Supporting Information

Discovery of new selenoureido analogs of 4-(4-fluorophenylureido)benzenesulfonamide as carbonic anhydrase inhibitors

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Experimental protocols.

Chemistry. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR, ^{19}F NMR, ^{77}Se-NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doublets. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230-400 mesh ASTM) as the stationary phase and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. HPLC was performed by using a Waters 2690 separation module coupled with a photodiode array detector (PDA Waters 996) and as column a Nova-Pak C18 4 μm 3.9 mm × 150 mm (Waters), silica-based reverse phase column. Sample was dissolved in acetonitrile 10%, and an injection volume of 45 μL was used. The mobile phase, at a flow rate of 1 mL/min, was a gradient of water + trifluoroacetic acid (TFA) 0.1% (A) and acetonitrile + TFA 0.1% (B), with steps as follows: (A%:B%), 0−10 min 90:10, 10−25 min gradient to 60:40, 26:28 min isocratic 20:80, 29−35 min isocratic 90:10. TFA 0.1% in water as well in acetonitrile was used as counterion. All compounds reported here were >96% HPLC pure. The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), purchased from Sigma-Aldrich (Milan - Italy), and mQ water 18 MΩ, obtained from Millipore's Simplicity system (Milan-Italy). The high resolution mass spectrometry analysis (HRMS) were performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with

an electrospray ionization source (ESI). The analysis were carried out in positive ion mode observing the cluster of the protonated molecules $[M+H]^+$ using a proper dwell time acquisition to achieve 60,000 units of resolution at Full Width at Half Maximum (FWHM). The elemental composition of each compound was formulated on the basis of the measured accurate mass of the most intense signal of the considered cluster. They were accepted only results with an attribution error Delta less than 5 ppm and a not integer RDB (double bond/ring equivalents) value, in order to consider only the protonated species. Stock solutions of analytes were prepared in acetone at 1.0 mg mL^{-1} and stored at 4°C. Working solutions of each analyte were freshly prepared by diluting stock solutions in a mixture of mQ H_2O/ACN 1/1 (v/v) up to a concentration of 1.0 μg mL^{-1} The mass spectra of each analyte were acquired by introducing, via syringe pump at 10 μL min^{-1}, of the its working solution.

General procedure for the synthesis of *N*-formyl compounds 2a-i. [16] The appropriate aromatic amine (**1a-i**) (1.0 mmol) and anhydrous $ZnCl_2$ (0.1 mmol) were placed under N_2 atmosphere and treated dropwise with formic acid (3.0 mmol) with constant stirring for 10 min. This mixture was heated at 70°C and the progress of reaction was monitored by TLC. When the reaction was completed, the mixture was cooled to r.t. and diluted with ethyl acetate. The organic layer was washed with a saturated solution of Na_2CO_3, water, brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the resulting crude product was purified by silica gel column chromatography to obtain the titled *N*-formyl derivate (**2a-i**). Experimental were in agreement with reported data. [32]

General procedure for the synthesis of isoselenocyanate 3a-i. [17-20] The appropriate formamide **2a-i** (1.5 mmol) was dissolved in DCM (5 mL) and treated with Et_3N (6.4 mmol) and 4Å molecular sieves. Then a solution of triphosgene (0.8 mmol) in DCM (2 mL) was added drop-wise for a period of 1 h followed by reflux for 2.5 h. Then selenium powder (3.0 mmol) was added and the resulting

mixture was refluxed for 4-7 h; conventional work-up and silica gel column chromatography afforded the titled isoselenocyanate **3a-i**. Experimental were in agreement with reported data. [17-20]

General procedure for the synthesis of selenoureido derivatives 7-21. [3] The appropriate isoselenocyanate **3a-i** (1.0 mmol) was dissolved in ACN (5 mL) and treated with the corresponding benzenesulfonamide **4-6** (1.0 mmol). The mixture was stirred overnight at r.t, quenched with H_2O and the readily formed precipitate was collected by filtration and dried on air to afford the titled selenourea **7-21**.

3-(3-Phenylselenoureido)benzenesulfonamide **7** was obtained according to the above reported general procedure using compound **3a** (0.09 g, 0.5 mmol). Yield 53%, 0.094 g; orange solid, M.p.180-183°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.44 (1H, brs, N*H*, exchange with D_2O), 10.33 (1H, brs, N*H*, exchange with D_2O),7.43-7.38 (4H, m), 7.42 (2H, brs, NH_2, exchange with D_2O), 7.24 (1H, t, *J*= 6.8), 6.98 (2H, d, *J*= 6.8), 6.40 (2H, d, *J*= 6.8); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.2 (*C*=Se), 145.2, 141.4, 130.8, 1329.8, 129.7, 129.2, 126.4, 125.5, 125.3, 123.0, 122.6; HRMS *m/z* $[M+H]^+$ calcd for C13H14N3O2SSe, 355.9966; found, 355.9972.

