Carbonic Anhydrase Inhibitors: Clash with Ala65 as a Means for Designing Inhibitors with Low Affinity for the Ubiquitous Isozyme II, Exemplified by the Crystal Structure of the Topiramate Sulfamide Analogue†

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Received July 10, 2006

The sulfamide analogue of the antiepileptic drug topiramate is a 210 times less potent inhibitor of isozyme II of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) compared to topiramate but effectively inhibits isozymes CA VA, VB, VII, XIII, and XIV (K_I in the range of 21−35 nM). Its weak binding to CA II is due to a clash between one methyl group of the inhibitor and Ala65 and may be exploited for the drug design of compounds with lower affinity for this ubiquitous isozyme, as Ala65 is unique to CA II. As shown by X-ray crystallography, the sulfamide analogue binds to CA II with the deprotonated sulfamide moiety coordinated to Zn(II) and with the organic scaffold making an extended network of hydrogen bonds with Thr199, Gln92, His94, Asn62, and Thr200. Its binding to this isozyme is more similar to that of topiramate and quite different from that of the topiramate cyclic sulfate analogue RWJ-37947.

Introduction

Inhibitors of zinc enzyme carbonic anhydrases (CAs, EC 4.2.1.1) have various clinical applications as diuretic, antiglaucoma, antiobesity, and antitumor drugs.[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.[2−6] In all of them, the inhibitor is bound 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: the sulfonamides, the sulfamates, and the sulfamides, possessing the general formula $RXSO_2NH_2$, where X is nothing, O, or NH.[1−6] X-ray crystal stuctures 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 just one sulfamide, the simple derivative $H_2NSO_2NH_2$.[10]

Recently, Maryanoff's group published two reports[16,17] in which a comparison of the sulfamate and sulfamide zinc binding groups in the design of CAIs has been performed. The conclusion of these studies in which 5 pairs of sulfamates and the corresponding sulfamides were investigated for inhibition of isozyme II was that in contrast to the sulfamate moiety, which generally leads to potent CAIs, the corresponding sulfamides are much less inhibitory, these results being in contradiction with earlier data from our group on a much larger series of sulfamide/sulfamate pairs, which have been assayed as inhibitors of four physiologically relevant isoforms, CA I, II, IX, and XII.[18,19] In order to resolve this contradiction and to better understand the drug design of sulfamide-based CAIs, we investigated the inhibition of 10 CA isozymes (i.e., CA I, II, IV, VA, VB, VII, IX, XII, XIII, and XIV) with the sulfamate antiepileptic drug topiramate[20] (for which we reported earlier the X-ray crystal structure in complex with CA II)[9b] and its corresponding sulfamide analogue. These are two of the key compounds also used in the analysis of Maryanoff's group.[16,17] Furthermore, we report the X-ray crystal structure of the topiramate sulfamide analogue in complex with CA II, showing that it is not the sulfamide zinc binding group that is responsible for the weaker inhibition observed with this compound but the fact that one of its moieties is involved in strong clashes with some amino acid residues and more precisely with the methyl group of Ala65, a residue not known up to now to be important for the binding of inhibitors within the CA II active site. These results allow us to hypothesize a novel means for designing potent CAIs that eventually do not inhibit the ubiquitous isoform II, as CA II is the only isozyme possessing Ala in position 65, all other human CAs (hCAs) having different amino acid residues in this position.[1] Indeed, most side effects of the classical sulfonamide drugs used clinically as CAIs, such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4**, dorzolamide **5**, and brinzolamide **6** (Chart 1), are mainly due to inhibition of this ubiquitous isozyme in organs other than the target one(s).[1−6] Designing inhibitors with weaker CA II binding may thus be useful for obtaining compounds targeting isoforms involved in tumorigenesis (such as CA IX and XII)[21] or in adipogenesis (CA VA and CA VB)[22] with fewer side effects compared to the derivatives in clinical use/investigation at this moment.[21,22]

† The X-ray coordinates of the hCA II−sulfamide **8** adduct are available in the PDB with the code 2H15.

