New sulfonamides containing organometallic-acylhydrazones: synthesis, characterisation and biological evaluation as inhibitors of human carbonic anhydrases

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ABSTRACT
A series of organometallic acylhydrazones was prepared, incorporating Re(CO)₃, Mn(CO)₃ and ferrocenyl moieties, which were subsequently reacted with amino-sulfonamides in order to obtain carbonic anhydrase (CA, EC 4.2.1.1) inhibitors possessing organometallic moieties in their molecules. The new derivatives were investigated as inhibitors of four human (h) CA isoforms with pharmaceutical applications, such as the cytotoxic hCA I, II and VII and the mitochondrial hCA VA. An interesting inhibitory profile against these isoforms was obtained, with some of these metal complexes acting as subnanomolar or low nanomolar inhibitors. They were also thoroughly characterised from the chemical point of view, making them of interest for further developments in the field of metal complexes of sulfonamides with CA inhibitory action.

1. Introduction
Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) are clinically used for several decades as diuretics, antiglaucoma agents, anti-obesity drugs, and more recently, a number of studies showed that CA inhibition has profound antitumor effects by inhibition of hypoxia-inducible isoforms CA IX and XII, overexpressed in many hypoxic tumors. Several proof-of-concept studies demonstrated the involvement of some CA isoforms in neuropathic pain and arthritis, with inhibitors of the sulfonamide/coumarin types demonstrating significant effects in vivo, in animal models of these diseases. This is obviously due to the fact that at least 15 different α-class CA isoforms are present in humans, and many of them are drug targets for the treatment or prevention of this large variety of pathologies. Thus, the field of drug design, synthesis and in vivo investigations of various types of CAIs is a highly dynamic one, with a large number of interesting new chemotypes acting on these widespread enzymes constantly emerging. Among the clinically used sulfonamide CAIs are acetazolamide (AAZ), methazolamide (MZA), ethoxzolamide (EZA), brinzolamide (BRZ) and dorzolamide (DRZ) – (Figure 1). Saccharin (SAC) is a sweetener widely used in beverages and food.

Coordination compounds of sulfonamides with CA inhibitory properties in which the sulfonamides act as ligands to various transition or main group metal ions, leading to sulfonamide metal complexes were also investigated for their interactions with these enzymes. Originally investigated for obtaining transition metal complexes of acetazolamide AAZ, methazolamide MZA, and ethoxzolamide EZA (the main sulfonamide, clinically used drugs belonging to this class of pharmacological agents), this approach was subsequently extended to a large set of primary and secondary aromatic/heterocyclic sulfonamides, also including the clinical drugs saccharin (SAC), brinzolamide (BRZ) and dorzolamide (DRZ). Other sulfonamides possessing a diverse scaffold but effective CA inhibitory properties were also included in such studies together with metal ions which may add a supplementary pharmacological activity, such as Pt(II), Pd(II) and Ru(II) for the antitumor effects, Zn(II) for the anticancer action, Al(III) for antacid properties, Co(II), Ag(I) and Cu(II) for antifungal activity. Imaging tumors overexpressing some CA isoforms (e.g. CA IX and XII) with sulfonamide complexes incorporating isotopes of metal ions which emit positrons (for PET imaging), such as Ga(III), In(III) or Cu(II) were also investigated, allowing interesting developments in the field. On the other hand, the organometallic complexes also incorporating sulfonamide CAIs as ligands were less investigated, although some rhenium(I) and ruthenium(II) derivatives were recently reported.

Here we explored the possibility to prepare organometallic-acylhydrazone incorporating Re(CO)₃, Mn(CO)₃ and ferrocenyi moieties, which were reacted with amino-sulfonamide in order to obtain CAIs possessing organometallic moieties in their molecules.

