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Carbonic anhydrase inhibitors: E7070, a sulfonamide anticancer agent, potently inhibits cytosolic isozymes I and II, and transmembrane, tumor-associated isozyme IX

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Abstract—E7070 [*N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide] is an anticancer drug candidate under clinical development for the treatment of several types of cancers. We prove here that this compound also acts as a potent carbonic anhydrase (CA) inhibitor. Similarly to the clinically used drugs acetazolamide, methazolamide and topiramate, E7070 showed inhibition constants in the range of 15–31 nM against isozymes I, II and IX, being slightly less effective as a CA IV inhibitor (K_i of 65 nM). The X-ray crystal structure of the adduct of hCA II with E7070 revealed unprecedented interactions between the inhibitor and the active site, with three different conformations of the chloroindole fragment of the inhibitor interacting with different amino acid residues/water molecules of the enzyme. A superimposition of these conformations with those of other sulfonamide/sulfamate CA inhibitors indicated that similar regions of the hCA II active site could be involved in the interaction with inhibitors.

1. Introduction

Sulfonamides possess many types of biological activities, and representatives of this class of pharmacological agents are widely used in clinic as antibacterial, hypoglycemic, diuretic, anti-hypertensive and antiviral drugs among others.[1,2] Recently, a host of structurally novel sulfonamide derivatives have been reported to show substantial antitumor activity in vitro and/or in vivo.[1–9] All these derivatives incorporate in their molecules a common chemical motif of aromatic/heterocyclic sulfonamide, but there are a variety of mechanisms of their antitumor action, such as inhibition of tubulin polymerization, cell cycle perturbation in the G1 phase, carbonic anhydrase (CA) inhibition, functional suppression of the transcriptional activator NF-Y, angiogenesis (matrix metalloproteinase or integrin α2) inhibition, and so on.[1–9] Among such compounds selected via elaborate screenings of compound libraries or obtained through computer-based drug design, E7070 (**1**) is one of the most potent anticancer sulfonamides ever reported.[8,10–12] This statement is based on the significant in vivo efficacy of **1** against various human tumor xenografts in nude mice, for example colon carcinomas HCT116, LS174T, SW620 and HCT15 and lung carcinomas LX-1 and PC-9.[8,10] In particular, this compound caused complete tumor regression in the xenografts of HCT116 and LX-1 following its intravenous administration at 25 mg/kg daily for 8 days.[10] Currently, **1** is under clinical evaluation (phase I trials in Japan and phase II trials in Europe and the United States), thus far demonstrating some objective antitumor responses in patients with colorectal cancer, non-small cell lung cancer, and so on.[11–14]

The precise mechanism of action has not been determined, but **1** is considerably different from conventional anticancer drugs in clinical use with respect to its effects on the cell cycle and its tumor type selectivity. In HCT116, one of the most sensitive cancer cell lines, **1** was found to suppress the expression of cyclin E and the

