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4-[*N*-(Substituted 4-pyrimidinyl)amino]benzenesulfonamides as inhibitors of carbonic anhydrase isozymes I, II, VII, and XIII

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ABSTRACT

A series of 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides were designed and synthesised. Their binding potencies as inhibitors of selected recombinant human carbonic anhydrase (hCA) isozymes I, II, VII, and XIII were measured using isothermal titration calorimetry and the thermal shift assay. To determine the structural features of inhibitor binding, the crystal structures of several compounds in complex with hCA II were determined. Several compounds exhibited selectivity towards isozymes I, II, and XIII, and some were potent inhibitors of hCA VII.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are prominent targets of drug design, as previously reviewed.[1] A well-known class of CA inhibitors is aromatic/heterocyclic sulfonamides that have been studied for the development of antiglaucoma, antitumour, antiobesity, and anticonvulsant drugs.[2,3] Despite the progress achieved in the development of isozyme-specific inhibitors,[4–7] there remains a great need of new potent CA inhibitors with one or more of the following advantages: improved activity, selectivity, solubility, and reduced toxicity and side effects.

Significant progress has been made in the understanding of quantitative structure–activity relationships of CA inhibitors.[3,8] However, most correlations were drawn based on inhibition measurements. It is important to extend the binding measurements by using biophysical thermodynamic techniques in addition to inhibition measurements.[9] Here, we use isothermal titration calorimetry (ITC) and the thermal shift assay (TSA, also called ThermoFluor®, differential scanning fluorimetry) to measure inhibitor binding to CAs. ITC has been routinely used to measure ligand–protein binding thermodynamics.[10,11] However, it has difficulty determining weak (millimolar) or extremely tight (subnanomolar) dissociation constants and is quite time and protein consuming. TSA is a rapid and relatively low protein consuming screening method for identification of specific binders (hit compounds) used in the pharmaceutical industry.[12,13] The method is based on protein melting temperature (T_m) shifts upon ligand binding where the T_m is observed by following intrinsic or extrinsic fluorescence changes upon protein unfolding induced by heat. Determination of the binding reactions using two techniques reduces the uncertainty of the measurements.

Several 4-(2-substituted amino-4-pyrimidinylamino)benzenesulfonamides have been shown to possess significant affinity for hCA II and hCA IV.[14] Continuing the search for more potent and selective hCA inhibitors among heterocyclic moieties containing sulfonamides,[15] here we present the synthesis of 4-*N*-(4-pyrimidinyl)aminobenzenesulfonamides and the results of their binding to human recombinant CA (hCA) isozymes hCA I, hCA II, hCA VII, and hCA XIII. To increase the interaction of the tail pyrimidine moiety with the hydrophilic part of the active site of CA, we have designed compounds with electron-withdrawing groups in the pyrimidine moiety (Fig. 1). Isozyme hCA VII is highly expressed in the brain

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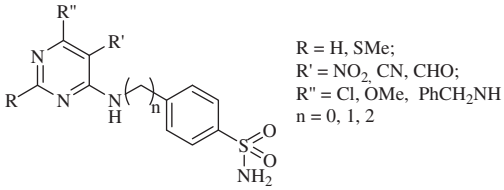


Figure 1. The general structure of 4-*N*-(4-pyrimidinyl)aminobenzenesulfonamides.

and inhibitors have been designed for it.[16,17] Isozyme hCA XIII has also been recently characterized and shown possibly to have a role in maintaining the acid–base balance in the kidneys and the gastrointestinal and reproductive tracts.[18] Several of our compounds show selectivity towards hCA XIII over other isozymes and may be useful for the development of therapeutic compounds.

2. Results and discussion

2.1. Chemistry

A series of benzene sulfonamide derivatives were designed and synthesised (Fig. 1, Scheme 1). Pyrimidines with chloro substituents in positions 2, 4, and 6 possess high reactivity towards various nucleophiles. Extensive work has been done in this area to gain control over sequential introduction of various functionalities on the pyrimidine templates by using halopyrimidines as electrophiles.[19–25] Therefore, for the synthesis of the target compounds reported here, the nucleophilic substitution of the corresponding 4,6-dichloropyrimidines **1–3** was applied. The choice was also suggested by the reports of successful synthesis of 4-[*N*-(4,6-substituted 1,3,5-triazin-2-yl)amino]benzenesulfonamides using regioselective nucleophilic substitution of cyanuric chloride.[26,27]

The desired monosubstitution products **5–7** were formed when compounds **1–3** were allowed to react with an equivalent amount of the corresponding 4-(amino substituted)benzenesulfonamides **4a–c** in the presence of potassium carbonate or triethylamine. It should be noted that the reaction of **1** and **2** with 4-(aminometyl)- or 4-(2-aminoethyl)benzenesulfonamides (**4b,c**) to give **5b,c** and **6b** proceeded at room temperature, whereas the best results of syntheses **5a**, **6a,c**, and **7a–c** were obtained at the reflux temperature of tetrahydrofuran (THF). The substitution of the second chlorine group in **5a–c** and **6a** with benzylamine required elevated temperatures: compounds **8a–c** and **9a** were synthesised by heating reagents at 100 °C in dimethylformamide for 3–12 h. For the synthesis of **10b,c**, an economic 'one-pot' method from **1** has been accomplished. Compound **11b** was synthesised by treatment of **5b** with water in THF solution at room temperature.

2.2. Binding

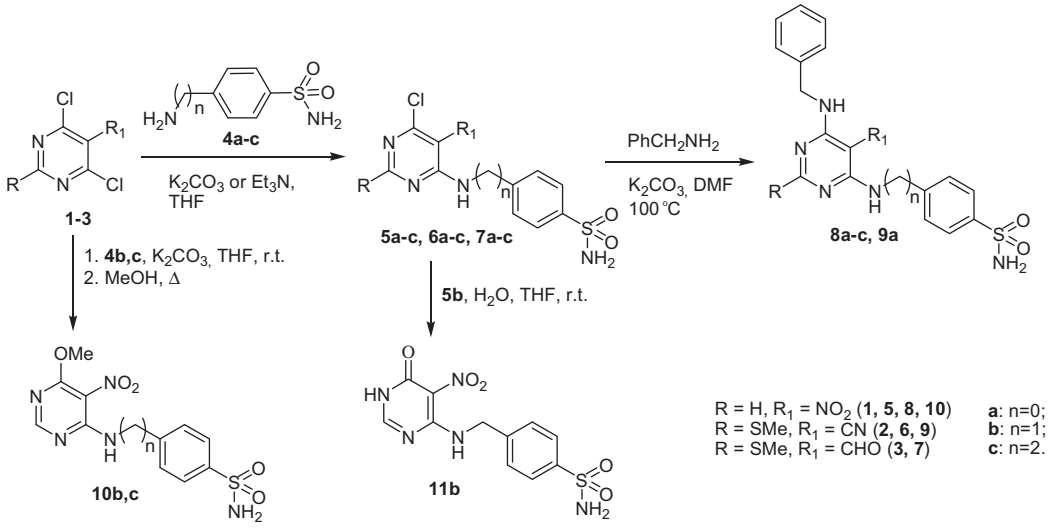
The compounds shown in Figure 1 and Scheme 1 are good binders of CAs. To determine compound affinity and specificity towards the four tested CA enzymes (hCA I, hCA II, hCA VII, and hCA XIII), compound binding was studied using two biophysical equilibrium techniques: TSA and ITC. The binding constants obtained by the two methods are listed in Table 1.

