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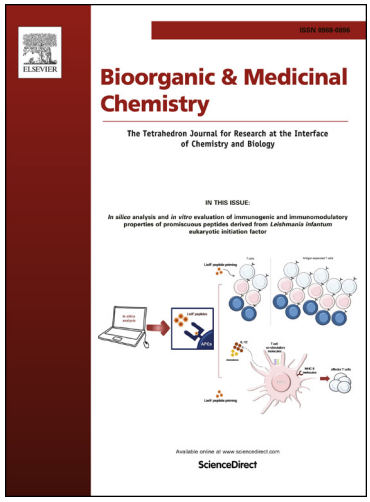

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Benzimidazole design, synthesis, and docking to build selective carbonic anhydrase VA inhibitors

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Abstract

The similarity of human carbonic anhydrase (CA) active sites makes it difficult to design selective inhibitors for one or several CA isoforms that are drug targets. Here we synthesize a series of compounds that are based on 5-[2-(benzimidazol-1-yl)acetyl]-2-chloro-benzenesulfonamide (**1a**) which demonstrated picomolar binding affinity and significant selectivity for CA isoform five A (VA), and explain the structural influence of inhibitor functional groups to the binding affinity and selectivity. A series of chloro-substituted benzenesulfonamides bearing a heterocyclic tail, together with molecular docking, was used to build inhibitors that explore substituent influence on the binding affinity to the CA VA isoform.

Keywords

Carbonic anhydrase isozyme I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV;fluorescent thermal shift assay; ThermoFluor®; sulfonamide; *N*-alkylated benzimidazole, imidazole, indoline, 3,4-dihydro-*2H*-quinoline;CA inhibitor; docking.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton. This simple reaction is essential for many physiological processes including pH regulation, respiration, electrolyte secretion, bone resorption, calcification, tumorigenesis, and biosynthetic reactions, which require bicarbonate as a substrate. Consequently, malfunction of these enzymes is often related to various diseases, and CA isozymes are interesting therapeutic targets whose inhibition could be used to treat a range of disorders including glaucoma, anemia, oxidative stress, cancer, epilepsy, edema, sterility, osteoporosis, obesity, etc[1–5].

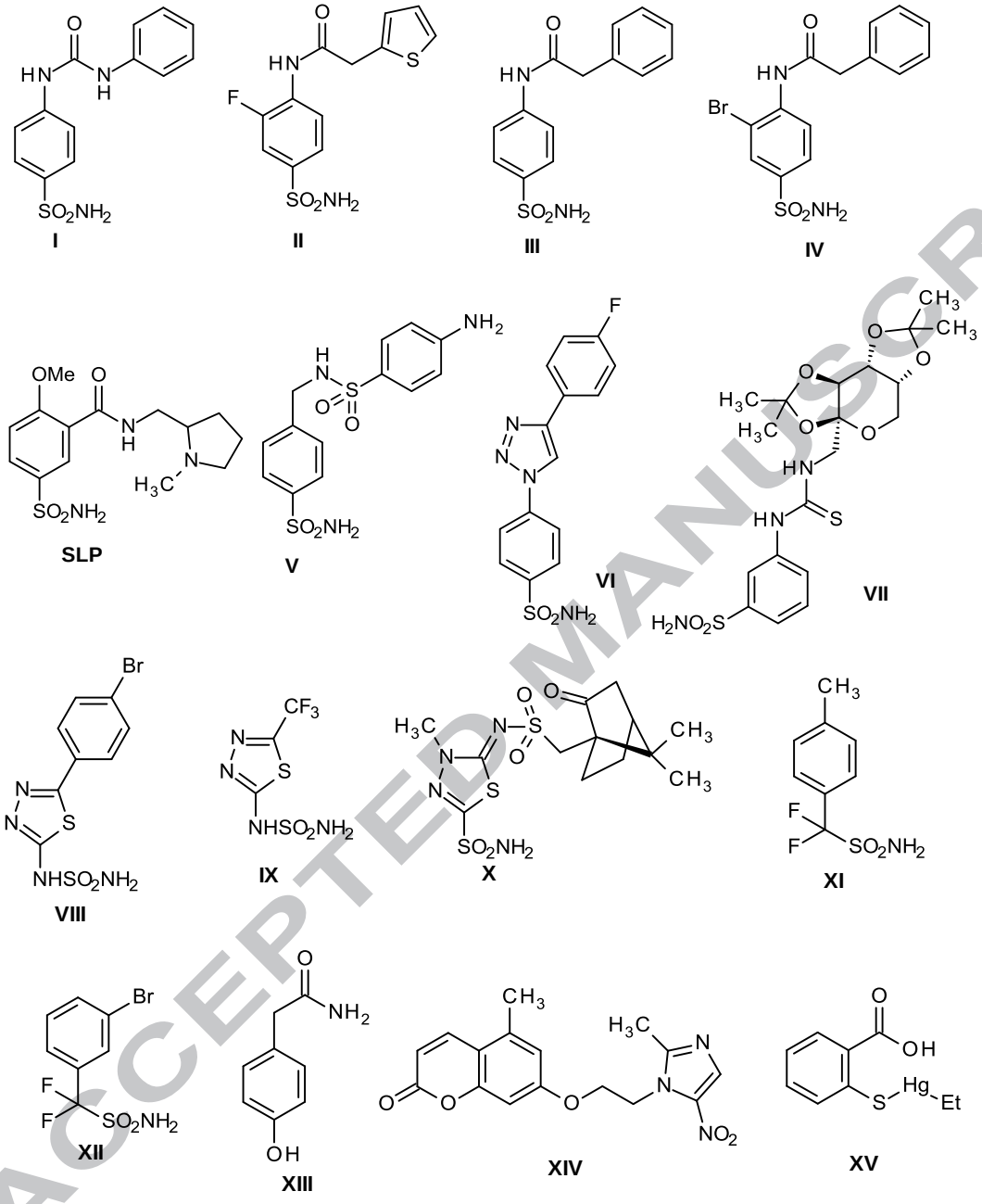
There are 12 catalytically active human CA isoforms, which differ in their kinetic properties, tissue distribution, and cellular localization[3,6]. In the design of CA inhibitors as drugs it is of crucial importance to inhibit only the target isoform with as little as possible effect on remaining CAs to avoid possible side effects. However, it is difficult to achieve desired selectivity due to high similarity of the active sites in CA isoforms[1,4,7].

Two CA isoforms in human, CA VA and CA VB, are expressed only in the mitochondria[8]. The CA VA and CA VB are involved in ureagenesis[9], gluconeogenesis [10], and lipogenesis[11–14]. Recently it was demonstrated by using an electrochemical method of wiring mitochondria that the mitochondrial CA VA and CA VB play an important role in the metabolism regulation[15].

The main class of CA inhibitors contain the primary sulfonamide group, or isosteres of this moiety, such as the sulfamate and the sulfamide[1]. Clinical use of sulfonamides such as an antiepileptic drug Topiramate (TPR)[16] exhibited a significant loss of body weight as a side effect in obese patients. A study of Topiramate showed the reduction of body weight gain in both lean and obese rats[17] and similar results were observed with Zonisamide (ZNS)[18]. Subsequently TPM was shown as an effective inhibitor of several CA isoforms[19,20], also for the mitochondrial CAs[21,22]. It has been demonstrated that ZNS is a more potent inhibitor of CA VA than CA II [23,24].

Numerous CA V inhibitors have been synthesized and their inhibitory activity against CAs has been determined. Below is shown a brief overview of the compounds synthesized and demonstrated as selective CA VA and CA VB inhibitors. In this short review, we have not included anionic inhibitors. The CA VA and CA VB inhibitory activities of compounds have been measured of Supuran group by the stopped-flow CO_2 hydration assay. The examples of some selective CA VA and CA VB inhibitors are shown in Figure 1.

Figure 1. Literature examples of the chemical structures of compounds that exhibit selectivity by inhibiting CA VA or CA VB stronger than CA I and CA II.



Daniela Vullo et al.[22] reported first inhibition study of the murine CA V with a series of aromatic and heterocyclic sulfonamides. Acylated sulfanilamides and ureido benzenesulfonamides (example **I**) showed higher affinity for CA V than for the other investigated isozymes (CA I, CA II and bCA IV).

Özlen Güzelet al.[25] prepared a series of aromatic/heterocyclic sulfonamides incorporating phenacetyl, pyridylacetyl, and thienylacetyl tails. All compounds were selective CA VA and CA VB inhibitors over CA I and CA II. The authors distinguish three of the most selective CA VA and CA VB inhibitors (*N*-(2-fluoro-4-sulfamoyl-phenyl)-2-(2-thienyl)acetamide (**II**), 2-phenyl-*N*-(4-sulfamoylphenyl)acetamide (**III**), and *N*-(2-bromo-4-sulfamoyl-phenyl)-2-phenyl-acetamide (**IV**)) over CA II. Unfortunately, later studies showed that compound **III** is not selective to CAs V[26–29].
Isao Nishimori et al.[21] focused on CA VB inhibition with a library of sulfonamides/sulfamates, that are clinically used (acetazolamide (AZM), methazolamide, ethoxzolamide (EZA), dichlorophenamide, dorzolamide, brinzolamide, benzolamide, TPM, sulpiride, and indisulam). None of these compounds showed better inhibitory activity toward CA VA than CA II but several compounds exhibited selectivity toward CA VB (for example, **SLP** and **V**).

Sally-Ann Poulsen et al.[30] synthesized and investigated 4-(4-phenyltriazole-1-yl)-benzenesulfonamide derivatives as inhibitors of CA VA and CA VB. Several of them possessed selectivity toward CA VA or CA VB. The best of them is represented by example **VI**.

Jean-Yves Winum et al.[31] synthesized and assayed a series of aromatic/heterocyclic sulfonamides incorporating fructopyranose–thioureido tails showing excellent CA VII inhibitory activity, distinguishing compound **VII**, which shows selectivity to CA VA.

Fatma-Zohra Smaine et al.[32] synthesized a small series of 2-substituted-1,3,4- thiadiazole-5-sulfamides. All compounds are selective to CA VA and CA VB over CA I, CA II, and CA IV. Several examples (**VIII** and **IX**) that possessed the best selectivity ratio K_i(CA II)/K_i(CA VA) are shown in Figure 1.

Alfonso Maresca et al.[33] prepared a series of (R)-/(S)-10-camphorsulfonyl-substituted aromatic/heterocyclic sulfonamides. All compounds are selective to CA VA and CA VB over CA I, CA II but one the best is thiadiazolesulfonamide **X**.

Alessandro Cecchi et al.[34] assayed a series of aromatic/heteroaromatic/polycyclic difluoromethanesulfonamides as inhibitors of CA I, CA II, CA VA, and CA IX. Several derivatives (examples **XI** and **XII)** showed selectivity for CA VA.

Rohan A. Davis et al.[35] investigated the enzyme inhibition characteristics of a natural product based phenolic library against a CA I, CA II, CA VA, and CAVB. Most of these compounds are selective to CA VA and CA VB over CA I and CA II with selectivity ratios in the range of 120–3800. Authors identified 2-(4-hydroxyphenyl)acetamide (**XIII**) as one of the best CA VA and CA VB selective inhibitors.

Adeline Bonneauet al.[36] reported the synthesis of two coumarin derivatives incorporating a nitroazole moiety. These compounds and 3-cyano-7-hydroxy-coumarin were assessed for their ability to inhibit the enzymatic activity of all human CAs. The mitochondrial isoforms CA VA and CA VB were inhibited efficiently with K_is in the range of 0.38–2.63 μM whereas CA I, CA II, CA IV, and CA XIII were not inhibited significantly. The best inhibitor is represented as example **XIV** shown in Figure 1.

