Appliance of the piperidinyl-hydrazidoureido linker to benzenesulfonamide compounds: synthesis, *in vitro* and *in silico* evaluation of potent carbonic anhydrase II, IX and XII inhibitors

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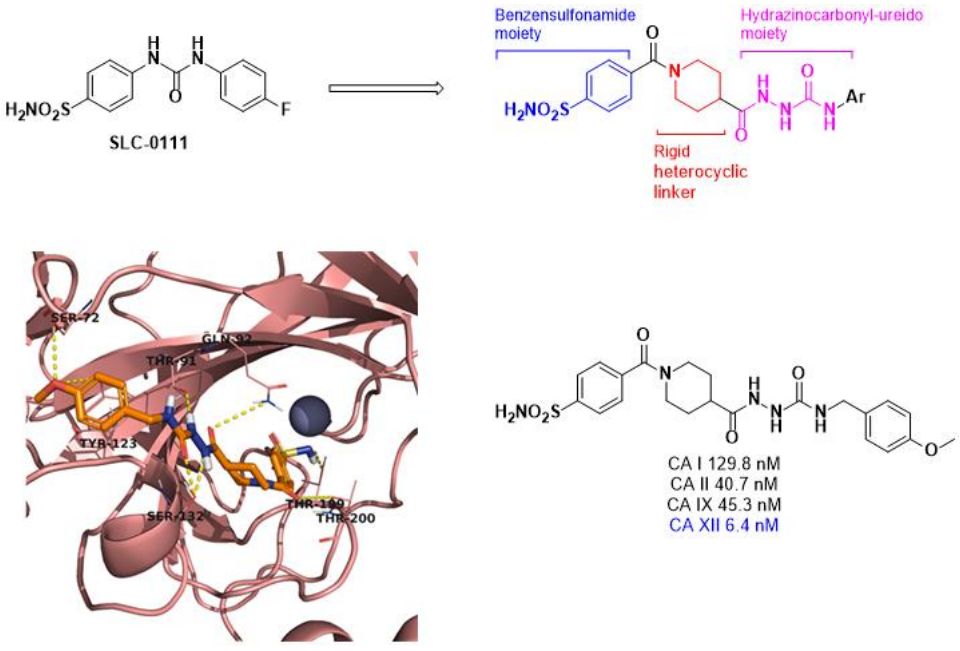
Highlights

* Hydrazidoureidobenzensulfonamides were designed and synthesized.
* Sulfonamides were assayed *in vitro* as human Carbonic Anhydrase (CA) isoforms inhibitors.
* Synthesized compounds show weak activity against off-target CA I isoform
* Some sulfonamides are CA II/IX/XII selective inhibitors representing leads for pharmacological applications.
* Docking revealed that CA-selective inhibition is related to distinguished interactions.

Keywords: metalloenzyme; inhibitor; sulfonamides; antitumor; selectivity.

Graphical abstract



Abstract

Herein we report on a new series of hydrazidoureidobenzensulfonamides investigated as inhibitors of the cytosolic human (h) hCA I and II isoforms, as well as the transmembrane, tumor-associated enzymes hCA IX and XII. The reported derivatives contain a 4-substituted piperidine fragment in which the hydrazidoureido linker has been involved as spacer between the benzenesulfonamide fragment which binds the zinc ion from the active site, and the tail of the inhibitor.

Depending on the substitution pattern at the piperidine ring, low nanomolar inhibitors were detected against hCA II, hCA IX and hCA XII, making the new class of sulfonamides of interest for various pharmacologic applications.

1. Introduction

Carbonic anhydrases (CA) are a superfamily of metalloenzymes that catalyze the reversible conversion of carbonic anhydride to bicarbonate ions and protons [1]. This simple reaction is spontaneous at high pH values, but it is slow at pH values of 7.5, that is typically the physiological pH in most of the organisms [2]. For this reason, all the organisms need CAs for being able to convert the CO_2, that is usually formed in metabolic reactions, in a water-soluble product. These water-soluble products, as ion bicarbonate and proton, have an important buffering activity so CAs have a crucial role in pH regulation [3]. Currently, 15 different isoforms have been described in humans, 12 of which are catalytically active: CAs I-IV, CA VA-VB, CA VI, CA VII, CA IX and CAs XII-XIV [1]. The active hCAs have different catalytic efficiencies and a different cellular localization. These isoforms have been grouped in four different classes depending on localization: hCAs I, II, III, VII, and XIII are in the cytosol, hCAs IV, IX, XII, and XIV are membrane-associated, and hCAs VA and VB are present in mitochondria, whereas hCA VI is present in saliva and milk [4]. In the last years, hCA IX and XII became an interesting target for the development of new antiproliferative compounds, due to their important role in cancer cell survival. In hypoxic conditions, hCA IX and XII play an important role in the control of the intracellular and extracellular pH. The reversible hydration of CO_2 to proton and bicarbonate ion mediated by CA IX and CA XII regulates pH and promotes tumor progression, so that a selective inhibition of these isoforms may have interesting clinical implications [5]. SLC-0111 (Figure 1) is a selective CA IX inhibitor and is currently in Phase Ib/II clinical trials in combination with gemcitabine (administered i.v.) in subjects affected by metastatic pancreatic ductal adenocarcinoma, overexpressing CA IX/XII [6]. The crucial features of SLC-0111 may be the presence of the ureido linker between the benzensulfonamide moiety and the tail of the inhibitor. The ureido linker improves flexibility of the tails in order to adopt different orientation to better interact

with the amino acid residues at the active site of the enzyme. [7-8]. In 2015 we reported a new series aryl sulfamates as carbonic anhydrase inhibitors based on SLC-0111 scaffold, bearing piperazinyl-ureido moiety (Figure 1, compounds A) [9]. The most important modification at the SLC-0111 scaffold was the incorporation of the ureido moiety into a rigid heterocyclic system, the N-substituted piperazine ring. Piperazine ring is broadly used in drug discovery and piperazine derivatives are well-known to produce compounds with different pharmacological activities, and recently it has been used in the development of CA inhibitors [10,11]. In 2010 Liu L. et al proved the importance of a carbonyl thioureido moiety to improve the selectivity against the cancer related isoform hCA IX (Figure 1, compounds B). In their work, the docking analysis showed that the polar sulfur atom and of thioureido group and the oxygen from the carbonyl group can form hydrogen bonds with Asn62 and Gln67 of hCA IX [1].

Here we report a new series of benzensulfonamide derivatives (Figure 1) that were designed and tested for their inhibitory activity against hCA I, II, IX, XII. To better understand the importance of a rigid heterocyclic scaffold, the piperazine ring was substituted with a piperidine. Furthermore, a hydrazinocarbonyl-ureido moiety was introduced as tail of inhibitors. The NH group of the hydrazide moiety may provide a supplementary H-bond donator which can better interact with the aminoacidic residues in the hydrophilic region of the active site.

