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Azidothymidine (AZT) "clicked" into 1,2,3-Triazoles: First Report on Carbonic Anhydrase-Telomerase Dual Hybrid Inhibitors

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Abstract. Cancer cells rely on the enzyme Telomerase (EC 2.7.7.49) to promote cellular immortality. Telomerase inhibitors (i.e. azidothymidine-**AZT**) can represent promising antitumor agents, although showing high toxicity when administered alone. Better outcomes were observed within a multi pharmacological approach instead. In this context we exploited the validated antitumor targets Carbonic Anhydrases (CAs; EC 4.2.1.1) IX and XII to attain the first *proof of concept* on CA-Telomerase dual hybrid inhibitors. Compounds **1b**, **7b**, **8b** and **11b** showed good *in vitro* inhibition potency against the CAs IX and XII, with K_I values in the low nanomolar range, and strong antitelomerase activity in PC-3 and HT-29 cells (IC_{50} values ranging from 5.2 to 9.1 μM). High resolution X-ray crystallography on selected derivatives in adduct with hCA II as model study allowed to determine their binding modes and thus to set the structural determinants necessary for further development of compounds selectively targeting the tumoral cells.

Keywords: Carbonic Anhydrase Inhibitors; AZT; Telomerase Inhibitors; Cancer; Molecular hybrids; Click Chemistry; Triazole.

INTRODUCTION

Eukaryotic cells do possess limited replicative potential as progressive shortage of the chromosome ends (i.e. the telomeres) takes place after every duplication cycle.[1] Once the critical physical limits are reached, cellular senescence programs, i.e. apoptosis, are triggered.[2] Such an effect is properly referred as the "Hayflick limit", who firstly reported experimentally the finite capacity of normal cells to replicate.[1,3]

State of-the-art knowledge on telomeres accounts for rather complicated and highly dynamic structures which are evolutionarily conserved among the eukaryotic cells.[4] Human telomeres are composed by repetitive, non-coding hexameric nucleotide repeats in complex with the telomere associated proteins (i.e. the shelterin proteins) and the telomerase.[5-8] The former are mainly responsible in maintaining the telomere structure and its signalling functions, whereas the latter in synthetizing new telomeric DNA strands from its own RNA template.[4,5] This enzyme is normally highly active in adult germline and stem cells, whereas it is poorly or not expressed at all in the somatic ones.[9,10] Besides the canonical function of telomere elongation, the telomerase enzymes (EC 2.7.7.49) were also found to act as transcriptional regulators of the Wnt/β-catenin signalling pathway, thus suggesting a role in determining cell growth, differentiation and apoptosis *via* a non-telomeric dependent manner too.[11-13]

The majority of malignant tumors in humans were demonstrated to depend from the telomerase activity, which resulted increased when compared to the non-tumorigenic counterpart cells.[14] As a matter of fact the catalytic subunit of the telomerase enzyme (i.e. hTERT) was found over-expressed in several tumors [15-18] and its regulatory role in metastatic events was also proved.[19] In light of such data the telomerase is properly considered a tumor marker,[20] and still it is taken into consideration as rational target for developing potent and effective anticancer drugs.[15,20-22]

By making use of the DNA polymerase activity of the telomerase, nucleoside and nucleotide analogues have been extensively investigated as potential inhibitors.[23] In particular chain-terminator reverse transcriptase inhibitors have been explored as antitumor agents.[23] The first study of this type

was conducted by Blackburn in 1994 on the ciliated protozoan *Tetrahymena thermophila* which is quite rich in telomeres.[24] Such studies revealed that azidothymidine (**AZT**) was able to decrease the *de novo* telomere addition, thus resulting in telomeres shortening.[24] Further studies showed that in spite of the low affinity of **AZT** for mammalian DNA polymerases, its triphosphate derivative (**AZT-TP**) was incorporated into telomeric region of eukaryotic genome through a process mediated by the telomerases.[25,26] The efficiency of **AZT** in affecting tumor growth was properly assessed [27-29] and its association with cisplatin, paclitaxel or 5-fluorouracil showed synergistic interactions. [30,31] Although such promising results were obtained, **AZT** was dropped as antitumor drug for its potential tumorigenic properties and to the tardiness of the drug to be fully functional, which may expose patients to dangerous side effects.[32] Various drawbacks are associated with the use of telomerase inhibitors for cancer therapy.[33] The tardiness to take action is the most critical issue, as cellular senescence is induced only when telomeres have reached their critical length, and thus implying that such agents do require appropriate time to become effective.[32,33] Induction of cellular senescence by telomeric dysfunction may also result in activation of oncogenes and/or silencing of tumor suppressor genes, thus promoting malignant transformations to occur instead.[34] In addition, the use of inhibitors of the telomerases may interfere with highly proliferative cells such germlines and stem cells.[10,22] For all these reasons the use of telomerase inhibitors (i.e. **AZT,** Imetelstat, BIBR1532 and antisense molecules) for the management of cancer is better envisaged within a polypharmacologically based approach and the metalloenzyme Carbonic Anhydrase (CA; EC 4.2.1.1) IX is well suited.[35-37] CA IX (and marginally CA XII) is selectively over-expressed in hypoxic solid tumors, it actively participates in a complex pH regulation machinery tuned to warrant cancer cells survival within a metabolic driven pH dysregulated environment.[37-40] The paramount importance of CA IX in regulating proton dynamics by means of the **equation 1** was conclusively demonstrated and that allowed to validate such an enzyme as druggable target for the management of hypoxic tumors.[38,39]

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$ **Equation 1**

A recent contribution on the active involvement of CA IX in tumor physiology demonstrated such an enzyme to provide the H^+ ions needed by the matrix metalloproteinase 14 (MMP14) to perform proteolytic cleavage of collagen which in turn determines tumor invasiveness.[41] In this context, during the last years great interests have been turned to the CA IX "interactome".[42-45] A significant study conducted on HEK-293 cells showed that the ARM and/or HEAT-repeat domains are a feature of CA IX interacting partners.[45] The majority of such proteins belong to the nuclear-cytoplasmatic trafficking machinery, such as XPO1 exportin and TNPO 1 importin, and were found to interact with the CA IX C terminal region.[45] These results strongly suggested that CA IX may take a role as cell-surface signal transducer by undergoing nuclear translocation. This is in agreement with confocal immunofluorescence spectroscopy experiments which showed nuclear distribution of CA IX in several cell lines, with a marked localization when experimental hypoxic conditions were established.[45]

In consideration of the robust antitumor effects observed when the Telomerase and the CA IX were targeted, the research herein reported is aimed to obtain CA-Telomerase dual small molecule inhibitors (CAI-TI) able to: *i*) efficiently bind to the CA IX (XII) enzymes which are assumed as discriminant feature between tumor and normal cells; *ii*) exert their antitumoral activity by inhibition of both the CA IX (or XII) and Telomerase. As consequence appropriate CAI-TI molecules will have the potential to achieve therapeutic performances far superior to the ones reached when co-administration of single therapeutic agents are considered. To the best of our knowledge this is the first report on CA-TI dual hybrid compounds designed to target two crucial players in cancer progression.

RESULT AND DISCUSSION

Design and Synthesis of Compounds.

The hybridization strategy was performed by exploiting the versatile "click chemistry" approach, which allows to merge efficiently single chemical entities and thus granting easy access to wide molecular diversities.[46,47] In this study we performed a Copper-catalyzed Azide–Alkyne Cycloaddition (CuAAC) between the azide of the reverse transcriptase inhibitor **AZT** with the terminal alkyne pendant installed on various CAIs scaffolds (**Figure 1**). Our interest in establishing such a chemical connection was mainly based on: *i*) the rapid and regioselective formation of the 1,4-disubstituted-1,2,3-triazole ring under mild reaction conditions;[47,48] *ii*) the 1,2,3-triazole is among the most commonly used scaffolds in Medicinal Chemistry in the last decade, since it is a bioisostere of the amide group and it shows good tolerance to metabolic processes as well as to pH fluctuations.[49,50] In addition, the abundancy of electrons within the triazole ring allows it to establish H-bonds and π-π stacking interactions with biological targets and thus ensuring additional stabilization of the adducts formed.[49,50]

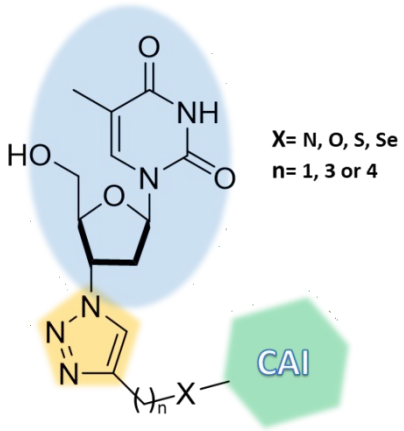


Figure 1. Schematic representation of the synthetized hybrids consisting of a CAI portion linked to AZT through the 1,2,3-triazole ring.

The synthesis and characterization data of the appropriate alkyne precursors **1a-3a, 5a-10a**, **14a-20a** and **4c**, reported in **Table 1**, are descripted within the Experimental Section. Both classical

(i.e. sulfonamides) and non-classical (i.e. coumarins and sulfocoumarins) CAIs have been included in our study. In particular, sulfonamide-based compounds **6a**, **9a**, **10a** and coumarin-based compounds **14a**, **18a** and **19a** are new.

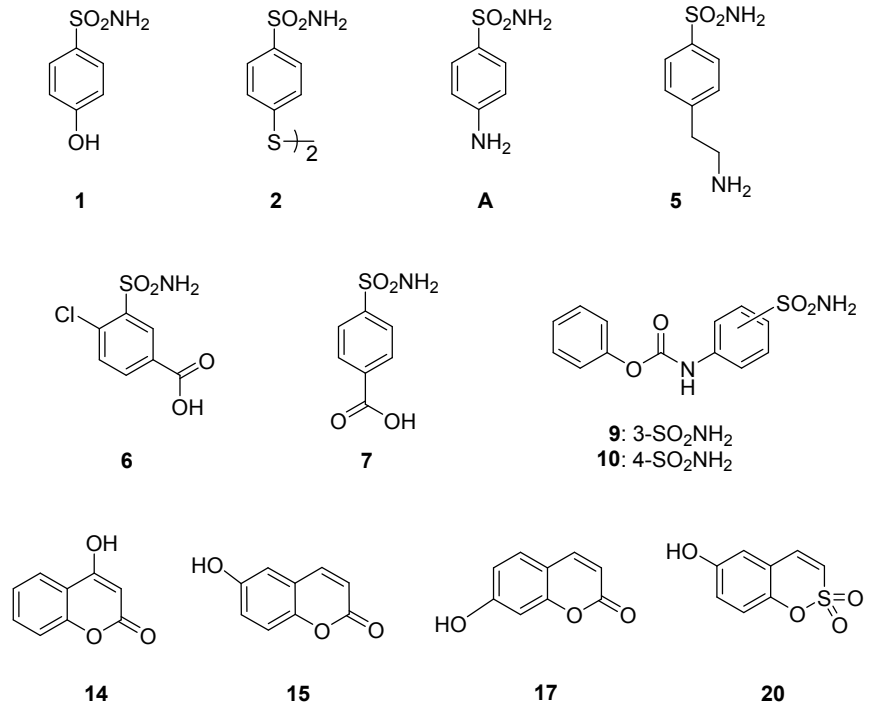
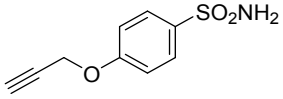
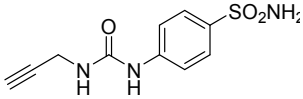
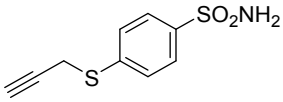
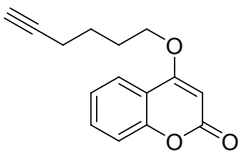
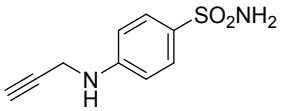
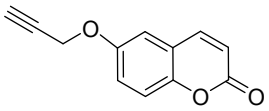
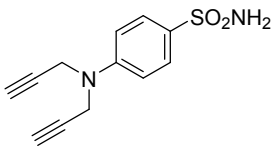
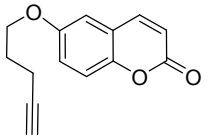
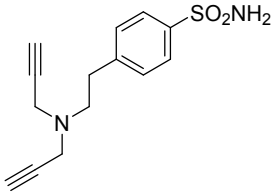
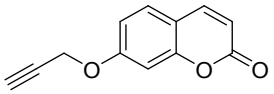
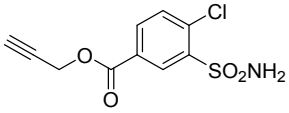
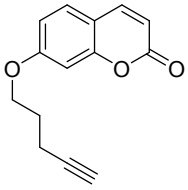
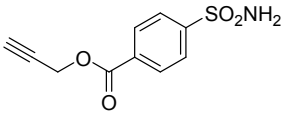
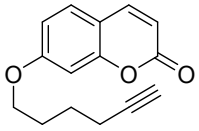
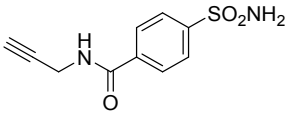
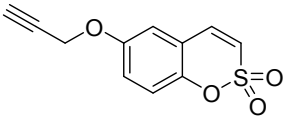
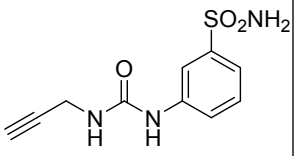


Figure 2. Substrates for the synthesis of alkynes **1b-3b, 5b-10b, 14b-20b, 4d and 13e.**

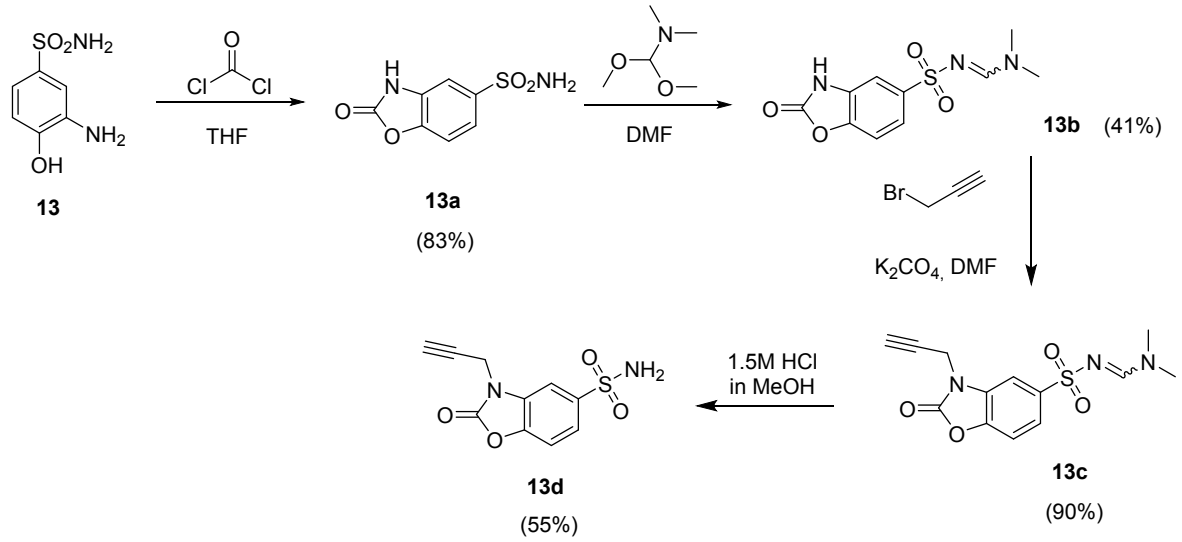
Table 1. Reagents and conditions for the synthesis of compounds **1a-3a, 5a-10a**, **14a-20a**, **4c**, **13d.**

