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Hypoxia-Targeting Carbonic Anhydrase IX Inhibitors by a New Series of Nitroimidazole-Sulfonamides/Sulfamides/Sulfamates

Marouan Rami,[†] Ludwig Dubois,[‡] Nanda-Kumar Parvathaneni,^{†,‡} Vincenzo Alterio,[§] Simon J. A. van Kuijk,[‡] Simona Maria Monti,[§] Philippe Lambin,[‡] Giuseppina De Simone,[§] Claudiu T. Supuran,^{||} and Jean-Yves Winum^{*,†}

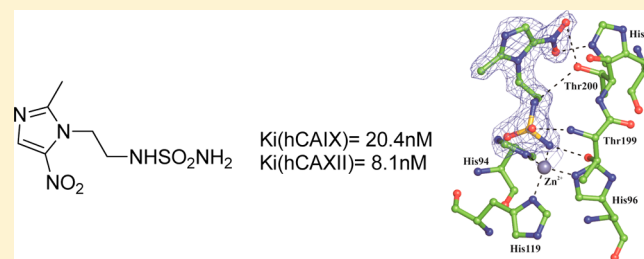
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ABSTRACT: A series of nitroimidazoles incorporating sulfonamide/sulfamide/sulfamate moieties were designed and synthesized as radio/chemosensitizing agent targeting the tumor-associated carbonic anhydrase (CA) isoforms IX and XII. Most of the new compounds were nanomolar inhibitors of these isoforms. Crystallographic studies on the complex of hCA II with the lead sulfamide derivative of this series clarified the binding mode of this type of inhibitors in the enzyme active site cavity. Some of the best nitroimidazole CA IX inhibitors showed significant activity *in vitro* by reducing hypoxia-induced extracellular acidosis in HT-29 and HeLa cell lines. *In vivo* testing of the lead molecule in the sulfamide series, in cotreatment with doxorubicin, demonstrated a chemosensitization of CA IX containing tumors. Such CA inhibitors, specifically targeting the tumor-associated isoforms, are candidates for novel treatment strategies against hypoxic tumors overexpressing extracellular CA isozymes.



INTRODUCTION

Hypoxia is a strong oncogenic phenotype occurring in all solid tumors which is responsible for enhanced malignancy and is associated with resistance to ionizing radiation and chemotherapy. This inherent feature provides significant opportunities for drug discovery especially for specific tumor-targeting agents.^{1,2}

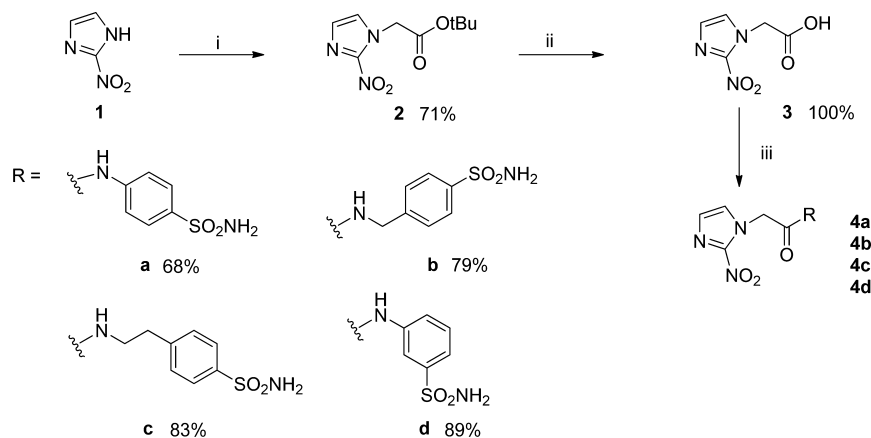
Among the proteins whose expression is induced by hypoxia, via the hypoxia inducible factor 1 α (HIF-1 α), membrane-associated carbonic anhydrase IX (CA IX, (CA, EC 4.2.1.1))³ is the most strongly induced one in human cancer cells.⁴ CA IX is also the most active CA isoform for the CO₂ hydration reaction, playing a major role in regulating the tumor acid–base balance.^{4,5} It is strongly overexpressed in a broad range of tumor types, and its expression negatively correlates with the prognosis of cancer patients. Moreover, this isoform exhibits a very limited expression in normal tissues, thus its inhibition may lead to significantly fewer side effects compared to classical anticancer agents in clinical use.⁴

CA IX is now a validated antitumor target, and its inhibition with antibodies, sulfonamide, and coumarin inhibitors has been undoubtedly proven to reverse the effect of tumor acidification, leading to the inhibition of the cancer cells growth *in vivo*.^{4,6–10}

To improve the potency of cancer treatment with CA inhibitors, we have recently focused our attention on the development of dual drugs able to inhibit selectively CA IX and to enhance hypoxic tumor sensitization toward therapy. To this aim, we decided to utilize nitroimidazole scaffolds as they are well-known as radiosensitizers and have been subject of investigation for several decades with a number of such derivatives being clinically used.¹¹ Thus, the present article deals with the development of novel nitroimidazole compounds whose properties have been optimized by conjugation with pharmacophoric moieties targeting CA IX. In particular, we report here the synthesis and carbonic anhydrase inhibitory activity of a panel of sulfonamide/sulfamide/sulfamate derivatives containing 2- or 5-nitroimidazole moieties. The crystallographic structures of the lead sulfamide derivative in adduct with isoform II was studied. Validation in *in vitro* studies identified compounds that selectively inhibit CA IX activity, thereby reducing hypoxia-induced extracellular acidosis. From this inhibitor library a lead compound was taken forward into *in vivo*, demonstrating to be able to chemosensitize tumors

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Scheme 1^a

^aReagents and conditions: (i) 1 equiv of 2-nitroimidazole, 1 equiv of *tert*-butyl bromoacetate, 4 equiv of potassium carbonate, MeCN, RT, 1 night; (ii) cocktail of trifluoroacetic acid/water/thioanisole 95/2.5/2.5 v/v, rt, 1 night; (iii) 1 equiv of 4-dimethylaminopyridine (DMAP), 1 equiv of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N,N*-dimethylacetamide (DMA), RT, 2 days.

(HT-29 colorectal carcinoma) to clinically available treatment schedules.

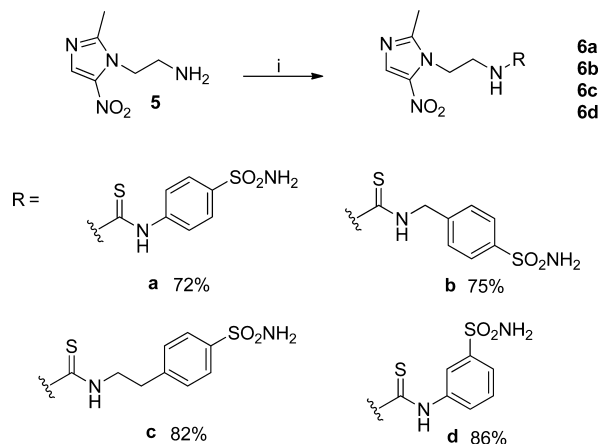
RESULTS AND DISCUSSION

Several studies have emphasized that specific inhibitors of CA IX activity are promising to pursue for their tumor-specific therapeutic properties also in combination with conventional therapies.^{3–5} A different approach to sensitize tumors is the use of nitroimidazole, although high clinical toxicity has been observed upon therapeutic efficacy.^{11,12} An important requisite for newly designed compounds is therefore the more specific targeting toward the hypoxic tumor regions using lower doses.

On the basis of these considerations, the rationale of this work for designing new CAIs was to incorporate in the same scaffold two different functionalities: (i) the sulfonamide/sulfamate/sulfamide one which is responsible for binding to the Zn(II) within the CA active site and hence inhibition of the enzyme and (ii) a nitroimidazole moiety which is responsible for radiosensitization in tumors (such moiety is found in agents such as Pimonidazole which has proved to be an effective and nontoxic hypoxia marker for several human squamous cell carcinomas). A comparable approach has been in fact reported earlier by one of our groups¹³ by combining benzenesulfonamide and aromatic nitro derivatives in the same molecule. Indeed, the derivatives of this type reported by D'Ambrosio et al.¹³ were highly effective CA IX/XII inhibitors.