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10.1021/jm060807n CCC: $33.50
Published on Web 11/09/2006

Chart 1. Chemical structures of Clinically Used Sulfonamides **1**−**6** and CA Inhibitors **7**−**9** Investigated in the Paper

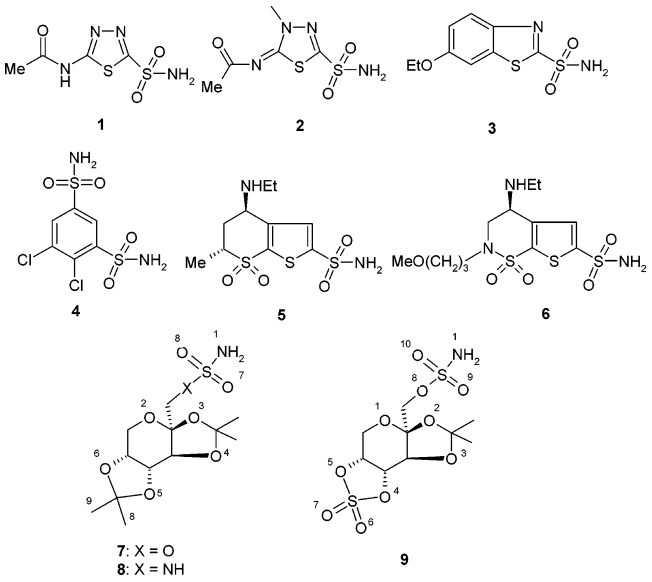

Table 1. Inhibition Data with the Clinically Used Sulfonamides **1**−**6**, the Clinically Used Sulfamate **7** (Topiramate), and the Topiramate Sulfamide Analogue **8** against Isozymes I−XIV, with Literature Data for hCA II Inhibition with the Cyclic Sulfate Analogue of Topiramate (compound **9**) Provided for Comparison

	K_I [b] (nM)								
isozyme[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
hCA I[c]	250	50	25	1200	50000	nt	250	3450	nt
hCA II[c]	12	14	8	38	9	3	10	2135	36[e]
hCA IV[c]	74	6200	93	15000	8500	nt	4900	941	nt
hCA VA[c]	63	65	25	630	42	50	63	32	nt
hCA VB[c]	54	62	19	21	33	30	30	21	nt
hCA VII[c]	2.5	2.1	0.8	26	3.5	2.8	0.9	35	nt
hCA IX[d]	25	27	34	50	52	37	1590[f]	4580	nt
hCA XII[d]	5.7	3.4	22	50	3.5	3.0	3.8	1875	nt
mCA XIII[c]	17	19	nt	23	18	nt	47	30	nt
hCA XIV[c]	41	43	25	345	27	24	1460	25	nt

[a] h = human; m = murine isozyme. [b] Errors in the range of 5−10% of the reported value (from three different assays). nt = not tested (no data available). [c] Human (cloned) isozymes by the CO_2 hydration method. [d] Catalytic domain of human cloned isozyme by the CO_2 hydration method.[23] [e] IC_{50} value (from ref 14) against red cell purified hCA II. [f] K_I of 1590 nM[30a] against the full length enzyme and of 58 nM against the catalytic domain of hCA IX.[9c]

Results

CA Inhibition Studies. Table 1 shows inhibition data of 10 CA isozymes (i.e., CA I, II, IV, VA, VB, VII, IX, XII, XIII, and XIV) obtained by a stopped-flow assay[23] monitoring the physiological reaction (CO_2 hydration to bicarbonate) with the clinically used sulfonamides **1**−**6**, the antiepileptic sulfamate topiramate **7**, and its sulfamide analogue **8**.[20] Literature[14] inhibition data on the cyclic sulfate analogue of topiramate **9**, RWJ-37947 (available only against CA II), for which the X-ray crystal stucture was reported by Recacha et al.,[14] are also provided in Table 1, as they are important for the analysis of the new data reported here.

X-ray Crystallography. Crystallographic parameters and refinement statistics for the hCA II−**8** complex are shown in Table 2, whereas Table 3 presents hydrogen bonds and other contacts of three hCA II−inhibitor adducts: with compound **8** reported here, with topiramate **7**, whose structure was previously reported by us,[9b] and with the topiramate close analogue (the cyclic sulfate **9**), whose crystal structure was reported by Recacha et al. (PDB code 1EOU).[14] Comparison of the major interactions of these three structurally similar inhibitors with the hCA II active site is important for better understanding the drug design of sulfamate/sulfamide CAIs.

Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−**8** Adduct

parameter	value
X-ray source	Enhance Ultra
wavelength (Å)	1.5418
space group	$P2_1$
cell parameters	
a (Å)	42.1
b (Å)	41.5
c (Å)	72.2
β (deg)	104.2
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]	18.6
R_{free} (%)	22.5
rmsd of bonds from ideality (Å)	0.009
rmsd of angles from ideality (deg)	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] $R_{factor} = \sum|F_o - F_c|/\sum F_o$; R_{free} calculated with 5% of data.

