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BRIEF ARTICLE

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Anticonvulsant 4-Aminobenzenesulfonamide Derivatives with Branched-Alkylamide Moieties: X-ray Crystallography and Inhibition Studies of Human Carbonic Anhydrase Isoforms I, II, VII, and XIV†

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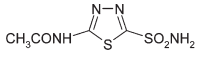
Supporting Information

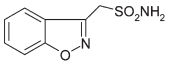
ABSTRACT: Aromatic amides comprising branched aliphatic carboxylic acids and 4-aminobenzenesulfonamide were evaluated for their inhibition of carbonic anhydrase (CA) isoforms. Of the most anticonvulsant-active compounds (**2**, **4**, **13**, **16**, and **17**), only **13**, **16**, and **17** were potent inhibitors of CAs VII and XIV. Compounds **9**, **14**, and **19** inhibited CA II, while **10** and **12** inhibited all isoforms. Structural studies suggest that differences in the active sites' hydrophobicity modulate the affinity of the inhibitors.

INTRODUCTION

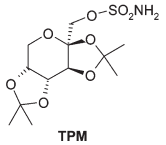
Epilepsy is one of the most common neurological disorders.[1] Triggering mechanisms by which seizures form remain unclear but are related to a rapid change in ionic composition, including an increase of intracellular potassium concentration and pH shifts within the brain.[2,3] The pH buffering of extra- and intracellular spaces is mainly carried out by the CO_2/HCO_3^- system, and this equilibrium is regulated by carbonic anhydrase (CA, EC 4.2.1.1).[2−4] Some CA inhibitors (CAIs) such as the antiepileptic drugs (AEDs) acetazolamide AAZ,[5] zonisamide ZNS,[6] topiramate TPM,[7] and lacosamide LAC[8] are used clinically for the treatment of epilepsy, migraine and other CNS disorders.[9]



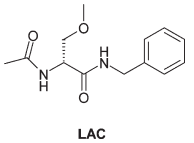
AAZ



ZNS



TPM



LAC

However, the link between CA inhibition and seizures is poorly understood primarily because many CA isoforms (such as CAs I, II, III, IV, VB, VII, VIII, X, XI, XII, and XIV) are present in the brain.[10−13] Several, such as CAs II, VII and XIV, have been noted for their contributions to epileptiform activity.[10−13] Indeed, the CA metalloenyzmes are ubiquitous, present in prokaryotes and eukaryotes, being encoded by five distinct, evolutionarily unrelated gene families (the α-, β-, γ-, δ-, and ζ-CAs), and catalyze the reversible hydration of carbon dioxide to bicarbonate and protons.[3,4] As this reaction is involved in many physiological processes, it is not surprising that in mammals there are 16 α-CA isozymes with different catalytic activity, subcellular localization, tissue distribution, physiological/pathological roles, and susceptibility to inhibitors.[3,4] Among them, there are five cytosolic catalytically active (CAs I−III, VII, and XIII), five membrane-bound (CAs IV, IX, XII, XIV, and XV), two mitochondrial (CAs VA and VB), one secreted (CA VI), and three acatalytic (CAs VIII, X, and XI) forms.[3,4] CA XV is not present in primates but is found in other vertebrates, such as rodents, birds, and fish.[3,4] These isoforms are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, epileptogenesis, tumorigenesis, and many other physiologic or pathologic processes.[3,4]

Several studies have investigated the design/synthesis of sulfonamide/sulfamate CAIs as potential anticonvulsants.[14−16] Most of these compounds have been shown to be low nanomolar inhibitors of CA isoforms, such as CAs II, VII, and XIV, but no correlations have been drawn as to which isoform(s) inhibition is related to potent anticonvulsant activity in vivo.[14−16] We report here a study on the inhibition of the CA isoforms thought to be involved in epileptogenesis, i.e., CAs I, II, VII, and XIV, with a series of benzenesulfonamides CAIs incorporating branched aliphatic carboxamide moieties in the para position of the aromatic ring. Furthermore, the X-ray crystal structures of adducts of three such

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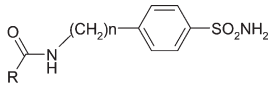
Table 1. Inhibition of CAs I, II, VII, and XIV with 1−21 by a Stopped Flow CO_2 Hydrase Assay[21]



