# 1,2-Benzisothiazole Derivatives Bearing 4-, 5- or 6-alkyl/aryl-carboxamide Moieties Inhibit Carbonic Anhydrase Isoform IX (CAIX) and Cell Proliferation under Hypoxic Conditions.

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**ABSTRACT:** Three novel series of 1,2-benzisothiazole derivatives have been developed as inhibitors of carbonic anhydrase isoform IX. Compounds **5c** and **5j**, tested in vitro on the human colon cell line HT-29, blocked the growth of cells cultured under chemically-induced hypoxic conditions, displaying a specific activity against cancer cells characterized by CAIX up-regulation. Moreover, a synergistic activity of **5c** with SN38 (the active metabolite of irinotecan) and 5-fluorouracil on cell proliferation under hypoxic conditions was demonstrated.

## INTRODUCTION

Solid tumors represent a major health problem, as their prevalence and incidence worldwide is growing at an alarming rate. Actually, according to the International Agency for Research on Cancer (IARC), 21.4 million new cancer cases and 13.2 million cancer related deaths are predicted throughout the world by 2030.[1] Suitable prevention measures and high-quality screenings for early-detection help to curb the global burden, but these strategies should necessarily be complemented by an improvement in cancer treatments, to achieve effective and safe clinical protocols for cancer patients. Chemotherapy and radiotherapy have been accompanying over time by novel molecularly targeted drugs, addressing both oncogenic pathways in cancer cells and angiogenic pathways in tumour endothelial cells.[2-4] However, despite an initial significant response, most of the available compounds still show poor performances as the abnormality of the tumor microenvironment impairs their distribution in the tumor tissues, thus providing the driving force for drug fecklessness and resistance. Therefore, focusing on the tumor microenvironment stands up as the current imperative strategy to improve the therapeutic efficacy of both the existing and the forthcoming anticancer agents.[5,6]

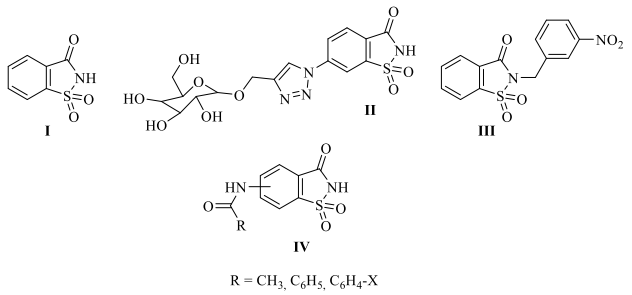
Most solid tumors contain hypoxic regions, arising from an imbalance between the oxygen demand of rapidly proliferating cancer cells and the ability of vasculature to meet these needs. To adapt to the impaired environment, cancer cells modulate their gene expression pattern and the resulting hypoxia-triggered metabolism leads to the extensive production of lactate, protons, and carbon dioxide. Being incompatible with cell survival and proliferation, these acidic metabolites are secreted in the microenvironment thanks to a complex pH regulating machinery, which maintains suitable cell pH values by ruling transmembrane ions flux.

A key component of this machinery is represented by carbonic anhydrase IX (CAIX), a hypoxia-induced zinc-enzyme whose active site, facing the extracellular space, catalyzes efficiently the conversion of carbon dioxide to bicarbonate ions and protons. While the former are imported inside the tumor cell, to modulate intracellular pH values, protons remain outside the plasma membrane feeding microenvironment acidosis. This latter, in turn, contributes to tumor progression via multiple effects including up-regulation of angiogenic factors and proteases, increased invasion and impaired immune functions. Playing a pivotal pH-regulating function, CAIX represents a key survival factor for tumor cells.[7-9] Its ectopic expression is strongly associated with several types of solid tumors and often correlated to poor prognosis, due to aggressive growth, malignant progression and poor treatment response of cancer cells. Indeed, through an increase in extra-cellular acidosis, CAIX confers resistance to anticancer treatments like chemotherapy, radiotherapy and the newer molecularly targeted compounds. Accordingly, this enzyme represents an excellent drug target for an up-to-date cancer therapy as its inhibition, ruining distinctive features of tumor microenvironment, can not only hit cancer cells but also enhance the activity of anticancer drugs such as antiangiogenic compounds.[10]

