



## HYCO-3, a dual CO-releaser/Nrf2 activator, reduces tissue inflammation in mice challenged with lipopolysaccharide

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### ABSTRACT

Oxidative stress and inflammation are predominant features of several chronic diseases. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a major arbiter in counteracting these insults via up-regulation of several defensive proteins, including heme oxygenase-1 (HO-1). HO-1-derived carbon monoxide (CO) exhibits anti-inflammatory actions and can be delivered to tissues by CO-releasing agents. In this study we assessed the pharmacological and anti-inflammatory properties of HYCO-3, a dual activity compound obtained by conjugating analogues of the CO-releasing molecule CORM-401 and dimethyl fumarate (DMF), an immunomodulatory drug known to activate Nrf2. HYCO-3 induced Nrf2-dependent genes and delivered CO to cells *in vitro* and tissues *in vivo*, confirming that the two expected pharmacological properties of this agent are achieved. In mice challenged with lipopolysaccharide, orally administered HYCO-3 reduced the mRNA levels of pro-inflammatory markers (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) while increasing the expression of the anti-inflammatory genes ARG1 and IL-10 in brain, liver, lung and heart. In contrast, DMF or CORM-401 alone or their combination decreased the expression of pro-inflammatory genes but had limited influence on anti-inflammatory markers. Furthermore, HYCO-3 diminished TNF- $\alpha$  and IL-1 $\beta$  in brain and liver but not in lung and heart of Nrf2<sup>-/-</sup> mice, indicating that the CO-releasing part of this hybrid contributes to reduction of pro-inflammation and that this effect is organ-specific. These data demonstrate that the dual activity of HYCO-3 results in enhanced efficacy compared to the parent compounds indicating the potential exploitation of hybrid compounds in the development of effective anti-inflammatory therapies.

### 1. Introduction

Inflammation is a process that characterizes acute responses to infection and tissue injury but is also associated with potential damage, especially when occurring persistently during chronic conditions [1–4]. This is why tissues have adapted to the stress inflicted by inflammatory challenges by inducing a series of proteins that serve to quench these

effects and restore tissue homeostasis [5,6]. There are specific transcription factors that work in coordination to either promote (NF- $\kappa$ B) or counteract inflammation, with nuclear factor erythroid 2-related factor 2 (Nrf2) representing a major control step in the antioxidant/anti-inflammatory response [6–10]. Nrf2 up-regulates a battery of cytoprotective genes with multiple functions, including heme oxygenase-1, which degrades heme to generate biliverdin (subsequently transformed

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ARG1, arginase 1; CO, carbon monoxide; COHb, carbonmonoxy hemoglobin; CO-RMs, CO-releasing molecules; EPF, ethyl prop-2-yn-1-yl fumarate; GCLC, glutamate cysteine ligase C; GCLM, glutamate cysteine ligase M; Hb, hemoglobin; HYCOs, HYbrid CO-releasing molecules; HMOX1, heme oxygenase-1 gene; HO-1, heme oxygenase-1 protein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; M1, pro-inflammatory macrophages; M2, anti-inflammatory macrophages; MCP-1/CCL2, monocyte chemoattractant protein-1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6, interleukin-6; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase

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into bilirubin) and carbon monoxide (CO), bioactive products that combat oxidative stress and dampen the production of pro-inflammatory molecules [7,8,11–13]. Importantly, Nrf2 and HO-1 are also involved in the macrophage switch from a pro- (M1) to an anti-inflammatory phenotype (M2), the latter being implicated in the resolution of inflammation and repair and regeneration of tissues [14,15]. Thus, inducers of Nrf2 or HO-1 have been investigated in animal studies and were shown to protect tissues from several types of insults, indicating an interest in using these targets for the discovery of novel drugs [13,16–18]. Indeed, compounds that target Nrf2 have been approved by the USA Food and Drug Administration (for example, dimethylfumarate “DMF-Tecfidera” for multiple sclerosis) or are under investigation for the treatment of inflammatory disease [19–22].

Our group has discovered more than a decade ago CO-releasing molecules (CO-RMs) as agents that deliver precise quantities of CO to biological systems and that are capable of mimicking the beneficial and anti-inflammatory activities of HO-1 [23–25]. More recently, we have synthesized new hybrid molecules (HYCOs) that contain an Nrf2 inducing part coordinated to a CO-releasing part, with the intention of creating compounds that donate CO to provide a first defensive action while simultaneously activating Nrf2 and associated genes for longer-lasting cytoprotective effects. We initially worked on hybrids containing fumaric esters as Nrf2 activators and cobalt-based CO-RMs [26,27], providing the first proof of principle that such kind of compounds are chemically feasible and can actually be generated. The choice of cobalt carbonyl complexes was dictated mainly by the easiness of synthesis and handling, although we were aware that cobalt is not a preferential metal for biological applications. We showed that cobalt-based HYCOs increase Nrf2 and HO-1 *in vitro* and reduce nitrite, a marker of inflammation, in microglia cells and macrophages stimulated with lipopolysaccharide (LPS) [26,27]. In addition, we determined that they generally deliver negligible amounts of CO to cells but that some of them can increase carbonmonoxy hemoglobin (COHb) *in vivo* [27]. In an effort to improve the CO delivery capacity of HYCOs and utilize metals different from cobalt, we have now prepared new compounds containing ruthenium or manganese carbonyls, the latter being more biologically compatible. In this study we report the pharmacological characterization of HYCO-3, a hybrid obtained by conjugating analogues of DMF and CORM-401, which displays a manganese metal center and is capable of liberating up to three molecules of CO [28–30]. We found that HYCO-3 exhibits significant CO releasing properties in cells and *in vivo* and exerts anti-inflammatory activities in organs of mice challenged with LPS. This effect is accompanied by modulation of the macrophages' profile, which display a more pronounced M2 phenotype.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Dimethyl fumarate (DMF), lipopolysaccharide (from *E. Coli* serotype 0127:B8) and all other reagents were purchased from Sigma Aldrich (France) unless otherwise specified. CORM-401 and ethyl prop-2-yn-1-yl fumarate (EPF) were synthesized in our laboratories as previously described [26–28,30]. RPMI-1640 medium, fetal bovine serum (FBS) and L-glutamine were from Lonza, while penicillin, streptomycin and Dulbecco Phosphate Buffer Solution (DPBS) were purchased from Life Technologies (Aubin, France). Antibodies were purchased from Enzo Life Sciences (HO-1 rabbit polyclonal), Cell Signaling Technology ( $\beta$ -actin mouse monoclonal) and Santa Cruz Biotechnology (Nrf2 clone C-20 rabbit polyclonal and Lamin A/C clone N-18 goat polyclonal). Nuclear Extract Kit for the isolation and preparation of nuclear extracts was from Active Motif (Paris, France). The CO sensitive probe COP-1 was kindly provided by Prof. Christopher Chang from the University of California, Berkeley [31].

