Carbonic anhydrase inhibitors: Gd(III) complexes of DOTA- and TETA-sulfonamide conjugates targeting the tumor associated carbonic anhydrase isozymes IX and XII

Marouan Rami,[a] Jean-Louis Montero,[a] Ludwig Dubois,[b] Philippe Lambin,[b] Andrea Scozzafava,[c] Jean-Yves Winum*[a] and Claudiu T. Supuran*[c]

Received (in Montpellier, France) 22nd March 2010, Accepted 28th April 2010
DOI: 10.1039/c0nj00214c

Gd(III)-sulfonamide complexes incorporating macrocyclic rings of the DOTA/TETA type have been prepared and assayed for the inhibition of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). The cytosolic isoform, CA I, was poorly inhibited, whereas cytosolic CA II and transmembrane, tumor-associated CA IX and XII were inhibited in the low nanomolar range by the Gd(III) complexes. Magnetic susceptibility and relaxivity measurements proved that the Gd(III) complexes have comparable parameters to those of clinically used MRI contrast agents like Dotarem, Prohance and Omniscan in aqueous solution. Some Gd(III) complexes were investigated for the inhibition of extracellular tumor acidification in two cell lines overexpressing CA IX, the colorectal HT-29 cell line and the cervical HeLa carcinoma cell line. In both tumor types, a slight but significant reduction of tumor acidosis was detected. Gd(III)-sulfonamide conjugates may thus be of interest for developing imaging techniques or novel treatment strategies for the management of hypoxic tumors overexpressing extracellular CA isozymes such as CA IX and XII.

Introduction

α-Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in higher vertebrates, including humans.[1,2] 16 isozymes have been characterized to date that differ in their sub-cellular localization, catalytic activity and susceptibility to different classes of inhibitors. There are cytosolic isozymes (CA I, CA II, CA III, CA VII and CA XIII), membrane bound examples (CA IV, CA IX, CA XII and CA XIV), and mitochondrial (CA VA and CA VB) and secreted (CA VI) isoforms.[3–9] Three acatalytic forms, called CA-related proteins (CARPs) (CARP VIII, CARP X and CARP XI) are also known.[1] In humans, CAs are present in a large variety of tissues, such as the gastrointestinal tract (GI), the reproductive tract, the nervous system, kidneys, lungs, skin and eyes.[1,2,6,10–12] Most CAs are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$), which is the only physiological reaction in which they are involved.[1]

Many CA isoforms are involved in critical physiological processes such as respiration and acid–base regulation, electrolyte secretion, bone resorption, calcification and biosynthetic reactions, which require bicarbonate as a substrate (lipogenesis, gluconeogenesis and ureagenesis).[1] Two of them, CA IX and CA XII, are predominantly associated with and overexpressed in many tumors, being involved in critical processes connected with cancer progression and responses to therapy.[1–3,11–14] CA IX is confined to a few normal tissues (stomach and body cavity lining) but it is ectopically induced and highly overexpressed in many solid tumor types through strong transcriptional activation by hypoxia, accomplished *via* the hypoxia inducible factor-1 (HIF-1) transcription factor, as shown schematically in Fig. 1.[1,3–5,12–14]

The expression of CA XII was detected in all tumor categories, although the mean staining intensity was weaker than for CA IX in all groups, except renal clear cell carcinomas.[11] These two enzymes are multidomain proteins with the CA domain situated outside the cell and their CO_2 hydrase catalytic activity being medium-to-high for CA XII and very high for CA IX.[8,15] The X-ray crystal structure of CA IX has only recently been reported our group,[15] allowing for interesting drug design campaigns. Sulfonamides and their isosteres (sulfamates and sulfamides) among others, constitute a well-known class of CA inhibitors (CAIs), with some derivatives, such as acetazolamide (**AAZ**) or ethoxzolamide (**EZA**), having been used clinically for a long time for the management of CA mis-balances different from cancer-related examples.[1–7]

Many new inhibitors of both CA IX and XII have been reported in recent years when connections between these CA isozymes and hypoxic tumors began to emerge,[1–10] with many of these new generation inhibitors being specifically designed for targeting tumor-associated CAs. Indeed, classical CAIs, such as **AAZ** and **EZA**, indiscriminately inhibit all CA isoforms,[1] and this may constitute a disadvantage when only tumor-associated examples must be targeted.

