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Optimization of heterocyclic substituted benzenesulfonamides as novel carbonic anhydrase IX inhibitors and their structure activity relationship

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ABSTRACT

In this study, starting from a lead compound discovered by virtual screening, a series of novel heterocyclic substituted benzenesulfonamides were designed and synthesized as new carbonic anhydrase IX (CA IX) inhibitors. Some compounds exhibited potent inhibitory effects against CA IX (in the low nanomolar range) as well as high selectivity against other carbonic anhydrase isozymes (CA I and CA II). The most potent and selective compound **27** could inhibit CA IX in the subnanomolar level with IC_{50} of 0.48 nM, which increased the potency by about 40-fold against CA IX compared with the lead compound **26**, and presented more than 10^3 fold selectivity over CA I and CA II. The structure–activity relationship (SAR) based on the docking experiments further elucidated the effects of the compounds on the bioactivity and selectivity.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes, which are the most widely spread biological catalysts all over the phylogenetic tree and encoded by five evolutionarily unrelated gene families: α-, β-, γ-, δ-, and ζ-CAs [1,2]. These enzymes catalyze the reversible hydration of CO_2 to HCO_3^- and H^+. Furthermore, CAs are involved in some pathological pathways and thus are viable targets for the treatment of glaucoma, cancer, obesity, and epilepsy [1–8]. Recently, two tumor-associated membrane CA isoforms (CA IX and CA XII) have drawn much attention [9]. Many reports are published studying the roles of CA IX in tumor physiology, such as the control of tumor pH and influence on other processes in the cell microenvironment that promote cell proliferation, invasion, or metastasis. The expression of CA IX is remarkably up-regulated via the hypoxia inducible factor-1 (HIF-1) transcription factor. The over-expression of CA IX will induce the pH imbalance in tumor tissue, and contribute significantly to the extracellular acidification of solid tumors. Thus, CA IX has been a promising target for hypoxic tumor treatment [10–12].

Up to now, many CA IX inhibitors are reported, such as hydroxamates, mercaptophenols, metal-complex anions and sulfonamides [3,13,14]. Among them, the most important class is sulfonamides derivatives. There are around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, such as clinically used sulfonamides AAZ, MZA, IND, and EZA (Chart 1) [15,16]. The negative charge of the deprotonated nitrogen in the sulfonamide group ($-SO_2NH_2$) can coordinate with the positively charged Zn^{2+}; the presence of one proton on the coordinated nitrogen atom also satisfies the hydrogen bond donor (Chart 2) [17–19].

However, design of therapeutic agents targeting at CAs is confronted by the presence of a large number of isoforms in humans, and the selectivity of the existing inhibitors is not optimal for any specific isozymes [20–23].

Through molecular docking, visual inspection and in vitro inhibition assay, we discovered a heterocyclic substituted benzenesulfonamides lead compound **26** against CA IX in our previous work (paper in submission). It exhibits an IC_{50} of 18.5 nM and poor inhibitory efficiency against CA I, CA II (Chart 2). Based on this lead compound, we designed a series of structurally optimized derivatives, which were tested to display improved bioactivity and selectivity.

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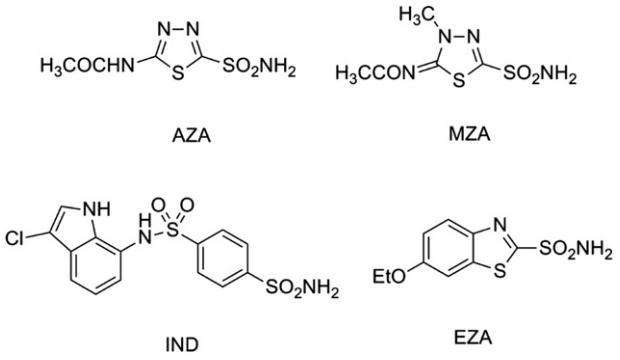


Chart 1. Clinically used sulfonamides as CAs inhibitors.

2. Results and discussion

2.1. Structure-based SAR and docking analysis

By inspecting the protein structure of CA IX (PDB ID: 3IAI) and the virtual action mode of the hit compound, several clues were obtained for the structure optimization. Sulfonamides A of compound **26** can bind with Zn^{2+} in the active site. Apart from chelating with Zn^{2+}, the coordinated sulfonamide nitrogen atom forms a hydrogen bond with the hydroxyl group of Thr199, and one of the oxygen atoms of the sulfonamide moiety forms hydrogen bond with the backbone amide of Thr199 as well. These hydrogen bonds contribute to the binding of these inhibitors bind to the active site. At the hydrophobic region, the benzene moieties can interact with Val121, Val143 and Leu198. While at the hydrophilic part of the pocket, the linker NH could forms hydrogen bonds with the side chains of His64. Besides the key sulfonamide group for Zn^{2+} coordination, other structural modifications could also potentially affect the interactions between the hydrophobic residues and hydrophilic residues of CA IX and inhibitors, as indicated by molecular modeling.

There are two sulfonamides in the hit compound **26**, and the type of the inhibitors has been reported by C. T. Supuran [24] (Chart 3). Firstly, to identify which sulfonamide coordinated to Zn^{2+}, the sulfonamide A was replaced by other substituents such as $-Cl$, $-CF_3$ and $-OCH_3$, while sulfonamide B was retained. This led to compounds **15**–**18**. The position of sulfonamide on benzene might also affect the activity. Therefore, compounds **19**–**23** with sulfonamide B switched to the *meta* position were developed. Biological activity evaluation showed that **15**–**23** had much poor inhibitory activity against CA IX, CA I and CA II (Table 1), therefore sulfonamide B at *meta* or *para* position couldn't coordinate with Zn^{2+} in CAs active site, and sulfonamide A in hit compound **26** should be the key sulfonamide. (Chart 4).

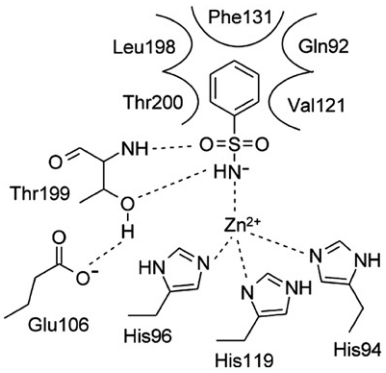


Chart 2. The binding mode of the benzenesulfonamide with the CA II active site.

