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Original article

Structure-based screening for the discovery of new carbonic anhydrase VII inhibitors

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ABSTRACT

Among the different mammalian isoforms of Carbonic Anhydrase, the hCA VII is mainly expressed in the brain where it is involved in several neurological diseases. Thereby hCA VII has been validated as an attractive target for the discovery of selective inhibitors for the treatment of epilepsy and neurological pain. To identify new chemical entities as carbonic anhydrase inhibitors (CAIs) targeting hCA VII, we used a structure-based approach. By means of LigandScout software we built pharmacophore models from crystal structures of two well-known CAIs in complex with hCA VII. A merged pharmacophore hypothesis has been obtained. Subsequently, a focused library of compounds was screened against pharmacophore model and the most interesting hits were docked into the crystal structure of hCA VII. As a result, we identified new compounds displaying significant CA inhibitory effects in the nanomolar range.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes highly abundant in all mammalian tissues. There are sixteen human CA isoforms [1] with peculiar cellular localization and distribution in organs and tissues. These various CAs are characterized by different molecular features, oligomeric arrangement and kinetic properties [2]. CAs catalyze the intercorvertion of carbon dioxide into bicarbonate ion and proton, regulating, in this way, a remarkable range of fundamental metabolic pathways (gluconeogenesis, lipogenesis and ureagenesis). As well as this, they are responsible for the pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, bone resorption and calcification [3–6]. Furthermore, this family of enzymes is involved in some pathological pathways, resulting interesting target for the identification of inhibitors (CAIs) that are useful tools for the treatment of glaucoma, cancer, obesity, and epilepsy [3,7–14]. However, the development of CA inhibitors (CAIs) possessing high potency and selectivity against specific isoforms still represents an attractive strategy to obtain newer compounds acting toward this "old target", thus avoiding side effects and improving therapeutic safety [15,16].

Among the different mammalian isoforms, the hCA VII is one of the least investigated cytosolic CA isoforms being isolated from a human genomic library by Montgomery et al. in 1991 [17]. The hCA VII sequence is characterized by 263 aminoacids and shows 50%, 56%, and 49% identity with hCA I, hCA II and hCA III, respectively [17]. However, in contrast to hCA II, which is widely spread in human tissues, CA VII isoform has a limited distribution being mainly expressed in the cortex, hippocampus and thalamus regions within the mammalian brain and in other organs including the stomach, duodenum, colon, liver and skeletal muscle of mice [18,19]. The hCA VII is currently considered to be involved in the mechanism of GABAergic excitation and in generating seizures [10,20]. Recently, its involvement in neuropathic pain control has also been proposed though the mechanism is not completely known [18,19]. This could represent an interesting pharmacological mechanism for the designing of new pain killers useful for therapeutic applications in central nervous system pathologies [21,22].

Crystallographic characterization of the recombinant hCA VII enzyme form was produced using *Escherichia coli* expression system.

Abbreviations: CAs, carbonic anhydrases; CAIs, carbonic anhydrase inhibitors; HBA, H-bond acceptors; HBD, H-bond donors; H, hydrophobic group.

