



Therapeutic effects of CO-releaser/Nrf2 activator hybrids (HYCOs) in the treatment of skin wound, psoriasis and multiple sclerosis

Zeina El Ali^{a,b}, Anthony Ollivier^c, Sylvie Manin^{a,b}, Michael Rivard^c, Roberto Motterlini^{a,b,*,1}, Roberta Foresti^{a,b,**,1}

^a Inserm U955, Equipe 12, Créteil, 94010, France

^b University Paris Est, Faculty of Medicine, Créteil, 94010, France

^c University Paris Est, ICMPE (UMR 7182), CNRS, UPEC, 94320, Thiais, France

ARTICLE INFO

Keywords:

Heme oxygenase-1 (HO-1)
Carbon monoxide (CO)
CO-Releasing molecules (CO-RMs)
Nrf2
Inflammation
Hybrid molecules

ABSTRACT

Carbon monoxide (CO) produced by heme oxygenase-1 (HO-1) or delivered by CO-releasing molecules (CO-RMs) exerts anti-inflammatory action, a feature also exhibited by the nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the stress response. We have recently developed new hybrid molecules (HYCOs) consisting of CO-RMs conjugated to fumaric esters known to activate Nrf2/HO-1. Here we evaluated the biological activities of manganese (Mn) and ruthenium (Ru)-based HYCOs in human monocytes and keratinocytes *in vitro* as well as *in vivo* models of inflammation. The effects of HYCOs were compared to: a) dimethyl fumarate (DMF), a known fumaric ester used in the clinic; b) a CO-RM alone; or c) the combination of the two compounds. Mn-HYCOs donated CO and up-regulated Nrf2/HO-1 *in vitro* more efficiently than Ru-HYCOs. However, irrespective of the metal, a strong reduction in anti-inflammatory markers in monocytes stimulated by LPS was observed with specific HYCOs. This effect was not observed with DMF, CO-RM alone or the combination of the two, indicating the enhanced potency of HYCOs compared to the separate entities. Selected HYCOs given orally to mice accelerated skin wound closure, reduced psoriasis-mediated inflammation and disease symptoms equalling or surpassing the effect of DMF, and ameliorated motor dysfunction in a mouse model of multiple sclerosis. Thus, HYCOs have potent anti-inflammatory activities that are recapitulated in disease models in which inflammation is a prominent component. Prolonged daily administration of HYCOs (up to 40 days) is well tolerated in animals. Our results clearly confirm that HYCOs possess a dual mode of action highlighting the notion that simultaneous Nrf2 targeting and CO delivery could be a clinically relevant application to combat inflammation.

1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2), the master regulator of the stress response, is strongly implicated in the defence of tissues against oxidative and inflammatory stress [1–3]. This action is due to Nrf2-mediated regulation of detoxifying, anti-inflammatory and antioxidant genes, which together coordinate the response of the organism to repair tissue and re-establish cell homeostasis. In particular, genes involved in the synthesis of glutathione (glutamate-cysteine ligases), removal of free radicals and antioxidant activities (NADPH-quinone oxidoreductase-1, heme oxygenase-1 and glutathione peroxidase) as well as export and transport of xenobiotics are all under

control of Nrf2 [4]. The importance of Nrf2 has been corroborated in a number of animal studies and was underlined in the last few years with notable examples of its important role in human diseases. For instance, it was shown that a dysfunctional Nrf2 and consequent oxidative stress is a mechanistic feature of pachyonychia congenita, a disease characterized by debilitating skin lesions [5]. It was also reported that impaired activity of Nrf2 is a driver mechanism in the premature aging disorder Hutchinson-Gilford progeria syndrome, as the authors of this study elegantly demonstrated in human skin fibroblasts that the progerin protein sequesters Nrf2 in the nucleus and prevents correct Nrf2 transcriptional activity [6]. Of interest, reactivation of the Nrf2 pathway using the pharmacological inducer sulforaphane or the

* Corresponding author. Faculty of Medicine, University Paris Est Créteil, 8 Rue de Général Sarrail, 94000, Créteil, France.

** Corresponding author. Faculty of Medicine, University Paris Est Créteil, 8 Rue de Général Sarrail, 94010, Créteil, France.

E-mail addresses: roberto.motterlini@inserm.fr (R. Motterlini), roberta.foresti@inserm.fr (R. Foresti).

¹ These authors contributed equally to this work.

chemopreventive drug Oltipraz, respectively improved skin lesions in pachyonychia congenita and aging defects in prematurely aged cells [5,6], indicating the usefulness of targeting Nrf2 for therapeutic applications to mitigate human skin disorders and other types of disease. Indeed, dimethyl fumarate (DMF) is another pharmacological Nrf2 activator that is clinically used for the treatment of relapsing multiple sclerosis and psoriasis [7–9]. Intriguingly, recent investigations described endogenously derived molecules that block inflammation via Nrf2-mediated activation. These include itaconate in lipopolysaccharide (LPS)-stimulated macrophages [10], and urolithin A, a major metabolite produced by the gut microbiota from polyphenolic substances found in berries and pomegranate [11]. The identification of these intracellular Nrf2 modulators is providing novel scaffolds for synthetic analogues that may be promising future anti-inflammatory agents [10,11].

Heme oxygenase-1 (HMOX1), the inducible isoform of heme oxygenase (HO-1) that converts heme to carbon monoxide (CO), iron and biliverdin, is one of the downstream genes controlled by Nrf2 and is alone endowed with unique protective properties that explain in part the beneficial effects of Nrf2 activation [12–14]. The antioxidant function of biliverdin and its reduced product bilirubin, the signalling and anti-inflammatory action of CO and the up-regulation of the iron-sequestering protein ferritin are known to contribute to the protection afforded by HO-1 [15–23]. Our past efforts to exploit the HO-1 pathway for therapeutic purposes resulted in the discovery of CO-releasing molecules (CO-RMs), transition metal carbonyls that deliver CO to cells and tissues *in vivo* and exhibit strong anti-inflammatory activity [24–29]. More recently, we also designed and synthesized new hybrid molecules termed HYCOs. These compounds with a dual mode of action were obtained from the conjugation of Nrf2 inducers with specific CO-RMs, and were conceived as compounds that can release CO for short-term protection while also stimulating Nrf2 accumulation for a longer lasting defensive approach [30–33]. We first showed that it was chemically feasible to combine a CO-RM (cobalt-based carbonyl) with a fumaric ester derivative as prototypical electrophile with Nrf2-inducing properties and that, although these initial compounds potently induced Nrf2 and HO-1, they were less efficient in donating CO to cells [30]. We then prepared a series of HYCOs in which cobalt-based carbonyls were hybridized with different Nrf2/HO-1 inducers and tested them *in vitro* and *in vivo* [31]. From this group we identified two HYCOs with considerable capacity to increase Nrf2/HO-1 as well as deliver CO to cells and *in vivo* [31]. Moving from HYCOs in which the less pharmacologically attractive cobalt carbonyls were replaced by more biocompatible manganese (Mn) or ruthenium (Ru) CO-RMs, we recently described the synthesis of these compounds [33]. Furthermore, we characterized the anti-inflammatory activities of a Mn-based complex (HYCO-3) in wild type and Nrf2 KO mice during inflammation induced by LPS [32]. This last study clearly demonstrated that replacement of cobalt with manganese-based CO-RMs improves the ability of HYCOs to deliver CO to cells. We also found that HYCO-3 given orally to mice counteracted LPS-mediated inflammation in several organs and that reduction of some inflammatory markers was dependent on the CO-releasing part of the hybrid while others relied on Nrf2 activation [32].

The current study focuses on the biological and pharmacological characterisation of the latest series of Mn- and Ru-containing HYCOs [33] in human THP-1 monocytes and keratinocytes cell lines and different inflammatory models of the skin and multiple sclerosis. Here we report our findings on the efficient pro-healing, psoriasis-reducing properties of selected HYCOs and their effect in mitigating symptoms of multiple sclerosis.

2. Material and methods

2.1. Reagents

RPMI-1640 medium, fetal bovine serum (FBS) and L-glutamine were

from Lonza (Levallois Perret, France), while penicillin, streptomycin and Dulbecco Phosphate Buffer Solution (DPBS) were purchased from Life Technologies (les Ulis, France). Dimethyl fumarate (DMF), Dulbecco's Modified Eagle Medium (DMEM), lipopolysaccharide (LPS, from E. Coli serotype O127:B8) and all other reagents were purchased from Sigma Aldrich (Saint Quentin Fallavier, France) unless otherwise specified. Antibodies were purchased from Abcam (HO-1 monoclonal ab13248), Ozyme (NF- κ B/p65 subunit rabbit monoclonal #3033S) and Santa Cruz Biotechnology (Nrf2 H-300 rabbit polyclonal sc-13032 and β -actin mouse monoclonal sc-47778). The CO sensitive probe COP-1 was kindly provided by Prof. Christopher Chang from the University of California, Berkeley [30,34,35].

2.2. Synthesis of CORM-401, manganese(Mn)- and ruthenium(Ru)-based HYCOs

CORM-401 was synthesized in our laboratories as previously reported [30,31,35,36]. “CO-releaser/Nrf2 activator” hybrids containing either manganese (HYCO-3, HYCO-7, HYCO-13) or ruthenium (HYCO-6 and HYCO-11) were prepared starting from commercially available $Mn_2(CO)_{10}$ and $[RuCl_2(CO)_3]_2$ purchased from Alfa Aesar (Thermo Fisher, les Ulis, France). The synthesis, purification and analytical characterization of HYCOs were performed following protocols recently described by our group [33]. For the *in vitro* experiments using cultured cells and reconstructed epidermis, HYCOs were solubilised in dimethyl sulfoxide (0.1% DMSO in complete medium) and incubated at final concentrations between 0.5 and 100 μ M. For the *in vivo* mouse models, HYCOs were solubilised in DMSO/sesame oil (5:100 vol/vol) and orally administered at the doses reported (see below).

2.3. Cell culture and reconstructed human epidermis

Human monocytic cells (THP1), human immortalized keratinocytes (HaCaT) and normal human epidermal keratinocytes (NHEK) were used in the study. THP1 were cultured in RPMI 1640-Glutamax medium supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% fetal bovine serum. HaCaT cells were cultured in DMEM supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% fetal bovine serum. NHEK were cultured in Keratinocyte-SFM medium (Thermo Fisher) supplemented with 0.25 ng/ml epidermal growth factor, 25 μ g/ml pituitary extract and 25 μ g/ml gentamycin. All 3 types of cells were grown at 37 °C in a humidified 5% CO₂ until confluent and used for the various assays described below. Reconstructed human epidermis from normal human keratinocytes was cultured on an inert polycarbonate filter at the air-liquid interface following instructions by the manufactures (Bioalternatives, Gençay, France) and used as an *in vitro* system to reproduce a “psoriasis-like” phenotype after treatment with a mixture of inflammatory cytokines as described below.