Compounds **24** and **25** are designed from hit compound **26**, by retaining sulfonamide A and replacing sulfonamide B with amino (compound **24**) and *t*-butyl (compound **25**) respectively. Their CA IX inhibition IC_{50} (18.5–28.3 nM) are at the same level with the hit compound **26**. So the substituents *R* almost doesn't affect the inhibition. This agrees with the previous observation that sulfonamide A in these compounds is the key groups coordinated with Zn^{2+}.

Compound **27** with the 3-sulfonamide and 4-methyl group at benzene was synthesized from hit compound **26**. It has a high inhibitory effect against CA IX (IC_{50} of 0.48 nM) and the selectivity over cytosolic ones (CA I and CA II) exceeded 10^3 times. This shows that 4-methyl contributed deeply to the inhibition and selectivity against CA IX. Compound **28**, with the 4-sulfonamide and 3-methyl group at benzene, shows about 6 times higher inhibitory activity toward CA IX (IC_{50} of 3.3 nM) than the hit **26**. But the CA IX selectivity ratio of compound **28** over the cytosolic ones (CA I and CA II) is poorer than that of compound **26**, which was at the same level with the clinical drug AZA. From Table 1, the selectivity ratio of AZA and EZA between CA IX and CA II is in the range of 4.9–19.3, which prohibited it from clinical trials as CA IX inhibitors.

In order to rationalize the affinity between these novel inhibitors and Zn^{2+} in CA IX catalytic sites, we studied their binding modes within the active binding site of CA IX (PDB: 3IAI) by using molecular docking method. Poor inhibition effect ($IC_{50} > 10$ μM) of compounds **15**–**23** for CA IX may result from the steric hindrance, which affects the access of sulfonamides B to the Zn^{2+} (Table 1). Instead, sulfonamides A of compounds **24**–**25** can bind with Zn^{2+} in the active site. In common with compound 26, the coordinated sulfonamide nitrogen atom forms a hydrogen bond with the hydroxyl group of Thr199, and one of the oxygen atoms of the sulfonamide moiety forms hydrogen bond with the backbone amide of Thr199 as well. At the hydrophobic region, the benzene moieties interact with Val121, Val143 and Leu198, and small hydrophobic groups such as methyl contribute remarkably to the higher affinity. So, by adding methyl at the *ortho* position of the left benzenesulfonamide, two potent CA IX inhibitors (compounds **27**, **28** with IC_{50} of 0.48, 3.3 nM) are obtained. While at the hydrophilic part of the pocket, the linker NH forms hydrogen bonds with the side chains of His64, Asn62 (compound **27**) and leads to the high affinities with CA IX (Fig. 1).

The differences of affinity of some inhibitors for the different isozymes have been evidenced [25]. CA II is the most susceptible to inhibition by sulfonamides, whereas CA I has generally a lower affinity for this type of inhibitors and a much larger one for the inorganic anions, such as cyanide, cyanate, thiocyanate. Therefore, we focused on the selectivity with CA IX over CA II. As for the high selectivity of compound **27**, superposition of the CA II and CA IX in Fig. 2 clearly reveals that the amino acid residue 131 of the active sites is different. This residue is known to be very important for the binding of sulfonamide inhibitors to CA II [26]. The phenyl group of phenylalanine locates in the CA II might prevent the interaction with compound **27**, while CA IX has a valine at the position, whose side chain is much smaller in terms of sterics. Consequently, compound **27** can bind CA IX much better than CA II.

2.2. Synthesis of the target compounds

The target compounds **15**–**28** were synthesized according to Scheme 1. The starting material 2,3-dihydrophthalazine-1,4-dione **1** reacted with phosphorus oxybromide in 1,2-dichloroethane to yield 1,4-dibromophthalazine **2** [27], which further reacted with different sulfanilamide in the presence of Na_2CO_3 in DMF to afford 4-((4-bromophthalazin-1-yl)amino)benzenesulfonamide **3** and **4**. By coupling **3** (or **4**) with suitable phenylboronic acid derivatives, the corresponding products **15**–**23** were obtained. As for the

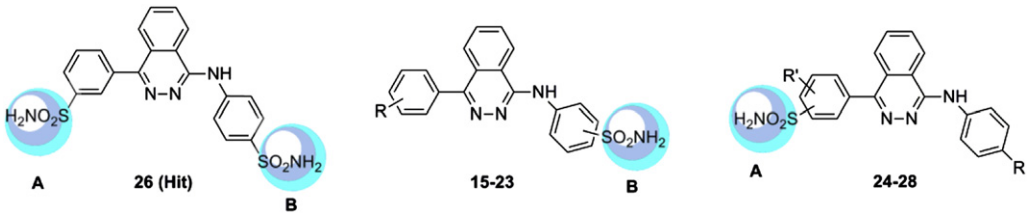


Chart 3. The structures of lead compound 26 and designed two novel series of compounds.

compounds **24**–**26**, three different aniline derivatives were used to react with compound **1** in DMF, and the products **12**–**14** coupled with the corresponding benzenesulfonamide boronates **10**, **11** [28] and 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamide in DME and water. Chlorosulfonation of 3-bromotoluene and 4-bromotoluene at 0 °C followed by ammonolysis using aqueous ammonium hydroxide in dioxane afforded sulfonamide (**8**, **9**) in good yield. Boronates **10** and **11** were obtained by palladium-catalyzed borylation of aryl bromide **8**, **9** with bis(pinacolato) diboron. Compounds **27** and **28** were obtained similarly to compounds **24**–**26** by Suzuki Coupling. Biological activity of compounds **15**–**28** and positive samples AZA, EZA against CA I, CA II, CA IX were studied and shown in Table 1.