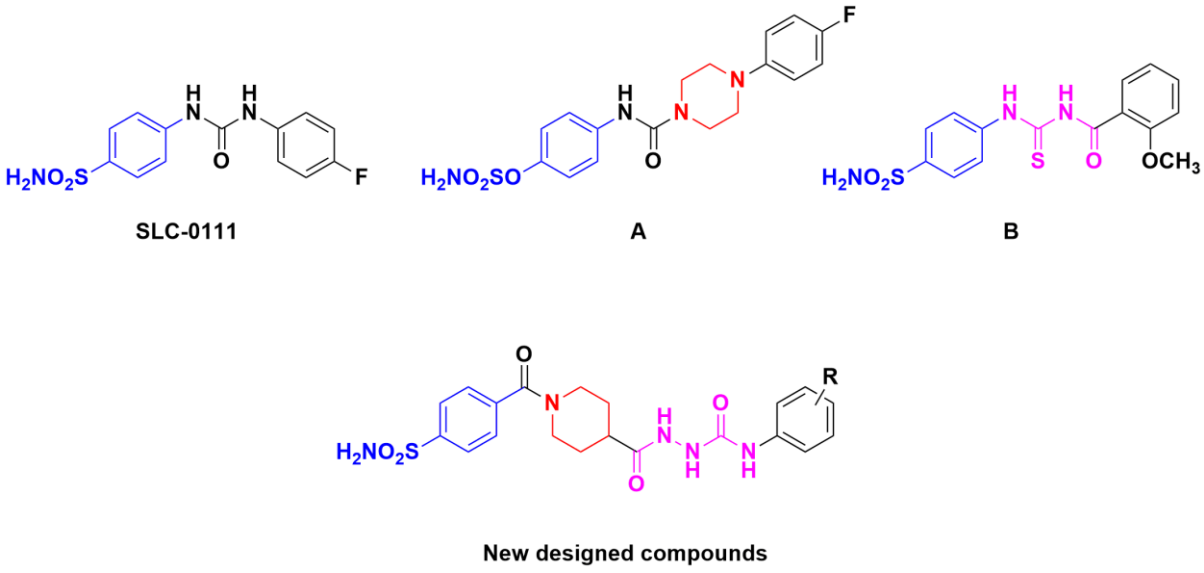
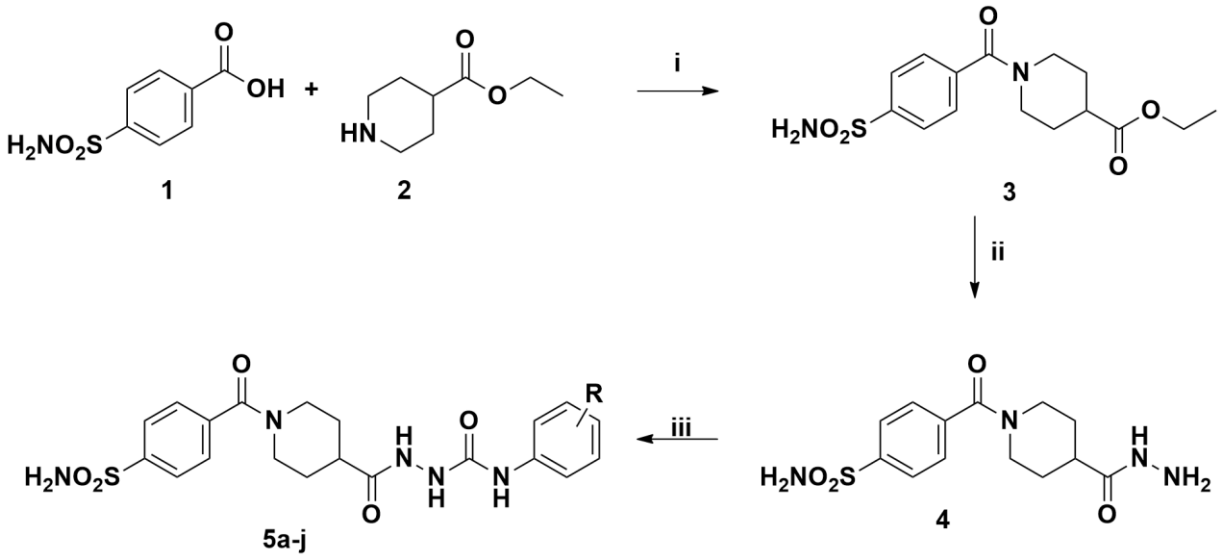


Figure 1. CA inhibitors discussed in the manuscript and design of new benzenesulfonamide derivatives reported here.

2. Results and discussion

2.1. Chemistry

The synthetic pathway to obtain these piperidinobenzoyl sulfonamides starts with the preparation of ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate **3** by amide coupling between ethyl piperidine-4-carboxylate **2** and 4-sulfamoylbenzoic acid **1** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) as coupling agent. The reaction was performed in dry acetonitrile solution (CH_3CN), in the presence of 1-hydroxybenzotriazole hydrate (HOBt). The resulting ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate **3** was treated with hydrazine hydrate in ethanol to obtain the corresponding hydrazidopiperidinocarbonylbenzensulfonamide **4**. Finally, the target compounds were obtained by reaction between intermediate **4** and substituted aryl and benzyl isocyanates to obtain the corresponding thioureas **5a-j**. The assigned structures were confirmed by analytical and spectral data and are consistent with results of reported studies [9-12].



Scheme 1. General synthetic procedure for sulfonamides subsets **5a-j**. Reagents and conditions: (i) EDCI, HOBt, dry CH_3CN r.t. 12h; (ii) $NH_2NH_2 \cdot H_2O$, absolute EtOH, reflux 3h; substituted isocyanates absolute EtOH, reflux 6h.

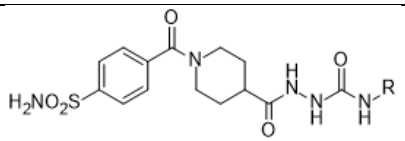
2.2. Carbonic anhydrase inhibition

The inhibitory activity against hCA I, hCA II, hCA IX and hCA XII of sulfonamide derivatives **5a-j** was tested by a stopped flow CO_2 hydrase assay using acetazolamide as standard inhibitor [13].

As reported in Table 1 the off-target isoform hCA I was inhibited by all the derivatives **5a-j** with a variety of potency. The 4-fluorophenyl derivative **5f** resulted to be the best compound of the series with a K_i of 60.6 nM. The substitution of the 4-fluorine group with a methyl group slightly reduced the inhibitory activity (**5b** Ki 81.7 nM). The introduction of one or two chlorine atoms, such as in compounds **5h** and **5g** led to reduction of activity (**5h** Ki 259.4 nM, **5g** Ki 217 nM) while the absence of substituent on the aryl-ureido ring restored the activity (**5a** Ki 93.3 nM). Furthermore, the introduction of benzyl and 4-(methoxy)benzyl groups such as in derivatives **5i** (Ki 77.8 nM) and **5j**

(Ki 129.8 nM) partially restored the activity. Overall, the reported compounds showed weak activity against hCA I, which is important for the development of selective CA inhibitors with potentially antiproliferative activity.

