Bioorg. Med. Chem. 94 (2023) 117467

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of sulfonamides incorporating a biotin moiety: Carbonic anhydrase inhibitory effects, antiproliferative activity and molecular modeling studies

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ARTICLE INFO

Keywords:
Carbonic anhydrase
Biotin
Sulfonamide
Antitumor agent
Glioblastoma
Triple-negative breast cancer
Pancreatic carcinoma

ABSTRACT

Sulfonamides constitute an important class of classical carbonic anhydrase (CA, EC 4.2.1.1) inhibitors. Herein we have accomplished the conjugation of biotin with an ample number of sulfonamide motifs with the aim of testing them *in vitro* as inhibitors of the human carbonic anhydrase (hCA) isoforms I and II (cytosolic isozymes), as well as hCA IX and XII (transmembrane, tumor-associated enzymes). Most of these newly synthesized compounds exhibited interesting inhibition profiles, with activities in the nanomolar range. The presence of a 4-F-C_6H_4 moiety, also found in SLC-0111, afforded an excellent selectivity towards the tumor-associated hypoxia-induced hCA isoform XII with an inhibition constant (K_I) of 4.5 nM. The 2-naphthyl derivative was the most potent inhibitor against hCA IX (K_I = 6.2 nM), 4-fold stronger than AAZ (K_I = 25 nM) with very good selectivity. Some compounds were chosen for antiproliferative activity testing against a panel of 3 human tumor cell lines, one compound showing anti-proliferative activity on glioblastoma, triple-negative breast cancer, and pancreatic carcinoma cell lines.

1. Introduction

Carbonic anhydrases (CA) catalyze the reversible CO_2 hydration in blood and tissues via a coordinated metal ion, typically Zn(II).[1] This physiological reaction plays an essential role in functions such as respiration, pH homeostasis, ion transport, gluconeogenesis, bone resorption, and fluid secretion.[2–4] CAs dysregulation is linked to many diseases, such as obesity, neuropathic pain, glaucoma, sleep apnea, epilepsy, cancer, or Alzheimer's disease, among others.[5] In humans, 15 CA isoforms have been identified differing in tissue distribution as well as in subcellular localization and catalytic activity; among them, CA IX and CA XII, which are the target isoforms herein, are membrane-bound enzymes.[6] hCA I and hCA II, distributed in the cytosol, and considered herein as off-target enzymes, are widely expressed in the gastrointestinal tract, eyes, or erythrocytes.[7] On the contrary, hCA IX is poorly expressed in healthy tissues;[8–10] similarly, a very low expression of hCA XII is found in colon, kidney, prostate, lung, etc.[10–12].

Cancer progression creates a hypoxic environment, producing extracellular acidification of hypoxic tumors, further promoting tumor progression and metastasis.[13] Transmembrane hCA IX is associated with tumor progression and invasion by regulating intracellular and extracellular pH under hypoxic conditions, including cervical, brain, neck, lung, colon, breast, and bladder cancers.[14] Furthermore, an increase in tumor aggression and resistance in hypoxic tumors is usually observed due to their reduced response to classical anticancer therapies.[15] For that reason, hCA IX is currently considered as an attractive anticancer target. hCA XII is also upregulated in many aggressive tumors.[16–19] Selective blockade of hCA IX and XII activities has been reported to reduce tumor growth, and to affect its capacity to expand, by minimizing angiogenesis and metastasis.[20,21].

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https://doi.org/10.1016/j.bmc.2023.117467
Received 23 July 2023; Received in revised form 2 September 2023; Accepted 4 September 2023
Available online 7 September 2023

Sulfonamides and their bioisosteres (e.g. sulfamates, sulfamides), potent inhibitors of CAs, are the most widely investigated derivatives in this context;[22] however, one common disadvantage is their usual lack of selectivity among the different CAs isoforms. Compound SLC-0111 (Fig. 1A), an ureido-derived sulfonamide, is the most advanced candidate, being in Phase Ib/II clinical trials for the treatment of advanced hypoxic solid tumors.[23] This compound has been used as a startpoint in the design of selective antitumor drugs, minimizing the side effects of classical CA inhibitors (CAI), like acetazolamide (AAZ), methazolamide (MZA), topiramate, and dichlorphenamide (DCP), in clinical use to treat glaucoma, seizures or edema.[6,24].

Vitamins B_{12}, folic acid, biotin (vitamin H, Fig. 1B), and riboflavin are crutial micronutrients involved in cell division processes, and are particularly relevant in cancer cells. Overexpression of vitamin receptors in cancer cells, particularly biotin receptors (BRs) (e.g. colon, renal, leukemia, lung, ovarian, breast, and glioblastoma)[25,26] has stimulated the development of drugs conjugated to biotin as a selective drug carrier with anticancer activity.[27–29].

It has been demonstrated that modifications of the carboxylic acid group of biotin (Fig. 1) do not compromise its affinity for BRs.[29] Herein, we describe the design, synthesis and evaluation of a new series of sulfonamides derived from biotin, as a cancer-targeting scaffold.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds **2a-l**, **4** and **6** reported herein was performed as highlighted in Scheme 1, coupling the carboxylic acid of biotin with sulfonamides **1a-d** (commercially available) and with **1e-l**,[30,31] **3**[32] and **5**[33] (prepared as reported earlier) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in anhydrous DMF as solvent. For compound **2c** the conditions were different (see the experimental section).

To obtain compound **9** with good yield (72% Scheme 2), conversion of the carboxylic acid moiety of biotin into the corresponding acid chloride was accomplished with thionyl chloride ($SOCl_2$), and subsequently, coupling with 4-amino-3-chlorobenzenesulfonamide **8** using DMF as solvent, and *N,N*-diisopropylethylamine (DIPEA) as base. Moreover, compound **12** was obtained by reaction of the mixed anhydride intermediate **10** of biotin with sulfonamide **11** previously prepared.

In the design of target molecules **17a-d**, a 1,2,3-triazole scaffold as a central tether to connect the two bioactive groups was included, as shown in Scheme 3. The benzenesulfonamide azides **13a-b** were prepared from sulfonamides **1a-b** through a diazotization reaction using conc. HCl, $NaNO_2$ and NaN_3, while **13c-d** were prepared from the corresponding chlorosulfonic bromides. The compounds **13a-d** were finally subjected to click reaction with alkyne intermediate **14** in the presence of sodium ascorbate and $Cu(CH_3COO)_2$ in MeOH/THF (1:3) as solvent; deprotection in acid medium followed by coupling with biotin afforded the final target products **17a-d** (Scheme 3) by using the same conditions as those depicted in Scheme 1. On the other hand, conversion of biotin into the corresponding acyl azide **18** using DPPA and Et_3N in anhydrous THF, allowed Curtius rearrangement at refluxing conditions in toluene as solvent to give the isocyanate intermediate **19**; nucleophilic addition of sulfonamides **1c** and **1d** afforded the ureas **20c** and **20d** respectively (Scheme 4).

All derivatives were purified by precipitating or by silica gel chromatography eluting with MeOH/DCM gradients and fully characterized by ^1H NMR, ^{13}C NMR and mass spectra (ESI-MS).

2.2. Carbonic anhydrase inhibition

The synthesized compounds were tested against the cytosolic isoforms hCA I and hCA II as well as the transmembrane tumor-associated isoforms hCA IX and hCA XII, by a stopped-flow kinetic assay;[34] acetazolamide (AAZ) and SLC-0111[35] were used as standard CAIs. The inhibition constants (K_Is) are shown in Table 1.

The cytosolic hCA I was effectively inhibited by most compounds with the inhibition constant (K_I) values ranging from 42.0 to 625.8 nM (AAZ: K_I = 250.0 nM), whereas the tertiary amides **2 h-k** showed a weak inhibition (K_I = 1633, 997.2, 1005 and 724 nM, respectively).

The other cytosolic isoform hCA II was strongly inhibited by few compounds with K_Is ranging between 0.65 and 10.8 nM, all of them were found to be more potent hCA II inhibitors compared to the standard AAZ. Compound **12** (K_I = 0.65 nM) was 18-fold stronger than AAZ (K_I = 12 nM) whereas compound **17a** with a 1,2,3-triazole moiety (K_I = 4.3 nM) was found to be 2-fold stronger than AAZ against hCA II. Some others showed moderate inhibitory properties (K_I = 20.1–91.5 nM), whereas compounds **2 h-j**, **17b** and **17c** afforded low inhibition against hCA II with K_I values of 1367 nM, 217.6 nM, 275.3 nM, 362.1 nM and 220.1 nM respectively.

The tumor-associated isoform hCA IX was significantly inhibited by most derivatives in the nanomolar range (K_I = 6.2–129.4 nM) with nine compounds being more potent hCA IX inhibitors than the standard AAZ. The most potent inhibitor against this isoform was found to be the compound **2 k** with naphthyl substitution (K_I = 6.2 nM), 4 and 7-fold stronger than AAZ (K_I = 25 nM) and SLC-0111, respectively.

The other tumor-associated isoform hCA XII, was also effectively inhibited by most derivatives (K_I = 2.1–63.9 nM). The 4-fluorophenyl substituted derivative (**2i**), the urea **20c** and **12** exhibited the best inhibition amongst all compounds with K_I values of 4.5, 5.0 and 2.1 nM respectively, with similar potencies to that of AAZ and SLC-0111.

Compound **2e** was the only one which showed very low inhibitory activity against all hCAs isoforms. This is probably due to the steric hindrance provided by the greater proximity of the amide substituents compared to compounds **2f** and **2 g**, limiting the binding of **2e** to the enzyme.

As pointed out by data in Table 1, secondary amides **2a-d**, **4** and **6** exhibited good inhibition profiles against all tested hCAs and no marked isoform selectivity was detected; in addition, including a triazole group in the structure (compounds **17a-d**) did not significantly improve selectivity towards hCAIX/XII over hCA I and hCA II. Notably, the introduction of a second substituent for obtaining tertiary amides significantly shifted the selectivity toward hCAIX/XII inhibition over hCA I and hCA II. Remarkably, the presence of 4-F-C_6H_4 (**2i**) afforded an excellent selectivity towards hCA XII over hCA II (hCA II/ hCA XII = 48) and hCA I (hCA I/ hCA XII = 221). For 2-Naph (**2k**), the most potent

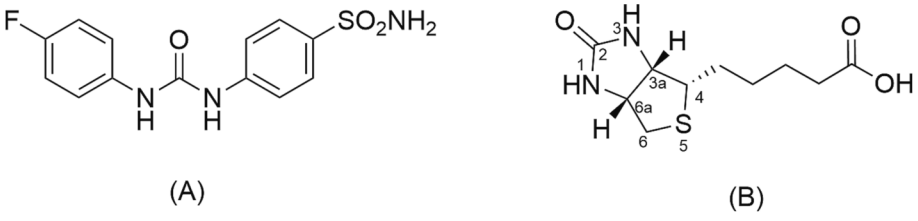
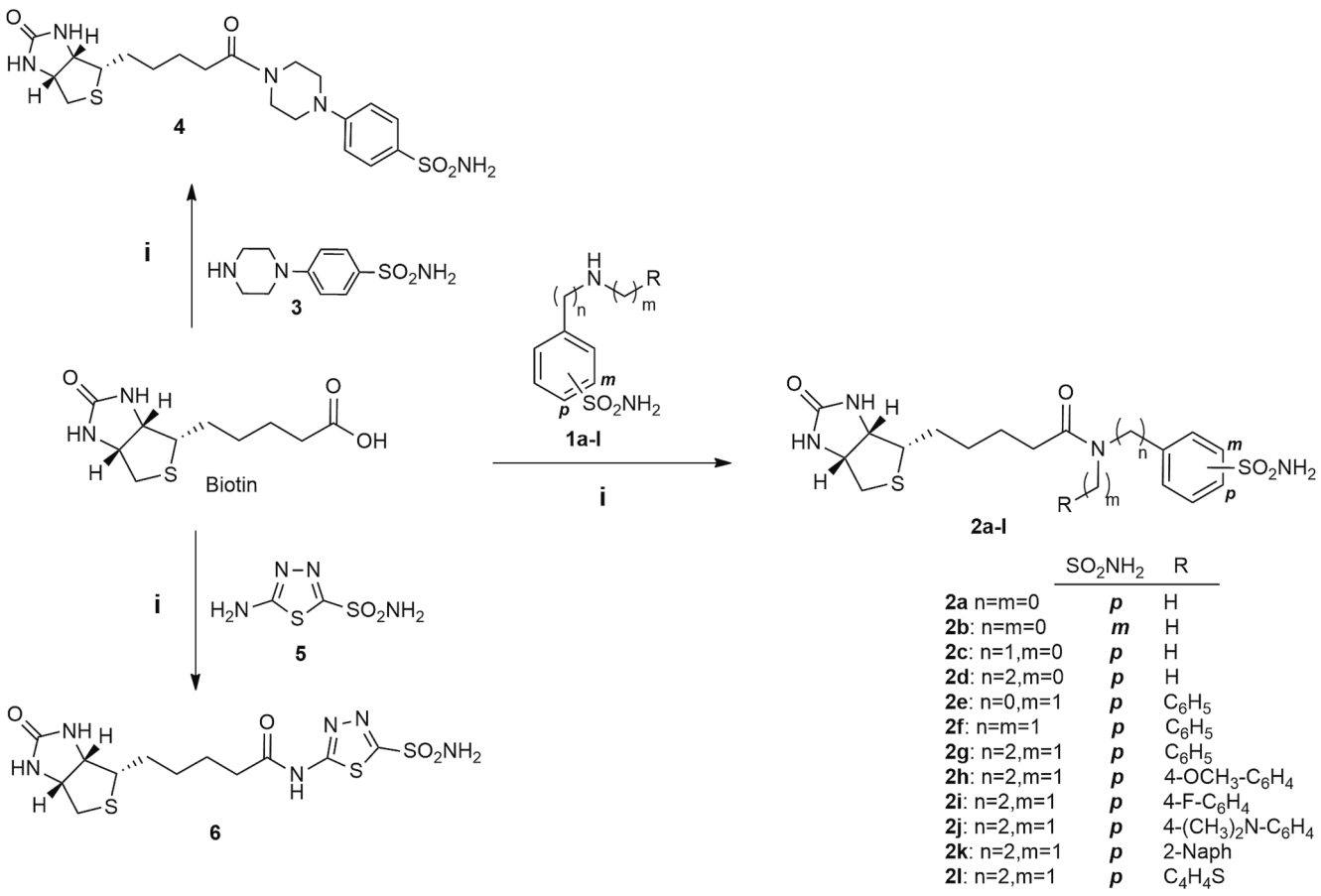
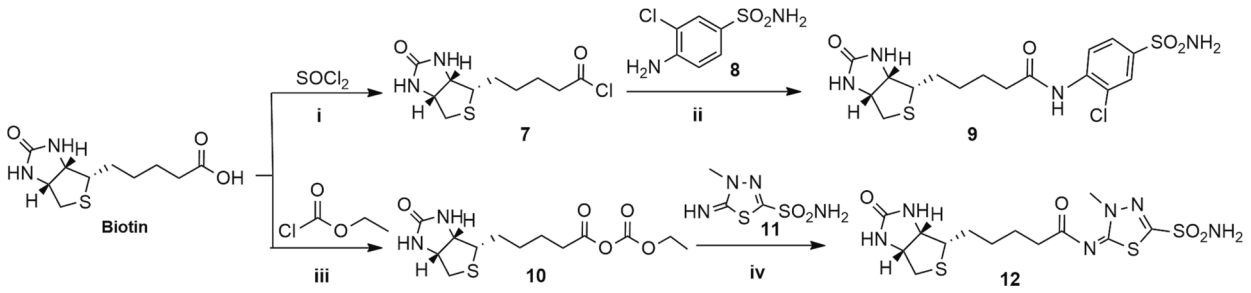


