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Discovery of New Sulfonamide Carbonic Anhydrase IX Inhibitors Incorporating Nitrogenous Bases

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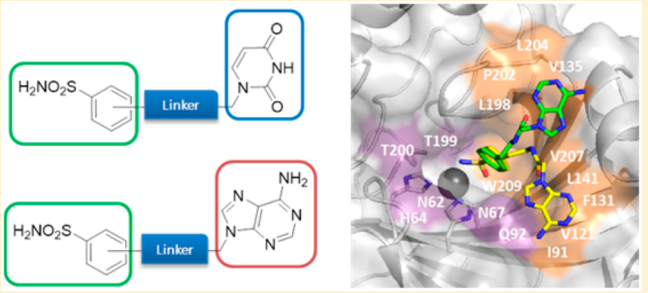
Supporting Information

ABSTRACT: Incorporation of the purine/pyrimidine moieties as tails to classical benzenesulfonamide scaffolds afforded two series of human (h) carbonic anhydrase (CA, EC 4.2.1.1) inhibitors. The compounds were designed according to the molecular hybridization approach, in order to modulate the interaction with different CA isozymes and exploit the antitumor effect of uracil and adenine derivatives in parallel and synergic mode to the inhibition of the tumor-associated hCA IX. The sulfonamides were investigated as inhibitors of four isoforms, cytosolic hCA I/II and transmembrane hCA IV/IX. The inhibitory profiles were dependent on the length and positioning of the spacer connecting the two pharmacophores. X-ray crystallography demonstrated the binding mode of an inhibitor to hCA II and hCA IX-mimic. Compounds endowed with the best hCA IX inhibitory efficacy were evaluated for antiproliferative activity against HT-29 colon cancer cell lines. The *in vitro* results suggest multiple mechanisms of action are responsible for the compounds' cytotoxic efficacy.



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Human carbonic anhydrase (CA, EC 4.2.1.1) isoform IX (hCA IX) is a validated anticancer drug target also recognized as a marker of tumor hypoxia and prognostic factor for several types of cancers.[1−4] CAs catalyze the reversible hydration of carbon dioxide to bicarbonate ion and a proton. This reaction is essential for a variety of physiological processes in humans, including respiration and transport of CO_2/HCO_3^-, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, and calcification.[5,6] In hypoxic tumors, the activity of extracellular hCA IX, whose expression is induced via the hypoxia inducible factor-1α (HIF-1α), maintains the external pH, supporting an acidic extracellular microenvironment suited for hypoxic tumor cell survival and proliferation, but detrimental to normal cells.[1] Since hCA IX plays a crucial role in pH regulation in several tumors (e.g., breast, brain, colorectal, etc.) and expression is limited in normal tissues, this isozyme has become an attractive target for the design of antineoplastic therapies.[3] Hence, it is not surprising that hCA IX has been the main focus of the past decade of research over other hCA isoforms. hCA IX belongs to the α-class CAs found in vertebrates, being a membrane-associated isoform.[1] Conversely, hCA I and II are ubiquitous, cytosolic isoforms and are drug targets for many diseases (such as cerebral and retinal edema, glaucoma, epilepsy, and probably altitude sickness).[7,8] Considering cancer therapies, hCA I and II are off-targets and are responsible for most of the side effects of nonselective inhibitors.[5,6] The membrane-associated isoform hCA IV, which shares with hCA IX the extra-cellular localization, is a drug target for retinitis pigmentosa and stroke, in addition to glaucoma, together with hCA II.[7,8]

The sulfonamides and their bioisosteres, such as sulfamates and sulfamides, are the most investigated class of hCA inhibitors (CAIs) and have been in clinical use for more than 70 years for the treatment of glaucoma, epilepsy, and obesity and as diuretics.[5] Since most of them are isoform nonspecific, a major issue in the therapeutic antitumor applications of hCA IX sulfonamide-based inhibitors is the lack of selectivity among the

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human isozymes because of the risk of a plethora of unwanted side effects.[5,9]

