

Synthesis and biological evaluation of sulfonamide-based compounds as inhibitors of carbonic anhydrase from *Vibrio cholerae*

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Abstract

This study reports our continued efforts to identify inhibitors capable of targeting carbonic anhydrases (CAs) expressed in bacteria. Based on previously identified chemotypes, we designed and synthesized new analogs that were screened toward the α , β , and γ classes encoded in *Vibrio cholerae* (Vch). The K_i values measured in the stopped-flow hydrazide assay revealed that very simple structural modifications might induce a relevant impact on the inhibitory effects as well as the selectivity profile over ubiquitous human isozymes (hCA I/II). Unfortunately, the best active VchCA inhibitors demonstrated a dramatic loss of hCA II selectivity when compared to previously reported compounds. Among the new series of sulfonamides, several molecules proved to be about sevenfold more potent against VchCA γ than the reference compound acetazolamide, thus furnishing new insights for further development of inhibitors targeting CAs expressed in bacteria.

KEYWORDS

carbonic anhydrases, enzyme inhibitors, sulfonamides, *Vibrio cholerae*

1 | INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) play a pivotal role in balancing CO₂ concentration as well as its hydration in HCO₃⁻ and H⁺ species. It has been clearly understood that CAs regulate physiological processes in a large series of living organisms.^[1-7] Among them, CAs are considered crucial for the growth, survival, and pathogenicity of several bacteria involved in human diseases, such as *Brucella suis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and so on.^[8] It has been suggested that bacterial CAs are localized into the cytoplasm and periplasmic space regulating crucial functions. Specifically, CAs promote bacterial toxin generation and

adaptation in specific tissues on host organisms. Therefore, the inhibition of bacterial CA activity might offer therapeutic opportunities to develop antimicrobial agents.^[6,9-12]

CA activity catalyzes the production of bicarbonate, which is a relevant factor regulating the transcription of cholera toxin and other proteins linked to the colonization and pathogenicity of the Gram-negative bacterium *V. cholerae*, provoking the related gastro-intestinal disease cholera.

There are three classes of CAs α , β , and γ encoded by *V. cholerae*, indicated as VchCA α , VchCA β , and VchCA γ , characterized by different molecular structures. The α -CA class generally assumes an

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active monomeric or dimeric structure, whereas the β -CA class is active as dimeric, tetrameric, or octameric structures. Finally, the γ -CA class is characterized by an active trimeric organization. For VchCA α , VchCA β , and VchCA γ classes, the X-ray crystallography furnished structural information exclusively for VchCA β , whereas no crystal structures of VchCA α and VchCA γ proteins are available so far. As expected, VchCA β assumes a tetrameric structure with four active sites composed of two cysteines and one histidine residue (namely, Cys42, Cys101, and His98) coordinating a zinc ion. Moreover, the dyad composed of aspartate and arginine residues (Asp44 and Arg46) regulates the opening and closing of the catalytic site. This process is finely tuned by pH value related to bicarbonate concentration. The pH value higher than 8.0 generates the salt bridge between Arg46 and Asp44, thus generating the active form type-I, for which the zinc ion results coordinated by water/hydroxide ion, whereas the type-II form is characterized by the zinc coordination mediated by aspartate in place of water/hydroxide ion at pH lower than 8.0. Different from VchCA β , in the other two classes, VchCA α and VchCA γ , the zinc ion is classically coordinated by three histidine residues in combination with a fourth histidine, which assumes the well-known pivotal role of the proton shuttle, thus assisting the CO₂ hydration process.

To reduce the cholera illness and treat the spreading antimicrobial resistance, emerging therapeutic approaches recognize the inhibition of VchCAs as an intriguing opportunity to impair the cholera toxin expression as well as the bacterial adaption in the intestinal environment. To date, several CA inhibitors (CAIs) have been screened for VchCA α , VchCA β , and VchCA γ .^[13] Different chemotypes demonstrated efficacy in the nanomolar range and relevant selectivity over human carbonic anhydrases (hCAs) belonging to the α -class. Sulfonamides are the most investigated CAIs; in the deprotonated form the sulfonamide moiety acts as a metal binder against the zinc ion of VchCAs, thus preventing the catalytic cycle of CAs as demonstrated for acetazolamide (AAZ, **1**)

and ethoxzolamide (EZA, **2**) displayed in Figure 1.^[9,14,15] The abovementioned binding mode has been demonstrated by several co-crystal adducts of sulfonamides in complex with α -classes^[16–20]; moreover, docking simulations suggested the network of interactions for sulfonamides with VchCA α , VchCA β , and VchCA γ .^[21–23] Several benzoxaboroles^[24] (e.g., compound **3**, Figure 1) also proved to be potent inhibitors against VchCA γ over VchCA α , VchCA β , and hCA I/II isozymes (**3**). Finally, dithiocarbamates, coumarins, and carboxylic acids inhibited bacterial CAs through a distinct mode of interaction.^[25,26]

In our previous study, we have reported a computational approach to identify VchCA inhibitors culminating in the discovery of *N*-(4-sulfamoylbenzyl)biphenyl-4-carboxamide (Figure 1, compound **4**) as a small molecule capable of reducing CA activity of all three classes expressed in *V. cholerae* in the nanomolar range in stopped-flow hydrazase assay^[22] and marked selectivity over hCA I/hCA II isoforms. To collect information about its binding mode, we have also described its hypothetical orientation into the VchCA β cavity through docking simulations (Figure 2). As a result, we have visualized the canonical contacts displayed by sulfonamide-based CAIs for which R-SO₂NH₂ functionality is anchored to the zinc ion coordinated by Cys42, His98, and Cys101 residues, whereas the aromatic ring of the benzenesulfonamide fragment established π - π interaction with Tyr83. Notably, the -NH-group of the amide spacer appeared to make an H-bond interaction with the oxygen atom of the Gly102 backbone. The best affinity measured for this inhibitor (K_i = 96.5 nM) has been rationalized by the additional hydrophobic interactions with a cluster of aminoacidic residues Thr105, Ala106, Ile108, Pro111, and Ala139 that were localized in the sub-pocket beyond the middle portion of the CA cavity. These data were consistent with previous molecular/dynamic studies on various sulfonamides targeting VchCAs.^[27]

Based on the study mentioned above, we decided to extend here the structure–affinity relationships (SARs) introducing new moieties able to occupy hydrophobic/hydrophilic pocket subsites. Therefore,