Fig. 1. Chemical structures of the CA inhibitor SLC-0111 (A) and of biotin.



	SO_2NH_2	R
2a n=m=0	*p*	H
2b: n=m=0	*m*	H
2c: n=1,m=0	*p*	H
2d: n=2,m=0	*p*	H
2e: n=0,m=1	*p*	C_6H_5
2f: n=m=1	*p*	C_6H_5
2g: n=2,m=1	*p*	C_6H_5
2h: n=2,m=1	*p*	4-OCH_3-C_6H_4
2i: n=2,m=1	*p*	4-F-C_6H_4
2j: n=2,m=1	*p*	4-$(CH_3)_2N$-C_6H_4
2k: n=2,m=1	*p*	2-Naph
2l: n=2,m=1	*p*	C_4H_4S

Scheme 1. Reagents and conditions: (**i**) EDCl, DMAP, dry DMF, rt.



Scheme 2. Reagents and conditions: (**i**) SO_2Cl, rt 30 min; (**ii**) DIPEA, dry DMF, rt overnight; (**iii**) Et_3N, dry DMF, 0 °C - rt 1 h; (**iv**) dry DMF, 0 °C - rt overnight.

inhibitor against hCA IX, a very good selectivity over hCA I (hCA I/ hCA IX = 117) was achieved.

2.3. *In vitro antiproliferative activity*

The biological activity of the best CAI candidates was assessed *in vitro* on different human cancer cells representing glioblastoma (U87MG), triple-negative breast cancer (MDA-MB-231), and pancreatic carcinoma (PANC-1) expressing hCAIX/XII.[36,37] Tumor cells were treated under hypoxic conditions with increasing concentrations of compounds **2i**, **2j** and **2 k** for 48 h and the effect on cell proliferation was quantified and compared with AAZ and SLC-0111.

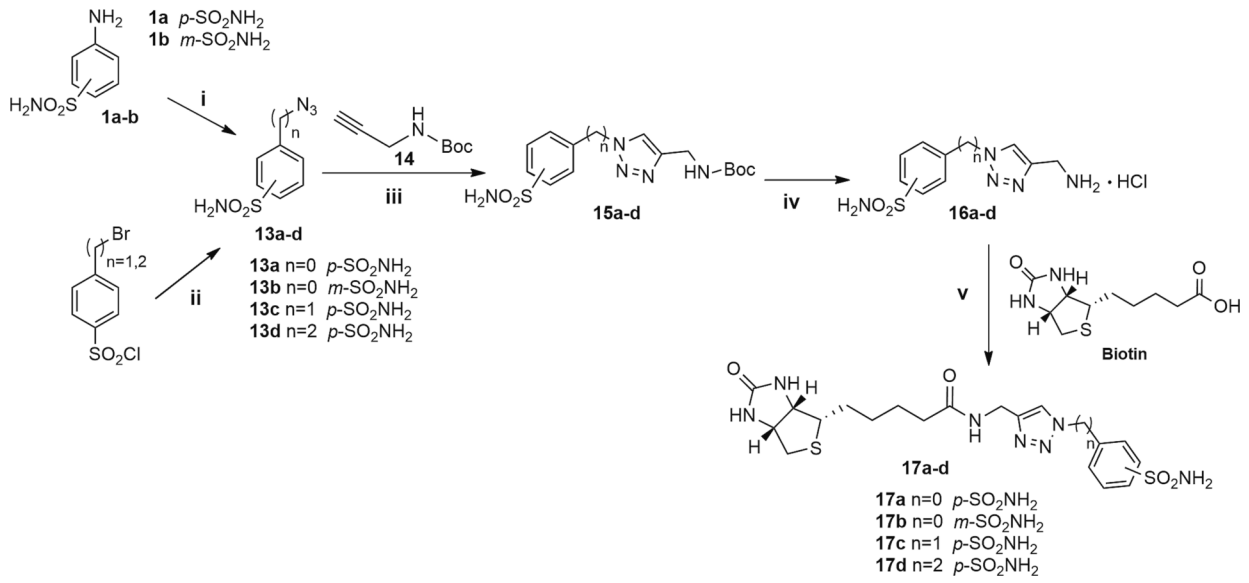
As shown in Fig. 2, no significant effect was exerted by compound **2i** nor AAZ on glioblastoma cells, proliferation being affected only by treatment with **2j** at the highest concentration (300 μM), and by **2 k** at 100 μM.

When assessed on MDA-MB-231 triple-negative breast cancer cells, no significant effect was observed by **2i**, **2j** and AAZ at all the concentrations (Fig. 3). Indeed, compound **2k** and SLC-0111 exerted a significant anti-proliferative effect reducing cell proliferation at 39% for **2k** and 62% for SLC-0111 at the highest dose tested.

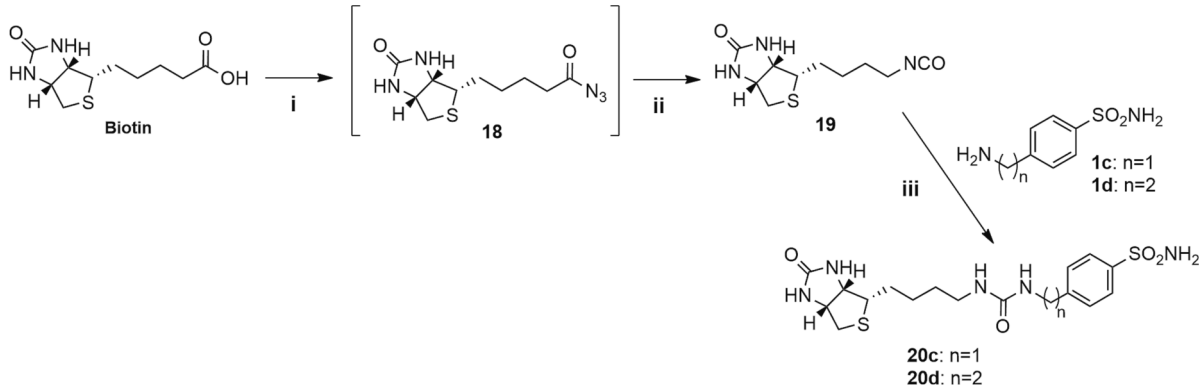
Finally, when tested in PANC-1 cells, compounds **2j**, **2k** and SLC-0111 displayed a significant antiproliferative effect at the highest doses evaluated reducing cell proliferation at 36%, 61% and 64%, respectively (Fig. 4). Also in this case, no significant inhibitory effect was observed when pancreatic cancer cells were treated with **2i** or AAZ.

2.4. *Molecular modeling studies*

Molecular docking was carried out to predict the binding properties of compounds **2i**, **6**, **17a** and **20d** within the active site of the off-targets CA I and II and the tumor-associated isoforms CA IX and XII and to explore thoroughly the correlation between their structural features and the inhibitory activity against these enzymes. The selected compounds share the common CAI scaffold and the biotin moieties that are connected by amide, triazole ring and the urea linkers, thereby representing



Scheme 3. Reagents and conditions: **(i)** 6 N HCl, $NaNO_2$ (1.2 equiv.), −5°C, 30 min; NaN_3 (1.6 equiv.), −5°C, 3 h; **(ii)** NH_4OH, THF, 0 °C - rt, 2 h; NaN_3, dry DMF, rt, overnight; **(iii)** Na-ascorbate, $Cu(CH_3COO)_2$, MeOH/THF 1:3, 36 °C overnight; **(iv)** 3 N HCl in MeOH; **(v)** DIPEA, EDCl, DMAP, dry DMF, 0 °C - rt 48 h.



Scheme 4. Reagents and conditions: **(i)** DPPA, Et_3N, dry THF, 0 °C - rt overnight; **(ii)** toluene, reflux overnight; **(iii)** dry ACN, rt.

all the derivative types in Table 1. As reported in the literature,[38–41] docking solutions found the benzenesulfonamide or 1,3,4-thiadiazole-2-sulfonamide group bound to the zinc ion through the deprotonated nitrogen atom of the $SONH^-$ moiety (Figs. 5 and 6). Moreover, the benzenesulfonamide/1,3,4-thiadiazole-2-sulfonamide forms two H-bonds between the T199 and the SO_2NH^- group (T199 O—H···NH– and T199 N—H···O═S), and is further stabilized by van der Waals (vdW) contacts occurring between the aromatic/heterocyclic ring and A121/V121 (CA I/CA II, IX and XII), V143, L198 and W205 (Figs. 5 and 6). Generally, the biotin moiety is oriented towards the hydrophilic half of all investigated hCA isoforms engaging hydrogen bonds with different residues based on the ligand: hCA I E71/Q92/K170 are involved in the interactions with the biotin NH and C═O of **2i** and **20d**/**6**/**17a**, (Fig. 5 A-D) while hCA II N62, D130, F131 interacts with **6**, **17a** and **20d** (Fig. 5 E-G).

Hydrophobic contacts contribute to the overall stabilization of the ligands binding modes. Both in hCA I and II the 4-fluorophenyl moiety of the dual-tailed inhibitor **2i** accommodates in the pocket lined by A135/V135, P201, P202, Y204/L204 and W5 while a *T*-shaped π-π stacking interaction occurs between the triazole ring of **17a** and the phenyl ring of the peculiar hCA II F131.

In the tumor-associated isoform CA IX active site, while the biotin C═O group of ligands **2i** and **17a** forms a three-center H-bond with the guanidinium group of the peculiar R60 (Fig. 6A and C), the 4-fluorophenyl moiety of ligand **2i** is located in a wide lipophilic pocket formed by L91, V131, L135, P201 and P202, undertaking a wide network of vdW contacts (Fig. 6A). Instead, the NH and C═O groups of the biotin pendant of ligands **6** and **20d** engage two H-bonds with the sidechain COO^- and backbone NH of D132 and V131, respectively (Fig. 6B and D), and the urea linker C═O group of **20d** is in H-bond distance with the sidechain NH_2 of Q92 (Fig. 6D).

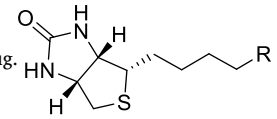
In the CA XII active site, the dual-tailed inhibitor **2i** places the 4-fluorophenyl moiety in a cleft defined by W5, P201 and P202, engaging H-bond interactions with the linker and biotin C═O groups with the sidechain NH_2 and OH of N62 and T91, respectively (Fig. 6E). In the case of ligands **6**, **17a** and **20d** the amidic/urea linker and biotin C═O groups are in H-bond distance with the sidechain NH_2 and the charged sidechain NH_3^+ of Q92 and the peculiar K67, respectively (Fig. 6F-H).