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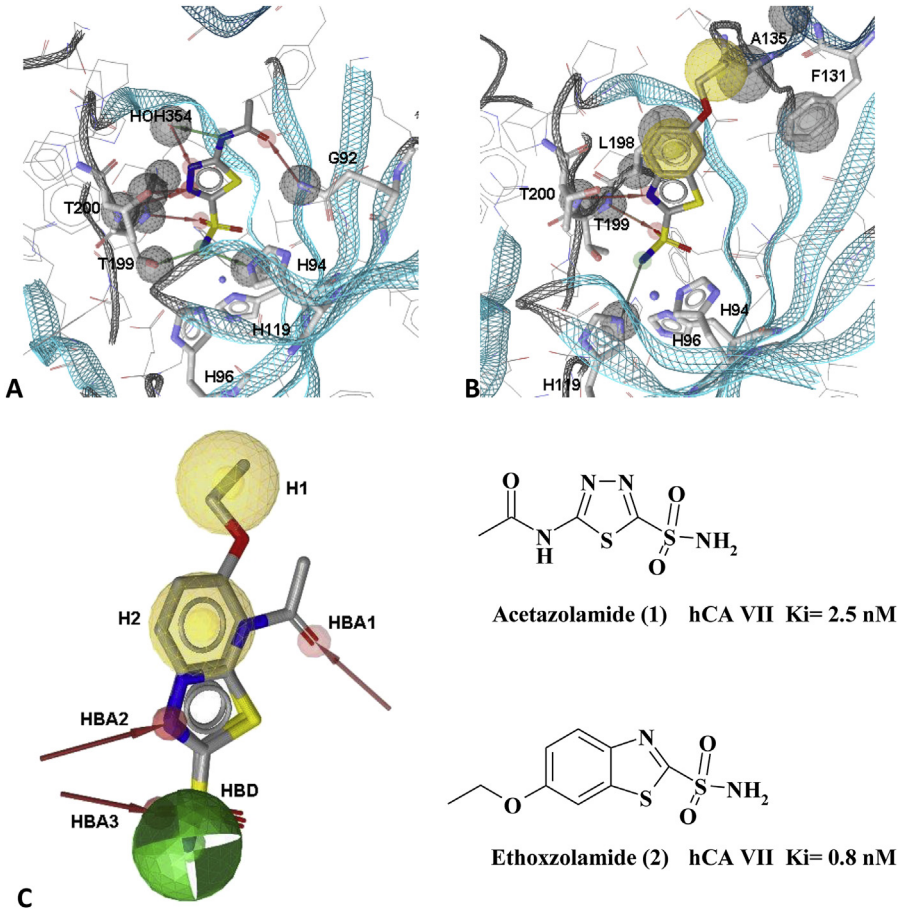

Fig. 1. Structure-based pharmacophore model generated using LigandScout [44] from the X-ray crystal structure of A) acetazolamide (**1**) and B) ethoxzolamide (**2**) in complex with CA VII (PDB: 3ML5 and 3MDZ, respectively). Hydrophobic groups (light yellow spheres), H-bond donors (green arrow), H-bond acceptors (red arrow) and excluded volumes (gray spheres) are shown. C) Representation of the merged pharmacophore model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The mutant isozyme, called MhCA VII, contained two amino acid substitutions (the cysteine residues in position 183 and 217 were mutated into serines) that avoided crystallization problems caused by the mixture of structurally different reduced and oxidized enzyme forms [23]. These structural studies demonstrated that hCA VII consists of a central 10-stranded β-sheet surrounded by several helices and additional β-strands. The active site is located in a conical cavity, with the catalytic zinc ion at the bottom coordinated by three histidine residues [23].

It is well known that sulfonamides (RSO_2NH_2) acting as CAIs inhibit enzymatic activity through the coordination of zinc ion and additional specific contacts with crucial aminoacidic residues [24–32]. Particularly, the deprotonated form of the sulfonamide group establishes strong interaction between zinc ion and specific residues in a depth area of the catalytic site [33]. Furthermore, the isoform selectivity is conditioned by the different interactions of R tail in the region adjacent to the catalytic site [2]. So the identification of new potent and selective CAIs could result from the optimization of binding recognition into the catalytic site of targeted isoform.

We have been actively engaged in identifying new CAIs [22,26,34] and recently reported the synthesis and biological characterization of new sulfonamide derivatives that were able to chelate zinc ion and demonstrated high inhibitory effects in a nanomolar range toward selected hCA isoforms [3,27,35]. By means of crystallographic studies we described the main interactions established within a catalytic pocket of ubiquitous hCA II [27]. Moreover, docking experiments suggested the pattern of interactions established with some selected CA isoforms [27] such as hCA VII, hCA IX, and hCA XIV. Importantly, several of our developed sulfonamides displayed in Fig. 1 proved to be potent CAIs showing the highest selectivity toward hCA VII isoform. Therefore, we suggested that these selective inhibitors could be promising lead compounds to identify new hCA VII inhibitors for the treatment of neurological diseases.

This paper reports a structure-based computational approach aimed to disclose new chemical entities targeting hCA VII isoform. The compounds emerged from this study were tested as CAIs towards a set of druggable isoforms.

