New structural features of isatin dihydrothiazole hybrids for selective carbonic anhydrase inhibitors

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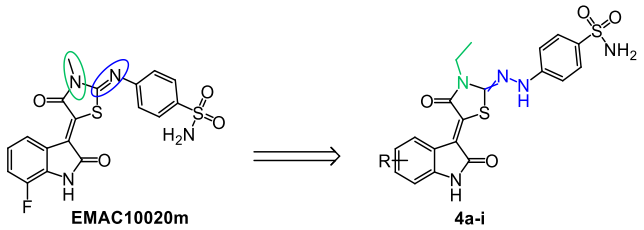
ABSTRACT: Chemotherapeutic agents have remained the first-line treatment option for advanced-stage cancers when surgery or radiation therapy is not viable. Human carbonic anhydrase (hCA) isoforms IX and XII have been validated as anticancer targets. In particular, hCA IX is overexpressed in several solid tumor cells. As a result, selective isoform inhibitors with high potency and low toxicity are sought after. Pursuing our investigation on new scaffolds as hCA selective inhibitors, a new series of isatin thiazolidinone hybrids has been designed and synthesized. Their biological activity and selectivity toward hCA I, hCA II, hCA IX, and hCA XII were investigated. The results revealed an inhibitory activity in the nanomolar range on carbonic anhydrases IX and XII, and the nature of substitution in positions 3 and 5 of thiazolidinone appears to be crucial for the compounds' selectivity. Docking experiments have been applied to predict the binding mode of these new, promising derivatives.

KEYWORDS. Enzyme inhibitors, human carbonic anhydrase, docking experiments, structural elucidation

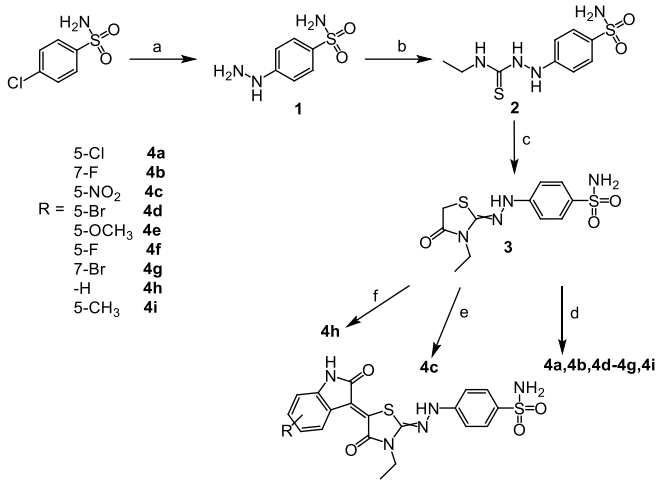
Isatin and thiazolidinone moieties can be found in several biologically active molecules such as antibacterial [1], HIV1 reverse transcriptase inhibitors [2, 3], anti-oxidants [4], antifungal [5-7], and anticancer molecules [8-10]. Since cancer is one of the leading causes of death in the world, the interest of the medicinal chemistry community is focused on identifying and developing novel effective anticancer drugs. Moreover, cancer is a multifactorial disease that, in most cases, requires a combination drug treatment. Therapeutic approaches must consider cancer cells' most characteristic features such as the abilities for uncontrolled growth and to invade tissues and the peculiarity to survive in a hypoxic environment, a condition connected with the metastatic phenotype of tumor cells [11]. The presence of a low concentration of O_2 in the cell environment causes a modification of the pH homeostasis which is modulated by different factors [11].

