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Carbonic anhydrase inhibitors. Interaction of the antitumor sulfamate EMD 486019 with twelve mammalian carbonic anhydrase isoforms: Kinetic and X-ray crystallographic studies ☆

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ABSTRACT

The new antitumor sulfamate **EMD 486019** was investigated for its interaction with twelve catalytically active mammalian carbonic anhydrase (CA, EC 4.2.1.1) isozymes, hCA I – XIV. Similarly to 667-Coumate, a structurally related compound in phase II clinical trials as steroid sulfatase/CA inhibitor with potent antitumor properties, **EMD 486019** acts as a strong inhibitor of isozymes CA II, VB, VII, IX, XII, and XIV (K_Is in the range of 13–19 nM) being less effective against other isozymes (K_Is in the range of 66–3600 nM against hCA I, IV, VA, VI, and mCA XIII, respectively). The complete inhibition profile of 667-Coumate against these mammalian CAs is also reported here for the first time. Comparing the X-ray crystal structures of the two adducts of CA II with **EMD 486019** and 667-Coumate, distinct orientations of the bound sulfamates within the enzyme cavity were observed, which account for their distinct inhibition profiles. CA II/IX potent inhibitors belonging to the sulfamate class are thus valuable clinical candidates with potential for development as antitumor agents with a multifactorial mechanism of action.

Inhibitors of zinc enzyme carbonic anhydrases (CAs, EC 4.2.1.1) have clinical applications as diuretic, antiglaucoma, antiobesity, or antitumor drugs/diagnostic tools.[1–6] Various CA isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target different isozymes of the 15 presently known in humans (CA I- CA XIV, but there are two CA V type isoforms, CA VA and CA VB).[2–6] In all of them, the inhibitor is bound usually as anion to the catalytically critical Zn(II) ion, also participating in extensive hydrogen bond networks and van der Waals interactions with amino acid residues both in the hydrophobic and hydrophilic halves of the enzyme active site, as shown by X-ray crystallographic studies of such enzyme-inhibitor complexes.[7–15] Three main classes of potent CA inhibitors (CAIs) were described so far: the sulfonamides, the sulfamates, and the sulfamides, possessing the general formula R-X-SO_2NH_2, where X is nothing, O or NH, respectively.[1–6] X-ray crystal structures are available for many adducts of several isozymes (i.e., CA I, II, IV, V, XII, and XIV) [7–15] mostly with sulfonamides, with several sulfamates (including the simplest one, sulfamic acid)[10] and with few sulfamides, such as the simple derivative $H_2NSO_2NH_2$ [10a] and the topiramate–sulfamide analogue.[10b] A number of such derivatives are clinically used drugs, such as acetazolamide (**AAZ**), methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate (**TPM**), zonisamide, sulpiride, sulthiame, celecoxib, and valdecoxib among others.[1–3] Other compounds are in clinical development, such as the antitumor sulfonamide indisulam and 667-Coumate (**CMT**), a compound acting both as a potent steroid sulfatase and as a CA inhibitor.[1]

CA inhibitors (CAIs) are mainly used in therapy as diuretics and antiglaucoma agents but some of them also show marked anticonvulsant, antiobesity, and antitumor effects.[1,2,5–11] However, most of the presently available compounds in clinical use show undesired side effects due to the indiscriminate inhibition of CA isoforms other than the target ones.[1,2,5–14] Thus, many new CAI classes are being developed in the search of isozyme-selective compounds as potential drugs with less side effects.[1–5,11–15]

In this work, we report a detailed inhibition study of all 12 catalytically active mammalian CA isoforms (i.e., CA I, II, III, IV, VA, VB, VI, VII. IX, XII, XIII, and XIV, of human—h or murine—m origin) with a new sulfamate compound, **EMD 486019**, as well as with its closely related analogue in phase II clinical trials as an antitumor drug, 667-coumate (**CMT**). The high resolution X-ray crystal structure for the adduct of **EMD 486019** with the ubiquitous and physiologically dominant isoform hCA II was also obtained and is presented here. Comparison of the X-ray crystal structures of the adducts of **EMD 486019** with that of the hCA II—**CMT** complex

☆ The coordinates of the hCA II—EMD 486019 adduct have been deposited in PDB, ID code 3DD8.

