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Nanoscale enzyme inhibitors: Fullerenes inhibit carbonic anhydrase by occluding the active site entrance

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ABSTRACT

We investigated a series of derivatized fullerenes possessing alcohol, amine, and amino acid pendant groups as inhibitors of the zinc enzymes carbonic anhydrases (CAs, EC 4.2.1.1). We discovered that fullerenes bind CAs with submicromolar—low micromolar affinity, despite the fact that these compounds do not possess moieties normally associated with CA inhibitors such as the sulfonamides and their isosteres, or the coumarins. The 13 different mammalian CA isoforms showed a diverse inhibition profile with these compounds. By means of computational methods we assessed the inhibition mechanism as being due to occlusion of the active site entrance by means of the fullerene cage (possessing dimension of the same order of magnitude as the opening of the enzyme cavity, of 1 nm). The pendant moieties to the fullerene cage make interactions with amino acid residues from the active site, among which His64, His94, His96, Val121, and Thr200. Fullerenes thus represent a totally new class of nanoscale CA inhibitors which may show applications for targeting physiologically relevant isoforms, such as the dominant CA II and the tumor-associated CA IX.

1. Introduction

Fullerene (C_{60}) and its derivatives represent nanoscale carbon materials with unique photo-, electro-chemical, and physical properties which render them particularly interesting for various biomedical and biotechnological applications.[1–6] Indeed, many such derivatives are reported to possess significant antiviral activity due to the inhibition of HIV-1 aspartic protease[2,3] with one such compound (Fullevir, the sodium salt of fullerene-polyhydropolyamino-caproic acid) being used clinically for the treatment of HIV infection.[2c] Furthermore, due to their extended π-conjugation, fullerenes absorb visible light, with a high triplet yield and can generate reactive oxygen species upon illumination, suggesting a possible role of this class of derivatives in photodynamic therapy.[4,5] Depending on the different functional groups with which fullerenes are derivatized (mainly for imparting water solubility and also different properties such as affinity for a target protein), these compounds are shown to effectively photoinactivate either pathogenic bacteria or malignant cancer cells.[4,5] In addition fullerenes are also shown to possess antioxidant and free-radical scavenging properties,[4–6] with potential applications as radioprotective[4] membrane tropic or anti-arthritis agents.[5,6]

Nanomedicine, that is, the application of nanotechnology to medicine, is not only a dynamic research field but also a powerful tool for designing innovative therapeutic and imaging approaches in cancer,[4a,b,d,7–10] and ocular[11] or infectious diseases.[4c] The first nanodrug in clinical use, pegylated liposomal doxorubicin, has had an important clinical success in the managements of various forms of tumors such as ovarian and breast cancers, lymphomas, and Kaposi sarcoma.[12] Indeed, many types of nanoscale materials have been explored in the last few years for drug delivery purposes, in order to address problems associated with limitations of conventional drug therapies such as poor solubility and biodistribution, lack of selectivity, and unfavorable pharmacokinetics.[4–12] In addition, nanomedicine has been exploited for its possibility to design tumor-targeted nanocarriers that are able to deliver radionuclides in a selective manner only to the tumor tissue, as well as for improving the efficacy and safety of cancer imaging and therapy methods.[4–12]

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is one of the targets recently shown to hold promise for designing novel approaches for therapy and imaging of hypoxic tumors.[13,14] Indeed, two of its isozymes, CA IX and XII (of the 16 presently known in

