Bio-organometallic Chemistry

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[(Cp-R)M(CO)$_3$] (M = Re or ^{99m}Tc) Arylsulfonamide, Arylsulfamide, and Arylsulfamate Conjugates for Selective Targeting of Human Carbonic Anhydrase IX**

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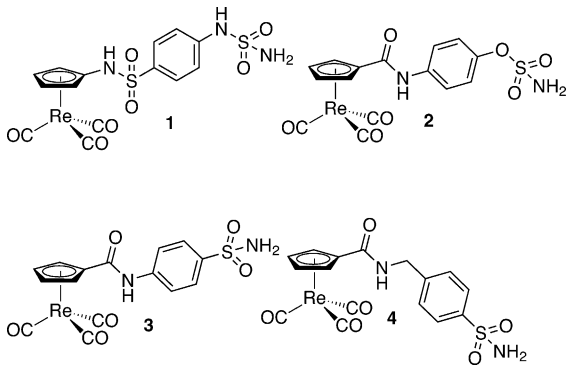
Diagnosis and treatment of diseases requires molecules designed for targeting specific receptors. Since nature has developed a realm of functionally and structurally similar receptors, selectivity for a specific target is a key criterion for inhibitors.[1] Inhibitors are typically organic but Meggers et al. showed that high selectivity does not only depend on intermolecular interactions but also on a directed 3D arrangement of different functionalities as exemplified with organometallic protein kinase inhibitors.[2–4] Inert complexes offer the opportunity to populate biologically relevant space. Thus, bio-organometallic complexes are versatile chemical probes[2,5–8] as pioneered by for example, Jaouen and coworkers who developed ferrocene-based, selective estrogen receptor inhibitors.[9–11] Replacing a phenyl ring in for example, tamoxifen by [(Cp-R)Re(CO)$_3$] (CP = cyclopentadienyl) resulted in retention of high affinity for the estrogen receptor.[12] These organometallic compounds have therapeutic potential but their use for in vivo diagnosis is limited since radionuclides such as ^{18}F for PET (positron emission tomography) cannot be introduced without alteration of chemical authenticity.

Given that Cp-based complexes can replace phenyl rings without affecting the bioactivity, identical compounds for combined therapy and noninvasive diagnosis are desirable for theragnostics.[13,14] Rhenium and technetium belong to the same triad. Whereas Re-based compounds can be used for therapy, their ^{99m}Tc homologous serve as imaging agents in single photon emission computed tomography (SPECT).[15–17] We recently introduced a strategy for the aqueous syntheses of [(Cp-R)^{99m}Tc(CO)$_3$] ("R" = target-specific moiety). In this report, we present Re bio-organometallic carbonic anhydrase inhibitors (CAI) with nanomolar affinities for specific CA subtypes. The ^{99m}Tc complexes have been prepared and complement the Re congeners as identical, diagnostic agents.

Carbonic anhydrases (CAs) are Zn enzymes catalyzing the formation of carbonic acid from CO_2 and water.[18] In mammals, 16 different membrane-bound, cytosolic or mitochondrial isozymes are known.[18,19] CAs are attractive from a pharmaceutical point of view because of hypoxia-induced overexpression of hCA IX and hCA XII (hCA = human carbonic anhydrase) in many malignancies, including cancer.[18,20–23] Therefore, CAs are targets for cancer diagnosis and therapy. However, the large number of isozymes and diffuse localization impede selective accumulation of inhibitors.[19] Several fluorescent sulfonamide- or sulfamate-based CAIs, radio-iodinated monoclonal antibodies[24–27] but only one ^{99m}Tc-labeled inhibitor have been reported.[28]

Following the concept of extended 3D space population, we synthesized four new arylsulfonamide, -sulfamide, and -sulfamate based CAIs with the [(Cp-R)M(CO)$_3$]-motif (M = Re or ^{99m}Tc) and evaluated their affinity to CA isoforms (Scheme 1). Compounds **2** and **3** were structurally characterized (see the Supporting Information).

Binding studies of complexes **1**–**4** with 12 CA isozymes showed inhibition constants in the low nanomolar range for some of the isoforms (Table 1). Such low K_i values are uncommonly rare for bio-organometallic compounds.[30] The hCA II, VI, VII, IX, XII, and XIII are considered as sulfonamide- and sulfamate-avid isoforms[18] which is consis-



Scheme 1. Structures of cyclopentadienyl-based CAIs.