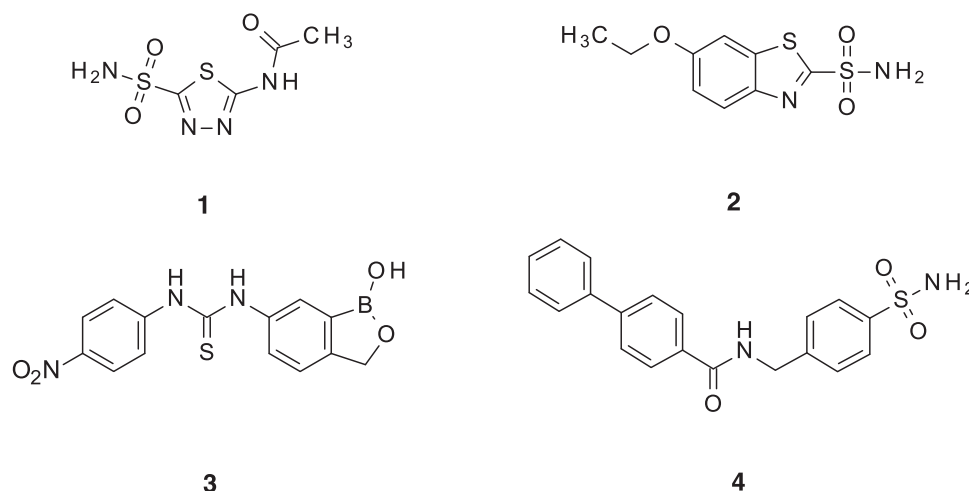


FIGURE 1 Chemical structures of well-known VchCA inhibitors: acetazolamide (AAZ, **1**), ethoxzolamide (EZA, **2**), 1-(1-hydroxy-3H-2,1-benzoxaborol-6-yl)-3-(4-nitrophenyl)thiourea (**3**), *N*-(4-sulfamoylbenzyl)-(1,1'-biphenyl)-4-carboxamide (**4**).

we first designed a small series of *N*-[(4-sulfamoylphenyl)methyl]carboxamides in which we investigated various hydrophobic tails having distinct electronic and/or steric features. To confirm that the hydrogen bond contact with Gly102 is necessary to increase the VchCA β affinity, we subsequently carried out a further structural modification by introducing an additional methylene bridge as a linking group between benzenesulfonamide moiety and amide function; thus, we designed a small series of corresponding *N*-[(4-sulfamoylphenyl)ethyl]carboxamides. Moreover, we extended our exploration focusing our interest on a different linking group that might offer the ability to establish additional H-bond interactions within the distinct VchCA cavities. This article describes the synthetic

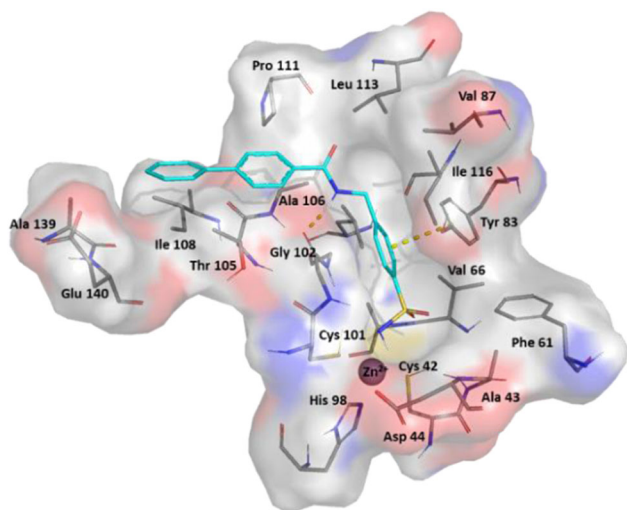


FIGURE 2 Proposed binding mode of the *N*-(4-sulfamoylbenzyl)-(1,1'-biphenyl)-4-carboxamide (**4**) docked into the VchCA β cavity.²² H-bond contact (Gly102) and π - π interaction (Tyr83) are represented by yellow dashed lines.

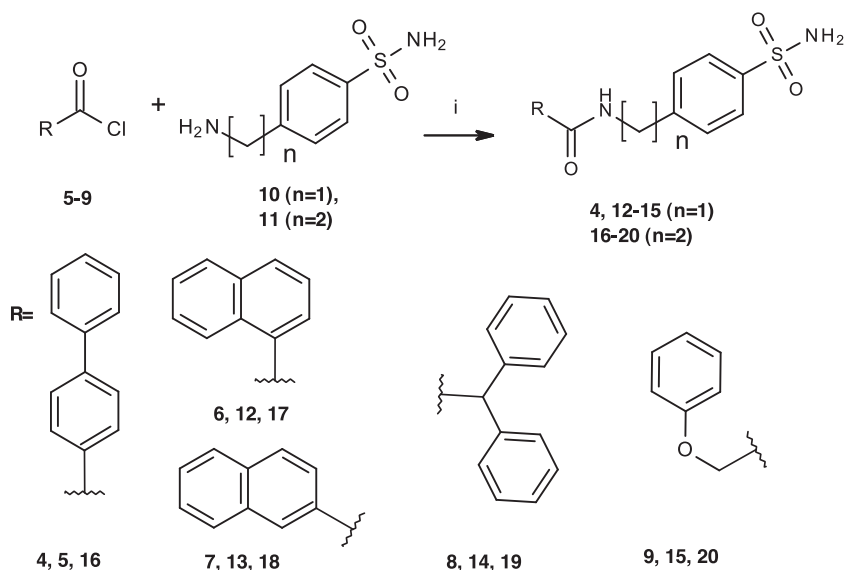
procedures and biochemical testing of this series of sulfonamide compounds for improving our knowledge of SAR of agents toward VchCAs.