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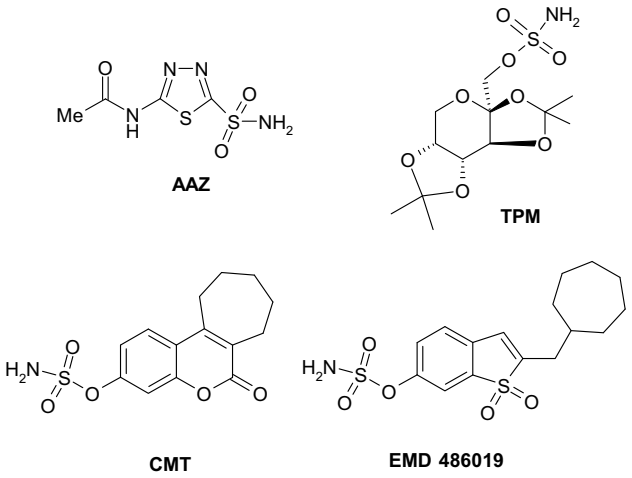
reported earlier by Potter's group[13b] allow us to draw some interesting conclusions regarding the drug design of sulfamate CAIs.

The following should be noted regarding the CA inhibition data of the three sulfamates (**TPM, CMT**, and **EMD 486019**) and standard sulfonamide CA inhibitor **AAZ** presented in Table 1[16]: (i) **EMD 486019** behaves as a potent inhibitor of isozymes hCA II, hCA VB, hCA VII, hCA IX, hCA XII, and hCA XIV, showing inhibition constants in the low nanomolar range (K_Is of 13–19 nM). The compound is also a medium potency mCA XIII inhibitor (K_I of 66 nM), and a weak inhibitor of hCA I, hCA IV, hCA VA, and hCA VI (K_Is in the range of 654–3600 nM). As most sulfonamides and sulfamates,[1–3,15e] the compound is a very weak hCA III inhibitor (Table 1); (ii) 667-Coumate presents a distinct inhibition profile as compared to the new sulfamate **EMD 486019**. Thus, **CMT** is a potent inhibitor of isoforms hCA II, hCA IV, hCA VII, hCA IX, and hCA XII, with inhibition constants in the range of 12–34 nM. On the other hand, all other isozymes (e.g., hCA I, hCA VA, hCA VB, hCA VI, mCA XIII, and hCA XIV) were weakly inhibited by this compound, with K_Is in the range of 653–3450 nM, whereas hCA III is inhibited in the millimolar range (Table 1); (iii) Topiramate (**TPM**), a clinically used sulfamate as antiepileptic drug,[4,6] behaves as a potent inhibitor of isoforms II, VB, VII, and XII (K_Is in the range of 0.9–30 nM), it is a medium potency inhibitor against hCA VA, hCA VI, hCA IX, and mCA XIII (K_Is in the range of 45–63 nM), and weakly inhibits hCA I, hCA III, hCA IV, and hCA XIV (K_Is in the range of 250–7.8 × 10^5nM); (iv) acetazolamide **AAZ**, the classical inhibitor of CAs, in clinical use since 1954,[1–3] behaves as a potent inhibitor against isoforms II and VI-XIII (K_Is in the range of 2.5–25 nM), is a medium potency inhibitor against hCA IV, hCA VA, and VB, as well as hCA XIV, with K_Is in the range of 41–74 nM. It has a rather weak affinity only for hCA I (K_I of 250 nM) and very weak affinity for hCA III (K_I of 2 × 10^5 nM).

The two structurally related sulfamates **CMT** and **EMD 486019** behave thus quite similarly against the following two groups of isozymes: hCA I, hCA III, hCA VA, and hCA VI (both are weak inhibitors); hCA II, VII, IX, and XII (both are strong inhibitors). However, there are net differences between them regarding their interactions with: hCA IV (**CMT** is a potent inhibitor, whereas **EMD 486019** is a weak one); hCA VB and hCA XIV (**CMT** is a weak inhibitor, whereas **EMD 486019** is a potent one); and mCA XIII (**CMT** is a weak inhibitor, **EMD 486019** a medium potency one). Thus, except for isozyme IV, much better inhibited by **CMT**, the sulfamate **EMD 486019** behaves always as a better CAI as compared to the corresponding behavior (against the same isozyme) of **CMT** (against hCA XII the two compounds may be considered equipotent). Considering all compounds investigated here, **EMD 486019** is a quite potent hCA II/hCA XII inhibitor, with potencies in the same range as **AAZ** and **TPM** (similarly also to **CMT**), it is the best hCA VB/hCA IX/hCA XIV inhibitor among the four derivatives considered here, but it is a much weaker inhibitor of hCA VA and hCA VI as compared to **AAZ** and **TPM**. Thus, the inhibition profile of **EMD 486019** is completely different from those of the clinically used derivatives **AAZ** and **TPM**, being more similar to that of **CMT**, but distinct of it. **EMD 486019** generally behaves as a better inhibitor of most isozymes (as compared to **CMT**), except for hCA IV.