Figure 2 shows several typical CA melting curves, determined by TSA in the presence of increasing concentration of a ligand. The curves were fit as previously described[9] and the resultant melting temperatures (T_m) were plotted as a function of added ligand concentration (Fig. 3). These dosing curves were analyzed as previously described[9] yielding the binding constants at 37 °C.

ITC is a method more widely used for determination of binding affinity between proteins and low molecular weight ligands than TSA, but ITC consumes more protein and time than TSA. To obtain a more reliable affinity ranking of the compounds, both methods were used as extensively as possible. Figure 4 shows a typical ITC raw data curve. Figure 5 shows several ITC integrated data curves comparing **5a** binding to all tested CAs, whereas Figure 6 compares several compound binding to hCA XIII. The compounds bound stoichiometrically to each tested CA isoform, as seen from the ITC curves.

Despite some discrepancy between TSA and ITC data and the tendency to overestimate the K_b by TSA as compared to the ITC method, a number of correlations between compound structure and binding energetics can be drawn from Table 1. Compounds **5a** and **11b** appear to bind hCA XIII significantly stronger than any other tested CA. Compounds **7b** and **10b** bound hCA I significantly stronger than other tested CAs. Compound **8c** had selectivity towards hCA II over other three CAs.

The 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides can be divided into several groups according to their affinity towards the four tested CAs. Compounds **5a**, **6b**, **6c**, **7b**, **7c**, **10b**, **10c**, and **11b** were the most potent, with affinities in the submicromolar range. Compounds **6a**, **8a**, **8b**, and **8c** had affinities in the micromolar range. Compound **9a** was the least potent and its affinity was in the millimolar range towards all four CAs.



Scheme 1.

Table 1
Dissociation constants determined by TSA and ITC in μM at 37 °C

Compound	hCA I		hCA II		hCA VII		hCA XIII	
	TSA	ITC	TSA	ITC	TSA	ITC	TSA	ITC
5a	0.13	0.26	0.09	0.17	0.13	0.77	0.002	0.014
6a	1.4	20	0.07	0.22	0.83	1.7	0.091	0.12
6b	0.1	ND	0.17	ND	0.1	ND	0.14	ND
6c	0.33	ND	0.42	ND	0.1	ND	0.10	ND
7a	1.0	2.8	0.17	0.32	4.0	ND	ND	ND
7b	0.007	0.083	0.024	0.043	0.1	0.10	0.028	0.13
7c	0.1	0.48	0.11	0.35	1.0	0.77	0.033	0.189
8a	0.07	ND	0.17	ND	10	ND	0.5	ND
8b	0.025	ND	0.1	ND	4.2	ND	0.33	ND
8c	0.63	ND	0.016	ND	1.4	ND	0.5	ND
9a	100	ND	100	ND	3300	ND	100	ND
10b	0.013	0.11	0.05	0.056	0.83	0.83	0.067	0.24
10c	0.067	0.28	0.07	0.15	0.13	0.44	0.13	0.23
11b	0.17	ND	0.2	ND	0.25	ND	0.02	ND
AZM	1.4	0.78	0.017	0.018	ND	ND	0.050	0.065
TFM	0.05	ND	0.13	0.091	0.036	0.029	0.020	0.027

ND—not determined mostly due to insufficient compound solubility.
AZM—acetazolamide, TFM—trifluoromethanesulfonamide.
Average standard deviations for both methods were about ±25%.

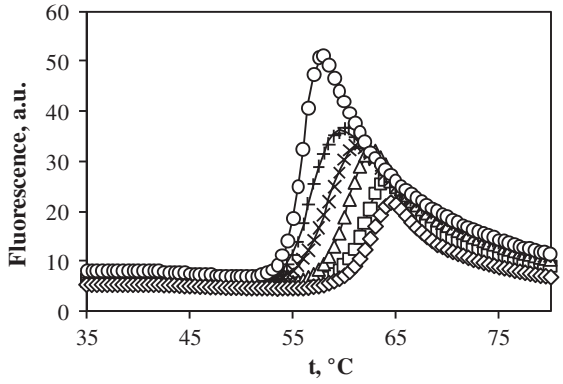


Figure 2. TSA data of **10c** binding to hCA II. Increasing ligand concentrations elevate melting temperatures (○—no ligand, +—3 μM, ×—7 μM, △—17 μM, □—59 μM, ◇—200 μM ligand; protein concentration 10 μM).

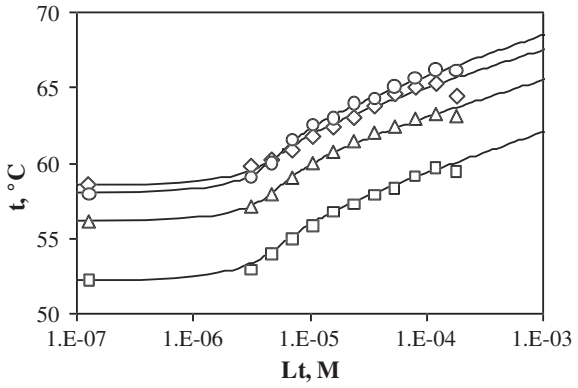


Figure 3. 10c binding to hCAs by TSA. Symbols (○—hCA I, △—hCA II, □—hCAVII, ◇—hCA XIII) show experimental values, corresponding solid lines were fit according to the model.

2.3. Crystallography

Crystal structures of compounds **5a**, **7a**, **7b**, **8b**, **10b**, **10c**, and **11b** bound to hCA II were determined by X-ray crystallography.

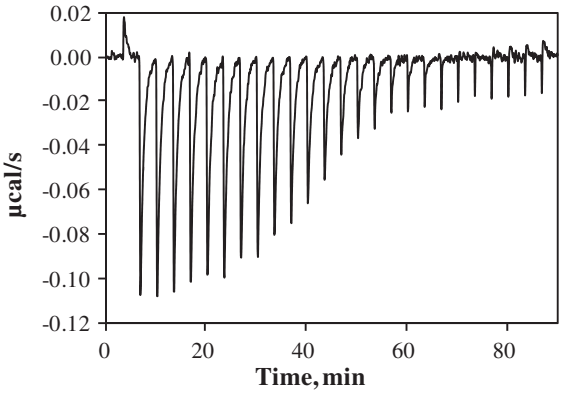


Figure 4. Example of raw ITC data—**10c** binding to hCA VII.