### 2.2. Synthesis of HYCO-3 and HYCO-6

The steps for the synthesis of the hybrid molecules HYCO-3 and HYCO-6 is reported in [Supplementary Fig. 1](#). All reactions were performed under an atmosphere of argon. Solvents and reagents were used without further purification. Dimanganese decacarbonyl ( $Mn_2(CO)_{10}$ ) was purchased from Strem. 2-(methylamino)ethanol was purchased from Alfa Aesar. Bromopentacarbonylmanganese(I) ( $Mn(CO)_5Br$ ) [32] and (*E*)-4-chloro-4-oxo-2-butenic acid ethyl ester [33] were prepared according to the literature procedures and spectral properties were in accordance with those reported. For the *in vitro* experiments in cultured cells, HYCOs were solubilized in dimethyl sulfoxide, while for the *in vivo* experiments HYCO-3 was solubilized in sesame oil.

### 2.3. Cell culture

BV2 mouse microglia cells were kindly donated by Professor Rosario Donato (University of Perugia) and Dr Adjanie Patabendige (Institute of Infection & Global Health, Liverpool) and were cultured as previously described by our group [34,35]. Briefly, cells were grown in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml) and 2 mM L-glutamine at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>. When they reached 80% confluence, cells were seeded onto 24-well or 6-well plates to perform further experiments.

### 2.4. Cell cytotoxicity assay

The viability of BV2 microglia cells was assessed by measuring the amount of lactate dehydrogenase (LDH) released from damaged cells in the medium using a Cytotoxicity Detection Kit (LDH) (Roche Applied Science). Briefly, cells were seeded in 24-well plates and treated for 24 h with HYCO-3, HYCO-6, CORM-401, EPF or DMF at different concentrations (0.5, 1, 5 and 10  $\mu$ M). X-100 Triton solution (2%) prepared in medium was used as a positive control to cause 100% cytotoxicity. At the end of the incubation, plates were centrifuged at 300g for 5 min and 100  $\mu$ l of supernatant were collected and transferred to a 96-well plate. After addition of the reaction mixture of the LDH kit, absorbance was measured at 485 nm using an Appliskan filter-based multimode microplate reader (Thermo Scientific). Data were expressed as % of the values obtained with X-100 Triton.

### 2.5. Determination of nitrite production

Nitrite production, an index of inflammation in response to LPS, was assessed in the cell culture supernatants using the Griess reagent assay as previously described [26,36]. Briefly, BV2 microglia cultured in 24-well plates were stimulated for 24 h with 0.5  $\mu$ g/ml LPS in the absence or presence of HYCO-3, CORM-401 or EPF at different concentrations. At the end of the incubation, plates were centrifuged at 3000g for 5 min and 50  $\mu$ l of each cell-free supernatant was mixed with the same volume of the Griess reagent and the concentration of nitrite was determined by measuring the absorbance at 540 nm.

### 2.6. Animal studies and experimental protocols

Male C57 BL/6J mice were obtained from Janvier Laboratories (France). On arrival, all animals were placed on a standard diet and allowed to acclimatize for at least two weeks on a 12 h light/dark cycle before any experiment was performed. C57BL6J Nrf2 knockout mice (*Nrf2*<sup>-/-</sup>), originally developed by Professor M. Yamamoto [37], were obtained from RIKEN BRC (Tsukuba, Japan) and bred in our animal facilities alongside wild-type littermates (C57BL6J Nrf2<sup>+/+</sup>). Mice were initially challenged with an intraperitoneal injection of either LPS (5 mg/kg) or saline (control group) and after 3 days they were administered by oral gavage with 40 mg/kg of one of the following

compounds: HYCO-3; DMF; CORM-401 or DMF plus CORM-401. Control groups received the vehicle (sesame oil). Mice were sacrificed 6 h after treatment and liver, brain, heart, lung and blood were collected for further analysis. 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).

## 2.7. Detection of CO release from HYCOs and CO accumulation in cells

The release of CO from HYCOs *in vitro* was assessed spectrophotometrically by measuring the conversion of deoxyhemoglobin to carbonmonoxy hemoglobin (COHb) using a method previously described [38]. Briefly, five microliters of mouse blood were transferred to the bottom of 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 after addition of HYCOs (5.5  $\mu$ M final concentration). To assess the amount of endogenous CO accumulated in cells after treatment with HYCO-3 and HYCO-6, a fluorescence probe sensitive to CO (COP-1) was used as previously described [26]. Briefly, BV2 microglia cells were initially suspended in DPBS, then treated with HYCOs (1–5  $\mu$ M) for 15 min at 37 °C and finally incubated for 30 min with 1  $\mu$ M COP-1. Intracellular fluorescence was measured using a CyAn™ ADP LX7 Analyzer (Beckman Coulter) with a pulse processing speed up to 70,000 events per second and results analyzed using FlowJo software.

## 2.8. Assessment of blood carbonmonoxy hemoglobin (COHb) levels in vivo

Blood (5  $\mu$ l) collected at different time points from the mice tail vein following the various treatments described in the experimental protocol above was added to a cuvette containing 4.5 ml of deoxygenated tris (hydroxymethyl) aminomethane solution and spectra recorded as reported above. The percentage of COHb was then calculated based on the absorbance at 420 and 432 nm with the reported extinction coefficients for mouse blood [38].

