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Carbonic anhydrase inhibitors. Interaction of the antiepileptic drug sulthiame with twelve mammalian isoforms: Kinetic and X-ray crystallographic studies☆

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Abstract—Sulthiame, a clinically used antiepileptic, was investigated for its interaction with 12 catalytically active mammalian carbonic anhydrase (CA, EC 4.2.1.1) isoforms. The drug is a potent inhibitor of CA II, VII, IX, and XII (K_Is of 6–56 nM), and a medium potency inhibitor against CA IV, VA, VB, and VI (K_Is of 81–134 nM). The high resolution crystal structure of the hCA II-sulthiame adduct revealed a large number of favorable interactions between the drug and the enzyme which explain its strong low nanomolar affinity for this isoform and may also be exploited for the design of effective inhibitors incorporating sultam moieties.

Sulthiame (4-sulfamoyl-phenyl-[1,2]thiazinane-1,1-dioxide) is a clinically used antiepileptic drug (AED) since 1964.[1–3] Although it exerts remarkable anticonvulsant effects, being the drug of choice in adult patients with benign epilepsy with centrotemporal spikes and for symptomatic focal epilepsies of children,[1,2] sulthiame is a poorly investigated pharmacological agent. In fact, only one study of 1964[3] reports the carbonic anhydrase (CA, EC 4.2.1.1) inhibitory effects of this primary sulfonamide, and more recently, in 1994, the unique pharmacokinetic study of sulthiame in epileptic patients has been published.[4] There are however increasing evidences regarding the efficacy of sulthiame in some forms of epilepsy which do not respond to other AED, such as for example its use in epileptic patients also affected by Rett syndrome.[1,2]

Some other CA inhibitors (CAIs) then sulthiame, such as among others acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, and dichlorophenamide **4**, were used with little success as anticonvulsants in the treatment of epilepsy,[5–7] as they led to the development of tolerance after an initial period of efficacy.[5a] The clinically used topically acting antiglaucoma sulfonamides dorzolamide **5** and brinzolamide **6** were never investigated, as far as we know, for anticonvulsant activity. However, the links between CAIs and seizures are quite intricate, since two newer AEDs, that is, the sulfamate topiramate **7**[8] and the sulfonamide zonisamide **8**,[9] possess strong CA inhibitory activity against a large number of physiologically relevant CA isoforms among the 15 presently known in humans.[5,6] Indeed, several CA isozymes (such as CA II, VB, VII, XIV, etc.) have been pointed out for their contribution to epileptiform activity,[5,6,10,11] but much research is warranted in this field in order to better understand which are the real targets of the anticonvulsant CAIs and what structure–activity relationship (SAR) rules govern this class of pharmacological agents.

Tanimukai et al.[3] reported in 1964 that sulthiame **9** is a potent inhibitor of the human red blood cell CA

