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**Vasorelaxing Effects and Inhibition of Nitric Oxide in Macrophages
by New Iron-Containing Carbon Monoxide-Releasing Molecules
(CO-RMs)**

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List of non-standard abbreviations: CO-RMs, carbon monoxide-releasing molecules; deoxy-Mb, deoxymyoglobin; DMSO, dimethylsulfoxide; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MbCO, carbonmonoxy myoglobin; iCO-RMs, inactive CO-RMs.

Abstract

Carbon monoxide-releasing molecules (CO-RMs) are a class of organometallo carbonyl complexes capable of delivering controlled quantities of CO gas to cells and tissues thus exerting a broad spectrum of pharmacological effects. Here we report on the chemical synthesis, CO releasing properties, cytotoxicity profile and pharmacological activities of four novel structurally-related iron-allyl carbonyls. The major difference among the new CO-RMs tested was that three compounds (CORM-307, CORM-308 and CORM-314) were soluble in dimethylsulfoxide (DMSO), whereas a fourth one (CORM-319) was rendered water-soluble by reacting the iron-carbonyl with hydrogen tetrafluoroborate. We found that despite the fact all compounds liberated CO, CO-RMs soluble in DMSO caused a more pronounced toxic effect both in vascular and inflammatory cells as well as in isolated vessels. More specifically, iron carbonyls soluble in DMSO released CO with a fast kinetic and displayed a marked cytotoxic effect in smooth muscle cells and RAW 247.6 macrophages despite exerting a rapid and pronounced vasorelaxation *ex-vivo*. In contrast, CORM-319 that is soluble in water and liberated CO with a slower rate, preserved smooth muscle cell viability, relaxed aortic tissue and exerted a significant anti-inflammatory effect in macrophages challenged with endotoxin. These data suggest that iron carbonyls can be used as scaffolds for the design and synthesis of pharmacologically active CO-RMs and indicate that increasing water solubility and controlling the rate of CO release are important parameters for limiting their potential toxic effects.

Key words: carbon monoxide, vasorelaxation, inflammation, carbon monoxide-releasing molecules (CO-RMs), macrophages.

1. Introduction

Carbon monoxide (CO) produced in mammals by constitutive (HO-2) and inducible (HO-1) heme oxygenase enzymes is a ubiquitous signaling molecule playing a central role in the restoration of cellular homeostasis during vascular, inflammatory and metabolic disorders [1]. Endogenous CO modulates blood vessel tone [2], differentially affects the proliferation of various cells [3,4], mitigates inflammation [2] and exerts beneficial effects against a variety of pathophysiological processes that alter the function of organs and tissues [2]. The multifactorial activities of this gas dovetail with the innate cytoprotective actions of HO-1, a redox-sensitive inducible protein that responds and adapts to conditions of intense oxidative and nitrosative stress [2,5,6]. The essential contribution of endogenous CO in the regulation of cellular processes is best typified in HO-1 deficient organisms as both mice and humans lacking this enzyme die prematurely due to a high susceptibility to oxidative stress, impaired vascular function, hematologic derangements and the ensuing multiorgan failure [7,8]. From a pharmacological viewpoint, compelling results demonstrating the feasibility of mimicking the protective action of the HO-1/CO pathway have been obtained by administering CO gas *in vivo* [2]. Indeed, brief exposure of mice and rats to CO gas at doses that do not compromise oxygen transport to tissues results in remarkable beneficial effects against graft rejection and vascular injury [9], pulmonary hypertension [10], ischemia-reperfusion damage [11] and a wide array of disorders characterized by a strong inflammatory component [2]. Notably, the identification and development of CO-releasing molecules (CO-RMs), a class of compounds that liberate CO, clearly opened a concrete opportunity to exploit this small molecular gas for pharmacological applications [12].

The discovery that transition metal carbonyls function as CO-RMs in a number of *in vitro*, *ex vivo* and *in vivo* models confirmed the notion that delivery of CO is attainable by storing this gas in a stable chemical form (carbonyl complex), which can then supply CO to cells or tissues in a more controllable fashion [13]. Carbonyl complexes containing manganese (CORM-1) and ruthenium (CORM-2) were the first compounds identified for this purpose and, despite their limited solubility in water (i.e. CORM-1 and CORM-2 are fully soluble in dimethylsulfoxide), they showed promising CO-dependent pharmacological effects including vessel relaxation *in vitro* and inhibition of acute hypertension *in vivo* [13]. Our group then developed the first prototypic water-soluble CO-releasing metal carbonyl (tricarbonylchloro(glycinato)ruthenium(II) or CORM-3) which proved to be very stable in aqueous solutions but rapidly liberated CO in contact with biological components or in the presence of a prototypic acceptor such as myoglobin [14]. To date CORM-3 is the best characterized and most widely used metal carbonyl as we and others demonstrated the potential of using this compound therapeutically for the treatment of disorders such as cardiac and renal ischemic injury [14,15], lethal sepsis [16], post-operative ileus [17], rheumatoid arthritis [18] hemorrhagic stroke [19], platelet aggregation, vascular thrombosis and vascular inflammation [20-22]. In recent years, different classes of CO-RMs containing a variety of transition metals such as rhenium, iron, manganese, cobalt and tungsten have been synthesized [23-28]. Although these compounds possess the ability to release CO in solution either spontaneously, by photodissociation or enzymatic reactions, a comparative analysis of their bioactive and pharmacological effects in relation to their solubility properties and cytotoxic profile has not been carried out. Our group has recently focused on the synthesis of CO-RMs containing either iron or manganese based on the rationale that these two metals, unlike the ruthenium found in CORM-2

and CORM-3, are naturally present in biological systems and could therefore render CO-releasing compounds more biologically compatible [29-32]. Here we studied the effects of newly synthesized CO-RMs containing an iron-allyl moiety by specifically analyzing the CO releasing properties in relation to their biochemical, biological and pharmacological parameters including solubility, cytotoxicity profile as well as vasodilatory and anti-inflammatory activities *in vitro*.

2. Materials and Methods

2.1. Synthesis of CORM-307 ($[Fe(CO)_3I(\eta^3-C_3H_5)]$)

This compound was prepared using a method previously described [33]. Briefly, a mixture of allyl iodide (6.65 g) and $Fe(CO)_5$ (6.0 g) was stirred for 40 h at 45°C in an air atmosphere. The mixture was then evaporated to dryness, and the residue extracted with hexane. The hexane was cooled to -78°C and the precipitate collected. This procedure was repeated until washings were colorless, yielding a red-brown crystalline solid (6.2 g, 60% yield).

2.2. Synthesis of CORM-308 ($[Fe(CO)_3Br(\eta^3-C_3H_5)]$)

This compound was prepared using a method previously described [34]. Briefly, a mixture of 1-bromoprop-2-ene (3.03 g) and $Fe_2(CO)_9$ (9.1 g) in hexane (20 ml) was stirred for 2 h at 40 °C in an air atmosphere. After filtration, the dark red solution was then concentrated and placed in the freezer. The yellow-brown solid was collected. The procedure was repeated with the mother liquor to provide a second crop of product. The crystalline product was then re-crystallized from pentane to afford a yellow-brown product (2.4 g, 37 % yield).

2.3. Synthesis of CORM-314 ($[Fe(CO)_3Cl(\eta^3-C_3H_5)]$)

This compound was prepared using a method previously described [34]. Briefly, a mixture of allyl chloride (1.91 g) and $Fe_2(CO)_9$ (9.1g) in hexane (20 ml) was stirred for 2 h at 40°C in an air atmosphere. After filtration, the dark red solution was then concentrated, then exposed to air for a few minutes and placed in the freezer. The yellow solid was collected. The procedure was

repeated with the mother liquor to provide a second crop of product. The crystalline product was then re-crystallized from pentane to afford a yellow product (1.1 g, 20 % yield).