2.4. Cytotoxicity assays

Confluent cells grown in 24 well plates were treated with the different HYCOs (0.5–100 μ M) and incubated for 24 h at 37 °C in a humidified 5% CO₂. DMSO 100% was used as a positive control. For experiments using HaCaT, cell viability was assessed by the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Briefly, after treatment cell plates were centrifuged at 300 \times g for 5 min and the medium carefully replaced with 400 μ l of 0.5 g/l MTT prepared in fresh medium. Plates were incubated at 37 °C for 2 h, followed by centrifugation at 300 \times g for 5 min. The medium was replaced with 400 μ l pure DMSO and the plate incubated at 37 °C for 10 min. Living cells with MTT produced a dark blue formazan product which was quantified spectrophotometrically at 560 nm and results expressed as the percentage of cell viability. For experiments using THP1, cell viability was assessed by a Cytotoxicity Detection Kit (Roche Applied

Science, Meylan, France) to measure lactate dehydrogenase (LDH) released from damaged cells using X-100 Triton solution (2%) as a positive control (100% cytotoxicity). Briefly, at the end of the incubation period, cell plates were centrifuged at $300 \times g$ for 5 min and 100 μ l cell-free supernatant transferred to a 96-well plate. A reaction mixture was added to the supernatant and the plate was incubated in the dark at room temperature with gentle shaking for 10 min. Absorbance was measured at 485 nm. With both assays, results were expressed as the percentage of cell viability.

2.5. Detection of intracellular CO *in vitro*

CO accumulated in cells after treatment with HYCOs was assessed using a fluorescence probe sensitive to CO (COP-1) as previously described [30]. Briefly, THP1 or HaCaT cells were initially suspended in DPBS, then treated with HYCOs (1–5 μ M) for 15 min at 37 °C in the dark, washed and finally incubated for 30 min with 1 μ M COP-1. Fluorescence was then measured by flow cytometry and results were expressed as the mean fluorescence intensity (MFI) calculated as the difference between the MFI of treated cells and the MFI of COP-1 background.

2.6. Measurement of reactive oxygen species (ROS)

Intracellular ROS production was measured using the specific dye 2'-7'-dichlorodihydrofluorescein diacetate (H2DCFDA) purchased from Interchim Bioscience (Montluçon, France). Briefly, confluent THP1 cells were loaded for 30 min in the dark with H2DCFDA before incubation with either DMSO (vehicle) or HYCOs at a final concentration of 5 μ M. At different times after treatment (0.5, 1 and 3 h), the intensity of the green DCF fluorescence was measured by flow cytometry (excitation/emission at 495 nm/529 nm). Results were expressed as the mean of fluorescence intensity.

2.7. Intracellular glutathione assay

The 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) colorimetric assay was used for the measurement of reduced glutathione (GSH). Briefly, THP1 were seeded in 12-well plates and incubated with 5 μ M HYCOs or dimethyl fumarate (DMF, positive control) for 1, 4 and 24 h. At the end of the incubation period, cells were initially washed with PBS, then lysed in 600 μ l of 2% (w/v) 5-sulfosalicylic acid solution and transferred to a 1.5 ml microtube, and finally centrifuged for 5 min at $10,000 \times g$. An aliquot of the supernatant (500 μ l) was then reacted with 0.5 ml DTNB solution (0.3 M sodium phosphate buffer, 10 mM EDTA and 0.2 mM DTNB) and after 5 min the absorbance was read using a spectrophotometer at 412 nm.

2.8. Luminex and ELISA assays

THP1 cells were challenged with 1 μ g/ml lipopolysaccharide (LPS) for 24 h in the presence of DMSO (vehicle) or 5 μ M HYCO-3, HYCO-6, HYCO-7, HYCO-13, DMF, CORM-401 or DMF + CORM-401. TNF- α , IL-6, IL-8, IL-1 α , IL-1 β and IL-10 were measured in the supernatants using a human magnetic Luminex screening assay as recommended by the manufacturer (R&D Systems, Rennes, France). Similarly, human epidermal keratinocytes (NHEK) or reconstructed human epidermis were treated with 2 μ M HYCO-6, HYCO-13, DMF or a positive control (JAK inhibitor, 10 μ M) for 24 h and then stimulated with a cytokine mixture (IL-17, oncostatin M and TNF- α at 3 ng/ml each) to simulate a psoriasis-like inflammation in the presence of the test compounds for an additional 48 h. After incubation, culture supernatants were collected for assessing IL-8 release using an ELISA kit assay (R&D Systems).

2.9. Western blot analysis

THP1 or HaCaT cells were washed in cold PBS before lysis in lysis buffer (20 mM Tris at pH = 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 1% Triton, 25 mM β -glycerophosphate, 1 mM Na_3VO_4 , 2 mM sodium pyrophosphate, 10% glycerol, 1 mM PMSF, 1% mammalian protease inhibitor). The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C. Equal amounts of denaturated proteins were loaded onto 10% SDS-PAGE gel and transferred on PVDF membrane Amersham (Dutcher, Brumath, France). Membranes were then incubated with antibodies directed against Nrf2, HO-1 or NF- κ B while β -actin was used as a loading control. Immunoreactive bands were detected by chemiluminescence (ECL solution, Amersham). Images were captured with GeneSys software in a G:Box F3 imagery station (Syngene).

2.10. Scratch test

HaCaT cells were grown to confluence in 6-well dishes, scratched with a 1 ml tip and wound closure was followed over time. After the scratch, cells were treated with DMSO, HYCO-6 or HYCO-13 at 5 μ M and 10 μ M. Closure was monitored under an inverted microscope Olympus XI70 (magnification $\times 2$) at T0, 24 h, and 48 h and images were taken for analysis. Quantitative assessment of wound area was performed using the Image J software. Area at T0 was considered as 100% and ratios of Time X area/T0 area were calculated to determine the percentage of wound closure.

2.11. Assessment of blood carbonmonoxy hemoglobin (COHb) levels *in vivo*

The levels of blood COHb in mice were measured as an index of CO release from HYCOs after oral administration *in vivo* using a method previously described [37] and modified by our group [29,31]. Briefly, blood (5 μ l) collected at different time points from the mice tail vein following treatments with HYCOs was transferred to a sealed cuvette containing a small magnetic bar and 4.5 ml tris(hydroxymethyl) aminomethane solution (20 mM) previously deoxygenated with sodium dithionite. The solution in the cuvette was gently mixed on a magnetic stirrer and absorbance spectra between 400 and 500 nm were recorded over time using a JASCO spectrophotometer. The percentage of COHb was then calculated based on the absorbance at 420 and 432 nm with the reported extinction coefficients for mouse blood [37].

2.12. *In vivo* model of skin wound

Male C57 BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animals were housed and allowed to acclimatize for at least two weeks on a 12 h light/dark cycle and fed according to federal guidelines. All experiments were performed in compliance with INSERM guidelines for the use of animals and approved by the institutional review board at Paris-Est Creteil Val de Marne University (project no. COMETH 16090). On the day of the experiment, mice were anesthetized by intraperitoneal injection of 80 μ l of ketamine (100 mg/l)/rompun (4 mg/ml) and shaved. Once the skin was exposed, two full-thickness circular wounds (6 mm in diameter) were made on either side of the dorsal midline by excising skin and panniculus carnosus using disposable biopsy punches as previously described [38]. Mice were then administered by oral gavage with 200 μ l HYCO-6 (40 mg/kg) or vehicle (DMSO/sesame oil, 1:10 vol/vol) daily for 14 days. Wounds were left uncovered and photographed every day for assessing the closure of the wounds over time using ImageJ software.

2.13. *In vivo* model of imiquimod-induced psoriasis

A mouse psoriasis model induced by imiquimod on the back of the animals and the internal face of the right ear was used in this study. Briefly, Balb/c female mice (6–8 weeks old from Janvier Labs) were

shaved and 3.5 mg of imiquimod were topically applied from day 1 to day 7 in the morning. Starting from day 1, mice were orally administered with 20 mg/kg (twice daily) HYCO-3, HYCO-6 or DMF while control untreated mice received the vehicle (DMSO/sesame oil, 1:10 vol/vol). Back skin thickness was recorded every day by a caliper and the following clinical scores were recorded: erythema, scaling and epidermis thickness.

2.14. *In vivo* model of multiple sclerosis

Remitting/relapsing experimental allergic encephalitis (EAE) in mice, an inflammatory model that mimics multiple sclerosis in humans, was used as previously described [39]. Briefly, female Swiss Jackson Laboratory (Bar Harbor, USA) mice (7–9 weeks old) were immunized with 100 µg proteolipid protein (PLP₁₃₉₋₁₅₁) emulsified in complete Freund's adjuvant (CFA) with 2 mg/ml heat-killed *Mycobacterium tuberculosis* to make a 1:1 emulsion. Mice were then weighed and injected subcutaneously in both flanks with 100 µl emulsion followed 2 h later by an intraperitoneal injection of 0.2 ml pertussis toxin (200 ng/mouse). Starting from the day of PLP injection, mice were treated once or twice a day for 40 days by oral gavage with tested compounds at the following doses: HYCO-3 (25 mg/kg once, 50 mg/kg once or 25 mg/kg twice), HYCO-13 (25 mg/kg once, 50 mg/kg once or 25 mg/kg twice) or DMF (25 or 50 mg/kg once). Animals were observed daily by measuring their body weight and assessing clinical signs of EAE. They were assigned one of the following clinical grades by an observer who was blinded to the treatment: 0, no obvious sign of motor dysfunction; 1, limp or flaccid tail; 2, limp tail and weakness; 3, severe paralysis of legs; 4, complete hind leg and partial front leg paralysis; and 5, complete paralysis or death.

2.15. Statistical analysis

Data analyses were performed using GraphPad Prism software version 5.0 (San Diego, CA). Data are expressed as mean ± S.E.M and One-way ANOVA post hoc Bonferroni multiple comparison tests were applied. Significance was accepted at $p \leq 0.05$.

3. Results

3.1. HYCOs deliver CO to human monocytes and keratinocytes while activating the Nrf2/HO-1 axis

Fig. 1 shows the chemical structure of a series of manganese (Mn)-based (in blue) and ruthenium (Ru)-based (in red) hybrid compounds (HYCOs), which were synthesized by conjugating the Nrf2 activator ethyl fumaric ester to two types of CO-releasing molecules (CO-RMs). An analogue of CORM-401, an orally active compound that delivers CO with high efficiency *in vitro* and *in vivo* [29,32,35,36,40–42], was used to synthesize Mn-HYCOs (HYCO-3, HYCO-7 and HYCO-13), whereas a Ru-CORM formed the basis for the synthesis of Ru-HYCOs (HYCO-6 and HYCO-11) (see chemical structures in Fig. 1). The conjugation of fumaric ester to the CO-RM moiety was achieved by the introduction of different linkers as previously described [33]. We first assessed whether these compounds are capable of delivering CO to human THP1 and HaCaT cells *in vitro*. As shown in Fig. 2, intracellular CO accumulation significantly increased in a concentration-dependent manner after treatment with 0.5–5 µM Mn-HYCOs while at 10 µM the cellular CO content either started to fall (HYCO-3) or reached a plateau (HYCO-7 and HYCO-13). In contrast, in cells treated with Ru-HYCOs (0.5–10 µM) we observed either no increase in intracellular CO (i.e. HYCO-11) or much lower CO levels in the case of HYCO-6 compared to Mn-HYCOs. These data clearly indicate that the CO-RM moiety within the Mn-HYCOs complexes is much more effective in causing promoting CO accumulation in human monocytes and keratinocytes than the analogous Ru-CORM moiety present in HYCO-6 and HYCO-11, confirming

previous data on BV2 microglia cells [32]. It has to be noted that all HYCOs tested did not cause any visible cytotoxic effect up to 5 µM (Supplementary Fig. 1); only monocytes and especially keratinocytes treated with HYCO-3 and HYCO-7 at 10 µM exhibited a slight decrease in cell viability which became significant at concentrations of 20 µM and above. Overall, our results indicate that these compounds can be used at concentrations between 0.5 and 10 µM without significant toxic effects in human THP1 and HaCaT cells.

