Benzoxaboroles: New Potent Inhibitors of the Carbonic Anhydrases of the Pathogenic Bacterium *Vibrio cholerae*

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ABSTRACT: A series of urea/thiourea substituted benzoxaboroles was investigated for the inhibition of the three carbonic anhydrases encoded by *Vibrio cholerae* (VchCA α , VchCA β , and VchCA γ). In particular, benzoxaborole derivatives were here first assayed for the inhibition of a γ -class CA, extending the panel of CA classes that benzoxaboroles efficiently target beyond α and β . Inhibition profiles demonstrated that VchCA α was significantly more inhibited compared to VchCA γ and, in turn, more efficiently modulated than VchCA β . Among the many selective benzoxaborole ligands detected against VchCA α over the off-target hCA II, compound 18, a *p*-NO₂-phenylthiourea derivative, even exhibited a fully selective inhibition profile against the three VchCAs over hCA II. A comprehensive ligand/target interaction study was performed *in silico* for all three VchCA isoforms providing the first molecular modeling investigation with inhibitors of a γ -class CA to the best of our knowledge. The present study reinforces the rationale behind the use of benzoxaboroles as innovative antibacterial agents with a new mechanism of action, furnishing suggestions for the rational design of new potent and selective inhibitors targeting *V. cholerae* CAs over human off-target ones.

KEYWORDS: Carbonic anhydrase, inhibitors, benzoxaborole, *Vibrio cholerae*, *in silico* study

Cholera is a highly contagious acute diarrheal disease due to a Gram-negative bacillus, *Vibrio cholerae*. Morbidity and mortality associated with this disease remain a significant problem in the developing world. Even if the prognosis is good with appropriate treatment, with a mortality rate of <0.2%, without treatment, the mortality rate can be as high as 50–70%.¹ The number of worldwide deaths due to the infection is estimated between 21 000 and 143 000.² Among the *Vibrio cholerae* species, we can distinguish two toxigenic strains belonging to the serogroups O1 or O139 responsible for cholera.^{1,3} The treatment of this disease involves immediate rehydration (preferably) with oral rehydration solutions containing salt and glucose or with rice-based rehydration solutions and/or isotonic solutions intravenously to compensate for the massive hydro-electrolytic losses observed in individual patients. The adjunctive treatment is based on the oral administration of antibiotics such as tetracyclines, fluoroquinolones, and macrolides. Besides, this procedure is beneficial in moderate to severe cases. Two oral inactivated cholera vaccines are available, WC/rBS (licensed in the USA) and BivWC (used in endemic areas), inoculated in 2 or 3 doses and providing 60–85% protection for 2–3 years.^{1–4} However, there are strains of *V. cholerae* resistant to tetracycline, and sometimes polyresistant to various antibiotics.^{5–7}

To assist the colonization of human hosts, *V. cholerae* secretes virulence factors, among which the main is the cholera toxin (CT). It is now established that the production of cholera toxin is essential for the prospect of acquiring the potential to cause epidemics by a serogroup. This has become particularly evident since the emergence of serogroup O139. The effects of cholera toxin are massive diarrhea, but the microorganism must first have escaped many of the host's nonspecific defense mechanisms to colonize the small intestine successfully. Therefore, enterotoxigenic strains of *V. cholerae* must survive the acidity of the stomach, colonize the intestine, and excrete sufficient cholera toxin to produce the diarrheal response associated with cholera.^{6–8} New treatment therapies that target virulence genes, toxin production, and colonization by *V. cholerae*, either alone or in combination with current therapies, could be beneficial in reducing the global health

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Figure 1. Ribbon representation of (A) the tetrameric type II VchCA β (pdb 5CXK) [13]; (B) α -CA from *H. pylori* as monomer in complex with the CAI acetazolamide (pdb 4YGF);²⁰ (C) active trimer of the γ -CA from *T. thermophilus* (pdb 6IVE) in adduct with phosphate.²¹

burden caused by this pathogen, as alternatives to antibiotics become increasingly needed.^{5,6}