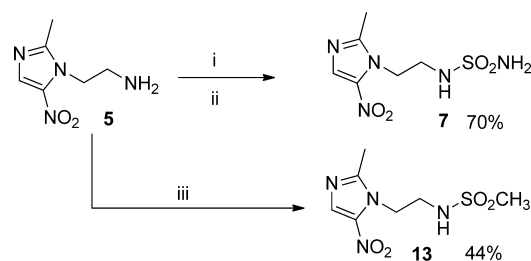
Chemistry. Different libraries of inhibitors were synthesized by incorporating 2- or 5-nitroimidazole moieties. Starting from the 2-nitroimidazole **1**, we introduced the acetic acid moiety on position 1 of the imidazole ring by reacting *tert*-butyl bromoacetate on **1** in basic medium followed by deprotection of the resulting ester **2** by trifluoroacetic acid to give compound **3**. The latter was then reacted with different amino-benzenesulfonamide derivatives using carbodiimide as a coupling agent to give compounds **4** in good yield (Scheme 1).^{10,11}

The synthesis of the 5-nitroimidazole series was realized by following classical strategies used for the preparation of CA inhibitors. Starting from the amine analogue of metronidazole **5**, we reacted different benzene sulfonamide isothiocyanates to lead to the series of thiourea compounds **6** (Scheme 2).^{14,15}

Scheme 2^a

^aReagents and conditions: (i) 1 equiv of 1-(2-aminoethyl)-2-methyl-5-nitroimidazole dihydrochloride monohydrate, 1 equiv SCN-Ph-SO₂NH₂, 2 equiv of triethylamine, MeCN, RT, 1 h.

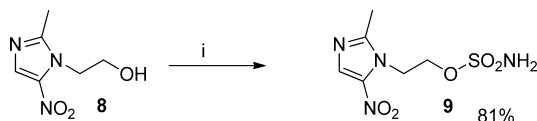
Sulfamide derivative **7** and methylsulfonamide analogue **13** (Scheme 3) were obtained from the same starting material **5** by a procedure previously reported by this group.¹⁶ Sulfamide analogue **9** was prepared starting from the metronidazole **8**

Scheme 3^a

^aReagents and conditions: (i) 1 equiv of 1-(2-aminoethyl)-2-methyl-5-nitroimidazole dihydrochloride monohydrate, 4 equiv of triethylamine, 1 equiv of chlorosulfonylisocyanate, 1 equiv of *tert*-butanol, CH₂Cl₂, rt, 1 h; (ii) trifluoroacetic acid-CH₂Cl₂ 7:3 v/v, RT, 6 h; (iii) 2 equiv of methane sulfonyl chloride, 2 equiv of triethylamine, CH₂Cl₂.

using a direct sulfamoylation procedure as described previously (Scheme 4).¹⁷

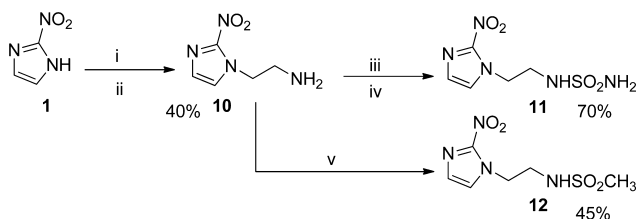
Scheme 4^a



^aReagents and conditions: (i) 1 equiv of 2-methyl-5-nitro-1-imidazoleethanol, *N,N*-dimethylacetamide, 3 equiv sulfamoyl chloride, RT, 1 night.

Compounds **11** and **12** were obtained as the same manners as compounds **7** and **13** starting from compound **10** obtained as previously described by Hoigebazar et al.¹⁸ (Scheme 5).

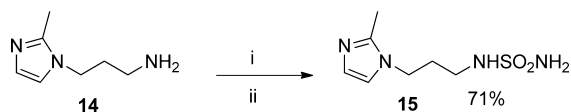
Scheme 5^a



^aReagents and conditions: (i) 1 equiv of 2-nitroimidazole, dry DMF, 1 equiv K_2CO_3 , 1.5 equiv *tert*-butyl 2-bromoethylcarbamate; (ii) trifluoroacetic acid– CH_2Cl_2 8:2 v/v, RT, 2 h; (iii) 4 equiv of triethylamine, 1.5 equiv of chlorosulfonylisocyanate, 1.5 equiv of *tert*-butanol, CH_2Cl_2 , rt, 12 h; (iv) trifluoroacetic acid– CH_2Cl_2 8:2 v/v, RT, 2 h; (v) 2 equiv of methane sulfonyl chloride, 2 equiv of triethylamine, CH_2Cl_2 .

Sulfamide **15** was prepared from the commercially available compound **14** following the sulfamoylation process used for compound **7** (Scheme 6).

Scheme 6^a



^aReagents and conditions: (i) 4 equiv of triethylamine, 1.5 equiv of chlorosulfonylisocyanate, 1.5 equiv of *tert*-butanol, CH_2Cl_2 , rt, 12 h; (ii) trifluoroacetic acid– CH_2Cl_2 8:2 v/v, RT, 2 h.

The new compounds were characterized extensively by spectral and physicochemical methods which confirmed their structures

CA Inhibition Assays. All compounds reported here were assayed as inhibitors of four physiologically relevant CA isoforms, the cytosolic hCA I and II (h = human isoform), and the transmembrane, tumor-associated hCA IX and XII using the CO_2 hydrase assay (Table 1).¹⁹ The clinically employed sulfonamide acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) has been used as standard in these measurements for comparison reasons.

Analysis of these inhibition data shows a very interesting inhibition profile for this newly designed series of compounds. Indeed, all compounds, except **12** and **13** which were prepared

as negative control molecules, acted as effective inhibitors against isoforms hCA I, II, IX, and XII.

Against the abundant, cytosolic isoform hCA I, the new compounds reported here behaved as medium/weak potency inhibitors, with K_i s in the range of 79 nM to 9.57 μ M. For the benzenesulfonamides **4** and **6**, the main factor influencing hCA I inhibition was the substitution pattern of the benzenesulfonamide and the length of the linker between this and the rest of the molecule. Indeed, for compounds of subseries **4**, the sulfanilamide derivative **4a** was a much weaker hCA I inhibitor compared to the congeners with a larger linker ($n = 1$ and 2) between the benzenesulfonamide functionality and the nitroazole-acetamido moiety. Indeed, the derivatives **4b** and **4c** having these longer linkers, as well as the metanilamide derivative **4d**, were much more effective hCA I inhibitors (K_i s of 79–107 nM) compared to the sulfanilamide derivative **4a** (K_i of 3203 nM). In the case of the thioureas **6**, the SAR is slightly different in the sense that the least effective derivative was the homosulfanilamide derivative **6b** (K_i of 483 nM), whereas the remaining ones (**6a**, **6c**, and **6d**) showed a similar behavior of medium potency inhibitors (K_i of 79–105 nM). Finally, sulfamates, sulfamides, and methylsulfonamide **7**, **9**, and **11–15**, were either very weak hCA I inhibitors or devoid of such an activity (Table 1).

The physiologically dominant off-target isoform hCA II was highly inhibited by most of the new sulfonamides reported here (**4b–4d**, **6a–6d**), which had K_i s in the range of 2.9–7.4 nM (better than that of AAZ of 12 nM). Compounds in the sulfamide and sulfamate series were medium potency inhibitor (K_i s of 33.8–58 nM) except **7**, which showed activity in the same range as AAZ. Derivatives **12** and **13** were not inhibitory also against this isoform (Table 1). Again, SAR is straightforward, as for the discussion of hCA I inhibition above. For amides **4**, the length of the spacer between the benzenesulfonamide and nitroazole functionalities was the main factor influencing activity, whereas for thioureas **6**, all substitution patterns were equal in generating potent hCA II inhibitors. The aliphatic derivatives **7–15** possessing a ZBG were effective or medium potency hCA II inhibitors, whereas those without such a moiety (the methylsulfonamides) were ineffective as hCA II inhibitors.