2. Experimental
2.1. Materials
All manipulations were conducted under an N₂ atmosphere using Schlenk techniques. The compounds (η5-C₅H₄CHO)Re(CO)₃, (η5-C₅H₄CHO)Mn(CO)₃, 2 or 4-(hydrazincarbonyl)benzenesulfonamide and 4-(3-hydrayzyl-3-oxopropy1)amino)benzenesulfonamide were prepared according to published procedures. Ferrocene carboxaldehyde (98%), sulfanilamide (99%), 4-sulfamoylbenzoic acid (97%), methazolamide (MZA), metha-zolamide (MZA), ethoxzolamide (EZA), brinzolamide (BRZ) and dorzolamide (DRZ) – (Figure 1)1-3. Saccharin (SAC) is a sweetener widely used in beverages and food.
methyly-2-(aminosulfonyl)benzoate (98%) and CF$_3$COOH (99%) were obtained from Sigma-Aldrich and used without additional purification. Solvents such as CH$_2$Cl$_2$, hexane, acetone, EtOH, DMSO, and THF were obtained commercially and purified using standard methods. Infrared spectra were recorded in solid state (KBr pellet) on a Jasco FT-IR 4600 spectrophotometer. $^1$H NMR spectra were measured on a Bruker spectrometer model ASCEND TM 400 MHz. All NMR spectra are reported in parts per million (ppm), $\delta$ relative to tetramethylsilane (Me$_4$Si), with the residual solvent proton resonances used as internal standards. Coupling constants ($J$) are reported in Hertz (Hz), and integrations are reported as number of protons. The following abbreviations were used to describe the peak patterns: $s$ = singlet, $d$ = doublet, $t$ = triplet, and $m$ = multiplet. Mass spectra were obtained on a Shimadzu model QP5050A GC-MS at the Laboratorio de Servicios Analíticos, Pontificia Universidad Católica de Valparaíso. Elemental analyses were measured on a Perkin Elmer CHN 2400."
2.2.5. \([(\eta^5-C_5H_5)CH=CH=CH=CH=CH]=N-NH(C(O)=C_5H_4-4-SO_2NH_2)]Mn(CO)_3 (2b)

This compound was prepared according to the general procedure described above, using in this case: \((\eta^5-C_5H_5)CH=CH=CH=CH=CH=CH=CH=CH=N\)Mn(CO)_3 (77 mg, 0.33 mmol) and 4-(hydrazinocarbonyl)benzenesulfonamide (72 mg, 0.33 mmol). Yellow solid, yield 83% (118 mg, 0.28 mmol). IR (KBr, cm\(^{-1}\)): 3233–3008 (vNH/Ar=O); 2025 (vMn=CO); 1593 (vC=N); 1567 (vC=O); 1539 (vC=S); 1336 (vC=N). ^1H NMR (DMSO-d_6): \(\delta\) 4.92 (s, 0.5H, C_5H_4); 4.93 (s, 0.5H, C_5H_4); 5.28 (s, 0.5H, C_5H_4); 5.63 (s, 1.5H, C_5H_4); 7.39 (s, 2H, C_5H_4); 7.94 (s, 2H, J = 8.2Hz, Ar–H); 8.04 (d, 2H, J = 8.2Hz, Ar–H); 8.10 (s, 0.25H, CH=N); 8.13 (s, 0.75H, CH=N); 11.90 (s, 0.25H, NH); 11.99 (s, 0.75H, NH). Mass spectrum (m/z): 429 [M^+]\(^+\), 345 [M^+ – C_3O]. Anal. (%) Calc. for C_18H_17N_3O_3SFe: C, 52.57; H, 2.83 and N, 9.78.