In the human body, the members of carbonic anhydrases enzyme family are involved in pH regulation and other physiological functions [12, 13]. These proteins are ubiquitous metalloenzymes that contain zinc ion in the active site [14], where the reversible hydration of carbon dioxide into bicarbonate ions and protons is catalyzed [15, 16]. In mammals more than 16 different isoforms of hCAs are found, differing for their sequence, tissue localization, expression, and activity [17-19]. Since these enzymes are involved in several physiological and pathological metabolic pathways, their role as drug targets has been investigated [13, 19, 20]. In particular, isoforms IX and XII play key roles in proliferation, cell differentiation, and pH regulation. Furthermore, isoform IX is overexpressed in different types of tumors [19, 21]. For this reason, hCA IX and XII inhibitors have been deeply explored as anticancer agents [13, 22-28]. Both represent attractive targets for selective inhibition compared with other hCA isoforms due to their relatively limited expression in normal tissues. These two isoforms are transmembrane and not cytosolic. Their selective inhibition could reduce the potential for off-target effects and toxicity. The benzene-sulfonamide moiety chelates the zinc ion in the active site [17] and represents the widest class of hCA inhibitors. Unfortunately, although they are very active, such inhibitors suffer from a lack of selectivity. However, according to structural differences in the outer part of the diverse hCA isoforms catalytic site, the benzene-sulfonamide scaffold can be decorated to improve isozyme selectivity and activity by adding structural features capable of different interactions with specific and less conserved residues among isozymes. According to this information and following the previous studies conducted by our group [18, 29-31], we explored the effect of the introduction of diverse substituents on the activity and selectivity towards hCA I, II, IX, and XII. All compounds **4a-i** have been characterized and submitted to biological tests on hCAs. In addition, docking experiments were carried out to predict ligand-protein interactions. Thiazolidinones were condensed with different isatins and the benzene-sulfonamide moiety to acquire more information on the structure-activity relationship. With respect to our previously reported isatin-thiazolidinone hybrids, a hydrazone spacer was inserted between the thiazolidinone and the benzene-sulfonamide moieties to increase the length between the zinc-binding group (ZBG), essential for the interaction with the enzyme, and the heterocyclic tail represented by the thiazolidinone and the isatin scaffolds. In addition, to further explore the role and structural requirements of the tail, an ethyl group was introduced on the thiazolidinone nitrogen. Position 5 of the thiazolidinone ring was decorated with nine different isatins (Scheme 1).



Scheme 1. Comparison of previous and new synthesized series of isatin dihydrothiazole derivatives.

The **4a-i** series was synthesized according to a versatile synthetic approach involving four steps (Scheme 2).



Scheme 2. Reaction conditions: a) hydrazine monohydrate, mw, 40 minutes; b) ethyl isothiocyanate, ethanol, reflux, 5h; c) ethyl bromo acetate, ethanol, reflux, 4 h; d) appropriate isatin, morpholine, MeOH, reflux; e) 5-nitroisatin, sodium acetate ethanol, reflux; f) appropriate isatin, sodium acetate, acetic anhydride and acetic acid.

However, since two regioisomers could be obtained during the thiazolidinone synthesis, NMR spectroscopy investigations were used to determine the molecular structure of compound **3**. The chemical shifts of the ^{13}C-NMR spectrum are reported in Table S1 in the Supporting Information (SI). The number of carbon atoms agrees with the signals present in the spectra. The signals at 12.77 and 37.94 ppm were assigned to the methyl (C3b) and methylene (C3a) moieties of the ethyl group, whereas the signal at 33.36 agreed with the methylene (C5) of thiazolidinone. Additionally, the quaternary carbons at 149.82 and 150.41 ppm were located at positions 2 and 2a, respectively. The signal at 170.93 was consistent with the carbonyl group of the amide. HMBC experiments were carried out to clearly define the nature of the obtained regio-isomer. The spectrum is depicted in Figure 1. It can be observed that H-3a was correlated with both C4 and C2 (green, Figure 1), whereas NH showed cross-peaks with C2 (in blue, Figure 1). These correlations could be observed only when the ethyl tail bound the nitrogen in position 3 of thiazolidinone. Concerning the geometry of the double bond formed by the condensation of the thiazolidinone with the isatin moiety, the Z configuration was attributed, according to the literature[32]. Unfortunately, it was not possible to determine the configuration of the double bond at position 2 of the thiazolidinone ring. Thus, although a single geometrical isomer was formed, both Z and E diastereomers were considered.

