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RESEARCH ARTICLE

# Computational investigation of the selectivity of salen and tetrahydrosalen compounds towards the tumor-associated hCA XII isozyme

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**Abstract**

In previous work, 14 salen and tetrahydrosalen compounds have been synthesized and tested in enzyme inhibition assays against cytosolic human carbonic anhydrase isozymes I and II (hCA I and II) and tumor-associated isozymes IX and XII (hCA IX and XII). These compounds show selectivity against hCA XII over hCA I, II and IX. In this study, molecular modeling and docking studies were applied to understand this preference of the compounds for hCA XII. Most likely, the compounds can displace the zinc-bound water molecule of hCA XII to form a direct interaction with the $Zn^{2+}$ ion. In the other isozymes, the compounds might not be able to displace the water molecule nor are they expected to interact with the $Zn^{2+}$ ion.

**Keywords**

Carbonic anhydrase inhibitor, docking, salen, tetrahydrosalen

**History**

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## Introduction

The human carbonic anhydrases (hCAs) are $Zn^{2+}$ containing enzymes that interconvert carbon dioxide and bicarbonate (EC 4.2.1.1)[1–4]. This very simple reaction is of great importance in physiology and homeostasis[1–4]. Among many other functions, hCAs control the acidity of the environment and they deliver bicarbonate to biosynthetic pathways or to carrier systems involved in the transport of essential compounds. As such, these enzymes are (putative) drug targets in various pathophysiological processes.

Many catalytically active hCA isoforms are present in humans (hCA I–VII, IX and XII–XIV)[1–4]. Of special interest are the tumor-associated hCA IX and XII isoforms. These enzymes have an extracellular catalytic domain and are anchored to the cell by a transmembrane domain. The hCA IX and XII isoforms are up-regulated in tumors of the kidney, lung, breast and brain[1,5–8]. Furthermore, inhibition of the hCA IX and XII isozymes in animal tumor models induced a decrease in cell proliferation[9].

Various inhibitors of hCA IX and XII have been synthesized by our group[10–12]. Most likely, these compounds inhibit the catalytic site by binding directly to the $Zn^{2+}$ ion. However, phenol and polyamine carbonic anhydrase inhibitors can form complexes with the zinc-bound water molecule instead of a direct interaction with the $Zn^{2+}$ ion[13–15].

In a recent study, 14 salen and tetrahydrosalen compounds were synthesized and tested in enzyme inhibition assays against the cytosolic hCA I and II and the tumor-associated hCA IX and XII[16]. These derivatives showed selectivity towards hCA XII over the other three tested isoforms. One of the compounds showed an $IC_{50}$ value in the lower nanomolar region (37 nM) and at least 95-fold selectivity over the other isoforms.

In this investigation, docking studies were performed to suggest possible binding modes for these ligands and to understand the high selectivity and inhibition of hCA XII compared to the other isoforms.

## Materials and methods

### Preparation of ligand files

Compounds were built in 3D using MOE (version 2013.08, Chemical Computing Group Inc., Montreal, Canada). Compounds **1B–6B** have two nitrogen atoms that can be protonated at physiological pH values, while compounds **1A–8A** cannot. In addition, these alkaline nitrogen atoms could be too close to each other for double-protonation to occur. Therefore, only one of the nitrogen atoms of compounds **1B–6B** was protonated. Finally, the molecules were energy minimized using the MMFF94x force field.

### Preparation of protein files

Crystal structures of hCA I in complex with topiramate (3LXE; 1.90 Å), hCA II in complex with 2,5-dihydroxybenzoic acid (4E3D; 1.60 Å) and resorcinol (4E49; 1.45 Å), hCA IX in complex with acetazolamide (3IAI; 2.20 Å) and hCA XII also in complex with acetazolamide (1JD0; 1.50 Å) were obtained from the Protein Data Bank (PDB server). Chain A was retained when more than one chain was present. The cocrystallized

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inhibitor, the $Zn^{2+}$ ion and, if present, the zinc-bound water molecules were retained and all other water molecules, buffers and ions were deleted. Hydrogen atoms were added using the ''protonate 3D'' tool of MOE and the system was energy minimized using the AMBER99 force field. Subsequently, all protein structures were superposed on the hCA I structure using their Cα-atoms with MOE.