3-(3-(4-Fluorophenyl)selenoureido)benzenesulfonamide carboxamide **8** was obtained according to the above reported general procedure using compound **3b** (0.1 g, 0.5 mmol). Yield 52%, 0,097 g; white solid, M.p. 187-190°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.38 (1H, brs, N*H*, exchange with D_2O), 10.31 (1H, brs, N*H*, exchange with D_2O), 7.86 (1H, s), 7.71 (1H, d, *J*=7.96),7.65 (1H, d, *J*=7.91), 7.55 (1H, t, *J*=7.87), 7.45-7.42 (2H, m), 7.44 (2H, brs, NH_2, exchange with D_2O), 7.24 (2H, apt); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.6 (*C*=Se), 160.7 (d, *J*= 242.32), 145.2, 141.3, 136.7, 129.9, 129.2, 128.1 (d, *J*= 8.44), 123.1, 122.7, 116.3 (d, *J*= 22.75); ^{19}F-NMR (DMSO-d_6, 376 MHz): -116.68; HRMS *m/z* $[M+H]^+$ calcd for C13H13FN3O2SSe, 373.9872; found, 373.9866.

4-(3-Phenylselenoureido)benzenesulfonamide **9** was obtained according to the above reported general procedure using compound **3a** (0.09 g, 0.5 mmol). Yield 55%, 0.098 g; white solid, M.p. 181-183°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.50 (1H, brs, N*H*, exchange with D_2O), 10.40 (1H, brs, N*H*, exchange with D_2O), 7.79 (2H, d, *J*=8.54), 7.65 (2H, d, *J*=8.51), 7.46-7.38 (4H, m), 7.36 (2H, brs, NH_2, exchange with D_2O), 7.24 (1H, t, *J*=7.12); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.1 (*C*=Se), 143.8, 140.8, 140.4, 129.5, 127.0, 126.3, 125.4, 124.9; HRMS *m/z* $[M+H]^+$ calcd for C13H14N3O2SSe, 355.9966; found, 355.9975.

4-(3-(4-Fluorophenyl)selenoureido)benzenesulfonamide **10** was obtained according to the above reported general procedure using compound **3b** (0.1 g, 0.5 mmol). Yield 53%, 0.099 g; white solid, M.p. 187-190°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.38 (2H, brs, N*H*, exchange with D_2O), 7.80 (2H, d, *J*= 8.65), 7.64 (2H, d, *J*=8.35), 7.46 (2H, m), 7.35 (2H, brs, NH_2, exchange with D_2O), 7.23 (2H, apt); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.4 (*C*=Se), 160.7 (d, *J*= 242.58), 143.8, 140.9, 136.9, 128.1 (d, *J*= 7.90), 127.1, 125.0, 116.2 (d, *J*= 22.76); ^{19}F-NMR (DMSO-d_6, 376 MHz): -116.83; HRMS *m/z* $[M+H]^+$ calcd for C13H13FN3O2SSe, 373.9872; found, 373.9868.

4-(3-(2-Fluorophenyl)selenoureido)benzenesulfonamide **11** was obtained according to the above reported general procedure using compound **3c** (0.1 g, 0.5 mmol). Yield 72%, 0,134 g; white solid, M.p. 188-191°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.50 (1H, brs, N*H*, exchange with D_2O), 10.20 (1H, brs, N*H*, exchange with D_2O), 7.83 (2H, d, *J*= 8.66), 7.67 (2H, d, *J*=8.65), 7.49 (1H, td, *J*= 7.85, J^3= 1.58), 7.38 (2H, brs, NH_2, exchange with D_2O), 7.33 (2H, m), 7.25 (1H, td, *J*= 7.67, J^3= 1.52); ^{13}C-NMR (DMSO-d_6, 100 MHz): 181.5 (*C*=Se), 157.7 (d, *J*= 247.36), 143.6, 141.1, 130.3, 127.1, 125.4, 125.3, 117.0 (d, *J*= 19.80); ^{19}F-NMR (DMSO-d_6, 376 MHz): -120.49; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 295; HRMS *m/z* $[M+H]^+$ calcd for C13H13FN3O2SSe, 373.9872; found, 373.9865.