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Table 1: Inhibition data of complexes **1**–**4** against selected CA isozymes in comparison with acetazolamide (AZA).

Compound	K_i [nM][a]				
	1	**2**	**3**	**4**	AZA
CAI	39	2570	4590	2775	250
CAII	15.1	27.4	35.5	25.3	12
CAIV	467	32.9	41.1	33.8	74
CAVA	481	113	124	109	63
CAVB	305	105	104	102	54
CAVI	497	10.6	22.1	14.5	11
CAVII	360	7.6	21.3	10.1	2.5
CAIX	43	3.7	5.2	7	25
CAXII	6.7	4.5	6.9	4.4	5.7
CAXIII	482	62.1	78.5	56.8	17
CAXIV	20.1	4.1	7.9	5.4	41
CAXV	313	28.4	36.8	12.5	72

[a] The data was determined by the CA-catalyzed hydration of CO_2.

tent with the observed values for inhibitors **1**–**4**. Our K_i values for the various isoforms compare well to those of the acetazolamide (AZA) standard and are for some isoforms even better. In addition, distinct selectivity for hCA IX, XII, and XIV over the other isoforms was observed (Figure 1). The K_i ratios (hCA II/hCA IX) of 4.0 (for **4**) or 7.0, respectively

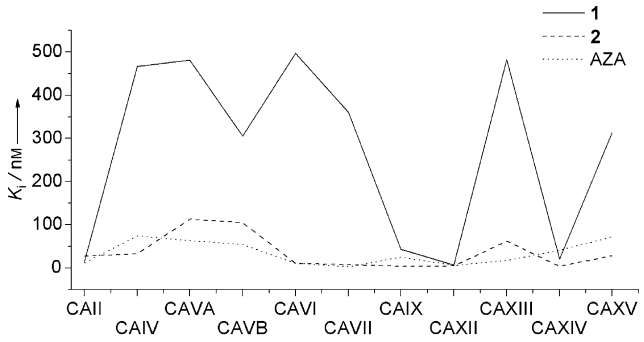

Figure 1. Graphical representation of K_i values of compounds **1** and **2** for selected isozymes of hCAs.

(for **2** and **3**), indicate a clear preference for hCA IX, overexpressed in certain tumors, over the physiologically dominant hCA II. Compound **1** showed the most pronounced selectivity pattern (solid line in Figure 1) and strongly favors hCA II, IX, and XIV. Compounds **3** and **4** behave similarly to **2**. Data are shown in the Supporting Information. In contrast, K_i values of the AZA standard do not show such a distinct preference pattern for any of the isoforms. It has only a slightly higher affinity for hCA II over hCA IX (hCA II/hCA IX = 0.5).

K_i values for **1**–**4** are, thus, in the same range as found for organic inhibitors[18] but much better than those reported for the aforementioned organometallic CA inhibitors,[28,29] indicating smaller and more compact [(Cp-R)M(CO)$_3$] (M = Re or ^{99m}Tc) to be favored over bulky complexes.

CAs are amongst the most effective catalysts in biological systems ($k_{cat}/K_M > 10^8\,M^{-1}$).[19] Due to increased carbonic acid formation, the high activity of hypoxia-induced, overexpressed CAs leads to a remarkable extracellular pH drop in tumor tissue (to around 6 as compared to 7.4 in normal tissues).[18,28,31,32] Consequently, strong CA inhibition will reduce acidification. To confirm this hypothesis, hCA IX-induced extracellular acidification was quantified before and after exposure to the inhibitors **3** and **4**. The three different human carcinoma cell models cervical adenocarcinoma HeLa, breast adenocarcinoma MDA MB 231 and colon adenocarcinoma HT29 were studied. This panel of cell lines was assessed by western blot for the levels of hCA IX expression under normoxic and hypoxic conditions (Figure 2). The HeLa and HT29 carcinoma cells showed

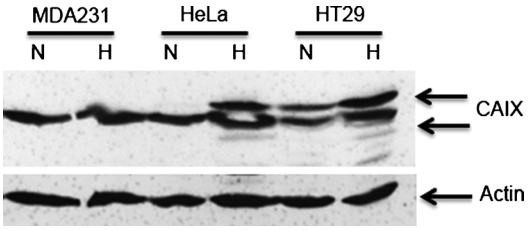

Figure 2. Western blot analysis of hCA IX expression in normoxic N (21% O_2, 24 h) and hypoxic H (1% O_2, 24 h) MDA MB 231, HeLa, and HT29 carcinoma cells using a polyclonal hCA IX antibody. Actin was used as loading control.

elevated levels of hCA IX under hypoxic conditions while MDA MB 231 did not. Only HT29 cells expressed hCA IX under normoxic conditions, albeit at a lower amount than under reduced oxygen levels. This basal expression in HT29 cells is not surprising, and might be caused by the cluster-shaped growth of these cells.

