Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different substituted 4-substituted-ureido-benzenesulfonamides correlate to inhibitor potency

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Table S1. Data collection and Final Model Statistics for the hCA II – (**1-5**) adducts

PDB accession number	**3N4B**	**3N0N**	**3N3J**	**3N2P**	**3MZC**
Compound	**1**	**2**	**3**	**4**	**5**
Data-collection statistics					
Temperature (K)	100	100	100	100	100
Wavelength (Å)	1.5418	1.5418	1.5418	1.5418	1.5418
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters (Å,º)	*a* =42.5 *b* =41.4 *c* =72.0 *β* =104.1	*a* =42.4 *b* =41.5 *c* =72.0 *β* =104.2	*a* =42.3 *b* =41.4 *c* =71.8 *β* =104.2	*a* =42.3 *b* =41.3 *c* =72.0 *β* =104.2	*a* =42.5 *b* =41.5 *c* =72.0 *β* =104.1
Total theoretical reflections	32224	39029	38826	29334	39139
Total measured reflections	30065	36024	36469	28366	35773
Resolution (Å) [a]R_{sym} (%) I/σ(I) Completeness Redundancy	19.9-1.6 (1.66-1.6) * 6.7 (55.0) 13.0 (3.5) 93.3 (90.1) 7.0 (7.1)	20.0-1.5 (1.55-1.5) 7.6 (37.4) 19.2 (5.1) 92.3 (87.4) 7.1 (6.9)	19.9-1.5 (1.55-1.5) 9.0 (17.7) 40.7 (3.3) 93.8 (97.3) 2.3 (2.1)	19.9-1.65 (1.7-1.65) 5.7 (53.9) 12.8 (3.8) 96.7(93.5) 7.0 (6.8)	19.9-1.5 (1.55-1.5) 4.1 (25.3) 24.4 (4.9) 91.4 (87.9) 3.8 (3.9)
Final Model Statistics					
[b]R_{cryst} (%) [c]R_{free}(%)	14.1 17.0	14.6 17.4	15.3 17.4	15.2 17.6	15.6 18.7
Residue Nos.	3-261	3-261	3-261	3-261	4-261
[d]No. of protein atoms	2133	2104	2087	2085	2085
No. of drug atoms	21	25	23	23	19
No. of H_2O molecules	258	308	359	218	306
R.m.s.d bond lengths (Å) bond angles (º)	0.010 1.3	0.009 1.4	0.009 1.3	0.012 1.4	0.010 1.4
Ramachandran statistics (%) Most favored, additionally allowed and generously allowed regions	 89.4 10.1 0.5	 87.6 12.0 0.5	 87.1 12.4 0.5	 88.9 10.6 0.5	 88.9 10.6 0.5
Average B factors (Å^2) Main-, side-chain Inhibitor, solvent	 14.2,18.6 14.8,29.2	 12.8,17.8 20.8,27.6	 15.1, 19.8 17.6, 29.4	 16.8,22.5 18.1,30.3	 13.4,17.8 16.4,29.1

[a]$R_{sym} = \Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle$. [b]$R_{cryst} = (\Sigma ||Fo| - |Fc|| / \Sigma |F_{obs}|) \times 100$. [c]R_{free} is calculated in same manner as R_{cryst}, except that it uses 5% of the reflection data omitted from refinement. [d]Includes alternate conformations. *Values in parenthesis represent highest resolution bin

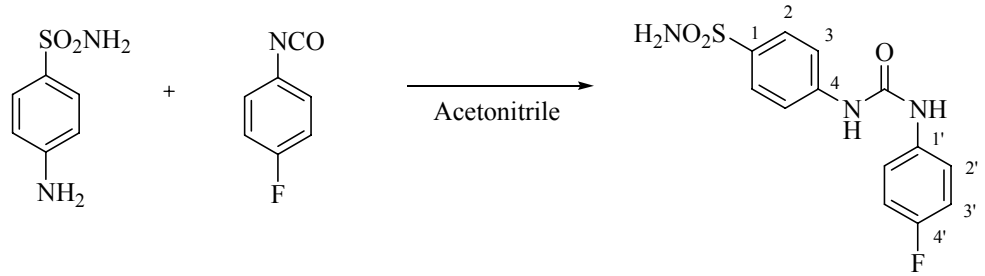
Chemistry

^{1}H, ^{13}C and ^{19}F spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Infrared spectra were recorded on a Perkin Elmer Spectrum R XI spectrometer as solids on KBr plates. Melting points (m.p.) were measured in open capillary tubes, unless otherwise stated, using a Büchi Melting Point B-540 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} aluminium backed plates. Elution of the plates was carried out using ethyl acetate/petroleum ether as eluting system. Visualization was achieved with UV light at 254 nm, by dipping into a ninhydrin TLC stain solution and heating with a hot air gun. Flash column chromatography was carried out using silica gel (obtained from Aldrich Chemical Co., Milan, Italy) as the adsorbent according to the procedure of Still et al (1). The crude product was introduced into the column as a solution in the same elution solvent system. Solvents and chemicals were used as supplied from Aldrich Chemical Co., Milan, Italy.