[a] *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. E-mail: jean-yves.winum@univ-montp2.fr*
[b] *Maastricht Radiation Oncology (MaastRO) Lab, GROW-School for Oncology and Developmental Biology, University of Maastricht, Maastricht, The Netherlands*
[c] *Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy. E-mail: claudiu.supuran@unifi.it*

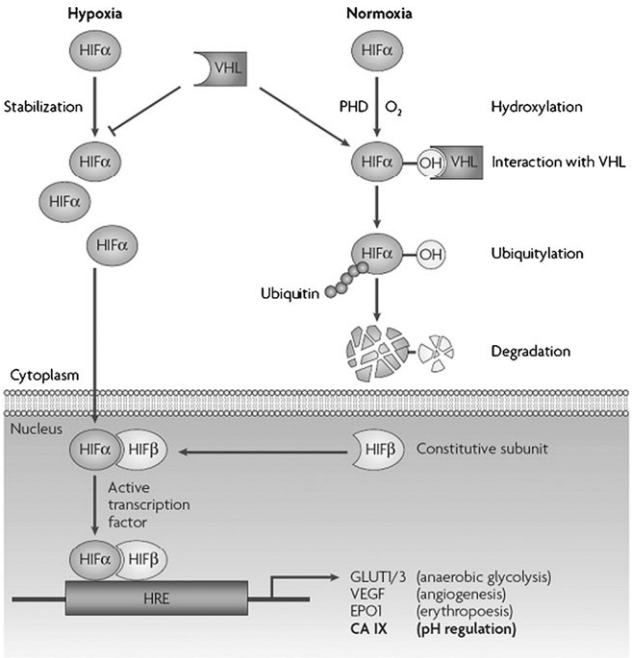
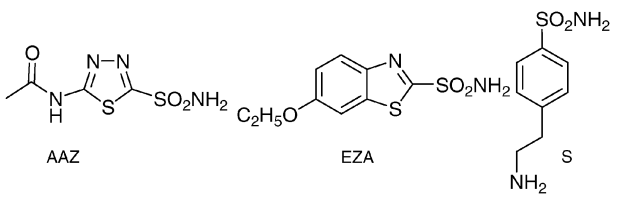


Fig. 1 The mechanism of hypoxia-induced gene expression mediated by the HIF transcription factor, leading to CA IX overexpression in hypoxic tumors (HRE = hypoxia responsive element; PHD = prolyl hydroxylase; VHL = von Hippel–Lindau protein).[1]



Recently our group reported some interesting CA IX inhibitors (possessing fluoresceinthioureido moieties in their molecules) that were critical for assessing the *in vivo* role of CA IX/XII in tumor acidification processes.[16–18] Such inhibitors were shown to bind to CA IX only under hypoxia *in vivo* in cell cultures or in animals bearing human tumors, followed by a reduction of extracellular pH acidification in the tumor tissues,[16–18] properties which may be exploited for designing diagnostic tools for the treatment or imaging of hypoxic tumors.[16–20] Indeed, we[16–18] and Neri's group[19,20] recently published proof of concept studies showing the possibility of using CA IX/XII inhibitors both for the imaging and treatment of hypoxic tumors.

Continuing our studies in the design of CA IX/XII inhibitors, we report here conjugates of sulfonamides incorporating macrocyclic rings that can bind gadolinium(III), a metal ion possessing interesting properties for the magnetic resonance imaging (MRI) of tumors and other diseases.[21–23] Indeed, paramagnetic lanthanide(III) complexes are used in two major classes of MRI applications as contrast agents: the well-established class of Gd-based agents and the emerging class of chemical exchange saturation transfer (CEST) agents.[24] A Gd-based complex increases the water signal by enhancing the longitudinal relaxation rate of water protons, whereas CEST agents decrease water signal as a consequence of the transfer of saturated magnetization from the exchangeable protons of the agent.[24] Here, we report that Gd(III)-incorporating conjugates of sulfonamides show a strong inhibition of the tumor-associated CA isoforms CA IX and XII, decrease tumor acidifications in cell cultures of HT-29 colorectal and HeLa cervical cancer cell lines, and can also be used for MRI purposes, making this class of conjugates of particular interest for further studies.

Experimental

Synthesis

Sulfonamide ligands **1a–c** and **2a–c** were synthesized as previously described by our group.[25] A solution of Gd(III) chloride hexahydrate (1.1 mmol) in de-ionized water (2 ml) was added dropwise to a solution of ligand **1a–c** (1 mmol) or **2a–c** (1 mmol) dissolved in water (50 ml). The pH of the aqueous solution was adjusted and maintained at pH 6.8 by the constant addition of 2N NaOH. The solution was vigorously stirred at room temperature and the formation of the gadolinium complex monitored by ESI-MS. After 2 h, the solution was concentrated to approximately 10 ml and acetonitrile (50 ml) was added. The precipitate was filtered, washed with water and ether, and dried over P_2O_5 to give the expected gadolinium complexes (**3a–c**) or (**4a–c**) quantitatively as a white solid.