2 | RESULTS AND DISCUSSION

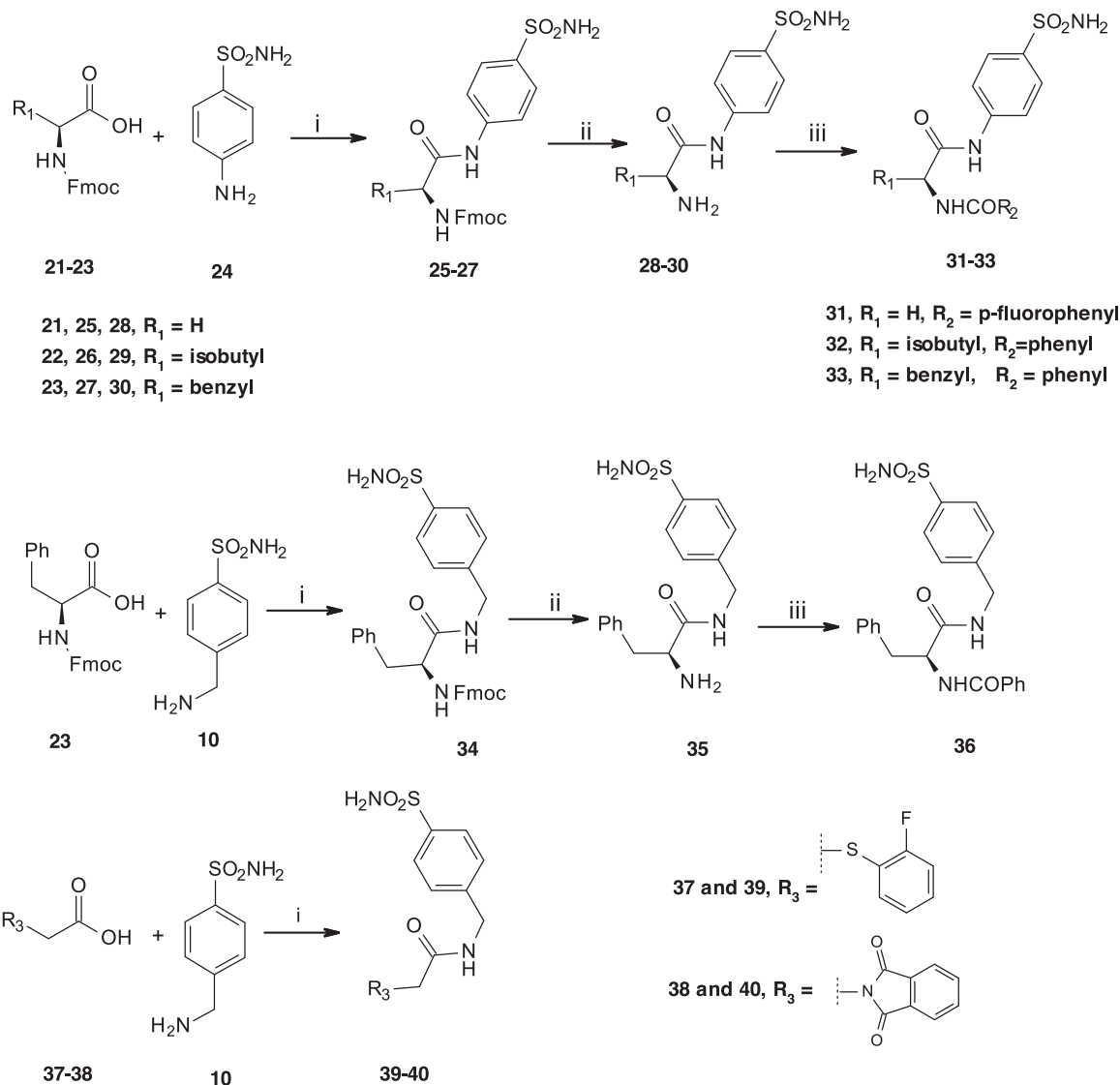
2.1 | Chemistry

In our optimization strategy for compound *N*-(4-sulfamoylbenzyl)-(1,1'-biphenyl)-4-carboxamide (**4**), we initially focused our attention on the effect of the substitution of the (1,1'-biphenyl)-4-carbonyl moiety with different hydrophobic fragments, including naphthyl, 2,2-diphenylmethane, 2-phenoxyethyl groups. Second, we synthesized an additional subset of homologous derivatives by a one-carbon atom homologation of the 4-(aminomethyl)benzenesulfonamide portion. As drawn in Scheme 1, the target compounds were easily obtained by a coupling reaction between the alkyl/aryl chlorides **5–9** and 4-(aminomethyl)benzenesulfonamide (**10**, $n = 1$) or 4-(aminoethyl)benzenesulfonamide (**11**, $n = 2$) in alkaline medium following a similar procedure employed for **4**.^[22]

Keeping in mind the crucial role of the amide functionality as an anchoring group within the backbone and a polar side chain of pivotal residues, in the third round of our SAR exploration, we moved our interest to a further set of analogs possessing an additional bond acceptor/donor moiety combined with aryl tail and/or alkyl branching groups. The synthetic route to obtain these sulfonamide-based compounds is shown in Scheme 2. The synthetic route started from the preparation of 4-aminobenzenesulfonamide-derived compounds **31–33**, which were obtained by coupling Fmoc-amino acids **21–23** with 4-aminobenzenesulfonamide **24** following a multistep procedure. In detail, the three F-moc protected amino acids glycine (**21**, $R_1 = H$), L-leucine (**22**, $R_1 = \text{isobutyl}$) or L-phenylalanine (**23**, $R_1 = \text{benzyl}$)



SCHEME 1 Reagents and conditions: (i) 4-(aminomethyl)benzenesulfonamide ($n = 1$) or 4-(aminoethyl)benzenesulfonamide ($n = 2$), DIPEA, DCM/DMF (2:1, v/v), rt, 2–6 h.



SCHEME 2 Synthesis of the target compounds **39** and **40**. Reagents and conditions: (i) HATU or HBTU, DIPEA, DMF, rt, overnight; (ii) 2% (v/v) DBU solution in DMF, rt, 20 min; (iii) DMF/DCM (50%, v/v), rt; 1 h.

reacted with **24** to furnish the corresponding three intermediates **25–27**, which were deprotected to give amines **28–30**. Finally, the amines **28–30** were coupled with selected aroyl chlorides to afford the target 4-aminobenzenesulfonamide-derived compounds **31–33**. For a comparison purpose, we also explored the replacement of 4-aminobenzenesulfonamide moiety with the 4-(aminomethyl)benzenesulfonamide one, thus preparing the homolog **36**, that contains an additional methylene linking group to respect parent compound **33**. The designed compound **36** was prepared following the previously described procedure via the formation of the Fmoc-protected **34** and subsequently amine **35**. Finally, the starting material 4-(aminomethyl)benzenesulfonamide (**10**) readily reacted with suitable acids **37** and **38** to give the target compounds **39** and **40** (Scheme 2) bearing the 2-fluorophenyl-sulfanyl and isindoline-dione tail, respectively. The chemical structures of synthesized compounds were confirmed by spectroscopic measurements and elemental analyses.