General procedure for the preparation of compounds (1-5).

4-Aminobenzenesulfonamude (sulfanilamide) **A** (2.90 mmols) was dissolved in acetonitrile (20-30 mL) and then treated with a stoichiometric amount of aryl/alkyl isocyanates **6-10**. The mixture was stirred at room temperature or heated at 50 °C for 2 h, until completion (TLC monitoring). The heavy precipitate formed was filtered-off, washed with diethyl ether (100 ml) and dried *under vacuo.*

Synthesis of 4-{[(4'-fluorophenyl)carbamoyl]amino}benzenesulfonamide (1).

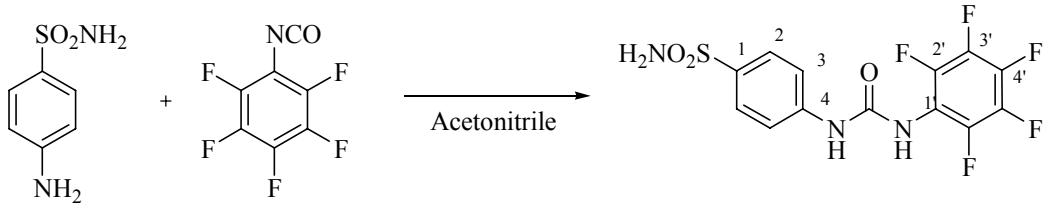


A **B** **1**; Yield (55.5 %)

Sulfanilamide **A** (0.50 g; 2.90 mmols) was treated with 4-fluorophenyl isocyanate **B** (0.40 g; 2.90 mmols) and the reaction was stirred at r.t. for 2 days, treated as described in the general procedure previously reported to give **1** as a white solid in 55.5 % yield.

4-{[(4'-Fluorophenyl)carbamoyl]amino}benzenesulfonamide (1): m.p. 242-243 ºC; silica gel TLC R_f 0.53 (ethyl acetate/petroleum ether 33 %); ν_{max} (KBr) cm^{-1}, 3338 (N-H urea), 1697 (C=O urea), 1593 (aromatic); δ_H (400 MHz, DMSO-d_6) 7.17 (2H, t, *J* 9.0, 2 x 2'-H), 7.24 (2H, s, SO_2NH_2), 7.51 (2H, dd, *J* 9.0 4.8, 2 x 3'-H), 7.64 (2H, d, *J* 8.8, 2 x 3-H), 7.76 (2H, d, *J* 8.8, 2 x 2-H), 8.86 (1H, s, N*H*), 9.09 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 158.5 (d, $J_{C\text{-}F}$ 237, C-4'), 153.3 (C=O urea), 143.7, 137.8, 136.6 (d, $^4J_{C\text{-}F}$ 3, C-1'), 127.7, 121.2 (d, $^3J_{C\text{-}F}$ 7, C-2'), 118.4, 116.3 (d, $^2J_{C\text{-}F}$ 22, C-3'); δ_F (376.5 MHz, DMSO-d_6) –121.0 (1F, s).

Synthesis of 4-{[(pentafluorophenyl)carbamoyl]amino}benzenesulfonamide (2).

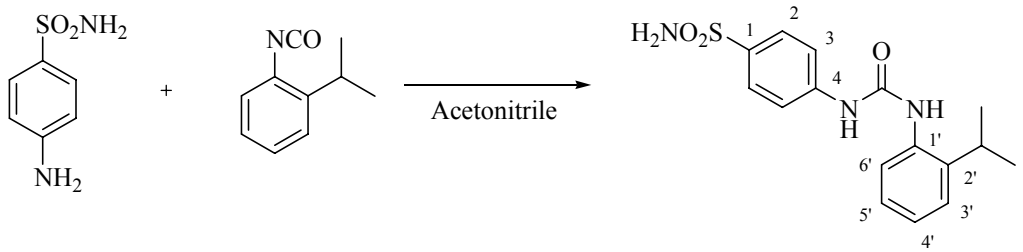


A **C** **2**; Yield (97.7 %)

Sulfanilamide **A** (0.50 g; 2.90 mmols) was treated with pentafluorophenyl isocyanate **C** (0.60 g; 2.90 mmols) and the reaction was stirred r.t. for 1 day, treated as described in the general procedure previously reported to give **2** as a white solid in 97.7 % yield.