3a: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 728.3 (M + H)$^+$, 750.3, (M + Na)$^+$, 726.2 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{23}H_{34}GdN_6O_9S$ 728.1349, found 728.11346; **3b**: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 742.15 (M + H)$^+$, 764.16, (M + Na)$^+$, 740.27 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{24}H_{36}GdN_6O_9S$ 742.1505, found 742.1508; **3c**: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 714.25 (M + H)$^+$, 736.21 (M + Na)$^+$, 712.19 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{22}H_{32}GdN_6O_9S$ 714.1192, found 714.1196; **4a**: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 778.30 (M + Na)$^+$, 778.21 (M + Na)$^+$, 754.35 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{25}H_{38}GdN_6O_9S$ 756.1662, found 756.1665; **4b**: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 770.07 (M + H)$^+$, 792.19 (M + Na)$^+$, 768.12 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{26}H_{40}GdN_6O_9S$ 770.1818, found 770.1816; **4c**: mp > 205 °C, MS (ESI$^+$/ESI$^-$) *m*/*z*: 742.21 (M + H)$^+$, 764.11, (M + Na)$^+$, 740.33 (M – H)$^-$; HRMS (M + H)$^+$ calc. for $C_{24}H_{36}GdN_6O_9S$ 742.1505, found, 742.1507.

Magnetic susceptibility measurements

Magnetic susceptibility data were collected using a Quantum Design MPMS-XL SQUID magnetometer working in the temperature range 1.8–350 K up to 5 T. The magnetic data were corrected for the sample holder and the diamagnetism contributions calculated from Pascal's constants.[26]

Relaxivity MRI protocols

For each compound, 10 samples were prepared with gadolinium concentrations ranging from 0 to 1 mM in steps of 0.1 mM. Measurements were performed on a 7 T Bruker Biospec 70/30 USR MRI system (Bruker Biospin GmbH, Ettlingen, Germany)

interfaced to an AVANCE II console. A BGA12-S mini-imaging gradient system (maximum gradient strength 720 mT m^{-1}, slew rate 6000 T m^{-1} s^{-1}) was used, and images were acquired using a 7.2 cm inner diameter quadrature volume resonator.

Longitudinal relaxation rates ($R_1 = 1/T_1$) were determined using a series of inversion recovery measurements with increasing inversion times of 200, 500, 750, 1000, 1500, 2000, 2500 and 3500 ms. Repetition and echo times were 7500 ms and 8.2 ms, respectively. Transverse relaxation rates ($R_2 = 1/T_2$) were measured using a multi-slice, multi-echo spin echo sequence, with a repetition time of 7500 ms, echo times ranging from 10 to 120 ms and an echo interval of 10 ms. For all images, 5 slices were recorded with a slice thickness of 1 mm and an interslice distance of 1.5 mm. The field-of-view was 7 × 7 cm^2 and the acquisition matrix was 256 × 256, resulting in a spatial resolution of 0.27 × 0.27 × 1.0 mm^3.

Regions of interest were drawn manually in MRIcro.[27] All further data processing was performed in Matlab (The MathWorks, Natick, MA). R_1 values were determined by non-linear curve fitting of the inversion recovery signal intensity function (eqn 1):[28]

$$S = S_0(1 - 2e^{-T_I R_1} + e^{-T_R R_1}) \quad (1)$$

using the Levenberg–Marquardt optimization algorithm. S_0 is a scaling factor including proton density, pre-amplifier gain and echo time. T_I and T_R are the inversion and repetition times, respectively. R_2 values were determined analogously by non-linear fitting of the spin echo signal intensity function (eqn 2):

$$S = Se^{-T_E R_2} \quad (2)$$

Here, S_0 is a scaling factor depending on proton density, pre-amplifier gain and repetition time, and T_E is the echo time. The longitudinal relaxivity, r_1, and transverse relaxivity, r_2, were given by the slope of a linear fit of R_1 and R_2 *vs.* gadolinium concentration, respectively.

CA inhibition

An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalysed CO_2 hydration activity.[29] Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at an absorbance maximum of 557 nm with 20 mM HEPES (pH 7.5) as the buffer and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), to follow the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction 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 and de-ionized water, and dilutions down to 0.01 nM were undertaken thereafter with further distilled and de-ionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay in order to allow for the formation of the E-I complex. The inhibition constant (K_I) was obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[25] and represent the mean from at least three different determinations. K_I is the equilibrium constant for the dissociation of the E-I complex.