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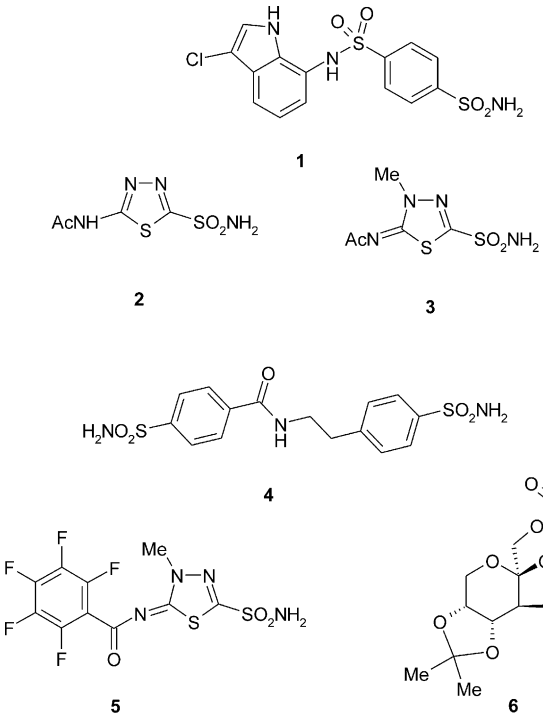
phosphorylation of CDK2, both of which are essential for the G1 to S transition.[8] A more detailed mechanistic study by Fukuoka et al.[15] clarified that **1** disrupted cell-cycle progression at multiple points, including both G1/S and G2/M transitions, in a human non-small cell lung cancer cell line A549. In their experiments using A549 and its **1**-resistant subline A549/ER, the compound was shown to inhibit pRb phosphorylation, to reduce the protein expression of cyclin A, cyclin B1, CDK2 and CDC2, and to suppress CDK2 catalytic activity with the induction of p53 and p21 proteins only in parental (drug sensitive) A549 cells. All these observations suggest that **1** belongs to a new class of cell cycle inhibitors affecting multiple cell-cycle checkpoints. More recently, DNA microarray analysis has further revealed that the drug treatment at 8 μM for 12 h altered at least 3-fold the expression levels of 60 transcripts; 58 of these were down-regulated, and the remaining two were up-regulated.[16,17] An observation of particular interest is profound transcriptional repression of subsets of energy metabolism and cell-cycle genes, including mitochondrial NAD(P)$^+$-dependent malic enzyme, DNA polymerase α and cyclin H, all of which are also significantly down-regulated by **1** in common in several other cancer cell lines (T. Owa, unpublished data).

Although the gene expression analysis has actually provided an important insight into the mode of action of **1**, it is also critical to identify a cellular protein target(s) of **1** for understanding accurately its anticancer mechanism(s). A most recent report by Oda et al. has described that cytosolic malate dehydrogenase (cMDH) is a specific binding protein and apparently a primary cellular target of **1** on the basis of quantitative proteomic analysis.[18] Independent of this study, we have investigated whether **1** shows inhibitory activity against CAs as this compound is a primary sulfonamide fitting for a general formula of CA inhibitors.[2,5] These enzymes play a key role in a multitude of physiological and physiopathological processes, including tumorigenesis.[2,5,6,19] Recently, at least two tumor-associated CA isozymes have been identified (CA IX and CA XII)[19] among the fourteen isozymes isolated in humans.[2,5,6] Of these, CA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia that involves HIF-1 binding to a hypoxia response element in the *CA9* gene promoter.[19] CA XII is present in various normal tissues and overexpressed in some tumors. It is also induced by hypoxia, but the underlying molecular mechanism remains undetermined. Both CA IX and CA XII are negatively regulated by von Hippel Lindau tumor suppressor protein and their expression in renal cell carcinomas is related to inactivating mutation of *VHL* gene.[19] The high catalytic activity of these two isozymes supports their role in acidification of tumor microenvironment that facilitates acquisition of metastatic phenotype.[19] Therefore, modulation of extracellular tumor pH via inhibition of CA activity represents a promising approach to anticancer therapy. Indeed, novel sulfonamide/sulfamate CA inhibitors with nanomolar activity against the tumor-associated isozyme IX have been reported by this group,[20–22] together with classical sulfonamide drugs such as acetazolamide **2** and methazolamide **3**. On the other hand, recently developed topically acting antiglaucoma sulfonamides such as the bis-sulfonamide **4**[23] and the perfluorophenyl derivative of methazolamide **5**[24] or the sulfamate antiepileptic drug topiramate **6**[25] have not been investigated for their interactions with the tumor-associated isozyme CA IX.



Here, we report a detailed CA inhibition study of **1** against four isozymes, the cytosolic CA I and II, the membrane-bound isozyme IV, as well as the transmembrane, tumor-associated isozyme CA IX. An X-ray crystallographic study for the hCA II–**1** adduct has also been performed, and is reported here. The structure of this adduct evidenced unprecedented interactions between the inhibitor and the enzyme active site and may be useful for the drug design of more active agents belonging to this class of pharmacological agents.

2. Chemistry and CA inhibition

1 was prepared as previously reported.[8] The other sulfonamides/sulfamates (**2–6**) used as reference compounds were either commercially available (**2**, **3** and **6**) or prepared as previously reported (**4** and **5**).[23,24] Inhibition data against four CA isozymes with the six compounds **1–6** are shown in Table 1.