2.2 | CA inhibition

The inhibitory effects of the newly synthesized compounds were measured for VchCA α , - β , and - γ classes by means of a stopped-flow CO₂ hydrase assay as previously reported for prototype **4**.^[22] In Table 1, we collected the K_i values for target compounds **12–20**, **29**, **31–33**, **36**, and **39–40** as well as prototype **4** and acetazolamide (AAZ, **1**), which were used as reference compounds. The K_i values measured for the physiologically ubiquitous hCA I and hCA II were included for comparison purposes, thus leading to information on the selectivity profile over human isoforms. The following SAR consideration may be recovered.

Considering the VchCA α class, the first subset of studied compounds, sulfonamides **12–20** were generally effective inhibitors possessing K_i values in the range of 9.3–76.3 nM. In detail, the naphthyl derivatives **12** and **13** demonstrated the same potency as

TABLE 1 Inhibitory effects toward VchCA α , - β , and - γ classes for benzenesulfonamides **4**, **12–20**, **29**, **31–33**, **36**, **39–40**, and AAZ (**1**)

K_i (nM) ^a					
Compounds	hCA I	hCAII	VhCA α	VhCA β	VhCA γ
4 ^b	2113	919.7	11.6	95.6	174.6
12	138.7	9.7	9.3	938.5	349.4
13	794.5	54.8	9.4	2133	605.1
14	207.1	21.9	75.3	1900	422.8
15	89.9	8.2	23.3	902.4	285.9
16	8907	485.7	58.7	919.9	224.6
17	397.8	48.7	76.3	890.6	273.4
18	404.2	57.1	47.2	895.4	851.6
19	453.0	49.1	33.4	532.5	598.3
20	540.8	36.2	63.0	2567	400.0
29	86.9	5.4	323.5	237.0	770.9
31	162.7	3.1	9.2	311.6	70.0
32	4.7	2.5	690.2	642.4	87.7
33	45.0	2.8	9.7	691.8	65.0
36	510.7	216.5	8.6	511.0	737.1
39	537.4	316.3	8.8	172.1	631.4
40	714.5	8.1	8.6	93.0	488.3
AAZ (1)	250.0	12.1	6.8	451	473

^aMean from three different assays using a stopped-flow technique (errors were in the range of $\pm 5\%$ – 10% of the reported values).

^bMancuso et al.^[22]

parent compound **4**, whereas the diphenylmethyl derivative **14** turned out to be 6.5-fold less active than compound **4**; the introduction of the phenoxy moiety provided a slight reduction of affinity for compound **15** (K_i value of 23.3 nM). Compared to the *N*-[(4-sulfamoylphenyl)methyl]carboxamide series **4**, **12**, **13**, **15**, the homologous **16–18** and **20** demonstrated low inhibitory effects; on the contrary, the compound **19** was about twofold more potent than corresponding analog **14** (K_i values 33.4 vs. 75.3 nM). Interestingly, among the second subset of sulfonamides, compounds **31**, **33**, **36**, and **39–40** inhibited VchCA α class with K_i values equivalent to those of **4** and reference compound **1**, whereas sulfonamide-derivative **32** (R^1 = isobutyl, K_i value of 690.2 nM) and its amine precursor **29** (K_i value of 323.5 nM) demonstrated poor affinity toward VchCA α class. This 75-fold decrease in affinity for sulfonamide-derivative **32** (R^1 = isobutyl, K_i value of 690.2 nM) to respective unsubstituted analog **31** (R^1 = H, K_i value of 9.2 nM) reflected that the presence of a hydrophobic branched-chain was poorly tolerated; whereas the introduction of the aromatic moiety did not affect the VchCA α of sulfonamide-derivative **33** (R^1 = benzyl, K_i value of 9.7 nM) when compared to unsubstituted analog **31** (R^1 = H, K_i value of 9.2 nM).

All new studied compounds were generally poor inhibitors against VchCA β class, they demonstrated K_i values in the range of 93.0–2567 nM and revealed a flat SAR; the best active inhibitor was the 2-(1,3-dioxo-2,3-dihydro-1*H*-isindol-2-yl)-*N*-[(4-sulfamoylphenyl)methyl]acetamide (**40**, K_i value of 93.0 nM), suggesting that there are necessary uncommon and compulsory structural requirements for profitable recognition into VchCA β cavity. Taken together, these data suggested that performed structural modifications induced a significant loss of efficacy in affinity toward this β -class, except for sulfonamide **40**, which demonstrated comparable activity to respect compound **4** and higher than AAZ (**1**). Unfortunately, inhibitor **40** failed to display selectivity over hCA I/II when compared with compound **4**. Regarding the VchCA γ , for *N*-[(4-sulfamoylphenyl)methyl]carboxamide derivatives **12–15** and *N*-[(4-sulfamoylphenyl)ethyl]carboxamides **16–20**, the simple replacement of biphenyl moiety of **4** was generally detrimental for activity. As shown in Table 1, only compounds **16** and **17** displayed comparative activity with respect to sulfonamide **4**. A poor affinity was also found for sulfonamides **29**, **36**, **39–40**. Interestingly, the introduction of additional H-bond donor/acceptor functionalities associated with an appropriate length of the aminoacidic-derived linker resulted in the best VchCA γ inhibitors **31–33**, for which an increase in potency occurred, giving K_i values ranging from 65.0 to 87.7 nM, which resulted in better inhibitors to respect reference compounds **4** and **1**.

The studied compounds were generally poor inhibitors of hCA I with K_i values spanning from 45.0 to 794.5 nM concentration, with exception of compound **32** (K_i value of 4.7 nM); this result indicated that these hydrophobic substituents (R_1 = isobutyl and R_2 = phenyl) are favorable for interaction with hCA I cavity. With exception of compounds **16**, **36**, and **39**, the other sulfonamide-based compounds were active hCA II inhibitors; these data evidenced a flat SAR, thus suggesting that these structural modifications are generally well tolerated during the recognition process within the active site.