Here, we also present the detailed X-ray crystallographic structure of the hCA II—**EMD 486019** adduct, and its comparison with the hCA II—**CMT** adduct reported earlier by Potter's group (at a resolution of 1.95 Å).[13b,17] Crystallographic refinement of the hCA II—**EMD 486019** adduct was performed at a final resolution of 1.90 Å. Crystals of the adduct were isomorphous with those of the native protein,[17] allowing for the determination of the crystallographic structure by difference Fourier techniques. The refined structure presents a good geometry with r.m.s.d. from ideal bond lengths and angles of 0.009 Å and 1.2°, respectively. The overall quality of the model was excellent with all residues in the allowed regions of the Ramachandran plot. Refinement statistics are summarized in Table 2. Inspection of the electron density maps at various stages of

Table 1
Inhibition data with the clinically used compounds **AAZ**, **TPM,** the clinical candidate **CMT,** and **EMD 486019,** against isozymes CAI—XIV



Isozyme*	K_I** (nM)			
	AAZ	**TPM**	**CMT**	**EMD 486019**
hCAI[a]	250	250	3450	3600
hCAII[a]	12	10	21[c]	14
hCAIII[a]	2.0 × 10^5	7.8 × 10^5	7.0 × 10^5	7.4 × 10^5
hCAIV[a]	74	4900	24	842
hCAVA[a]	63	63	765	682
hCAVB[a]	54	30	720	18
hCAVI[a]	11	45	653	654
hCAVII[a]	2.5	0.9	23	19
hCAIX[b]	25	58	34	18
hCAXII[b]	5.7	3.8	12	13
mCAXIII[a]	17	47	1050	66
hCAXIV[a]	41	1460	755	13

* h, human; m, murine isozyme.
** Errors in the range of 5–10% of the reported value (from 3 different assays).
[a] Human (cloned) isozymes, by the CO_2 hydration method.
[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[16]
[c] IC_{50} value of 25 nM reported by Potter's group.[13b]

Table 2
Crystallographic parameters and refinement statistics for the hCA II—**EMD 486019** adduct

Parameter	Value
X-ray source	Enhance Ultra
Wavelength (Å)	1.5418
Space group	P21
Cell parameters	*a* = 42.1 Å
	b = 41.5 Å
	c = 72.2 Å
	(3 = 104.23°
No of total reflections	46,588
No. of unique reflections	18,403
Completeness (%)[a]	96.0(89.3)
$\langle I/\sigma(I)\rangle$	10.8 (4.0)
Resolution range (Å)	20.00 – 1.90
R-merge (%)[b]	8.0 (21.1)
R-factor (%)[c]	20.0
R-free (%)	25.0
Rmsd of bonds from ideality (Å)	0.009
Rmsd of angles from ideality (°)	1.2

[a] Values in parentheses relate to the highest resolution shell (2.00–1.90).
[b] R-merge = $\sum|I_i - \langle I\rangle|/\sum I_i$.
[c] R-factor = $\sum|F_o - Fc|/\sum F_o$; *R-free* calculated with 5% of data.

the refinement showed features compatible with the presence of one molecule of inhibitor bound to the active site (Fig. 1). These maps are well defined for all the moieties of the inhibitor (data not shown).