## 2.9. RNA isolation and real time PCR (q-PCR)

Total RNA was extracted and purified from liver, brain, heart and lung tissues using an RNeasy mini kit following the instructions provided by the manufacturer (Qiagen). Isolated RNA was reverse transcribed into cDNA by using SuperScript® III Reverse Transcriptase kits (Life Technologies) following standard protocols. The expression of different genes was analyzed by Real Time Quantitative PCR (qRT-PCR) using an ABI 7000 sequence detection system (Applied Biosystems) with Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Life Technologies). The samples were analyzed in duplicates in 96-well plate in Real-time PCR System. The fold changes in gene expression were normalized to 2 reference genes: 18S rRNA and Hypoxanthine Phosphoribosyltransferase 1 (HRPT1). The primer sequences for each gene were as follow: HMOX-1, forward (5'-GGCCACCAAGGAGGTACA CAT-3' and reverse (5'-TGGGGCATAGACTGGGTTCT-3'); GCLC, forward (5'-CATCATCAATGGGAAGGAAG-3') and reverse (5'-CATTAGT TCTCCAGATGCTCTC-3'); GCLM, forward (5'-GACCCGAAAGAAGCTG CTC-3') and reverse (5'-AGCCTTAGACTTGATGATTCC-3'); NQO1, forward (5'-AGCCAATCAGCGTTCGGTAT-3') and reverse (5'-GTAGTT GAATGATGTCTTCTCTGAATGG-3'); TNF- $\alpha$ , forward (5'-CCACCACGC TCTTCTGTCTACTGAACCT-3') and reverse (5'-GTGGGCTACAGGCTTG TCACTCG-3'); IL-1 $\beta$ , forward (5'-TGAGAATGACCTGTTCTTTGAAG TTG-3') and reverse (5'-GACAGCCCAGGTCAAAGGTTT-3'); IL-6, forward (5'-ACAAAGCCAGAGTCTCCAGAGATACAG-3') and reverse (5'-TGAATTGGATGGTCTTGGTCCCTTAGCCAC-3'); iNOS, forward (5'-TGGAGCGAGTTGTGATTGC-3') and reverse (5'-GGTCGTAATGTC

CAGGAAGTAGGT-3'); ARG1, forward (5'-CATGGGCAACCTGTGTCCTT -3') and reverse (5'-CGATGTCTTTGGCAGATATGCA-3'); MCP1, forward 5'-CCAGCCTACTCATTGGGAT-3' and reverse (5'-GGCCTGCTG TTCACAGTT-3').

## 2.10. Western blotting

Cultured cells or tissues were lysed in lysis buffer [20 mM Tris pH 7.4, 137 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA) pH 7.4, 1% Triton, 25 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), cocktail phosphatases inhibitors]. The homogenates were centrifuged at 15,000 rpm for 10 min at 4 °C and equal amounts of denaturated protein (20  $\mu$ g) were loaded onto 10% SDS-PAGE gel and transferred on polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked with Tris Buffer Saline supplemented with 5% milk for 1 h at room temperature. Membranes were then incubated overnight with primary antibodies and after three washing (Tris/Tween solution) membranes were incubated for 1 h with secondary anti-mouse antibody.  $\beta$ -actin (Santa Cruz Biotechnology, USA) was used as a loading control. Immunoreactive bands were detected by chemiluminescence (ECL solution, Amersham Biosciences, France) and a densitometric analysis to quantify protein expression was performed using the Image J software.

## 2.11. Serum parameters of tissue damage

Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase levels (ALP) and lactate dehydrogenase (LDH) activities were measured in the blood collected from mice using an automated analyzer and commercially available assays at the diagnostic laboratory of the Hematology Clinic of the Hôpital Henri-Mondor, Creteil.

## 2.12. Histological examination and immunofluorescence staining

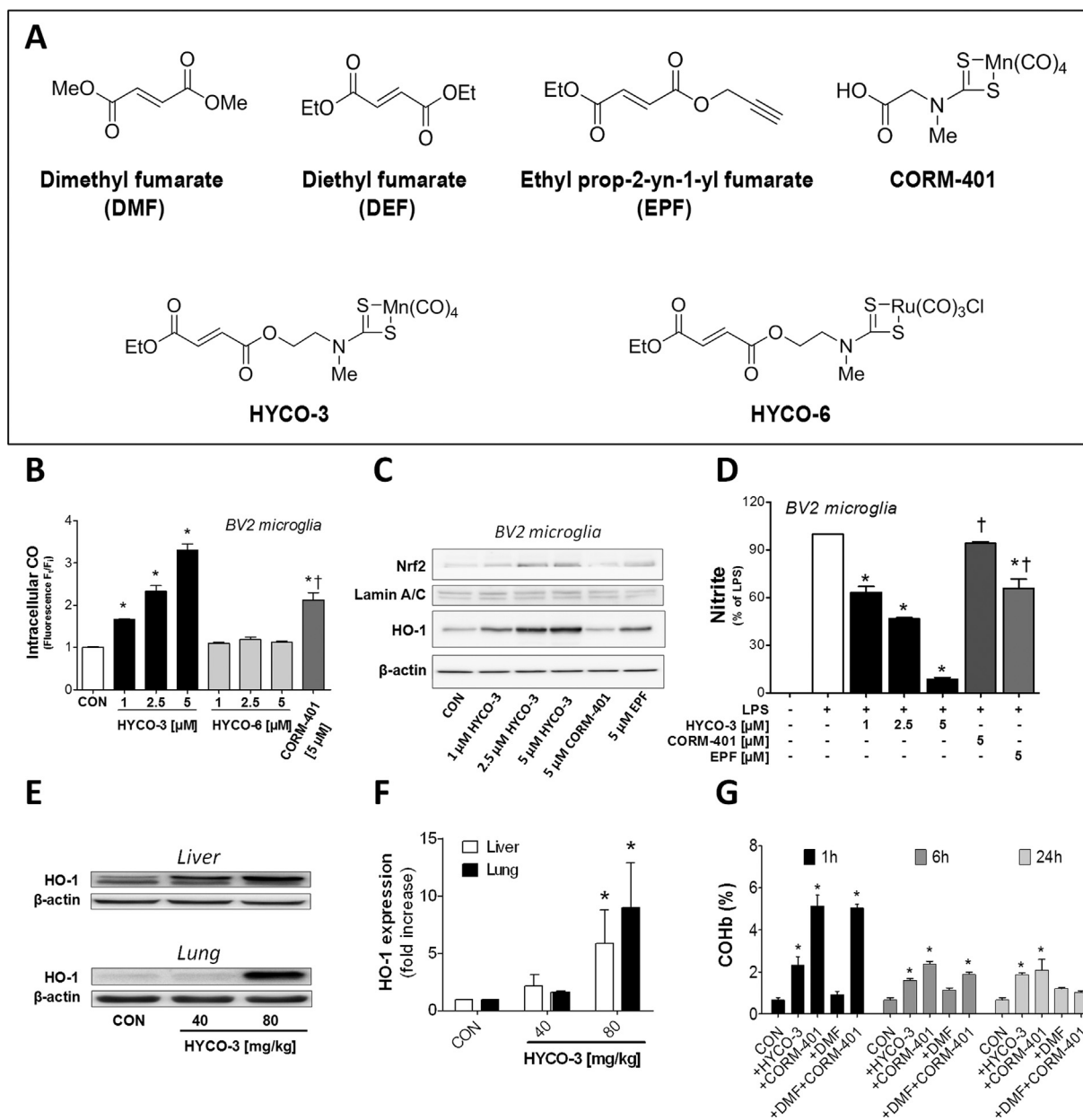
Liver, brain, heart and lung specimens were initially fixed in 10% formalin and paraffin-embedded. Tissue sections (4  $\mu$ m) were then prepared and stained with hematoxylin-eosin for routine and blind examination by a pathologist. Immunofluorescence was carried out on paraffin-embedded liver and brain tissue sections. After antigen retrieval in citrate buffer pH = 6 for brain sections, immunodetection of MAC3 was performed using primary rat monoclonal anti CD107b, (Pharmingen 550292) at 1:12 dilution and immunodetection of CD206 was performed using primary rat monoclonal anti MR5D3 (GeneTex GTX42263) at 1:200 dilution and a secondary goat anti rat -Alexa488 (LIFE A11006) at 1:300 dilution; both revealed by a secondary goat anti rat -Alexa488 (Life Technologies A11006) at 1:300 dilution. Immunodetection of CD68 in the liver was performed using a primary rabbit polyclonal anti-CD68 antibody (Abcam ab125512) at 1:500 dilution and a secondary biotinylated goat anti-rabbit antibody (Dako E0432) at 1:100 dilution, revealed by Fluoresceine-Avidine-DCS (Vector Laboratories A2011) at 1:100 dilution. After antigen retrieval in EDTA pH = 9 for liver sections, immunodetection of ARG1 was performed using a primary rabbit polyclonal anti-ARG1 (Genetex GTX109242) at 1:900 dilution, revealed by a secondary goat anti rabbit -Alexa488 (Life Technologies IFE A11034) at 1:300 dilution. After antigen retrieval in EDTA pH = 9 for liver and brain sections, immunodetection of iNOS was performed using a primary rabbit polyclonal anti-iNos (Arigo ARG56509) at 1:100 dilution and revealed by a secondary goat anti rabbit-Alexa488 (Life Technologies A11034) at 1:200 dilution. Sections were covered with Invitrogen ProLong Gold Antifade with DAPI (ThermoFisher Scientific P36941). The number of MAC3, CD206, CD68, ARG1, iNOS and expressing cells was quantified from 15 random fields analyzing at least 1100 cells (magnification X400) using Image J software. Results were expressed as MAC3, CD206,