1-10 & 12-19, n = 0; **11, 20**, n = 1; **21**, n = 2

			K_i (nM)[a]			
compd	R	MES ED_{50}[b] (μmol/kg)	CA I	CA II	CA VII	CA XIV
1	*t*-Bu	165 (89−338)	5900	2500	7200	730
2	$(Et)_2CH$	37 (24−54)	4160	2810	861	643
3	$(i\text{-}Pr)_2CH$	NA	2440	237	8370	660
4	*n*-Pr(Me)CH	40 (27−57)	1710	220	605	644
5	(*i*-Pr)(*n*-Pr)CH	139 (80−213)	1850	190	581	682
6	(*i*-Pr)(*sec*-Bu)CH	NA	101	106	53	66
7	(*n*-Pr)(*sec*-Bu)CH	115 (83−141)	4260	233	5150	610
8	$(i\text{-}PrCH_2)(Et)CH$	56 (28−86)	737	104	64	62
9	$(Et)(Me)CHCH_2$	61 (44−81)	9.4	10.3	54	78
10	(*n*-Bu)(Et)CH	80 (55−109)	9.7	10.2	5.0	5.5
11	$(Et)_2CH$	87 (57−120)	82	143	71	82
12	$PhCH_2$	55 (29−86)	7.6	9.0	52	46
13	Me(Et)CH	30 (18−41)	2280	339	65	78
14	Et(*t*-Bu)CH	NA	3590	9.6	49	771
15	*i*-Pr(*t*-Bu)CH	58 (26−128)	3050	258	556	7.3
16	$t\text{-}BuCH_2$	35 (20−65)	2100	3500	62	71
17	(*n*-Pr)(Et)CH	41 (17−66)	1560	321	60	6.5
18	(*sec*-Bu)(Et)CH	NA	2495	142	54	6.9
19	(*n*-Pr)(*t*-Bu)CH	NA	3830	10.1	6.5	70
20	*t*-Bu	NA	339	28.6	5.8	65
21	$(Et)_2CH$	NA	407	33.8	51	684
AAZ[c]		239 (174−327)[c]	250	12	2.5	41
ZNS[d]		100 (84−120)	56	35	117	5250
TPM[d]		10 (6−15)	250	10	0.9	1460

[a] Mean from three different assays. Errors were in the range ±5−10% of the reported value. [b] MES: maximal electroshock test in rats (po). The intervals in parentheses stand for 95% confidence interval. Data are taken from ref 17. [c] AAZ: acetazolamide. Data in mice (ip) taken from ref 5. [d] ZNS: zonisamide. TPM: topiramate. Data are taken from ref 20.

compounds with the physiologically dominant isoform CA II have led to new insights for the design of potent inhibitors of these metalloenzymes.

RESULTS AND DISCUSSION

Chemistry and CA Inhibition. The synthesis and characterization of the benzenesulfonamides **1**−**21** CAIs were reported recently by Hen et al.[17] These compounds incorporate lipophilic moieties in the form of carboxamides with various aliphatic tails (Table 1), substituting the 4-amino moiety of sulfanilamide (**1**−**10**, **12**−**19**), homosulfanilamide (**11**, **20**), or *p*-aminoethylbenzenesulfonamide (**21**). The rationale for preparing these compounds was the valproic acid amides of aromatic/heterocyclic sulfonamide reported by Masereel et al.,[14a] of type $(n\text{-}Pr)_2CH\text{-}CONH\text{-}A\text{-}SO_2NH_2$ (A = aromatic/heterocyclic moiety) that had been shown to possess significant anticonvulsant activity.[14,17]

Table 1 shows **1**−**21** to act as inhibitors of all investigated isoforms but with a potency varying from the low nanomolar to micromolar, depending on the substitution pattern of the aliphatic chain present in the R moiety. The following structure−activity relationship (SAR) can be drawn from the inhibition data in Table 1:

(i) The isoform CA I is inhibited with potencies of 7.6−5900 nM by the series of sulfonamides investigated, with good inhibition observed with derivatives **9**, **10**, and **12** (K_I of 7.6−9.7 nM), which incorporate two highly branched aliphatic chains (present in **9** and **10**) and the benzyl moiety (present in **12**). Other compounds, including **6**, **8**, **11**, **20**, and **21**, showed moderate CA I inhibitory action, with inhibition constants of 82−407 nM. Compounds **11**, **20**, and **21** are the only representatives possessing a methylene or ethylene linker between the benzenesulfonamide and RCONH moieties, unlike **1**−**10** and **12**−**19**, which are sulfanilamide derivatives. Thus, by comparision of **1** with its homologue **20**, both incorporating the *tert*-butyl moiety, an increase in inhibition of 17.4 was observed for **20** with the additional methylene group. This observation was also true for the pair **2** and **21** and the pair **11** and **21** with an increase of inhibition of 10.2 and 4.9, respectively. This enhancement of activity for the compounds incorporating a CH_2 or CH_2CH_2 linker may be explained by the fact that the bulky pivaloyl or valproyl moieties are accommodated more easily toward the exit of the active site (for the longer compounds) than in the active site.[18] In addition **1**−**5**, **7**, and **13**−**19** showed weak CA I inhibition, with K_I of 1.56−5.90 μM, indicating that small changes in the nature of the R moiety lead to large changes of inhibitory activity.

