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 van Kuijk, Simona Maria Monti, Philippe Lambin, Giuseppina de Simone, Claudiu Supuran, Jean-Yves Winum

To cite this version:
Marouan Rami, Ludwig Dubois, Nanda-Kumar Parvathaneni, Vincenzo Alterio, Simon van Kuijk, et al.. Hypoxia-Targeting Carbonic Anhydrase IX Inhibitors by a New Series of Nitroimidazole-Sulfonamides/Sulfamides/Sulfamates. Journal of Medicinal Chemistry, American Chemical Society, 2013, 56 (21), pp.8512-8520. 10.1021/jm4009532. hal-03543866

HAL Id: hal-03543866
https://hal.archives-ouvertes.fr/hal-03543866
Submitted on 26 Jan 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Hypoxia-Targeting Carbonic Anhydrase IX Inhibitors by a New Series of Nitroimidazole-Sulfonamides/Sulfamides/Sulfamates

Citation for published version (APA):

Document status and date:
Published: 14/11/2013

DOI:
10.1021/jm4009532

Document Version:
Publisher's PDF, also known as Version of record

Document license:
Taverne

Please check the document version of this publication:
• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.
Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:
www.umlib.nl/taverne-license

Take down policy
If you believe that this document breaches copyright please contact us at: repository@maastrichtuniversity.nl providing details and we will investigate your claim.

Download date: 26 janv., 2022
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,*†

†Institut des Biomolécules Max Mousseron (IBMM) UMR 5247 CNRS-UM1-UM2 Bâtiment de Recherche Max Mousseron, Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l’Ecole Normale, 34296 Montpellier Cedex, France
‡Department of Radiation Oncology (MAASTRO Lab), GROW—School for Oncology and Developmental Biology, Maastricht University Medical Centre, Universiteitszinge 50/23, PO Box 616, 6200 MD, Maastricht, The Netherlands
§Istituto di Biostrutture e Bioimmagini—CNR, via Mezzocannone 16, 80134 Naples, Italy
‖Istituto di Chimica dei Materiali Inorganici, CNR, via Mezzocannone 16, 80134 Naples, Italy

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. Crystalllographic 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 1α (HIF-1α), 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 crystalllographic 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

Received: June 25, 2013
Published: October 15, 2013
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. A different approach to sensitize tumors is the use of nitroimidazole, although high clinical toxicity has been observed upon therapeutic efficacy. 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 groups by combining benzenesulfonamide and aromatic nitro derivatives in the same molecule. Indeed, the derivatives of this type reported by D’Ambrosio et al. 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, we introduced the acetic acid moiety on position 1 of the imidazole ring by reacting tert-butyl bromoacetate on in basic medium followed by deprotection of the resulting ester to give compound 3. The latter was then reacted with different amino-benzensulfonamide derivatives using carbodiimide as a coupling agent to give compounds 4 in good yield (Scheme 1).

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, we reacted different benzene sulfonamide isothiocyanates to lead to the series of thiourea compounds (Scheme 2).

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

---

**Scheme 1**

![Diagram](image1)

**Scheme 2**

![Diagram](image2)

**Scheme 3**

![Diagram](image3)
using a direct sulfamoylation procedure as described previously (Scheme 4).\textsuperscript{17}

Scheme 4\textsuperscript{a}

\[
\begin{align*}
8 & \overset{\text{Reagents and conditions: (i) 1 equiv of 2-methyl-5-nitro-1-imidazolylthanol, N,N-dimethylacetamide, 3 equiv sulfamoyl chloride, RT, 1 night.}}{\longrightarrow} \\
9 & \quad 81\%
\end{align*}
\]

\textsuperscript{a}Reagents and conditions: (i) 1 equiv of 2-methyl-5-nitro-1-imidazolylthanol, 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.\textsuperscript{18} (Scheme 5).

Scheme 5\textsuperscript{a}

\[
\begin{align*}
11 & \overset{\text{Reagents and conditions: (i) 1 equiv of 2-nitroimidazole, dry DMF, 1 equiv K_2CO_3, 1.5 equiv tert-butyl 2-bromoethylcarbamate; (ii) trifluoroacetic acid–CH_2Cl_2 8.2 v/v, RT, 2 h; (iii) 4 equiv of triethylamine, 1.5 equiv of chlorosulfonylisocyanate, 1.5 equiv of tert-butanol, CH_2Cl_2, rt, 12 h; (iv) trifluoroacetic acid–CH_2Cl_2 8.2 v/v, RT, 2 h; (v) 2 equiv of methane sulfonyl chloride, 2 equiv of triethylamine, CH_2Cl_2.}}{\longrightarrow} \\
12 & \quad 45\%
\end{align*}
\]