2. Results and discussion

To accomplish our project addressed to the identification of new hCA VII inhibitors, we planned a computational study encompassing various steps. Starting from crystal data we generated 3D pharmacophore models describing the main chemical features shared by several hCA VII inhibitors. By merging the developed pharmacophore models we obtained a new hypothesis that has been used to carry out a virtual screening on compound libraries in order to take out new hypothetical hCA VII inhibitors. Finally the binding poses of ligands have been predicted using a docking approach.

2.1. Generation of structure-based pharmacophore models

Our study began with the use of LigandScout software, that is a tool able to create pharmacophores from structure-based complex data and to include some excluded volume spheres regarding areas inaccessible to any potential ligand, thus reflecting possible steric restrictions. As input for structure-based pharmacophore generation, the 3D structures of CA VII bound with the well-known sulfonamide inhibitors such as acetazolamide (**1**) (PDB: 3MDZ) and ethoxzolamide (**2**) (PDB: 3ML5) were chosen. These data are the only crystal complexes currently available on PDB.

Acetazolamide (**1**) and ethoxzolamide (**2**) display K_i values at nanomolar concentration and are characterized by hCA VII selectivity over hCA II.

By means of this automated structure-based model generation, we obtained 3D pharmacophore hypotheses representing the main interactions between enzyme and inhibitors **1** or **2**. In Fig. 1 (panel A) the generated model for acetazolamide (**1**) is shown. It consists of seven hydrogen bonding features including: (i) four H-bond acceptors which bind residues G92, T199, T200, and a water molecule (HOH354); (ii) three H-bond donors pointed towards H94, T199, and a water molecule (HOH354). The panel B (Fig. 1) depicts the generated model for ethoxzolamide (**2**). This five feature hypothesis contains: (i) two H-bond acceptor features which made H-bond with residues T199 and T200; (ii) one H-bond donor interacting with the H119 and (iii) two hydrophobic spheres which project into a hydrophobic pocket defined by A135, F131 and L198. Finally, we found that the two pharmacophoric hypotheses shares eight excluded volumes.

Later, a new structure-based pharmacophore model was generated by superimposing the two structure-based hypotheses and removing the overlapped chemical features (Fig. 1, panel C). Since HOH354 is not a conserved molecule, we eliminated the two related H-bond features. Furthermore, we created a global sphere collecting the H-bond donor features generated from amino group of the two sulfonamide moieties. The final merged pharmacophore model, consisting of three H-bond acceptors (HBA1-3), one H-bond donor (HBD) and two hydrophobic regions (H1−H2), is depicted in panel C.

To validate this pharmacophore model we selected a test set of already reported hCA VII inhibitors which displayed a different degree of inhibitory effects. The best fit method was used to map the chemical functions of each compound and select the most suitable alignment among all conformations. The obtained fit values of test set of 20 CA inhibitors are listed in Table S1 (Supplementary data). Overall these results revealed a good prediction power of our final merged model.

Fig. 2 displays the alignment of the pharmacophoric hypothesis with four inhibitors taken out of the test set. For these selected compounds we reported experimental K_i values of inhibitory effects toward hCA VII and calculated best fit values. Among them we retrieved two sulfonamide derivatives such as celecoxib and indapamide which are well-known marketed anti-inflammatory and diuretic agent, respectively. Celecoxib is anti-inflammatory agent, while indapamide is diuretic agent. On the basis of the results obtained by analyzing test set data, we might suppose that molecules having fit value over 3.0, should be considered active CAIs. Thereby this model appears able to successfully filter the most active compounds from a series of CA inhibitors, thus having good discriminatory power.

2.2. Virtual screening

Encouraged by the results of model validation we performed a virtual screening on commercial available database. At the beginning of our virtual screening workflow we created a focused library retrieving from ZINC database only molecules (6313) containing sulfonamide moiety as crucial structural requirement for CA inhibition.

Geometric fit values were calculated for every hit compound based on how the chemical substructures well mapped onto the pharmacophore features.

The criterion for screening for further validation was the high fit values, which indicate good matches; therefore, 299 compounds having fit value over 3.0 were selected and among them, we found 22 already reported CA inhibitors. This result revealed a good predictive capability of our pharmacophore model. We scored out these well-known CA inhibitors as well as racemic compounds. Finally, to achieve chemical diversity we decided to screen the hit compounds through visual inspection.