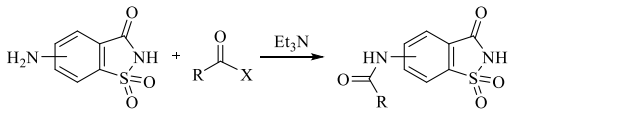
A huge amount of CAIX inhibitors (CAIs) have been described throughout years, most of them represented by aromatic/heterocyclic sulfonamide derivatives. In particular, those bearing the primary sulfonamide functional group turned out to be the most effective ones, thus imposing this fragment as the key structural element of CAIs. Actually, the nitrogen atom of a primary sulfonamide, once in its deprotonated form, is able to coordinate the zinc ion in

the active site of the enzyme, thus subtracting it from the catalytic machinery of CAIX, which becomes inhibited. The functional efficacy of this structural fragment is maintained even when it is stiffened into small heterocyclic cores, like isothiazoles. The pioneering study of Klebe and co-workers[11] demonstrated the ability of the benzo[*d*]isothiazol-3(2*H*)-one-1,1-dioxide (saccharin, **I**, Chart 1) to inhibit efficiently the target CA IX, and additional authors confirmed recently this result in differently substituted saccharin derivatives (compounds **II** and **III**, Chart 1).[12,13] Moving from these outcomes, and considering that the benzo[*d*]isothiazol-3(2H)-one-1,1-dioxide is used as sweetener in the everyday life, hence it is toxicologically safe, this scaffold can rightfully be considered a key intriguing intermediate for the obtainment of novel effective and potentially safe CAIX inhibitors. Prompted by this speculation, and exploiting our synthetic expertise in the benzo[*d*]isothiazol-3(2*H*)-one-1,1-dioxide core,[14,15] we designed and synthesized three different series of saccharin derivatives of general formula **IV** (Chart 1), to develop as drug candidates for the treatment of solid tumours *via* CAIX inhibition. Differently substituted alkyl- and aryl-amido groups were inserted in positions 4, 5 and 6 of the heterocyclic scaffold, to start investigating their contribution to both the efficacy and the selectivity toward different carbonic anhydrase isoenzymes of the designed compounds.

**Chart 1.** Carbonic Anhydrase Inhibitors




**Scheme 1.** Synthesis of Benzo[*d*]isothiazol-3(2*H*)one-1,1-dioxide derivatives **4a**,**b**,**h**, **5b-f**,**h**,**j**, and **6a-g**.




## RESULTS AND DISCUSSION

**Chemistry.** The target inhibitors, **4a**,**b**,**h**, **5b-f**,**h**,**j** and **6a-g**, were synthesized as illustrated in Scheme 1. The starting 4-, 5-, and 6-aminobenzo[*d*]isothiazol-3(2*H*)-one-1,1-dioxide derivatives, **1-3**, were prepared according to synthetic methods previously described by Rose[16] and Saary et al.[17] Once obtained, the key intermediates **1-3** afforded the aroyl-substituted compounds by treatment with the suitable aryl chloride, in anhydrous toluene and in the presence of triethylamine. Derivative **5j**, bearing an acetylamino substituent on the distal phenyl ring, was synthesized by reaction of the amino compound **2** with acetic anhydride, at 100 °C.