Substrate[a]	X-Alkynyl	Product (%)[b]	Substrate[a]	X-Alkynyl	Product (%)[b]
1	X=Br	 **1a** (47)	**10**	X=NH_2	 **10a** (60)
2	X=Cl	 **2a** (83)	**14**	X=Cl	 **14a** (80)
A	X=Br	 **3a** (33)	**15**	X=Br	 **15a** (65)

A	X=Br	 **4c** (10)	**15**	X=Cl	 **16a** (79)
5	X=Br	 **5a** (70)	**17**	X=Br	 **17a** (73)
6	X=OH	 **6a** (40)	**17**	X=Cl	 **18a** (71)
7	X=OH	 **7a** (38)	**17**	X=Cl	 **19a** (80)
7	X=NH_2	 **8a** (82)	**20**	X=Br	 **20a** (85)
9	X=NH_2	 **9a** (65)			

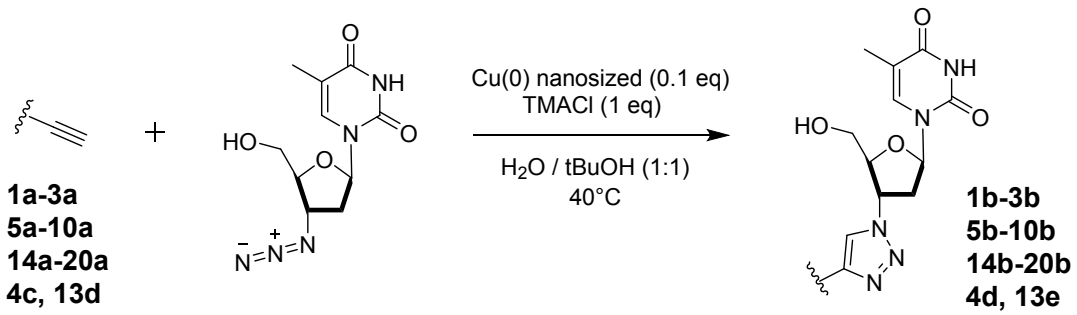
[a] Reported in **Figure 2**; [b]Yields refer to isolated products.

Compounds **13d**, here reported for the first time, was obtained through a multistep synthetic approach, reported in **Scheme 1**.



Scheme 1. Synthesis of compounds **1b-3b, 5b-10b, 14b-20b, 4d and 13e.** Yields are reported in brackets.

The final compounds **1b-3b, 5b-12b, 14b-20b, 4d** and **13e**, reported in **Figure 3**, were obtained by performing the CuAAC by using Cu (0) nanosized, tetramethyl ammonium chloride (TMACl) as a phase transfer agent in *t*BuOH/H_2O 1/1 as solvent at 40 °C (**Scheme 2)**.



Scheme 2. Synthesis of compounds **1b-3b, 5b-10b, 14b-20b, 4d and 13e.** Yields are reported in brackets.

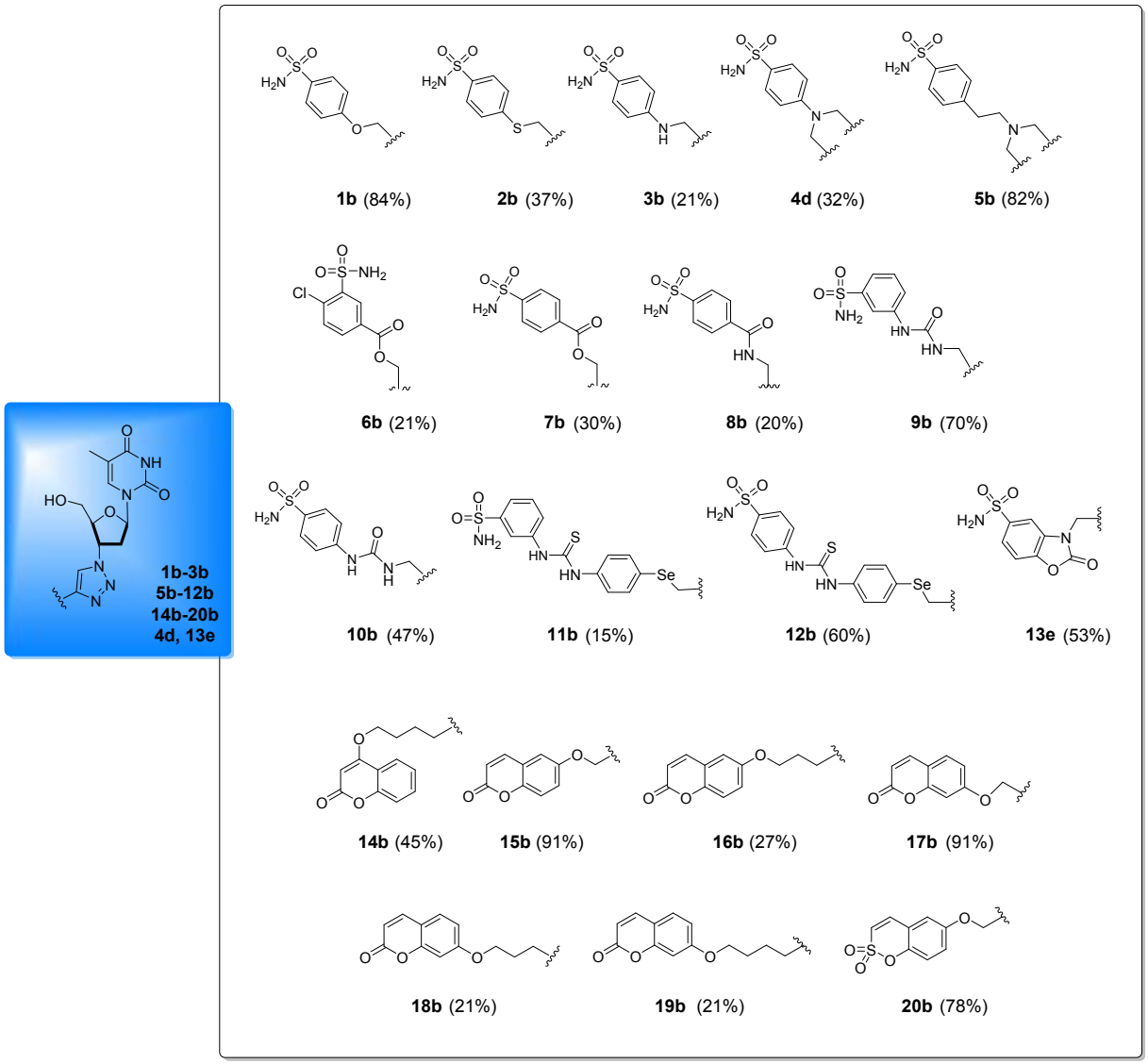
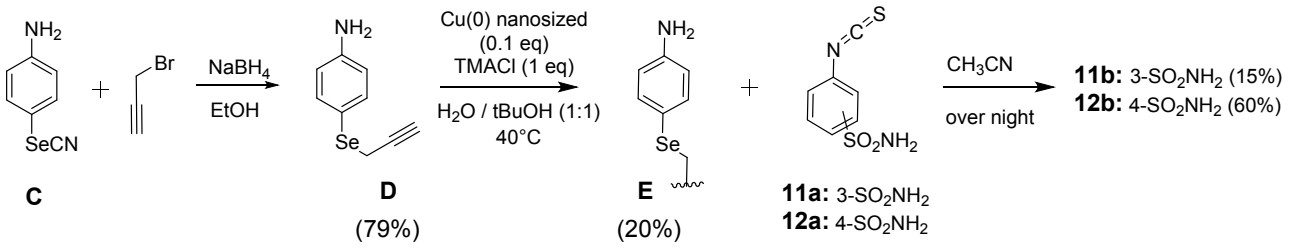


Figure 3. Chemical structures of compounds **1b-3b, 5b-12b, 14b-20b, 4d** and **13e**. Yields are reported in brackets and are referred to the final coupling reaction.



Scheme 3. Synthesis of compounds **11b** and **12b.** Yields are reported in brackets.

The synthesis of compounds **11b** and **12b** are reported separately (**Scheme 3**), as for these compound CuAAC was not performed as last reaction step. The synthesis started with the preparation of compound **D**, bearing the terminal alkyne pendant, obtained reducing 4-selenocyanatoaniline **C** with $NaBH_4$ and treating it *in situ* with propargyl bromide. The CuAAC reaction between the azide of **AZT** and the terminal alkyne of **D** was then performed, to afford the common intermediate **E**, which was subsequently reacted with 3-isothiocyanatobenzenesulfonamide or 4-isothiocyanatobenzenesulfonamide, to afford compound **11b** and **12b**, respectively. All final compounds were obtained in good yields and with high purity grade (i.e. ≥ 95 %) as determined by HPLC. Structural characterization of both intermediates and final compounds was assessed by means of ^{1}H NMR and ^{13}C NMR as well as high resolution mass spectra (HRMS).

To the best of our knowledge the **AZT**-coumarin derivative **17b** was previously reported in the literature as part of a set of compounds intended to be used for their fluorescent properties. No biological applications were reported in such a study.[51] In addition ester-triazole-linked triterpenoid–**AZT** conjugates were also reported.[52] Cytotoxic analysis of these hybrids and their triterpenoid precursors revealed moderate to good cytotoxic activities against two human tumor cell lines (KB, Hep-G2).[52] However, no detailed studies on the specific targets responsible for the anticancer effects were conducted.

Carbonic Anhydrase Inhibition Profiling.

The library of compounds obtained **1b-3b, 5b-12b, 14b-20b, 4d** and **13e** was evaluated for the inhibition properties against the human expressed (h) CAs I, II, VA, VB, VII, IX and XII isoforms by means of the stopped flow technique applied to the CO_2 hydrase assay.[53] The inhibition data, compared to those of the standard sulfonamide inhibitor acetazolamide (**AAZ**) are reported in **Table 2**.

Table 2. Inhibition data of hCA I, hCA II, hCA VA, hCA VB, hCA VII, hCAIX and hCA XII with compounds **1b-3b, 5b-12b, 14b-20b, 4d** and **13e** and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a Stopped flow CO_2 hydrase assay.[53]

	K_I (nM)*						
	hCA I	**hCA II**	**hCAVA**	**hCAVB**	**hCAVII**	**hCA IX**	**hCA XII**
1b	4666.7	9.3	59.1	141.3	51.6	6.2	78.9
2b	4037.5	7.7	57.3	52.6	31.0	653.3	61.6
3b	>10000	32.9	64.6	52.6	329.8	488.6	74.4
4d	>10000	8.5	57.3	45.9	383.5	6557.1	74.0
5b	>10000	70.7	59.4	42.9	281.1	8047.1	74.0
6b	85.5	7.7	3217.2	22.6	688.5	240.1	40.4
7b	28.0	1.3	4795.3	54.2	48.5	3.7	7.0
8b	483.0	13.4	1469.3	29.0	9.5	85.5	7.8
9b	289.7	6.3	3243.0	47.8	9.4	>10000	8.4
10b	93.1	8.2	437.8	37.9	9.4	267.6	38.9
11b	92.8	73.2	3972.2	45.0	66.0	373.2	9.0
12b	62.3	5.6	6258.9	46.7	21.8	>10000	7.1
13e	8771.0	21.3	3651.5	28.0	38.0	>10000	8.1
14b	>10000	>10000	1725.8	44.0	0.7	>10000	8.7
15b	>10000	>10000	57.8	161.0	9.3	6557.1	3.6
16b	>10000	>10000	666.8	40.1	0.7	21.2	9.4
17b	>10000	>10000	179.4	151.5	9.4	4885.7	3.5
18b	>10000	>10000	301.8	42.7	0.6	2948.3	40.4
19b	>10000	>10000	531.2	43.9	0.6	>10000	8.9
20b	>10000	>10000	172.4	54.6	10.5	5852.3	2.8
AAZ	250.0	12.1	63.0	54.0	2.5	25.8	5.7

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

As reported above in **Table 2** the compound series was investigated on the most relevant hCA isoforms such as the ubiquitous hCAs I and II, the mitochondrial expressed hCAs VA and VB, the abundantly central nervous system (CNS) expressed hCA VII and tumor associated hCA IX and XII. The structure-activity relationships (SARs) for the titled compounds are below discussed:

i) Overall the compound series screened *in vitro* against the ubiquitous hCAs I and II showed preferential inhibition in the low nanomolar range for the latter. In both cases the coumarin and sulfocoumarin based derivatives (i.e. **14b-20b**) resulted ineffective (i.e. K_Is > 10000 nM) as in agreement with previously reported data.[54,55] The isosteric ethers **1b** and **2b** resulted low micromolar inhibitors of the hCA I with the latter being just 1.2-fold more potent inhibitor (K_Is of 4666.7 and 4037.5 nM respectively). Interestingly the same kinetic profile for both compounds was retained for the hCA II isoform too, although the kinetic data were in the low nanomolar range (K_Is of 9.3 and 7.7 nM, respectively). Further manipulations on the scaffold of the type reported in compounds **3b, 4d** and **5b** resulted detrimental for the hCA I (K_Is > 10000 nM). As for the isoform II the introduction of a *N* atom as in **3b** and **5b** determined enhancement of the K_I values (32.9 and 70.7 nM, respectively) which were re-aligned to the previous ones when the *N,N*-bis-substituted aniline moiety was introduced instead (K_I of **4d** 8.5 nM). Compound **7b** was the most potent inhibitor among the series against both the hCA I and II (K_Is of 28.0 and 1.3 nM, respectively). Variations of the sulfonamide position (i.e. **6b**) or of the linker connection (i.e. **8b**) badly affected the potencies (see **Table 2**). Noteworthy the switch of the sulfonamide moiety from 3- to 4-position as in **9b** to **10b** and **11b** to **12b** resulted in decrease of the inhibition values for the hCA I. As for the hCA II a similar profile was observed only for **11b** and **12b,** whereas the opposite was obtained for the regioisomers **9b** and **10b** (i.e K_Is of 6.3 and 8.2 nM, respectively). Finally, compound **13e** showed excellent discrimination between the isoforms tested, being 411.8-fold more potent against the hCA II over the I.