4-{[(Pentafluorophenyl)carbamoyl]amino}benzenesulfonamide (2): m.p. 251-253 ºC; silica gel TLC R_f 0.49 (ethyl acetate/petroleum ether 33 %); ν_{max} (KBr) cm^{-1}, 3329 (N-H urea), 1656 (C=O urea), 1597 (aromatic); δ_H (400 MHz, DMSO-d_6) 7.23 (2H, s, SO_2NH_2), 7.61 (2H, d, *J* 8.8, 2 x 3-H), 7.74 (2H, d, *J* 8.8, 2 x 2-H), 8.65 (1H, s, N*H*), 9.48 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 152.9 (C=O urea), 144.0 (m, $J_{C\text{-}F}$ 239, C-2'), 143.3, 139.6 (m, $J_{C\text{-}F}$ 248, C-4'), 138.5, 138.2 (m, $J_{C\text{-}F}$ 249, C-3'), 127.8, 118.8, 114.7 (ddd, $^2J_{C\text{-}F}$ 23, $^3J_{C\text{-}F}$ 14, $^4J_{C\text{-}F}$ 4, C-1'); δ_F (376.5 MHz, DMSO-d_6) –146.2 (2F, dd, ^3J 24, ^4J 5.1, 2 x 2'-F), –159.2 (2F, t, ^3J 23, 2 x 4'-F), –164.0 (1F, dd, ^3J 23.3, ^4J 5.0, 2 x 3'-F).

Synthesis of 4-{([(2'-isopropylphenyl)amino]carbonyl)amino}benzenesulfonamide (3).

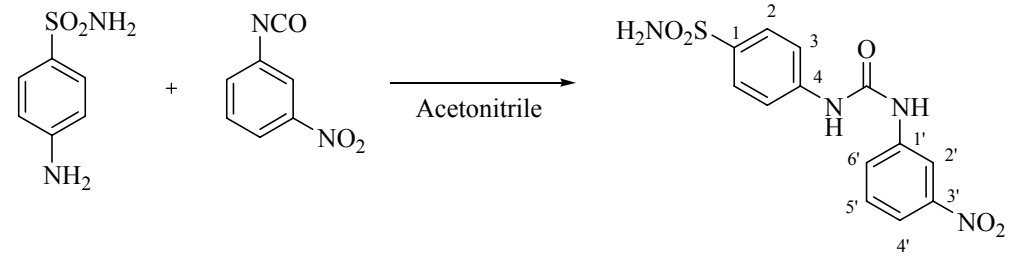


A **D** **3**; Yield (48.7 %)

Sulfanilamide **A** (0.50 g; 2.90 mmols) was treated with 2-isopropylphenyl isocyanate **D** (0.47 g; 2.90 mmols) and the reaction was stirred at r.t. for 6 h, treated as described in the general procedure previously reported to give **3** as a white solid in 48.7 % yield.

4-{([(2'-Isopropylphenyl)amino]carbonyl)amino}benzenesulfonamide (3): m.p. 226-227 ºC; silica gel TLC R_f 0.65 (ethyl acetate/petroleum ether 33 %); ν_{max} (KBr) cm^{-1}, 3361 (N-H urea), 2966 (C–H aliphatic), 1676 (C=O urea), 1592 (aromatic); δ_H (400 MHz, DMSO-d_6) 1.24 (6H, d, *J* 6.8, 2 x CH_3), 3.19 (1H, sept, *J* 6.8, C*H*), 7.14 (1H, ddd, *J* 7.9 7.6 1.6, 4'-H), 7.19 (ddd, *J* 7.9 6.8 1.2, 5'-H), 7.23 (2H, s, SO_2NH_2), 7.34 (1H, dd, *J* 7.6 1.6, 3'-H), 7.65 (2H, d, *J* 8.8, 2 x 3-H), 7.68 (1H, dd, *J* 6.8 1.2, 6'-H), 7.76 (2H, d, *J* 8.8, 2 x 2-H), 8.11 (1H, s, N*H*), 9.37 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 153.8 (C=O urea), 144.0, 140.8, 137.6, 136.0, 127.8, 126.7, 126.3, 125.3, 124.8, 118.2, 27.8 (*C*H), 24.1 (2 x CH_3).