Table 3. Hydrogen Bonds and Contacts of Sulfamide **8**, Topiramate **7**,[9b] and RWJ-37947 **9**[14] in Complex with hCA II

8	hCA II residue	distance (Å)
N1	Zn	1.8
O8	Zn	3.0
O7	N Thr199	2.7
N1	Oγ1 Thr199	2.6
O2	Oγ1 Thr200	2.8
O5	Nϵ2 His94	3.3
O6	Nδ2 Asn62	2.7
C9	w176	2.9
C8	Cβ Ala65	3.0
O4	Nϵ2 Gln92	3.3
C8	Nδ2 Asn67	3.3
topiramate **7**	hCA II residue	distance (Å)
N1	Zn	1.9
O7	N Thr199	2.8
O4	Nϵ2 Gln92	2.8
O2	w1134	3.0
O2	Oγ1 Thr200	2.8
O3	w1134	2.8
O6	Nδ2 Asn62	3.0
C8	Cβ Ala65	3.5
C8	Nδ2 Asn67	3.5
RWJ−37947 **9**	hCA II residue	distance (Å)
N1	Zn	2.1
O10	Zn	3.1
O9	N Thr199	2.9
N1	Oγ1 Thr199	2.5
O1	Nϵ2 His94	3.5
C8	Nδ2 Asn62	3.4
C8	Cβ Ala65	5.0
O3	Nϵ2 Gln92	3.3
O1	Nϵ2 Gln92	3.1
C8	Nδ2 Asn67	3.1
C8	Oδ1 Asn67	3.4

The electron density map of **8** bound to the active site of hCA II is shown in Figure 1, whereas the stereoview of the complex (only inhibitor and main residues involved in the binding) and the schematic representation of all interactions of **8** with active site amino acid residues and the metal ion are

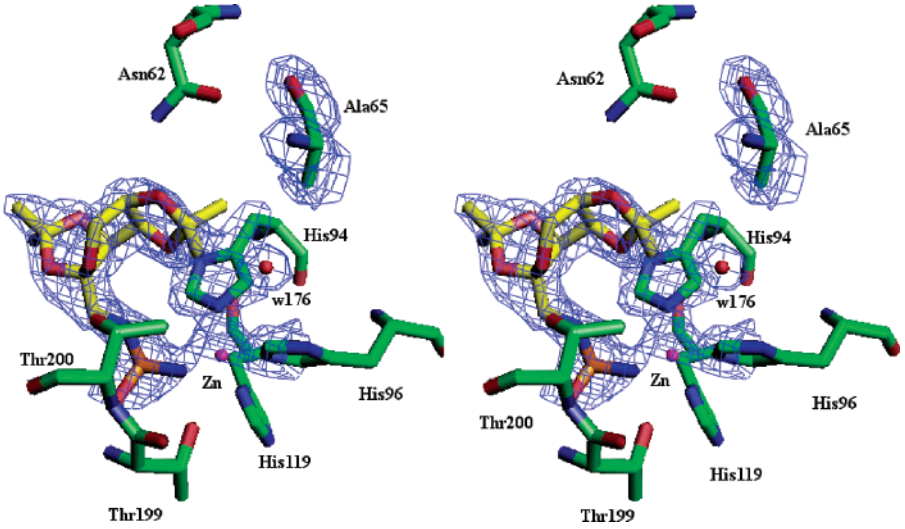

Figure 1. Stereoview of simulated annealing omit $|2F_o - F_c|$ electron density map of inhibitor **8** (in yellow) bound within the hCA II active site at the 1σ contour level. The electron densities of the Zn(II) ion (magenta sphere), zinc ligands (His94, His96, and His119), Thr199, Thr200, Asn62, Ala65, and Wat176 are also shown.