The main method applied for the design of isoform-selective CA inhibitors (CAIs) is the tail approach. This technique modulates the moieties appended to the aromatic/heterocyclic ring in the scaffold of the zinc-binding group (ZBG) in order to selectively promote interactions with isoform unique residues at the entrance of the active site cavity.[10−12] The extensive application of the tail approach over the last few decades has greatly enriched the database of CAIs, though eliciting only a rather limited number of derivatives showing relevant isoform-selectivity inhibition profiles (among the sulfonamide CAIs). However, in this context, recent studies from our group led to the discovery and clinical development of SLC-0111, a sulfonamide CAI that successfully completed Phase I clinical trials for the treatment of advanced, metastatic hypoxic tumors overexpressing hCA IX/XII, being scheduled for Phase II trials later this year.[13,14]

The purine and pyrimidine scaffolds are among the most abundant N-based heterocycles in nature and have been involved in many metabolic and cellular processes.[15,16] The pyrimidine and purine moieties are considered privileged structures in drug discovery with a wide array of synthetic accessibility and ability to confer drug-like properties to the compound libraries based on them.[15−18] Thus, nitrogenous bases such as uracil and adenine are components of a number of useful drugs and are associated with many biological, pharmaceutical, and therapeutic activities.[15−18] In particular, pyrimidines and purines were widely used as antitumor pharmacophores in medicinal chemical research, considering their ability to interfere with DNA function in diverse manners.[15,16] Modified pyrimidine nucleosides were among the first chemotherapeutic agents introduced into the medical treatment of cancer.[17] Antiviral and antitumor actions are two of the most widely reported activities of uracil analogues.[15] Moreover, many pyrimidine-like scaffolds have been developed that exhibit potent antitumor cytotoxic activity *in vitro* against different human cell lines, interfering with DNA synthesis in the case of tumors or RNA/DNA synthesis in antiviral activity.[18] Hence, reported here are two novel series of derivatives combining the benzenesulfonamide ZBG pharmacophore with uracil and adenine scaffolds. The compounds were investigated for their inhibition of cytosolic hCA I and II and transmembrane hCA IV and IX. In addition, X-ray crystallography demonstrates the binding mode of a nitrogenous base within the active site of hCA II and hCA IX-mimic. Finally, the most effective compounds were evaluated for their antiproliferative activity against HT-29 colon cancer cell lines.

The rationale of the presented work is the incorporation of the purine/pyrimidine pharmacophore as the tail of a classical CAI with a benzenesulfonamide ZBG scaffold (Figure 1). Uracil and adenine moieties were used to modulate the interaction with the different CA isozymes and exploit their intrinsic antitumor effect in parallel and synergic to the inhibition of hCA IX. Indeed, molecular hybridization, which covalently combines two or more drug pharmacophores into a single molecule, have proven to be an effective tool for designing novel entities as potent antitumor agents.[19−21] It should also be stressed that purine/pyrimidine scaffolds confer drug-like properties to the compounds in which they are present as well as enhanced solubility in water due to their polar nature.[15] The uracil and adenine moieties were incorporated at various positions of the benzenesulfonamide scaffold by means of different length spacer of the ether, amide, and bioisostere triazole types, to elicit diverse positioning of the

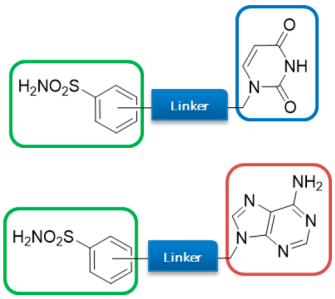

Figure 1. Design approach to nitrogenous base-bearing sulfonamides.

purine and pyrimidine groups within the CAs binding pockets. The bioisosteric amide/triazole substitution was pursued due to the anticancer activity of 1,2,3-triazoles and their derivatives reported in the literature in addition to the *in vivo* stability of this linker.[12,22] It should be stressed that uracil and adenine were appended at the compounds' tails through the N1 and N9 moieties, respectively, in order to maintain the pharmacophore and the connection by which such nitrogenous bases are incorporated in nucleotides and nucleic acids.[15,16]