Taken together these results evidenced that among the first series of sixteen sulfonamides no compound resulted in a more potent and selective inhibitor when compared to the previously reported parent compound **4**. These data revealed that the introduction of several hydrophobic substituents in place of diphenyl moiety of prototype **4** was generally poorly tolerated or dramatically detrimental to VchCA affinity for the first series *N*-[(4-sulfamoylphenyl)ethyl]carboxamide derivatives **12–20**. Despite the small size of the additional methylene group, this modification dramatically reduced the VchCA β affinity, presumably due to the different ability to form the requested hydrogen bonding interaction with the carbonyl oxygen atom of the Gly102 backbone within the narrow VchCA β cavity. An enhanced VchCA γ affinity measured for sulfonamides **31–33** corroborated the hypothesis that the presence of additional H-bond donor/acceptor moieties was preferred for the recognition of the active site. Based on these SAR considerations, we can speculate that both hydrophobic interactions and polar contacts with the region of the entrance and the middle area of the VchCA cavity might play a crucial role in improving enzymatic α , β , γ -VchCA affinity and selectivity over human isoforms.

3 | CONCLUSION

In conclusion, a small series of sulfonamide-based compounds was designed and synthesized. By collecting the data on VchCA inhibition, we found that binding requirements for these classes of CAs are extremely mandatory to produce inhibitory effects and/or selectivity over hCA I/II isoforms. The collected SAR information could extend our knowledge of the hydrophobic as well as polar interactions controlling the recognition process into the cavity of the CAs encoded in *V. cholerae*.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

To carry out the synthesis of designed compounds, we employed reagents and solvents purchased from common commercial suppliers and they were used without further purification. A Buchi B-545 apparatus (BUCHI Labortechnik AG) was used to determine the melting points. The purity of synthesized compounds was detected by combustion analysis (C, H, N), which was carried out on a Carlo Erba Model 1106-Elemental Analyzer; the collected data confirmed a $\geq 96\%$ purity. Thin-layer chromatography (TLC) was employed to monitor the reaction progress by using glass-backed TLC silica gel 60G plates (0.25 mm) with fluorescence indicator F254. Therefore, all chromatograms were visualized by UV light ($\lambda = 254/366$ nm); in several cases, the reaction progress was monitored by staining with ninhydrin solution. Proton and carbon NMR spectra (see the Supporting Information) were measured in DMSO- d_6 with a Varian Gemini 500 spectrometer (Varian Inc.). The chemical shifts were quoted in δ (ppm); the coupling constants (J) were reported in hertz (Hz). The multiplicity was reported by using standard abbreviations: brs = broad singlet, d = doublet, m = multiplet, s = singlet, t = triplet. All exchangeable protons were confirmed by the addition of D₂O.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure for the synthesis of amides 12–20

To a stirring solution of 4-(2-aminomethyl)benzenesulfonamide hydrochloride **10** (280 mg, 1.25 mmol, 1 mol. Eq.) or 4-(2-aminoethyl)benzenesulfonamide **11** (250 mg, 1.25 mmol, 1 mol. Eq.) in DCM/DMF (6 ml, 2:1, v/v), *N,N*-diisopropylethylamine (DIPEA) (544 μ l, 3.13 mmol, 2.5 mol. Eq.) and the suitable acyl chloride **5–9** (1 mmol, 0.8 mol. Eq.) were added. The reaction was stirred at 25°C for a particular time and monitored by TLC in 10% MeOH in DCM. The disappearance of the starting material revealed the reaction completion, then the organic solvent was removed under a vacuum. The resulting residue was dissolved in H₂O (10 ml)

and extracted with EtOAc (3 \times 10 ml). The organic layers were collected and dried over Na₂SO₄, then the combined fractions were concentrated until dry under reduced pressure. The crude product was crystallized with a mixture of Et₂O and EtOH giving compounds **12–20** as powder. The ¹H-NMR spectra measured for compounds **12** and **14** were in good agreement with those reported in the literature.^[28,29]

N-[4-Sulfamoylphenyl)methyl]naphthalene-1-carboxamide (12)

Yield: 70%; mp: 211–212°C; ¹H-NMR (500 MHz, DMSO- d_6): (δ) 4.61 (d, $J = 6.0$ Hz, 2H, CH₂), 7.34 (bs, 2H, NH₂), 7.53–7.62 (m, 5H, Ar-H), 7.69 (d, $J = 6.98$ Hz, 1H, Ar-H), 7.83 (d, $J = 8.18$, 2H, Ar-H), 8.02 (d, $J = 8.04$, 1H, Ar-H), 8.17–8.24 (m, 1H, Ar-H), 9.18 (t, $J = 6.0$ Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO- d_6): 42.8 (CH₂), 125.5, 125.8, 126.3, 126.8, 127.3, 128.1, 128.8, 130.3, 130.5, 133.7, 124.8, 143.2, 144.2 (C4'), 169.2 (C=O); Anal. for C₁₈H₁₆N₂O₃S: C, 63.51%, H, 4.74%, N, 8.23%. Found: C, 63.48%; H, 4.78%; N, 8.20%.

N-[4-Sulfamoylphenyl)methyl]naphthalene-2-carboxamide (13)

Yield: 55%; mp: decomposition at 400°C; ¹H-NMR (500 MHz, DMSO- d_6): (δ) 4.58 (d, $J = 5.9$ Hz, 2H, CH₂), 7.33 (s, 2H, NH₂), 7.54 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.58–7.64 (m, 2H, Ar-H), 7.79 (d, $J = 8.7$ Hz, 2H, Ar-H), 7.96–8.05 (m, 4H, Ar-H), 8.55 (s, 1H, Ar-H), 9.45 (t, $J = 5.9$ Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO- d_6): 42.6 (CH₂), 124.4, 125.9, 126.9, 127.8, 128.1, 129.0, 131.6, 132.3, 134.3, 142.8, 144.0 (C4'), 166.5 (C=O); Anal. for C₁₈H₁₆N₂O₃S: C, 63.51%, H, 4.74%, N, 8.23%. Found: C, 63.48%; H, 4.77%; N, 8.27%.

2,2-Diphenyl-*N*-[4-sulfamoylphenyl)methyl]acetamide (14)

Yield: 72%; mp: 196–197°C; ¹H-NMR (500 MHz, DMSO- d_6): (δ) 4.37 (d, $J = 5.8$ Hz, 2H, CH₂), 5.03 (s, 1H, CH), 7.22–7.29 (m, 10H, Ar-H), 7.30 (s, 2H, NH₂), 7.38 (d, $J = 8.3$, 2H, Ar-H), 7.73 (d, $J = 8.3$, 2H, Ar-H), 8.87 (t, $J = 5.8$ Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO- d_6): 42.2 (CH₂), 56.8 (CH), 125.9, 127.0, 127.7, 128.5, 128.8, 140.5, 143.7 (C4'), 171.5 (C=O); Anal. for C₂₁H₂₀N₂O₃S: C, 66.30%, H, 5.30%, N, 7.36%. Found: C, 66.26%; H, 5.31%; N, 7.38%.

