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Carbonic anhydrase inhibitors. Zonisamide is an effective inhibitor of the cytosolic isozyme II and mitochondrial isozyme V: solution and X-ray crystallographic studies

Giuseppina De Simone,[a,*] Anna Di Fiore,[a] Valeria Menchise,[a] Carlo Pedone,[a] Jochen Antel,[b] Angela Casini,[c] Andrea Scozzafava,[c] Michael Wurl[b] and Claudiu T. Supuran[c,*]

[a]*Dipartimento di Chimica Biologica-Sezione Biostrutture and Istituto di Biostrutture e Bioimmagini-CNR, University of Naples "Federico II", via Mezzocannone 16, 80134 Naples, Italy*
[b]*Solvay Pharmaceuticals Research Laboratories, Hans Böckler-Allee 20, D-30173 Hannover, Germany*
[c]*Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy*

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Abstract—The antiepileptic drug zonisamide was considered to act as a weak inhibitor of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) (with a K_I of 4.3 μM against the cytosolic isozyme II). Here we prove that this is not true. Indeed, testing zonisamide in the classical assay conditions of the CO_2 hydrase activity of hCA II, with incubation times of enzyme and inhibitor solution of 15 min, a K_I of 10.3 μM has been obtained. However, when the incubation between enzyme and inhibitor was prolonged to 1 h, the obtained K_I was of 35.2 nM, of the same order of magnitude as that of the clinically used sulfonamides/sulfamates acetazolamide, methazolamide, ethoxzolamide and topiramate (K_Is in the range of 5.4–15.4 nM). The inhibition of the human mitochondrial isozyme hCA V with these compounds has been also tested by means of a dansylamide competition binding assay, which showed zonisamide and topiramate to be effective inhibitors, with K_Is in the range of 20.6–25.4 nM. The X-ray crystal structure of the adduct of hCA II with zonisamide has also been solved at a resolution of 1.70 Å, showing that the sulfonamide moiety participates in the classical interactions with the Zn(II) ion and the residues Thr199 and Glu106, whereas the benzisoxazole ring is oriented toward the hydrophobic half of the active site, establishing a large number of strong van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Leu198, Thr200, Pro202.

1. Introduction

Zonisamide, **ZNS** (1,2-benzisoxazole-3-methanesulfonamide), is a widely used antiepileptic drug.[1,2] In vitro studies with cultured neurons showed that zonisamide blocks repetitive firing of voltage-sensitive sodium channels and reduces voltage-sensitive T-type calcium currents without affecting L-type calcium currents.[1,2] Such a complicated mechanism of action may explain its efficacy in patients resistant to other antiepileptic drugs, whereas its pharmacokinetic profile is favorable for clinical use since the drug is rapidly and completely absorbed and has a long half-life (63–69 h), which allows twice- or once-daily dosing.[1,2] Being an unsubstituted sulfonamide, zonisamide has also been investigated for the inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) by its discoverers,[3,4] being concluded that although it binds significantly to erythrocytes[5] (where two CA isozymes, CA I and II are highly abundant)[6,7] its CA inhibitory properties are rather weak, and thus, this phenomenon does not play any role in the anticonvulsant activity of the drug.[1–4] Indeed, most inhibitors of CAs belong to the sulfonamide or sulfamate class of compounds,[6–10] with several derivatives such as acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, or topiramate **TPM** being clinically used for the treatment or prevention of a variety of disorders such as glaucoma, acid–base disequilibria, epilepsy, and other minor neuromuscular disorders, or

* Corresponding authors. Tel.: +39 081 2534579; fax: +39 081 2536642 (G.D.S.); tel.: +39 055 4573005; fax: +39 055 4573385 (C.T.S.); e-mail addresses: gmg@chemistry.unina.it; claudiu.supuran@unifi.it

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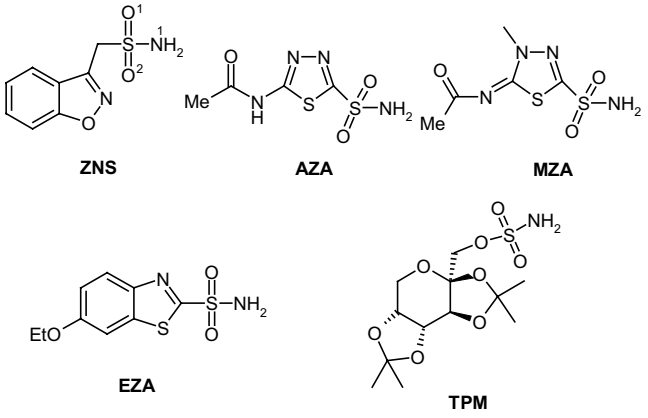
as diuretics, etc., whereas many other compounds are in clinical investigation as antiobesity or anticancer drugs.[11–15]