**CAs inhibitory activity.** We started our functional evaluation by testing the synthesized inhibitors, **4a**,**b**,**h**, **5b-f**,**h**,**j** and **6a-g**, for their efficacy against the target human CAIX. As reported in Table 1, all the compounds proved to inhibit the enzyme exhibiting potency levels in the nanomolar/high nanomolar range. In the 4-substituted sub-series, compounds **4a**,**b**,**h**, the best inhibitory activity resided in the benzamido derivatives, **4a**,**b**, as the presence of a bulky and stiff biphenyl ring, like in **4h** ($K_i$ 1186 nM), determined an almost 5-fold reduction of potency with respect to the hit, **4a** ($K_i$ 223 nM). Moving from the hit, the insertion of an electron-withdrawing substituent in position meta of the pendant phenyl ring gave rise to a remarkable increase in efficacy and compound **4b** ($K_i$ 31,2 nM) turned out to be the most effective inhibitor of this sub-series. A similar pattern of structure-activity relationships was observed with compounds belonging to the 5-substituted sub-series. Actually, also in this case, the biphenyl-substituted compound, **5h** ($K_i$ 231 nM), was less effective than both the benzamido derivatives, **5b-f**, and the acetylamino derivative, **5j**. Regarding compounds **5b-f**, the insertion of electron-withdrawing substituents on the distal phenyl ring contributed more favorably to the inhibitory efficacy than the presence of electron-donating ones. Compare for example **5f**, bearing a methoxy group in position para ($K_i$ 74 nM), with **5d**, carrying a fluoro atom in the same position of the ring ($K_i$ 27.2 nM). Significantly, among the electron-withdrawing atoms inserted on the ring, the best result in terms of potency was obtained with a fluoro atom, like in **5d**. On the contrary, replacement of the fluoro atom with a chloro one gave rise to a modest reduction in the inhibitory efficacy of the resulting compound. A remarkable activity against hCAIX was observed also with compound **5j** ($K_i$ 30.4 nM), characterized by the presence of the small acetylamino substituent. Derivatives belonging to the 6-substituted sub-series, **6a-g**, were generally less active than the corresponding regioisomers, 4 and 5. Actually, they all showed $K_i$ values in the high nanomolar range, thus revealing that this particular substitution pattern on the benzisothiazole core is the less effective one for a fruitful interaction with the catalytic site of hCAIX. Moving from the progenitor, **6a** ($k_i$ 220 nM), neither the insertion of electron-donating groups, like methyl and methoxy (**6f**,**g**), nor the presence of electron-withdrawing atoms, like fluoro, bromo and chloro on the distant phenyl ring (**6b-e**), contributed significantly to the observed inhibitory activity, as **6b-g** turned out to be either equipotent or even slightly less effective than **6a**. Also in this subseries, the presence of a chloro atom in the para position of the distal ring had the most detrimental effect on the functional efficacy, as compound **6e** raised its activity to the low micromolar range.

**Table 1.** Inhibitory Activity of Benzo[*d*]isothiazole Derivatives **4a**,**b**,**h**, **5b-f**,**h**,**j** and **6a-g** against Human CA Isozymes I, II, VII, IX and XII.

| | | $K_i$ (nM) | | | | |
|---|---|---|---|---|---|---|
| **Compound** | **R** | **hCAI** | **hCAII** | **hCAVII** | **hCAIX** | **hCAXII** |
| **4a** | $C_6H_5$ | >50000 | 184 | n.t.[a] | 223 | 910 |
| **4b** | $C_6H_4$-m-Br | 8365 | 593 | >50000 | 31.2 | 58.9 |
| **4h** | $C_6H_4$-p-$C_6H_5$ | >50000 | 910 | n.t.[a] | 1186 | 89.8 |
| **5b** | $C_6H_4$-m-Br | 8120 | 3925 | 273 | 31.5 | 41.4 |
| **5c** | $C_6H_4$-m-Cl | 763 | 589 | 94.5 | 67.9 | 38.7 |
| **5d** | $C_6H_4$-p-F | 7697 | 6880 | 705 | 27.2 | 35.9 |
| **5e** | $C_6H_4$-p-Cl | >50000 | 2300 | n.t.[a] | 86.2 | 78.5 |
| **5f** | $C_6H_4$-p-$OCH_3$ | 929 | 790 | 609 | 74.0 | 52.6 |
| **5h** | $C_6H_4$-p-$C_6H_5$ | >50000 | 82.9 | n.t.[a] | 231 | 76.8 |
| **5j** | $CH_3$ | 8914 | 1480 | >50000 | 30.4 | 670 |
| **6a** | $C_6H_5$ | >50000 | 7270 | n.t.[a] | 220 | 9.4 |
| **6b** | $C_6H_4$-m-Br | >50000 | 837 | 735 | 665 | 459 |
| **6c** | $C_6H_4$-m-Cl | >50000 | 6020 | 534 | 233 | 413 |
| **6d** | $C_6H_4$-p-F | >50000 | 908 | 810 | 543 | 537 |
| **6e** | $C_6H_4$-p-Cl | >50000 | 4670 | n.t.[a] | 1920 | 8.7 |
| **6f** | $C_6H_4$-p-$OCH_3$ | >50000 | 6945 | n.t.[a] | 205 | 8.9 |
| **6g** | $C_6H_4$-p-$CH_3$ | >50000 | 411 | 355 | 539 | 804 |
| **Saccharin** | | 18540 | 5950 | 10 | 103 | 633 |