Fabrizio Carta et al.[37] investigated a series of non-sulfonamide inhibitors of human CAs consisting of pyridine-*N*-oxide-2-thiophenol, thiobenzoic acid, thimerosal, two oxime

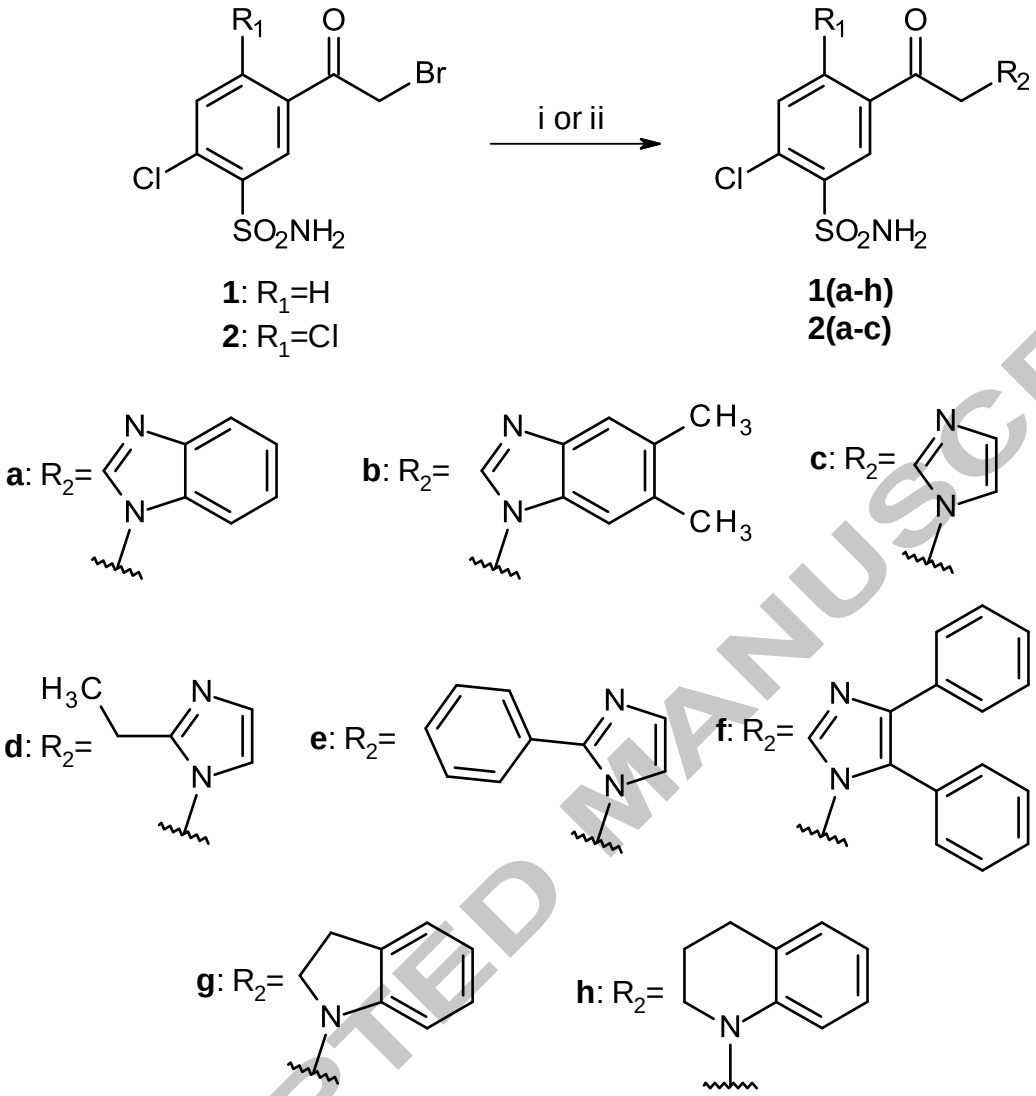
derivatives, 2-hydroxyquinoline, and coumaphos. The majority of compounds possessed no inhibitory activity to off-target isoform CA II, but inhibited the two mitochondrial isoforms CA VA and CA VB. Unfortunately, significant selectivity to these isoforms was not observed (example **XV**).

In our previous work, the synthesis and binding to human CAs of *N*-alkylated benzimidazoles[38–40] has been described. Several compounds were highly selective for CA VA, especially 5-[2-(benzimidazol-1-yl)acetyl]-2-chloro-benzenesulfonamide (**1a).** This compound exhibited 0.25 nM *observed* affinity to CA VA and bound from 1200 to 800,000 times stronger than to the remaining off-target CAs (K_ds were 200,000-303nM). This was highly unexpected because, for example, 2-chloro-5-[(2-methyl-1*H*-benzimidazol-1-yl)acetyl]benzenesulfonamide (**1i**) that is different from **1a** by only one methyl group showed more than 1000 times lower binding affinity to CA VA than to **1a**. Therefore, here we explore the influence of substituents for binding affinity to CA VA and synthesized a number of **1a** analogs, a series of chloro-substituted benzene sulfonamides, bearing heterocyclic tail. Furthermore, molecular modeling was used in order to understand how such small changes in the inhibitor structure so significantly influences the binding affinity to CA VA.

2. Results and discussion

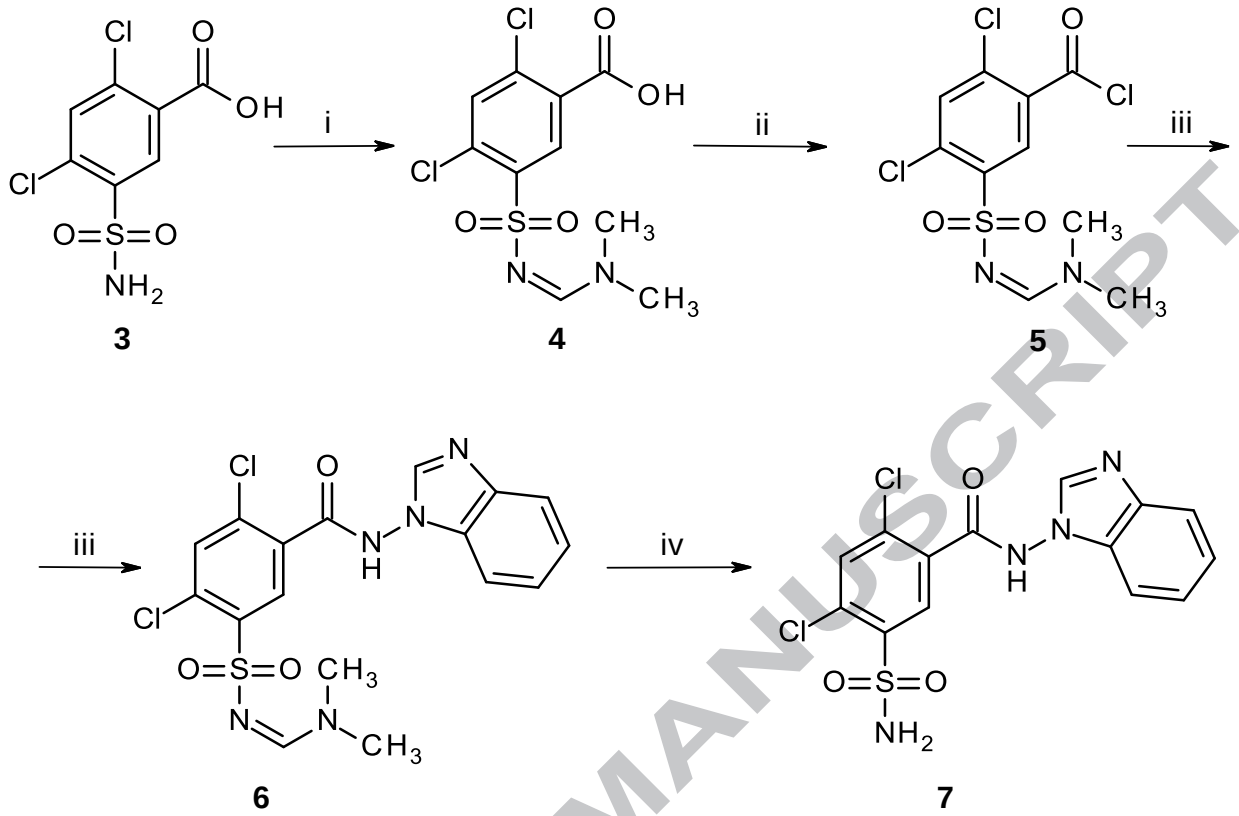
2.1. Chemistry

A series of 2-chloro- and 2,4-dichlorobenzenesulfonamides bearing different heterocyclic moieties were designed and synthesized. As shown in Scheme 1, the synthesis of target *N*-alkylated heterocycle derivatives **1(a-h**) and **2(a-c)** was achieved by alkylation of appropriate heterocycle with 5-(bromoacetyl)-2-chlorobenzenesulfonamide (**1**) and 5-(bromoacetyl)-2,4-dichlorobenzenesulfonamide (**2**). *N*-alkylation was carried out in the presence of NaOAc in THF at room temperature. It should be noted that the dialkylation of the benzimidazole/imidazole ring can be avoided by using of slight excess of heterocycle. An excess of 1,2,3,4-tetrahydroquinoline and indoline was used in several cases instead of mentioned base for the synthesis of **1g** and **1h.**



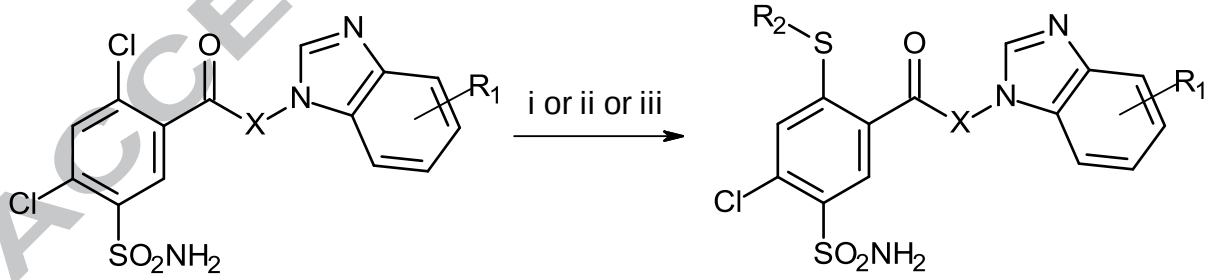
Scheme 1. Synthesis of compounds **1(a-h**) and **2(a-c).** Reagents and conditions: (i) benzimidazole or imidazole (1.5 eq), NaOAc, THF, room temperature, 24 h (for **1(a-f**) and **2(a-c)**); (ii) 1,2,3,4-tetrahydroquinoline or indoline (2 eq), THF, room temperature, 48 h (for **1g** and **1h**).

First attempt to obtain amide **7** by direct acylation of 1-aminobenzimidazole with acyl chloride (2,4-dichloro-5-sulfamoyl-benzoyl chloride) was unsatisfactory. Using pyridine as a base and performing the reaction in boiling THF led to formation of an inseparable mixture of products. It is presumable that the acylation of sulfonamide group occurred. Therefore it was decided to protect sulfonamide group with an *N*,*N*-dimethylaminomethylidene residue.The protection let us to avoid the formation of byproducts and to enhance the ability of compound **5** to acylation reaction. The treatment of 2,4-dichloro-5-sulfamoyl-benzoic acid (**3**) with dimethylformamide and $SOCl_2$ resulted methylidene **4**. The protected sulfonamide **4** was converted to acyl chloride**5** followed by successful amide **6** formation using pyridine in THF at 80°Candremoval of the sulfonamide-protecting group using NaOH (aq) as shown in Scheme 2.



Scheme 2. Synthesis of compound **7**. Reagents and conditions: (i) DMF, $SOCl_2$, -10°C, then r.t., 2h; (ii) $SOCl_2$, toluene, reflux, 2h; (iii) 1-aminobenzimidazole, pyridine, THF 80°C, 3h, then overnight at r.t.; (iv) 2M NaOH(aq), r.t, 48h, then 2M HCl(aq).

2-chloro-4,5-disubstituted benzenesulfonamides (compounds **8-11**) were obtained from compounds **2a, 2b,** and **7** by using appropriate thiol in methanol or DMSO in the presence of Et_3N as depicted in Scheme 3.



