, followed by reflux for 4 h. After cooling to room temperature the precipitate was collected by filtration, washed with methanol (4 × 2 ml) and dried at temperatures gradually increasing to 100 °C.

*4.1.7.3. 4-(Methylhydrazino)-3-pyridinesulfonamide (**25**)*

Starting from methylhydrazine the title compound **25** was obtained (1.6 g, 79%); m.p. 211–212 °C dec.; IR (KBr) 3335, 3285, 3245, 3185 (SO_2NH_2 and NH–NH), 1635, 1585 (C=N and C=C), 1320, 1150 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.18 (s, 3H, NCH_3), 5.13 (s, 2H, HN–NH), 6.76 (d, $J = 5.9$ Hz, 1H, H-5), 7.27 (s, 2H, SO_2NH_2), 8.24 (d, $J = 5.9$ Hz, 1H, H-6), 8.74 (s, 1H, H-2) ppm. Anal. ($C_6H_{10}N_4O_2S$) C, H, N.

*4.1.7.4. 4-Hydrazino-3-pyridinesulfonamide (**26**)*

Starting from hydrazine hydrate the title compound **26** was obtained (1.6 g, 85%); m.p. 202–203 °C dec.; IR (KBr) 3365, 3315, 3270, 3165 (SO_2NH_2 and NH–NH_2), 1635, 1595 (C=N and C=C), 1315, 1135 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.69 (s, 2H, N–NH_2), 7.22 (d, $J = 5.9$ Hz, 1H, H-5), 7.52 (s, 3H, SO_2NH_2 and NH–N), 8.24 (s, $J = 5.9$ Hz, 1H, H-6), 8.46 (s, 1H, H-2) ppm. Anal. ($C_5H_8N_4O_2S$) C, H, N.

*4.1.7.5. 4-[2-(4-Sulfamoylphenyl)ethylamino]-3-pyridinosulfonamide (**27**) (Method C).*

A solution of triethylamine (2.13 g, 21 mmol), 4-(2-aminoethyl) benzenesulfonamide (2.0 g, 10 mmol) and 4-chloro-3-pyridinesulfonamide (1.93 g, 10 mmol) in methanol (25 ml) was stirred at reflux for 8 h. After cooling to room temperature water (40 ml) was added and stirred for 3 h. The crude product **27** was collected by filtration, washed with water, dried and purified by crystallization from methanol. Yield 2.6 g (73%), m.p. 249–251 °C dec.; IR (KBr) 3415, 3335, 3255 (SO_2NH and NH), 1600, 1570 (C=N and C=C), 1350, 1320, 1150 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.96 (t, $J = 4.9$ Hz, 2H, CH_2Ph), 3.52 (t, $J = 4.9$ Hz, 2H, N–CH_2), 6.50 (s, 1H, NH), 6.83 (d, J = 5.6 Hz, 1H, H-5, pyrid.), 7.36 (s, 4H, 2× SO_2NH_2), 7.47 (d, $J = 7.6$ Hz, 2H, H-3 and H-5, $PhSO_2$), 7.75 (d, $J = 7.6$ Hz, 2H,

H-2 and H-6, $PhSO_2$), 8.21 (d, $J = 5.6$ Hz, 1H, H-6, pyrid.), 8.49 (s, 1H, H-2, pyrid.) ppm. Anal. ($C_{13}H_{16}N_4O_4S_2$) C, H, N.

*4.1.8. Procedure for the preparation of 4-(N′-heteroarylhydrazino)-3-pyridinosulfonamides (**31**–**33**)*

A mixture of **26** (0.76 g, 4 mmol) and the appropriate 3-methylthia-1,4,2-ditiazine **28**, **29** or **30** (4 mmol) in anhydrous methanol (35 ml) was stirred at reflux until the evolution of MeSH had ceased (14–36 h) (Caution: because of high toxicity, MeSH should be trapped in aqueous NaOH solution). After cooling to room temperature the precipitate was collected by filtration, washed with methanol (4×2 ml) and dried. In this manner the following 3-pyridinesulfonamides were obtained.