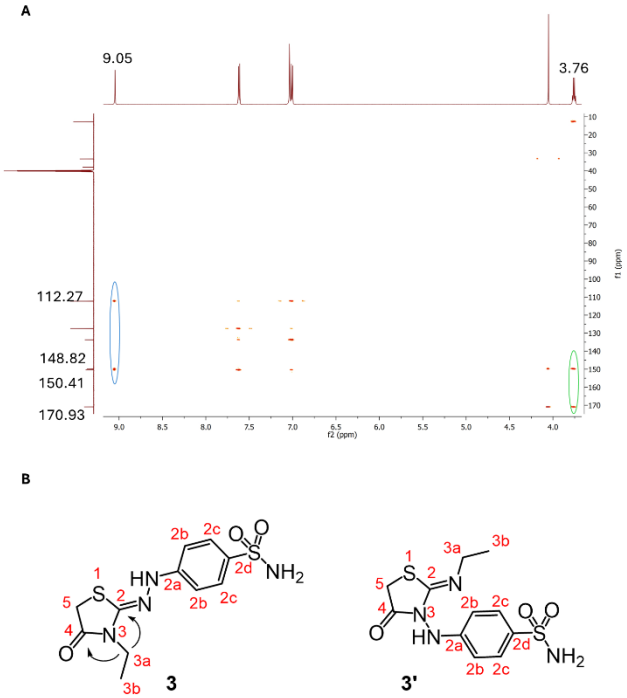


Figure 1. (A) HMBC spectrum of compound **3**. On the horizontal trace, the ^{1}H-NMR spectrum is reported, and on the vertical the ^{13}C-NMR spectrum. (B) Two cyclization options. The first (1) is the observed: the hydrazone proton is coupled to C2, 2a, and 2b (blue), while the proton on 3a is coupled to C4 and C2.

To understand how the introduced structural novelties affect the activity and selectivity, a biochemical evaluation on hCA I, II, IX, and XII was carried out, using the known nonselective hCA inhibitor acetazolamide (AAZ) as a control. Results are reported in Table 1. All compounds showed promising inhibitory activity towards hCA IX and XII isoenzymes in the nanomolar range. Compounds **4d**, **4g**, and **4h** were found to be the most active or selective on isoforms IX and XII within the series. Comparing compound **4h** with the unsubstituted indolinone compound previously reported[18], where a methyl substituent was present in the position 3 of the thiazolidinone ring, the introduction of the bulkier ethyl group seemed to increase the isozyme selectivity. However, the nature and position of the substituents on the indolinone nucleus also play relevant roles in determining the activity and selectivity of the new derivatives. Nevertheless, the insertion of the hydrazone spacer might also be beneficial for activity and selectivity, at least in the cases of compounds **4d** and **4h**. Altogether these data suggest that since the active site entrances of hCA IX and hCA XII are wider than those of hCA I and hCA II isoforms, both the size and position of the indolinone/thiazole cap moiety are relevant for selectivity. Regarding the selectivity ratio between hCA I and hCA IX, none of the new compounds exhibited a higher selectivity when compared with the reference AAZ. Conversely, when the ratio hCA II/IX is considered, most of the compounds were more selective than the reference. In particular, the introduction of a 5-nitro group on the indolinone lead to the most selective compound **4c**. AAZ is also more efficient when the ratio hCA I/XII is evaluated, but some substitutions on the indolinone ring led to comparable results (7-Br>5-NO_2>5-Br> 5Cl). All derivatives **4a-i**, with the exception of compound **4b**, possess a higher selectivity ratio hCA II/XII with respect to AAZ (Table2), indicating the 5-Cl indolinone as the best

substitution (5-Cl>7-Br>5-NO_2>5-Br>5-CH_3>H>5-F>5-OCH_3). As a general observation, the introduction of the ethyl substituent, combined with the hydrazone spacer, appears to be favorable for the inhibition of the hCA XII isoform.

Table 1 4a-i inhibitory activity toward hCA I, II, IX and XII

		Ki (nM)			
	R	**I**	**II**	**IX**	**XII**
4a	5-Cl	831.1	3931	377.8	34.7
4b	7-F	27.8	41.5	436.9	58.7
4c	5-NO_2	1702	4029	364.4	51.1
4d	5-Br	299.7	724.1	223.9	9.7
4e	5-OCH_3	96.9	280.4	140.3	27.9
4f	5-F	53.9	174.7	292.2	9.6
4g	7-Br	325.7	803.9	264.6	9.7
4h	-H	68.9	460.7	44.7	23.4
4i	5-CH_3	17.6	236.6	46.7	8.8
AAZ		250.0	12.1	25.7	5.7

Table 2 4a-i hCA isoform selectivity ratio I/IX, II/IX, I/XII, and II/XII

		Selectivity ratio			
	R	**I/IX**	**II/IX**	**I/XII**	**II/XII**
4a	5-Cl	2.19	10.40	23.95	113.28
4b	7-F	0.06	0.09	0.47	0.70
4c	5-NO_2	4.67	11.05	33.30	78.84
4d	5-Br	1.33	3.23	30.89	74.64
4e	5-OCH_3	0.69	1.99	3.47	10.05
4f	5-F	0.18	0.59	5.61	18.19
4g	7-Br	1.23	3.03	33.57	85.52
4h	-H	1.54	10.30	2.94	19.68
4i	5-CH_3	0.37	5.06	2.00	26.88
AAZ		9.72	0.04	43.85	2.12