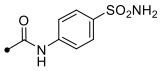
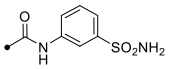
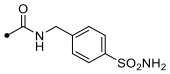
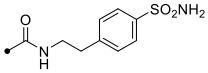
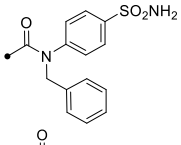
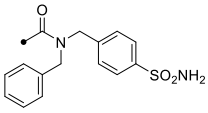
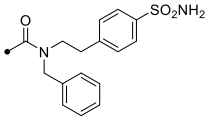
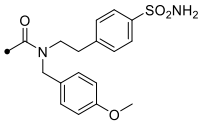
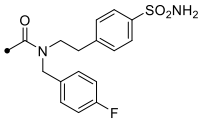
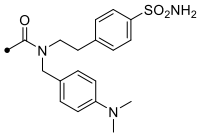
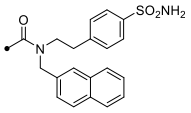
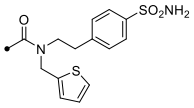
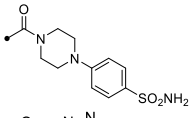
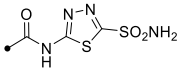
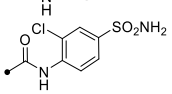
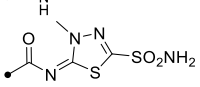
Derivatives **2i**, **6**, **17a** and **20d** were also investigated for their binding properties within the sodium-dependent multivitamin transporter protein (SMVT), considered to be the main uptake system for biotin.[42–48] No experimental 3D coordinates for SMVT are reported, thus the predicted model of the transport protein was retrieved from Alphafold Protein Structure database[49] and used after the proper preparation procedures in docking experiments. Induced fit, followed by MM-GBSA-based refinement calculations, were performed to evaluate more reliable the ΔG binding values of the ligand-target complexes.

Docking studies found biotin to interact with residues Q80, S81, Y99

Table 1

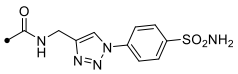
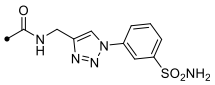
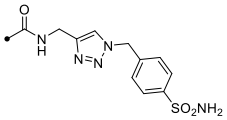
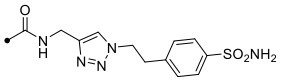
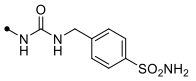
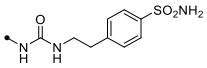
Inhibition data of human CA isoforms I, II, IX and XII with compounds synthesized using AAZ as standard drug.



cmpd	R	K_I (nM)[a]			
		hCA I	hCA II	hCA IX	hCA XII
2a		52.1	33.4	72.1	8.6
2b		423.2	46.8	60.8	19.2
2c		113.6	85.7	48.9	15.2
2d		42.0	38.5	54.1	22.0
2e		4452	8954	245.8	124.2
2f		65.7	6.1	14.0	36.8
2g		598.2	56.2	32.4	24.1
2h		1633	1367	129.4	49.7
2i		997.2	217.6	24.3	4.5
2j		1005	275.3	62.1	33.5
2k		724.3	55.1	6.2	14.8
2l		85.2	8.3	17.5	6.7
4		64.7	42.9	33.5	63.9
6		189.5	20.1	15.8	8.3
9		115.3	8.4	19.7	52.4
12		92.1	0.65	8.7	2.1

(*continued on next page*)

Table 1 (*continued*)

cmpd	R	K_I (nM)[a]			
		hCA I	hCA II	hCA IX	hCA XII
17a		68.2	4.3	11.6	9.8
17b		625.8	362.1	62.7	46.1
17c		452.2	220.1	82.3	52.0
17d		71.9	42.9	22.0	28.9
20c		94.6	10.8	29.5	5.0
20d		554.2	91.5	45.7	12.9
AAZ	–	250	12.0	25.0	5.7
SLC-0111	–	5080	960	45	4.5

a. Mean from 3 different assays, by a Stopped-flow technique (errors were in the range of ± 5–10 % of the reported values).

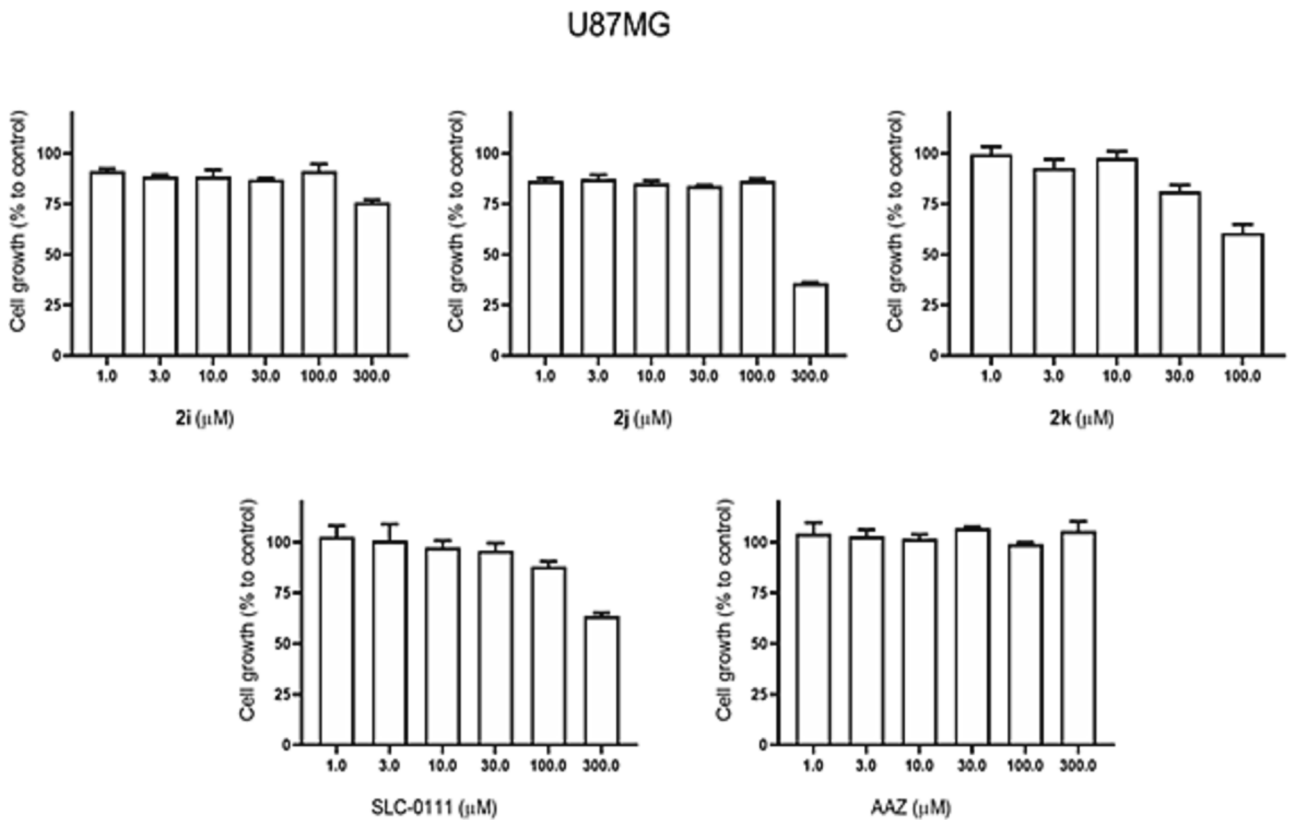


Fig. 2. Cell proliferation of human glioblastoma cells treated for 48 h with compounds **2i**, **2j**, **2k**, SLC-0111 or AAZ under hypoxic conditions. Data are the mean ± SEM and represent the percentage of cell proliferation with respect to the control (vehicle-treated) group.

and Y156. In particular, the NH group of biotin is in H-bond distance with the sidechain OH and backbone NH of S81 (S81 O—H···O═C- and S81 N—H···O═C) and with the sidechain OH of Y156 (Y156 O—H…H—N), while the free carboxylic function serves as H-bond acceptor from the sidechains NH_2 and OH of Q80 and Y99, respectively. Furthermore, vdW interactions take part in the ligand-target complex stabilization with residues F79, Q80, S81, V88, Y99, Y156, M266, M267, L270, Q301, L305, L424, I428 and F431 (Fig. 7A).

Despite the lack of a free COOH group, the biotinylated derivatives **2i**, **6**, **17a** and **20d** were able to dock the binding site, all locating the biotin moiety in the same area of the binding pocket, albeit the bicycle is rotated by about 90º with respect to that of the vitamin.

As described for the biotin, the ureido C═O is in H-bond distance with the sidechain OH and backbone NH of S81, while the NH group

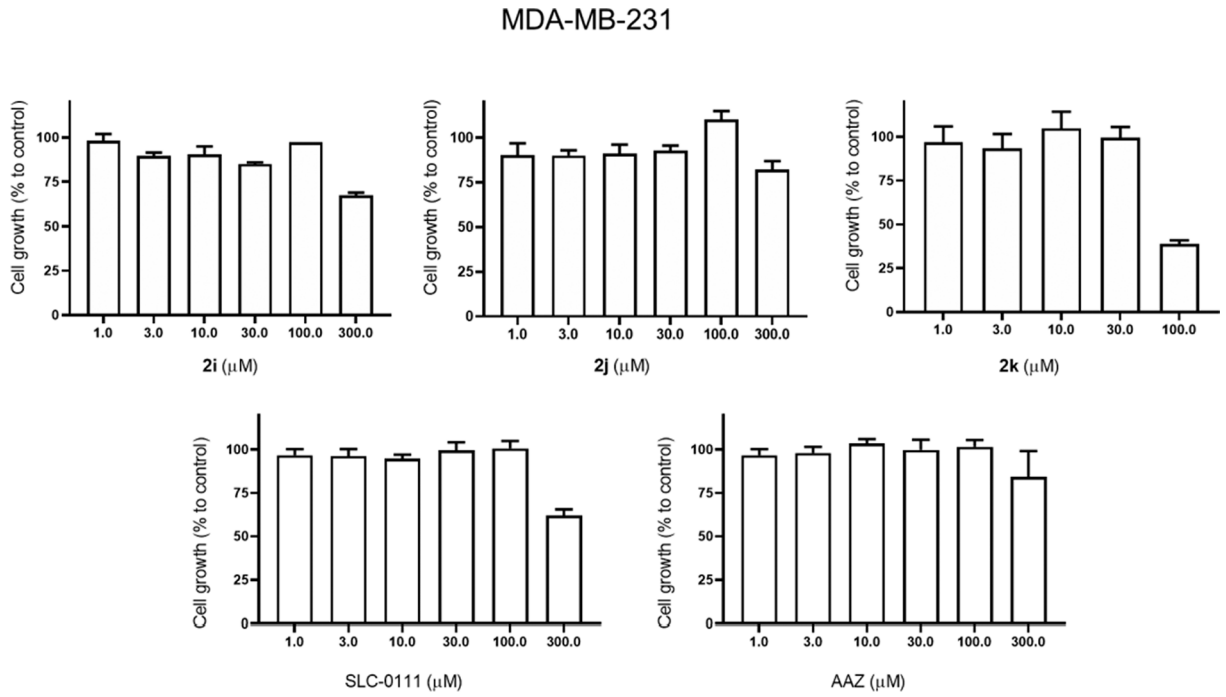


Fig. 3. Cell proliferation of human triple-negative breast cancer cells treated for 48 h with compounds **2i**, **2j**, **2k**, SLC-0111 or AAZ under hypoxic conditions. Data are the mean ± SEM and represent the percentage of cell proliferation with respect to the control (vehicle-treated) group.