The triazole derivatives (**11−12**, **15−16**) were prepared through analogue synthetic strategies both for the uracil and adenine scaffold, consisting in copper-catalyzed azide−alkyne cycloadditions (CuAAC), better known as "Click Chemistry", between N1-propargyluracil (**8**) and N9-propargyladenine (**14**) and freshly prepared azides of sulfanilamide and metanilamide (Scheme 1).[12,23]

Conversely, different synthetic pathways had to be designed for the amide-bearing derivatives considering the diverse reactivity of these nitrogenous bases. For uracil, it was feasible to obtain the 1-carboxymethyl derivative (**2**) in one step with good yields by reacting uracil with bromoacetic acid in KOH(aq) environment. The carboxy intermediate was activated in situ with 1-hydroxy-7-azabenzotriazole (HOAt) or *N*-hydroxysuccinimmide (NHS) in the presence of 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP) and subsequently coupled with different amino benzenesulfonamides to give the uracil bearing compounds **3−7**.

The synthetic pathway planned for adenine involves the selective and fast protection of its amino group with *N*,*N*-dimethylformamide diethyl acetal (**17**), followed by S_N1 reaction with the chloroacetamido derivatives of amino-benzenesulfonamides (**18−21**) (Scheme 2). The obtained intermediates bearing the N-protected adenine (**22−25**) were evaluated for their CA inhibitory activity and thereafter submitted to an acidic treatment to free the sulfonamide group and produce the planned adenine-incorporating derivatives (**26−29**). Finally, compound **32** was obtained coupling the N-protected 4-hydroxybenzenesulfonamide (**31**) with the 2-bromoethyl derivative of NH_2-protected adenine (**30**) and subsequently removing both the protecting groups in acidic media.

The CA inhibitory activities of compounds **3−7**, **11−12**, **15−16**, **22−29**, and **32**, in addition to acetazolamide (AAZ) as standard inhibitor, were measured against four isoforms hCA I II, IV, and IX by a stopped flow CO_2 hydrase assay.[24] The following structure−activity relationships (SARs) were obtained from the inhibition data reported in Table 1:

(i) The inhibitory profile of the screened derivatives against the four CA isoforms was dependent on the length and positioning of the spacer at the benzenesulfonamide scaffold more than on the nature of both the spacer and the nitrogenous