These data clearly indicate that **1** acts as a strong CA inhibitor against the four investigated isozymes CA I, II, IV and IX, with potencies comparable to those of the reference sulfonamide/sulfamate drugs **2–6**, some of which have been in clinical use for a long period as diuretics, antiglaucoma agents, antiepileptics, and so on.[1–3,5,6] Against the red blood isozyme CA I, **1** is the

most potent inhibitor in this series of test compounds, with an inhibition constant of 31 nM. Compounds **2**, **5** and **6** exhibit almost the same potency as CA I inhibitors, with K_i values in the range of 250–270 nM, whereas **3** and **4** show inhibition constants more similar to that of **1**, in the range of 40–50 nM. Against the most abundant and rapid isozyme, CA II, **1** shows the same level of inhibition as **2** and **3**, with K_i values in the range of 12–15 nM, whereas compounds **4**–**6** are more effective inhibitors, with K_i values in the range of 1.5–5 nM. The same level of inhibition as that of **2** is observed for **1** against the membrane-associated isozyme CA IV. These two compounds possess K_i values in the range of 65–70 nM, whereas the other ones investigated here (**3**–**6**) are slightly more effective CA IV inhibitors, with inhibition constants in the range of 8–54 nM. The tumor-associated isozyme CA IX is also efficiently inhibited by sulfonamides **1**–**5**, which show K_i values in the range of 18–29 nM, whereas the sulfamate **6** is a less effective inhibitor with an inhibition constant of 58 nM. Thus, it may be concluded that CAs also constitute target proteins of **1**, leading to a hypothesis that the CA inhibition may in part explain its multifactorial mechanisms of antitumor action.

Structure and activity/gene expression relationship studies of a series of **1**-related antitumor sulfonamides have so far clarified that the sulfamoyl group ($-SO_2NH_2$) of **1** is not an essential functionality for the in vitro growth inhibitory activity against cancer cells.[8,17] However, there is still a possibility that the CA inhibitory properties of **1** may contribute, at least in part, to its in vivo efficacy. The isozymes most abundant and considered to play a critical physiological/pathological role,[5–7] such as CA II and CA IX, exhibit the highest affinity for **1**, with an inhibition profile quite similar to those of the clinical drugs **2** and **3**. Interestingly enough, **2** has been shown to reduce the invasion capacity of renal cell carcinoma lines with high expression of CAs II, IX and XII in a model culture system[26] and display synergistic effects with other chemotherapeutic agents (e.g., cisplatin and melphalan) in animal models.[27] Based on these findings, the CA inhibition of **1** might be considered as a positive factor for the clinical strategy of anti-metastasis and combination therapies.

Table 1. CA inhibition data with E7070 (**1**) and reference sulfonamide/sulfamate inhibitors **2**–**6** against isozymes I, II, IV and IX

No.	Inhibitor	K_i (nM)[a]			
		hCA I[b]	hCA II[b]	bCA IV[c]	hCA IX[d]
1	E7070	31	15	65	24
2	Acetazolamide	250	12	70	25
3	Methazolamide	50	14	36	27
4		40	5	13	18
5		270	1.5	8	29
6	Topiramate	250	5	54	58

[a] Errors in the range of ±10% of the reported value.
[b] Human cloned isozymes, esterase assay method.[28]
[c] Isolated from bovine lung microsomes, esterase assay method.[28]
[d] Human cloned isozyme (catalytic domain), CO_2 hydrase assay method.[29,30]

3. Crystallography

The hCA II–**1** adduct obtained by co-crystallization, was subjected to detailed X-ray crystallography. The programs SHELX97[31] and O[32] were used to build the model and to compute the Fourier maps. The last refinement cycle yielded a final R factor of 0.21 ($R_{free} = 0.25$) with a final temperature factor of the inhibitor atoms ranging between 8.5 and 24.0 Å^2. The final number of water molecules was 195 and the final rmsd's from ideal geometry for bond lengths and angles were