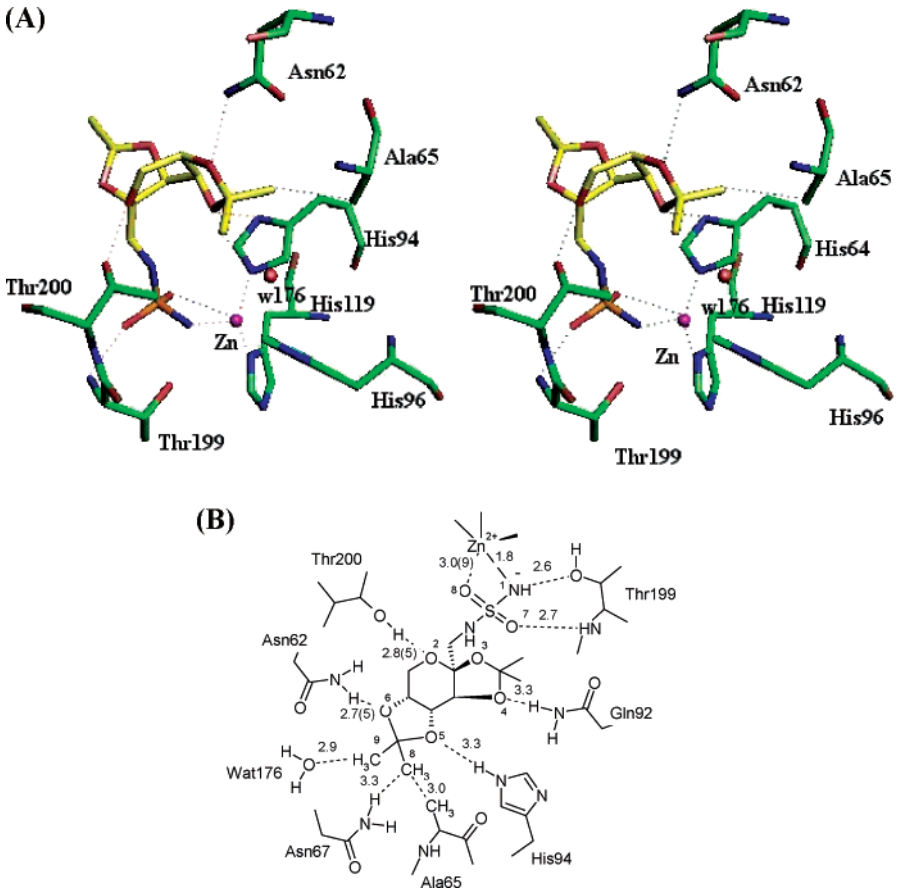

Figure 2. hCA II−**8** complex: (A) stereoview of the zinc coordination sphere and neighboring amino acid residues involved in the binding of the inhibitor (in yellow), with hydrogen bonds and clash interactions shown as dotted lines; (B) detailed schematic representation of all interactions in which **8** participates when bound to the hCA II active site (distances in Å).

presented in Figure 2. Figure 3 shows a superposition of the hCA II−**8** adduct with that of the hCA II−topiramate **7** complex, whereas Figure 4 shows the superposition of the hCA−**8** with the hCA II−**9** adduct.

Discussion

CA Inhibition Studies. The data of Table 1 show that, similar to the clinically used compounds **1**−**7**, the topiramate sulfamide analogue **8** acts as an inhibitor of all 10 investigated CA isozymes but with an inhibition profile quite different from that of all other inhibitors tested earlier and particularly very different from that of topiramate **7**. Thus, **8** is a weak hCA I inhibitor (K_I of 3.45 μM), whereas topiramate **7** behaved as a medium potency inhibitor of this isoform (K_I of 250 nM, similar to that of acetazolamide **1**).[9b] However, the most interesting data are those against hCA II, the main and most ubiquitous CA isozyme in humans.[1−6] As reported by Maryanoff's group,[16,17] we confirm that **8** is a much weaker CAI compared to topiramate

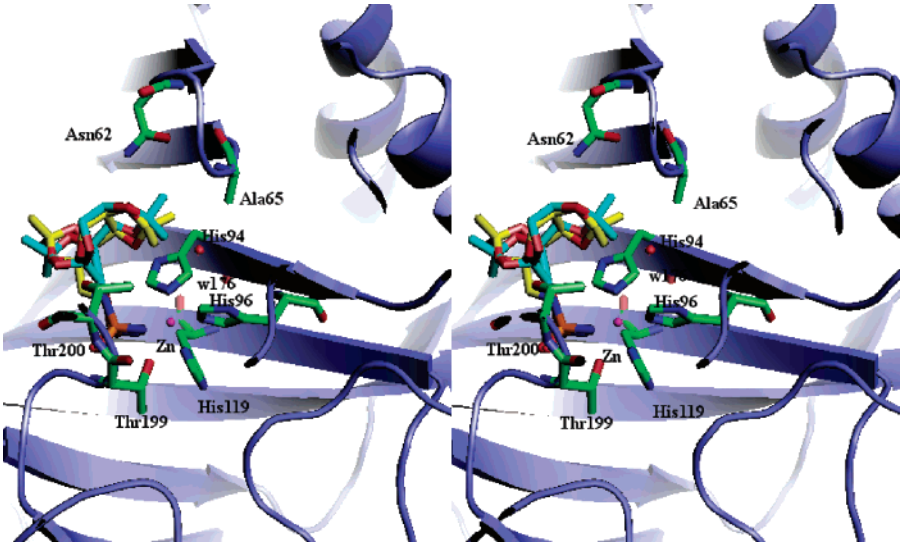

Figure 3. Stereoview of the superposition of the hCA II−topiramate **7** (light-blue) and hCA II−**8** (yellow) adducts (the Zn(II) ion is the magenta sphere). The protein molecules are superimposed by least-squares fitting of the first amino acid (His4) and the last (Phe260) aminoacid residue (rmsd = 0.27). The two zinc binding groups are highly superposable, whereas the main differences between the two adducts concern the C8 and C9 methyl groups and the corresponding dioxolane rings, which show different orientation in the hCA II−**8** adduct because of steric hindrance and clash with the methyl group of Ala65.