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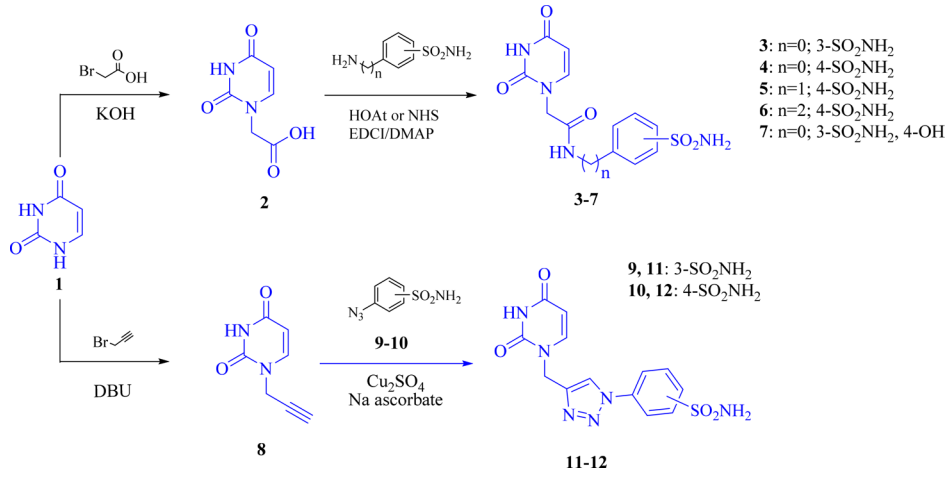
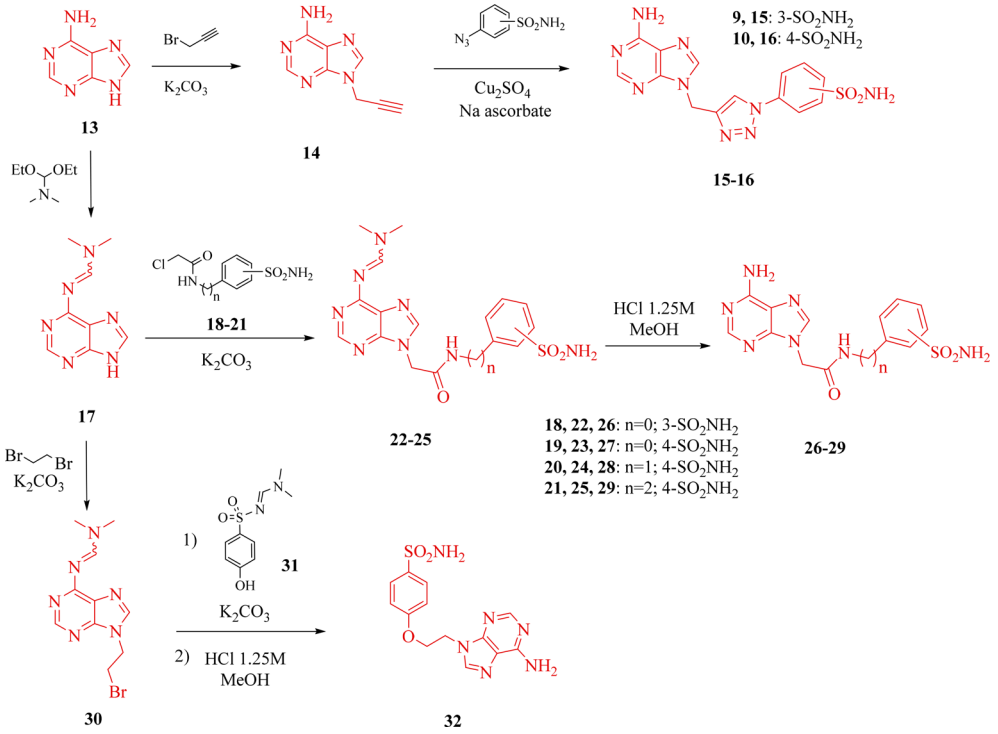
Scheme 1. General Synthetic Procedure for Uracil Derivatives 2−12



Scheme 2. General Synthetic Procedure for Adenine Derivatives 15−32



base. In this context, the *para*-substitution at the benzenesulfonamide scaffold was more favorable than meta (**3**, **7**, **11**, **15**, **22**, and **26**) in inducing effective CA inhibitory properties. Moreover, in the case of the amidic linker at position 4, a direct or two carbon spacer between the amide and the benzene ring led to better inhibitory profiles over a single carbon atom connection in the uracil derivatives (**4** and **6**) and to a lesser extent for adenines (**23**, **25**, **27**, and **29**).

(ii) Weak hCA I inhibitory potency can be ascribed to most of the adenine- and uracil-bearing compounds reported, with K_Is ranging between 256.2 and 9840.2 nM. Within the pyrimidine subset, derivatives **4**, **6**, and **12**, incorporating a direct or two carbon atoms spacer amide or triazole linker at the 4 position of the ZBG scaffold, respectively, were weak nanomolar inhibitors (K_Is of 645.5, 658.9, and 256.2 nM). The adenines showed a similar tendency such that the derivatives with the privileged spacer were the best hCA I inhibitors, whereas the *m*-substituted **15**, **22**, and **26** did not inhibit the isozyme to 10 μM. It should be stressed the better effectiveness of the free NH_2-bearing adenines (**27**−**29**) compared to the N-protected derivatives (**23**−**25**). Furthermore, compounds **12** (first series) and **16** (second series), incorporating the triazole linker at the 4-position, were the best hCA I inhibitors.