Table 1. Inhibition data of human CA isoforms hCA I, II, IX and XII with sulfonamides **5a-j** reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay (errors were in the range of ±5–10% of the reported values) [13].



Compound	R	Ki (nM)			
		hCAI	hCAII	hCAIX	hCAXII
5a	phenyl	93.3	29.3	16.5	22.8
5b	4-methylphenyl	81.7	22.0	8.1	36.7
5c	2,6-dimethylphenyl	363.6	62.1	25.1	51.2
5d	2,4-dimethoxyphenyl	321.8	19.8	23.2	15.6
5e	naphthyl	521.5	101.3	26.7	60.6
5f	4-fluorophenyl	60.6	12.1	2.1	24.0
5g	3-chlorophenyl	217.9	5.1	37.8	44.6
5h	2,6-dichlorophenyl	259.4	68.8	28.9	16.4
5i	benzyl	77.8	48.2	40.0	20.1
5j	4-methoxybenzyl	129.8	40.7	45.3	6.4
AAZ	-	250	12.5	25	5.7

The cytosolic isoform hCA II was inhibited by all ureido derivatives with Ki in the 5.1-101.3 nM range. The phenyl derivative **5a** showed Ki of 29.3 nM and the introduction of a substituent in 4-position, such as a fluorine atom (**5f** Ki 12.1 nM) or a methyl group (**5b** Ki 22.0 nM) led to increase in activity. The introduction of a second methyl group to give the 2,6-dimethylphenyl derivative **5c** reduced the inhibitory activity (Ki 62.1 nM), while the introduction of a chlorine atom into 3-position (**5g** Ki 5.1 nM) led to increase in activity. The presence of two chlorine atoms as in the 2,6-dichlorophenyl derivative **5h** led to considerable decrease in activity (Ki 68.8 nM) comparable with the 2,6-dimethylphenyl analog **5b.** It is possible to speculate that the substitution at both 2 and 6 positions could be important to reduce the activity against hCAII. Furthermore, the replacement of

the phenyl group with a benzyl group (**5i** Ki 48.2 nM) or with a naphthyl group (**5e** Ki 101.3 nM) reduced the inhibitory activity.

On the first cancer-related isoform hCA IX, all the compounds showed good inhibitory activity. The phenyl derivative **5a** showed a Ki of 16.5 nM, while the replacement of the phenyl ring with a benzyl group (**5i** Ki 40.0 nM) or with a 4-methoxybenzyl group (**5j** Ki 45.3 nM) resulted in about 4-fold reduction of activity. Furthermore, the replacement of the phenyl group with a naphthyl group also reduced the activity (**5e** Ki 26.7 nM). Remarkably, the introduction of substituents at 4-position of the phenyl ring of **5a** led to increased activity. For example, the 4-fluorophenyl derivative **5f** (Ki 2.1 nM) resulted to be 8-fold more active than **5a** as well as the 4-methylphenyl derivative **5b** (Ki 8.1 nM) was 2-fold more active than **5a**. The introduction of two chlorine atoms to obtain the 2,6-dichlorophenyl derivative **5h** led to reduction in activity (Ki 28.9 nM) as well as the replacement of the chlorine atoms with methyl groups (**5c** Ki 25.1 nM). Interestingly both **5h** and **5c** were about 3-fold more selective against hCA IX than hCA II confirming the importance of the substitution at the 2 and 6 position to obtain selective hCA IX inhibition.

Concerning hCA XII, this isoform was inhibited by ureido derivatives with Ki in the 6.4-60.6 nM range. The 4-methoxybenzyl derivative **5j** resulted the best compound of the series, with a Ki of 6.4 nM and it was about 7-fold more selective against hCA XII respect to both hCA II and hCA IX. Removing the 4-methoxy group to give the benzyl derivative **5i** (Ki 20.1 nM) led to reduction in activity. The replacement of the benzyl group with the phenyl group to give **5a** (Ki 22.8 nM) did not change the activity, whereas the introduction of a naphthyl group led to a 3-fold reduction of activity (**5e** Ki 60.6 nM). The introduction of two chlorine atoms on the aryl ring of **5a** to give the 2,6-dichlorophenyl derivative **5h** increased the activity (Ki 16.4 nM). On the contrary the replacement of the chlorine groups with methyl groups (**5c** Ki 51.2 nM) resulted in decrease of activity. Unlike the inhibitory activity showed against hCA IX, 4-fluorophenyl derivative (**5f** Ki 24.0 nM) and 4-methylphenyl (**5b** Ki 36.7 nM) resulted about 11-fold and 4-fold less active against hCA XII respectively while the 2,4-dimethoxyphenyl derivative **5d** showed Ki 15.6, comparable with the 2,6-dichlorophenyl derivative **5h**.

2.3. Molecular Docking

The most active compound against each isoform, except for hCA I for the poor activity, was selected for further investigations to understand their possible binding mode to the target. As a result, compounds **5f**, **5g** and **5j** were docked in hCA II (PDBID: 4g0c), hCA IX (PDBID: 3iai), hCA XII (PDBID: 1jd0). Moreover, to better rationalize the structure-activity relationship, additional compounds were docked to figure out the interactions with the targets driving the inhibitory

efficiency. The sulfonamide group of all compounds fits deeply into the active sites with the negatively charged nitrogen coordinating the zinc ion, and the NH^- and S=O moieties being in H-bond distance with hydroxyl group and backbone NH of Thr199, respectively [14].

The 3-chloro-substituted compound **5g**, the best hCA II inhibitor, showed other five key interactions to the target (Figure 2A): the three oxo groups of the ligand establish as many H-bonds with Gln92, Asn67 and Asn62, with the latter receiving another H-bond by the hydrazide group. A further interaction between the chlorine atom of the ligand and Lys170 stabilizes the binding of the derivative tail. To corroborate this, the 2,6-dichloro-substituted compound **5h** was also docked in hCA II (Figure 2B).