2.2.6. \([(\eta^5-C_5H_5)CH=CH=CH=CH=CH=N\)Mn(CO)_3 (2c)

This compound was prepared according to the general procedure described above, using in this case: \((\eta^5-C_5H_5)CH=CH=CH=CH=CH=CH=CH=CH=N\)Mn(CO)_3 (77 mg, 0.33 mmol) and 4-(3-hydrazinyl-3-oxopropyl)amino)benzenesulfonamide (86 mg, 0.33 mmol). Brown solid, yield 77% (121 mg, 0.26 mmol). IR (KBr, cm\(^{-1}\)): 3369–3080 (vNH/N=CH); 2960 (vC=O); 1925 (vMn=CO); 1602 (vC=N); 1327 (vC=O). ^1H NMR (DMSO-d_6): \(\delta\) 2.45 (t, 0.9H, J = 6.8Hz, CH_2CO); 2.81 (t, 1.1H, J = 6.8Hz, CH_2CO); 3.36 (m, 2H, CH_2NH); 5.11 (s, 2H, C_5H_4); 5.50 (s, 1.1H, C_5H_4); 5.54 (s, 0.9H, C_5H_4); 6.44 (m, 1H, NH); 6.64 (m, 2H, CH–H); 6.92 (s, 2H, NH); 7.51 (d, 2H, J = 7.9Hz, Ar–H); 7.62 (s, 0.6H, CH=N); 7.83 (s, 0.4H, CH = N); 11.34 (s, 0.6H, NH); 11.36 (s, 0.4H, NH). Mass spectrum (m/z): 472 [M^+]\(^+\), 388 [M^+ – C_3O]. Anal. (%) Calc. for C_18H_17N_3O_3SFe: C, 52.87; H, 4.88 and N, 12.33; found: C, 52.86; H, 4.89 and N, 12.37.

2.3. CA inhibition studies
An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isoforms for CO_2 hydration reaction. Phenol red (at a concentration of 0.2mM) was 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 ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. 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 5–10s of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionised water and dilutions up to 1mM were done thereafter with the assay buffer. Enzyme and inhibitor solutions were pre-incubated together for 15 min (standard assay at room temperature) prior to assay, in order to allow for the formation of the enzyme-inhibitor complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier. All CAs were recombinant proteins produced as reported earlier by our group.

3. Results and discussion
3.1. Synthesis and characterisation of organometallic-acylhydrazones containing sulfonamide fragments
The preparation of these new family of organometallic-acylhydrazones described in the experimental section involved the synthesis of the appropriate organic acylhydrazide precursor 2-(hydrazinecarbonyl)benzenesulfonamide, 4 (hydrazinecarbonyl)benzenesulfonamide and 4-(3-hydrazinyl-3-oxopropyl)amino)benzenesulfonyl amide, which were prepared according to published procedures. The organometallic-acylhydrazones containing sulfonamide moieties were obtained as described in Scheme 1, following the same procedure reported for some organic analogues, that is, by the condensation reaction of the appropriate acylhydrazide and the corresponding formyl organometallic complex in anhydrous EtOH. All compounds were isolated in good yields (71–83%) as solids, after crystallisation from THF/hexane mixture. These products are air-stable and slightly soluble in most polar organic solvents (e.g. CH_2Cl_2, acetone, CH_3CN).
In all cases, the infrared spectral analysis of these compounds showed the characteristic absorption corresponding stretching vibration of the ν(C = N) bond in the range of 1605–1537 cm⁻¹ in KBr disk. Similar ν(C = N) frequency values have been previously reported for other organometallic-acylhydrazones derived from ferrocenyl21 and cymantrenyl groups22. The absence of the band assigned to the aldehyde carbonyl group of organometallic complexes confirmed the formation of the organometallic Schiff bases. Moreover, all compounds showed the expected absorption bands for the νN–H, νCO, and νSO₂ stretches in the ranges of 3369–3080 cm⁻¹, 1683–1635 cm⁻¹ and 1343–1321 cm⁻¹, respectively. In addition, the spectra for 1c, 2c, and 3c exhibited an absorption band for νCsp³–H at ~2950 cm⁻¹. Furthermore, the IR spectra of cyrhetrenyl (1a–c) and cymantrenyl (2a–c) acylhydrazones revealed the presence of terminal metal carbonyl groups in the region of 2027–1912 cm⁻¹.23–25 A strong molecular ion was shown in the mass spectrum of each organometallic-acylhydrazones, in addition to the detection of notable successive losses of CO ligands for the cyrhetrenyl (1a–c) and cymantrenyl (2a–c) derivatives. The elemental analysis data determined for all compounds are in agreement with their proposed formulas.