Table 2. Statistics of data collection and refinement for the hCAII–**1** adduct

	hCA II–**1** complex
Resolution range (Å)	40–1.9
Space group	$P2_1$
Unit cell (Å, ° for β)	$a = 42.2$, $b = 41.2$, $c = 71.9$, $\alpha = 90$, $\beta = 104.2$, $\gamma = 90$
Highest resolution shell (Å)	1.95–1.90
No. of reflections	19,363
Completeness (%)	91.2
Mean I/σ_I	24.3 (2.2)
R_{sym} (%)	8.4 (48.4)
Refined residues	261
Refined water molecules	195
Resolution range in refinement (Å)	20–1.9
R_{cryst} ($F_o > 4\sigma F_o$; F_o)	21.1, 19.9
R_{free} ($F_o > 4\sigma F_o$)	25.0
Rms deviations	
Bond lengths (Å)	0.02
Bond angles (°)	1.8
Average B value (Å^2)	24.0
Ramachandran plot	
Most favored (%)	99.5
Generously allowed (%)	0.5
Disallowed (%)	0.0

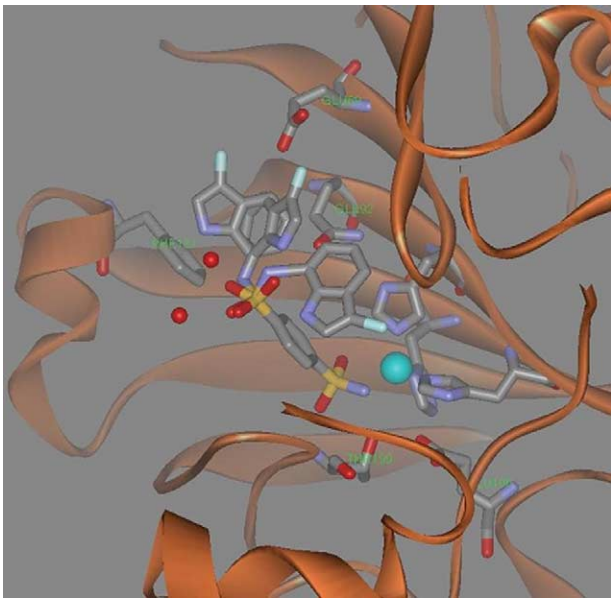


Figure 1. The hCA II–**1** adduct. The zinc ion (blueish sphere), its three protein ligands (His 94, 96 and 119), the three conformations of the inhibitor molecule bound within the active site (CPK colors) and amino acid residues in its neighborhood (Phe 131; Glu 69; Gln 92, etc.) are evidenced.

0.02 Å and 1.8°, respectively. The statistics of data collection and refinement are shown in Table 2. A final refinement resolution of 1.9 Å has been achieved.[33]

The structure refinement allowed us to evidence the spatial arrangement of the inhibitor within the active site of the enzyme (Fig. 1). The schematic, detailed representation of the interactions of **1** with the metal ion and amino acid residues/water molecules present in the hCA II active site are shown in Figure 2.

As seen from these figures, three different conformations of **1** bound within the hCA II active site have been evidenced, with three different electron densities for the terminal part of E7070 being observed. These conformations regard only the terminal, chloroindole fragment of the molecule, which is rather flexible and allows the three different spatial arrangements, whereas the benzenesulfonamide head binds unequivocally to the active site in one conformation, similarly to other sulfonamide CA inhibitors for which the three-dimensional structures have been reported in complexes with different CA isozymes.[34–38] Thus, the ionized sulfonamide moiety of **1** has replaced the hydroxyl ion/water molecule coordinated to Zn(II) in the native enzyme (Zn–N distance of 1.87 Å), as in other hCA II–sulfonamide complexes for which the X-ray structures have been reported (Fig. 2A).[34–38] An important note is that the Zn–N bond is appreciably shortened in this complex as this distance is usually around 1.95–2.10 Å. This shortening may be considered as a first factor favoring the high affinity of **1** for hCA II, as already documented recently for the structurally related adduct of **4** with hCA II.[38] The Zn(II) ion remains in its stable tetrahedral geometry, being coordinated, in addition to the sulfonamidate nitrogen of **1**, by the imidazolic nitrogens of His 94, His 96 and His 119. The proton attached to the sulfonamidate nitrogen atom of the inhibitor also makes a hydrogen bond with the hydroxyl group of Thr 199, which in turn accepts a hydrogen bond from