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

Scheme 6\textsuperscript{a}

\[
\begin{align*}
14 & \overset{\text{Reagents and conditions: (i) 4 equiv of triethylamine, 1.5 equiv of chlorosulfonylisocyanate, 1.5 equiv of tert-butanol, CH_2Cl_2, rt, 12 h; (ii) trifluoroacetic acid–CH_2Cl_2, 8.2 v/v, RT, 2 h.}}{\longrightarrow} \\
15 & \quad 71\%
\end{align*}
\]

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\textsubscript{2} hydrase assay (Table 1).\textsuperscript{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 \(K_s\) in the range of 79 nM to 9.57 \(\mu\)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 (\(K_s\) of 79–107 nM) compared to the sulfanilamide derivative 4a (\(K_s\) 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 (\(K_s\) of 483 nM), whereas the remaining ones (6a, 6c, and 6d) showed a similar behavior of medium potency inhibitors (\(K_s\) 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 \(K_s\) 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 (\(K_s\) 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 isozyme over CA II, the selectivity obtained for these series is comparable or better with those of all the clinically used CA
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. 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. Previously, we have demonstrated that inhibitor binding to CA IX requires both its expression under ambient air, irrespective of the CA IX expression levels. The 5-nitroimidazole series was selected for further investigation based on the more favorable toxicity profile compared with 2-nitroimidazoles. 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. 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 and subsequently exposed to chemotherapy. 7-treated (P < 0.01) or doxorubicin-treated (P < 0.001) mice demonstrated a significantly reduction in tumor growth, with an average time to reach 4× starting volume (T4×SV) of 25.43 and 23.39 days for 7 and doxorubicin, respectively, compared to vehicle treatment only (14.07 days). T4×SV was further enhanced (40.66 days, P < 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. Derivative 7 sensitizes tumors toward radiotherapy and doxorubicin-based chemo-

<table>
<thead>
<tr>
<th>compd</th>
<th>hCA I*</th>
<th>hCA II*</th>
<th>hCA IX†</th>
<th>hCA XII†</th>
<th>K_i hCA II/K_i hCA IX</th>
<th>K_i hCA II/K_i hCA XII</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAZ</td>
<td>250</td>
<td>12</td>
<td>25</td>
<td>5.6</td>
<td>0.48</td>
<td>2.14</td>
</tr>
<tr>
<td>4a</td>
<td>3203</td>
<td>330</td>
<td>70</td>
<td>64</td>
<td>4.71</td>
<td>5.15</td>
</tr>
<tr>
<td>4b</td>
<td>107</td>
<td>37</td>
<td>7.9</td>
<td>8.1</td>
<td>4.68</td>
<td>4.56</td>
</tr>
<tr>
<td>4c</td>
<td>79</td>
<td>4.8</td>
<td>8.0</td>
<td>6.7</td>
<td>0.6</td>
<td>0.71</td>
</tr>
<tr>
<td>4d</td>
<td>101</td>
<td>3.8</td>
<td>7.3</td>
<td>8.0</td>
<td>0.52</td>
<td>0.47</td>
</tr>
<tr>
<td>6a</td>
<td>105</td>
<td>5.5</td>
<td>7.3</td>
<td>8.0</td>
<td>0.75</td>
<td>0.68</td>
</tr>
<tr>
<td>6b</td>
<td>483</td>
<td>7.4</td>
<td>7.2</td>
<td>7.7</td>
<td>1.02</td>
<td>0.96</td>
</tr>
<tr>
<td>6c</td>
<td>79</td>
<td>2.9</td>
<td>8.3</td>
<td>8.5</td>
<td>0.35</td>
<td>0.34</td>
</tr>
<tr>
<td>6d</td>
<td>84</td>
<td>6.6</td>
<td>7.8</td>
<td>7.6</td>
<td>0.84</td>
<td>0.86</td>
</tr>
<tr>
<td>7</td>
<td>9576</td>
<td>10.1</td>
<td>20.4</td>
<td>8.1</td>
<td>0.49</td>
<td>1.24</td>
</tr>
<tr>
<td>9</td>
<td>4435</td>
<td>33.8</td>
<td>8.3</td>
<td>8.9</td>
<td>4.07</td>
<td>3.79</td>
</tr>
<tr>
<td>10</td>
<td>9120</td>
<td>58</td>
<td>41</td>
<td>38</td>
<td>1.41</td>
<td>1.52</td>
</tr>
<tr>
<td>12</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>8718</td>
<td>52</td>
<td>21</td>
<td>37</td>
<td>2.47</td>
<td>1.40</td>
</tr>
</tbody>
</table>

*Selectivity ratios for the inhibition of the tumor-associated (CA IX and XII) over the cytosolic (CA II) isozyme are also reported. †Errors in the range of ±5–10% of the reported value from three different determinations. ‡Full length, cytosolic isoform. §Catalytic domain, recombinant enzyme.