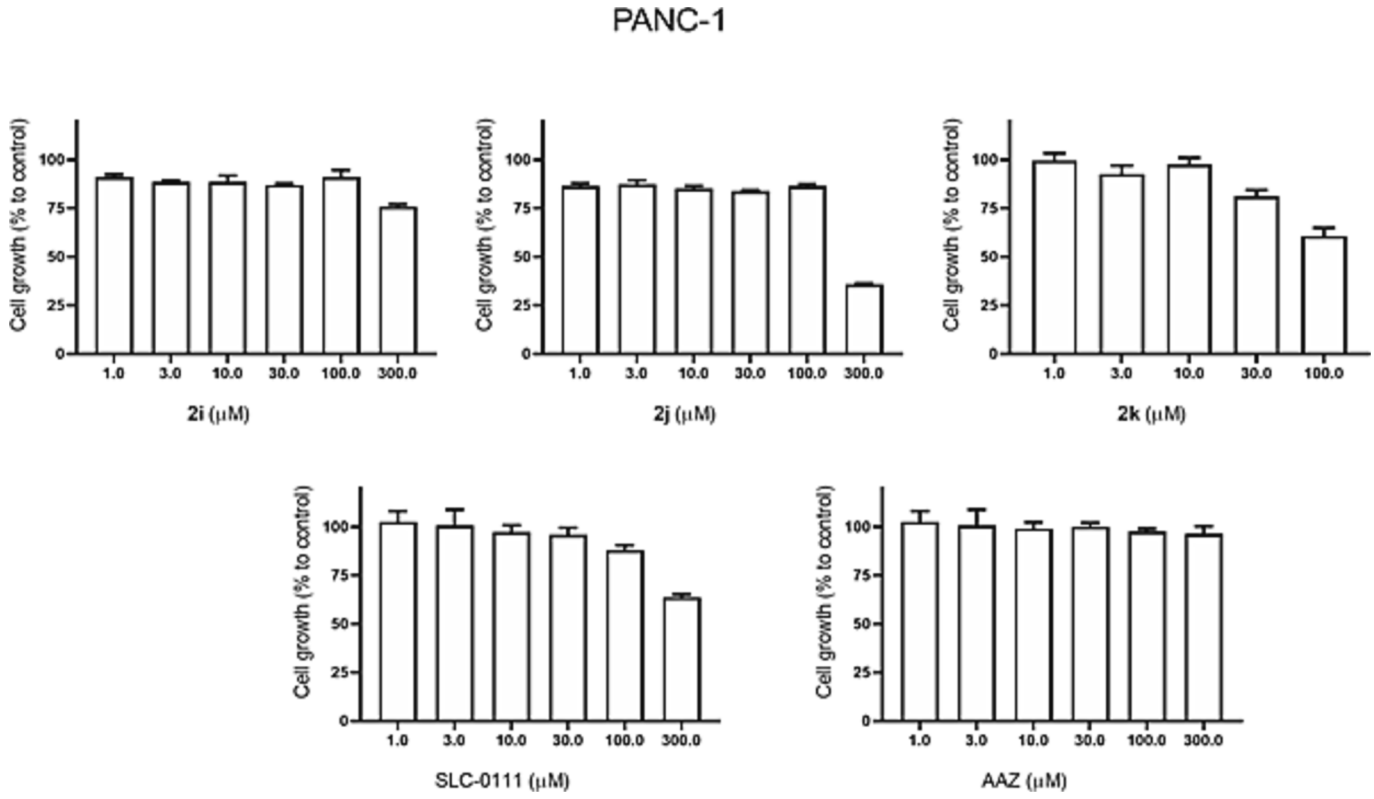


Fig. 4. Cell proliferation of human pancreatic cancer cells treated for 48 h with compounds **2i**, **2j**, **2k**, SLC-0111 or AAZ under hypoxic conditions. Data are the mean ± SEM and represent the percentage of cell proliferation with respect to the control (vehicle-treated) group.

engages an H-bond with the backbone carbonyl oxygen atom of F79.

The amidic C═O forms an H-bond with the NH_2 sidechain of Q301 (Fig. 7B, compound **2i**) or with the sidechain OH of Y156 (Fig. 7C,E, compounds **6** and **20d**). Moreover, the triazole ring of ligand **17a** interacts through an H-bond and a π-π stacking interaction with Y156 (Fig. 7D). All the investigated ligands orient the aromatic sulfonamide moiety towards an area defined by polar residues (Q80, Q214 and S304) and the aromatic ring is stabilized by hydrophobic contacts with A84, V88 and L305. Specifically, the benzyl tail of compound **2i** makes vdW contacts with residues Y99, L102, Y106, M266, M267, L305 and I428,

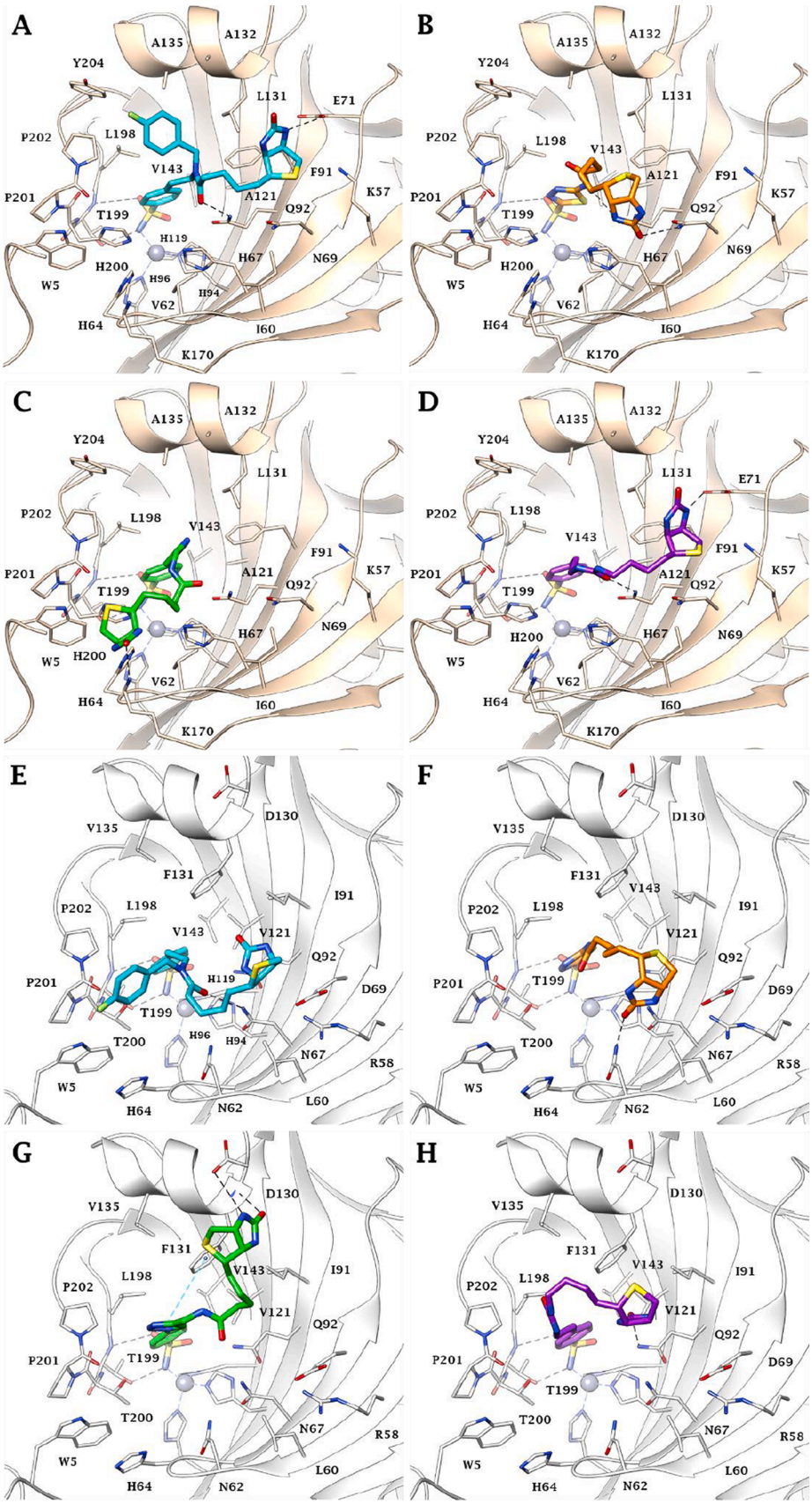


Fig. 5. Predicted binding mode of ligands **2i** (cyan), **6** (orange), **17a** (green), **20d** (purple), within the hCA I (orange: A-D) and hCA II (white; E-H) active site. H-bonds and π-π stacking interactions are depicted as black and cyan dashed lines, respectively.

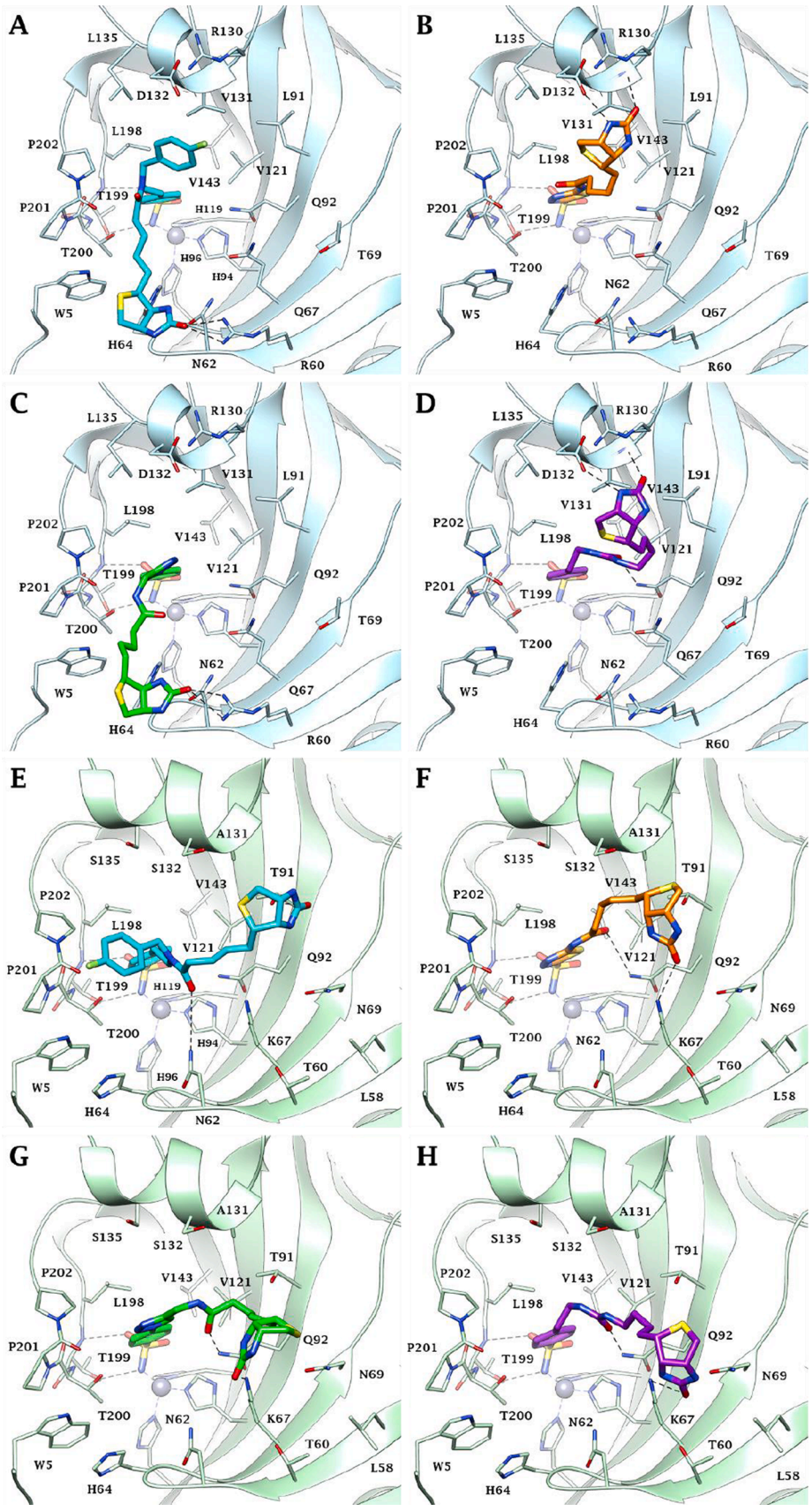


Fig. 6. Predicted binding mode of ligands **2i** (cyan), **6** (orange), **17a** (green), **20d** (purple), within the hCA IX (blue: A-D) and hCA XII (green; E-H) active site. H-bonds are depicted as black dashed lines.