[a]n.t.: not tested. Errors ± 10% (from 3 different assays, data not shown)

Once screened against the target enzyme, all the synthesized compounds were investigated for their selectivity against structurally related CA isoforms, namely hCAI, hCAII, hCAVII and hCAXII (Table 1). CAs are highly expressed in a variety of healthy tissues, where they take part to many physiological processes including respiration, acid-base balance, and ions transport.[18] Accordingly, to be exploited as a drug candidate, the ideal CAIX inhibitor should possess a functional profile as selective as possible against the target enzyme, in order to avoid any side effects arising from the interaction with multiple CA isozymes in different tissues.

None of the tested compounds showed any relevant inhibitory efficacy against hCAI. On the contrary, they all displayed diverse activities against isozymes II and VII, although remaining preferential inhibitors of hCAIX. In particular, focusing on the 5-substituted subseries, which proved to inhibit the target enzyme better than the 4- and 6-isomers, the selectivity ratios against the II and VII isoforms ranged from 9 to 250 and 1.4 and 26, respectively. Significantly, most of the compounds proved to inhibit hCAXII, showing $K_i$ values in the nanomolar/low nanomolar range. Even though this specific isozyme is present to a large extent in normal tissues, like colon, kidney, rectum, esophagus and prostate, its expression is increased in many solid, hypoxic tumors.[18] Accordingly, its inhibition cannot be considered an undesired event as it should, in principle, strengthen the antitumor activity resulting from the block of CAIX.[19] While compounds belonging to the 4-substituted sub-series showed a moderate inhibitory preference for hCAIX, those of the 6-substituted sub-series turned out to be generally more effective against isozyme XII rather than IX. Derivatives **5b-f**,**h** proved to inhibit equally the two enzymes demonstrating that, among all the compounds obtained modifying the benzisothiazole heterocyclic core, those distinguished by a 5-substitution pattern offer the best guarantee of being effective as anti-tumor agents.

**Anti-proliferative activity.** Representative examples of the benzisothiazole derivatives, **4b**, **5c** and **5j**, chosen among those possessing the best inhibitory profile, were tested on human colon adenocarcinoma cell line HT-29, to investigate their ability to block cell proliferation in the presence of $CoCl_2$. This chloride is able to simulate the conditions of hypoxia by inhibiting prolyl hydroxylation of the oxygen-dependent degradation domain of HIF-1α and its

subsequent interaction with the protein onco-suppressive Von Hippel-Lindau, without affecting cell proliferation.[20] After 72-h exposure, tested compounds alone did not significantly inhibit the proliferation of HT-29 cells at any drug concentrations. On the contrary, the 72-hour exposure of **5c** and **5j** in combination with $CoCl_2$ significantly inhibited, in a concentration-dependent manner, the proliferation of HT-29, with an $IC_{50}$ of 44.10±8.4 and 50.97±0.049 μM, respectively (Table 2).