3. Conclusion

In this paper, some potent CA IX inhibitors with high selectivity against other carbonic anhydrase isozymes (CA I and CA II) were designed and synthesized by structural optimization of a previously discovered lead compound. Compound **27** is the most potent CA IX inhibitor with an IC_{50} of 0.48 nM and exhibits higher inhibitory effects over CA I and CA II. At the same time, the structure–activity relationship and CA IX selectivity for the newly synthesized compounds are analyzed by docking analysis, in which the introduction of methyl group on benzene can improve the activity and selectivity efficiently due to the favorable interactions with some specific amino acid residues of CA IX catalytic sites. The hit compounds with novel scaffold discovered in this work lay the foundation for further development of therapeutic candidates for cancer treatment.

4. Chemistry

All starting materials and reagents were of analytic grade and used without further purification. ^{1}H NMR was measured on a Bruker AV-500 spectrometer with chemical shifts reported in ppm (in Aceton-d_6/DMSO-d_6/$CDCl_3$, TMS as an internal standard). Mass spectra were measured on a HP 1100 LC–MS spectrometer. Melting points were determined by an X-6 micro-melting point apparatus and uncorrected.

Table 1
Inhibitory activities of the compounds against CA I, CA II and CA IX.

Compounds	IC_{50}(nM)			Selectivity ratios	
	CA I	CA II	CA IX	I/IX	II/IX
15	$>10^4$	$>10^4$	$>10^4$	/	/
16	$>10^4$	$>10^4$	$>10^4$	/	/
17	$>10^4$	$>10^4$	$>10^4$	/	/
18	$>10^4$	$>10^4$	3930	/	/
19	$>10^4$	$>10^4$	$>10^4$	/	/
20	$>10^4$	$>10^4$	$>10^4$	/	/
21	$>10^4$	$>10^4$	690	/	/
22	$>10^4$	$>10^4$	$>10^4$	/	/
23	220.2 ± 13.1	61.8 ± 3.1	$>10^4$	/	/
24	$>10^4$	146.5 ± 6.8	28.3	$>10^2$	5.3
25	$>10^4$	$>10^4$	29.0	$>10^2$	$>10^2$
26	$>10^4$	455.1 ± 3.2	18.5	$>10^3$	24.5
27	$>10^4$	547.0 ± 34.9	0.48	$>10^4$	1139.7
28	85.7 ± 4.1	26.8 ± 9.0	3.3	25.7	8.1
EZA	67.6	4.8	0.25	270.6	19.3
AZA	509.6	37.5	7.7	66.0	4.9

4.1. Synthesis

4.1.1. Synthesis of 1,4-dibromophthalazine (*2*)

Phosphorus oxybromide (861 mg, 3.0 mmol) was added to a stirred suspension of 2,3-dihydrophthalazine-1,4-dione (162 mg, 1.0 mmol) in 1,2-dichloroethane (5 mL). The mixture was heated to 100 °C for 18 h and poured into water (100 mL) upon cooling to rt The aqueous layer was neutralized with saturated Na_2CO_3 solution and concentrated to give a residue, which was washed with water to afford the product as a white solid. Yield: 88%, m.p. 160.1–161.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.37–8.33 (m, 2H), 8.12–8.08 (m, 2H). GC–MS (EI): *m*/*z* calc. 285.8, *m*/*z* found 285.8.

4.1.2. Synthesis of 4-((4-bromophthalazin-1-yl)amino) benzenesulfonamide (*3*)

To a mixture of 1,4-dibromophthalazine (286 mg, 1.0 mmol) and 4-aminobenzenesulfonamide (172 mg, 1.0 mmol) in DMF (10 mL) was added potassium carbonate (276 mg, 2.0 mmol). The reaction was heated at 90 °C overnight. The reaction mixture was distilled under reduced pressure to remove most of the DMF, and the remaining residue was partitioned between CH_2Cl_2 (250 mL) and saturated ammonium chloride solution (50 mL). The water layer was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and evaporated under reduced pressure. Purification was performed by flash chromatography on silica gel (CH_2Cl_2) to give the title compound as a yellow crystal. Yield: 20%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.83 (s, 1H), 8.35 (d, J = 8.0 Hz, 1H), 8.10–8.05 (m, 1H), 8.00–7.95 (m, 2H), 7.67 (d, J = 8.8 Hz, 2H), 6.60 (d, J = 8.8 Hz, 2H), 5.93 (s, 2H). LC-MS (ESI): $C_{14}H_{12}BrN_4O_2S$ calc. 379.9, found 380.0.

4.2. General procedure for compounds *15*–*23*

4.2.1. 4-((4-(4-Chlorophenyl)phthalazin-1-yl) amino) benzenesulfonamide (*15*)

A mixture of 4-((4-bromophthalazin-1-yl)amino)benzenesulfonamide (189 mg, 0.5 mmol), 4-chlorophenylboronic acid (76 mg, 0.5 mmol) and sodium carbonate (106 mg, 1.0 mmol) in ethanol (3 mL), water (3 mL) and toluene (10 mL) was degassed with Ar for 10 min. Then tetrakis(triphenylphosphine)palladium (30 mg, 0.025 mmol) was added. The reaction mixture was stirred at 80 °C under nitrogen for 8 h. After cooling to rt, the reaction mixture was distilled under reduced pressure to remove the solvent, and extracted with ethyl acetate (3 × 5 mL), water (5 mL). The organic layer was washed with an aqueous saturated solution of $NaHCO_3$ (2 × 5 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. Purification was effected by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide

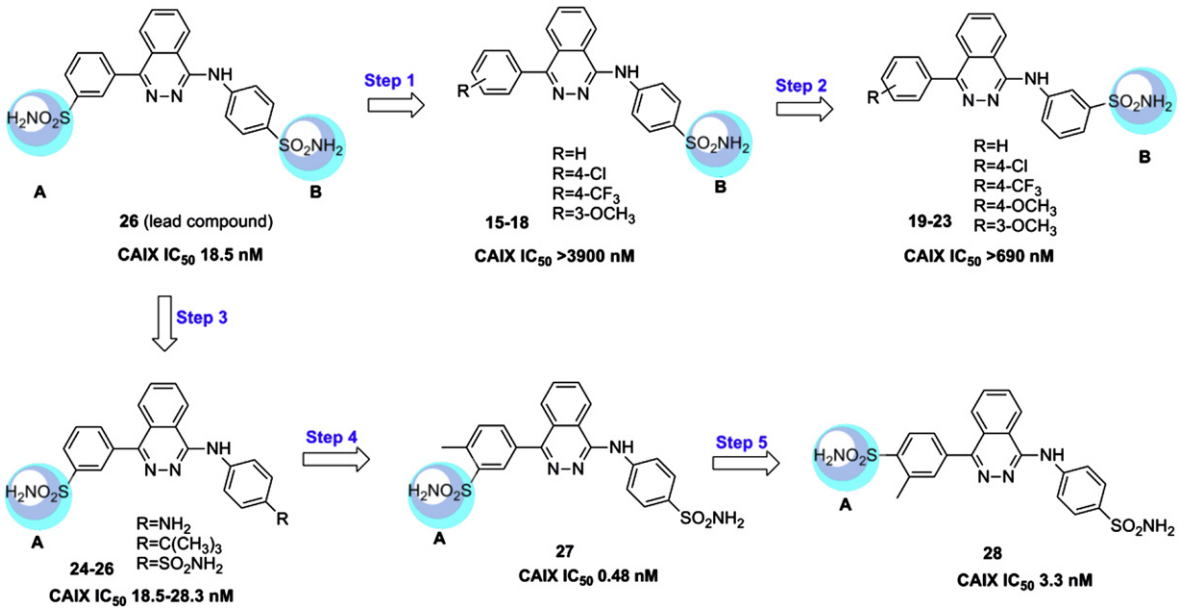


Chart 4. Novel designed benzenesulfonamides from structural optimization of the hit compound.

the title compound as a pale yellow solid. Yield: 35%, m.p. 230.8–231.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.63 (s, 1H), 8.66–8.64 (m, 1H), 7.87 (d, J = 8.4 Hz, 2H), 7.84 (t, J = 8.4 Hz, 2H), 7.77–7.75 (m, 1H), 7.55 (s, 4H), 6.70 (d, J = 8.4 Hz, 2H), 5.32 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ153.06, 149.68, 149.43, 135.16, 134.87, 133.35, 131.84, 129.28, 129.17, 128.60, 128.30, 127.51, 127.04, 126.98, 126.72, 113.01. HRMS (ESI): $C_{20}H_{16}ClN_4O_2S$ calc. 411.0683, found 411.0676.

4.2.2. 4-((4-(4-(Trifluoromethyl)phenyl)phthalazin-1-yl)amino) benzenesulfonamide (**16**)

According to the general procedure, compound **3** was treated with 4-(trifluoromethyl)phenylboronic acid and reacted for 10 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **16**. Yellow solid, yield: 38%, m.p. 206.1–207.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.90 (s, 1H), 8.47–8.45 (m, 1H), 8.09 (t, J = 7.2 Hz, 1H), 8.01–7.98 (m, 2H), 7.96 (d, J = 7.2 Hz, 2H), 7.88 (d, J = 7.6 Hz, 1H), 7.71 (d, J = 8.8 Hz, 2H), 7.68–7.66 (m, 1H), 6.62–6.60 (m, 2H), 5.93 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.08, 149.44, 138.60, 135.22, 133.44, 132.00, 131.90, 130.97, 129.27, 129.16, 128.62, 128.26, 127.39, 126.95, 126.76, 126.00, 113.01. HRMS (ESI): $C_{21}H_{16}F_3N_4O_2S$ calc. 445.0946, found 445.0943.

4.2.3. 4-((4-Phenylphthalazin-1-yl)amino)benzenesulfonamide (**17**)

According to the general procedure, compound **3** was treated with phenylboronic acid and reacted for 4 h. Then the crude product was purified by flash chromatography on silica gel using CH_2Cl_2 to provide compound **17**. Pale yellow solid, yield: 33%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.61 (s, 1H), 8.65 (d, J = 8.4 Hz, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 4.8 Hz, 2H), 7.61–7.58 (m, 2H), 7.58–7.55 (m, 3H), 6.69 (d, J = 8.4 Hz, 2H), 4.11 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.03, 150.74, 149.48, 135.14, 134.47, 133.70, 133.28, 132.51, 132.00, 129.94, 129.28, 128.59, 127.20, 126.71, 113.03. HRMS (ESI): $C_{20}H_{17}N_4O_2S$ calc. 377.1072, found 377.1080.

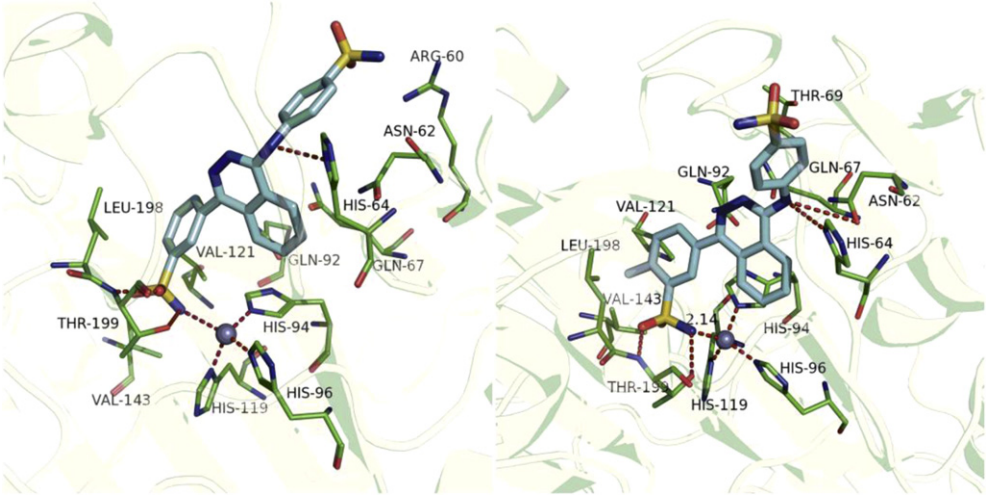


Fig. 1. The potential binding modes of compounds **26** (left), and **27** (right) against CA IX (PDB code: 3IAI). Protein and the key residues were colored in light pink and green respectively, and the docked molecules were colored in cyan. The Gray sphere represented the zinc ions. The hydrogen bonds and coordinate interactions were shown with red dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