2a: X=CH_2, R_1=H
2b: X=CH_2, R_1=CH_3
7: X=NH, R_1=H

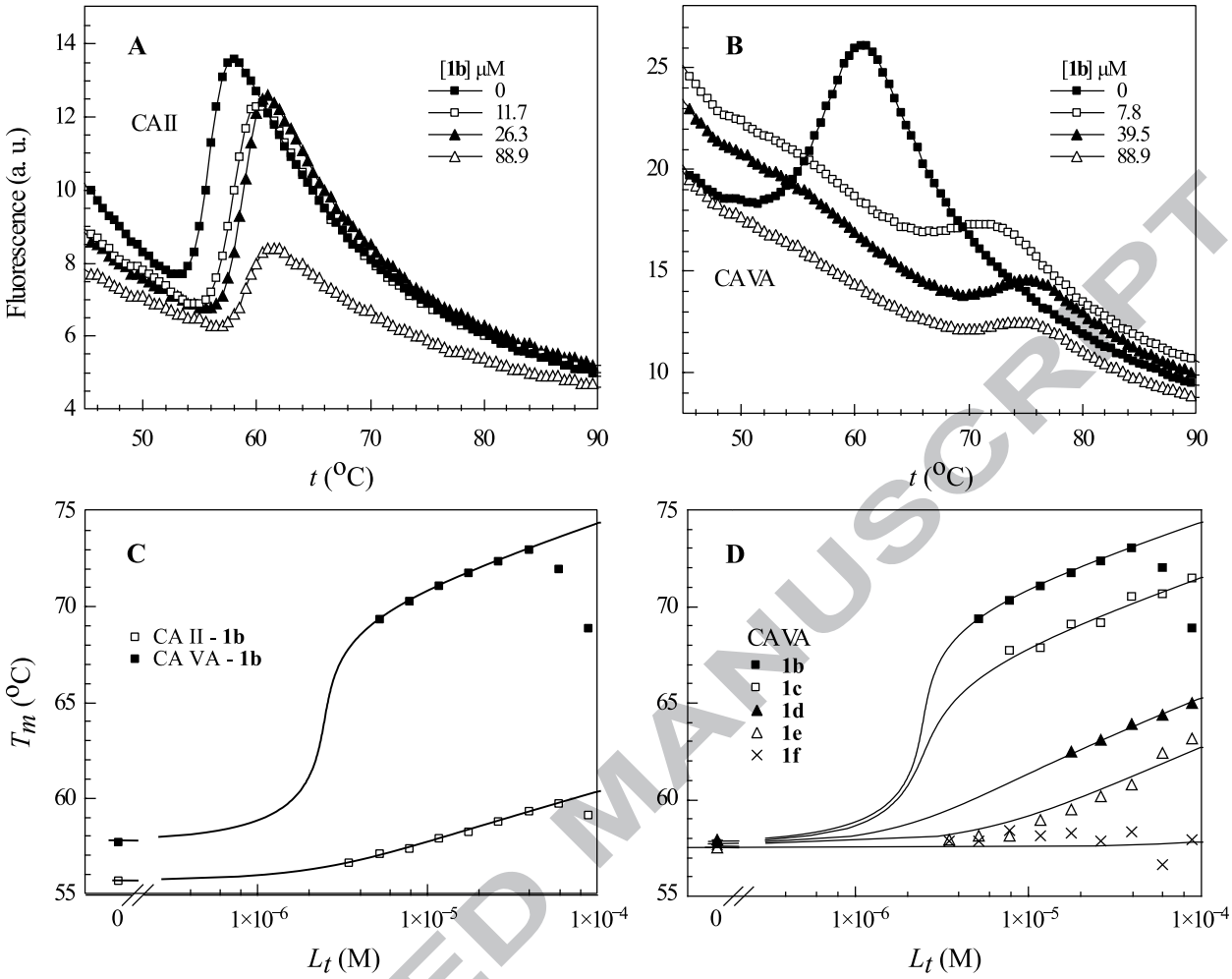
8: X=CH_2, R_1=H, R_2=$(CH_2)_2$Ph
9: X=CH_2, R_1=CH_3, R_2=Ph
10: X=NH, R_1=H, R_2=Ph
11: X=NH, R_1=H, R_2=cyclohexyl

Scheme 3. Synthesis of 2-chloro-4,5-disubstituted benzenesulfonamides (compounds **8-11)**. Reagents and conditions: (i) 2-phenylethanethiol, Et_3N, DMSO, r.t., 24 h; (ii) thiophenol, Et_3N, MeOH, reflux, 3 h; (iii) thiophenol or cyclohexanethiol, Et_3N, DMSO, r.t., 7 days.

2.2. Binding studies

Compound binding affinities towards all 12 human CAs were determined by the fluorescent thermal shift assay (FTSA). All observed K_d values are listed in Table 1. Several examples of affinity determination by FTSA are shown in Figure 2.

Figure 2. Thermal shift assay (differential scanning fluorimetry) data where top panels show protein melting curves observed via fluorescence of extrinsic probe (compound **1b** with CA II, Panel A, and CA VA, Panel B) and bottom panels show the melting temperature (T_m) dependence inhibitor dosing curves. Panel C shows selective binding of **1b** towards CA VA over CA II while Panel D compares the dosing curves of **1b**, **1c**, **1d**, **1e**, and **1f**. Lines were fit according to the model[62]. Measurements were performed at pH7.0 in 50 mM sodium phosphate buffer containing 100 mM NaCl.



It has been previously shown that **1a** exhibited exceptional affinity and selectivity towards CA VA and therefore we investigated the binding mode of **1a** analogs by changing substituents in the 4th and 5th position of the benzene ring bearing the sulfonamide group. The *N*-alkylated benzimidazoles **1i** and **1j** (Fig. 3) bearing methyl or ethyl substituent in the 2nd position of the benzimidazole ring have a also been previously reported [38–40].

The influence of the substituent in 5th position on CA binding affinity

It was discovered that certain small modifications of the compound structure cause large changes to the CA binding, while most small structural changes did not significantly affect the CA affinity. Newly synthesized compound **1b**, containing benzimidazole moiety with two methyl groups, bound to CA VA with K_d = 0.769 nM, similar to **1a**, and maintained high selectivity against other CA isoforms.

The replacement of the benzimidazole moiety in **1a** with imidazole in **1c** significantly reduced the binding potency to CA VA (53.3 times). The affinity to the other CAs compared to **1a**

remained similar, except the increased binding to CA IV (8.63 times) and the decreased binding to CA I and CA VB (10 and 3 times, respectively). Introduction of the methyl substituent in the 2nd position of the benzimidazole ring (compound **1i**) strongly reduced affinity to CA VA (333 nM, 1330 times) while the affinity to other CAs remained similar or slightly lower (200000-260 nM).

The insertion of the ethyl group (**1j**) reduced the binding potency to CA VA 2670 times, whereas affinity to the other CAs remained similar. The same trend is observed with compounds containing imidazole fragment (**1c** and **1d**). 2-ethyl substituted imidazole (**1d**) compared to unsubstituted (**1c**) showed 34.1 times lower affinity to CA VA, while changes between affinities to remaining CAs were similar with exception of CA IV (affinity diminished 133 times). The introduction of the phenyl substituent in the 2nd position of the imidazole ring (compound **1e**) reduced the binding potency to CA VA 107 times. A strong decrease in all CA affinities (K_d = 15400 nM - 200 000 nM) was observed when the imidazole ring was enriched with phenyl groups in 4th and 5th positions (**1f**).

The selectivity to CA VA disappeared when the benzimidazole moiety was replaced with structurally similar indoline (**1g** and **IND**) or 3,4-dihydro-*2H*-quinoline (**1h**) fragment. In **1g**, it may be noted that selectivity to CA IV (K_d = 154 nM) increased (other CAs were inhibited from 4.06 to 1300 times less).

The influence of substituent in the 4th position on CA binding affinity

It was known that a ligand's binding affinity depends on the acidity of the sulfonamide group[2]. Therefore, electron-withdrawing groups that increase the sulfonamide acidity, such as chlorine and carbonyl groups, also increase the binding affinity.

Comparison of three pairs of compounds, unsubstituted and substituted with chlorine in the 4th position of benzenesulfonamide (**1c/2c**, **1a/2a**, **1b/2b)** showed that the introduction of chlorine substituent increased the binding affinities to many CAs, especially to the CA III (from 3.6 to 20 times). The largest gain in the binding affinity was observed in compound pairs bearing imidazole fragment (**1c/2c**), while the smallest – in compound pairs bearing 5,6-dimethylbenzimidazole moiety (**1b/2b**). However, an inverse effect was observed for CA VA. 4-chloro substituted compounds (**2(a-c)**) possessed from 2.08 to 7.27 times diminished binding affinity as compared to chloro unsubstituted compounds (**1(a-c)**). The selectivity towards CA VA remained.

It was interesting to investigate the influence of larger substituents that should interact with the hydrophobic active site amino acids to improve binding. The introduction of 4-phenethylsulfanyl substituent (**8**) enhanced the binding to many CAs, especially to CA I, CA IX, CA XII, and CA XIV (5.78, 29.3, 7.29, and 15.2, respectively) as compared to the parent compound **1a**. The binding to CA VA decreased 28.6 times, to CA VI - 15.6 times. It should be mentioned that insertion of 4-chloro substituent (**2a**) enhanced binding affinities to other CAs (CA II, CA III, and CA VII - 26.5, 12 and 7 times respectively). The introduction of 4-phenylsulfanyl substitutuent (**9**) led to a decrease of the binding affinity to all CAs as compared to the parent compound **1a**. The largest decrease in affinity was observed to CA IV, CA VA, CA VB, and CA VI (60.0, 59.1, 50.0, and 29.4 times, respectively). Although the introduction of the substituents reduced selectivity of **8** and **9** toward CA VA, but it still remained.

The influence of the linker (NH instead of CH_2) in compounds 7, 10, and 11

When linker carbon atom (**2a**) was replaced with the nitrogen atom (**7**) the affinities to CA VA, CA I, and CA VI decreased (11.5, 40.0, and 142 times, respectively), whereas binding to other CAs remained unchanged. The NH linker-containing compound **10** possessed slightly decreased binding affinity to CA VA (1.69 times) as compared to the structurally related compound **9** bearing CH_2 linker, whereas the binding affinity to the majority of CAs increased from 4 to 35 times, especially toward CA IV – 6000 times.

The replacement of the chlorine substituent in the 4^{th} position (compound **7**) with cyclohexylsulfanyl substituent (compound **11**) increased the affinity to CA VA 10.2 times, while the replacement with phenylsulfanyl substituent (compound **10**) exhibited an opposite effect where the binding to CA VA decreased 3.7 times compared to **7**. Moreover, the introduction of the cyclohexylsulfanyl substituent (**11**), improved the binding to CA I and CA XII (90 and 66.7 times, respectively) more than of the phenylsulfanyl substituent (**10**) (8.4 and 6.7 times, respectively) as compared to the parent compound **7**.

Compound selectivity toward CA VA

Many of the newly synthesized **1a** analogs (**1b**, **1c**, **2a**, **2b**, **2c**, **8**, **9**, **7**, and **11**) retained high or moderate selectivity towards CA VA (Fig.2, Panels A, B, and C). Substituents in the 2^{nd} position of the benzimidazole/imidazole ring were not suitable to achieve selectivity to CA VA (**1d**, **1e**, and previously synthesized **1i** and **1j**) (Fig. 2, Panel D). The bulky 4,5-diphenyl imidazole derivative **1f**, and compounds with indoline (**1g**) or 3,4-dihydro-*2H*-quinoline (**1h**) fragments instead of the benzimidazole ring also are not suitable for selective binders to CA VA. The compound **10** affinity to CA VA is smaller than to CA IV. Affinities of all studied compounds to CA isoforms were compared to conventionally used acetazolamide and indapamide as control inhibitors.

Intrinsic binding affinities

In the design of selective inhibitors of a specific CA isoform, the structure-activity relationship requires the characterization of *intrinsic* thermodynamics, which often is significantly different from the *observed* (Fig. 4). In this series of compounds, the intrinsic binding affinity is significantly greater than the observed, but the tendency remains essentially the same. The CA VA interaction with **1b** (K_d = 0.8 nM) is a little stronger than compound **11** binding to CA XIV (K_d = 0.83 nM), but intrinsic K_d for CA VA and **1b** binding becomes weaker (K_d = 0.008 nM) than CA XIV with **11** (K_d = 0.005 nM) (Table 2).