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mammals)[13,14] are predominantly found in tumor cells and show a restricted expression in normal tissues.[15] By efficiently hydrating carbon dioxide to protons and bicarbonate, these CAs contribute significantly to the extracellular acidification of solid tumors.[13–15] CA IX and XII are overexpressed in many such tumors in response to the hypoxia inducible factor (HIF) pathway,[15] and are inhibited by aromatic/heterocyclic sulfonamides and sulfamates. The inhibitors, however, generally do not show specificity for the inhibition of these tumor-associated isoforms versus the remaining CA isozymes (CA I–VII and XIII–XV) found in mammals.[13–15] CAs are in fact involved in numerous physiological and pathological processes, including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, 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, and many other such processes in humans.[13,16–21] In addition to the established role of the sulfonamide/sulfamate CA inhibitors (CAIs) as diuretics and antiglaucoma drugs, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, and antiinfective drugs.[13] Many of the mammalian CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[13–19] However critical barriers to the design of CAIs as therapeutic agents are related to the high number of isoforms in humans (i.e., 16 CAs, of which 13 have catalytic activity), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[13] Only very recently nanoscale CAIs have been reported by this group.[20] By decorating gold(0) nanoparticles with sulfonamides incorporating lipoic acid tails, we obtained nanoscale sulfonamide CAIs (with a diameter of 3 nm) which selectively inhibited the tumor-associated isoform CA IX over the cytosolic ubiquitous isozymes CA I and II. We also recently reported a totally novel class of CAIs, the coumarins,[22] which bind CAs in a zinc-independent manner, by anchoring, in hydrolyzed form, as 2-hydroxycinnamic acids, at the entrance of the active site and occluding it.

With a diameter of about 1 nm, which is the same as the opening of the active site entrance of most CA isozymes,[21] fullerene, the archetypical building block in nanoscience, might be an interesting candidate to be investigated for its interaction with CAs. In fact, neither fullerene nor any of its derivatives have been studied so far for their interaction with CAs. We investigate here whether some derivatized fullerenes may show CA inhibitory activity against all mammalian isoforms, that is, CA I–XV, binding in a similar manner as the coumarins.

2. Results and discussion

2.1. Chemistry

A series of derivatized fullerenes, compounds **1–7** have been included in this study.[23,24] These compounds incorporate either an aromatic alcohol (compound **1**) or aromatic amine/amino acid scaffolds (**2–7**) in their molecules (Chart 1). The annealing of the alcohol/amine/amino acid scaffold to the fullerene cage is achieved through cyclopropyl or cyclohexyl units.

These compounds have been chosen to be investigated for their interactions with CAs for at least two reasons:

(i) the presence of polar moieties in the molecules of derivatized fullerenes, such as the hydroxy, amino, or carboxy ones, leads to enhanced water solubility of these derivatives (a limitation of fullerene-based derivatives is constituted just by their poor water solubility),[1,4,6,24] and

(ii) the same moieties were shown to interact with amino acid residues at the entrance of the CA active site cavity, both in sulfonamide/sulfamate inhibitors[21,25,26] and in the coumarin inhibitors or the CA activators (CAAs), which bind in the same active site region as the coumarins.[22,27,28] It should be also stressed that many amino acids and amines, such as L-/D-histidine, L-/D-phenylalanine, and D-tryptophan, act as potent CAAs and their X-ray crystal structure in adduct with isozymes CA I and/or II has been reported by one of our groups.[27,28] Thus, the presence of the amino acid/amine moieties as pendant moieties on the fullerene cage may offer a relevant strategy for obtaining derivatized fullerenes that can interact with the CA active site.

2.2. CA inhibition

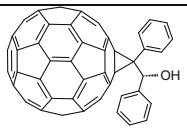
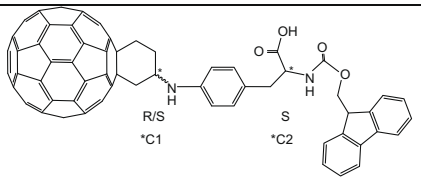
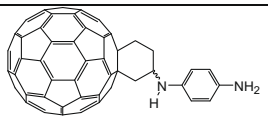
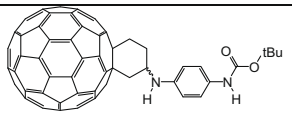
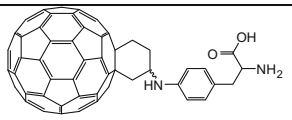
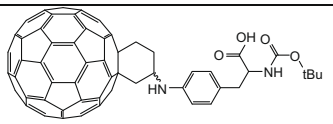
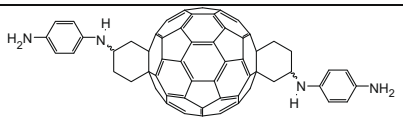
Inhibition data for compounds **1–7** against all 13 catalytically active mammalian (h = human, m = murine) CA isoforms, CA I–IV, VA, VB, VI, VII, IX, XII–XV, are presented in Table 1. The data have been obtained by a stopped-flow technique monitoring the physiological reaction catalyzed by these enzymes, that is, CO_2 hydration to bicarbonate and protons.[29]