One of the key physiological reactions for the life cycle of most organisms, including bacteria, is the hydration/dehydration of CO₂. This critical reaction is connected with numerous metabolic pathways, such as the biosynthetic processes requiring CO₂ or HCO₃[−] and biochemical pathways, including pH homeostasis, secretion of electrolytes, and transport of CO₂ and bicarbonate among others.⁹ The reversible hydration of CO₂ is catalyzed by essential enzymes carbonic anhydrases (CAs), which belong to a superfamily of zinc metalloenzymes. *Vibrio cholerae* encodes carbonic anhydrases belonging to α -CA, β -CA, and γ -CA classes (VchCA α , VchCA β , and VchCA γ)^{10–15} that play an important role in the supplementation of CO₂ and bicarbonate for the bacterial cellular metabolism and pH homeostasis.^{16–18} These three CAs could represent potential targets for the development of anti-infectives targeting *V. cholerae* colonization.¹⁹ The 3D structure of VchCA β was solved by X-ray crystallography in 2015 revealing a tetrameric type II β -CA (Figure 1A).¹³ Structural information was not collected to date for VchCA α and VchCA γ . In contrast, the structures of α - and γ -class CAs from other bacteria, such as *Helicobacter pylori* or *Thermus thermophilus*, were reported^{20,21} and depicted in Figure 1B–C.

In our current research studies, searching for new chemotypes for the design of carbonic anhydrase, we develop new inhibitors in the benzoxaborole series, which demonstrated very potent inhibitory activity against human CAs isoforms as well as fungi and parasites CAs.^{22–27} Therefore, the purpose of our work is to provide new information about the potential of benzoxaboroles as potential inhibitors of the three CAs-classes of *Vibrio cholerae*, with a main focus on VchCA γ , as no benzoxaborole derivative has been assayed to date for this class of CAs. Additionally, a thorough interaction study *in silico* is here reported with benzoxaborole derivatives and the three VchCA isoforms. To the best of our knowledge, we described here the first molecular modeling investigation with inhibitors of a γ -class CA.

Inhibition data of benzoxaboroles 1–23 against VchCAs of α -, β -, and γ -classes were measured by a stopped flow CO₂ hydase assay²⁸ and are shown in Table 1. Acetazolamide (AAZ) a clinically used sulfonamide inhibitor, was used as standard. Tavaborole (TVB), a benzoxaborole commercially used as topical antifungal medication, was included in the inhibition assay (Figure 2).

Inhibitory profiles were displayed as inhibition constants (K_i s) in comparison with those against the main physiological human isoform hCA II, off-target in this study (Table 1). The

Table 1. Inhibition Data of hCA II, VchCA α , VchCA β , and VchCA γ with Benzoxaboroles 1–23, TVB, and the Standard Sulfonamide Inhibitor AAZ by a Stopped Flow CO₂ Hydase Assay²⁸

Cpd	X	R	K_i (nM) ^a			
			VchCA α	VchCA β	VchCA γ	hCA II
1			319	70313	2837	8180
2			142	8866	818	504
3			165	21830	1168	590
4	O	CH ₂ Ph	90	42143	1874	439
5	O	CH ₂ -(3-Cl,5-CH ₃ -Ph)	53	61318	718	276
6	O	Ph	117	56351	705	730
7	O	4-Cl-Ph	56	19532	650	707
8	O	CH ₂ -fur-2-yl	164	79946	1229	841
9	O	4-F-Ph	48	28046	644	480
10	O	4-CF ₃ -Ph	46	55124	957	456
11	O	2,4,6-Cl-Ph	76	84459	895	272
12	O	2-OCH ₃ ,5-CH ₃ -Ph	556	67286	410	89
13	O	4-COCH ₃ -Ph	103	65084	1128	797
14	S	CH ₂ CH ₂ Ph	102	18372	554	1547
15	S	4-CH ₃ -Ph	109	2586	1762	1253
16	S	napht-2-yl	603	25994	2974	1148
17	S	4-OCH ₃ -Ph	165	2297	874	1250
18	S	4-NO ₂ -Ph	80	933	687	>10 ⁴
19	S	CH ₂ Ph	96	30408	3955	1305
20	S	4-F-Ph	112	6350	544	1500
21	S	CH ₂ -fur-2-yl	220	31939	1996	2230
22	S	4-CF ₃ -Ph	72	1253	1919	1838
23	S	Ph	1204	19128	834	1625
TVB			157	97278	841	462
AAZ			6.8	451	473	12

^aMean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5 –10% of the reported values).

Figure 2. Structure of benzoxaboroles evaluated against VchCAs and standard CAI.

following structure activity relationship can be worked out on the basis of data from Table 1.