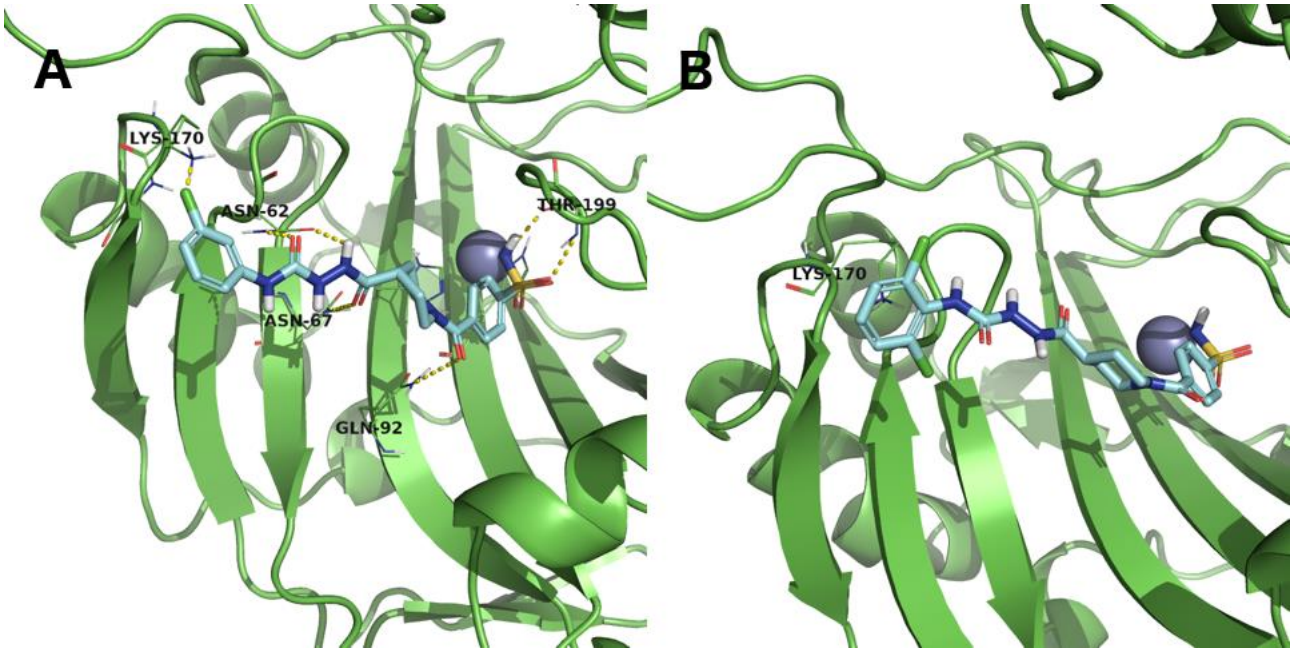


Figure 2. A) Compound **5g** best docking pose in hCA II (PDBID:4g0c); B) Compound **5h** best docking pose in hCA II (PDBID: 4g0c)

The 2,6-dichlorophenyl ring binds in a position not optimal for the interaction with Lys170. The absence of this interaction could be the reason behind the **5g**/**5h** difference in activity.

A pool of interactions presents in the best docking pose of the 4-fluorophenyl derivative **5f** to hCA IX could justify its potent inhibition against this tumor-associated CA (Figure 3A).

The binding of the ligand tail to the target is stabilized by the Arg89 - F atom H-bond. Four additional H-bonds occur between the linker and Arg130, Gln92 and Gln67 side chains. The 4-methylphenyl derivative **5b**, the second most active hCA IX inhibitor in the study, showed a superimposable binding mode with **5f**, except for the absence of the Arg89 - F atom H-bond that decreases the hCA IX inhibition efficiency of **5b** when compared to the halo-substituted compound (Figure 3B).

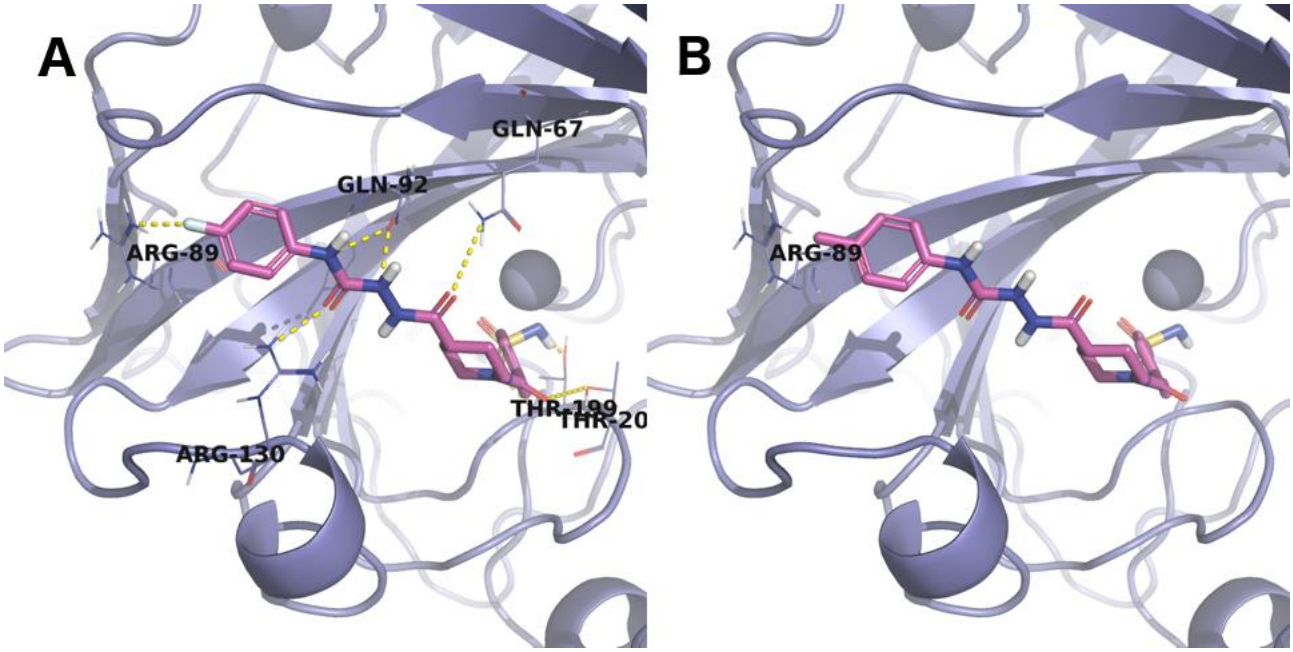


Figure 3. A) Compound **5f** best docking pose in hCA IX (PDBID: 3iai); B) Best docking pose of compound **5b** in hCA IX (PDBID: 3iai)

Compound **5j**, the best hCA XII inhibitor here, forms an extended H-bonds network with the target in the best docked orientation with the second tumor-associated isoform (Figure 4A). While the linker establishes four H-bonds with Gln92, Thr91, Ser132 and Thr200, the outer methoxy group receives two H-bonds by the OH moieties of Ser72 and Tyr123. When the benzyl derivative **5i** was docked in hCA XII (Figure 4B), the best binding pose showed the same set of interactions of **5j**, except for the absence of the H-bonds between the ligand tail and the target, which decreases its hCA XII inhibition activity.