The following should be noted regarding the inhibition data given in Table 1:

(i) Several CA isozymes were potently inhibited by all fullerenes **1–7**, with inhibition constants in the submicromolar range (K_Is of 0.61–0.93 μM), among which are the tumor-associated hCA XII, and the transmembrane ones hCA XIV and mCA XV. The structure–activity relationship (SAR) is quite straightforward, with all derivatized fullerenes **1–7** showing good activity. For example, the best inhibitor against hCA XII was the *p*-phenylenediamine derivative **3** (K_I of 0.61 μM). The protection of the amino group with the Boc moiety, as in **4**, or the bis-phenylenediamine **7** led to a slightly diminished activity compared to **3**. The same is true for the bulkier compounds **2, 5**, and **6** as well as for the alcohol **1**, but these derivatives also showed considerable hCA XII inhibitory activity. In the case of isozyme hCA XIV, the best inhibitor was the alcohol **1**, although the differences among inhibitory activity of all these compounds were rather small.

(ii) The only secreted isoform (in saliva and milk) hCA VI showed a more intricate inhibition profile with the fullerenes **1–7**. Indeed, the less bulky compounds **1** and **3** were submicromolar inhibitors (K_Is of 0.87–0.89 μM), the bisamine **7** had a K_I of 1.04 μM, whereas the remaining compounds were less inhibitory, with K_Is in the range of 5.91–8.50 μM (Table 1). In this case it may be stated that the compounds with more compact moieties (such as **1**, **3**, and **7**) were the best inhibitors whereas the bulkiest one (**2**, incorporating a Fmoc-protecting group) was the most ineffective one.

(iii) A large number of CA isozymes such as hCA I, II, III, VII, and XIII (cytosolic CAs) and the tumor-associated transmembrane and tumor-associated CA IX were inhibited by all fullerenes **1–7** in the low micromolar range, with inhibition constants typically in the range of 7–15 μM (Table 1). For example, the widely expressed, low activity hCA I and the very catalytically ineffective hCA III showed a very flat SAR with fullerenes **1–7**, being inhibited *t* with K_Is of 7.57–9.63 μM, and of 8.23–9.58 μM, respectively. The ubiquitous and catalytically very efficient hCA II (the physiologically dominant isozyme)[13] was best inhibited by the bisamine **7** (K_I of 6.73 μM) whereas the worst inhibitor was the alcohol **1** (K_I of 16.6 μM). hCA VII was slightly less susceptible to this

Compound No.	Structure
1	
2	
3	
4	
5	
6	
7	
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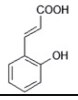
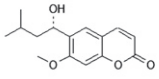
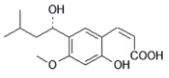
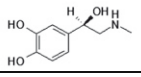
9	
10	
11	
12	
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Chart 1. Fullerene derivatives **1**–**7** and some non-fullerene CA inhibitors/activators **8**–**13**.

class of inhibitors compared to hCA I, with compounds **1–7** possessing K_Is in the range of 9.73–12.59 μM. The best hCA VII inhibitor was the very bulky, Fmoc-substituted Phe derivative **2**. The tumor-associated hCA IX showed inhibition constants with fullerenes **1–7** in the range of 7.93–14.32 μM. The best hCA IX inhibitors were the alcohol **1** and the bulky amino acid **2**, whereas the least effective one was the Boc-substituted Phe derivative **6**. Thus, even for a small series of derivatives relatively minor structural changes influence the CA inhibitory activity significantly, which is promising in designing a totally new class of inhibitors.