For all complexes, the ¹H NMR spectra showed the presence of a sharp singlet in the range of 8.43–7.62 ppm, and it was assigned to the iminic proton. These results are in agreement with the values reported for organometallic Schiff bases.26–28. Moreover, ¹H NMR spectra for 1a–c and 2a–c showed sets of resonances in the region of 6.26–4.92 ppm, which are ascribed to the protons of the cyrhetrenyl and cymantrenyl moieties.29 On this regard, the ferrocenyl derivatives 3a–c exhibited resonances around δ 4.69–4.32 due to the non-equivalent alpha and beta protons containing in the substituted Cp ring and a singlet in the region of 4.26–4.18 ppm, which was assigned to the proton resonances of the unsubstituted cyclopentadienyl group. For all compounds, the multiplets observed between 8.06 and 6.63 ppm were assigned to the hydrogen atoms of the CpH₅ ring. As per literature reports, the broad singlet observed at 7.52–6.91 ppm was assigned to the hydrogen nuclei of the SO₂NH₂ group.18,30,31

The presence of the –NH–CO– group registered as a broad singlet in the range of 12.03–11.05 ppm. Similar δ have been reported for other organometallic hydrazones.21,22,32 On this regard, it is an important remark that the chemical shifts of the –NH– resonance showed a dependence on the presence of the organometallic moiety bound to the iminic entity. In fact, the downfield shift observed for the cyrhetrenyl (1a–c) and cymantrenyl (2a–c) derivatives (Δδ~0.5) compared with ferrocenyl analogues (3a–c) can be related to the electron-withdrawing properties of the (°CH₂H₅)M(CO)₃ moieties,23,34 which produce a deshielding of the NH resonance, thus suggesting that the nature of the organometallic framework modifies the degree of electronic delocalization on the –C(H)=N–NH– unit. We have found similar results for ferrocenyl and cyrhetrenyl hydrazones19,35 and 1,3,4-thiadiazoles36. In the case of the acylhydrazones 1c, 2c and 3c, additional signals were observed at 6.44 ppm, 3.36 ppm and 2.80–2.42 ppm, respectively. These resonances were attributed to the presence of the –CH₂CH₂NH– unit.17

It is important to mention that acylhydrazones can form four isomers owing to the presence of amide (–CONH–) and azomethine groups (–CH=N–) in their structure.38,39. The geometrical isomers (E/Z) originate from the azomethine group and rotamer (cis/trans) formation is due to the restricted rotation of the amide group (see Figure 2). However, the survey of the literature reveals that the N-acyl hydrazones synthesised from aromatic carbaldheyde are essentially planar and exist completely in the form of geometric (E)-configuration about the C=N bond due to steric hindrance on the imine bond. Therefore, we discarded the formation of Z, cis and Z, trans isomers.

Based on ¹H NMR data, the organometallic-acylhydrazones (1a–c), (2a–c) and (3a–c) reported in this work exist as a mixture of cis/trans isomers in DMSO-d₆ solutions. On this regard, ¹H NMR spectra for all compounds show resonances for the CO–NH and CH=N group protons are present in double sets and the signal intensity ratio is ~0.6 cis: 0.4 trans. This splitting signals pattern was also observed for cyclopentadienyl (C₅H₅)₂ and ethyl (–CH₂CH₂–) protons. The cis isomer predominates because of a hindered rotation around the CO–NH bond.19 Similar results have been reported for organic-acylhydrazones derived from other benzzenesulfonamide derivatives.