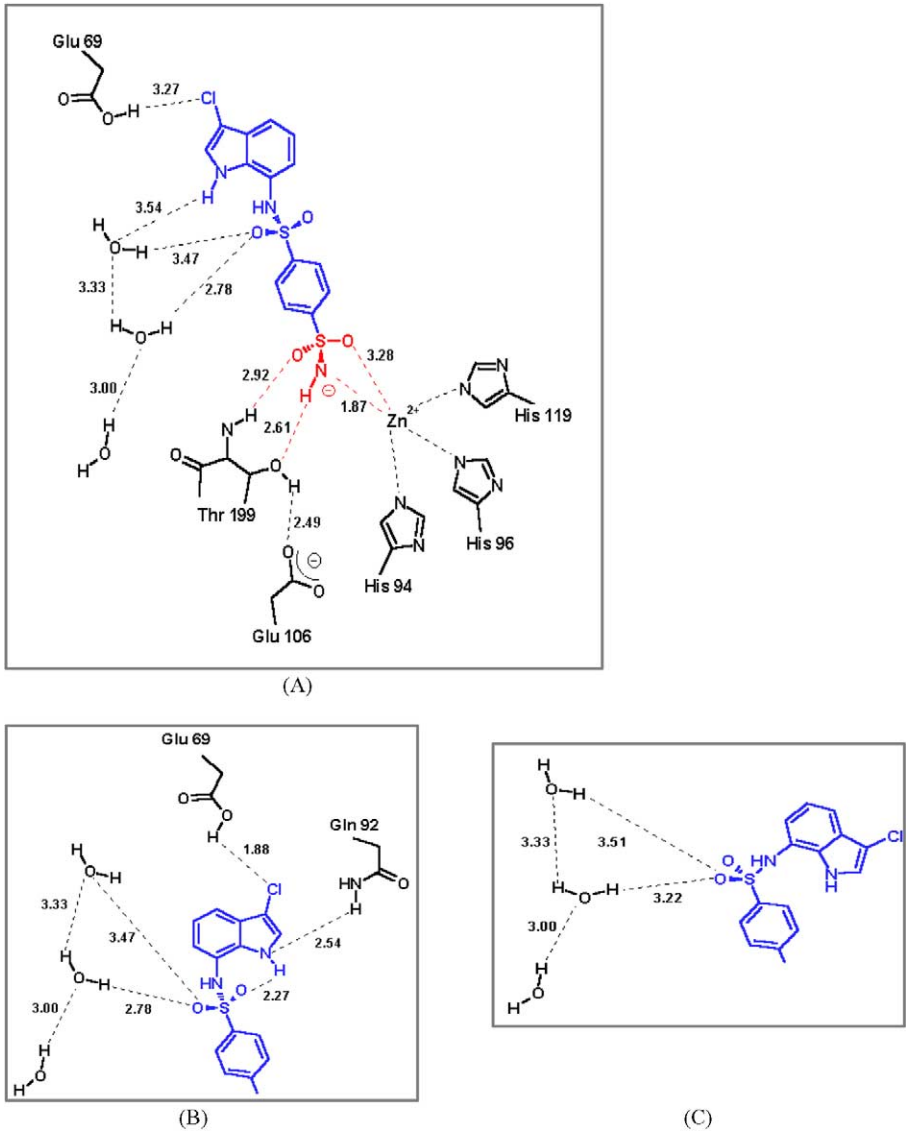


Figure 2. Schematic representation of **1** bound within the hCA II active site (figures represent distances in Å). The three conformations of the chloroindole fragment of the inhibitor bound within the active site and its interactions with amino acid residues/water molecules are detailed in A, B and C. The interactions of the $-C_6H_4-SO_2NH_2$ moiety of **1** with hCA II is the same for the three conformations A–C.

