Bioorganic & Medicinal Chemistry 18 (2010) 7357–7364

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Indapamide-like benzenesulfonamides as inhibitors of carbonic anhydrases I, II, VII, and XIII

Edita Čapkauskaitė [a,b], Lina Baranauskienė [a], Dmitrij Golovenko [c], Elena Manakova [c], Saulius Gražulis [c], Sigitas Tumkevičius [b], Daumantas Matulis [a,*]

[a] Laboratory of Biothermodynamics and Drug Design, Institute of Biotechnology, Graičiūno 8, Vilnius LT-02241, Lithuania
[b] Department of Organic Chemistry, Faculty of Chemistry, Vilnius University, Naugarduko 24, Vilnius LT-03225, Lithuania
[c] Laboratory of Protein–DNA Interactions, Institute of Biotechnology, Graičiūno 8, Vilnius LT-02241, Lithuania

ARTICLE INFO

Article history:
Received 27 May 2010
Revised 2 September 2010
Accepted 7 September 2010
Available online 15 September 2010

Keywords:
Carbonic anhydrase isozymes I, II, VII, and XIII
Isothermal titration calorimetry
Thermal shift assay
ThermoFluor®
Sulfonamides
X-ray crystallography

ABSTRACT

A series of novel 2-chloro-5-[(1-benzimidazolyl- and 2-benzimidazolylsulfanyl)acetyl]benzenesulfonamides were designed and synthesized. Their binding to recombinant human carbonic anhydrase (hCA) isozymes I, II, VII, and XIII was determined by isothermal titration calorimetry and thermal shift assay. The designed S-alkylated benzimidazole derivatives exhibited stronger binding than the indapamide-like N-alkylated benzimidazoles, with the K_d reaching about 50–100 nM with drug-targeted hCAs VII and XIII. The cocrystal structures of selected compounds with hCA II were determined by X-ray crystallography, and structural features of the binding event were revealed.

1. Introduction

Carbonic anhydrases (CAs, also known as carbonate dehydratases, EC 4.2.1.1) are ubiquitous zinc-containing metalloenzymes present in prokaryotes and eukaryotes.[1] They catalyze the hydration of carbon dioxide to bicarbonate and the corresponding dehydration of bicarbonate in acidic medium, to regenerate CO_2.[2] CA isoforms are found in a variety of tissues, where they participate in numerous important biological processes such as pH balance, CO_2 homeostasis, respiration, carbon dioxide and ion transport, ureagenesis, gluconeogenesis, lipogenesis, electrolyte secretion, bone resorption, calcification and tumorigenicity.[1,3–5]

Many of these isozymes are important targets for the design of inhibitors with clinical applications. The most prominent class of CA inhibitors consists of aromatic/heterocyclic sulfonamides, which have been studied for their use as antiglaucoma, antitumor, antiobesity or anticonvulsant drugs.[6,7] However, there are several critical problems in the design of CA inhibitors with pharmacological applications: the high number of isoforms (12 active forms in human), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity for the presently available inhibitors.[8–10]

Inhibitor binding potency depends on the acidity of the sulfonamide group. Therefore, inhibitors with electron-withdrawing groups that increase the sulfonamide acidity, such as chlorine and carbonyl groups, also increase inhibitor potency. Substituents on the benzene ring could also improve inhibitor selectivity. Despite the progress made in the understanding of quantitative structure–activity relationships of CA inhibitors,[7,11] most correlations have been drawn based on activity-inhibition measurements. However, it is important to also measure binding by using biophysical thermodynamic techniques.[12] Here, we report the use of isothermal titration calorimetry (ITC) and 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.[13,14] However, it is not appropriate for determining weak (millimolar) or extremely tight dissociation constants (subnanomolar K_ds require displacement ITC[15]) and consumes extensive amounts time and protein. In contrast, TSA is a rapid screening method used in pharmaceutical industry for the identification of specific binders and requires lesser amounts of protein.[16,17] The method is based on the protein melting temperature (T_m) shift that occurs upon ligand binding; the T_m is observed by following intrinsic or extrinsic fluorescence changes upon heat-induced protein unfolding. The employment of two techniques to determine binding reactions reduces the uncertainty of the measurements.

* Corresponding author.
E-mail address: matulis@ibt.lt (D. Matulis).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.09.016

Indapamide (Fig. 1) has been used for the treatment of patients with hypertension and type-2 diabetes. The beneficial effect might be due to its potent inhibition of CA isoforms present in kidneys and blood vessels, which would thus explain both the blood pressure lowering effects as well as the organ-protective activity of the drug.[18] It has been reported that the K_is of indapamide to a series of CAs are as follows: 0.23 nM with hCA VII, 51,900 nM for hCA I, 2520 nM with hCA II, and 13 nM with murine CA XIII.[19] Our ITC and TSA measurements confirm that hCA I is poorly inhibited by indapamide. However, the potency towards hCA VII is only about 300 (TSA) to 1800 (ITC) nM. This discrepancy may be due to the different nature of the techniques employed. Therefore, we believe that it is important to confirm the potency of inhibitors by numerous techniques to avoid technique-specific effects.

Several our synthetic compounds reported here, **3l**, **3m**, and **3n**, were found to be more potent than indapamide towards the tested CAs, making them potential candidates for further development.

2. Results and discussion

2.1. Chemistry

A series of substituted benzene sulfonamides were designed as CA inhibitors. The chemistry employed for the synthesis of the compounds reported is shown in Scheme 1.

2-Substituted benzimidazoles **2a–i** were prepared from 1,2-benzenediamine and the appropriate carboxylic acids according to a Phillips procedure.[20] The physical characteristics of the 2-substituted benzimidazoles corresponded with the reported data.[20–24] Compound **2j** was synthesized from 2-(chloromethyl)-1*H*-benzimidazole via nucleophilic substitution of the chloride with morpholine.[25] 2-Methylthiobenzimidazole (**2k**) was obtained from 1,3-dihydro-2*H*-benzimidazole-2-thione (**2l**)[26] according to a previously reported procedure.[27] Synthesis of 5-(2-bromoacetyl)-2-chlorobenzenesulfonamide (**1**) was performed from commercially available 1-(4-chlorophenyl)ethanone as reported previously.[28,29]

N-Alkylation of **2a–k** with 5-(bromoacetyl)-2-chlorobenzenesulfonamide (**1**) to give N-substituted benzimidazole derivatives **3a–k** was carried out in the presence of sodium acetate in tetrahydrofuran at room temperature (Scheme 1). Using sodium methoxide as a base and performing the reaction in boiling methanol led to an inseparable mixture of products. It should be noted that better results in the N-alkylation reaction were obtained when a slight excess of the corresponding benzimidazole **2a–k** was used. Conversely, employing an excess of compound **1** results in the dialkylation of the benzimidazole ring.

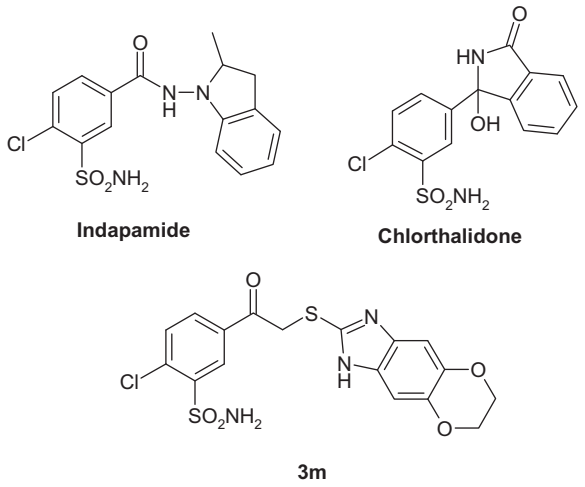


Figure 1. The chemical structure of indapamide, chlorthalidone, and **3m**.