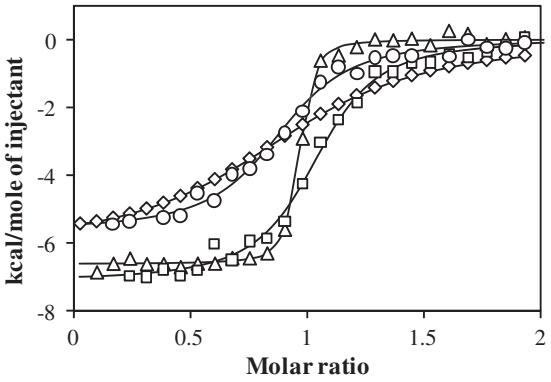


Figure 5. Integrated ITC data—comparison of **5a** binding to hCA I (○), hCA II (□), hCA VII (◇), and hCA XIII (△).

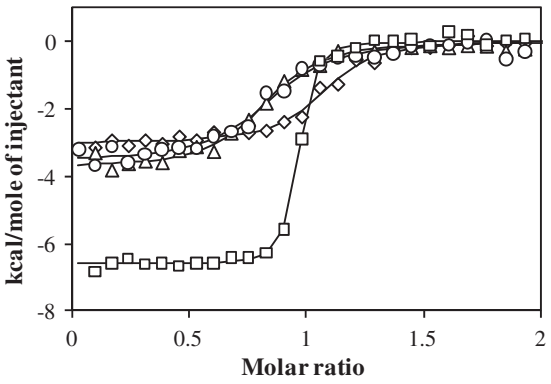


Figure 6. Integrated ITC data—comparison of **5a** (□), **6a** (◇), **7c** (△), and **10c** (○) binding to hCA XIII.

All crystallographically characterized ligands (Fig. 7, Table 2) can be divided into three groups according to the length of the linker connecting the pyrimidine and benzene rings: **5a** and **7a** have a short linker between the benzene and pyrimidine rings consisting of just the amino group; **7b**, **8b**, **10b**, and **11b** have a longer linker with a methylene group attached to amino group; the ligand **10c** linker is the longest and has two connecting methylene groups.

The benzenesulfonamide ring of all inhibitors in all crystal structures is found in the same orientation. The ring is fixed by sulfonamide bound to catalytic Zn. Rotations of the ring are restricted by van der Waals contacts, with residues forming an active site cavity. Side chains of Val121 and Thr200 check the mobility from

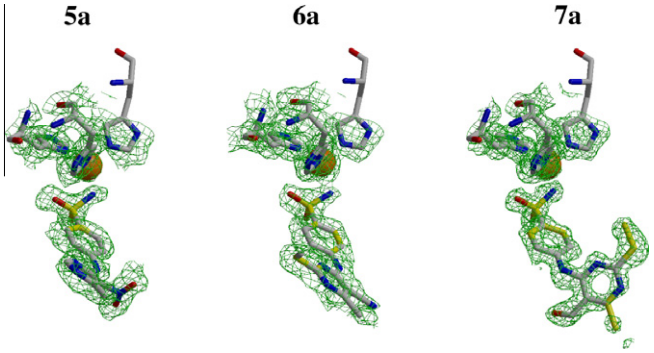


Figure 7. View of **5a**, **6a**, and **7a**, located in the active center of hCA II. The Zn atom, coordinated by His94, His96, and His119, is shown as an orange sphere. The electron density map, contoured at 0.8σ. The pictures are generated using MOLSCRIPT,[38] Raster3D,[39] and BOBSCRIPT.[40]

the sides, whereas Leu198 supports the ring plane. The pyrimidine ring is observed in three different orientations in the crystal structures.

Compounds **5a**, **8b**, **10b**, **10c**, and **11b** make an intramolecular hydrogen bond-like connection between the nitro group at the C(5) atom of the pyrimidine ring and the amino group of the linker. Figure 8 shows the arrangement of **10b** in the active site of hCA II with closest protein–ligand interatomic distances as green lines. Electron density of the pyrimidine ring in ligands **5a**, **8b**, and **10c** is shifted towards the nitro group due to its electron-withdrawing effect. Negative difference electron density was found in complexes with ligands **5a**, **7a**, and **7b** containing Cl at the C(6) position of the pyrimidine ring, which could be explained by partial hydrolysis (or radiation damage) of the Cl group.

Compounds that contain Cl in the pyrimidine ring, especially **5b** and **5c**, would hydrolyze in aqueous solution by exchanging Cl for an OH group due to the adjacent electron-withdrawing nitro group. Some hydrolysis was also observed with other Cl-containing compounds in crystal structures after prolonged exposure to aqueous solution during compound soaking. However, the hydrolysis was only partial, and it should not occur in ITC and TSA binding assays because compound solutions were freshly prepared and the assays lasted about 1 h.

The structure of hCA II complexed with compound **7a** is solved with 1.15 Å resolution (see Table 2 for refinement statistics). An excellent electron density for the inhibitor is visible in a composite omit map. The pyrimidine ring of **7a** is fixed by hydrophobic interaction with Pro202, Val135, Trp5, His64, and Phe131. Similar orientation of the pyrimidine ring as in **7a** is observed in the crystal structure of hCAII complexed with inhibitor **11b**, which belongs to the second group of ligands with the medium length linker. Similarly to **7a**, the pyrimidine ring of **11b** appears to be trapped in this orientation mostly by the van der Waals interactions with Pro202, Phe131, and Val135. In both crystal structures, the DMSO molecule, which is obtained from the stock solution of the inhibitor, is found between Phe131 and Gln92. Interestingly, compound **11b** has an alternative non-specific binding site near the N-terminus of the protein. The second binding site could be a result of crystal packing, because thermodynamic data do not support the binding of two molecules of this inhibitor by hCAII.

Figure 9 shows the position of several superimposed ligands. The pyrimidine rings of ligands **5a**, **7b**, **8b**, and **10b** form a plane,