Thus, although Masuda and Karasawa[3] reported that **ZNS** is a weak inhibitor of the major red cell isozyme hCA II, with an inhibition constant of 4.3 μM, some of the side effects of this drug, such as kidney stones,[16] oligohydrosis, and fever,[17,18] as well as the fact that it has been reported that the drug is highly bound to erythrocytes,[5] are typical of a potent CA inhibitor.[8] Furthermore, a recent patent[19] claimed that zonisamide is effective for reducing weight in obese subjects and for treating eating disorders, such as binge-eating disorder, bulimia nervosa, or anorexia nervosa. Zonisamide has been employed in a clinical study conducted in 60 subjects to evaluate the short-term efficacy and safety in the treatment of obesity.[19] The group treated with **ZNS** lost on average, more bodyweight than the placebo group (5.98% vs 1.09%) during the 16-week period of the study and 17/30 subjects in the treated group and 3/30 in the vehicle group were observed to have lost greater than 5% weight. **ZNS** is thus similar to topiramate **TPM**, another antiepileptic drug showing potent antiobesity effects,[20,21] which have been explained recently by us as due to inhibition of the CA isozymes involved in lipogenesis, that is, hCA II and V.[11,22,23] Indeed, in a previous study we showed by means of solution and X-ray crystallographic studies that TPM is a low nanomolar inhibitor of hCA II, appreciably inhibiting other physiologically relevant CA isozymes too.[12,22] These were some of the reasons that prompted us to (re)investigate the interaction of zonisamide with hCA II and hCA V, the two isozymes involved in lipogenesis and other metabolic processes, by means of solution and X-ray crystallographic studies.

2. Chemistry and CA inhibition

The clinically used compounds **ZNS–TPM** and dansylamide are commercially available (from DaiNippon, Sigma-Aldrich or Johnson & Johnson) and were used without further purification. Human recombinant isozymes hCA II and V have been obtained in our laboratory as reported earlier.[23]

Table 1. hCA II and V inhibition data with **ZNS–TPM** by means of a stopped-flow method (with hCA II, for the CO_2 hydrase activity of the enzyme)[24] and by means of a spectrophotometric dansylamide binding assay (against hCA V)[25]

Compound	K_I[a] (nM)	
	hCA II[b]	hCA V[c]
ZNS	35.2	20.6
AZA	12.3	1.8
MZA	15.4	2.5
EZA	5.4	2.0
TPM	10.3	25.4

[a] Mean value from three different determinations.
[b] Enzyme (10 nM) and inhibitor were incubated for 15 min prior to assay, except for **ZNS** which was incubated for 1 h. When **ZNS** was incubated for 15 min, the corresponding K_I obtained was of 10.3 μM.
[c] Enzyme (0.5 μM) and inhibitor were incubated for 1 h prior to assay.

Two assay methods[24,25] have been employed here for determining the inhibition constants of the above mentioned compounds against hCA II and hCA V: a stopped-flow assay for the CO_2 hydration activity of hCA II,[24] and a dansylamide competition binding assay for hCA V.[25] As far as we know, this is the first report of assaying CA V inhibitors with this method, which has generally been used for measuring the inhibition of hCA II and some of its mutants.[25] As seen from data of Table 1, in contrast with that reported earlier,[3] **ZNS** is an effective hCA II inhibitor, possessing an inhibition constant of 35.2 nM, of the same order of magnitude as the other clinically used derivatives **AZA**, **MZA**, **EZA**, or **TPM**; which were slightly more effective inhibitors (K_Is in the range of 5.4–15.4 nM). However, an important difference has been observed between **ZNS** and all the other CA inhibitors investigated here. Thus, in the classical inhibition assay using the CO_2 hydrase activity of diverse CA isozymes developed by Khalifah,[24] enzyme and inhibitor solutions are incubated for a standard period of time, which is of 15 min. In the case of **ZNS**, such a standard incubation time led to a weak inhibition, with a measured K_I of 10.3 μM, which is comparable to that reported by Masuda and Karasawa (4.3 μM) who concluded that this compound behaves as a weak CA II inhibitor.[3] We have performed thereafter inhibition assay after longer incubation periods of the enzyme and inhibitor solutions (of 30–60 min) and observed that the measured K_I decreased constantly till reaching a plateau after 60 min incubation period (data not shown). At the end of that period a constant K_I with the value reported in Table 1 has been obtained, proving that **ZNS** is a much more effective hCA II inhibitor as originally reported.[3] This phenomenon is on the other hand not observed with the other sulfonamides/sulfamates investigated up to now. Presumably, **ZNS** shows a very slow rate of association with the enzyme active site, unlike the other sulfonamides/sulfamates investigated here. In fact such association/dissociation rates have been investigated kinetically by Maren, who showed that the rate of association of inhibitors (k_{on}) with the active site of hCA II may vary between 3.10^{-3} and 3.10^{7} $L\,M^{-1}s^{-1}$, whereas the dissociation rate of the E–I complex (k_{off}) is generally of the same order of magnitude for very

