Enzyme inhibition

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Carborane-Based Carbonic Anhydrase Inhibitors**

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Human carbonic anhydrases (CAs) are zinc metalloenzymes that play an important role in many physiological processes. To date, 15 human CA isozymes with different subcellular localization and tissue expression profiles have been identified. Vast experimental evidence also suggests the involvement of CAs in various pathological processes (e.g., tumorigenicity, obesity, and epilepsy). Many CA isozymes are thus recognized as diagnostic and therapeutic targets.[1]

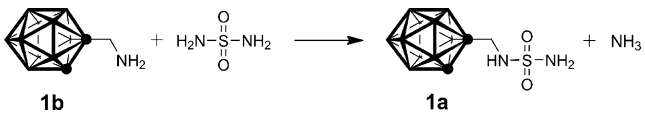
About 30 CA inhibitors are used clinically, for example, as anti-glaucoma drugs (targeting CAII, CAIV, and CAXII), anti-convulsants (targeting CAII, CAVII, and CAXIV) and anti-obesity agents (targeting CAVA and CAVB).[2] Recently, other isozymes, namely the neuronal CAVII and CAXIV, and the cancer-associated forms CAIX and CAXII, have been validated as targets for inhibitor development.[3]

The traditional CA inhibitors contain a sulfonamide or sulfamide moiety that coordinates the zinc cation located in the CA catalytic site.[4] Most of the currently used CA inhibitors lack selectivity, and their use causes numerous unwanted side effects. A current challenge is the design of compounds that can inhibit specific isozymes. Although the conical active-site clefts of different human CA isoezymes are conserved, variations exist in the amino acid residues at the entrance to the active site. As a result of their differing in shape and hydrophobicity, these surface pockets can be exploited to design specific inhibitors.[5,6]

Structural analysis of CAII in complex with numerous inhibitors revealed two general binding modes, each involving a distinct site within the enzyme active site cavity.[7] This led us to hypothesize that CA inhibitors could be designed more effectively based on three-dimensional scaffolds rather than flat structures.

Carboranes, icosahedral clusters containing boron, carbon, and hydrogen are bulky pharmacophores used to replace various hydrophobic structures in biologically active molecules.[8,9] The 12-vertex carboranes increase the in vivo stability and bioavailability of biologically active molecules and enhance the hydrophobic interactions between them and their receptors.[10] They are an abiotic species that are very stable towards catabolism and degradation by enzymes and thus the use of boron clusters as components of new pharmacological agents has been increasing.[11–15]

With the help of manual molecular docking into the active site of CAII, we designed **1a**, which contains a sulfamide group connected to a carborane cluster intended to optimally fill the enzyme active site. The length of the linker between the sulfamide group and carborane cluster was chosen based on comparison with the structures of isoquinoline sulfonamide inhibitors.[7] Attachment of the sulfamide moiety was accomplished by using a transamination reaction between aminomethylcarborane **1b** and sulfamide (Scheme 1).



Scheme 1. Preparation of **1a** by heating **1b** with sulfamide in dioxane. Vertices represent BH, black spheres CH or C if substituted.

Compound **1a** showed inhibitory activity toward CAII (K_i value of 0.7 μM) and showed almost 2-fold higher activity toward the tumor-associated isoform CAIX (K_i of 0.38 μM).

The crystal structure of **1a** in complex with CAII determined at 1.35 Å resolution (PDB code 4 MDG) confirmed that the inhibitor binds in the enzyme active site as predicted (Figure 1) and revealed key interactions responsible for inhibitor binding and enzyme inhibition.