Using the same reaction conditions employed for the syntheses of **3a–k**, thiones **2l–o** reacted with compound **1** to form the corresponding S-alkylated products **3l–o**. Structural assignments were made based upon their ^{1}H and ^{13}C NMR spectra. In the ^{1}H NMR spectra of N-alkylated benzimidazoles **3a–k**, a singlet was observed that corresponded to the NCH_2CO group in the region of 5.97–6.08 ppm. The corresponding ^{1}H signal for the SCH_2CO group of compounds **3l–o** was observed at 5.02–5.16 ppm; in comparison to the NCH_2CO group, the signal is shifted upfield due to the lower electronegativity of the sulfur atom. An analogous difference in the chemical shifts for the corresponding carbons was also observed in the ^{13}C NMR spectra: for compounds **3a–k**, the NCH_2 signal appears in the region 50.53–55.96 ppm, whereas the SCH_2 signal for compounds **3l–o** is observed at 39.66–40.25 ppm. The structure of **3m** is shown in Figure 1.

Thione **2m** was prepared according to a previously reported procedure.[30] The synthesis of 5-bromo-1,3-dihydro-2*H*-benzimidazole-2-thione (**2n**) was accomplished via the bromination of 2-nitroaniline[31] and subsequent reduction and cyclization.[32] 1,3-Dihydro-2*H*-imidazo[4,5-*c*]quinoline-2-thione (**2o**) was obtained by the reaction of 3,4-quinolinediamine[33] with carbon disulfide.

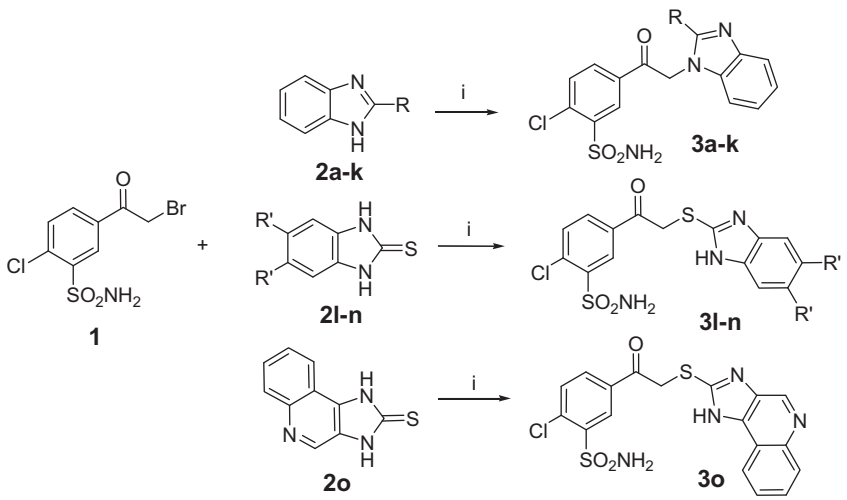
2.2. Binding studies

The binding affinities of indapamide-like benzenesulfonamides to recombinant human carbonic anhydrase isozymes I, II, VII, and XIII were determined by thermal shift assay and isothermal titration calorimetry. Figure 2 shows typical thermal shift assay data for inhibitor **3f** binding to hCA II. Panel A shows fluorescence profiles of CA heat-induced denaturation. The midpoints of the unfolding transitions are plotted in panel B, giving compound dosing curves as a function of the concentration of added compound. Figure 3 shows typical ITC data for compound **3l** binding to hCA II. Panel A shows a raw ITC data curve, and Panel B shows the integrated ITC curve. Fitting both TSA and ITC dosing curves yielded the binding constants for the binding of all tested compounds to the CAs. The observed dissociation constants for compounds **3a–o** are listed in Table 1.

The S-alkylated imidazo derivatives **3l–o** have dissociation constants in the range of 0.02–3.1 μM and bind stronger than N-alkylated benzimidazoles **3a**–**k**, which have dissociation constants in the range of 0.3–40 μM, as determined by TSA. Compound **3l** bound hCA VII more tightly than other CAs. However, compounds **3m**–**o** bound CA II most strongly (Table 1).

There was some systematic discrepancy between TSA and ITC data observed with hCA II and hCA VII, which was not observed with the other two CAs. Both ITC and TSA experiments were repeated several times, and the discrepancy appeared to be out of the range of the standard deviations for both methods.

Indapamide inhibition constants (K_i) have been previously determined for most CA isozymes.[19] K_is for CAs I, II, VII, and XIII were reported to equal 51.9, 2.52, 0.00023, and 0.013 μM, respectively. These numbers differ from our values measured by ITC and TSA (Table 1). CAs I and II appear to bind indapamide slightly tighter than previously determined, whereas CAs VII and XIII appear to bind indapamide significantly weaker than previously determined. The largest difference was observed for hCA VII. Our determined K_d value is about 1000 times weaker than previously determined. Despite a general agreement between the three techniques,[12] quite often there is a discrepancy between activity and binding measurements, likely due to the significant differences between the techniques: ITC is done at constant temperature, thermal shift assay is performed by heating the sample, and the inhibition measurements involve stop-flow kinetics, the only method to follow enzymatic activity.



a: R=H; **h**: R=$CHMe_2$;
b: R=Me; **i**: R=CH_2CHMe_2;
c: R=CH_2OH; **j**: R=$CH_2N(CH_2CH_2)_2O$;
d: R=CH_2Ph; **k**: R=SMe;
e: R=CH_2Me; **l**: R'=H;
f: R=$(CH_2)_2Me$; **m**:R'+R'=OCH_2CH_2O;
g: R=$(CH_2)_3Me$; **n**: R'=Br, R'=H.

Scheme 1. Synthesis of compounds **3a**–**o**. Reagents and conditions: (i) NaOAc (1.15 equiv), THF, room temperature, 24 h.

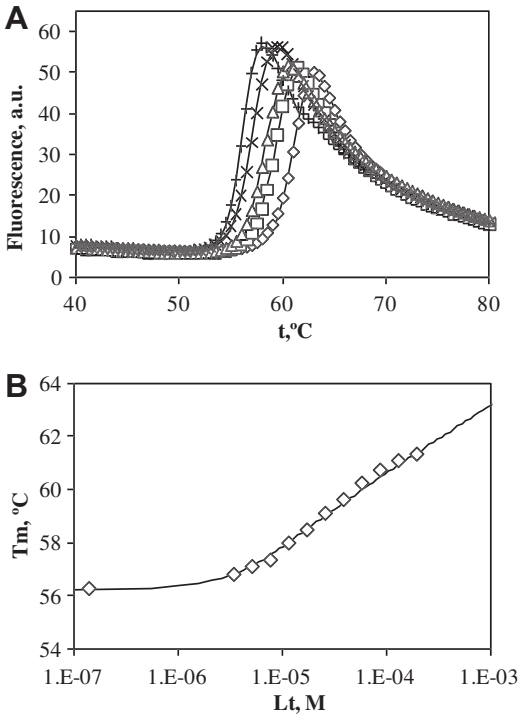


Figure 2. Thermal shift assay data of compound **3f** binding to hCA II. Panel A—raw data: addition of different compound concentrations increases the protein melting temperature (◇—200 μM, □—39 μM, △—17 μM, ×—7 μM, and **+**—no ligand). Panel B—integrated compound dosing curve.