Synthesis of 4-{[(3'-nitrophenyl)carbamoyl]amino}benzenesulfonamide (4).

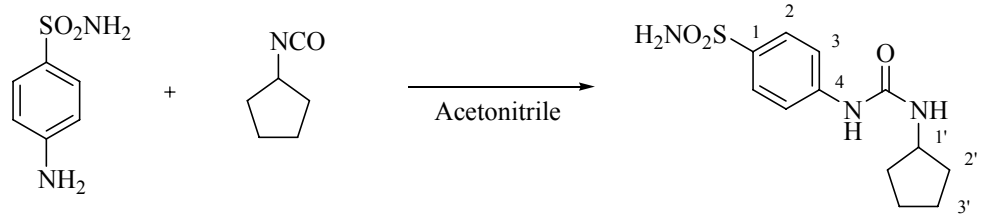


A **E** **4**; Yield (44.3 %)

Sulfanilamide **A** (0.50 mg; 2.90 mmols) was treated with 3-nitrophenyl isocyanate **E** (0.47 g; 2.90 mmols) and the reaction was stirred at r.t. for 1 day, treated as described in the general procedure previously reported to give **4** as a yellow solid in 44.3 % yield.

4-{[(3'-Nitrophenyl)carbamoyl]amino}benzenesulfonamide (4): m.p. 246-248 ºC; silica gel TLC R_f 0.39 (ethyl acetate/petroleum ether 33 %); ν_{max} (KBr) cm^{-1}, 3370 (N-H urea), 1709 (C=O urea), 1592 (aromatic); δ_H (400 MHz, DMSO-d_6) 7.23 (2H, s, SO_2NH_2), 7.59 (1H, dd, *J* 8.4 8.0, 5'-H), 7.65 (2H, d, *J* 9.0, 2 x 3-H), 7.73 (1H, ddd, *J* 8.4 2.0, 0.8, 6'-H), 7.76 (2H, d, *J* 9.0, 2 x 2-H), 7.86 (1H, ddd, *J* 8.0 2.4 0.8, 4'-H), 8.58 (1H, appt, *J* 2.2, 2'-H), 9.25 (1H, s, N*H*), 9.35 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 153.2 (C=O urea), 149.1, 143.3, 141.6, 138.3, 131.1, 127.7, 125.5, 118.8, 117.6, 113.3.

Synthesis of 4-[(cyclopentylcarbamoyl)amino]benzenesulfonamide (5).



A **F** **5**; Yield (68.8 %)

Sulfanilamide **A** (0.50 g; 2.90 mmols) was treated with cyclopentyl isocyanate **F** (0.32 g; 2.90 mmols) and the reaction was stirred at 50 ºC for 2 h, treated as described in the general procedure previously reported to give **5** as a white solid in 68.8 % yield.

4-[(Cyclopentylcarbamoyl)amino]benzenesulfonamide (5): m.p. 224-226 ºC; silica gel TLC R_f 0.57 (ethyl acetate/petroleum ether 33 %); ν_{max} (KBr) cm^{-1}, 3328 (N-H urea), 3055 (C-H aliphatic), 1684 (C=O urea), 1591 (aromatic); δ_H (400 MHz, DMSO-d_6) 1.41 (2H, m, 2 x 3'-H_{ax}), 1.58 (2H, m, 2 x 2'-H_{ax}), 1.67 (2H, m, 2 x 2'-H_{eq}), 1.88 (2H, m, 2 x 3'-H_{eq}), 3.98 (1H, six, *J* 6.8, 1'-H), 6.35 (1H, d, *J* 6.8, N*H*), 7.18 (2H, s, SO_2NH_2), 7.55 (2H, d, *J* 8.8, 2 x 3-H), 7.70 (2H, d, *J* 8.8, 2 x 2-H), 8.68 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 155.3 (C=O urea), 144.6, 136.8, 127.7, 117.6, 51.8 (C-1'), 33.7 (C-2'), 24.1 (C-3').

CA inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity by the method of Khalifah.[22] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as 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, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations.