The sulfamide moiety of **1a** proved to be the anchoring group that completes the coordination sphere of Zn^{2+} in the active site and makes the polar interactions with Thr199 that are typical of other CA inhibitors.[4] An additional polar interaction is a hydrogen bond between a linker NH group and the side chain Oγ of Thr200 (Figure 1a). Further interactions of the inhibitor with the active site cavity are mediated through van der Waals interactions between the carborane cluster and amino acid residues Gln92, His94, Phe131, Leu198, and Thr200. For a complete list of interactions, see Table S1 in the Supporting Information. Compound **1a** fills the proximal active site cavity of CAII, but

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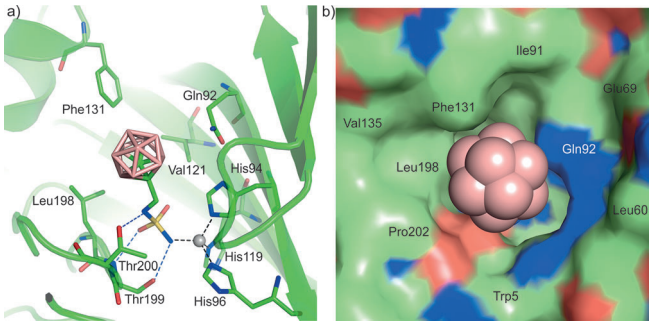

Figure 1. Crystal structure of CAII in complex with **1a**. a) The protein is shown as a ribbon diagram; residues involved in interactions with the Zn^{2+} ion (gray sphere) and **1a** are shown as stick representations. C green, B pink, S yellow, O red, and N blue atoms are shown. Polar interactions are represented by blue dashed lines; Zn^{2+} ion coordination is shown as black dashed lines. b) Top view into the active site, shown as a surface representation. **1a** is shown as a space filling model.

some side pockets at the entrance of the active site could potentially be targeted by cluster substituents (Figure 1b).

Next, we considered several modifications and prepared a series of novel boron cage compounds combining various carborane clusters with a polar sulfamide moiety (Figure 2). Compounds **2a**–**10a** were prepared in high yields using the transamination reaction depicted in Scheme 1.

Our pilot series of ten CA inhibitors includes the parent compounds **1a** and **4a**, which contain one alkyl sulfamide group and icosahedral *ortho*- and *meta*-carborane units, respectively. A structurally similar, charged derivative based on the $[CB_{11}H_{12}]^-$ cage was also prepared (**6a**). *Closo*-carboranes containing either hydrophobic (**2a** and **3a**) or hydrophilic (second sulfamide moiety; **5a**) substituents and their charged 11-vertex *nido* counterparts (**7a**–**10a**) complete this structurally diverse series.

The compounds were tested for inhibition of the human CA isozymes CAII and CAIX using a colorimetric enzymatic assay. Most of the compounds tested displayed good CA inhibition profiles, with K_i values in the low micromolar or submicromolar range (Table 1). Almost all of the compounds showed selectivity for the cancer-specific CAIX isozyme over the more abundant variant CAII.

Analysis of crystal structures of CAII in complex with **4a** and **7a** (PDB codes 4MDL and 4MDM) confirmed that these compounds are positioned similarly in the enzyme active site to **1a**. In fact, **4a** binds to CAII identically to **1a** in terms of conformation and interactions (Figure 3a).

Compound **7a** binds CAII differently and, unlike **1a** and **4a**, does not form a hydrogen bond between its linker NH group and the side chain Oγ of Thr200 (Figure 3b). The distance between the position of the linker NH group in **1a** and **7a** is 0.18 Å. The *nido*-carborane cluster interacts with His64, Gln92, His94, Leu198, Thr200, and Pro201 (for a complete list of interactions see Table S1 in Supporting Information).