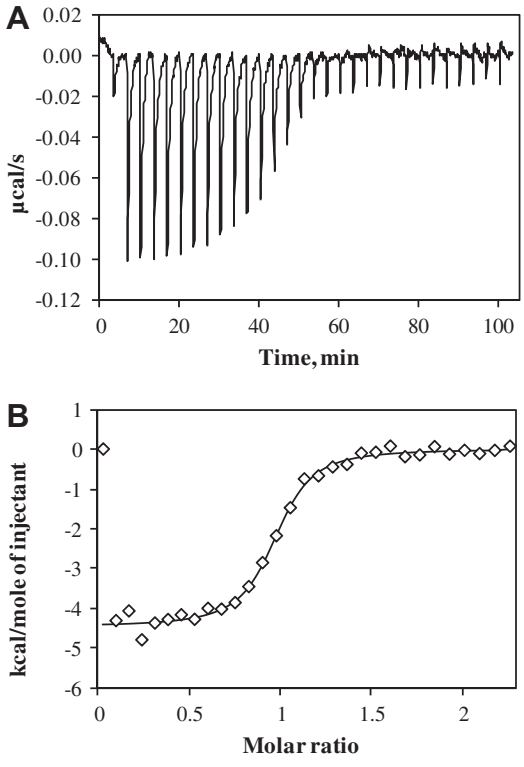


Figure 3. ITC data of **3l** binding to hCA II. Panel A—raw data, panel B—integrated data.

2.3. Crystallography

To determine the structural features of compound binding to CAs, the structures of compounds **3m**, **3n**, and **3o** bound to hCA II were solved by X-ray crystallography (Fig. 4). Comprehensive structural analysis would require comparison of binding data with structural data for all tested CAs. However, crystallization of other CAs bound with the compounds has been unsuccessful to date. The

Table 1
Dissociation constants (in μM) determined by thermal shift assay and for several compounds revised by isothermal titration calorimetry (ITC values given in brackets) at 37 °C

	hCA I	hCA II	hCA VII	hCA XIII
3a	11	1.6	1.0	0.7
3b	10	2.0	2.5	0.4
3c	7.1	1.0	0.4	0.3
3d	8.3	1.6	3.3	1.0
3e	7.1	0.6	1.7	0.4
3f	10	0.8	2.5	1.4
3g	10	0.5	3.3	0.4
3h	4.5	0.7	2.0	0.4
3i	3.3	0.4	1.0	2.9
3j	40	4.2	10.0	12.5
3k	5.0	1.0	1.7	1.1
3l	1.0 (1.3)	0.1 (0.1)	0.03 (0.1)	0.2 (0.1)
3m	3.1 (2.2)	0.02 (0.18)	0.1 (1.1)	0.1 (0.3)
3n	1.3 (1.4)	0.06	0.1	0.1 (0.2)
3o	1.3	0.03	0.1	0.1
Indapamide	10	0.3 (0.2)	0.3 (1.8)	0.1 (0.2)

Average standard deviations for both methods were below 25%.

data refinement statistics for the crystal structures are listed in Table 2.

The analysis of hCA II in complex with **3m–o** revealed a conserved network of bonds commonly formed upon binding of a sulfonamide moiety. The positions of Zn^{2+}, His94, His96, and His119 are similar to observed in previously reported structures. The 2-chlorobenzensulfonamide ring of the compounds is fixed by steric constraints imposed by residues Gln92, Leu198, Val121 and Val143. The chlorine atom is found in a hydrophobic pocket formed by residues Leu141, Val143, Val207, Val121 and Leu198. The orientation of the 2-chlorobenzensulfonamide ring within the hCAII active site is identical to previous reports of chlorthalidone and indapamide (Fig. 5).[19,34]

The linker between the chlorobenzensulfonamide and the second benzimidazole ring is an important part of ligand binding to hCA II. The S atom likely accepts the hydrogen bond from the Nδ2 atom of Asn62 (Fig. 4). The carbonyl group participates in hydrogen bond with Nε2 of Gln92 (**3m**, **3n**) or in two hydrogen bonds with Oγ1 of Thr200 and Nε2 of His64 (**3o**). The only multiring system of **3m** has additional direct and water mediated hydrogen bonds with the protein (Fig. 4). The conformation of inhibitors **3n** and **3m** is similar. Such conformation of inhibitors **3n** and **3m** could be maintained through the hydrophobic contacts which their large aromatic multiring systems make with the protein pocket. The structural data show that hCAII-bound ligands **3m–o** have larger hydrogen bond pattern and bigger area of hydrophobic interactions than indapamide (Table 3).

3. Conclusions

A series of novel benzenesulfonamides with benzimidazole moiety have been synthesized and evaluated against four hCA isoforms using isothermal titration calorimetry and thermal shift assay. The S-alkylated benzimidazole derivatives **3l–o** bind stronger than indapamide-like N-alkylated benzimidazoles **3a–k**. The S-alkylated benzimidazole K_ds reach about 50–100 nM with CAs VII and XIII.

4. Experimental

4.1. Syntheses

Melting points of the compounds were determined in open capillaries on a Thermo Scientific 9100 Series 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) in DMSO-d_6 using residual DMSO signals (2.52 and 40.21 ppm for ^{1}H and ^{13}C NMR spectra, respectively) as the internal standard. TLC was performed with Silica Gel 60 F254 aluminum plates (Merck) and visualized with UV light. High-resolution mass spectra (HRMS) were recorded on a Dual-ESI Q-TOF 6520 mass spectrometer (Agilent Technologies).

4.1.1. 1,3-Dihydro-2*H*-imidazo[4,5-*c*]quinoline-2-thione (2o)

To a solution of 3,4-quinolinediamine (100 mg, 0.628 mmol) in methanol (5 ml) a solution of sodium hydroxide (0.028 g, 0.691 mmol) and carbon disulfide (0.053 g, 0.691 mmol) in water (1 ml) was added dropwise. The reaction mixture was heated at reflux for 3 h and then stirred at rt for 24 h. The reaction mixture was acidified with 0.5 M HCl, solvents evaporated under reduced pressure and the residue suspended in water. The precipitate obtained was filtered off and washed with water to give compound **2o**. Yield 79%, mp 324–326 °C. IR ν cm^{-1}: 3044 (NH), 1498 (C=S).

