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Carbonic anhydrase inhibitors: Copper(II) complexes of polyamino-polycarboxylamido aromatic/heterocyclic sulfonamides are very potent inhibitors of the tumor-associated isoforms IX and XII

Marouan Rami,[a] Jean-Yves Winum,[a,*] Alessio Innocenti,[b] Jean-Louis Montero,[a] Andrea Scozzafava[b] and Claudiu T. Supuran[b,*]

[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*
[b]*Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Room 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy*

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Abstract—Reaction of EDTA/DTPA dianhydride with aromatic/heterocyclic sulfonamides afforded a series of derivatives incorporating polyaminopolycarboxylate tails and benzenesulfonamide or 1,3,4-thiadiazole-2-sulfonamide heads. These compounds have been used as ligands to prepare Cu(II) complexes. Both parent sulfonamides as well as their copper complexes behaved as potent inhibitors of four carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic CA I and II, and transmembrane CA IX and XII. Some Cu(II) complexes showed subnanomolar affinities and some selectivity for the inhibition of the tumor-associated isoforms IX and XII and might be used as PET hypoxia markers of tumors.

Although sulfanilamide **SA** and acetazolamide **AZA** metal complexes were used for the gravimetric determination of silver for a long time,[1] only recently such sulfonamide coordination compounds were investigated in more detail by our and Borras' groups.[2–5] Indeed, many transition metal ion acetazolamide complexes were synthesized by the two groups and their 3D structure determined by X-ray crystallography, allowing a detailed understanding of the multiple possible interactions between this sulfonamide and transition di-/tri-valent cations.[2–5] However, the most interesting property of such derivatives was their unexpectedly strong inhibitory activity against the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[2] Thus, metal complexes of the various clinically used sulfonamide CA inhibitors (CAIs), such as acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, and saccharin **SAC** with different main group and transition metal ions, were shown to act as very powerful inhibitors of these enzymes (more precisely of isoforms CA I and II), being generally 10–100 times more effective as compared to the parent sulfonamides from which they were synthesized.[2–6] This was thereafter explained[6] as being due to a dual inhibition of the enzyme by the metal complexes, by means of both sulfonamide anions (which bind to the catalytic Zn(II) ion within the CA active site) as well as metal cations (which bind to critical histidine residues for the catalytic cycle, such as His64, within the enzyme active site).[6–11] Metal complexes of such ligands as the sulfonamides mentioned above were also shown to be not very stable in aqueous solution,[6] being easily dissociated to metal ions and sulfonamidate anions which both bind to different binding sites within the enzyme cavity, explaining thus the enhanced inhibitory power of the complexes as compared to the parent sulfonamides.[2–7]

Keywords: Carbonic anhydrase; Sulfonamide; Metal complex; Cu(II); Tumor-associated isoform; CA IX; Isozyme selective inhibitor.
* Corresponding authors. Tel.: +39 055 4573005; fax: +39 055 4573385; e-mail addresses: winumj@univ-montp2.fr; claudiu.supuran@unifi.it

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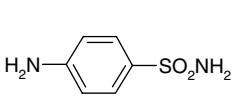

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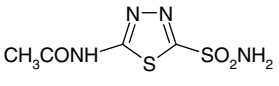

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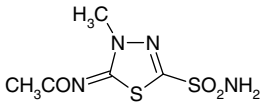
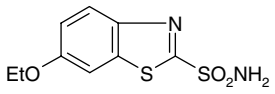
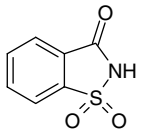


