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Carbonic Anhydrase Inhibitors: Design of Membrane-Impermeant Copper(II) Complexes of DTPA-, DOTA-, and TETA-Tailed Sulfonamides Targeting the Tumor-Associated Transmembrane Isoform IX

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The synthesis and carbonic anhydrase (CA, EC 4.2.1.1) inhibitory activity of two series of aromatic sulfonamides and their Cu^{II} derivatives, incorporating metal-complexing moieties of the DTPA, DOTA, and TETA type are reported. The new compounds were designed in such a way as to possess high affinity for Cu^{II} ions, exploiting four pendant carboxylate moieties in the DTPA derivatives, as well as the cyclen/cyclam macrocyles, and three pendant acetate moieties in the DOTA and TETA derivatives. The new derivatives showed modest inhibition of the cytosolic isoform CA I (K_I values in the range of 66–2130 nM), were better CA II inhibitors (K_I values in the range of 21–360 nM), and excellent inhibitors of the tumor-associated isoform CA IX (K_I values in the range of 4.1–110 nM), with selectivity ratios for the inhibition of the tumor (CA IX) over the cytosolic (CA II) isozyme in the range of 10.74–20.88 for the best derivatives. Copper complexes were more inhibitory than the corresponding ligand sulfonamides, and showed membrane impermeability, thus, having the possibility to specifically target the transmembrane CA IX that has an extracellular active site. Incorporation of radioactive copper isotopes in this type of CA inhibitor may lead to interesting diagnostic/therapeutic applications for such compounds.

Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes, encoded by at least five distinct, evolutionarily unrelated gene families: the α-CAs (in prokaryotes from the bacteria domain, algae, cytoplasm of green plants, and vertebrates—with 15 isozymes presently known in humans), the β-CAs (predominantly in bacteria, fungi, algae, and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (in archaea and some bacteria), the δ-CAs, found so far only in marine diatoms, and the recently characterized ζ-CAs, which are cadmium enzymes also present in marine diatoms.[1–8] These enzymes catalyze the reversible hydration of carbon dioxide to bicarbonate and protons by means of a metal-hydroxide ($Lig^3M^{2+}(OH)^-$) mechanism, but at least the α-CAs possess other catalytic activities (esterase, phosphatase, cyanate/cyanamide hydrase, etc.).[5] In the α-, γ-, and δ-CA classes, Lig_3 is always constituted by three His residues, and M is Zn^{II} for all classes but may also be Fe^{II} for the γ-CAs.[6] The β- and ζ-CAs have the active site metal ion coordinated by two Cys and one His residue, whereas the fourth ligand may be either a water molecule/hydroxide ion, or a carboxylate from a conserved aspartate residue in some (but not all) β-CAs.[2,3–6] However, the metal hydroxide catalytic mechanism seems to be also valid for these enzymes,[3,4] as the coordinated aspartate is replaced during the catalytic cycle by a water molecule that then generates the strong nucleophilic zinc/cadmium hydroxide species (the β-CAs are strictly zinc enzymes, whereas the active site metal ion in ζ-CAs may be Cd^{II} or Zn^{II}, as shown recently in a very elegant study by Morel's group).[2]

CAs are involved in numerous physiological and pathological processes, including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in humans, as well as the growth and virulence of various fungal/bacterial pathogens.[1,3–4,9–11] In addition to the established role of carbonic anhydrase inhibitors (CAIs) as diuretics and antiglaucoma drugs, it has recently emerged that CAIs could have potential as novel antiobesity, anticancer, or anti-infective drugs.[3,4] Many of the CA isozymes involved in these processes

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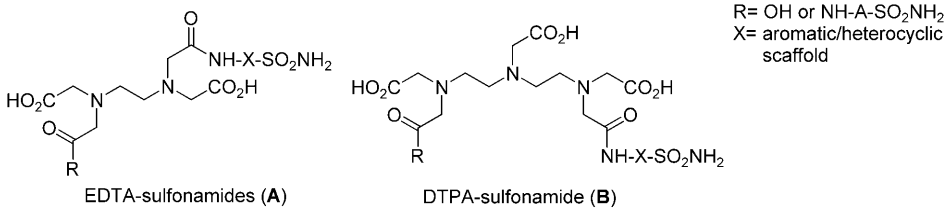
are important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[3,4] Two main classes of CAIs are known: the metal-complexing anions, and the unsubstituted sulfonamides and their bioisosteres (sulfamates, sulfamides), which bind to the metal ion of the enzyme either by substituting the nonprotein zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal-bipyramidal species.[3,4] However a critical problem in the design of CAIs is related to the high number of isoforms, their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors.[3–5]



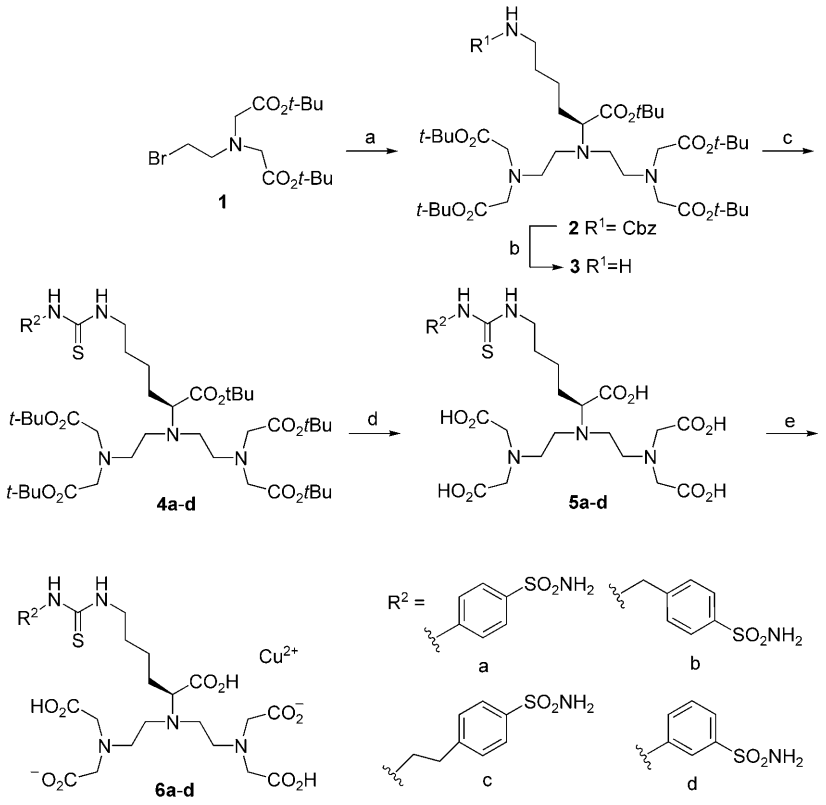
Human CA IX (hCA IX) is an extracellular, transmembrane isoform, which was recently shown to constitute a novel and interesting target for the anticancer therapy because of its overexpression in many cancer tissues and not in their normal counterparts.[3,12–18] Its expression is strongly induced by the hypoxia present in many tumor tissues, and correlated with a bad response to classical chemo- and radiotherapies.[3,12] CA IX was shown to acidify the extratumoral medium leading both to the acquisition of metastasic phenotypes and to chemoresistance with many anticancer drugs, these processes were reverted by the inhibition of enzyme catalytic activity with sulfonamide inhibitors.[15] The development of selective CA IX inhibitors might thus be useful for highlighting the exact role of CA IX in hypoxic cancers,[3,12–19] to control the pH imbalance of tumor cells, and lead to novel diagnostic or therapeutic applications for the management of such tumors.[3,12] Although many sulfonamide/sulfamate/sulfamide potent CA IX inhibitors have been reported,[3] few of them show an acceptable level of selectivity for inhibiting the transmembrane tumor-associated target isoform IX over the cytosolic, house keeping isozymes hCA I and II.[3,12–19] Considering the extracellular localization of the target CA isoform and the fact that we already obtained membrane-impermeant CAIs designed by various approaches,[3,20] we report herein the preparation, enzyme inhibitory activity, and membrane penetration studies of a novel class of copper-containing sulfonamides that show excellent CA IX inhibitory properties and selectivity for the inhibition of the tumor-associated isoform over hCA I and II.