In our pilot series of carborane-based CA inhibitors, we exploited four different carborane cluster types. The effect of cluster type on inhibition can be deduced from comparison of

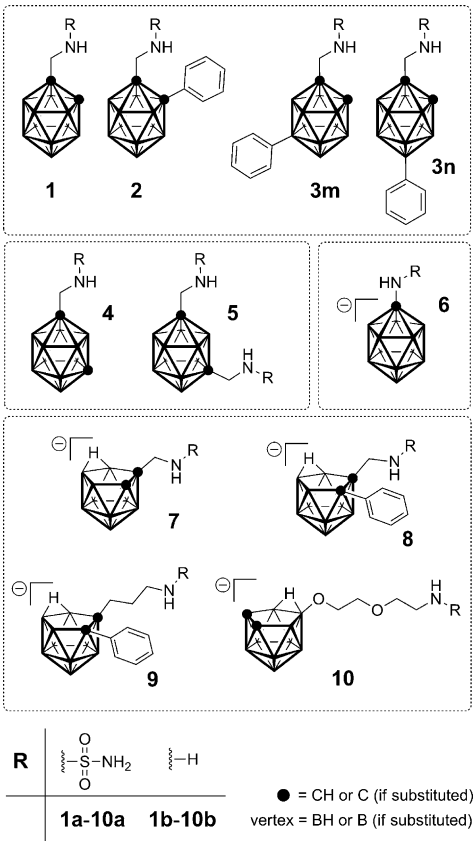

Figure 2. Structures of compounds used in this study. Amino groups attached to the anionic clusters (**6b**–**10b**) are protonated, forming zwitterions. **3** was prepared as an inseparable mixture of two isomers (termed **3m** and **3n**). The substituent in **10b** is located at boron B(10) sitting in the open face and not at a carbon as in **7**.

Table 1: In vitro inhibition of selected carbonic anhydrase isozymes.

Compound	K_i (CAII) [μM]	K_i (CAIX) [μM]	Selectivity Index[a]
1a	0.70 ± 0.14	0.38 ± 0.11	1.8
2a	348 ± 113	161 ± 37	2.2
3ma + **3na**	0.51 ± 0.08	0.43 ± 0.17	1.2
4a	1.16 ± 0.24	1.12 ± 0.20	1.0
5a	0.38 ± 0.14	0.23 ± 0.04	1.7
6a	8.57 ± 2.24	2.20 ± 0.48	3.9
7a	6.79 ± 2.01	5.09 ± 2.38	1.3
8a	9.00 ± 2.23	2.32 ± 1.02	3.9
9a	2.71 ± 0.47	0.15 ± 0.06	18.1
10a	132 ± 19	26.04 ± 2.93	5.1

[a] Selectivity index is the ratio between K_i (CAII) and K_i (CAIX).

the K_i values obtained for **1a**, **4a**, and **7a**. The *o*-carborane derivative **1a** has slightly better inhibitory potency toward CA isozymes than the *m*-carborane derivative **4a**. This could result from the presence of a slightly acidic CH group in the position vicinal to the substituted carbon in **1a**. Compound **7a** (containing 7,8-*nido*-carborane) is a less efficient inhibitor of CA isozymes than the *closo*-carborane derivatives; however, it shows a slightly different binding mode. As revealed by the

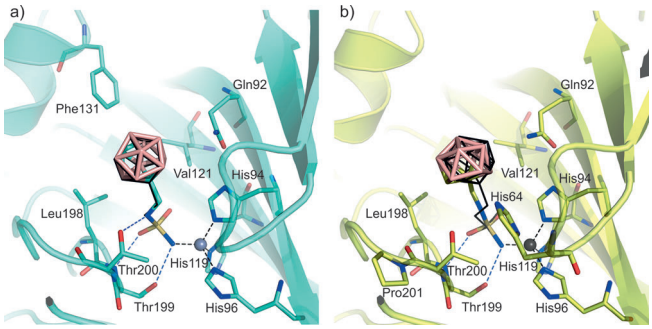

Figure 3. Crystal structure of CAII in complex with **4a** (a) or **7a** (b). The protein is shown as ribbon diagram; residues involved in interactions are shown as stick representations. Polar interactions are represented by dashed blue lines; Zn^{2+} ion coordination is represented by dashed black lines. The binding mode of **1a** (black line) is superimposed on the crystal structures.

co-crystal structure, the *nido* cluster **7a** has the possibility to adjust its position within the active site, which confers a potential advantage in that it can accommodate additional substituents that might affect selectivity toward a particular CA isozyme.