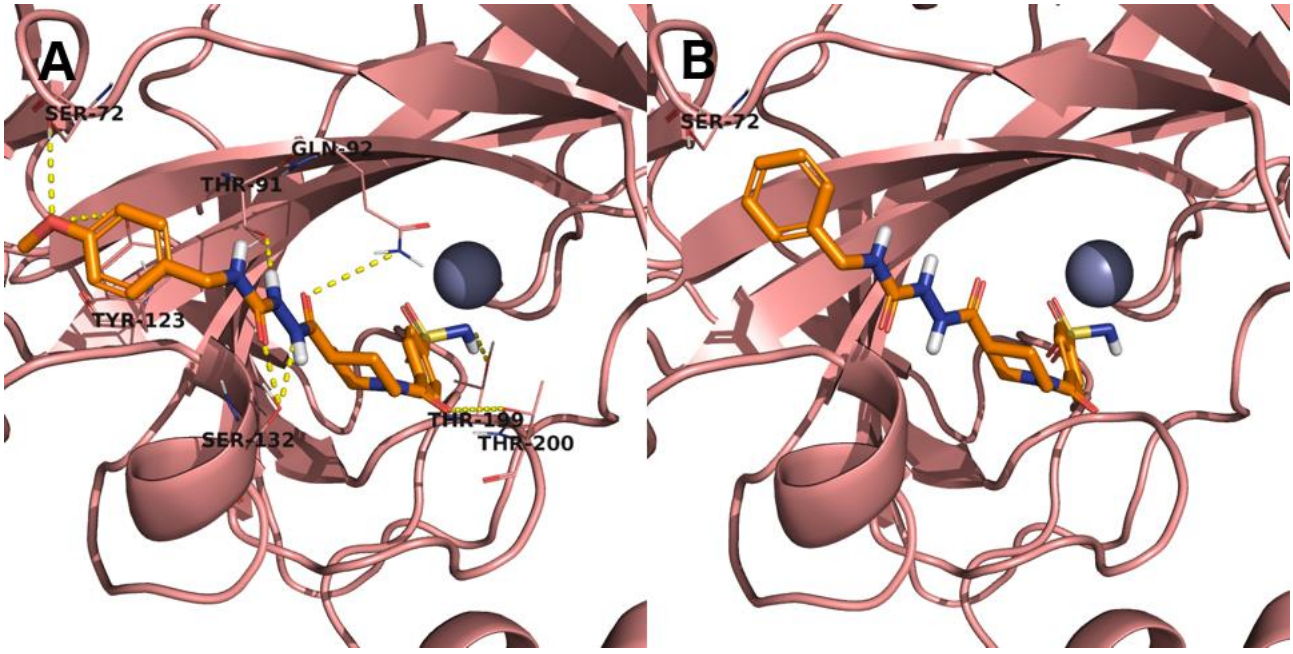


Figure 4. A) Best docking pose of compound **5j** in hCA XII (PDBID: 1jd0); B) Best docking pose of compound **5i** in hCA XII (PDBID: 1jd0)

3. Conclusions

A new series of benzensulfonamides endowed with piperidinyl-hydrazidoureido linker were designed and synthesized as hCAs inhibitors. Benzenesulfonamide derivatives **5a-j** showed generally poor activity against the cytosolic hCA I isoform (in the range of 60.6-521.5 nM). Conversely, against the other isoforms they showed different activities and a variable spectrum of selectivity. Compound **5g**, bearing the 3-chlorophenyl group was the most active and selective compound of the series against the hCA II isoform with a Ki of 5.1 nM and being less than 7 and 8-folds active against isoforms IX and XII respectively. Similar behavior but against isoform hCA IX was shown by derivative **5f** (Ki = 2.1 nM), 5 and 11 folds more active on this isoform than II and XII respectively. Compound **5j**, bearing a 4-(methoxy)benzyl tail, was the best active sulfonamide on the isoform hCA XII with a Ki of 6.4 nM. Moreover, molecular docking analysis revealed the probable key interactions that could explain the good activity and selectivity of these compounds.

4. Experimental

4.1. Chemistry

All commercially available solvents and reagents were used without further purification. NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA, USA). The chemical shifts (δ) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard, and the spectra were recorded in hexadeuteriodimethylsulphoxide (DMSO-d_6). Infrared spectra were recorded on a Vector 22 spectrometer (Bruker, Bremen, Germany) in Nujol mulls. The main bands are given in $cm^{-}1$. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing MAT 95 instrument (Finnigan, Waltham, MA, USA) with BE geometry. Melting points (mp) were determined with a SMP1 Melting Point apparatus (Stuart Scientific, Stone, UK) and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. The purity of the tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Department of Chemical and Pharmaceutical Sciences of the University of Ferrara with a MT-5 CHN recorder elemental analyzer (Yanagimoto, Kyoto, Japan) and the values found were within 0.4% of theoretical values.

*4.1.1. Ethyl 1-(4-sulfamoylbenzoyl)piperidine-4-carboxylate (**3**).* 4-(Aminosulfonyl)benzoic acid (**1**) (4.2 g, 20 mmol), EDCI (3.9g, 22 mmol) and HOBt (2.7 g, 20 mmol) were dissolved in anhydrous CH_3CN (100 mL). The resulting mixture was stirred at rt for 30 minutes, then ethyl isonipecotate (**2**) (3.1 g, 20 mmol) was added. The mixture was stirred at rt for 12 hours. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (30 mL) and washed sequentially with water (2 x 10 mL), saturated $NaHCO_3$ aqueous solution (2 x 10 mL), 10% aqueous citric acid (2 x 10 mL) and brine (2 x 10 mL). The organic layer was dried over anhydrous sodium sulfate (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was tritured with isopropyl ether (iPr_2O) and the formed solid was filtered off, dried and used in the next step without further purification. Yield 77% M.p. 155-156 °C. ESIMS (m/z): 341 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.17 (t, *J* = 7.5 Hz, 3H, CH_3), 1.52 (m, 2H, CH_2), 1.78-1.92 (m, 2H, CH_2), 2.63-2.95 (m, 2H, CH_2), 3.09-3.43 (m, 2H, CH_2), 4.07 (q, *J* = 7.5 Hz, 2H, CH_2), 4.32 (m, 1H, CH), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.0 Hz, 2H, Ar), 7.85 (d, *J* = 8.0 Hz, 2H, Ar). IR (Nujol) 3328, 3225, 1732, 1596 cm^{-1}. Anal. Calcd for $C_{15}H_{20}N_2O_5S$ (340.39) %C 52.93, %H 5.92, %N 8.23, found %C 52.99, %H 5.90, %N 8.26.