2.4. Synthesis of CORM-319 ($[Fe(CO)_4(\eta^3-C_3H_4Me)]BF_4$)

This compound was prepared using a method previously described [35,36]. Briefly, in a Schlenk tube, $Fe_2(CO)_9$ (0.91 g) was placed with a stirrer bar and a dropping funnel attached. The flask was filled with a CO atmosphere, before a solution of 3-buten-2-ol (0.18 g) in diethylether (25 ml) was added dropwise with stirring. The solution was allowed to stir overnight. The next morning the yellow solution was syringed into another Schlenk tube to remove it from precipitate. In a conical flask a solution of HBf_4 (48% aqueous solution, 0.90 g) in acetic anhydride (7 ml) was prepared at 0 °C. This solution was then added to the reaction mixture resulting in an immediate precipitation of a cream solid, which was collected by filtration and dried under high vacuum (0.45 g, 58 % yield).

2.5. Reagents and preparation of solutions

Stock solutions of CORM-307, 308 and 314 (in DMSO) as well as CORM-319 (in distilled water) were prepared freshly on the day of the experiments and used within 1 min of preparation. Solutions of the inactive forms of each compound (iCO-RMs), which do not release CO and were used as negative controls, were also prepared. For this purpose, CORM-319 was depleted of CO by adding the compound to the cell culture medium (see below) and leaving it for at least 24 h at room temperature in a 5% CO_2 humidified atmosphere. The additional inactive CO-RMs were prepared in DMSO and left for at least 48 h to decompose. The iCO-RMs solutions were finally bubbled with a stream of nitrogen to remove the residual CO present in the solution.

Lipopolysaccharide (LPS - *E. Coli* serotype 026:B6) was obtained from Sigma. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

2.6. Cell culture

Rat aortic smooth muscle cells (A7r5) and murine RAW264.7 monocyte macrophages were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence as previously described [13,37].

2.7. Determination of CO release using the myoglobin assay

The release of CO from iron-containing CO-RMs was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) as previously reported [13,14,38-40]. A small aliquot of a stock CO-RM solution (to give a final concentration of CO-RM: 10, 20 and 40 μM) was added to 1 ml deoxy-Mb solution (final concentration of Mb: 53 μM) in phosphate buffer and changes in the Mb spectra were recorded over time. The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient = 15.4 M⁻¹ cm⁻¹) and plotted over time.

2.8. Detection of CO release from CO-RMs using an amperometric CO sensor

The spontaneous release of CO from the selected CO-RMs was measured using a CO-sensitive electrode (World Precision Instrument, Stevenage, Herts, UK). Technical details of this electrode have been reported previously by our group [41,42]. The electrode was immersed into distilled

water containing 0.1 M phosphate buffer (pH=7.4) and equilibrated for 30 min prior to addition of CO-RMs (100 μ M) and changes in current were continuously recorded over time. The experiments were conducted at 37 °C and the solutions were maintained at the desired temperature using a Grant W6 thermostat (Cambridge).

2.9. Cell viability assays

Cell viability was determined in macrophages and aortic smooth muscle cells using an Alamar Blue assay kit and LDH kit and carried out according to the manufacturer's instructions (Serotec, UK) as previously reported by us [43]. The alamar blue assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised (blue) form to a reduced (red) form. The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control. Lactate dehydrogenase (LDH) released in the medium was also measured and used as an index of cellular damage. Since the data on LDH release were very similar to the ones obtained with the Alamar Blue assay, only results based on the second method are presented.

2.10. Nitrite assay

Macrophages were exposed for 24 hr to LPS (1 μ g/ml) in the presence or absence of CO-RMs (10, 50 and 100 μ M) and nitrite levels were determined at the end of the incubation. Experiments were also conducted in the presence of negative controls (iCO-RMs) to verify whether the effects observed were only due to CO liberated by CO-RMs or caused by other parts of the molecules. After incubation, nitrite levels were determined using the Griess method as previously described

[37,44]. This measurement is an indication of NO production following stimulation of inducible nitric oxide synthase (iNOS). Briefly, the medium from treated cells cultured in 24 well plates was removed and placed into a 96 well plate (50 μ l per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0 μ M to 300 μ M in cell culture medium).

2.11. Aortic ring preparation

Transverse ring sections were prepared from thoracic aortas of male adult Sprague Dawley rats (350 g) as previously described [45,46]. After removal of superficial fat and connective tissue, the aorta was cut into rings and mounted in organ baths containing normal Krebs-Henseleit solution with the following composition (mM): (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 22, glucose 11, K^+ EDTA 0.03, CaCl_2 2.5). The bath solution was maintained at 37 $^\circ\text{C}$ and bubbled continuously with a mixture of 95% O_2 and 5% CO_2 . Aortic rings were equilibrated for 30 min at a resting tension of 2 g and pre-contracted with KCl (100 mM) before specific experimental protocols were initiated. Responses were recorded isometrically via a force- transducer connected to a Power Lab recording system (AD Instruments, UK). The extent of vasorelaxation over time elicited by a single addition of CO-RMs (100 μ M) was assessed in aortic rings pre-contracted with phenylephrine (1 μ M) and compared with the effect produced by the inactive forms (iCO-RMs). Vasorelaxation was expressed as a reduction (in percentage) of the initial contraction produced by phenylephrine.

2.12. Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with Bonferroni test.

Differences were considered to be significant at $P < 0.05$.

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3. Results

3.1. Detection of CO release from iron-containing CO-RMs

The chemical structures of CORM-307, CORM-308, CORM-314 and CORM-319 are represented in Figure 1A. While CORM-307, 308 and 314 represent examples of an isostructural series of iron carbonyl complexes of the general formula $[\text{Fe}(\text{allyl})\text{X}(\text{CO})_3]$ with the halide ligand $\text{X} = \text{Cl}, \text{Br}, \text{or I}$ and a facial arrangement of the three CO ligands, CORM-319 is distinct from these by the presence of a fourth carbonyl ligand instead of the halide, which leads to a trans OC-Fe-CO orientation of two of the carbonyl groups. The halide-containing compounds are soluble in DMSO, while CORM-319 is soluble in water. We tested the ability of these metal carbonyl complexes to release CO by measuring the conversion of deoxy-Mb to MbCO over time [13,14,38,41]. We report in Figure 1B the maximal MbCO value reached for each CO-RM at three different concentrations. As shown all CO-RMs (10, 20 and 40 μM) liberated CO in a concentration-dependent manner. The amount of MbCO formed suggests that approximately one mole of CO is released per mole of compound at all concentrations used.

3.2. Time-course of CO release and vascular activities of iron-containing CO-RMs

The conversion of deoxy-Mb to MbCO can be followed over time and the amount of MbCO quantified by measuring the changes in the absorption spectra of this protein. As shown in Figure 2A, addition of 40 μM CORM-307 to a Mb solution rapidly changes the deoxy-Mb spectrum into spectra typical of MbCO. Mb appears to be fully saturated with CO within 5 min as all the spectra recorded at subsequent times overlap to this initial spectrum. The quantification of MbCO over time is reported in Figure 2B and shows that the inactive compound (iCORM-307) released