To explore a greater diversity of carborane clusters, we also prepared **6a**, which contains a 1-carbadodecaborate ion $[CB_{11}H_{12}]^-$. The removal of one methylene group from the linker and the presence of a negatively charged cluster led to a decrease in efficiency but a substantial increase in selectivity index compared to **1a**, **4a**, and **7a**. This demonstrates that a relatively subtle change in structure can strongly influence inhibitor interactions with a particular CA isozyme.

In our initial molecular design, we hypothesized that tuning compound selectivity to a certain CA isozyme could be achieved by cluster substitutions. In our series, we exploited a phenyl ring as a substituent in synthetically accessible positions on the cluster. Analysis of the crystal structure of **1a** in complex with CAII suggested that a phenyl ring in the *ortho* position on the *closo*-carborane cluster would have steric clashes with amino acids lining the entrance to the enzyme active site. Indeed, this detrimental effect on inhibitory efficiency was illustrated by the 400-fold greater K_i value obtained for inhibition of CAII with **2a**. On the other hand, the phenyl substituent placed in the *meta*-position, as in **3ma** and **3na**, seems not to experience any steric clash with the bottom of the active site, and the inhibitory potencies toward CAII and CAIX remain virtually unchanged, with K_i values close to those of parent compound **1a**.

The *nido*-carborane cluster was substituted with a phenyl ring in the *ortho* position, leading to **8a**. We did not observe a loss of inhibitory potency as a result of the addition of a phenyl ring in this position. Derivative **8a** shows only a slightly increased K_i value for CAIX, while the K_i value for CAII is lower than that of parent compound **7a**. The smaller *nido* cluster is apparently able to adjust its position in the active site cavity; the phenyl ring can thus be accommodated in a side pocket. Inspection of the crystal structure of CAII in complex with **7a** suggests that a pocket formed by Ile91 and Phe131 (Figure 1b) would be accessible for substituent binding. In CAIX, this pocket contains Leu91 and Val131 and is more spacious.

In **5a**, an additional sulfamide group was attached by a methylene linker to the *meta* position of the *meta*-carborane cluster. This increased the inhibitory potency compared to that of the parent *m-closo*-carborane by a factor of approximately 2. This increase can be attributed to the presence of two sulfamide groups that can anchor the compound into the catalytic site of the enzyme.

The effect of the linker connecting the cluster to the sulfamide group can be deduced from comparison of the inhibitory properties of **8a** and **9a**, and **7a** and **10a**; two pairs of compounds with the same carborane cage but with linkers differing in length and nature.

Compound **8a** contains the methylene linker designed in the initial docking. Increasing the length of the aliphatic linker to a propylene moiety improved the inhibitory potency of **9a** and made it almost 5-fold more selective toward CAIX. Increasing the length of the cluster-sulfamide linker by two methylene groups probably allows accommodation of the *nido* cluster with its phenyl substituent in side pockets at the entrance of the enzyme active site (Figure S3 in the Supporting Information). With a CAIX/CAII selectivity index of 18.1, Compound **9a** showed the highest selectivity observed within the entire series.

For the parental *nido* cluster, we therefore tested an even longer, 7-atom linker (**10a**). This elongation, however, substantially decreased the inhibitory efficiency of the compound compared to that of the cluster bearing a short methylene linker (**7a**). A possible explanation for this decrease, based on this compound's similarity to ionic metallacarboranes bearing a similar linker, is that complexation with sodium cations may lead to a rigid, crown-ether-like arrangement.[16] This would decrease the compound's solubility and its ability to interact with the catalytic cleft. Our observations suggest that both the length and the nature of the cluster-to-sulfamide linker are crucial for inhibitory efficacy and should be carefully tuned in future design.