CD68, ARG1 or iNOS positive stained cells/total cells.

### 2.13. Metabolic stability of HYCOs and pharmacokinetic studies

To assess the metabolic stability of HYCO-3 and HYCO-6, standard plasma and microsomes assays were performed *in vitro*. For the stability in plasma, 2  $\mu$ l of HYCOs (100  $\mu$ M stock solution prepared in DMSO) were mixed with 198  $\mu$ l of mouse plasma/PBS (1:1 volume). At different time points, 70  $\mu$ l of the mixture were added to 175  $\mu$ l of ice-cold acetonitrile followed by 3 min vortex and 1 min sonication. Plasma

proteins were sedimented by centrifugation (15,000g for 5 min at 4 °C) and the supernatants analyzed by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). For the stability in tissue, 2  $\mu$ l of HYCOs stock solution were added to 198  $\mu$ l of PBS containing liver microsomes (0.5 mg/ml) and 3 mM MgCl<sub>2</sub> and the reaction initiated by adding 1 mM NADPH after 3 min of pre-incubation. At different time points, 200  $\mu$ l of ice-cold acetonitrile were added to stop the reactions. Testosterone, well known to be metabolized by cytochromes P450, was used as a positive control and a control incubation was also performed in the absence of NADPH. The microsomal mixtures



**Fig. 1.** HYCO-3 induces Nrf2/HO-1 expression and simultaneously releases CO *in vitro* and *in vivo*. **A.** Chemical structure of dimethyl fumarate (DMF), diethyl fumarate (DEF), ethyl prop-2-yn-1-yl fumarate (EPF), CORM-401, HYCO-3 and HYCO-6. **B.** Detection of intracellular CO after 6 h incubation of BV2 microglia cells with different concentrations of HYCO-3, HYCO-6 and CORM-401. **C.** HYCO-3 induces Nrf2 and HO-1 expression in a concentration-dependent manner in BV2 microglia cells. The effect of HYCO-3 on protein expression was measured by western blotting after 6 h incubation and compared to cells treated with either EPF or CORM-401 alone, the two chemical moieties that are hybridized to form HYCO-3. **D.** HYCO-3 reduces nitrite production in BV2 microglia cells challenged with lipopolysaccharide (LPS). Nitrite was measured in the medium of cells incubated for 24 h and results were compared to cells treated with either EPF or CORM-401 alone. **E, F.** HYCO-3 induces HO-1 expression *in vivo*. Mice were administered HYCO-3 (40 and 80 mg/kg) by oral gavage and HO-1 expression was measured in lung and liver after 6 h. **G.** Time-course of blood carbonmonoxy hemoglobin (COHb) levels in mice after oral administration of 40 mg/kg HYCO-3, CORM-401, DMF or a combination of DMF + CORM-401. All results are expressed as the mean  $\pm$  S.E.M. n = 3–5 animals per group. \*p < 0.01 versus control group (CON); †p < 0.01 versus control HYCO-3 (5  $\mu$ M).

were finally centrifuged and the supernatants analyzed by LC-MS/MS. To assess the bioavailability of HYCOs *in vivo*, CD-1 mice were administered with the compounds (40 mg/kg in DMSO/sesame oil) and sacrificed after 10, 30, 60 and 120 min. Blood was collected by cardiac puncture and 400  $\mu$ l plasma were mixed with 1 ml of acetonitrile. Samples were centrifuged as described above and the supernatants analyzed by LC-MS/MS. Finally, to assess the biodistribution of CO *in vivo*, mice orally administered with either vehicle (sesame oil), HYCO-3 (40 mg/kg) or CORM-401 (40 mg/kg) were sacrificed after 6 h and liver and heart tissues were initially placed in ice-cold potassium phosphate buffer (1:4 w/v) and stored at  $-80^{\circ}\text{C}$  until analysis. The detection of CO content in tissues was determined by the method of Vreman and colleagues [39]. Briefly, tissues were diced in an 0.1 M ice-cold phosphate buffer (pH 7.4, 1:4 by weight) and sonicated and 40  $\mu$ l of this suspension was added to CO-free sealed vials containing 5  $\mu$ l of 30% (w/v) sulfosalicylic acid and incubated for 30 min on ice. CO released into the vial headspace was quantified by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, CA, USA).