We then assessed the ability of HYCOs to increase the expression of Nrf2 and HO-1 proteins, knowing that HYCO-3 and HYCO-6 have been already shown to induce these two pathways in BV2 microglia [32]. We found that THP1 and HaCaT cells exposed to 0.5–10 µM Mn-HYCOs for 6 h resulted in a concentration-dependent increase in Nrf2 and HO-1 protein expression with some exceptions for HO-1 expression, which started to decrease at 10 µM with HYCO-6 in THP1 and with HYCO-3 and HYCO-13 in HaCaT cells (Fig. 3A and B). In comparison, neither CORM-401 nor a combination of DMF plus CORM-401 at 5 µM each showed any induction of Nrf2 and HO-1 whereas only DMF at 100 µM (positive control) strongly enhanced both Nrf2 and HO-1 protein levels in the two cell types (Supplementary Fig. 2A). In the case of Ru-HYCOs, both HYCO-6 and HYCO-11 were able to increase, albeit to different degrees, Nrf2 protein expression in a concentration-dependent manner in THP1 and HaCaT cells but HYCO-6 increased HO-1 protein expression only at 5 µM in THP1 cells. In addition, HYCO-11 completely failed to induce HO-1 in both cell types (Fig. 3A and B). When looking at HO-1 changes over time in THP1 cells, we also observed that the marked increase in protein expression at 6 h in cells exposed to 5 µM HYCO-3 was maintained in later times (18 and 24 h), whereas in the case of all other HYCOs HO-1 expression gradually returned to basal levels by 24 h (Supplementary Fig. 2B). Similar data were obtained in HaCaT keratinocytes (data not shown). In addition, in these kinetic studies we found that in both THP1 and HaCaT cells Nrf2 activation always preceded HO-1 induction. These data demonstrate that, as in the case of CO delivery, Mn-HYCOs are more potent than Ru-HYCOs in activating the Nrf2/HO-1 axis in the two types of human cells tested. Because HYCO-11 was the only compound incapable of either releasing CO or stimulating HO-1 protein expression, we decided to continue our investigation with the remaining four HYCOs.

3.2. Differential anti-inflammatory actions by HYCOs in LPS-stimulated human monocytes cells

We next evaluated whether the different HYCOs affected the release of inflammatory markers by THP1 cells challenged with LPS. As shown in Fig. 4, the increased production of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β induced by LPS was significantly reduced by HYCO-6, HYCO-7 and HYCO-13, the latter clearly showing the most potent effect. HYCO-13 was also the only compound to markedly reduce IL-8 production and, together with HYCO-6, the most effective in reducing IL-1α. In contrast, HYCO-3 failed to reduce the production of any pro-inflammatory markers after LPS stimulation and in the case of TNF-α and IL-1α we found that HYCO-3 further increased the levels of these two cytokines. THP1 cells challenged with LPS in the presence of HYCOs displayed similar or even better cell viability compared to LPS alone (data not shown). In addition, HYCOs did not affect the expression of NF-κB in LPS stimulated-cells (Supplementary Figs. 3A and 3B). At the concentrations used (5 µM), neither DMF nor CORM-401 or the combination of the two compounds had any effect on LPS-induced production of cytokines. It has to be noted that the levels of the anti-inflammatory marker IL-10 in THP1 cells markedly increased after treatment with LPS and all four HYCOs were able to significantly reduce the production of this cytokine with similar efficacy (Fig. 4).

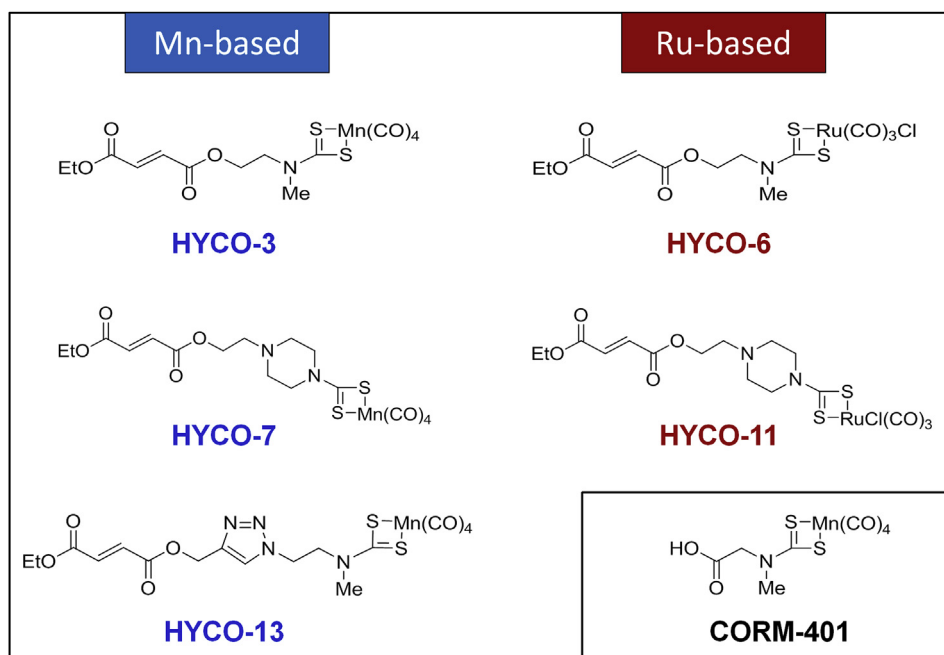


Fig. 1. Chemical structure of Mn-based and Ru-based HYCOs. HYCO-3, HYCO-7 and HYCO-13 (in blue) were synthesized by conjugating the Nrf2 activator ethyl fumaric ester to CORM-401, a Mn-based CO-releasing moiety. In contrast, a Ru-based CO-releasing molecule hybridized to the fumaric ester was used to synthesize HYCO-6 and HYCO-11 (in red). The chemical structure of CORM-401, which was also tested for comparison in our *in vitro* and *in vivo* experiments, is also shown. Details about the synthesis of these compounds are reported in Materials and Methods. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Effect of HYCOs on ROS production and glutathione levels in human monocytes

To investigate whether HYCOs change the redox status of cells, we assessed if these compounds influence ROS and glutathione levels in monocytes. While HYCO-3 and DMF did not cause any change in cellular ROS, exposure of THP1 cells to HYCO-6, HYCO-7 and HYCO-13 resulted in a time-dependent increase in ROS production (Fig. 5). The relative amount of ROS induced by these three HYCOs was significantly increased although much lower (3-fold) than that produced by the same concentrations of TBHP, a standard oxidant known to generate free radicals and used here as a positive control. We also found that total glutathione levels did not significantly change over time in THP1 cells treated with HYCOs except for a transient decrease by HYCO-13 at 4 h and an increase by HYCO-6 after 24 h incubation.

3.4. HYCOs deliver CO *in vivo* and display therapeutic benefits in models of skin wound, psoriasis and multiple sclerosis

We next moved to *in vivo* models of skin disease and inflammation to assess the potential therapeutic effects of HYCOs. We first evaluated the ability of HYCOs to liberate CO into the blood circulation after oral administration to mice by measuring changes in the levels of carbon-monoxo hemoglobin (COHb). As shown in Supplementary Fig. 4, COHb levels significantly increased after treatment with all HYCOs, including HYCO-6, which elicited a very low accumulation of CO *in vitro*. The peak of COHb was observed 1 h after administration of the compounds, with HYCO-13 displaying the highest increase (from 0.4 to 5%). After 6 and 24 h, COHb remained significantly high ($\approx 2\%$) in mice treated with HYCO-3, HYCO-13 and HYCO-6 but returned to basal levels within 24 h after treatment with HYCO-7. These results, together with the above characterization of the biological activities of HYCOs *in vitro*, indicate that although Mn-HYCOs appear to fulfil the requirements of “CO releasers/Nrf2 activators” better than Ru-HYCOs, we cannot exclude any potential benefit *in vivo* also by HYCO-6. Considering that HYCO-3 showed a mixed profile *in vitro* as it induced Nrf2 and HO-1 in THP-1 cells but only Nrf2 in keratinocytes and released CO *in vivo*, we decided to keep HYCO-6 within our portfolio of compounds to be tested also *in vivo*.

Therefore, we next evaluated the comparative effects of HYCO-13

and HYCO-6 in models of skin wound *in vitro* and *in vivo*. As shown in Fig. 6A, the scratch test conducted on HaCaT cells revealed that both HYCOs were able to stimulate keratinocytes proliferation thus accelerating the closure of the scratched area; their effect was significantly better than either DMF, CORM-401 or the combination of the two compounds. In addition, daily oral administration of HYCO-6 but not HYCO-13 (data not shown) markedly accelerated the closure of the wound over time in mice following skin injury (Fig. 6B). Topical application of the compounds was not effective in reducing wound size (not shown). When we assessed the effect of HYCO-6 and HYCO-13 in psoriasis-like models, we found that: 1) both HYCOs significantly reduced IL-8 production in reconstructed human epidermis challenged with a psoriasis-like cytokines mix but only HYCO-6 was effective in reducing this inflammatory marker in human keratinocytes (Fig. 7A); 2) parameters of skin psoriasis induced by imiquimod, such as increased erythema, epidermis thickness and scaling, were markedly attenuated in mice treated with HYCO-6 and HYCO-13 (Fig. 7B, C and 7D). Notably, in both models the beneficial effects of HYCOs were comparable or better than the effect exerted by DMF. Finally, in Fig. 8 we show the efficacy of two Mn-HYCOs (HYCO-3 and HYCO-13) during a period of 40-day treatment in a mouse model of multiple sclerosis (EAE). The peak of disease was significantly diminished by the highest dose of DMF but less so by HYCO-3 or HYCO-13. However, we found that HYCO-3 administered at 25 mg/kg twice a day orally was effective as DMF given at 50 mg/kg daily in reducing the clinical signs of the disease during the remittance phase. In addition, the relapse of the disease after treatment with HYCO-3 was delayed compared to the DMF group. HYCO-13 showed instead efficacy at all the doses tested (25 or 50 mg/kg daily as well as 25 mg/kg administered twice a day), with the 25 mg/kg dose administered twice a day also showing a significant delay in relapse compared to the DMF group (Fig. 8). Data showing that treatment with HYCOs did not affect changes in body weight over time indicate that mice well tolerated the composition and doses of the compounds administered.