The tumor-associated transmembrane isoforms hCA IX and hCA XII were both potently inhibited by all sulfonamides series **4** and **6** (except **4a**) reported here, which showed inhibition constants <10 nM, more precisely, in the range of 7.2–8.3 nM against hCA IX, and of 6.7–8.5 against hCA XII, respectively. Sulfamides **7**, **11**, and **15** and sulfamate **9** displayed also high potency at nanomolar levels for these isoforms. SAR for hCA IX inhibition is similar to what is discussed above for hCA I and II inhibition, whereas in the case of hCA XII, all compounds (except those without a ZBG, i.e., **12** and **13**) were highly effective inhibitors, proving that all substitution patterns explored here led to highly effective CAIs.

Selectivity ratios for inhibiting hCA IX over hCA II and hCA XII over hCA II were in the range of 0.4–5. The inhibition profiles of CA II and IX (or CA II and XII) were generally rather similar for all the series of compounds; indeed, only inhibitors **4a**, **4b**, and **9** were shown to have a moderate CA IX/XII selectivity. Nevertheless, considering the difficulty to obtain small compounds with a better affinity for the tumor-associated isozymes over CA II, the selectivity obtained for these series is comparable or better with those of all the clinically used CA

Table 1. hCA I, II, IX, and XII Inhibition Data with Compounds 4–15 and Standard Inhibitor Acetazolamide AAZ by a Stopped-Flow, CO₂ Hydration Assay Method^{19,a}

compd	K_i (nM) ^b				selectivity ratios	
	hCA I ^c	hCA II ^c	hCA IX ^d	hCA XII ^d	K_i hCA II/ K_i hCA IX	K_i hCA II/ K_i hCA XII
AAZ	250	12	25	5.6	0.48	2.14
4a	3203	330	70	64	4.71	5.15
4b	107	37	7.9	8.1	4.68	4.56
4c	79	4.8	8.0	6.7	0.6	0.71
4d	101	3.8	7.3	8.0	0.52	0.47
6a	105	5.5	7.3	8.0	0.75	0.68
6b	483	7.4	7.2	7.7	1.02	0.96
6c	79	2.9	8.3	8.5	0.35	0.34
6d	84	6.6	7.8	7.6	0.84	0.86
7	9576	10.1	20.4	8.1	0.49	1.24
9	4435	33.8	8.3	8.9	4.07	3.79
11	9120	58	41	38	1.41	1.52
12	>20000	>20000	>20000	>20000		
13	>20000	>20000	>20000	>20000		
15	8718	52	21	37	2.47	1.40

^aSelectivity ratios for the inhibition of the tumor-associated (CA IX and XII) over the cytosolic (CA II) isozyme are also reported. ^bErrors in the range of ± 5 –10% of the reported value from three different determinations. ^cFull length, cytosolic isoform. ^dCatalytic domain, recombinant enzyme.

inhibitors which have selectivity ratios for the inhibition of CA II over CA IX <1.

In Vitro Extracellular Acidification Tests. To investigate the efficacy of different dual targeting compounds, their effects on hypoxia-induced extracellular acidification was evaluated by measuring changes in pH before and after exposure to the dual compounds.²⁰ For this purpose, HeLa cervical and HT29 colorectal carcinoma cells were grown under ambient air or lowered oxygen concentrations and exposed to compounds in 0.1 and 1 mM concentrations. HeLa demonstrated increased CA IX expression upon hypoxia, while HT29 also had a high expression under ambient air.²⁰ Previously, we have demonstrated that inhibitor binding to CA IX requires both its expression and its activation and this was only observed exclusively during hypoxia, irrespective of the CA IX expression levels.^{9,20} The 5-nitroimidazole series was selected for further investigation based on the more favorable toxicity profile compared with 2-nitroimidazoles.¹² For all compounds, a concentration of 1 mM resulted in a significant reduction ($P < 0.05$) in hypoxia-induced extracellular acidosis, while the effect on cells exposed to ambient air was negligible (Figure 1). Only for 7 a significant ($P < 0.05$) dose-dependent effect was observed, while a lower concentration was not effective for the other compounds. For 6c, a strong alkalization was observed only for the HeLa cells. Previously, we have shown that for the single targeting compound 13 and 15, only 15 was able to reduce the extracellular acidification.²¹ These data indicated that the efficacy of the dual targeting compound is attributed to the CA IX inhibiting moiety. On the basis of these and the current results, 7 was selected as the lead compound for further in vivo investigations.

In Vivo Tests. To investigate whether 7 exerted a chemosensitizing effect, HT-29 tumor bearing mice were treated with 7 according to previously described strategies^{9,10,21} and subsequently exposed to chemotherapy. 7-treated ($P < 0.01$) or doxorubicin-treated ($P < 0.001$) mice demonstrated a significant reduction in tumor growth, with an average time to reach 4 \times starting volume (T4 \times SV) of 25.43 and 23.39 days for 7 and doxorubicin, respectively, compared to vehicle treatment only (14.07 days). T4 \times SV was further enhanced (40.66 days, P

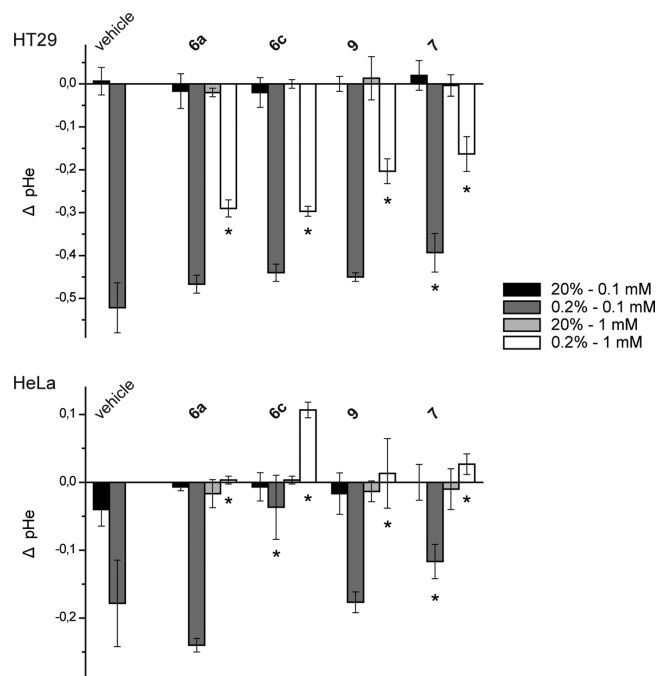


Figure 1. Extracellular acidification of cells exposed to normoxia (20%) or hypoxia (0.2%) upon treatment with dual targeting compounds (0.1 or 1 mM). Data are expressed as the difference between medium pH values ($\Delta pHe = pH$ after incubation – pH before incubation) and show the mean \pm SD of at least three independent experiments. Asterisks indicate significant difference ($*P < 0.05$).