**Table 2.** Anti-proliferative activities on HT-29 cancer cells of compounds **4b**, **5c**,**j**, alone or in combination with $CoCl_2$ to simulate hypoxia conditions.

| Compound | $IC_{50}$ (μM) | |
|---|---|---|
| | w/o $CoCl_2$ | with $CoCl_2$ |
| **4b** | >100 | >100 |
| **5c** | >100 | 44.10±8.4 |
| **5j** | >100 | 50.97±0.049 |

Moreover, derivative **5c** showed a strong synergistic activity when tested in combination with two well-known chemoterapic agents, SN-38 (the active metabolite of irinotecan) and 5-FU. The 72-h exposure of HT-29 cells to SN-38 and 5-FU, under the same hypoxic conditions, determined an IC50 of 0.029±0.012 μM and 10.95±2.73 μM, respectively. The concurrent combination of **5c** with Sn-38 or 5-FU determined a high synergistic effect (CI<1 and DRI>1) for marked antiproliferative activity (>70% of affected cells) (Tables 3 and 4). To evaluate the level of interaction (synergistic, additive or antagonist) between compound **5c** and 5-FU or SN38, the method proposed by Chou[21] was followed. Briefly, synergism or antagonism for **5c** plus 5-FU or SN38 has been calculated on the basis of the multiple drug-effect equation, and quantitated by the combination index (CI), where CI<1, CI=1 and CI>1 indicate synergism, additive effect and antagonism, respectively. Based on the classic isobologram, the CI value has been calculated from the following equation (1):

$$CI = [(D)_1/(Dx)_1] + [(D)_2/(Dx)_2] \quad (1)$$

As an example, at the 90% inhibition level (see table 3), $(Dx)_1$ and $(Dx)_2$ are the concentrations of compound **5c** and 5-FU or SN-38, respectively, that induce a 90% inhibition of cell proliferation; $(D)_1$ and $(D)_2$ are the concentrations of compound **5c** and 5-FU or SN-38 in combination that also inhibits cell proliferation by 90% (isoeffective as compared with the single drugs alone). The dose-reduction index (DRI, equations (2) and (3)) defines the degree of dose reduction that is possible in combination for a given degree of effect as compared with the concentration of each drug alone:

$$(DRI)_1=(Dx)_1/(D)_1 \quad (2)$$

$$(DRI)_2 = (Dx)_2/(D)_2 \quad (3)$$

In other words, the DRI represents the theoretical magnitude of concentration reduction allowed for each drug when given in synergistic combination in vitro to achieve the same effect as that obtained with the concentration of each single agent. Indeed, in the case of 5-FU or SN-38, it could be possible to reduce the concentration of the drug in vitro more than 90-fold or 30-fold (see table 4), respectively, when the drug is combined with compound **5c** to obtain the same 90% level of cytotoxic effects under hypoxic conditions. Of course, synergism and related reductions of drug concentrations is highly desirable for high level of inhibition of cell proliferation (>70%) in order to obtain a clinical advantage such as the reduction of tumor mass or the control of neoplastic disease.

**Table 3.** Synergistic activity of **5c** and SN-38 or 5-FU association expressed as combination index (CI) values at 70%, 80% and 90% inhibition of HT-29 cell proliferation under hypoxic conditions.

| Affected Cell Fraction (%) | Combination Index (CI) | |
|---|---|---|
| | 5c+SN-38 | 5c+5-FU |
| **70%** | 1.186 | 0.621 |
| **80%** | 0.522 | 0.508 |
| **90%** | 0.167 | 0.493 |

**Table 4.** Synergistic activity of **5c** and SN-38 or 5-FU association expressed as dose reduction index (DRI) values for each drug at 70%, 80% and 90% inhibition of HT-29 cell proliferation under hypoxic conditions.

| Affected Cell Fraction (%) | Dose Reduction Index (DRI) | | | |
|---|---|---|---|---|
| | 5c+SN-38 | | 5c+5-FU | |
| | **5c** | **SN-38** | **5c** | **5-FU** |
| **70%** | 32.2870 | 0.866 | 2.3710 | 5.0170 |
| **80%** | 31.9150 | 2.0390 | 2.2470 | 15.8840 |
| **90%** | 31.3650 | 7.3950 | 2.0730 | 89.9520 |

## CONCLUSIONS

Thanks to the earliest studies of Klebe and co-workers,[11] the 1,2-benzisothiazole heterocyclic core (saccharin, Chart 1), is now considered a privileged scaffold for the obtainment of effective and potentially safe inhibitors of the key anticancer target CAIX.