### Docking studies

Docking studies were performed using the GOLD Suite package (version 5.2, Cambridge Crystallographic Data Centre, Cambridge, UK) with and without the zinc-bound water molecule. Compounds **1A–8A** and **1B–6B** were docked into all five protein structures (25 dockings per ligand) and scored with the ChemScore scoring function. The binding pocket was defined as all residues within 13 Å of a centroid (*X*: −17.071, *Y*: 35.081, *Z*: 43.681; this point corresponds to the location of atom CAL of topiramate in the 3LXE structure).

For docking studies with the water molecule, we used the zinc-bound water present in the hCA II structures 4E3D and 4E49. The hCA I, IX and XII structures do not have a zinc-bound water molecule. Since the structures (especially the $Zn^{2+}$-binding histidine residues) superposed well, we used the zinc-bound water from 4E3D in the docking studies.

## Results and discussion

### Inhibition of the cytosolic hCA I and II and the tumor-associated hCA IX and XII enzymes by compounds 1A–8A and 1B–6B

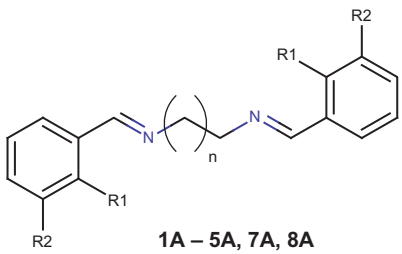
Salen and tetrahydrosalen derivatives **1A–8A** and **1B–6B** have been synthesized by Supuran et al. (see Table 1 for the ligand structures)[16]. Subsequently, these compounds were tested in enzyme inhibition assays against the cytosolic hCA I and II and the tumor-associated hCA IX and XII (Table 1)[16].

All compounds show low inhibition of hCA I ($IC_{50} > 100\,\mu M$). However, several derivatives show $IC_{50}$ values in the low micromolar range for hCA II and IX or even in the low nanomolar range for hCA XII (Table 1).

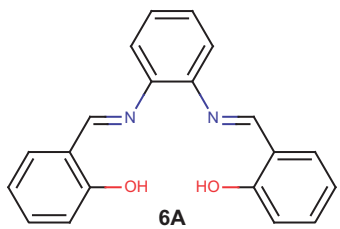
Compounds **7A** and **8A** show high selectivity towards hCA XII over the other isozymes. The $IC_{50}$ values of these two compounds for hCA I, II and IX are $>100\,\mu M$. Compounds **1A** and **1B** also show selectivity towards hCA XII over the other isozymes. However, the selectivity towards hCA XII over hCA II is smaller compared to compounds **7A** and **8A**.

Compound **3B** shows the lowest obtained $IC_{50}$ value ($IC_{50}$: 37 nM for hCA XII; see Table 1). It also shows low $IC_{50}$ values for hCA II and IX (3.53 and 4.37 μM, respectively), but the selectivity ratio against hCA XII is high (95-fold and 118-fold, respectively).

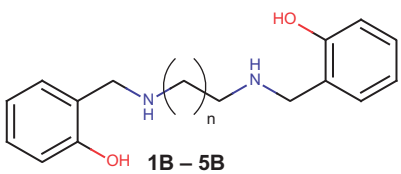
Table 1. Inhibition of the cytosolic hCA II and tumor-associated hCA IX and XII enzymes by compounds **1A–8A** and **1B–6B**.