Table 2
X-ray crystallographic data collection and refinement statistics

Compound:	**5a**	**7a**	**7b**	**8b**	**10b**	**10c**	**11b**
PDB ID	3M40	3MHO	3M5E	3MHM	3MHL	3M3X	3MHI
Temperature	100 K	100 K	100 K	100 K	100 K	100 K	100 K
Spacegroup	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell (Å)	a = 42.18, b = 41.16, c = 72.48, $\alpha = \gamma$ = 90, β = 104.54	a = 42.13, b = 41.21, c = 72.08, $\alpha = \gamma$ = 90, β = 104.30	a = 42.42, b = 41.49, c = 72.43, $\alpha = \gamma$ = 90, β = 104.49	a = 42.25, b = 41.17, c = 72.24, $\alpha = \gamma$ = 90, β = 104.10	a = 41.71, b = 40.68, c = 70.72, $\alpha = \gamma$ = 90, β = 104.13	a = 42.04, b = 41.05, c = 71.80, $\alpha = \gamma$ = 90, β = 104.39	a = 42.24, b = 41.24, c = 71.95, $\alpha = \gamma$ = 90, β = 104.43
Resolution, Å (final shell)	1.60	1.15	1.70	1.50	1.90	1.77	1.70
Unique reflections (total)	30,141 (176,541)	85,194 (566,937)	27,116 (314,528)	37,716 (154,392)	18,388 (280,542)	26,749 (299,614)	26,639 (251,427)
Completeness (%) overall (final shell)	94.4 (90.2)	99.9 (99.7)	100.0 (100.0)	97.3 (96.1)	100.0 (100.0)	98.3 (97.6)	99.9 (99.3)
I/σ_I overall (final shell)	10.2 (6.4)	20.3 (4.1)	32.8 (13.0)	25.4 (5.8)	37.9 (7.5)	50.5 (17.8)	31.2 (10.7)
R_{merge} overall (final shell)	16.1 (20.2)	6.7 (32.3)	5.4 (11.7)	3.3 (15.7)	7.6 (25.2)	3.9 (7.9)	4.5 (18.5)
Number of atoms	2410	2468	2488	2500	2202	2504	2437
Number of solvent molecules	234	291	332	294	120	295	282
Number of bound buffer molecules	0	2	0	0	0	0	0
Test set size	10%	10%	10%	10%	10%	10%	10%
R_{cryst} (R_{free})	15.2 (22.4)	17.5 (19.2)	16.4 (20.2)	13.3 (18.8)	16.2 (22.1)	16.2 (22.1)	16.2 (21.5)
RMS bonds/angles	0.026/1.947	0.006/1.182	0.011/1.304	0.028/2.117	0.023/1.993	0.028/2.197	0.029/2.234
Average B-factors (Å^2)	14.491	11.401	12.68	15.419	25.434	16.159	14.065
Main chain:	11.6688	9.461	13.2454	12.427	23.7181	13.2707	11.1913
Side chains:	14.2259	10.726	11.441	15.0619	26.1502	15.7152	13.8444
Solvent:	28.0151	20.6237	24.1527	26.1515	32.7987	27.4072	22.7711
Ions:	6.32	5.53	4.98	7.67	16.33	7.11	9.3
Co-factors:	20.8162	12.7227	17.7765	31.499	34.152	34.3517	28.8634

$R_{merge} = \sum_h \sum_{i=1}^{n_h} |\langle I_h \rangle - I_{hi}| / \sum_h \sum_{i=1}^{n_h} |I_{hi}|$, where I_{hi} is an intensity value of the ith measurement of reflection $\boldsymbol{h}$, $\boldsymbol{h} = (h, k, l)$, sum $\sum_h$ runs over all measured reflections, and $\langle I_h \rangle$ is an average measured intensity of the reflection $\boldsymbol{h}$. The $n_{\boldsymbol{h}}$ is the number of measurements of reflection $\boldsymbol{h}$.

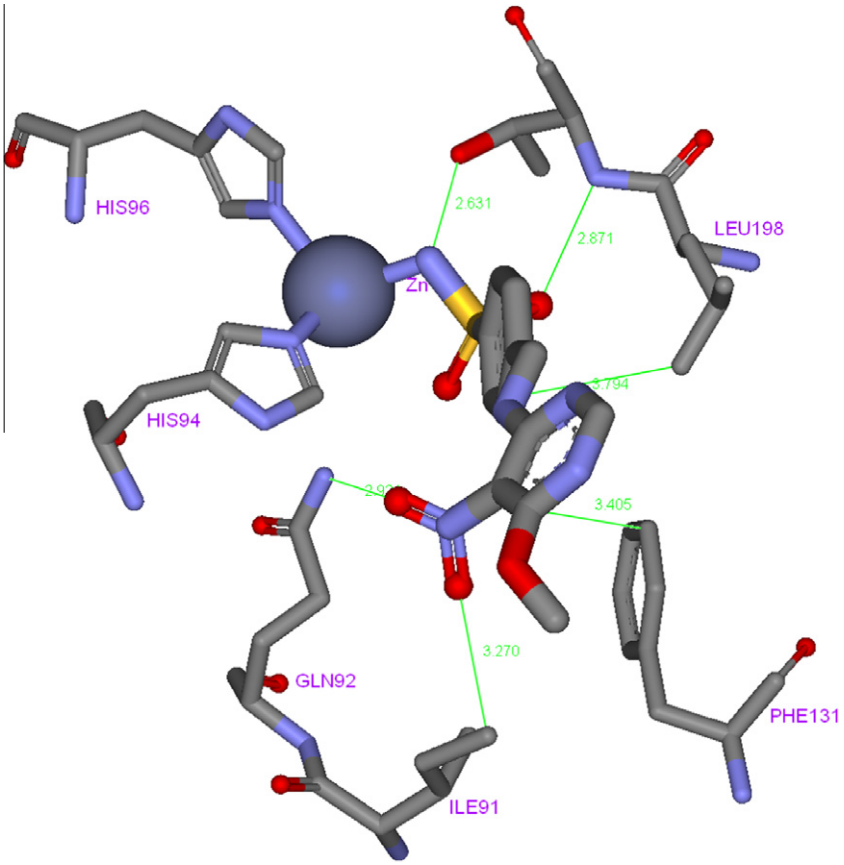


Figure 8. The view of **10b** bound to the active site of hCA II. Several selected distances between the ligand and protein atoms, shown as lines. The picture was generated using Accelrys DS visualiser.

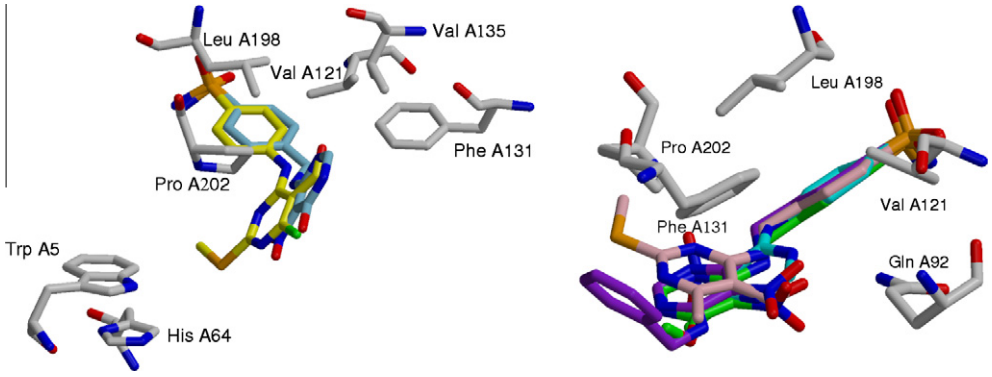


Figure 9. Left panel: View of compounds **7a** (yellow) and **11b** (blue) located in the active center of hCAII. Right panel: Compounds **5a** (cyan), **7b** (pink), **8b** (purple), and **10b** (green) bound in active center of hCAII. Protein residues are shown in gray. The pictures are generated using MOLSCRIPT,[38] Raster3D,[39] and BOBSCRIPT [40].

that is, nearly orthogonal to that formed by **7a** and **11b**. This conformation is fixed in the protein active center by a stacking interaction with Phe131 and by a hydrogen bond between the amino group of Gln92 and a nitro or formyl group at C(5) of the pyrimidine ring (in **5a** it is the N(3) atom of the pyrimidine). Phe131 restrains the rotation of ligands. An inferred intramolecular hydrogen bond between the nitro group and the amino group of the linker can render molecules more rigid because it can prevent rotation of the pyrimidine ring.