### 2.14. Statistical analysis

Data analyses were performed using GraphPad Prism software version 6.0 (San Diego, CA). Data were expressed as mean  $\pm$  S.E.M and One-way ANOVA combined with Bonferroni multiple comparison tests were used for statistical significance ( $p \leq 0.05$ ).

## 3. 3. results

### 3.1. HYCO-3 delivers CO and induces the protein expression of HO-1/Nrf2 *in vitro*

Fig. 1A shows the chemical structure of the fumaric acid derivatives dimethylfumarate (DMF), diethylfumarate (DEF) and ethyl prop-2-yn-1-yl fumarate (EPF) as well as CORM-401, a CO-releasing molecule capable of liberating 3 equivalents of CO [28,30,35]. Fig. 1A also reports the structure of HYCO-3 and HYCO-6, newly synthesized hybrid compounds composed of the Nrf2 inducing part (fumaric ester) conjugated to a CO-releasing molecule containing manganese or ruthenium, respectively. It has to be noted that the choice of fumaric esters is based on the fact that DMF and several fumaric ester derivatives have been reported to induce HO-1 and/or Nrf2-dependent genes [40,41]. We initially tested *in vitro* if these hybrid molecules could liberate CO to hemoglobin (Hb). Upon incubation with Hb, HYCO-3 or HYCO-6 caused an increase in carbonmonoxy hemoglobin (COHb) (Supplementary Fig. S2A and B). We then evaluated if the compounds caused CO accumulation in BV2 microglia cells and observed that HYCO-3, but not HYCO-6, elicited a concentration-dependent increase in intracellular CO (Fig. 1B). Interestingly, CO levels in microglia cells were higher after exposure to 5  $\mu\text{M}$  HYCO-3 compared to 5  $\mu\text{M}$  CORM-401 (Fig. 1B). Since HYCO-6 appeared to be a poor CO releaser in cells, we continued our investigation focusing on HYCO-3. Microglia cells did not show evident signs of toxicity after exposure to 0.5–5  $\mu\text{M}$  HYCO-3; however, at 10  $\mu\text{M}$  HYCO-3 caused significant cell damage (Supplementary Fig. S2C). We then evaluated if the second expected activity of HYCO-3, namely, activation of Nrf2/HO-1, was also achieved. Fig. 1C shows that non-toxic concentrations of HYCO-3 induced an increase in Nrf2 nuclear levels and HO-1 expression, which was accompanied by higher heme oxygenase activity in BV2 cells (Supplementary Fig. S2D). In contrast, CORM-401 at the highest concentration used (5  $\mu\text{M}$ ) did not change Nrf2 or HO-1 expression, while 5  $\mu\text{M}$  EPF augmented Nrf2 and HO-1 levels, although to a lower extent compared to the same concentration of HYCO-3 (Fig. 1C). In microglia cells challenged with LPS, HYCO-3 significantly reduced the production of the pro-inflammatory mediator nitric oxide (NO, measured as nitrite) and this effect was more powerful than that elicited by 5  $\mu\text{M}$  EPF or CORM-401, which did not affect nitrite levels (Fig. 1D). These data

indicate that HYCO-3 is a hybrid molecule with potent pharmacological properties that are based on enhanced CO delivery, Nrf2/HO-1 activation and anti-inflammatory activity.

### 3.2. HYCO-3 increases HO-1 protein expression and COHb *in vivo*

We next administered HYCO-3 orally to mice and confirmed after 6 h that HO-1 expression was up-regulated in the liver and lung (Fig. 1E and F), although we were unable to detect HO-1 protein in the brain or heart (1 mouse over 3 responded) at this time point (data not shown). We also measured COHb *in vivo* at different times after oral administration of HYCO-3 and a series of additional compounds. For these experiments HYCO-3 was compared to the same doses of CORM-401 alone (the CO-releasing part of HYCO-3), DMF alone (to mimic the Nrf2/HO-1 inducing part of HYCO-3) or the combination of DMF + CORM-401 (the two components of HYCO-3 given as separate molecules). As shown in Fig. 1G, COHb in blood was significantly increased by HYCO-3, CORM-401 and DMF + CORM-401 1 h following gavage of the compounds. The effect was more pronounced in the case of CORM-401 alone or DMF + CORM-401. COHb remained elevated at 6 h in all cases and was still higher than control 24 h after administration of HYCO-3 or CORM-401. Markers of tissue injury (AST, ALT, ALP for liver damage and LDH) were determined in plasma after gavage of HYCO-3 alone (6 and 24 h) or in combination with LPS (6 h, as for the protocol described below). At 6 h post-administration AST and LDH were not significantly elevated, while ALT and ALP were normal (Table 1). At 24 h all values were similar to the control group. No major increases in tissue injury were also observed when HYCO-3 was given after LPS challenge.

This set of findings demonstrates that HYCO-3 reproduces *in vivo* the HO-1 inducing activity and CO-releasing properties that were observed *in vitro*. The data also highlight the fact that oral administration of HYCO-3 and CORM-401 leads to their rapid absorption and maintenance of their pharmacological properties.