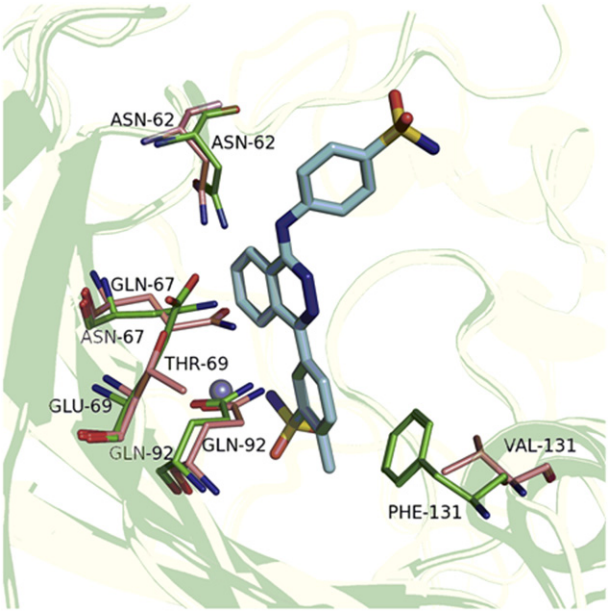


Fig. 2. Superpositions of the crystal structures of the CA II and the CA IX. The binding poses of the compound **27** (cyan stick) were predicted by the molecular docking. The pink structure was CA IX (PDB code: 3IAI); the green structure was CA II (PDB code: 3R16); Gray spheres represented the zinc ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2.4. 4-((4-(3-Methoxyphenyl)phthalazin-1-yl)amino) benzenesulfonamide (**18**)

According to the general procedure, compound **3** was treated with 3-methoxyphenylboronic acid and reacted for 4 h. Then the crude product was purified by flash chromatography on silica gel using CH_2Cl_2 to provide compound **18**. Pale yellow solid, yield: 29%, m.p. 208.5–209.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.79 (s, 1H), 8.45 (d, J = 7.6 Hz, 1H), 8.00–7.92 (m, 2H), 7.72 (t, J = 8.4 Hz, 3H), 7.50 (t, J = 8.0 Hz, 1H), 7.18–7.14 (m, 3H), 6.61 (d, J = 8.8 Hz, 2H), 5.91 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.71, 153.05, 150.57, 149.48, 135.76, 135.14, 133.28, 132.48, 132.00, 131.90, 130.21, 129.28, 129.16, 128.60, 127.24, 126.67, 122.17, 113.01, 55.75. HRMS (ESI): $C_{21}H_{19}N_4O_3S$ calc. 407.1178, found 407.1171.

4.2.5. 3-((4-(4-Methoxyphenyl)phthalazin-1-yl)amino) benzenesulfonamide (**19**)

According to the general procedure, compound **4** was treated with 4-methoxyphenylboronic acid and reacted for 4 h. Then the crude product was purified by flash chromatography on silica gel using CH_2Cl_2 to provide compound **19**. Yellow solid, yield: 20%, m.p. 257.5–258.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.67 (s, 1H), 8.69–8.66 (m, 1H), 7.87–7.81 (m, 3H), 7.62–7.56 (m, 6H), 7.45 (d, J = 7.6 Hz, 1H), 7.39 (s, 1H), 6.83 (d, J = 8.0 Hz, 1H), 4.11 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.28, 150.61, 149.36, 133.91, 132.33, 131.32, 129.30, 127.99, 127.44, 126.53, 125.77, 116.83, 116.46, 114.38, 113.73, 111.56, 55.76. HRMS (ESI): $C_{21}H_{19}N_4O_3S$ calc. 377.1072, found 377.1070.

4.2.6. 3-((4-(4-Chlorophenyl)phthalazin-1-yl)amino) benzenesulfonamide (**20**)

According to the general procedure, compound **4** was treated with 4-chlorophenylboronic acid and reacted for 8 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **20**. Brown solid, yield: 28%, m.p. 189.1–190.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.04 (s, 1H), 8.47 (d, J = 7.2 Hz, 1H), 8.37 (d, J = 8.0 Hz, 1H), 8.12 (t, J = 7.2 Hz, 1H), 8.04–7.96 (m, 3H), 7.72 (d, J = 7.6 Hz, 1H), 7.21–7.13 (m, 4H), 6.75 (t, J = 3.2 Hz, 1H), 5.55 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.19, 148.78, 143.67, 135.45, 134.95, 134.62, 133.51, 133.27, 131.87, 129.89, 129.20, 128.18, 127.64, 127.14, 126.83, 118.23, 114.10, 111.73. HRMS (ESI): $C_{20}H_{16}ClN_4O_2S$ calc. 411.0683, found 411.0686.

4.2.7. 3-((4-(4-(Trifluoromethyl)phenyl)phthalazin-1-yl)amino) benzenesulfonamide (**21**)

According to the general procedure, compound **4** was treated with 4-(trifluoromethyl)phenylboronic acid and reacted for 10 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **21**. Brown solid, yield: 33%, m.p. 176.7–176.9 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.11 (s, 1H), 8.37 (d, J = 6.8 Hz, 1H), 8.11 (t, J = 7.2 Hz, 1H), 8.03–7.96 (m, 3H), 7.72–7.54 (m, 2H), 7.21–7.13 (m, 4H), 6.75 (d, J = 7.2 Hz, 1H), 5.55 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 149.71, 143.32, 136.19, 135.14, 134.61, 132.00, 131.90, 131.00, 129.81, 129.29, 129.17, 128.82, 128.17, 127.12, 126.07, 124.41, 124.37, 117.76, 113.37, 111.15. HRMS (ESI): $C_{21}H_{16}F_3N_4O_2S$ calc. 445.0946, found 445.0941.