2-Phenoxy-*N*-[4-sulfamoylphenyl)methyl]acetamide (15)

Yield: 65%; mp: 178–179°C; ¹H-NMR (500 MHz, DMSO- d_6): (δ) 4.41 (d, $J = 5.9$ Hz, 2H, NHCH₂), 4.57 (s, 2H, OCH₂), 6.95–7.05 (m, 3H, Ar-H), 7.28–7.37 (m, 4H, Ar-H and NH₂), 7.40 (d, $J = 8.06$ Hz, 2H, Ar-H), 7.75 (d, $J = 8.06$ Hz, 2H, Ar-H), 8.74 (t, $J = 5.9$ Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO- d_6): 41.95 (NHCH₂), 67.29 (OCH₂), 115.11, 121.62, 125.98, 127.83, 129.85, 142.95, 143.75 (C4'), 157.99 (C1'', phenoxy), 168.40 (C=O); Anal. for C₁₅H₁₆N₂O₄S: C, 56.24%, H, 5.03%, N, 8.74%. Found: C, 56.27%; H, 5.10%; N, 8.70%.

N-[2-(4-Sulfamoylphenyl)ethyl]-(1,1'-biphenyl)-4-carboxamide (16)

Yield: 80%; mp: 294–295°C; ¹H-NMR (500 MHz, DMSO- d_6): (δ) 2.95 (t, $J = 7.1$ Hz, 2H, CH₂), 3.50–3.58 (dd, $J_1 = 5.4$ Hz, $J_2 = 7.1$ Hz, 2H, CH₂), 7.29 (s, 2H, NH₂), 7.38–7.52 (m, 5H, Ar-H), 7.68–7.79 (m, 6H, Ar-H), 7.91 (d, $J = 8.2$ Hz, 2H, Ar-H), 8.63 (t, $J = 5.4$ Hz, 1H, NH);

¹³C-NMR (126 MHz, DMSO-*d*₆): 34.8 (CH₂), 40.5 (NHCH₂), 126.2, 127.0, 127.3, 128.3, 128.5, 129.6, 133.8, 139.7, 142.5, 143.1, 144.3 (C4'), 166.3 (C=O); Anal. for C₂₁H₂₀N₂O₃S: C, 66.30%, H, 5.30%, N, 7.36%. Found: C, 66.26%; H, 5.31%; N, 7.40%.

N-[2-(4-Sulfamoylphenyl)ethyl]naphthalene-1-carboxamide (17)

Yield: 80%; mp: 211–212°C; ¹H-NMR (500 MHz, DMSO-*d*₆): (δ) 2.98 (t, *J* = 6.9 Hz, 2H, CH₂), 3.60–3.64 (dd, *J*₁ = 5.6 Hz, *J*₂ = 6.9 Hz, 2H, CH₂), 7.32 (s, 2H, NH₂), 7.49–7.54 (m, 6H, Ar-H), 7.78 (d, *J* = 8.12 Hz, 2H, Ar-H), 7.94–7.99 (m, 3H, Ar-H), 8.61 (t, *J* = 5.6 Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO-*d*₆): 34.5 (CH₂), 40.6 (NHCH₂), 124.7, 125.2, 125.5, 126.1, 126.5, 128.0, 129.1, 129.5, 132.8, 132.8, 142.0, 143.6 (C4'), 168.4 (C=O); Anal. for C₁₉H₁₈N₂O₃S: C, 64.39%, H, 5.12%, N, 7.90%. Found: C, 64.35%; H, 5.08%; N, 7.86%.

N-[2-(4-Sulfamoylphenyl)ethyl]naphthalene-2-carboxamide (18)

Yield: 90%; mp: 239–240°C; ¹H-NMR (500 MHz, DMSO-*d*₆): (δ) 2.97 (t, *J* = 7.2 Hz, 2H, CH₂), 3.54–3.61 (dd, *J*₁ = 5.5 Hz, *J*₂ = 7.2 Hz, 2H, CH₂), 7.28 (s, 2H, NH₂), 7.45 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.55–7.63 (m, 2H, Ar-H), 7.75 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.90 (d, 1H, Ar-H), 7.94–8.03 (m, 3H, Ar-H), 8.41 (s, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO-*d*₆): 35.4 (CH₂), 40.8 (NHCH₂), 124.6, 126.2, 127.2, 127.8, 128.0, 128.1, 128.3, 129.3, 129.6, 132.3, 132.6, 134.6, 142.5, 144.6 (C4'), 166.7 (C=O); Anal. for C₁₉H₁₈N₂O₃S: C, 64.39%, H, 5.12%, N, 7.90%. Found: C, 64.37%; H, 5.09%; N, 7.94%.

2,2-Diphenyl-*N*-[2-(4-sulfamoylphenyl)ethyl]acetamide (19)

Yield: 75%; m.p.: 212–214°C; ¹H-NMR (500 MHz, DMSO-*d*₆): (δ) 2.79 (t, *J* = 6.9 Hz, 2H, CH₂), 3.35 (m, 2H, CH₂), 4.90 (s, 1H, CH), 7.21–7.31 (m, 14H, NH₂, Ar-H), 7.68 (d, *J* = 8.0 Hz, 2H, Ar-H), 8.35 (t, *J* = 5.1 Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO-*d*₆): 34.4 (CH₂), 56.6 (NHCH₂), 125.7, 126.6, 128.2, 128.6, 129.2, 140.4, 142.1, 143.7 (C4'), 171.1 (C=O); Anal. for C₂₂H₂₂N₂O₃S: C, 66.98%, H, 5.62%, N, 7.10%. Found: C, 66.94%; H, 5.66%; N, 7.08%.