Results and Discussion

Chemistry

A general approach for the preparation of water-soluble sulfonamides incorporating polyamino-polycarboxylate tails (of the EDTA or DTPA type, **A** and **B**), and of their metal complexes containing Zn^{II}, Cu^{II}, or other divalent metal ions has been reported earlier by our group.[20] Both mono- and symmetrical bis-sulfonamides of types **A** and **B** were prepared, together with their Zn^{II} and Cu^{II} metal complexes, which showed excellent inhibitory activity of isoforms CA I, II, and IV.[20b,c] More recently, some of these derivatives were also investigated for their interaction with the tumor-associated isoforms CA IX and XII,[20a] with some Cu^{II} complexes of sulfonamides **A** and **B** showing subnanomolar affinities and some selectivity for the inhibition of the tumor-associated isoforms IX and XII over the cytosolic forms. Their use as positron emission tomography (PET) hypoxia markers for tumors overexpressing these CAs has also been contemplated.[20a] Indeed, copper radiopharmaceuticals have recently emerged as interesting tools for imaging (or even treatment) of cardiac diseases and tumors by means of PET because of the appropriate radiochemical properties of ^{64}Cu (half life of 12.7 h, emission of positrons of 0.655 MeV, and an EC of 41%),[21] its ease of production, and favorable and versatile complexation chemistry, which allows the facile synthesis of different types of such derivatives, targeting various proteins/tissues/organs.[21–25]

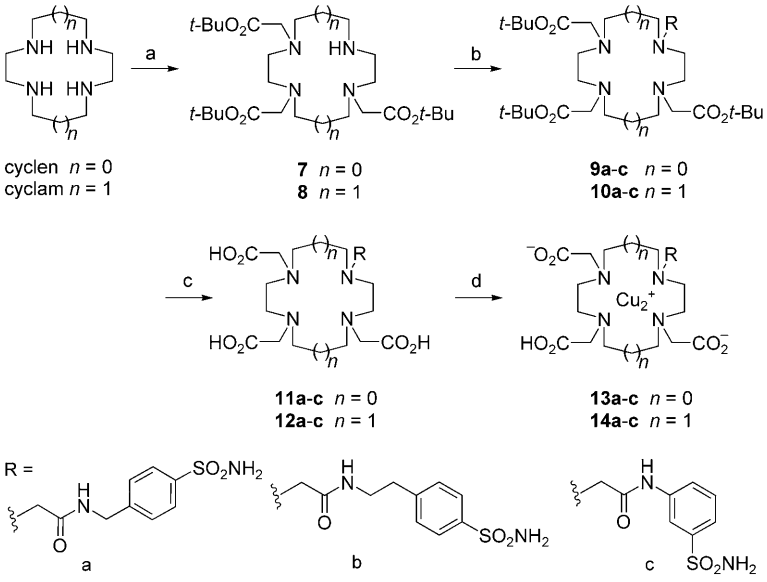
The copper-sulfonamide derivatives with CA inhibitory properties reported earlier[20] incorporated EDTA and/or DTPA functionalized sulfonamides as ligands of types **A** and **B**, and although the stability constants for the Cu^{II}-EDTA complexes (log $K_{stab} = 18.7$) and Cu^{II}-DTPA complexes (log $K_{stab} = 21.4$) are high, it has been observed that such metal complexes are not stable enough in biological samples for long enough periods, as they easily lose the complexed Cu^{II} ions.[21,22] Similar effects were also observed for our Cu^{II} complexes incorporating ligands of types **A** and **B**. To correct these defects in the potent CAI incorporating Cu^{II} ions, which have potential to be used as diagnostic tools/radiotherapeutics for the management of hypoxic tumors, we herein propose alternative approaches for designing Cu^{II}-containing CAIs with enhanced stability. Two synthetic approaches were explored: 1) preparation of different DTPA derivatives of compounds **B** was previously reported,[20] in which the central N-CH_2COOH moiety is derivatized by incorporation of aromatic sulfonamide scaffolds leaving the four lateral tetraacetate pendant groups free for the interaction with metal ions (Scheme 1). Such compounds would in principle possess higher stability when complexed to metal(II) ions as compared to derivatives of type **B**, in which the one or two derivatized lateral carboxamides cannot interact easily with cations, as compared to the corresponding carboxylate moieties; 2) use of tetraazamacrocycles ligands incorporating pendant arms, which use both the macrocyclic and chelate effects to enhance stability of the metal complex (Scheme 2).[21,26] We decided to use the cyclam and cyclen chemistry, as Cu^{II} complexes incorporating these macrocycles were thoroughly investigated and shown to be more stable as compared to the



Scheme 1. Synthesis of DTPA sulfonamide derivatives and their Cu^{II} complexes. Reagents and conditions: a) 2.2 equiv of **1**, 1.1 equiv of H-Lys(*Z*)-O*t*Bu, phosphate buffer (2 M, pH 8) MeCN 1.1/1 *v/v*, 82 °C, 3 days; b) Ammonium formate, 5% Pd/C, EtOH, reflux, 2 h; c) R_2-NCS, MeCN, 82 °C, overnight; d) TFA/H_2O/thioanisole 95/2.5/2.5 *v/v/v*, RT, overnight; e) 1.1 equiv copper (II) chloride dihydrate, DMSO, RT, 1 h.

tives, and log K_{stab} = 22.7 for DOTA derivatives, respectively).[21,26]