In this work we presented three novel series of saccharin derivatives, bearing suitably substituted alkyl- and arylamido groups in positions 4, 5, or 6 of the nucleus, which

demonstrated to inhibit the target protein showing $K_i$ values in the nanomolar range. The 5-substitution pattern turned out to be the most effective one as the resulting compounds, besides inhibiting the target hCAIX, proved to block efficiently even the enzymatic activity of the correlated isozyme hCAXII, whose expression is increased in many solid, hypoxic tumors.

A preliminary evaluation of selected synthesized compounds on the human cell line HT-29, chosen as an example of solid tumor, revealed the promising anti-proliferative activity of the novel derivatives (Table 2). Significantly, compounds **5c** and **5j** turned out to be active on cells cultured under chemically-induced hypoxic conditions, showing $IC_{50}$ values in the micromolar range. At the same time, they left unaltered those under normoxic conditions, thus demonstrating a specific activity against cells characterized by CAIX up-regulation. Compound **5c** displayed also a strong synergistic activity on colon cancer cell proliferation when tested, under hypoxic conditions, in combination with irinotecan and 5-fluorouracil, both widely used in the clinical practice to treat colorectal cancer. Moreover, a preliminary in vivo pilot study,[21] performed on nude mice harbouring a HT-29 tumor xenograft, suggested the possible safe tolerability profile of compound **5c**. Indeed, administered at the daily dose of 25 mg/kg and 50 mg/kg i.p., respectively, for 16 days, the compound gave no signs of weight loss or evident toxicity at the clinical inspection (data not shown).

The observed selectivity of action, combined with a functional efficacy in the micromolar range and a safe tolerability profile, suggest that these compounds could be safely and effectively administered in combination with chemotherapeutic and anti-angiogenic agents, used at lower doses (as suggested by the DRI), to ruin tumor microenvironment and guarantee the effectiveness of the conventional anticancer agents. Accordingly, they are worth developing to obtain novel drug candidates for the combination treatment of hypoxic solid tumors.

## EXPERIMENTAL SECTION

**Chemistry.** Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Routine $^1$H-NMR spectra were recorded in DMSO-$d_6$ solution on a Varian Gemini 200 spectrometer operating at 200 MHz. Evaporation was performed in vacuo (rotary evaporator). Analytical TLCs were carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Purity of the target inhibitors, **4a**,**b**,**h**, **5b-f**,**h**,**j** and **6a-g**, was determined by HPLC analysis, using a Merck Hitachi D-7000 liquid chromatograph (UV detection at 242 nm) and a Discovery C18 column (250 mm x 4.6 mm, 5 µm, Supelco), with a gradient of water and methanol and a flow rate of 1.5 mL/min. All the compounds showed percent purity values ≥95%. Acetic anhydride and the suitably substituted aroyl chlorides, used to obtain the target inhibitors, were from Alfa Aesar, Aldrich and Fluka. The 4-, 5-, and 6-aminosaccharin, exploited to prepare the target inhibitors, were obtained as reported previously.[16,17]

**General Procedure for the Synthesis of N-(1,1,3-trioxo-2,3-dihydrobenzo[*d*]isothiazol-4- or 5- or 6-yl)benzamides 4a,b,h, 5b-f,h, and 6a-g.** A solution of the appropriate benzo[*d*]isothiazole derivative, **1**, **2** or **3** (0.100 g, 0.50 mmol) in anhydrous toluene was added of the suitable benzoyl chloride (0.50 mmol) and triethylamine (0.50 mmol), and refluxed under stirring until the disappearance of the starting material (TLC analysis). The crude so obtained was evaporated to dryness, then water was added and the resulting compound, separated as a white solid, was collected by filtration, purified through crystallization from the suitable solvent and characterized with physiochemical and spectroscopic data (Supporting Information, Tables 1-6).