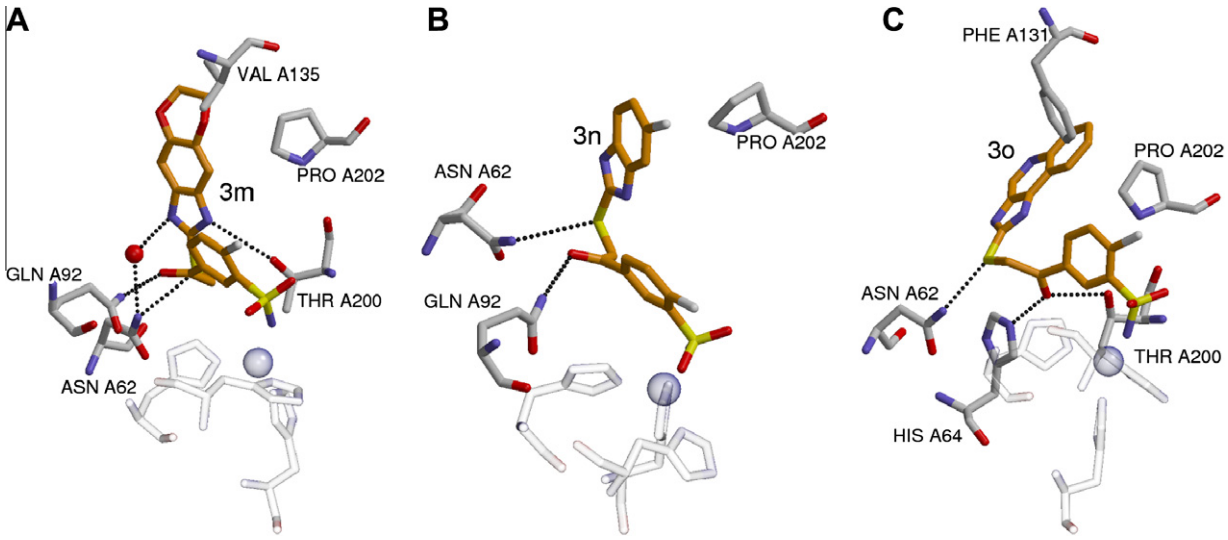


Figure 4. View of compounds **3m** (a), **3n** (b), and **3o** (c) bound in the active center of hCA II. The Zn atom, His94, His96, and His119 are shown as transparent, inhibitors are shown in orange. Picture is generated using MOLSCRIPT[44] and Raster3D.[45]

Table 2
X-ray crystallographic data collection and refinement statistics

Compound	**3m**	**3n**	**3o**
PDB ID	3M67	3M96	3MYQ
Temperature, K	100	100	100
Space group	$P2_1$	$P2_1$	$P2_1$
Unit cell	a = 41.9722, b = 41.0069, c = 71.6806 Å, $\alpha = \gamma$ = 90, β = 104.4303	a = 42.0496, b = 40.9205, c = 71.4681 Å, $\alpha = \gamma$ = 90, β = 104.1442	a = 41.9796, b = 40.9099, c = 71.6071 Å, $\alpha = \gamma$ = 90, β = 104.3360
Resolution, Å (final shell)	40.66–1.80 (1.90–1.80)	28.88–1.40 (1.48–1.40)	28.84–1.35 (1.42–1.35)
Reflections unique (total)	22,019 (165,478)	46,297 (431,844)	50,807 (131,001)
Completeness (%) overall (final shell)	99.3 (99.0)	99.2 (98.1)	98.1 (97.0)
I/σ_I overall (final shell)	31.5 (7.0)	22.9 (4.4)	16.9 (3.6)
R_{merge} overall (final shell)	0.052 (0.222)	0.095 (0.253)	0.057 (0.229)
B(iso) from Wilson, (Å^2)	19.3	14.3	13.5
Refinement			
Number of atoms[a]	2294	2362	2436
Number of solvent molecules	158	239	264
Number of bounded buffer molecule atoms	8	8	20
Test set size, %	10	10	10
R_{cryst} (R_{free})	0.181 (0.228)	0.180 (0.213)	0.139 (0.181)
RMS bonds/angles	0.027(2.061)	0.032 (2.587)	0.024 (2.187)
Average B factors (Å^2), total	17.3231	12.9173	13.454
Main chain	19.0262	15.857	16.252
Side chains	25.8868	24.8892	26.458
Solvent	10.01	7.21	8.29
Ions	30.665	25.6413	22.9765
Cofactors	31.5714	21.6448	24.6336
Inhibitor	31.5714	21.6448	24.6336

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

[a] Including atoms in alternative positions.

^{1}H NMR δ ppm: 7.67 (2H, m, $C_{7,8}$-H), 8.04 (1H, d, J = 9.3 Hz, C_9-H), 8.30 (1H, d, J = 9.6 Hz, C_6-H), 8.77 (1H, s, C_4-H), 13.18 (1H, br s, NH), 13.80 (1H, br s, NH). ^{13}C NMR δ ppm: 115.36, 122.15, 126.44, 127.59, 128.40, 130.21, 133.82, 135.04, 144.48, 169.00. HRMS calcd for $C_{10}H_7N_3S$ ([M+H]$^+$): 202.0433, found: 202.0434.

4.1.2. General procedure for the synthesis of 3a–k

A mixture of the corresponding 2-substituted benzimidazole (**2a–k**) (0.832 mmol), compound **1** (200 mg, 0.640 mmol) and sodium acetate (60.3 mg, 0.736 mmol) in tetrahydrofuran (5 ml) was stirred at rt for 24 h. The reaction mixture was poured into water. The precipitate was filtered off, washed with water and then with diethyl ether.

4.1.2.1. 5-(1*H*-Benzimidazol-1-ylacetyl)-2-chlorobenzenesulfonamide (3a). Yield 83%, mp 196–198 °C. IR ν cm^{-1}: 3382 (NH_2), 1705 (C=O). ^{1}H NMR δ ppm: 6.08 (2H, s, CH_2CO), 7.23–7.26 (2H, m, $C_{5',6'}$-H), 7.51–7.57 (1H, m, $C_{7'}$-H), 7.68–7.71 (1H, m, $C_{4'}$-H), 7.91 (2H, s, NH_2), 7.95 (1H, d, J = 8.1 Hz, C_3-H), 8.19 (1H, s, $C_{2'}$-H), 8.35 (1H, dd, J = 2.1 Hz, J = 8.1 Hz, C_4-H), 8.55 (1H, d, J = 2.1 Hz, C_6-H). ^{13}C NMR δ ppm: 51.60, 111.39, 119.97, 122.37, 123.19, 128.75, 133.06, 133.56, 133.90, 135.26, 136.54, 142.32, 143.59, 145.53, 192.80. HRMS calcd for $C_{15}H_{12}ClN_3O_3S$ ([M+H]$^+$): 350.0361, found: 350.0367.

4.1.2.2. 2-Chloro-5-[(2-methyl-1*H*-benzimidazol-1-yl)acetyl] benzenesulfonamide (3b). Yield 84%, mp 266–268 °C. IR ν cm^{-1}: 3393, 3285 (NH_2), 1704 (C=O). ^{1}H NMR δ ppm: 2.44 (3H, s, CH_3), 6.07 (2H, s, CH_2CO), 7.15–7.18 (2H, m, $C_{5',6'}$-H), 7.47–7.49 (1H, m, $C_{7'}$-H), 7.56–7.58 (1H, m, $C_{4'}$-H), 7.90 (2H, s, NH_2), 7.98 (1H, d, J = 8.4 Hz, C_3-H), 8.40 (1H, d, J = 8.4 Hz, C_4-H), 8.58 (1H, s, C_6-H). ^{13}C NMR δ ppm: 14.01, 50.73, 110.66, 118.80, 122.06, 122.22, 128.92, 132.97, 133.80, 133.87, 136.51, 136.65, 142.35, 142.88, 153.34, 192.94. HRMS calcd for $C_{16}H_{14}ClN_3O_3S$ ([M+H]$^+$): 364.0517, found: 364.0518.