(iii) Isoform II was greatly affected by most sulfonamide-bearing nitrogenous bases, with K_Is spanning in the subnanomolar to medium nanomolar range (0.85−889.2 nM), except for compound **7**, incorporating the spacer at the 3-position of the scaffold and an additional OH group in *para*, which inhibited hCA II in the micromolar range (K_I of 4357.7 nM). Further details may be inferred from the results reported for hCA I, but two orders of magnitude below. Indeed, the *para*-substituted compounds **4**, **6**, and **12** were the most potent within the uracil series, showing K_I values ranging between 0.85 and 42.1 nM. The equally substituted adenines, both N-protected (**23**−**25**) and not

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Table 1. Inhibition Data of Human CA Isoforms I, II, IV and IX with Sulfonamides 3−7, 11−12, 15−16, 22−29, and 32 and the Standard Inhibitor Acetazolamide (AAZ) by a Stopped Flow CO_2 Hydrase Assay[24]

	K_I (nM)[a]				Selectivity Ratio[b]
Cmpd	hCA I	hCA II	hCA IV	hCA IX	hCA II/hCA IX
3	7966.7	704.3	2940.3	3121.2	0.23
4	645.5	17.7	469.6	25.7	0.69
5	1565.7	495.4	1180.7	405.2	1.2
6	658.9	42.1	1780.0	46.0	0.91
7	9840.2	4357.7	2296.5	439.2	9.9
11	4871.0	388.1	299.5	460.3	0.84
12	256.2	0.85	386.7	4.8	0.18
15	>10000	655.3	311.1	1310.1	0.5
16	410.9	5.6	211.4	1.9	2.9
22	>10000	756.2	3837.7	2805.5	0.27
23	966.3	30.7	395.4	40.0	0.77
24	4721.4	87.0	1164.6	165.8	0.52
25	1962.6	57.8	1974.3	112.7	0.51
26	>10000	889.2	3347.0	428.5	2.1
27	666.6	8.4	156.1	16.9	0.5
28	977.1	59.2	277.4	45.2	1.3
29	811.8	8.2	2246.9	11.4	0.72
32	552.6	78.0	173.5	29.9	2.6
AAZ	250	12	74	25	0.48

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values). [b]Selectivity as determined by the ratio of K_I for hCA II isoform relative to hCA IX.

(**16**, **27**−**29**, and **32**) within the second series (K_Is in the range of 30.7−87.0 nM and 5.6−59.2 nM, respectively) confirmed the efficacy of this substitution pattern, being the most active within their subsets. A methylene linker between the amide and benzene ring consistently reduced the CA inhibition efficacy in the uracil series and slightly influenced the adenines. Again, the triazoles **12** (K_I of 0.85 nM) and **16** (K_I of 5.6 nM) possessed the most efficient hCA II inhibitory properties.

(iv) The reported hCA IV inhibitory profiles of the sulfonamides deviate from the general tendency observed for the other isoforms tested. Beside the 3-substituted amides **3**, **7**, **22**, and **26** that showed ineffective inhibition (K_Is in the range 2296.5−3837.7 nM), compounds **4**, **23**, and **27**, which incorporate the amide group directly appended at the benzene ring, were the most efficient amide-bearing compounds, regardless of the uracil or adenine moiety present in their molecules. Inversely, the amides of methylaminobenzenesulfonamides (**5**, **24**, and **28**) were better hCA IV inhibitors than the ethylaminobenzenesulfonamide derivatives (**6**, **25**, and **29**), with the biggest extent in case of the free amine adenines **28** and **29**, with a ratio approaching 10 (K_Is of 277.4 and 2246.9 nM). The uracil (**11**−**12**) and adenine (**15**−**16**) compounds bearing a triazole linker inhibited hCA IV in the medium nanomolar range, regardless of the positioning at the benzene ring (K_Is in the range 211.4−386.7 nM). Finally, the ether **32** exhibited the second best hCA IV inhibition profile with a K_I of 173.5 nM.