The new DTPA derivatives of the first approach mentioned above were prepared according to Scheme 1. Commercially available N^6-Cbz-L-Lys *tert*-butyl ester was reacted with bromide **1** in acetonitrile to yield the dialkylated intermediate **2** as described earlier by Anelli et al.[27] Cleavage of the Cbz moiety by hydrogenolysis afforded the DTPA--aminopentaester **3**. The amino group of **3** was derivatized subsequently by reaction with the corresponding 4-sulfamoylphenylisothiocyanates,[28] leading to the thioureido sulfonamides **4a–d**. The *tert*-butyl ester groups of **4a–d** were then removed using TFA in the presence of thioanisole as scavenger, leading to the sulfonamide ligands **5a–d**, which incorporate the DTPA scaffold with four free lateral acetate groups available for complexation of metal ions. Indeed, complexation of **5a–d** with Cu^{2+} salts gave the target complexes **6a–d**. The four sulfonamide scaffolds incorporated in these derivatives, that is, sulfanilamide, homosulfanilamide, 4-aminoethylbenzenesulfonamide, and metanilamide were earlier shown by this group to lead to effective CA IX inhibitors when incorporated in various scaffolds.[3,29]



Scheme 2. Synthesis of cyclen and cyclam sulfonamide derivatives and their Cu^{II} complexes. Reagents and conditions : a) 2.7 equiv of *tert*-butyl bromoacetate, K_2CO_3, MeCN, RT; b) 1 equiv of R-Br, K_2CO_3, MeCN, 80–82 °C; c) trifluoroacetic acid/H_2O/thioanisole 95/2.5/2.5 *v/v/v*, RT, overnight; d) 1.1 equiv of copper (II) chloride dihydrate, DMSO, RT.

Compounds obtained by the second approach, incorporating DOTA and TETA scaffolds were synthesized as outlined in Scheme 2. Starting from the tetraazamacrocycles cyclen or cyclam, alkylation reactions with halogenoacetic acid *tert*-butyl esters were performed at room temperature leading to the trisubstituted tetraazamacrocyles **7** and **8** in good yields (Scheme 2). These later compounds were then reacted with 2-bromo-*N*-(4-sulfamoylphenyl)acetamides (prepared by reaction of bromoacetyl chloride and aminobenzenesulfonamides in the presence of triethylamine)[30] leading to compounds **9** and **10**, which were subsequently hydrolyzed with a cocktail of TFA/water/thioanisole to give the corresponding tricarboxylic acids **11** and **12** incorporating the macrocycle and pendant acetate groups (for complexation of metal ions) as well as benzenesulfonamide tails (which bind the zinc ion within the CA active site).[1,3] Complexation reactions of these intermediates with Cu^{2+} salts led to the metal chelates **13a–c** and **14a–c**. Again

corresponding, less rigid acyclic chelating agents (their stability constants were shown to be log K_{stab} = 21.9 for TETA derivative

the benzenesulfonamide scaffolds leading to effective CA IX inhibitors were used for the preparation of these new compounds.[29] The new derivatives and their metal complexes reported herein were extensively analyzed by spectroscopic (^{1}H- and ^{13}C NMR) and high resolution MS procedures, which confirmed the proposed structures (see Experimental Section for details).

Carbonic anhydrase inhibition

The data in Table 1 show that the sulfonamide ligands **5a–d**, **11a–c**, and **12a–c**, as well as their corresponding Cu^{II} complexes **6a–d, 13a–c**, and **14a–c**, generally act as very potent inhibitors of all three CA isozymes investigated, that is, the cytosolic, ubiquitous house-keeping human CA I and II,[1,3] as well as the transmembrane, tumor-associated hCA IX.[3,12–18] The following structure–activity relationships can be elucidated from the data:

1) Against the slow cytosolic isoform hCA I, highly abundant in the blood and gastrointestinal tract,[32] the new CAIs reported herein showed moderate inhibitory activity (Table 1). Thus, the DTPA derivatives **5a–c** showed inhibition constants in the range of 167-2100 nM, whereas their Cu^{II} complexes were more inhibitory, with K_I values in the range of 125–490 nM. It may be observed that CA I inhibitory activity increased from the sulfanilamide to the homosulfanilamide and to the 4-aminoethylbenzenesulfonamide scaffolds with the increasing spacer size between the benzenesulfonamide functionality and the derivatized amino group, both for the ligands and the corresponding Cu^{II} complexes, whereas the metanilamide derivatives (**5d** and **6d**) showed a weaker activity as compared to the 4-substituted benzenesulfonamides mentioned above (an exception is the Cu^{II} complex **6a**, which is also a weak CA I inhibitor). For the macrocycle-scaffold containing CAIs, the CA I inhibition constants were in the range of 88–2130 nM for ligands **11** and **12**, and in the range of 66–380 nM for the Cu^{II} complexes **13** and **14**, respectively (Table 1). Considering the sulfonamide scaffold incorporated in these derivatives, this time the best CA I inhibitors in each subseries incorporated the metanilamide scaffold (**11c** and **12c**, as well as the corresponding Cu^{II} complexes) followed by the 4-aminoethylbenzenesulfonamide derivatives **11b**, **12b**, and the corresponding metal complexes, whereas the homosulfanilamide derivatives **11a**, **12a**, **13a**, and **14a** were less active as inhibitors of this isoform. The derivatives incorporating the 12-membered macrocycles **11** and **13** were generally more active than the corresponding derivatives incorporating the 14-membered macrocycles, of types **12** and **14** (the only exception is **12b**, which is slightly more active than **11b**).

Table 1. Inhibition of CA isoforms hCA I, II, and IX.[a]

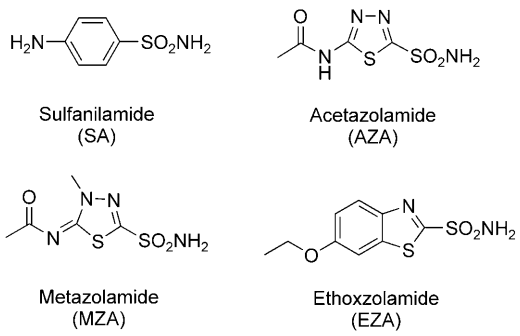
Inhibitor	K_I[b] (nM) hCA I[c]	hCA II[c]	hCA IX[d]	Selectivity ratio K_I(hCA II)/K_I(hCA IX)
5a	530	89	54	1.64
5b	237	39	8.6	4.53
5c	167	97	37	2.62
5d	2100	59	28	2.10
6a	490	60	40	1.50
6b	127	21	6.6	3.18
6c	125	44	6.9	6.37
6d	280	54	4.5	12.00
11a	970	298	110	2.71
11b	425	720	41	17.56
11c	88	290	27	10.74
12a	2130	360	96	3.75
12b	390	267	36	7.41
12c	367	280	20	14.00
13a	193	55	8.7	6.32
13b	66	81	8.9	9.10
13c	78	25	7.5	3.33
14a	380	355	17	20.88
14b	363	99	5.8	17.06
14c	325	74	4.1	18.04
SA	25000	240	294	0.81
AZA	250	10	25	0.40
MZA	50	14	27	0.51
EZA	25	8	34	0.23

[a] By sulfonamides **5a–d**, **11a–c**, **12a–c** and the corresponding Cu^{II} complexes **6a–d**, **13a–c**, **14a–c**. Clinically used CA inhibitors SA, AZA, MZA, and EZA were used as standards. [b] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[31] [c] Human, recombinant isozymes. [d] Catalytic domain of human, cloned isoform.[28–30]