Tables

Table 1. The observed dissociation constants of compound binding to human recombinant CA isoforms I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV as determined by the fluorescent thermal shift assay (values listed for 37 °C, experiments performed at pH 7.0).

CA / Cpd	**Dissociation constants K_d (nM) for CA isoforms**											
	CA I	**CA II**	**CA III**	**CA IV**	**CA VA**	**CA VB**	**CA VI**	**CA VII**	**CA IX**	**CA XII**	**CA XIII**	**CA XIV**
1a	11 100	1 560	200 000	719	0.25	3 330	714	1 000	772	2 080	667	303
1b	7 140	588	80 000	3 330	0.77	500	2 000	1 670	625	313	769	192
1c	111 000	1 540	200 000	83.3	13.3	10 000	1250	1 180	2 040	3 030	1 280	588
1d	26 300	1 720	200 000	11 100	454	3 700	4350	2 630	1250	2 860	625	526
1e	50 000	833	50000	2 220	1430	313	6670	2 220	667	9 520	454	154
1f	200 000	20 000	200 000	200 000	200 000	200 000	200 000	200 000	1 5400	200 000	34 500	16 700
1g	5 000	1 000	200 000	154	1 250	833	3 850	4 170	1 000	1 670	625	250
1h	2 700	1 250	200 000	3 330	400	222	10 000	3 330	400	2 500	526	333
1i	10 000	2 000	200 000	952	333	4 000	5 000	2 500	1 020	6 060	400	260
1j	7 140	556	125 000	1 330	667	1 670	2 500	1 670	571	1 820	400	213
2a	5 000	303	16 700	1 110	1.82	1000	1 410	143	370	3 330	714	125
2b	7 690	526	22 200	2 860	4.54	2500	4 000	714	1 110	4 000	1 090	833
2c	28 600	417	10 000	1 560	27.8	2630	2 000	125	400	1 050	263	125
7	200 000	200	5000	500	20.8	66.7	200 000	125	286	3 330	1 000	111
8	1 920	500	90 900	2 500	7.14	2000	11 100	1 670	26.3	286	294	20.0
9	20 000	2 860	200 000	200 000	45.4	25000	58 800	10 000	714	14 300	6 670	625
10	23 800	385	33300	33.3	76.9	100	200 000	286	10.5	500	1 670	10.0
11	2 220	133	16700	250	2.04	22.2	50 000	34.5	4.00	50.0	400	0.83
IND	18 000	530	28000	77.0	670	59.0	710	250	430	2000	290	63.0
AZM	1 400	38.0	200 000	100	1000	310	310	17.0	20.0	130	50.0	11.0

The standard deviation of the FTSA measurements is ±1.6-fold in K_d. The K_d for compounds **1a, 1i**,and **1j** binding to CAs are taken from our previously published data [38–40]indapamide (**IND**) and acetazolamide (**AZM**) from[40]. The values of 200 000 mean that no binding has been detected and the K_d is equal or above 200 000.

Table 2. Intrinsic dissociation constants of compound binding to human recombinant CA isoforms determined by FTSA (values listed for 37 °C). The pK_as of the water molecule bound to catalytically active Zn in the active center of CA are listed below CA isoform and the pK_as of compound sulfonamide group are listed next to the compound number.

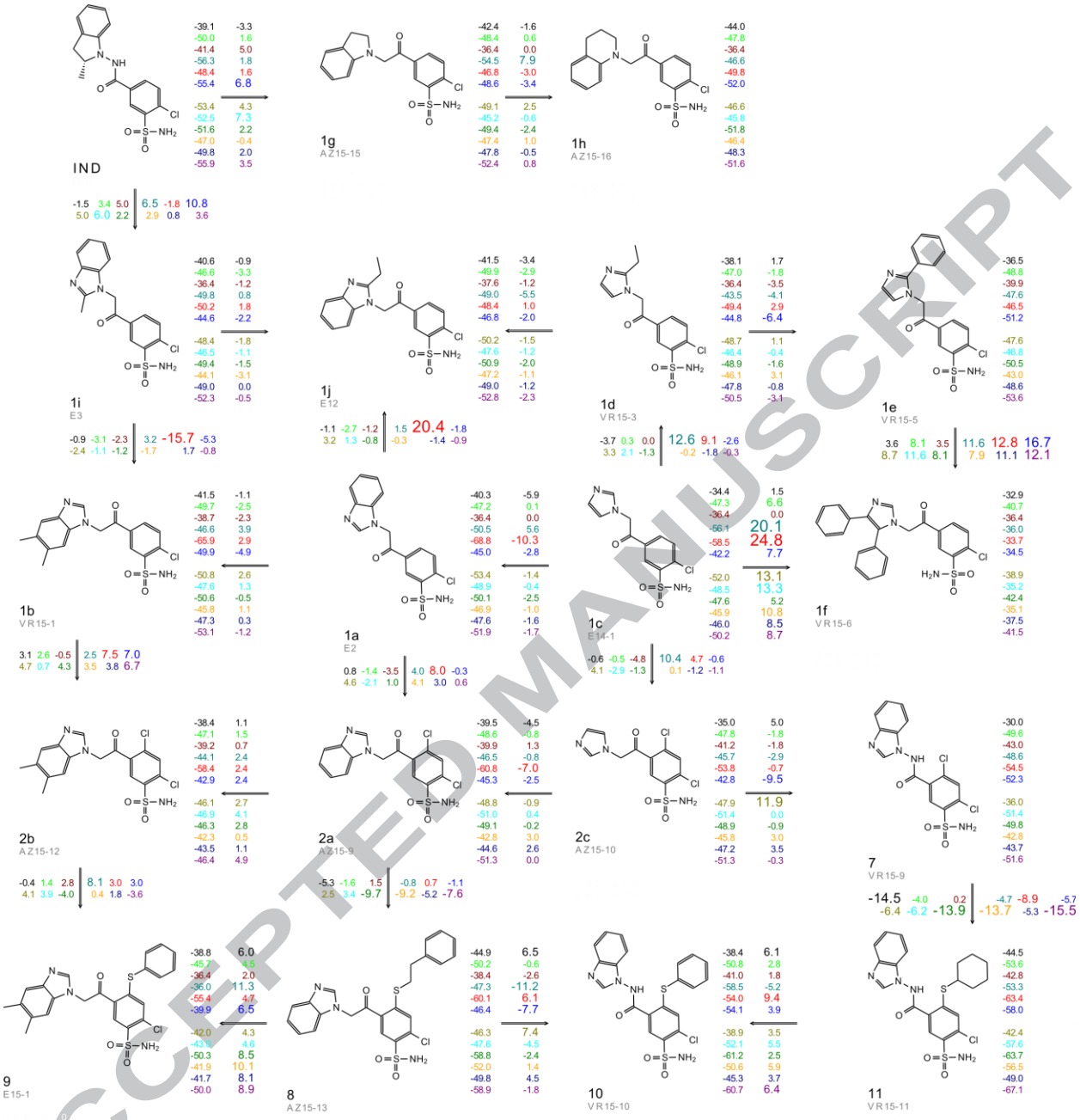
CA (pK_a) / **Cpd** (pK_a)	**Dissociation constants K_{d_intr} (nM) for CA isoforms**											
	CA I (8.1)	**CA II** (6.9)	**CA III** (6.5)	**CA IV** (6.6)	**CA VA** (7.3)	**CA VB** (7.0)	**CA VI** (6.0)	**CA VII** (6.8)	**CA IX** (6.6)	**CA XII** (6.8)	**CA XIII** (8.0)	**CA XIV** (6.8)
1a (8.8)	161	11.1	750	3.07	0.003	25.9	1.01	5.84	3.64	12.8	9.46	1.83
1b (8.8)	104	4.19	300	14.2	0.008	3.89	2.84	9.73	2.95	19.1	10.9	1.16
1c (8.8)	1612	11.0	750	0.36	0.14	77.7	1.77	6.87	9.63	18.6	18.2	3.55
1d (8.8)	382	12.3	750	47.5	4.72	28.8	6.17	15.4	5.90	17.5	8.87	3.18
1e (8.8)	725	5.93	187	9.49	14.8	2.43	9.46	13.0	3.15	58.3	6.45	0.93
1f (8.8)	2901	142	750	854	2079	1554	284	1167	72.6	1225	489	101
1g (8.8)	72.5	7.12	750	0.66	13.0	6.48	5.46	24.3	4.72	10.2	8.87	1.51
1h (8.8)	39.2	8.90	750	14.2	4.16	1.73	14.2	19.5	1.89	15.3	7.47	2.01
1i (8.8)	145	14.2	750	4.07	3.46	31.1	7.09	14.6	4.79	37.1	5.68	1.57
1j (8.8)	104	3.96	469	5.68	6.93	13.0	3.55	9.75	2.70	11.1	5.68	1.29
2a (8.3)	222	6.60	191	14.5	0.06	23.8	6.11	2.55	5.35	62.4	31.0	2.31
2b (8.3)	341	11.5	255	37.3	0.14	59.4	17.4	12.8	16.0	74.9	47.2	15.4
2c (8.3)	1268	9.07	115	20.4	0.88	62.6	8.68	2.23	5.78	19.7	11.4	2.31
7 (8.3)	8875	4.36	57.3	6.53	0.66	1.58	868	2.23	4.13	62.4	43.4	2.05
8 (8.8)	27.9	3.56	341	10.7	0.07	15.5	15.8	9.73	0.12	1.75	4.17	0.12
9 (8.8)	290	20.3	750	854	0.47	194	83.4	58.4	3.37	87.5	94.6	3.77
10 (8.8)	345	2.74	125	0.14	0.80	0.78	284	1.67	0.05	3.06	23.6	0.06
11 (8.8)	32.2	0.95	62.5	1.07	0.02	0.17	70.9	0.20	0.02	0.31	5.68	0.005
IND (8.8)	261	3.77	105	0.33	6.96	0.46	1.01	1.46	2.03	12.2	4.11	0.38
AZM(7.0)	651	8.67	24025	13.7	333	77.2	14.1	3.18	3.03	25.5	22.7	2.13

Intrinsic parameters describe the binding energy without the influence of protonation. It is known that CA can bind sulfonamide only when Zn^{2+}-bound hydroxide in the active site of CA is protonated and the sulfonamide of the compound is deprotonated. In these experimental conditions only a small fraction of CA and compound can interact (the pK_a values of CA isoforms and sulfonamide groups of every compound are shown in Table 2 in the brackets near each isozyme and compound number). The fractions differ due to different pK_as. For example, the difference between $\Delta_b G_{obs}$s of compound **1h** and **2a** binding to CA I is 1.85, while the difference of $\Delta_b G_{intr}$ values is 5.7. Thus, only the intrinsic parameters should be used in drug design when the influence of every functional group of the ligand is being evaluated. Moreover, compound **2a** binds to CA II with affinity $\Delta_b G_{obs}$ = -38.7 kJ/mol, while **1b** with $\Delta_b G_{obs}$= -37.0 kJ/mol. The intrinsic parameters showed that the compound **1b** is more potent than **2a** ($\Delta_b G_{intr}$ are -49.7 kJ/mol and -48.6 kJ/mol, respectively). The same discrepancies between the observed and intrinsic parameters can be seen for **2a** and **8** binding to CA II, **9** and **7** binding to CA IX, etc.

Figure 3 shows the map of benzensulfonamide analogs and the correlation of structural changes with the intrinsic Gibbs energies of binding. Similar compounds are arranged near each other that small structural changes could be compared. The values near the compounds show Gibbs energies of binding, and values near the arrows show $\Delta_b G$ differences between compounds. The larger numbers show larger differences. Colors describe CA isoforms.

Figure 3. A map is shown with the inhibitor chemical structures and the intrinsic Gibbs energies of binding ($\Delta_b G_{intr}$) to all 12 recombinant human CA isoforms in different colors listed above the map. The $\Delta_b G_{intr}$ are shown on the right side of compound and the differences in binding energies ($\Delta\Delta_b G_{intr}$) between compounds of similar chemical structure are shown above and below the arrow connecting the neighboring structures.