significantly much less CO compared to the active one. The vasoactive properties of CORM-307 were evaluated using the isolated aortic ring preparation and the data are shown in Figure 2C. Addition of 100 μ M CORM-307 produced a profound vasorelaxation over time and a similar result was obtained with the inactive compound (iCORM-307). The vasorelaxation mediated by both CORM-307 and iCORM-307 was gradual and progressive reaching a maximum at 60 min after addition of the compounds to the organ bath. These data suggest that the vasodilatation mediated by CORM-307 cannot be ascribed solely to the liberated CO as the CO-depleted compound itself can partially contribute to the observed effect. It is important to note that CORM-307 caused a sustained and progressive vasodilatation over time with no re-contraction of the vessel after the treatment. This suggests that ensuing tissue damage may have occurred and we examined this possibility by assessing the viability of rat smooth muscle cells incubated for 24 h with increasing concentrations of CORM-307. As shown in Figure 2D, concentrations of CORM-307 as low as 10 μ M caused a complete loss in cell viability. The inactive compound, although less toxic, also significantly decreased cell viability (44%) at 100 μ M. Although with some quantitative differences (see Figures 3 and 4), similar profiles on the rate of CO release, vasodilatation and viability of smooth muscle cells were obtained with CORM-308 (Figures 3A, 3B, 3C and 3D) and CORM-314 (Figures 4A, 4B, 4C and 4D). However, iCORM-308 and iCORM-314 exerted less vasodilatation compared to their active counterparts. In general, the iron-carbonyl complexes soluble in DMSO (CORM-307, COMR-308 and CORM-314) are good and fast releasers but possess a certain degree of toxicity which can be revealed both in vascular tissue and smooth muscle cells in culture. Interestingly, a rather different profile was observed with CORM-319, a water-soluble iron-containing CORM. As shown in Figures 5A and 5B, CORM-319 released CO to myoglobin with a slower rate compared to the other CO-RMs. The

experiment using the CO electrode reveals that CORM-319 releases some CO spontaneously in buffer solution (see *inset* in Figure 5B). The CO spontaneously released with this method is hard to quantify but from the curve fitted to the spectral MbCO data we calculate that the half-life of CORM-319 is around 18 min. Predictably, the inactive compound (iCORM-319) did not release any detectable CO to Mb (Figure 5B). The water-soluble CORM-319 caused a rapid and concentration-dependent vasorelaxation in pre-contracted isolated aortic rings. In fact, as shown in Figure 5C, the vasodilatation reached a maximum 10 min after addition of CORM-319; this was quantified as $21.4\pm 6.1\%$ at $12.5\ \mu\text{M}$, $39.1\pm 7.7\%$ at $50\ \mu\text{M}$ and $57.1\pm 2.7\%$ at $100\ \mu\text{M}$ CORM-319. Since $100\ \mu\text{M}$ iCORM-319 produced only a slight and significantly less relaxation ($6.8\pm 3.2\%$, $p<0.05$), the data strongly suggest that CO liberated from this iron-carbonyl complex is responsible for the observed effect. In addition, the fact that the vessels promptly re-contracted after the maximal relaxation was achieved is indicative of a much less toxic effect on the vascular tissue compared to the DMSO-soluble compounds tested. This is supported by the data shown in Figure 5D showing that rat smooth muscle cells incubated for 24 h with increasing concentrations of CORM-319 (10 , 50 and $100\ \mu\text{M}$) are significantly more viable than cells treated with CORM-307 (see Figure 2D), CORM-308 (see Figure 3D) or CORM-314 (see Figure 4D).

3.3. *Effect of iron-containing CO-RMs on cell viability and LPS-mediated nitrite production in macrophages*

As shown in Figure 6, iron-containing CO-RMs affected the viability of macrophages followed a profile similar to that obtained with smooth muscle cells, CORM-319 being significantly less toxic than the DMSO-soluble compounds. Specifically, the calculated IC_{50} (the concentration at which 50% of the cells are not viable) was $9.1\ \mu\text{M}$ for CORM-307 (Figure 6A), $11.9\ \mu\text{M}$ for

CORM-308 (Figure 6B), 23.6 μM for CORM-314 (Figure 6C) and 797 μM for CORM-319 (Figure 6D). Therefore, the water-soluble CORM-319 appears to be approximately 40 times less toxic than iron-containing carbonyls that are not soluble in water. Since CORM-319 was the most promising iron-containing compound tested, we investigated its potential anti-inflammatory activity. We have previously reported on the biological properties of CORM-2 and CORM-3, two carbonyl complexes containing ruthenium as transition metal, and their ability to significantly reduce both TNF- α and nitrite production in macrophages stimulated with LPS [37]. As shown in Figure 7, CORM-319 significantly attenuated LPS-induced nitrite production in a concentration-dependent manner. This effect appeared to be dependent on the CO released by the compounds since the inactive form (iCORM-319) did not have any effect on nitrite levels. It is important to note that the concentrations which showed an effective reduction in LPS-mediated nitrite production (10, 50 and 100 μM) did not cause any toxic effect in macrophages (see Figure 6D).

4. Discussion

The emerging evidence that CO serves as signaling and cytoprotective mediator within mammalian organisms prompted scientists to develop pharmacological approaches for the therapeutic use of this gas. Transition metal carbonyls capable of liberating CO in biological systems known as CO-releasing molecules (CO-RMs) represent an alternative to CO gas inhalation as they can be designed to deliver CO in a controlled and physiological fashion [47,48]. The present study reports on a new class of iron carbonyl complexes that function as CO-RMs in cells and tissues revealing a differential cytotoxic as well as pharmacological profile of these compounds depending on their solubility properties and kinetic of CO release. Specifically, CORM-307, CORM-308 and CORM-314 that are soluble in DMSO release CO with a fast kinetic and display a marked cytotoxic effect in vascular and inflammatory cells despite exerting pronounced vasorelaxation *ex-vivo*. In contrast, CORM-319 that is soluble in water and liberates CO with a slower rate, preserves smooth muscle cell viability, relaxes aortic tissue and exerts a significant anti-inflammatory effect in endotoxin-stimulated macrophages. These data suggest that iron carbonyls can be used as scaffolds for the design and synthesis of pharmacologically active CO-RMs and indicate that increasing water solubility and controlling the rate of CO release are important parameters for limiting their potential toxic effects.

Different classes of transition metal-containing CO-RMs have been synthesized in the last decade following the discovery that both DMSO- (CORM-2) and water-soluble (CORM-3) ruthenium carbonyls exert a variety of CO-mediated pharmacological effects *in vitro* and *in vivo* including vasorelaxation, protection against ischemia as well as anti-apoptotic and anti-inflammatory activities [13,14,16,37,49,50]. The majority of these compounds have been

designed with the main objective of producing stable CO-RMs which could then be triggered to liberate CO by a given stimulus in solution. For instance, certain metal carbonyls are known to be light sensitive and several newly synthesized CO-RMs containing either iron or manganese have been shown to release CO upon irradiation with either visible or UV light [24,25,51]. Other metal carbonyls possess different properties since liberation of CO and the rate of CO release can be triggered by various mechanisms involving interaction with the solvent, loss or substitution of ligands present in the metal center, oxidation of the metal, the presence of a prototypic CO acceptor in the vicinity or a combination of any of these [26,28,52,53]. Irrespective of the amount and kinetic of CO release, which we are learning to be modulated by different characteristics of the CO-RM scaffold, it is evident that many other properties need to be considered for the optimization of pharmacologically active molecules. These include the choice of the metal, stability of the complex, its solubility and the degree of reactivity of the metal with intracellular components other than the target(s). These important aspects will aid the development of compounds with reduced side effects, limited toxicity and improved efficacy. Therefore, the type of screening for the identification of novel CO-RMs is not trivial and should include not only assays for the quantification of CO release but also appropriate *in vitro* and/or *in vivo* tests for the assessment of CO-mediated pharmacological effects coupled with their cytotoxicity profile.