Table 1
Inhibition of mammalian carbonic anhydrase (CA, EC 4.2.1.1) isozymes with fullerenes **1–7**, by a stopped-flow CO_2 hydrase assay[29]

Isozyme	K_I[a] (μM)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
hCA I	7.57	8.74	9.63	9.54	8.82	9.31	7.95
hCA II	16.6	8.21	9.38	8.75	9.05	9.38	6.73
hCA III	8.90	9.37	8.77	8.91	9.58	9.42	8.23
hCA IV	3.66	5.27	6.44	11.5	7.04	12.3	94.5
hCA VA	49.7	60.8	51.8	50.3	111	94.4	85.9
hCA VB	38.3	34.4	40.6	27.7	87.6	28.6	64.5
hCA VI	0.87	8.5	0.89	5.91	5.98	6.07	1.04
hCA VII	10.31	9.73	9.82	10.58	10.6	10.94	12.59
hCA IX	7.93	9.19	12.9	11.07	13.4	14.32	12.87
hCA XII	0.73	0.79	0.61	0.66	0.67	0.76	0.73
hCA XIII	7.57	8.15	8.75	8.78	7.64	8.73	8.71
hCA XIV	0.77	0.78	0.93	0.89	0.9	0.88	0.84
mCA XV	0.82	0.86	0.9	0.89	0.83	0.81	0.82

[a] Errors in the range of ±5% of the reported data from three different assays; h = human, m = murine isoforms; Stopped flow, CO_2 hydrase assay technique.[29]

(iv) The extracellular isoform hCA IV, similar to hCA VI, shows a very typical and unique inhibition profile with these compounds (K_Is in the range of 3.66–94.5 μM). Thus, the bis-amine **7** is a very ineffective hCA IV inhibitor (K_I of 94.5 μM), whereas compounds **1–3** are effective ones (K_Is of 3.66–6.44 μM). Thus, the difference of activity between the two structurally related compounds **3** and **7** is important, with the monoamine **3** being 14.7 a better hCA IV inhibitor compared to the bis-derivative **7**. The remaining compounds **4–6** showed an intermediate activity between these extremes, with K_Is in the range of 7.04–12.30 μM (Table 1).

(v) The two mitochondrial isoforms hCA VA and hCA VB were the least inhibited ones by the fullerenes **1–7**. Indeed, these compounds showed inhibition constants in the range of 27.7–111 μM against these two enzymes.

All these data clearly show that each of the 13 catalytically active CA isozymes has a particular inhibition profile with fullerenes **1–7**. Furthermore, the SAR for this small series of derivatives is rather complex, with a range of inhibition constants from the submicromolar to submillimolar, which makes this class of derivatives of particular interest in developing nanoscale CAIs.

2.3. Docking studies and inhibition mechanism

Although fullerenes **1–7** contain amino and amino acyl moieties, typically found in CAAs[27,28] and not in CAIs,[13] these compounds do not activate but inhibit these enzymes. Nevertheless this is not so surprising, as recently, we have discovered a non-zinc-mediated CA inhibition mechanism, illustrated in Figure 1 for the simple coumarin **8** which is converted to the 2-hydroxycinnamic acid **9**.

Thus, our working hypothesis was that fullerenes amphiphiles, with their bulky cage and through the polar moieties found in pendant tails attached to them, may interact with the entrance of the CA active site in a manner which may resemble the binding of coumarins[22] (such as compounds **9** and **11** formed from **8** and **10**, respectively), or amines (such as L-adrenaline **12** and histamine **13**)[27,28] to CAs. Amino acids bind in the same active site region as amine CAAs to the various CA isoforms.[27,28] In fact, the coumarin and CAA binding sites to CAs are the same, as shown in Figure 2 where a superposition of the two hydrolyzed coumarins[22] and two activators bound to CA II, as determined by X-ray crystallography,[27,28] are presented. Indeed, these two classes of compounds (with different enzymatic activities, as inhibitors or activators of