ii) Despite the high degree of similarity between the mitochondrial expressed hCAs VA and VB, the kinetic profile of the majority of the tested compounds **1b-20b** accounted for the preferential inhibition of the latter. The ether derivative **1b** was the only sulfonamide bearing compound among

the series which showed selective inhibition of the hCA VA over the VB up to 2.4-fold. The substitution of the ethereal oxygen in **1b** with a sulphur or a nitrogen instead, as in compounds **2b** and **3b** respectively, suppressed any isoform selectivity, that was maintained when *N,N*-disubstitution (i.e. **4d**) or elongation (i.e. **5b**) was applied (see **Table 2**). As for the remaining sulfonamide derivatives **6b-12b** and **13e**, their K_I values against the hCA VA were all in the micromolar range with compound **10b** being the most potent among them (K_I of 437.8 nM). The same compounds were more effective in inhibiting the second mitochondrial expressed hCA as they showed medium nanomolar K_I values. The derivatives **6b**, **13e** and **8b** were the most effective against the hCA VB and their K_I values resulted up to 2.4-fold lower when compared to the reference **AAZ** (see **Table 2**). Interesting kinetic data were observed for the coumarin containing CAIs. The 4-alkyl substituted derivative **14b** resulted quite effective in inhibiting the hCA VB with a Selectivity Index (SI; K_I hCA VA/ hCA VB) of 39.2. Relocation of the chain to 7-position of the coumarin ring as in **19b** didn't change the kinetic profile but heavily reduced the SI for the preferential inhibition of the VB isoform (see **Table 2**). Regioisomeric effects on kinetics were also evident for compounds **15b** and **17b**. As reported in **Table 2** the 6-methylenesubstituted coumarin derivative **15b** resulted 2.8-fold stronger inhibitor of the hCA VA over the VB. The preferential inhibition for the former was lost when the chain in **15b** was moved to the adjacent 7-position as in **17b** (See **Table 2**). Interestingly the same swapping position as in compounds **16b** and **18b** did not altered the SI, which was in favour of the hCA VB for both derivatives, affected its intensity as it resulted halved. Finally, the sulfocoumarin prodrug **20b** also reported preferential inhibition for the hCA VB isoform with K_I values of 172.4 and 54.6 nM, respectively.

iii) As for the central nervous system (CNS) expressed hCA VII, the majority of the compounds tested resulted low nanomolar inhibitors. On considering the SARs it is worth noting that the ethers **1b** and **2b** showed K_I values within the medium nanomolar range (51.6 and 31.0 nM, respectively). The introduction of a nitrogen atom instead (i.e. compounds **3b** and **4d**) or a tertiary amine with an alkyl spacer (i.e. compound **5b**) spoiled the inhibition potency against the hCA VII

and thus raising the inhibition values up to the high nanomolar range (See **Table 2**). Interestingly the ester linkage seems to affect the inhibition potency for this isoform as demonstrated by the kinetic data for both compounds **6b** and **7b**. As a matter of fact the insertion of the amide, as in compound **8b**, or the ureido linker (i.e. **9b** and **10b**) resulted in a sensible enhancement of the hCA VII inhibition potency as reported in **Table 2** for the corresponding K_I values which are all comprised in the low nanomolar range (i.e. 9.5, 9.42 and 9.4 nM for **8b-10b,** respectively). Interesting results were obtained for the seleno containing compounds **11b** and **12b** as the regioisomer effect on kinetics was clearly observed. As reported in **Table 2** the para substituted benzenesulfonamide derivative **12b** was 3.0-fold more potent inhibitor of the hCA VII when compared to the meta one **11b** (K_Is of 21.8 and 66.0 nM, respectively). Finally, among the sulfonamide containing CAIs is the 2-oxo-2,3-dihydrobenzo[*d*]oxazole derivative **13e** which resulted a medium hCA VII nanomolar inhibitor with a K_I value of 38.0 nM. As for the coumarin containing CAI moieties, the regioisomeric substitution seems to be ineffective on the kinetic profile of such compounds against the hCA VII isoform. As reported in **Table 2**, compounds **14b-19b** resulted low nanomolar inhibitors and among them the 6- and 7-methylene substituted derivatives **15b** and **17b** were the less effective when compared to compounds bearing longer alkyne chain between the CAI portion and the **AZT** scaffold (**14b**, **16b**, **18b** and **19b**).

iv) A very interesting inhibitory profile can be observed for all the synthetized compounds **1b-12b, 14b-20b** and **13e** against the tumor associated isoforms hCA IX and XII. In general, all of them acted as low nanomolar inhibitors of CA XII, with K_I values ranging from 2.8 to 78.9 nM. As for CA IX, the different CAI moiety inserted within the scaffold (sulfonamide or coumarin) as well as the substitution patterns, both turned out to deeply influence the inhibition potency against this isoform. Three main groups can be delineated on the basis of the observed K_I values against CA IX. To the first group belong compounds which efficiently inhibit both tumor associated isoforms, such as compounds **1b**, **7b**, **8b** and **16b** (K_I values < 100 nM against CA IX) and compounds **2b**, **3b**, **6b**, **10b** and **11b** (K_I values < 1000 nM against CA IX). Except for compound **16b**, which is a 6-

substituted coumarin derivative, all the compounds belonging to this group are sulfonamide based derivatives, in which only one **AZT** moiety is present within the scaffold. In the second group (K_I values < 10000 nM against CA IX) we can include di-substituted sulfonamide based compounds **4d** and **5b**, in which two **AZT** moieties were "clicked" to the di-propargyl aminobenzensulfonamide and ethylaminobenzensulfonamide, respectively. In particular, ethylaminobenzensulfonamide derivative **5b** proved to be 1.23 fold less potent against CA IX then the shorter analogue **4d** (**Table 2**). In the second group we can also enumerate coumarin based compounds **15b**, **17b** and **18b** and sulfocoumarin compound **20b**, which inhibited CA IX in the micromolar range (K_I values ranging from 2948.3 nM to 6557.1 nM). Interestingly, these compounds strongly inhibited CA XII in the low nanomolar range (**Table 2**). Finally, in the third group (K_I values > 10000 nM against CA IX) we can find compounds which selectively inhibited CA XII over CA IX. In particular, 4 and 7-substituted coumarins **14b** and **19b**, both bearing a four methylene alkyne chain between the coumarin scaffold and the **AZT** moiety, showed to be ineffective against CA IX in the concentration range considered, whereas a strong inhibition of CA XII can be observed (K_I values of 8.7 nM and 8.9 nM for **14b** and **19b**, respectively). Meta-substituted ureido compound **9b**, para-substituted thioureido compound **12b** and 2-oxo-2,3-dihydrobenzo[*d*]oxazole-5-sulfonamide compound **13e** proved to be inactive in CA IX inhibition too (K_I values > 10000 nM). Again, a strong CA XII inhibition can be observed for all the compounds. Noteworthy, comparing homologous compounds such as meta- and para-substituted ureido compounds **9b** and **10b** and thioureido compounds **11b** and **12b**, the crucial impact of the regioisomery on the inhibition potency against CA IX can be appreciated, one isomer being about 30 fold more potent than the other. In particular, the meta-substituted ureido compound **10b** proved to be more potent than the para-analogue **9b**, whereas for the seleno-containing thioureido compounds **11b** and **12b**, the meta analogue **11b** showed to be the most potent.

Co-Crystallographic Studies.

In light of the promising K_I values observed against the tumor associated isoforms CA IX and XII, the binding modes of compounds **1b** and **3b** within the hCA II, used as a model study, was determined by means of X-ray experiments. The electron density maps of both **1b**-hCA II and **3b**-hCA II adducts accounted for both ligands placed well-ordered within the enzymatic cleft with their sulfonamide moieties deep buried up to the bottom of the cavity and coordinated to the zinc (II) ion in the canonical tetrahedral geometry. Again the additional interaction between the sulfonamidic oxygen with the T199 residue was conserved (**Figures 4** and **5**).[56]

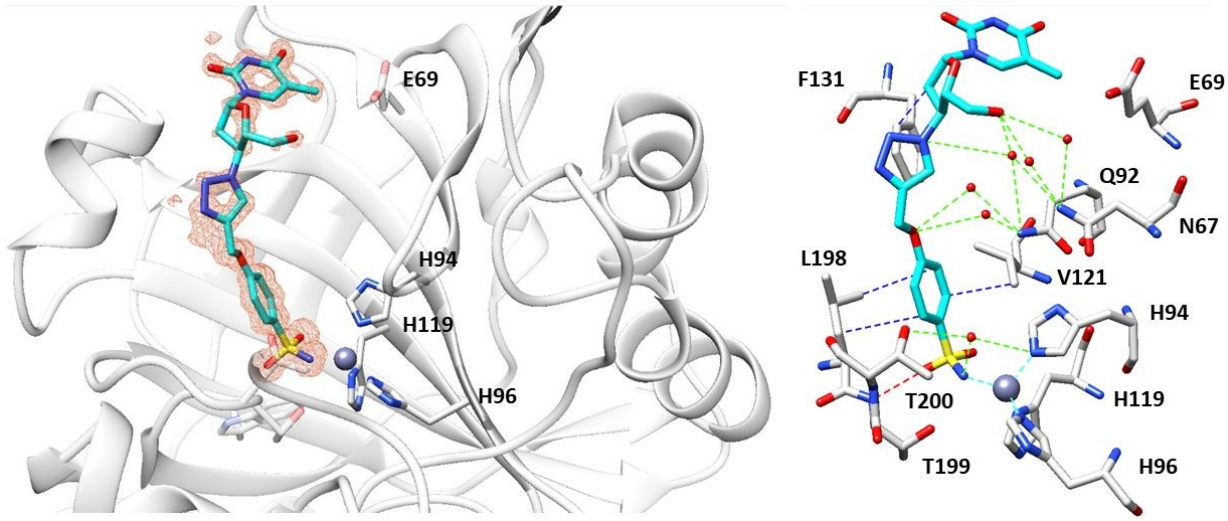


Figure 4. Inhibitor **1b** bound within the active site of hCA II at 1.1 Å resolution and showing the σA-weighted |Fo−Fc| map contoured at 2.5 σ. Ligand **1b** is shown in cyan. Hydrogen bonds, van der Waals interactions and Water Bridges are shown and labelled in red, blue and green, respectively. Residues involved in the binding of inhibitors are also shown. PDB access code **6YPW**.

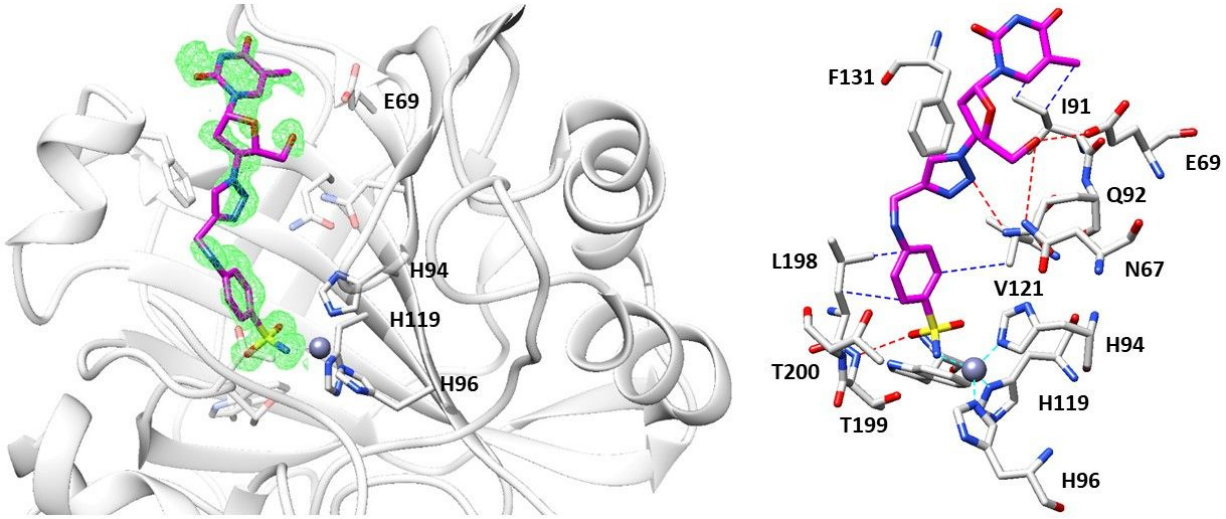


Figure 5. Inhibitor **3b** bound within the active site of hCA II at 1.3 Å resolution and showing the σA-weighted |Fo−Fc| map contoured at 2.5 σ. Ligand **3b** is shown in magenta. Hydrogen bonds, van der Waals interactions are shown and labelled in red and blue. Residues involved in the binding of inhibitors are also shown. PDB access code **6WKA.**

The ligands backbones of **1b** and **3b** are stabilized within the hCA II cavity site by means of a network of hydrogen bonds and van der Waals interactions with substantial differences of the tails orientations as clearly showed when superposition of two structures was performed as in **Figure 6**.

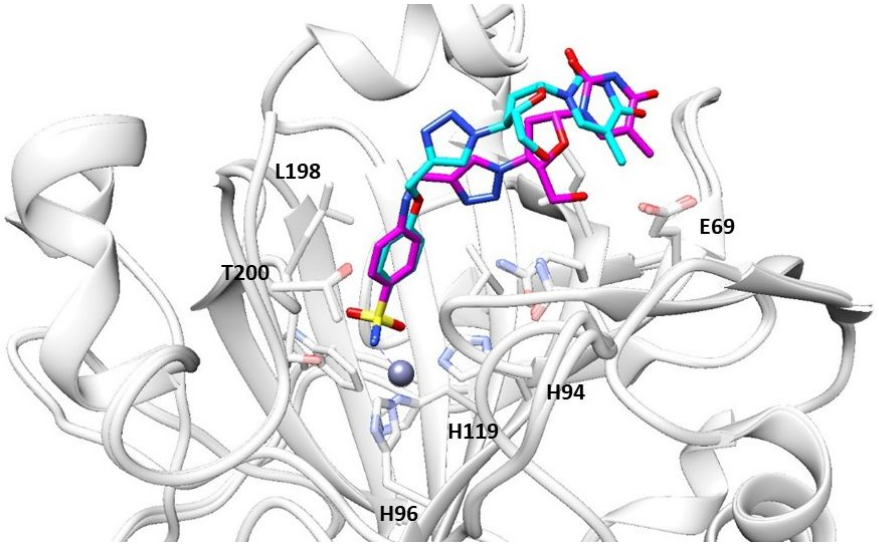


Figure 6. Superposition of inhibitors **1b** and **3b** bound in the active site of hCA II. Ligand **1b** is shown in cyan and **3b** in magenta.