Co-Crystallization and X-ray data collection of HCA II drug complexes. Co-crystals for each of the four HCA II - drug complexes were obtained using the hanging drop vapor diffusion method *(1)*. 10 µl drops (0.2 mM HCA II; 0.4 mM drug 1; 0.1 % dimethyl sulfoxide (DMSO); 0.8 M sodium citrate; 50 Mm Tris-HCl; pH 8.0) were equilibrated against precipitant solution (1.6 M sodium citrate; 50 mM Tris-HCl; pH 8.0) at room temperature (~20 ºC). Useful crystals were observed 4 days after the crystallization setup. A crystal was cryoprotected by quick immersion into 25% glycerol precipitant solution and flash-cooled by exposing it to a gaseous stream of nitrogen at 100K. The X-ray diffraction were obtained using an R-AXIS IV^{++} image plate system with Osmic Varimax HR optics and a Rigaku RU-H3R Cu rotating anode operating at 50 kV and 22 mA. The detector-crystal distance was set to 80 mm. The oscillation steps were 1° with a 6 min exposure per image. Indexing, integration, and scaling were performed using HKL2000 (2).

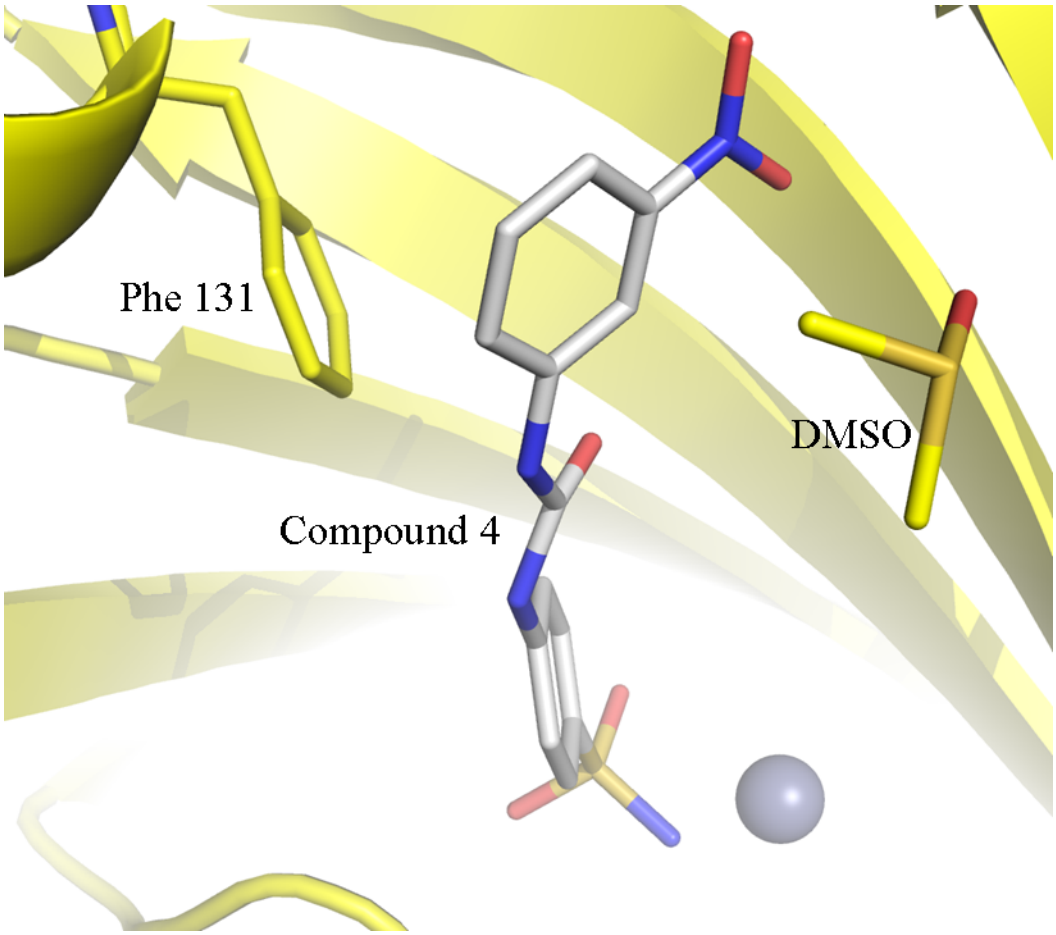


Fig. S1: Hydrophobic interactions for compound **4** are observed between the aromatic ring of the nitrophenyl group and a dimethyl sulfoxide (DMSO) C1 carbon atom. The ring is also in a partial stacking interaction with Phe131, which-includes N9, C8 and O8 of the urea linker.

References

1. McPherson, A. (1982) Preparation and Analysis of Protein Crystals, 1st Ed., Wiley, New York.

3. Otwinowski, Z., and Minor, W. (1997) Processing of x-ray diffraction data collected in oscillation mode, *Methods Enzymol.* 276, 307–326.