diverse classes of sulfonamides.[26] This may explain the very different inhibition constants reported earlier for **ZNS**,[3] as compared to our data, and also may explain the side effects of this antiepileptic, some of which are typical of strong CA inhibitors, such as acetazolamide, methazolamide, or topiramate (for this last compound the same type of side effects, that is, kidney stones,[16,27] oligohydrosis,[18,28] or metabolic acidosis,[29] have been reported in rare cases).[21,30] However, side effects of a drug may sometimes lead to novel applications, as for the antiobesity actions of topiramate[20,21] and zonisamide,[19] which we hypothesize to be at least partly due to inhibition of the CA isozymes involved in lipogenesis, that is, hCA II and V.[11] In order to check this hypothesis, we tested the hCA V inhibitory activity of these derivatives (and other standard, clinically used compounds such as **AZA**, **MZA**, and **EZA**) against the recombinant full length mitochondrial isozyme hCA V (Table 1). In order to avoid the kinetic effects observed with the CO_2 hydrase method discussed above, we selected the dansylamide competition binding assay,[25] for which longer incubation times are employed, that is, around 1 h, which presumably assures the right equilibration between the enzyme and the inhibitor in the E–I complex. Indeed, data of Table 1 show that **AZA**, **MZA**, and **EZA** are very potent hCA V inhibitors, with inhibition constants in the low nanomolar range (of 1.8–2.5 nM) whereas zonisamide and topiramate are slightly less potent, but anyhow, very effective inhibitors, with K_Is in the range of 20.6–25.4 nM.

3. X-ray crystallography

To assess the molecular basis responsible for the high affinity of **ZNS** toward hCA II, we solved the crystal structure of the hCA II–**ZNS** complex, which was prepared and crystallized using experimental conditions previously reported for other sulfonamide/sulfamate CA inhibitors.[31–36] The three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to those of the native enzyme[37] and refined using the CNS program.[38] The statistics for data collection and refinement are summarized in Table 2.[39]

Inspection of the electron density in the enzyme active site, at various stages of the crystallographic refinement, showed features compatible with the presence of one inhibitor molecule bound within the active site (Fig. 1). The zonisamide structure conformed perfectly to the shape of this electron density (Fig. 2). The binding of the inhibitor to the enzyme active site did not significantly perturb the enzyme structure, even in the close proximity of the ligand. As a matter of fact, a r.m.s. deviation value of 0.33 Å, over the entire Cα atoms of hCA II-zonisamide complex with respect to the unbound enzyme, was calculated. Similarly, interactions between the protein and Zn(II) ion were entirely preserved in the complex.

Several polar and hydrophobic interactions stabilized the inhibitor within the hCA II active site, as clearly illustrated in Figure 3. According to this figure, the inhibitor molecule shows a spatial arrangement similar to that observed in other hCA II-sulfonamide/sulfamate complexes for which the X-ray structure has been

Table 2. Crystal parameters, data-collection and refinement statistics for the hCA II–**ZNS** complex

Crystal parameters	
Space group	$P2_1$
Unit-cell parameters (Å,°)	$a = 42.28$
	$b = 41.81$
	$c = 72.35$
	$\beta = 104.59$
Data collection statistics (20.00–1.70 Å)	
Temperature (K)	100
Completeness (%)	89.1 (75.2)
R-sym[a]	0.04 (0.18)
I/σ(I)	13.1 (2.17)
Refinement statistics (20.00–1.70 Å)	
R-factor[b] (%)	18.8
R-free[b] (%)	22.0
r.m.s.d. from ideal geometry:	
Bond lengths (Å)	0.005
Bond angles (°)	1.80
Number of protein atoms	2060
Number of water molecules	306
Number of inhibitor atoms	14
Average *B* factor	12.66

Values in parentheses refer to the highest resolution shell, 1.80–1.70 Å.
[a] $R\text{-sym} = \Sigma|I_i - \langle I\rangle|/\Sigma I_i$; over all reflections.
[b] $R\text{-factor} = \Sigma|F_o - F_c|/\Sigma F_o$; *R*-free calculated with 5% of data withheld from refinement.