(v) The targeted tumor-associated isoform hCA IX was potently inhibited by most sulfonamides reported with binding affinities comparable to hCA II. Therefore, the general tendencies described above were also applicable for this isoform. The 3-substituted derivatives were shown to act as the weakest hCA IX inhibitors, regardless of the nature of the tails (K_Is in the range of 428.5−3121.2 nM). The data in Table 1 demonstrate triazoles **12** and **16** as the most efficacious in inhibiting the isozyme (K_Is of 4.8 and 1.9 nM). The addition of a carbon atom spacer between the amide moiety and the benzene ring decreased the hCA IX inhibition for the uracil bearing derivative **5** (from 25.7 to 405.2 nM) and adenines **24** (from 40.0 to 165.8 nM) and **28** (from 16.9 to 45.2 nM). Conversely, the increase of the spacer to two carbon atoms improved the inhibitory potency, mostly for the NH_2-free adenine **29** (K_I of 11.4 nM). It is worth mentioning the K_Is reported for the free adenines **27**−**29** (K_Is in the range 40.0−165.8 nM) were lower than the N-protected ones **23**−**25** (K_Is in the range 16.9−45.2 nM). Compound **32** bearing the adenine moiety through an ether linker was a potent hCA IX inhibitor, with a K_I of 29.9 nM.

Some of the reported derivatives (e.g., **5**, **7**, **16**, **26**, **28**, and **32**) exhibited a preferential inhibitory profile for the tumor-associated isoform hCA IX over the ubiquitous hCA II. Noteworthy, more than two-fold IX/II selectivity was observed for adenines **26** and **32**. We focus on the three-fold selectivity shown by the potent triazole **16** and on the 10-fold IX/II selective efficacy of 3-amido-4-hydroxy uracil bearing compound **7** though it exerted its action only in the high nanomolar range.

Crystal structures of hCA II and hCA IX-mimic were each solved in complex with compound **29** to a resolution of 1.6 Å (Figures 2 and 1S and Table 1S, Supporting Information). The

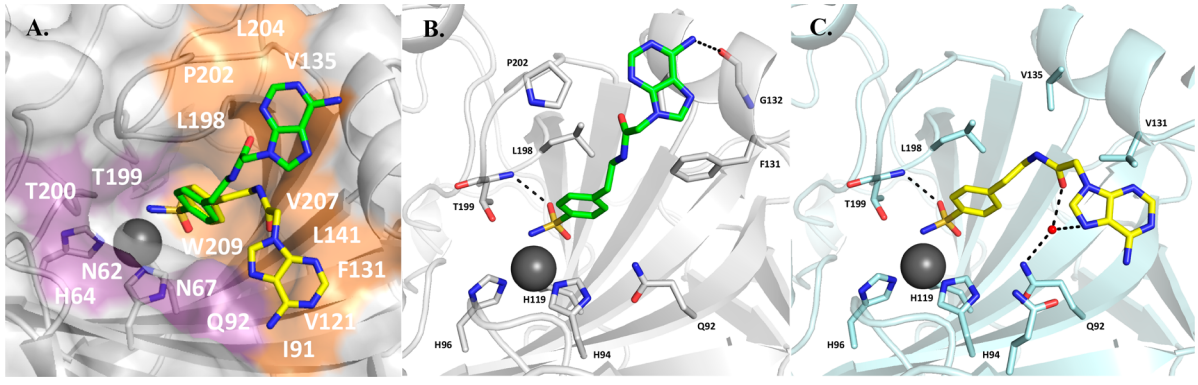

Figure 2. (A) Superimposition of compound **29** in complex with hCA II and hCA IX-mimic. The overlay is shown in a surface representation of hCA II (gray) with inhibitor shown as sticks (green in hCA II and yellow in hCA IX-mimic). Hydrophobic and hydrophilic active site residues are labeled and colored orange and purple, respectively. (B) hCA II (gray) and (C) hCA IX-mimic (pale cyan) in complex with **29** (green and yellow, respectively). Hydrogen bonds are shown as black dashes.