2) The rapid, housekeeping, ubiquitous isoform CA II[1–3,32] was more efficiently inhibited by these compounds compared to the slow isoform CA I discussed above. Thus, the DTPA derivatives **5a–d** showed inhibition constants in the range of 39–97 nM, whereas the corresponding copper complexes were better inhibitors, with K_I values in the range of 21–60 nM (Table 1). The best CA II inhibitors incorporated the homosulfanilamide scaffold (**5b** and **6b**), followed by those incorporating the metanilamide, sulfanilamide, and *p*-aminoethylbenzenesulfonamide scaffolds (for ligands **5**). For the copper complexes the order of activity was slightly different, with the 4-aminoethylbenzenesulfonamide **6c** complex being more active than the corresponding metanilamide **6d** and sulfanilamide **6a** derivatives. For the macrocycles-containing subseries of CAIs reported herein, the inhibitory activity of the ligands **11** and **12** was much lower than that of the DTPA derivatives **5** mentioned above, in the range of 267–720 nM. On the other hand, the copper complexes **13** and **14** were better CA II inhibitors compared to the corresponding sulfonamide ligands, with K_I values in the range of 25–355 nM. SAR was similar with that discussed above for isoform CA I for both the sulfonamide scaffold and the macrocycle ring, with the cyclen derivatives being generally slightly more efficient CA II inhibitors compared to the corresponding cyclam ones (Table 1).

3) The high activity, transmembrane tumor-associated[3,12–18] isoform CA IX was inhibited by the ligands and Cu^{II} complexes reported herein, as previously reported for structurally related compounds incorporating EDTA and different DTPA scaffolds.[20a] Thus, the acyclic derivatives **5** showed K_I values in the range of 8.6–54 nM, whereas their Cu^{II} complexes were again better inhibitors, with K_I values in the range of 4.5–40 nM. The best sulfonamide component was homosulfanilamide, followed by metanilamide, 4-aminoethylbenzenesulfonamide, and sulfanilamide for the ligands **5**, whereas for the Cu^{II} complexes

the order of activity was different, with the metanilamide derivative **6d** being the best inhibitor, followed by the homosulfanilamide **6b** and 4-aminoethylbenzenesulfonamide **6c** complexes, which showed very similar activity, with the sulfanilamide complex **6a** the least active CA IX inhibitor. The cyclic ligands **11** and **12** again showed a weaker inhibitory activity against CA IX as compared to the acyclic ones discussed above (K_I values in the range of 20–110 nM), whereas the corresponding Cu^{II} complexes were very effective inhibitors (K_I values in the range of 4.1–17 nM). Thus, the best sulfonamide warhead was the metanilamide one, both for ligands and copper complexes, whereas the bulkier, 14-membered derivatives **12** and **14** were generally better CA IX inhibitors compared to the corresponding 12-membered ring compounds **11** and **13**, respectively (except for **13a**, which is a better inhibitor than **14a**). This is probably due to the fact that the CA IX active site seems to be larger than that of CA I and II, as shown by homology modeling,[16] as the X-ray crystal structure of CA IX is not yet known. It was also observed that many of the reported new compounds and their metal complexes show much better CA IX inhibitory activities compared to clinically used sulfonamides such as sulfanilamide SA, acetazolamide AZA, methazolamide MZA, or ethoxzolamide EZA (Table 1).



4) A major problem regarding most of the clinically used CAIs, such as those mentioned above, regards their lack of selectivity for the inhibition of the tumor-associated isoform CA IX over the highly active cytosolic CA II.[1,3,12–18] Indeed, as seen from the data contained in Table 1, the four clinically used CAIs SA, AZA, MZA, and EZA showed selectivity ratios for the inhibition of the cytosolic isoform CA II over the transmembrane CA IX in the range of 0.23–0.81, and therefore are better CA II than CA IX inhibitors. As seen from the data of Table 1, all compounds reported in the present paper show a selectivity ratio for the inhibition of the cytosolic isoform CA II over the transmembrane isoform CA IX >1, in some cases with values as high as 12.00–20.88. This means that some of these derivatives are 12–21-fold more selective for CA IX than CA II, which makes this type of CAIs highly interesting for investigation as CA IX-selective inhibitors. Some of the most CA IX-selective inhibitors were the copper-TETA complexes **14a–c** (selectivity ratios of 17.06–20.88) and the ligands **6d**, **11b**, **11c**, and **12c** (selectivity ratios of 10.74–17.56).

Membrane permeability studies

As CA IX is a transmembrane isoform with an extracellular active site,[3,12–18] it is possible to specifically inhibit its activity with membrane-impermeant compounds, as already reported by this group using CAIs incorporating cationic pyridinium derivatives.[33] As blood contains high amounts of the cytosolic isozymes CA I and II (150 μM hCA I, and 20 μM hCA II, but not the membrane-associated CA IX)[32] we investigated the penetrability of sulfonamide CAIs through membranes using red blood cells (RBCs) as an experimental model. The experiments were performed with freshly isolated human blood. RBCS were incubated with millimolar concentrations of sulfonamide inhibitors, such as acetazolamide AZA, the sulfonamide ligands **5**, **11**, or **13**, and their copper complexes of type **6**, **13**, and **14** (Table 2). It may be observed that incubation of both AZA and sulfonamide ligands led to inhibitor saturation of the two isozymes present in erythrocytes after short periods of incubation (30–180 min) (Table 2). This is obviously due to the high diffusibility through membranes of these inhibitors. On the contrary, the copper complexes were only detected in negligible amounts within the RBCs, probably due to contaminations with membranes, proving that the these copper-containing CAIs are unable to penetrate biological membranes. Even after incubation times as long as 24 h (data not shown), only minute traces of copper-containing CAIs were detected in the supernatants (due to the possible contaminations mentioned above). These experiments show that these copper-containing CAIs are totally membrane-impermeant, which is a highly desirable feature for a compound that should selectively inhibit only the extracellular CA IX. Thus, by incorporating radioactive copper isotopes in their molecules, compounds of the type reported herein constitute interesting candidates to be investigated both for the imaging and treatment of tumors overexpressing CA IX.

Table 2. Levels of sulfonamide CAIs in red blood cells.[a]

Inhibitor	[sulfonamide] μM[b]		
	$t=30$ min	$t=60$ min	$t=180$ min
AZA	140 ± 4	160 ± 8	163 ± 6
5b	121 ± 3	145 ± 4	166 ± 5
11c	89 ± 6	121 ± 5	137 ± 4
12c	104 ± 8	138 ± 7	156 ± 5
6d	0.2 ± 0.05	0.4 ± 0.03	0.5 ± 0.04
13b	0.1 ± 0.02	0.3 ± 0.06	0.4 ± 0.05
14c	0.5 ± 0.05	0.6 ± 0.04	0.5 ± 0.02

[a] 30, 60, and 180 min after exposure of 10 mL of blood to solutions of sulfonamides (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide in RBCs has been determined enzymatically[31] as reported earlier.[33] [b] Mean ± standard deviation (from three determinations).[9]

Conclusions

The synthesis and CA inhibitory activity of two series of aromatic sulfonamides and their Cu^{II} derivatives, incorporating

metal-complexing moieties of the DTPA, DOTA, and TETA type are reported in this paper. The new compounds were designed in such a way as to possess high affinity for Cu^{II} ions, exploiting four pendant carboxylate moieties in the DTPA derivatives, as well as the cyclen/cyclam macrocyles and three pendant acetate moieties in the DOTA and TETA derivatives. The new derivatives showed modest inhibition of the cytosolic isoform CA I (K_I values in the range of 66–2130 nM), were better CA II inhibitors (K_I values in the range of 21–360 nM), and excellent inhibitors of the tumor-associated isoform CA IX (K_I values in the range of 4.1–110 nM), with selectivity ratios for the inhibition of the tumor (CA IX) over the cytosolic (CA II) isozyme in the range of 10.74–20.88 for the best derivatives. Copper complexes were more inhibitory than the corresponding sulfonamide ligands, and showed membrane impermeability, thus having the possibility to specifically target the transmembrane CA IX, which has an extracellular active site. Incorporation of radioactive copper isotopes in this type of CA inhibitor may lead to interesting diagnostic/therapeutic applications for such compounds.