The benzylamino moiety at position C(6) of the pyrimidine in **8b** is packed tightly between residues 130–132 and residues 235–236 of the symmetrically equivalent protein molecule, so this conformation could be a result of crystal packing. This conformation is considered to be dominant, albeit the electron density is forked at the first methylene group of the linker. This suggests that there might be another orientation of the pyrimidine ring and benzylamino moiety, but with our present data, it could not be modeled with certainty.

Compound **10c** has the longest linker with two CH_2 groups, and therefore higher intrinsic flexibility could be expected. Nevertheless, it is resolved very well in the crystal structure of the corresponding complex with hCA II. It appears that in the binding of this ligand, hydrophobic interactions play the most important role. Therefore, a nitro group is exposed to bulk solvent without contacts with the protein. The linker and the pyrimidine ring adopt a conformation that places them equidistantly between hydrophobic

side chains (Pro202, Val135, and Phe131). Orientation of the pyrimidine ring of **10c** differs from that of other inhibitors.

To determine structural features of compound selectivity towards particular CA isozymes, we would need to obtain co-crystal structures of the compounds with all CAs and not just with hCA II. Further development of more potent and more selective compounds through various techniques, including crystallography with other CAs, is underway.

3. Conclusion

A series of 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides bind CAs with submicromolar to nanomolar potency. Several compounds exhibited selectivity towards one CA out of four tested CAs: **5a** and **11b** towards hCA XIII, **7b** and **10b** towards hCA I, and **8c** towards hCA II. Compound **7b** was the most potent but was non-selective towards hCA VII.

4. Experimental

4.1. Synthesis

Melting points were determined in open capillaries on a Thermo Scientific 9100 Series apparatus and are uncorrected. IR spectra were run on a Perkin-Elmer FT-IR spectrophotometer Spectrum BX II in KBr. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova spectrometer (300 and 75 MHz, respectively) using residual DMSO signals (2.52 ppm and 40.21 ppm for ^{1}H and ^{13}C NMR spectra, respectively) as internal standards. Elemental analyzes were performed at the Elemental Analysis Laboratory of the Department of Organic Chemistry of Vilnius University. TLC was performed with silica gel 60 F254 aluminum plates (Merck) and visualized with UV light. For column chromatography, silica gel 60 (0.040–0.063 mm) (Merck) was used. High-resolution mass spectra (HRMS) were recorded on an Dual-ESI Q-TOF 6520 mass spectrometer (Agilent Technologies).

Synthesis of the starting materials **1**–**3** was accomplished as previously described.[28,29]

4.1.1. 4-[*N*-(6-Chloro-5-nitropyrimidin-4-yl)amino]benzenesulfonamide (5a)

A mixture of 0.20 g (1.03 mmol) of 4,6-dichloro-5-nitropyrimidine (**1**), 0.18 g (1.03 mmol) of 4-aminobenzenesulfonamide (**4a**), and 278 μL (2 mmol) of Et_3N in 10 mL THF was refluxed for 16 h. Solvent was removed under reduced pressure and the residue was purified by column chromatography using EtOAc/*n*-hexane (1:1 v/v) as the eluent to give 0.24 g (71%) of compound **5a**. Mp 200 °C (dec). IR: 3375, 3340, 3270 cm^{-1} (NH, NH_2). ^{1}H NMR (DMSO-d_6) δ (ppm): 7.38 (s, 2H, NH_2), 7.72–7.85 (m, 4H, Ar–H), 8.61 (s, 1H, CH), 10.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 117.9, 125.8, 126.8, 140.8, 141.5, 152.6, 155.5, 156.7. HRMS calcd for $C_{10}H_8ClN_5O_4S$ $[M-H]^-$ = 327.9913, found 327.9912.

4.1.2. 4-{[*N*-(6-Chloro-5-nitropyrimidin-4-yl)amino]methyl}benzenesulfonamide (5b)

A mixture of 0.291 g (1.5 mmol) of 4,6-dichloro-5-nitropyrimidine (**1**), 0.335 g (1.5 mmol) of 4-(aminomethyl)benzenesulfonamide hydrochloride (**4b**) and 0.345 g (2.5 mmol) of anhydrous K_2CO_3 in 15 mL THF was stirred at room temperature for 8 h. The solution was filtered to remove inorganic salts, solvent was evaporated under reduced pressure and the residue was purified by column chromatography using ethyl acetate:*n*-hexane (1:1 v/v) as eluent to give 0.394 g (76%) of compound **5b**. Mp 166 °C. IR: 3294, 3370 cm^{-1} (NH, NH_2). ^{1}H NMR (DMSO-d_6) δ (ppm): 4.76 (d, J = 5.9 Hz, 2H, CH_2), 7.34 (s, 2H, NH_2), 7.50 (d, J = 8.3 Hz, 2H, Ar–H), 7.78 (d, J = 8.3 Hz, 2H, Ar–H), 8.46 (s, 1H, CH), 9.11 (t, 1H, NH, J = 5.9 Hz). ^{13}C NMR (DMSO-d_6) δ (ppm): 44.7, 126.5, 128.1, 128.9, 142.9, 143.5, 151.7, 155.1, 158.9. Anal. Calcd for $C_{11}H_{10}ClN_5O_4S$: C, 38.43; H, 2.93. Found: C, 38.54; H, 2.86.

4.1.3. 4-{2-[*N*-(6-Chloro-5-nitropyrimidin-4-yl)amino]ethyl}benzenesulfonamide (5c)

Compound **5c** was synthesised by the procedure described for compound **5b**. Yield 64%. Mp 189 °C. IR: 3267, 3349, 3396 cm^{-1} (NH, NH_2). ^{1}H NMR (DMSO-d_6) δ (ppm): 2.97 (t, J = 7.2 Hz, 2H, CH_2) 3.69–3.76 (m, 2H, CH_2), 7,33 (s, 2H, NH_2), 7.43 (d, J = 8.2 Hz, 2H, Ar–H), 7.77 (d, J = 8.2 Hz, 2H, Ar–H), 8.49 (s, 1H, CH), 8.57 (t, J = 5.5 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 34.7, 43.0, 126.4, 128.8, 129.9, 143.0, 143.8, 151.5, 154.9, 158.9. Anal. Calcd for $C_{12}H_{12}ClN_5O_4S$: C, 40.28; H, 3.38. Found: C, 40.46; H, 3.59.

4.1.4. 4-[*N*-(6-Chloro-5-cyano-2-methylthiopyrimidin-4-yl)amino]benzenesulfonamide (6a)

To a solution of 0.22 g (1 mmol) of 4,6-dichloro-2-methylthiopyrimidine-5-carbonitrile (**2**) and 0.17 g (1 mmol) of 4-aminobenzenesulfonamide (**4a**) in 10 mL of THF 278 μL (2 mmol) Et_3N was added dropwise. The reaction mixture was refluxed for 24 h. Then solvent was removed under reduced pressure and the residue was purified by column chromatography using diethyl ether as eluent to give 0.30 g (84%) of compound **6a**. Mp 217 °C (dec). IR: 3326, 3272 (NH, NH_2), 2222 cm^{-1} (CN). ^{1}H NMR (DMSO-d_6) δ (ppm): 2.47 (s, 3H, SCH_3), 7.36 (s, 2H, NH_2), 7.80 (m, 4H, Ar–H), 10.44 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.6, 87.6, 114.3, 124.1, 126.8, 140.9, 141.0, 160.2, 162.1, 175.5. HRMS calcd for $C_{12}H_{10}ClN_5O_2S_2$ $[M+H]^+$ = 356.0037, found 356.0033.