4.1.2.3. 2-Chloro-5-{[2-(hydroxymethyl)-1*H*-benzimidazol-1-yl]acetyl}benzenesulfonamide (3c). Yield 49%, mp 253–255 °C. IR ν cm^{-1}: 3385, 3285 (OH, NH_2), 1706 (C=O). ^{1}H NMR δ ppm: 4.69 (2H, d, J = 4,2 Hz, CH_2O), 5.67 (1H, br s, OH), 6.07 (2H, s, CH_2CO), 7.21–7.24 (2H, m, $C_{5',6'}$-H), 7.52–7.54 (1H, m, $C_{7'}$-H), 7.63–7.66 (1H, m, $C_{4'}$-H), 7.91 (2H, s, NH_2), 7.95 (1H, d, J = 8.1 Hz, C_3-H), 8.38 (1H, d, J = 7.8 Hz, C_4-H), 8.56 (1H, s, C_6-H). ^{13}C NMR δ ppm: 50.87, 57.51, 111.00, 119.61, 122.38, 123.08, 128.85, 133.04, 133.62, 133.92, 136.52, 136.96, 142.29, 154.70, 192.55. HRMS calcd for $C_{16}H_{14}ClN_3O_4S$ ([M+H]$^+$): 380.0466, found: 380.0472.2

4.1.2.4. 5-[(2-Benzyl-1*H*-benzimidazol-1-yl)acetyl]-2-chlorobenzenesulfonamide (3d). Yield 30%, mp 128–130 °C. IR ν cm^{-1}: 3401 (NH_2), 1705 (C=O). ^{1}H NMR δ ppm: 4.26 (2H, s, CH_2Ph), 6.05 (2H, s, CH_2CO), 7.20–7.25 (m, 7H, $C_{5',6'}$-H, C_6H_5), 7.45 (d, 1H, J = 6.9 Hz, $C_{7'}$-H), 7.62 (1H, d, J = 6.9 Hz, $C_{4'}$-H), 7.89 (2H, s, NH_2), 7.95 (1H, d, J = 8.1 Hz, C_3-H), 8.31 (1H, d, J = 8.4 Hz, C_4-H), 8.47 (1H, s, C_6-H). ^{13}C NMR δ ppm: 33.43, 50.68, 111.01, 119.24, 122.22, 122.55, 127.19, 128.88, 129.07, 129.62, 132.85, 133.64, 133.88, 136.48, 136.67, 137.43, 142.26, 142.99, 154.84, 192.39. HRMS calcd for $C_{22}H_{18}ClN_3O_3S$ ([M+H]$^+$): 440.0830, found: 440.0837.

4.1.2.5. 2-Chloro-5-[(2-ethyl-1*H*-benzimidazol-1-yl)acetyl]benzenesulfonamide (3e). Yield 70%, mp 245–247 °C. IR ν cm^{-1}: 3281 (NH_2), 1705 (C=O). ^{1}H NMR δ ppm: 1.29 (3H, t, J = 7.5 Hz, CH_3), 2.78 (2H, q, J = 7.5 Hz, CH_2), 6.07 (2H, s, CH_2CO), 7.17–7.19 (2H, m, $C_{5',6'}$-H), 7.47–7.50 (1H, m, $C_{7'}$-H), 7.60–7.62 (1H, m, $C_{4'}$-H), 7.88 (2H, s, NH_2), 7.98 (1H, d, J = 8.4 Hz, C_3-H), 8.41 (1H, d, J = 8.4 Hz, C_4-H), 8.58 (1H, s, C_6-H). ^{13}C NMR δ ppm: 12.16, 20.43, 50.53, 110.75, 118.85, 122.17, 122.40, 128.93, 132.96, 133.81, 133.82, 136.44, 136.67, 142.37, 142.48, 157.47, 193.01. HRMS calcd for $C_{17}H_{16}ClN_3O_3S$ ([M+H]$^+$): 378.0674, found: 378.0677.

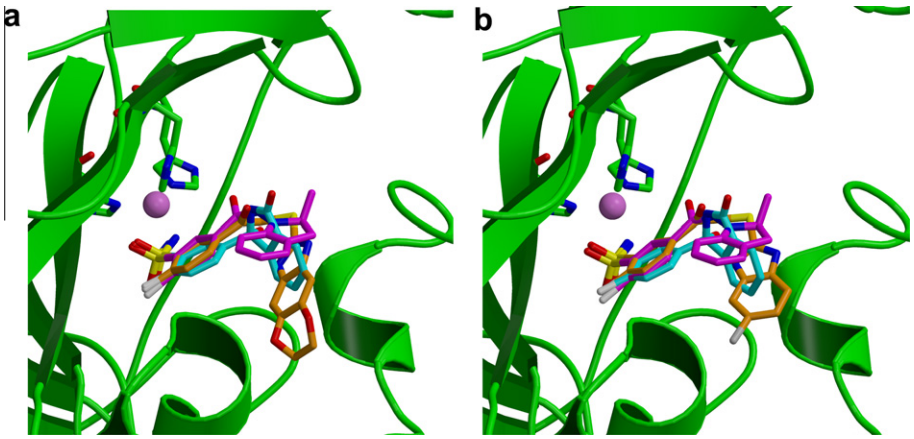


Figure 5. Superposition of the hCA II-indapamide [PDB ID 3BL1] (magenta), hCA II-chlorthalidone [PDB ID 3F4X] (cyan) with hCA II-2-chlorobenzenesulfonamide adducts (orange) **3m** (a) or **3n** (b). Zinc ion is shown as pink sphere, His94, His96, His119, and protein secondary structures are in green.

Table 3
The comparison of ligand (**3m**–**o**) and indapamide interaction to hCA II

Inhibitor	Number of hydrogen bonds (excluding those of sulfonamide moiety)	Buried surface area in the hCAII-inhibitor complex, (Å^2)
Indapamide	1	655.1
3m	4	728.0
3n	2	719.5
3o	3	709.2

4.1.2.6. 2-Chloro-5-[(2-propyl-1*H*-benzimidazol-1-yl)acetyl] benzenesulfonamide (3f). Yield 73%, 247–249 °C. IR ν cm^{-1}: 3304 (NH_2), 1706 (C=O). ^{1}H NMR δ ppm: 0.97 (3H, t, J = 7.5 Hz, CH_3), 1.78 (2H, sextet, J = 7.5 Hz, CH_2), 2.75 (2H, t, J = 7.5 Hz, CH_2), 6.07 (2H, s, CH_2CO), 7.13–7.20 (2H, m, $C_{5',6'}$-H), 7.45–7.47 (1H, m, $C_{7'}$-H), 7.59–7.62 (1H, m, $C_{4'}$-H), 7.88 (2H, s, NH_2), 7.98 (1H, d, J = 8.4 Hz, C_3-H), 8.42 (1H, dd, J = 1.8 Hz, J = 8.4 Hz, C_4-H), 8.59 (1H, d, J = 1.8 Hz, C_6-H). ^{13}C NMR δ ppm: 14.48, 20.97, 28.87, 50.54, 110.73, 118.96, 122.06, 122.26, 128.93, 132.95, 133.86, 136.43, 136.66, 142.38, 142.86, 156.36, 192.99. HRMS calcd for $C_{18}H_{18}ClN_3O_3S$ ([M+H]$^+$): 392.0830, found: 392.0834.