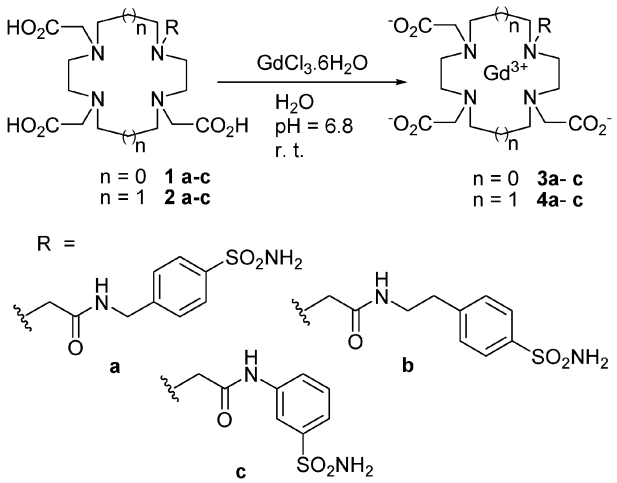
Cell culture and pH experiments

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. Hypoxic conditions were maintained in a hypoxic culture chamber (MACS VA500 micro-aerophilic workstation, Don Whitley Scientific, Shipley, UK) consisting of an atmosphere of 0.2% O_2, 5% CO_2 and residual N_2. In parallel, normoxic dishes were incubated in air with 5% CO_2. The pH of the culture medium was immediately measured at the end of each experiment and the data expressed as delta pH (pH after incubation − pH before incubation). The Gd compounds were dissolved in PBS containing 10% DMSO at a 100 mM concentration and diluted in culture medium to a final concentration of 1 mM just before adding to the cells. The cells were incubated with the compounds for 24 h during normoxia or hypoxia. In parallel, 4-aminoethylbenzene-sulfonamide (**S**), a compound investigated earlier and shown to decrease tumor acidification,[16] was used as a positive control in these experiments.

Results and discussion

Chemistry

Many sulfonamide/sulfamate/sulfamide CAIs have been reported as inhibitors of both CA IX and XII in recent years, in the search for derivatives with selectivity for tumor-associated enzymes over other CAs involved in crucial physiological processes.[1–7] The compounds specifically designed for targeting tumor-associated isoforms may be classified as follows: (i) sulfonamides tagged with fluorescent or other moieties (*e.g.*, complexed metal ions, stable free radicals, *etc.*), which make them useful for imaging purposes;[16–18] (ii) positively- or negatively-charged compounds that cannot cross plasma membranes due to their charged character and thus inhibit selectively only extracellular CAs, among which are CA IX and XII;[16,30] (iii) hypoxia-activatable compounds that exploit the reducing conditions of hypoxic tumors to convert an inactive prodrug into an active CAI;[31] (iv) sugar-containing sulfonamides/sulfamates/sulfamides that, due to their highly hydrophilic character, do not easily cross membranes and thus possess an enhanced affinity for extracellular CAs, such as CA IX and XII;[32–34] (v) diverse chemotypes other than the sulfonamides and their bioisosters, such as phenols, coumarins and other compounds, have recently been investigated as alternative CAIs to classical types of inhibitor.[35] Among the different approaches mentioned above, we have observed that sulfonamides incorporating macrocyclic rings of the DOTA and TETA type, as well as their Cu(II) complexes, show excellent inhibitory activity and selectivity for tumor-associated CAs over cytosolic isoforms CA I and II.[25] We thus decided to prepare the Gd(III) derivatives of previously reported sulfonamides **1** and **2** in



Scheme 1 The synthesis of Gd complexes **3a–c** and **4a–c**.

order to investigate their interaction with these CA isoforms (Scheme 1). This is the first detailed study of lanthanide-containing CAIs; these compounds being the first conjugate CAIs to be investigated as potential MRI agents.

The 12-membered ring DOTA and 14-membered ring TETA, known to easily complex lanthanide(III) ions[22–24] were chosen as starting materials. These macrocycles also contain four pendant arms substituting the four endocyclic nitrogen atoms, three of which are of the methylcarboxylate type (for effective binding of the Gd(III) ions) and one of which is derivatized by means of aromatic sulfonamides, such as homosulfanilamide (**3a** and **4a**), 4-aminoethylbenzenesulfonamide (**3b** and **4b**) or metanilamide (**3c** and **4c**), which will assure an interaction with the enzyme.[1,10] Indeed, sulfonamides in their deprotonated form complex Zn(II) ions from the CA active site and usually bind with low nanomolar affinity to many CA isoforms.[1–10]