In conclusion, our results suggest that carborane-based compounds are promising lead structures for the development of inhibitors of CA isozymes. Our experiments demonstrated that various types of hydrophobic, space-filling carborane clusters can be accommodated in the CA active site and that substitution with an appropriately attached sulfamide group and other substituents leads to compounds with high selectivity for the cancer-specific CAIX isozyme over the widespread CAII isozyme. Crystal structures confirmed our hypothesis that three-dimensional scaffolds could be efficiently used in CA inhibitors and provided structural information that can be applied to the structure-based design of specific CAIX inhibitors.

Experimental Section

Experimental procedures including detailed synthetic procedures for the sulfamide derivatives and their precursors are described in the Supporting Information. Briefly, the sulfamide compounds were prepared from their respective amine or ammonium carborane derivatives (**1b**–**10b**). Compounds **1b**[17] and **6b**[18] were prepared

according to known procedures. **4b** and **5b** were prepared by lithiation of 1,7-carborane, reaction with bromomethyl phthalimide, and removal of the phtalimido group by hydrazine hydrate.[19–22] Derivatives **2b** and **3b** were synthesized similarly, but the final steps involved reduction with $NaBH_4$ and subsequent hydrolysis. Although this pathway has been described as failing for 1-aminomethyl-*o*-carboranes,[20] we have demonstrated the feasibility of this approach for compounds **2b** and **3b** bearing methylene connectors. During synthesis of **3b**, an equilibrium mixture of two isomers formed after lithiation of 9-C_6H_5-1,2-$C_2B_{10}H_{11}$ and subsequent reaction with bromomethyl phthalimide as a result of the presence of two available CH sites with similar reactivity. The isolated mixture of the 9- and 12-phenyl isomers (in a nearly 1:1 ratio) could not be separated by liquid chromatography either as phthalimido-protected amines or after deprotection. This isomeric mixture was therefore used for the synthesis of sulfamide derivatives **3ma** and **3na**. Derivatives **8b** and **9b** were obtained using hydrazine hydrate to convert 1-alkylphthalimido-2-phenyl-*closo*-1,2-carboranes into their respective 11-vertex *nido*-$[C_2B_9H_{12}]^-$ ion species, as described in a previous report.[17] For the preparation of **7b**, we used a new straightforward one-step deboronation[8]/amination of 1-$BrCH_2$-1,2-$C_2B_{10}H_{11}$. The reaction proceeds even with 24 % aqueous NH_4OH, producing a mixture of the target *nido*-derivative **7b** along with two other boron-substituted isomeric species (7-CH_3-9-NH_3-1,7-$C_2B_9H_{10}$, 7-CH_3-11-NH_3-1,7-$C_2B_9H_{10}$) in lower yield (ca. 35 %). Compound **7b** could be isolated in good yield (62 %) by column chromatography. Compound **10b** was prepared by cleavage of the dioxane derivative 10-$O(CH_2CH_2)_2O$-$C_2B_9H_{11}$ with ammonia, as described in a previous report.[23]

Addition of the sulfamide end group was accomplished with high yields by heating the respective amines with sulfamide in dioxane (see Scheme 1). Potassium carbonate was used for the deprotonation of the ammonium groups or to release the respective amines from their hydrochlorides in situ. Nevertheless, the attempted synthesis of 1-H_2NSO_2NH-$(CH_2)_2$-2-C_6H_5-*closo*-1,2-$C_2B_{10}H_{10}$ and $[7$-H_2NSO_2NH-$(CH_2)_2$-8-C_6H_5-*nido*-7,8-$C_2B_9H_{10}]^-$ (potential members of the series with intermediate chain lengths; the amines prepared analogously to a published procedure[22]) failed owing to quantitative elimination of the substituent in the reaction with sulfamide, thus resulting in only the starting phenyl carborane.

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