4.1.2.7. 5-[(2-Butyl-1*H*-benzimidazol-1-yl)acetyl]-2-chlorobenzenesulfonamide (3g). Yield 74%, mp 221–223 °C. IR ν cm^{-1}: 3363, 3302 (NH_2), 1705 (C=O). ^{1}H NMR δ ppm: 0.90 (3H, t, J = 6.9 Hz, CH_3), 1.35–1.41 (2H, m, CH_2), 1.71–1.75 (2H, m, CH_2), 2.76 (2H, t, J = 6.9 Hz, CH_2), 6.07 (2H, s, CH_2CO), 7.16 (2H, br s, $C_{5',6'}$-H), 7.46 (1H, d, J = 6.6 Hz, $C_{7'}$-H), 7.60 (1H, d, J = 6.6 Hz, $C_{4'}$-H), 7.88 (2H, s, NH_2), 7.98 (1H, d, J = 8.1 Hz, C_3-H), 8.42 (1H, d, J = 7.2 Hz, C_4-H), 8.58 (1H, s, C_6-H). ^{13}C NMR δ ppm: 14.46, 22.48, 26.57, 29.62, 50.53, 110.72, 118.92, 122.06, 122.25, 128.91, 132.94, 133.84, 133.87, 136.43, 136.65, 142.37, 142.81, 156.50, 193.01. HRMS calcd for $C_{19}H_{20}ClN_3O_3S$ ([M+H]$^+$): 406.0987, found: 406.0990.

4.1.2.8. 2-Chloro-5-[(2-isopropyl-1*H*-benzimidazol-1-yl)acetyl] benzenesulfonamide (3h). Yield 65%, 265–267 °C. IR ν cm^{-1}: 3339 (NH_2), 1711 (C=O). ^{1}H NMR δ ppm: 1.28 (6H, d, J = 6 Hz, $(CH_3)_2$), 3.12–3.27 (1H, m, CH), 6.10 (2H, s, CH_2CO), 7.17 (2H, br s, $C_{5',6'}$-H), 7.46 (1H, d, J = 7.2 Hz, $C_{7'}$-H), 7.61 (1H, d, J = 6.6 Hz, $C_{4'}$-H), 7.88 (2H, s, NH_2), 7.98 (1H, d, J = 8.1 Hz, C_3-H), 8.44 (1H, d, J = 6.9 Hz, C_4-H), 8.58 (1H, s, C_6-H). ^{13}C NMR δ ppm: 27.16, 30.88, 55.20, 115.61, 123.78, 126.89, 127.08, 133.67, 137.69, 138.60, 138.65, 140.98, 141.43, 147.12, 147.44, 165.84, 197.91. HRMS calcd for $C_{18}H_{18}ClN_3O_3S$ ([M+H]$^+$): 392.0830, found: 392.0837.

4.1.2.9. 2-Chloro-5-[(2-isobutyl-1*H*-benzimidazol-1-yl)acetyl] benzenesulfonamide (3i). Yield 75%, 223–225 °C. IR ν cm^{-1}: 3316 (NH_2), 1704 (C=O). ^{1}H NMR δ ppm: 0.96 (6H, d, J = 6.6 Hz, $(CH_3)_2$), 2.11–2.24 (1H, m, CH), 2.67 (2H, d, J = 7.2 Hz, CH_2), 6.07 (2H, s, CH_2CO), 7.12–7.20 (2H, m, $C_{5',6'}$-H), 7.43–7.45 (1H, m, $C_{7'}$-H), 7.60–7.62 (1H, m, $C_{4'}$-H), 7.87 (2H, s, NH_2), 7.97 (1H, d, J = 8.1 Hz, C_3-H), 8.43 (1H, dd, J = 1.5 Hz, J = 8.1 Hz, C_4-H), 8.58 (1H, d, J = 1.5 Hz, C_6-H). ^{13}C NMR δ ppm: 23.09, 27.65, 35.73, 50.60, 110.80, 118.97, 122.08, 122.24, 128.92, 132.94, 133.85, 136.34, 136.66, 142.38, 142.93, 155.75, 192.89. HRMS calcd for $C_{19}H_{20}ClN_3O_3S$ ([M+H]$^+$): 406.0987, found: 406.0994.

4.1.2.10. 2-Chloro-5-{[2-(4-morpholinylmethyl)-1*H*-benzimidazol-1-yl]acetyl}benzenesulfona-mide (3j). Yield 63%, 199–201 °C. IR ν cm^{-1}: 3435 (NH_2), 1703 (C=O). ^{1}H NMR δ ppm: 2.24 (4H, br s, $(CH_2)_2$), 3.10 (4H, br s, $(CH_2)_2$), 3.79 (2H, s, CH_2N), 6.01 (2H, s, CH_2CO), 7.19–7.26 (2H, m, $C_{5',6'}$-H), 7.53–7.56 (1H, m, $C_{7'}$-H), 7.62–7.65 (1H, m, $C_{4'}$-H), 7.91 (2H, s, NH_2), 7.98 (1H, d, J = 8.4 Hz, C_3-H), 8.43 (1H, dd, J = 1.8 Hz, J = 8.1 Hz, C_4-H), 8.60 (1H, d, J = 1.8 Hz, C_6-H). ^{13}C NMR δ ppm: 50.57, 53.42, 55.96, 66.20, 110.89, 119.50, 122.17, 123.00, 128.82, 133.09, 133.46, 134.48, 136.35, 137.60, 142.40, 142.57, 151.50, 192.47. HRMS calcd for $C_{20}H_{21}ClN_4O_4S$ ([M+H]$^+$): 449.1045, found: 449.1049.

4.1.2.11. 2-Chloro-5-{[2-(methylsulfanyl)-1*H*-benzimidazol-1-yl]acetyl}benzenesulfonamide (3k). Yield 75%, 239–240 °C. IR ν cm^{-1}: 3362, 3271 (NH_2), 1710 (C=O). ^{1}H NMR δ ppm: 2.71 (3H, s, CH_3), 5.97 (2H, s, CH_2CO), 7.14–7.23 (2H, m, $C_{5',6'}$-H), 7.48–7.51 (1H, m, $C_{4'}$-H), 7.59–7.62 (1H, m, $C_{7'}$-H), 7.85 (2H, br s, NH_2), 7.97 (1H, d, J = 8.4 Hz, C_3-H), 8.42 (1H, d, J = 8.4 Hz, C_4-H), 8.56 (1H, s, C_6-H). ^{13}C NMR δ ppm: 15.25, 50.86, 110.35, 118.25, 122.34, 122.41, 128.82, 133.11, 133.65, 133.77, 136.85, 137.66, 142.47, 143.52, 153.69, 192.20. HRMS calcd for $C_{16}H_{14}ClN_3O_3S_2$ ([M+H]$^+$): 396.0238, found: 396.0237.