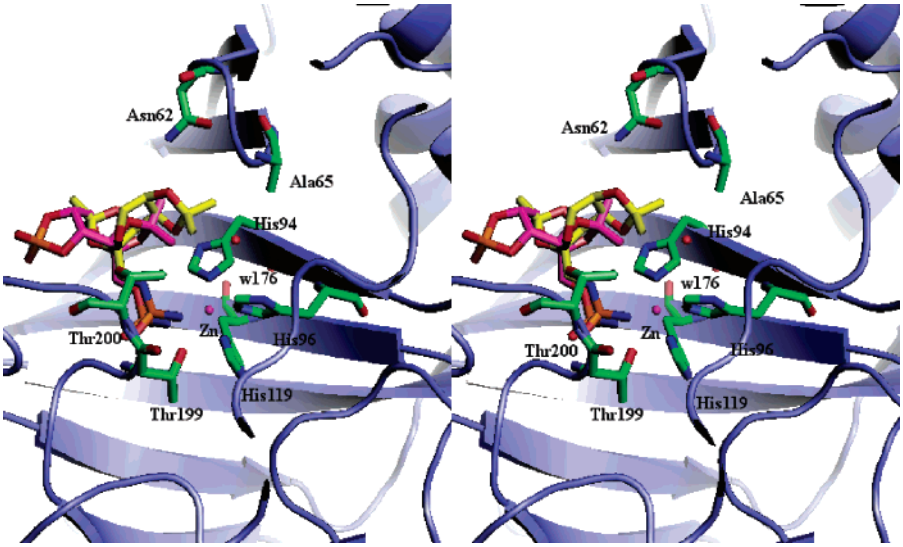

Figure 4. Superposition of the hCA II−RWJ-37947 **9** adduct (PDB code 1EOU, magenta)[14] with the hCA II−**8** adduct (PDB code 2H15, yellow). The two inhibitors show very different orientations and conformations when bound within the enzyme active site. The two zinc binding groups are less superposable compared to the structures shown in Figure 3.

7 against this isozyme, by a factor of 213.5 (Table 1). However, our data are very different from those of Maryanoff's group,[16,17] who reported by means of two different assays an inhibition constant of 650 μM by a pH shift CO_2 hydration method[16] and a binding affinity constant of 25 μM by a thermofluor method.[17] Obviously, the differences between these two values are already very high, and only the second one compares well with our K_I of 2.135 μM, obtained by a stopped-flow method monitoring the CO_2 hydration reaction. We estimate that the first data set of Maryanoff et al.[16] may be technically flawed by the presence of zinc complexing agents in their buffer and will consider only the second data set in our discussion, which is approximately 10 times higher than data obtained by us. It should be mentioned that sulfamide $H_2NSO_2NH_2$ (the lead compound for obtaining organic sulfamide CAIs) was indeed reported to be a very weak CA II inhibitor, with a K_I of 1130 μM.[24] It is hard to believe that the presence of a large organic moiety such as the derivatized fructose group of **8** (and **7**), which interacts extensively with many amino acid residues from the active site (see ref 9b and later in the text), leads to only an approximately 2 times enhancement of the inhibitory power (if one considers the K_I of 650 μM a correct value) of **8** over $H_2NSO_2NH_2$. We shall explain, when discussing the X-ray crystal structure of the hCA II−**8** adduct compared to those of the hCA II−**7**/**9** adducts, the structural basis for the diminished hCA II inhibition observed with sulfamide **8** compared to the closely structurally related sulfamates **7** and **9**, which showed inhibition constants against hCA II in the range 10−36 nM.[9b,14]

The data of Table 1 also show that sulfamide **8** is a weak inhibitor of isozymes hCA IV, hCA IX, and hCA XII (all of them membrane-bound), with inhibition constants in the range 941−4580 nM. Again, the differences with topiramate **7** are noteworthy, since the sulfamate compound is a very weak hCA IV inhibitor (K_I of 4900 nM), a rather weak hCA IX inhibitor (K_I of 1590 nM), but an extremely potent hCA XII inhibitor (K_I of 3.8 nM) (Table 1). On the other hand, compound **8** is an