4-(3-(3-(Trifluoromethyl)phenyl)selenoureido)benzenesulfonamide **12** was obtained according to the above reported general procedure using compound **3d** (0.125 g, 0.5 mmol). Yield 62%, 0.131 g; white solid, M.p. 195-198°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.65 (2H, brs, N*H*, exchange with D_2O), 7.90 (1H, s), 7.83 (2H, d, *J*= 8.67), 7.78 (1H, d, *J*= 7.92), 7.76 (2H, d, *J*= 8.70), 7.59 (2H, m),7.39 (2H, brs, NH_2, exchange with D_2O); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.6 (*C*=Se), 143.4, 141.7, 141.1, 130.6, 130.2, 129.9, 129.5, 127.3, 126.3, 125.0, 123.6, 122.6, 122.2; ^{19}F-NMR (DMSO-d_6, 376 MHz): -61.18; HRMS *m/z* $[M+H]^+$ calcd for C14H13F3N3O2SSe, 423.9840; found, 423.9833.

4-(3-(2-Chlorophenyl)selenoureido)benzenesulfonamide **13** was obtained according to the above reported general procedure using compound **3e** (0.108 g, 0.5 mmol). Yield 50%, 0.097 g; yellow solid, M.p. 198-201°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.47 (1H, brs, N*H*, exchange with D_2O), 10.23 (1H, brs, N*H*, exchange with D_2O), 7.83 (2H, d, *J*= 8.32), 7.70 (2H, d, *J*= 8.27), 7.58 (1H, d, J= 7.28), 7.52 (1H, d, *J*= 7.14), 7.39 (2H, brs, NH_2, exchange with D_2O), 7.40 (2H, m); ^{13}C-NMR (DMSO-d_6, 100 MHz): 181.1 (*C*=Se), 143.4, 141.1, 137.9, 131.9, 131.3, 130.6, 129.4, 128.4, 127.1, 125.3; HRMS *m/z* $[M]^+$ calcd for C13H12ClN3O2SSe, 388.9574 ; found, 388.9581.

4-(2-(3-Phenylselenoureido)ethyl)benzenesulfonamide **14** was obtained according to the above reported general procedure using compound **3a** (0.09 g; 0.5 mmol). Yield 60%, 0.115 g; white solid, M.p. 210-213°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.02 (1H, s, N*H*, exchange with D_2O), 8.14 (1H, brs, N*H*, exchange with D_2O), 7.81 (2H, d, *J*= 8.37), 7.48 (2H, d, *J*= 7.98), 7.39(2H, m), 7.36 (2H, brs, NH_2, exchange with D_2O), 7.22 (3H, m), 3.83 (2H, brs), 3.02 (2H, t, *J*= 7.38); ^{13}C-NMR (DMSO-d_6, 100 MHz): 179.4 (*C*=Se), 144.3, 143.1, 139.2, 130.1, 130.0, 126.7, 126.2, 124.9, 48.7, 35.2; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 216; HRMS *m/z* $[M+H]^+$ calcd for C15H18N3O2SSe, 384.0279 ; found, 384.0284.

4-(2-(3-(2-Fluorophenyl)selenoureido)ethyl)benzenesulfonamide **15** was obtained according to the above reported general procedure using compound **3c** (0.1 g, 0.5 mmol). Yield 92%, 0.184 g; white solid, M.p. 202 - 205°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 9.74 (1H, brs, N*H*, exchange with D_2O), 8.18 (1H, brs, N*H*, exchange with D_2O), 7.81 (2H, d, *J*= 8.20), 7.38 (2H, d, *J*= 8.20), 7.45 (3H, m), 7.34 (2H, brs, NH_2, exchange with D_2O), 7.25 (1H, m), 3.82 (2H, q, *J*= 5.23), 3.01 (2H, t, *J*= 7.35); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.9 (*C*=Se), 157.2 (d, *J*= 246.73), 144.2, 143.1, 130.0, 129.9, 129.1 (d, *J*= 7.72), 127.0 (d, *J*= 13.01), 126.7, 125.4, 117.2 (d, *J*= 20.14), 48.8, 35.2; ^{19}F-NMR (DMSO-d_6, 376 MHz): -121.28; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 207; HRMS *m/z* $[M+H]^+$ calcd for C15H17FN3O2SSe, 402.0185 ; found, 402.0177.

4-(2-(3-(3-(Trifluoromethyl)phenyl)selenoureido)ethyl)benzenesulfonamide **16** was obtained according to the above reported general procedure using compound **3d** (0.125 g, 0.5 mmol). Yield 65%, 0.146 g; orange solid, M.p. 202-205°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.19 (1H, brs, N*H*, exchange with D_2O), 8.48 (1H, brs, N*H*, exchange with D_2O), 7.85 (1H, s), 7.83 (2H, brs), 7.58 (2H, m), 7.51 (3H, m), 7.36 (2H, brs, NH_2, exchange with D_2O), 3.89 (2H, q, *J*= 5.70), 3.06 (2H, t, *J*= 7.25); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.4 (*C*=Se), 144.3, 143.2, 140.8, 130.9, 130.1, 128.5, 126.8, 122.1, 121.1, 48.6, 35.1; ^{19}F-NMR (DMSO-d_6, 376 MHz): -61.26; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 225; HRMS *m/z* $[M+H]^+$ calcd for C16H17F3N3O2SSe, 452.0153 ; found, 452.0146.