4. Discussion

We present in this work the *in vitro* and *in vivo* characterization of Mn- and Ru-based HYCOs, hybrid complexes composed of a Nrf2 inducing part (fumaric ester derivative) and a CO-releasing moiety. The

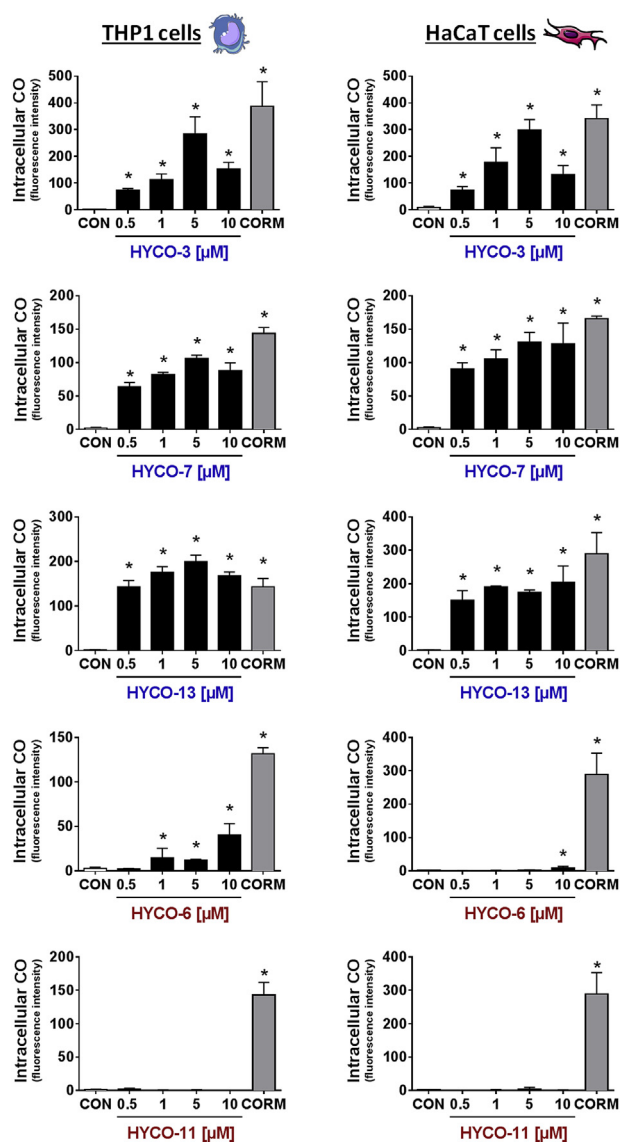


Fig. 2. HYCOs deliver CO to cells *in vitro*. THP1 (left panel) and HaCaT cells (right panel) resuspended in DPBS were treated with HYCOs at different concentrations for 15 min, washed and then incubated in the presence of COP-1 for an additional 30 min. CORM-401 (CORM, 10 μM) was used for comparison and control cells (CON) were treated with vehicle (DMSO). Increased mean fluorescence intensity was indicative of intracellular CO accumulation. Results are expressed as the mean ± S.E.M. of n = 3 independent experiments per each HYCO tested. **p* < 0.01 vs. CON group.

compounds were evaluated in relation to their metal component but were also compared to DMF, an approved fumaric ester-based drug used in the clinic, to a CO-releasing molecule alone (CORM-401) or the combination of the two. For the *in vivo* studies we focused on inflammatory models in which DMF is already known to exert significant protection. We found that Mn-HYCOs donated CO more efficiently to cells and consistently up-regulated Nrf2/HO-1 compared to Ru-HYCOs. However, irrespective of the metal present in the molecule, several HYCOs strongly reduced the production of inflammatory markers in human monocytes stimulated by LPS. When orally administered *in vivo*, HYCO-6 accelerated skin wound closure, HYCO-6 and HYCO-13 reduced psoriasis symptoms equalling or surpassing the effect of DMF, and HYCO-3 and HYCO-13 significantly ameliorated scores of motor dysfunction in the EAE mouse model that mimics human multiple sclerosis, with HYCO-13 showing efficacy at all doses tested. Overall, these findings in pre-clinical models clearly support Nrf2 targeting and

CO delivery for clinical applications and emphasize the therapeutic utility of selected HYCOs to ameliorate the outcome of diseases in which inflammation is a prominent feature.

We recently reported on the preparation of Mn- and Ru-HYCOs and shown that they release CO to haemoglobin in cuvette and also to microglia cells in culture [33]. We report here in THP1 and HaCaT human cell lines that exposure to Mn-HYCOs resulted in a higher CO intracellular accumulation compared to Ru compounds. This could be due to a different ability of HYCOs to penetrate cells, although we favour the idea that a fast CO release in the culture medium by Ru carbonyls will prevent these compounds from effectively donating CO to cells. It is also possible that Ru-HYCOs can release CO only to an appropriate acceptor with very high affinity for CO (for example, haemoglobin) or present in high quantities. This is a valid hypothesis, which is supported by our data demonstrating that oral administration of the Ru-based HYCO-6 to mice augmented blood COHb at 1, 6 and 24 h, unequivocally indicating that the compound can donate CO *in vivo*. We have also reported previously that the Ru-containing CORM-2 and CORM-3, unlike other CO-RMs, do not spontaneously release CO in aqueous solutions as assessed by a CO electrode [43] but still provide pharmacological effects in biological systems that involves CO [25,27].

In conjunction with a more pronounced CO donating capacity by Mn-HYCOs compared to Ru-HYCOs, we also observed that the Mn compounds induced a consistent and stronger activation of Nrf2 and HO-1, indicating that penetration of HYCOs is an important factor explaining their effects since the molecules have to enter the cell for activation of Nrf2. Curiously, the Ru-HYCOs activated Nrf2 without necessarily inducing HO-1 and this effect was especially evident in HaCaT cells, suggesting that mechanisms other than Nrf2 are important for HO-1 induction (for example the repressor BACH1) [44]. Indeed, we observed that some HYCOs caused a mild initial increase of intracellular ROS production, which may contribute to modulation of Nrf2/HO-1. In kinetic studies we demonstrated that Nrf2 activation always preceded HO-1 expression in both THP1 and HaCaT cells. Furthermore, a long-lasting HO-1 up-regulation up to 24 h was observed only in cells treated with HYCO-3, while at this time point HO-1 had already returned to basal levels in the presence of the other HYCOs. These results could be once again a consequence of compounds penetrating differently or having a different metabolism within cells. We note that the modifications from HYCO-3 to HYCO-7 or HYCO-13 (Mn-HYCOs) and from HYCO-6 to HYCO-11 (Ru-HYCOs) employing nitrogen-containing linkers were carried out to increase their water solubility. Even though these modifications did not render the compounds water soluble, the behaviour of the molecules might have been influenced by the linkers once coming in contact with culture media or biological fluids *in vivo*.

In THP1 cells challenged with LPS several HYCOs strongly reduced the production of a number of inflammatory markers, highlighting the potent anti-inflammatory action of these complexes. The inhibition of pro-inflammatory cytokines was not due to toxicity and was independent from down-regulation of the pro-inflammatory factor NF-κB, since viability was not affected and NF-κB was still highly expressed in THP1 cells exposed to LPS in the presence of HYCOs. HYCO-13 was the most effective compound but, surprisingly, also HYCO-6 was efficient despite displaying lower CO donating and HO-1 inducing capacity compared to Mn-HYCOs *in vitro*. These effects may be linked to the ability of HYCO-6 to raise glutathione levels and these results indicated to us the importance of keeping HYCO-6 for *in vivo* testing. Likewise, HYCO-3 was also kept in our screening assay because we showed previously that this compound significantly diminished pro-inflammatory markers in different organs of mice treated with LPS [32] even though this compound did not really decrease inflammation in THP1 cells. Importantly, HYCOs were also more powerful than an equal concentration of CORM-401 alone, DMF alone or the two molecules combined, suggesting that incubating cells with the hybrids as a single molecule achieves stronger efficacy than the two separate entities. This

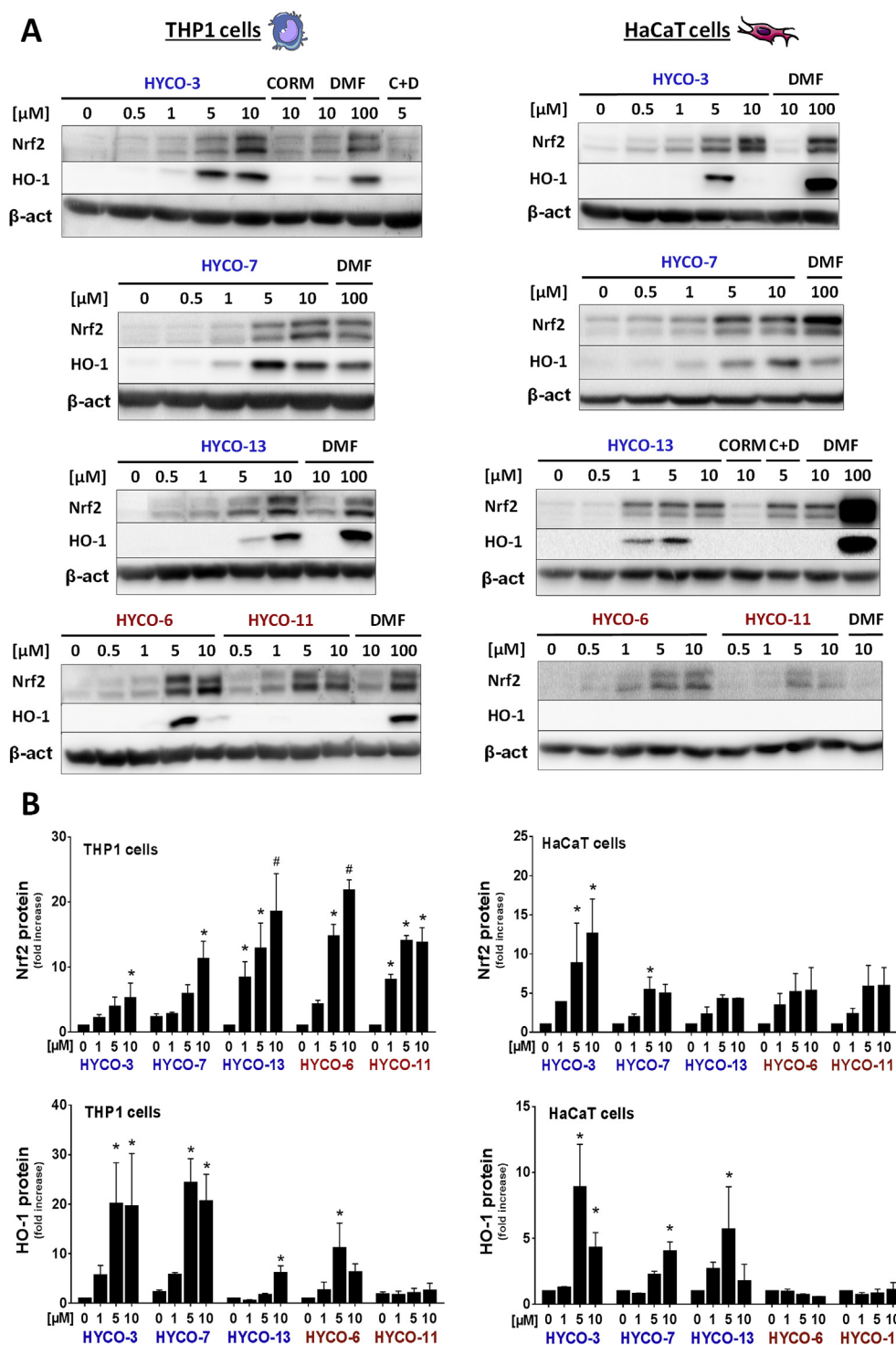



Fig. 3. HYCOs induce Nrf2 and HO-1 protein expression *in vitro*. THP1 (left panel) and HaCaT cells (right panel) were treated with HYCOs at the concentrations indicated for 6 h. CORM-401 (CORM, 10 μM), dimethylfumarate (DMF, 10 or 100 μM) or a combination of the two compounds (C + D, 5 μM each) were used for comparison. Nrf2 and HO-1 protein expression were measured by Western blotting at the end of the incubation (β-actin used as a loading control). Representative blotting of experiments conducted in triplicate are shown in A while the densitometric analysis is shown in B. Densitometric bands of Nrf2 and HO-1 were normalized to β-actin and data expressed as fold-increase compared to untreated cells (0 μM). Results are expressed as the mean ± S.E.M. of n = 3 independent experiments. *p < 0.05 vs. 0 μM; #p < 0.01 vs. 0 μM.

is in line with our original hypothesis for the design of HYCOs with dual pharmacological activity [30–33] as we observed that: 1) CORM-401 alone increases CO in cells but does not induce Nrf2/HO-1; 2) DMF alone used at the same concentrations of HYCOs does not activate Nrf2/HO-1 and, predictably, does not increase intracellular CO.