MZA



EZA



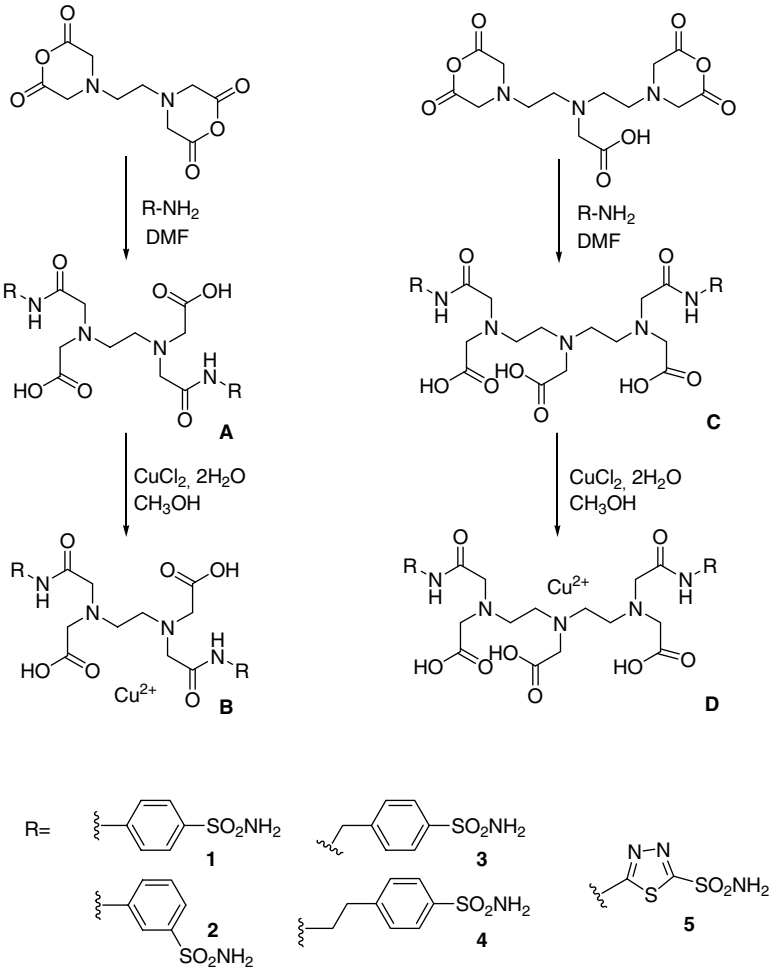
SAC

The strong in vitro enzyme inhibitory properties of such sulfonamide metal complexes[2–6] against CA I and II, as well as their excellent in vivo activity as intraocular pressure (IOP) lowering agents in animal models of glaucoma,[7–9] led to further investigations of such enzyme inhibitors. Indeed, in addition to the metal complexes of the rather simple aromatic/heterocyclic sulfonamides **SA**–**SAC** mentioned above, new ligands were designed incorporating benzenesulfonamide or 1,3,4-thiadiazole-sulfonamide heads as well as polyamino-polycarboxylate moieties present in classical metal-complexing agents such as EDTA, DTPA, IDA (iminodiacetic acid), etc.[7b,9,10] These metal-complexing moieties (EDTA, DTPA, IDA, etc.) were incorporated in the molecules of sulfonamide ligands used to prepare the metal complexes in order to both enhance the stability of the metal complex itself, as well as the CA inhibitory properties of such larger molecules, which presumably may lead to additional favorable interactions when bound within the enzyme cavity, a hypothesis largely confirmed by our studies.[7b,9,10] Some Zn(II) and Cu(II) metal complexes of such sulfonamides incorporating polyamino-polycarboxylated tails have also been reported, which indeed showed very good in vitro CA inhibitory activity against isoforms CA I, II, and IV, and some of them were also effective in vivo IOP lowering agents in hypertensive rabbits as a glaucoma animal model.[7b,9] Srivastava's group[10] proposed then that such 'two-prong' inhibitors may also lead to the design of isozyme selective CAIs, due to the presence of various histidine residues on the surface of different CA isoforms (an observation initially reported by our group)[11] which can interact with the metal ions present in the complex inhibitor. However, this observation has not been confirmed thereafter, since the metal ion of all copper complexes investigated up to now by X-ray crystallography binds only to His64 in CA II and His200 in CA I, irrespective of the length of their spacers separating the sulfonamide head of the IDA–Cu(II) moiety.[10b] In addition, the weak electron density reported as a third binding site for such sulfonamide metal complexes was found at the interface between two protein molecules, toward the amino terminal part of the enzyme, and might be due to the very high concentration of copper complex (1.4–2 mM) present in the crystallographic experiments,[10b] which are not at all comparable with the experiments reported here in which micro-nanomolar concentrations of inhibitor are used (see later in the text).