4.1.5. 4-{[*N*-(6-Chloro-5-cyano-2-methylthiopyrimidin-4-yl)amino]methyl}benzenesulfonamide (6b)

A mixture of 0.22 g (1 mmol) 4,6-dichloro-2-methylthiopyrimidine-5-carbonitrile (**2**), 0.22 g (1 mmol) of 4-(aminomethyl)benzenesulfonamide hydrochloride (**4b**) and 0.28 g (2 mmol) of anhydrous K_2CO_3 in 8 mL THF was stirred at room temperature for 12 h and then poured into 50 mL of water. The precipitate was filtered off and recrystallised to give 0.14 g (38%) of compound **6b**. Mp 140 °C (from methanol). IR: 3340, 3250 (NH, NH_2), 2222 cm^{-1} (CN). ^{1}H NMR (DMSO-d_6) δ (ppm): 2.37 (s, 3H, SCH_3), 4.69 (d, J = 5.8 Hz, 2H, CH_2), 7.35 (s, 2H, NH_2), 7.51 (d, J = 8.1 Hz, 2H, Ar–H), 7.78 (d, J = 8.1 Hz, 2H, Ar–H), 9.13 (t, J = 5.8 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.5, 44.9, 85.7, 114.6, 126.4, 128.3, 143.2, 149.5, 161.1, 161.4, 175.3. Anal. Calcd for $C_{13}H_{12}ClN_5O_2S_2$: C, 42.22; H, 3.27. Found: C, 42.43; H, 3.42.

4.1.6. 4-{2-[*N*-(6-Chloro-5-cyano-2-methylthiopyrimidin-4-yl)amino]ethyl}benzenesulfonamide (6c)

A mixture of 0.220 g (1 mmol) of 4,6-dichloro-2-methylthiopyrimidine-5-carbonitrile (**2**), 0.200 g (1 mmol) of 4-(aminoethyl)benzenesulfonamide (**4c**) and 0.144 g (1.04 mmol) of anhydrous K_2CO_3 in 10 mL THF was refluxed for 3 h, then cooled to room temperature and poured into 70 mL of water. The precipitate was filtered off, dried in air and recrystallised to give 0.280 g (73%) of compound **6c**. Mp 213 °C (from methanol). IR: 3366, 3264 (NH, NH_2), 2218 cm^{-1} (CN). ^{1}H NMR (DMSO-d_6) δ (ppm): 2.53 (s, 3H, SCH_3), 2.97 (t, J = 7.1 Hz, 2H, CH_2), 3.65–3.72 (m, 2H, CH_2), 7.33 (s, 2H, NH_2), 7.43 (d, J = 8.2 Hz, 2H, Ar–H), 7.77 (d, J = 8.2 Hz, 2H, Ar–H), 8.61 (t, J = 6.1 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.6, 34.8, 43.0, 85.3, 114.6, 126.5, 129.9, 142.9, 143.9, 161.1, 161.3, 175.3. Anal. Calcd for $C_{14}H_{14}ClN_5O_2S_2$: C, 43.80; H, 3.68. Found: C, 44.12; H, 3.59.

4.1.7. 4-[*N*-(6-Chloro-5-formyl-2-methylthiopyrimidin-4-yl) amino]benzenesulfonamide (7a)

Compound **7a** was synthesized from 4,6-dichloro-2-methylthiopyrimidine-5-carbaldehyde (**3**) and 4-aminobenzenesulfonamide (**4a**) according to a procedure described for the synthesis of **6a**. The reaction time was 36 h, yield 30%. Mp was 227 °C (from methanol). IR: 3318, 3236 (NH, NH_2), 1638 cm^{-1} (CHO). ^1H NMR (DMSO-d_6) δ (ppm): 2.57 (s, 3H, SCH_3), 7.38 (s, 2H, NH_2), 7.89 (m, 4H, Ar–H), 10.25 (s, 1H, NH), 11.26 (s, 1H, CHO). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.9, 105.9, 115.8, 123.2, 127.3, 140.4, 140.9, 158.4, 164.5, 191.6. Anal. Calcd for $C_{12}H_{11}ClN_4O_3S_2$: C, 40.17; H, 3.09. Found: C, 40.27; H, 3.16.

4.1.8. 4-{[*N*-(6-Chloro-5-formyl-2-methylthiopyrimidin-4-yl)amino]methyl}benzenesulfonamide (7b)

A mixture of 0.22 g (1 mmol) of 4,6-dichloro-2-methylthiopyrimidin-5-carbaldehyde (**3**), 0.22 g (1 mol) of 4-(aminomethyl)benzenesulfonamide hydrochloride (**4b**) and 0.28 g (2.1 mmol) of anhydrous K_2CO_3 in 8 mL THF was refluxed for 10 h, cooled to room temperature and poured into 50 mL of water. Resulting precipitate was filtered off, dried in air and recrystallised to give 0.26 g (70%) of compound **7b**. Mp 195 °C (from 2-propanol). IR: 3480, 3276 (NH, NH_2), 1648 cm^{-1} (CO). ^1H NMR (DMSO-d_6) δ (ppm): 2.39 (s, 3H, SCH_3), 4.83 (d, J = 6.2 Hz, 2H, CH_2), 7.33 (s, 2H, NH_2), 7.50 (d, J = 8.3 Hz, 2H, Ar–H), 7.78 (d, J = 8.3 Hz, 2H, Ar–H), 9.82 (t, J = 6.2 Hz, 1H, NH), 10.18 (s, 1H, CHO). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.6, 44.5, 105.3, 126.5, 128.3, 143.3, 143.5, 160.1, 164.1, 176.1, 190.5. Anal. Calcd for $C_{13}H_{13}ClN_4O_3S_2$: C, 41.88; H, 3.51. Found: C, 41.87; H, 3.58.

4.1.9. 4-{2-[*N*-(6-Chloro-5-formyl-2-methylthiopyrimidin-4-yl) amino]ethyl}benzenesulfonamide (7c)

Compound **7c** was synthesized from 4,6-dichloro-2-methylthiopyrimidine-5-carbaldehyde (**3**) and 4-(aminoethyl)benzenesulfonamide (**4c**) according to a procedure described for the synthesis of **7b**. The reaction time was 3 h, yield 61%. Mp was 182 °C (from 2-propanol). IR: 3256 (NH, NH_2), 1646 cm^{-1} (CO). ^1H NMR (DMSO-d_6) δ (ppm): 2.56 (s, 3H, SCH_3), 3.01 (t, J = 7.2 Hz, 2H, CH_2), 3.79–3.86 (m, 2H, CH_2), 7.34 (s, 2H, NH_2), 7.46 (d, J = 8.0 Hz, 2H, Ar–H), 7.78 (d, J = 8.0 Hz, 2H, Ar–H), 9.38 (t, J = 5.8 Hz, 1H, NH), 10.13 (s, 1H, CH). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.6, 35.0, 42.4, 105.0, 126.5, 129.9, 143.0, 143.8, 160.1, 164.2, 176.2, 190.7. HRMS calcd for $C_{14}H_{15}ClN_4O_3S_2$ $[M+H]^+$ = 387.0347, found 387.0340.