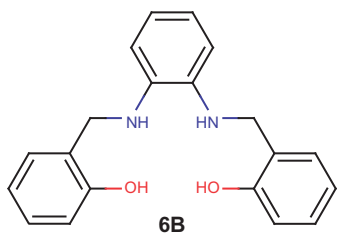

**1A – 5A, 7A, 8A**




**6A**




**1B – 5B**




**6B**

| | | | | Ki (μM) | | | Ratio/selectivity* | | |
|---|---|---|---|---|---|---|---|---|---|
| Cpd | *N* | R1 | R2 | hCA II | hCA IX | hCA XII | hCA II/hCA IX | hCA II/hCA XII | hCA IX/hCA XII |
| **1A** | 1 | OH | H | 1.72 | >100 | 0.28 | hCA II | 6.14 | hCA XII |
| **2A** | 2 | OH | H | 4.19 | 15.7 | 0.95 | 0.27 | 4.41 | 16.53 |
| **3A** | 3 | OH | H | 3.92 | 10.5 | 0.88 | 0.37 | 4.45 | 11.93 |
| **4A** | 4 | OH | H | 3.13 | 4.47 | 0.54 | 0.70 | 5.80 | 8.28 |
| **5A** | 5 | OH | H | 3.60 | 4.35 | 0.43 | 0.83 | 8.37 | 10.12 |
| **6A** | – | – | – | 3.42 | 2.45 | 1.17 | 1.40 | 2.92 | 2.09 |
| **7A** | 1 | OH | $OCH_3$ | >100 | >100 | 1.49 | – | hCA XII | hCA XII |
| **8A** | 5 | $NO_2$ | H | >100 | >100 | 15.6 | – | hCA XII | hCA XII |
| **1B** | 1 | – | – | 3.69 | >100 | 0.17 | hCA II | 21.71 | hCA XII |
| **2B** | 2 | – | – | 0.39 | 3.37 | 0.04 | 0.12 | 9.75 | 84.25 |
| **3B** | 3 | – | – | 3.53 | 4.37 | 0.037 | 0.81 | 95.41 | 118.11 |
| **4B** | 4 | – | – | 3.39 | 4.19 | 0.17 | 0.81 | 19.94 | 24.65 |
| **5B** | 5 | – | – | 0.24 | 2.51 | 0.047 | 0.10 | 5.11 | 53.40 |
| **6B** | – | – | – | 0.31 | 0.22 | 0.37 | 1.41 | 0.84 | 0.59 |

All compounds show $IC_{50} > 100\,\mu M$ for hCA I. Data are derived from Carradori et al.[16]

*The ratio between the $IC_{50}$ values of the two hCA enzymes is calculated. Otherwise, the enzyme for which the compound shows the lowest $IC_{50}$ value is given.

Increasing the spacer length of compound **1A** (to yield series **1A–5A**) the inhibition values for hCA II and hCA XII (Table 1) do not change markedly. In contrast, longer spacer lengths appear to be favorable for the inhibition of hCA IX as the inhibition value decreases from >100–4.35 μM (Table 1).

The effect of increasing the spacer length of compound **1B** (to yield series **1B–5B**) is less obvious. For hCA II, compounds **2B** and **5B** seem to have the correct spacer length, while for hCA XII compounds **2B**, **3B** and **5B** seem to have the proper spacer length. For hCA IX, all compounds seem to fit well except for compound **1B**.

Compounds of series **1B–6B** show higher inhibition of hCA IX and XII compared to compounds **1A–6A** (see couples **2B/2A**, **3B/3A**, **5B/5A** and **6B/6A**), thus the presence of an imine group in the compounds is not well tolerated by these two enzymes (Table 1), because the C=N double bond both changes the conformation of the molecule and decreases the basicity of the nitrogen atom. The nitrogen atoms of compounds **1B–6B** can be protonated at physiological pH values, whereas the nitrogen atoms of derivatives **1A–6A** cannot. This charge might improve the binding interactions of **1B–6B** compared to compounds **1A–6A**.

## Comparisons of the binding pockets of hCA I, II, IX and XII

The binding pockets of hCA I, II, IX and XII have been compared to identify differences that could result in the experimentally derived inhibition values. To this end, crystal structures of the enzymes (hCA I: 3LXE; hCA II: 4E3D and 4E49; hCA IX: 3IAI; hCA XII: 1JD0) have been superposed on their Cα-atoms. The structures superpose well (RMSD: 1.157 Å over 238 residues).

The crystal structure of hCA IX contains the residues before the conserved Trp5 (Gly1–Trp5), while this region is not present in the crystal structures of the other isozymes. This region is important since it could line the binding pocket and influence the binding mode of larger ligands (for example compounds **5A**, **5B** and **8A**).

The binding pocket is also largely conserved (Table 2), although there are differences that could influence ligand binding. In hCA I, residues His67, Phe91 and His200 are the most important differences with respect to the other CA isozymes. The His67 residue present in hCA I shows different hydrogen bonding and charge properties compared to the flexible Asn67 of hCA II, the flexible and longer side chain containing Gln67 of hCA IX and the flexible and cationic Lys67 of hCA XII (Table 2). His200 is located close to the $Zn^{2+}$ ion and Trp5 residue. As a result Trp5 has a different conformation in hCA I compared to the other crystal structures. His200 could adopt a conformation where it projects into the binding site and hinders the interaction of ligands with the $Zn^{2+}$ ion and Thr199 residue. Finally, the Thr200His mutation disables any hydrogen bonds that the ligands could form with Thr200 present in hCA II, IX and XII. Especially, the His67 and His200 residues might be responsible of the high $IC_{50}$ values ($IC_{50} > 100$ μM) of the compounds for the hCA I isozyme.