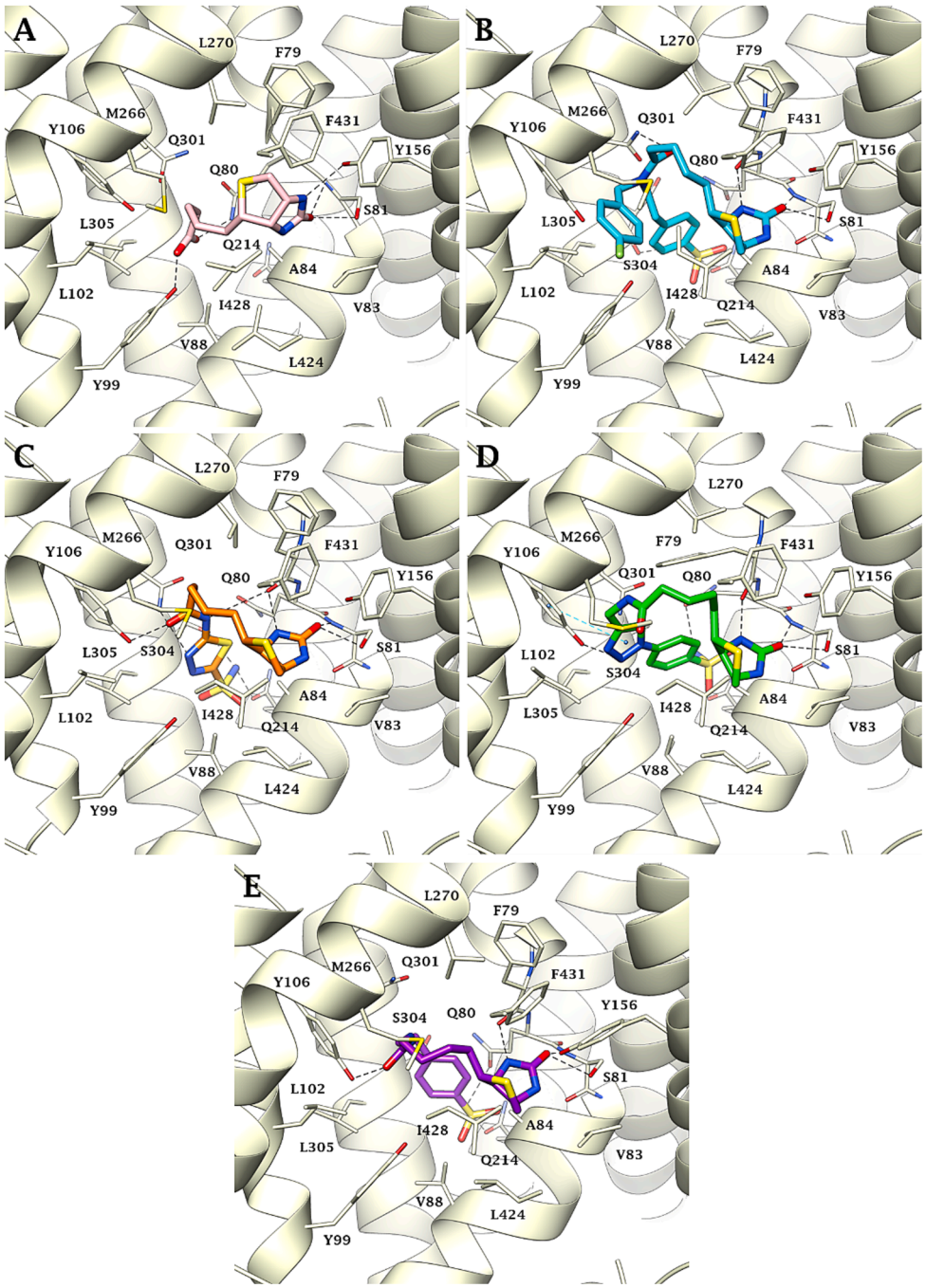


Fig. 7. Predicted binding mode of biotin (pink) and ligands **2i** (cyan), **6** (orange), **17a** (green), **20d** (purple), within the SMTV (yellow: A-E) binding pocket. H-bonds and π-π stacking interactions are depicted as black and cyan dashed lines, respectively.

while the sulfonamide NH_2 engages two H-bonds with the backbone C═O and sidechain C═O of S304 and Q214, respectively (Fig. 7B). In derivative **6**, the nitrogen atom in position 4 of the 1,3,4-thiadiazole ring is in H-bond distance with the sidechain NH_2 of Q301, sulfonamido group interacts via hydrogen bonds with the sidechain OH and C═O of S304 and Q214 (Fig. 7C, compound **2i**) and with the sidechain C═O of Q80 (Fig. 7D,E, compounds **17a** and **20d**), while derivative **20d** is involved in an additional H-bond with the sidechain C═O of Q214 (Fig. 7E).

The calculated ΔG binding values for **2i**, **6**, **17a** and **20d** in complex with SMVT (-78.77, −55.05, −74.61 and −67.85 kcal/mol, respectively), point out the higher affinity for the biotin transporter of the biotinylated derivatives over the biotin-SMVT complex (−23.48 kcal/mol). Such evidence could explain the ability of these compounds to be competitive in the transport with respect to biotin.

3. Conclusions

Herein, a new series of sulfonamides derived from biotin were reported. All the sulfonamide derivatives were investigated as inhibitors of four physiologically relevant CA isoforms, cytosolic off-target isoforms hCA I, II and tumor-associated transmembrane isoforms IX and XII. Many nanomolar inhibitors were detected against all isoforms among the secondary amides. Notably, the introduction of a second substituent for obtaining tertiary amides significantly shifted the selectivity toward hCAIX/XII inhibition over hCA I and hCA II. Remarkably, the presence

of 4-F-C_6H_4 (**2i**) afforded an excellent selectivity towards the tumor-associated hypoxia-induced hCA isoform XII over hCA II and over hCA I. The 2-Naph (**2k**) was the most potent inhibitor against hCA IX (K_I = 6.2 nM), 4-fold stronger than AAZ (K_I = 25 nM) with very good selectivity over hCA I. Some compounds were chosen for antiproliferative activity testing against a panel of 3 human tumor cell lines and compound **2k** displayed the most efficacious anti-proliferative activity on these tumor cells. Finally, the inhibitory profiles of the synthesized derivatives were further investigated by carrying out docking studies to predict the binding properties of compounds **2i**, **6**, **17a** and **20d** within the active site of the off-targets CA I and II, the tumor-associated isoforms CA IX and XII and the SMVT transporter protein.

4. Materials and methods

4.1. Chemistry

4.1.1. General

Solvents and chemicals were obtained from commercial sources and used as received. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 aluminum-backed plates. Elution of the plates was carried out using as indicated in each compound. Visualization was achieved with UV light at 254 nm by dipping into a ninhydrin TLC stain solution and heating with a hot air gun. Flash column chromatography was carried out using silica gel. 1H, ^{13}C, and ^{19}F spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm), and the coupling constants (*J*) are expressed in hertz (Hz). Mass spectra (MS) were recorded on a mass spectrometer with a Q-TOF micro mass analyzer using the ESI technique.

4.1.2. General procedure for the preparation of compounds *2a-d, 4* and *6*

The corresponding sulfonamide (1.16 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (1.5 mL) and then biotin (1.10 mmol, 0.95 equiv.), EDC hydrochloride (1.86 mmol, 1.6 equiv.) and finally 4-(dimethylamino)piridine (catalytic amount) were added. The resulting mixture was stirred at room temperature under N_2 during the time indicated below, until completion (TLC monitoring). After that, the crude reaction was partitioned between EtOAc (20 mL) and water (20 mL). A precipitate appeared in water, it was filtrated and the solid was washed with EtOAc (10 mL), water (10 mL) and petrol ether (10 mL). For compound **2c** the conditions used are indicated below.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[4-(Aminosulfonyl)phenyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2a). Sulfanilamide **1a** was used. The reaction took place for 3 h. White solid; 24% yield; R_f 0.40 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 10.22 (s, 1H, NHCO), 7.73 (s, 4H, Ar-H), 7.24 (s, 2H, SO_2NH_2), 6,44 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin), 4.14 (m, 1H, CHCH, biotin), 3.12 (m, 1H, CHS, biotin), 2.82 (dd, 1H, *J* = 4.8 Hz, *J* = 12.5 Hz, CH_2S, biotin), 2.57 (d, 1H, *J* = 12.5 Hz, CH_2S, biotin), 2.34 (t, 2H, *J* = 7.1 Hz, CH_2CO, biotin), 1.62–1.36 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 171.8 (C═O, amide), 162.8 (C═O urea, biotin), 142.2 (C, Ar-C*ipso*), 138.1 (C-SO_2NH_2), 126.6 (2C, Ar-C), 118.5 (2C, Ar-C), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 36.2, 28.2, 28.1, 24.9 (4C, aliphatic, biotin); *m/z* (ESI +): 399.2 $[M + H]^+$, 397.1 $[M-H]^-$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[3-(Aminosulfonyl)phenyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2b). 3-Aminobenzenesulfonamide **1b** was used. The reaction took place for 3 h. White solid; 37% yield; R_f 0.38 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 10.20 (s, 1H, NHCO), 8.17 (brs, 1H, Ar-H), 7.72 (m, 1H, Ar-H), 7.47 (m, 2H, Ar-H), 7.35 (s, 2H, SO_2NH_2), 6.45 (s, 1H, NHCONH, biotin), 6.38 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, *J* = 5.0 Hz, *J* = 7.6 Hz, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.12 (m, 1H, CHS, biotin), 2.82 (dd, 1H, *J* = 5.0 Hz, *J* = 12.4 Hz, CH_2S, biotin), 2.57 (d, 1H, *J* = 12.4 Hz, CH_2S, biotin), 2.33 (t, 2H, *J* = 7.4 Hz, CH_2CO, biotin), 1.62–1.36 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 171.6 (C═O, amide), 162.8 (C═O urea, biotin), 144.5 (C, Ar-C*ipso*), 139.6 (C-SO_2NH_2), 129.3, 121.9, 120.0, 116.0 (4C, Ar-C), 61.1, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 36.2, 28.2, 28.1, 25.0 (4C, aliphatic, biotin); *m/z* (ESI +): 399.2 $[M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]methyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2c). The 4-(aminomethyl)benzenesulfonamide **1c** (0.89 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (1 mL) and then DIPEA (2.16 mmol, 2.4 equiv.), biotin (0.85 mmol, 0.95 equiv.) and PyBOP (0.98 mmol, 1.1 equiv.) were added. The resulting mixture was stirred at room temperature under N_2 overnight, until completion (TLC monitoring). After that, the crude reaction was partitioned between 1 M aq. HCl (20 mL) and EtOAc (3x20 mL). The water was filtrated and the solid was washed with water (30 mL) to give **2c**. White solid; 41% yield; R_f 0.22 (MeOH 10%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.41 (t, 1H, *J* = 5.23 Hz, NHCO), 7.75 (m, 2H, Ar-H), 7.40 (m, 2H, Ar-H), 7.31 (s, 2H, SO_2NH_2), 6.40 (m, 2H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin; 2H, CH_2NH), 4.12 (dd, 1H, *J* = 4.4 Hz, *J* = 7.6 Hz, CHCH, biotin), 3.09 (m, 1H, CHS, biotin), 2.82 (dd, *J* = 5.1 Hz, *J* = 12.5 Hz, CH_2S, biotin), 2.57 (d, 1H, *J* = 12.5 Hz, CH_2S, biotin), 2.15 (t, 2H, *J* = 7.6 Hz, CH_2CO, biotin), 1.54 (m, 4H, $2CH_2$, biotin), 1.32 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 172.3 (C═O, amide), 162.7 (C═O urea, biotin), 143.9 (C, Ar-C*ipso*), 142.5 (C-SO_2NH_2), 127.4 (2C, Ar-C), 125.6 (2C, Ar-C), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 41.6 (CH_2NH), 39.8 (C-4, tetrahydrothiophene, biotin), 35.1, 28.2, 28.0, 25.2 (4C, aliphatic, biotin); *m/z* (ESI +): 413.2 $[M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2d). 4-(2-Aminoethyl)benzenesulfonamide **1d** was used. The reaction took place for 4 h. White solid; 66% yield; R_f 0.26 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.90 (t, 1H, *J* = 5.35 Hz, NHCO), 7.73 (m, 2H, Ar-H), 7.38 (m, 2H, Ar-H), 7.30 (s, 2H, SO_2NH_2), 6.43 (s, 1H, NHCONH, biotin), 6.37 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin), 4.11 (m, 1H, CHCH, biotin), 3.28 (m, 2H, CH_2CH_2NH), 3.08 (m, 1H, CHS, biotin), 2.83 (dd, 1H, *J* = 5.2 Hz, *J* = 12.3 Hz, CH_2S, biotin), 2.77 (t, 2H, *J* = 7.1 Hz, CH_2CH_2NH), 2.57 (d, 1H, *J* = 12.3 Hz, CH_2S, biotin), 2.07 (t, 2H, *J* = 7.4 Hz, CH_2CO, biotin), 1.52 (m, 4H, $2CH_2$, biotin), 1.26 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 172.1 (C═O, amide), 162.8 (C═O urea, biotin), 143.8 (C, Ar-C*ipso*), 142.0 (C-SO_2NH_2), 129.1 (2C, Ar-C), 125.6 (2C, Ar-C), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 39.6 (CH_2NH), 35.1 (C, aliphatic, biotin), 34.8, (CH_2CH_2NH), 28.2, 28.0, 25.3 (3C, aliphatic, biotin); *m/z* (ESI +): 427.2 $[M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]-1-piperazinyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (4). Piperazine-1-sulfonamide **3** was used. The reaction took place for 3.5 h. White solid; 66% yield; R_f 0.21 (MeOH 10%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.63 (m, 2H, Ar-H), 7.08 (s, 2H, SO_2NH_2), 7.03 (m, 2H, Ar-H), 6.44 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.59 (s, 4H, pyperizine), 3.10 (m, 1H, CHS, biotin), 2.81 (dd, 1H, *J* = 5.3 Hz, *J* = 12.6 Hz, CH_2S, biotin), 2.56 (d, 1H, *J* = 12.6 Hz, CH_2S, biotin), 2.35 (t, 2H, *J* = 7.3 Hz, CH_2CO, biotin), 1.56 (m, 4H, $2CH_2$, biotin), 1.35 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 170.8 (C═O, amide), 162.8 (C═O urea, biotin), 152.6 (C, Ar-C*ipso*), 133.2 (C-SO_2NH_2), 127,1 (2C, Ar-C), 113.9 (2C, Ar-C), 61.0, 59.2, 55.5 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 47.3, 46.9, 44.3, 40.5 (4C, piperazine), 39.8 (C-4, tetrahydrothiophene, biotin), 32.0, 28.3, 28.1, 24.8 (4C, aliphatic, biotin); *m/z* (ESI +): 468.2 $[M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[5-(Aminosulfonyl)-1,3,4-thiadiazol-2-yl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (6). Zolamide **5** was used. The reaction took place for 5 h. White solid; 60% yield; R_f 0.26 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 12.96 (s, 1H, NHCO), 8.32 (s, 2H, SO_2NH_2), 6.45 (s, 1H, NHCONH, biotin),

6.37 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.11 (m, 1H, CHS, biotin), 2.82 (dd, 1H, $J = 5.2$ Hz, $J = 12.3$ Hz, CH_2S, biotin), 2.57 (d, 1H, $J = 12.3$ Hz, CH_2S, biotin), 2.52 (m, 2H, CH_2CO, biotin), 1.69–1.23 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.3 (C═O, amide), 164.3 (C-NH), 162.8 (C═O urea, biotin), 161.1 (C-SO_2NH_2), 61.0, 59.2, 55.3 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 42.4, 34.6, 28.0, 24.4 (4C, aliphatic, biotin); *m/z* (ESI +): 407.1 $[M + H]^+$.