Before examining the effect of HYCOs in mice, we first determined blood COHb levels as an index of CO delivery after oral gavage of the Mn-HYCOs and HYCO-6. All HYCOs caused an increase in COHb with maximal levels measured at 1 h after treatment. HYCO-13 induced the highest increase in blood COHb reaching ~5%, which is well below the safety limits indicated by the FDA in trials using CO gas (12–14%

COHb). The superior ability of HYCO-13 to donate CO *in vivo* may be correlated to its chemical modification that was introduced to facilitate water solubility compared to HYCO-3 and HYCO-7. We then examined HYCO-6 and HYCO-13 as representatives of Ru- and Mn-HYCOs in wound healing. HYCO-13 and HYCO-6 accelerated wound closure in a scratch assay *in vitro* and their effect was again more pronounced than that of CORM-401 or DMF alone or the combination of the two. In contrast, HYCO-6 given orally was the only compound accelerating wound closure in a skin wound model *in vivo*. It needs to be pointed out that HYCOs applied topically did not ameliorate wound closure (data not shown). This may be due to inefficient absorption and/or the dose

THP1 cells 

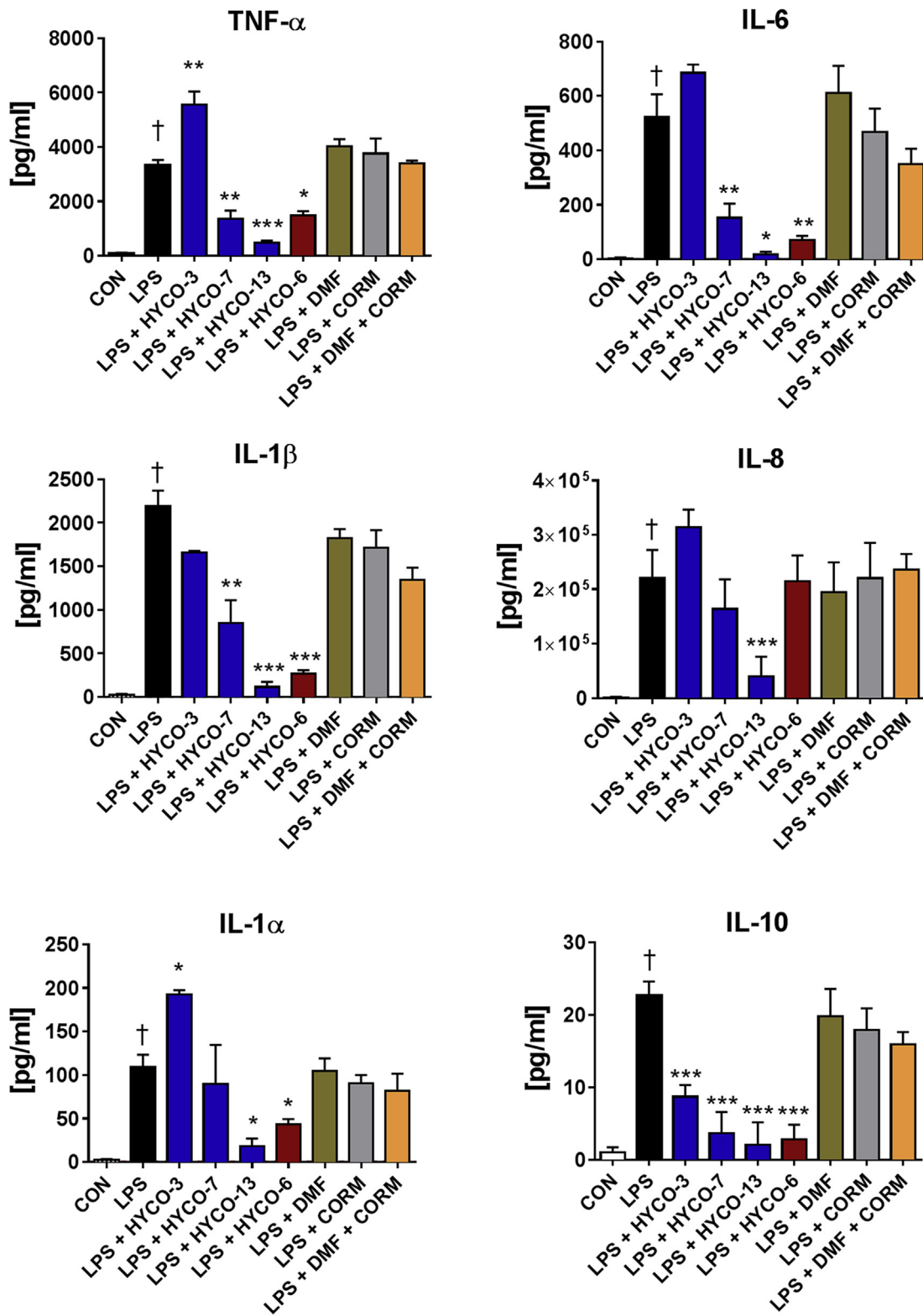

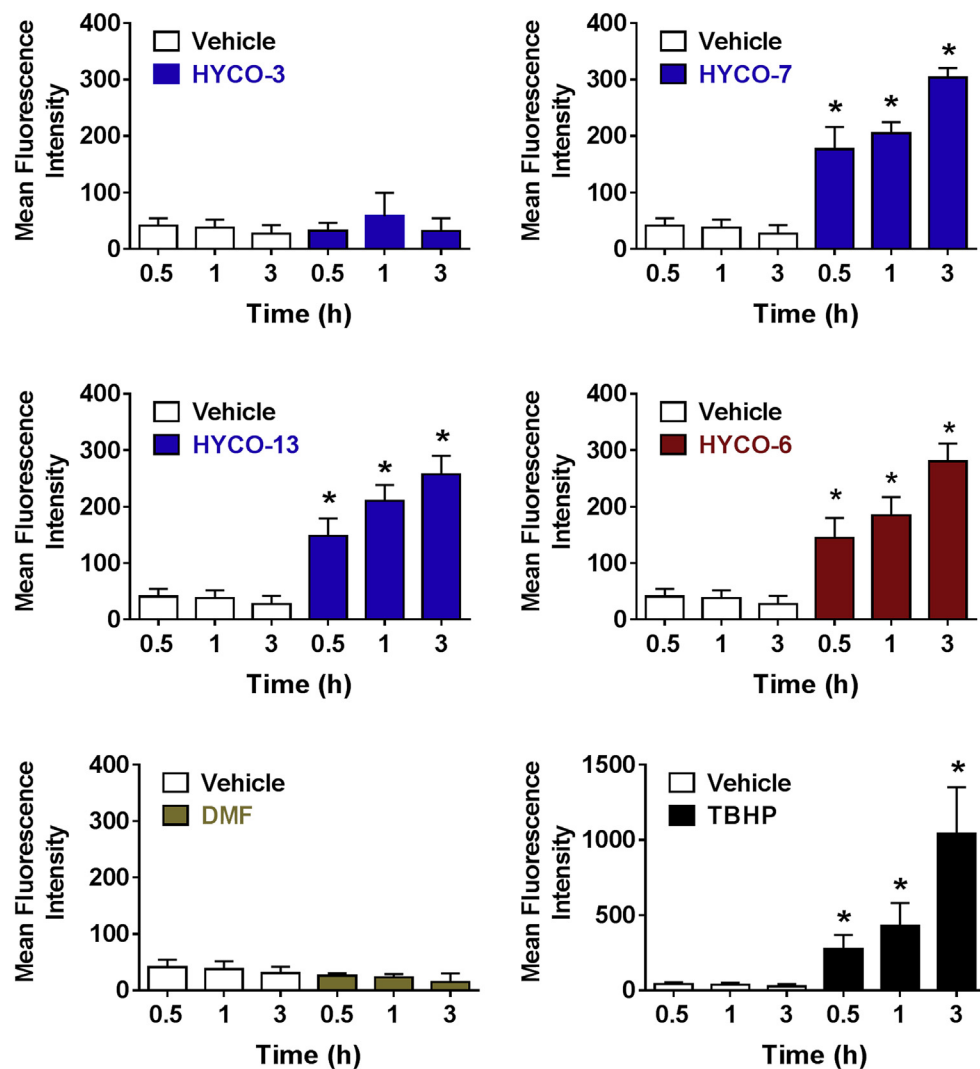


Fig. 4. Effect of HYCOs on the production of inflammatory cytokines in THP1 cells challenged with lipopolysaccharide (LPS). THP1 cells were incubated with LPS (1 µg/ml) in the presence of DMSO (CON), HYCO-3, HYCO-6, HYCO-7, HYCO-13, dimethyl fumarate (DMF), CORM-401 (CORM) or DMF + CORM-401 (5 µM each, final concentration). After 24 h incubation, the amount of various cytokines released in the medium was quantified by the Luminex assay. Results represent the average of 3 to 6 independent experiments and are expressed in pg/ml. †p < 0.05 vs. control; *p < 0.05 vs. LPS; **p < 0.01 vs. LPS; ***p < 0.001 vs. LPS.