Experimental Section

General methods

TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art.1.05554). Spots were visualized at 254 nm under UV illumination, or by ninhydrin solution spraying. Melting points were determined on a Büchi Melting Point 510 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Brüker DRX-400 spectrometer using [D_6]DMSO as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in Hertz. Electron ionization mass spectra (10 eV) were recorded in positive or negative mode on a Water MicroMass ZQ. The syntheses of compounds **2** and **3** have been described previously.[27]

General procedure for the preparation of compounds (4): 1 equiv of compound (**3**), prepared as previously described,[7] was mixed with 1 equiv of the corresponding isothiocyanatophenylsulfonamide[28] in ACN. The mixture was refluxed overnight then concentrated under vacuum. The residue was purified by silica gel column chromatography using DCM–MeOH 95–5 as eluent.

***N*^{6}-[(4-Sulfamoylphenyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl] amino]ethyl]-L-lysine 1,1-dimethylethyl ester (4a)**: Yield 61%; mp: 145–147 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.28–1.59 (m, 51H), 2.53–2.76 (m, 8H), 3.25 (t, 1H, J = 7.2 Hz), 3.38 (s, 8H), 3.48 (m, 2H), 7.30 (s, 2H), 7.66 (d, 2H, J = 8.0 Hz), 7.74 (d, 2H, J = 8.0 Hz), 8.03 (s, 1H), 8.77 (s, 1H); MS (ESI$^+$) m/z: 959.42 [M + H]$^+$, 981.58 [M + Na]$^+$.

***N*^{6}-[(4-Sulfamoylphenylmethyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethyl ethoxy)-2-oxoethyl]amino]ethyl]-L-lysine 1,1-dimethylethyl ester (4b)**:Yield 56%; mp: 133–135 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.39–1.40 (m, 51H), 2.59–2.70 (m, 8H), 3.15 (d, 2H, J = 5.30 Hz), 3.35–3.37 (m, 11H), 7.31 (s, 2H), 7.4 (d, 2H, J = 8 Hz), 7.74 (d, 2H, J = 8 Hz); MS (ESI$^-$/ESI$^+$) m/z: 971.62 [M−H]$^-$, 973.7 [M + H]$^+$, 995.62 [M + Na]$^+$.

***N*^{6}-[(4-Sulfamoylphenylethyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxo ethyl]amino]ethyl]-L-lysine 1,1-dimethylethyl ester (4c)**: Yield 70%; mp: 138–140 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.39–1.40 (m, 51H), 2.59–2.70 (m, 8H), 2.86 (t, 2H, J = 7.20 Hz), 3.15–3.17 (td, 2H, J = 5.2 Hz), 3.36 (m, 11H), 7.3 (s, 2H), 7.39 (d, 2H, J = 8 Hz); MS (ESI$^-$/ESI$^+$) m/z: 985.65 [M−H]$^-$, 987,67 [M + H]$^+$, 1009.76 [M + Na]$^+$.

***N*^{6}-[(3-Sulfamoylphenyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl] amino]ethyl]-L-lysine 1,1-dimethylethyl ester (4d)**: Yield: 59%; mp 147–149 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.39–1.40 (m, 51H), 2.6–2.69 (m, 8H), 3.2 (t, 1H, J = 7.2 Hz), 3.36 (m, 8H), 3.41 (m, 2H), 7.36 (s, 2H), 7.44–7.49 (m, 2H), 7.68 (m, 1H), 7.90 (s, 1H), 9.68 (s, 1H); MS (ESI$^+$) m/z 959.73 [M + H]$^+$, 981.69 [M + Na]$^+$.

General procedure for the preparation of compounds (5): 250 mg of *tert*-butyl ester (**4**) were dissolved in 5 mL of a cocktail of trifluoroacetic acid-water-thioanisole 95/2.5/2.5. The mixture was stirred overnight at RT, and then concentrated under vacuum. The residue was co-evaporated several times with Et_2O until precipitation. The solid was filtered and washed several times with methylene chloride and ACN.

***N*^{6}-[(4-Sulfamoylphenyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl] amino]ethyl]-L-lysine (5a)**: Yield 76%; mp: 110–112 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.25–1.58 (m, 6H), 2.07 (s, 2H), 3.02 (m, 4H), 3.09 (m, 4H), 3.55(m, 11H), 4.49 (s, 1H), 7.26 (s, 2H), 7.68–7.73 (m, 4H); ^{13}C NMR (101 MHz, [D_6]DMSO) δ = 23.57, 43.42, 49.70, 50.47, 54.14, 63.94, 121.49, 126.19, 138.26, 142.87, 170.55, 172.60, 180.20; MS (ESI$^-$/ESI$^+$) m/z: 677.0 [M−H]$^-$, 679.21 [M + H]$^+$, 701.21 [M + H]$^+$.

***N*^{6}-[(4-Sulfamoylphenylmethyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethyl ethoxy)-2-oxoethyl] amino]ethyl]-L-lysine (5b)**: Yield 93%; mp: 103–104 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.32–1.71 (m, 6H), 2.06 (s, 2H), 2.91–3.01 (m, 8H), 3.49 (m, 10H), 3.73 (s, 2H), 4.72 (s, 1H), 7.3 (s, 2H), 7.41 (d, 2H, J = 8 Hz), 7.74 (d, 2H, J = 8 Hz); ^{13}C NMR (101 MHz, [D_6]DMSO) δ = 23.61, 27.62, 28.41, 43.27, 49.70, 51.07, 54.59, 54.67, 63.90, 125.55, 127.35, 142.37, 143.96, 140.77, 143.93, 157.48, 172.23, 172.45, 180.52; MS (ESI$^-$/ESI$^+$) m/z: 691.25 [M−H]$^-$, 693.29 [M + H]$^+$, 715,39 [M + Na]$^+$, 731.16 [M + K]$^+$.