4.2.8. 3-((4-Phenylphthalazin-1-yl)amino)benzenesulfonamide (**22**)

According to the general procedure, compound **4** was treated with phenylboronic acid and reacted for 4 h. Then the crude product was purified by flash chromatography on silica gel using CH_2Cl_2 to provide compound **22**. Yellow solid, yield: 26%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.67 (s, 1H), 8.69–8.66 (m, 1H), 7.87–7.81 (m, 3H), 7.62–7.56 (m, 6H), 7.45 (d, J = 7.6 Hz, 1H), 7.39 (s, 1H), 6.83 (d, J = 8.0 Hz, 1H), 4.11 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 151.19, 150.20, 149.69, 143.67, 135.40, 134.54, 133.90, 133.42, 130.47, 129.97, 129.80, 129.09, 127.81, 127.29, 126.84, 117.68, 113.40, 111.19. HRMS (ESI): $C_{20}H_{17}N_4O_2S$ calc. 377.1072, found 377.1070.

4.2.9. 3-((4-(3-Methoxyphenyl)phthalazin-1-yl)amino) benzenesulfonamide (**23**)

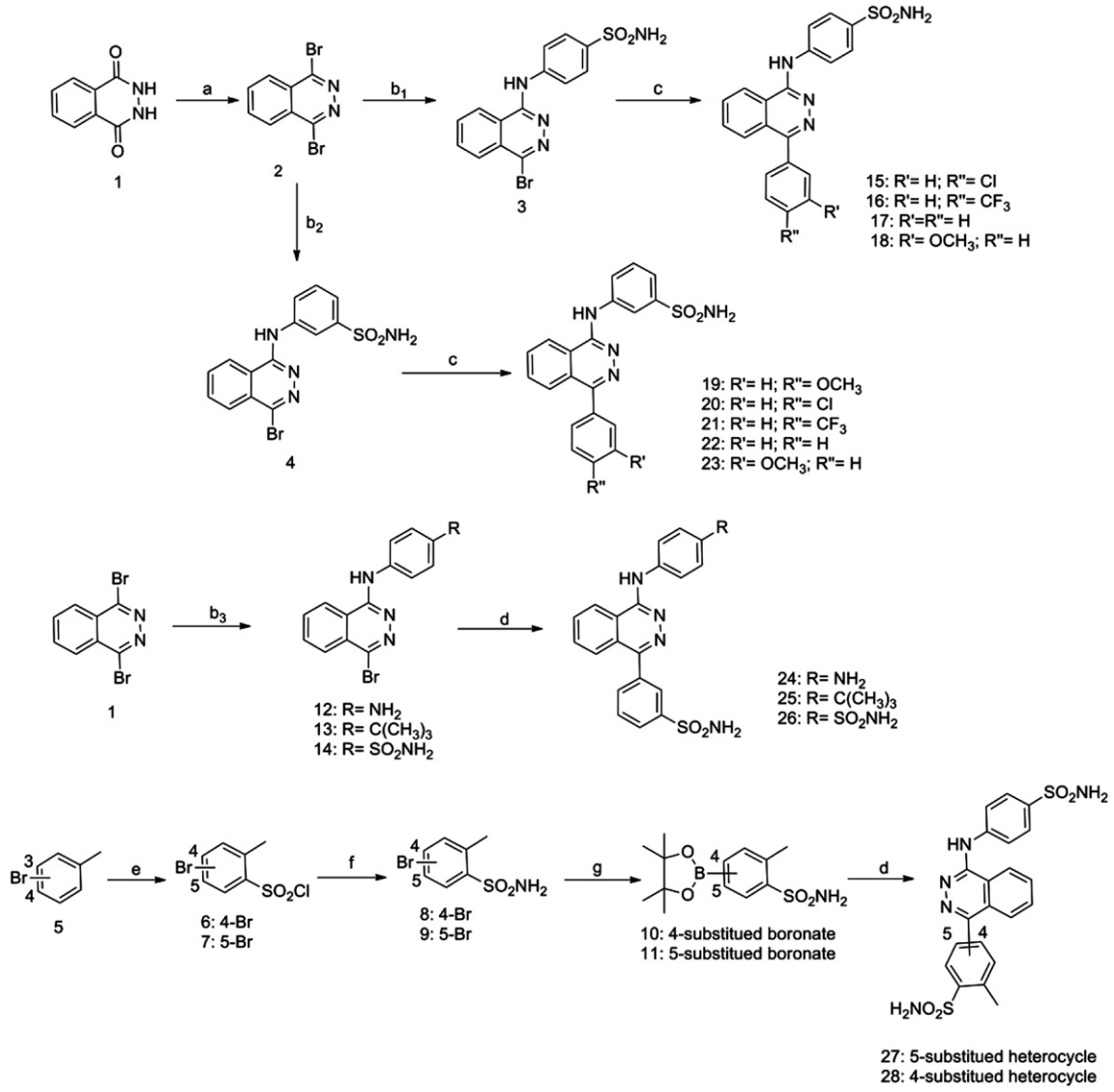
According to the general procedure, compound **4** was treated with 3-methoxyphenylboronic acid and reacted for 4 h. Then the crude product was purified by flash chromatography on silica gel using CH_2Cl_2 to provide compound **23**. Pale yellow solid, yield: 35%, m.p. 245.0–245.4 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.98 (s, 1H), 8.48 (d, J = 7.6 Hz, 1H), 8.03–7.95 (m, 2H), 7.76 (d, J = 7.6 Hz, 1H), 7.51 (t, J = 8.0 Hz, 1H), 7.20 (d, J = 7.6 Hz, 2H), 7.18–7.15 (m, 4H), 6.76–6.74 (m, 1H), 5.52 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.72, 151.05, 150.02, 149.70, 143.65, 135.70, 135.43, 133.45, 130.25, 129.80, 127.82, 127.35, 126.80, 122.20, 117.67, 115.71, 115.41, 113.38, 111.17, 55.77. HRMS (ESI): $C_{21}H_{19}N_4O_3S$ calc. 407.1178, found 407.1180.

4.2.10. Synthesis of 4-bromo-2-methylbenzenesulfonyl chloride (**6**) and 5-bromo-2-methylbenzensulfonyl chloride (**7**)

Chlorosulfonic acid (696 mg, 6.0 mmol) was added slowly to a cold solution (0 °C) of 3-bromotoluene (170 mg, 1.0 mmol) (or 4-bromotoluene (170 mg, 1.0 mmol)), in $CHCl_3$ (10 mL). The reaction was allowed to proceed for 4 h with stirring at 0 °C prior to pouring onto crushed ice (250 g). The resulting mixture was extracted with $CHCl_3$ (3 × 150 mL). The combined $CHCl_3$ extracts were washed with water (3 × 100 mL) and dried with Na_2SO_4, and the solvent was removed in vacuum to yield the arylsulfonyl chloride product (**6** or **7**), which was used in next step without purification.