Figure 1 shows the new inhibitor to fill the entire active site cavity of hCA II, coordinating to the Zn(II) ion by means of the nitrogen atom of the deprotonated sulfamate moiety, similarly to **CMT** or other sulfamate/sulfonamide/sulfamide inhibitors for which the X-ray structures were resolved earlier.[1–5,16] In particular, the Zn–NH distance in the hCA II—**EMD 486019** adduct is of 1.93 Å, whereas in the **CMT** adduct, the same distance is of 2.15 Å.[13b] This may already be a reason why **EMD 486019** is a slightly better hCA II inhibitor (K_I of 14 nM) as compared to **CMT** (K_I of 21 nM). In fact this shorter distance is a confirmation that there is a better ionic (coordinative) interaction between the Zn^{2+} cation and the sulfamate anion in the first complex as compared to the second one.

As in all other CA II—sulfamate complexes investigated up until now, the coordinated NH moiety of the inhibitor also participates in a strong hydrogen bond with the OH moiety of Thr199, of 2.78 Å (Fig. 2). In the hCA II—**CMT** adduct this bond was much shorter, of 2.42 Å, which is in fact atypical, as in most complexes of hCA II with sulfonamides/sulfamates/sulfamides this bond is always around 2.6–2.8 Å.[1–14,10,17,18] One of the oxygen atoms (S=O) of the sulfamate moiety of **EMD 486019** also participates in a second hydrogen bond (of 2.87 Å) with the NH backbone nitrogen of Thr199 (in the **CMT** complex the corresponding distance is of 3.02 Å). A second oxygen of the same moiety is at about 3.20 Å away from the Zn(II) ion, being "semicoordinated" to the metal ion (the same is true for the **CMT** adduct, the distance being of 3.19 Å, and all other sulfonamides, sulfamates, and sulfamides investigated up to now). Thus, the sulfamate moieties of the two inhibitors **EMD 486019** and **CMT** bind similarly to the Zn(II) ion