***N*^{6}-[(4-Sulfamoylphenylethyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxo ethyl]amino]ethyl]-L-lysine (5c)**: Yield 89%; mp 107–108 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.25–1.72 (m, 6H), 2.06 (s, 2H), 2.85–3.05 (m, 10H), 3.36–3.38 (m, 2H), 3.49 (m, 8H), 3.59–3.60 (m, 2H), 3.87–3.88 (m, 1H), 7.3 (s, 2H), 7.4 (d, 2H, J = 8 Hz), 7.73 (d, 2H, J = 8 Hz).^{13}C NMR (101 MHz, [D_6]DMSO) δ = 23.57, 27.37, 27.41, 34.62, 44.60, 49.93, 50.73, 54.48, 54.88, 63.87, 125.68, 129.08, 141.97, 143.65, 158.10, 171.69, 172.36, 180.28; MS (ESI$^-$/ESI$^+$) m/z 691.25 [M−H]$^-$, 693.29 [M + H]$^+$, 715.39 [M + Na]$^+$, 731.16 [M + K]$^+$.

***N*^{6}-[(3-Sulfamoylphenyl)thioureido]-*N*^{2},*N*^{2}-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl] amino]ethyl]-L-lysine (5d)**: Yield 94%; mp: 111–113 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.35- 1.67 (m, 6H), 2.08 (s, 2H), 2.99–3.05 (m, 8H), 3.51–3.52 (m, 11H), 7.34 (s, 2H), 7.45 (m, 2H), 7.77 (m, 1H), 8.13 (s, 1H);^{13}C NMR (101 MHz, [D_6]DMSO) δ = 23.69, 26.77, 28.0, 44.48, 49.36, 51.35, 54.99, 63.88, 119.26, 124.74, 125.83, 128.9, 140.77, 143.93, 171.88, 172.38, 180.63; MS (ESI$^-$/ESI$^+$) m/z: 679.31 [M−H]$^-$, 679.31 [M + H]$^+$, 701.21 [M + Na]$^+$, 717.30 [M + K]$^+$.

General procedure for the preparation of copper complexes (6): 1 equiv of compound (**5**) and 1.1 equiv of copper(II) chloride dihydrate were stirred in DMSO at RT. The complexation was monitored by electrospray mass spectrometry until completion of the reaction (1 h). The complex was then precipitated with a mixture of 50–50

DCM–Et_2O. The solid was filtered, washed several times with acetone and ACN, and dried under vacuum.

Complex 5a–Cu^{2+} (6a): Yield 79%; mp: 145–147 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.03–1.23 (m, 6H), 2.07 (s, 2H), 3.28–3.62 (m, 19H), 7.20 (s, 2H), 7.62–7.67 (m, 4H); MS (ESI^-) *m/z:* 738.25 $[M-H]^-$.

Complex 5b–Cu^{2+} (6b): Yield 86%; mp: 107–108 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.12–1.37 (m, 6H), 2.07 (s, 2H), 2.51–2.54 (m, 8H), 3.39–3,42 (m, 13H), 7.23–7.48 (m, 6H). MS (ESI^-) *m/z:* 752.11 $[M-H]^-$.

Complex 5c–Cu^{2+} (6c): Yield 91%; mp: 165–167 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.10–1.69 (m, 6H), 2.06 (s, 2H), 2.51–2.53 (m, 10H), 3.53–3.60 (m, 13H), 7.25–7.34 (m, 4H), 7.71–7.72 (m, 2H); MS (ESI^-) *m/z*: 766.25 $[M-H]^-$.

Complex 5d–Cu^{2+} (6d): Yield 88%; mp: 121–123 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.07–1.39 (m, 6H), 2.07 (s, 2H), 2.51–2.54 (m, 8H), 3.45–3.52 (m, 11H), 7.29–7.46 (m, 6H); MS (ESI^-) *m/z*: 738.10 $[M-H]^-$.

General procedure for the synthesis of compounds (7) and (8): *tert*-Butylbromoacetate (2.7 equiv) diluted in ACN was added dropwise to 1 equiv of cyclen or cyclam and 3 equiv of potassium carbonate in ACN (or DCM for cyclam). The reaction was stirred overnight at RT, and then filtered. The filtrate was concentrated under vacuum, and the resulting residue purified on silica gel column chromatography using DCM–MeOH 98–2 as eluent.

1,4,7-Tris(carbo*tert*-butoxymethyl)-1,4,7,10-tetraazacyclododecane (7): Yield 73%; mp: 181–183 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.42 (s, 9H), 1.43 (s, 18H), 2.08 (s, 1H), 2.67 (m, 8H), 2.83 (s, 4H), 2.96 (s, 4H), 3.40 (s, 6H); MS (ESI^+) *m/z*: 515.49 $[M+H]^+$, 537.41 $[M+Na]^+$.

1,4,8-Tris(carbo*tert*-butoxymethyl)-1,4,8,11-tetraazacyclotetradecane (8): Yield 67%; mp: 183–185 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.41 (s, 9H), 1.43 (s, 18H), 1.54 (m, 2H), 1.75 (m, 2H), 2.08 (s, 1H), 2.50–2.53 (m, 4H) , 2.64 (s, 4H), 2.85 (m, 2H), 3.01 (m, 2H), 3.12 (s, 2H), 3.20 (s, 2H), 3.31 (s, 2H), 3,38 (s, 4H); MS (ESI^+) *m/z*: 543.42 $[M+H]^+$, 565.41 $[M+Na]^+$.

General procedure for the synthesis of compounds (9) and (10): 1 equiv of potassium carbonate and 1 equiv of 2-bromo-*N*-(sulfamoylphenyl)acetamide were added to a solution of 1 equiv of (**7**) or (**8**) in ACN.[30] The mixture was refluxed overnight and then filtered. The filtrate was concentrated under vacuum to give a residue, which was purified by silica gel column chromatography using DCM–MeOH 95–5 as eluent.

1,4,7-Tris(carbo*tert*butoxymethyl)-10-[(4-sulfamoylphenylmethyl)-aminocarboxymethyl]-1,4,7,10-tetraazacyclododecane (9a): Yield 66%; mp: 104–106 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.38 (s, 18H), 1.43 (s, 9H), 3.35 (s, 24H), 4.36 (m, 2H), 7.35 (s, 2H), 7.42 (d, 2H, *J* = 8 Hz), 7.72 (d, 2H, *J* = 8 Hz), 8.78 (t, 1H, *J* = 5.81 Hz); MS (ESI^+) *m/z* 741.39 $[M+H]^+$, 763.43 $[M+Na]^+$.

1,4,7-Tris(carbo*tert*butoxymethyl)-10-[(4-sulfamoylphenylethyl)-aminocarboxy methyl]-1,4,7,10-tetraazacyclododecane (9b): Yield 57%; mp > 205 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.43 (s, 18H), 1.44 (s, 9H), 2.83 (m, 2H), 3.35 (s, 26H), 7.32 (s, 2H), 7.34 (d, 2H, *J* = 8 Hz), 7.71 (d, 2H, *J* = 8 Hz), 8,28 (t, 1H, *J* = 5.8 Hz); MS (ESI^+) *m/z:* 755.44 $[M+H]^+$, 777.44 $[M+Na]^+$.