### 3.3. Effects of HYCO-3 on COHb levels after LPS challenge *in vivo*

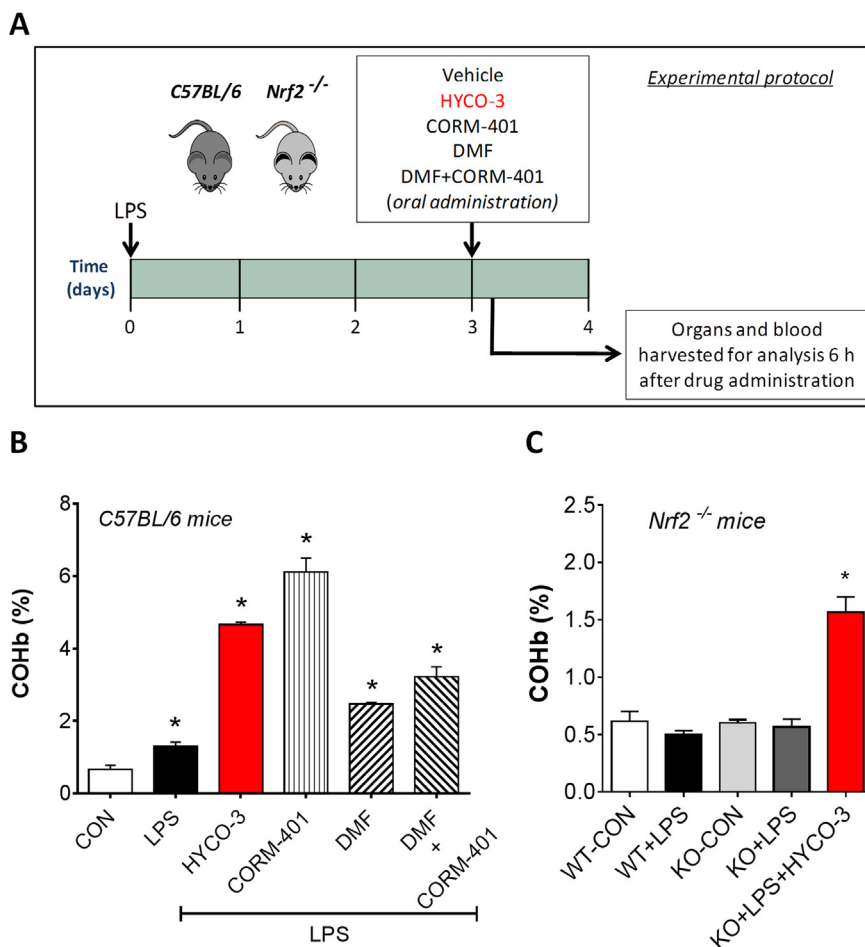
Based on our initial findings *in vitro* and *in vivo*, we established a model of inflammation elicited by i.p. injection of LPS to study the potential anti-inflammatory activity of HYCO-3 in mice. In preliminary experiments we tested LPS at different doses (3, 5 and 10 mg/kg) and determined the mRNA expression of TNF- $\alpha$  and IL-1 $\beta$  in different organs collected after three days. We found that LPS at 5 mg/kg elicited a marked increase in these two pro-inflammatory markers and this dose was chosen for our subsequent studies. We used C57BL/6, Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice in which LPS was given on day 0; three days later HYCO-3, DMF, CORM-401 or DMF + CORM-401 (all compounds at the dose of 40 mg/kg) were administered orally and liver, brain, heart and lung were collected after 6 h (Fig. 2A). At this time point we also collected blood and measured COHb. The percentage of COHb was increased by LPS + HYCO-3, LPS + CORM-401, LPS + DMF and LPS + DMF + CORM-401 in C57BL/6 mice (Fig. 2B), although for this last group COHb levels at 6 h were lower than those observed with LPS + CORM-401. This was

**Table 1**

Markers of tissue injury in plasma following oral administration of HYCO-3 in untreated mice or mice challenged with lipopolysaccharide (LPS).

GROUP	AST	ALT	ALP	LDH
CON	192 $\pm$ 17	60 $\pm$ 1	20 $\pm$ 7	538 $\pm$ 2
HYCO-3 (6 h)	243 $\pm$ 50	77 $\pm$ 9	7 $\pm$ 2	738 $\pm$ 85
HYCO-3 (24 h)	181 $\pm$ 60	61 $\pm$ 26	12 $\pm$ 10	482 $\pm$ 44
LPS (6 h)	115 $\pm$ 3	32 $\pm$ 1	33 $\pm$ 1	500 $\pm$ 4
LPS + HYCO-3 (6 h)	239 $\pm$ 71	79 $\pm$ 22	2 $\pm$ 0.3	646 $\pm$ 147

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.



**Fig. 2. HYCO-3 increases blood COHb levels after challenge with LPS.** A. Scheme of the experimental protocol utilized in this study. Mice were challenged with a single intraperitoneal injection of lipopolysaccharide (LPS, 5 mg/kg) or saline (control group, CON). After 3 days, animals received by oral gavage either vehicle (sesame oil) or 40 mg/kg of the following: HYCO-3, CORM-401, DMF or a combination of DMF + CORM-401. Blood samples and tissues were collected 6 h later for biochemical and histological analysis. B. Blood COHb levels in LPS-challenged mice treated with the different compounds described in (A). C. Blood COHb levels in C57BL/6 or Nrf2-deficient (Nrf2<sup>-/-</sup>) mice challenged with LPS and then treated with HYCO-3 (40 mg/kg orally). Results are expressed as the mean  $\pm$  S.E.M. of n = 3–5 animals per group. \*p < 0.01 versus control group (CON) in this figure.

an unexpected result since it was not evident in mice receiving DMF + CORM-401 in the absence of LPS challenge (Fig. 1G); it is thus possible that the inflammatory state induced by LPS modified the absorption and responses to the pharmacological compounds used in this study. In fact, COHb was also mildly raised by LPS and while DMF only slightly raised COHb levels in untreated mice (Fig. 1G), it more than doubled this parameter after LPS challenge (Fig. 2B). In the case of Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice we found that COHb was not changed by LPS but was increased by LPS+HYCO-3, although to a smaller extent compared to the same treatment in C57BL/6 mice (Fig. 2C).

### 3.4. HYCO-3 induces antioxidant Nrf2-dependent genes in vivo in LPS challenged mice

We further determined the expression of HMOX1, NAD(P)H:quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase C (GCLC) and glutamate cysteine ligase M (GCLM), four Nrf2-dependent genes implicated in anti-oxidant/anti-inflammatory responses, in the organs of mice collected as described above (Table 2). Interestingly, we found that these genes were modulated in an organ-dependent manner. Specifically, HYCO-3 up-regulated (approximately 2–3 fold) the expression of all four genes in the brain, DMF decreased their expression while CORM-401 and DMF + CORM-401 did not change their levels (Fig. 3A). In the liver HYCO-3 induced HMOX1 and partially recovered the expressions of NQO1, GCLC and GCLM that were decreased below control by LPS (Fig. 3C). In contrast, DMF, CORM-401 or DMF + CORM-401 did not increase HMOX1 mRNA expression nor restored NQO1, GCLC or GCLM levels. HYCO-3 strongly up-regulated the four genes in the heart (from 4 to 15 fold, Supplementary Fig. S3A), DMF and CORM-401 alone did not change their expression, while the combination of

DMF + CORM-401 mainly affected HMOX1. Finally, HYCO-3 induced HMOX1 in the lung but did not change the expression of NQO1, GCLC or GCLM (Supplementary Fig. S3C). In this organ DMF only increased NQO1 and GCLM. In Nrf2<sup>-/-</sup> mice HYCO-3 completely lost the ability to induce the four genes in the brain (Fig. 3B); however, HMOX1, but not the other genes, was increased by the compound in the other organs, albeit to a lesser extent (Fig. 3D, Supplementary Fig. S3B and D). Thus, HYCO-3 up-regulates a series of protective genes in an Nrf2-dependent manner in the organs examined. The only exception was HMOX1, which was still induced by HYCO-3 in Nrf2<sup>-/-</sup> mice, suggesting that control of this gene expression is not solely dependent on Nrf2 and that the compound may have additional targets among transcription factors that regulate the stress response. We note that the DMF + CORM-401 did not reproduce the results obtained with HYCO-3 despite being the combination of compounds that mostly resembles HYCO-3.