Gd(III) complexes **3** and **4** were prepared from the corresponding macrocyclic ligands **1** and **2** by a reaction with Gd(III) salts under controlled pH conditions, as reported in the literature.[21–24] Gd(III)-sulfonamide conjugates **3** and **4** were characterized by standard procedures that established their purity (of >99%) and stability in the conditions of the experiments performed *in vitro* and *ex vivo* (see later in the text). The absence of free gadolinium in the final compounds was confirmed by the detection of free Gd(III) using a xylenol orange indicator.[23]

Magnetic properties and susceptibility measurements

It was determined by means of magnetic measurements that complexes **3b** and **4b**, as with all the Gd(III) derivatives investigated so far,[21–24] present a strong paramagnetic behavior (Fig. 2 and Table 1). The temperature dependencies of χT and $1/c$ measured for these two compounds are shown Fig. 2. For both compounds, the χT values at room temperature were equal to 7.869 and 7.870 emu K mol^{-1}, which corresponds to the calculated value for the one Gd^{3+} ion (7.875 emu K mol^{-1}) per molecule of complex.[24]

Table 1 summarizes the ionic longitudinal and transverse relaxivities for compounds **3b** and **4b**, as determined at 7 T and

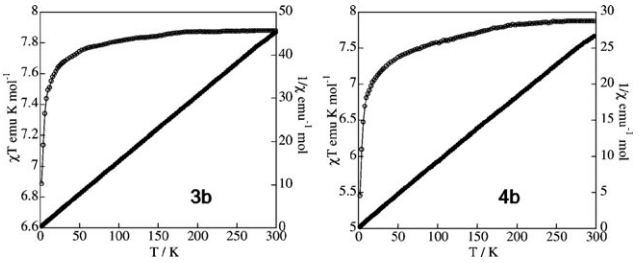


Fig. 2 Magnetic susceptibility measurements for compounds **3b** and **4b**.

Table 1 Longitudinal (r_1) and transverse (r_2) relaxivities at 7 T and 20 °C for Gd(III) complexes **3b** and **4b**

	r_1/mM_{Gd}^{-1} s^{-1}	r_2/mM_{Gd}^{-1} s^{-1}
3b	3.6 ± 0.2	12.7 ± 1.1
4b	3.2 ± 0.1	21.8 ± 0.9

20 °C. The relaxivities of both compounds are in line with the known values of clinically-used MRI contrast agents like Dotarem, Prohance and Omniscan in aqueous solution.[22–24] They are typical of compounds incorporating this paramagnetic ion, making these derivatives suitable for MRI purposes.[24]

Carbonic anhydrase inhibition

Inhibition data of four physiologically relevant CA isozymes, the cytosolic, ubiquitous CA I and II (off-targets), and the tumor-associated CA IX and XII, with compounds **1–4** are shown in Table 2. The inhibition of standard sulfonamides **AAZ**, **EZA** and **S** are also included in Table 2 for comparison. The data for ligands **1** and **2** were reported earlier.[25] The following should be noted regarding CA inhibition with the gadolinium complexes investigated here.

(i) The slow cytosolic isoform hCA I was not effectively inhibited by Gd(III) complexes **3** and **4**, which showed inhibition constants in the range 380–7700 nM. This is a positive feature for a compound which must show selectivity for the transmembrane isoforms and not inhibit too effectively the cytosolic examples, such as CA I. It is, however, interesting to note that the bulkier TETA derivatives were generally better hCA I inhibitors compared to the corresponding DOTA complexes. Furthermore, Gd(III) complexes **3** were generally weaker inhibitors compared to corresponding ligands **1** (except **3a**) for the DOTA derivatives, whereas they were more inhibitory in the case of TETA complexes **4** (compared with **2**). It is rather difficult to rationalize these data as no X-ray crystal structures of the adducts of such compounds with any CA isoform are known at present.

(ii) The physiologically dominant cytosolic isoform hCA II was, on the other hand, effectively inhibited by all complexes **3** and **4** (K_I values of 2.0–17.7 nM), except **3c**, which was a medium potency inhibitor (K_I of 110 nM, Table 2). In this case, metal complexes **3** and **4** were much more effective hCA II inhibitors compared to the corresponding ligands from which they were obtained of type **1** and **2** (which showed inhibition constants in the range 267–720 nM).[25] Again, it is difficult to rationalize these results without detailed X-ray

Table 2 The inhibition of human (h)CA isozymes I, II, IX and XII with sulfonamides **1** and **2**, and their corresponding Gd(III) complexes **3** and **4**, by a stopped-flow, CO_2 hydrase assay.[29] Data for the standard sulfonamide CAIs **AAZ**, **EZA** and **S** are also included for comparison