Sulfonamide metal complexes have been assayed up to now only for the inhibition of three CA isoforms (i.e., CA I, II, and IV)[2–9] of the 16 presently known in mammals.[12] Many of these isoforms were shown to be involved in a host of physiological and pathological processes, such as among others: gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity, and the growth and virulence of various pathogens.[12] Thus, in the search of derivatives showing some specificity for various CA isoforms, and more precisely the tumor-associated ones CA IX and XII,[13] we report here the preparation and inhibition assay of some Cu(II) complexes of aromatic/heterocyclic sulfonamides incorporating EDTA and DTPA tails. In addition, such copper(II) derivatives with potent CA IX/XII inhibitory activity might also be important for developing positron emission tomography (PET) imaging agents for tumor hypoxia. Indeed, recently, Cu(II)-diacetyl-bis(N^4-methylthiosemicarbazone), a compound with no CA inhibitory activity, was shown to be a valuable PET hypoxia marker in some but not all tumor types.[14] As hypoxia is present in many tumors, where it triggers a strong overexpression of CA IX and XII via the HIF-1α transcription factor activation,[15,16] compounds containing Cu(II) and also possessing a high affinity for these CA isozymes might be even more useful than Cu(II)-diacetyl-bis(N^4-methylthiosemicarbazone) as PET imaging agents.

It should be also stressed that the levels of CA IX and XII in most organs are rather low, whereas hypoxia triggers a very strong overexpression of these enzymes,[15] which attain quite high concentrations in such tumors. Being absent (or present in very low concentrations) within the normal tissues, and abundant in tumors, as shown by extensive immunohistological work from Pastorekova's and Harris' groups among others,[15,16] CA IX and XII become very attractive targets for both diagnosis and treatment of hypoxic tumors.[12,13]

Sulfonamides **A1**–**A5** and **C1**–**C5** were prepared as previously described[7b,9] by reacting EDTA dianhydride or DTPA dianhydride with amino-sulfonamides such as sulfanilamide, metanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide, and 5-amino-1,3,4-thiadiazole-2-sulfonamide (Scheme 1). The copper complexes **B1**–**B5** and **D1**–**D5** were then obtained by reacting the corresponding bis-sulfonamides (as carboxylate disodium salts) with Cu(II) chloride (Scheme 1) as reported earlier by this group.[7b,9,17] The ligands were re-



Scheme 1. Preparation of sulfonamides **A1**–**A5** and **C1**–**C5** and their corresponding Cu(II) complexes **B1**–**B5** and **D1**–**D5**.

ported earlier,[9] whereas the Cu(II) complexes are new compounds, except for **B5** and **D5** which were obtained in the previous study.[9] However, all these compounds (ligands **A1**–**A5** and **C1**–**C5** and metal complexes **B1**–**B5** and **D1**–**D5**) were never assayed for the inhibition of the tumor-associated isoforms CA IX and XII. Their testing for the inhibition of CA I, II, and IV has been reported earlier by using an esterase assay,[9] whereas here we used a stopped-flow, CO_2 hydration assay for reinvestigating the inhibition of the physiological reaction with all these derivatives.[18]

Data of Table 1 show that bis-sulfonamides **A1**–**A5** and **C1**–**C5**, as well as their Cu(II) complexes **B1**–**B5** and **D1**–**D5**, generally act as very potent inhibitors of all four CA isozymes investigated here, the cytosolic, ubiquitous human CA I and II,[12] as well as the transmembrane, tumor-associated hCA IX and hCA XII.[13,15] The following structure–activity relationship (SAR) data are evident: (i) against hCA I, the EDTA bis-sulfonamides **A1**–**A5** show effective inhibitory activity, with K_I-s in the range of 8.6–9.4 nM except for **A3** which is a medium potency inhibitor (K_I of 438 nM). The corresponding metal complexes are on the other hand much better inhibitors, with K_I-s in the range of 1.7–7.4 nM. It is rather difficult to rationalize the behavior of the homosulfanilamide derivative **A3** as a weaker inhibitor, but probably this compound has a clash with some amino acid(s) from the hCA I active site, a situation we recently evidenced for inhibitors of both hCA II[19] and hCA I.[20] Work is in progress in our laboratories to better understand the interactions between these inhibitors and various CAs by means of X-ray crystallography. The DTPA bis-sulfonamides **C1**–**C5** were on the other hand medium potency hCA I inhibitors, with K_I-s in the range of 386–578 nM, except for **C4** which is a potent inhibitor (K_I of 9.5 nM). The copper complexes **D1**–**D5** were stronger inhibitors as compared to the corresponding parent bis-sulfonamides, with K_I-s in the range of 6.5–144 nM (Table 1). It is thus clear that the more compact EDTA moiety generally leads to better hCA I inhibiting bis-sulfonamides as compared to the bulkier DTPA moiety, probably due to the restricted hCA I active site as compared to that of isoforms hCA II, IX, and XII. Indeed, some bulky amino acid residues (among others His200 and His67) are found only in isoform I[10,12,19,20]; (ii) against the ubiquitous hCA II, the EDTA-derived bis-sulfonamides **A1**–**A5** generally behaved as very potent inhibitors, with K_I-s in the range of 0.77–11.3 nM. The corresponding copper complexes were also good inhibitors (generally better than the parent sulfonamides, except for **B3** and **B4** which are