---

Figure 1. Extracellular acidification of cells exposed to normoxia (20%) or hypoxia (0%) upon treatment with dual targeting compounds (0.1 or 1 mM). Data are expressed as the difference between medium pH values (ΔpHe = pH after incubation − pH before incubation) and show the mean ± SD of at least three independent experiments. Asterisks indicate significant difference (*P < 0.05).
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 agents or nitroimidazole-based drugs.12

**X-ray Crystallography.** To determine the molecular features responsible for 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 threedimensional 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 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 enzyme 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 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 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 4x 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).](Image 161x624 to 464x749)
(2-Nitro-imidazol-1-yl) Acetic Acid (3). Compound 2 (2.5 g) 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-d6, 400 MHz) δ 5.21 (s, 2H), 7.21 (d, 1H, J = 1.01 Hz), 7.64 (d, 1H, J = 1.01 Hz), 5.13 (d, 2H, J = 8.2 Hz), 7.77 (d, 1H, J = 8.2 Hz), 10.7 (s, 1H). 13C (DMSO-d6, 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 + H]+, 348.07 [M + Na]+, 364.17 [M + K]+, 673.18 [2M + Na]+.

General Procedure for the Preparation of Compounds (4a–d).

The aminooxybenzene sulfonamide (1.17 mmol, 1 equiv), 4-dimethylaminopropylamine (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-Nitro-imidazol-1-yl)-N-(4-sulfamoylbenzyl)acetamide (4a).

Yield 68%; mp 163–165 °C. 1H NMR (DMSO-d6, 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), 7.77 (d, 2H, J = 8.2 Hz), 7.77 (d, 2H, J = 8.2 Hz), 10.7 (s, 1H). 13C (DMSO-d6, 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 + H]+, 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 [C12H14N5O5S]+, 326.0559; found, 326.0563.

2-(2-Nitro-imidazol-1-yl)-N-(4-sulfamoylphenyl)acetamide (4b).

Yield 83%; mp 181–183 °C. 1H NMR (DMSO-d6, 400 MHz) δ 4.37 (d, 2H, J = 5.7 Hz), 5.22 (s, 2H), 7.18 (s, 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). 13C (DMSO-d6, 101 MHz) δ 41.81, 51.55, 106.87, 125.58, 127.39, 138.91, 142.66, 149.92, 156.82, 168.85. MS (ESI+/ESI−) [M + H]+, 348.15 [M − H]−, 362.17 [M + Na]+. HRMS (ESI) [M + H]+ calculated for [C13H11N5O5S]+, 326.0559; found, 326.0563.

2-(2-Nitro-imidazol-1-yl)-N-(2-sulfamoylpropyl)acetamide (4c).

Yield 89%; mp 139–141 °C. 1H NMR (DMSO-d6, 400 MHz) δ 2.80 (t, 2H, J = 8.2 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), 10.7 (s, 1H). 13C (DMSO-d6, 101 MHz) δ 34.59, 51.49, 125.64, 127.38, 128.74, 129.09, 142.05, 144.32, 147.95, 165.02. MS (ESI+/ESI−) [M + H]+, 348.15 [M − H]−, 374.07 [M + Cl]−, 713.16 [2M + Cl]−, 340.15 [M + H]+, 362.17 [M + Na]+. HRMS (ESI) [M + H]+ calculated for [C12H10N5O5S]+, 307.0716; found, 340.0723.

2-(2-Nitro-imidazol-1-yl)-N-[2-(4-sulfamoylphenyl)ethyl]acetamide (4d).

Yield 79%; mp 195–197 °C. 1H NMR (DMSO-d6, 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). 13C (DMSO-d6, 101 MHz) δ 52.16, 116.10, 120.72, 121.92, 127.59, 128.86, 129.67, 136.87, 142.74, 164.87, 167.75. MS (ESI+/ESI−) [M + H]+, 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 [C17H12N5O5S]+, 326.0569; 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 98:2 v/v as eluent.

N-(4-Sulfamoylphenyl)-N-(2-aminoethyl)-2-methyl-5-nitroimidazolyl Thiourea (6a). Yield 72%; mp 186–188 °C. 1H NMR...
in methylene chloride (30% volume) and stirred at room temperature for one hour.

HRMS (ESI) [M + H]+ calculated for [C6H12N5O4S]+, 250.0610; found, 250.0616.