Table 2. Residues forming the binding pockets of hCA I, II, IX and XII.

| hCA I | hCA II | hCA IX | hCA XII |
|---|---|---|---|
| Trp5 | Trp5 | Trp5 | Trp5 |
| Glu58 | Arg58 | Arg59 | Leu58 |
| Ile60 | Leu60 | Arg60 | Thr60 |
| Val62 | Asn62 | Asn62 | Asn62 |
| His64 | His64 | His64 | His64 |
| Ser65 | Ala65 | Ser65 | Ser65 |
| His67 | Asn67 | Gln67 | Lys67 |
| Asn69 | Glu69 | Thr69 | Asn69 |
| Phe91 | Ile91 | Leu91 | Thr91 |
| Gln92 | Gln92 | Gln92 | Gln92 |
| Ala121 | Val121 | Val121 | Val121 |
| Leu131 | Phe131 | Val131 | Ala131 |
| Ala135 | Val135 | Leu135 | Ser135 |
| Leu141 | Leu141 | Leu141 | Leu141 |
| Val143 | Val143 | Val143 | Val143 |
| Leu198 | Leu198 | Leu198 | Leu198 |
| Thr199 | Thr199 | Thr199 | Thr199 |
| His200 | Thr200 | Thr200 | Thr200 |
| Tyr204 | Leu204 | Ala204 | Asn204 |

Almost all ligands showed the lowest $IC_{50}$ values for the tumor-associated hCA XII (Table 1). Considerable differences do exist between the active site of hCA XII and the active sites of the other isoforms (Table 2). These will influence ligand binding by steric interactions as well as long range electrostatic interactions.

## Binding interactions of the compounds with hCA I, II, IX and XII

Compounds **1A–8A** and **1B–6B** have been docked into the crystal structures of hCA I (PDB: 3LXE), hCA II (PDB: 4E3D and 4E49), hCA IX (PDB: 3IAI) and hCA XII (PDB: 1JD0) to understand the inhibition data. Docking studies were performed with and without a $Zn^{2+}$-bound water molecule.

### *Binding interactions with the tumor-associated hCA XII*

Docking studies of compounds **1A–8A** and **1B–6B** into the active site of hCA XII, without the $Zn^{2+}$-bound water molecule, resulted in similar binding poses for the ligands. One of the aromatic rings is positioned close to the $Zn^{2+}$ ion within the hydrophobic pocket formed by Val121, Leu141, Val143, Leu198, Val207 and Trp209. With the exception of Val121, all these residues are conserved in the four isozymes (Table 2). The ligands interact with the $Zn^{2+}$ ion through the aromatic hydroxyl group. The other aromatic moiety forms hydrophobic interactions with Trp5 and hydrogen bonding interactions with Pro201 are also observed. In several binding poses, the other aromatic moiety is able to form hydrogen bonds with the side chain of Trp5. Interestingly, the ligand hydroxyl group that interacts with the $Zn^{2+}$ ion adopts a similar location as the oxygen atom of the $Zn^{2+}$-bound water molecule

A representative binding pose of compound **3B**, with the lowest obtained inhibition value for hCA XII (37 nM) and the highest selectivity over the other isozymes, is shown in Figure 1. Compound **3A**, which has a nitrogen atom involved in a double bond, shows a higher inhibition value for hCA XII (880 nM). The increase in $IC_{50}$ value due to the presence of this further unsaturation might be related to the diminished flexibility of the ligand. As a result, it could be more difficult for the ligand to adapt to the binding site and pick-up binding interactions. Alternatively, the positive charge of **3B** may interact with the flexible and protonatable His64 (proton shuttle; distance ~4.1 Å).

Several docked poses of the flexible compound **5A** have been obtained for hCA XII. One of the aromatic groups is located close to the $Zn^{2+}$ ion and the hydroxyl group is situated at a similar position as the zinc-bound water molecule. The other part of the molecule adopts different docked poses. In one pose, the compound forms hydrogen bonds with Trp5 and Asn62, while the aromatic group forms hydrophobic interactions with His64. In two other poses, the ligand forms only hydrogen bonds with Trp5 and/or Pro201 (Figure 2A).