*4.1.3. General procedure for the preparation of compounds **2e-l***

The corresponding sulfonamide (0.77 mmol, 0.95 equiv.) was dissolved in anhydrous DMF (1.5 mL) and then biotin (0.81 mmol, 1.0 equiv.), EDC hydrochloride (1.29 mmol, 1.6 equiv.) and finally 4-(dimethylamino)piridine (catalytic amount) were added. The resulting mixture was stirred at room temperature under N_2 overnight, until completion (TLC monitoring). After that, the crude reaction was partitioned between water (15 mL) and EtOAc (3 × 20 mL); the combined organic fractions were further extracted with brine (2 × 10 mL). The combined organic fractions were dried over Na_2SO_4, filtrated and the filtrate was concentrated to dryness. Then, the residue was purified by column chromatography using the eluant indicated in each case. The compound **2 l** was purified by precipitating it in water.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[4-(Aminosulfonyl)phenyl]-2-oxohexahydro-*N*-(phenylmethyl)-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2e). 4-[(Phenylmethyl)amino]benzenesulfonamide **1e** was used. Column chromatography (MeOH 2%-7%/CH_2Cl_2) gave **2e** as a white solid; 27% yield; R_f 0.24 (MeOH 10%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.53 (m, 2H, Ar-H), 7.34 (s 2H, SO_2NH_2), 7.32 (brs, 3H, Ar-H'), 7.25 (m, 2H, Ar-H') 6.63 (m, 2H, Ar-H), 6.39 (s, 1H, NHCONH, biotin), 6.35 (s, 1H, NHCONH, biotin), 4.33 (m, 2H, CH_2N), 4.28 (m, 1H, CHCH, biotin), 4.07 (m, 1H, CHCH, biotin), 3.01 (m, 1H, CHS, biotin), 2.80 (dd, 1H, $J = 5.1$ Hz, $J = 12.34$ Hz, CH_2S, biotin), 2.55 (d, 1H, $J = 12.3$ Hz, CH_2S, biotin), 2.11 (m, 2H, CH_2CO, biotin), 1.45–1.36 (m, 4H, $2CH_2$, biotin), 1.20 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 171.3 (C═O, amide), 162.7 (C═O urea, biotin), 152.7 (C, Ar-C*ipso*), 139.1, 129.6 (C-SO_2NH_2; C, Ar-C'*ipso*), 128.4, 127.2, 126.9, 124.4, 110.9 (9C, Ar-C, Ar-C'), 61.0, 59.2, 55.3 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 45.8 (CH_2N), 39.8 (C-4, tetrahydrothiophene, biotin), 35.1, 27.9, 27.8, 24.0 (4C, aliphatic, biotin); *m/z* (ESI +): 489.30 $[M + H]^+$, 511.30 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]methyl]-2-oxohexahydro-*N*-(phenylmethyl)-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2f). 4-[2-[(Phenylmethyl)amino]methyl]benzenesulfonamide **1f** was used. Column chromatography (MeOH 2%-8%/CH_2Cl_2) gave **2f** as a white solid. 57% yield; R_f 0.26 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.78 (m, 2H, Ar-H), 7.35 (m, 2H, SO_2NH_2; 5H, Ar-H'), 7.20 (m, 2H, Ar-H), 6.42 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.57 (m, 2H, CH_2N), 4.52 (m, 2H, CH_2N), 4.29 (m, 1H, CHCH, biotin), 4.10 (m, 1H, CHCH, biotin), 3.06 (m, 1H, CHS, biotin), 2.81 (dd, 1H, $J = 5.3$ Hz, $J = 12.61$ Hz, CH_2S, biotin), 2.56 (d, 1H, $J = 12.6$ Hz, CH_2S, biotin), 2.38 (m, 2H, CH_2CO, biotin), 1.64–1.26 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 171.8 (C═O, amide), 162.7 (C═O urea, biotin), 149.6, 143.0, 142.0 (C-SO_2NH_2; C, Ar-C*ipso*; C, Ar-C'*ipso*), 129.1, 127.6, 125.8, 124.7, 111.4 (9C, Ar-C, Ar-C'), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 50.1, 46.5 ($2CH_2N$), 40.1 (C-4, tetrahydrothiophene, biotin), 33.3, 29.6, 28.2, 25.0 (4C, aliphatic, biotin); *m/z* (ESI +): 503.3 $[M + H]^+$, 525.3 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-2-oxohexahydro-*N*-(phenylmethyl)-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2 g). 4-[2-[(Phenylmethyl)amino]ethyl]benzenesulfonamide **1 g** was used. Column chromatography (MeOH 3%-5%/CH_2Cl_2) gave **2 g** as a yellow wax. 19% yield; R_f 0.40 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.31 (m, 2H, Ar-H; 5H, Ar-H'; 2H, SO_2NH_2), 6.42 (s, 1H, NHCONH, biotin), 6.36 (d, 1H, $J = 4.7$ Hz, NHCONH, biotin), 4.54 (m, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.12 (m, 1H, CHCH, biotin), 3.45 (m, 2H, CH_2CH_2N), 3.08 (m, 1H, CHS, biotin), 2.89 (m, 1H, CH_2S, biotin), 2.81 (m, 2H, CH_2CH_2N), 2.57 (dd, 1H, $J = 5.7$ Hz, $J = 12.4$, CH_2S, biotin), 2.28 (m, 2H, CH_2CO, biotin), 1.66–1.21 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.0 (C═O, amide), 162.6 (C═O urea, biotin), 142.8, 142.0 (C, Ar-C*ipso*; C-SO_2NH_2), 137.8 (C, Ar-C'*ipso*), 129.0, 128.4, 127.2, 126.5, 125.7 (9C, Ar-C, Ar-C'), 60.9, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 47.9, 46.9 ($2CH_2$-N), 39.8 (C-4, tetrahydrothiophene, biotin), 32.2, 30.6, 28.1, 28.0, 24.8 (5C, aliphatic); *m/z* (ESI +): 517.20 $[M + H]^+$, 539.20 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-*N*-[[4-(metoxyphenyl)methyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2 h). 4-[2-[[(4-Methoxyphenyl)methyl]amino]ethyl]benzenesulfonamide **1 h** was used. Column chromatography (MeOH 2%-4%/CH_2Cl_2) gave **2 h** as a white solid. 30% yield; R_f 0.45 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.39 (m, 2H, Ar-H), 7.29 (m, 2H, SO_2NH_2), 7.23 (m, 1H, Ar-H'), 6.79 (m, 3H, Ar-H'), 6.40 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.51 (m, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.11 (m, 1H, CHCH, biotin), 3.73 (m, 3H, OCH_3), 3.46 (m, 2H, CH_2CH_2N), 3.08 (m, 1H, CHS, biotin), 2.89 (m, 1H, CH_2S, biotin), 2.82 (m, 2H, CH_2CH_2N), 2.57 (dd, 1H, $J = 4.9$ Hz, $J = 12.4$, CH_2S, biotin), 2.28 (m, 2H, CH_2CO, biotin), 1.66–1.22 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.0 (C═O, amide), 162.6 (C═O urea, biotin), 159.3 (C-OMe), 142.8, 142.0 (C, Ar-C*ipso*; C-SO_2NH_2), 139.5 (C, Ar-C'*ipso*), 129.4, 129.0, 125.7, 118.4, 112.4 (8C, Ar-C, Ar-C'), 60.9, 59.2, 55.3 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 54.9 (OCH_3), 47.9, 46.9 ($2CH_2N$), 39.8 (C-4, tetrahydrothiophene, biotin), 33.0, 31.6, 28.1, 28.0, 24.8 (5C, aliphatic); *m/z* (ESI +): 547.20 $[M + H]^+$, 569.20 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-*N*-[[4-(fluorophenyl)methyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2i). 4-[2-[[(4-Fluorophenyl)methyl]amino]ethyl]benzenesulfonamide **1i** was used. Column chromatography (MeOH 3%-4%/CH_2Cl_2) gave **2i** as a white solid; 43% yield; R_f 0.40 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.39 (m, 2H, Ar-H), 7.20 (m, 2H, SO_2NH_2; 4H, Ar-H'), 6.39 (s, 1H, NHCONH, biotin), 6.35 (s, 1H, NHCONH, biotin), 4.52 (m, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.12 (m, 1H, CHCH, biotin), 3.44 (m, 2H, CH_2CH_2N), 3.08 (m, 1H, CHS, biotin), 2.88 (m, 1H, CH_2S, biotin), 2.81 (m, 2H, CH_2CH_2N), 2.57 (dd, 1H, $J = 4.1$ Hz, $J = 12.3$ Hz, CH_2S, biotin), 2.27 (m, 2H, CH_2CO, biotin), 1.52 (m, 4H, $2CH_2$, biotin), 1.30 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 172.1 (C═O, amide), 162.6 (C═O urea, biotin), 160.0 (C-F), 142.7, 142.0 (C, Ar-C*ipso*; C-SO_2NH_2), 133.9 (C, Ar-C'*ipso*), 129.0, 128.5, 125.7, 115.0 (8C, Ar-C, Ar-C'), 60.9, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 47.9, 46.6 ($2CH_2N$), 39.8 (C-4, tetrahydrothiophene, biotin), 32.9, 31.6, 28.1, 28.0, 24.8 (5C, aliphatic); *m/z* (ESI +): 535.2 $[M + H]^+$, 557.1 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-*N*-[[4-(dimethylamino)phenyl]methyl]-2-oxohexahydro-1-*H*-thieno[3,4-*d*]imidazol-4-yl}pentanamide (2j). 4-[2-[[[4-(Dimethylamino)phenyl]methyl]amino]ethyl]benzenesulfonamide **1j** was used. Column chromatography (MeOH 2%-5%/CH_2Cl_2) gave **2j** as a yellow solid; 36% yield; R_f 0.48 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.37 (m, 2H, Ar-H), 7.30 (m, 2H, SO_2NH_2), 7.04 (m, 2H, Ar-H'), 6.68 (m, 2H, Ar-H'), 6.43 (m, 1H, NHCONH, biotin), 6.37 (s, 1H, NHCONH, biotin), 4.39 (m, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.12 (m, 1H, CHCH, biotin), 3.40 (m, 2H, CH_2CH_2N), 3.09 (m, 1H, CHS, biotin), 2.86 (m, 6H, $2CH_3$; 1H, CH_2S, biotin), 2.80 (m, 2H, CH_2CH_2N), 2.57 (d, 1H, $J = 12.3$ Hz, CH_2S, biotin), 2.33 (m, 1H, CH_2CO, biotin), 2.21 (m, 1H, CH_2CO, biotin), 1.66–1.24 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 171.8 (C═O, amide), 162.7 (C═O urea, biotin), 149.6 (C—$N(Me)_2$), 143.0, 142.0 (C, Ar-C*ipso*; C-SO_2NH_2), 129.1, 127.6, 125.8, 124.7, 112.4 (9C, Ar-C, Ar-C'), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 50.1, 46.5 ($2CH_2N$), 40.2 ($2CH_3$), 40.1 (C-4, tetrahydrothiophene, biotin), 33.0, 31.8, 29.6, 28.2, 25.0 (5C, aliphatic); *m/z* (ESI +): 560.2 $[M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl]ethyl]-*N*-(1-naphthalenylmethyl)-2-oxohexahydro-1-*H*-thieno[3,4-*d*]

imidazol-4-yl}pentanamide (2 k). 4-[2-[(2-Naphthalenylmethyl) amino]ethyl]benzenesulfonamide **1 k** was used. Column chromatography (MeOH 2%-5%/CH_2Cl_2) gave **2k** as a white solid. 51% yield; R_f 0.52 (MeOH 15%/CH_2Cl_2).