The diverse spatial orientations of the tail sections must be ascribed to the replacement of the ethereal oxygen in **1b** with the nitrogen atom instead as in **3b** which is the only structural difference among them. The tail in **1b** is located towards the hydrophobic half of the catalytic cleft with the F131 residue acting as the major clipping point. The adduct is further stabilized by a network of hydrogen bonds which connects the inner face of the inhibitor to the opposite hydrophilic half of the enzymatic cleft by means of bridged water molecules (**Figures 4** and **6**). As for the compound **3b** tail, it resulted laid towards the hydrophilic section of the enzymatic cavity and directly stabilized by means of hydrogen bonds to the aminoacidic residues N67, E69 and Q92 (**Figures 5** and **6**). Such results were in agreement with the previously discussed CA kinetic data which showed the strongly stabilized compound **1b** being 3.7-fold more potent inhibitor against the hCA II when compared to **3b**.

Telomerase Activity Assay.

As mentioned above, **AZT** is known to be a potent telomerase inhibitor.[57,58] To check whether our compounds can affect telomerase, we incubated PC3 and HT-29 cells with the most potent CA IX and XII **CAI-TI** compounds **1b**, **7b**, **8b** or **11b** and measured telomerase activity. Results of Telomerase Repeated Amplification Protocol (TRAP) assay showed that all the tested compounds suppressed telomerase in both PC3 and HT-29 cells (**Figure 7A and B**). Telomerase activity in PC3 cells was higher than in HT-29. Compounds **1b** and **11b** demonstrated the strongest antitelomerase activity, while **7b** and **8b** appeared to be less potent.

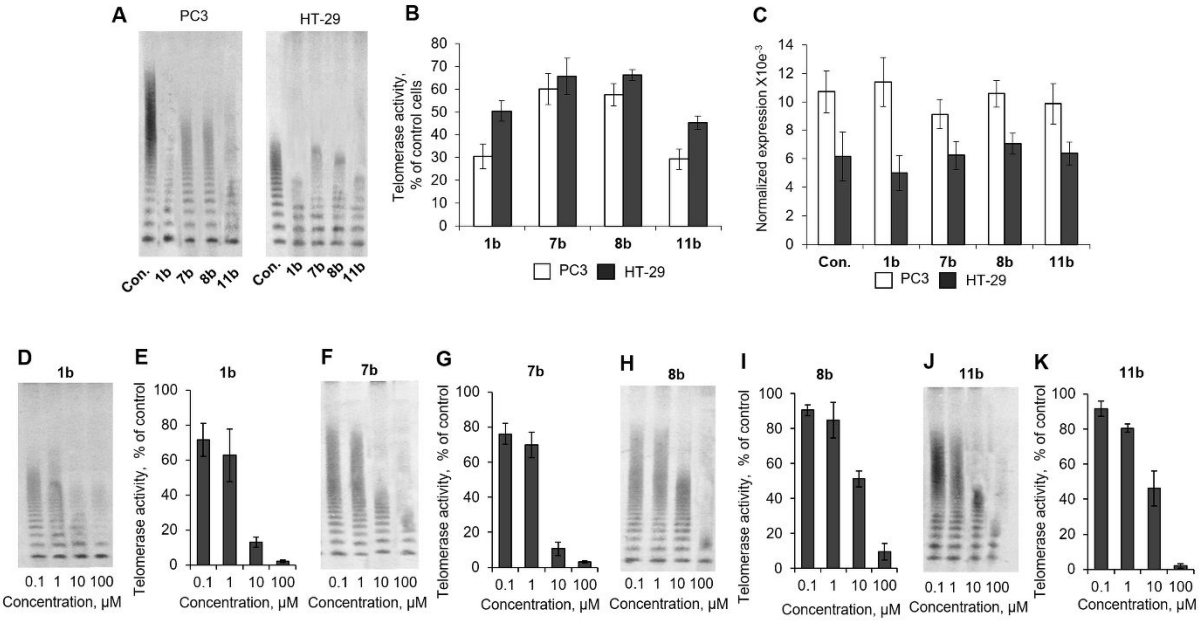


Figure 7. Suppression of telomerase activity by **CAI-TI** compounds. **(A)** Representative TRAP gel electrophoresis for PC3 or HT-29 cells incubated with 20 μM **CAI-TI** for 48 h. **(B)** Quantification of TRAP for living cells. **(C)** hTERT expression in incubated PC3 or HT-29 cells. Levels of hTERT mRNA were normalized relative to the levels of reference 18S RNA. **(D, F, H, J)** Representative TRAP gel electrophoresis for cell lysates treated with different concentrations of **CAI-TI**. **(E, G, I, K)** Quantification of TRAP for treated cell lysates. One representative TRAP gel of total four for each of experiment is shown. The results are presented as the mean±standard error of the mean (SEM). Con., control intact cells.

Telomerase activity is strongly regulated by the expression of its catalytic subunit hTERT and inhibition of its expression can be one of the ways of how **CAI-TI** suppress telomerase in cells.[59] We investigated hTERT expression in cells incubated with **CAI-TI**. In general, hTERT expression in PC3 cells was higher than in HT-29, which corresponds to increased telomerase activity in such cells (**Figure 7C**). We found that the compounds have no effect on hTERT gene expression in both types of cells. Another possible way of telomerase inhibition is the binding of substance to hTERT

protein subunit.[60] As it shown in **Figure 7A**, PC3 cells have more active telomerase, that is why their lysates were used for telomerase testing in cell-free experiments. All the compounds demonstrated dose-dependent activity to inhibit telomerase within the rage of concentrations 0.1 – 100 μM (**Figure 7D–K**). IC_{50} and IC_{90} values for each compound are shown in **Table 3**. Compounds **1b** and **11b** had the lowest IC_{50}, that is in accordance to telomerase inhibition in living cell.

Table 3. Determined IC_{50} and IC_{90} values for telomerase inhibitors (**CAI-TI)**.

	IC_{50}, μM*	**IC_{90}, μM***
1b	5.2	40.0
7b	6.0	31.8
8b	9.1	60.3
11b	5.6	42.8

* Mean from 4 different assays, by a RTQ-TRAP (errors were in the range of ± 5 % of the reported values).

CONCLUSIONS

To the best of our knowledge this work is the *proof-of-concept* study about the concomitant use of CAIs and TIs merged within the same molecular scaffold and able to act on two validated targets for the management of cancer. Molecular hybridization is a powerful tool in Medicinal Chemistry, with extensive and several successful applications reported so far.[61] Herein, a series of 20 **CAI-TI** of the **AZT** type compounds has been synthetized and fully characterized. Then, inhibition potencies against the two designed targets have been assessed. CA inhibition data against seven hCA isoforms revealed that all the titled compounds **1b-3b**, **5b-12b**, **14b-20b**, **4d** and **13e** strongly inhibit hCA XII, whereas some of them (**1b-3b**, **6b-8b**, **10b, 11b**, and **16b**) showed medium-high inhibition potency against hCA IX.

The evaluation of telomerase activity in cell lysates or in cells incubated with the CA IX and XII most potent inhibitors **1b**, **7b**, **8b** and **11b** showed their strong antitelomerase properties, which rely on the ability to suppress processivity of the enzyme rather than the suppression of hTERT expression.

High resolution X-ray crystallography on compounds **1b** and **3b** in adduct with hCA II as model study allowed to properly assess their binding mode. In particular we: *i*) highlighted the crucial role played by a single heteroatom in determining CA isoform selectivity by means of diverse space orientation of the tail; *ii*) firstly determined molecular features of the **CAI-TI** molecules which may be useful to address CA selectivity once proper chemical manipulation is operated.

Overall the preliminary results obtained in this study fully sustained our strategy and gave us a strong background to further proceed in developing *ad hoc* designed **CAI-TI** molecules which will be considered in appropriate tumor cell lines.

EXPERIMENTAL PROTOCOLS

Chemistry.

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich (Milan, Italy), Alfa Aesar (Milan, Italy) and TCI (Milan, Italy). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance spectra (^{1}H-NMR: 400 MHz; ^{13}C-NMR: 100 MHz) were recorded in DMSO-d_6 using an Avance III 400 MHz spectrometer (Bruker, Milan, Italy). Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doublets. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D_2O.

The purity of the final compounds was determined in high purity grade (i.e. ≥ 95 %) by HPLC using an Agilent 1200 liquid chromatography system composed by autosampler, binary pumps, column oven and diode-array detector (LC-DAD) operating in UV range (210-400 nm). The operating conditions were reported within the Supporting Information file.

The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), purchased from Sigma–Aldrich and mQ water 18 MΩ cm, obtained from Millipore's Simplicity system (Milan, Italy). The high resolution mass spectrometry (HRMS) analysis was performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI). The accurate mass measure was carried out by introducing, via syringe pump at 10 μL min^{-1}, the sample solution (1.0 μg mL^{-1} in mQ water: acetonitrile 50:50), and the signal of the positive ions was acquired. The proposed experimental conditions allowed to monitoring the protonated molecules of studied compounds ([M+H]$^{+}$ species), that they were measured with a proper dwell time to achieve 60,000 units of resolution at Full Width at Half Maximum (FWHM).

Synthesis of final compounds 1b-3b, **5b-12b**, **14b-20b**, **4d and 13e and their intermediates.**

General procedure A: The proper alkyl halide (1.2 eq.) was added to a suspension of either **1**, **14**, **15**, **17-20** (0.5 g, 1.0 eq.) and K_2CO_3 (2.0 eq.) in dry DMF (4 mL) under N_2 atmosphere. The mixture was stirred at r.t or 60° or 100°C, depending on the alkyl halide, until consumption of starting material (5 h, TLC monitoring). The reaction mixture was cooled at r.t. and quenched with slush. The mixture was extracted with EtOAc (x 3) and the combined organic layers were washed with H_2O and brine solution, then dried over Na_2SO_4, filtered-off and concentrated under vacuum.

General procedure B. The proper carbamate derivative **9** or **10** (0.5 g, 1 eq.) was dissolved in EtOH and propargylamine (1.2 eq.) was added. The reaction was refluxed for 16h, then cooled at r.t. and quenched with slush. The mixture was extracted with EtOAc (x 3), dried over Na_2SO_4, filtered-off and concentrated under vacuum, to afford a solid that was purified by silica gel column chromatography, eluting with 60% EtOAc/Hx.

General Procedure C. To a suspension of azidonucleoside **AZT** (1.1 eq. or 2.2 eq.) in H_2O/t-BuOH 1/1 (4 mL) the appropriate alkyne (0.12 g, 1.0 eq.) was added at r.t., followed by copper(0) nanosized (0.1 eq.) and TMACl (1.0 eq.). The suspension was stirred at 40°C until starting materials were consumed (TLC monitoring), then diluted with MeOH (20 mL), and filtered through Celite 521®. The solvent was evaporated, affording to a residue that was triturated from EtOAc, to give a white powder.

4-(Prop-2-ynyloxy)benzenesulfonamide (1a). Compound **1a** was synthetized according to the **general procedure A** using 4-hydroxybenzenesulfonamide **1** and propargyl bromide 80% in toluene at 60°C. Purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford the titled compound **1a** as a white powder. 47% yield; δ_H (400 MHz, DMSO-d_6) 3.67 (1H, br s, C*H*), 4.94 (2H, br s, CH_2), 7.17 (2H, d, J = 7.2, Ar-*H*), 7.28 (2H, s, exchange with D_2O, SO_2NH_2), 8.01 (2H, d, J = 7.2, Ar-*H*). Experimental in agreement with reported data.[62]

4-((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methoxy)benzenesulfonamide (1b). Compound

1b was obtained according to the **general procedure A** using **1a** as starting material, to afford the title compound **1b** as a light yellow solid: 84% yield; δ_H (400 MHz, DMSO-d_6): 1.85 (3H, s, C*H*$_3$), 2.73 (2H, m, C*H*$_2$), 3.70 (2H, m, C*H*$_2$), 4.27 (1H, q, *J*= 3.5, C*H*), 5.29 (2H, s, C*H*$_2$), 5.34 (1H, br t, exchange with D_2O, O*H*), 5.45 (1H, m, C*H*), 6.46 (1H, t, *J*= 6.5, C*H*), 7.24 (4H, m, overlapped signals, 2 x Ar*H*, exchange with D_2O, SO_2NH_2), 7.80 (2H, d, *J*= 8.8, Ar*H*), 7.86 (1H, s, C*H*), 8.50 (1H, s, C*H*), 11.36 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6):13.1, 38.0, 55.2, 60.3, 62.0, 84.5, 85.4, 110.5, 115.6, 125.4, 128.5, 137.1, 137.4, 143.2, 151.3, 161.2, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{19}H_{23}N_6O_7S$= 479.1343, found 479.1336.

4-(Prop-2-ynylthio)benzenesulfonamide (2a). $NaBH_4$ (23 mg, 0.60 mmol, 3.0 eq.) was added portion-wise to a freshly prepared solution of solution of 4,4′-disulfanediyldibenzenesulfonamide **2** (75 mg, 0.20 mmol, 1.0 eq.) in EtOH (2 mL) at r.t. under a N_2 atmosphere. After 2 h, propargyl chloride (0.42 mmol, 2.1 eq.) was slowly added and the reaction mixture was stirred at r.t. for 3 h, until complete consumption of the starting material was observed by TLC. The reaction was quenched by addition of saturated NH_4Cl aqueous solution (2 mL) and diluted with EtOAc (5 mL), The layers were separated and the aqueous layer was extracted with EtOAc (2 × 5 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The crude material was purified by silica gel flash chromatography to afford the titled compound **2a** as a white solid. 83% yield; δ_H (400 MHz, DMSO-d_6): 3.22 (1H, t, *J* = 2.6, C*H*), 4.02 (2H, d, *J* = 2.6, C*H*$_2$), 7.37 (2H, s, exchange with D_2O, SO_2NH_2), 7.56 (2H, dd, *J* = 2.0, 6.7, Ar-*H*), 7.79 (2H, dd, *J* = 2.0, 6.7, Ar-*H*). Experimental in agreement with reported data.[63]

4-(((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)thio)benzenesulfonamide (2b). Compound **2b** was obtained according to the **general procedure C** using **2a** as starting material, to afford the title compound **2b** as a white solid: 37% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.69 (2H, m, C*H*$_2$), 3.64 (2H, m, C*H*$_2$), 4.19 (1H, q, *J*= 3.5, C*H*), 4.45 (2H, s, C*H*$_2$), 5.38 (2H,

m, overlapped signals, 1 x C*H*, exchange with D_2O, 1 x O*H*), 6.43 (1H, t, *J*= 6.5, C*H*), 7.36 (2H, s, exchange with D_2O, SO_2NH_2), 7.57 (2H, d, *J*= 8.4, Ar*H*), 7.76 (2H, d, *J*= 8.4, Ar*H*), 7.85 (1H, s, C*H*), 8.29 (1H, s, C*H*), 11.35 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 27.0, 37.9, 60.2, 61.6, 84.8, 85.5, 110.5, 124.2, 127.1, 127.7, 137.1, 141.9, 142.1, 144.1, 151.3, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{19}H_{23}N_6O_6S_2$= 495.1115, found 495.1118.