THP1 cells 

A



B

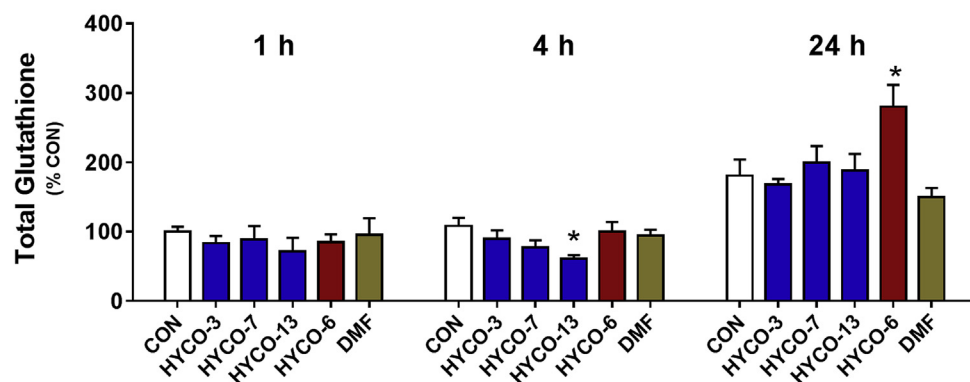


Fig. 5. Effect of HYCOs on intracellular ROS production and glutathione levels. (A) THP1 cells were loaded initially with the fluorescent dye 2'-7'- dichlorodihydrofluorescein diacetate (H2DCFDA) and then incubated in the presence of DMSO (vehicle) HYCO-3, HYCO-6, HYCO-7, HYCO-13 or dimethyl fumarate (DMF) (5 μ M final concentration each). *Tert*-butyl hydroperoxide (TBHP, 5 μ M) was used as a positive control. After 0.5, 1 or 3 h, cells were collected and the mean fluorescent intensity was measured by flow cytometry. Results represent the average of 3 independent experiments. *p < 0.05 vs. vehicle. (B) THP1 cells were treated for 1, 4 or 24 h with the compounds tested in A (5 μ M each). Cells were then collected for the assessment of total glutathione. Results are expressed as the mean \pm S.E.M. of n = 6 independent experiments. *p < 0.05 vs. CON.

of HYCOs used for topical application. However, we did not perform a dose-dependent study to exclude the possibility that the compounds do not work topically, nor were we able to assess whether HYCOs can penetrate and deliver CO to the skin under these conditions. Concomitantly, HYCO-6 and HYCO-13 decreased the production of IL-8

in *in vitro* models of psoriasis and were very efficient in reducing erythema, epidermis thickness and scaling in mice treated with imiquimod to induce psoriasis. The effect was stronger with HYCOs than with DMF, supporting the dual activity advantage of our compounds in this model. Finally, the effects of Mn-based HYCO-3 and HYCO-13 were

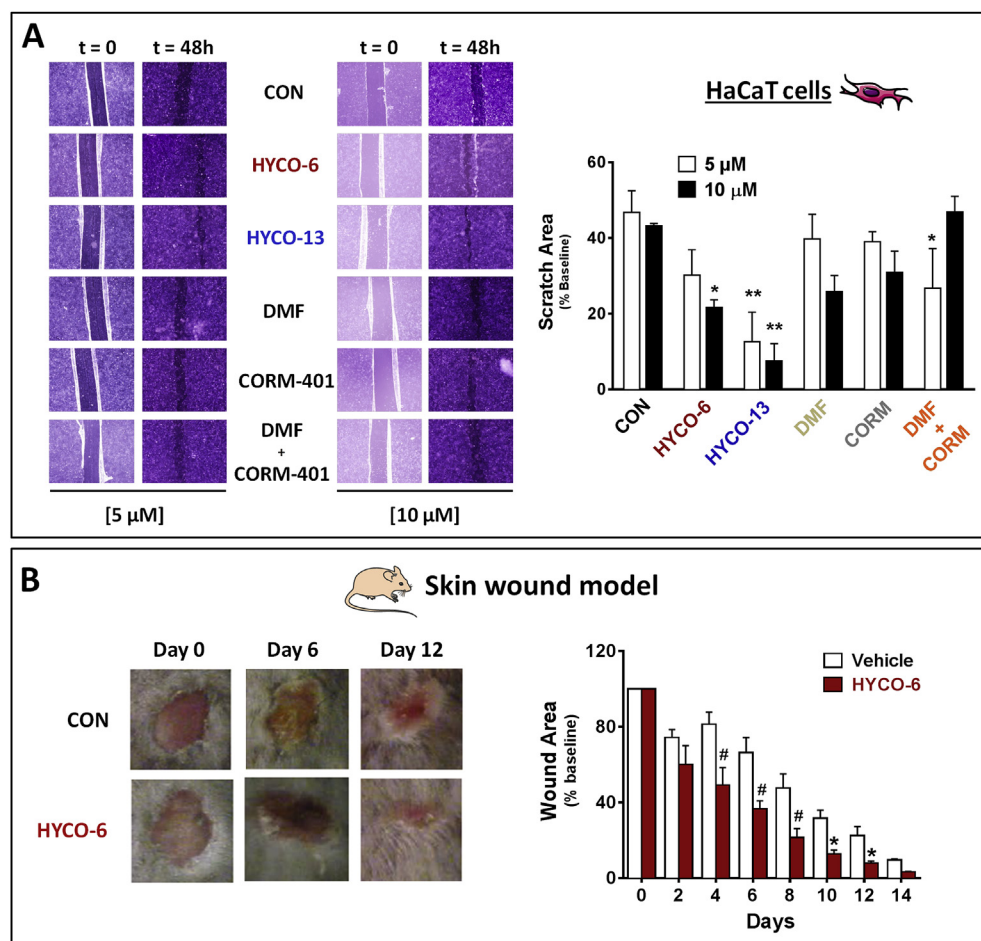


Fig. 6. HYCOs accelerate skin wound healing *in vitro* and *in vivo*. (A) Representative pictures (left panel) of the scratch test conducted on HaCaT keratinocytes treated with 5 or 10 μM HYCO-6, HYCO-13, dimethyl fumarate (DMF), CORM-401 or DMF + CORM-401. Control is represented by cells treated with DMSO (vehicle). The scratch area (right panel) was calculated after 48 h of treatment and expressed as percentage of baseline (time 0, 100%). Results represent the mean \pm S.E.M. of n = 4 independent experiments. *p < 0.05 vs. CON; **p < 0.01 vs. CON. (B) Representative pictures of skin wounds taken at day 0, 6 and 12 after the injury in mice treated with vehicle (DMSO-sesame oil) or HYCO-6 (40 mg/kg daily by oral gavage). Wound areas were then calculated by Image J software at different days after skin injury. Results represent the mean \pm S.E.M. of n = 8 independent experiments. *p < 0.05 vs. time 0; #p < 0.01 vs. time 0.

compared to the effect elicited by DMF in the multiple sclerosis model in mice. This is the same pre-clinical model used to prove that DMF was a useful therapeutic candidate before the progression of the compound to human clinical trials. As expected, DMF significantly attenuated motor dysfunction in the peak phase of the disease and the highest dose also ameliorated this parameter during the remission phase. We observed that HYCO-3 and HYCO-13 (25 mg/kg) given twice a day were not as efficient as DMF in diminishing motor dysfunction at peak phase but decreased this parameter more effectively at the end of the relapse phase compared to DMF. We speculate that the regular daily boosts of CO from HYCOs may be responsible for this effect since other CO-RMs and CO gas have already exhibited therapeutic potential in multiple sclerosis [39,45]. We also note that HYCOs were given to mice by oral gavage every day for seven days in the psoriasis model and for 40 days in the multiple sclerosis model without evident toxicity, which is a promising outcome for the long-term use of these compounds.

In conclusion, we showed in this work that HYCOs possess strong anti-inflammatory activity *in vitro* and their effects translated into therapeutic effectiveness in three *in vivo* models in which inflammation is a predominant factor. Since little is known on the chemical reactivity of metal carbonyl complexes within biological systems, our investigation highlights a concept that we started to elaborate recently [29,32] on the methodologies to characterize the pharmacological properties of CO-RMs, HYCOs and in general, compounds containing metal carbonyls. That is, a comprehensive and detailed set of *in vitro* and *in vivo* assays must be developed and carried out by interested laboratories to fully understand the behaviour and the potential therapeutic activity of these prototypical molecules that links their structure and ability to release CO to desired pharmacological effects in animal models of disease.

Author disclosure statement

Patent applications have been filed by the Société d'Accélération de Transfert Technologique (SATT)-Ile de France Innovation for the HYCOs technology with Michael Rivard, Anthony Ollivier, Roberto Motterlini and Roberta Foresti as inventors.

Authors' contribution

ZEA performed experiments and analyzed the data. AO and MR synthesized HYCOs and read the manuscript. SM performed experiments, analyzed the data and revised the manuscript. RM and RF conceived and designed the study, performed experiments, analyzed the data and wrote the manuscript.

Acknowledgements

The authors would like to thank Dr Rachid Souktani and Cecile Lecoite of the animal facility platform for their assistance, Mathieu Surenaud for help with the Luminex technique and Aurelie Guguin and Adeline Henry of the flow cytometry platform. The authors also thank Bioalternatives (Gençay, France) and Imavita (Ramonville St. Agne, France) for their help with the psoriasis models and Washington Biotechnology (USA) for their assistance with the multiple sclerosis model. This work was supported by grants from the Agence National de la Recherche (Project CO-HEAL) and the Société d'Accélération de Transfert Technologique (SATT)-Ile de France Innovation (Project HYCOs).