< 0.05) upon combination of 7 with doxorubicin (Figure 2). None of the treatment schedules caused observable toxicity assessed by body weight loss. Furthermore, recently we have demonstrated that 7 also exerted a radiosensitizing effect in a CA IX dependent manner. A significantly higher sensitization enhancement ratio (SER) was observed upon pretreatment with 7 before radiotherapy compared to vehicle pretreatment only for CA IX expressing tumors.²¹ Derivative 7 sensitizes tumors toward radiotherapy and doxorubicin-based chemo-

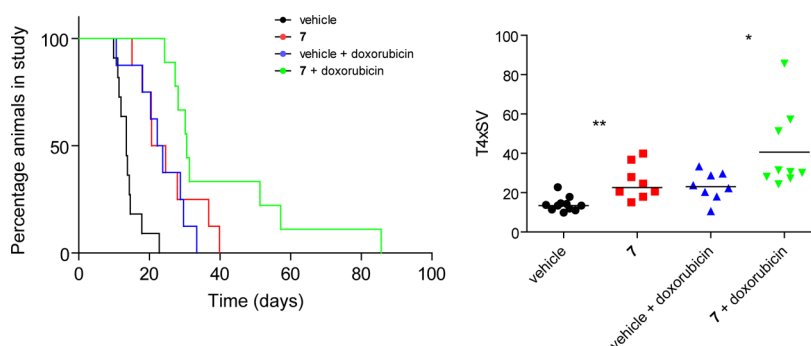


Figure 2. 7 (10 mg/kg 3 days) and doxorubicin (5 mg/kg at day 3) were injected intravenously when tumors reached a volume of 250 mm³. The treatment schedule was repeated for three consecutive weeks. Tumor growth was monitored until reaching 4× the volume at treatment time (T4×SV). Data represent the mean ± SD of 8–11 independent animals. Asterisks indicate statistical significance (**P* < 0.05; ***P* < 0.01).

therapy, indicating the potential utility of nitroimidazole-based CA IX targeting compounds as novel anticancer agents. The sensitization effects of the “dual drug” were markedly higher compared with single CA IX targeting agents⁹ or nitroimidazole-based drugs.¹²

X-ray Crystallography. To determine the molecular features responsible of the CA inhibitory properties of the dual drug 7, the crystal structure of such molecule in complex with the cytosolic dominant isoform hCA II has been solved. Crystals of the hCA II–7 adduct were isomorphous with those of the native protein,²² allowing for the analysis of the three-dimensional structure by difference Fourier techniques. Data collection and refinement statistics are shown in Table 2.

Inhibitor binding did not generate significant changes in hCA II structure, indeed, the rmsd for the superposition of the corresponding C α atoms between the native enzyme and the enzyme–inhibitor adduct was 0.3 Å. The analysis of the electron density maps showed the presence of one inhibitor molecule bound within the active site. The main protein–inhibitor interactions are schematically depicted in Figure 3. Analysis of this figure reveals that the tetrahedral coordination geometry of the Zn²⁺ ion and the key hydrogen bonds between the sulfamide moiety of the inhibitor and the enzyme active site are all retained with respect to other hCA II–sulfamide complexes solved so far.^{23,24} In particular, the ionized sulfamide NH[−] group coordinates to Zn²⁺ ion and donates a hydrogen bond to Thr199OG1, while one of the two sulfamide oxygens accepts a hydrogen bond from the backbone NH group of Thr199. An additional H-bond interaction is observed between the Thr200OG1 atom and the second nitrogen atom of the sulfamide moiety. The imidazole ring of the inhibitor is located in the middle of the active site cavity, with the nitro group oriented toward the hydrophilic part,²⁵ establishing strong direct hydrogen bond interactions with residues Thr200 and His64, stabilized in its *in* conformation (Figure 3). The inhibitor binding is further stabilized by additional water mediated hydrogen bonds and van der Waals interactions with residues of enzyme active site.

CONCLUSION

We designed and synthesized a new class of CA IX inhibitors containing nitroimidazole moieties. These molecules showed efficacy *in vitro* for the inhibition of extracellular tumor acidification in two cell lines overexpressing CA IX, namely the colorectal HT-29 and the cervical HeLa carcinoma cell lines. In both tumor types, a significant reduction of tumor acidosis was detected. Moreover, one of the lead molecules, the sulfamide

Table 2. Crystal Parameters, Data Collection, and Refinement Statistics^a

Crystal Parameters	
space group	P2 ₁
<i>a</i> (Å)	42.17
<i>b</i> (Å)	41.22
<i>c</i> (Å)	71.95
γ (deg)	104.22
no. of independent molecules	1
Data Collection Statistics	
resolution (Å)	20.00–1.85
wavelength (Å)	1.54178
temperature (K)	100
<i>R</i> _{merge} (%) ^b	4.7 (13.2)
mean <i>I</i> / σ (<i>I</i>)	25.7 (6.6)
total reflections	94974
unique reflections	20161
redundancy (%)	4.7 (2.4)
completeness (%)	97.2 (82.2)
Refinement	
resolution (Å)	20.00–1.85
<i>R</i> _{factor} (%) ^c	16.3
<i>R</i> _{free} (%) ^c	19.6
RMSD from ideal geometry	
bond lengths (Å)	0.011
bond angles (deg)	1.7
no. of protein atoms	2177
no. of water molecules	199
no. of inhibitor atoms	16
average <i>B</i> factor (Å ²)	
all atoms	14.1
protein atoms	13.6
inhibitor atoms	20.1
water molecules	22.9

^aValues in parentheses refer to the highest resolution shell (1.92–1.85 Å). ^b $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. ^c $R_{\text{factor}} = \frac{\sum_{hkl} ||F_o(hkl)| - |F_c(hkl)||}{\sum_{hkl} |F_o(hkl)|}$; *R*_{free} calculated with 5% of data withheld from refinement.

derivative 7, was demonstrated to enhance sensitization toward radiotherapy and chemotherapy with doxorubicin *in vivo*. The X-ray crystal structure of the hCA II–7 adduct was also solved, showing that the binding of 7 in the middle of the enzyme active site cavity was mainly driven by the canonical interactions of the sulfamide group, two H-bond interactions between the nitro group of 7 and residues His64 and Thr200,

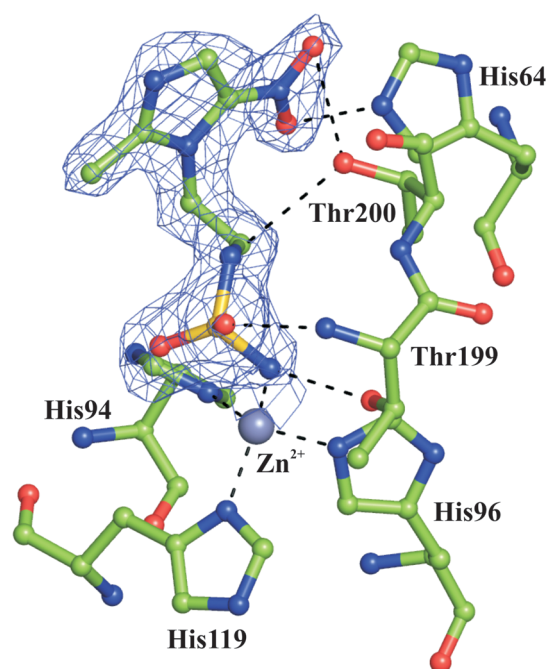


Figure 3. Active site region in the hCAII-7 complex. Active site Zn^{2+} coordination and direct hydrogen bonds between inhibitor and enzyme residues are reported as dashed lines. The σ -A weighted $2F_o - F_c$ simulated annealing omit map (at 1.0σ) relative to the inhibitor molecule is also shown.

as well as several water mediated hydrogen bonds and hydrophobic interactions. Altogether, these data indicate the potential utility of nitroimidazole compounds and open the way to their use as novel anticancer agents targeting the tumor-associated CA isoforms IX and XII.

EXPERIMENTAL SECTION

Chemistry. General. All reagents and solvents were of commercial quality and used without further purification unless otherwise specified. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art. no. 1.05554). Spots were visualized under 254 nm UV illumination or by ninhydrin solution spraying. Melting points were determined on a Büchi Melting Point 510 and are uncorrected. 1H and ^{13}C NMR spectra were recorded on Bruker DRX-400 spectrometer using $DMSO-d_6$ as solvent and tetramethylsilane as internal standard. For 1H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in hertz. Electron Ionization mass spectra were recorded in positive or negative mode on a Water MicroMass ZQ. All compounds that were tested in the biological assays were analyzed by high-resolution ESI mass spectra (HRMS) using on a Q-ToF I mass spectrometer fitted with an electrospray ion source in order to confirm the purity of >95%.

tert-Butyl-(2-nitroimidazol-1-yl) Acetate (2). A solution of *tert*-butylbromoacetate (17.7 mmol, 1 equiv) in 10 mL of acetonitrile was added dropwise to a mixture of 2-nitroimidazole **1** (17.7 mmol, 1 equiv) and anhydrous potassium carbonate (70.74 mmol, 4 equiv) in 20 mL of acetonitrile. The mixture was stirred one night at room temperature and concentrated under vacuum. The residue was purified by chromatography on silica gel using a mixture $CH_2Cl_2/MeOH$ 95/5 as eluent to give the expected compound as white powder in 71% yield; mp 95–97 °C. 1H NMR ($DMSO-d_6$, 400 MHz) δ 1.46 (s, 9H), 4.99 (s, 2H), 7.06 (d, 1H, $J = 1.01$ Hz), 7.17 (d, 1H, $J = 1.01$ Hz). MS (ESI⁺/ESI⁻) m/z 226.15 [M - H]⁻, 262.13 [M + Cl]⁻, 250.20 [M + H]⁺.