(ii) The physiologically dominant isoform CA II was inhibited by **1**−**21** with K_I of 9.0−3500 nM. The best inhibitors were **9**, **10**, **12**, **14**, and **19**−**21**, which showed inhibition constants of 9.0−33.8 nM, of the same order of magnitude as the clinically used antiepileptic drugs AAZ, ZNS, and TPM (Table 1). Except for **12**, which incorporated the benzyl moiety, the other compounds possess a variety of diversely branched and different lengths of aliphatic chains. As for the inhibition of CA I discussed above, **20** and **21**, incorporating the homosulfanilamide and 4-aminoethylbenzenesulfonamide scaffolds, respectively, were more active than the corresponding shorter sulfanilamide derivatives **1** and **2** (by a factor of 87.4 and 83.1, respectively). A group of derivatives, including **3**−**8**, **11**, **13**, **15**, **17**, and **18**, behaved as medium potency CA II inhibitors, with K_I of 106−339 nM, whereas **1**, **2**, and **16** were the least effective inhibitors, with K_I of 2500−3500 nM (Table 1).

(iii) The brain-specific isoform CA VII was strongly inhibited by most of the compounds. Compounds **6**, **8**−**14**, and **16**−**21**, showed good inhibitory activity, with K_I of 5.0−71 nM, with **2**, **4**, **5**, and **15** exhibiting medium efficiency CAIs (K_I of 556−861 nM), whereas **1**, **3**, and **7** were weak inhibitors, with K_I of 5.10−8.37 μM (Supporting Information Table 1). The best CA VII inhibitor was **10** (which was also among the best CA I and CA II inhibitors) together with the simple pivaloyl derivative of homosulfanilamide **20** (which was 1241 times a better inhibitor compared to the structurally related **1**, which has just one CH_2 moiety less than **20**).

(iv) The transmembrane isoform CA XIV was also inhibited significantly by **1**−**21**. The best inhibitors were **6**, **8**−**13**, and **15**−**20** with K_I of 5.5−78 nM. As for the other isoforms discussed here, the R present in these derivatives includes the benzyl and the highly ramificated aliphatic moieties, of a rather variable nature. The remaining derivatives (**1**−**5**, **7**, **14**, and **21**) were less effective CA XIV inhibitors, with potencies of 643−771 nM. It is clear that minor changes in the inhibitor scaffold (R moiety within the tail or

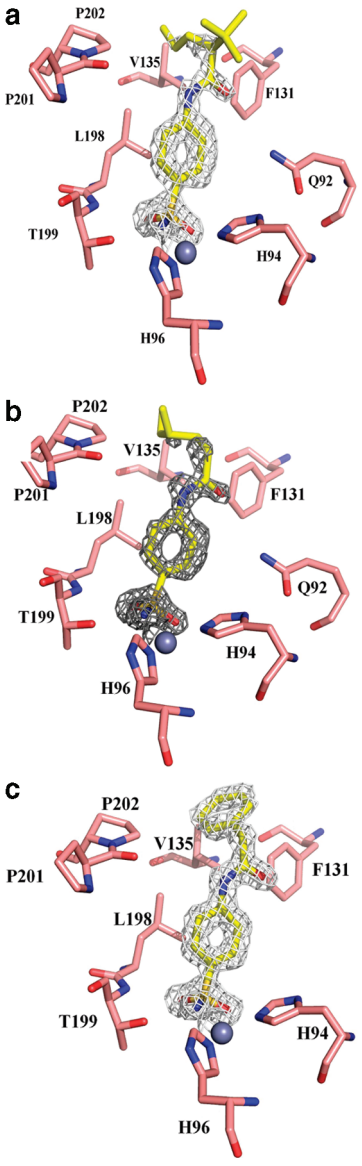


Figure 1. Stick representation of hCA II active site with (a) **19**, (b) **9**, and (c) **12**. The active-site zinc is depicted as a gray sphere. The electron density is represented by a σ-weighted $2F_o - F_c$ Fourier map (gray mesh) at 1.2σ cutoff. Amino acids are as labeled.