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☆ The coordinates of the hCA II-sulthiame adduct have been deposited in PDB, ID code 2Q1Q.
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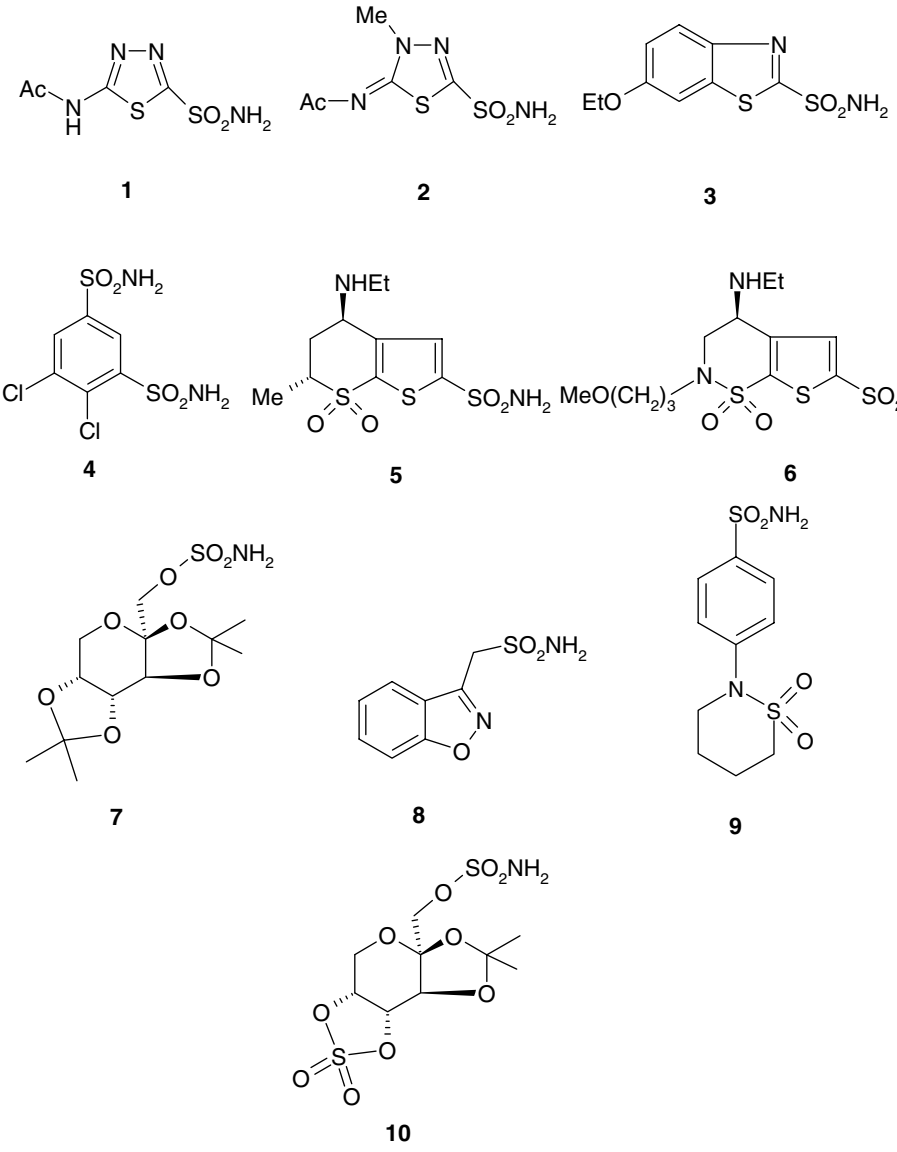

(presumably the physiologically dominant isoform hCA II), with an IC_{50} of 6.4 nM (the CA isozymes started to be distinguished in the early 70 s, so that in the period when this study was published[3] it was generally accepted that blood contains just one CA isozyme, which is not true).[5,6] No other inhibition data were thereafter reported with this drug. Here we investigate the in vitro inhibition of all 12 catalytically active mammalian isozymes (CA I—XIV) with sulthiame **9** and compare its activity with that of the clinically used sulfonamides/sulfamate **1**–**8** and **10**. We also report the high resolution X-ray crystal structure of the hCA II-sulthiame adduct. These data may thus fill a gap in the literature regarding the CA inhibitory properties of a clinically used AED on one hand, and might be useful for the drug design of CAIs targeting brain CA isoforms involved in epileptogenesis on the other one.

Inhibition data of the mammalian isoforms (h, human; m, mouse enzyme) hCA I, hCA II, hCA III, hCA IV, hCA VA, hCA VB, hCA VI, hCA VII, hCA IX, hCA XII, mCA XIII, and hCA XIV with the clinically used compounds **1**–**10** are shown in Table 1 (all isozymes were recombinant ones, being obtained in-house as reported earlier).[12–15,16a] Some inhibition data for the clinically used derivatives **1**–**8** are also reported here for the first time, as they were not available in the literature.