Significantly different inhibition profiles were measured with benzoxaborole 1–23 and tavorole against VchCA isoforms α (K_i s in a medium to high nM range), β (K_i s in a medium to high μ M range), and γ (K_i s in a high nM to low μ M range). In detail, the CO₂ hydration activity of VchCA α was the most affected by the tested benzoxaboroles derivatives that showed K_i s in the range 46–603 nM. Compound 23 only acted as a micromolar inhibitor (K_i of 1.2 μ M). This makes VchCA α even more inhibited by these benzoxaboroles than hCA II, the physiologically most relevant among hCAs (K_i s ranging from 89 nM to 10 μ M). The most effective VchCA α inhibitors were the halogenated ureido compounds 5, 7, and 9–11 with K_i s spanning between 46 and 76 nM. Among the thioureido inhibitors, the trifluoromethylphenyl derivative 22 also showed an inhibitory activity of 72 nM against this α -CA, while the other halo-compound 20 displayed a K_i above 100 nM. Among thioureas, a comparable K_i was shown by the nitrophenyl derivate 18 (K_i of 80 nM). The detachment of the aryl ring from the (thio)ureido linker by a 1 or 2 carbon units spacer produced a slight enhancement in the inhibitory action against VchCA α . This occurs with ureas 4 and 6 (K_i s of 90 and 117 nM, respectively), and it is even more evident with thioureas 14, 19, and 23 (K_i s of 102, 96, and 1204 nM, respectively). The 10-fold decrease of CAI efficacy measured swapping the oxygen atom of 6 to a sulfur, as in 23, suggests a loss of favorable ligand/target interactions or clashes induced by the S atom. The addition of substituents to the ring or, as aforesaid, its separation from the thioureido spacer, appears to restore favorable binding contacts to the target and thus K_i s in a medium nanomolar range. It should also be stressed that several (thio)ureido substitutions (e.g., in 6, 8, 12, 13–16, 20, 21, and 23) did not produce a relevant breakthrough in the binding to VchCA α with respect to the simple benzoxaborole 1, its 6-NO₂ (2), and 6-NH₂ (3) derivatives and tavorole that shows K_i s between 142 and 319 nM.

Intriguingly, VchCA γ was markedly more inhibited than VchCA β with K_i s in the range 410–3955 nM. This is a relevant result as VchCA γ is the first γ -CA ever tested for inhibition by benzoxaborole derivatives. This widens the set of CA classes potentially affected by such a CAI scaffold and, as a result the chorus of pathogens can be hit pharmacologically with benzoxaboroles (i.e., those encoding for γ -class CAs). The SAR against VchCA γ significantly differs from that illustrated for VchCA α . A rather flat inhibition trend was observed on the whole, with most inhibitors displaying K_i values in the narrow span 544–1996 nM. The ureido derivative 12 harboring a 2-OCH₃, 5-CH₃-phenyl group solely went below 500 nM as far as the K_i value against VchCA γ is concerned. On the other hand, the simple benzoxaborole 1 and thioureas 16 and 19 only exhibited K_i s against this isozyme above 2.5 μ M (2.8, 2.9, and 3.9 μ M, respectively). In other words, most derivatization of the benzoxaborole scaffold of 1 increased VchCA γ inhibition, solely except for a naphthyl (16) or benzyl (19) tail within the thioureas subset. Of note, again the spacing of the outer aromatic ring by the linker had a detrimental effect on VchCA γ inhibition within the urea subset (K_i more than doubled from 6 to 4) and partially in the thiourea subset (K_i almost increased 5-fold from 23 to 19), as the further elongation of the linker up to 2-carbon atoms (14) brought back the K_i to a medium nanomolar value (554 nM).

VchCA β was the least inhibited by benzoxaboroles 1–23 among the isoforms encoded by *V. cholerae*. In fact, most inhibitors displayed K_i s spanning in the range 6350–84459 nM. Thioureas 18 and 22 were the two best VchCA β inhibitors, solely reporting K_i s approaching 1 μ M (933 and 1253 nM, respectively). Right after in terms of inhibition potency are again two thioureido compounds, namely the *p*-methyl and *p*-methoxy phenyl 15 and 17 (K_i s of 2.6 and 2.3 μ M). It is of interest that, against VchCA β , thioureido derivatives showed a better inhibitory effectiveness on the whole with respect to ureas 4–13. In fact, the remaining thioureas showed K_i s not overcoming the value of 31.9 μ M (21), whereas most ureido derivatives reported inhibition constants in the range 42.1–84.4 μ M, except for the *p*-halo compounds 7 and 9 (K_i s of 19.5 and 28.0 μ M). Interestingly, when the unsubstituted benzoxaborole 1 was again among the weakest VchCA β inhibitors, its substitution in position 5, as in tavorole, even worsened the affinity to the target producing the worst K_i value of the study against the β -class isozyme.