Replacement of the hydroxyl group of compound **5A** by a nitro group yields compound **8A**. The latter compound has an approximately 36-fold higher $IC_{50}$ value for hCA XII compared to compound **5A**. One nitro group is located close to the $Zn^{2+}$ ion

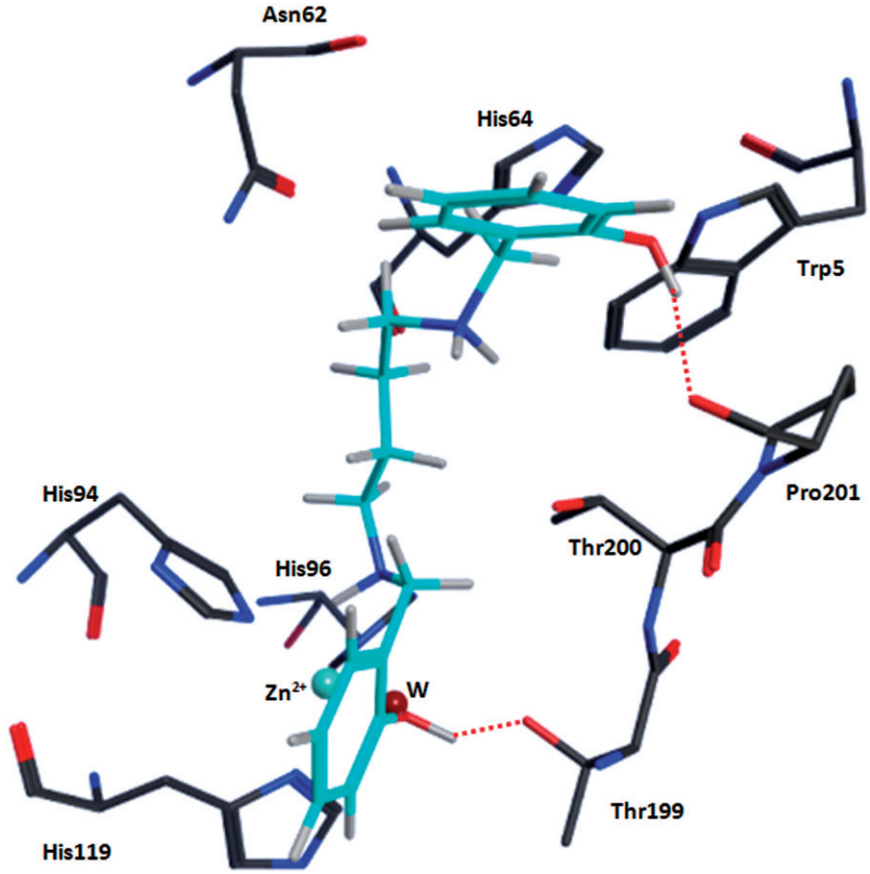



Figure 1. A representative docked pose of compound **3B** (turquoise) within the active site of hCA XII. One of the ligand's hydroxyl group is located at the position of the zinc-bound water molecule and interacts both with $Zn^{2+}$ as well as the side chain of Thr199. The other hydroxyl group of the ligand forms hydrogen bonds (red-dotted lines) with the backbone carbonyl group of Pro201 while the phenyl group forms hydrophobic interactions with Trp5.