4-(2-(3-(4-Bromophenyl)selenoureido)ethyl)benzenesulfonamide **17** was obtained according to the above reported general procedure using compound **3f** (0.131 g, 0.5 mmol). Yield 60%, 0.138 g; white solid, M.p. 207-210°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.02 (1H, brs, N*H*, exchange with D_2O), 8.28 (1H, brs, N*H*, exchange with D_2O), 7.82 (2H, d, *J*= 8.21), 7.54 (2H, d, *J*= 8.72), 7.48 (2H, d, J= 8.12), 7.35 (2H, brs, NH_2, exchange with D_2O), 7.21 (2H, d, *J*= 8.33), 3.83 (2H, brs), 3.03 (2H, t, *J*= 7.27); ^{13}C-NMR (DMSO-d_6, 100 MHz): 179.9 (*C*=Se), 144.3, 143.1, 138.9, 132.7,

130.1, 127.0, 126.7, 118.2, 48.7, 35.0; HRMS *m/z* $[M+H]^+$ calcd for C15H17BrN3O2SSe, 461.9381 ; found, 461.9390.

4-(2-(3-(2-Chlorophenyl)selenoureido)ethyl)benzenesulfonamide **18** was obtained according to the above reported general procedure using compound **3e** (0.108 g, 0.5 mmol). Yield 73%, 0.152 g; white solid, M.p. 206 - 209°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 9.74 (1H, brs, N*H*, exchange with D_2O), 8.15 (1H, brs, N*H*, exchange with D_2O), 7.81 (2H, d, *J*= 8.20), 7.56 (1H, d, *J*= 7.57), 7.47 (3H, m), 7.38 (2H, m), 7.35 (2H, brs, NH_2, exchange with D_2O), 3.82 (2H, brs), 3.02 (2H, t, *J*= 7.14); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.9 (*C*=Se), 144.3, 143.1, 136.7, 131.3, 130.9, 130.7, 130.0, 129.2, 128.5, 126.7, 48.7, 35.3; HRMS *m/z* $[M+H]^+$ calcd for C15H17ClN3O2SSe, 417.9887; found, 417.9895.

4-(2-(3-(2-Methoxyphenyl)selenoureido)ethyl)benzenesulfonamide **19** was obtained according to the above reported general procedure using compound **3g** (0.106 g, 0.5 mmol). Yield 55%, 0.113 g; white solid, M.p. 200 - 203°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 9.42 (1H, brs, N*H*, exchange with D_2O), 7.88 (1H, brs, N*H*, exchange with D_2O), 7.80 (2H, d, *J*= 8.04), 7.46 (2H, d, *J*= 8.00), 7.41 (2H, d, *J*= 7.55), 7.33 (2H, brs, NH_2, exchange with D_2O), 7.28 (1H, t, *J*= 7.82), 7.11 (1H, d, *J*= 8.16), 6.96 (1H, t, *J*= 7.58), 3.81 (4H, m), 2.99 (2H, t, *J*= 7.29); ^{13}C-NMR (DMSO-d_6, 100 MHz): 179.5 (*C*=Se), 154.1, 144.3, 143.0, 130.0, 128.3, 128.2, 127.4, 126.7, 121.1, 113.1, 56.4, 48.6, 35.4; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 197; HRMS *m/z* $[M+H]^+$ calcd for C16H20N3O3SSe, 414.0385 ; found, 414.0391.

4-(2-(3-(4-Iodophenyl)selenoureido)ethyl)benzenesulfonamide **20** was obtained according to the above reported general procedure using compound **3h** (0.154 g, 0.5 mmol). Yield 81%, 0.206 g; white solid, M.p. 209-211°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 9.99 (1H, brs, N*H*, exchange with

D_2O), 8.27 (1H, brs, N*H*, exchange with D_2O), 7.83 (2H, d, *J*= 8.18), 7.70 (2H, d, *J*= 8.59), 7.49 (2H, d, *J*= 8.12), 7.35 (2H, brs, NH_2, exchange with D_2O), 7.09 (2H, d, *J*= 8.06), 3.84 (2H, brs), 3.03 (2H, t, *J*= 7.19); ^{13}C-NMR (DMSO-d_6, 100 MHz): 179.8 (*C*=Se), 144.3, 143.1, 139.3, 138.5, 130.1, 127.1, 126.7, 90.5, 48.7, 35.0; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 224; HRMS *m/z* $[M+H]^+$ calcd for C15H17IN3O2SSe, 509.9246 ; found, 509.9254.