(2-Nitroimidazol-1-yl) Acetic Acid (3). Compound **2** (2.5g) was dissolved in 20 mL of a cocktail of TFA–water–thioanisole 95:2.5:2.5 and stirred at room temperature for one night. The mixture was then concentrated under vacuum and coevaporated several times with diethyl ether until formation of a powder. After filtration, the precipitate was washed with dichloromethane and acetonitrile to give quantitatively the expected product; mp 143 °C (decomposition). 1H NMR ($DMSO-d_6$, 400 MHz) δ 5.21 (s, 2H), 7.21 (d, 1H, $J = 1.01$ Hz), 7.64 (d, 1H, $J = 1.01$ Hz). ^{13}C ($DMSO-d_6$, 101 MHz) δ 50.65, 127.69, 128.44, 168.56, 168.57. MS (ESI⁺/ESI⁻) m/z 170.12 [M - H]⁻, 341.05 [2M - H]⁻, 194.14 [M + Na]⁺.

General Procedure for the Preparation of Compounds (4a–d). The aminoalkylbenzene sulfonamide (1.17 mmol, 1 equiv), 4-dimethylaminopyridine (1.17 mmol, 1 equiv), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1.17 mmol, 1 equiv) were added to a solution of compound **3** (1.17 mmol, 1 equiv) in 8 mL of *N,N*-dimethylacetamide. The mixture was stirred two days at room temperature, then diluted with ethyl acetate and washed three times with water. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under vacuum. The residue was then purified by chromatography on silica gel using methylene chloride–methanol 98:2 v/v as eluent.

2-(2-Nitroimidazol-1-yl)-N-(4-sulfamoylphenyl)acetamide (4a). Yield 68%; mp 163–165 °C. 1H NMR ($DMSO-d_6$, 400 MHz) δ 5.36 (s, 2H), 7.24 (d, 1H, $J = 1.01$ Hz), 7.28 (s, 2H), 7.67 (d, 1H, $J = 1.01$ Hz), 7.70 (d, 2H, $J = 8.9$ Hz), 7.77 (d, 2H, $J = 8.9$ Hz), 10.7 (s, 1H). ^{13}C ($DMSO-d_6$, 101 MHz) δ 52.20, 118.70, 126.81, 127.57, 128.84, 138.76, 141.18, 144.79, 165.02. MS (ESI⁺/ESI⁻) m/z 324.09 [M - H]⁻, 359.92 [M + Cl]⁻, 649.15 [2M - H]⁻, 685.01 [2M + Cl]⁻, 348.14 [M + Na]⁺. HRMS (ESI) [M + H]⁺ calculated for $[C_{11}H_{12}N_5O_5S]^+$, 326.0559; found, 326.0563.

2-(2-Nitroimidazol-1-yl)-N-(4-sulfamoylbenzyl)acetamide (4b). Yield 83%; mp 181–183 °C. 1H NMR ($DMSO-d_6$, 400 MHz) δ 4.37 (d, 2H, $J = 5.7$ Hz), 5.22 (s, 2H), 7.18 (d, 1H, $J = 1.01$ Hz), 7.35 (s, 2H), 7.43 (d, 2H, $J = 8.4$ Hz), 7.67 (d, 1H, $J = 1.01$ Hz), 7.76 (d, 2H, $J = 8.4$ Hz), 9.15 (t, 1H, $J = 6.06$ Hz). ^{13}C ($DMSO-d_6$, 101 MHz) δ 41.81, 51.55, 106.87, 125.58, 127.39, 138.91, 142.66, 142.99, 156.82, 165.88. MS (ESI⁺/ESI⁻) m/z 338.15 [M - H]⁻, 374.22 [M + Cl]⁻, 713.16 [2M + Cl]⁻, 340.15 [M + H]⁺, 362.17 [M + Na]⁺. HRMS (ESI) [M + H]⁺ calculated for $[C_{12}H_{14}N_5O_5S]^+$, 340.0716; found, 340.0723.

2-(2-Nitroimidazol-1-yl)-N-[2-(4-sulfamoylphenyl)ethyl]acetamide (4c). Yield 89%; mp 139–141 °C. 1H NMR ($DMSO-d_6$, 400 MHz) δ 2.80 (t, 2H, $J = 6.9$ Hz), 3.16 (m, 2H), 5.07 (s, 2H), 7.18 (d, 1H, $J = 1.01$ Hz), 7.30 (s, 2H), 7.40 (d, 2H, $J = 8.2$ Hz), 7.61 (d, 1H, $J = 1.01$ Hz), 7.74 (d, 2H, $J = 8.2$ Hz), 8.46 (t, 1H, $J = 5.6$ Hz). ^{13}C ($DMSO-d_6$, 101 MHz) δ 34.59, 51.49, 125.64, 127.38, 128.74, 129.09, 142.05, 143.39, 144.87, 165.57. MS (ESI⁺/ESI⁻) m/z 352.19 [M - H]⁻, 388.07 [M + Cl]⁻, 354.12 [M + H]⁺, 376.09 [M + Na]⁺, 729.21 [2M + Na]⁺. HRMS (ESI) [M + H]⁺ calculated for $[C_{13}H_{16}N_5O_5S]^+$, 354.0872; found, 354.0873.

2-(2-Nitroimidazol-1-yl)-N-(3-sulfamoylphenyl)acetamide (4d). Yield 79%; mp 195–197 °C. 1H NMR ($DMSO-d_6$, 400 MHz) δ 5.35 (s, 2H), 7.24 (d, 1H, $J = 1.01$ Hz), 7.38 (s, 2H), 7.52 (m, 2H), 7.65 (m, 1H), 7.67 (d, 1H, $J = 1.01$ Hz), 8.14 (s, 1H), 10.81 (s, 1H). ^{13}C ($DMSO-d_6$, 101 MHz) δ 52.16, 116.10, 120.72, 121.92, 127.59, 128.86, 129.67, 138.67, 144.72, 164.87, 167.75. MS (ESI⁺/ESI⁻) m/z 324.24 [M - H]⁻, 360.18 [M + Cl]⁻, 685.13 [2M + Cl]⁻, 326.24 [M + H]⁺, 348.07 [M + Na]⁺, 364.17 [M + K]⁺, 673.18 [2M + Na]⁺. HRMS (ESI) [M + H]⁺ calculated for $[C_{11}H_{12}N_5O_5S]^+$, 326.0559; found, 326.0553.

General Procedure for the Preparation of Compounds (6a–d). Isothiocyanate (0.76 mmol, 1 equiv) was added to a solution of the commercially available compound **5** (0.76 mmol, 1 equiv) in 10 mL of acetonitrile. The reaction was stirred for one hour at room temperature and then filtered. The filtrate was concentrated under vacuum and the residue obtained purified by chromatography on silica gel using methylene chloride–methanol 95:5 as eluent.