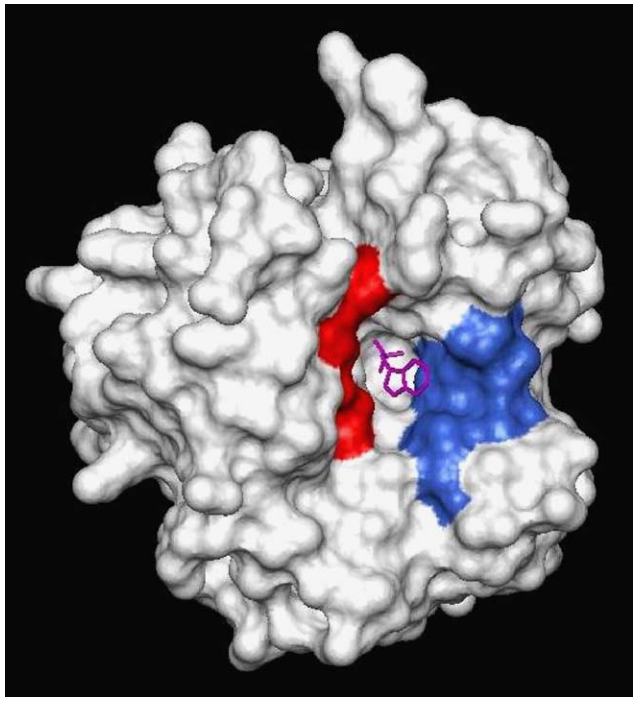


Figure 1. Solvent accessible surface of hCA II in its complex with zonisamide. The hydrophobic half of the active site cleft is shown in cyan while the hydrophilic one in red. The inhibitor molecule is shown in magenta.

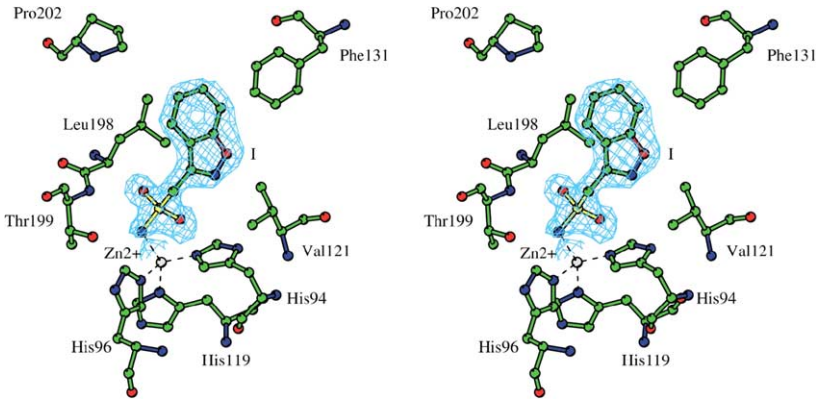


Figure 2. Stereo view of the active site region in the hCA II–**ZNS** complex. The inhibitor (labeled I) is shown associated with simulated annealing omit $|2F_o-F_c|$ electron density map,[38] computed at 1.70 Å and contoured at 1.0 σ.

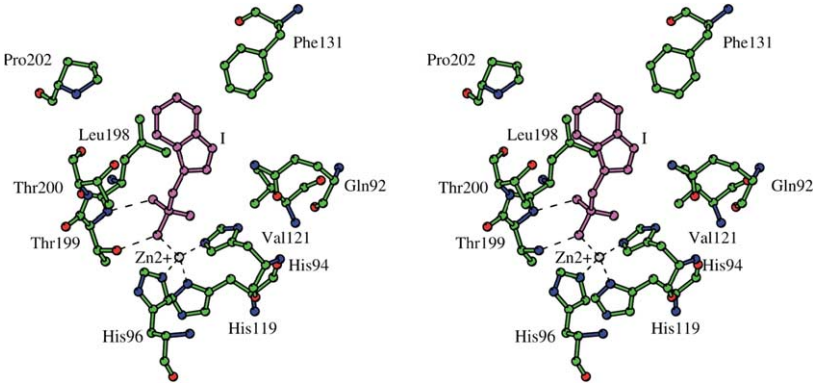


Figure 3. Stereo view of the active site region in the hCA II–**ZNS** complex showing the residues participating in recognition of the inhibitor molecule, reported in magenta. Hydrogen bonds and the active site Zn(II) ion coordination are also shown (dotted lines).