Based on previous experience and through a preliminary screening of a group of iron indenyl carbonyl compounds that were tested for their CO-releasing properties, we observed that introducing water solubility significantly reduces the cytotoxic effect of CO-RMs [30]. In this study we utilized a similar class of iron complexes to assess more in detail this aspect and explore a possible correlation between the differential solubility of iron carbonyls in hydrophobic and hydrophilic solutions, their toxicity profile and CO-mediated pharmacological actions. These

CO-RMs were selected for comparison as they are structurally related and liberate the same total amount of CO to myoglobin. In line with our predictions, we found that among the iron-allyl carbonyls tested, the ones soluble in DMSO (CORM-307, CORM-308 and CORM-314) caused a much more significant cytotoxic effect on cultured cells compared to the one soluble in water (CORM-319). In smooth muscle cells, treatment with DMSO-soluble CO-RMs at concentrations as low as 10 μ M resulted in complete loss of viability whereas (60%) were still viable after treatment with 100 μ M CORM-319. In addition, in isolated aortic rings both DMSO- and water-soluble CO-RMs induced a marked vasorelaxation but with a totally different kinetic. While CO-RMs soluble in DMSO progressively relaxed vessels over time reaching 90% relaxation at 1 h without subsequent re-contraction, the water-soluble CORM-319 elicited significant concentration-dependent relaxation followed by a rapid regain in contractility within 20 min. The inability of dilated vessels to recontract after addition of CO-RMs soluble in DMSO is indicative of an ensuing vascular damage and may be due to the potent toxic effect caused by these compounds on smooth muscle cells. However, it should be noted that viability of smooth muscle cells was assessed 24 h after exposure to CO-RMs while the effect observed in vessels occurred within 1 h of treatment.

Thus, iron carbonyl CO-RMs soluble in DMSO promote a marked toxic effect in cells and tissues of vascular origin. The results obtained with CO-RMs depleted of CO (iCO-RMs) are rather instructive and helpful in understanding the possible causes of toxicity as they show that iCO-RMs soluble in DMSO cause far less smooth muscle cytotoxicity than their active counterparts. In the case of iCORM-308 and iCORM-314, their better cytotoxic profile is also accompanied by decreased vascular damage as indicated by maintenance of contractility following a transient vasodilatory effect elicited by these two compounds. At first, one could

argue that CO is the factor responsible for the observed vascular damage as DMSO-soluble CO-RMs could be more toxic by releasing CO with a very rapid kinetic compared to CORM-319, which liberates CO at a much slower rate. However, this is unlikely since all iCO-RMs soluble in DMSO still significantly decreased cell viability within the range of 10-50 μM while the water-soluble CORM-391 and iCORM-391 did not. In addition, previous results have clearly shown that at these concentrations CO gas is not toxic to cells or vascular tissues [54], and for many “fast” and “slow” water-soluble CO-RMs tested so far toxicity *in vitro* can be observed only at concentrations above 100 μM [55]. The higher toxicity of the DMSO-soluble iron carbonyl complexes might be due to a more efficient cellular uptake, but this needs to be confirmed by assessing the lipophilicity or by measuring the precise amount of internalization of these compounds. It cannot be excluded a priori that the presence of iron, after liberation of CO, would favour some Fenton reaction activities inside the cells and consequently the increased production of reactive oxygen species (ROS) could contribute to the toxic effect and the lack in regaining contractility we observed [46,56]. This is supported also by circumstantial evidence showing that an increased production of ROS can be detected in cells treated with ruthenium-based CORM-2, which is soluble in DMSO [57,58].

The experiments conducted on macrophages further support the idea that iron-carbonyls solubilised in DMSO are inherently more toxic. First of all, it is interesting to note that compared to smooth muscle cells, RAW264.7 are significantly more resistant to the toxic effect elicited by all iron-containing CO-RMs. In fact, while smooth muscle cells treated with 10 μM DMSO-soluble CO-RMs for 24 h exhibited 100% loss in cell viability, more than 50% viable macrophages were observed after exposure to 10 μM CORM-307, 308 or 309. Moreover, the IC_{50} for the water-soluble CORM-319 is markedly increased from ≈ 100 μM in smooth cells to

797 μM in macrophages. When macrophages were challenged with LPS, we found that CORM-319, but not iCORM-319, inhibited nitrite production in a concentration-dependent manner demonstrating that CO is the major component responsible for this anti-inflammatory effect. These data confirm that within the range of concentrations 10 to 100 μM , both CORM-319 itself and CO liberated from it are clearly not toxic. Thus, compared to previously published reports, our present study reveals a marked improvement in the synthesis of iron carbonyls for pharmacological applications highlighting the importance of increasing their solubility in water in order to limit cytotoxic side effects [25,30,59]. Several newly synthesized CO-RMs based on iron metal carbonyls have been recently reported by different groups who have primarily focussed on generating compounds that can liberate CO in solution by exploiting the chemical reactivity of metal-CO bond towards various mechanisms of action of CO release [25,27,30,32,59]. However, and in view of the data presented here, the intrinsic toxicity of these iron-containing CO-RMs is a major obstacle to overcome; thus, efforts should be made to carefully assess the cytotoxic profile in different cell types and tissues every time a novel CO-RM is synthesized.

In conclusion, the results presented here disclose important information on the viability of water-soluble iron carbonyls as CO-RMs since we identified that vasodilatory and anti-inflammatory effects are strictly related to the extent and rate of CO liberation without any contribution of the ensuing cytotoxic activity observed for similar compounds soluble in DMSO.

Acknowledgments

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Figure Legends

Figure 1 Chemical structure of iron-containing CO-RMs and quantification of CO release. (A) CORM-307, CORM-308 and CORM-314 are iron tricarbonyl complexes containing different halides (I, Br and Cl, respectively) and are soluble in DMSO, whereas CORM-319 is an iron tetracarbonyl complex which is soluble in water. (B) CO-RMs (10, 20 and 40 μM) were added to a solution containing deoxy-myoglobin and the formation of carbonmonoxy myoglobin (MbCO) was determined over time. The reported values are the maximal MbCO reached for each CO-RM (see Materials and Methods). Bars represent the mean \pm S.E.M. of experiments performed in duplicate.

Figure 2. Biochemical and pharmacological properties of CORM-307. (A) Absorption spectra of carbonmonoxy myoglobin (MbCO, *open symbols*) formed over time after addition of CORM-307 (40 μM) to a phosphate buffer solution containing deoxy-myoglobin (*filled squares*) (see Materials and Methods). Note: the Mb becomes fully saturated with CO within 5 min (see Soret peak at 540 nm) and all the spectra recorded at subsequent times overlap the initial spectrum. (B) Quantification of MbCO formed over time after reaction of deoxy-Mb with 40 μM CORM-307 or its inactive counterpart (iCORM-307). (C) Vasorelaxing effects of CORM-307. Pre-contracted rat aortic rings were exposed to a bolus addition of 100 μM CORM-307 or iCORM-307 and changes in tension were recorded over time. Data represent the mean \pm S.E.M. of 5 independent experiments and are expressed as a percentage of the initial contraction to phenylephrine. (D) Cytotoxicity profile of CORM-307. Cell viability was assessed 24 hr after exposure of rat smooth muscle cells to CORM-307 or iCORM-307 (10-100 μM) using the Alamar Blue assay (see Materials and Methods). Results represent the mean \pm S.E.M. of 5

independent experiments. Bars are not visible when the cell viability is 0%. * indicates $P < 0.05$ vs. control (0 μM).

Figure 3. Biochemical and pharmacological properties of CORM-308. **(A)** Absorption spectra of carbonmonoxy myoglobin (MbCO, *open symbols*) formed over time after addition of CORM-308 (40 μM) to a phosphate buffer solution containing deoxy-myoglobin (*filled squares*) (see Materials and Methods). Note: the Mb becomes fully saturated with CO within 5 min (see Soret peak at 540 nm) and all the spectra recorded at subsequent times overlap the initial spectrum. **(B)** Quantification of MbCO formed over time after reaction of deoxy-Mb with 40 μM CORM-308 or its inactive counterpart (iCORM-308). **(C)** Vasorelaxing effects of CORM-308. Pre-contracted rat aortic rings were exposed to a bolus addition of 100 μM CORM-308 or iCORM-308 and changes in tension were recorded over time. Data represent the mean \pm S.E.M. of 5 independent experiments and are expressed as a percentage of the initial contraction to phenylephrine. **(D)** Cytotoxicity profile of CORM-308. Cell viability was assessed 24 hr after exposure of rat smooth muscle cells to CORM-308 or iCORM-308 (10-100 μM) using the Alamar Blue assay (see Materials and Methods). Results represent the mean \pm S.E.M. of 5 independent experiments. * indicates $P < 0.05$ vs. control (0 μM).