4-(2-(3-(Naphthalen-2-yl)selenoureido)ethyl)benzenesulfonamide **21** was obtained according to the above reported general procedure using compound **3i** (0.116 g, 0.5 mmol). Yield 82%, 0.177 g; white solid, M.p. 208 - 210°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.19 (1H, brs, N*H*, exchange with D_2O), 8.30 (1H, brs, N*H*, exchange with D_2O), 7.92 (2H, d, *J*= 6.92), 7.85 (3H, d, *J*= 8.17), 7.74 (1H, s), 7.52 (4H,m), 7.36 (3H, brs), 3.89 (2H, brs), 3.07 (2H, t, *J*= 7.27); ^{13}C-NMR (DMSO-d_6, 100 MHz): 179.7 (*C*=Se), 144.4, 143.1, 136.8, 134.2, 131.8, 130.1, 129.6, 128.5, 128.4, 127.4, 126.7, 126.5, 124.8, 122.2, 48.8, 35.2; ^{77}Se-NMR (DMSO-d_6, 76 MHz): 221; HRMS *m/z* $[M+H]^+$ calcd for C19H20N3O2SSe, 434.0436 ; found, 434.0443.

General procedure for the synthesis of thioureido derivative 22. [3] A solution of 4-fluroroaniline **1b** (1 mmol) in H_2O was treated with thiophosgene (1.5 mmol) at r.t. The mixture was stirred for 2 h, was extracted with chloroform and the organic layers were dried over Na_2SO_4 and filtered. The solvent was evaporated under vacuo to afford the corresponding thioisocyanate which was immediately dissolved in ACN and treated with sulphanilamide **5**. The mixture was quenched with H_2O and the precipitate formed was collected by filtration, dried on air to afford the titled thioureido derivate **22**.

4-(3-(4-Fluorophenyl)thioureido)benzenesulfonamide **22** was obtained according to the above reported general procedure using compound **1i** (0.056 g, 0.5 mmol). Yield 62%, 0.101 g; white solid, M.p. 185-188 °C (187°C; Lit. [21]); ^{1}H-NMR (DMSO-d_6, 400 MHz): 10.11 (1H, brs, N*H*,

exchange with D_2O), 10.30 (1H, brs, N*H*, exchange with D_2O), 7.80 (2H, d, *J*= 8.77), 7.72 (2H, d, *J*= 8.78), 7.52 (2H, m), 7.32 (2H, brs, N*H*, exchange with D_2O), 7.23 (2H, t, *J*= 8.87); ^{13}C-NMR (DMSO-d_6, 100 MHz): 180.9 (*C*=Se), 160.2 (d, *J*= 241.6) , 143.5, 140.0, 136.4, 127.2, 127.1 (d, *J*= 8.36), 123.6, 116.1 (d, *J*= 22.52), ^{19}F-NMR (DMSO-d_6, 76 MHz): -117.54; HRMS *m/z* $[M+H]^+$ calcd for C13H13FN3O2S2, 326.0428 ; found, 326.0425.

Carbonic Anhydrase Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as a pH indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as the buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of 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 (0.1 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least squares methods using PRISM 3, as reported earlier,[33-37] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in house as reported earlier.[33-37]

GPx like activity measurements. GPx-like catalytic activity was measured through DTT oxidation test and NADPH/GR-coupled assay according to literature procedures. [28-30]

X-ray crystallography. hCA II protein was purified as previously described. [38] The protein was concentrated to about 10 mg/mL and set up in SD-2 crystallization plates (Molecular Dimensions) with the following ratio of protein plus reservoir plus micro-seeds: 250 nL + 225 nL + 25 nL. The plate was incubated at 8 ºC and the reservoir conditions consisted of 2.6 to 3.0 M ammonium sulfate with 0.1 M Tris buffer at pH 8.0 to pH 8.5. Dry compound was added to the crystallization drops after crystals had formed and several days before data were collected. 360 frames of one degree oscillation were obtained from the MX-1 beamline of the Australian Synchrotron. The data were indexed using XDS [39] or DIALS [40] and scaled using Aimless. [41] Molecular replacement was done using Phaser [42] using 4cq0 as the initial starting model. The model was manually rebuilt using Coot [43] and refined using Refmac. [44] The compound was placed in density using the program Afitt (OpenEye Scientific Software) and further refined using Refmac.

Cell culture and treatments

Human prostate cancer cell line PC3, human breast cancer cell line MDA-MB-231, and human colon cancer cell line HT-29 were obtained from American Type Culture Collection (Rockville, MD). PC3, MDA-MB-231 and HT-29 were cultured in DMEM high glucose with 20% FBS in 5% CO_2 atmosphere at 37° C. Media contained 2 mM L-glutamine, 1% essential amino acid mix, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Sigma, Milan, Italy). Cells were plated in 96-wells cell culture ($1 \cdot 10^4$/well) and, 24 h after, treated with the tested compounds (0-300 µM) for 48 h. Low oxygen conditions were acquired in a hypoxic workstation (Concept 400 anaerobic incubator, Ruskinn Technology Ltd., Bridgend, UK). The atmosphere in the chamber consisted of 0.1% O2 (hypoxia), 5% CO_2, and residual N_2. In parallel, normoxic (20% O_2) dishes were incubated in air with 5% CO_2.