As mentioned above, the measured inhibition of VchCA α by benzoxaboroles 1–23 is even greater than that previously reported against hCA II. In fact, the selectivity index (SI) calculated for VchCA α over hCA II (Table 2) is chiefly above 1, solely except for compound 12, which is instead more active against hCA II.

A computational protocol consisting of joint docking and MM-GBSA and MD simulations was undertaken to explore in-depth the inhibitory profiles of benzoxaboroles 1–23 toward VchCA α , VchCA β , and VchCA γ .

Table 2. Selectivity Index (SI) of Benzoxaboroles 1–23, TVB, and AAZ Calculated as CA II/VchCA K_i s Ratio

Cpd	X	R	Selectivity Index (SI) over hCA II		
			VchCA α	VchCA β	VchCA γ
1			25.6	0.1	2.9
2			3.5	0.1	0.6
3			3.6	<0.1	0.5
4	O	CH ₂ Ph	4.9	<0.1	0.2
5	O	CH ₂ -(3-Cl,5-CH ₃ -Ph)	5.2	<0.1	0.4
6	O	Ph	6.2	<0.1	1.0
7	O	4-Cl-Ph	12.6	<0.1	1.1
8	O	CH ₂ -fur-2-yl	5.1	<0.1	0.7
9	O	4-F-Ph	10.0	<0.1	0.7
10	O	4-CF ₃ -Ph	9.9	<0.1	0.5
11	O	2,4,6-Cl-Ph	3.6	<0.1	0.3
12	O	2-OMe,5-CH ₃ -Ph	0.2	<0.1	0.2
13	O	4-COCH ₃ -Ph	7.7	<0.1	0.7
14	S	CH ₂ CH ₂ Ph	15.2	0.1	2.8
15	S	4-CH ₃ -Ph	11.5	0.5	0.7
16	S	napht-2-yl	1.9	<0.1	0.4
17	S	4-OCH ₃ -Ph	7.6	0.5	1.4
18	S	4-NO ₂ -Ph	>125.0	>10.7	>14.6
19	S	CH ₂ Ph	13.6	<0.1	0.3
20	S	4-F-Ph	13.4	0.2	2.8
21	S	CH ₂ -fur-2-yl	10.1	0.1	1.1
22	S	4-CF ₃ -Ph	25.5	1.5	1.0
23	S	Ph	1.3	0.1	1.9
TVB			2.9	<0.1	0.5
AAZ			1.8	<0.1	<0.1

Figure 3. Predicted binding mode of **1** (green) and **10** (blue) within VchCA α active site. (A) Tetrahedral (t); (B and C) trigonal bipyramid (p) coordinated poses of compounds **1** and **10**, respectively. H-bonds and π - π interactions are shown as black and blue dashed lines, respectively. Water molecules are represented as red spheres.

Figure 4. Predicted binding mode for **1** and **18** within the type-I VchCA β active site. Tetrahedral (t) coordinated poses of compounds (A) **1** and (B) **18**. H-bonds and π - π interactions are shown as black and blue dashed lines, respectively. Water molecules are represented as red spheres. The labels of amino acids from different chains are colored differently.

Benzoxaboroles were shown to act as zinc-binder CAIs against hCA II in the form of their conjugated Lewis base. Compounds **1**–**4**, **6**, **12**, **19**, and **23** were observed in the tetrahedral anionic $\text{B}(\text{OH})_2^-$ form as tetra- and/or penta-coordinated around the metal atom in the hCA II active site.²² A subsequent *in silico* study showed that the same benzoxaborole derivatives can only exhibit a tetrahedral coordination geometry around the Zn^{2+} in the narrower active site of β -CAs from fungi.²³ Thus, docking studies were performed considering the benzoxaborole $\text{B}(\text{OH})_2^-$ anion form.

Representative ligands were submitted to QM geometry optimization (B3LYP/6-31G $^{*+}$) and ESP charges computation prior to docking the molecules into the homology-built models of VchCA α , VchCA β , and VchCA γ and verifying the adducts stability by MD simulations. In fact, the pdb database only contains the 3D coordinates for VchCA β that however is present in the type II or closed form of the enzyme (PDB 5CXK)¹³ and thus not suitable for docking aims. The HM models were respectively developed using as templates the solved coordinates of α -CA from *Photobacterium profundum* (5HPJ), type-I β -CA from *Pseudomonas aeruginosa* (6D2N) and γ -CA from *Escherichia coli* (3TIO). The templates show the highest sequence identity percentage with the targets, namely 53%, 52%, and 61%, respectively (Figures S1–S9, Tables S1–S3, Supporting Information). The best scored HM-built models were used to figure out the binding mode of

representative benzoxaboroles within VchCAs active sites. ΔG binding energies were computed by a MM-GBSA method, and the stability of the predicted ligand/target adducts was evaluated by 100 ns MD. The structure of the three investigated CA isoforms was stable during the computation with the backbone atom RMSDs exhibiting small fluctuations over the course of the dynamic. As well, all docked binding orientations showed a significant stability along the MD, with the Zn coordination being firmly maintained (Figures S10–S12, Supporting Information).