Data of Table 1 show that the AED sulfonamide sulthiame **9** acts as an inhibitor of all 12 CA isozymes investigated here, with a variable efficacy, similarly to the other clinically used sulfonamides/sulfamate **1**–**8**. Thus, sulthiame is a highly effective CA II, VII, IX, and XII inhibitor, with inhibition constants in the range of 6–56 nM. Our data are in good agreement with the earlier published data[3] on the inhibition of red blood cell CA with this drug, although the assay methods in the two studies are rather different (both of them however monitor the physiological reaction catalyzed by these enzymes).[17] The best inhibition was achieved against hCA II and VII (K_I of 6–7 nM), isozymes for which the drug was active in the low nanomolar range, similarly to the clinically used compounds **1**–**3** and **5**–**7** (K_Is in the range of 3–14 nM against hCA II, and 0.9–43 nM against hCA VII, respectively). A special

Table 1. Inhibition data with the clinically used derivatives acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4**, dorzolamide **5**, brinzolamide **6**, topiramate **7**, zonisamide **8**, sulthiame **9**, and RWJ-37947 **10**, against CA isozymes I—XIV by a stopped-flow technique monitoring the CO_2 hydration reaction.[17]

Isozyme*	K_I (nM)**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**[a]	**10**
hCA I[b]	250	50	25	1200	50000	45000	250	56	374	nt
hCA II[b]	12	14	8	38	9	3	10	35	7[e]	36 [f]
hCA III[a,b]	2.10^5	7.10^5	$1.1.10^6$	$6.8.10^5$	$7.7.10^5$	$1.1.10^5$	$7.8.10^5$	$2.2.10^6$	$6.3.10^5$	nt
hCA IV[b]	74	6200	93	15000	8500	3950[a]	4900	8590[a]	95	nt
hCA VA[b]	63	65	25	630	42	50	63	20	81	nt
hCA VB[b]	54	62	19	21	33	30	30	6033[a]	91	nt
hCA VI[b]	11	10	43	79	10	0.9	45	89[a]	134	nt
hCA VII[b]	2.5	2.1	0.8	26	3.5	2.8	0.9	117[a]	6	nt
hCA IX[c]	25	27	34	50	52	37	58[d]	5.1	43	nt
hCA XII[c]	5.7	3.4	22	50	3.5	3.0	3.8	11000	56	nt
mCA XIII[b]	17	19	50[a]	23	18	10	47[a]	430[a]	1450	nt
hCA XIV[b]	41	43	25	345	27	24	1460	5250[a]	1540	nt

Data for compounds **1**–**8** and **10** are from the literature.[12–16] When they were not available, the compounds have been tested in the same conditions as **9** and the data are indicated as new.

[a] New data reported here for the first time.

[b] Full length, recombinant enzyme.

[c] Catalytic domain.

[d] The data against the full length enzyme is of 1590 nM.

[e] Lit.[3] IC_{50} for human red cell enzyme (presumably CA II) is 6.4 nM, in strong agreement with our data.

[f] From Ref. 16b.

* h, human; m, murine isozyme.

** Errors in the range of ±5% of the reported data from three different assays.

mention should be made regarding the CA VII inhibition data, since this isoform is predominantly present in the brain,[11] and its inhibition was correlated to changes in pH and ionic (K^+ and Cl^-) composition within the cells expressing it, phenomena which were demonstrated to be responsible for epileptogenesis.[5,11] Indeed, strong CA VII/CA II inhibition may be one of the main factors characterizing compounds acting as anticonvulsants in this class of pharmacological agents, since, as observed from data in Table 1, all derivatives **1**–**9** except zonisamide **8** against hCA VII are very effective inhibitors of these isozymes (K_Is in the range of 0.8–35 nM). It is of course difficult to explain why zonisamide is a less effective hCA VII inhibitor (K_I of 117 nM) while still possessing excellent anticonvulsant properties. However, it may be observed that zonisamide **8** appreciably inhibits many CA isozymes, in the low nanomolar range, such as CA II, CA VA, and CA IX, also possessing moderate-potent CA VII inhibitory activity. These data tentatively prompt us to propose that compounds with effective CA II and CA VII inhibitory activity might show good anticonvulsant action and thus lead to novel AEDs.