effective inhibitor of CA isozymes VA, VB, VII, XIII, and XIV, with K_I values in the range 21−35 nM. Thus, Maryanoff et al.'s hypothesis that the sulfamide group is inapproriate for designing potent CAIs[16,17] is wrong, since several isozymes (such as CA VA, CA VB, CA VII, CA XIII, and CA XIV) are highly inhibited by the topiramate sulfamide analogue **8** (as well as by other sulfamides, as we have shown earlier),[18,19] although this compound is indeed a weak CA I, II, IV, IX, and XII inibitor. Furthermore, the particular inhibition profile exhibited by **7** against various CA isozymes (which is, we stress again, very different from that of any other CAI investigated up to now, belonging to the sulfonamide, sulfamate, or sulfamide class[1−6]) might probably be exploited for designing CAIs with weaker binding to the ubiquitous isozyme CA II. Indeed, most side effects of the clinically used sulfonamides **1**−**6**[1−6] (and probably also those of topiramate **7**)[25] are probably due to inhibition of CA II, which is present in high amounts in many tissues/organs in which its targeting is undesirable. Designing compounds with weaker affinity for this isozyme but with a potent effect in inhibiting isoforms involved in particular physiological/pathological processes (such as CA VA, CA VB, CA IX, CA XII, or CA XIII, among others)[21,22,26] may lead to compounds with better efficacy and reduced side effects compared to the presently available sulfonamides/sulfamates.

X-ray Crystallography. Crystallographic refinement of the hCA II−**8** adduct was performed at a final resolution of 1.9 Å. Crystals of the adduct were isomorphous with those of the native protein,[27] allowing for the determination of the crystallographic structure by difference Fourier techniques. The refined structure presents a good geometry with rmsd 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 the refinement showed features compatible with the presence of one molecule of inhibitor bound to the active site (Figure 1). These maps are well defined for all the moieties of the inhibitor **8** except the 8-methyl group. A poorer definition was observed for this moiety, which is sterically hindered by the presence of the methyl group of Ala65 and the carboxamide side chain of Asn67. Thus, as for the hCA II−**9** adduct, the case in which it was also difficult to accommodate the inhibitor within the active site,[14] we experienced the same problem regarding the C8 and to a smaller extent the C9 methyl groups of **8**, which show several close contacts with Ala65, Asn67, and Wat176 (Figure 2 and Table 3). The structure of hCA II in the enzyme−inhibitor complex exhibited only minor differences when compared to that of the native protein,[27] as shown by the rmsd calculated over all the Cα atoms between the hCA II−**8** complex and the unbound enzyme (rmsd of 0.37 Å). Interactions between the protein and the Zn^{2+} ion were entirely preserved in the adduct (data not shown). A careful analysis of the three-dimensional structure of the complex revealed a compact binding between the inhibitor and the enzyme active site, similar to what was reported earlier for the structurally related sulfamate **7** in complex with hCA II,[9b] with the tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the XSO_2NH_2 moiety of the inhibitor and enzyme active site all retained with respect to other hCA II−sulfonamide/sulfamate complexes structurally characterized so far (Figure 2).[7−15] In particular, the ionized nitrogen atom of the sulfamide group of **8** is coordinated to the zinc ion at a distance of 1.8 Å, much shorter than the corresponding distance in the topiramate **7** adduct (1.9(7) Å) or the RWJ-37947 **9** adduct (2.1 Å) (Table 3). The N1 nitrogen is also hydrogen-bonded to the hydroxyl group of Thr199 (N−Thr199OG = 2.7 Å), which in turn interacts with the Glu106OE1 atom (2.5 Å, data not shown). The inhibitor O7 atom is hydrogen-bonded to the backbone amide of Thr199 (ThrN−O2 = 2.7 Å), whereas the O8 atom is 3.0(9) Å away from the catalytic Zn^{2+} ion, being considered weakly coordinated to the metal ion.[7−15] All these interactions have also been observed in the adducts of hCA II with topiramate **7** and RWJ-37947 **9**, but the corresponding distances are slightly different (Table 3). The second NH group of **8**, similar to the corresponding oxygen atom of topiramate,[9b] does not participate in any interaction with the protein or the metal ion, unlike the corresponding atom of RWJ-37947, which interacts with the endocyclic oxygen O1.[14] This is another important difference between the inhibitors discussed here. (It must be mentioned that the original numbering from Recacha et al.[14] has been maintained for **9**, the numbering from Casini et al.[9b] for the topiramate adduct, whereas the numbering of atoms in the sulfamide derivative **8** discussed here was adapted from the corresponding topiramate numbering. However, there are some discrepancies between these numbering systems, which can be followed more easily by looking at the structures of these derivatives in which the crystallographic numbering was included.)