2.3. Docking

After visual inspection the remaining 34 hits were submitted to a docking simulation using the genetic optimization for ligand docking (GOLD) software package with GoldScore scoring function. These computational studies were carried out using the crystal structure of hCA VII extracted from the structure of the complex hCA VII/acetazolamide from the RCSB Protein Data Bank (PDB: 3ML5).

On the basis of gold fitness score and ability of these compound to coordinate the zinc ion of the catalytic site, nine molecules were further selected. Finally, considering commercial availability and their price, the 4-bromo-2-chloro-*N*-[4-(sulfamoylmethyl)phenyl] benzenesulfonamide (**3**) and *N*-[4-(sulfamoylmethyl)phenyl]cyclohex-3-ene-1-carboxamide (**4**) were selected for further studies.

Fig. 3 displays the putative binding modes of hit compounds **3** and **4** (panel A and B, respectively). As drawn in the figure the sulfonyl group of compound **3** and **4** coordinates Zn^{2+} in the active site and establishes hydrogen bond interactions with the crucial residues T199 and T200.

In the region located above catalytic site, both **3** and **4** are able to create a hydrogen bond interaction with residue Q92 as it has been found for acetazolamide (**1**, see Fig. 1A). Furthermore, **3** and **4** occupy the hydrophobic area which has been highlighted in the pharmacophore model generated from crystal complex with inhibitor ethoxzolamide (**2**, see Fig. 1B). As indicated in Fig. 3 the substituted phenyl ring of compound **3** interacts with hydrophobic residues G1, V121, F131, and L198 (Panel A), whereas the cyclohexene moiety of **4** is involved in hydrophobic interaction with residues V121, F131, A135, L141, and L198 (Panel B). In the Supplementary data the alignment of **3** and **4** onto the 3D merged six features hypothesis has been reported (with a high best fit value of 5 and 4.5, respectively).

2.4. Biological evaluation of the selected compounds

Considering the promising results of virtual screening and docking studies, we chose to purchase **3** and **4** from Sigma−Aldrich (Milan, Italy) and to perform biochemical study. These compounds have been evaluated for their ability to inhibit human carbonic anhydrases.

The assayed compounds **3** and **4** showed K_i values towards hCA VII in the nanomolar range with K_i value of 62.9 nM and K_i value of 39.4 nM, respectively. Compounds **3** and **4** were also proved to be active CA inhibitors towards two other druggable isoforms such as hCA IX and hCA XIV (see Table 1).

3. Conclusions

We were able to demonstrate that a virtual screening approach based on the combined use of pharmacophore modeling and