*4.1.2. 4-(4-(Hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide (**4**).* A mixture of **3** (4.9 g, 15 mmol) and hydrazine monohydrate (2.5 mL, 45 mmol) in EtOH was refluxed overnight. After cooling, the formed precipitate was filtered off, washed with water (3 x 10 mL), dried and used in the next step without further purification. Yield 78% M.p. 191-192 °C. ESIMS (m/z): 327 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.54 (m, 2H, CH_2), 1.73-1.84 (m, 2H, CH_2), 2.35-2.81 (m, 2H, CH_2), 3.04-3.48 (m, 2H, CH_2), 4.14 (s, 2H, NH_2) 4.44 (m, 1H, CH), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.99 (s, 1H, NH). IR (Nujol) 3309, 3208, 1614 cm^{-1}. Anal. Calcd for $C_{13}H_{18}N_4O_4S$ (326.37) %C 47.84, %H 5.56, %N 17.17, found %C 47.90, %H 5.54, %N 17.21.

*4.1.3. General procedure for the preparation of benzenesolfonamides (**5a-j**)*

A mixture of 4-(4-(hydrazinecarbonyl)piperidine-1-carbonyl)benzenesulfonamide **4** (0.326 g, 1 mmol) and the appropriate isocyanate (1 mmol) in EtOH (5 mL) was refluxed overnight. After cooling, the formed precipitate was filtered off, washed with Et_2O (3 x 10 mL) and recrystalized from EtOH.

*4.1.3.1. N-Phenyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5a**).* Yield 72%, m.p. >250 °C. ESIMS (m/z): 446 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.55 (br s, 2H, CH_2), 1.71-1.84 (br s, 2H, CH_2), 2.53-2.88 (br s, 2H, CH_2), 3.08-3.49 (br s, 2H, CH_2), 4.43 (br s, 1H, CH), 6.93 (d, *J* = 7.0 Hz, 1H, Ar), 7.23 (m, 2H, Ar), 7.40 (m, 1H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.0 Hz, 2H, Ar), 7.85 (m, 1H, Ar), 7.87 (d, *J* = 7.5 Hz, 2H, Ar), 7.96 (s, 1H, NH), 8.67 (s, 1H, NH), 9.65

(s, 1H, NH). IR (Nujol) 3265, 1667, 1563 cm^{-1}. Anal. Calcd for $C_{20}H_{23}N_5O_5S$ (445.49) %C 53.92, %H 5.20, %N 15.72, found %C 53.88, %H 5.19, %N 15.76.

*4.1.3.2. 2-(1-(4-Sulfamoylbenzoyl)piperidine-4-carbonyl)-N-(p-tolyl)hydrazinecarboxamide (**5b**).* Yield 82%, m.p. 233-234°C. ESIMS (m/z): 460 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.55 (br s, 2H, CH_2), 1.69-1.84 (br s, 2H, CH_2), 2.21 (s, 3H, CH_3), 2.51-2.88 (br s, 2H, CH_2), 3.08-3.49 (br s, 2H, CH_2), 4.44 (br s, 1H, CH), 7.03 (d, *J* = 8.5 Hz, 2H, Ar), 7.29 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.90 (s, 1H, NH), 8.56 (s, 1H, NH), 9.65 (s, 1H, NH). IR (Nujol) 3332, 3228, 1599 cm^{-1}. Anal. Calcd for $C_{21}H_{25}N_5O_5S$ (459.52) %C 54.89, %H 5.48, %N 15.24, found %C 54.94, %H 5.46, %N 15.19.

*4.1.3.3. N-(2,6-Dimethylphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5c**).* Yield 85%, m.p. 212-213 °C. ESIMS (m/z): 474 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.56 (br s, 2H, CH_2), 1.70-1.86 (br s, 2H, CH_2), 2.14 (s, 6H, CH_3), 2.54-2.84 (br s, 2H, CH_2), 3.07-3.50 (br s, 2H, CH_2), 4.44 (br s, 1H, CH), 7.02 (m, 3H, Ar), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.5 Hz, 2H, Ar), 7.81 (s, 1H, NH), 7.85 (s, 1H, NH), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 9.65 (s, 1H, NH). IR (Nujol) 3323, 3224, 1611 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_5O_5S$ (473.55) %C 55.80, %H 5.75, %N 14.79, found %C 55.85, %H 5.76, %N 14.75.

*4.1.3.4. N-(2,4-Dimethoxyphenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5d**).* Yield 84%, m.p. >250 °C. ESIMS (m/z): 506 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.56 (br s, 2H, CH_2), 1.70-1.86 (br s, 2H, CH_2), 2.54-2.84 (br s, 2H, CH_2), 3.07-3.50 (br s, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.44 (br s, 1H, CH), 6.44 (m, 1H, Ar), 6.58 (s, 1H, NH), 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.82 (d, *J* = 8.0 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.38 (s, 1H, NH), 9.71 (s, 1H, NH). IR (Nujol) 3332, 3212, 1615 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_5O_7S$ (505.54) %C 52.27, %H 5.38, %N 13.85, found %C 52.20, %H 5.40, %N 13.89.

*4.1.3.5. N-(Naphthalen-2-yl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5e**).* Yield 71%, m.p. 233-234 °C. ESIMS (m/z): 496 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.59 (br s, 2H, CH_2), 1.73-1.88 (br s, 2H, CH_2), 2.56-2.90 (br s, 2H, CH_2), 3.09-3.52 (br s, 2H, CH_2), 4.46 (br s, 1H, CH), 7.44 (s, 2H, NH_2), 7.55 (m, 5H, Ar), 7.80 (m, 1H, Ar), 7.88 (d, *J* = 8.5 Hz, 2H, Ar), 7.91 (m, 1H, Ar), 8.05 (d, *J* = 8 Hz, 2H, Ar), 8.32 (s, 1H, NH), 8.75 (s, 1H, NH), 9.80 (s, 1H, NH). IR (Nujol) 3222, 1611 cm^{-1}. Anal. Calcd for $C_{24}H_{25}N_5O_5S$ (495.55) %C 58.17, %H 5.08, %N 14.13, found %C 58.22, %H 5.10, %N 14.09.

*4.1.3.6. N-(4-Fluorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5f**).* Yield 65%, m.p. 209-210 °C. ESIMS (m/z): 464 $(M+H)^+$. ^1H NMR (DMSO-d_6): δ 1.55 (br s, 2H, CH_2) 1.72-1.81 (br s, 2H, CH_2), 2.52-2.87 (br s, 2H, CH_2), 3.08,

3.51 (br s, 2H, CH_2), 4.43 (br s, 1H, CH), 7.11 (d, *J* = 8.5 Hz, 2H, Ar), 7.36 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.57 (d, *J* = 8.0 Hz, 2H, Ar), 7.88 (d, *J* = 8.5 Hz, 2H, Ar), 7.92 (s, 1H, NH), 8.63 (s, 1H, NH), 9.58 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.9 (2C), 44.0, 49.7 (2C), 118.1 (2C), 129.0 (2C), 130.3 (2C), 130.4 (2C), 139.1, 142.6, 147.6, 158.6, 159.5, 170.9, 176.3. IR (Nujol) 3311, 1612 cm^{-1}. Anal. Calcd for $C_{20}H_{22}FN_5O_5S$ (463.48) %C 51.83, %H 4.78, %N 15.11, found %C 51.87, %H 4.79, %N 15.07.