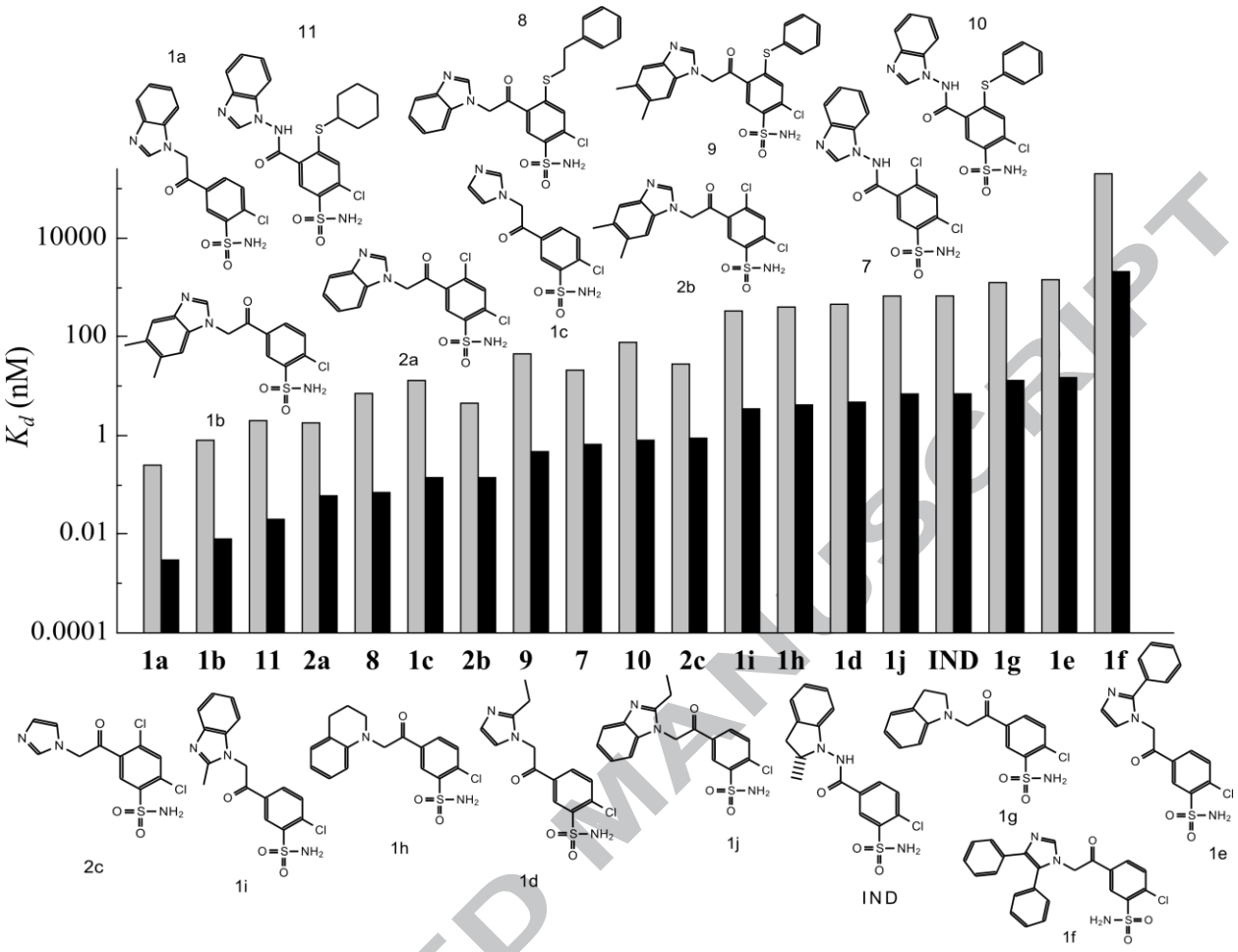
CA I, CA II, CA III, CA IV, CA VA, CA VB, CA VI, CA VII, CA IX, CA XII, CA XIII, CA XIV.



The largest difference was found between compound **1c** and **1f** binding to CA VA. Bulky hydrophobic group added to tail moiety significantly diminished the binding affinity. These two compounds have the same influence to CA IV. However, small hydrophobic group also highly reduced compound's binding affinity to CA VA as we can see in comparison of compounds **1a** and **1j** interaction to this isoform. It was interesting finding that the functional group added to the *para* position strongly diminished the binding affinity and the chlorine atom (compound **2a**) reduced the affinity similarly to the long 4-phenethylsulfanyl substituent (compound **8**).

Figure 4 shows the differences of intrinsic and observed dissociation constants when studied compounds bind to CA VA. The affinity decreases when substituent in *para* position is added. However, the potency of binding becomes even lower when tail in *meta* position changes. Bars are grouped from highest to lowest affinity of studied ligand binding to CAs (intrinsic parameters). The observed parameters fluctuated slightly. It means that the protonation effects influence binding. Thus, only intrinsic parameters should be used when thermodynamics of binding are compared.

Figure 4. Comparison of the observed (grey) and intrinsic (black) dissociation constants of the compound binding to CA VA, shown by arranging the compounds in the order of decreasing intrinsic affinity. Intrinsic affinities are approximately 100 fold stronger than the observed ones, but the order of compounds would change if only the observed constants would be compared. The data are listed in Tables 1 and 2.

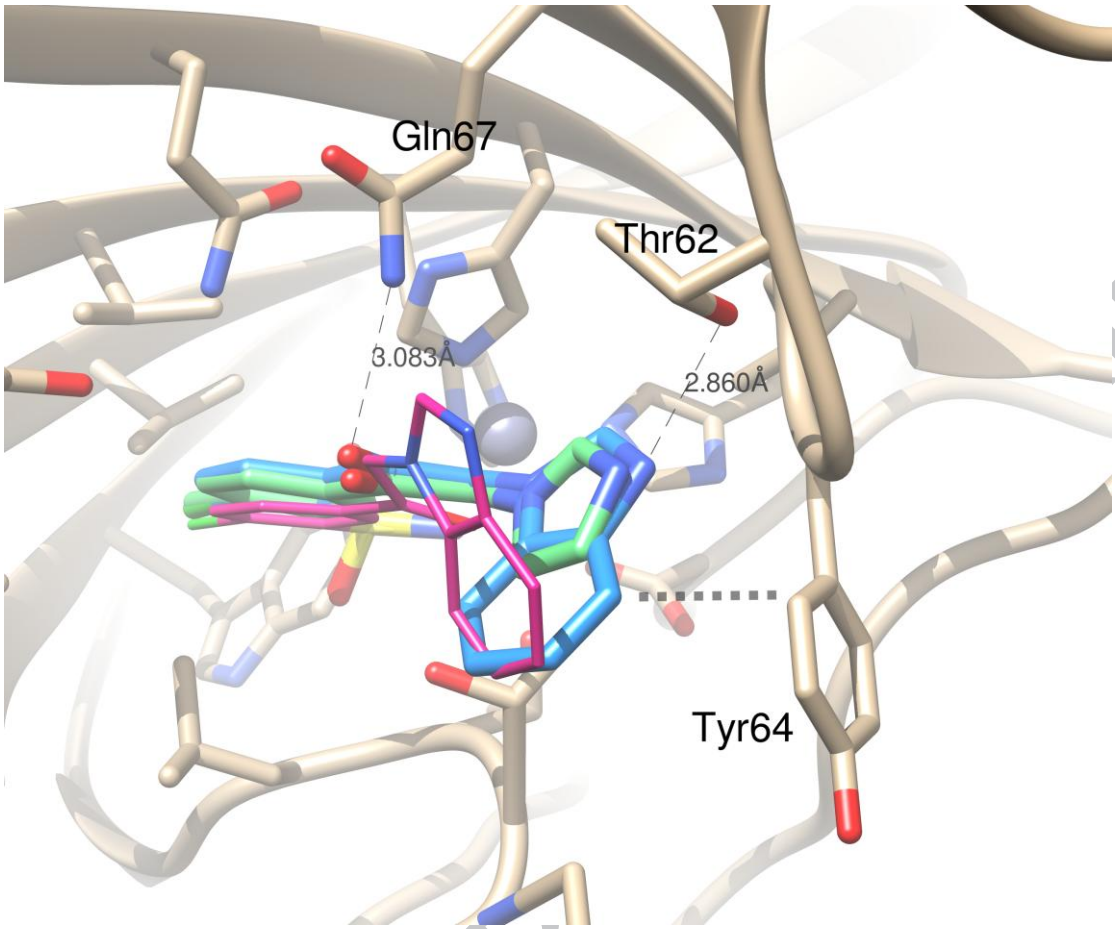


2.3. Docking studies

Superposition of **1a** conformation in complex with CA II on the homology model of CA VA does not clearly explain the **1a** selectivity towards CA VA. For this reason, it is reasonable to assume that **1a** may bind to CA VA in a different binding mode compared to CA II. One possible reason for the observed binding mode in CA II is that **1a** forms a hydrogen bond, and also an edge-to-face interaction between the ligand's aromatic ring and one of alternative conformations of His64. This conformation of His64 side chain, labeled as B in the PDB file, is pointing towards the ligand. It is impossible to form the same interactions in CA VA since in CA VA the histidine is replaced by a much larger tyrosine. A docking experiment should give insights into the putative binding mode in CA VA. We performed a docking of **1a** ligand into CA VA active site, in order (1) to observe if the docking indeed yields the binding mode which is different from the **1a**/CA II complex and if so, (2) to verify if the trends seen in the binding affinities of various ligands to CA VA can be explained by the putative binding mode.

The **1a** docked to CA VA is shown in Figure 5. The best docked conformation exhibits two hydrogen bonds between the ligand and the receptor: between the benzimidazole nitrogen and hydroxyl of Thr62, and between the carbonyl of the ligand and the side chain of Gln67. Indeed, this conformation corresponds to a different benzenesulfonamide-CO bond rotamer compared to the **1a**/CA II complex (PDB ID: 3M98) (Fig. 5). Importantly, the carbonyl of the ligand is in a *cis* and *trans* position with respect to the sulfonamide group in **1a**/CA II and **1a**/CA VA, respectively. The carbonyl in **1a**/CA II complex does make hydrogen bonds with Thr200 and with one of His64 alternative conformations visible in the 1.5 Å resolution X-ray structure (not shown). However, the benzimidazole nitrogen apparently is not making hydrogen bonds with the CA II (the nearest Asn62 nitrogen is at a 3.44 Å distance and at an unfavorable angle). Notably, in the **1a**/CA complex, the benzimidazole is likely to benefit from the edge-to-face aromatic-aromatic interaction with Tyr64 (in CA VA), and in CA II (His64), but with a very different geometry (the side chain pointing toward the Zn in CA II and away from Zn in CA VA).

Figure 5. The docking of compounds**1a** (blue) and **1c** (green) into the CA VA homology model (beige). The side chains of the residues within 4 Å from the ligand are shown. Compound **1a** forms two hydrogen bonds (thin dashed lines) with Thr62 and Gln67 (the residues are numbered according to the homologous CA II). A thick dashed line shows a possible aromatic-aromatic interaction between the benzene ring of the benzimidazole fragment of **1a**and the Tyr64 benzene ring. The superposed **1a** from the complex with CA II is shown as thin purple sticks.



Because of a larger number of hydrogen bonds formed, notably the extra H-bond with the benzimidazole nitrogen in CA VA, the docking offers an explanation of selectivity of the **1a** ligand towards CA VA. Since there are no experimental **1a**/CA VA structures available, the proposed binding conformation could be additionally verified by theoretically exploring the interactions between the receptor and the substituted compounds.

For example, removal of the nitrogen atom from the benzimidazole substituent when going from **1a** to **1g** and **1h** would lead to losing of the hydrogen bond to Thr62 in CA VA. This is consistent with a significant decrease of the binding affinity towards CA VA for the **1g** and **1h**. Since the above-mentioned hydrogen bond is absent in the **1a**/CA II complex, the binding affinity towards CA II is essentially similar for all three ligands (Table 1).