1,4,7-Tris(carbo*tert*butoxymethyl)-10-[(3-sulfamoylphenyl)aminocarboxymethyl] −1,4,7, 10-tetraazacyclododecane (9c): Yield 65%; mp: 152–154 °C; ^{1}H RMN (400 MHz, [D_6]DMSO) δ = 1.38 (s, 18H), 1.44 (s, 9H), 3.35 (s, 24H), 7,50 (m, 6H), 10.41 (s, 1H); MS (ESI^+) *m/z*: 727.87 $[M+H]^+$, 749.42 $[M+Na]^+$.

1,4,8-Tris(carbo*tert*-butoxymethyl)-11-[(4-sulfamoylphenylmethyl)-aminocarboxy methyl]-1,4,8,11-tetraazacyclotetradecane (10a): Yield 59%; mp: 42–44 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.38 (s, 9H), 1.388 (s, 9H), 1.39 (s, 9H), 2.57 (t, 2H, *J* = 7.1 Hz), 2.65 (t, 2H, *J* = 7.1 Hz), 3.01 (m, 2H), 3.15 (m, 4H), 3.18 (m, 2H), 3.35 (s, 16H), 4.36 (d, 2H, *J* = 5.94 Hz), 7.29 (s, 2H), 7.40 (d, 2H, *J* = 8 Hz), 7.39 (d, 2H, *J* = 8 Hz), 8.57 (t, 1H, *J* = 5.8 Hz); MS (ESI^-/ESI^+) *m/z*:767.5 $[M-H]^-$, 803.5 $[M+Cl]^-$, 769.5 $[M+H]^+$.

1,4,8-Tris(carbo*tert*-butoxymethyl)-11-[(4-sulfamoylphenylethyl)-aminocarboxy methyl]-1, 4,8,11-tetraazacyclotetradecane (10b): Yield 58%; mp: 69–71 °C; ^{1}H RMN (400 MHz, [D_6]DMSO) δ = 1.39 (m, 31H), 2.59 (s, 8H), 2.80 (m, 2H), 2.90 (m, 2H), 3.18 (m, 4H), 3.35 (s, 12H), 7.29 (s, 2H), 7.38 (d, 2H, *J* = 8.0 Hz), 7.72 (d, 2H, *J* = 8.0 Hz), 8.03 (t, 1H, *J* = 5.8 Hz); MS (ESI^-/ESI^+) *m/z*: 781.5 $[M-H]^-$, 817.4 $[M+Cl]^-$, 783.5 $[M+H]^+$.

1,4,8-Tris(carbo*tert*-butoxymethyl)-11-[(3-sulfamoylphenyl)aminocarboxy methyl]-1,4,8,11-tetraazacyclotetradecane (10c): Yield 65%; mp: 61–63 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 1.31 (s, 9H), 1.37 (s, 9H), 1.39 (s, 9H), 1.51–1.55 (m, 2H), 2.57–2.61 (m, 2H), 2.60–2.62 (m, 12H), 2.70–2.72 (m, 4H), 3.17 (s, 4H), 3.22 (s, 2H), 3.25 (s, 2H), 7.37 (s, 2H), 7.50–7.52 (m, 2H), 7.63–7.66 (m, 1H), 8.15 (s, 1H), 10.20 (s, 1H); MS (ESI^-/ESI^+) *m/z*: 753.5 $[M-H]^-$, 755.5 $[M+H]^+$.

General procedure to prepare compounds (11) and (12): 250 mg of compound (**9**) or (**10**) was dissolved in a cocktail of trifluoroacetic acid-water-thioanisole 95/2.5/2.5. The mixture was stirred overnight at RT then concentrated under vacuum. The residue was coevaporated several times with Et_2O until precipitation. The solid was filtered and washed several times with DCM and ACN.

1,4,7-Tris(carboxymethyl)-10-[(4-sulfamoylphenylmethyl)aminocarboxymethyl]-1,4,7, 10-tetraazacyclododecane (11a): Yield 89%; mp > 205 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 3.06–3.16 (m, 16H), 3.63 (s, 6H), 3.82 (s, 2H), 4.39 (s, 2H), 7.34 (s, 2H), 7.47 (d, 2H, *J* = 8 Hz), 7.76 (d, 2H, *J* = 8 Hz), 8.92 (s, 1H); ^{13}C NMR (101 MHz, [D_6]DMSO) δ = 41.87, 48.81, 48.89, 50.36, 50.43, 53.19, 54.01, 64.95, 115.66, 118.63, 125.8, 127.8, 142.77, 171.32, 171.34, 171.36; MS (ESI^-/ESI^+) *m/z*: 571.12 $[M-H]^-$, 773.3 $[M+H]^+$, 595.3 $[M+Na]^+$, 611.23 $[M+K]^+$.

1,4,7-Tris(carboxymethyl)-10-[(4-sulfamoylphenylethyl)aminocarboxymethyl]-1,4,7,10-tetraazacyclododecane (11b): Yield 89%; mp: 193–195 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 2.81 (m, 2H), 3.04–3.16 (m, 16H), 3.57 (s, 6H), 3.72 (s, 2H), 3.89 (s, 2H), 7.33 (s, 2H), 7.40 (d, 2H, *J* = 8 Hz), 7.73 (d, 2H, *J* = 8 Hz), 8.54 (s, 1H); ^{13}C NMR (101 MHz, [D_6]DMSO) δ 34.67, 48,67, 50.36, 52.98, 53.88, 54.68, 64.89, 125.66, 129.2, 142.09, 143.34, 158.26, 171.39, 171.53, 173.31; MS (ESI^-/ESI^+) *m/z*: 585.2 $[M-H]^-$, 587.3 $[M+H]^+$, 609.3 $[M+ Na]^+$, 625.3 $[M+K]^+$.

1,4,7-Tris(carboxymethyl)-10-[(3-sulfamoylphenyl)aminocarboxymethyl]-1,4,7, 10-tetraazacyclododecane (11c): Yield 91%; mp: 110–112 °C; ^{1}H NMR (400 MHz, [D_6]DMSO) δ = 3.10–3.16 (m, 16H), 3.73 (s, 6H), 3.81 (s, 2H), 7.41 (s, 2H), 7.52 (m, 2H), 7.81 (m, 1H), 8.07 (m, 1H), 10.53 (s, 1H); ^{13}C NMR (101 MHz, [D_6]DMSO) δ = 49.81, 49.93, 53.78, 53.82, 55.15, 55.23, 64.96, 116.14, 122.46, 125.87, 128.96, 138.83, 144.57, 158.13, 166.92, 167.92, 170.70; MS (ESI^-/ESI^+) *m/z*: 557.2 $[M-H]^-$, 559.3 $[M+H]^+$, 597.2 $[M+K]^+$.