To minimize potential cytotoxicity, acidification experiments were performed at a 0.5 mM of the Re complexes **3** and **4** (Figure 3). Hypoxic incubation of HeLa and HT29 cells led

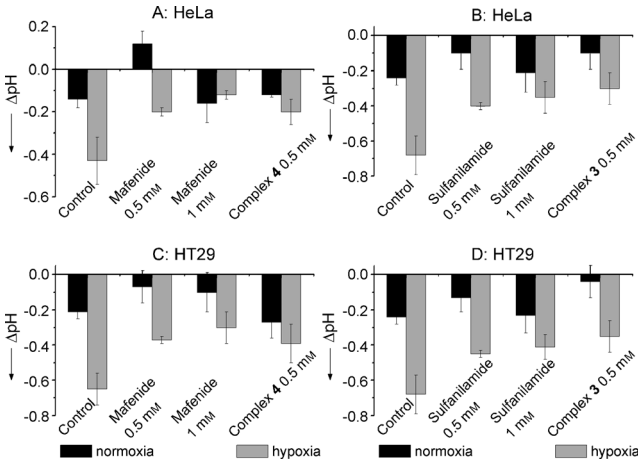

Figure 3. Effect of the reference sulfonamides and compounds **3** and **4** on the reduction of extracellular acidification in HeLa and HT29 cell lines. Cells were incubated for 24 h. The data show the mean ± standard deviation of 4 replicates and are plotted as difference between pH_e values [ΔpH = pH(after incubation) − pH(before incubation)] measured in the absence (control) or in the presence of the sulfonamides.

to the expected extracellular acidification by around 0.5 to 0.7 pH units, caused by overexpression of hCA IX. Under normoxic conditions, a pH drop of only about 0.1 for HeLa (no overexpression) and 0.2 for HT29 (slight expression) was found.

The presence of the reference inhibitors mafenide and sulfanilamide under hypoxia caused a significant reduction of acidification; more pronounced for HeLa than for HT29 cells (Figure 3A compare with 3C). Complexes **3** and **4** showed a distinct reduction of extracellular acidification, in hypoxia comparable or even better than the standards, confirming CA IX inhibition. The normoxic extracellular pH was not significantly affected by incubation with either compound, indicating specific interaction with hCA IX. In agreement with the lack of hCA IX expression, MDA MB 231 cells did not show a pH difference for cells grown under normoxia or hypoxia.

To assess the binding mode of the Re-based CAIs with proteins, crystals of hCA II were grown with the hanging drop vapor diffusion method. The crystals were soaked with the respective inhibitor and gave diffraction data until 1.2 Å resolution. The electron density of the complete complex **4** could be traced in the binding pocket of hCA II. An electron density map is shown in Figure 4A. The binding mode is reminiscent to the arrangement as seen in other hCA II structures.[33,34] Avvaru et al. defined two binding pockets I and II for elongate hCA II inhibitors.[35] Complex **4** binds in the hydrophobic pocket II (see the Supporting Information). The deprotonated nitrogen of the arylsulfonamide terminus coordinates to the Zn atom in the active site (Figure 4B). The major conformation of the $[(Cp)Re(CO)_3]$ unit, including the amide linker, does not interact with either the protein or water molecules. However, there is a hydrophobic interaction between the $[(Cp)Re(CO)_3]$ moiety and the hydrophobic part of Phe131, Leu198, and Pro202. Further details are given in the Supporting Information.