Table 1. Inhibition of CA isoforms hCA I, II, IX, and XII with sulfonamides **A1**–**A5**, **C1**–**C5** and their corresponding Cu(II) complexes **B1**–**B5**, **D1**–**D5**, as well as clinically used inhibitors

Inhibitor	K_I[a] (nM)			
	hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
A1	9.0	10.7	7.8	3.2
B1	1.7	7.8	3.7	1.4
A2	9.0	11.3	7.9	4.8
B2	7.4	1.4	3.9	2.0
A3	438	0.89	7.3	3.0
B3	4.3	1.2	6.2	1.3
A4	9.4	0.77	8.2	2.8
B4	4.6	2.6	4.1	1.2
A5	8.6	11.0	8.5	2.1
B5	2.0	5.6	6.4	0.7
C1	386	46	8.4	4.4
D1	111	1.2	8.1	2.1
C2	578	69	9.6	6.8
D2	12.2	1.4	9.2	3.5
C3	575	62	8.7	4.0
D3	144	33	4.5	1.7
C4	9.5	11.4	7.5	3.9
D4	6.5	2.9	5.5	1.0
C5	487	13.4	8.3	2.8
D5	62	4.5	4.8	0.6
SA	25000	240	294	37
AZA	250	10	25	5.7
MZA	50	14	27	3.4
EZA	25	8	34	22
SAC	18540	5950	103	633

[a] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[20]
[b] Human, recombinant isozymes.
[c] Catalytic domain of human cloned isoforms.[13,21]

slightly less effective than **A3** and **A4**, respectively), with K_I-s in the range of 1.2–7.8 nM. The DTPA bis-sulfonamides **C1**–**C5** were on the other hand less effective hCA II inhibitors as compared to the corresponding EDTA derivatives, with K_I-s in the range of 11.4–69 nM. However, all their copper complexes **D1**–**D5** showed enhanced hCA II inhibitory properties, with K_I-s in the range of 1.2–33 nM (Table 1); (iii) both the EDTA- as well as DTPA-based sulfonamides **A1**–**A5** and **C1**–**C5** showed excellent hCA IX inhibitory activity, with inhibition constants in the range of 7.3–9.6 nM, whereas all their corresponding copper complexes **B1**–**B5** and **D1**–**D5** were even better inhibitors, with K_I-s in the range of 3.7–9.2 nM (Table 1). Thus, for hCA IX which has a larger active site as compared to hCA I and II,[21] all these sulfonamide ligands and their Cu(II) complexes showed a very compact behavior of extremely potent CAIs, a rather positive feature for compounds possibly useful to target hypoxic tumors in which CA IX is usually highly overexpressed[13,15,16]; (iv) an even better inhibition profile has been observed for hCA XII[22] with the derivatives reported here. Thus, the ligands **A1**–**A5** and **C1**–**C5** showed inhibition constants in the range of 1.2–6.8 nM, whereas the corresponding copper derivatives **B1**–**B5** and **D1**–**D5** in the range of 0.6–3.5 nM. Again the behavior of these derivatives was very compact, all of them being extremely potent CAIs, as already reported earlier for many sulfonamide complexes investigated by us,[22] such as **AZA**, **MZA**, **EZA**, etc. However, here are the first ever reported subnanomolar hCA XII inhibitors (e.g., **B5** and **D5**). It may be also observed that generally the EDTA derivatives and their copper complexes are slightly better CAIs as compared to the corresponding DTPA derivatives and their metal complexes (Table 1). It should also be noted that the compounds investigated here of type **A1**–**A5**, **C1**–**C5**, **B1**–**B5**, and **D1**–**D5** are generally much better CAIs than the clinically used drugs **AZA**, **MZA**, **EZA**, **SAC**, etc. The observed specificity for inhibiting CA IX/XII over the cytosolic isoforms with the compounds reported here is probably due to the fact that the copper-polyamino-polycarboxylate tails interact in a distinct manner with the active site cavities of these isoforms, as shown already for some structurally related derivatives by Christianson's group[10b]; (v) one of the important issues regarding the design of CAIs regards the selectivity of such compounds for the inhibition of the target isoform over that of the ubiquitous ones CA I and II.[23] As seen from data of Table 1, clinically used sulfonamides such as **AZA**, **MZA**, **EZA** do indiscriminately inhibit both the cytosolic isoforms CA I and II as well as the tumor-associated ones CA IX and XII with rather similar potency. Furthermore, as observed from Table 2, many times the selectivity ratios for inhibiting the tumor-associated isoform CA IX over the cytosolic ubiquitous one CA II are in the range of 0.23–0.51 for these compounds, meaning that all of them are better CA II than CA IX inhibitors. However, data of Table 2 also show that some of the compounds investigated here show good selectivity ratios for the inhibition of the transmembrane over the cytosolic isozymes. The most tumor-associated CA selective such inhibitors are **C1**, **C3**, and the copper complex of this derivative, **D3**, which showed excellent selectivity ratios for the inhibition of CA IX over CA I, CA XII over CA I and II (in the range of 10.4–143.7), and acceptable ones for the inhibition of CA IX over CA II, in the range of 5.4–7.3.