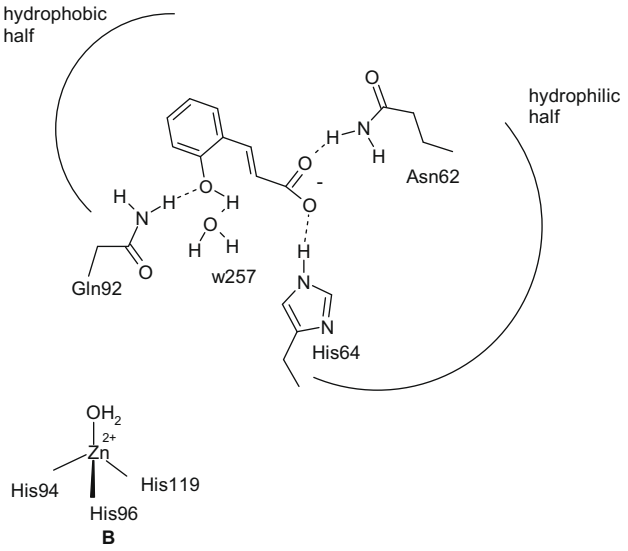

Figure 1. CA inhibition with hydrolyzed coumarin **8**. The 2-hydroxycinnamic acid **9** formed by active site mediated hydrolysis of **8** occludes the entrance of the active site cavity, interacting both with hydrophilic and hydrophobic amino acid residues.[22] The inhibitor does not interact with the catalytically crucial Zn(II) ion which is coordinated by three His residues and a water molecule.

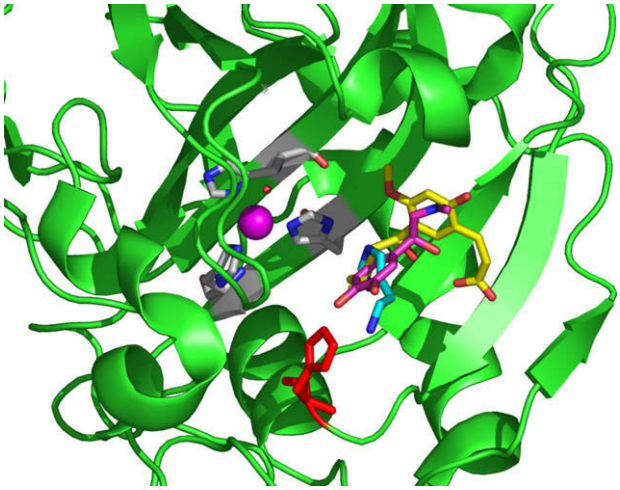

Figure 2. Superposition of hCA II adducts of hydrolyzed coumarin **10** (yellow, compound **11**) with L-adrenaline (magenta) **12** and histamine (blue sky) **13**. The coumarin and CAA binding sites are the same, at the entrance of the active site cavity. The catalytic Zn(II) ion is the violet sphere. Its three His ligands are shown in CPK colors, whereas in red is shown His64, an amino acid playing an important role in catalysis as proton shuttle residue.[13]

the physiologic CO_2 hydration reaction, respectively) bind at the entrance of the active site and interact with a large number of amino acid residues both from the hydrophilic and from the hydrophobic halves of it.[13,21,27–29]

As we were unable for the moment to crystallize a CA isozyme in complex with one of the fullerenes **1–7**, we performed in silico docking studies in order to investigate the interactions between fullerenes and amino acid residues within the CA active site.

The Glide-Induced Fit Docking (IFD) method was used in our docking simulations which provide a full flexibility to the active site residues and docked ligands.[30,31] It should be noted that the crystal water molecules and the Zn(II) ion were kept throughout the docking simulations, and thus, the used model represents an

accurate description of the biological environment in the enzyme when it interacts with fullerenes **1–7**. Two CA isozymes (CA II and CA IX) were used in these docking studies.

For both CA isoforms, the fullerene binds in similar active site region, as shown in Figure 3. Docking binding scores for fullerenes 1–7 within the CA II and IX active sites varied between −6.02 kcal/mol and −9.36 kcal/mol (i.e., from micromolar to submicromolar binding affinity, Table 2), which is within the same range as those obtained experimentally (Table 1).