	K_I/nM[a]			
Compound	hCA I	hCA II	hCA IX	hCA XII
1a	970	298	110	14
1b	425	720	41	38
1c	88	290	27	39
2a	2130	360	96	17
2b	390	267	36	40
2c	367	280	20	17
3a	680	5.5	34	89
3b	1600	9.5	27	38
3c	7700	110	98	38
4a	560	13	32	20
4b	380	2.0	37	67
4c	750	17.7	40	55
AAZ	250	12	25	5.7
EZA	25	8	34	22
S	21000	160	33	3.2

[a] Errors in the range ±5% of the reported data from three different assays.

crystal structures of the complexes of hCA II with this type of inhibitor. Both DOTA-Gd(III) and TETA-Gd(III) conjugates lead thus to very effective hCA II inhibitors. This enzyme is, in fact, a drug target itself for obtaining diuretics, antiglaucoma and/or antiobesity agents.[1]

(iii) The transmembrane, tumor-associated enzyme CA IX was also inhibited by all Gd(III) complexes reported here, with K_I values in the range 27–98 nM (Table 2). The metal complexes are thus more effective hCA IX inhibitors than their parent ligands, from which they have been prepared. Both the DOTA and TETA derivatives showed similar efficacy, the main factor influencing the inhibitory activity being the sulfonamide head group present in these molecules. Indeed, the 4-substituted benzenesulfonamide derivatives (**3a**, **3b**, **4a** and **4b**) were slightly better inhibitors compared to the 3-substituted benzenesulfonamides (**3c** and **4c**) for both sub-series. It should be noted that these metal complexes show the same CA IX inhibitory activity as the clinically-used sulfonamides **AAZ** and **EZA**.

(iv) hCA XII is also inhibited by Gd(III) complexes **3** and **4** reported here, with K_I values in the range 20–89 nm, but the complexes are generally weaker inhibitors compared to the corresponding sulfonamide from which they were prepared.

Thus, both tumor-associated isoforms hCA IX and XII are effectively inhibited by the Gd(III)-sulfonamide conjugates reported here, but these compounds do not act as selective inhibitors for tumor-associated CAs, as hCA II is also highly inhibited by most of them. However, as cytosolic CAs seem to be also involved in tumorigenesis,[36] we decided to investigate the activity of some of these Gd(III) derivatives *in vitro* in cell cultures of hypoxic tumors overexpressing CAs. On the other hand, the presence of the Gd(III) ions in the new compounds reported here may lead to membrane-impermeable derivatives, as reported earlier by our group for positively-charged sulfonamides.[37] Thus, *ex vivo*, the reported Gd(III)-sulfonamide conjugates may prevalently inhibit only the transmembrane isoforms, among which are also the tumor-associated examples CA IX and XII.

Reduction of tumor acidosis

As shown in Fig. 3, two of the effective hCA IX/XII inhibitors detected here, *i.e.*, **3b** and **4b**, were investigated for the inhibition of extracellular tumor acidification in two cell lines: the colorectal HT-29 and cervical HeLa carcinoma cell lines, both of which overexpress high amounts of CA IX under hypoxic conditions through the HIF-1 activation cascade described in Fig. 1. **S** was used as a control in these experiments, being shown earlier[16–18] to reduce extracellular acidosis due to CA IX inhibition.

It may be observed that in both cell lines, the three investigated sulfonamides reduced acidosis only under hypoxic conditions when CA IX/XII were overexpressed, due to the binding of the inhibitor within the enzyme active site. In the blank experiments, the medium was acidified by about 0.48 pH units for the HT-29 cell line and by about 0.30 pH units for the HeLa cell line. Inhibition of the enzyme by sulfonamides lead to the slight but significant ($P < 0.01$) effect of reducing this acidosis by around 0.10–0.20 pH units. Complex **4b** was the most effective compound against HT-29, whereas **S** was the most effective against HeLa cells. Although modest, this effect might be enhanced by using the complementary targeting of the tumors by means of a combination therapy, in which the CAI is combined either with radiation or with other antitumor