2-Phenoxy-*N*-[2-(4-sulfamoylphenyl)ethyl]acetamide (20)

Yield: 85%; mp: 177–178°C; ¹H-NMR (500 MHz, DMSO-*d*₆): (δ) 2.83 (t, *J* = 7.2 Hz, 2H, CH₂), 3.39–3.41 (dd, *J*₁ = 5.4 Hz, *J*₂ = 7.2 Hz, 2H, CH₂), 4.49 (s, 2H, OCH₂), 6.92–6.99 (m, 3H, Ar-H), 7.29–7.32 (m, 4H, Ar-H and NH₂), 7.37 (d, *J* = 8.05 Hz, 2H, Ar-H), 7.73 (d, *J* = 8.05 Hz, 2H, Ar-H), 8.18 (t, *J* = 5.4 Hz, 1H, NH); ¹³C-NMR (126 MHz, DMSO-*d*₆): 35.7 (CH₂), 39.8, (NHCH₂), 67.0 (OCH₂), 115.6, 122.1, 126.6, 130.0, 130.4, 143.0, 144.4 (C4'), 158.6 (C1'', phenoxy), 168.6 (C=O); Anal. for C₁₆H₁₈N₂O₄S: C, 57.47%, H, 5.43%, N, 8.38%. Found: C, 57.51%; H, 5.45%; N, 8.34%.

4.1.3 | General procedure for the synthesis of benzamides 31–33 and 36

To a solution of *N*-α-Fmoc-protected amino acids (1 mmol, 1 mol. Eq.) (21–23) in dry dimethylformamide (DMF) (2 ml), 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium-

3-oxide-hexafluorophosphate (HATU) (441 mg, 1.16 mmol, 1 mol. Eq.) was added in different time at 0°C, then the mixture was vigorously stirred at 450 rpm. Then *N,N*-diisopropylethylamine (DIPEA) (505 μl, 2.9 mmol, 2.5 mol. Eq.) and *p*-aminobenzenesulfonamide (24) (250 mg, 1.45 mmol, 1.25 mol. Eq.) or 4-(2-aminomethyl)benzenesulfonamide hydrochloride (10) (332 mg, 1.45 mmol, 1.25 mol. Eq) were added. The reaction mixture was vigorously stirred at room temperature overnight. Water (10 ml) was added, and the mixture was extracted with EtOAc (3 × 10 ml). The organic phase was washed with acidic water (pH = 4–5), dried with Na₂SO₄, and concentrated until dry under reduced pressure. The crude product was sonicated with Et₂O to give the intermediates 25–27 and 34 as a white powder, which was used for the next reaction step without further purification. The *N*-α-Fmoc-protected intermediates 25–27 and 34 were treated with 2% (v/v) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dimethylformamide (DMF, 2.5 ml). The reaction mixture was stirred at room temperature for 20 min. After the starting material has disappeared, the crude product was concentrated in vacuo and then subjected to sonication with an *n*-hexane (Hex)/dichloromethane (DCM) solution (50%, v/v) to provide the free amines 28–30 and 35 as a white powder. The structural characterization of amines has been already reported by other authors.^[30–32] To a stirred solution of 28–30 or 36 (1 mol. Eq.) in DMF/DCM (50%, v/v), the suitable aryl chloride (0.8 mol. Eq.) was added and the reaction mixture was placed in ice bath. The resulted mixture was stirred at room temperature for 1 h; then the organic solvent was evaporated in vacuo, thus giving a residue that was purified by flash chromatography (eluting with 0–20% v/v of MeOH in DCM). The final crude product was sonicated with petroleum ether/ethanol (50%, v/v) to obtain the desired compounds 31–33 and 36 as a white powder.

4-Fluoro-*N*-[2-oxo-2-[(4-sulfamoylphenyl)amino]ethyl]-benzamide (31)

Yield: 33%; m.p.: 269–271°C; ¹H-NMR (DMSO-*d*₆): (δ) 4.10 (d, *J* = 5.87 Hz, 2H, CH₂), 7.26 (bs, 2H, NH₂), 7.53–7.96 (m, 6H, ArH), 9.04 (t, *J* = 5.87 Hz, 1H, NH), 10.42 (s, 1H, NH). Anal. for C₁₅H₁₄FN₃O₄S: C, 51.28%; H, 4.02%; N, 11.96%; Found: C, 51.24%; H, 3.96%; N, 11.92%.

N-[1-Oxo-3-phenyl-1-[(4-sulfamoylphenyl)amino]propan-2-yl]benzamide (32)

Yield: 32%; m.p.: 163–165°C; ¹H-NMR (DMSO-*d*₆): 2.89–3.15 (m, 2H, CH₂), 4.83–4.89 (m, 1H, CH), 7.16–7.31 (m, CH, ArH), 7.41–7.55 (m, 5H, ArH), 7.77 (bs, 2H, NH₂), 7.79–7.84 (m, 3H, ArH), 7.95–7.98 (m, 1H, ArH), 8.81 (d, *J* = 7.68 Hz, 1H, NH), 10.56 (s, 1H, NH). ¹³C-NMR (126 MHz, DMSO-*d*₆): 37.58 (CH₂), 56.53 (CH), 112.8, 119.4, 120.3, 126.9, 127.2, 127.9, 128.0, 128.6, 128.7, 129.6, 130.5, 131.9, 134.2, 138.4, 139.0, 142.2, 152.4, 167.1 (C=O), 171.5 (C=O). Anal. for C₂₂H₂₁N₃O₄S: C, 62.40%; H, 5.00%; N, 9.92%. Found: C, 62.23%; H, 5.13%; N, 9.83%.

N-[4-Methyl-1-oxo-1-[(4-sulfamoylphenyl)amino]pentan-2-yl]benzamide (33)

Yield: 48%; m.p.: 179–180°C; ¹H-NMR (500 MHz, DMSO-*d*₆): (δ) 0.91–0.96 (m, 6H, CH₃), 1.54–1.60 (m, 1H, CH), 1.72–1.86 (m, 2H, CH₂), 4.63–4.69 (m, 1H, CH), 7.25 (bs, 2H, NH₂), 7.45–7.56 (m, 3H, ArH), 7.74–7.80 (m, 4H, ArH), 7.90–7.92 (m, 2H, ArH), 8.63–8.68 (m, 1H, NH), 10.46 (bs, 1H, NH). ¹³C-NMR (126 MHz, DMSO-*d*₆): 21.9, 23.5, 25.0, 53.3, 119.3, 127.1, 128.1, 128.7, 131.9, 134.4, 138.9, 142.4, 167.1 (C=O), 172.5 (C=O). Anal. for C₁₉H₂₃N₃O₄S: C, 58.59%; H, 5.95%; N, 10.79%. Found: C, 58.48%; H, 5.99%; N, 10.83%.