Cell viability assay

PC3, MDA-MB-231 and HT-29 cell viability was evaluated by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Cells were plated and treated as described. Post-treatments, after extensive washing, 1 mg/ml MTT was added into each well and incubated for 30 minutes at 37 °C. After washing, the formazan crystals were dissolved in 150 µl DMSO. The absorbance was measured at 550 nm. Experiments were performed in quadruplicate on at least three different cell batches.

Statistical analysis

Results were expressed as mean ± S.E.M. and the analysis of variance was performed by one way ANOVA. A Bonferroni's significant difference procedure was used as post-hoc comparison. *P* values of less than 0.05 were considered as significant. Data were analyzed using the "Origin® 9.1" software.

Table S1. Summary of Data Collection and Atomic Model Refinement Statistics for compounds **10, 14** and **22**

PDB	5ULN	5UMC	5WEX
compound	**22**	**14**	**10**
Space group	$P2_1$	$P2_1$	$P2_1$
Cell dimensions			
a, b, c	42.5, 41.6, 72.2	42.5, 41.5, 72.3	42.5, 41.5, 72.4
alpha, beta, gamma	90, 104.5, 90	90, 104.4, 90	90, 104.4, 90
Resolution (Å)	41.6 - 1.35	41.5 - 2.15	41.1 - 1.26
Resolution-high (Å)	1.37 - 1.35	2.21 - 2.15	1.28 - 1.26
Rmerge	0.042 (0.284)	0.225 (0.793)	0.076 (1.1.75)
Rpim	0.017 (0.124)	0.112 (0.396)	0.034 (0.511)
CC ½	1.000 (0.948)	0.978 (0.755)	0.999 (0.609)
I/sigI	31.3 (6.3)	5.7 (2.0)	10.6 (2.0)
Completeness (%)	94.0 (61.5)	99.3 (92.4)	96.9 (93.4)
Redundancy	7.3 (6.1)	5.0 (4.8)	6.8 (6.0)
Refinement			
resolution (Å)	41.6-1.35	41.5 - 2.15	41.1 - 1.26
unique reflections	48038	12801	60631
Rwork/Rfree (%)	10.6 / 14.3	19.6 / 26.2	11.8/ 14.7
# atoms	2586	2252	2464
Protein	2230	2101	2196
metal (Zn)	1	1	1
ligand	21	22	21
water	327	95	237
B-factors (Å2)	13.8	20.2	17.0
protein	12.9	20.4	16.4
metal (Zn)	5.7	12.3	8.2
ligand	17.1	25.4	23.2
water	27.3	20.1	29.6
r.m.s. deviations			
Bond length (Å)	0.015	0.014	0.015
Bond angle (°)	1.783	1.664	1.766

Figure S1. Difference Electron Density Map of Compound **10** within the hCA II Catalytic Site