Sulthiame **9** is also an effective inhibitor of the two tumor-associated isozymes CA IX and XII (Table 1), similarly to derivatives **1**–**7**. The behavior of sulthiame against hCA IX closely parallels that of all these standard CAIs (also that of zonisamide **8** which has the most distinct inhibition profile among the investigated derivatives discussed here, but this compound is also the only aliphatic sulfonamide). Against hCA XII sulthiame **9** is slightly less effective an inhibitor as compared to derivatives **1**–**3** and **5**–**7**, being similar to the only other benzenesulfonamide derivative in the investigated series, that is, dichlorophenamide **4**.[16c] Sulthiame **9** acts as a medium potency inhibitor against isoforms hCA IV, hCA VA, hCA VB, and hCA VI, with K_Is in the range of 81–134 nM (Table 1). Against hCA I, a highly abundant isozyme in the blood and gastrointestinal tract,[5,6] sulthiame acts as a modest inhibitor (K_I of 374 nM), similarly to acetazolamide **1** and topiramate **7**. Two isozymes which showed lower affinity for **9** were mCA XIII and hCA XIV, which were inhibited with K_Is in the range of 1.45–1.54 μM. As all sulfonamide/sulfamate CAIs, hCA III was also the least susceptible isoform to inhibition with sulthiame, with a K_I of 630 μM.[5,6] This is clearly due to the presence of a very bulky amino acid residue just in the middle of the active site cavity (Phe198) of this isozyme, which interferes with the binding of inhibitors.[5,6] All these data clearly show that sulthiame **9** has a unique inhibition profile among all clinically used CAIs, with the drug possessing high affinity for the two isozymes presumably involved in epileptogenesis (hCA II and VII), and also for the tumor associated isoforms CA IX and XII. Thus, in addition to its use as an AED, the potent in vitro CA IX/XII inhibition properties of the compound warrant the study of its use as a potential antitumor agent.[15]

In order to better understand the effective hCA II inhibitory activity of **9** and also to learn some lessons for the drug design of new CAIs, we report the high resolution X-ray crystal structure of the hCA II-sulthiame adduct (Table 2).[18] The three-dimensional structure of the enzyme was very similar to that of hCA II without any ligand bound,[19] as judged by an rms deviation for Cα atoms of 0.35 Å only. Examination of the initially calculated electron density maps in the active-site region showed the clear evidence for the binding of sulthiame

Table 2. Crystallographic parameters and refinement statistics for the hCA II-sulthiame complex

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 42.19Å
	b = 41.28 Å
	c = 72.07 Å
	β = 104.29°
Data collection statistics (20.0–1.90 Å)	
No. of total reflections	62612
No. of unique reflections	19099
Completeness (%)[a]	98.84 (93.9)
F2/sig (F2)	7.60 (1.80)
R-sym (%)	7.20 (25.0)
Refinement statistics (20.0–1.90 Å)	
R-factor (%)	19.7
R-free (%)[b]	25.1
Rmsd of bonds from ideality (Å)	0.009
Rmsd of angles from ideality (°)	1.30

[a] Values in parenthesis relate to the highest resolution shell (2.00–1.90).
[b] Calculated using 5% of data.

within the active site cavity. The electron density of all moieties of the inhibitor is in fact very well defined (Fig. 1).

The tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the SO_2NH_2 moiety of sulthiame and enzyme active site are all retained with respect to other hCA II-sulfonamide/sulfamate/sulfamide complexes structurally characterized so far (Figs. 1 and 2).[5,16c,8,9,19] In particular, the ionized nitrogen atom of the sulfonamide group of **9** is coordinated to the zinc ion at a distance of 1.98 Å. This nitrogen atom is also hydrogen bonded to the hydroxyl group of Thr199 (N···Thr199OG = 2.67 Å), which in turn interacts with the Glu106OE1 atom (2.50 Å, data not shown). One oxygen atom of the coordinated sulfamoyl moiety is hydrogen bonded to the backbone amide of Thr199 (ThrN···O2 = 2.92 Å), whereas the second oxygen atom of this moiety is 3.01 Å away from the catalytic Zn^{2+} ion, being considered as weakly coordinated to the metal ion.[5,8,9,19] All these interactions have also been observed in the adducts of hCA II with topiramate **7** and zonisamide **8**, but the corresponding distances are of course different.[8,9] The phenyl-[1,2]thiazinane-1, 1-dioxide scaffold of sulthiame is accommodated perfectly within the active site channel, being oriented toward the hydrophilic half of it, and participating to a host of favorable interactions with various amino acid residues and water molecules (Figs. 1 and 2). It should be noted the particular conformation of this scaffold (Fig. 1), with the planes of the two rings perpendicular to each other, a situation never observed before in other CA—inhibitor adducts. The endocyclic SO_2N moiety (the sultam one) of **9** also participates in four strong hydrogen bonds and at least two Van der Waals interactions, which probably contribute to the great stabilization of the enzyme-inhibitor adduct and explain the very effective inhibitory properties of the compound against this isoform (Figs. 1 and 2). Thus, one oxygen atom of this moiety makes two hydrogen bonds with the terminal $CONH_2$ group of Gln92 and a water molecule, Wat179 (of 2.62 and 3.08 Å, respectively) as well as a van der Waals contact with the Oδ1 atom of Asn67 (data not shown) of 3.34 Å. The second oxygen of the sultam moiety participates to two hydrogen bonds with two different water molecules, Wat177 and Wat70

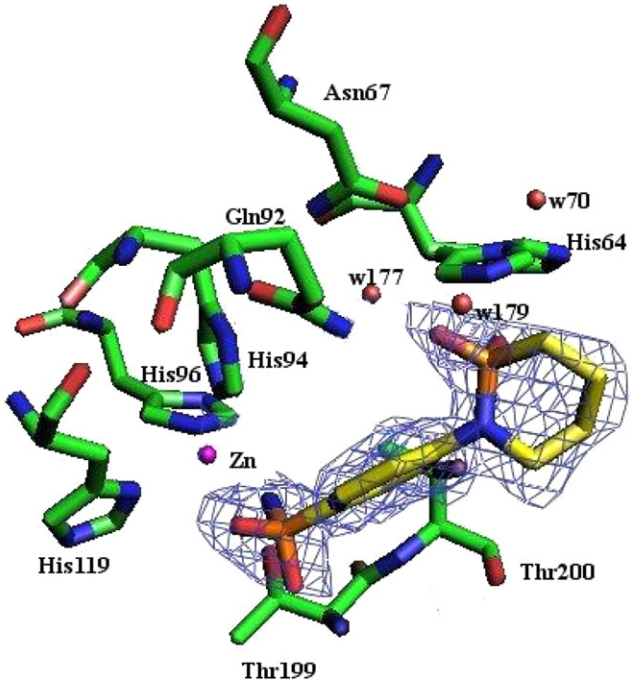

Figure 1. Electron density map of sulthiame (in yellow) bound within the hCA II active site. The Zn(II) ion of the enzyme, its three histidine ligands (His94, 96 and 119), residues involved in the binding of the inhibitor (Thr199, Thr200, Asn67, His 64—double conformations, and Gln92) as well as three water molecules participating in hydrogen bonds with the endocyclic SO_2 moiety of the inhibitor are also shown.