N-[1-Oxo-3-phenyl-1-[(4-sulfamoylbenzyl)amino]propan-2-yl]benzamide (36)

Yield: 67%; m.p.: 229–230°C; ¹H-NMR (500 MHz, DMSO-*d*₆): 3.01–3.16 (2H, CH₂), 4.35–4.37 (2H, CH₂), 4.71–4.75 (1H, CH), 7.15–7.18 (m, 1H, ArH), 7.24–7.27 (m, 2H, ArH), 7.29 (bs, 2H, NH₂), 7.32–7.36 (m, 4H, ArH), 7.42–7.53 (m, 3H, ArH), 7.72 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.81 (d, *J* = 8.5 Hz, 2H, Ar-H), 8.63 (d, *J* = 8.4 Hz, 1H, NH), 8.67 (d, *J* = 6.0 Hz, 1H, NH). ¹³C-NMR (126 MHz, DMSO-*d*₆): 37.6 (CHCH₂Ph), 42.3 (NHCH₂Ph), 55.6 (CH), 126.0, 126.7, 127.8, 127.9, 128.5, 128.6, 129.6, 131.7, 134.5 (C=O), 138.8, 143.0, 143.9, 166.8 (C=O), 171.9 (C=O). Anal. for C₂₃H₂₃N₃O₄S: C, 63.14%; H, 5.30%; N, 9.60%; Found: C, 63.28%; H, 5.18%; N, 9.72%.

4.1.4 | General procedure for the synthesis of acetamides 39 and 40

To a solution of *N*-phthaloylglycine (37) (205 mg, 1 mmol, 1 mol. Eq.) or [(2-fluorophenyl)sulfanyl]acetic acid (38) (186 mg, 1 mmol, 1 mol. Eq.) in *N,N*-dimethylformamide (DMF) (2 ml), *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uranylhexafluorophosphate (HBTU) (379 mg, 1 mmol, 1 mol. Eq.) was added. The mixture was stirred at room temperature for 15 min. Then DIPEA (505 μl, 2.9 mmol, 2.5 mol. Eq.) and 4-aminomethylbenzenesulfonamide hydrochloride 10 (223 mg, 1 mmol, 1 mol. Eq.) were added. The reaction mixture was left overnight at room temperature and then quenched with H₂O (10 ml) and extracted with EtOAc (3 × 10 ml). The organic phase was washed with saturated NaCl solution, dried over Na₂SO₄, and concentrated until dryness under reduced pressure. The crude was purified by crystallization from EtOH and furnished the desired final compounds 39 and 40 as white powder.

2-[(2-Fluorophenyl)sulfanyl]-*N*-[(4-sulfamoylphenyl)methyl]-acetamide (39)

Yield: 58%; m.p. 196–198°C; ¹H-NMR (500 MHz, DMSO-*d*₆): 3.74 (s, 2H, CH₂), 4.36 (d, *J* = 6.0 Hz, 2H, CH₂), 7.16–7.25 (m, 2H, ArH), 7.27–7.30 (m, 3H, ArH), 7.31 (s, 2H, NH₂), 7.43–7.49 (m, 1H, ArH), 7.71 (d, *J* = 8.9 Hz, 2H, ArH), 8.72 (t, *J* = 6.0 Hz, 1H, NH). ¹³C-NMR (126 MHz, DMSO-*d*₆): 35.9 (SCH₂C=O), 42.5 (NHCH₂Ph), 115.9, 123.0, 125.5, 128.8, 130.9, 143.1, 143.6, 156.23, 161.16 (C=O), 168.2 (C=O). Anal. for C₁₅H₁₅FN₂O₃S: C, 55.89%; H, 4.69%; N, 8.69%; Found: C, 55.91%; H, 4.38%; N, 8.55%.

2-(1,3-Dioxoisindolin-2-yl)-*N*-(4-sulfamoylbenzyl)acetamide (40)

Yield: 78%; m.p. 174–175°C; ¹H-NMR (500 MHz, DMSO-*d*₆): 4.28 (s, 2H, CH₂), 4.36 (d, *J* = 5.9 Hz, 2H, CH₂), 7.31 (s, 2H, NH₂), 7.42 (d, *J* = 8.2 Hz, 2H, ArH), 7.78 (d, *J* = 8.2 Hz, 2H, ArH), 7.83–7.85 (m, 2H, ArH), 7.89–7.91 (m, 2H, ArH), 8.84 (t, *J* = 5.9 Hz, 1H, NH). ¹³C-NMR (126 MHz, DMSO-*d*₆): 40.7 (NHCH₂Ph), 42.4 (NCH₂CO), 123.6, 126.2, 127.9, 132.2, 135.0, 143.1, 143.6, 166.9 (C=O), 168.0 (C=O). Anal. for C₁₇H₁₅N₃O₅S: C, 54.69%; H, 4.05%; N, 11.25%; Found: C, 54.76%; H, 3.98%; N, 11.47%.

4.2 | Biological assay

4.2.1 | Preparation of the CAs from bacteria

The α, β, and γ- CAs were obtained accordingly to the previously reported by our groups.^[33] The GeneArt Company (Invitrogen), which specialized in gene synthesis, designed the genes encoding for the bacterial α, β, and γ- CAs. The BL21 DE3 competent cells (Agilent) were transformed with the expression vector pET15-b containing the gene encoding for one of the three CA-classes. Then bacterial cells were induced with 1 mM IPTG and, after 30 min, treated with 0.1 M ZnCl₂. After 4 h, cells were harvested and disrupted by sonication at 4°C. After centrifugation at 12,000g for 45 min, the supernatant was incubated with His Select HF nickel affinity gel resin (Sigma) equilibrated in lysis buffer for 30 min. The protein was eluted with the wash buffer containing 200 mM imidazole. Collected fractions were dialyzed against 50 mM Tris/HCl, pH 8. At this stage of purification, the protein was at least 95% pure.

4.2.2 | CA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO₂ hydrase activity. Phenol red (0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH = 7.5) or Tris (pH = 8.3) as buffers, and 20 mM Na₂SO₄ or 20 mM NaClO₄ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM to determine the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5%–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of tested inhibitor (10 mM) were prepared in distilled-deionized water, then dilutions up to 0.01 nM were done with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature before assay, to allow for the formation of the E-I complex. The inhibition constants (K_i) were obtained by nonlinear least-squares methods using PRISM 3 and represent the mean from at least three different determinations. CA isoforms were recombinant ones, as reported earlier by this group.^[34]

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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