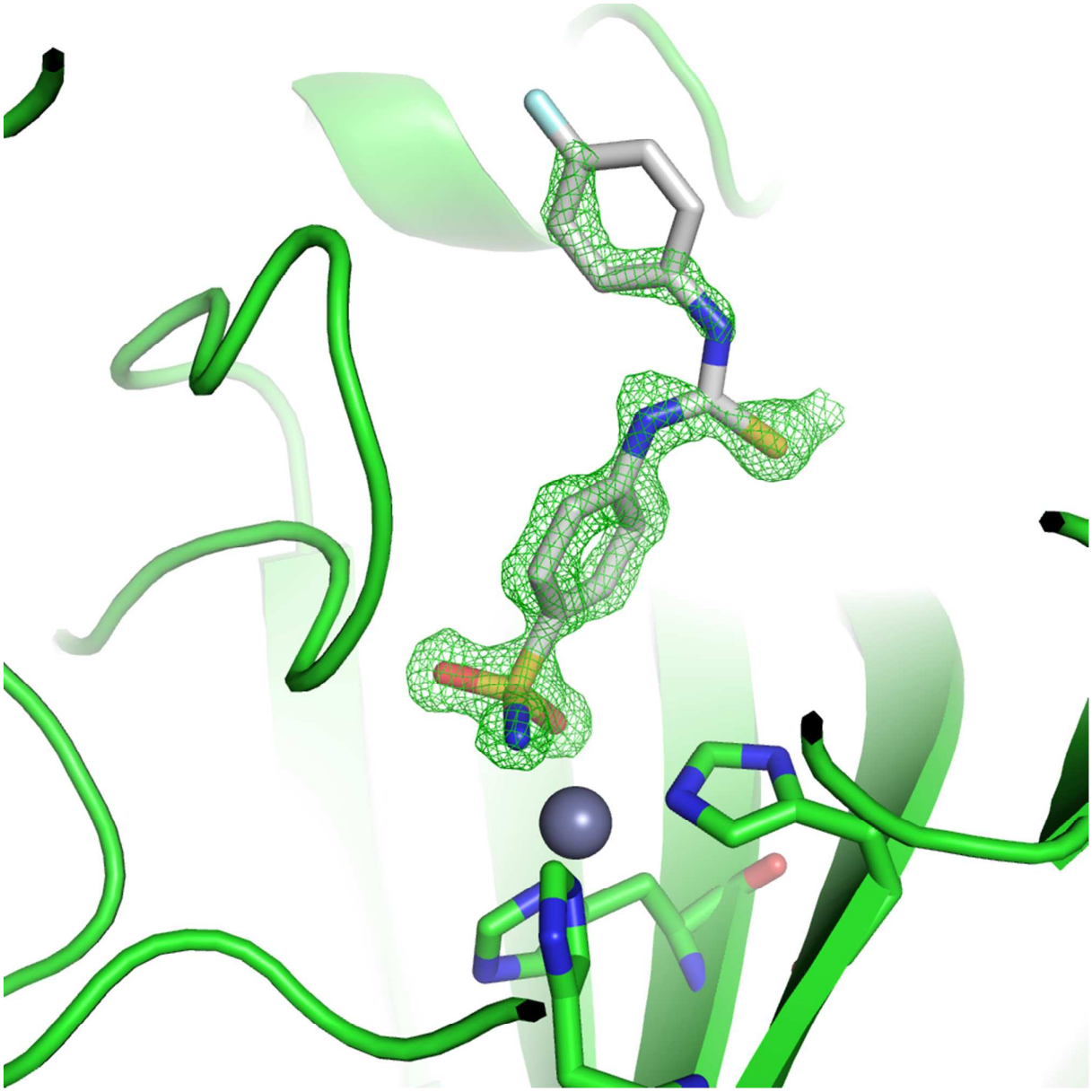


Figure S2. Difference Electron Density Map of Compound **22** within the hCA II Catalytic Site