Despite the structural differences between VchCA α and hCA II (i.e., the hCA II α -helix structural motif residues ranging from 129 to 135 are replaced in VchCA α by a short coil formed by residues 128–132), benzoxaborole derivatives share similar interaction features within the bacterial and human isozymes.

In fact, the predicted binding mode for the unsubstituted benzoxaborole **1** in VchCA α strictly resembles the interaction mode within hCA II,²² and two main binding solutions, featured by almost the same interaction energy values with the target, were found with different coordination geometry, namely a tetrahedral (t, Figure 3A) and a trigonal bipyramid (p, Figure 3B). In the tetracoordinated form, the fourth coordination position about the metal ion is occupied by one hydroxyl group on the boron atom, that also forms a H-bond with the T189 side chain (95% stable along the MD); the other B–OH is involved in a bifurcated H-bond with the T189

Figure 5. Predicted binding poses of **1** and **20** in the VchCA γ active site: (A) **p1** and (B) **p2** of derivative **1** and (C) compound **20**. H-bonds and π - π interactions are shown as black and blue dashed lines, respectively. Water molecules are represented as red spheres. The labels of amino acids from different chains are colored differently.

Figure 6. Alternative view of the (A) **p1** and (B) **p2** interaction modes of **1** and (C) **20** in the VchCA γ active site. H-bonds and π - π interactions are shown as black and blue dashed lines, respectively. Water molecules are represented as red spheres. The labels of amino acids from different chains are colored differently.

backbone NH (92%) and the side chain hydroxyl group of T190 (81%). In the pentacoordinated pose, the cyclic oxygen atom additionally binds the Zn²⁺, and similar H-bonds as above only occur with T189, involving the residue side chain OH (97%) and the backbone NH (94%) groups, respectively. Moreover, a 65% stable water bridged H-bond occurs between the coordinating OH and H79. The position of the heterocycle in both orientations is further stabilized by hydrophobic interactions with residues from the lipophilic half of the binding cavity (V125, V135, L133, L188, and P192).

The introduction of a bulky substituent at the 6 position of the benzoxaborole scaffold, as in derivative **10**, the most potent VchCA α inhibitor, only enables a p-like binding mode, with the formation of H-bonds of stability comparable to those of derivative **1**. The 4-CF₃-phenyl-ureido pendant accommodates into the pocket formed by W23, H79, and P191, establishing π - π (43%) and VdW interactions (Figure 3C). A water bridged H-bond network occurring between the ureido oxygen atom and the N77 (53%) and Q82 (32%) side further stabilizes the adduct.

The binding site of the active VchCA β dimer, as the other members of the β -CAs family, locates at the interface and is composed by amino acids from the two monomers forming the

dimer (Figure 4). The zinc ion is coordinated by a His and two Cys residues belonging to the same monomer.

The *in silico* insights showed that the narrow active site of VchCA β can accommodate benzoxaborole derivatives only as stably tetracoordinated (**t**) around the Zn(II) ion (Figure 4). The binding orientation is stabilized by multiple interactions with Y83: a Y83-OH $\cdots$ O-B interaction involving the second OH group of the ligand (that not coordinated to the Zn²⁺) and the hydroxyl group of the residue (67%); a π - π stacking interaction between the benzene ring of **1** and the phenyl ring of the amino acid (75%). In addition, the same OH group donates a H-bond to the D44 side chain carboxylate (69%) and is in H-bond contact with G100 (43%) through a bridged water molecule whereas the benzoxaborole endocyclic oxygen acts as an acceptor forming another H-bond with the Q33 side chain (58%). Moreover, the binding pose is further stabilized by vdW interactions with F61, V66, I116, and L120 (Figure 4A). However, the dynamic behavior of this adduct highlighted an overall reduced stability of the pose over the 100 ns long simulation (Figure S11, Supporting Information). This might probably be related to the enclosed, restricted active site of VchCA β , when compared to other β -CAs (e.g., Can2),²³ that prevents a consistent pose stabilization and induces the lower

inhibitory profile of the benzoxaboroles against VchCA β compared to VchCA α , VchCA γ , and Can2.