*4.1.3.7. N-(3-Chlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5g**).* Yield 79%, m.p. 231-232 °C. ESIMS (m/z): 480 $(M+H)^+$. ^{1}H NMR (DMSO-d_6): δ 1.55 (br s, 2H, CH_2), 1.76-1.91 (br s, 2H, CH_2), 2.53, 2.88 (br s, 2H, CH_2), 3.08, 3.51 (br s, 2H, CH_2), 4.44 (br s, 1H, CH), 7.20 (s, 1H, Ar), 7.34 (m, 3H, Ar) 7.42 (s, 2H, NH_2), 7.56 (d, *J* = 7.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.59 (s, 1H, NH), 9.67 (s, 1H, NH), 9.93 (s, 1H, NH). IR (Nujol) 3309, 3183, 3094, 1680 cm^{-1}. Anal. Calcd for $C_{20}H_{22}ClN_5O_5S$ (479.94) %C 50.05, %H 4.62, %N 14.59, found %C 50.09, %H 4.58, %N 14.64.

*4.1.3.8. N-(2,6-Dichlorophenyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5h**).* Yield 78%, m.p. 220-221 °C. ESIMS (m/z): 514 $(M+H)^+$. ^{1}H NMR (DMSO-d_6): δ 1.55 (br s, 2H, CH_2), 1.72-1.83 (br s, 2H, CH_2), 2.84-3.05 (br s, 2H, CH_2), 3.48-4.33 (br s, 2H, CH_2), 4.43 (br s, 1H, CH), 7.27 (d, *J* = 8.0 Hz, 1H, Ar), 7.42 (s, 2H, NH_2), 7.47 (d, *J* = 8.0 Hz, 2H, Ar), 7.56 (d, *J* = 7.0 Hz, 2H, Ar), 7.87 (d, *J* = 8.0 Hz, 2H, Ar), 8.29 (s, 1H, NH), 9.03 (s, 1H, NH), 9.73 (s, 1H, NH). IR (Nujol) 3316, 3216, 1614 cm^{-1}. Anal. Calcd for $C_{20}H_{21}Cl_2N_5O_5S$ (514.38) %C 46.70, %H 4.11, %N 13.62, found %C 46.75, %H 4.10, %N 13.66.

*4.1.3.9. N-Benzyl-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5i**).* Yield 70%, m.p. 246-247 °C. ESIMS (m/z): 446 $(M+H)^+$. ^{1}H NMR (DMSO-d_6): δ 1.52 (br s, 2H, CH_2), 1.72-1.89 (br s, 2H, CH_2), 2.45, 2.85 (br s, 2H, CH_2), 3.06, 3.49 (br s, 2H, CH_2), 4.43 (br s, 1H, CH), 4.72 (s, 2H, CH_2), 7.19 (m, 1H, Ar), 7.22 (m, 4H, Ar), 7.43 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 8.35 (s, 1H, NH), 9.25 (s, 1H, NH), 9.78 (s, 1H, NH). IR (Nujol) 3225, 1659 cm^{-1}. Anal. Calcd for $C_{21}H_{25}N_5O_5S$ (459.52) %C 53.92, %H 5.20, %N 15.72, found %C 53.98, %H 5.18, %N 15.68.

*4.1.3.10. N-(4-Methoxybenzyl)-2-(1-(4-sulfamoylbenzoyl)piperidine-4-carbonyl)hydrazinecarboxamide (**5j**).* Yield 70%, m.p. >250 °C. ESIMS (m/z): 490 $(M+H)^+$. ^{1}H NMR (DMSO-d_6): δ 1.52 (br s, 2H, CH_2), 1.67-1.82 (br s, 2H, CH_2), 2.44, 2.85 (br s, 2H, CH_2), 3.05, 3.48 (br s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 4.12 (s, 2H, CH_2), 4.43 (br s, 1H, CH), 6.71 (s, 1H, NH), 6.84 (d, *J* = 8.5 Hz, 2H, Ar), 7.14 (d, *J* = 8.5 Hz, 2H, Ar), 7.42 (s, 2H, NH_2), 7.55 (d, *J* = 8.5 Hz, 2H, Ar), 7.72 (s, 1H, NH), 7.86 (d, *J* = 8.5 Hz, 2H, Ar), 9.49 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 42.9, 43.1, 43.2 (2C), 45.2 (2C), 58.2, 116.7 (2C), 129.0 (2C), 130.4 (2C), 131.4 (2C), 135.5, 142.6, 147.8, 161.2,

161.3, 170.9, 176.8. IR (Nujol) 3383, 3306, 3214, 1613 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_5O_6S$ (489.54) %C 53.98, %H 5.56, %N 14.33, found %C 54.03, %H 5.58, %N 14.28.

4.2. Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity [13]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mMHepes (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 inhibitor (0.1 mM) were prepared in 10% DMSO aqueous solution and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier [15-17] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier [18-20].

4.3. Molecular Docking

Molecular Docking simulations were carried out as previously described [21] using rDock [22]. The crystal structures of CAII (pdb 4G0C), CAIX (pdb 3IAI) and CAXII (PDBID: 1jd0) were retrieved from RCSB Protein Data Bank web server (http://www.rcsb.org/). Proteins preparation was executed using HTMD (HighThroughput MD) tool [23] to add hydrogens, ionize side chain of amino acids at physiological pH using propKa, deleting water molecules and co-crystallized small molecules (AAZ included). 3D ligands were prepared using an in-house python script developed using RDKit toolkit [24] and minimized using MMFF94 forcefield. Considering the high conservation of the binding mode of the sulfonamide moiety of co-crystallized compounds a tethered docking was executed [24] to enforce the partial binding modes of the sulfamates group. The ‘dock_solv’ rDock protocol was used, this protocol allows a full docking search but using the desolvation scoring function. Docking grid with radius of 10.0 Å was centered on the co-crystalized ligand. Number of poses was set to 10. Docking protocol validation was described in our previous paper [21]. It contemplates the re-docking of the co-crystallized ligand AAZ and the evaluation of the RMSD with the crystallographic pose.

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Conflict of interest

The authors declare no conflict of interest.

References

[1] C.T. Supuran. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat. Rev. Drug Discov. 7 (2008) 168–181.

[2] D. Neri, C.T. Supuran. Interfering with pH regulation in tumors as a therapeutic strategy. Nat. Rev. Drug Discov. 10 (2011) 767–777.

[3] K.S. Smith, C. Jakubzick, Whittam TS, Ferry JG. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. Proc. Natl. Acad. Sci. USA. 96 (1999) 15184-15189.