400 MHz) δ 3.73 (s, 3H), 2.87 (m, 2H), 1.43 (t, 2H, J = 5.08 Hz), J = 6.2 Hz), 3.73 (s, 1H), 7.37 (d, 2H, J = 8.2 Hz), 7.52 (s, 1H), 6.73 (s, 1H), 7.74 (d, 2H, J = 8.2 Hz), 8.03 (s, 1H), 8.15 (s, 1H).1H NMR (DMSO-d6, 100 MHz) δ 13.58, 30.64, 42.70, 45.45, 125.52, 127.23, 133.17, 138.61, 142.48, 151.41, 184.44. MS (ESI+) m/z 399.23 [M + H]+, 411.16 [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 [C40H46N9O12S]3+, 399.0909; found, 399.0912.

3.3 mol, 1.5 equiv) was added at room temperature to a solution of 2-nitro imidazole (2.2 mmol, 1 equiv) in 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–methyl alcohol 9:1 as eluent. The pure Boc protected compound was then dissolved in 20% trifluoroacetic acid–methylene chloride (10 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-d6, 400 MHz) δ 3.33 (t, 2H, J = 6.0 Hz), 6.46 (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−/ESI−) m/z 157.09 [M + H]+, 313.35 [2M + H]+.

N-(4-Sulfamoylphenethyl)-N-(2-aminoimethyl)-2-methyl-5-nitroimidazolyl thiourea (6b). Yield 86%; mp 75−77 °C. 1H NMR (DMSO-d6, 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), 6.73 (s, 1H), 7.74 (d, 2H, J = 8.2 Hz), 8.03 (s, 1H).1H NMR (DMSO-d6, 100 MHz) δ 13.58, 30.64, 45.45, 125.65, 129.05, 133.18, 138.61, 142.48, 151.41, 184.44. MS (ESI+−) m/z 413.06 [M + H]+, 433.02 [M + Na]+, 829.09 [2M + H]+, 847.21 [2M + Na]+, 411.06 [M − H]−, 447.20 [M + Cl]−, 822.99 [2M − H]−. HRMS (ESI) [M + H]+ calculated for [C40H46N9O12S]3+, 413.1066; found, 413.1069.

N-(3-Sulfamoylphenyl)-N-(2-aminoimethyl)-2-methyl-5-nitroimidazolyl thiourea (6d). Yield 75%; mp 66−68 °C. 1H NMR (DMSO-d6, 400 MHz) δ 2.43 (s, 3H), 3.91 (m, 2H), 4.49 (t, 2H, J = 5.08 Hz), 3.73 (s, 2H), 5.71 (m, 1H), 5.75 (s, 1H), 5.77 (m, 1H), 7.81 (s, 1H), 7.93 (m, 1H), 8.04 (s, 1H), 9.94 (s, 1H).1H NMR (DMSO-d6, 100 MHz) δ 13.31, 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]+, 833.12 [M − H]−, 419.09 [M − Cl]−, 767.26 [2M − H]−. HRMS (ESI) [M + H]+ calculated for [C26H17N7O4S]2+, 385.0753; found, 385.0746.

N-(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 122−125 °C. 1H NMR (DMSO-d6, 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), 8.11 (s, 1H).1H NMR (DMSO-d6, 100 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 [C14H15N4O2S]3+, 250.0610; found, 250.0616.

N-(2-Methyl-5-nitroimidazol-1-yl)ethyl)sulfamate (9). Sulfa-
moyl 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-d6, 400 MHz) δ 2.45 (s, 3H), 4.35 (t, 2H, J = 5.05 Hz), 7.57 (s, 2H), 8.06 (s, 1H).13C (DMSO-d6, 100 MHz) δ 14.04, 44.98, 57.21, 133.10, 138.52, 152.81. 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 [C12H5N8O4S3]+, 251.0450; found, 251.0456.

2-Nitro-imidazol-1-yl)ethylenimine (10). tert-Butyl 2-

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 NaNO3 (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 mM 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 achieved in a hypoxic workstation (Ruskin 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.504

**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 at the end of each experiment as previously described.505

**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. Diffraction intensities were processed using the HKL2000 crystallographic data reduction package (Denoz/Scalepack).28 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)26 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 >3σ in stereochemistry maps that demonstrated appropriate hydrogen-bonding geometry. Final crystallographic Rmerge and Rfree 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).

**AUTHOR INFORMATION**

**Corresponding Author**

*Phone: 33-467-147234. E-mail: jean-yves.winum@univ-montp2.fr.*

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work has been funded with the support of the EU 6th framework Program (Euroxy project ref 2003-S02932), the EU 7th framework (Metoxia project ref 2008-222741), the KWF UM2012-5394, the NGI Life Science Pre-Seed grant 93613002, and also the CNRS/CRN (CoproINTER program, grant no. 131999).

**REFERENCES**