1,4,8-Tris(carboxymethyl)-11-[(4-sulfamoylphenylmethyl)aminocarboxymethyl]-1,4,8, 11-tetraazacyclotetradecane (12a): Yield

92%;mp: 59–61 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.88–2.89 (m, 4H), 3.21–3.22 (m, 8H), 3.6 (s, 8H), 3.81 (s, 4H), 3.89 (s, 4H), 4.41(d, 2H, J = 5.58 Hz), 7.36 (s, 2H, J = 8 Hz), 7.74 (d, 2H, J = 8 Hz), 7.77 (d, 2H, J = 8 Hz), 9.12 (t, 1H, J = 5.8 Hz); ^{13}C NMR (101 MHz, [D$_6$]DMSO) δ = 21.64, 21.7, 41.93, 50.42, 50.66, 51.51, 51.96, 52.2, 52.44, 52.84, 54.03, 64.94, 125.76, 127.7, 142.62, 142.87, 158.27, 166.82, 169.27, 171.01; MS (ESI$^-$/ESI$^+$) m/z: 599.2 [M−H]$^-$, 601.3 [M + H]$^+$, 639.3 [M + K]$^+$.

1,4,8-Tris(carboxymethyl)-11-[(4-sulfamoylphenylethyl)aminocarboxymethyl]-1,4,8, 11-tetraazacyclotetradecane (12b): Yield 95%; mp: 111–113 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 1.73 (m, 4H), 2.82 (m, 4H), 2.88 (m, 4H), 3.0 (m, 8H), 3.29 (m, 2H), 3.36 (td, 2H, J = 7.32 Hz), 3.38 (s, 2H), 3.44 (s, 6H), 7.35 (s, 2H), 7.4 (d, 2H, J = 8.0 Hz), 7.73 (d, 2H, J = 8.0 Hz), 8.36 (t, 1H); ^{13}C NMR (101 MHz, [D$_6$]DMSO) δ = 22.1, 34.67, 50.94, 51.01, 54.34, 54.42, 55.99, 64.89, 125.63, 129.12, 142.05, 143.44, 157.79, 169.73, 169.82, 170.73; MS (ESI$^-$/ESI$^+$) m/z: 613.3 [M- H]$^-$, 615.13 [M + H]$^+$, 653.3 [M + K]$^+$.

1,4,8-Tris(carboxymethyl)-11-[(3-sulfamoylphenyl)aminocarboxymethyl]-1,4,8,11-tetra azacyclotetradecane (12c): Yield 95%; mp: 107–109 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.75 (m, 2H), 2.84 (m, 2H), 2.88 (m, 4H), 2.95 (m, 4H), 3.04 (m, 4H), 3.12–3.16 (m, 4H), 3.38 (s, 2H), 3.41 (s, 2H), 3.43 (s, 2H), 3.45 (s, 2H), 7.43 (s, 2H), 7.51 (m, 2H), 7.78 (m, 1H), 8.25 (s, 1H), 10.36 (s, 1H); ^{13}C NMR (101 MHz, [D$_6$]DMSO) δ = 22.14, 22.45, 50.0, 50.11, 51.08, 51.11, 52.2, 52.27, 55.05, 55.22, 64.97, 116.41, 120.56, 122.28, 129.5, 139.02, 144.65, 157.96, 168.28, 168.10, 171,62; MS (ESI$^-$/ESI$^+$) m/z: 585.3 [M−H]$^-$, 587.3 [M + H]$^+$, 625.2 [M + K]$^+$.

General procedure for the preparation of copper complexes (13) and (14): 1 equiv of compound (**11**) or (**12**) and 1.1 equiv of copper (II) chloride dihydrate were stirred in DMSO at RT. The complexation was monitored by electrospray mass spectrometry until completion of the reaction (2 h). The complex was then precipitated with a mixture of 50–50 DCM-Et$_2$O. The solid was filtered, washed several times with acetone and ACN, and dried under vacuum.

Complex 11a–Cu^{2+} (13a): Yield 78%; mp: 170 °C decomp.; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.54 (m, 24H), 4.47 (s, 2H), 7.07 (s, 2H), 7.29 (m, 2H), 7.67 (m, 2H), 7.97 (s, 1H); MS (ESI$^-$/ESI$^+$) m/z: 632.2 [M−H]$^-$, 634.3 [M + H]$^+$, 656.3 [M + Na]$^+$.

Complex 11b–Cu^{2+} (13b): Yield 71%; mp: 58–60 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.07 (m, 4H), 2.54 (s, 24H), 7.26 (s, 2H), 7.36 (m, 2H), 7.69 (m, 2H), 7.84 (s, 1H); MS (ESI$^-$/ESI$^+$) m/z: 646.3 [M−H]$^-$, 648.3 [M + H]$^+$, 670.3 [M + Na]$^+$, 686.3 [M + K]$^+$.

Complex 11c–Cu^{2+} (13c): Yield 84%; mp: 79–81 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.53 (m, 24H), 7.29 (s, 2H), 7.41 (m, 2H), 7.54 (m, 2H), 7.74 (s, 1H). MS (ESI$^-$/ESI$^+$) m/z: 618.2 [M−H]$^-$, 620.2 [M + H]$^+$, 642.2 [M + Na]$^+$.

Complex 12a–Cu^{2+} (14a): Yield 85%; mp: 72–74 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.53 (m, 30H), 7.34 (s, 2H), 7.44 (m, 2H), 8.0 (m, 2H), 9.21 (s, 1H); MS (ESI$^-$/ESI$^+$) m/z: 660.3 [M−H]$^-$, 662.3 [M + H]$^+$.

Complex 12b–Cu^{2+} (14b): Yield 45%; mp: 56–58 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.54 (m, 32H), 7.33 (s, 2H), 7.76 (m, 2H), 7.86 (m, 2H),9.22 (s, 1H); MS (ESI$^-$/ESI$^+$) m/z: 674.3 [M−H]$^-$, 676.3 [M + H]$^+$.

Complex 12c–Cu^{2+} (14c): Yield 78%; mp: 137–139 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO) δ = 2.53 (m, 28H), 7.53 (s, 2H), 7.71 (m, 2H), 8.28 (m, 1H), 8.77 (m, 1H), 13.5 (s, 1H); MS (ESI$^-$/ESI$^+$) m/z 646.2 [M−H]$^-$, 648.3 [M + H]$^+$, 770.3 [M + Na]$^+$.

CA inhibition assay

An applied photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity.[31] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5) or TRIS (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (to maintain constant ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were made thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at RT 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, as reported earlier,[28–31] and represent the mean from at least three different determinations.

Penetrability through red blood cell membranes

Freshly isolated human red cells (10mL) thoroughly washed several times with TrisHCl buffer (pH 7.40, 5 mM) and centrifuged for 10 min were treated with 25 mL of sulfonamide inhibitor (2 mM). Incubation was at 37 °C with gentle stirring, for periods of 30 min–24 h. After the incubation the RBCs were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer to eliminate all unbound inhibitor. The cells were then lysed in 25 mL of distilled water, centrifuged for eliminating membranes, and other insoluble impurities. The obtained solution was heated at 100 °C for 5 min (to denature CAs) and sulfonamides possibly present have been assayed in each sample enzymatically,[31] as described earlier.[33] The amount of sulfonamide present in the lysate has been evaluated based on hCA II inhibition measured by the CO_2 hydrase stopped-flow method of Khalifah,[31] as described above. Standard inhibition curves have been obtained previously for each sulfonamide–metal complex, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate.[33]

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