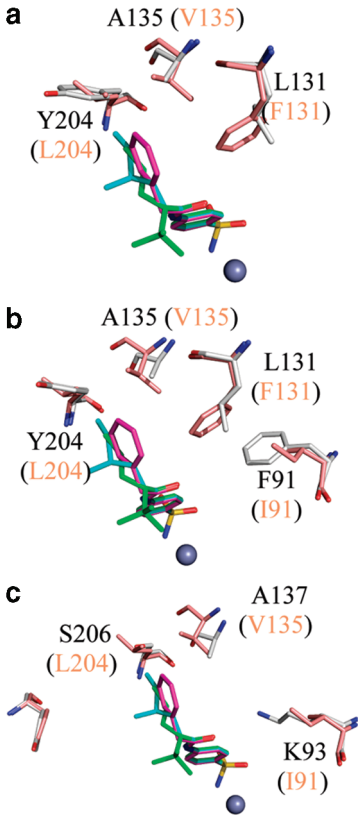


Figure 2. Stick representation of (a) hCA XIV, (b) hCA I, and (c) hCA VII active sites superimposed onto hCA II (salmon) complexed with **19** (green), **9** (cyan), and **12** (pink). The active-site zinc is depicted as a black sphere. Amino acids are as labeled. hCA II residues are labeled in parentheses.

the spacer between the benzenesulfonamide and remaining part of the molecules, i.e., n = 0, 1, or 2) lead to important changes of inhibitory activity.

X-ray Crystallography. The crystal structures of CA II complexed with **9**, **12**, and **19** were determined (Figure 1) with maximum resolution ranges of 1.50−1.65 Å, using a protocol previously described by us (refer to Supporting Information text and Table S1 for details).[18] All three compounds were well ordered and refined with occupancies between 0.7 and 1.0, with *B*-factors that were comparable to that of the solvent within the active site. The compounds were buried deep into the active site, displacing the catalytic zinc-bound solvent, such that the sulfonamide amine nitrogen binds directly to the zinc atom of CA II (distance, ∼2.0 Å). Hence, the overall $Zn(N)_4$ coordination can be described as a distorted tetrahedron.[19] Protruding from the active site, the tails of the compounds are stabilized predominantly by hydrophobic residues. All three structures are at a van der Waals distance from the side chains of Val135, Phe131, Leu198, and Pro202 (Figure 1 and Supporting Information Figure 1). Hence, **9**, **12**, and **19** bury approximately the same amount of protein surface area of 138.6, 124.7, and 124.3 Å^2, respectively. Compound **12** exhibits the lowest average *B* of 12.8 Å^2 compared to **19** and **9** whose average *B*-factors are 24.7 and 21.7 Å^2, respectively (Table 1). Also, **19** refines with a lower occupancy of 0.7 than the other two compounds and the bifurcated hydrophobic tail is less ordered and more solvent exposed at the mouth of the active site. However, all these compounds have very similar inhibitory action against hCA II, with K_I of 9.0−10.3 nM.

Structure−Activity Relationships. Although the overall structures of CAs I, II, VII, and XIV are similar, there are several differences in the type and spatial arrangement of amino acids within the active sites (Figure 2). Of particular note are the three hydrophobic residues (Phe131, Val135, and Leu204) on the surface of CA II that play an important stabilizing role when interacting with the longer hydrophobic termini R groups of sulfonamide inhibitors. The weaker binding (K_I) of **9**, **12**, and **19** with CA XIV compared to CA II can be directly attributed to these amino acid differences, which are Leu131, Ala135, and Tyr204 in CA XIV. The overall reduction in hydrophobicity of the active site appears to have a significant effect and reduces the affinity of these compounds to CA XIV (Figure 2a, Table 1).

The amino acids differences between CAs I and II in the regions where the R groups of **9** and **12** interact seem to compensate each other. The hydrophobicity decreases with

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Phe131 and Val135 in CA II replaced with Leu131 and Ala135, respectively, in CA I but increases with Ile91 and Leu204 in CA II replaced with Phe91 and Tyr204, respectively, in CA I, and these compensatory changes may account for why the two compounds show similar affinities for CAs I and II. Of note is that the R group of **19** is less hydrophobic than **12** or **9**. Thus, the reduced modulation in hydrophobicity of amino acids (F131L, V135A, and L204Y) in CA I that are in proximity to **19** may cause a cumulative effect and weaken its K_I to CA I compared to CA II with **9** and **12** (Figure 2b, Table 1).