### 3.5. HYCO-3 reduces the pro-inflammatory profile and up-regulates anti-inflammatory genes in LPS-challenged mice

We next focused on the expression of pro-inflammatory genes and compared the effect of HYCO-3 with that of the other compounds (Table 2). In C57BL/6 mice HYCO-3 considerably decreased the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) induced by LPS challenge in all the organs collected (Fig. 4A, C, Supplementary Fig. S4A and C). Inducible nitric oxide synthase (iNOS) was also generally reduced except in the heart, where HYCO-3 up-regulated its expression (Fig. 4A, C, Supplementary Fig. S4A and C). The other compounds elicited a response similar to that of HYCO-3, although they diminished iNOS more efficiently in the brain and the heart. In Nrf2<sup>-/-</sup> mice we observed interesting outcomes. First,

**Table 2**  
Summary of tissue-specific response to HYCO-3 in C57BL/6 and Nrf2-deficient (Nrf2<sup>-/-</sup>) mice for Nrf2-dependent and inflammatory genes.

	C57BL/6 mice												Nrf2 <sup>-/-</sup> mice											
	Brain		Liver		Heart		Lung		Brain		Liver		Heart		Lung									
	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3	LPS	HYCO-3								
<b>1. Nrf2-dependent genes</b>																								
<i>HMOX1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
<i>NQO1</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
<i>GCLC</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
<i>GCLM</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
<b>2. Pro-inflammatory genes</b>																								
<i>TNF-α</i>	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++								
<i>IL-1β</i>	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++								
<i>IL-6</i>	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++								
<i>iNOS</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
<i>MCP1</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
<b>3. Anti-inflammatory genes</b>																								
<i>IL-10</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
<i>ARG1</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								

Changes in the expression of genes were categorized into: - (no significant changes compared to control untreated mice), down (down-regulation compared to controls), + (between 2- and 5-fold induction compared to control), ++ (between 5- and 10-fold induction compared to control) and +++ (> 10-fold induction compared to untreated controls).

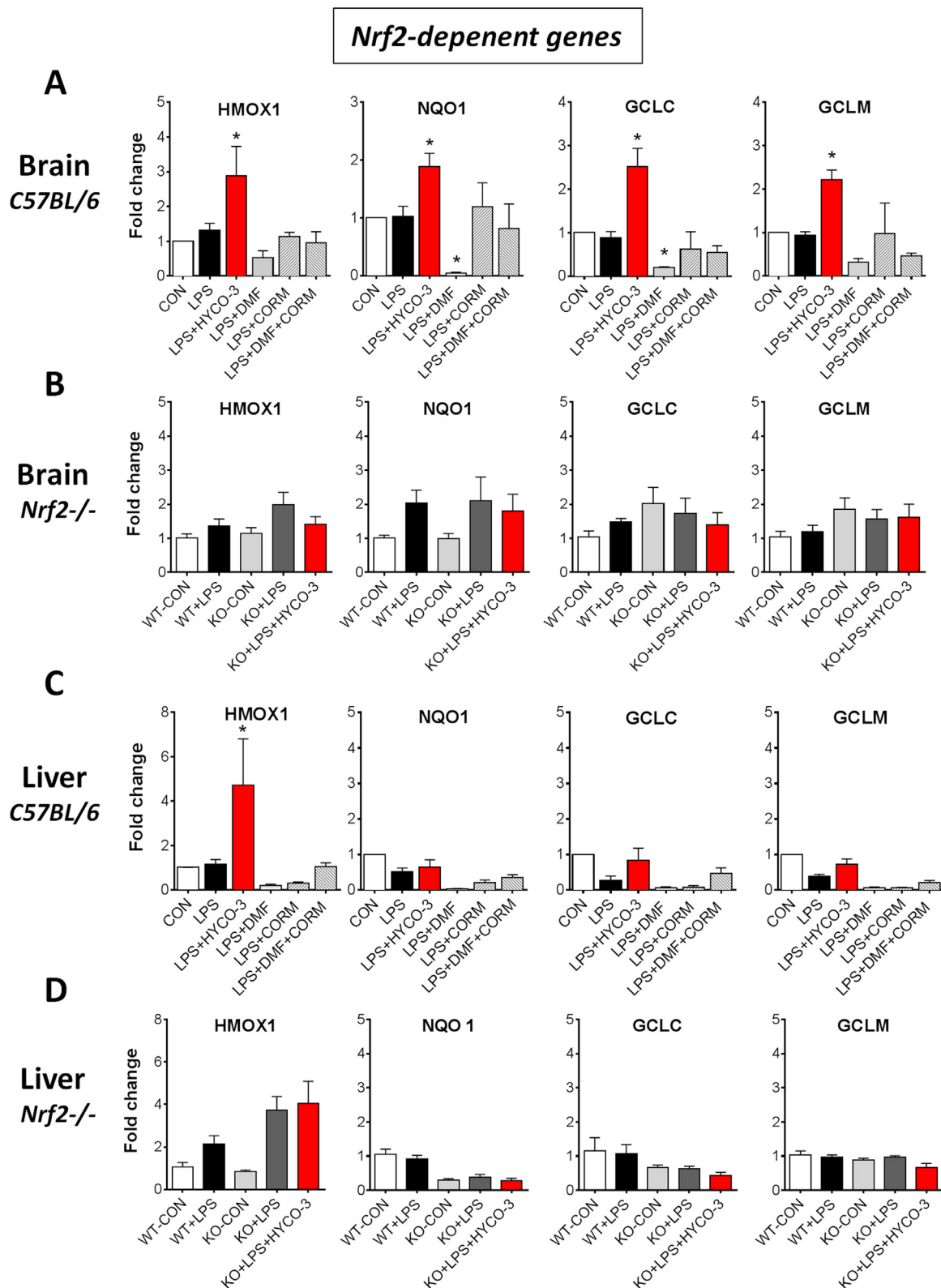
and in accordance with previous findings [42], deficiency of Nrf2 rendered the mice more susceptible to LPS as the up-regulation of pro-inflammatory genes was exacerbated in most organs compared to wild type mice (Fig. 4B, D, Supplementary Fig. S4B and D); second, the extent of increase in pro-inflammatory factors was reduced in Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> animals compared to C57BL/6 mice (Fig. 4 and Supplementary Fig. S4), considering that some organs (liver, heart) hardly exhibited an activation of specific cytokines (IL-6) after LPS, at least at the time point we examined in our study; third, HYCO-3 did not decrease TNF-α and IL-1β in the heart or lung (Supplementary Fig. S4B and D) but was still able to reduce the expression of these cytokines in the brain and the liver (Fig. 4B and D), suggesting a contribution of the CO-releasing part of the molecule to this anti-inflammatory effect.