4-(Prop-2-ynylamino)benzenesulfonamide (3a). Propargyl bromide 80% in toluene (1.2 eq.) was added to a suspension of sulfanilamide **A** (0.5 g, 1.0 eq.) and pyridine (1.2 eq.) in dry DMF (2 ml) under N_2 atmosphere and the mixture was stirred at 70°C o.n. (TLC monitoring). The reaction was quenched with H_2O (10 ml) and extracted with EtOAc (3 x 15 ml). The combined organic layers were washed with H_2O (3 x 15 ml) and brine (3 x 15 ml), dried over anhydrous Na_2SO_4, filtered-off and concentrated under vacuum to give a solid that was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford the desired product **65** as a yellow solid. 33 % yield; δ_H (400 MHz, DMSO-d_6): 3.13 (1H, t, *J* = 2.4, C*H*), 3.97 (2H, dd, *J*= 2.4, 6.0, CH_2), 6.73 (2H, d, *J* = 8.8, Ar-*H*), 6.76 (1H, br t, exchange with D_2O, N*H*), 6.98 (2H, br.s, exchange with D_2O, SO_2NH_2), 7.59 (2H, d, *J* = 8.8, Ar-*H*). Experimental in agreement with reported data.[64]

4-(((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)benzenesulfonamide (3b). Compound **3b** was obtained according to the **general procedure C** using **3a** as starting material, to afford the title compound **3b** as a white solid. 21% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 2.69 (2H, m, CH_2), 3.68 (2H, m, CH_2), 4.23 (1H, q, *J*= 3.5, C*H*), 4.40 (2H, d, *J*= 5.7, CH_2), 5.32 (1H, br t, 1H, exchange with D_2O, O*H*), 5.38 (1H, m, C*H*), 6.45 (1H, t, *J*= 6.5, C*H*), 6.74 (2H, d, *J*= 8.8, 2x Ar-*H*), 6.92 (1H, t, J=5.7, exchange with D_2O, N*H*), 6.98 (2H, s, exchange with D_2O, SO_2NH_2), 7.55 (2H, d, *J*= 8.8, 2x Ar-*H*), 7.84 (1H, s, C*H*), 8.24 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6):12.2, 36.9, 37.9, 59.1, 60.7, 83.8, 84.5, 110.1, 111.2, 122.6,

127.2, 130.4, 136.3, 145.2, 150.4, 150.9, 163.7; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{19}H_{24}N_7O_6S$= 478.1503, found 478.1508.

4-(diProp-2-ynylamino)benzenesulfonamide (4c). Sulfanilamide **A** (0,5 g, 1.0 eq.) was dissolved in DMF and the solution was cooled to 0°C. Then, dimethoxy-*N*,*N*-dimethylmethanamine (1.2 eq.) was added. The solution was stirred at r.t. until consumption of starting material (2h). The reaction was quenched with DCM and precipitate formed was filtered-off, dried to afford *N*'-((4-aminophenyl)sulfonyl)-*N,N*-dimethylformimidamide **4a** which was used for the next step without further purification. *N*'-((4-aminophenyl)sulfonyl)-N,N-dimethylformimidamide **4a** (1.0 eq.) was solubilized in dry DMF and K_2CO_3 (3.0 eq.) was added. Then, propargyl bromide 80% in toluene (4.0 eq.) was added and the mixture was stirred at 80°C o.n. until consumption of starting material. Then the reaction was quenched with H_2O (20 ml) and extracted with EtOAc (3 x 15 ml). The combined organic layers were washed with H_2O (3 x 15 ml) and brine(3 x 15 ml), dried over Na_2SO_4, filtered-off and concentrated under vacuum to give a residue (**4b**) that was suspended in isopropylamine in a sealed tube and stirred at r.t. o.n. The solvent was removed in vacuo obtaining a residue that was purified by silica gel column chromatography eluting with 50% ethyl acetate in *n*-hexane to afford a sticky residue which was triturated from Et_2O to afford the titled compound **4c** as a white powder: 10% yield; δ_H (400 MHz, DMSO-d_6): 3.21 (2H, t, *J* = 2.4, 2x C*H*), 4.29 (4H, d, *J* = 2.4, 2 x CH_2), 7.03 (2H, d, *J* = 8.8, Ar-*H*), 7.08 (2H, s, exchange with D_2O, SO_2NH_2), 7.70 (2H, d, *J* = 8.8, Ar-*H*). Experimental in agreement with reported data.[65]

4-(bis((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)benzenesulfonamide (4d). Compound **4d** was obtained according to the **general procedure C** using **4c** as starting material, to afford the title compound **4d** as a light yellow solid. 32% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (6H, s, 2 x CH_3), 2.69 (4H, m, 2 x CH_2), 3.68 (4H, m, 2 x CH_2), 4.21 (2H, q, *J*= 3.8, 2 x C*H*), 4.79 (4H, s, 2 x CH_2), 5.35 (2H, br t, exchange with D_2O, 2 x O*H*), 5.40 (2H, m, 2 x C*H*), 6.45 (2H, t, *J*= 6.4, 2 x

C*H*), 7.03 (4H, m, overlapped signals, 2 x Ar*H*, exchange with D_2O, SO_2NH_2), 7.60 (2H, d, *J*= 8.9, 2 x Ar*H*), 7.85 (2H, s, 2 x C*H*), 8.28 (2H, s, 2 x C*H*), 11.38 (2H, br s, exchange with D_2O, 2 x N*H*). δ_C(100 MHz, DMSO-d_6): 12.2, 37.9, 45.4, 59.1, 60.7, 83.8, 84.5, 110.1, 111.2, 122.6, 127.2, 130.4, 136.3, 145.2, 150.4, 150.9, 163.7; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{32}H_{39}N_{12}O_{10}S$= 783.2627, found 783.2632.

4-(2-(di-Prop-2-ynylamino)ethyl)benzenesulfonamide (5a). Propargyl bromide (80% in toluene) (2 eq.) and DIPEA (1.7 eq.) were added to a stirred solution of 4-(2-aminoethyl) benzensulfonamide **5** (0.5 g, 1.0 eq.) in CH_3CN (8 ml) under N_2 atmosphere. The mixture was stirred at r.t. o.n. until consumption of starting material (TLC monitoring). The solvent was removed under reduced pressure and the obtained residue was portioned between H_2O and EtOAc followed by extraction with EtOAc (3 x 15 ml). The combined organic layers were washed with H_2O(3 x 15 ml) and brine(3 x 15 ml), then dried over Na_2SO_4, filtered-off and concentrated under vacuum to afford compound **5a** as a dark oil. 70 % yield; δ_H (400 MHz, DMSO-d_6): 2.75 (2H, t, *J* = 6.8, CH_2), 2.84 (2H, t, *J* = 6.8, CH_2), 3.21 (2H, br t, 2 x C*H*), 3.44 (4H, d, *J* = 2.0, 2 x CH_2), 7.37 (2H, s, exchange with D_2O, SO_2NH_2), 7.45 (2H, d, *J* = 8.4, Ar-*H*), 7.76 (2H, d, *J* = 8.4, Ar-*H*); δ_C (100 MHz, DMSO-d_6): 32.6, 41.5, 53.3, 75.8, 79.1, 125.6, 129.1, 141.9, 144.4; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{14}H_{17}N_2O_2S$= 277.1005, found 277.1009. Experimental in agreement with reported data.[66]

4-(2-(bis((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethyl)benzenesulfonamide (5b). Compound **5b** was obtained according to the **general procedure C** using **5a** as starting material, to afford the title compound **5b** as a white solid. 82% yield; δ_H (400 MHz, DMSO-d_6): 1.85 (6H, s, 2 x CH_3), 2.72 (6H, m, overlapped signals, 3 x CH_2), 2.93 (2H, t, *J*= 7.2, CH_2), 3.70 (4H, m, 2 x CH_2), 3.80 (4H, s, 2 x CH_2), 4.24 (2H, q, *J*= 3.5, 2 x C*H*), 5.40 (4H; m, overlapped signals, 2 x C*H*, exchange with D_2O, 2 x O*H*), 6.48 (2H, t, *J*= 6.4, 2 x C*H*), 7.32 (2H, br s, exchange with D_2O, SO_2NH_2), 7.41 (2H, d, *J*= 8.3, 2x Ar-*H*), 7.75 (2H, d, *J*= 8.3, 2x Ar-*H*), 7.87 (2H, s, 2 x C*H*), 8.22 (2H, s, 2 x C*H*),

11.40 (2H, br s, exchange with D_2O, 2 x N*H*). δ_C(100 MHz, DMSO-d_6): 13.1, 33.4, 37.9, 48.2, 54.7, 60.1, 61.6, 84.8, 85.5, 110.5, 124.5, 126.5, 130.0, 137.2, 142.6, 144.6, 145.7, 151.4, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{34}H_{43}N_{12}O_{10}S$= 811.2940, found 811.2951.

Prop-2-yn-1-yl 4-chloro-3-sulfamoylbenzoate (6a). To a stirring solution of 4-chloro-3-sulfamoylbenzoic acid **6** (1 eq.) in dry DMF, EDC HCl (1.2 equiv) was added at 0°C. After 30 min, propargyl alcohol (1.2 eq.) and DMAP (1.2 eq.) were added. The mixture was stirred at room temperature under N_2 for an additional 3 h, until consumption of starting material. The reaction was quenched with slush and extracted with EtOAc (x3). The organic extract was washed with saturated aqueous $NaHCO_3$, water and brine, dried over Na_2SO_4, filtered-off and concentrated under vacuum. The crude was purified by flash silica chromatography (40% EtOAc/Hx) to afford the title compound **6a** as a white solid. 40% yield; δ_H (400 MHz, DMSO-d_6): 3.68 (1H, t, *J*= 2.4, C*H*), 5.05 (2H, d, *J*= 2.5, CH_2), 7.86 (3H, m, 1x Ar-*H*, 2x SO_2NH_2), 8.16 (1H, dd, *J*= 2.2, 8.2, Ar-*H)*, 8.56 (1H, d, *J*= 2.1, Ar-*H*); δ_C(100 MHz, DMSO-d_6): 53.6, 79.0, 79.3, 129.0, 130.6, 133.4, 134.5, 136.7, 142.4, 165.5; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{15}H_{16}N_3O_2$= 270.1237, found 270.1237.

(1-((2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl 4-chloro-3-sulfamoylbenzoate (6b). Compound **6b** was obtained according to the **general procedure C** using **6a** as starting material, to afford the title compound **6b** as a white solid. 21% yield; δ_H (400 MHz, DMSO-d_6): 1.83 (3H, s, CH_3), 2.69 (2H, m, CH_2), 3.67 (2H, m, CH_2), 4.22 (1H, q, *J*= 3.9, C*H*), 4.76 (2H, s, CH_2), 5.29 (1H, br t, 1H, exchange with D_2O, O*H*), 5.38 (1H, dt, *J*= 8.3, 5.29, C*H*), 6.43 (1H, t, *J*= 6.5, C*H*), 7.47 (2H, s, exchange with D_2O, SO_2NH_2), 7.86 (2H, m, 2x Ar-*H*), 7.98 (1H, s, C*H*), 8.10 (1H, s, Ar-*H*), 8.38 (1H, s, C*H*), 11.3 (1H, br s, exchange with D_2O, N*H*). δ_C (100 MHz, DMSO-d_6):13.1, 27.5, 38.0, 60.4, 61.6, 84.8, 85.3, 110.5, 111.6, 116.9, 123.1, 124.6, 137.1, 142.0, 143.2, 151.3, 153.8, 163.2, 164.6, 167.1; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{22}ClN_6O_8S$= 541.0903, found 541.0899.

Prop-2-ynyl 4-sulfamoylbenzoate (7a). To a stirring solution of 4-sulfamoylbenzoic acid **7** (2.0 g, 9.9 mmol) in dry DMF (40 mL) were successively added propargyl alcohol (1.17 mL, 19.8 mmol, 2.0 eq.), Et_3N (2.8 mL, 19.9 mmol, 2.0 eq.), and EDC HCl (1.9 g, 9.9 mmol, 1.0 eq.). The solution was stirred at room temperature under N_2 for an additional 4 h. The mixture was then concentrated under reduced pressure and ethyl acetate (40 mL) was added. The organic extract was washed with saturated aqueous $NaHCO_3$ (40 mL) and back-extracted with ethyl acetate (40 mL). The organic layers were combined and washed with brine (40 mL), dried over Na_2SO_4, filtered, and evaporated. The crude oil was purified by flash silica chromatography (50% EtOAc/Hx) to afford the title compound **7a** as a white crystalline solid. 38% yield; δ_H (400 MHz, DMSO-d_6): 3.63 (1H, t, *J*= 2.4, C*H*), 4.97 (2H, d, *J*= 2.8, CH_2), 7.55 (2H, brs, exchange with D_2O, SO_2NH_2), 7.98 (4H, m, 4x Ar*H*); δ_C(100 MHz, DMSO-d_6): 53.0, 78.1, 78.3, 126.2, 130.0, 131.7, 148.3, 164.0. Experimental in agreement with reported data.[67]

(1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl 4-sulfamoylbenzoate (7b). Compound **7b** was obtained according to the **general procedure C** using **7a** as starting material, to afford the title compound **7b** as a white solid. 30% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 2.74 (2H, m, CH_2), 3.71 (2H, m, CH_2), 4.28 (1H, m, C*H*), 5.32 (1H, t, *J*= 5.1, exchange with D_2O, O*H*), 5.42 (1H, m, C*H*), 5.50 (2H, s, CH_2), 6.46 (1H, t, *J*= 6.6, C*H*), 7.60 (2H, s, exchange with D_2O, SO_2NH_2), 7.85 (1H, s, C*H*), 8.00 (2H, d, *J*= 8.4, 2x Ar-*H*), 8.18 (2H, d, *J*= 8.5, 2x Ar-*H*), 8.49 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.3, 38.0, 59.3, 60.5, 61.7, 84.8, 85.3, 110.5, 125.7, 127.3, 131.2, 133.0, 137.1, 142.8, 149.1, 151.3, 164.6, 165.4; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{23}N_6O_8S$= 507.1293, found 507.1294.