Docking experiments have been carried out to obtain more information about the compounds putative binding mode and to understand how to further modify the scaffold for more selective compounds. According to a previously validated protocol, the compounds were docked on isoforms IX, XII and CA II. [30, 33] Docking results show that these compounds can reach the hCAIX and hCAXII catalytic sites. Specifically, the benzosulfonamide moiety can chelate the zinc ion in the deepest part of the catalytic pocket. A representation of the putative binding mode of one of the most interesting compounds, **4d**, on hCA IX is reported in Figure 2. Due to the possible formation of two diastereoisomers, docking experiments were performed considering both ZE and ZZ configurations of compounds. The **4dZZ** compound (Figure 2, panels A-B) is predicted to enter the cavity, reach the active site of hCA IX isoform, chelate the zinc ion, and establish several interactions in the residues in the entry cavity, such as hydrogen bonds with Trp9 and Thr200. Additionally, the binding is stabilized by hydrophobic contacts with Val, Leu, and Pro residues of hydrophobic region close to the catalytic site. Considering **4dZE** derivative (Figure 2, panels C-D), docking studies revealed its capacity to enter the active site and to chelate the zinc ion. As observed with **4dZZ**, also the binding mode of **4dZE** is stabilized by several interactions, in particular, the polar contacts with Gln92 and Thr200 residues, π–π stacking with His94, and halogen bond with the Arg129 residue. Similarly, the interactions with hCA XII of compounds **4dZZ** and **4dZE** were investigated by docking, and the results are depicted in Figure 3. Since the access to the active site differs from each considered isoform, the presence of a bulky cap portion, in the ligand structure, might lead to different interactions between the ligand itself and this enzyme portion. As observed in hCA IX, also in hCA XII, both **4d** diastereomers can bind the zinc ion, and the complex is stabilized by a network of hydrogen bonds with Thr198 and Lys69 residues of the enzyme (Figure 3, panels A-B). More specifically, due to the position assumed for **4dZE** the nitrogen of the hydrazone moiety is available to form a hydrogen bond with Gln89 (Figure 3, panels C-D).

Docking experiments have been performed on the hCA II to better understand the selectivity of **4d** and rationalize the biological results. In agreement with the biochemical data, **4d** does not efficiently bind the hCA II active site (Figure S1). This is probably, due to the narrowed cavity being partially obstructed by the presence of Phe131. Indeed, the presence of the large cap, represented by the thiazolidinone isatin hybrid, does not allow the compound to assume the proper orientation to coordinate the zinc ion. Conversely, the active site entrance of hCA IX and XII, is broader because hCA II's Phe131 is substituted by smaller residues, Val and Ala in IX and XII, respectively. Considering these findings, it can be assumed that, due to the relative rigidity of both the ligand and the protein, this aminoacid substitution is determined to affect the selectivity. Indeed, the lack of conformational adaptability of the isatin thiazolidinone condensed cap and the isatin decoration, might lead to the preferential inhibition of isoforms IX and XII compared with the isozyme II. QikProp software was applied to carry out in silico ADMET predictions[34] to evaluate the drug-likeness of the compounds. Part of the results are reported in Tables S2 and S3. Most of the compounds exhibited a good, predicted drug-like profile.

Biological results corroborate our hypothesis that the combination of the isatin thiazolidinone scaffold with the benzene-sulfonamide moiety might represent a valid strategy to pursue selectivity and activity toward the tumor-associated isoforms of hCA, IX and XII. In fact, most of the compounds exhibited inhibitory activity towards hCA IX and XII in the nM range. Furthermore, the substitution pattern appeared to be determinant to address isozyme selectivity, and this aspect deserves further investigation. Both bromine and chlorine substituents in position 5 of the indolinone moiety benefit the selectivity toward the IX and XII isoforms. Nevertheless, the bromine substitution appeared more promising for the activity toward the XII isozyme in comparison with chlorine. 5-fluoro and 5-methyl derivatives were the most active towards hCA XII. The former exhibited a good selectivity towards hCA XII with respect to the other isoforms, while the latter was also active toward the hCA I isoform. Probably, the balance between activity and selectivity resides in a blend of electronic and steric factors of the indolinone thiazolidinone hybrid cap. Thus, further investigation on similar derivatives, such as the introduction of bulkier and more complex moieties in place of the ethyl substituent in position three of thiazolidinone, is needed to clearly define the structure-activity relationships for the optimal activity and selectivity profile. Although the optimal substitution pattern has yet to be discovered, the overall results are extremely encouraging and motivate us to further explore similar scaffolds to develop benzene-sulfonamide based hCA inhibitors with higher potency and selectivity.