The endocyclic sugar oxygen of **8** (O2) makes a hydrogen bond of 2.8(5) Å with the hydroxyl moiety of Thr200 (the same interaction is present in the topiramate adduct, where the distance is 2.8(4) Å) (Table 3). In contrast, the corresponding oxygen atom of compound **9** does not interact with Thr200 but makes a much weaker van der Waals interaction (of 3.5 Å) with the NH moiety of His94. Thus, it is already clear that topiramate **7** and its sulfamide derivative **8** bind to hCA II in a diverse conformation and in active site regions compared to RWJ-37947 **9**. This is clearly illustrated in the superpositions of the three structures of Figures 3 and 4.

Three oxygen atoms of the dioxolane rings of **8**, i.e., O4, O5, and O6, participate in three hydrogen bonds with Gln92, His94, and Asn62, respectively (Figure 2B). One of them (involving O5) was not observed in the topiramate adduct,[9b] whereas the other two are present in both adducts (Table 3), although the distances between the corresponding pairs of atoms are rather different. Thus, the distance between O6 and the amide nitrogen of Asn62 is in the range 2.7−3.0 Å for the two adducts of hCA II with **8** and **7**, respectively, whereas the interaction with Gln92 is a strong one in the case of topiramate (distance of 2.8 Å) and much weaker in the topiramate sulfamide adduct (distance of 3.3 Å). Only one of the oxygen atoms of RWJ-37947 **9**, O3 (corresponding to O4 in the topiramate numbering), participates in this type of interaction with Gln92 (distance of 3.3 Å; see Table 3).

However, the most important differences in the structures of the adducts of topiramate **7** and its sulfamide analogue **8** in complex with hCA II regard the C8 and C9 methyl groups of the inhibitors. In the case of the topiramate adduct, the C8 group is in van der Waals contacts (distances of 3.5 Å;[9b] see Table 3) with the methyl group of Ala65 and the carboxamide moiety of Asn67. For the topiramate sulfamide **8** adduct the corresponding distances are much shorter (in the range 3.0−3.3 Å), leading to an important clash between the C8 methyl and the methyl side chain of Ala65. In fact, this was the main reason that we experienced difficulties in fitting the electron density of the inhibitor **8** in this region of the active site, which is sterically hindered by the presence of these amino acid side chains (Ala65 and Asn67) and also by the presence of the second

methyl group of the inhibitor (C9) and a water molecule with which it is in close contact (Wat176−C9 = 2.9 Å) (Figures 1 and 3). The electron density of this water molecule (Wat176) is very clearly defined in the electron density maps, as shown in Figure 1. These repulsive interactions were not observed in the hCA II−topiramate **7** adduct, and they constitute the only explanation regarding the important differences of activity between the two compounds, with topiramate being approximately 210 times a better hCA II inhibitor compared to its sulfamide analogue (Table 1).

Superpositions of the three X-ray crystal structures of compounds **7**−**9** in adducts with hCA II (Figures 3 and 4) clearly illustrate the following two important findings. (i) Although differing only by the replacement of an oxygen atom with an NH moiety and although binding rather similarly (in the same conformation and the same region of the active site) to the enzyme, topiramate **7** and its sulfamide analogue **8** show very different inhibitory capacity against hCA II. The first compound is a potent hCA II inhibitor, whereas the second one is a weak one, and this difference of activity is due to the clash between the C8 methyl group of the sulfamide inhibitor with Ala65 (and not because the sulfamide moiety is not a good zinc binding group compared to the sulfamate one, as claimed by Maryanoff et al.).[16,17] (ii) On the other hand, in the case of the topiramate adduct, the C8 methyl makes favorable van der Waals interactions with the methyl group of Ala65; the sulfamide **8** and the structurally related sulfamate **9** bind in a very different manner to the hCA II active site, a situation also evidenced earlier for the hCA II−topiramate adduct.[9b,28] Thus, resolving the X-ray crystal structures of even very closely structurally related inhibitors (such as compounds **7**−**9** discussed here) is highly relevant for understanding in detail the drug design of such enzyme inhibitors.