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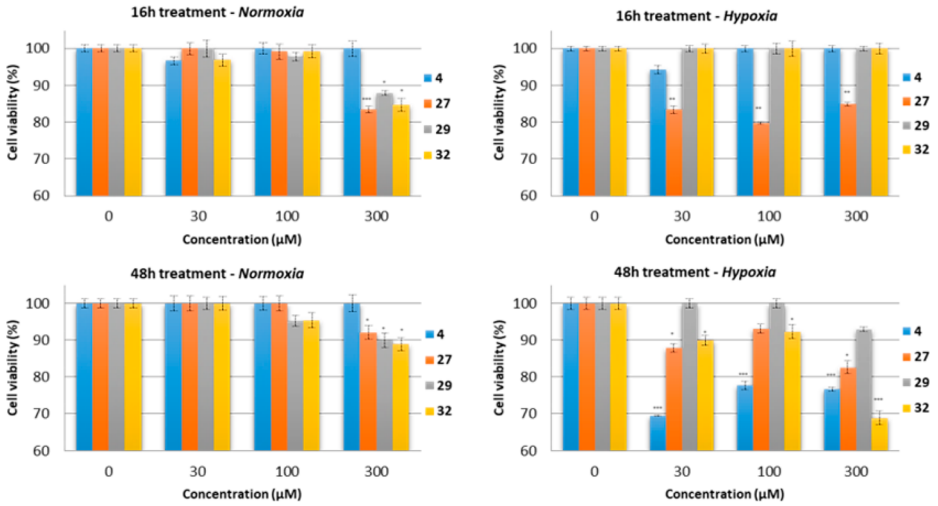

Figure 3. HT-29 cells (1×10^4/well) were treated with compounds **4**, **27**, **29**, and **32** (30−300 μM). Incubation was allowed for 16 and 48 h in normoxic (20% O_2) and hypoxic conditions (0.1% O_2). Control condition was arbitrarily set as 100%, and values are expressed as the mean ± SEM of three experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 in comparison to control (0 μM).

benzenesulfonamide ZBG was observed to displace the zinc-bound solvent, binding directly to the catalytic zinc. The orientation of this pharmacophore was conserved in both isoforms (Figure 2A) with a hydrogen bond between the oxygen of the sulfonamide and the amide of T199 (3.1 Å) (Figure 2B,C). The tail of compound **29** therefore dictates the differences in binding between CA isoforms. In complex with hCA II, the free amine of the adenine ring in **29** forms a weak hydrogen bond with the carbonyl oxygen of G132 (3.5 Å). The tail is further supported by van der Waals interactions with residues Q92, F131, V135, L198, and P202 (Figure 2B). In CA IX-mimic, the decrease in steric hindrance of residue V131 in relation to F131 of hCA II allows the rotation of the adenine ring to form two hydrogen bonds with an active site solvent molecule that is anchored to Q92 (3.0 Å). The bonds between the carbonyl oxygen and N7 of adenine in compound **29** with this water molecule are 2.6 and 3.1 Å, respectively. Similar to hCA II, binding of **29** in the hCA IX active site is further stabilized by van der Waals interactions with residues Q67, V131, V135, and L198 (Figure 2C).

Compound **29** exhibited approximately 80- and 200-fold selectivity for both hCA II and hCA IX over hCA I and hCA IV, respectively (Table 1). The hCA I active site contains larger, aromatic residues H67, F91, H200, and Y204 that may cause steric hindrance with the nitrogenous base of the compound tail. In hCA II and hCA IX-mimic, side chains of the α-helix-containing residues 126−136 stabilize the tail of the inhibitor through hydrogen bonds and van der Waals interactions. However, in CA IV, this helix is replaced by a loop that extends away from the active site, decreasing the number of possible interactions with the inhibitor.