N-(4-Sulfamoylphenyl)-N-((2-aminoethyl)-2-methyl-5-nitroimidazolyl) Thiourea (6a). Yield 72%; mp 186–188 °C. 1H NMR

(DMSO- d_6 , 400 MHz) δ 2.42 (s, 3H), 3.91 (m, 2H), 4.5 (t, 2H, $J = 5.68$ Hz), 7.3 (s, 2H), 7.46 (d, 2H, $J = 8.7$ Hz), 7.71 (d, 2H, $J = 8.7$ Hz), 8.05 (s, 2H), 10.0 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 13.81, 42.87, 44.86, 122.46, 126.30, 133.19, 138.66, 139.14, 141.88, 151.35, 180.88. MS (ESI⁺/ESI⁻) m/z 385.17 [M + H]⁺, 407.07 [M + Na]⁺, 769.22 [2M + H]⁺, 383.21 [M - H]⁻, 419.18 [M + Cl]⁻, 767.16 [2M - H]⁻. HRMS (ESI) [M + H]⁺ calculated for [C₁₃H₁₇N₆O₄S₂]⁺, 385.0753; found, 385.0756.

N-(4-Sulfamoylbenzyl)-*N*-((2-aminoethyl)-2-methyl-5-nitroimidazolyl) Thiourea (**6b**). Yield 82%; mp 67–69 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.37 (s, 3H), 2.87 (m, 2H), 4.43 (t, 2H, $J = 5.18$ Hz), 4.70 (br s, 2H), 7.32 (s, 2H), 7.34 (d, 2H, $J = 8.4$ Hz), 7.68 (s, 1H), 7.75 (d, 2H, $J = 8.4$ Hz), 8.03 (s, 1H), 8.15 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 13.84, 30.64, 42.70, 45.44, 125.52, 127.23, 133.17, 138.61, 142.48, 151.41, 181.44. MS (ESI⁺/ESI⁻) m/z 399.23 [M + H]⁺, 421.16 [M + Na]⁺, 797.08 [2M + H]⁺, 819.26 [2M + Na]⁺, 397.10 [M - H]⁻, 433.09 [M + Cl]⁻, 795.33 [2M - H]⁻. HRMS (ESI) [M + H]⁺ calculated for [C₁₄H₁₉N₆O₄S₂]⁺, 399.0909; found, 399.0912.

N-(4-Sulfamoylphenylethyl)-*N*-((2-aminoethyl)-2-methyl-5-nitroimidazolyl) thiourea (**6c**). Yield 86%; mp 75–77 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.35 (s, 3H), 2.83 (m, 2H), 3.62 (m, 2H), 3.82 (m, 2H), 4.41 (m, 2H), 7.31 (s, 2H), 7.37 (d, 2H, $J = 8.2$ Hz), 7.52 (s, 1H), 7.63 (s, 1H), 7.74 (d, 2H, $J = 8.2$ Hz), 8.03 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 13.78, 30.64, 45.4, 125.65, 129.05, 133.18, 138.54, 142.03, 143.45, 151.42, 180.83. MS (ESI⁺/ESI⁻) m/z 413.06 [M + H]⁺, 435.02 [M + Na]⁺, 825.09 [2M + H]⁺, 847.21 [2M + Na]⁺, 411.06 [M - H]⁻, 447.20 [M + Cl]⁻, 822.99 [2M - H]⁻, 859.26 [2M + Cl]⁻. HRMS (ESI) [M + H]⁺ calculated for [C₁₅H₂₁N₆O₄S₂]⁺, 413.1066; found, 413.1069.

N-(3-Sulfamoylphenylethyl)-*N*-((2-aminoethyl)-2-methyl-5-nitroimidazolyl) Thiourea (**6d**). Yield 75%; mp 66–68 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.43 (s, 3H), 3.91 (m, 2H), 4.49 (t, 2H, $J = 5.68$ Hz), 7.39 (s, 2H), 7.51 (m, 1H), 7.55 (s, 1H), 7.57 (m, 1H), 7.81 (s, 1H), 7.93 (m, 1H), 8.04 (s, 1H), 9.94 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 13.81, 42.82, 44.99, 120.29, 121.39, 126.62, 129.09, 133.18, 138.65, 139.51, 144.35, 151.37, 181.28. MS (ESI⁺/ESI⁻) m/z 385.23 [M + H]⁺, 406.94 [M + Na]⁺, 791.19 [2M + Na]⁺, 383.12 [M - H]⁻, 419.09 [M + Cl]⁻, 767.26 [2M - H]⁻. HRMS (ESI) [M + H]⁺ calculated for [C₁₃H₁₇N₆O₄S₂]⁺, 385.0753; found, 385.0746.

N-[2-(2-Methyl-5-nitroimidazol-1-yl)ethyl]sulfamide (**7**). A solution of chlorosulfonyl isocyanate (4.59 mmol, 1.2 equiv) and *tert*-butanol (4.59 mmol, 1.2 equiv) in 2 mL of methylene chloride (prepared *ab initio*) was added to a solution of **5** (3.83 mmol, 1 equiv) and triethylamine (30.63 mmol, 4 equiv) in 10 mL of methylene chloride. The mixture was stirred at room temperature for one hour, then diluted with ethyl acetate and washed with water. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The residue was purified by chromatography on silica gel using methylene chloride–methanol 98:2 as eluent. This intermediate was then diluted in a solution of trifluoroacetic acid in methylene chloride (30% volume) and stirred at room temperature for 6 h. The mixture was then concentrated under vacuum and coevaporated several times with diethyl ether to give the expected sulfamide as a white powder. Overall yield 70%; mp 122 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.52 (s, 3H), 3.26 (m, 2H), 4.37 (t, 2H, $J = 5.81$ Hz), 6.65 (s, 2H), 6.86 (s, 1H), 8.1 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 14.03, 41.8, 46.0, 132.68, 138.26, 151.65. MS (ESI⁺/ESI⁻) m/z 250.19 [M + H]⁺, 272.34 [M + Na]⁺, 499.32 [2M + H]⁺, 249.09 [M - H]⁻, 284.12 [M + Cl]⁻, 533.14 [2M + Cl]⁻. HRMS (ESI) [M + H]⁺ calculated for [C₆H₁₂N₃O₄S]⁺, 250.0610; found, 250.0616.

N-[2-(2-Methyl-5-nitroimidazol-1-yl)ethyl]sulfamate (**9**). Sulfamoyl chloride (5.25 mmol, 3 equiv) was added to a solution of the commercially available compound **8** (1.75 mmol, 1 equiv) in *N,N*-dimethylacetamide. The mixture was stirred at room temperature for one night, then diluted with ethyl acetate and washed three times with water. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under vacuum. The residue was purified by chromatography on silica gel using methylene chloride–methanol 9:1 as eluent. Yield 81%; mp 166–168 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.45 (s, 3H), 4.35 (t, 2H, $J = 5.05$ Hz), 4.61 (t, 2H, $J = 5.05$

Hz), 7.57 (s, 2H), 8.06 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 14.04, 44.98, 57.21, 133.10, 138.32, 151.82. MS (ESI⁺/ESI⁻) m/z 250.3 [M + H]⁺, 272.32 [M + Na]⁺, 521.30 [2M + Na]⁺, 770.16 [3M + Na]⁺. HRMS (ESI) [M + H]⁺ calculated for [C₆H₁₁N₄O₅S]⁺, 251.0450; found, 251.0456.

2-(2-Nitroimidazol-1-yl)ethylamine (**10**). *tert*-Butyl 2-bromocarbamate (3.3 mmol, 1.5 equiv) was added at room temperature to a solution of 2-nitroimidazole (2.2 mmol, 1 equiv) and K₂CO₃ (2.2 mmol, 1 equiv) in 3 mL of DMF. The reaction mixture was stirred at room temperature overnight then filtered, and the filtrate was concentrated under vacuum. The solid obtained was dissolved with ethyl acetate and washed with water. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on silica column chromatography using methylene chloride–methanol 9:1 as eluent. The pure Boc protected compound was then dissolved in 20% trifluoroacetic acid–methylene chloride (16 equiv TFA) solution and stirred at room temperature for 2 h. The reaction mixture was then concentrated in vacuo and coevaporated with methanol. The pure expected compound (under trifluoroacetate salt) was then obtained after precipitation in diethyl ether and filtration. Yield 40%; mp 166–168 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 3.33 (t, 2H, $J = 6.0$ Hz), 4.64 (t, 2H, $J = 6.0$ Hz), 7.23 (d, 1H, $J = 0.6$ Hz), 7.65 (d, 1, $J = 0.5$ Hz), 8.17 (s, 2H). MS (ESI⁺) m/z 157.09 [M + H]⁺, 313.35 [2M + H]⁺.