The fullerene cage was observed to be located at the entrance of the CA active site when interacting with both CA isozymes, CA II and IX, thus occluding the entrance of substrates or other molecules within the cavity. Indeed, with an opening of around 1 nm,[25,27] the CA active site possesses the same dimensions as the fullerene cage itself.[1,23,24] The amino acid residues involved in these interactions were His64, His94, His96, Val121, and Thr200 (CA I numbering system, see Fig. 4). The docking studies showed that hCA II was best inhibited by compound **5**, whereas the worst inhibitor was the fullerene **1** (Table 2), which was found also experimentally to be the worst inhibitor of this isozyme. Thus, there is a good agreement between the experimental and computational studies regarding the interaction of hCA II/IX with fullerenes **1–7**.

Compound **6** showed good inhibition against isozyme hCA IX. Its binding to this isoform is represented in Figure 5, as obtained by the docking procedure. Although compound **6** has similar binding scores with hCA II and IX, the location of the fullerene cage showed different locations within these isozyme active sites. In hCA II, the fullerene cage was located more outwards within the active site compared to its binding to the hCA IX active site (Fig. 6). Since the binding scores were similar for these two isozymes, this is a confirmation that the pendant groups present in the fullerene molecule have an important role in the inhibition of the diverse CA isozymes. Thus, the main in silico finding is that the fullerene cage binds at the entrance of the CA active site and occludes it, inhibiting the enzymes in a manner similar to that of the coumarins, the recently discovered CAIs.[22] A second significant finding is that the pendant groups present in the fullerene derivative strongly influence the interaction of the inhibitor with amino acid residues found at the entrance of the active site cavity. As these residues are generally different between the various CA isozymes, this can lead to isoform-selective CAIs. This has been observed experimentally in the small library of derivatives investigated here and confirmed by means of the docking studies.

3. Conclusions

We report here that derivatized fullerenes amphiphiles bind to CAs with submicromolar-low micromolar affinity, although these compounds do not possess moieties normally associated with CA inhibitors such as the sulfonamides and their isosteres or the coumarins. The 13 different mammalian CA isoforms (CA I–XV) showed diverse inhibition profiles with these compounds. By means of computational methods we assessed the inhibition mechanism as being due to occlusion of the active site by means of the fullerenne cage (possessing dimension of the same order of magnitude with the opening of the enzyme cavity, of 1 nm) whereas the pendant moieties to the fullerene cage interact with amino acid residues from the active site, among which are His64, His94, His96, Val121, and Thr200. Fullerenes thus represent a totally new class of nanoscale CA inhibitors which may show applications for targeting physiologically relevant isoforms, such as the dominant CA II and the tumor-associated CA IX, which represent established drug targets.

4. Experimental protocols

4.1. Chemistry

Fullerenes **1–7** have been reported earlier by one of our groups[23,24] and were of >99% purity.

4.2. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[29] Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_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 the inhibitor (0.1 mM) were prepared in distilled–deionized water and dilutions up to

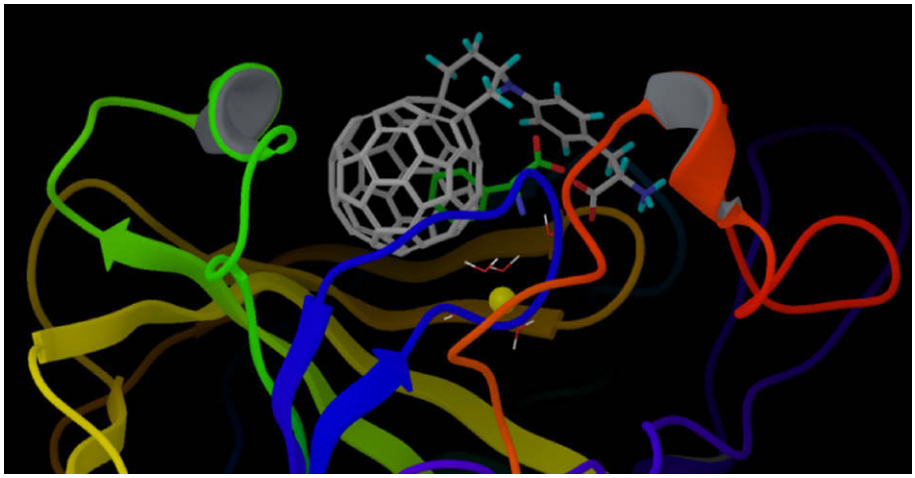

Figure 3. Binding of fullerene **5** to hCA II (shown as ribbon representation of the amide backbone). The catalytic Zn(II) ion is the yellow sphere at the center of the molecule. The hCA II—L-Phe adduct (from PDB file 2FMZ)[27b] has been used for the docking.