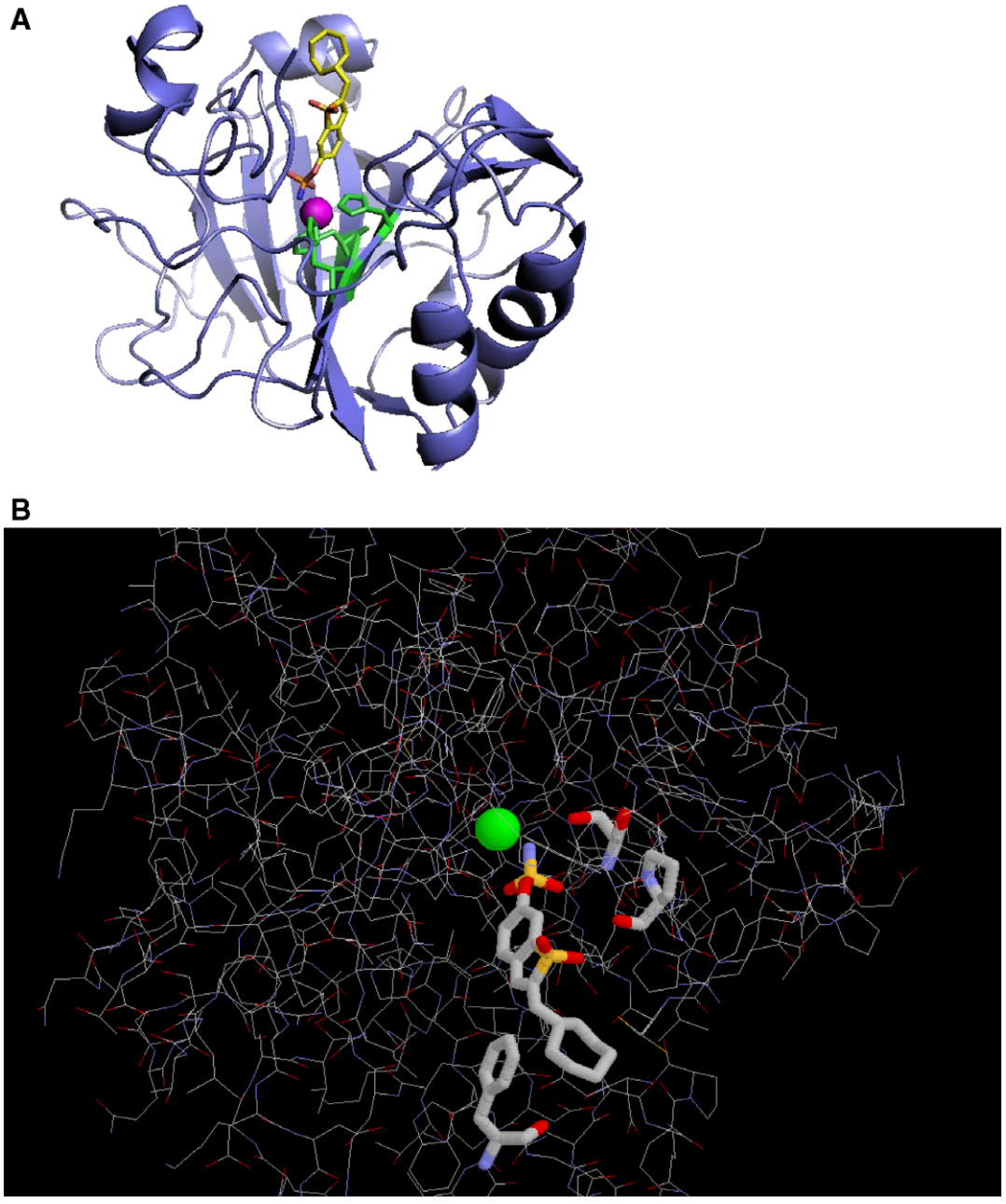


Figure 1. (A) Overall structure of the adduct of hCA II with **EMD 486019**. The protein is represented in the ribbon form, with the Zn(II) ion (central violet sphere), its three histidine ligands (His94, 96, and 119, in green) and the inhibitor molecule (in yellow) shown as sticks. (B) The protein represented as stick model, the Zn(II) ion (green sphere), the inhibitor (stick, CPK colors) and residues Thr199, Pro201, and Phe131 evidenced (CPK colors).

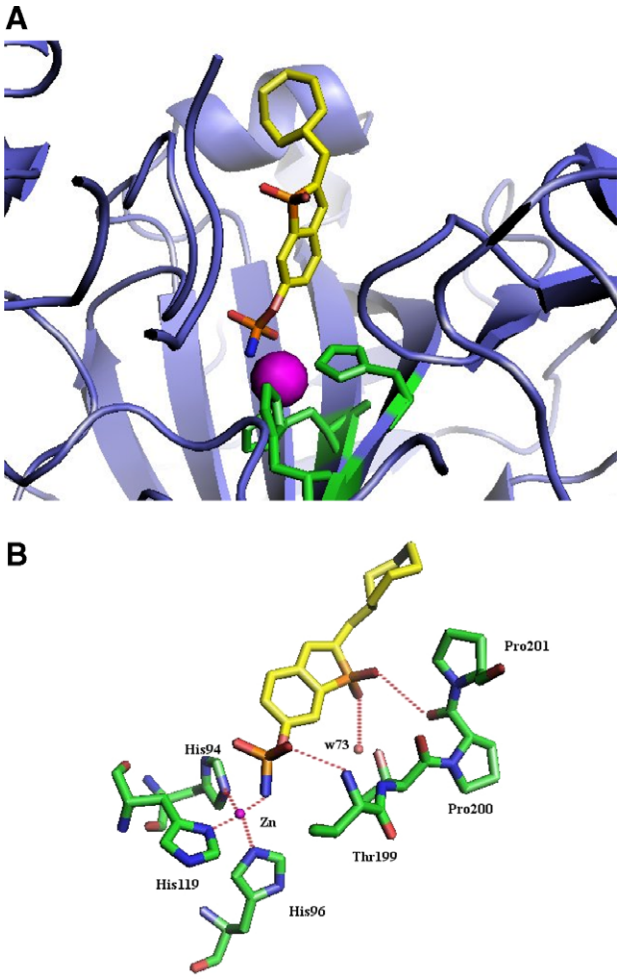


Figure 2. (A) Detailed view of the hCA II active site complexed to **EMD 486019.** (B) Binding of the inhibitor to the Zn(II) ion and hydrogen bonds/dipole-dipole interactions in which **EMD 486019** participates with residues 199–201 and a water molecule (w73), within the enzyme active site cavity.

and Thr199 within the enzyme active site, although there are some differences regarding the length of the hydrogen bonds and the coordination bond in which they are involved.