4.2.11. Synthesis of 4-bromo-2-methylbenzenesulfonamide (**8**) and 5-bromo-2-methylbenzenesulfonamide (**9**)

An aqueous solution of NH_4OH (15 mL of 30% w/v) was added to a cold solution of the arylsulfonyl chloride (**6** or **7**, 268 mg, 1.0 mmol) in dioxane (10 mL) at 0 °C with vigorous stirring, and the reaction was allowed to proceed for 6 h at 0 °C with stirring. The insoluble material was removed by filtration, washed with water



Scheme 1. The route for the synthesis of compounds **15**–**28**. Reagents and reaction conditions: (a) $POBr_3$, $C_2H_4Cl_2$, 100 °C, 18 h; (b1–b3) 4-Aminobenzenesulfonamide, 3-Aminobenzene- sulfonamide, aniline derivatives, K_2CO_3, DMF, 90 °C, 12 h; (c) Arylboronic acid, Na_2CO_3, ethanol, water, toluene, $Pd(PPh_3)_4$, 80 °C, 8 h; (d) Arylboronic acid pinacol ester, K_2CO_3, DME, H_2O, $Pd(PPh_3)_4$, reflux, 16 h; (e) Chlorosulfonic acid, $CHCl_3$, 0 °C, 4 h; (f) NH_4OH, dioxane, 0 °C, 6 h; (g) Bis(pinacolato)diboron, KOAc, $PdCl_2$(dppf), dioxane, reflux, 16 h.

(3 × 10 mL). The organic layer was concentrated and recrystallized with 50% acetone in hexane to yield the product.

4-*Bromo*-2-*methylbenzenesulfonamide* (**8**). White solid, yield: 85%, m.p. 176–177 °C ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.76 (d, J = 8.4 Hz, 1H), 7.65 (s, 1H), 7.60 (dd, J_1 = 2.0, J_2 = 8.8 Hz, 1H), 7.52 (s, 2H), 2.58 (s, 3H).

5-*Bromo*-2-*methylbenzenesulfonamide* (**9**). White solid, yield: 90%, m.p. 164–165 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.95 (d, J = 2.0 Hz, 1H), 7.70 (dd, J_1 = 2.0, J_2 = 8.0 Hz, 1H), 7.56 (s, 2H), 7.36 (d, J = 8.0 Hz, 1H), 2.54 (s, 3H).

*4.2.12. The synthesis of 2-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzenesulfonamide(**10**) and 2-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzenesulfonamide (**11**)*

To a mixture of 4-bromo-2-methylbenzenesulfonamide **8** (249 mg, 1.0 mmol) or 5-bromo-2-methylbenzenesulfonamide **9** (249 mg, 1.0 mmol), bis(pinacolato)diboron (381 mg, 1.5 mmol), and KOAc (392 mg, 4 mmol) in 10 mL of dioxane was added $PdCl_2$(dppf) (40 mg, 0.05 mmol). After bubbling nitrogen for 15 min, the reaction mixture was heated up to reflux for 16 h under nitrogen. Subsequently the mixture was cooled to room temperature and filtered through Celite. The filtrate was concentrated and the residue was purified by flash chromatography on silica gel eluted with 0–40% ethyl acetate in hexane to give compounds **10** and **11** as a white solid.

2-*Methyl*-4-(4,4,5,5-*tetramethyl*-1,3,2-*dioxaborolan*-2-*yl*)*benzenesulfonamide* (**10**). White solid, yield: 62%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.86 (d, J = 8.0 Hz, 1H), 7.63 (d, J = 4.8 Hz, 2H), 7.45 (s, 2H), 2.60 (s, 3H), 1.31 (s, 12H).

2-*Methyl*-5-(4,4,5,5-*tetramethyl*-1,3,2-*dioxaborolan*-2-*yl*)*benzenesulfonamide* (**11**). White solid, yield: 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.18 (s, 1H), 7.74 (d, J = 7.6 Hz, 1H), 7.41 (s, 1H), 7.39 (s, 2H), 2.62 (s, 3H), 1.31 (s, 12H). General Procedure for compounds 24–27.

*4.2.13. General procedure for the synthesis of 3-(4-((4-aminosulfamoylphenyl)amino) phthalazin -1-yl) benzenesulfonamide (**24**)*

A mixture of *N*-(4-bromophthalazin-1-yl)benzene-1,4-diamine (157 mg, 0.5 mmol), 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-

yl)benzenesulfonamide (142 mg, 0.5 mmol) and potassium carbonate (138 mg, 1.0 mmol) in DME (20 mL), water (10 mL) was degassed with Ar for 10 min. Then tetrakis(triphenylphosphine) palladium (30 mg, 0.025 mmol) was added. The mixture was heated to reflux for 12 h under Ar atmosphere. Subsequently, the mixture was cooled to rt and diluted with dichloromethane and washed with brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash chromatography on silica gel eluted with 0–5% methanol in dichloromethane to give the title compound as a brown solid. Yield: 73%, m.p. 266.1–267.2 °C. ^{1}H NMR (400 MHz, DMSO-*d*6) δ (ppm): 9.02 (s, 1H), 8.63 (d, J = 8.4 Hz, 1H), 8.12 (s, 1H), 7.94–8.02 (m, 2H), 7.90 (t, J = 8.0 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.76 (t, J = 7.6 Hz, 1H), 7.49 (s, 1H), 7.47 (s, 2H), 6.62 (d, J = 8.4 Hz, 2H), 4.92 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 152.92, 151.32, 145.34, 144.85, 138.20, 133.31, 132.59, 132.00, 129.64, 129.53, 127.10, 125.90, 125.84, 125.53, 124.45, 123.22, 118.54, 114.22. HRMS (ESI): $C_{20}H_{18}N_5O_2S$ calc. 392.1181, found 392.1177.