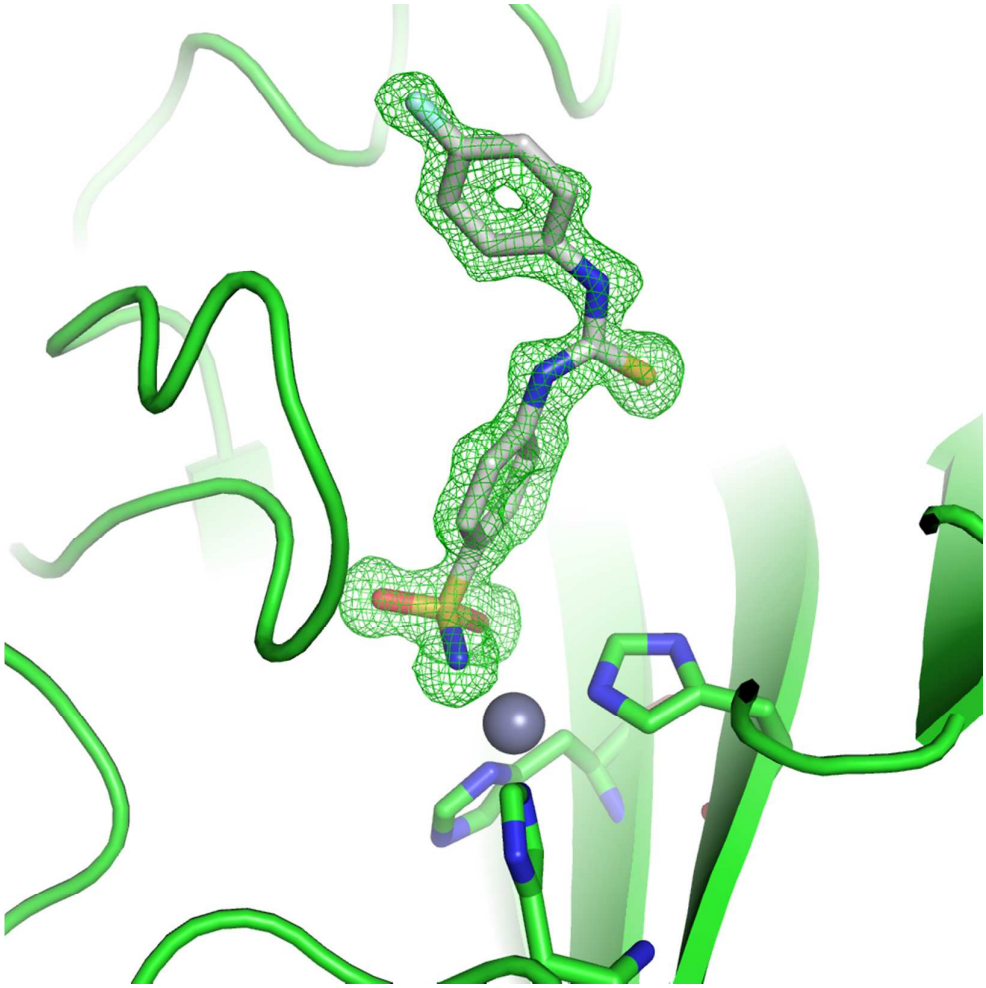
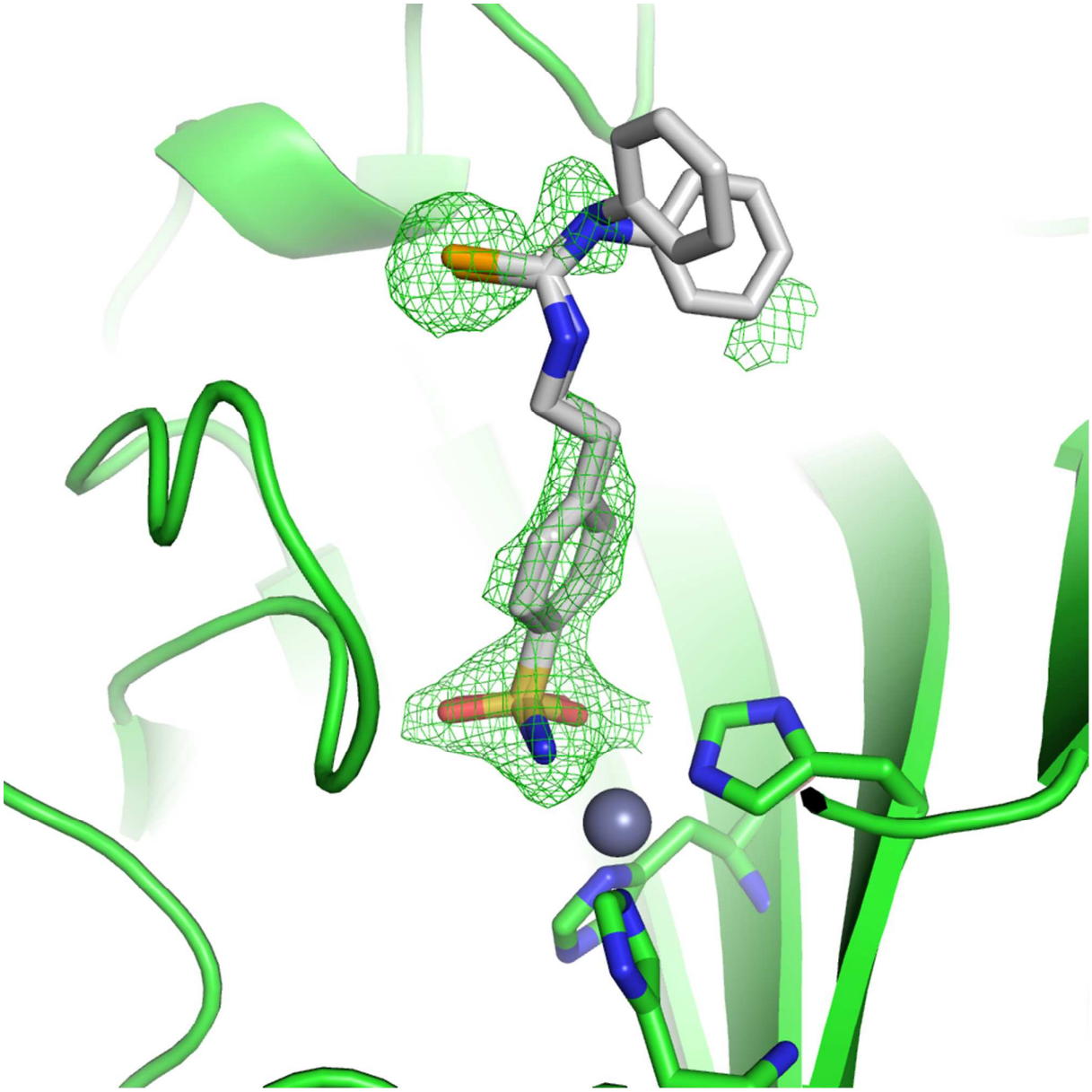


Figure S3. Difference Electron Density Map of Compound **14** within the hCA II Catalytic Site



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