Table 2
Computational binding energies of fullerenes **1–7** to CA II and CA IX

Compound	CA II Glide/IFD docking score (kcal/mol)	CA IX Glide/IFD docking score (kcal/mol)
1	−6.66	−7.90
2	−8.32	−8.84
3	−6.76	−6.02
4	−7.31	−6.03
5	−9.14	−6.63
6	−7.95	−8.47
7	−6.95	−6.49

0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min—24 h at room temperature (15 min) or upto 24 h at 4 °C prior to assay, in order to allow for the formation of the E–I complex or for the eventual slow association of the inhibitor with the enzyme. With all incubation times the same K_I-s were obtained (within the limits of the experimental errors of this method, that is, ±5% of the reported K_I value), proving that the complexation of the fullerene with the CA active site is a rapid process. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[1,6,7] and represent the mean from at least three different determinations.

4.3. In silico docking studies

Crystal structures of CAs in complex with activators/inhibitors (pdb code 2FMZ (for hCA II)[27b] and 3IAI (for hCA IX)[25] were used for the docking calculations. Explicit water molecules and metal ions from the X-ray structure were kept for all the calculations. Before the docking simulations, the complexes were submitted to the protein preparation module of Schrodinger.[30] Fullerenes were constructed using the Schrodinger's Maestro module and then geometry optimization was performed for these ligands using Polak–Ribiere conjugate gradient (PRCG) minimization (0.0001 kJ $Å^{-1}$ mol^{-1}, convergence criteria). Protonation states of ligands and residues were tested using LigPrep and Protein Preparation modules under Schrodinger package[30] at neutral pH (experimentally the compounds have been tested at pH of 7.4). The Glide-XP (extra precision) (v5.0)[31] combined with Induced Fit Docking (IFD) have been used for the docking calculations. IFD uses the Glide docking program to account for the ligand flexibility and the refinement module and the Prime algorithm to account for flexibility of the receptor. Schrodinger's IFD protocol model uses the following steps

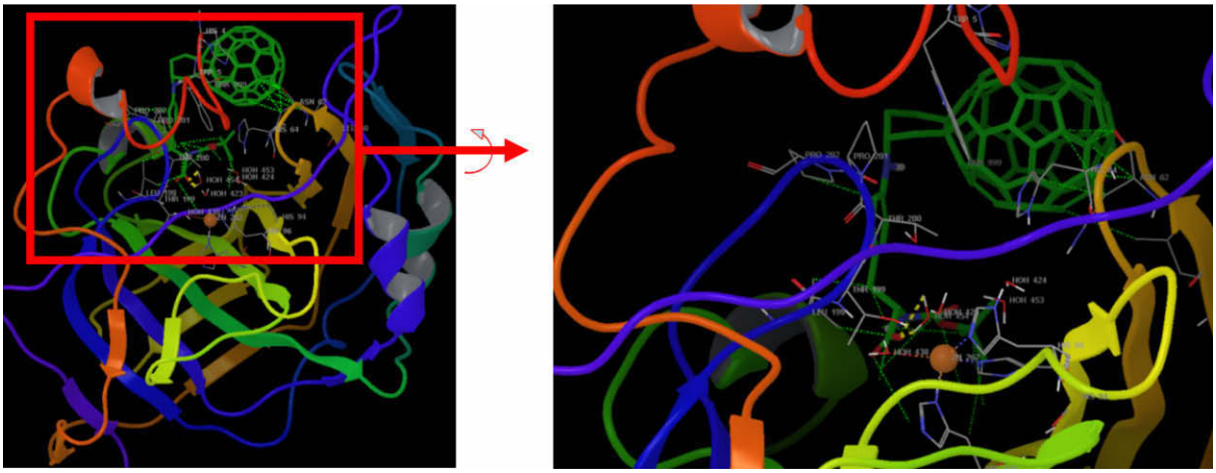

Figure 4. Binding of fullerene **4** to hCA II as obtained by the induced fit docking method. Yellow and green dashed bonds show H-bonds and close-van der Waals contacts, respectively. The catalytic Zn(II) ion is shown as orange spheres. The hCA II—L-Phe adduct (PDB file 2FMZ)[27b] has been used for the docking.