***N*-(Prop-2-ynyl)-4-sulfamoylbenzamide (8a).** To a stirring solution of 4-sulfamoylbenzoic acid **7** (2.0 g, 9.9 mmol) and propargylamine (0.64 mL, 9.9 mmol, 1.0 eq.) in dry DMF (40 mL) were successively added N-hydroxybenzotriazole monohydrate (0.94 g, 6.6 mmol, 0.6 eq.),

diisopropylethylamine (1.7 mL, 9.9 mmol, 1.0 eq.), and HBTU (3.8 g, 9.9 mmol, 1.0 eq.). The deep yellow solution was stirred at room temperature under N_2 for 1 h when found complete by TLC. The mixture was concentrated under reduced pressure and ethyl acetate (40 mL) was added. The organic extract was washed with water (40 mL) and back-extracted with EtOAc (x3). The organic extracts were combined and washed with brine (50 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated to a crude white solid. Recrystallization from hot methanol:water (9:1) afforded the title compound **8a** as a white crystalline solid. 82% yield; δ_H (400 MHz, DMSO-d_6): 3.12 (1H, t, *J*= 2.4, C*H*), 4.05 (2H, d, *J*= 5.6, 2.8, CH_2), 7.45 (2H, brs, exchange with D_2O, SO_2NH_2), 7.92 (4H, m, 4x Ar*H*), 9.09 (1H,t, *J*= 5.6, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 29.0, 73.8, 81.7, 126.4, 128.6, 137.3, 147.1, 164.6. Experimental in agreement with reported data.[67]

N-((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)-4-sulfamoylbenzamide (8b). Compound **8b** was obtained according to the **general procedure C** using **8a** as starting material, to afford the title compound **8b** as a white solid. 20% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 2.71 (2H, m, CH_2), 3.69 (2H, m, CH_2), 4.25 (1H, t, *J*= 4.6, C*H*), 4.59 (2H, s, CH_2), 5.30 (1H, m, exchange with D_2O, O*H*), 5.38 (1H, m, C*H*), 6.45 (1H, t, *J*= 6.6, C*H*), 7.51 (2H, s, exchange with D_2O, SO_2NH_2), 7.84 (1H, s, C*H*), 7.94 (2H, m, 2x Ar-*H*), 8.07 (2H, d, *J*= 8.5, 2x Ar-*H*), 8.24 (1H, s, C*H*), 9.25 (1H, br s, exchange with D_2O, N*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 35.9, 38.0, 60.1, 61.7, 84.8, 85.4, 110.5, 123.6, 126.5, 128.9, 137.1, 137.9, 145.9, 147.2, 151.3, 164.6, 166.0; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{24}N_7O_7S$= 506.1452, found 506.1453.

3-(3-(prop-2-yn-1-yl)ureido)benzenesulfonamide (9a). Compound **9a** was synthetized according to the **general procedure B** using phenyl (3-sulfamoylphenyl)carbamate **9** as starting material. White solid, 65% yield; δ_H (400 MHz, DMSO-d_6): 3.14 (1H, t, *J*= 2.4, C*H*), 3.93 (2H, dd, *J*= 2.2, 5.7, CH_2), 6.59, (1H, t, *J*= 5.7, exchange with D_2O, N*H*), 7.34 (2H, brs, exchange with D_2O, SO_2NH_2), 7.40 (1H,

dt, *J*= 8.2, 1.9, Ar*H*), 7.45 (1H, d, *J*= 7.8, Ar*H*), 7.57 (1H, dt, *J*= 8.1, 1.6, Ar*H*), 8.02 (1H, t, *J*= 2.0, Ar*H*), 8.98 (1H, brs, N*H*); δ_C(100 MHz, DMSO-d_6): 29.7, 73.7, 82.8, 115.6, 119.3, 121.5, 130.2, 141.5, 145.5, 155.5; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{10}H_{12}N_3O_3S$= 254.0594, found 254.0592.

3-(3-((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)ureido)benzenesulfonamide (9b). Compound **9b** was obtained according to the **general procedure C** using **9a** as starting material, to afford the title compound **9b** as a yellow solid. 70% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.72 (2H, m, C*H*$_2$), 3.68 (2H, m, C*H*$_2$), 4.24 (1H, q, *J*= 4.1, C*H*), 4.41 (2H, d, *J*= 5.6, C*H*$_2$), 5.30 (1H, t, *J*= 5.2, exchange with D_2O, O*H*), 5.39 (1H, m, C*H*), 6.45 (1H, t, *J*= 6.6, C*H*), 6.75 (1H, t, *J* = 5.7, exchange with D_2O, N*H*), 7.33 (2H, s, exchange with D_2O, SO_2NH_2), 7.42 (2H, m, 2x Ar-*H*), 7.56 (1H, d, *J* = 8.2, Ar-*H*), 7.84 (1H, s, C*H*), 8.03 (1H, d, *J*= 2.0, Ar-*H*), 8.19 (1H, s, C*H*), 8.96 (1H, br s, exchange with D_2O, N*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 35.7, 38.0, 60.0, 61.6, 84.7, 85.3, 110.5, 115.5, 119.1, 121.4, 123.3, 130.1, 137.1, 141.7, 145.4, 146.6, 151.3, 155.7, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{25}N_8O_7S$= 521.1561, found 521.1556.

4-(3-(prop-2-yn-1-yl)ureido)benzenesulfonamide (10a). Compound **10a** was synthetized according to the **general procedure B** using phenyl (4-sulfamoylphenyl)carbamate **10** as starting material. White solid, 60% yield; δ_H (400 MHz, DMSO-d_6): 3.14 (1H, t, *J*= 2.4, C*H*), 3.37 (2H, d, *J*= 2.4, C*H*$_2$), 6.67, (1H, brt, exchange with D_2O, N*H*), 7.19 (2H, brs, exchange with D_2O, SO_2NH_2), 7.58 (2H, d, *J*= 8.2, 2x Ar*H*), 7.69 (2H, d, *J*= 8.2, 2x Ar*H*), 9.04 (1H, brs, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 29.7, 73.8, 82.8, 118.0, 127.7, 137.3, 144.2, 155.5; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{10}H_{12}N_3O_3S$= 254.0594, found 254.0593.

4-(3-((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)ureido)benzenesulfonamide (10b).

Compound **10b** was obtained according to the **general procedure C** using **10a** as starting material, to afford the title compound **10b** as a yellow solid. 47% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.71 (2H, m, C*H*$_2$), 3.68 (2H, m, C*H*$_2$), 4.24 (1H, q, *J*= 3.9, C*H*), 4.41 (2H, d, *J*= 5.6, C*H*$_2$), 5.30 (1H, t, *J*= 5.2, exchange with D_2O, O*H*), 5.39 (1H, dt, *J*= 8.3, 5.4, C*H*), 6.45 (1H, t, *J*= 6.6, C*H*), 6.84 (1H, t, *J* = 5.7, exchange with D_2O, N*H*), 7.18 (2H, s, exchange with D_2O, SO_2NH_2), 7.59 (2H, d, *J* = 8.9, 2x Ar-*H*), 7.71 (2H, d, *J* = 8.8, Ar-*H*), 7.84 (1H, s, C*H*), 8.20 (1H, s, C*H*), 9.04 (1H, brs, exchange with D_2O, N*H*), 11.4 (1H, brs, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 35.7, 38.0, 60.0, 61.6, 84.7, 85.3, 110.5, 117.8, 123.2, 127.6, 137.8, 141.5, 144.3, 146.4, 151.3, 155.6, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{25}N_8O_7S$= 521.1561, found 521.1554.

4-(prop-2-yn-1-ylselanyl)aniline (D). 4-selenocyanatoaniline **C** (1 eq.) was dissolved in EtOH and $NaBH_4$ (4 eq.) was added. The reaction was stirred for 20 min. Then, propargyl bromide (1.2 eq.) was added and the reaction stirred until consumption of starting material. The reaction was quenched with NH_4Cl satured solution, extracted with EtOAc (x3), dried over Na_2SO_4, filtered and concentrated in reduced pressure to give desired product **D** as yellow solid. 79% yield; δ_H (400 MHz, DMSO-d_6): 2.26 (1H, s, C*H*), 3.34 (2H, d, *J*= 2.4, C*H*$_2$), 3.76 (2H, brs, exchange with D_2O, N*H*$_2$), 6.59 (2H, d, *J*= 8.3, 2x Ar*H*), 7.44 (2H, d, *J*= 8.3, 2x Ar*H*); δ_C(100 MHz, DMSO-d_6): 13.7, 71.9, 81.7, 115.8, 116.2, 136.8, 147.2; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_9H_{10}NSe$= 211.9973, found 211.9969.

1-(4-(4-(((4-aminophenyl)selanyl)methyl)-1H-1,2,3-triazol-1-yl)-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (E). Synthetized according to the **general procedure C** using **4-(prop-2-yn-1-ylselanyl)aniline D** as starting material. Yellow solid. 20% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.69 (2H, m, C*H*$_2$), 3.67 (2H, m, C*H*$_2$), 4.04 (2H, s, C*H*$_2$), 4.16 (2H, m, 2x C*H*), 5.34 (3H, brs, exchange with D_2O, 1x O*H,* 2x *NH*$_2$), 6.43 (1H, t, *J*= 6.6, C*H*), 6.49 (2H, d, *J* = 7.9, 2x Ar*H*), 7.13 (2H, d, *J* = 7.9, 2x Ar*H*), 7.84 (1H, s, C*H*), 7.96 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.2, 22.5,

38.0, 60.0, 61.5, 84.7, 85.3, 110.5, 113.5, 115.4, 123.6, 136.9, 137.1, 146.1, 149.2, 151.3, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{19}H_{23}N_6O_4Se$= 479.0942, found 479.0932.

3-(3-(4-(((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)selanyl)phenyl)thioureido)benzenesulfonamide (11b). Compound **11b** was obtained reacting compound **E** (1 eq.) dissolved in CH_3CN with 3-isothiocyanatobenzenesulfonamide **11a** (1.1 eq.). The reaction was stirred o.n., then quenched with H_2O, assisting to the formation of a yellow precipitate, that was filtered to afford the crude product. Purified by silica gel column chromatography, eluting with 8% MeOH/DCM, to obtain the title compound **11b** as a white solid. 15% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.72 (2H, m, C*H*$_2$), 3.62 (2H, m, C*H*$_2$), 4.14 (1H, s, C*H*), 4.23 (2H, s, C*H*$_2$), 5.31 (1H, m, exchange with D_2O, O*H*), 5.38 (1H, m, C*H*), 6.38 (1H, t, *J*= 6.3, C*H*), 7.49 (8H, m, exchange with D_2O, 2x SO_2NH_2, 4x Ar-*H* , 2x Ar-*H*,), 7.75 (1H, d, *J* = 7.9, Ar-*H*), 7.84 (1H, s, C*H*), 8.00 (1H, s, Ar-*H*), 8.11 (1H, s, C*H*), 10.16 (2H, brs, exchange with D_2O, 2x N*H*), 11.37 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 20.9, 37.9, 60.0, 61.6, 84.7, 85.3, 110.5, 121.4, 122.3, 123.6, 125.1, 126.2, 127.7, 129.8, 133.4, 137.1, 139.2, 140.9, 145.1, 145.5, 151.3, 164.6, 180.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{26}H_{29}N_8O_6S_2Se$= 693.0812, found 693.0822.

4-(3-(4-(((1-(2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)selanyl)phenyl)thioureido)benzenesulfonamide (12b). Compound **12b** was obtained reacting compound **E** (1 eq.) dissolved in CH_3CN with 4-isothiocyanatobenzenesulfonamide **12a** (1.1 eq.). The reaction was stirred o.n., then quenched with H_2O, assisting to the formation of a yellow precipitate, that was filtered to afford the crude product. Purified by silica gel column chromatography, eluting with 8% MeOH/DCM, to obtain the title compound **12b** as a white solid. 60% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, C*H*$_3$), 2.69 (2H, m, C*H*$_2$), 3.67 (2H, m, C*H*$_2$), 4.18

(1H, dd, *J* = 3.2, 5.7, C*H*), 4.28 (2H, s, CH_2), 5.31 (1H, t, *J* = 5.3, exchange with D_2O, O*H*), 5.34 (1H, m, C*H*), 6.43 (1H, t, *J*= 6.6, C*H*), 7.33 (2H, s, exchange with D_2O, SO_2NH_2), 7.50 (4H, m, 4x Ar-*H*), 7.72 (2H, d, *J* = 8.7, 2x Ar-*H*), 7.80 (2H, d, *J* = 8.7, 2x Ar-*H*), 7.84 (1H, s, C*H*), 8.11 (1H, s, C*H*), 10.10 (1H, brs, exchange with D_2O, N*H*), 10.15 (1H, brs, exchange with D_2O, N*H*), 11.39 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 20.9, 38.0, 60.0, 61.5, 84.7, 85.3, 110.5, 123.5, 125.1, 126.3, 127.1, 128.3, 133.3, 137.1, 139.2, 140.3, 143.5, 145.5, 151.3, 164.6, 180.3; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{26}H_{29}N_8O_6S_2Se$= 693.0812, found 693.0819.

2-oxo-2,3-dihydrobenzo[d]oxazole-5-sulfonamide (13a). A solution of 3-amino-4-hydroxybenzenesulfonamide **13** (2.76 g, 1.0 eq) in dry THF (90 mL) was treated with drop-wise phosgene solution (~20% in toluene, 1.2 eq) at 0 °C then reaction was warmed to rt and stirred over night. After the consumption of the starting material (TLC monitoring) the reaction was quenched with slush, and acidified with 1M aqueous solution of HCl, extracted with EtOAc (3 x 20 ml) and the combined organic layers were washed with H_2O (3 x 20 ml), dried over Na_2SO_4, filtered and concentrated in reduced pressure to give desired product as a brown solid. 83% yield; δ_H (400 MHz, DMSO-d_6) 7.41 (2H, s, exchange with D_2O, SO_2NH_2), 7.49 (1H, d, *J* = 8.4, Ar-*H*), 7.52 (1H, d, *J* = 1.9, Ar-*H*), 7.60 (1H, dd, *J* = 1.9, 8.4, Ar-*H*), 12.03 (1H, s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6): 108.2, 110.5, 121.0, 131.6, 140.8, 146.3, 155.1; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_7H_7N_2O_4S$= 215.0121, found 215.0122.

N,N-dimethyl-N'-((2-oxo-2,3-dihydrobenzo[d]oxazol-5-yl)sulfonyl)formimidamide (13b). A solution of **13a** (6.27g, 1.0 eq) in DMF (5 ml) was cooled to 0 °C then treated with *N*,*N*-dimethylformamide dimethyl acetal (1.2 eq). The reaction continued until the consumption of starting material (TLC monitoring). The reaction was quenched with slush to obtain a precipitate that was filtered and washed with water (3 x 5 ml) and dried under vacuum to afford **13 b** as white solid. 41% yield; δ_H (400 MHz, DMSO-d_6) 2.93 (3H, t, *J* = 0.6, CH_3), 3.17 (3H, t, *J* = 0.6, CH_3), 7.43 (2H, m, 2x Ar-*H*), 7.55 (1H, dd, *J* = 1.8, 8.4, Ar-*H*), 8.25 (1H, s, C*H*), 12.01 (1H, s, exchange with D_2O, N*H*);

δ_C (100 MHz, DMSO-d_6) 35.9, 41.8, 108.3, 110.5, 121.3, 131.6, 139.6, 146.3, 155.1, 160.7; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{10}H_{12}N_3O_4S$= 270.0543, found 270.0538.