4.1.3. General procedure for the synthesis of 3l–o

A mixture of the corresponding benzimidazole-2-thione **2l–o** (0.320 mmol), compound **1** (100 mg, 0.320 mmol) and sodium acetate (30.2 mg, 0.368 mmol) in tetrahydrofuran (3 ml) was stirred at rt for 24 h. The reaction mixture was poured into water. The precipitate was filtered off, and subsequently washed with water and then diethyl ether.

4.1.3.1. 5-[(1*H*-Benzimidazol-2-ylsulfanyl)acetyl]-2-chlorobenzenesulfonamide (3l). Yield 91%, mp 167–169 °C. IR ν cm^{-1}: 3379, 3327, 3266 (NH_2, NH), 1673 (C=O). ^{1}H NMR δ ppm: 5.07

(2H, s, CH_2CO), 7.13–7.24 (2H, m, $C_{5',6'}$-H), 7.42–7.43 (2H, m, $C_{4',7'}$-H), 7.85 (2H, s, NH_2), 7.89 (1H, d, J = 8.7 Hz, C_3-H), 8.33 (1H, d, J = 8.1 Hz, C_4-H), 8.56 (1H, s, C_6-H), 12.67 (1H, br s, NH). ^{13}C NMR (DMSO-d_6 with three drops TFA) δ ppm: 41.50, 114.01, 125.28, 129.05, 133.02, 133.80, 134.46, 136.67, 142.37, 150.87, 191.57. HRMS calcd for $C_{15}H_{12}ClN_3O_3S_2$ ([M+H]$^+$): 382.0081, found: 382.0088.

4.1.3.2. 2-Chloro-5-[(6,7-dihydro-1*H*-[1,4]dioxino[2,3-*f*]benzimidazol-2-ylsulfanyl)acetyl]ben-zenesulfonamide (3m). Yield 89%, mp 219–221 °C. IR ν cm^{-1}: 3348, 3293 (NH_2, NH), 1686 (C=O). ^{1}H NMR δ ppm: 4.22 (4H, s, $O(CH_2)_2O$), 5.02 (2H, s, CH_2CO), 6.89 (2H, s, $C_{4',9'}$-H), 7.84 (2H, s, NH_2), 7.88 (1H, d, J = 8.1 Hz, C_3-H), 8.30 (1H, dd, J = 8.4 Hz, J = 2.1 Hz, C_4-H), 8.56 (1H, d, J = 1.8 Hz, C_6-H), 12.32 (1H, br s, NH). ^{13}C NMR (DMSO-d_6 with three drops TFA) δ ppm: 41.81, 64.72, 101.02, 127.79, 129.03, 132.99, 133.76, 134.25, 136.72, 142.37, 143.32, 148.82, 191.44. HRMS calcd for $C_{17}H_{14}ClN_3O_5S_2$ ([M+H]$^+$): 440.0136, found: 440.0144.

4.1.3.3. 5-{[(5-Bromo-1*H*-benzimidazol-2-yl)sulfanyl]acetyl}-2-chlorobenzenesulfonamide (3n). Yield 64%, mp 188–190 °C. IR ν cm^{-1}: 3367, 3327, 3278 (NH_2, NH), 1690 (C=O). ^{1}H NMR δ ppm: 5.10 (s, 2H, CH_2CO), 7.27 (d, J = 8.4 Hz, 1H, $C_{5'}$-H), 7.39 (d, J = 8.4 Hz, 1H, $C_{7'}$-H), 7.63 (s, 1H, $C_{4'}$-H), 7.87 (s, 2H, NH_2), 7.90 (d, J = 8.4 Hz, 1H, C_3-H), 8.33 (d, J = 8.1 Hz, 1H, C_4-H), 8.56 (s, 1H, C_6-H), 12.73 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, with three drops TFA) δ ppm: 67.72, 115.12, 115.84, 117.25, 125.70, 129.05, 133.00, 133.78, 134.83, 136.36, 137.69, 140.43, 142.30, 151.98, 192.42. HRMS calcd for $C_{15}H_{11}BrClN_3O_3S_2$ ([M+H]$^+$): 461.9164, found: 461.9170.

4.1.3.4. 2-Chloro-5-[(1*H*-imidazo[4,5-*c*]quinolin-2-ylsulfanyl)acetyl]benzenesulfonamide (3o). Yield 92%, mp 218–220 °C. IR ν cm^{-1}: 3406, 3310 (NH_2, NH), 1693 (C=O). ^{1}H NMR δ ppm: 5.16 (2H, s, CH_2CO), 7.64–7.75 (2H, m, $C_{7',8'}$-H), 7.85 (2H, s, NH_2), 7.92 (1H, d, J = 8.4 Hz, C_3-H), 8.07–8.09 (1H, m, $C_{9'}$-H), 8.23–8.26 (1H, m, $C_{6'}$-H), 8.38 (1H, dd, J = 8.4 Hz, J = 1.8 Hz, C_4-H), 8.60 (1H, d, J = 1.8 Hz, C_6-H), 9.08 (1H, s, $C_{4'}$-H), 13.88 (1H, br s, NH).^{13}C NMR (DMSO-d_6 with three drops TFA) δ ppm: 40.54, 117.23, 121.76, 123.30, 126.51, 129.07, 129.54, 129.62, 132.05, 132.64, 133.08, 133.79, 134.89, 135.08, 136.48, 142.36, 158.46, 192.12. HRMS calcd for $C_{18}H_{13}ClN_4O_3S_2$ ([M+H]$^+$): 433.0190, found: 433.0192.

4.2. Protein preparation

Expression and purification of hCA I, hCA II, hCA VII, and hCA XIII was previously described: hCA I in,[12] hCA II in,[35] and hCA VII and XIII in.[36]

4.3. Determination of compound binding to CAs

4.3.1. Thermal shift assay

Thermal shift assay experiments were performed in a Corbett Rotor-Gene 6000 (QIAGEN Rotor-Gene Q) instrument using 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 the final DMSO concentration at 2%. The applied heating rate was 1 °C/min. Data analysis was performed as previously described.[12,17]

4.3.2. Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC instrument (Microcal, Inc., Northampton, USA) with 5–20 μM protein solution in the cell and 50–200 μM of the ligand solution in the syringe. A typical experiment consisted of 25–30 injections (10 μl each) within 3–4 min intervals. Variation of the time period between injections enabled the detection and elimination of possible kinetic effects that may distort the binding data. Experiments were carried out at 37 °C in a phosphate buffer containing 50 mM NaCl at pH 7.0 and with a final DMSO concentration between 0.5% and 2%.

4.4. Crystallography

4.4.1. Crystallization

Crystals of hCAII were prepared by sitting drop method. Protein was concentrated to 20–60 mg/ml by ultrafiltration in a buffer containing 20 mM Na-Hepes pH 7.5 and 50 mM NaCl. A crystallization drop was prepared by mixing equal volumes of protein solution and crystallization buffer. Crystallization buffers were prepared by mixing the following solutions: 1 M Na-Bicine, pH 9, to a concentration 0.1 M; 2.7 M Na-Malonate pH 7–7.55 to a final concentration range of 1.5–2.2 M; and 3.5 M ammonium sulfate to a concentration from 0 to 0.2 M. Crystals appeared in a few days and belong to the space group $P2_1$. Crystals were soaked for several days with the 0.5 mM inhibitor solution in a reservoir buffer.