Figure 4. Biochemical and pharmacological properties of CORM-314. **(A)** Absorption spectra of carbonmonoxy myoglobin (MbCO, *open symbols*) formed over time after addition of CORM-314 (40 μM) to a phosphate buffer solution containing deoxy-myoglobin (*filled squares*) (see Materials and Methods). Note: the Mb becomes fully saturated with CO within 5 min (see Soret peak at 540 nm) and all the spectra recorded at subsequent times overlap the initial spectrum. **(B)**

Quantification of MbCO formed over time after reaction of deoxy-Mb with 40 μ M CORM-314 or its inactive counterpart (iCORM-314). (C) Vasorelaxing effects of CORM-314. Pre-contracted rat aortic rings were exposed to a bolus addition of 100 μ M CORM-314 or iCORM-314 and changes in tension were recorded over time. Data represent the mean \pm S.E.M. of 5 independent experiments and are expressed as a percentage of the initial contraction to phenylephrine. (D) Cytotoxicity profile of CORM-314. Cell viability was assessed 24 hr after exposure of rat smooth muscle cells to CORM-314 or iCORM-314 (10-100 μ M) using the Alamar Blue assay (see Materials and Methods). Results represent the mean \pm S.E.M. of 5 independent experiments. Bars are not visible when the cell viability is 0%. * indicates $P < 0.05$ vs. control (0 μ M).

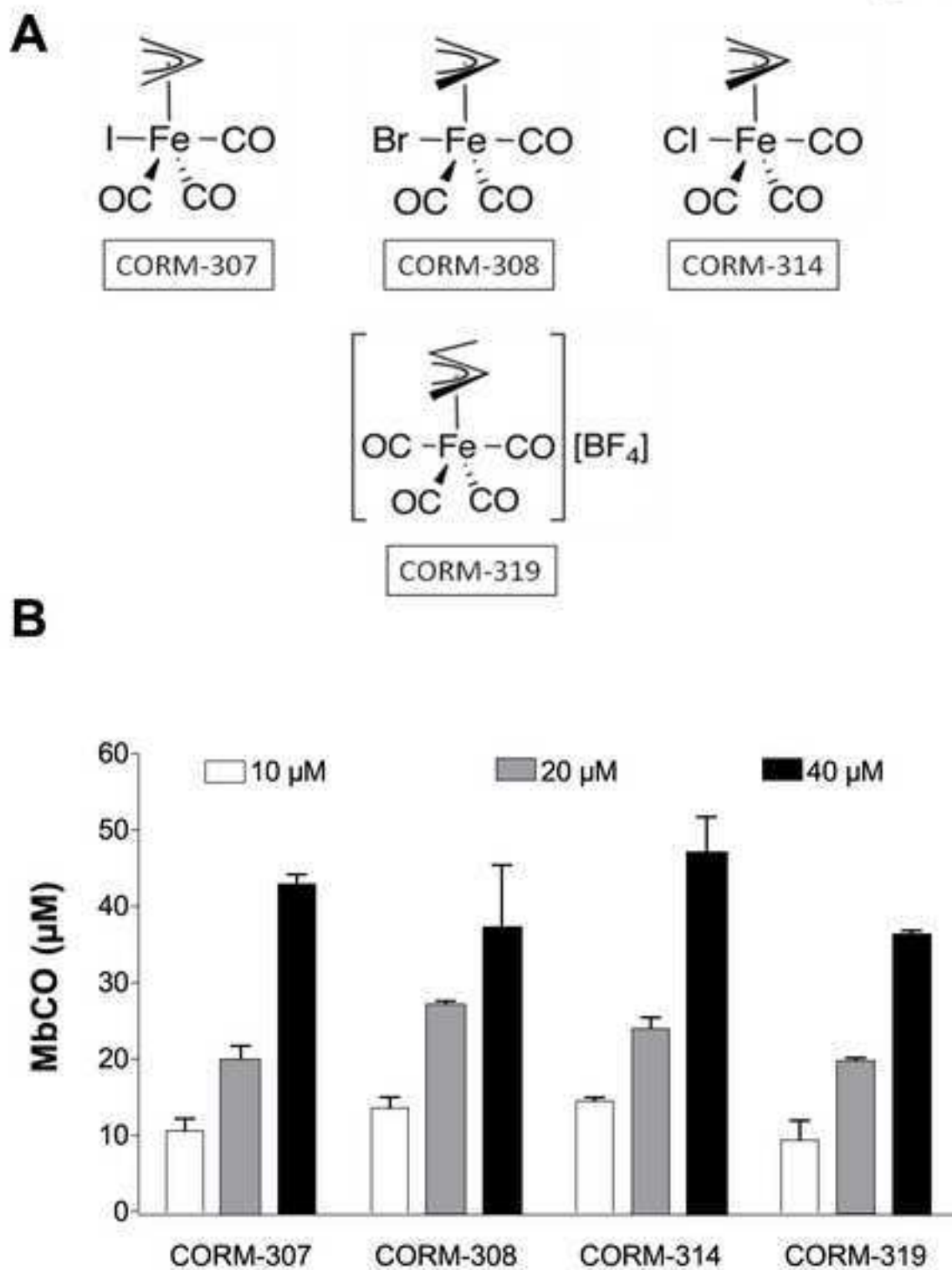
Figure 5. Biochemical and pharmacological properties of CORM-319. (A) Absorption spectra of carbonmonoxy myoglobin (MbCO, *open symbols*) formed over time after addition of CORM-319 (40 μ M) to a phosphate buffer solution containing deoxy-myoglobin (*filled squares*) (see *Materials and Methods*). (B) Quantification of MbCO formed over time after reaction of deoxy-Mb with 40 μ M CORM-319 or its inactive counterpart (iCORM-319). *Inset:* Detection of CO release from CORM319 using an amperometric CO electrode. Changes in current over time is reported after addition of 100 μ M CORM-319 to a phosphate buffer solution (pH=7.4) (C) Vasorelaxing effects of CORM-319. Pre-contracted rat aortic rings were exposed to a bolus addition of 100 μ M CORM-319 or iCORM-319 and changes in tension were recorded over time. Data represent the mean \pm S.E.M. of 5 independent experiments and are expressed as a percentage of the initial contraction to phenylephrine. (D) Cytotoxicity profile of CORM-319. Cell viability was assessed 24 hr after exposure of rat smooth muscle cells to CORM-319 or iCORM-319 (10-