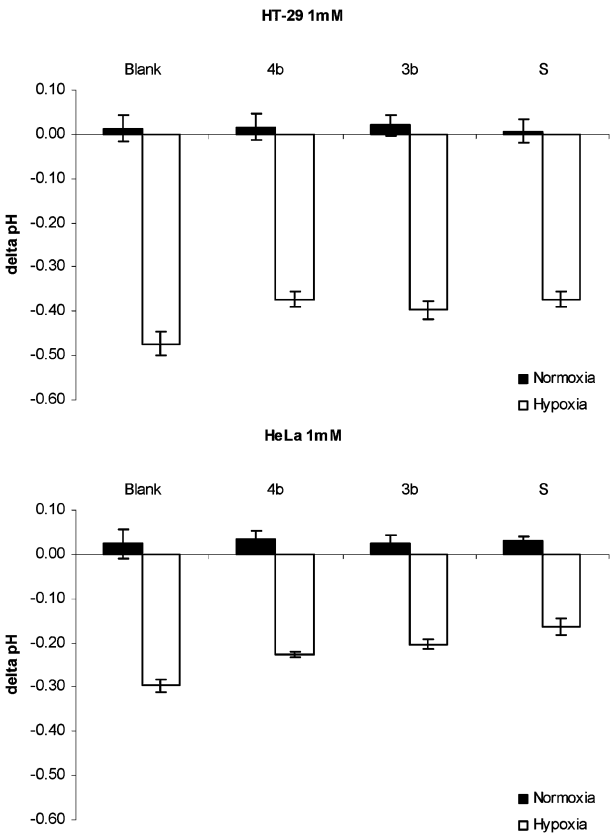


Fig. 3 The decrease of tumor cell extracellular acidosis in HT-29 and HeLa cell cultures treated with Gd(III) sulfonamide conjugates **4b**, **3b** and **S** as a standard[16] under normoxic and hypoxic conditions.

agents possessing a different mechanism of action.[19] Studies are in progress in several laboratories to demonstrate the efficacy of such a combination therapy of CAIs with other antitumor agents or radiation.

Conclusion

We have reported here for the first time that Gd(III)-sulfonamide complexes incorporating the macrocyclic ring systems DOTA or TETA are remarkable inhibitors of both tumor-associated CA isozymes CA IX and XII, being able to reduce tumor acidosis *in vitro*. This new class compound thus promises applications in the diagnostic and treatment of hypoxic tumors, which are largely non-responsive to classical chemo- and radiotherapy.

Acknowledgements

This research was financed in part by a grant from the 6th Framework Programme of the European Union (DeZnIT project, to C. T. S. and A. S.) and by a 7th Framework Programme of the European Union grant (METOXIA). The authors thank Prof. Joulia Larionova (Institut Charles Gerhardt, Montpellier, France) for performing the magnetic measurements and Dr Marlies Oostendorp (University of Maastricht, The Netherlands) for determining the relaxivity values.