4.4.2. Data collection and structure determination

Diffraction data from the complexes of hCAII with **3a**, **3e**, **3m**, and **3n** were collected at the EMBL X12 and X13 beam lines at the DORIS storage ring (DESY, Hamburg, Germany). MOSFLM[37,38] and SCALA[39] were used for image processing. Initial phases were obtained by molecular replacement with the protein moiety from PDB entry 3HLJ[12] as an initial model. Structures were refined using REFMAC,[40] and COOT[41] was used for model inspection. Atomic coordinates of ligands were generated by DSVisualizer 1.7[42] (Accelrys). Topology and parameters for structure refinement were generated by LIBREFMAC.[43] Data collection and refinement statistics are presented in Table 2. Coordinates and structure factors were deposited under PDB ID 3M67 (**3m**), 3M96 (**3n**), 3M98 (**3a**), and 3MYQ (**3o**).

Acknowledgments

The project was supported in part by EEA and Norway Grants 2004-LT0019-IP-1EEE and the Lithuanian Government. E. Capkauskaite and L. Baranauskienė acknowledge the Lithuanian Science Council for a Student Research Fellowship Award. Crystal diffraction data were collected at the EMBL/DESY, Hamburg. D. Golovenko's access to the measurement facilities was funded from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 226716. E. Manakova's and S. Grazulis' travels to DESY, Hamburg were supported by 2004-LT0019-IP-1EEE and the Research Council of Lithuania. We thank our local contacts at the EMBL beamlines, Dr. Gleb Bourenkov and Dr. Michele Cianci, for their help with beamline operation. We thank Dr. Fernando Ridoutt for his help with the beamline cryosystems.

References and notes

1. Sly, W. S.; Hu, P. Y. *Annu. Rev. Biochem.* **1995**, *64*, 375.
2. Mikulski, R. L.; Silverman, D. N. *Biochim. Biophys. Acta* **2010**, *1804*, 422.
3. Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168.
4. Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **2007**, *15*, 4336.
5. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199.
6. Pastorekova, S.; Parkkila, S.; Zavada, J. *Adv. Clin. Chem.* **2006**, *42*, 167.
7. Clare, B. W.; Supuran, C. T. *Expert Opin. Drug Metab. Toxicol.* **2006**, *2*, 113.
8. *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 1–376. and references cited therein.
9. Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146.

10. Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Patents* **2006**, *16*, 1627.
11. Krishnamurthy, V. M.; Kaufman, G. K.; Urbach, A. R.; Gitlin, I.; Gudiksen, K. L.; Weibel, D. B.; Whitesides, G. M. *Chem. Rev.* **2008**, *108*, 946.
12. Baranauskienė, L.; Hilvo, M.; Matulienė, J.; Golovenko, D.; Manakova, E.; Dudutienė, V.; Michailovienė, V.; Torresan, J.; Jachno, J.; Parkkila, S.; Maresca, A.; Supuran, C. T.; Gražulis, S.; Matulis, D. *J. Enzyme Inhib. Med. Chem.* doi:10.3109/14756360903571685.
13. Freire, E. *Drug Discovery Today: Technol.* **2004**, *1*, 295.
14. Velazquez-Campoy, A.; Ohtaka, H.; Nezami, A.; Muzammil, S.; Freire, E. *Curr. Protoc. Cell Biol.* **2004**, *17*, 1934.
15. Velazquez-Campoy, A.; Freire, E. *Nat. Protoc.* **2006**, *1*, 186.
16. Todd, M.; Salemme, F. R. *Genet. Eng. News* **2003**, *23*, 28.
17. Matulis, D.; Kranz, J. K.; Salemme, F. R.; Todd, M. J. *Biochemistry* **2005**, *44*, 5258.
18. Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 641.
19. Temperini, C.; Cecchi, A.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2009**, *52*, 322.
20. Phillips, M. A. *J. Chem. Soc.* **1928**, 2393.
21. Feitelson, B. N.; Rothstein, R. *J. Chem. Soc.* **1958**, 2426.
22. Pool, W. O.; Harwood, H. J.; Ralston, A. W. *J. Am. Chem. Soc.* **1937**, *59*, 178.
23. Nishiwaki, T. *J. Chem. Soc.* **1968**, 428.
24. Hancock, K.; Lochte, H. L. *J. Am. Chem. Soc.* **1939**, *61*, 2448.
25. Bloom, A.; Day, A. R. *J. Org. Chem.* **1939**, *4*, 14.
26. Zhivotova, T. S.; Gazaliev, A. M.; Fazylov, S. D.; Aitpaeva, Z. K.; Turdybekov, D. M. *Zhurnal Organicheskoi Khimii.* **2006**, *42*, 460.
27. Hoggarth, E. *J. Chem. Soc.* **1949**, 3311.
28. Oelschlager, H. *Justus Liebigs. Ann.* **1961**, *641*, 81.
29. Fujikura, T.; Miigata, K.; Hashimoto, S.; Imai, K.; Takenaka, T. *Chem. Pharm. Bull.* **1982**, *30*, 4092.
30. Krasso, A.; Ramuz, H. U.S. Patent 4,599,347, 1986.
31. Fuchs, W. *Monatsh. Chem.* **1915**, *36*, 113.
32. Ermakova, Z. I.; Gricenko, A. N.; Zhuravlov, S. V. *Khim. Get. Soed.* **1974**, *2*, 202.
33. Galen, P. J. M.; Nissen, P.; Wijngaarden, I.; IJzerman, A. P.; Soudjin, W. *J. Med. Chem.* **1991**, *34*, 1202.
34. Temperini, C.; Cecchi, A.; Scozzafava, A.; Supuran, C. T. *Org. Biomol. Chem.* **2008**, *6*, 2499.
35. Cimmperman, P.; Baranauskiene, L.; Jachimoviciute, S.; Jachno, J.; Torresan, J.; Michailoviene, V.; Matuliene, J.; Sereikaite, J.; Bumelis, V.; Matulis, D. *Biophys. J.* **2008**, *95*, 3222.
36. Sudzius, L.; Baranauskiene, L.; Golovenko, D.; Matuliene, J.; Michailovienė, V.; Torresan, J.; Jachno, J.; Sukackaite, R.; Manakova, E.; Grazulis, S.; Tumkevicius, S.; Matulis, D. *Bioorg. Med. Chem.* **2010**, *18*, 7413.
37. Leslie, A. G. W. *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography* **1992**, *26*.
38. Leslie, A. G. W. *Acta Crystallogr., Sect D* **2006**, *62*, 48.
39. Collaborative Computational Project, Number 4. 'The CCP4 Suite: Programs for Protein Crystallography'. *Acta Crystallogr., Sect D* **1994**, *50*, 760.
40. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr., Sect D* **1997**, *53*, 240.
41. Emsley, P.; Cowtan, K. *Acta Crystallogr., Sect D* **2004**, *60*, 2126.
42. Discovery Studio Visualizer 2.0 Free Life Science Visualizer, 2009.
43. Vagin, A. A.; Steiner, R. A.; Lebedev, A. A.; Potterton, L.; McNicholas, S.; Long, F.; Murshudov, G. N. *Acta Crystallogr., Sect D* **2004**, *60*, 2184.
44. Kraulis, P. J. *J. Appl. Crystallogr.* **1991**, *24*, 946.
45. Merritt, E. A.; Bacon, D. J. *Methods Enzymol.* **1997**, *277*, 505.