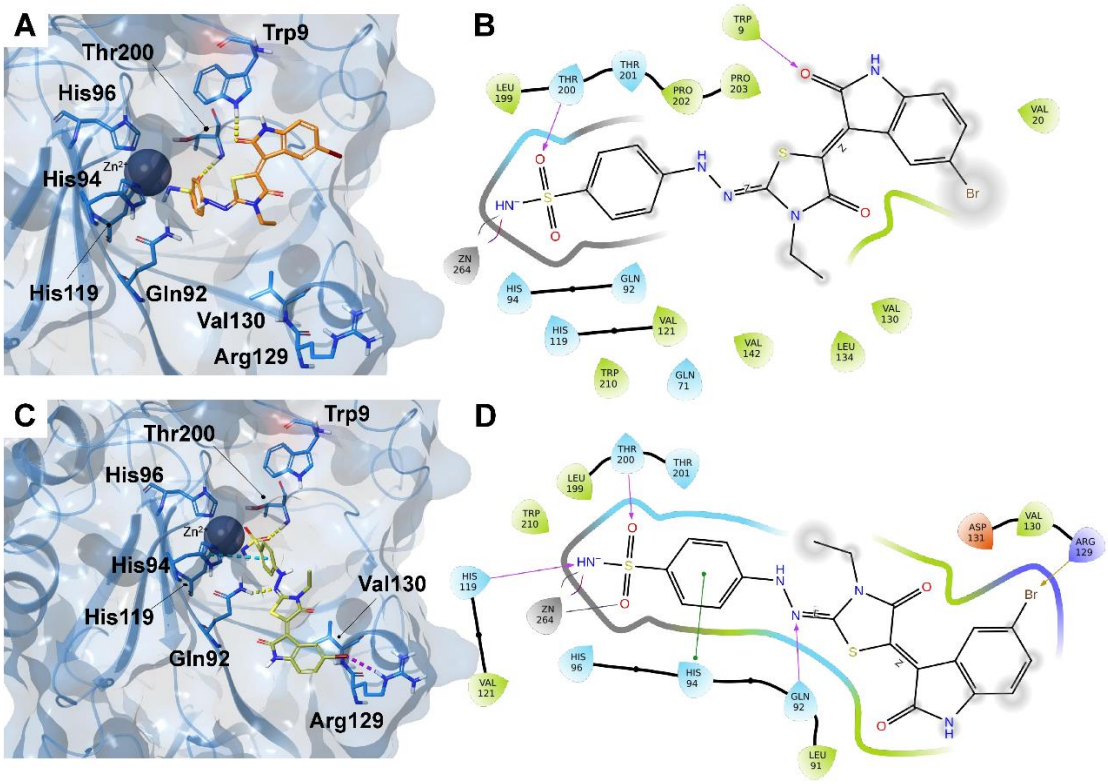


Figure 2. Representation of the putative binding mode of **4dZZ** (orange) and **4dZE** on hCA IX (crystal 5FL4). (A) 3D view of interactions between **4dZZ** and active site of hCA IX. (B) 2D representation of ligand-enzyme interactions. (C) **4dZE** and interactions with residues of active site. (D) 2D representation of compound **4dZE** with the enzyme.