But how relevant are our findings for the design of better drugs based on CAIs? Clearly, clash interactions between an inhibitor and an amino acid residue within the active site, which may prevent the strong binding of the inhibitor, may be useful for designing compounds with selectivity for some isoforms, provided that the interacting residue is present only in isoforms that should be not inhibited. This is especially important for families of enzymes with many isoforms, such as the CAs (in humans 15 CAs are known, 12 of which are catalytically active),[1−6] in which one isozyme is ubiquitous and its inhibition may be deleterious. This happens to be the case with CA II, which is an extremely abundant housekeeping enzyme in most cells in humans.[1−6] On the other hand, it is ever more obvious that selective inhibition of isoforms other than CA II, which are involved in specific physiologic/pathologic processes (such as CA IX and XII involved in tumorigenesis[21] or CA VA and CA VB involved in adipogenesis[22]) or show a restricted localization only in some tissues/organs (such as CA VA,[22] CA VII,[29] or CA XIII[26]), may lead to drugs with less severe side effects. A close look at the amino acid sequences of all known human CAs[1] show that only CA II has Ala in position 65, whereas this amino acid is Ser in CA I, CA IV, CA VB, CA VII, CA IX, CA XII, and CA XIII, Thr in CA III, CA VI, and CA XIV, and Leu in CA VA. We do not want to imply that just one amino acid substitution may change the binding affinity of an inhibitor for the active site, since it is clear that isozymes possessing Ser65, such as hCA I, hCA IV, hCA IX, or hCA XII, are only slightly inhibited by sulfamide **8**, whereas other isoforms possessing the same amino acid, such as CA VB, CA VII, or CA XIII, are quite well inhibited by this compound. What we stress here is that by resolving the X-ray crystal structure of hCA II in adduct with **8**, we discovered an amino acid residue that is unique to the hCA II active site and whose clashing with some moieties of an inhibitor may constitute a means for designing more selective CAIs, with weaker binding to this ubiquitous isozyme but preserving a strong affinity for other isoforms. As far as we know, this is the first example in which CA II-sparing inhibitors are evidenced, and this fact is explained at the molecular level. Our findings may thus be quite useful in designing compounds with less CA II inhibitory activity but that maintain strong inhibition of clinically relvant isoforms such as CA VA, CA VB, CA IX, CA XII, CA XIII, or CA XIV among others.

Conclusion

The sulfamide analogue of the antiepileptic sulfamate topiramate is roughly 210 times a less potent inhibitor of isozyme II of CA compared to topiramate but effectively inhibits isozymes CA VA, VB, VII, XIII, and XIV (K_I values in the range 21−35 nM). Its weak binding to CA II is due to a clash between one methyl group of the inhibitor and Ala65 and might be exploited to obtain compounds with lower affinity for this ubiquitous isozyme. Ala65 is an amino acid residue unique only to CA II, an isoform whose inhibition is sometimes detrimental to the activity of inhibitors targeting isozymes involved in tumorigenesis, lipogenesis, or glaucoma formation. The sulfamide analogue of topiramate otherwise binds in the usual way to CA II, similar to other sulfonamide and sulfamate inhibitors investigated earlier. Thus, the deprotonated sulfamide moiety coordinates to Zn(II), and the organic scaffold participates in an extended network of hydrogen bonds with Thr199, Gln92, His94, Asn62, and Thr200. Its binding to this isozyme is more similar to that of topiramate and quite different from that of the topiramate cyclic sulfate analogue RWJ-37947. Exploiting the unfavorable interaction between the methyl group of Ala65 (unique to CA II among the many mammalian CA isoforms described up to now) and moieties present in a newly dsigned CA inhibitor (such as derivative **8** investigated here) might thus lead to compounds with lower affinity for the ubiquitous isoform CA II and to drug candidates possessing fewer side effects.[35]

Experimental Section

Materials. Sulfonamides **1**−**6** and sulfamate **7** are commercially available compounds (from Sigma-Aldrich or Johnson & Johnson), whereas the sulfamide derivative **8** was prepared as reported in the literature.[20] The 10 CA isozymes used in the experiments were recombinant ones obtained and purified as reported earlier by this group.[21,22,26,29,30]

CA Inhibition Assay. An Applied Photophysics (Oxford, U.K.) 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 and 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction.[23] 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 (10 mM) were prepared in distilled−deionized water with 10−20% (v/v) DMSO (which is not inhibitory at these concentrations), 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 and represent the mean from at least three different determinations.[23]

X-ray Crystallography. The hCA II−**8** complex was cocrystallized at 4 °C by the hanging drop vapor diffusion method.[9b] 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 **8** 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 KM4 CCD sapphire detector using Cu Kα radiation (1.5418 Å). Data were processed with MOSFLM and the CCP4 suite.[31,32] The structure was analyzed bythe difference Fourier technique, using the PDB file 1BV3[27] as the starting model for the refinement. Electron density maps ($2F_o - F_c$) and ($F_o - F_c$) were calculated with the REFMAC5 program[33] and displayed using the graphic program O.[34] The final model of the complex had an *R* factor of 18.6%, R_{free} of 22.3%, for 18 403 reflections in the resolution range 20.0−1.9 Å with an rmsd from standard geometry of 0.009 Å in bond lengths and 1.2° in bond angles.

Acknowledgment. This research was financed in part by a grant of the 6th Framework Programme of the European Union (EUROXY project) and by an Italian FIRB project (Grant MIUR/FIRB RBNE03PX83_001). Special thanks are given to Dr. Giuseppina De Simone (University of Naples, Italy) for helpful discussions.

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JM060807N