Replacement of the benzimidazole in **1a** with imidazole in **1c** should lead to the loss of aromatic-aromatic interaction with Tyr64 in CA VA (Fig. 3). This is essentially consistent with the observed 50-fold decrease of the binding affinity towards CA VA for **1c** compared to **1a**. In CA II, since the benzimidazole in **1a** does not participate in many aromatic-aromatic interactions, the binding affinity stays essentially the same.

According to the model in Figure 5, addition of reasonably small groups attached to the benzimidazole in **1b** neither creates clashes with the CA VA receptor, nor adds any new important, hence their binding affinities do not significantly change compared to **1a**. A similar rationale can be applied for the CA II receptor. However, substitution of the fused benzene ring in benzimidazole by two phenyl rings in **1f** would cause large clashes with the receptor in both CA VA and CA II, strongly negatively affecting the binding affinities.

Inspection of interactions in Figure 3 also suggests that the introduction of the methyl or ethyl substituent in the 2nd position of benzimidazole moiety in **1i** and **1j** causes sterical clashes in both binding modes, leading to a decrease of the binding affinity towards CA II and CA VA.

The clashes are apparently more easily avoided by some side chain adjustment in **1i**(**1j**)/CA II because the additional alkyl group in **1a** is close to the bulk solvent, and in **1i**(**1j**)/CA VA it is pointing inside the protein, hence it more significantly lowers the binding to CA VA compared to CA II (Table 1).

An important consideration is the fact that **1a** binding modes towards CA II and CA VA correspond to the different rotamers of the COR group with respect to the scaffold consisting of the benzene ring and the sulfonamide group. If new substitutions were introduced into the benzene ring in the *ortho*-position with respect to the COR group, this would cause different stability between the different rotamers.

For example, compound **2a** differs from **1a** by only one atom: the former has a chlorine atom on the benzene ring next to the -COR substituent, while the latter contains hydrogen. It has been shown that the *trans* rotamer is predominant compared to *cis* in *ortho*-chlorobenzaldehyde and *ortho*-chloroacetophenone[41,42]. Hence, we might expect that the conformer where the chlorine atom and the carbonyl oxygen are in the *trans* position is more stable (and therefore binds better) compared to the *cis* position. The **2a** compound is likely to have the same conformation as with **1a** in the complex with CA II. Therefore, the chlorine atom that replaces the hydrogen atom in **2a** will be in the *trans* position with respect to the carbonyl group, and this will not negatively impact the binding to the receptor. The actual 5-fold increase of the binding affinity to CA II when going from **1a** to **2a** can be explained by the increase of the acidity of sulfonamide group due to the electron-withdrawing effect of the chlorine substituent, therefore indirectly improving the binding affinity. In the complex with CA VA, the decrease of the binding affinity from 0.25 nM (**1a**) to 1.82 nM (**2a**) (Table 1) is consistent with the chlorine substituent in the 4th position in **2a** being in the less stable *cis* geometry, predicted by docking (consistent with the conformation of **1a** in Fig.5). It is also feasible that adding the chlorine substituent in the 4th position of benzene ring with respect to sulfonamide group could cause the conformation of the ligand bound to the CA VA receptor to flip from *cis* to *trans*. Forcing a ligand into the *trans* conformation would lead to the loss of the hydrogen bond as well as the aromatic-aromatic interaction, therefore causing a decrease of the binding affinity.

3. Conclusions

A series of selective CA VA inhibitors, based on a library of new 5-[2-(benzimidazol-1-yl)acetyl]-2-chloro-benzenesulfonamide (**1a**) analogs, a series of chloro substituted benzene sulfonamides, bearing a heterocycle tail were synthesized. The influence of substituents on the binding affinity to the CA VA was investigated. The computational modeling of the **1a** bound to

the CA VA suggested a different binding mode from the one observed in the **1a**/CA II complex. The ligand in the proposed binding mode forms several new interactions the CA VA receptor, which are not present in CA II, allowing to interpret CA II/CA selectivity of **1a,** and to predict the effect of modifications of **1a** scaffold. Benzimidazole ring is more favorable than the imidazole due to aromatic-aromatic interaction with Tyr64 of CA VA. The nitrogen atom of the benzimidazole/imidazole ring is also important and it forms hydrogen bonds between the nitrogen atom and the hydroxyl of Thr62 in CA VA. When the nitrogen atom is absent, for example, in the case of indoline or the 3,4-dihydro-*2H*-quinoline substituents (compounds **1g**, **1h**, **IND**), the selectivity towards CA VA disappears. Substituent in the 2nd position of the benzimidazole moiety is not desirable due to the sterical clashes that significantly diminish binding affinity to CA VA. The introduction of a substituent in the 4th position slightly reduces the selectivity towards CA VA due to the less stable *cis* rotamer in CA VA, as predicted by the computational modeling. An introduction of small group to benzimidazole (except in the 2nd position) does not substantially affect the binding affinity to CA VA, as the modification of the linker than CH_2 replaced with NH.

4. Experimental

4.1. Syntheses

Compounds 5-(2-bromoacetyl)-2-chloro-benzenesulfonamide (**1**) and 5-(2-bromoacetyl)-2,4-dichloro-benzenesulfonamide (**2**) were prepared by reduction of a nitro group of appropriate 1-(4-chloro-3-nitro-phenyl)ethanone[43] and 1-(2,4-dichloro-5-nitro-phenyl)ethanone[44] according procedure reported in [43]. An amino group was converted via diazonium salt to sulfonamide group and alfa bromination was carried out as described in [45] to afford corresponding 5-(2-bromoacetyl)-2-chloro-benzenesulfonamide (**1**)[45] and 5-(2-bromoacetyl)-2,4-dichloro-benzenesulfonamide (**2**) [44]. Starting compound 1-(4-chloro-3-nitrophenyl)ethanone is commercially available and 1-(2,4-dichloro-5-nitro-phenyl)ethanone was prepared by introducing of a nitro group into commercially available 1-(2,4-dichlorophenyl)ethanone according method reported in [46].

All starting materials and reagents were commercial products and were used without further purification. Melting points of the compounds were determined in open capillaries on a Thermo Scientific 9100 Series and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a (400 and 100 MHz, respectively) spectrometer in DMSO-d_6 using residual DMSO signals (2.52 ppm and 40.21 ppm for ^{1}H and ^{13}C NMR spectra, respectively) as the internal standard. TLC was performed with silica gel 60 F_{254} 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). The purity of final compounds was verified by HPLC to be >95% using the Agilent 1290 Infinity instrument with a Poroshell 120 SB-C18 (2.1 mm × 100 mm, 2.7 μm) reversed-phase column. Analytes were eluted using a linear gradient of water/methanol (20 mM ammonium formate in both phases) from 60:40 to 30:70 over 12 min, then from 30:70 to 20:80 over 1 min, and then 20:80 over 5 min at a flow rate of 0.2 mL/min. UV detection was at 254 nm.

General procedure for the synthesis of 1(a-f), **2(a-c)**.

A mixture of the appropriate bromoacetophenone **1** or **2** (0.500 mmol), appropriate benzimidazole or imidazole (0.750 mmol), and sodium acetate (49.2 mg, 0.600 mmol) in tetrahydrofuran (3 ml) was stirred at room temperature for 24 h. The reaction mixture was poured into water. The precipitate was filtered off, washed with water, and then with diethyl ether.

5-[2-(benzimidazol-1-yl)acetyl]-2-chloro-benzenesulfonamide (**1a)** was synthesized as previously described [38].

2-chloro-5-[2-(5,6-dimethylbenzimidazol-1-yl)acetyl]benzenesulfonamide (1b). The product was purified by flash chromatography over silica gel with EtOAc then EtOAc:MeOH (2:1), R_f = 0.80. Yield 41%, mp 247-249 °C. ^{1}H NMR δ ppm: 2.29 (3H, s, CH_3), 2.31 (3H, s, CH_3), 6.01 (2H, s, CH_2CO), 7.33 (1H, s, $C_{7'}$-H), 7.45 (1H, s, $C_{4'}$-H), 7.89 (2H, s, SO_2NH_2), 7.95 (2H, d, *J* = 8.4 Hz, C_3-H), 8.03 (1H, s, $C_{2'}$-H), 8.35 (1H, dd, *J* = 8.4 Hz, *J* = 2.0 Hz, C_4-H), 8.55 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 20.3, 20.5, 51.3, 111.1, 119.8, 128.6, 130.3, 131.4, 132.8, 133.2, 133.6, 133.8, 136.2, 142.1, 142.2, 144.4, 192.7. HRMS calcd. for $C_{17}H_{16}ClN_3O_3S$ [$(M+H)^+$]: 378.0674, found: 378.0679.

2-chloro-5-(2-imidazol-1-ylacetyl)benzenesulfonamide (1c). The product was purified by chromatography on a column of silica gel with EtOAc:MeOH (2:1), R_f = 0.52. Yield 54%, mp 228-230°C. ^{1}H NMR δ ppm: 5.78 (2H, s, CH_2CO), 6.94 (1H, s, $C_{4'}$-H), 7.13 (1H, s, $C_{5'}$-H), 7.60 (1H, s, $C_{2'}$-H), 7.87 (2H, s, SO_2NH_2), 7.91 (1H, d, *J* = 8.0 Hz, C_3-H), 8.26 (1H, dd, *J* = 8.4 Hz, *J* = 2.0 Hz, C_4-H), 8.50 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 53.2, 121.3, 128.4 (2C), 132.8, 133.0, 133.8, 136.1, 138.8, 142.2, 192.8. HRMS calcd. for $C_{11}H_{10}ClN_3O_3S$ [$(M+H)^+$]: 300.0204, found: 300.0200.

2-chloro-5-[2-(2-ethylimidazol-1-yl)acetyl]benzenesulfonamide (**1d).** The product was purified by flash chromatography over silica gel with EtOAc then EtOAc:MeOH (2:1), R_f = 0.70. Yield 32%, mp 223-225°C. ^{1}H NMR δ ppm: 1.14 (3H, d, *J* = 7.2 Hz, CH_3), 2.48-2.53 (2H, m, CH_2, superposed with DMSO), 5.74 (2H, s, CH_2CO), 6.81 (1H, d, *J* = 1.2 Hz, $C_{4'}$-H), 7.00 (1H, d, *J* = 1.2 Hz, $C_{5'}$-H), 7.86 (2H, s, SO_2NH_2), 7.92 (2H, d, *J* = 8.0 Hz, C_3-H), 8.29 (1H, dd, *J* = 8.4 Hz, *J* = 2.0 Hz, C_4-H), 8.52 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 12.5, 19.5, 52.4, 121.4, 126.4, 128.5, 132.7, 133.2, 133.7, 136.2, 142.2, 149.8, 192.9. HRMS calcd. for $C_{13}H_{14}ClN_3O_3S$ [$(M+H)^+$]: 328.0517, found: 328.0518.

2-chloro-5-[2-(2-phenylimidazol-1-yl)acetyl]benzenesulfonamide (1e). The product was purified by chromatography on a column of silica gel with EtOAc, R_f = 0.39. Yield 29%, mp 131-132°C. ^{1}H NMR δ ppm: 5.87 (2H, s, CH_2CO), 7.07 (1H, d, *J* = 0.8 Hz, $C_{4'}$-H), 7.28 (1H, d, *J* = 1.2 Hz, $C_{5'}$-H), 7.37-7.44 (3H, m, Ph-H), 7.45-7.51 (2H, m, Ph-H), 7.85 (2H, s, SO_2NH_2), 7.89 (2H, d, *J* = 8.4 Hz, C_3-H), 8.26 (1H, dd, *J* = 8.4 Hz, *J* = 2.0 Hz, C_4-H), 8.48 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 54.0, 123.9, 128.2, 128.4(2C), 129.0, 129.1, 131.1, 132.8, 133.2, 133.4, 136.5, 142.2, 147.7, 192.8. HRMS calcd. for $C_{17}H_{14}ClN_3O_3S$ [$(M+H)^+$]: 376.0517, found: 376.0522.