the carboxylic group of Glu 106 (Fig. 2A). One of the oxygen atoms of the coordinated sulfonamidate moiety makes a hydrogen bond with the backbone amide of Thr 199 (O–H distance of 2.92 Å), whereas the other one is semi-coordinated to the catalytic Zn(II) ion (O–Zn distance of 3.28 Å). These interactions are generally seen in all complexes of hCA II with sulfonamide/sulfamate inhibitors.[34–38] The benzenedisulfonamide part of **1** lies in the hydrophobic part of the active site cleft, where it makes van der Waals interactions with the side chains of Val 135, Phe 131, Leu 204, Pro 202, Trp 209, Val 121, Leu 198 and Thr 200. One oxygen of the secondary sulfonamide moiety participates in two hydrogen bonds with two active site water molecules (Fig. 2A–C). In two of the adopted conformations (A and B), these hydrogen bonds are identical, one of 2.78 Å and the other one of 3.47 Å, whereas for the conformation C, two weaker bonds are formed, of 3.22 and 3.51 Å, respectively. Regarding the most notable and unprecedented interactions evidenced in this complex, the chloroindole fragment of the inhibitor (which lies in the hydrophilic half of the active site) adopts three different conformations, in which it interacts with *different* amino acid residues and water molecules present in its neighborhood. Thus, in conformation A (Fig. 2A), the endocyclic NH group of the inhibitor serves as a hydrogen donor to the same water molecule that was mentioned to form a hydrogen bond with the secondary sulfonamide group. Furthermore, the chlorine atom is also engaged in a weak hydrogen bond with the COOH group of Glu 69. In the second conformation of **1** (Fig. 2B), the presence of an intramolecular hydrogen bond between the second oxygen atom of the secondary sulfonamide moiety and the endocyclic NH group is evident. The same NH group of the inhibitor is then involved in another hydrogen bond (of 2.54 Å) with the $CONH_2$ group of Gln 92, a residue known to play a critical role for the binding of sulfonamide/sulfamate inhibitors.[25,34–38] The chlorine atom forms this time a shorter (1.88 Å) hydrogen bond with the COOH group of Glu 69. Finally, the third conformation of **1** bound to hCA II (Fig. 2C) involves only a network of four hydrogen bonds in which one oxygen atom of the secondary sulfonamide moiety and three water molecules participate.

In order to better understand the significance of our findings, that is why **1** is a unique and strong CA inhibitor possessing three different conformations when bound within the hCA II active site, we superimposed these conformations of **1** on those of three hCA II–