The predicted interaction mode for compound **18**, the most effective VchCA β inhibitor here, highlighted how the flexibility of the (thio)ureido linker allows the pendant in position 6 to accommodate in the lipophilic pocket formed by A106, L113, and I116 (Figure 4B). MD simulations pointed out that the persistence of the H-bonds involving the zinc binding group is enhanced by 10–15%, as well as the pose stability over the MD, with respect to derivative **1** (Figure S11, Supporting Information). The lodging of the X ureido atom in the inner portion of the aforesaid lipophilic cleft can support the more effective inhibitory action of thioureido over ureido derivatives. The *p*-NO₂ group in **18** (and presumably the CF₃ in **22**, the second most potent VchCA β inhibitor) favors water bridged H-bonds with residues at the outer rim of the binding cavity such as D109 (38%).

Likewise, the active site of the trimer VchCA γ , as other γ -CAs, locates at the interface and is composed by amino acids belonging to two distinct monomers (Figures 5 and 6). In the case of VchCA γ , the zinc ion is coordinated by three His residues belonging to distinct chains. Unlike VchCA β , the active site of VchCA γ is roomy enough to accommodate two alternative stable pentacoordinated orientations (**p1** and **p2**) for benzoxaborole **1** (Figures 5, 6, and S7, Supporting Information). In **p1**, the dual coordination around Zn²⁺ occurs by both OH groups of the ligand, with one also involved in a direct and a water mediated H-bond with the phenolic group of the Y166 (74%) and Q59 side chain (48%). The other OH and the endocyclic oxygen atom are water bridged with the V66 backbone C=O (37%) and the D46 (32%) side chain carboxylate (Figures 5A and 6A). Additionally, the benzene ring of **1** is sandwiched between H68 and T86, forming π – π (56%) and π –alkyl interactions with these residues. In **p2**, the endocyclic oxygen atom and an OH group of the ligand coordinate around the zinc ion. The **p2** binding orientation is stabilized by two H-bonds with the phenolic group of Y166 and the amide side chain of Q59 (72% and 61% stable, respectively). The other OH and the cyclic oxygen atom are involved in 42% and 21% stable water bridges with the V66 backbone C=O and R44 side chain (Figures 5B and 6B). Moreover, it is in hydrophobic contact with T67, H68, and H94 (Figure 5B and 6B). From an energetical point of view, the **p2** binding mode is favored over **p1** (dG –52.34 kcal/mol vs –46.83 kcal/mol). Interestingly, the 6-ureido derivatives bind in the active site by a **p2**-like mode (Figure 6C), conserving a similarly stable H-bond network as described for derivative **1**. The 4-F-phenylureido group of inhibitor **20**, the most active against VchCA γ , extends toward the lipophilic half of the active site, interacting through a π – π interaction (45%) and VdW contacts with L103, M121, and F158. Further, the ureidic NH are commonly implicated in a water bridge with the S162 side chain (approximately 55% stable).

In conclusion, a series of urea/thiourea substituted benzoxaboroles was investigated for the inhibition of the three CAs from *Vibrio cholerae* (VchCA α , VchCA β , and VchCA γ). In this study, benzoxaborole derivatives were first assayed to inhibit a γ -class CA, widening the panel of CA classes significantly affected by such a type of CAIs. The inhibitory activity was more intense against VchCA α compared to VchCA γ , which, in turn, showed an inhibition more potent respect to VchCA β . Selective benzoxaborole derivatives were detected against VchCA α with a high selective index against

the off-target hCA II. A compound was identified, namely the *p*-NO₂-phenylthiourea **18**, which exhibited a selective inhibitory action against all VchCAs over hCA II.

Additionally, an exhaustive *in silico* ligand/target interaction study was carried out with all three VchCA isoforms, including, to the best of our knowledge, the first molecular modeling investigation with inhibitors of a γ -class CA. The computational analysis showed that benzoxaborole derivatives adopt both a tetrahedral and trigonal bipyramidal zinc-coordination in complex with VchCA α and two alternative pentacoordinated geometries around the zinc ion in VchCA γ active site. In contrast, the active site of VchCA β , narrower than that of the other VchCAs as well as of other β -CAs (i.e., Can2 from *C. neoformans*), was able to accommodate benzoxaborole derivatives bound to the zinc ion only by a tetrahedral geometry, with a lower stability emerging as a result of the 100 ns long MD.