Some selected compounds (**4**, **27**, **29**, and **32**), chosen among those possessing the best CA inhibitory profile, were tested (30−300 μM) to evaluate their effects on viability of human colon cancer HT-29 cells. Measurements were performed after 16 and 48 h incubation in both normoxic and hypoxic conditions. The efficacy of tested compounds is represented in Figure 3. After 16 h of incubation in normoxic conditions, **27**, **29**, and **32** were significantly active (300 μM), inducing cell mortality by about 10−15%. Hypoxia conditions favored the efficacy of compound **27** at all concentration tested. Incubation prolonged up to 48 h highlighted the efficacy of **4**, **27**, and **32**, particularly in hypoxic conditions (25−30% cell viability decrease). Compound **4** showed the best potency. It is worth commenting on the trend of the cytotoxic effect shown by the screened derivatives. Indeed, compounds **27**, **29**, and **32** showed comparable cytotoxic effects both after 16 and 48 h in normoxia, although a mildly worsened effect might be observed after 48 h. It is reasonable to ascribe the weak observed effect to alternative, not yet identified, mechanisms of action beyond hCA IX inhibition (hCA IX is not upregulated in these cells),[25] which are likely to be related to the adenine pharmacophore.

In hypoxic conditions, derivative **27** uniquely maintained its antiproliferative activity after 16 h, whereas **29** and **32** totally lost the efficacy shown in normoxia, but after 48 h in hypoxia, the cytotoxic profile was distinctly different due to the strong upregulation of hCA IX. Indeed, the uracil-bearing **4**, which showed total inactivity up to now, aroused with the best efficacy herein reported, together with **32**, which was inactive after only 16 h of hypoxia. Conversely, **27** slightly lost its efficacy after a prolonged hypoxia, however maintaining better effectiveness than that shown after 48 h in normoxic conditions. Therefore, derivative **27** exhibits a cytotoxic efficacy that likely relies on both an adenine-related alternative mechanism of action and hCA IX inhibition. In contrast, since compound **32** and **4** show a great gain in efficacy after prolonged hypoxia, it is reasonable to ascribe their relevant antiproliferative activity to the inhibition of the overexpressed hCA IX.

In conclusion, we report the synthesis of two series of nitrogenous bases-bearing benzenesulfonamides obtained according to the molecular hybridization design approach. The incorporation of the purine/pyrimidine pharmacophore as the tail of a classical CAI with a benzenesulfonamide ZBG scaffold was considered both to modulate the interaction with CA isozymes and exploit an intrinsic antitumor effect in parallel and synergic to the inhibition of hCA IX. The compounds were investigated for their inhibition of cytosolic hCA I and II and transmembrane hCA IV and IX. In addition, X-ray crystallography demonstrates the binding mode of a nitrogenous base within the active site of hCA II and hCA IX-mimic. Finally, the most effective compounds were evaluated for their antiproliferative activity against HT-29 colon cancer cell lines. The analysis

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of the trend of the obtained results allowed us to ascribe the responsibility for the observed antiproliferative activities to either multiple, not yet identified mechanisms of action, or the inhibition of the tumor-associated hCA IX, depending on the treatment time and the applied hypoxic or normoxic conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00399.

Synthetic procedures, characterization of compounds, in vitro kinetic procedure, cells viability procedure, X-ray statistics, and difference electron density map of compound **29** with hCA II and hCA IX-mimic (PDF)

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Author Contributions

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

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CAI(s), carbonic anhydrase inhibitor(s); AAZ, acetazolamide; (h)CA, (human) carbonic anhydrase; K_I, inhibition constant; HOAt, 1-hydroxy-7-azabenzotriazole; NHS, *N*-hydroxysuccinimmide; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide; DMAP, 4-dimethylaminopyridine; DBU, 1,5-diazabiciclo(5.4.0)undec-5-ene

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