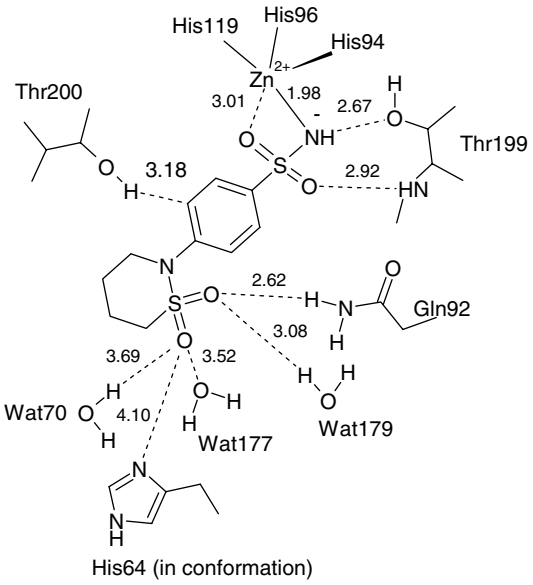

Figure 2. Detailed schematic representation for interactions in which sulthiame **9** participates when bound to the hCA II active site (figures represent distances in Å; dotted lines are hydrogen bonds or significant van der Waals contacts).

(of 3.52 and 3.69 Å, respectively), and a van der Waals contact with the Nε2 atom of the imidazole moiety of His64 (of 4.10 Å). It is interesting to note that this amino acid residue critical for the CA catalytic cycle is present in this adduct in its two conformations, the 'in' and 'out' one.[5,6] Usually in all other adducts with CAIs investigated earlier, only one of these conformations was present.[24] Thr200 also makes a strong van der Waals contact (of 3.18 Å) with a carbon atom of the phenyl moiety in meta to the zinc binding group of sulthiame (Fig. 2). It is thus obvious that the very characteristic sultam moiety present in sulthiame confers quite favorable features to this compound, orienting it in a way which allows many good interactions with hydrophilic amino acid residues and water molecules and leading to a great stability of the complex. It is interesting to note that the sultam group has never been exploited for the design of other CAIs than sulthiame, as far as we know.[25]

In order to better understand whether the active site binding region of CAIs acting as anticonvulsants/AEDs is the same or different, in Figure 3 we show the superpositions of the hCA II-sulthiame adduct with that of the hCA II-topiramate **7**[8] and that of the hCA II-RWJ-37947 **10**[16b] adducts (the topiramate adduct coordinates were not deposited in PDB and are available from the authors, whereas the RWJ-37947 PDB entry used was 1EOU).[16b] RWJ-37947 **10**, the cyclic sulfate analogue of topiramates was shown by Maryanoff's group to act as a potent CAI (only the CA II inhibition data were published) and as an effective anticonvulsant.[16b] Data of Figure 3a show that whereas topiramate **7** practically fills the entire active site cavity of hCA II,[8] sulthiame **9** binds oriented toward the hydrophilic half of the active site. Except for the zinc binding groups of the two inhibitors **7** and **10** which are superposable, no other parts of their molecules occupy the same region(s) within the enzyme cavity. But, as observed from Figure 3b, although structurally quite similar to topiramate **7**, the cyclic sulfate RWJ-37947 **10** binds in a completely different active site region of hCA II as compared to topiramate.[8,16b] On the other hand, the inhibitors **9** and **10** show some slight superposability (Fig. 3b) when bound to hCA II, due to the fact that the sugar moiety of **10** is oriented toward the hydrophilic half of the active site (where the scaffold of sulthiame **9** also binds), unlike the same group from topiramate **7** (see also Fig. 3a and Ref. 8) which binds more centrally and in a different orientation. The conclusions of these superposition/inhibition data are: (i) even for structurally very similar compounds (such as **7** and **10**) the binding mode of the inhibitor within the enzyme active site may be very different and non-intuitive,[8,16b] and (ii) there is no straightforward correlation between the inhibitor binding mode within the hCA II active site cavity and its anticonvulsant activity, since the three compounds investigated here (**7**, **9**, and **10**), which bind in quite distinct modes to hCA II, do show appreciable anticonvulsant activity and some of them (**7** and **9**) are clinically used as AEDs.

