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The effect of specific modifications of the amine ligands on the solubility, stability, CO release to myoglobin and whole blood, cell toxicity and haemolytic index of $[Mo(CO)_4(NR_3)_2]$ complexes

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ABSTRACT

A series of *cis*-$[Mo(CO)_4(amine)_2]$ complexes (NR_3 = morpholine **1**; 4-Me–piperazine, **2**; $H_2NCH_2CH_2NH_2$, **3**; $H_2NCH_2CH_2$–morpholine (**4**) $R_2NCH_2CH_2$–piperazine–4-Me (R = H, **5**); R = Me, **6**); $Me_2NCH_2CH_2NMe_2$, **7**) was prepared in good yields, in a one-step microwave-assisted synthesis. The X-ray diffraction structures of the complexes **4**, **5** and **6** are reported. The stability of the complexes **1**–**7** in aqueous, aerobic media was studied by UV–Vis spectrophotometry, RP-HPLC and gas chromatography at several pH values. Stability beyond 1 h requires bidentate ligands with at least one tertiary amine ligand and increases in the order **4** < **5** < **6**. Stability is approximately the same at pH 7.5 and pH 3.9 for **5** and **6** in solutions acidified with HCl. Acidification with CF_3COOH induces decomposition. The order of CO transfer rate to deoxy-Mb and haemoglobin in whole blood is **1** > **2** > **3** > **4** > **5** > **6** >> **7**, but it is much faster to whole blood. The haemolytic index of some compounds increases in a similar order: **1** < **2** < **5** < **6**; with the exception of **1**, the complexes are not toxic to RAW264.7 cells up to a concentration of 100 μM.

1. Introduction

Carbon Monoxide (CO) is an essential signalling molecule produced in the body by the action of the enzyme Heme Oxygenase (HO), which catabolises toxic heme released by dying cells. It was the second reported member of the group of simple mediators now called gaseotransmitters: NO, CO and H_2S [1]. Its endogenous production plays a very important role in innate immune defence and cytoprotection [2,3]. Accordingly, exposure of cells, tissues or rodents to exogenous CO produces important therapeutic results in a very broad range of medical indications, some of which do not have alternative treatments [4–7]. Although this finding already led to human clinical trials [8], the application of CO gas as a therapy, namely in humans, suffers from a number of shortcomings, as discussed elsewhere [9]. This fact was recognised soon after the first report on the therapeutic action of CO gas and led to the search for pro-drugs capable of delivering CO to biological systems and animals in safer and more easily controllable manners, compatible with its ambulatory use. Such pro-drugs were named CORMs or CO-Releasing Molecules, by Motterlini and Mann who reported the first examples based on transition metal carbonyls [10]. These CORMs, the dimethyl sulfoxide (DMSO) soluble $[Ru(CO)_3Cl_2]_2$ (CORM-2) and the water soluble $[RuCl(CO)_3$(glycinate)] (CORM-3) [11,12], have demonstrated their therapeutic properties in a broad variety of *in vivo* preclinical, animal model studies namely in cardiovascular disease, rejection of transplanted organs, acute lung, kidney and liver failure, cancer, sepsis and shock as recently reviewed [13,14]. They also recapitulated the action of CO gas in the same models with the important advantage of generating much lower values of carboxyhaemoglobin (COHb) in systemic circulation. Our early studies used air-sensitive zerovalent Mo carbonyls, like *fac*-$[Mo(CO)_3$(histidinate)]Na (ALF186) and $[Mo(CO)_5Br][NEt_4]$ (ALF062) in several animal models of disease with evident and extensive beneficial effects although producing higher COHb values than the CORM-2 and CORM-3 congeners [15–17]. These four molecules remained the only transition metal CORMs reported to be used *in vivo* until 2012. In spite of their encouraging results in proof-of-concept studies they lack a series of physical and chemical properties that are necessary to make their

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pharmaceutical use acceptable. As discussed elsewhere [9], the rational implementation of these favourable properties on a transition metal carbonyl complex implies building a suitable coordination sphere around the metal ion of choice. This construction comprises two layers: an inner coordination sphere that determines the chemistry around the central metal, and an outer coordination sphere that creates an acceptable ADME profile, and targets the diseased tissues. This methodology has led us to prepare $[Mo(CO)_3(CNCMe_2COOH)_3]$, an advanced drug-like CORM for the treatment of acute liver failure [18]. Other authors have also produced CORMs with many drug-like properties and rather sophisticated coordination spheres, like the vitamin B12 conjugate $[Re(CO)_2Br_2(H_2O)$(cyanocobalamine)] [19], and the esterase or phosphatase triggered CORMs $[Fe(CO)_3(\eta^4\text{-}C_6H_7OX)]$ (X = C(O)Me; P(O)$(OMe)_2$) [20–22] but they were never reported to be tested *in vivo*. Since CORMs are pro-drugs, the double sphere construction process must have in mind that at some point, ideally at the site of disease, CORMs must breakdown to release CO. In other words, there must be a balance between stability in circulation and instability in the tissues. The latter can only be tuned through *in vivo* studies since it depends strongly on targeting and pharmacokinetics, that is, the outer sphere. On the contrary, making complexes that are stable in circulation depends on the inner coordination sphere, relies on *in vitro* studies, and is the starting point in CORM design. Accordingly, CORM candidates must be stable to air, water and plasma. Moreover, they must present sufficient solubility in water and be devoid of haemolytic and cytotoxic properties. However, the still limited knowledge of the chemistry of metal carbonyl complexes in aqueous, aerobic and protein containing media makes the assembling of inner coordination spheres with these characteristics a rather difficult task with many unpredictable hurdles and assumptions. In fact, besides the kinetically inert d^6 tricarbonyl complexes of Tc(I) and Re(I), which have been intensively studied for radiopharmaceutical use [23], very few metal carbonyl complexes have been studied in aqueous biological media.