[4] C.T. Supuran, A. Scozzafava, J. Conway. Carbonic anhydrase: its inhibitors and activators. CRC press. (2004) ISBN 9780415306737

[5] S. Pastorekova, S. Parkkila, J. Zavada. Tumor-associated carbonic anhydrases and their clinical significance. Adv. Clin. Chem. 42 (2006) 167–216.

[6] Welichem Biotech Incorporated. Safety Study of SLC-0111 in Subjects with Advanced Solid Tumours. *NCT02215850*. https://clinicaltrials.gov/ct2/show/NCT03450018

[7] F. Pacchiano, F. Carta, P.C. McDonald, Y. Lou, D. Vullo, A. Scozzafava, S. Dedhar, C.T. Supuran. Ureido-Substituted Benzenesulfonamides Potently Inhibit Carbonic Anhydrase IX and Show Antimetastatic Activity in a Model of Breast Cancer Metastasis. J. Med. Chem. 54 (2011), 1896-1902.

[8] Y. Lou, P.C. McDonald, A. Oloumi, S.K. Chia, C. Ostlund, A. Ahmadi, A. Kyle, U. Auf dem Keller, S. Leung, D.G. Huntsman, B. Clarke, B.W. Sutherland, D. Waterhouse, M.B. Bally, C.D. Roskelley, C.M. Overall, A. Minchinton, F. Pacchiano, F. Carta, A. Scozzafava, N. Touisni, J.Y. Winum, C.T. Supuran, S. Dedhar. Cancer Res. 71 (2011) 3364-3376.

[9] C. Congiu, V. Onnis, A. Deplano, G. Balboni, N. Dedeoglu, C.T. Supuran Synthesis of sulfonamides incorporating piperazinyl-ureido moieties and their carbonic anhydrase I, II, IX and XII inhibitory activity. Bioorg. Med. Chem. Lett. 25 (2015) 3850-3853.

[10] N. Chiaramonte, S. Bua, M. Ferraroni, A. Nocentini, A. Bonardi, G. Bartolucci, M.C. Durante, L. Lucarini, D. Chiapponi, S. Dei, D. Manetti, E. Teodori, P. Gratteri, E. Masini, C.T. Supuran, M.N. Romanelli. 2-Benzylpiperazine: A new scaffold for potent human carbonic anhydrase inhibitors. Synthesis, enzyme inhibition, enantioselectivity, computational and crystallographic studies and in vivo activity for a new class of intraocular pressure lowering agents. Eur. J. Med. Chem. 151 (2018) 363-375.
[11] N. Chiaramonte, S. Bua, A. Angeli, M. Ferraroni, I. Picchioni, G. Bartolucci, L. Braconi, S. Dei, E. Teodori, C.T. Supuran, M.N. Romanelli. Sulfonamides incorporating piperazine bioisosteres as potent human carbonic anhydrase I, II, IV and IX inhibitors. Bioorg. Chem. 91 (2019) 103130.
[12] L. Liu, W. Wang, J. Huang, Z. Zhao, H. Li, Y. Xu. (2018). Novel benzoyl thioureido benzene sulfonamides as highly potent and selective inhibitors of carbonic anhydrase IX: Optimization and bioactive studies. Med. Chem. Comm. 9 (2018) 2100-2105.
[13] R.G. Khalifah. The carbon dioxide hydration activity of carbonic anhydrase. J. Biol. Chem. 246 (1971) 2561–2573.
[14] A. Nocentini, D. Moi, A. Deplano, S.M. Osman, Z.A. AlOthman, G. Balboni, C.T. Supuran, V. Onnis. Sulfonamide/sulfamate switch with a series of piperazinylureido derivatives: Synthesis, kinetic and in silico evaluation as carbonic anhydrase isoforms I, II, IV, and IX inhibitors. Eur. J. Med. Chem. 186 (2020) 111896.
[15] L. E. Kiss, H. S. Ferreira, L. Torrao, M. J. Bonifacio, P. N. Palma, P. Soares-da-Silva, D.A. Learmonth. Discovery of a Long-Acting, Peripherally Selective Inhibitor of Catechol-O-methyltransferase. J. Med. Chem. 53 (2010) 3396-3411.
[16] D. Vullo, S. Del Prete, A. Nocentini, S.M. Osman, Z. AlOthman, C. Capasso, M. Bozdag, F. Carta, P. Gratteri, C.T. Supuran, Dithiocarbamates effectively inhibit the b-carbonic anhydrase from the dandruff-producing fungus Malassezia globose. Bioorg. Med. Chem. 25 (2017) 1260–1265.
[17] Y. Entezari Heravi, S. Bua, A. Nocentini, S. Del Prete, A. A. Saboury, H. Sereshti, C. Capasso, P. Gratteri, C.T. Supuran. Inhibition of Malassezia globosa carbonic anhydrase with phenols. Bioorg. Med. Chem. 25 (2017) 2577-2582.
[18] A. Nocentini, A. Bonardi, P. Gratteri, B. Cerra, A. Gioiello, C.T. Supuran. Steroids interfere with human carbonic anhydrase activity by using alternative binding mechanisms. J. Enzyme Inhib. Med. Chem. 33 (2018)1453-1459.
[19] A. Nocentini, D. Moi, G. Balboni, V. Onnis, C.T. Supuran. Discovery of thiazolin-4-one-based aromatic sulfamates as a new class of carbonic anhydrase isoforms I, II, IV, and IX inhibitors. Bioorg. Chem. 77 (2018) 293-299.

[20] A. Bonardi, A.B. Vermelho, V. da Silva Cardoso, M.C. de Souza Pereira, L.L. da Silva, S. Selleri, P. Gratteri, C.T. Supuran, A. Nocentini. N-Nitrosulfonamides as Carbonic Anhydrase Inhibitors: A Promising Chemotype for Targeting Chagas Disease and Leishmaniasis. ACS Med. Chem. Lett. 10 (2018) 413-418.

[21] D. Moi, A. Nocentini, A. Deplano, G. Balboni, C. T. Supuran, V. Onnis. Structure-activity relationship with pyrazoline-based aromatic sulfamates as carbonic anhydrase isoforms I, II, IX and XII inhibitors: synthesis and biological evaluation. Eur. J. Med. Chem. 182 (2019) 111638.

[22] S. Ruiz-Carmona, D. Alvarez-Garcia, N. Foloppe, A.B. Garmendia-Doval, S. Juhos, P. Schmidtke, X. Barril, R.E. Hubbard, S.D. Morley. rDock: a fast, versatile and open source program for docking ligands to proteins and nucleic acids. PLoS Comput. Biol. 10 (2014) e1003571.

[23] S. Doerr, T. Giorgino, G. Martínez-Rosell, J.M. Damas, G. De Fabritiis, Highthroughput automated preparation and simulation of membrane proteins with HTMD. J. Chem. Theory Comput. 13 (2017) 4003-4011.

[24] RDKit, Cheminformatics and machine learning software. http://www.rdkit. Org (2013)