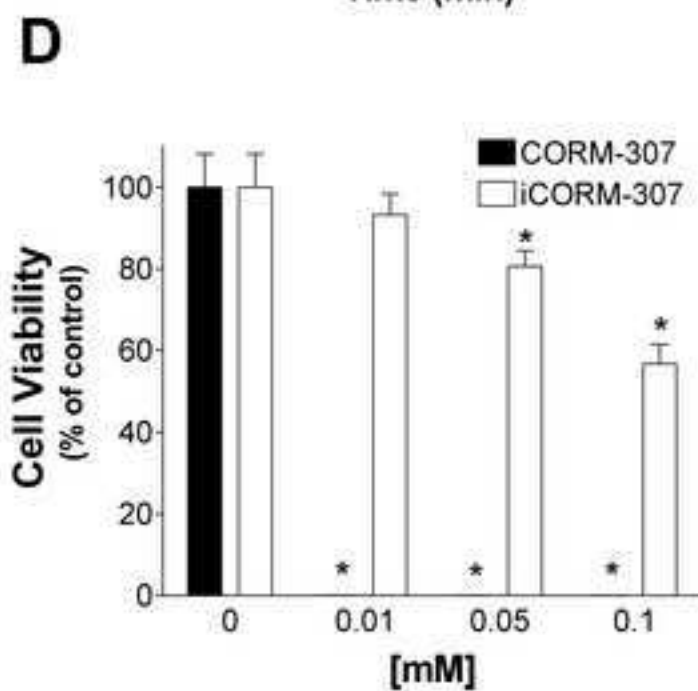
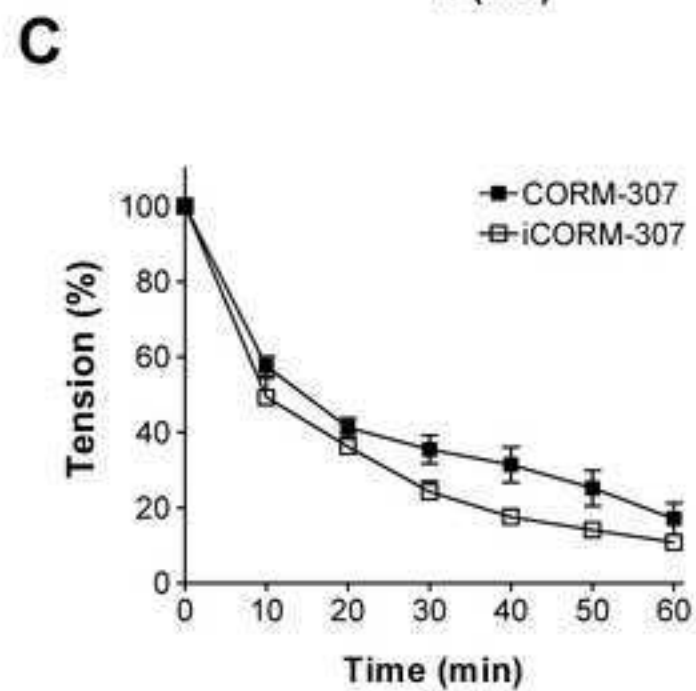
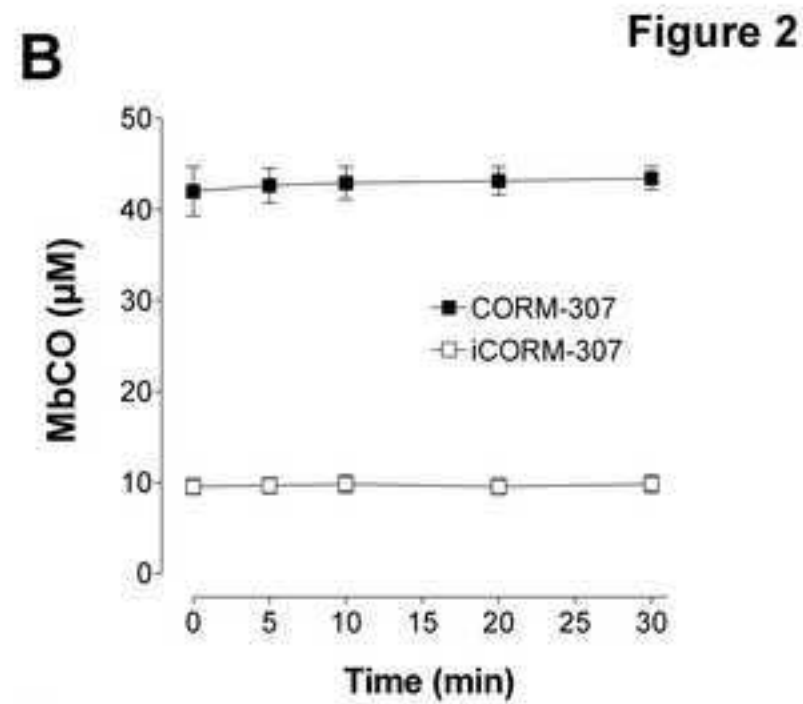
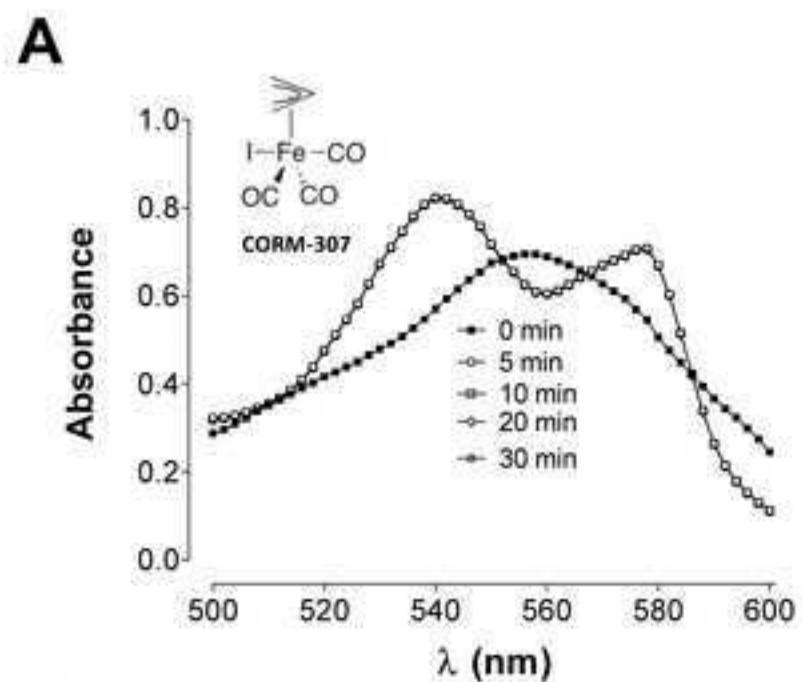
100 μM) using the Alamar Blue assay (see Materials and Methods). Results represent the mean \pm S.E.M. of 5 independent experiments. * indicates $P < 0.05$ vs. control (0 μM).

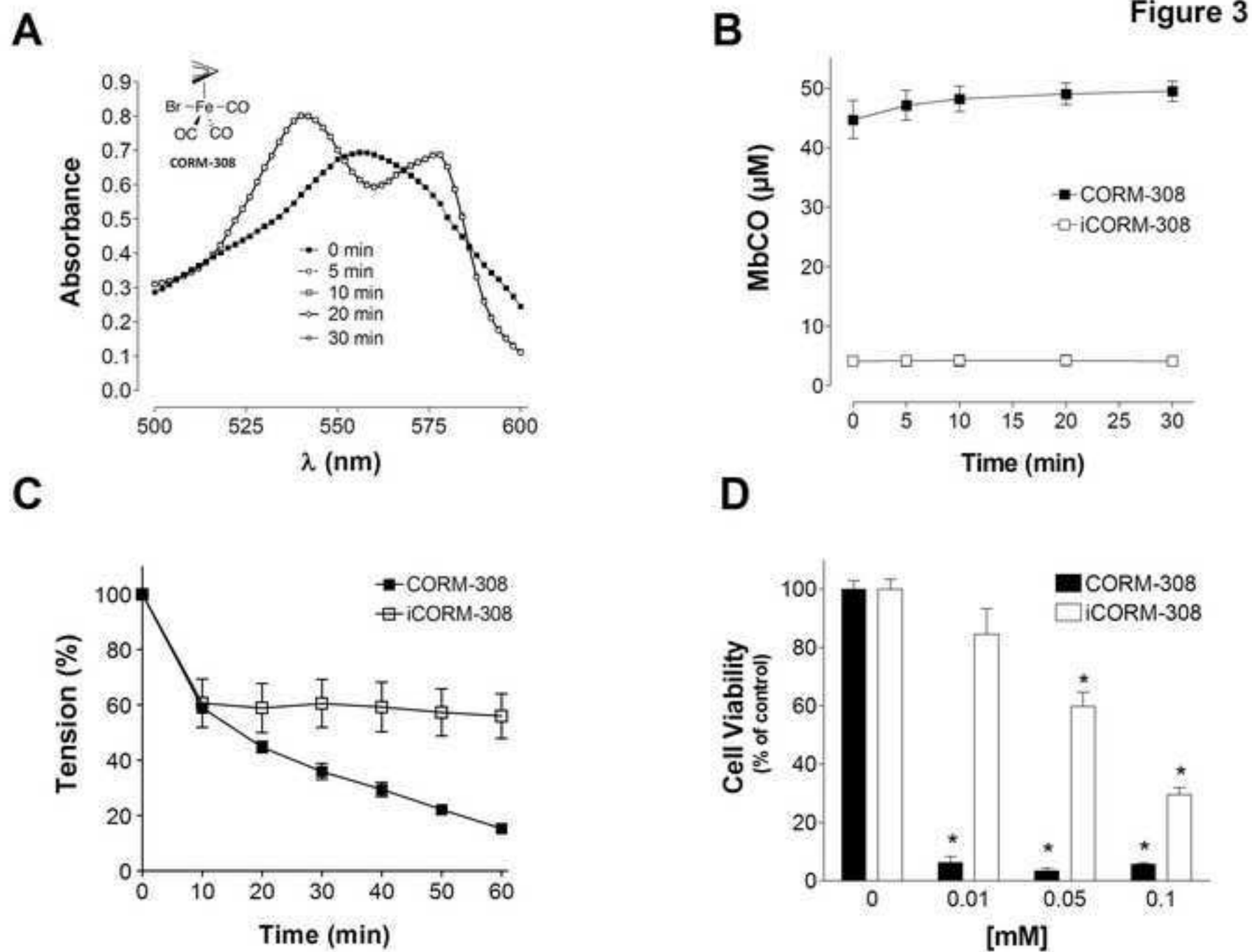
Figure 6. Effect of iron-containing CO-RMs on cell viability in RAW264.7 macrophages. Cell viability was assessed in macrophages 24 hr after exposure to increasing concentrations of CORM-307 (A), CORM-308 (B), CORM-314 (C) or CORM-319 (C). Viability was expressed as percentage of control and the reported IC_{50} for each active CO-RM was expressed as the concentration necessary to cause 50% of cell damage. Data represent the mean \pm S.E.M. of 6 independent experiments. Bars are not visible when the cell viability is 0%. * indicates $P < 0.05$ compared to controls (0 μM); † indicates $P < 0.05$ compared to inactive CO-RMs.