The two oxygen atoms of the thiophene-*S*,*S*-dioxide moiety of **EMD 486019** are also involved in two interactions (Fig. 2): a strong hydrogen bond (of 2.76 Å) with a water molecule (wat73) and a dipole-dipole interaction (of 3.28 Å), with the carbonyl oxygen of Pro201. In turn, the corresponding CO moiety of **CMT** makes only one weaker hydrogen bond involving the endocyclic oxygen atom of the coumarine cycle with Asn92 (of 3.60 Å).[15b] Thus, **EMD 486019** participates in several favorable interactions with various amino acid residues within the enzyme cavity, explaining its good inhibition profile against hCA II.

Similarly to **CMT**, the organic scaffold of **EMD 486019** participates to a large number of favorable hydrophobic (van der Waals) interactions (distance < 4.6 Å) with amino acid residues present in the hydrophobic half of the hCA II active site, among which Val121, Phe131, Val135, Leu141, Leu198, and Leu 204. One of these amino acids (the most important one in fact), Phe131, is shown in Figure 1B. The distance between one of its phenyl carbons and the 3-carbon atom of **EMD 486019** is in fact of 4.50 Å (data not shown). The cycloheptyl moiety of **EMD 486019** also makes several good van der Waals contacts. For example, the carbon atom labeled 4 in the compound structural formula is at 3.76 Å from one of the methyl groups of Leu204, an amino acid residue situated at the edge (entrance) of the active site cavity (data not shown). This is a very favorable interaction assuring a good complexation even of this tail of the inhibitor to the enzyme cavity.

Figure 3 shows two different orientations for the superposition of the two hCA II—sulfamate adducts, with **EMD 486019** (PDB entry 3DD8) and with **CMT** (PDB entry 1TTM).[13b] It may be observed that, as mentioned above, only the O–SO_2NH_2 moieties of the two inhibitors are readily superposable, whereas the organic scaffold of the two compounds present different orientations when bound to the enzyme cavity. In fact the **CMT** scaffold is almost perpendicular on the plane of the benzothiophene-*S*,*S*-dioxide moiety of **EMD 486019,** although the two compounds possess an identical phenylsulfamate functionality in their molecule. This last functionality (more precisely the substituted-phenyl fragment) are oriented quite differently in the two adducts (Fig. 3). There is also no superposition between the thiophene-*S*,*S*-dioxide moiety of **EMD 486019** and the coumarinyl moiety of **CMT**, nor between the seven-membered rings of the two inhibitors (Fig. 3). In fact the annulated cycloheptene ring present in **CMT** and the corresponding cycloheptylmethyl moiety of **EMD 486019** adopt very different conformations and bind in completely different regions of the enzyme active site. This has two important consequences for the drug design of CAIs: (i) it proves that rather bulky scaffolds (such as the coumarine one, present in **CMT**, or the benzothiophene-*S*,*S*-dioxide one, present in **EMD 486019**) are easily accommodated within the

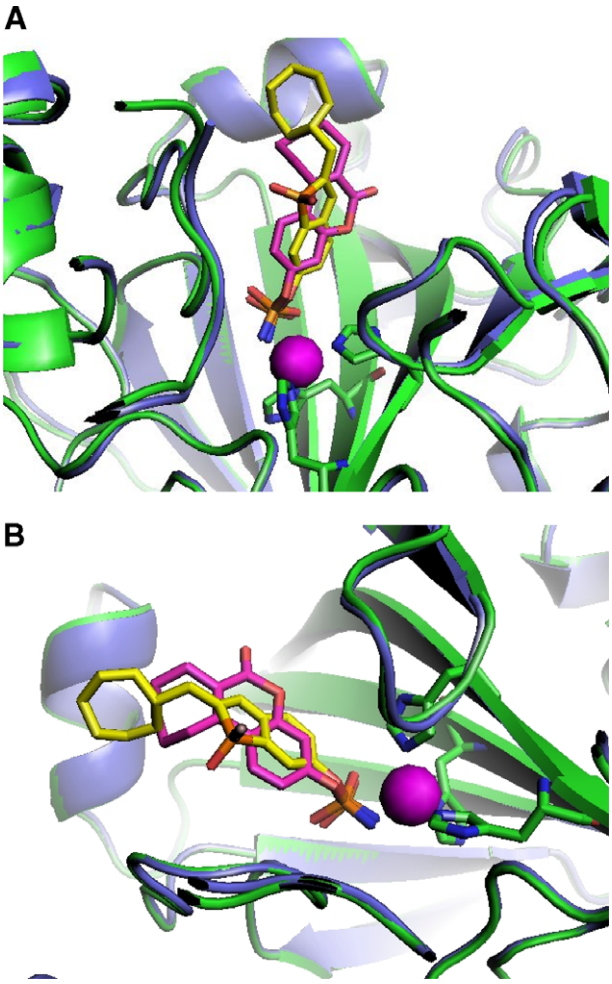


Figure 3. (A and B) Two orientations for the superposition of the hCA II—**EMD 486019** (in yellow) complex reported here with the hCA II—**CMT** (in magenta) complex reported by Potter's group.[13b] The protein backbone is represented in green for the first complex and in blue for the second one.