N-[2-(2-Nitroimidazol-1-yl)ethyl]sulfamide (**11**). Same protocol as for the synthesis of compound **7** starting from **10**. Overall yield 70%; mp 78–80 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 3.39 (q, 2H, $J = 6.2$ Hz), 4.46 (t, 2H, $J = 5.9$ Hz), 6.64 (s, 2H), 7.18 (d, 1H, $J = 1$ Hz), 7.23 (t, 1H, $J = 6.2$ Hz), 7.59 (d, 1H, $J = 1$ Hz). ^{13}C (DMSO- d_6 , 101 MHz) δ 41.85, 49.45, 127.63, 128.52. MS (ESI⁺/ESI⁻) m/z 236.15 [M + H]⁺. HRMS (ESI) [M + H]⁺ calculated for [C₅H₁₀N₅O₄S]⁺, 236.0453; found, 236.0456.

N-Methanesulfonyl 2-(2-Nitroimidazol-1-yl)ethylamine (**12**). Compound **10** (1 equiv) was suspended in methylene chloride and triethylamine (2 equiv 2.56 mmol) was added at 0 °C. Methanesulfonyl chloride (2 equiv 2.56 mmol) was added dropwise to the resulting solution. Reaction was monitored by TLC until completion. Then the reaction mixture was diluted with methylene chloride and washed with water. The organic phase was dried over sodium sulfate and concentrated under vacuum. The residue was purified on silica gel column chromatography (eluent methylene chloride–methanol 9:1 v/v) to afford the pure expected compound as a white powder. Yield 45%; mp 122–125 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.87 (s, 3H), 3.39 (q, 2H, $J = 6.2$ Hz), 4.47 (t, 2H, $J = 5.9$ Hz), 7.19 (d, 1H, $J = 1.0$ Hz), 7.24 (t, 1H, $J = 6.2$ Hz), 7.60 (d, 1H, $J = 1.0$ Hz). ^{13}C (DMSO- d_6 , 101 MHz) δ 128.52, 127.63, 49.45, 41.85, 39.64. MS (ESI⁺/ESI⁻) m/z 235.13 [M + H]⁺. HRMS (ESI) [M + H]⁺ calculated for [C₆H₁₁N₄O₄S]⁺, 235.0501; found, 235.0507.

N-Methanesulfonyl 1-(2-Aminoethyl)-2-methyl-5-nitroimidazole (**13**). Same protocol as for the synthesis of compound **12** starting from **5**. Yield 44%; mp 132–135 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.47 (s, 3H), 2.87 (s, 3H), 3.33 (q, 2H, $J = 6.2$ Hz), 4.34 (t, 2H, $J = 5.9$ Hz), 7.33 (t, 1H, $J = 6.4$ Hz), 8.05 (s, 1H). ^{13}C (DMSO- d_6 , 101 MHz) δ 151.72, 138.35, 133.17, 46.33, 41.66, 39.40, 14.16. MS (ESI⁺/ESI⁻) m/z 249.20 [M + H]⁺. HRMS (ESI) [M + H]⁺ calculated for [C₇H₁₃N₄O₄S]⁺, 249.0658; found, 249.0657.

N-[2-(2-Methylimidazol-1-yl)propyl]sulfamide (**15**). Same protocol as for the synthesis of compound **7** starting from **14**. Overall yield 71%; mp 133–135 °C. ^1H NMR (DMSO- d_6 , 400 MHz) δ 2.47 (s, 3H), 2.86 (m, 2H), 3.38 (q, 2H, $J = 6.1$ Hz), 4.46 (t, 2H, $J = 5.9$ Hz), 6.64 (s, 2H), 7.18 (d, 1H, $J = 1.0$ Hz), 7.23 (t, 1H, $J = 6.2$ Hz), 7.59 (d, 1H, $J = 1.0$ Hz). ^{13}C (DMSO- d_6 , 101 MHz) δ 151.72, 138.35, 133.17, 46.33, 41.66, 38.81, 14.16. MS (ESI⁺/ESI⁻) m/z 219.28 [M + H]⁺. HRMS (ESI) [M + H]⁺ calculated for [C₇H₁₃N₄O₂S]⁺, 219.0916; found, 219.0917.

CA Inhibition Assays. An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO₂ hydration activity.¹⁹ Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer and 20 mM Na₂SO₄ (for maintaining

constant the ionic strength), following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction were used for determining the initial 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 distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay to allow for the formation of the E–I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3 and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained in house as reported earlier.

Biological Assays. *Cells.* Exponentially growing colorectal (HT-29, ATCC HTB-38) and cervical (HeLa, ATCC CCL-2) carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Low oxygen conditions were acquired in a hypoxic workstation (Ruskinn INVIVO2 1000). The atmosphere in the chamber consisted of 0.2% O₂ (hypoxia), 5% CO₂, and residual N₂. In parallel, normoxic (20% O₂) dishes were incubated in air with 5% CO₂. pH of the culture medium was immediately measured at the end of each experiment as previously described.^{9,20}

Compounds Preparation. Compounds were dissolved in culture medium containing 1% DMSO at the indicated final concentrations just before addition to the cells. For the animal experiments, 7 was dissolved in NaCl 0.9% containing 1% DMSO to a final concentration of 10 mg/kg and injected intravenously via a lateral tail vein.

Animals. Cells were resuspended in Matrigel (BD Biosciences) and injected subcutaneously into the lateral flank of adult NMRI-nu mice (28–32 g). Intravenous 7 and doxorubicin treatment started at a tumor volume of 250 mm³ for 3 days (10 mg/kg daily) and at day 3 (5 mg/kg), respectively. This schedule was repeated during 3 weeks. Tumor growth was monitored until reaching 4× the volume at treatment time (T4×SV), and treatment toxicity was scored by body weight measurements. All experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethical Committee of the University (2008-025).

Statistics. All statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, 2009, California, USA). A nonparametric Mann–Whitney U test for small groups was used to determine the statistical significance of differences between two independent groups of variables. For all tests, a $P < 0.05$ was considered significant.

X-ray Studies. The hCA II–7 complex was obtained by adding a 5 molar excess of inhibitor to a 10 mg/mL protein solution in 20 mM Tris-HCl pH 8, 0.1% DMSO. Crystals of the complex were obtained using the hanging drop vapor diffusion technique. In particular 2 μL of complex solution and 2 μL of precipitant solution (1.4 M Na-citrate, 100 mM Tris-HCl pH 8.0) were mixed and suspended over a reservoir containing 1 mL of precipitant solution at 20 °C. Crystals grew within 3 days. A complete data set was collected at 1.85 Å resolution from a single crystal, at 100 K, with a copper rotating anode generator developed by Rigaku and equipped with Rigaku Saturn CCD detector. Prior to cryogenic freezing, crystals were transferred to the precipitant solution with the addition of 20% (v/v) glycerol. Diffracted intensities were processed using the HKL2000 crystallographic data reduction package (Denzo/Scalepack).²⁶ Crystal parameters and data processing statistics are summarized in Table 2. The structure of the complex was analyzed by difference Fourier techniques using hCA II crystallized in the P2₁ space group (PDB code 1CA2)²² as the starting model after deletion of nonprotein atoms. An initial round of rigid body refinement followed by simulated annealing and individual B factor refinement was performed using the program CNS.²⁷ Model visualization and rebuilding was performed using the graphics program O.²⁸ After an initial refinement, limited to the enzyme structure, a model for the inhibitor was easily built and introduced into the atomic

coordinates set for further refinement. Restraints on inhibitor bond angles and distances were taken from the Cambridge Structural Database,²⁹ and standard restraints were used on protein bond angles and distances throughout refinement. Water molecules were built into peaks $>3\sigma$ in $|F_o| - |F_c|$ maps that demonstrated appropriate hydrogen-bonding geometry. Final crystallographic R_{factor} and R_{free} values were 0.163 and 0.196, respectively. Statistics for refinement are summarized in Table 2.