**N-(1,1-dioxide-3-oxo-2,3-dihydrobenzo[*d*]isothiazol-5-yl)acetamide, 5j.** A solution of 5-aminobenzo[*d*]isothiazole **2** (0.100 g, 0.50 mmol) and acetic anhydride (0.047 mL, 0.50 mmol) was heated at T=100 °C under stirring until the disappearance of the starting material (TLC analysis). The volatiles were then removed in vacuo and the residue was diluted with water. The resulting white solid was collected by filtration, washed with water, recrystallized from the appropriate solvent and characterized with physio-chemical and spectroscopic data (Supporting Information, Tables 3 and 4).

**Biology. Reagents, proteins, and cell lines.** All the CA isoforms were recombinant enzymes obtained in-house as reported earlier.[22,23] Cell culture media McCoy's 5A, fetal bovine serum (FBS), L-glutamine, and antibiotics were from Gibco (Gaithersburg, MD, USA). $CoCl_2$ was purchased from Sigma (St. Louis, MO, USA); $CoCl_2$ was dissolved in a stock solution of 10 mM in sterile water for cell culture. Plastics were supplied by Sarstedt (Verona, Italy).

**CA inhibition assay.** An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed $CO_2$ hydration activity.[24] 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 inhibitors (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 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,[25,26] and represent the mean from at least three different determinations. Concentration of all the CA isofoms in the assay system varied between 7.8 and 13.6 nM.

**Proliferation assay.** Test compounds, **4b**, **5c** and **5j** were dissolved in a stock solution of 10 mM in 100% dimethylsulfoxide for in vitro studies. DMSO concentration in the control's media was the same used to make up the highest concentration of test compounds in growth media for the same experiment. In vitro chemosensitivity was tested on colorectal cancer HT-29 cell lines. Cells were plated in sterile 24-well plastic plates and treated for 72 h (using $10^3$ cells/well of cancer cells in 1 ml of medium) with added **4b**, **5c**, **5j** (range from 100 μM to 0.1 μM), SN-38 or 5-FU (0.001-50 μM) alone or in combination with 100 μM $CoCl_2$, or vehicle alone or in combination with 100 μM $CoCl_2$. To determine the effect of $CoCl_2$ alone on HT-29 cell proliferation, cells were treated with 100 μM $CoCl_2$ for 72 h.[20] The synergistic effect between **5c** and SN-38 or 5-FU was calculated with the method of Chou [21] based on the multiple drug–effect equation, and quantitated by the combination index (CI) and the dose reduction index (DRI), where CI<1 and DRI>1 indicate synergism. At the end of the experiment, cells were harvested with trypsin/EDTA, and viable cells were quantified using the automatic cell counter ADAM MC Digital B (Twin Helix, Milano, Italy). The data are presented as the percentage of vehicle-treated cells. The concentration of drugs that decreased cell count by 50% ($IC_{50}$) compared with controls was calculated by nonlinear fitting of experimental data. All experiments were repeated, independently, three times with at least nine samples for each concentration.

## ASSOCIATED CONTENT

**Supporting Information**. Tables 1-6, including physical and spectral data of compounds described. This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

## ACKNOWLEDGMENTS

C.L.M. acknowledges the University of Pisa for the financial support (PRA Project).

## ABBREVIATIONS

IARC, International Agency for Research on Cancer; CA, carbonic anhydrase; hCAI, human carbonic anhydrase isoform I; hCAII, human carbonic anhydrase isoform II; hCAVII, human carbonic anhydrase isoform VII; hCAIX, human carbonic anhydrase isoform IX; hCAXII, human carbonic anhydrase isoform XII; 5-FU, 5-fluorouracil; FBS, fetal bovine serum; DRI, dose reduction index; CI, combination index.

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