The above findings indicate that benzoxaboroles represent an interesting chemotype worth developing as innovative antibacterial agents since they possess a new mechanism of action and isoform selectivity preferentially against the bacterial expressed CAs. This study enriches the inhibitory profiles database for the different *Vibrio cholerae* CA-classes. Furthermore, it furnishes suggestions for the rational design of new potent and selective inhibitors targeting *V. cholerae* CAs over human off-target ones.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00403>.

Sequence alignment of VchCA α , VchCA β , and VchCA γ ; 3D representation and parameters of homology models of VchCA α , VchCA β , and VchCA γ and templates; supplemental modeling figure and materials and methods. (PDF)

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Notes

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ABBREVIATIONS

CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; AAZ, acetazolamide; TVB, tavorole

REFERENCES

- (1) Weil, A. A.; Ryan, E. T. Cholera: recent updates. *Curr. Opin. Infect. Dis.* **2018**, 31 (5), 455–461.
- (2) WHO World Health Organization. Available from: https://www.who.int/health-topics/cholera#tab=tab_1 (accessed on 2020-04-29).
- (3) Deen, J.; Mengel, M. A.; Clemens, J. D. Epidemiology of cholera. *Vaccine* **2020**, 38 (Suppl 1), A31–A40.
- (4) Clemens, J. D.; Nair, G. B.; Ahmed, T.; Qadri, F.; Holmgren, J. Cholera. *Lancet* **2017**, 390 (10101), 1539–1549.
- (5) Baker-Austin, C.; Oliver, J. D.; Alam, M.; Ali, A.; Waldor, M. K.; Qadri, F.; Martinez-Urtaza, J. *Vibrio* spp. infections. *Nat. Rev. Dis. Primers* **2018**, 4 (1), 8.
- (6) Narendrakumar, L.; Gupta, S. S.; Johnson, J. B.; Ramamurthy, T.; Thomas, S. Molecular Adaptations and Antibiotic Resistance in *Vibrio cholerae*: A Communal Challenge. *Microb. Drug Resist.* **2019**, 25 (7), 1012–1022.
- (7) Das, B.; Verma, J.; Kumar, P.; Ghosh, A.; Ramamurthy, T. Antibiotic resistance in *Vibrio cholerae*: Understanding the ecology of resistance genes and mechanisms. *Vaccine* **2020**, 38 (Suppl1), A83–A92.
- (8) Matson, J. S.; Withey, J. H.; DiRita, V. J. Regulatory networks controlling *Vibrio cholerae* virulence gene expression. *Infect. Immun.* **2007**, 75 (12), 5542–9.
- (9) Supuran, C. T.; Capasso, C. Biomedical applications of prokaryotic carbonic anhydrases. *Expert Opin. Ther. Pat.* **2018**, 28 (10), 745–754.
- (10) Del Prete, S.; Isik, S.; Vullo, D.; De Luca, V.; Carginale, V.; Scozzafava, A.; Supuran, C. T.; Capasso, C. DNA cloning, characterization, and inhibition studies of an α -carbonic anhydrase from the pathogenic bacterium *Vibrio cholerae*. *J. Med. Chem.* **2012**, 55 (23), 10742–8.

(11) Del Prete, S.; De Luca, V.; Scozzafava, A.; Carginale, V.; Supuran, C. T.; Capasso, C. Biochemical properties of a new α -carbonic anhydrase from the human pathogenic bacterium, *Vibrio cholerae*. *J. Enzyme Inhib. Med. Chem.* **2014**, 29 (1), 23–7.

(12) Del Prete, S.; Vullo, D.; De Luca, V.; Carginale, V.; Ferraroni, M.; Osman, S. M.; AlOthman, Z.; Supuran, C. T.; Capasso, C. Sulfonamide inhibition studies of the β -carbonic anhydrase from the pathogenic bacterium *Vibrio cholerae*. *Bioorg. Med. Chem.* **2016**, 24 (5), 1115–20.

(13) Ferraroni, M.; Del Prete, S.; Vullo, D.; Capasso, C.; Supuran, C. T. Crystal structure and kinetic studies of a tetrameric type II β -carbonic anhydrase from the pathogenic bacterium *Vibrio cholerae*. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2015**, 71 (Pt 12), 2449–56.