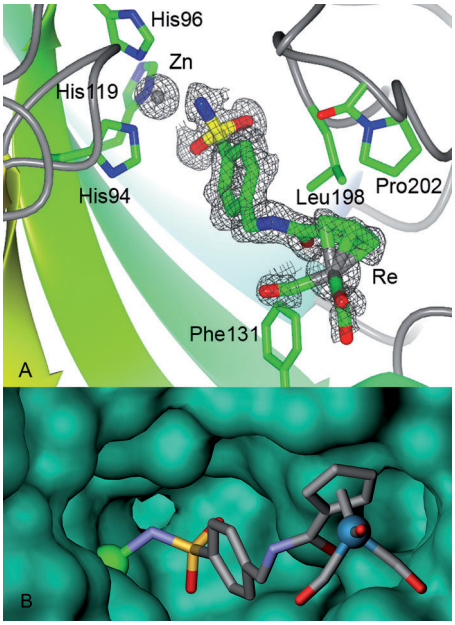

Figure 4. A) Crystal structure of **4** bound to hCA II (only the major conformation of **4** is shown). A) Electron density map of **4**, superimposed with the stick model of the inhibitor bound to the active site of hCA II. Hydrophobic interactions can be observed between the $[(Cp)Re(CO)_3]$ moiety and Phe131, Leu198, and Pro202. B) Detail of the binding cavity of hCA II with **4**. The Zn atom is shown as a green sphere.

Compounds **1**–**4** are strong organometallic CAIs. They follow the concept of increased and matched space occupation in the binding pocket.[3] To extend the concept from therapy to combined therapy and imaging, the preparation of the ^{99m}Tc homologues from water is mandatory.[6] $[(Cp\text{-}R)^{99m}Tc(CO)_3]$ type complexes are accessible from $(HCp\text{-}R)_2$ dimers and $[^{99m}TcO_4]^-$.[36–38] For extending this approach, we prepared the ^{99m}Tc analogs of **3** and **4**, namely. Their authenticity was assessed by comparing the HPLC retention times of **3**/**6** and **4**/**8** (Figure 5). The "one pot" reactions of $[^{99m}TcO_4]^-$ with ligands **5** and **7** (millimolar concentrations)

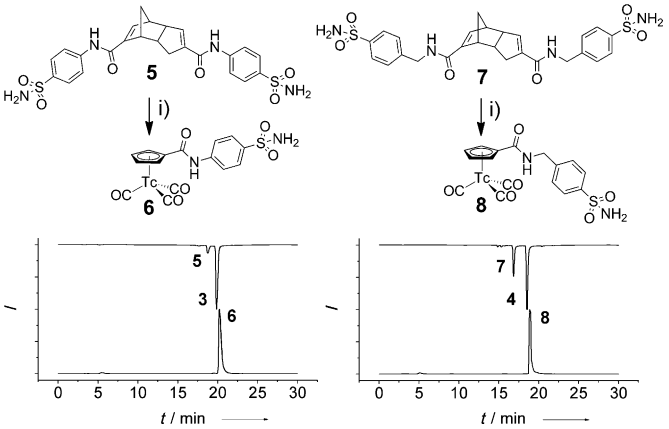

Figure 5. i) Isolink Kit, TcO_4^-. Superposition of HPLC traces. Top: UV trace showing ligands **5** and **7** and Re complexes **3** and **4**, respectively. Bottom: γ trace of the ^{99m}Tc complexes **6** and **8**.

gave nearly quantitative yields at 90 °C after 60–90 min. Based on these ^{99m}Tc syntheses, macroscopic amounts of the Re complexes **1**–**4** for therapy can be combined with homologous ^{99m}Tc compounds for accompanying diagnosis. It should be emphasized that the phenyl–$[(Cp\text{-}R)M(CO)_3]$ analogy is not limited to CAIs but can be extended to any pharmacophore with a corresponding structural subunit.

In conclusion, four new piano-stool-type Re complexes with pendent arylsulfonamides, -sulfamides, and -sulfamates were synthesized. They inhibit CAs with nanomolar affinities. Particularly strong inhibition was found for pharmaceutically relevant isozymes hCA IX and hCA XII. Compound **1** showed superior CA isoform selectivity than the AZA standard. Enhanced receptor selectivity because of better space occupation is a preponderance of bio-organometallic compounds. These CAIs are potential therapeutics for CA overexpression related diseases. For imaging purposes, the ^{99m}Tc homologues of **3** and **4** were synthesized. Inert bio-organometallic ^{99m}Tc and Re compounds behave biologically identical, thus, these potent CAIs can be applied in combination with each other, macroscopic amounts of Re for

therapy and microscopic amounts of the ^{99m}Tc analogs for diagnosis, according to the theragnostic concept.

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