N-((dimethylamino)methyl)-2-oxo-3-(prop-2-yn-1-yl)-2,3-dihydrobenzo[d]oxazole-5-sulfonamide (13c). Compound **13b** (2.0g, 1.0 eq) was treated with potassium carbonate (1.0 eq) in dry DMF (5ml) and the suspension was stirred at r.t. for 20 min. Then propargyl bromide (1.2 eq) was added and the reaction was stirred at r.t. until starting material was consumed (TLC monitoring). The reaction was quenched with slush and the precipitate formed was collected by filtration and washed with Et_2O (3 x 5 ml) and dried under vacuum to obtain desired compound as brown solid. 90% yield; δ_H (400 MHz, DMSO-d_6) 2.94 (3H, t, *J* = 0.7, C*H*$_3$), 3.19 (3H, t, *J* = 0.6, C*H*$_3$), 3.53 (1H, t, *J* = 2.5, C*H*), 4.84 (2H, d, *J* = 2.5, C*H*$_2$), 7.54 (1H, d, *J* = 8.4, Ar-*H*), 7.65 (1H, dd, *J* = 1.8, 8.4, Ar-*H*), 7.80 (1H, d, *J* = 1.8, Ar-*H*), 8.27 (1H, s, C*H*); δ_C(100 MHz, DMSO-d_6): 32.6, 36.0, 41.8, 77.0, 77.7, 108.5, 111.0, 122.1, 131.3, 140.1, 144.8, 153.8, 160.7; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{13}H_{14}N_3O_4S$= 308.0700, found 308.0698.

2-oxo-3-(prop-2-yn-1-yl)-2,3-dihydrobenzo[d]oxazole-5-sulfonamide (13d). Compound **13c** (3.4 g, 1.0 eq) was dissolved in a 1.5 M HCl in MeOH solution (30 ml) and the reaction was stirred at 60 °C in a sealed tube for 4 h, concentrated under vacuum to give a precipitate that was washed water (3x 5 ml) then with Et_2O (3 x 5 ml) and dried under vacuum to afford desired product as a brown solid. 55% yield; δ_H (400 MHz, DMSO-d_6) 3.53 (1H, t, *J* = 2.5, C*H*), 4.81 (2H, d, *J* = 2.5, C*H*$_2$), 7.49 (2H, s, exchange with D_2O, SO_2NH_2), 7.61 (1H, d, *J* = 8.4, Ar-*H*), 7.72 (1H, dd, *J* = 1.9, 8.4, Ar-*H*), 7.83 (1H, d, *J* = 1.9, Ar-*H*); δ_C(100 MHz, DMSO-d_6): 32.7, 35.0, 77.3, 108.2, 111.1, 122.0, 131.1, 141.3, 144.8, 153.9; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{10}H_9N_2O_4S$= 253.0278, found 253.0280.

3-((1-(2-(Hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl)-1H-1,2,3-triazol-4-yl)methyl)-2-oxo-2,3-dihydrobenzo[d]oxazole-5-sulfonamide (13e). Compound **13e** was obtained according to the **general procedure C** using **13d**

as starting material, to afford the title compound **13e** as a white solid. 53% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 2.70 (2H, m, CH_2), 3.68 (2H, m, CH_2), 4.07 (1H, m, C*H*), 4.22 (1H, d, *J* = 5.4, C*H*), 5.22 (2H, s, CH_2), 5.30 (1H, brt, exchange with D_2O, O*H*), 6.44 (1H, t, *J*= 6.6, C*H*), 7.46 (2H, s, exchange with D_2O, SO_2NH_2), 7.59 (1H, d, *J* = 8.4, Ar-*H*), 7.67 (1H, m, Ar-*H*), 7.78 (1H, s, Ar-*H*), 7.84 (1H, s, C*H*), 8.44 (1H, s, C*H*), 11.39 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6): 13.1, 37.9, 38.3, 60.3, 61.6, 84.8, 85.3, 108.1, 110.5, 110.8, 121.6, 124.3, 131.8, 137.0, 141.1, 142.1, 144.8, 151.3, 154.4, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{20}H_{22}N_7O_8S$= 520.1245, found 520.1236.

4-(hex-5-yn-1-yloxy)-2H-chromen-2-one (14a) was synthetized according to the **general procedure A** using 4-hydroxy-2H-chromen-2-one **14** as starting material and 6-chlorohex-1-yne as alkyl halide at 100°C. Compound **14a** obtained as white powder. 80% yield; δ_H (400 MHz, DMSO-d_6): 1.70 (2H, m, CH_2), 1.95 (2H, m, CH_2), 2.31 (2H, m, CH_2), 2.84 (1H, t, *J*=2.5, C*H*), 4.28 (2H, t, *J*=6.2, CH_2), 5.92 (1H, s, Ar-*H*), 7.39 (1H, d, *J*=7.8, Ar-*H*), 7.43 (1H, d, *J*=8.1, Ar-*H*), 7.69 (1H, t, *J*=8.1, Ar-*H*), 7.85 (1H, d, *J*=7.8, Ar-*H*); δ_C (100 MHz, DMSO-d_6): 18.4, 25.6, 28.1, 70.0, 72.5, 85.2, 91.5, 116.3, 117.5, 123.8, 125.2, 133.7, 153.8, 162.7, 165.9; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{15}H_{15}O_3$= 243.1016, found 243.1016.

1-(5-(hydroxymethyl)-4-(4-(4-((2-oxo-2H-chromen-4-yl)oxy)butyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (14b). Compound **14b** was obtained according to the **general procedure C** using **14a** as starting material, to afford the title compound **14b** as a white solid. 45% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 1.89 (2H, m, 2 x CH_2), 2.70 (2H, m, CH_2), 2.78 (2H, m, CH_2), 3.68 (2H, m, CH_2), 4.22 (1H, m, C*H*), 4.29 (2H, m, CH_2), 5.34 (2H, m, 1x C*H*, exchange with D_2O, 1x O*H*), 5.93 (1H, br s, Ar-*H*), 6.45 (1H, br t, C*H*), 7.41 (2H, m, 1x Ar-*H*, 1x C*H*), 7.70 (1H, t, *J*= 7.9, Ar-*H*), 7.86 (2H, m, 1x Ar-*H,* 1x C*H*), 8.13 (1H, s, Ar-*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d_6):13.2, 25.5, 26.2, 28.4, 38.0, 59.9, 61.7, 70.1, 84.8, 85.4, 91.4, 110.5, 116.2, 117.4, 122.5, 123.7, 125.1, 133.6, 137.1,

147.8, 151.3, 153.7, 162.6, 164.6, 165.9; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{25}H_{28}N_5O_7$= 510.1983, found 510.1989.

6-(Prop-2-ynyloxy)-2*H*-chromen-2-one (15a) was synthetized according to the **general procedure A** using 6-hydroxy-2H-chromen-2-one **15** as starting material and propargyl bromide 80% in toluene as alkyl halide. Reaction performed at room temperature. Compound **15a** obtained as a white powder: 65% yield; δ_H (400 MHz, DMSO-d_6) 3.64 (1H, br t, C*H*), 4.90 (2H, d, *J* = 2.1, CH_2), 6.54 (1H, d, *J* = 9.6, Ar-*H*), 7.30 (1H, dd, *J* = 2.9, 9.0, Ar-*H*), 7.38 (1H, d, *J* = 2.9, Ar-*H*), 7.41 (1H, d, *J* = 9.0, Ar-*H*), 8.06 (1H, d, *J* = 9.6, Ar-*H*); δ_C(100 MHz, DMSO-d_6): 56.0, 78.6, 78.9, 112.3, 116.8, 117.4, 119.2, 120.0, 144.0, 148.3, 153.4, 160.1; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{12}H_9O_3$= 201.0546, found 201.0543. Experimental in agreement with reported data.[68]

1-(5-(hydroxymethyl)-4-(4-(((2-oxo-2H-chromen-6-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (15b). Compound **15b** was obtained according to the **general procedure C** using **15a** as starting material, to afford the title compound **15b** as a white solid. 91% yield; δ_H (400 MHz, DMSO-d_6): 1.85 (3H, s, CH_3), 2.74 (2H, m, CH_2), 3.70 (2H, m, CH_2), 4.26 (1H, q, *J*= 3.5, C*H*), 5.25 (2H, s, CH_2), 5.45 (2H, m, overlapped signals, 1 x C*H*, exchange with D_2O, 1 x O*H*), 6.47 (1 H, t, *J*= 6.5, C*H*), 6.53 (1H, d, *J*= 9.6, Ar*H*), 7.33 (1H, dd, *J*= 2.8, 9.0, Ar*H*), 7.39 (1H, d, *J*= 9.0, Ar*H*), 7.48 (1H, d, *J*= 2.8, Ar*H*), 7.88 (1H, s, C*H*), 8.07 (1H, d, *J*= 9.6, Ar*H*), 8.54 (1H, s, C*H*), 11.37 (1H, br s, exchange with D_2O, N*H*). δ_C(100 MHz, DMSO-d_6):13.1, 38.0, 60.3, 61.6, 62.6, 84.8, 85.4, 110.5, 112.9, 117.5, 118.3, 120.1, 120.9, 125.4, 137.1, 143.4, 144.9, 148.9, 151.3, 155.2, 161.0, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{22}H_{22}N_5O_7$= 468.1514, found 468.1508.[69]

6-(pent-4-yn-1-yloxy)-2H-chromen-2-one (16a). Compound **16a** was synthetized according to the **general procedure A** using 6-hydroxy-2H-chromen-2-one **15** as starting material and 5-chloropent-1-yne as alkyl halide, at 100°C. Compound **16a** obtained as white powder. 79% yield; δ_H (400 MHz, DMSO-d_6): 1.94 (2H, m, CH_2), 2.38 (2H, dd, *J*= 4.9, 6.8, CH_2), 2.87 (1H, s, C*H*), 4.11 (2H, t, *J*=6.1,

C*H*$_2$), 6.53 (1H, d, *J*=9.6, Ar-*H*), 7.24 (1H, dd, *J*=2.6, 9.0, Ar-*H*), 7.34 (1H, d, *J*=2.4, Ar-*H*), 7.37 (1H, d, *J*=9.0, Ar-*H*), 8.04 (1H, d, *J*=9.6, Ar-*H*); δ_C(100 MHz, DMSO-d$_6$):15.6, 28.7, 67.7, 72.7, 84.6, 112.5, 117.6, 118.4, 120.3, 120.9, 145.1, 148.9, 155.9, 161.2; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{14}H_{13}O_3$= 229.0859, found 229.0861. Experimental in agreement with reported data.[70]

1-(5-(hydroxymethyl)-4-(4-(3-((2-oxo-2H-chromen-6-yl)oxy)propyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (16b). Compound **16b** was obtained according to the **general procedure C** using **16a** as starting material, to afford the title compound **16b** as a white solid. 27% yield; δ_H (400 MHz, DMSO-d$_6$): 1.84 (3H, s, C*H*$_3$), 2.14 (2H, q, *J*= 6.8, C*H*$_2$), 2.71 (2H, m, C*H*$_2$), 2.85 (2H, t, *J*= 7.5, C*H*$_2$), 3.69 (2H, m, C*H*$_2$), 4.12 (2H, t, *J*= 6.3, C*H*$_2$), 4.23 (1H, q, *J*= 4.1, C*H*), 5.34 (2H, m, 1x C*H*, exchange with D_2O, 1x O*H*), 6.45 (1H, t, *J*= 6.6, C*H*), 6.52 (1H, d, *J*= 9.5, 1x Ar-*H*), 7.25 (1H, dd, *J*=2.9, 9.0, 1x Ar-*H*), 7.32 (1H, d, *J*= 2.9, 1x Ar-*H*), 7.38 (1H, d, *J*= 8.9, 1x Ar-*H*), 7.85 (1H, s, C*H*), 8.04 (1H, d, *J*= 9.5, 1x Ar-*H*), 8.15 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H*); δ_C(100 MHz, DMSO-d$_6$):13.1, 22.5, 29.2, 38.0, 59.9, 61.6, 68.3, 84.8, 85.4, 110.5, 112.3, 117.4, 118.2, 120.1, 120.8, 122.5, 137.1, 144.9, 147.3, 148.7, 151.3, 155.8, 161.0, 164.1; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{24}H_{26}N_5O_7$= 496.1827, found 496.1830.

7-(Prop-2-ynyloxy)-2*H*-chromen-2-one (17a). Compound **17a** was synthetized according to the **general procedure A** using 7-hydroxy-2H-chromen-2-one **17** as starting material and propargyl bromide 80% in toluene as alkyl halide. Reaction performed at room temperature. Compound **17a** obtained as a white powder.73% yield; δ_H (400 MHz, DMSO-d$_6$) 3.69 (1H, t, *J*= 2.4, C*H*), 4.97 (2H, d, *J*= 2.4, C*H*$_2$), 6.36 (1H, d, *J*= 9.6, Ar-*H*), 7.03 (1H, dd, *J*= 2.4, 8.6, Ar-*H*), 7.09 (1H, d, *J*= 2.4, Ar-*H*), 7.70 (1H, d, *J*= 8.6, Ar-*H*), 8.04 (1H, d, *J*= 9.6, Ar-*H*). Experimental in agreement with reported data.[70]

1-(5-(hydroxymethyl)-4-(4-(((2-oxo-2H-chromen-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (17b). Compound **17b** was

obtained according to the **general procedure C** using **17a** as starting material, to afford the title compound **17b** as a white solid. 91% yield; δ_H (400 MHz, DMSO-d_6): 1.85 (3H, s, CH_3), 2.73 (2H, m, CH_2), 3.69 (2H, m, CH_2), 4.27 (1H, q, *J*= 3.5, C*H*), 5.32 (2H, s, CH_2), 5.36 (1H, t, *J*= 5.0, exchange with D_2O, O*H*), 5.46 (1 H, m, C*H*), 6.34 (1H, d, *J*= 9.5, Ar*H*), 6.47 (1 H, t, *J*= 6.5, C*H*), 7.07 (1H, dd, *J*= 2.4, 8.6, Ar*H*), 7.21 (1H, d, *J*= 2.4, Ar*H*), 7.69 (1H, d, *J*= 8.6, Ar*H*), 7.86 (1H, s, C*H*), 8.04 (1H, d, *J*= 9.5, Ar*H*), 8.53 (1H, s, C*H*), 11.38 (1H, br s, exchange with D_2O, N*H*). δ_C(100 MHz, DMSO-d_6): 13.1, 38.1, 60.4, 61.7, 62.6, 84.9, 85.5, 102.5, 110.6, 113.5, 113.6, 113.8, 125.7, 130.5, 137.2, 143.1, 145.2, 151.4, 156.2, 161.2, 162.0, 164.0; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{22}H_{22}N_5O_7$= 468.1514, found 468.1520.[51]

7-(pent-4-yn-1-yloxy)-2H-chromen-2-one (18a). Compound **18a** was synthetized according to the **general procedure A** using 7-hydroxy-2H-chromen-2-one **17** as starting material and 5-chloropent-1-yne as alkyl halide, at 100°C. Compound **18a** obtained as white powder. 71% yield; δ_H (400 MHz, DMSO-d_6): 1.95 (2H, m, CH_2), 2.38 (2H, m, CH_2), 2.88 (1H, d, *J*=1.8, C*H*), 4.18 (2H, t, *J*=6.0, CH_2), 6.33 (1H, d, *J*=9.4, Ar-*H*), 6.99 (1H, d, *J*=8.6, Ar-*H*), 7.04 (1H, s, Ar-*H)*, 7.67 (1H, d, *J*=8.5, Ar-*H*), 8.03 (1H, d, *J*=9.5, Ar-*H*); δ_C (100 MHz, DMSO-d_6): 15.5, 28.5, 67.8, 72.7, 84.5, 102.2, 113.4, 113.5, 113.7, 130.6, 145.3, 156.4, 161.3, 162.7; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{14}H_{13}O_3$= 229.0859, found 229.0857.