Penta-, tetra- and tricarbonyl complexes of group 6 metals with ligands like pyrrolidine, piperazine and morpholine [24], cyclohexylamine, pyridine and ethylenediamine [25], and ethylenediamine derivatives [26], have been known for a long time. Although piperazine, morpholine and polyamine ligands offer excellent possibilities for enhancing the water solubility of their hydrophobic metal carbonyl complexes, due to either protonation or formation of hydrogen bonds, such complexes were never tested in aqueous aerobic conditions. There is only one example of a study of potential CORMs of this type, namely that of the amino-acid complexes of the type $[Mo(CO)_5(H_2NCRR'CO_2R'')]$ [27]. The traditional synthesis of these amine complexes was done by refluxing solutions of $Mo(CO)_6$ with the corresponding ligand in benzene or toluene. Depending on the reaction time the corresponding mono- and disubstituted complex was obtained usually with contamination by each other. Access to these species became much easier when Hogarth and Ardon introduced a modified conventional microwave oven to synthesise molybdenum carbonyl complexes. This technique allows the synthesis of molybdenum tetracarbonyl complexes $[Mo(CO)_4L_2]$ with amine ligands in a much shorter time and with higher purity than the traditional reflux method [28–30]. The overheating of high boiling point solvents in open microwave systems (e.g. diglyme from 162 to 175 °C) leads directly to reaction acceleration and therefore less time for air decomposition of unstable intermediates [31].

Extending our previous work on zerovalent molybdenum carbonyl based CORMs, we now present our findings on a series of $[Mo(CO)_4(NR_3)_2]$ complexes bearing saturated amine ligands, and prepared by microwave-assisted synthesis. In this study we investigate the impact that specific ligand modifications made to the inner sphere of $[Mo(CO)_4(NR_3)_2]$ complexes have on the stability, solubility, CO release, haemolytic and cytotoxicity profiles of the respective complexes. The objective is to identify trends and guidelines for the future design of CORM scaffolds based on amine ligands for therapeutic use *in vivo*.

2. Results and discussion

2.1. Synthesis and characterisation

A series of *cis*-$[Mo(CO)_4$(amine)$_2]$ complexes (**1**–**7**; Scheme 1) was prepared in a one-step synthesis directly from $[Mo(CO)_6]$ and several saturated mono- and bidentate amines, by using microwave-assisted heating with an open reflux system. All complexes were obtained in less than 16 min reaction time, using a power of 700 W, as yellow crystalline solids in high purity and yields between 30 and 82%. Compounds **1** [24], **3** [25,30] and **7** [32] are well described and characterised in the literature. However, their preparation by this less conventional method led to excellent results, reducing both time consumption and the formation of secondary products, when compared to traditional reflux techniques.

The compounds **1** and **2** bearing the monodentate secondary amines morpholine and 1-methylpiperazine, respectively, proved slightly sensitive to air and moisture changing colour from yellow to brownish when kept in closed vials under air. On the other hand, compounds **3**–**7** are air stable for periods of days and can be manipulated on the bench without any inert atmosphere precautions. This first observation indicates that the use of bidentate saturated amines (N–N) improves the stability of $[Mo(CO)_4(N–N)]$ complexes to atmospheric conditions when compared to their monodentate analogues $Mo(CO)_4(NR_3)_2$.

The FT-IR spectra of **1**–**7** show a pattern of four distinct CO stretching frequencies in the 2021–1775 cm^{-1} range consistent with a *cis*-tetracarbonyl geometry. The N–H stretching frequencies were also observed for complexes 1–5, as sharp bands, between 3240 and 3370 cm^{-1}. The lowering of the N–H stretching frequency, when compared to free 1-methylpiperazine (3266 cm^{-1}) indicates that in compound **2** the ligand binds via the secondary amine, as drawn in Scheme 1, and not via the tertiary amine group.

Single crystals suitable for X-ray diffraction studies were obtained for complexes **4**, **5** and **6** by layering the reaction solution (diglyme/THF) with diethyl ether or *n*-hexane. The structures are represented in Fig. 1. Each complex has the metallic centre coordinated to four carbonyl groups and to a saturated bidentate nitrogen amine ligand via the amine of the side chain and through the tertiary amine of the morpholine or the piperazine ring, giving a slightly distorted octahedral geometry. Comparable crystal structures of the type $[Mo(CO)_4(NR_3)_2]$ where (NR_3) is a non-cyclam amine are limited to a few structures, namely $[Mo(CO)_4$(piperidine)$_2]$ (pip) [33], $[Mo(CO)_4$(diamino-monosaccharide)] (saccharide) [34], $[Mo(CO)_4((PhCH_2)HN(CH_2)_2NMe_2)]$ (Bn-en-Me_2) [26] and $[Mo(CO)_4$(TMEDA)] (TMEDA) (**7**) [32]. Selected bond lengths and bite angles are listed and compared in Table 1.

The Mo–N bond lengths in complexes **4** and **5** represent two extreme situations. On the one hand the Mo–N1 distances of 2.282(3) and 2.291(1) Å, respectively, are quite short for a Mo–N bond but comparable with the other Mo-(primary amine) bonds of the saccharide complex (2.284(3) Å). On the other hand, Mo–N2 distances are extraordinarily long. The values of 2.410(3) and 2.404(1) Å are the longest observed for Mo^0–amine bonds. This discrepancy between the two Mo–N distances was also observed in the corresponding octahedral $[Ni(N–N)_2X_2]$ complexes and interpreted as a consequence of steric constraints introduced by the