2-chloro-5-[2-(4,5-diphenylimidazol-1-yl)acetyl]benzenesulfonamide (**1f).** The product was washed with 2M HCl(aq), dried and then recrystallization was accomplished from toluene:MeOH (1:1). Yield 40%, mp 111-112°C. ^{1}H NMR δ ppm: 5.96 (2H, s, CH_2CO), 7.37-7.53 (10H, m, Ph-H), 7.87 (2H, d, *J* = 8.4 Hz, C_3-H), 7.89 (2H, s, SO_2NH_2), 8.17 (1H, dd, *J* = 8.4

Hz, *J* = 2.0 Hz, C_4-H), 8.43 (1H, d, *J* = 2.0 Hz, C_6-H), 8.36 (1H, s, $C_{2'}$-H). ^{13}C NMR δ ppm: 53.6, 126.0, 127.6, 127.7, 128.6, 129.4, 129.6, 129.9, 130.2, 130.5, 131.0, 131.3, 132.6, 132.9, 133.2, 137.0, 137.4, 142.3, 190.9. HRMS calcd. for $C_{23}H_{18}ClN_3O_3S$ [$(M+H)^+$]: 452.0830, found: 452.0836.

5-[2-(benzimidazol-1-yl)acetyl]-2,4-dichloro-benzenesulfonamide (2a). Recrystallization was accomplished from MeOH:H_2O (2:1) (twice). Yield 66%, mp 245-250°C (dec). ^{1}H NMR δ ppm: 5.95 (2H, s, CH_2), 7.20-7.32 (2H, m, $C_{5',6'}$-H), 7.56 (1H, dd, *J* = 6.8 Hz, *J* = 1.6 Hz, $C_{7'}$-H), 7.70 (1H, dd, *J* = 6.8 Hz, *J* = 1.6 Hz, $C_{4'}$-H), 7.92 (2H, s, SO_2NH_2), 8.11 (1H, s, C_3-H), 8.23 (1H, s,$C_{2'}$-H), 8.53 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.7, 111.1, 119.8, 122.2, 123.0, 130.3, 133.7, 134.8 (2C), 134.9, 135.3, 140.7, 143.4, 145.2, 194.6. HRMS calcd. for $C_{15}H_{11}Cl_2N_3O_3S$ [$(M+H)^+$]: 383.9971, found: 383.9973.

2,4-dichloro-5-[2-(5,6-dimethylbenzimidazol-1-yl)acetyl]benzenesulfonamide (2b). Recrystallization was accomplished from MeOH:H_2O (2:1) (twice). Yield 64%, mp 248-251°C. ^{1}H NMR δ ppm: 2.31 (3H, s, CH_3), 2.32 (3H, s, CH_3), 5.86 (2H, s, CH_2CO), 7.31 (1H, s, $C_{7'}$-H), 7.45 (1H, s, $C_{4'}$-H), 7.91 (2H, s, SO_2NH_2), 8.05 (1H, s, $C_{2'}$-H), 8.10 (1H, s, C_3-H), 8.51 (1H, s, C_6-H). ^{13}C NMR δ ppm: 20.3, 20.6, 53.6, 111.0, 119.9, 130.3, 130.4, 131.5, 133.4, 133.7, 134.7, 134.9, 135.3, 140.7, 142.1, 144.3, 194.7. HRMS calcd. for $C_{17}H_{15}Cl_2N_3O_3S$ [$(M+H)^+$]: 412.0284, found: 412.0279.

2,4-dichloro-5-(2-imidazol-1-ylacetyl)benzenesulfonamide (2c). Recrystallization was accomplished from MeOH:EtOAc (1:1). Yield 16%, mp 208-210°C. ^{1}H NMR δ ppm: 5.62 (2H, s, CH_2CO), 6.93 (1H, s, $C_{4'}$-H), 7.15 (1H, s, $C_{5'}$-H), 7.63 (1H, s, $C_{2'}$-H), 7.88 (2H, s, SO_2NH_2), 8.07 (1H, s, C_3-H), 8.43 (1H, s, C_6-H). ^{13}C NMR δ ppm: 55.4, 121.1, 128.5, 130.2, 133.6, 134.7, 135.0, 135.1, 138.7, 140.6, 194.9. HRMS calcd. for $C_{11}H_9Cl_2N_3O_3S$ [$(M+H)^+$]: 333.9814, found: 333.9818.

General procedure for the synthesis of (1g) and **(1h)**.

A mixture of 5-(2-bromoacetyl)-2-chlorobenzene-1-sulfonamide **1**(200 mg, 0.640 mmol) and the appropriate amine (1.30 mmol) in THF (4 ml) was stirred at room temperature for 48 h. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure.

2-chloro-5-(2-indolin-1-ylacetyl)benzenesulfonamide (1g). Recrystallization was accomplished from 2-PrOH:H_2O (5:1). Yield 51%, mp 195-198°C. ^{1}H NMR δ ppm: 2.96 (2H, t, *J* = 8.4 Hz, CH_2), 3.47 (2H, t, *J* = 8.4 Hz, CH_2), 4.76 (2H, s, CH_2CO), 6.48 (1H, d, *J* = 8.0 Hz, $C_{7'}$-H), 6.58 (1H, t, *J* = 7.6 Hz, $C_{5'}$-H), 6.95 (1H, t, *J* = 7,6 Hz, $C_{6'}$-H), 7.05 (1H, d, *J* = 7.2 Hz, $C_{4'}$-H), 7.80 (2H, s, SO_2NH_2), 7.85 (1H, d, *J* = 8.4 Hz, C_3-H), 8.24 (1H, dd, *J* = 8.0 Hz, *J* = 2.0 Hz, C_4-H), 8.50 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 28.6, 53.4, 55.2, 107.0, 117.7, 124.7, 127.5, 128.4, 129.6, 132.6, 133.1, 134.6, 135.7, 142.0, 152.3, 195.6. HRMS calcd. for $C_{16}H_{15}ClN_2O_3S$ [$(M+H)^+$]: 351.0565, found: 351.0569.

2-chloro-5-[2-(3,4-dihydro-2H-quinolin-1-yl)acetyl]benzenesulfonamide (1h). Recrystallization was accomplished from 2-PrOH:H_2O (5:1). Yield 84%, mp 210-213°C. ^{1}H NMR δ ppm: 1.91 (2H, quint, *J* = 6.4 Hz, CH_2), 2.74 (2H, t, *J* = 6.4 Hz, CH_2), 3.34 (2H, t, *J* = 5.6 Hz, CH_2), 4.93 (2H, s, CH_2CO), 6.34 (1H, d, *J* = 7.6 Hz, $C_{8'}$-H), 6.48 (1H, td, *J* = 7.2 Hz, *J* = 1.2 Hz, $C_{6'}$-H), 6.85 (1H, td, *J* = 7,6 Hz, *J* = 1.6 Hz, $C_{7'}$-H), 6.90 (1H, dd, *J* = 7.2 Hz, *J* = 1.2 Hz,

$C_{5'}$-H), 7.80 (2H, s, SO_2NH_2), 7.87 (2H, d, *J* = 8.4 Hz, C_3-H), 8.26 (1H, dd, *J* = 8.4 Hz, *J* = 2.0 Hz, C_4-H), 8.48 (1H, d, *J* = 2.0 Hz, C_6-H). ^{13}C NMR δ ppm: 22.3, 28.0, 50.1, 57.7, 110.8, 116.2, 122.3, 127.2, 128.3, 129.3, 132.6, 132.9, 134.5, 135.8, 142.0, 145.6, 196.3. HRMS calcd. for $C_{17}H_{17}ClN_2O_3S$ [$(M+H)^+$]: 365.0721, found: 365.0718.

Synthesisof 5-[2-(benzimidazol-1-yl)acetyl]-2-chloro-4-phenethylsulfanyl-benzenesulfonamide (8). The mixture of 5-[2-(benzimidazol-1-yl)acetyl]-2,4-dichloro-benzenesulfonamide (**2a**) (65.0 mg, 0.168 mmol), DMSO (1 mL), 2-phenylethanethiol(24.0 mg, 0.168 mmol) and Et_3N (17.6 mg, 0.175 mmol) was stirred at room temperature for 24 h. The brine was added to the mixture and product was extracted with EtOAc (3x5 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered and concentrated. Recrystallization was accomplished from EtOAc:MeOH (5:1). Yield 31%, mp 205-207°C. ^{1}H NMR δ ppm: 2.92 (2H, t, *J* = 7.2 Hz, $\underline{CH_2}$Ph), 3.33-3.40 (2H, m, CH_2S), 6.00 (2H, s, CH_2CO), 7.19-7.32 (7H, m, Ph-H, $C_{5',6'}$-H), 7.50 (1H, dd, *J* = 6.0 Hz, *J* = 2.8 Hz, $C_{7'}$-H), 7.69 (1H, dd, *J* = 5.6 Hz, *J* = 2.8 Hz, $C_{4'}$-H), 7.72 (1H, s, C_3-H), 7.83 (2H, s, SO_2NH_2), 8.15 (1H, s, $C_{2'}$-H), 8.66 (1H, s, C_6-H). ^{13}C NMR δ ppm: 33.0, 33.7, 52.0, 111.1, 119.8, 122.0, 122.8, 126.9, 128.8, 128.9, 129.0, 129.1, 130.1, 135.1, 135.4, 136.8, 140.0, 143.6, 145.3, 147.7, 193.3. HRMS calcd. for $C_{23}H_{20}ClN_3O_3S_2$ [$(M+H)^+$]: 486.0707, found: 486.0709.

Synthesis of 2-chloro-5-[2-(5,6-dimethylbenzimidazol-1-yl)acetyl]-4-phenylsulfanyl-benzenesulfonamide (9). The mixture of 2,4-dichloro-5-[2-(5,6-dimethylbenzimidazol-1-yl)acetyl]benzenesulfonamide (compound **2b**),MeOH (5 mL), thiophenol (121 mg, 1.10 mmol) and Et_3N (121 mg, 1.20 mmol) was refluxed for 3 h. MeOH was evaporated under reduced pressure and the resultant precipitate was washed with H_2O. Recrystallization was accomplished from acetone:MeOH (1:1). Yield 30%, mp 229-231°C. ^{1}H NMR δ ppm: 2.30 (3H, s, CH_3), 2.32 (3H, s, CH_3), 6.01 (2H, s, CH_2CO), 6.78 (1H, s, C_3-H), 7.36 (1H, s, $C_{7'}$-H), 7.46 (1H, s, $C_{4'}$-H), 7.60 (5H, br s, Ph-H), 7.81 (2H, s, SO_2NH_2), 8.04 (1H, s, $C_{2'}$-H), 8.72 (1H, s, C_6-H). ^{13}C NMR δ ppm: 20.3, 20.6, 51.8, 111.2, 119.8, 129.1, 129.2, 130.3, 130.6, 131.0, 131.1, 131.3, 131.4, 133.7, 135.3, 135.8, 137.5, 142.3, 144.5, 149.0, 193.2. HRMS calcd. for $C_{23}H_{20}ClN_3O_3S_2$ [$(M+H)^+$]: 486.0707, found: 486.0701.

Synthesis of 2,4-dichloro-5-[(dimethylamino)methyleneamino]sulfonyl-benzoic acid (4). $SOCl_2$ (2.0 ml 28 mmol) was added dropwise to a solution of 2,4-dichloro-5-sulfamoyl-benzoic acid **3** (1.50 g 5.56 mmol) in DMF (4.3 ml) at -10°C. The reaction mixture was stirred at room temperature for 2h and then poured on ice; the resulted precipitate was filtered off and washed with water until pH 7 was reached. Yield 77%, mp 254-255°C. ^{1}H NMR δ ppm: 2.94 (3H, s, CH_3N), 3.19 (3H, s, CH_3N), 7.92 (1H, s,C_3-H), 8.27 (1H, s,NC-H), 8.41 (1H, s,C_6-H). ^{13}C NMR δ ppm: 35.8, 41.5, 130.2, 132.1, 133.9, 134.6, 136.9, 139.1, 161.3, 165.2. HRMS calcd. for $C_{10}H_{10}Cl_2N_2O_4S$ [$(M+H)^+$]: 324.9811, found: 324.9814.