1-(5-(hydroxymethyl)-4-(4-(3-((2-oxo-2H-chromen-7-yl)oxy)propyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (18b). Compound **18b** was obtained according to the **general procedure C** using **18a** as starting material, to afford the title compound **18b** as a white solid. 21% yield; δ_H (400 MHz, DMSO-d_6): 1.84 (3H, s, CH_3), 2.13 (2H, m, CH_2), 2.70 (2H, m, CH_2), 2.86 (2H, m, CH_2), 3.69 (2H, m, CH_2), 4.21 (3H, m, 1x CH_2, 1x C*H*), 5.34 (2H, m, 1x C*H*, exchange with D_2O, 1x O*H*), 6.32 (1H, d, *J*= 9.47, 1x Ar-*H*), 6.45 (1H, m, C*H*), 7.01 (2H, m, 1x C*H*, 1x Ar-*H*), 7.66 (1H, m, Ar-*H*), 7.85 (1H, s, C*H*), 8.03 (1H, m, Ar-*H*), 8.15 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H);* δ_C(100 MHz, DMSO-d_6):13.2, 22.4, 29.1, 38.0,

59.9, 61.6, 68.4, 84.8, 85.4, 102.1, 110.5, 113.2, 113.3, 113.6, 122.6, 130.4, 137.1, 145.2, 147.3, 151.3, 156.3, 161.2, 162.7, 164.1; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{24}H_{26}N_5O_7$= 496.1827, found 496.2000.

7-(hex-5-yn-1-yloxy)-2H-chromen-2-one (19a). Compound **19a** was synthetized according to the **general procedure A** using 7-hydroxy-2H-chromen-2-one **17** as starting material and 6-chlorohex-1-yne as alkyl halide, at 100°C. Compound **19a** obtained as white powder. 80 % yield; δ_H (400 MHz, DMSO-d_6): 1.64 (2H, m, CH_2), 1.86 (2H, m, CH_2), 2.28 (2H, m, CH_2), 2.82 (1H, t, *J*=2.5, C*H*), 4.13 (2H, t, *J*=6.4, CH_2), 6.31 (1H, d, *J*=9.5, Ar-*H*), 6.97 (1H, dd, *J*=2.1, 8.6, Ar-*H*), 7.01 (1H, d, *J*=2.1, Ar-*H*), 7.65 (1H, d, *J*=8.6, Ar-*H*), 8.02 (1H, d, *J*=9.5, Ar-*H*); δ_C(100 MHz, DMSO-d_6):18.7, 25.9, 28.8, 68.8, 72.4, 85.3, 102.3, 113.3, 113.4, 113.7, 130.5, 145.3, 156.5, 161.3, 162.9; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{15}H_{15}O_3$= 243.1016, found 243.1012.

1-(5-(hydroxymethyl)-4-(4-(4-((2-oxo-2H-chromen-7-yl)oxy)butyl)-1H-1,2,3-triazol-1-yl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (19b). Compound **19b** was obtained according to the **general procedure C** using **19a** as starting material, to afford the title compound **19b** as a white solid. 21% yield; δ_H (400 MHz, DMSO-d_6): 1.83 (7H, m, 2x CH_2, 1x CH_3), 2.70 (2H, m, CH_2), 2.76 (2H, m, CH_2), 3.71 (2H, m, CH_2), 4.15 (2H, m, CH_2), 4.23 (1H, m, C*H*), 5.34 (2H, m, 1x C*H*, exchange with D_2O, 1x O*H*), 6.31 (1H, d, *J*= 9.4, Ar-*H*), 6.45 (1H, t, *J*= 6.5, C*H*), 6.96 (1H, m, Ar-*H*), 7.0 (1H, m, Ar-*H*), 7.64 (1H, d, *J*= 8.6, 1x Ar-*H*), 7.85 (1H, s, C*H*), 8.01 (1H, d, *J*= 9.4, 1x Ar-*H*), 8.11 (1H, s, C*H*), 11.4 (1H, br s, exchange with D_2O, N*H*). δ_C(100 MHz, DMSO-d_6):13.2, 25.6, 26.3, 28.9, 38.0, 59.9, 61.7, 68.9, 84.8, 85.4, 102.0, 110.5, 113.2, 113.4, 113.6, 122.4, 130.4, 137.1, 145.2, 147.8, 151.3, 156.3, 161.2, 162.7, 164.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{25}H_{28}N_5O_7$= 510.1983, found 510.1989.

6-Prop-2-ynyloxy-benzo-[e][1,2]-oxathiine 2,2-dioxide (20a). Compound **20a** was synthetized according to the **general procedure A** using 6-hydroxybenzo[e][1,2]oxathiine 2,2-dioxide **20** as starting material and propargyl bromide 80% in toluene as alkyl halide. Reaction performed at room

temperature. Compound **20a** obtained as a white powder, pure: 85% yield; δ_H (400 MHz, DMSO-d_6): 3.66 (1H, t, *J* = 2.4, C*H*), 4.90 (2H, d, *J* = 2.4, CH_2), 7.24 (1H, dd, *J* = 3.0, 9.0, Ar-*H*), 7.38 (1H, d, *J* = 3.0, Ar-*H*), 7.45 (1H, d, *J* = 9.0, Ar-*H*), 7.55 (1H, d, *J* = 10.3, Ar-*H*), 7.68 (1H, d, *J* = 10.3, Ar-*H*); δ_C(100 MHz, DMSO-d_6): 56.1, 78.8, 78.9, 115.2, 119.1, 119.6, 119.7, 123.3, 136.4, 145.0, 154.6; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{11}H_9O_4S$= 237.0216, found 237.0212. Experimental in agreement with reported data.[68]

1-(4-(4-(((2,2-dioxidobenzo[e][1,2]oxathiin-6-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione (20b). Compound **20b** was obtained according to the **general procedure C** using **20a** as starting material, to afford the title compound **20b** as a white solid. 78% yield; δ_H (400 MHz, DMSO-d_6): 1.85 (3H, s, CH_3), 2.74 (2H, m, CH_2), 3.70 (2H, m, CH_2), 4.26 (1H, q, *J*= 3.5, C*H*), 5.26 (2H, s, CH_2), 5.37 (1H, br t, exchange with D_2O, O*H*), 5.45 (1H, m, C*H*), 6.47 (1H, t, *J*= 6.5, C*H*), 7.29 (1H, dd, *J*= 3.0, 9.0, Ar*H*), 7.44 (1H, d, *J*= 9.0, Ar*H*), 7.47 (1H, d, *J*= 3.0, Ar*H*), 7.54 (1H, d, *J*= 10.3, Ar*H*), 7.68 (1H, d, *J*= 10.3, Ar*H*), 7.87 (1H, s, C*H*), 8.51 (1H, s, C*H*), 11.38 (1H, br s, exchange with D_2O, N*H*). δ_C(100 MHz, DMSO-d_6):13.1, 38.0, 60.3, 61.6, 62.7, 84.8, 85.4, 110.5, 115.7, 119.9, 120.4, 120.5, 124.0, 125.5, 137.1, 137.3, 143.3, 145.6, 151.4, 156.4, 164.9; ESI-HRMS (*m/z*) calculated for $[M+H]^+$ ion species $C_{21}H_{22}N_5O_8S$= 504.1184, found 504.1183.

CA inhibition.

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[53] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six

traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min for sulfonamide derivatives and 6 h for coumarin and sulfocoumarin derivatives 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, and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[53]

Co-crystallization and X-ray Data Collection.

Crystals of hCA II were obtained using the hanging drop vapor diffusion method using 24 well Linbro plate. 2 µl of 10 mg/ml solution of hCA II in Tris-HCl 20 mM pH 8.0 were mixed with 2 µl of a solution of 1.5 M sodium citrate, 0.1 M Tris pH 8.0 and were equilibrated against the same solution at 296 K. Crystals of the protein grew in one week. Afterwards hCAII crystals were soaked in 5mM inhibitor solution for 3 days. The crystals were flash-frozen at 100K using a solution obtained by adding 15% (v/v) glycerol to the mother liquor solution as cryoprotectant. Data on crystal of the complex with **1b** was collected using synchrotron radiation at the ID-11.2C beamline at Elettra (Trieste, Italy) with a wavelength of 1.000 Å and a Pilatus3_6M Dectris CCD detector. Data on crystal of the complex with **3b** was collected using synchrotron radiation at the MX1 beamline of the Australian Synchrotron. Data were integrated and scaled using the program XDS.[71]

Structure Determination.

The crystal structure of hCA II (PDB accession code: 3P58) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5.[72] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree

calculations. The initial |Fo - Fc| difference electron density maps unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40.[73] Refinements proceeded using normal protocols of positional, isotropic atomic displacement parameters alternating with manual building of the models using COOT.[74] Solvent molecules were introduced automatically using the program ARP.[75] The quality of the final models was assessed with COOT and RAMPAGE.[76] Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6YPW, 6WKA). Graphical representations were generated with Chimera.[77]

***In vitro* Telomerase Activity Assay.**

Human prostate cancer PC3 and human colorectal adenocarcinoma HT-29 cell lines (both from ATCC, Manassas, VA) were cultivated in RPMI-1640 cell media supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UK) at 37°C in the presence of 5% CO_2 and 95% humidity. Cell lines have been tested for mycoplasma contamination before the experiment using Mycoplasma Detection Kit PlasmoTest™ (InvivoGen, San Diego, CA). The most potent CA IX and XII inhibitors **1b**, **7b**, **8b** or **11b** were diluted to final concentration 20 μM and incubated with cells for 48 h. Telomerase activity was determined using the TRAP assay[15] with modifications previously described by us.[78,79] Briefly, cells were lysed in 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM 2-mercaptoethanol, 0.5% CHAPS and 10% glycerol (all from Sigma-Aldrich, St. Louis, MO) and centrifuged for 30 min at 12000xg. Supernatants were stored at −80 °C. The protein concentration of cell extracts was determined using BCA-1 Protein Assay Kit (Sigma-Aldrich, St. Louis, MO). For elongation reaction 5 μg of total protein and **CAI-TI** within the range of concentrations 0 – 100 μM were added to 30-μl of reaction mixture containing 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, 1 mM EGTA (all from Sigma-Aldrich, St. Louis, MO), 0.25 mM each dNTPs (Evrogen, Moscow, Russia) and Telomerase Substrate primer (TS-primer- AATCCGTCGAGCAGAGTT). Elongation was performed for 30 min at 37°C and 10

min at 96°C to inactivate the telomerase. Copy Extended primer 0.1 µl (CX-primer-CCCTTACCCTTACCCTTACCCTAA) and 2.5 Units of Taq-polymerase were added to the elongation mixture followed by the following PCR reaction: 94° C − 5 min; 30 cycles of 94° C − 30 s, 50 °C − 30 s, and 72° C − 40 s; and 72° C − 5 min. PCR product visualization was performed using 12% non-denaturing PAAG electrophoresis and TBE buffer. Ten microliters of each sample were added to each well of the gel comb. Gels were stained with SYBR Green I (Invitrogen, Grand Island, NY), photographed under UV light in a ChemiDoc™ XRS imaging system and analyzed using a GelAnalyzer 2010a. Statistical analysis involving the Student's t-test was implemented with the Statistica 6.0 software (StatSoft, Tulsa, OK). To determine IC_{50} and IC_{90} values (inhibitor concentration where the response is reduced by 50% and 90%, respectively) 1 µL of reaction mixture was subjected to Real-Time Quantitative Telomeric Repeat Amplification Protocol Assay (RTQ-TRAP) as described by Hou M. and co-authors.[80]

RNA isolation and real-time RT-PCR.

A previously described protocol was followed.[81] Briefly, total RNA from cells was extracted using a PureLink RNA Mini kit (Life Technologies, Carlsbad, CA). Five micrograms of total RNA were reverse-transcribed using the RevertAid RT Kit (Invitrogen, Grand Island, NY) in a 25 µl reaction mixture, followed by real-time RT-PCR using DTprime5 (DNA Technology, Protvino, Russia). The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Grand Island, NY) according to the manufacturer's recommendations using the following primers (5'-3'). hTERT sense: GTCCGAGGTGTCCCTGAGTA; hTERT antisense: CAGGGCCTCGTCTTCTACAG; 18S sense: GGATCCATTGGAGGGCAAGT; 18S antisense: ACGAGCTTTTTAACTGCAGCAA (all primers were from Evrogen, Moscow, Russia). Two temperature cycles for annealing/extension were used. The fluorescence was measured at the end of each annealing step, and the melting curve analysis was performed at the end of the reaction (after the 35th cycle), between 60°C and 95°C, to assess the quality of the final PCR products. The standard

curves indicating reaction effectiveness were generated using 4 serial dilutions (1:40, 1:80, 1:160 and 1:320) of total cDNAs. The relative level of hTERT mRNA was calculated using DTprime5 software. Levels of mRNA were normalized relative to the expression of the reference gene 18S. The data are presented as normalized mRNA levels of the studied genes, using the averaged expression values of the reference gene.

Statistics.

Telomerase Activity Assay and measurement of hTERT gene expression were performed in quadruplicate. Statistical analysis using Student's t-test was completed using Statistica 9.0 software (StatSoft, Tulsa, OK). Differences described as $p \leq 0.05$ were considered significant. The values of IC_{50} and IC_{90} were calculated using Prism 6 software (GraphPad, San Diego, CA) according to recommendations by Sebaugh J.L. and co-authors.[82]

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Telomerase assays and hTERT gene expression were performed by Dmitry D. Zhdanov (D.D.Z.) and Anna P. Kiryukhina (A.P.K.).

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Conflicts of Interest

The authors state no conflict of interest.

Abbreviation used

CA, carbonic anhydrase; CAI(s), carbonic anhydrase inhibitor(s); AAZ, acetazolamide; AZT, azidothymidine; TERT, telomerase reverse transcriptase; TERC, telomerase RNA component; RTQ-TRAP, Real-Time Quantitative Telomeric Repeat Amplification Protocol; RT, Reverse Transcriptase; EGTA, Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetracetic acid; PMSF, Phenylmethylsulfonylfluoride; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonium]-1-propanesulfonate hydrate.

ASSOCIATED CONTENT

Supporting information is available free of charge on the ACS Publications website: SMILES representation for compounds (CSV), Supporting Information file with Summary of Data Collection and Atomic Model Refinement Statistics, HPLC-DAD method for purity analysis and HPLC traces.

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Table of Contents graphic (TOC):