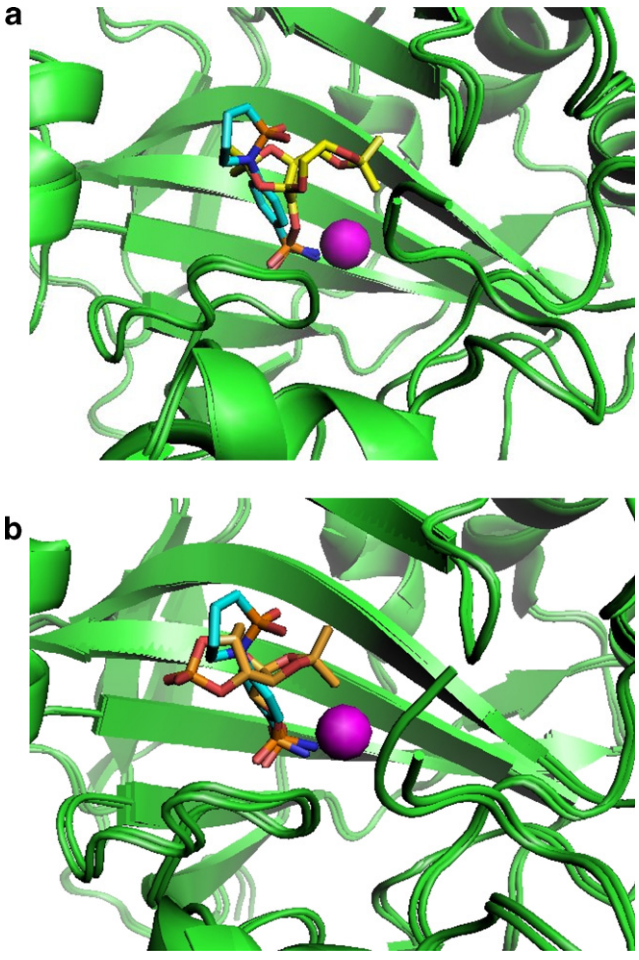

Figure 3. Superposition of the (a) sulthiame **9** and topiramate **7** X-ray crystal structures and (b) sulthiame **9** and RWJ-37947 **10** X-ray crystal structures. Sulthiame is represented as the stick model in blue, topiramate in yellow and RWJ-37947 in gold. The active site zinc ion is the violet sphere, and the amino acids lining the cavity are shown as ribbon diagram in green.

In conclusion, we investigated the interaction of the AED sulthiame with all the catalytically active mammalian CA isoforms. The drug is a potent inhibitor of CA II, VII, IX, and XII, and a medium potency inhibitor against CA IV, VA, VB, and VI. The high resolution crystal structure of the hCA II-sulthiame adduct revealed many favorable interactions between the drug and the enzyme which explain its strong low nanomolar affinity for this isoform but may also be exploited for the design of effective inhibitors incorporating sultam moieties. On the other hand, sulthiame binds in a different region of the enzyme active site cavity as compared to other anticonvulsants/AEDs such as topiramate or its cyclic sulfate analogue.

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17. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used. 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 or $NaClO_4$ (for maintaining constant the ionic strength- these anions are not inhibitory in the used concentration),[11–16] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. K_Is were obtained from Lineweaver-Burk plots, as reported earlier.[12–16]
18. The hCA II-sulthiame complex was crystallized as previously described by cocrystallization procedures.[19] Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using CuKα radiation (1.5418 Å). Data were processed with CrysAlis RED (Oxford Diffraction 2006).[20] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5,[21] model building and map inspections were performing using the COOT program.[22] The final model of the complex had an R-factor of 19.7% and R-free 25.1% in the resolution range 20.0–1.9 Å, with a rms deviation from standard geometry of 0.009 Å in bond lengths and 1.30° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[23] Coordinates and structure factors have been deposited with the Protein Data Bank (PDB accession code 2Q1Q). The crystallographic parameters and refinement statistics are summarized in Table 2.
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