Synthesis of *N*-(benzimidazol-1-yl)-2,4-dichloro-5-[dimethylaminomethyleneamino]sulfonyl-benzamide (6). Mixture of 2,4-dichloro-5-[(dimethylamino)methyleneamino]sulfonyl-benzoic acid **(4)** (326 mg, 1.00 mmol), toluene (0.6 ml) and $SOCl_2$ (1.20 ml, 16.7mmol) was refluxed for 2h. Excess $SOCl_2$ and toluene were removed under reduced pressure and the crude acid chloride **5** was used directly in the next step. The obtained 2,4-dichloro-5-[dimethylaminomethyleneamino]sulfonyl-benzoyl chloride **(5)** was added to mixture of 1-aminobenzimidazole (134 mg 1.01 mmol), pyridine (1 ml), and THF (1 ml). The reaction mixture was stirred at 80°C for 3h and obtained solution was stirred overnight

at room temperature. Then water (15 ml) was added and reaction mixture stirred additionally for some minutes. Precipitate was filtered off, washed with water, dried and recrystallized from large volume of acetic acid. Yield 66%, mp 157-158°C. ^{1}H NMR δ ppm: 2.97 (3H, s, CH_3N), 3.23 (3H, s, CH_3N), 7.31 (1H, t, *J* = 8.0 Hz, $C_{5'}$-H), 7.38 (1H, t, *J* = 7.6 Hz, $C_{6'}$-H), 7.51 (1H, d, *J* = 8.0 Hz, $C_{4'}$-H), 7.75 (1H, d, *J* = 8.0 Hz, $C_{7'}$-H), 8.08 (1H, s, C_3-H), 8.33 (1H, s,NC-H), 8.44 (1H, s, C_6-H), 8.49 (1H, s, $C_{2'}$-H), 12.32 (1H, s, NHCO). ^{13}C NMR δ ppm: 35.8, 41.6, 109.8, 120.5, 122.9, 124.1, 130.5, 132.8, 133.1, 133.5, 134.2, 135.4, 139.5, 141.5, 144.3, 161.3, 164.5. HRMS calcd. for $C_{17}H_{15}Cl_2N_5O_3S[(M+H)^+]$: 440.0345, found: 440.0349.

Synthesis of *N*-(benzimidazol-1-yl)-2,4-dichloro-5-sulfamoyl-benzamide (7). The mixture of ***N*-(benzimidazol-1-yl)-2,4-dichloro-5-[dimethylaminomethyleneamino]sulfonyl-benzamide (6)** (660mg, 1.49 mmol) and 2M NaOH(aq) (5 ml) was stirred at room temperature for 48h. To obtained solution 2M HCl(aq) was added until pH 7 was reached. The resulted precipitate was filtered, washed with water, dried and recrystallized from aqueous ethanol. Yield 76%, mp 258-259°C. ^{1}H NMR δ ppm: 7.31 (1H, t, *J* = 8.0 Hz, $C_{5'}$-H), 7.38 (1H, t, *J* = 8.0 Hz, $C_{6'}$-H), 7.51 (1H, d, *J* = 7.6 Hz, $C_{4'}$-H), 7.75 (1H, d, *J* = 8.0 Hz, $C_{7'}$-H), 7.92 (2H, s, NH_2SO_2), 8.14 (1H, s, C_3-H), 8.37 (1H, s, C_6-H), 8.49 (1H, s, $C_{2'}$-H), 12.36 (1H, s, NHCO). ^{13}C NMR δ ppm: 109.8, 120.5, 122.9, 124.1, 130.0, 133.0 133.1, 133.5, 133.8, 135.1, 140.8, 141.4, 144.2, 164.5. HRMS calcd. for $C_{14}H_{10}Cl_2N_4O_3S$ $[(M+H)^+]$: 384.9923, found: 384.9920.

General procedure for the synthesis of 10 and **11**. The mixture of ***N*-(benzimidazol-1-yl)-2,4-dichloro-5-sulfamoyl-benzamide (7)**(150 mg, 0.390 mmol), DMSO (1 mL), appropriate thiophenol (47.2 mg, 0.429 mmol) or cyclohexanethiol (49.8 mg, 0.429 mmol) and Et_3N (59.1 mg, 0.585 mmol) was stirred at room temperature under argon for a week. The brine was added to the mixture and product was extracted with EtOAc (3x5 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered and concentrated.

***N*-(benzimidazol-1-yl)-4-chloro-2-phenylsulfanyl-5-sulfamoyl-benzamide (10).** The product was purified by flash chromatography over silica gel with $CHCl_3$:MeOH (10:1). Yield 51%, mp 272-274°C. ^{1}H NMR δ ppm: 6.98 (1H, s, C_3-H), 7.30 (1H, t, *J* = 7.2 Hz, $C_{5'}$-H), 7.36 (1H, t, *J* = 7.2 Hz, $C_{6'}$-H), 7.50 (1H, d, *J* = 8.0 Hz, $C_{4'}$-H), 7.57-7.60 (3H, m, $C_{2'',4'',6''}$-H), 7.63-7.67 (2H, m, $C_{3'',5''}$-H), 7.75 (1H, d, *J* = 8.0 Hz, $C_{7'}$-H), 7.78 (2H, s, SO_2NH_2), 8.44 (1H, s, C_6-H), 8.48 (1H, s, $C_{2'}$-H), 12.44 (1H, s, CONH). ^{13}C NMR δ ppm: 109.9, 120.5, 122.9, 124.0, 129.5, 130.4, 130.6, 131.0 (2C), 133.6, 133.9, 135.1 (2C), 138.5, 144.3 (2C), 145.2, 165.6. HRMS calcd. for $C_{20}H_{15}ClN_4O_3S_2$ $[(M+H)^+]$: 459.0347, found: 459.0351.

***N*-(benzimidazol-1-yl)-4-chloro-2-cyclohexylsulfanyl-5-sulfamoyl-benzamide (11).** Theproduct was purified by flash chromatography over silica gel with $CHCl_3$:MeOH (10:1).Yield 22%, mp 231-233°C. ^{1}H NMR δ ppm: 1.25-1.34 (1H, m, Cy-H), 1.38-1.48 (4H, m, Cy-H), 1.59-1.62 (1H, m, Cy-H), 1.72-1.78 (2H, m, Cy-H), 1.96-2.00 (2H, m, Cy-H), 2.09 (1H, s, Cy-H), 3.71 (1H, br s, Cy-H), 7.31 (1H, t, *J* = 7.6 Hz, $C_{5'}$-H), 7.37 (1H, t, *J* = 7.6 Hz, $C_{6'}$-H), 7.55 (1H, d, *J* = 8.0 Hz, $C_{4'}$-H), 7.75 (1H, d, *J* = 8.0 Hz, $C_{7'}$-H), 7.77 (2H, s, SO_2NH_2), 7.85 (1H, s, C_3-H), 8.25 (1H, s, C_6-H), 8.45 (1H, s, $C_{2'}$-H), 12.20 (1H, s, CONH).^{13}C NMR δ ppm: 25.5, 25.7, 32.7, 45.0, 109.9, 120.5, 122.9, 123.9, 129.0, 131.6, 133.3, 133.5, 133.7, 138.4, 141.5, 141.9, 144.3, 166.0. HRMS calcd. for $C_{20}H_{21}ClN_4O_3S_2$ $[(M+H)^+]$: 465.0816, found: 465.0809.

4.2. Protein preparation

Expression and purification of CA III, IV, VA, VB, IX, and XIV has been previously described in[47], of CA I and CA VIin[48], CA II - [49], CA VII and CA XIII- [50], CA XII - [51].

4.3. Determination of compound binding to CAs

4.3.1. Fluorescent thermal shift assay

The fluorescent thermal shift assay (FTSA, ThermoFluor®) measurements were performed by following the temperature shift of protein denaturation curve as a function of the added ligand concentration in a Corbett Rotor-Gene 6000 (QIAGEN Rotor-Gene Q) instrument using the blue channel (excitation 365±20, detection 460±15 nm). The fluorescence of a protein unfolding was followed as a function of temperature due to reporting probe 8-anilino-1-naphthalene sulfonate (ANS),which binds to hydrophobic parts of a protein that expose when protein unfolds. The samples contained a constant concentration of protein, different concentrations of compound, 50 μM ANS in 50 mM phosphate buffer (pH 7.0) containing 100 mM NaCl and 2% (v/v) of DMSO. The applied heating rate was 1ºC/min. Data analysis was performed as previously described [52].

4.3.2. The pK_as of CAs and sulfonamide group of the compounds

The pK_a values of proteins and compounds **1a** and **1j** were taken from[40]. The pK_as of the other compounds could not be observed by low solubility, thus we stated that ionization constants for structurally similar compounds are the same. For the compounds **2a**, **2b**, **2c** and **7** pK_as were determined according to the NMR chemical shift[53]. Values were compared with pK_as calculated by Marvin, and differ only 0,02 – 0,1 units.

4.3.3. Intrinsic binding parameters

Sulfonamide binding to CA is linked to protonation reactions. It is known that the binding reaction occurs when sulfonamide group of compound is deprotonated and Zn^{2+}-bound hydroxide is protonated (water molecule is produced)[2,54].The intrinsic Gibbs energy change of this binding is:

$$\Delta_b G_{intr} = -RT \ln K_{b\_intr} = RT \ln K_{d\_intr}, \quad (1)$$

where R is the universal ideal gas constant, T– temperature, K_{b_intr} and K_{d_intr}– intrinsic binding and dissociation constants, respectively. The intrinsic binding constant (K_{b_intr}) is related to the observed binding constant (K_b) and the fractions of the protonated zinc hydroxy anion in active center of carbonic anhydrase (f_{CAZnH_2O}) and the deprotonated sulfonamide ($f_{RSO_2NH^-}$):

$$K_{b\_intr} = \frac{K_b}{f_{RSO_2NH^-} \times f_{CAZnH_2O}} \quad (2)$$

$$f_{RSO_2NH^-} = \frac{10^{pH-pK_{a\_RSO_2NH_2}}}{1+10^{pH-pK_{a\_RSO_2NH_2}}} \quad (3)$$

$$f_{CAZnH_2O} = 1 - \frac{10^{pH - pK_{a\_CAZnH_2O}}}{1 + 10^{pH - pK_{a\_CAZnH_2O}}} \quad (4)$$

The pK_as of the carbonic anhydrases (H_2O bound to the Zn^{2+} in the active center), sulfonamides and K_{d_intr} are listed in Table 2 and the intrinsic Gibbs energies of binding in Figure 4.

4.4. Computational docking details

The homology model for human CA VA was taken from SWISS-MODEL repository[55], based on the murine CA V template (PDB ID: 1dmx). The ligand-receptor complex was prepared for docking using UCSF Chimera, v. 1.10 [56]. The protein atoms were assigned CHARMM22 [57] parameters. The ligand atoms were typed by Discovery Studio Visualizer 3.5 (Accelrys Software Inc., San Diego, CA) using CHARMm[58] parameters. Vdock program was used for docking [59,60]. The coordinates of the ligand atoms for **1a** were taken directly from PDB ID 3m98 without reoptimization, unless otherwise noted. For the derivative ligands, necessary changes were made in the substituent, and the substituent was afterwards optimized with MMFF94s force field [61] using Avogadro program, v. 1.2.0 [62]. The influence of the solvent was approximated using the 4*r* distance-dependent dielectric approximation [63]. As in our previous study [64], the position of the sulfonamide nitrogen was fixed in space, and the dihedral angle between the chlorophenyl group and the sulfonamide group was fixed as well. Because of the presence of the alternative conformations of the His64 residue in CA II bound to **1a**, we permitted the side chain of the homologous Tyr64 in CA VA receptor to remain flexible during docking. This allowed a formation of aromatic-aromatic interactions between the receptor and the ligand (see Discussion for more details). To validate the used docking approach, we also performed a docking of **1a**into CA II receptor (PDB ID: 3m98) using similar protocol as with CA VA, resulting in reasonable 1.67 and 1.65 Å ligand heavy atom RMSDs from the X-ray structure, if the ligand coordinates were taken straight from the PDB file, or optimized using MMFF94s force field, correspondingly. Some deviation from the experimental structure was mostly due to the relatively poor ability of the force field to model a possibly strong histidine-aromatic interaction [65]. However, when docking into the CA VA receptor this was not essential, because the docking in CA VA was able to discover aromatic-aromatic interaction, differently from the CA II.

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