Isomer 1. δ_H (400 MHz, DMSO-d_6) 7.89 (m, 3H, naphtyl), 7.72 (m, 2H, Ar-H), 7.72 (s, 1H, naphtyl), 7.49 (m, 2H, naphtyl), 7.39 (m, 2H, Ar-H), 7.37 (m, 1H, naphtyl), 7.29 (s, 2H, SO_2NH_2), 6.40 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.71 (m, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.52 (m, 2H, CH_2CH_2N), 3.10 (m, 1H, CHS, biotin), 2.92 (m, 1H, CH_2S, biotin), 2.86 (m, 2H, CH_2CH_2N), 2.58 (d, 1H, $J = 13.5$ Hz, CH_2S, biotin), 2.32 (m, 2H, CH_2CO, biotin), 1.65–1.23 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.2 (C═O, amide), 162.7 (C═O urea, biotin), 142.9, 142.1 (C, Ar-C*ipso*; C-SO_2NH_2), 135.5 (C-2, napthyl), 132.8, 132.1 (C-4a, C-8a, napthyl), 129.1, 128.1, 127.6, 127.5, 126.2, 125.9, 125.7, 124.6 (9C, Ar-C, Ar-C'-), 60.9, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 47.9, 47.1 ($2CH_2N$), 39.8 (C-4, tetrahydrothiophene, biotin), 33.1, 31.7, 30.7, 28.2, 24.9 (5C, aliphatic); *m/z* (ESI +): 567.2 $[M + H]^+$, 589.2 $[M + Na]^+$, 1132.7 $[2\ M + H]^+$.

Isomer 2. δ_H (400 MHz, DMSO-d_6) 7.89 (m, 3H, naphtyl), 7.72 (m, 2H, Ar-H), 7.67 (s, 1H, naphtyl), 7.49 (m, 2H, naphtyl), 7.39 (m, 2H, Ar-H), 7.37 (m, 1H, naphtyl), 7.27 (s, 2H, SO_2NH_2), 6.37 (s, 1H, NHCONH, biotin), 6.32 (s, 1H, NHCONH, biotin), 4.71 (m, 2H, CH_2N), 4.25 (m, 1H, CHCH, biotin), 4.03 (m, 1H, CHCH, biotin), 3.52 (m, 2H, CH_2CH_2N), 3.01 (m, 1H, CHS, biotin), 2.92 (m, 1H, CH_2S, biotin), 2.78 (m, 2H, CH_2CH_2N), 2.58 (d, 1H, $J = 13.5$ Hz, CH_2S, biotin), 2.32 (m, 2H, CH_2CO, biotin), 1.65–1.23 (m, 6H, $3CH_2$, biotin); *m/z* (ESI +): 567.2 $[M + H]^+$, 589.2 $[M + Na]^+$, 1132.7 $[2\ M + H]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl] ethyl] -2-oxohexahydro-*N*-(2-thienylmethyl)-1-*H*-thieno [3,4-*d*] imidazol-4-yl} pentanamide (2 l). 4-[2-[(2-Thienylmethyl)amino]ethyl]benzenesulfonamide **1 l** was used. White solid; 67% yield; R_f 0.41 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.39 (m, 1H, CHS; 2H, SO_2NH_2), 7.30 (m, 2H, Ar-H), 7.04 (m, 1H, C*H*CHCH-S), 6.94 (m, 1H, C*H*CH-S), 6.42 (m, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.68 (m, 2H, CH_2N), 4.29 (m, 1H, CHCH, biotin), 4.12 (m, 1H, CHCH, biotin), 3.47 (m, 2H, CH_2CH_2N), 3.08 (m, 1H, CHS, biotin), 2.88 (m, 1H, CH_2S, biotin), 2.81 (m, 1H, CH_2CH_2N), 2.56 (d, 1H, $J = 12.4$ Hz, CH_2S, biotin), 2.38 (m, 1H, CH_2CO, biotin), 2.18 (m, 1H, CH_2CO, biotin), 1.51 (m, 4H, $2CH_2$, biotin), 1.29 (m, 2H, CH_2, biotin); δ_C (100 MHz, DMSO-d_6) 171.8 (C═O, amide), 162.7 (C═O urea, biotin), 142.8, 142.0, 140.9 (C, Ar-C*ipso*; C-SO_2NH_2; C—S), 129.0, 126.7, 125.9, 125.7, 125.5 (4C, Ar-C; 3C, thiophene), 61.0, 59.2, 55.4 (C-3a, C-6a, C-6, tetrahydrothiophene, biotin), 46.6, 42.7 ($2CH_2N$), 39.8 (C-4, tetrahydrothiophene, biotin), 33.0, 31.6, 28.1, 28.0, 24.8 (5C, aliphatic); *m/z* (ESI +): 523.2 $[M + H]^+$, 545.3 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[4-(Aminosulfonyl)-2-chlorophenyl] -2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (9). To biotin (0.82 mmol, 1.0 equiv.) was added $SOCl_2$ (38.5 mmol, 47 equiv.) and was stirred at room temperature under N_2 for 30 min. Solubilization of the solid was spontaneous. In seconds, the acid chloride precipitated as a white solid. Then, the volatiles were eliminated under N_2 to dryness to give **7**, which was directly used for the next step without any further purification.

To a solution of biotinyl chloride **7** (0.76 mmol, 1.0 equiv.) in anhydrous DMF (1.5 mL) were added 4-amino-3-chlorobenzenesulfonamide **8** (0.84 mmol, 1.1 equiv.) and DIPEA (0.84 mmol, 1.1 equiv.). The resulting mixture was stirred at room temperature under N_2 overnight, until completion (TLC monitoring). After that, the crude reaction was partitioned between EtOAc (20 mL) and water (20 mL). The water was filtrated and the solid was washed with EtOAc (10 mL), water (10 mL) afforded **9** as a brown solid. 72% yield; R_f 0.34 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.96 (d, 1H, $J = 8.5$ Hz, Ar-H), 7.86 (d, 1H, $J = 2.1$ Hz, Ar-H), 7.72 (dd, 1H, $J = 2.1$ Hz, $J = 8.5$ Hz, Ar-H), 7.45 (s, 2H, SO_2NH_2), 6.44 (s, 1H, NHCONH, biotin), 6.36 (s, 1H, NHCONH, biotin), 4.30 (m, 1H, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.11 (m, 1H, CHS, biotin), 2.81 (m, 1H, CH_2S, biotin), 2.57 (d, 1H, $J = 12.7$ Hz, CH_2S, biotin), 2.43 (t, 2H, $J = 6.9$ Hz, CH_2CO, biotin), 1.51 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 171.9 (C═O, amide), 162.7 (C═O urea, biotin), 140.8 (C-NH), 138.1 (C-SO_2NH_2), 126.8, 125.6, 125.5, 124.8 (4C, Ar-C), 61.0, 59.2, 55.4 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 35.6, 28.1, 28.0, 25.0 (4C, aliphatic, biotin); *m/z* (ESI +): 433.2 $[M + H]^+$, 455.2 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[5-(Aminosulfonyl)-3-methyl-1,3,4-thiadiazol-2(3*H*)-ylidene] -2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (12). To a solution of biotin (0.41 mmol, 1.0 equiv.) in anhydrous DMF (1 mL) was added Et_3N (0.92 mmol, 2.25 equiv.) and at 0 °C was slowly added ethyl chloroformate (0.43 mmol, 1.05 equiv.). The resulting mixture was stirred at room temperature under N_2 for 1 h. Then, a solution of 5-imino-4-methyl-1,3,4-thiadiazoline-2-sulfonamide **11** (0.39 mmol, 0.95 equiv.) in anhydrous DMF (1 mL) was slowly added to the anhydride at 0 °C. The resulting mixture was stirred at room temperature under N_2 overnight, until completion (TLC monitoring). After that, the crude reaction was partitioned between water (15 mL) and EtOAc (3 × 20 mL); the combined organic fractions were further extracted with brine (2 × 10 mL). The combined organic fractions were dried over Na_2SO_4, filtrated and the filtrate was concentrated to dryness. Then, the residue was purified by column chromatography using the eluant MeOH 1%-5%/CH_2Cl_2 to give **12** as a white solid. 22% yield; R_f 0.36 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.33 (s, 2H, SO_2NH_2), 6.41 (s, 1H, NHCONH, biotin), 6.33 (s, 1H, NHCONH, biotin), 4.29 (m, 1H, CHCH, biotin), 4.13 (m, 1H, CHCH, biotin), 3.92 (s, 3H, CH_3), 3.10 (m, 1H, CHS, biotin), 2.81 (m, 1H, CH_2S, biotin), 2.57 (m, 1H, CH_2S, biotin), 1.51 (m, 8H, $4CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 182.4 (C═O, amide), 164.1 (C═O urea, biotin), 162.7 (C═N), 157.6 (C-SO_2NH_2), 61.0, 59.2, 55.4 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 38.8 (CH_3), 38.2, 28.2, 28.0, 25.0 (4C, biotin-); *m/z* (ESI +): 421.2 $[M + H]^+$, 443.2 $[M + Na]^+$.

*4.1.4. Synthesis the target compounds **17a-d**; general procedure*

The corresponding azide **13a-d** (5.05 mmol, 1.0 equiv.) was dissolved in MeOH/THF (1 mL/3 mL) and the alkyne intermediate **14** (7.56 mmol, 1.5 equiv.), sodium ascorbate (5.05 mmol, 1.0 equiv.) and $Cu(CH_3COO)_2$ (0.51 mmol, 0.1 equiv.) were added and stirred at 36 °C overnight. The progress of the click reaction was monitored by TLC. On completion, the reaction mixture was concentrated under vacuum; the residue was dissolved in EtOAc (3 × 20 mL) and washed with water (20 mL), and then dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum.

After that, the treatment with HCl 3 M in MeOH (30 equiv.) afforded the compounds intermediate **16a-d**, which (0.63 mmol, 1.0 equiv.) were dissolved in anhydrous DMF (1.5 mL), then at 0 °C DIPEA (1.5 mmol, 2.4 equiv.), biotin (0.56 mmol, 0.9 equiv.), EDC hydrochloride (1.25 mmol, 2 equiv.) and DMAP (catalytic amount) were added. The resulting mixture was stirred at room temperature under N_2 for 48 h. On completion, the crude reaction was concentrated under vacuum, ice/water was added, and the solid precipitated was collected by filtration and washed with water (10 mL) to give the compounds **17a-d**.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-((Aminosulfonyl)phenyl)-1*H*-1,2,3-triazol-4-yl] methyl] -2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (17a). 4-[4-(Aminomethyl)-1*H*-1,2,3-triazol-1-yl] benzenesulfonamide hydrochloride **16a** was used. Yellow solid; 59% yield; R_f 0.27 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.69 (s, 1H, triazol), 8.39 (t, 1H, $J = 5.5$ Hz, NHCO), 8.11 (m, 2H, Ar-H), 7.99 (m, 2H, Ar-H), 7.51 (s, 2H, SO_2NH_2), 6.40 (s, 1H, NHCONH, biotin), 6.34 (s, 1H, NHCONH, biotin), 4.38 (d, 2H, $J = 5.5$ Hz, CH_2NH), 4.28 (m, 1H, CHCH, biotin), 4.10 (m, 1H, CHCH, biotin), 3.07 (m, 1H, CHS, biotin), 2.78 (dd, 1H, $J = 5.3$ Hz, $J = 12.57$ Hz, CH_2S, biotin), 2.54 (d, 1H, $J = 12.6$ Hz, CH_2S, biotin), 2.12 (t, 2H, $J = 7.3$ Hz, CH_2CO, biotin), 1.44 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.1 (C═O, amide), 162.7 (C═O urea, biotin), 146.6 (C-SO_2NH_2), 143.7 (C, Ar-