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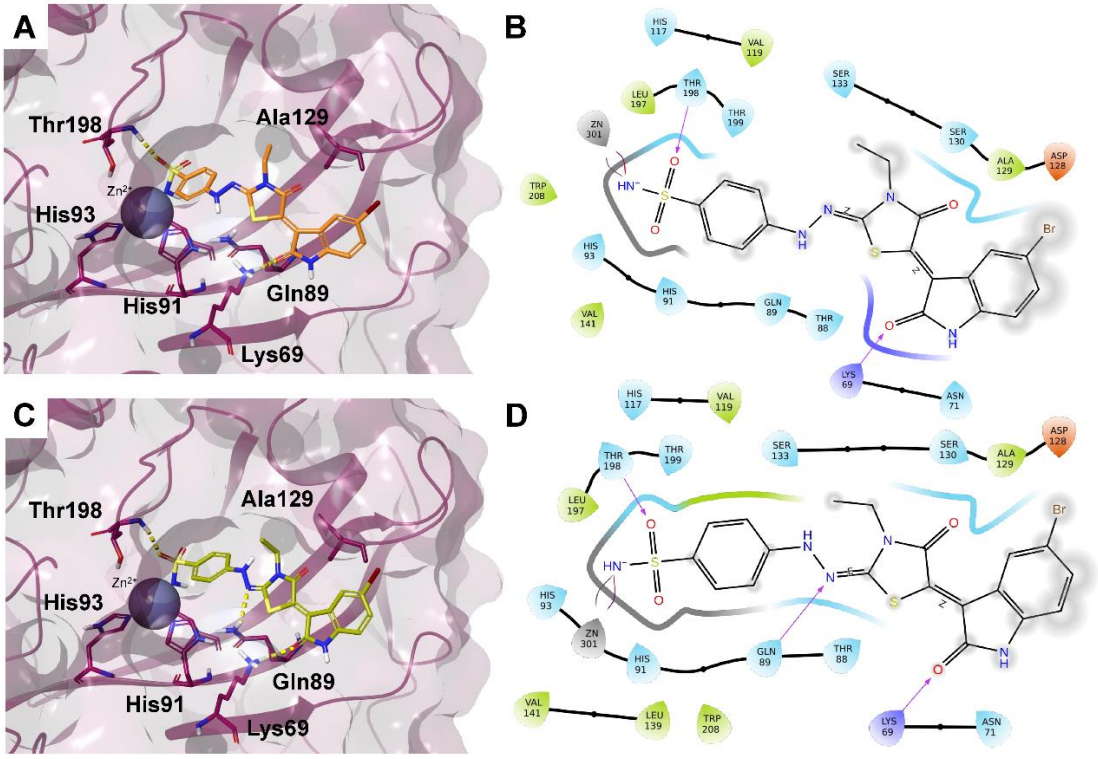

Figure 3. Representation of the putative binding mode of **4dZZ** (orange) and **4dZE** on hCA XII (crystal 5MSA). (A) 3D view of interactions between **4dZZ** and active site of hCA XII. (B) 2D representation of ligand-enzyme interactions. (C) **4dZE** and interactions with residues of active site; (D) 2D representation of compound **4dZE** with the enzyme.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

List Item Additional cxpcrimcntal dctails: matcrials, mcthods, synthcsis, charactcrization data (^{1}H and ^{13}C NMR, MS spectra of all thc ncwly synthesizcd compounds), molccular modcling, the-orctical prcdiction of drug-likc propcrtics, and biologica! assays (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

All experiments involving hazardous materials were conducted in compliance with relevant safety protocols and guidelines. Appropriate safety measures, including the use of protective equipment and fume hoods, were employed for both synthetic and biochemical study.

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ABBREVIATIONS

HIV: Human Immunodeficiency Virus; hCA: Human Carbonic Anhydrase; HMBC: Heteronuclear Multiple Bond Correlation; ADMET: Absorption, Distribution, Metabolism, Excretion, and Toxicity.

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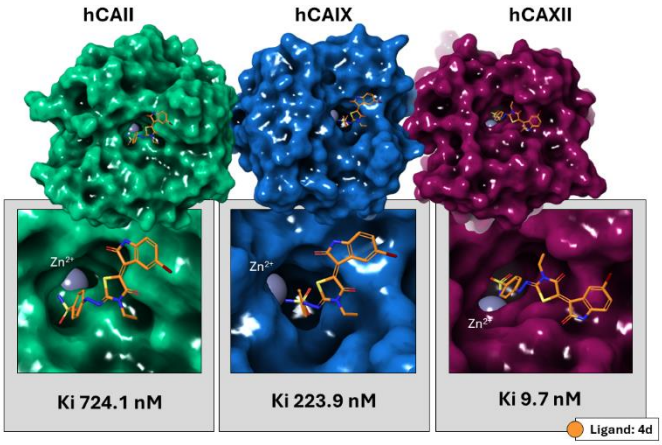
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Zn2+
Zn2+
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Ki 9.7 nM
Ligand: 4d