Figure 7. Effect of CO-RM-319 on LPS-stimulated nitrite production in RAW264.7 macrophages. Cells were exposed to 1 $\mu\text{g/ml}$ LPS in the presence of increasing concentrations of CORM-319 (10-100 μM) or its inactive counterpart (iCORM-319). Nitrite levels, an index of NO production, was assessed after 24 h exposure. Control cells were incubated with medium alone (open bars). Data represent the mean \pm S.E.M. of 6 independent experiments. * indicates $P < 0.05$ compared to LPS-treated cells.

Figure 1







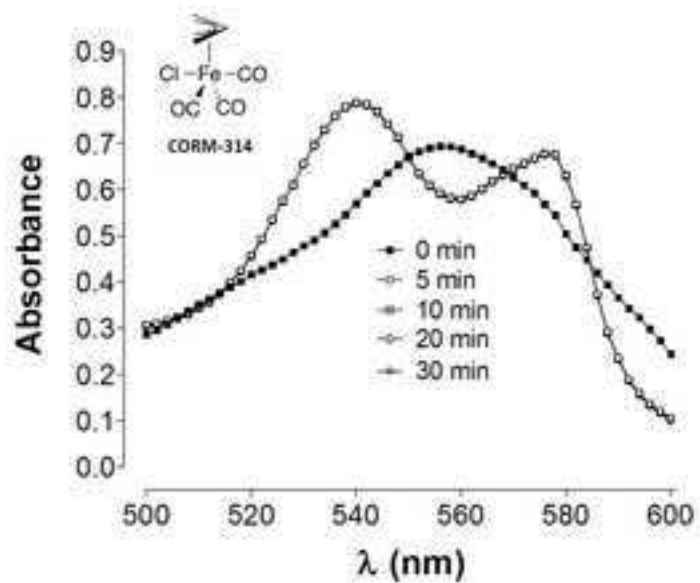
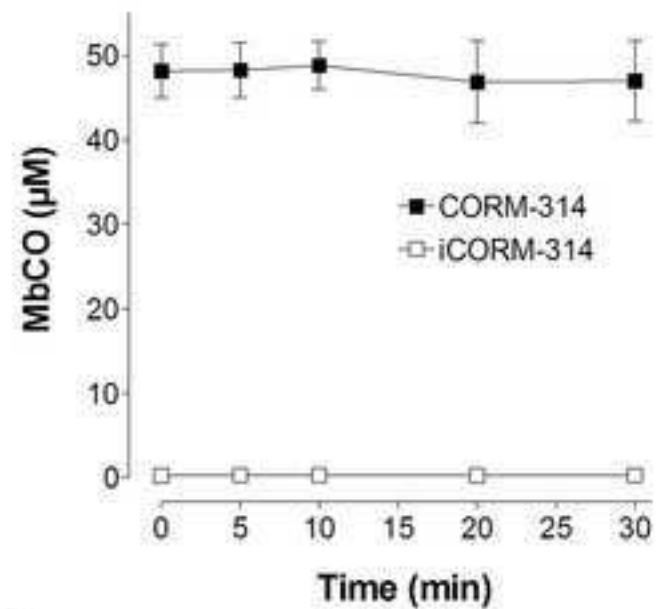
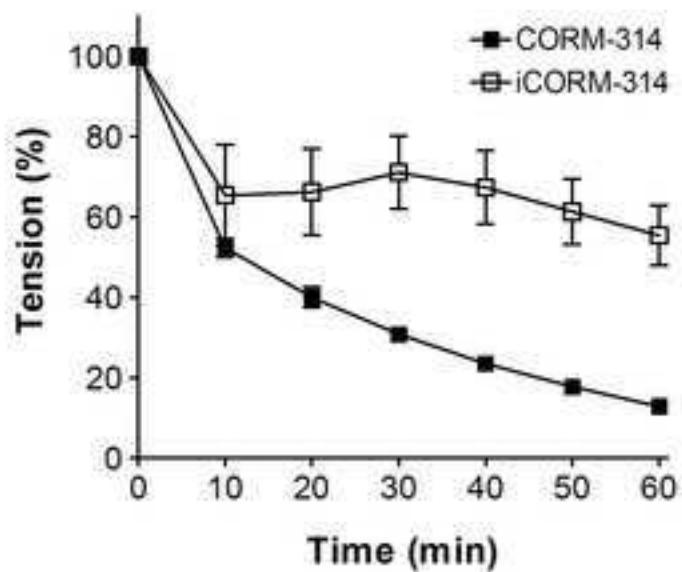
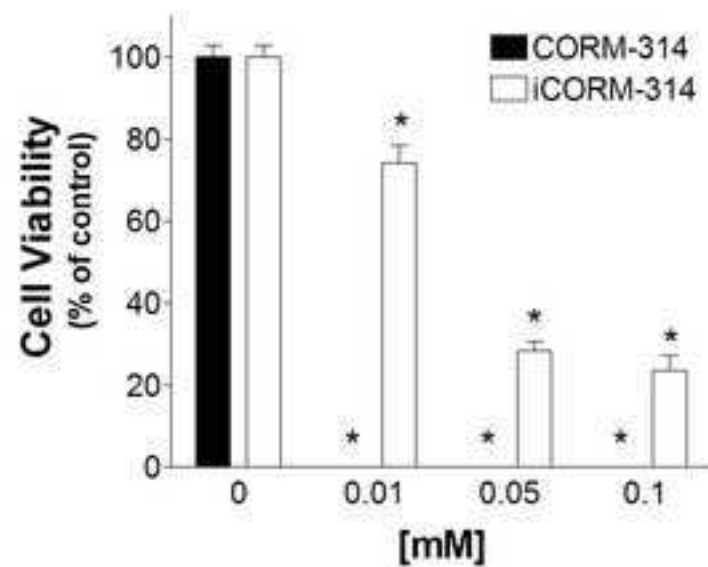
A**B**