4.1.10. 4-[*N*-(6-Benzylamino-5-nitro-4-yl)amino]benzenesulfonamide (8a)

A mixture of 0.120 g (0.364 mmol) 4-[*N*-(6-chloro-5-nitropyrimidin-4-yl)amino]benzenesulfonamide (**5a**), 40 μL (0.364 mmol) of benzylamine, and 0.055 g (0.40 mmol) of anhydrous K_2CO_3 in 5 mL DMF was heated at 100 °C for 12 h. Then cooled to room temperature and poured into 50 mL of water. Resulting precipitate was purified by column chromatography using ethyl acetate/*n*-hexane (1:1 v/v) as eluent to give 0.088 g (60%) of compound **8a**. Mp 195 °C. IR: 3347 cm^{-1} (NH). ^1H NMR (DMSO-d_6) δ (ppm): 4.84 (d, J = 6.0 Hz, 2H, CH_2), 7.25–7.37 (m, 7H, NH_2, Ph), 7.83 (s, 4H, Ar–H), 8.18 (s, 1H, CH), 9.91 (t, J = 6.0 Hz, 1H, NH), 11.03 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 45.0, 113.7, 124.8, 126.9, 127.6, 128.0, 129.0, 139.4, 140.9, 141.2, 156.1, 157.2, 159.9. Anal. Calcd for $C_{17}H_{16}N_6O_4S$: C, 50.99; H, 4.03. Found: C, 50.94; H, 4.23.

4.1.11. 4-{[*N*-(6-Benzylamino-5-nitropyrimidin-4-yl)amino] methyl}benzenesulfonamide (8b)

Compound **8b** was prepared according to a procedure described for the synthesis **8a**. The reaction time was 3 h, yield 34%. Mp was 162 °C. IR: 3339, 3273 cm^{-1} (NH, NH_2). ^1H NMR (DMSO-d_6) δ (ppm): 4.80 (d, J = 6.1 Hz, 2H, CH_2), 4.85 (d, J = 6.0 Hz, 2H, CH_2), 7.23–7.37 (m, 7H, Ph, NH_2), 7.51 (d, J = 8.2 Hz, 2H, Ar–H), 7.78 (d, J = 8.2 Hz, 2H, Ar=H), 8.04 (s, 1H, CH), 9.88 (t, J = 6.1 Hz, 1H, NH), 9.96 (t, J = 6.0 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 44.6, 44.9, 113.1, 126.4, 127.6, 128.0, 128.1, 129.0, 139.5, 143.2, 143.8, 157.4, 157.5, 160.0. Anal. Calcd for $C_{18}H_{18}N_6O_4S$: C, 52.17; H, 4.38. Found: C, 52.43; H, 4.59.

4.1.12. 4-{2-[*N*-(6-Benzylamino-5-nitropyrimidin-4-yl) amino]ethyl}benzenesulfonamide (8c)

Compound **8c** was prepared according to procedure described for the synthesis **8a**. The reaction time was 3 h, yield 35%. Mp was 211 °C. IR: 3274, 3335 cm^{-1} (NH, NH_2). ^1H NMR (DMSO-d_6) δ (ppm): 3.00 (t, J = 6.2 Hz, 2H, CH_2), 3.79–3.86 (m, 2H, CH_2), 4.80 (d, J = 5.9 Hz, 2H, CH_2), 7.22–7.35 (m, 7H, Ph, NH_2), 7.46 (d, J = 8.2 Hz, 2H, Ar–H), 7.77 (d, J = 8.2 Hz, 2H, Ar–H), 8.12 (s, 1H, CH), 9.49 (t, J = 5.8 Hz, 1H, NH), 9.86 (t, J = 5.9 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 35.1, 42.8, 44.9, 112.9, 126.5, 127.6, 128.0, 129.0, 129.9, 139.5, 142.9, 144.0, 157.4, 157.5, 160.2. HRMS calcd for $C_{19}H_{20}N_6O_4S$ $[M+H]^+$ = 429.1340, found 429.1339.

4.1.13. 4-[*N*-(6-Benzylamino-5-cyano-2-methylthiopyrimidin-4-yl)amino]benzenesulfonamide (9a)

Compound **9a** was synthesized from 4-[*N*-(6-chloro-5-cyano-2-methylthiopyrimidin-4-yl)amino]benzenesulfonamide (**6a**) and benzylamine according to procedure described for the synthesis of **8a**. Compound **9a** was purified by column chromatography using diethyl ether as eluent. The reaction time was 12 h, yield 54%. Mp was 194 °C. IR: 3417 (NH, NH_2), 2198 cm^{-1} (CN). ^1H NMR (DMSO-d_6) δ (ppm): 2.34 (s, 3H, SCH_3), 4.59 (s, 2H, CH_2), 7.24–7.34 (m, 7H, 2NH, Ph), 7.66 (m, 4H, Ar–H), 8.10 (s, 1H, NH). HRMS calcd for $C_{19}H_{18}N_6O_2S_2$ $[M+H]^+$ = 427.1005, found 427.1011.

4.1.14. 4-{[*N*-(6-Methoxy-5-nitropyrimidin-4-yl)amino] methyl}benzenesulfonamide (10b)

A mixture of 0.194 g (1 mmol) 4,6-dichloro-5-nitropyrimidine (**1**), 0.223 g (1 mmol) 4-(aminomethyl)benzenesulfonamide hydrochloride (**4b**) and 0.290 g (2.1 mmol) of anhydrous K_2CO_3 in 10 mL THF was stirred for 8 h at room temperature. The solution was filtered and the solvent was removed under reduced pressure. To the obtained residue 20 mL MeOH was added and the mixture was refluxed for 30 min. The hot solution was filtered and cooled to room temperature. The precipitate was filtered off and dried in air to give 0.20 g (57%) of compound **12b**. Mp 174 °C. IR: 3270, 3374 cm^{-1} (NH, NH_2). ^1H NMR (DMSO-d_6) δ (ppm): 4.00 (s, 3H, OCH_3), 4.80 (d, J = 6.1 Hz, 2H, CH_2), 7.33 (s, 2H, NH_2), 7.50 (d, J = 8.1 Hz, 2H, Ar–H), 7.78 (d, J = 8.1 Hz, 2H, Ar–H), 8.31 (s, 1H, CH), 9.14 (t, J = 6.1 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 44.8, 55.9, 116.7, 126.4, 128.0, 143.3, 143.8, 156.6, 159.3, 163.8. Anal. Calcd for $C_{12}H_{13}N_5O_5S$: C, 42.47; H, 3.86. Found: C, 42.29; H, 4.01.