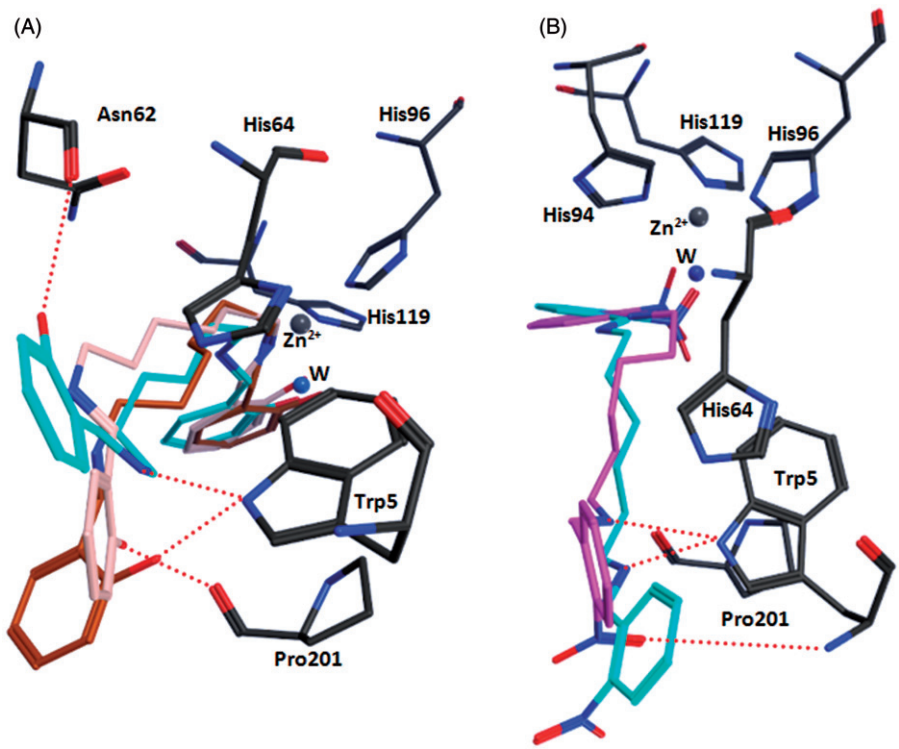



Figure 2. Docked poses of compounds **5A** (panel A) and **8A** (panel B) within the active site of hCA XII. Hydrogen bonds are indicated with dotted red lines.

and one of its oxygen atoms is located at a similar position as the zinc-bound water molecule. The other nitro group interacts with either Trp5 or Tyr20 (Figure 2B). It should be noted that these larger ligands (**5A** and **8A**) approach the region that is missing in the crystal structures of hCA I, II and XII (first 5 residues). Docking studies were repeated using the same protocol, but in the presence of the zinc-bound water molecule. The obtained docked poses were not able to interact with the water molecule.

### *Binding interactions with the other hCA isozymes*

Compounds **1A**–**8A** and **1B**–**6B** were docked into the binding pockets of hCA I, II and IX in the presence or absence of the

zinc-bound water molecule. In the absence of the water molecule, the ligands could adopt docked poses for hCA II and IX that are similar to hCA XII. However, differences do exist. The hydroxyl group of the ligands is located close to the water molecule, while for hCA XII the hydroxyl group adopted a similar location. The other end of the ligands could form hydrogen bonds with Trp5 and/or Pro201 of the hCA II and hCA IX structures. In addition, the ligands could form hydrophobic interactions with Phe131 of hCA II.

For the hCA I structure without the water molecule, no docking poses were obtained that could displace the zinc-bound water molecule. Again, docking interactions obtained for hCA I, II and IX structures with the zinc-bound water molecule showed no interactions with this water molecule.

*Interpretation of the SAR using the docking results*

It has proven difficult to precisely pinpoint structural features that are responsible for the selectivity of the compounds for the CA isozymes. Most likely, the selectivity of the compounds for hCA XII is caused by the displacement of the zinc-bound water molecule by the ligands (and form a direct interaction with the $Zn^{2+}$ ion) while forming hydrophobic interactions with Trp5 and hydrogen bonds with Pro201 (Figure 1). This is in strong contrast to polyamines and phenols, which interact with the zinc-bound water molecule and not with the $Zn^{2+}$ ion[13–15]. It seems that the ligands can less easily adopt docked poses that could displace the zinc-bound water molecule in hCA II and hCA IX. In hCA I, no direct interaction between the $Zn^{2+}$ ion and the ligands has been observed. This could explain the selectivity of the compounds for hCA XII and the high $IC_{50}$ values for hCA I.

Differences in inhibition values of the compounds observed for specific CA isozymes are more difficult to rationalize. The length and flexibility of the spacer influence the capability of the ligand to pick-up interactions with the active site residues. In addition, the charge of the ligands will definitively influence binding strength due to long-ranged electrostatic interactions.

## Conclusions

Salen and tetrahydrosalen compounds belong to a new proposed scaffold which display strong inhibition of tumor-associated hCA XII, weaker inhibition of hCA II and hCA IX and virtually no inhibition of hCA I (>100 μM). Rationalization of the inhibition proved difficult since long-ranged electrostatic interactions and entropy effects are important in ligand–protein interactions. Nevertheless, the high inhibition of hCA XII could be the result of the displacement of the zinc-bound water molecule and hence a direct coordination to the $Zn^{2+}$ ion. For hCA II and IX, the displacement of the water molecule seems to be more difficult, while it might be absent for hCA I.

## Declaration of interest

The authors report no declarations of interest. This work was supported by a grant from the 7th FP of EU (Metoxia).

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