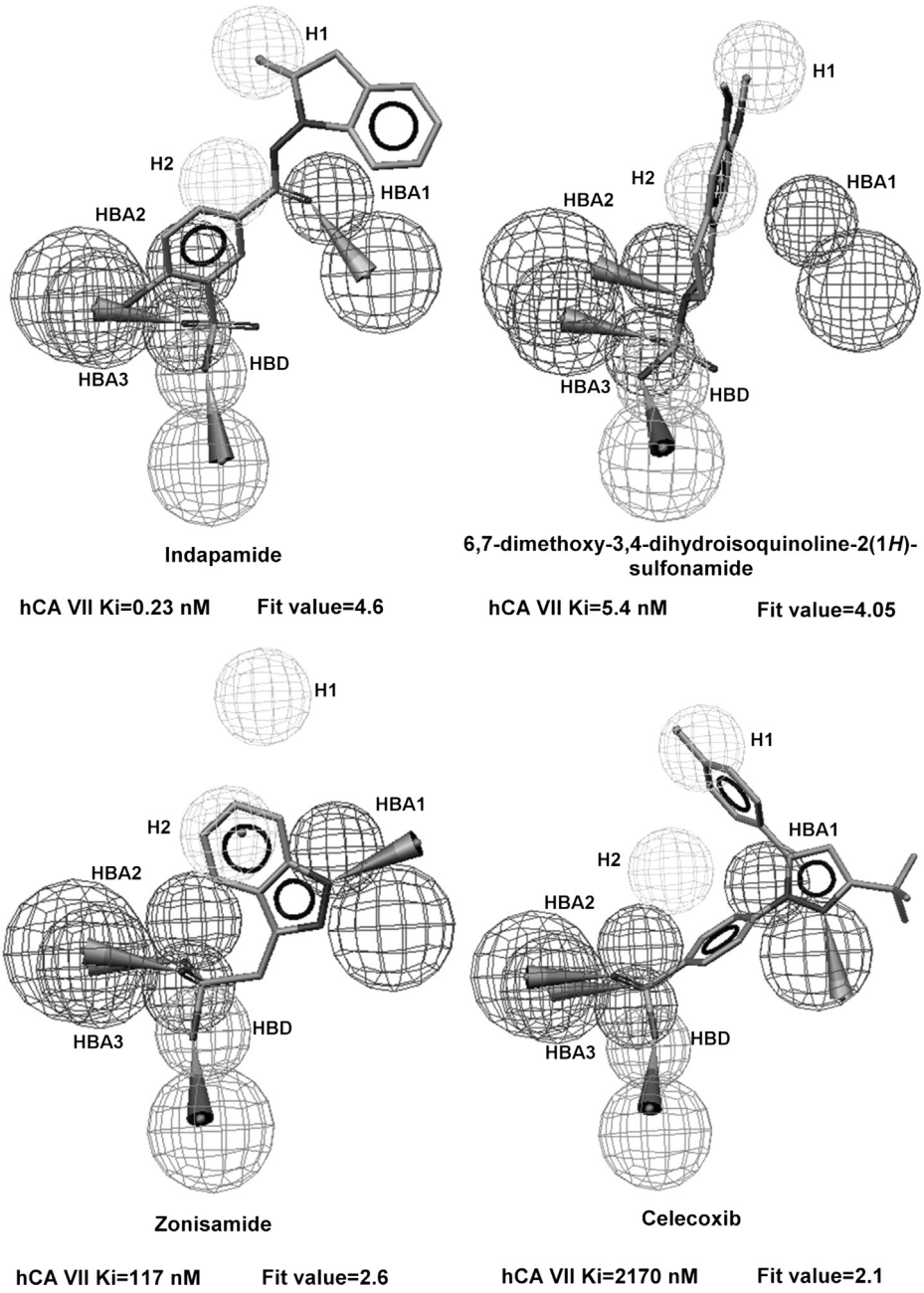

Fig. 2. Alignment of the pharmacophore model with four CAIs taken out of the test set of 20 molecules. Experimental K_i values toward hCA VII and calculated best fit values are reported.

docking procedure can be applied to discover novel scaffolds for carbonic anhydrase VII inhibitors. The two assayed molecules **3** and **4** exerted inhibitory properties against CA catalyzed CO_2 hydration activity and were effective in the nanomolar range toward hCA VII isoform.

4. Experimental section

4.1. Pharmacophore modeling

For the generation of structure-based pharmacophore models for CAIs, the software LigandScout was applied. As input for structure-based pharmacophore generation we selected 3D structures of CA VII bound with acetazolamide (3MDZ) and ethoxzolamide (PDB: 3ML5). Following this, a new structure-based pharmacophore was generated by superimposing the two structure-based hypotheses and removing the overlapped chemical features. This model was imported into Catalyst 4.11 [36] and used as input for HipHop algorithm to generate qualitative common feature-based pharmacophore model for CAIs. This pharmacophore was validated with a test set of known hCA VII inhibitors. The 20 compounds of the test set were generated using the 2D/3D editor sketcher in the Catalyst program and energy minimized to the closest local minimum using a molecular mechanics approach. To build conformational models of up to 250 conformers for each molecule, the best conformer generation option and a 10 kcal/mol energy cutoff were chosen.