In CA VII, the hydrophilic Ser206, Tyr22 and less hydrophobic Ala137 are Leu204, Phe20, and Val135 in CA II, respectively, and this reduced hydrophobicity could explain the weak K_I of **9** and **12** to CA VII compared to CA II. However, Lys93 in CA VII (which is the hydrophobic residue Phe in CA I, Ile in CA II, and Ala in CA XIV) can be modeled to hydrogen-bond with the carbonyl group of **19**. The presence of strong hydrophobicity at the inhibitor interaction regions of **9** and **12** (which are more hydrophobic) precludes Lys93 of CA VII from adopting this conformation (Figure 2c, Table 1). Therefore, the SARs between the CA isoforms clearly imply that small differences in the hydrophobicity of the CA isoform active sites modulate the affinity of the inhibitors' various aliphatic chains.

Anticonvulsant Activity. Table 1 lists the anticonvulsant activity and ED_{50} at the rat (po) maximal electroshock seizure (MES) test of the tested 4-aminobenzensulfonamide derivatives.[17,20] The most active compounds were **2**, **4**, **13**, **16**, and **17**. None of these compounds were potent inhibitors of CAs I and II; **13**, **16**, and **17** were potent inhibitors of CAs VII and XIV. In spite of their different chemical structures, the anticonvulsant-active compounds **10** and **12** inhibited all four tested CAs with **10** being the more potent inhibitor with K_i of 5.0−10.2 nM. On the other hand, potent CA II inhibitors like **14** and **19** lacked anticonvulsant MES activity.

The antiepileptic and CNS drug TPM inhibits CAs II and IV at 1−10 μM.[7] Although TPM CA inhibition is generally not considered to represent a significant anticonvulsant mechanism of the drug, the possibility remains that this inhibition may contribute to the anticonvulsant activity by modulation of pH-dependent voltage- and receptor-gated ion channels. Given that the excitatory effects of bicarbonate may occur in the hippocampus, where some forms of epilepsy originate, it can be hypothesized that CA inhibitors will block the excitation.[20]

In the investigated series of 4-aminobenzensulfonamide derivatives depicted in Table 1 there seems to be little correlation between anticonvulsant (MES) activity and inhibition of any of the four tested CAs. This is substantiated by the fact that two of the potent CA II inhibitors evaluated in the X-ray crystallography analysis were active as anticonvulsants (**9** and **12**) and one (**19**) was not.

CONCLUSIONS

A novel class of anticonvulsant aromatic amides obtained by the coupling of 4-aminobenzenesulfonamides or 4-alkylaminobenzenesulfonamides (alkyl = methyl or ethyl) with phenylacetic acid or branched aliphatic carboxylic acids were recently reported by us.[17] In the current study the most active compounds were **2**, **4**, **13**, **16**, and **17** and were more potent inhibitors of CAs VII and XIV than CAs I and II. The hydrophobicity differences in the CA isoforms studied clearly show that small differences in hydrophobic residues in the CA active sites modulate the affinity of the inhibitors with various aliphatic chains, but in this series of 4-aminobenzenesulfonamide derivatives there seems to be little correlation between anticonvulsant (MES) activity and inhibition of CA I, II, VII, or XIV. Since most AEDs including the CAIs AAZ, TPM, and ZNS have multiple mechanisms of action, there are many unknown issues regarding the correlation between CAs' inhibition and AEDs' activity, and therefore, resolving them is not so straightforward (because of too many CA isoforms). Still the current publication offers an advance in the resolution of this complicated issue.

EXPERIMENTAL PROTOCOLS

Compounds. Derivatives **1**−**21** were reported earlier by Hen et al.[17] Enzymes were recombinant proteins obtained as reported earlier by this group.[4,9,14,15]

CA Inhibition. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity.[21] The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver−Burk plots were obtained as reported earlier[9,14,15] and represented the mean from at least three different determinations.

X-ray Data Collection of CA II Drug Complexes. The X-ray diffraction data were collected using an R-AXIS IV^{++} image plate system on a Rigaku RU-H3R Cu rotating anode operating at 50 kV and 22 mA, using Osmic Varimax HR optics. The detector−crystal distance was set to 80 mm. The oscillation steps were 1° with a 5 min exposure per image. The structures were determined using standard protocols as describe in Supporting Information.

SAR Studies. The coordinates of hCAs I (PDB accession code 3LXE),[22] VII (PDB accession code 3MDZ, unpublished), and XIV (PDB accession code 1RJ5)[23] were superimposed onto the coordinates of CA II complexed with compounds **9**, **12**, and **19**.

ASSOCIATED CONTENT

Supporting Information. Structures, crystallographic data, additional results and discussion, and additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

[†]The atomic coordinates 3OY0, 3OQY, and 3OYS have been deposited in the Protein Data Bank.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; AAZ, acetazolamide; AED, antiepileptic drug; CNS, central nervous system; LAC, lacosamide; MES, maximal electroshock seizure; SAR, structure−activity relationship; TPM, topiramate; ZNS, zonisamide

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