In conclusion, we report here some polyamino-polycarboxylated sulfonamide derivatives incorporating EDTA and DTPA tails as well as benzenesulfonamide-/1,3,4-thiadiazole-5-sulfonamide heads, together

Table 2. Selectivity ratios for the inhibition of the tumor-associated (CA IX and XII) over the cytosolic (CA I and II) isozymes with selected CAIs reported here

Compound	Selectivity ratio			
	hCA I/hCA IX	hCA II/hCA IX	hCA I/hCA XII	hCA II/hCA XII
AZA	10	0.48	43.8	2.10
MZA	1.85	0.51	14.7	4.11
EZA	0.73	0.23	1.13	0.36
B4	1.1	0.63	3.8	2.1
C1	45.9	5.4	87.7	10.4
C3	66.0	7.1	143.7	15.5
D3	32.0	7.3	84.7	19.4
D4	1.1	0.52	6.5	2.9
D5	12.9	0.93	103.3	7.5

with their corresponding Cu(II) complexes. Both parent sulfonamides as well as the copper complexes behaved as potent inhibitors of four CA isoforms, the cytosolic CA I and II, and transmembrane, tumor-associated CA IX and XII. Some Cu(II) complexes showed subnanomolar affinities and some selectivity for the inhibition of the tumor-associated isoforms IX and XII over that of the cytosolic ones, and might be used as PET hypoxia markers of tumors.