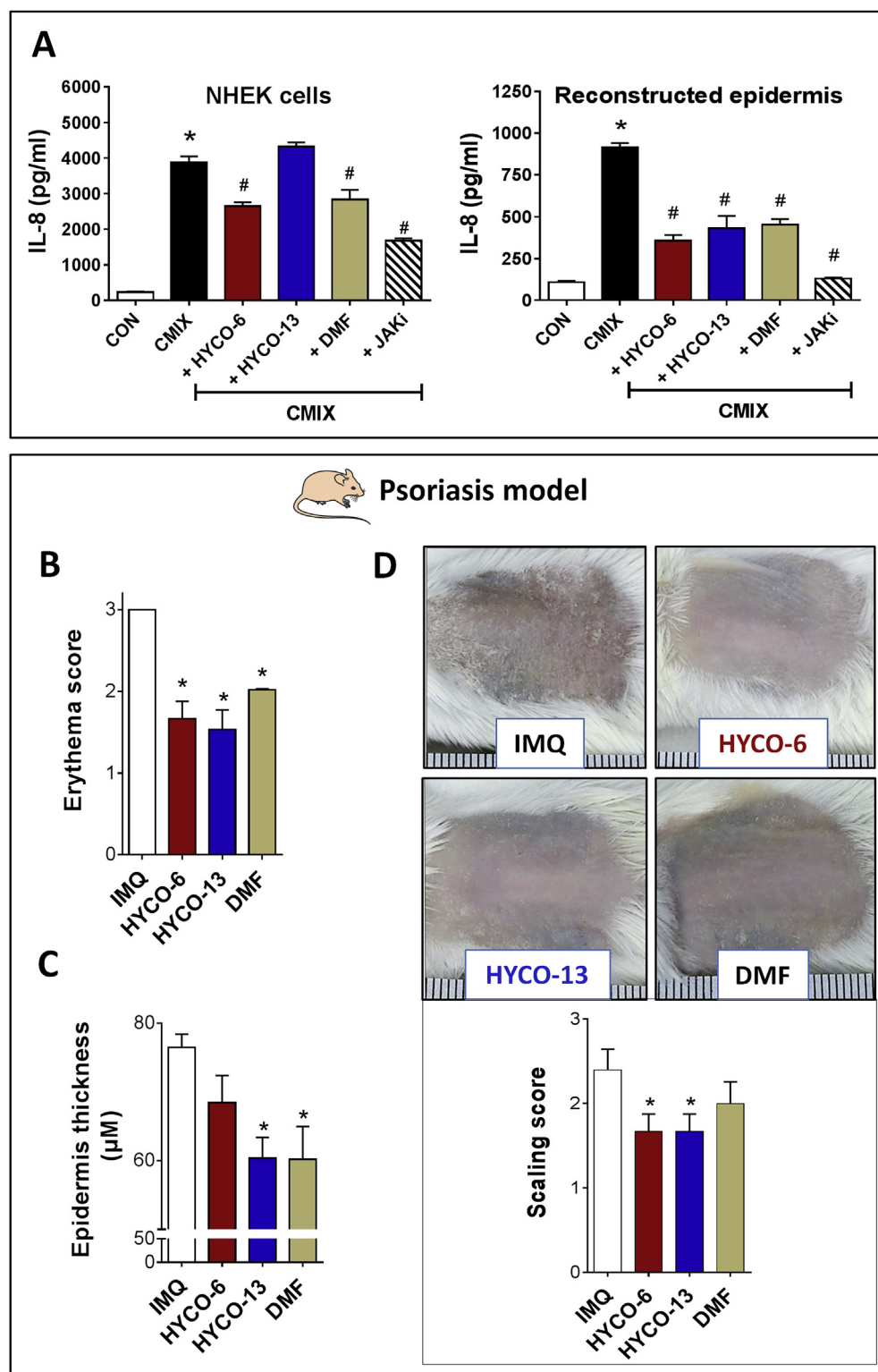



Fig. 7. HYCOs ameliorate psoriasis-like skin disease *in vitro* and *in vivo*. (A) Normal human epidermal keratinocytes (NHEK) and reconstructed human epidermis were treated with 2 μM HYCO-6, HYCO-13, dimethyl fumarate (DMF) or a positive control (Janus kinase inhibitor, JAKi 10 μM) for 24 h and then challenged with a cytokine mixture (CMIX) as described in Materials and Methods. IL-8 release was assessed by an ELISA assay. Results represent the mean ± S.E.M. of n = 8 independent experiments. *p < 0.05 vs. control (CON); #p < 0.05 vs. CMIX. (B, C, D) Psoriasis was induced in mice by treatment with imiquimod (IMQ) and then orally administered with 20 mg/kg (twice daily) HYCO-3, HYCO-6 or DMF while control mice received the vehicle as described in Materials and Methods. Erythema score (B), epidermis thickness (C) and scaling (D, see representative pictures and average score) were then evaluated after 3 days. Results represent the mean ± S.E.M. of n = 6 independent experiments. *p < 0.05 vs. IMQ.

Abbreviations

CO carbon monoxide
 COHb carbon monoxide hemoglobin
 CO-RMs carbon monoxide-releasing molecules
 DMF dimethylfumarate
 DMEM Dulbecco's Modified Eagle Medium
 DMSO dimethyl sulfoxide
 DPBS Dulbecco Phosphate Buffer Solution

DTNB 5,5' dithiobis-(2-nitrobenzoic acid)
 EAE experimental allergic encephalitis
 FBS fetal bovine serum
 H2DCFDA 2',7'- dichlorodihydrofluorescein diacetate
 GSH reduced glutathione
 HaCaT human immortalized keratinocytes
 HO-1 heme oxygenase-1
 HYCOs HYbrid CO-releasing molecules
 LDH lactate dehydrogenase

 Multiple sclerosis model

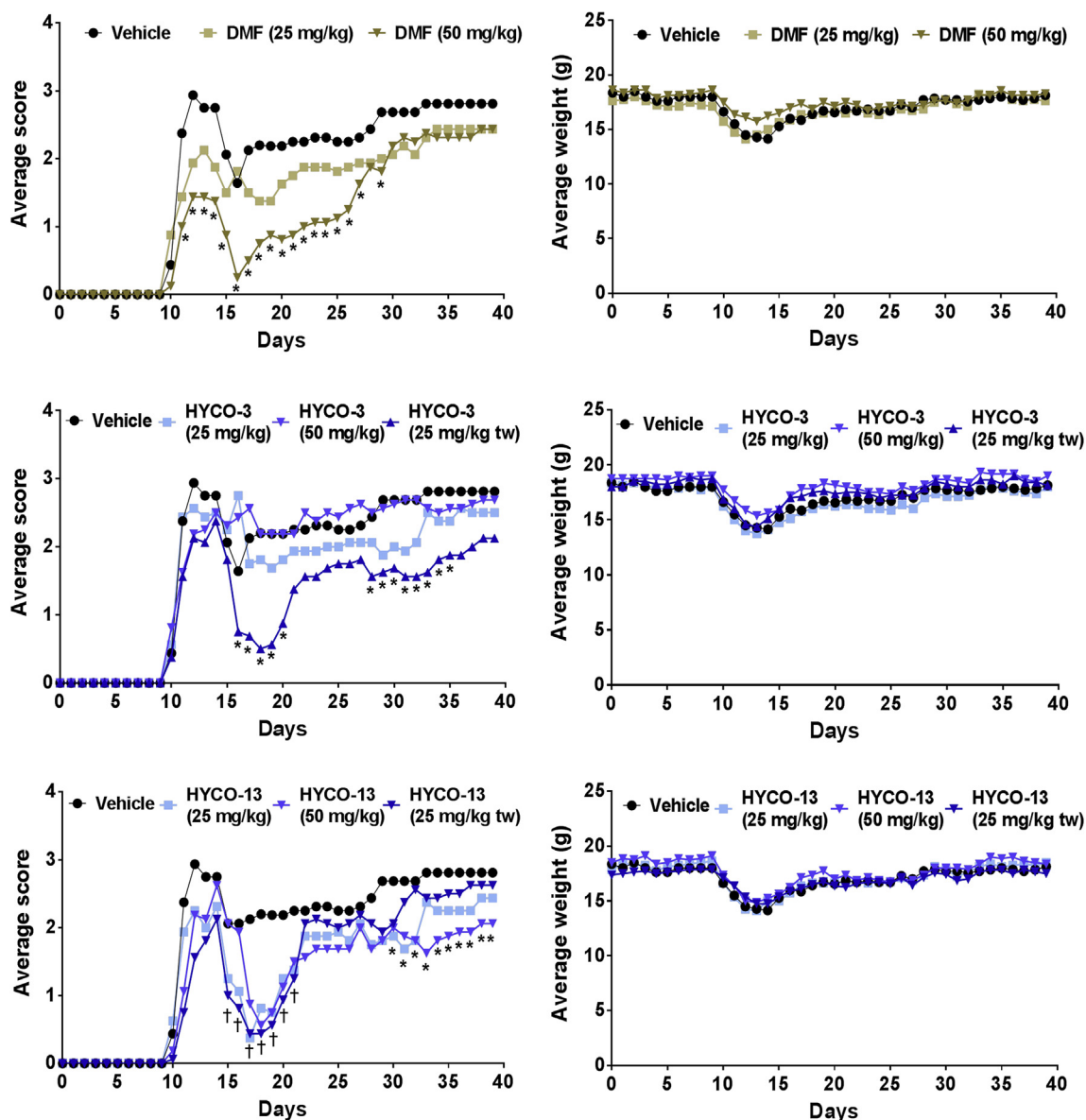


Fig. 8. HYCOs improve the clinical manifestations of multiple sclerosis. (A) A mouse model of experimental allergic encephalitis (EAE) that mimic multiple sclerosis in humans was used (see Materials and Methods) to assess the potential therapeutic effects of HYCOs. After the onset of the disease, mice were daily administered by oral gavage with dimethyl fumarate (DMF), HYCO-3 or HYCO-13 at the doses indicated for 40 days (“tw” refers to the drug given twice a day). The control group received vehicle (DMSO-sesame oil). The clinical signs of the disease were scored (*left panel*) in conjunction with measurements of body weight over time (*right panel*). Results represent the mean ± S.E.M. of n = 8 independent experiments. *p < 0.05 vs. vehicle.

LPS	lipopolysaccharide
Mn	manganese
NF-κB	nuclear factor kappa B
NHEK	normal human epidermal keratinocytes
Nrf2	nuclear factor (erythroid-derived 2)-like 2; reactive oxygen species (ROS)
Ru	ruthenium
THP1	human monocytic cells

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101521>.

References

- [1] S.K. Niture, J.W. Kaspar, J. Shen, A.K. Jaiswal, Nrf2 signaling and cell survival, *Toxicol. Appl. Pharmacol.* 244 (1) (2010) 37–42.
- [2] Q. Ma, Role of nrf2 in oxidative stress and toxicity, *Annu. Rev. Pharmacol. Toxicol.* 53 (2013) 401–426.
- [3] L. Baird, A.T. Dinkova-Kostova, The cytoprotective role of the Keap1-Nrf2 pathway, *Arch. Toxicol.* 85 (4) (2011) 241–272.
- [4] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an interface between redox and intermediary metabolism, *Trends Biochem. Sci.* 39 (4) (2014) 199–218.
- [5] M.L. Kerns, J.M. Hakim, R.G. Lu, Y. Guo, A. Berroth, R.L. Kaspar, P.A. Coulombe, Oxidative stress and dysfunctional NRF2 underlie pachyonychia congenita phenotypes, *J. Clin. Invest.* 126 (6) (2016) 2356–2366.
- [6] N. Kubben, W. Zhang, L. Wang, T.C. Voss, J. Yang, J. Qu, G.H. Liu, T. Misteli, Repression of the antioxidant NRF2 pathway in premature aging, *Cell* 165 (6) (2016) 1361–1374.