C*ipso*), 138.6 (C, triazol), 127.5 (2C, Ar-C), 121.2 (CH, triazol), 120.1 (2C, Ar-C), 60.9, 59.1, 55.3 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 34.9 (CH_2NH), 33.9, 28.1, 27.9, 25.0 (4C, aliphatic, biotin); *m/z* (ESI +): 480.4 $[M + H]^+$, 502.4 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[3-((Aminosulfonyl)phenyl)-1*H*-1,2,3-triazol-4-yl] methyl] -2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (17b). 3-[4-(Aminomethyl)-1*H*-1,2,3-triazol-1-yl] benzenesulfonamide hydrochloride **16b** was used. White solid; 21% yield; R_f 0.11 (MeOH 10%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.68 (s, 1H, triazol), 8.37 (m, 2H, Ar-H, NHCO), 8.11 (m, 1H, Ar-H), 7.90 (m, 1H, Ar-H), 7.79 (m, 1H, Ar-H), 7.56 (s, 2H, SO_2NH_2), 6.39 (s, 1H, NHCONH, biotin), 6.33 (s, 1H, NHCONH, biotin), 4.39 (d, 2H, $J = 5.2$ Hz, CH_2NH), 4.28 (m, 1H, CHCH, biotin), 4.11 (m, 1H, CHCH, biotin), 3.08 (m, 1H, CHS, biotin), 2.78 (m, 1H, CH_2S, biotin), 2.55 (d, 1H, $J =$ 12.3 Hz, CH_2S, biotin), 2.13 (t, 2H, $J = 7.1$ Hz, CH_2CO, biotin), 1.44 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.2 (C═O, amide), 162.8 (C═O urea, biotin), 146.7 (C-SO_2NH_2), 145.7 (C, triazol), 136.8 (C, Ar-C), 130.9 (C, Ar-C), 125.4 (C, Ar-C), 122.9 (C, Ar-C), 121.2 (CH, triazol), 117.0 (C, Ar-C), 61.0, 59.2, 55.4 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 39.8 (C-4, tetrahydrothiophene, biotin), 35.0 (CH_2NH), 34.0, 28.2, 28.0, 25.1 (4C, aliphatic, biotin); *m/z* (ESI +): 480.3 $[M + H]^+$, 502.3 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(((Aminosulfonyl)phenyl)methyl)-1*H*-1,2,3-triazol-4-yl] methyl] -2-oxohexadydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (17c). 4-[[4-(Aminomethyl)-1*H*-1,2,3-triazol-1-yl]methyl]benzenesulfonamide hydrochloride **16c** was used. Yellow solid; 89% yield; R_f 0.15 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.26 (t, 1H, $J = 5.1$ Hz, NHCO), 7.97 (s, 1H, triazol), 7.80 (m, 2H, Ar-H), 7.46 (m, 2H, Ar-H), 7.35 (s, 2H, SO_2NH_2), 6.39 (s, 1H, NHCONH, biotin), 6.34 (s, 1H, NHCONH, biotin), 5.65 (s, 2H, CH_2N), 4.30 (m, 1H, CHCH, biotin), 4.27 (d, 2H, $J = 5.6$ Hz, CH_2NH), 4.11 (m, 1H, CHCH, biotin), 3.08 (m, 1H, CHS, biotin), 2.81 (dd, 1H, $J = 5.2$ Hz, $J = 12.4$ Hz, CH_2S, biotin), 2.57 (d, 1H, $J = 12.4$ Hz, CH_2S, biotin), 2.08 (t, 2H, $J = 7.2$ Hz, CH_2CO, biotin), 1.44 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.0 (C═O, amide), 162.8 (C═O urea, biotin), 143.8 (C-SO_2NH_2), 139.9 (C, Ar-C*ipso*), 128.5 (C, triazol), 128.4 (2C, Ar-C), 126.1 (2C, Ar-C), 123.1 (CH, triazol), 61.0, 59.2, 55.3 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 52.1 (CH_2N), 39.8 (C-4, tetrahydrothiophene, biotin), 34.9 (CH_2NH), 34.1, 28.1, 27.9, 25.1 (4C, aliphatic, biotin); *m/z* (ESI +): 494.4 $[M + H]^+$, 516.3 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(((Aminosulfonyl)phenyl)ethyl)-1*H*-1,2,3-triazol-4-yl] methyl] -2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl}pentanamide (17d). 4-[[4-(Aminomethyl)-1*H*-1,2,3-triazol-1-yl]ethyl]benzenesulfonamide hydrochloride **16d** was used. White solid; 24% yield; R_f 0.42 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 8.26 (t, 1H, $J = 5.5$ Hz, NHCO), 7.88 (s, 1H, triazol), 7.72 (m, 2H, Ar-H), 7.38 (m, 2H, Ar-H), 7.29 (s, 2H, SO_2NH_2), 6.39 (s, 1H, NHCONH, biotin), 6.34 (s, 1H, NHCONH, biotin), 4.60 (t, 2H, $J = 7.2$ Hz, CH_2N), 4.29 (m, 1H, CHCH, biotin), 4.24 (d, 2H, $J = 5.5$ Hz, CH_2NH), 4.12 (m, 1H, CHCH, biotin), 3.22 (t, 2H, $J = 7.2$ Hz, CH_2CH_2N), 3.09 (m, 1H, CHS, biotin), 2.81 (dd, 1H, $J = 5.1$ Hz, $J = 12.4$ Hz, CH_2S, biotin), 2.57 (d, 1H, $J = 12.4$ Hz, CH_2S, biotin) 1.42 (m, 8H, $4CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 172.1 (C═O, amide), 162.9 (C═O urea, biotin), 145.8 (C-SO_2NH_2), 145.2 (C, Ar-C*ipso*), 142.3 (C, triazol), 129.3 (2C, Ar-C), 125.8 (2C, Ar-C), 122.8 (CH, triazol), 61.0, 59.2, 55.4 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 49.9 (CH_2N), 39.8 (C-4, tetrahydrothiophene, biotin), 35.4, 35.0, 34.1, 28.2, 28.0, 25.2 (CH_2NH; CH_2CH_2N; 4C, aliphatic, biotin); *m/z* (ESI +): 508.4 $[M + H]^+$, 530.4 $[M + Na]^+$.

4.1.5. General procedure for the preparation of compounds **20c** and **20d**

To a suspension of biotin (1.23 mmol, 1.0 equiv.) in anhydrous THF (8 mL) at 0 °C, Et_3N (2.21 mmol, 1.8 equiv.) and diphenylphosphoryl azide (DPPA, 1.85 mmol, 1.5 equiv.) were added. The resulting mixture was stirred at room temperature under N_2 overnight. After that, the crude reaction was concentrated under vacuum and ice/water was added, the excess of DPPA was eliminated by filtrating. Then, the filtrate was extracted with EtOAc (3 × 20 mL). The combined organic fractions were dried over Na_2SO_4, filtrated and the filtrate was concentrated to dryness to afford the acyl azide **18**. Compound **18** (0.54 mmol, 1.0 equiv.) in toluene (6 mL) was heated to reflux overnight. The solvent was evaporated to afford the isocyanate **19**, which was directly used for the next step without any further purification.

To a solution of isocyanate **19** (0.59 mmol, 1.0 equiv.) in anhydrous acetonitrile (6 mL) was added the corresponding sulfonamide (1.1 equiv.). The resulting mixture was stirred at room temperature under N_2 for the time indicated in each case. On completion, the crude reaction was concentrated under vacuum; then acidic water was added and filtered. The residue was purified by column chromatography using the eluant indicated in each case.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl] methyl] -*N*'-[2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazole-4-yl] }urea (20c). 4-(2-Aminomethyl)benzenesulfonamide hydrochloride **1c** was used, Et_3N (1.2 equiv.) was added and the reaction took place overnight. Column chromatography (MeOH 3%-8%/CH_2Cl_2) gave **20c** as a pink solid. 2% yield; R_f 0.27 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.74 (m, 2H, Ar-H), 7.39 (m, 2H, Ar-H), 7.30 (s, 2H, SO_2NH_2), 6.45 (s, 1H, NHCONH, biotin), 6.40 (m, 1H, NHCO), 6.37 (s, 1H, NHCONH, biotin), 6.01 (m, 1H, NHCO), 4.30 (m, 1H, CHCH, biotin), 4.25 (m, 2H, CH_2NH), 4.11 (m, 1H, CHCH, biotin), 3.09 (m, 1H, CHS, biotin), 3.00 (m, 2H, CH_2NH), 2.82 (dd, 1H, $J = 4.6$ Hz, $J = 12.5$ Hz, CH_2S, biotin), 2.57 (d, 1H, $J = 12.5$ Hz, CH_2S, biotin), 1.66–1.27 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 162.7 (C═O, urea, biotin), 158.0 (C═O urea), 145.4, 142.4 (C, Ar-C*ipso*; C-SO_2NH_2), 127.2, 125.6 (4C, Ar-C), 61.0, 59.2, 55.5 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 42.5 (*C*H_2NH), 39.8 (C-4, tetrahydrothiophene, biotin), 30.0, 28.0, 25.9 (4C, aliphatic, biotin); *m/z* (ESI +): 428.3 $[M + H]^+$, 450.3 $[M + Na]^+$.

(3a*S*, 4*S*, 6a*R*)-5-{*N*-[[4-(Aminosulfonyl)phenyl] ethyl] -*N*'-[2-oxohexahydro-1-*H*-thieno [3,4-*d*] imidazol-4-yl] }urea (20d). 4-(2-Aminoethyl)benzenesulfonamide **1d** was used. The reaction took place 72 h. Column chromatography (MeOH 2%-15%/CH_2Cl_2) gave **20d** as a white solid. 13% yield; R_f 0.19 (MeOH 15%/CH_2Cl_2); δ_H (400 MHz, DMSO-d_6) 7.73 (m, 2H, Ar-H), 7.37 (m, 2H, Ar-H), 7.27 (s, 2H, SO_2NH_2), 6.41 (s, 1H, NHCONH, biotin), 6.33 (s, 1H, NHCONH, biotin), 5.84 (t, 1H, $J = 5.5$ Hz, NHCO), 5.79 (t, 1H, $J = 5.5$ Hz, NHCO), 4.30 (m, 1H, CHCH, biotin), 4.12 (m, 1H, CHCH, biotin), 3.16 (m, 2H, CH_2NH), 3.09 (m, 1H, CHS, biotin), 2.96 (m, 2H, CH_2NH), 2.82 (dd, 1H, $J = 5.3$ Hz, $J = 12.6$ Hz, CH_2S, biotin), 2.74 (t, 2H, $J = 7.2$ Hz, CH_2CH_2NH), 2.57 (d, 1H, $J = 12.6$ Hz, CH_2S, biotin), 1.66–1.28 (m, 6H, $3CH_2$, biotin); δ_C (100 MHz, DMSO-d_6) 162.7 (C═O, urea, biotin), 158.0 (C═O, urea), 144.0 (C, Ar-C*ipso*), 141.9 (C-SO_2NH_2), 129.1, 125.6 (4C, Ar-C), 60.9, 59.2, 55.5 (C-3a, C-6, C-6a, tetrahydrothiophene, biotin), 40.5 (*C*H_2NH), 39.8 (C-4, tetrahydrothiophene, biotin), 35.9 (*C*H_2CH_2NH), 30.1, 28.0, 25.9 (4C, aliphatic, biotin); *m/z* (ESI +): 442.3 $[M + H]^+$, 464.3 $[M + Na]^+$.

4.2. CA inhibitory assay

An applied photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO_2 hydration activity.[15] Phenol red (at 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) 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 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 were used for determining the initial rate. 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 distilled-deionized water, and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room

temperature before assay in order to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, and the Cheng –Prusoff equation, as reported earlier[50,51] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier.[52,53].

4.3. Antiproliferative assay

Human glioblastoma U87MG cells and human triple-negative breast cancer cells MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS, while human pancreatic cancer cells PANC-1 were grown in RPMI plus 10% FBS. Cells were kept at the low passage, and checked for *Mycoplasma* negativity. Hypoxic culture conditions were realized in the presence of 1% O_2 and 5% CO_2. For cell proliferation assay the different cell lines were seeded 10.000 cells/well in 48-well plates and treated in 1% FBS with increasing concentrations of compounds **2i**, **2j**, **2k**, AAZ or SLC-0111. After 48 h of incubation at 37 °C with 1% O_2 and 5% CO_2, cell proliferation was assessed using Cell Counting Kit – 8 (CCK-8, Merck) protocol. Data are the mean ± SEM and represent the percentage of cell proliferation with respect to the control (vehicle-treated) group.

4.4. Molecular modeling studies

The crystal structures of CA I, II, IX, XII and of the model of SMVT[42] were downloaded from structure databases Protein Data Bank[54] (CA I: PDB code 2NMX;[38] CA II: PDB code 3 K34;[39] CA IX: PDB code 5FL4;[40] CA XII: PDB code 1JD0541), and Alphafold database.[49] The targets were prepared according to the Protein Preparation module in Maestro Schrödinger suite placing any missing side chains, assigning bond orders, adding hydrogens, deleting water molecules, and optimizing H-bonding networks. Finally, energy minimization with a Root Means Square Deviation (RMSD) value of 0.30 was applied using an Optimized Potential for Liquid Simulation (OPLS4) force field.[55–59] The 3D ligand structures were prepared by Maestro and evaluated for their ionization states at pH 7.3 ± 1.0 with Epik.[55a,b] The conjugate gradient method in Macromodel[55c] was used for energy minimization (maximum iteration number: 2500; convergence criterion: 0.05 Kcal/mol/Å^2). Grids for docking within the CA active site were centered in the centroid of the complexed ligand. Docking studies were carried out with the program Glide [55e] using the standard precision (SP) mode. Ligand binding site prediction in the SMVT Alphafold model was carried out using SiteMap module in Maestro Schrödinger suite.[55a] Then the Induced Fit Docking protocol in Schrödinger was used consisting of Glide SP docking[55e] followed by Prime[55f] refinement of the residue side chains within 5 Å by the ligand followed by a final Glide XP docking of the ligand into the receptor in the refined conformation. The docking grid was centered on the center of mass of the bound ligands. In the initial Glide SP docking, the vdW scaling was set to 0.7 for non-polar atoms of the receptor and 0.5 for those of the ligand. All the obtained complexes within an energy range of 30 kcal/mol from the best one were re-docked into the model. The best poses for each compound in SMVT were re-docked and scored for its binding free energies (ΔG bind) with the Prime MM-GBSA protocol using a VSGB solvation model and 3 Å protein flexibility around the ligands.[55f, 60] Figures were generated with Maestro and Chimera.[55,61].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

PB thanks the European Union (NextGenerationEU) and the Ministerio de Universidades of Spain for the award of a Grant Margarita Salas grant. R.R was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC IG 2019 – ID. 23151). Ó.L. thanks Grant PID2020-116460RB-I00 funded by MCIN/AEI/10.13039/501100011033.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2023.117467.

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