In order to confirm the existence of cis/trans-amide rotamers, we carried out ¹H NMR spectra of 1a measurements at several temperatures (Figure 3). Variable temperature (VT) ¹H NMR spectra showed that increasing the temperature within the range of 296–346 K led to coalescence of the –CONH– resonance of cis- and trans-amide rotamers. Similar results have been published previously by Barhoumi-Slimi and co-workers in organic-acylhydrazones derived from cinnamaldehyde and β-unsaturated aldehydes.42

Unfortunately, the low solubility of all the compounds in deuterated solvents precluded us to measure their ¹³C NMR and bidimensional NMR spectra to complement their characterisation.

3.2. CA inhibition studies

The sulfonamide containing organometallic-acylhydrazones have been evaluated in vitro as CAIs. Three cystolic human (h) isoforms (hCA I, II, and VII) and one mitochondrial (hCA VA) have been included for the screening, and the results revealed interesting selectivity profiles for some of the evaluated compounds. Inhibition data obtained with the standard stopped-flow CO₂
hydrase assay are compared to those of the standard sulfonamide inhibitor acetazolamide (AAZ)\(^43\)–\(^47\) (Table 1). Structure-activity relationships have been delineated dividing the compounds into three classes, depending on the organic portion responsible for the CA inhibition.

i. The 2-(hydrazinecarbonyl)benzenesulfonamide derivatives (series a) revealed to be ineffective in inhibiting hCA I (Ki > 10,000 nM) and showed poor activity against hCA II, with Ki values in the micromolar range (2595.6 < Ki < 10,000 nM), regardless of the different metal substituted Cp ring contained in them. On the other hand, the insertion of the sulfonamide group in ortho position of the aromatic ring turned out to be favourable for the inhibition of hCA VA and VII, which were strongly inhibited by all the compounds investigated here, with Ki values in the nanomolar range for the cychetrenyl 1a, cymantrenyl 2a and ferrocenyl 3a derivatives. Therefore, compounds 1a, 2a, and 3a were potent and selective hCA VA and VII inhibitors (over the cytosolic enzymes hCA I and II).

ii. The insertion of 4-(hydrazinecarbonyl)benzenesulfonamide fragment on the molecular scaffolds (series b) led to a dramatic enhancement of inhibition potency against hCA II, particularly for compounds 1b and 2b, which were 8-fold more potent than the ferrocenyl derivative 3b. A slight enhancement of potency against hCA I was also observed for the

Figure 2. General structure of possible Z/E geometrical isomers and cis/trans amide rotamers of organometallic-acylhydrazones.

Figure 3. NH region of \(^1\)H NMR spectra of complex 1a in DMSO-d\(_6\), registered at variable temperatures.
Table 1. Inhibition of human (h) CA isoforms hCA I, II, VA, and VII with acetazolamide (AAZ) and the organometallic derivatives reported here, by a stopped-flow, CO₂ hydrase assay.²,³

|           | hCA I | hCA II | hCA VA | hCA VII |
|-----------|-------|--------|--------|---------|
| Cmp       | Kᵢ (nM) |       |        |         |
| 1a        | >10000 | 2595.6 | 58.7   | 69.7    |
| 1b        | 595.4  | 0.48   | 69.5   | 74.2    |
| 1c        | 7736.8 | 6.59   | 35.7   | 9.6     |
| 2a        | >10,000| >10,000| 73.8   | 76.1    |
| 2b        | 373.6  | 0.47   | 30.3   | 77.7    |
| 2c        | 136.6  | 1.54   | 32.9   | 9.2     |
| 3a        | >10000 | 6624.2 | 66.3   | 77.8    |
| 3b        | 2817.3 | 4.02   | 34.2   | 27.2    |
| 3c        | 8090.9 | 18.2   | 21.8   | 9.5     |
| AAZ       | 250    | 12.1   | 63     | 2.5     |

Mean from 3 different assays. Errors were in the range of ±5–10% of the reported values (data not shown).

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