- [7] L. Kappos, R. Gold, D.H. Miller, D.G. Macmanus, E. Havrdova, V. Limmroth, C.H. Polman, K. Schmierer, T.A. Youstry, M. Yang, M. Eraksoy, E. Meluzinova, I. Rektor, K.T. Dawson, A.W. Sandrock, G.N. O'Neill, Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study, *Lancet* 372 (2008) 1463–1472.
- [8] P.J. Altmeyer, U. Matthes, F. Pawlak, K. Hoffmann, P.J. Frosch, P. Ruppert, S.W. Wassilew, T. Horn, H.W. Kreyse, G. Lutz, Antipsoriatic effect of fumaric acid derivatives. Results of a multicenter double-blind study in 100 patients, *J. Am. Acad. Dermatol.* 30 (6) (1994) 977–981.
- [9] K. Ghoreschi, J. Bruck, C. Kellerer, C. Deng, H. Peng, O. Rothfuss, R.Z. Hussain, A.R. Gocke, A. Respa, I. Glocova, N. Valtcheva, E. Alexander, S. Feil, R. Feil, K. Schulze-Osthoff, R.A. Rupec, A.E. Lovett-Racke, R. Dringen, M.K. Racke, M. Rocken, Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells, *J. Exp. Med.* 208 (11) (2011) 2291–2303.
- [10] E.L. Mills, D.G. Ryan, H.A. Prag, D. Dikovskaya, D. Menon, Z. Zaslona, M.P. Jedrychowski, A.S.H. Costa, M. Higgins, E. Hams, J. Szpyt, M.C. Runtsch, M.S. King, J.F. McGouran, R. Fischer, B.M. Kessler, A.F. McGettrick, M.M. Hughes, R.G. Carroll, L.M. Booty, E.V. Knatko, P.J. Meakin, M.L.J. Ashford, L.K. Modis, G. Brunori, D.C. Sevin, P.G. Fallon, S.T. Caldwell, E.R.S. Kunji, E.T. Chouchani, C. Frezza, A.T. Dinkova-Kostova, R.C. Hartley, M.P. Murphy, L.A. O'Neill, Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1, *Nature* 556 (7699) (2018) 113–117.
- [11] R. Singh, S. Chandrashekarappa, S.R. Bodduluri, B.V. Baby, B. Hegde, N.G. Kotla, A.A. Hiwale, T. Saiyed, P. Patel, M. Vijay-Kumar, M.G.I. Langille, G.M. Douglas, X. Cheng, E.C. Rouchka, S.J. Waigel, G.W. Dryden, H. Alatassi, H.G. Zhang, B. Haribabu, P.K. Vemula, V.R. Jala, Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway, *Nat. Commun.* 10 (1) (2019) 89.
- [12] J. Alam, D. Stewart, C. Touchard, S. Boinapally, A.M. Choi, J.L. Cook, Nrf2, a Cap'nCollar transcription factor, regulates induction of the heme oxygenase-1 gene, *J. Biol. Chem.* 274 (37) (1999) 26071–26078.
- [13] E. Balogun, M. Hoque, P. Gong, E. Killen, C.J. Green, R. Foresti, J. Alam, R. Motterlini, Curcumin activates the heme oxygenase-1 gene via regulation of Nrf2 and the antioxidant responsive element, *Biochem. J.* 371 (3) (2003) 887–895.
- [14] G.S. Drummond, J. Baum, M. Greenberg, D. Lewis, N.G. Abraham, HO-1 over-expression and underexpression: clinical implications, *Arch. Biochem. Biophys.* 673 (2019) 108073.
- [15] R. Stocker, Y. Yamamoto, A.F. McDonagh, A.N. Glazer, B.N. Ames, Bilirubin is an antioxidant of possible physiological importance, *Science* 235 (1987) 1043–1046.
- [16] J.E. Clark, R. Foresti, C.J. Green, R. Motterlini, Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress, *Biochem. J.* 348 (3) (2000) 615–619.
- [17] J.E. Clark, R. Foresti, P. Sarathchandra, H. Kaur, C.J. Green, R. Motterlini, Heme oxygenase-1-derived bilirubin ameliorates post-ischemic myocardial dysfunction, *Am. J. Physiol. Heart Circ. Physiol.* 278 (2) (2000) H643–H651.
- [18] R. Foresti, C.J. Green, R. Motterlini, Generation of bile pigments by heme oxygenase: a refined cellular stratagem in response to stressful insults, *Biochem. Soc. Symp.* 71 (71) (2004) 177–192.
- [19] I.A. Sammut, R. Foresti, J.E. Clark, D.J. Exon, M.J.J. Vesely, P. Sarathchandra, C.J. Green, R. Motterlini, Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1, *Br. J. Pharmacol.* 125 (7) (1998) 1437–1444.
- [20] L.E. Otterbein, F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, A.M. Choi, Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway, *Nat. Med.* 6 (4) (2000) 422–428.
- [21] G. Balla, H.S. Jacob, J. Balla, M. Rosenberg, K.A. Nath, F. Apple, J.W. Eaton, G.M. Vercellotti, Ferritin: a cytoprotective antioxidant stratagem of endothelium, *J. Biol. Chem.* 267 (1992) 18148–18153.
- [22] A. Ayer, A. Zarjou, A. Agarwal, R. Stocker, Heme oxygenases in cardiovascular health and disease, *Physiol. Rev.* 96 (4) (2016) 1449–1508.
- [23] R. Motterlini, R. Foresti, Heme oxygenase-1 as a target for drug discovery, *Antioxidants Redox Signal.* 20 (11) (2014) 1810–1826.
- [24] R. Motterlini, J.E. Clark, R. Foresti, P. Sarathchandra, B.E. Mann, C.J. Green, Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities, *Circ. Res.* 90 (2) (2002) e17–e24.
- [25] J.E. Clark, P. Naughton, S. Shurey, C.J. Green, T.R. Johnson, B.E. Mann, R. Foresti, R. Motterlini, Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule, *Circ. Res.* 93 (2) (2003) e2–e8.
- [26] M.L. Ferrandiz, N. Maicas, I. Garcia-Arnandis, M.C. Terencio, R. Motterlini, I. Devesa, L.A. Joosten, W.B. van den Berg, M.J. Alcaraz, Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis, *Ann. Rheum. Dis.* 67 (2008) 1211–1217.
- [27] R. Motterlini, R. Foresti, Biological signaling by carbon monoxide and carbon monoxide-releasing molecules (CO-RMs), *Am. J. Physiol. Cell Physiol.* 312 (3) (2017) C302–C313.
- [28] A. Yabluchanskiy, P. Sawle, S. Homer-Vanniasinkam, C.J. Green, R. Foresti, R. Motterlini, CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of hemorrhagic stroke, *Crit. Care Med.* 40 (2) (2012) 544–552.
- [29] L. Braud, M. Pini, L. Muchova, S. Manin, H. Kitagishi, D. Sawaki, G. Czibik, J. Ternacle, G. Derumeaux, R. Foresti, R. Motterlini, Carbon monoxide-induced metabolic switch in adipocytes improves insulin resistance in obese mice, *JCI Insight* 3 (22) (2018) e123485.
- [30] J.L. Wilson, S. Fayad-Kobeissi, S. Oudir, B. Haas, B.W. Michel, J.L. Dubois-Rande, A. Ollivier, T. Martens, M. Rivard, R. Motterlini, R. Foresti, Design and synthesis of novel hybrid molecules that activate the transcription factor Nrf2 and simultaneously release carbon monoxide, *Chemistry* 20 (45) (2014) 14698–14704.
- [31] A. Nikam, A. Ollivier, M. Rivard, J.L. Wilson, K. Mebarki, T. Martens, J.L. Dubois-Rande, R. Motterlini, R. Foresti, Diverse Nrf2 activators coordinated to cobalt carbonyls induce heme oxygenase-1 and release carbon monoxide in vitro and in vivo, *J. Med. Chem.* 59 (2) (2016) 756–762.
- [32] R. Motterlini, A. Nikam, S. Manin, A. Ollivier, J.L. Wilson, S. Djouadi, L. Muchova, T. Martens, M. Rivard, R. Foresti, HYCO-3, a dual CO-releaser/Nrf2 activator, reduces tissue inflammation in mice challenged with lipopolysaccharide, *Redox Biol.* 20 (2019) 334–348.
- [33] A. Ollivier, R. Foresti, Z. El Ali, T. Martens, H. Kitagishi, R. Motterlini, M. Rivard, Design and biological evaluation of manganese- and ruthenium-based hybrid CO-releasing molecules (HYCOs), *ChemMedChem* 14 (2019) 1684–1691.
- [34] B.W. Michel, A.R. Lippert, C.J. Chang, A reaction-based fluorescent probe for selective imaging of carbon monoxide in living cells using a palladium-mediated carbonylation, *J. Am. Chem. Soc.* 134 (38) (2012) 15668–15671.
- [35] S. Fayad-Kobeissi, J. Ratovonantenaina, H. Dabire, J.L. Wilson, A.M. Rodriguez, A. Berdeux, J.L. Dubois-Rande, B.E. Mann, R. Motterlini, R. Foresti, Vascular and angiogenic activities of CORM-401, an oxidant-sensitive CO-releasing molecule, *Biochem. Pharmacol.* 102 (2016) 64–77.
- [36] S.H. Crook, B.E. Mann, J.A.H.M. Meijer, H. Adams, P. Sawle, D. Scapens, R. Motterlini, [Mn(CO)₄(S₂CNMe(CH₂CO₂H))], a new water-soluble CO-releasing molecule, *Dalton Trans.* 40 (16) (2011) 4230–4235.
- [37] F.L. Rodkey, T.A. Hill, L.L. Pitts, R.F. Robertson, Spectrophotometric measurement of carboxyhemoglobin and methemoglobin in blood, *Clin. Chem.* 25 (8) (1979) 1388–1393.
- [38] A. Grochot-Przeczek, R. Lach, J. Mis, K. Skrzypek, M. Gozdecka, P. Sroczynska, M. Dubiel, A. Rutkowski, M. Kozakowska, A. Zagorska, J. Walczynski, H. Was, J. Kotlinowski, J. Drukala, K. Kurowski, C. Kieda, Y. Herauld, J. Dulak, A. Jozkowicz, Heme oxygenase-1 accelerates cutaneous wound healing in mice, *PLoS One* 4 (6) (2009) e5803.
- [39] P. Fagone, K. Mangano, C. Quattrocchi, R. Motterlini, R. Di Marco, G. Magro, N. Penacho, C.C. Romao, F. Nicoletti, Prevention of clinical and histological signs of proteolipid protein (PLP)-induced experimental allergic encephalomyelitis (EAE) in mice by the water-soluble carbon monoxide-releasing molecule (CORM)-A1, *Clin. Exp. Immunol.* 163 (3) (2011) 368–374.
- [40] P. Kaczara, R. Motterlini, G.M. Rosen, B. Augustynek, P. Bednarczyk, A. Szweczyk, R. Foresti, S. Chlopicki, Carbon monoxide released by CORM-401 uncouples mitochondrial respiration and inhibits glycolysis in endothelial cells: a role for mitoBK channels, *Biochim. Biophys. Acta* 1847 (10) (2015) 1297–1309.
- [41] J.L. Wilson, F. Bouillaud, A.S. Almeida, H.L. Vieira, M.O. Ouidja, J.L. Dubois-Rande, R. Foresti, R. Motterlini, Carbon monoxide reverses the metabolic adaptation of microglia cells to an inflammatory stimulus, *Free Radic. Biol. Med.* 104 (2017) 311–323.
- [42] R.N. Bhattacharjee, M. Richard-Mohamed, Q. Sun, A. Haig, G. Aboalsamh, P. Barrett, R. Mayer, I. Alhasan, K. Pineda-Solis, L. Jiang, H. Alharbi, M. Saha, E. Patterson, A. Sener, G. Cepinska, A.M. Jevnikar, P.P.W. Luke, CORM-401 reduces ischemia reperfusion injury in an ex vivo renal porcine model of the donation after circulatory death, *Transplantation* 102 (7) (2018) 1066–1074.
- [43] M. Desnard, R. Foresti, D. Morin, M. Dagoussat, A. Berdeux, E. Denamur, S.H. Crook, B.E. Mann, D. Scapens, P. Montravers, J. Boczkowski, R. Motterlini, Differential antibacterial activity against *Pseudomonas aeruginosa* by carbon monoxide-releasing molecules, *Antioxidants Redox Signal.* 16 (2) (2012) 153–163.
- [44] A.K. MacLeod, M. McMahon, S.M. Plummer, L.G. Higgins, T.M. Penning, K. Igarashi, J.D. Hayes, Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds, *Carcinogenesis* 30 (9) (2009) 1571–1580.
- [45] R.A. Chora, P. Fontoura, A. Cunha, T.F. Pais, S. Cardoso, P.P. Ho, L.Y. Lee, R.A. Sobel, L. Steinman, M.P. Soares, Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation, *J. Clin. Invest.* 117 (2) (2007) 438–447.