4.1.15. 4-{2-[*N*-(6-Methoxy-5-nitropyrimidin-4-yl)amino] ethyl}benzenesulfonamide (10c)

Compound **10c** was synthesized and purified by the procedure described for compound **10b**. Yield 47%. Mp 208 °C. IR: 3256, 3358 cm^{-1} (NH, NH_2). ^1H NMR (DMSO-d_6) δ (ppm): 2.98 (t, J = 7.2 Hz, 2H, CH_2), 3.75–3.81 (m, 2H, CH_2) 4.00 (s, 3H, OCH_3), 7.33 (s, 2H, NH_2), 7.45 (d, J = 8.2 Hz, 2H, Ar–H), 7.77 (d, J = 8.2 Hz, 2H, Ar–H), 8.38 (s, 1H, CH), 8.63 (t, J = 5.7 Hz, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 35.2, 42.9, 55.8, 120.7, 126.5, 129.9, 142.9, 144.0, 156.5, 159.4, 163.8. Anal. Calcd for $C_{13}H_{15}N_5O_5S$: C, 44.19; H, 4.28. Found: C, 44.25; H, 4.38.

4.1.16. 4-{[(5-Nitro-6-oxo-1,6-dihydro-4-pyrimidinyl) amino]methyl}benzenesulfonamide (11b)

To a solution of 0.160 g (0.466 mmol) of 4-{[*N*-(6-chloro-5-nitropyrimidin-4-yl)amino]methyl}benzenesulfonamide (**5b**) in

THF (5 mL) water (150 μL) was added. The mixture was left at room temperature for 3 days. The solid formed was filtered off, washed with THF (3 × 5 mL) and dried in an air to give 0.125 g (83%) of compound **11b**. Mp >300 °C (dec) IR: 1676 (CO), 3066, 3191, 3274, 3324 cm^{-1} (NH, NH_2). ^1H NMR (DMSO-d_6) δ (ppm): 4.86 (d, J = 6.2 Hz, 2H, CH_2), 7.33 (s, 2H, NH_2), 7.49 (d, J = 8.2 Hz, 2H, Ar–H), 7.78 (d, J = 8.2 Hz, 2H, Ar–H), 8.09 (s, 1H, CH), 10.0 (t, 1H, NH, J = 6.2 Hz), 12.47 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ (ppm): 45.1, 116.3, 126.4, 128.1, 143.4, 143.5, 152.7, 155.6, 158.9. HRMS calcd for $C_{11}H_{11}N_5O_5S$ $[M+H]^+$ = 326.0554, found 326.0554. Anal. Calcd for $C_{11}H_{11}N_5O_5S$: C, 40.61; H, 3.41. Found: C, 40.94; H, 3.64.

4.2. Construction of expression vectors and protein purification

Expression and purification of hCA I and hCA II were previously described: hCA I in Ref. 9 and hCA II in Ref. 30.

The hCA VII gene was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung (Berlin, Germany) in the pCMV-SPORT6 vector and cloned into the pET-15b vector (Novagen, Madison, WI) for expression. The nearly full-length CA VII nucleotide sequence encoding residues 3–264 was inserted into the BamHI site of the pET-15b vector fusing the His-tag sequence to the N-terminus of CA VII.

The hCA XIII gene was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung (Berlin, Germany) in the pOTB7 vector and cloned into the pET-15b vector for expression. The nucleotide sequence encoding full-length CA XIII (residues 1–262) was inserted into the pET-15b vector (Novagen, Madison, WI) via NcoI and XhoI sites. The cloning procedure resulted in the removal of the His-tag sequence from the vector, thus the pET-15b-CA XIII plasmid contains an open reading frame for full-length hCA XIII protein, with one additional methionine at the N-terminus.

Recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3). Expression plasmid-transformed cells were grown at 37 °C in LB media containing 0.1 mg/mL ampicillin and 60 μM $ZnSO_4$ to OD_{550} 0.5–0.8. After induction with 1 mM IPTG and 0.5 mM $ZnSO_4$, cells were cultured at 30 °C for 4 h. Cells were harvested by centrifugation and lysed by sonication. Soluble protein was purified using an affinity column (Ni^{+2} Chelating Sepharose, FF GE Healthcare Bio-Sciences, Uppsala, Sweden), followed by anion exchange chromatography (CM-Sepharose, FF, GE Healthcare Bio-Sciences, Uppsala, Sweden). Eluted protein was dialyzed against storage buffer (20 mM HEPES, pH 7.5, 50 mM NaCl) and stored at −80 °C.

4.3. Compound binding to CA

4.3.1. Isothermal titration calorimetry (ITC)

ITC experiments were performed using a VP-ITC instrument (Microcal, Inc.) using 5–20 μM protein solution in the cell and 50–200 μM ligand solution in the syringe. A typical experiment consisted of 25–30 injections 10 μL each within 3–4 min intervals. Experiments were carried out at 37 °C in phosphate buffer containing 50 mM NaCl at pH 7.0, with a final DMSO concentration of 0.5–2%.

4.3.2. Thermal shift assay (TSA)

TSA experiments were performed in the Corbett Rotor-Gene 6000 (QIAGEN Rotor-Gene Q) instrument using the blue channel (excitation 365 ± 20, detection 460 ± 15 nm). Samples contained 10 μM protein, 0–200 μM ligand, 50 μM solvatochromic dye ANS (8-anilino-1-naphthalene sulfonate), and phosphate buffer containing 50 mM NaCl at pH 7.0, with a final DMSO concentration of 2%. The applied heating rate was 1 °C/min.

4.4. Crystallography

4.4.1. Crystallization

hCA II was concentrated prior to crystallization to 20–60 mg/mL by ultrafiltration in 20 mM Na-Hepes pH 7.5 and 50 mM NaCl. Crystallization was started by mixing equal volumes of protein solution with reservoir buffer. Crystallization buffers were prepared by mixing 1 M Na-Bicine pH 9 to the concentration 0.1 M, 2.7 M Na-Malonate pH 7–7.55 to the final concentration ranged from 1.5 to 2.2 M, and 3.5 M ammonium sulfate to the final concentration ranged from 0 to 0.2 M. Crystals belonging to the $P2_1$ space group were grown over several days. Crystals were soaked in a 0.5–1.0 mM solution of the ligand of interest, prepared by mixing 100 mM ligand solution in DMSO with 50 μL of the reservoir buffer used in crystallization.

4.4.2. Data collection and structure determination

Diffraction data from all complexes of hCA II with inhibitors, except **11b**, were collected at the EMBL X11, X12, and X13 beam lines at the DORIS storage ring (DESY, Hamburg). The dataset of hCA II with **11b** was measured by the Institute of Biotechnology (Vilnius, Lithuania) with an RU-H3R diffractometer (Rugaku, Japan). MOSFLM[31,32] and SCALA[33] were used for image processing. Initial phases were obtained by molecular replacement with the protein moiety from PDB entry 3HLJ[9] as an initial model. Structures were refined using REFMAC[34] and COOT[35] was used for model inspection. Atomic co-ordinates of ligands were generated by DSVisualizer 1.7[36] (Accelrys). Topology and parameters for structure refinement were generated by LIBREFMAC.[37] Data collection and refinement statistics are presented in Table 2. Co-ordinates and structure factors were deposited in the RCSB Protein Data Bank and the PDB IDs are listed in Table 2.

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