References

1 C. T. Supuran, *Nat. Rev. Drug Discovery*, 2008, **7**, 168–181.
2 J.-Y. Winum, M. Rami, A. Scozzafava, J.-L. Montero and C. T. Supuran, *Med. Res. Rev.*, 2008, **28**, 445.
3 A. Cecchi and C. T. Supuran, *Curr. Pharm. Des.*, 2008, **14**, 699.
4 C. T. Supuran, *Curr. Pharm. Des.*, 2008, **14**, 601.
5 A. Scozzafava, A. Mastrolorenzo and C. T. Supuran, *Expert Opin. Ther. Pat.*, 2004, **14**, 667.
6 S. Pastorekova, S. Parkkila, J. Pastorek and C. T. Supuran, *J. Enzyme Inhib. Med. Chem.*, 2004, **19**, 199.
7 A. Scozzafava, A. Mastrolorenzo and C. T. Supuran, *Expert Opin. Ther. Pat.*, 2006, **16**, 1627.
8 M. Hilvo, L. Baranauskiene, A. M. Salzano, A. Scaloni, D. Matulis, A. Innocenti, A. Scozzafava, S. M. Monti, A. Di Fiore, G. De Simone, M. Lindfors, J. Janis, J. Valjakka, S. Pastorekova, J. Pastorek, M. S. Kulomaa, H. R. Nordlund, C. T. Supuran and S. Parkkila, *J. Biol. Chem.*, 2008, **283**, 27799.
9 C. T. Supuran and A. Scozzafava, *Expert Opin. Ther. Pat.*, 2002, **12**, 217.
10 C. T. Supuran, A. Scozzafava and A. Casini, *Med. Res. Rev.*, 2003, **23**, 146.
11 A. J. Kivelä, S. Parkkila, J. Saarnio, T. J. Karttunen, J. Kivelä, A. K. Parkkila, S. Pastoreková, J. Pastorek, A. Waheed, W. S. Sly and H. Rajaniemi, *Histochem. Cell Biol.*, 2000, **114**, 197.
12 C. C. Wykoff, N. J. Beasley, P. H. Watson, K. J. Turner, J. Pastorek, A. Sibtain, G. D. Wilson, H. Turley, K. L. Talks, P. H. Maxwell, C. W. Pugh, P. J. Ratcliffe and A. L. Harris, *Cancer Res.*, 2000, **60**, 7075.
13 M. C. Brahimi-Horn and J. Pouysségur, *Essays Biochem.*, 2007, **43**, 165.
14 M. Bartošová, S. Parkkila, K. Pohlodek, T. J. Karttunen, S. Galbavy, V. Mucha, A. L. Harris, J. Pastorek and S. Pastorekova, *J. Pathol.*, 2002, **197**, 314.
15 V. Alterio, M. Hilvo, A. Di Fiore, C. T. Supuran, P. Pan, S. Parkkila, A. Scaloni, J. Pastorek, S. Pastorekova, C. Pedone, A. Scozzafava, S. M. Monti and G. De Simone, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 16233.
16 E. Švastová, A. Hulíková, M. Rafajová, M. Zat'ovičová, A. Gibadulinová, A. Casini, A. Cecchi, A. Scozzafava, C. T. Supuran and J. Pastorek, *FEBS Lett.*, 2004, **577**, 439.
17 L. Dubois, K. Douma, C. T. Supuran, R. K. Chiu, M. A. M.J. van Zandvoort, S. Pastoreková, A. Scozzafava, B. G. Wouters and P. Lambin, *Radiother. Oncol.*, 2007, **83**, 367.
18 L. Dubois, N. G. Lieuwes, A. Maresca, A. Thiry, C. T. Supuran, A. Scozzafava, B. G. Wouters and P. Lambin, *Radiother. Oncol.*, 2009, **92**, 423.
19 J. K. J. Ahlskog, C. E. Dumelin, S. Trüssel, J. Marlind and D. Neri, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 4851.
20 J. K. Ahlskog, C. Schliemann, J. Mårlind, U. Qureshi, A. Ammar, R. B. Pedleym and D. Neri, *Br. J. Cancer*, 2009, **101**, 645.
21 S. Aime and P. J. Caravan, *J. Magn. Reson. Imaging*, 2009, **30**, 1259.
22 L. Lattuada and G. Lux, *Tetrahedron Lett.*, 2003, **44**, 3893.
23 A. Barge, G. Cravotto, E. Gianolio and F. Fedeli, *Contrast Media Mol. Imaging*, 2006, **1**, 184.
24 S. Aime, D. D. Castelli, S. G. Crich, E. Gianolio and E. Terreno, *Acc. Chem. Res.*, 2009, **42**, 822.
25 M. Rami, A. Cecchi, J.-L. Montero, A. Innocenti, D. Vullo, A. Scozzafava, J.-Y. Winum and C. T. Supuran, *ChemMedChem*, 2008, **3**, 1780.
26 E. A. L. Boudreaux and N. Mulay, *Theory and Applications of Molecular Paramagnetism*, John Wiley & Sons, New York, 1976, pp. 125–147.
27 C. Rorden and M. Brett, *Behav. Neurol.*, 2000, **12**, 191.
28 E. M. Haacke, R. W. Brown, M. R. Thompson and R. Venkatesan, Introductory Signal Acquisition Methods: Induction Decay, Spin Echoes, Inversion Recovery and Spectroscopy, in *Magnetic Resonance Imaging: Physical Principles and Sequence Design*, John Wiley & Sons, Inc., New York, 1999, pp. 111–138.
29 R. G. Khalifah, *J. Biol. Chem.*, 1971, **246**, 2561.
30 S. Pastorekova, A. Casini, A. Scozzafava, D. Vullo, J. Pastorek and C. T. Supuran, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 869.
31 G. De Simone, R. M. Vitale, A. Di Fiore, C. Pedone, A. Scozzafava, J. L. Montero, J. Y. Winum and C. T. Supuran, *J. Med. Chem.*, 2006, **49**, 5544.
32 B. L. Wilkinson, L. F. Bornaghi, T. A. Houston, A. Innocenti, C. T. Supuran and S.-A. Poulsen, *J. Med. Chem.*, 2006, **49**, 6539.
33 J. Y. Winum, C. Temperini, K. El Cheikh, A. Innocenti, D. Vullo, S. Ciattini, J. L. Montero, A. Scozzafava and C. T. Supuran, *J. Med. Chem.*, 2006, **49**, 7024.
34 J.-Y. Winum, S. A. Poulsen and C. T. Supuran, *Med. Res. Rev.*, 2009, **29**, 419.
35 A. Maresca, C. Temperini, H. Vu, N. B. Pham, S. A. Poulsen, A. Scozzafava, R. J. Quinn and C. T. Supuran, *J. Am. Chem. Soc.*, 2009, **131**, 3057.
36 P. Roy, E. Reavey, M. Rayne, S. Roy, M. Abed El Baky, Y. Ishii and C. Bartholomew, *FEBS J.*, 2010, **277**, 441.
37 A. Scozzafava, F. Briganti, M. A. Ilies and C. T. Supuran, *J. Med. Chem.*, 2000, **43**, 292.