(14) Del Prete, S.; Vullo, D.; De Luca, V.; Carginale, V.; Osman, S. M.; AlOthman, Z.; Supuran, C. T.; Capasso, C. Comparison of the sulfonamide inhibition profiles of the α -, β - and γ -carbonic anhydrases from the pathogenic bacterium *Vibrio cholerae*. *Bioorg. Med. Chem. Lett.* **2016**, 26 (8), 1941–6.

(15) Del Prete, S.; Vullo, D.; De Luca, V.; Carginale, V.; di Fonzo, P.; Osman, S. M.; AlOthman, Z.; Supuran, C. T.; Capasso, C. Anion inhibition profiles of α -, β - and γ -carbonic anhydrases from the pathogenic bacterium *Vibrio cholerae*. *Bioorg. Med. Chem.* **2016**, 24 (16), 3413–7.

(16) Abuaita, B. H.; Withey, J. H. Bicarbonate Induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. *Infect. Immun.* **2009**, 77 (9), 4111–20.

(17) Thomson, J. J.; Withey, J. H. Bicarbonate increases binding affinity of *Vibrio cholerae* ToxT to virulence gene promoters. *J. Bacteriol.* **2014**, 196, 3872–80.

(18) Cobaxin, M.; Martínez, H.; Ayala, G.; Holmgren, J.; Sjöling, A.; Sánchez, J. Cholera toxin expression by El Tor *Vibrio cholerae* in shallow culture growth conditions. *Microb. Pathog.* **2014**, 66, 5–13.

(19) Capasso, C.; Supuran, C. T. Inhibition of bacterial carbonic anhydrases as a novel approach to escape drug resistance. *Curr. Top. Med. Chem.* **2017**, 17, 1237–48.

(20) Modakh, J. K.; Liu, Y. C.; Machuca, M. A.; Supuran, C. T.; Roujeinikova, A. Structural basis for the inhibition of *Helicobacter pylori* α -carbonic anhydrase by sulfonamides. *PLoS One* **2015**, 10, No. e0127149.

(21) Wang, W.; Zhang, Y.; Wang, L.; Jing, Q.; Wang, X.; Xi, X.; Zhao, X.; Wang, H. Molecular structure of thermostable and zinc-ion-binding γ -class carbonic anhydrases. *BioMetals* **2019**, 32, 317–28.

(22) Alterio, V.; Cadoni, R.; Esposito, D.; Vullo, D.; Fiore, A. D.; Monti, S. M.; Caporale, A.; Ruvo, M.; Sechi, M.; Dumy, P.; Supuran, C. T.; De Simone, G.; Winum, J. Y. Benzoxaborole as a new chemotype for carbonic anhydrase inhibition. *Chem. Commun.* **2016**, 52 (80), 11983–11986.

(23) Nocentini, A.; Cadoni, R.; Del Prete, S.; Capasso, C.; Dumy, P.; Gratteri, P.; Supuran, C. T.; Winum, J. Y. Benzoxaboroles as Efficient Inhibitors of the β -Carbonic Anhydrases from Pathogenic Fungi: Activity and Modeling Study. *ACS Med. Chem. Lett.* **2017**, 8 (11), 1194–1198.

(24) Nocentini, A.; Cadoni, R.; Dumy, P.; Supuran, C. T.; Winum, J. Y. Carbonic anhydrases from *Trypanosoma cruzi* and *Leishmania donovani* chagasi are inhibited by benzoxaboroles. *J. Enzyme Inhib. Med. Chem.* **2018**, 33 (1), 286–289.

(25) Nocentini, A.; Supuran, C. T.; Winum, J. Y. Benzoxaborole compounds for therapeutic uses: a patent review (2010–2018). *Expert Opin. Ther. Pat.* **2018**, 28 (6), 493–504.

(26) Larcher, A.; Nocentini, A.; Supuran, C. T.; Winum, J. Y.; van der Lee, A.; Vasseur, J. J.; Laurencin, D.; Smietana, M. Bis-benzoxaboroles: Design, Synthesis, and Biological Evaluation as Carbonic Anhydrase Inhibitors. *ACS Med. Chem. Lett.* **2019**, 10 (8), 1205–1210.

(27) Langella, E.; Alterio, V.; D'Ambrosio, K.; Cadoni, R.; Winum, J. Y.; Supuran, C. T.; Monti, S. M.; De Simone, G.; Di Fiore, A. Exploring benzoxaborole derivatives as carbonic anhydrase inhibitors: a structural and computational analysis reveals their conformational

variability as a tool to increase enzyme selectivity. *J. Enzyme Inhib. Med. Chem.* **2019**, *34* (1), 1498–1505.

(28) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561.