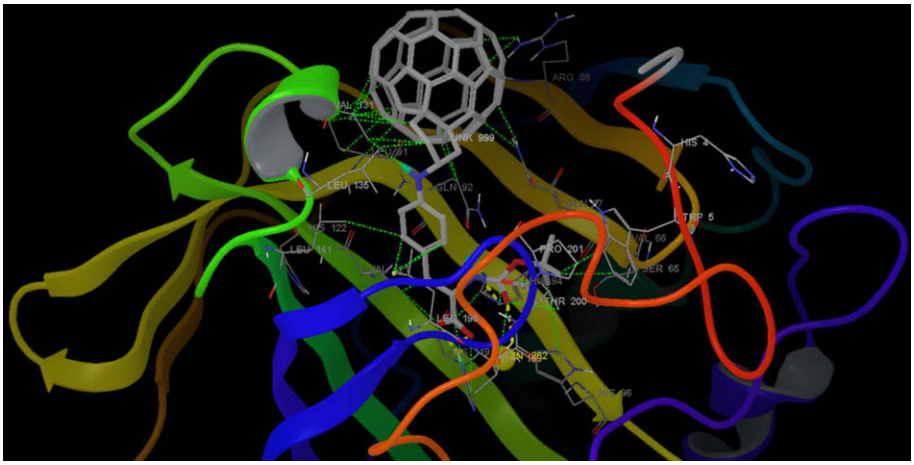

Figure 5. Binding of compound **6** to hCA IX. Yellow and green dashed bonds show H-bonds and close-van der Waals contacts, respectively. The catalytic Zn(II) ion is shown as the yellow sphere. The hCA IX—acetazolamide X-ray crystal structure has been used for the docking (PDB code 3IAI).[25]

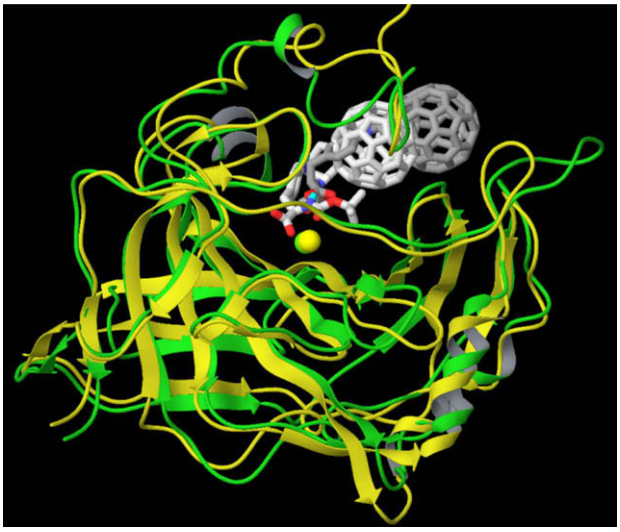

Figure 6. Superimposition of hCA II (shown in yellow colored ribbons) and hCA IX (shown in green colored ribbons) docking complexes of fullerene **6**. Dark colored fullerene shows the binding pose at hCA II and light colored fullerene shows the binding pose at hCA IX. The Zn(II) ion is shown by yellow (for hCA II) and green (for hCA IX) sphere, respectively.

(the description below id from the IFD user manual): (i) Constrained minimization of the receptor with an RMSD cutoff of 0.18 Å. (ii) Initial Glide docking of each ligand using a soft potentials (0.5 van der Waals radii scaling of non-polar atoms of ligands and receptor using partial charge cutoff of 0.15). (iii) Derived docking poses were refined using the Prime Induced Fit module of Schrodinger. Residues within the 5.0 Å of ligand poses were minimized in order to form suitable conformations of poses at the active site of the receptor. (iv) Glide re-docking of each protein–ligand complex.

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