solved.[31–36] In particular, the inhibitor molecule has the N1 atom of the sulfamoyl moiety coordinated in tetrahedral geometry to the Zn(II) ion of the enzyme active site (N1–Zn(II) = 1.99 Å). The Zn(II) ion is also coordinated to the imidazole groups of three histidine residues (His94, His96, and His119), as in the uninhibited enzyme.[37] The N1 atom of the inhibitor is also hydrogen bonded to the hydroxyl group of Thr199 (N1–ThrOG = 2.59 Å), which in turn interacts with the Glu106OE1 atom (2.58 Å), these interactions being observed in all adducts of sulfonamides/sulfamates with various CA isozymes.[31–36] Furthermore, the inhibitor O1 atom is hydrogen bonded to the backbone amide of Thr199 (ThrN–O1 = 2.76 Å), whereas the O2 atom was at a distance of 3.06 Å from the catalytic Zn(II) ion. The benzisoxazole ring of zonisamide was oriented toward the hydrophobic part of the active site cleft (Figs. 1 and 3), establishing a large number of strong van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Leu198, Thr200, Pro202, respectively. These amino acids have been already reported to establish important interactions with the organic scaffold of other CA inhibitors,[31–36] critically contributing to stabilize the inhibitor within the active site cleft. It is important to highlight that although the organic scaffold of zonisamide does not establish significant polar interactions with the enzyme, as otherwise observed for some other hCA II-sulfonamide/sulfamate complexes,[31,32] the large number of hydrophobic contacts mentioned above between the inhibitor and the enzyme can account for the good inhibitory properties of this molecule.

4. Conclusions

Although the literature data presented the antiepileptic drug zonisamide as a weak hCA II inhibitor (with a K_I of 4.3 μM), we prove here that this needs to be revised. Indeed, testing the compound in the classical assay conditions of the CO_2 hydrase activity (i.e., with incubation times of enzyme and inhibitor solution of 15 min) a K_I of 10.3 μM has been obtained. However, when the incubation between enzyme and inhibitor was prolonged to 1 h, the obtained K_I was of 35.2 nM, of the same order of magnitude as that of the clinically used sulfonamides/sulfamates acetazolamide, methazolamide, ethoxzolamide, and topiramate (K_Is in the range of 5.4–15.4 nM). The inhibition of the mitochondrial isozyme hCA V with these compounds has been also tested by means of a dansylamide competition binding

assay, which showed zonisamide and topiramate to be effective inhibitors, with K_Is in the range of 20.6–25.4 nM. The X-ray crystal structure of the adduct of hCA II with zonisamide has also been solved at a resolution of 1.70 Å, showing that the sulfonamide moiety participates in the classical interactions with the Zn(II) ion and the residues Thr199 and Glu106, whereas the benzisoxazole ring is situated in the hydrophobic half of the active site, establishing a large number of strong van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Leu198, Thr200, Pro202. On the basis of these results we suggest that zonisamide, similarly with topiramate may be used as the lead molecule for the design and further exploration of subtype selective CA inhibitors.

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39. Crystals of the hCA II–**ZNS** complex were obtained by cocrystallization as previously described. Data collection was carried out on a single crystal which was flash-frozen at 100 K, using Cu-Kα radiation from a rotating anode X-ray generator coupled with a SMART 1K CCD detector from Bruker. The crystal diffracted up to 1.70 Å resolution. Diffracted intensities were processed using the Saint program.[40] Crystal data and data-collection statistics are given in Table 2. The structure of the hCA II–**ZNS** complex was analyzed by difference Fourier techniques, using the PDB file 1CA2[37] as a starting model for refinement. Water molecules were removed from the starting model prior to structure factor and phase calculations. Fourier maps calculated with $3F_o-2F_c$ and F_o-F_c coefficients showed prominent electron density features in the active site region. After an initial refinement, limited to the enzyme structure (R-factor 0.281 and R-free 0.320), a model for the inhibitor was easily built and introduced

into the atomic coordinates set for further refinement, which proceeded to convergence with continuous map inspection and model updates. Final crystallographic *R*-factor and *R*-free values calculated for the 23,166 observed reflection (in the 20.00–1.70 Å resolution range) were 0.188 and 0.220, respectively. The refined model included 2,074 complex atoms, 14 atoms belonging to the inhibitor, and 306 water molecules. The r.m.s. deviations from the ideal value of bond lengths and bond angles[41] were 0.005 Å and 1.80°, respectively. The average temperature factor (*B*) for all atoms was 12.66 Å^2. The stereochemical quality of the model was assessed by Procheck.[42] The statistics for refinement are summarized in Table 2. The coordinates of the hCA II–**ZNS** adduct are available immediately from gmg@chemistry.unina.it or claudiu.supuran@unifi.it.

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