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17. Synthesis of derivatives **A** and **C**: 1 equiv of EDTA dianhydride or DTPA dianhydride was mixed with 2 equiv of amino-sulfonamide in DMF. The mixture was stirred at room temperature during the night. Methylene chloride was then poured in the mixture and the precipitate was filtered and washed several times with acetone and acetonitrile. In the case of compound, **A3**, **A5**, **C3**, and **C5** the starting amine (chlorohydrate form) was previously treated with 2 equiv of triethylamine. Synthesis of copper complexes **B** and **D**: 1 equiv of compound **A** or **C** was dissolved in methanol, and 1.1 equiv of $CuCl_2$, $2H_2O$ was added. The mixture was stirred for 15 min and evaporated. The residue is washed several times with acetone and chloroform and filtered. **A1**: yield: 84%; mp 193–195 °C; MS (ESI^+): 600.94 $[M+H]^+$, 623.16 $[M+Na]^+$; ^1H NMR (DMSO-d_6, 400 MHz): δ 3.25 (m, 4H), 3.90 (s, 4H), 3.93 (s, 4H), 7.67 (s, 4H), 8.15 (d, 4H, J = 9.1 Hz), 8.21 (d, 4H, J = 9.1 Hz), 10.86 (s, 2H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 42.13, 55.37, 58.01, 118.66, 126.66, 138.30, 141.63, 170.47, 173.04. **A2**: Yield: 83%; mp 179–181 °C; MS (ESI^+): 601.07 $[M+H]^+$, 623.04 $[M+Na]^+$, 639.03 $[M+K]^+$; ^1H NMR (DMSO-d_6, 400 MHz): δ 3.25 (m, 4H), 3.90 (s, 4H), 3.91 (s, 4H), 7.77 (s, 4H), 7.91 (m, 6H), 8.67 (s, 2H), 10.80 (s, 2H). ^{13}C NMR (DMSO-d_6, 101 MHz): δ 52.17, 55.54, 58.07, 116.18, 122.19, 129.55, 139.12, 144.62, 170.23, 173.12. **A3**: Yield: 84%; mp 110–112 °C; MS (ESI^+): 629.15 $[M+H]^+$, 650.99 $[M+Na]^+$; ^1H NMR (DMSO-d_6, 400 MHz): δ 3.16 (s, 4H), 3.36 (m, 4H), 3.71 (s, 4H), 3.75 (s, 4H), 7.73 (s, 4H), 7.82 (d, 4H, J = 8.33 Hz), 8.16 (d, 4H, J = 8.33 Hz), 9.21 (s, 2H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 45.37, 52.51, 56.12, 57.51, 125.83, 127.56, 142.55, 143.76, 170.87, 172.98. **A4**: Rdt: 81%; mp 175–177 °C; MS (ESI^+): 657.23 $[M+H]^+$, 679.20 $[M+Na]^+$.; ^1H NMR (DMSO-d_6, 400 MHz): δ 3.09 (m, 4H), 3.21 (m, 4H), 3.62 (m, 4H), 3.76 (m, 8H), 7.71(s, 4H), 7.80 (d, 4H, J = 8.2 Hz), 8.14 (d, 4H, J = 8.2 Hz), 8.56 (s, 2H).; ^{13}C NMR (DMSO-d_6, 101 MHz): δ 52.29, 55.45, 57.45, 125.83, 129.24, 143.77, 170.40, 172.57. **A5**: Rdt: 69%; mp 182–184 °C; MS (ESI^+): 639.85 $[M+Na]^+$. ^1H