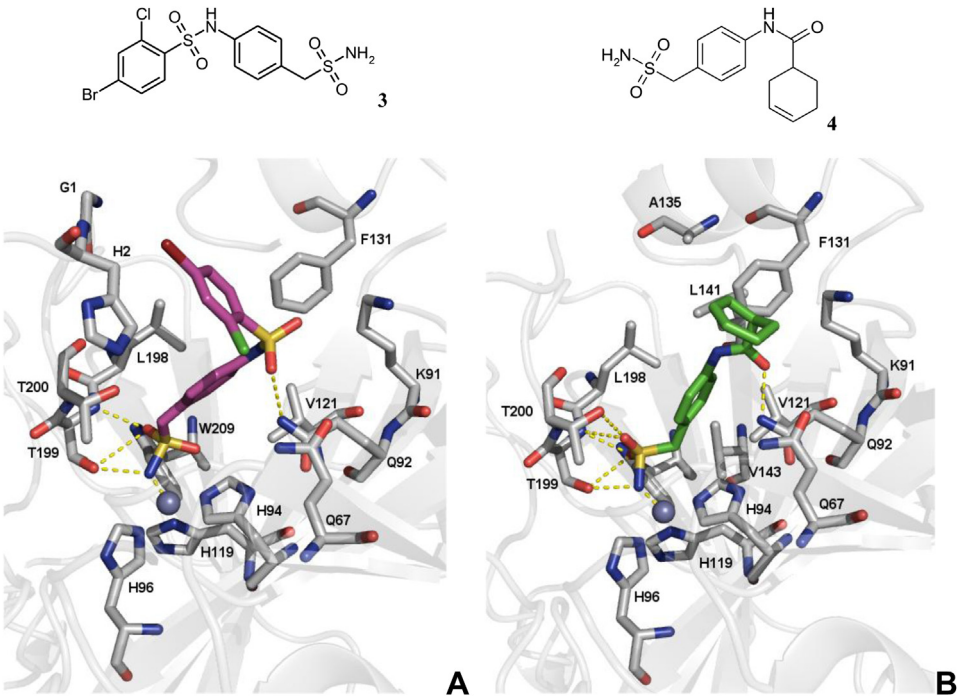

Fig. 3. View of **3** (magenta) and **4** (green) located in the active center of CA VII. Zn^{2+} is shown as a gray transparent sphere. The hydrogen bonds and coordinating interactions are shown with yellow dashed lines. The picture was generated using PyMOL [45]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Virtual screening of commercial libraries

VS experiments were conducted within Catalyst with the pharmacophore model as search queries applying the Best Flexible Search option. Conformational models for the commercial library compounds were created using the same procedure as applied for the compounds of test set. Only compounds that showed a best fit value major than 3.0, which were used as virtual screening queries, were retrieved as hits.

4.3. Docking studies

The crystal structures of hCA VII in complex with the inhibitor acetazolamide was retrieved from the RCSB Protein Data Bank (PDB: 3ML5). The ligand and water molecules were discarded, and the hydrogen atoms were added to protein with Discovery Studio 2.5.5 [37] The conformational behavior of simulated compounds was investigated by a MonteCarlo procedure (as implemented in the VEGA suite of programs which generated 1000 conformers by randomly rotating the rotors) [38]. All geometries obtained were stored and optimized to avoid high-energy rotamers. The 1000 conformers were clustered by similarity to discard redundancies; in this analysis, two geometries were considered nonredundant when they differed by more than 60° in at least one torsion angle. For each derivative, the lowest energy structure was then submitted to docking simulations.

The ligands minimized in this way were docked in their corresponding proteins by means of Gold Suite 5.0.1 from the Cambridge Crystallographic Data Centre (CCDC) [39].

The region of interest used by Gold was defined in order to contain the residues within 10 Å from the original position of the ligand in the X-ray structure. GoldScore was chosen as a fitness function. The standard default settings were used in all the calculations, and the ligands were submitted to 100 genetic algorithm runs. The "allow early termination" command was deactivated. Results differing by less than 0.75 Å in ligand-all atom rmsd were clustered together.

Table 1
K_i values (nM) against hCA I, hCA II, hCA VII, hCA IX and hCA XIV isoforms showed by compounds **3**–**4**, acetazolamide (**1**) and ethoxzolamide (**2**).

	K_i (nM)[a]				
	hCA I	hCA II	hCA VII	hCA IX	hCA XIV
3	8.6	6.3	62.9	66.0	19.4
4	8.9	73.2	39.4	53.2	7.6
Acetazolamide (**1**)[b]	250	12	2.5	25.0	41.0
Ethoxzolamide (**2**)[b]	25.0	8.0	0.8	34.0	2.5

[a] Recombinant full length hCA I, II VII and XIV and catalytic domain of hCA IX were used.
[b] Data are taken from Ref. [2].

4.4. CA inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 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 non-linear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained as reported earlier by this group [40–43].

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.071.

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