■ ASSOCIATED CONTENT

Accession Codes

Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 4MO8).

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Wilson, W. R.; Hay, M. P. Targeting hypoxia in cancer therapy. *Nature Rev. Cancer* **2011**, *11*, 393–410.
- (2) Bennewith, K. L.; Dedhar, S. Targeting hypoxic tumour cells to overcome metastasis. *BMC Cancer* **2011**, *11*, 504.
- (3) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nature Rev. Drug Discovery* **2008**, *7*, 168–181.
- (4) McDonald, P. C.; Winum, J.-Y.; Supuran, C. T.; Dedhar, S. Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. *Oncotarget* **2012**, *3*, 84–97.
- (5) Neri, D.; Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Rev. Drug Discovery* **2011**, *10*, 767–777.
- (6) Pacchiano, F.; Carta, F.; McDonald, P. C.; Lou, Y.; Vullo, D.; Scozzafava, A.; Dedhar, S.; Supuran, C. T. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. *J. Med. Chem.* **2011**, *54*, 1896–1902.
- (7) Touisni, N.; Maresca, A.; McDonald, P. C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J.-Y.; Supuran, C. T. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J. Med. Chem.* **2011**, *54*, 8271–8277.
- (8) Lou, Y.; McDonald, P. C.; Oloumi, A.; Chia, S.; Ostlund, C.; Ahmadi, A.; Kyle, A.; Auf dem Keller, U.; Leung, S.; Huntsman, D.; Clarke, B.; Sutherland, B. W.; Waterhouse, D.; Bally, M.; Roskelley, C.; Overall, C. M.; Minchinton, A.; Pacchiano, F.; Carta, F.; Scozzafava, A.; Touisni, N.; Winum, J.-Y.; Supuran, C. T.; Dedhar, S. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res.* **2011**, *71*, 3364–3376.
- (9) Dubois, L.; Peeters, S.; Lieuwes, N. G.; Geusens, N.; Thiry, A.; Wigfield, S.; Carta, F.; McIntyre, A.; Scozzafava, A.; Dogné, J.-M.; Supuran, C. T.; Harris, A. L.; Masereel, B.; Lambin, P. Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation. *Radiother. Oncol.* **2011**, *99*, 424–431.

- (10) Gieling, R. G.; Babur, M.; Mamnani, L.; Burrows, N.; Telfer, B. A.; Carta, F.; Winum, J.-Y.; Scozzafava, A.; Supuran, C. T.; Williams, K. J. Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts. *J. Med. Chem.* **2012**, *55*, 5591–600.
- (11) Liu, K.; Zhu, H. L. Nitroimidazoles as anti-tumor agents. *Anti-Cancer Agents Med. Chem.* **2011**, *11*, 687–691.
- (12) Overgaard, J.; Overgaard, M.; Nielsen, O. S.; Pedersen, A. K.; Timothy, A. R. A comparative investigation of nimorazole and misonidazole as hypoxic radiosensitizers in a C3H mammary carcinoma in vivo. *Br. J. Cancer* **1982**, *46*, 904–911.
- (13) D'Ambrosio, K.; Vitale, R. M.; Dogné, J. M.; Masereel, B.; Innocenti, A.; Scozzafava, A.; De Simone, G.; Supuran, C. T. Carbonic anhydrase inhibitors: bioreductive nitro-containing sulfonamides with selectivity for targeting the tumor associated isoforms IX and XII. *J. Med. Chem.* **2008**, *51*, 3230–3237.
- (14) Rami, M.; Cecchi, A.; Montero, J.-L.; Innocenti, A.; Vullo, D.; Scozzafava, A.; Winum, J.-Y.; Supuran, C. T. Carbonic anhydrase inhibitors: design of membrane-impermeant copper(II) complexes of DTPA-, DOTA-, and TETA-tailed sulfonamides targeting the tumor-associated transmembrane isoform IX. *ChemMedChem* **2008**, *3*, 1780–1788.
- (15) Rami, M.; Montero, J.-L.; Dubois, L.; Lambin, P.; Scozzafava, A.; Winum, J.-Y.; Supuran, C. T. Carbonic anhydrase inhibitors: Gd(III) complexes of DOTA- and TETA-sulfonamide conjugates targeting the tumor associated carbonic anhydrase isozymes IX and XII. *New J. Chem.* **2010**, *34*, 2139–2144.
- (16) D'Ambrosio, K.; Smaine, F. Z.; Carta, F.; De Simone, G.; Winum, J.-Y.; Supuran, C. T. Development of potent carbonic anhydrase inhibitors incorporating both sulfonamide and sulfamide groups. *J. Med. Chem.* **2012**, *55*, 6776–6783.
- (17) Winum, J.-Y.; Carta, F.; Ward, C.; Mullen, P.; Harrison, D.; Langdon, S. P.; Cecchi, A.; Scozzafava, A.; Kunkler, I.; Supuran, C. T. Ureido-substituted sulfamates show potent carbonic anhydrase IX inhibitory and antiproliferative activities against breast cancer cell lines. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4681–4685.
- (18) Hoigebazar, L.; Jeong, J. M.; Choi, S. Y.; Choi, J. Y.; Shetty, D.; Lee, Y. S.; Lee, D. S.; Chung, J. K.; Lee, M. C.; Chung, Y. K. Synthesis and characterization of nitroimidazole derivatives for ⁶⁸Ga-labeling and testing in tumor xenografted mice. *J. Med. Chem.* **2010**, *53*, 6378–6385.
- (19) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561–2573.
- (20) Dubois, L.; Douma, K.; Supuran, C. T.; Chiu, R. K.; van Zandvoort, M. A.; Pastoreková, S.; Scozzafava, A.; Wouters, B. G.; Lambin, P. Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors. *Radiother. Oncol.* **2007**, *83*, 367–373.
- (21) Dubois, L.; Peeters, S. G.; van Kuijk, S. J.; Yaromina, A.; Lieuwes, N. G.; Saraya, R.; Biemans, R.; Rami, M.; Parvathaneni, N. K.; Vullo, D.; Vooijs, M.; Supuran, C. T.; Winum, J.-Y.; Lambin, P. Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs. *Radiother. Oncol.* **2013**, *108*, 523–528.
- (22) Eriksson, A. E.; Jones, T. A.; Liljas, A. Refined structure of human carbonic anhydrase II at 2.0 Å resolution. *Proteins* **1988**, *4*, 274–282.
- (23) Winum, J.-Y.; Temperini, C.; El Cheikh, K.; Innocenti, A.; Vullo, D.; Ciattini, S.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: clash with Ala65 as a means for designing inhibitors with low affinity for the ubiquitous isozyme II, exemplified by the crystal structure of the topiramate sulfamide analogue. *J. Med. Chem.* **2006**, *49*, 7024–7031.
- (24) Di Fiore, A.; Monti, S. M.; Innocenti, A.; Winum, J. Y.; De Simone, G.; Supuran, C. T. Carbonic anhydrase inhibitors: crystallographic and solution binding studies for the interaction of a boron-containing aromatic sulfamide with mammalian isoforms I–XV. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3601–3605.
- (25) De Simone, G.; Alterio, V.; Supuran, C. T. Exploiting the hydrophobic and hydrophilic binding sites for designing carbonic anhydrase inhibitors. *Expert Opin. Drug Discovery* **2013**, *8*, 793–810.
- (26) Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **1997**, *276*, 307–326.
- (27) Brünger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *54*, 905–921.
- (28) Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr. Sect. A: Found. Crystallogr.* **1991**, *47*, 110–119.
- (29) Allen, F. H. The Cambridge Structural Database: a quarter of a million crystal structures and rising. *Acta Crystallogr., Sect. B: Struct. Sci.* **2002**, *58*, 380–388.