CA II active site, even when substituted with additional bulky moieties, that is, the $(CH_2)_5$ fragment of **CMT** which is part of the cycloheptene ring, and the cycloheptylmethyl moiety of **EMD 486019**, respectively, and (ii) these last fragments ("tails") of the two inhibitors lie in quite different parts of the enzyme active site (Fig. 3) and do not overlap at all, a fact explaining both the different inhibition profiles of the two compounds (since the entrance of the active site cavity is the region with least conserved amino acid residues among the 12 investigated CA isozymes)[1–3] and allowing us to hope that more isozyme-selective CAIs can be designed by changing just these fragments of the inhibitor molecule, that is, their tails. Indeed, it can be observed that among the four CAIs investigated here, **AAZ, TPM, CMT,** and **EMD 486019**, just the last two ones, possessing the bulkiest tails that also interact with amino acid residues at the entrance of the active site cavity (Fig. 3) show a certain degree of isozyme selective inhibition, as compared to the more compact, promiscuous inhibitors **AAZ** and **TPM** which inhibit most CA isozymes (except CA III and I) with K_Is of <75 nM (Table 1), probably because they are unable to make additional favorable (or unfavorable, clash)[10b] interactions with amino acid residues situated at the entrance of the active site.

In conclusion, the sulfamate **EMD 486019** was investigated for its interaction with twelve catalytically active mammalian isozymes, hCA I—XIV. Similarly to 667-Coumate, a structurally related sulfamate in phase II clinical trials as steroid sulfatase/CA inhibitor with potent antitumor properties, **EMD 486019** acts as potent inhibitor of several physiologically relevant isozymes, such as CA II, VB, VII, IX, XII, and XIV (K_Is in the range of 13–19 nM). The complete inhibition profile of 667-Coumate against these mammalian CAs was also reported here for the first time. Comparing the X-ray crystal structure of the CA II—**EMD 486019** complex with that of the 667-Coumate adduct, we revealed distinct orientations of the two sulfamates when bound within the enzyme cavity, although some interactions with amino acid residues near the zinc ion are preserved in both adducts. CA II/IX potent inhibitors belonging to the sulfamate class are thus valuable clinical candidates with potential for development as antitumor agents with a multifactorial mechanism of action.

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16. Khalifah, R. G. *J. Biol. Chem.* **1971,** *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. 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 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 inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[12–14] and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were in the range of 7.1–13 nM. [12–14].
17. The hCA II- **EMD 486019** complex was co-crystallized at 4 °C by the hanging drop vapor diffusion method.[10,18] Drops containing 5 μl of 10–20 mg/ml hCA II in 50 mM Tris–HCl buffer pH 7.7–7.8 were mixed with 5 μl of precipitant buffer (2.4–2.5 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl pH 7.7–7.8 and 1 mM sodium 4-(hydroxymercury)benzoate) with added 50 mM **EMD 486019** and equilibrated over a reservoir of 1 ml of precipitant buffer. Crystals were transferred into a cryoprotectant solution (20% ethylene glycol), mounted in nylon loop and exposed to a cold (100° K) nitrogen stream. Diffraction data were collected on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). Data were processed with MOSFLM and the CCP4 suite.[19,20] The structure was analyzed by difference Fourier technique, using the PDB file 1BV3[21] as starting model for the refinement. Electron density maps (2Fo-Fc) and (Fo-Fc) were calculated with the REFMAC5 program[22] and displayed using the graphic program O.[23] The final model of the complex had an R-factor of 20.0%, R-free 25.0%, for 18403 reflections in the resolution range 10.0–1.90 Å with a rms deviation from standard geometry of 0.009 Å in bond lengths and 1.2° in bond angles.
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