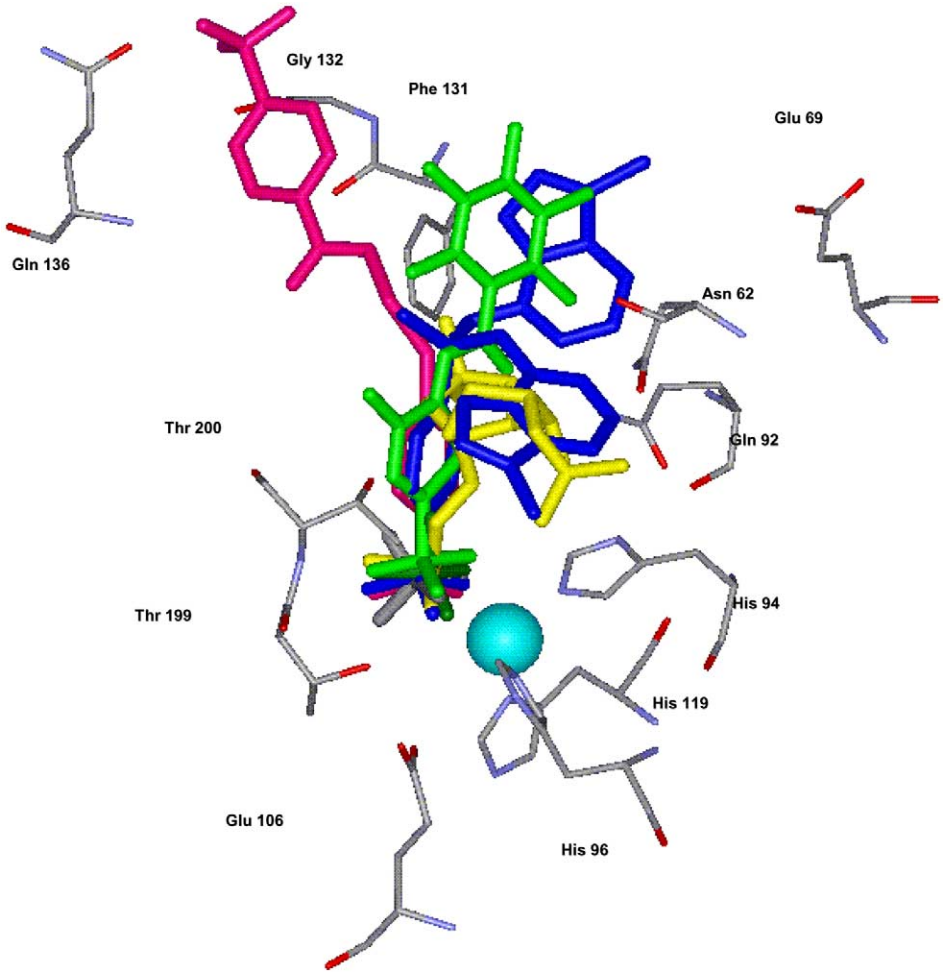


Figure 3. Superimposition of hCA II–inhibitor adducts: **1** (dark blue, two conformations A and B); **4** (magenta); **5** (green); and **6** (yellow). Amino acid residues important for binding of these inhibitors (such as Glu 106; Thr 199; Thr 200; Gln 136; Gly 132; Phe 131; Glu 69; Asn 62 and Gln 92), the Zn(II) ion and its three protein ligands (His 94, 96 and 119) are also evidenced (CPK colors).

inhibitor adducts for which the X-ray crystal structures have recently been reported by our group: the adducts of hCA II with compounds **4**–**6**[25,37,38] (Fig. 3). To our greatest surprise, we observed that conformation A is superimposable to that of the hCA II–**5** adduct, with the chloroindole ring of **1** assuming a steric arrangement very close to that of the perfluorobenzoyl ring of **5**. On the other hand, the second conformation of **1** (conformation B) overlaps quite well with **6** bound to hCA II, with the chloroindole ring of **1** occupying the same region of the active site as the sugar moiety of **6**. The third conformation (C) of **1** is in fact positioned between the conformations A and B (see Fig. 1) mentioned above and is not represented in Figure 3 for the sake of simplicity. It must also be mentioned that the benzenesulfonamide moieties of **1** and **4** (possessing the same head as **1**) bound to hCA II overlap each other completely. However, the tail of **4** adopts a totally different conformation, being oriented towards the hydrophobic half of the active site, whereas all the other inhibitors discussed here prefer to orient their tails towards the hydrophilic half.[39]

In conclusion, we prove here that E7070 (**1**), an anticancer sulfonamide under clinical development for the treatment of several types of cancers, also acts as a strong CA inhibitor. Similarly to the clinically used drugs **2**, **3** and **6**, **1** shows inhibition constants in the range of 15–31 nM against isozymes I, II and IX, being slightly less effective as a CA IV inhibitor (K_i of 65 nM). The X-ray crystal structure of the adduct of hCA II with **1** revealed unprecedented interactions between the inhibitor and the active site, with three different conformations of the chloroindole fragment of the inhibitor interacting with different amino acid residues/water molecules of the enzyme. A superimposition of these conformations with those of other sulfonamide/sulfamate CA inhibitors indicated that similar regions of the hCA II active site are involved in the interaction with such inhibitors. These data may be used for the design of novel CA inhibitors with antitumor activity or other biomedical applications.

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29. The cDNA of the catalytic domain of hCA IX was amplified by using PCR and specific primers for the vector pCAL-*n*-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-*n*-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo et al. (Wingo, T.; Tu, C.; Laipis, P. J.; Silverman D. N. *Biochem. Biophys. Res. Commun.* **2001**, *288*, 666). The

homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10 mM Hepes (pH 7.4), 10 mM Tris–HCl, 100 mM Na_2SO_4 and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[30]

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