NMR (DMSO-d_6, 400 MHz): δ 2.09 (s, 2H), 3.47 (m, 4H), 3.53 (s, 4H), 3.72 (s, 4H), 8.32(s, 4H). ^{13}C NMR (DMSO-d_6, 101 MHz): δ 45.56, 45.59, 161.16, 164.24, 172.01, 172.67. **B1**: Yield: 86%; mp 170–172 °C; MS (ESI^+): 661.96 $[M+H]^+$, 698.04 $[M+K]^+$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.06 (s, 2H), 2.06 (m, 4H), 3.41 (s, 4H), 3.90 (s, 4H), 7.57 (s, 4H), 8.44 (m, 8H). **B2**: Yield: 82%; mp 165–167 °C; MS (ESI^+): 661.96 $[M+H]^+$, 700.09 $[M+K]^+$.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.07 (s, 2H), 2,07 (m, 4H), 3.43 (s, 2H), 3.74 (m, 4H), 7.68 (m, 12H). **B3**: Yield: 83%; mp 163–165 °C; MS (ESI^-): 688.02 $[M-H]^-$.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.18 (s, 2H, N*H*), 1.18 (m, 4H), 2.07 (s, 4H), 3.04 (s, 4H), 3.43 (s, 4H), 7.04 (s, 4H), 8.0 (m, 4H). **B4**: Yield: 78%; mp 187–189 °C; MS (ESI^+): 718.16 $[M+H]^+$, 740.06 $[M+Na]^+$.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.07 (s, 2H), 3.38 (m, 20H), 7.27 (s, 4H), 7.9 (m, 8H). **B5**: Yield: 77%; mp 176-178 °C; MS (ESI^+): 677.92 $[M+H]^+$, 699.96 $[M+Na]^+$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.17 (m, 4H), 2.06 (s, 2H), 3.04 (s, 4H), 3.9 (s, 4H), 8.35 (s, 4H). **C1**: Yield: 89%; mp 145–147 °C; MS (ESI^+): 702.18 $[M+H]^+$, 724.22 $[M+Na]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.22 (m, 4H), 3.37 (m, 4H), 3.65 (s, 4H), 3.76 (s, 4H), 3.83 (s, 2H), 7.73 (s, 4H), 7.82 (d, 4H, J = 7.9Hz), 8.16 (d, 4H, J = 7.9 Hz), 8.68 (s, 2H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 49.64, 49.67, 51.69, 54.64, 56.77, 128.81, 125.37, 141.56, 143.31, 168.27, 169.88, 172.37.**C2**: Yield: 91%; mp 142–144 °C; MS (ESI^+): 702.12 $[M+H]^+$, 724.15 $[M+Na]^+$.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.30 (m, 4H), 3.47 (m, 4H), 3.88 (s, 4H), 3.91 (s, 4H), 3,97 (s, 2H), 7.87 (s, 4H), 7.88 (m, 6H), 8.71 (s, 2H), 10.77 (s, 2H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 50.37, 52.48, 55.31, 55.51, 58.11, 116.44, 120.51, 122.52, 129.41, 139.15, 144.54, 168.93, 169.88, 173.27. **C3**: Yield: 89%; mp 149–151 °C; MS (ESI^-): 728.11 $[M-H]^-$.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.89 (m, 4H), 3.01 (m, 4H), 3.32 (s, 4H), 3.34 (s, 4H), 3.42 (s, 2H), 4.33 (m, 4H), 7.32 (s, 4H), 7.42 (d, 4H, J = 8.1Hz), 7.74 (d, 4H, J = 8.1 Hz), 8.82 (s, 2H); ^{13}C NMR(DMSO-d_6, 101 MHz): δ 41.57, 45.29, 50.37, 52.19, 55.21, 55.45, 125.61, 127.46, 142.41, 143.65, 168.96, 170.62, 172.82. **C4**: Yield: 86%; mp 127–129°C; MS (ESI^+): 758.18 $[M+H]^+$, 780.16 $[M+Na]^+$, 796.28 $[M+K]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.81 (m, 8H), 2.93 (m, 8H), 3.23 (s, 4H), 3.33 (s, 4H), 3,38 (s, 2H), 7.31 (s, 4H), 7.38 (d, 4H, J = 8.1 Hz), 7.72 (d, 4H, J = 8.1 Hz), 8.20 (s, 2H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 35.85, 50.46, 52.41, 52.49, 55.22, 55.29, 58.14, 118.93, 126.57, 138.31, 141.67, 160.01, 170.09, 173.14. **C5**: Yield: 75%; mp 147–149 °C; MS (ESI^-): 716.04 $[M-H]^-$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.09 (s, 2H), 3.04 (m, 4H), 3.53 (s, 4H), 3.71 (s, 4H), 3.81 (s, 2H), 8.32 (s, 4H); ^{13}C NMR (DMSO-d_6, 101 MHz): δ 45.36, 45.4, 51.55, 55.31, 55.391, 161.48, 164.19, 171.51, 172.52, 172.97.

D1: Yield: 85%; mp 165–167 °C; MS (ESI^+): 819.16 $[M+H_2O+K]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.08 (s, 2H), 3.42 (m, 18H), 7.28 (s, 4H), 7.81 (m, 4H). **D2**: Yield: 84%; mp 174–176 °C; MS (ESI^+): 763.09 $[M+H]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.76 (s, 2H), 3.44 (m, 18H), 7.61 (s, 4H), 7.95 (m, 4H). **D3**: Yield: 81%; mp 171–173 °C; MS (ESI^+): 791.12 $[M+H]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.18 (s, 4H), 2.07 (s, 2H), 3.42 (m), 7.35 (s, 4H), 7.85 (m, 4H). **D4**: Yield: 71%; mp 160-162 °C; MS (ESI^+): 819.11 $[M+H]^+$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.07 (2H). 3.38 (m, 24H), 7.24 (s, 4H), 7.77 (m, 8H). **D5**: Yield: 82%; mp 180–182 °C; MS (ESI^+): 778.98 $[M+H]^+$, 800.95 $[M+Na]^+$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.07 (s, 2H), 3.06 (s, 4H), 3.16 (m,4H), 3.57 (m, 8H), 3.82 (s, 2H), 8.35 (s, 4H). The Cu(II) complexes **B** and **D** afforded elemental analysis (C, Cu, H, and N) within ±0.4% of the theoretical, calculated values for the proposed formulas (data not shown).

18. Khalifah, R. G.. *J. Biol. Chem.* **1971**, *246*, 2561; An applied photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[9] and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were: 9.2 nM for hCA I, 7.6 nM for hCA II, 12 nM for hCA IX, and 13 nM for hCA XII.[7–9]
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