4.2.14. 3-(4-((4-(tert-Butyl)phenyl)amino)phthalazin-1-yl) benzenesulfonamide (**25**)

According to the general procedure, compound **13** was treated with 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamide and reacted for 24 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **25**. Yellow solid, yield: 15%, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.43 (s, 1H), 8.80 (d, J = 8.4 Hz, 1H), 8.14 (s, 1H), 8.05 (t, J = 7.2 Hz, 1H), 7.95–8.00 (m, 2H), 7.89 (d, J = 8.8 Hz, 2H), 7.78 (t, J = 8.0 Hz, 1H), 7.52 (s, 2H), 7.49 (d, J = 7.2 Hz, 2H), 3.99 (s, 2H), 1.07(s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 152.70, 152.30, 145.24, 144.90, 138.37, 137.95, 133.37, 132.87, 132.20, 129.67, 127.16, 126.08, 125.92, 125.67, 125.43, 123.67, 121.57, 118.81, 34.48, 31.78. HRMS (ESI): $C_{24}H_{25}N_4O_2S$ calc. 433.1698, found 433.1703.

4.2.15. 3-(4-((4-Sulfamoylphenyl)amino)phthalazin-1-yl) benzenesulfonamide (**26**)

According to the general procedure, compound **14** was treated with 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenesulfonamide and reacted for 12 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **26**. Yellow solid, yield: 39%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.47 (d, J = 7.2 Hz, 1H), 8.07 (s, 1H), 8.03 (d, J = 8.0 Hz, 1H), 8.00–7.96 (m, 1H), 7.88 (d, J = 8.0 Hz, 1H), 7.80 (t, J = 7.6 Hz, 1H), 7.73–7.67 (m, 3H), 7.52 (s, 1H), 6.61 (d, J = 8.8 Hz, 2H), 5.92 (s, 2H). HRMS (ESI): $C_{20}H_{17}N_5O_4NaS_2$ calc. 478.0620, found 478.0624.

4.2.16. 2-Methyl-5-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl) benzenesulfonamide (**27**)

According to the general procedure, compound **3** was treated with compound **11** and reacted for 24 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **27**. Yellow solid, yield: 53%, m.p. 238.4–239.9 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.87 (s, 1H), 8.46 (d, J = 7.2 Hz, 1H), 8.09 (d, J = 1.6 Hz, 1H), 7.98–7.95 (m, 1H), 7.76 (dd, J_1 = 1.6, J_2 = 7.6 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 7.6 Hz, 1H), 7.54 (s, 2H), 6.61 (d, J = 9.2 Hz, 2H), 5.92 (s, 2H), 5.76 (s, 2H), 2.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.00, 149.51, 142.93, 137.82, 135.16, 133.38, 133.17, 133.07, 132.38, 128.61, 128.38, 127.42, 126.90, 126.82, 112.95, 20.22. HRMS (ESI): $C_{21}H_{20}N_5O_4S_2$ calc. 470.0957, found 470.0959.

4.2.17. 2-Methyl-4-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl) benzenesulfonamide (**28**)

According to the general procedure, compound **3** was treated with compound **10** and reacted for 24 h. Then the crude product was purified by flash chromatography on silica gel (eluted with 0–1% methanol in CH_2Cl_2) to provide compound **28**. Yellow solid, yield: 46%, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.46 (d, J = 7.2 Hz, 1H), 8.03 (d, J = 7.6 Hz, 1H), 7.99–7.96 (m, 1H), 7.70 (d, J = 8.0, 4H), 7.64 (s, 1H) 7.54 (s, 2H), 7.56 (s, 1H), 6.61 (d, J = 9.6 Hz, 2H), 5.91 (s, 2H), 2.68 (s, 3H). HRMS (ESI): $C_{21}H_{20}N_5O_4S_2$ calc. 470.0957, found 470.0956.

4.3. Biology assay

An Applied Photophysics stopped-flow instrument was used to test the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as the indicator, working at the absorbance maximum of 557 nm. The 10–20 mM HEPES (pH 7.5) or Tris (pH 8.3) buffer solutions with 20 mM Na_2SO_4 or 20 mM $NaClO_4$ was used as solvents. The rate of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s was monitored. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reactions have been used for determining the initial rates. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled–deionized water, and diluted up to 0.01 nM were performed thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at rt prior to the assay study, in order to allow for the formation of the E–I complex. The half maximal inhibitory concentration (IC_{50}) was obtained by nonlinear least-squares methods using PRISM 3 as reported earlier and represented the mean from at least three different determinations. CA isoforms were recombinant ones obtained as described previously [29,30].

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC. Solutions of substrate were prepared in anhydrous ethanol; the substrate concentrations were 1 mM at 25 °C. A molar absorption coefficient of 400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis in the conditions of the experiments (pH 7.5). Nonenzymatic hydrolysis rates were subtracted from the observed rates. The experiments were repeated three times at each inhibitor concentration. Stock solutions of the inhibitor (50 mM) were prepared (which was not inhibitory at these concentrations) and diluted up to 0.5 nM with DMSO. At least 8 different inhibitor concentrations have been used, ranging from 5 mM, 0.5 mM, 50 μM, 5 μM, 0.5 μM, 50 nM, 5 nM, 0.5 nM. Inhibitor and enzyme solutions were preincubated together for 15 min at rt prior to the assay study, to allow for the formation of the E–I complex. Enzyme concentrations were 1 ng/L for CA II, 50 ng/L for CA I [31].

4.4. Molecular modeling

The lead inhibitor, compound 26, was obtained through virtual screening in our previous work (unpublished). The protein structures of CA IX, and CA II were derived from PDB, with the PDB entries 3IAI and 3R16, respectively. Protein preparation was performed to optimize the protein structures using Glide module in Maestro 9.0 (Schrödinger Inc), and all the water molecules were removed. The grid-enclosing box was formed based on the ligand in the complex structure, and other parameters were set up by default. These structures of two compounds were' drawn using the

Builder tool and then optimized with Ligprep module in Maestro, both were docked into each protein mentioned above using Glide with standard precision (SP) approach, and a scaling factor of 1.0 was set to van der Waals (VDW) radii of those protein atoms with the partial atomic charges less than 0.25. For each protein, the best 20 binding poses of each compound ranked by GlideScore were remained for further inspection.

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