Figure 4

**C****D**

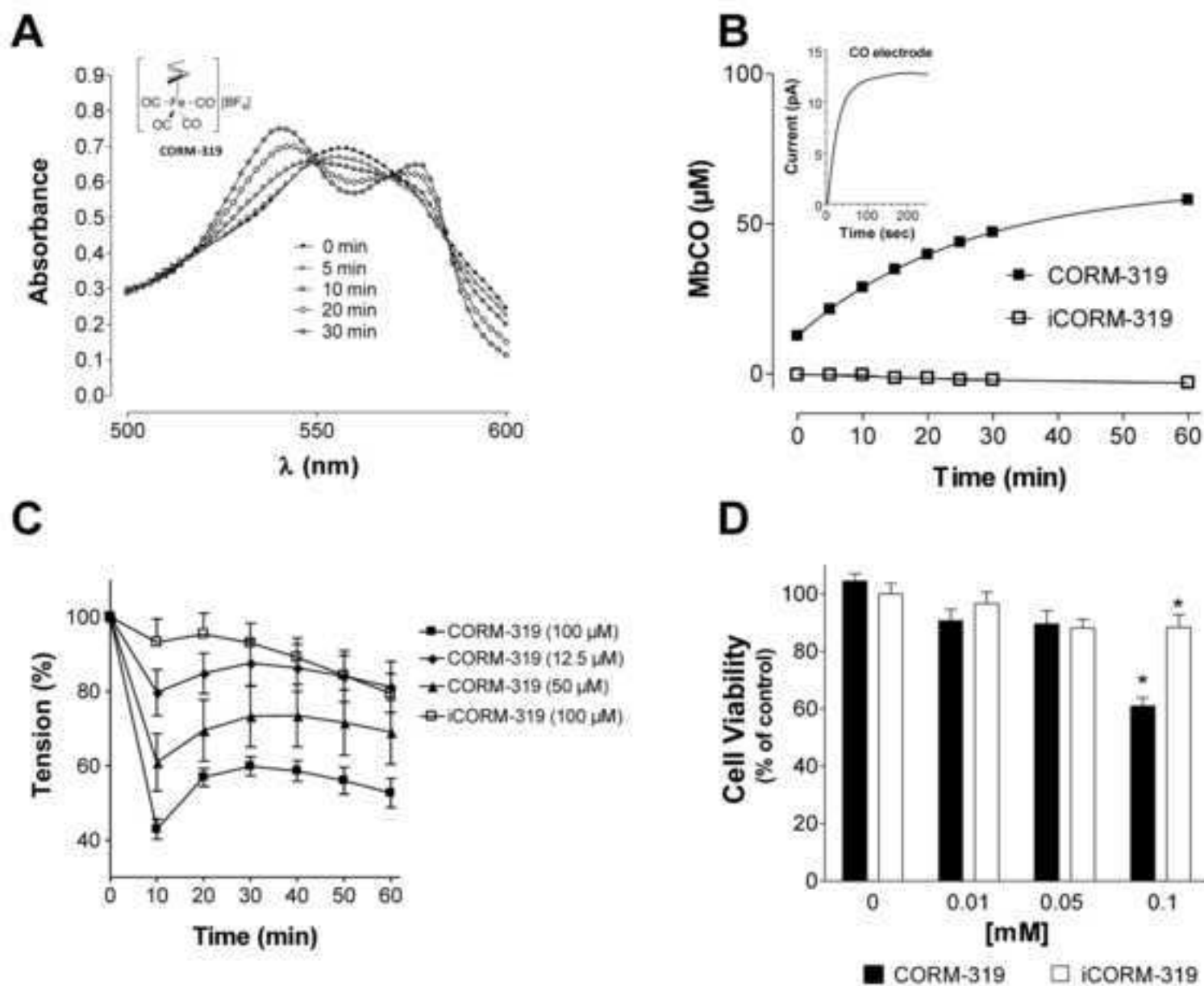


Figure 6

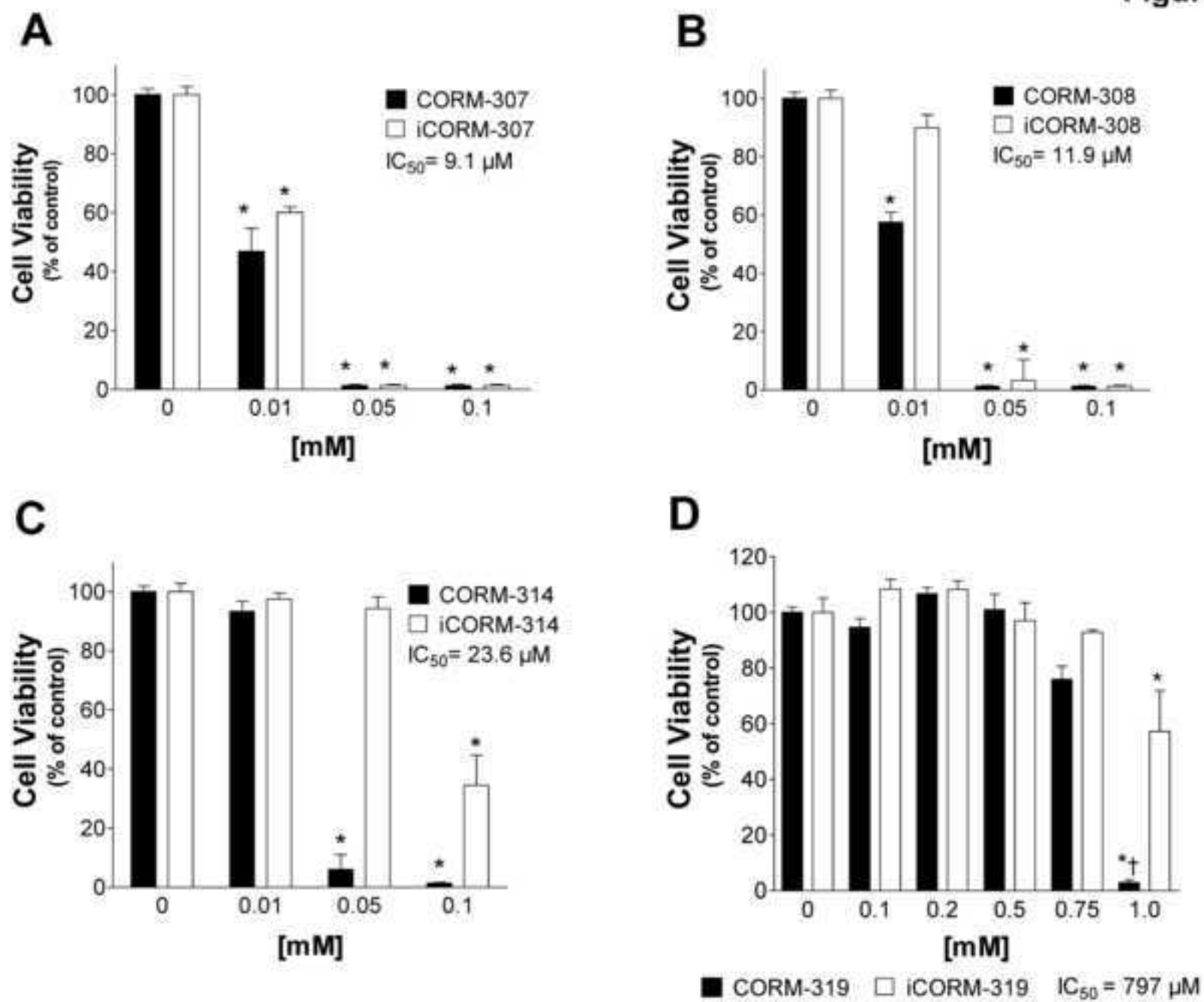


Figure 7