*4.1.8.1. 4-[N′-(6-Chloro-7-cyano-1,1-dioxo-1,4,2-benzodithiazin-3-yl) hydrazino]-3-pyridinesulfonamide (**31**).* Starting from 6-chloro-7-cyano-3-methylthio-1,4,2-benzodithiazine 1,1-dioxide **28** (1.22 g), the title compound **31** was obtained (1.6 g, 89%); m.p. 390–393 °C dec.; IR (KBr) 3340, 2280, 3210 (SO_2NH_2 and HN–NH), 2225 (C≡N), 1650, 1580 (C═N and C═C), 1360, 1150 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.42 (d, $J = 7.1$ Hz, 1H, H-5, pyridine), 8.03 (s, 1H, H-5, benzodithiazine), 8.09 (s, 2H, SO_2NH_2), 8.18 (d, $J = 7.1$ Hz, 1H, H-6, pyridine), 8.27 (s, 1H, H-8, benzodithiazine), 8.50 (s, 1H, H-2, pyridine), 10.80 (s, 1H, NH), 13.31 (br.s, 1H, NH) ppm. Anal. ($C_{13}H_9ClN_6O_4S_3$) C, H, N.

*4.1.8.2. 4-[N′-(6-Chloro-7-methyl-1,1-dioxo-1,4,2-benzodithiazin-3-yl)hydrazyno]-3-pyridinesulfonamide (**32**).* Starting from 6-chloro-7-methyl-3-methylthio-1,4,2-benzodithiazine 1,1-dioxide **29** (1.18 g), the title compound **32** was obtained (1.6 g, 92%); m.p. 321–322 °C dec.; IR (KBr) 3425, 3340, 3260, 3210 (SO_2NH_2 and HN–NH), 1650, 1570 (C═N and C═C), 1345, 1320, 1140 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.40 (s, 3H, CH_3), 7.43 (d, $J = 7.4$ Hz, 1H, H-5, pyridine), 7.65 (s, 1H, H-5, benzodithiazine), 7.84 (s, 1H, H-8, benzodithiazine), 8.10 (s, 3H, SO_2NH_2 and NH), 8.17 (d, $J = 7.4$ Hz, 1H, H-6, pyridine), 8.49 (s, 1H, H-2, pyridine), 10.94 (s, 1H, NH) ppm. Anal. ($C_{13}H_{12}ClN_5O_4S_3$) C, H, N.

*4.1.8.3. 4-[N′-(1,1-Dioxopyrido[4,3-e]-1,4,2-dithiazin-3-yl)hydrazino]-3-pyridinesulfonamide (**33**).* Staring from 3-methylthiopyrido[4,3-*e*]-1,4,2-dithiazine 1,1-dioxide **30** (0.99 g), the title compound **33** was obtained (1.4 g, 90%); m.p. 302–303 °C dec.; IR (KBr) 3370, 3270, 3220 (SO_2NH_2 and NH), 1665, 1620, 1580 (C═N and C═C), 1360, 1320, 1150 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.20 (s, 1H, NH), 7.45 (d, $J = 7.1$ Hz, 1H, H-5, pyridine), 7.59 (d, $J = 5.1$ Hz, 1H, H-5, pyridodithiazine), 8.11 (s, 2H, SO_2NH_2), 8.21 (d, $J = 7.1$ Hz, 1H, H-6, pyridine), 8.53 (s, 1H, H-2, pyridine), 8.60 (d, $J = 5.1$ Hz, H-6, pyridodithiazine), 8.88 (s, 1H, H-8, pyridodithiazine), 10.94 (s, 1H, NH) ppm. Anal. ($C_{11}H_{10}N_6O_4S_3$) C, H, N.

4.2. CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity [26]. Phenol red (at a concentration of 0.2 mM) has been 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 to 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 E–I complex. The inhibition constants were obtained by non-linear last-squares methods using PRISM 3, as reported earlier [16,27–30], and represent the mean from at least three different determinations.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2010.02.020.

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