Anti-inflammatory genes were also modified by administration of HYCO-3 in C57BL/6 mice (Table 2). Arginase 1 (ARG1), which expression was at or below control levels in LPS-challenged mice, was markedly increased after HYCO-3 treatment (Fig. 5A, C, Supplementary Fig. S5A and S5C). All the other compounds did not change ARG1 levels, apart from the combination of DMF + CORM-401 that restored ARG1 in the liver. Interleukin-10 (IL-10) was instead up-regulated by LPS and HYCO-3 administration considerably enhanced this effect in all organs, from a range of two to seven fold with LPS alone to approximately five to 13 fold with LPS+HYCO-3 (Fig. 5A and C, Supplementary Fig. S5A and C). DMF either decreased (in the brain, liver and heart) or did not change IL-10 expression (in the lung) compared to LPS alone. CORM-401 enhanced IL-10 expression only in the heart while the combination of DMF + CORM-401 increased IL-10 in the liver and the heart. Interestingly, in Nrf2<sup>-/-</sup> animals HYCO-3 was able to enhance ARG1 in the heart and lung (Supplementary Fig. S5B and D) but not in the brain or liver (Fig. 5B and D). IL-10 was markedly up-regulated by LPS in Nrf2<sup>-/-</sup> mice and the compound further increased IL-10 only in the lung.

Together with the pro-inflammatory profile described above, these data indicate that HYCO-3 effectively dampens the expression of inflammatory markers and exhibits a superior ability to up-regulate an anti-inflammatory profile compared to all the other compounds investigated.

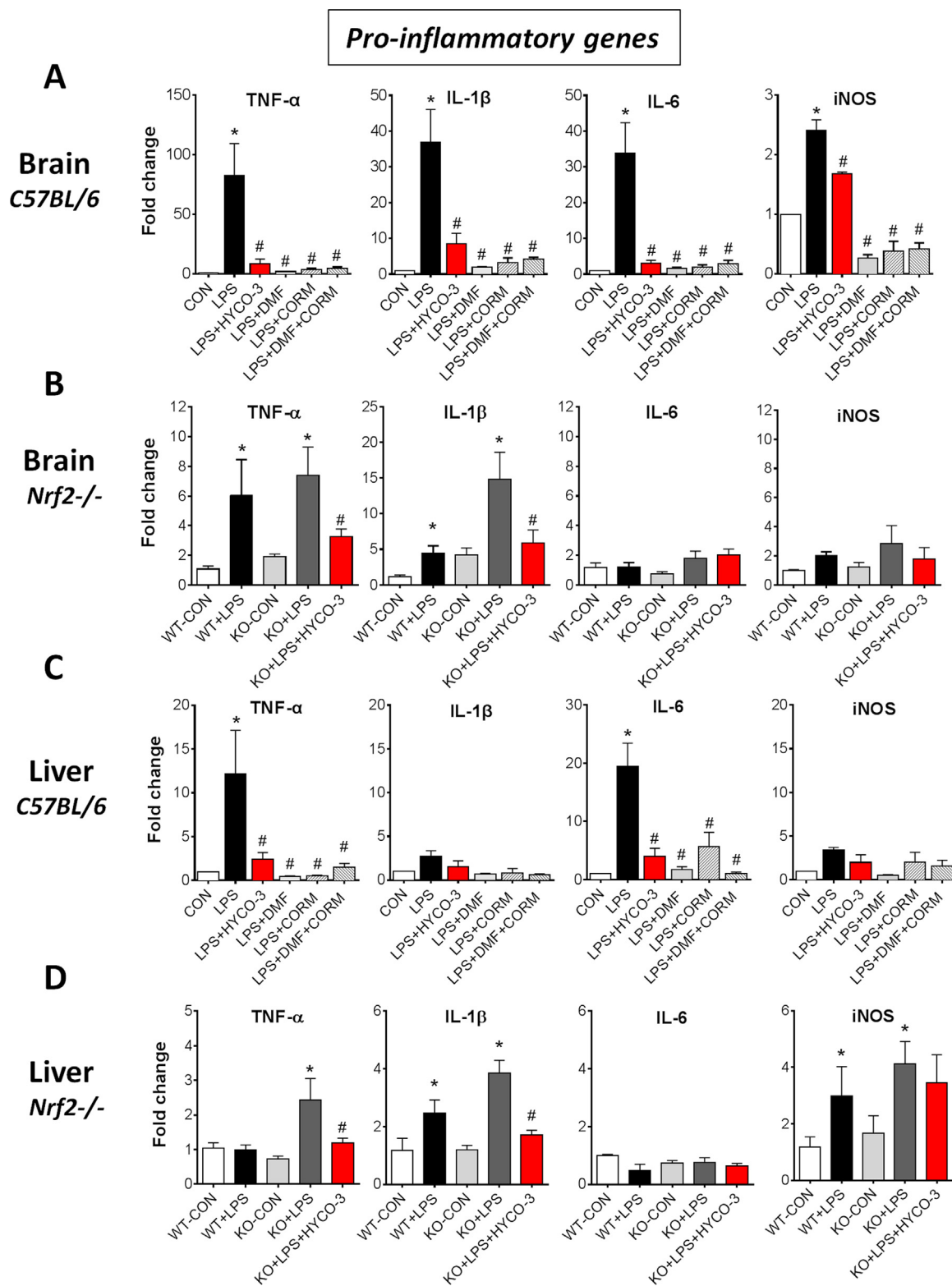
### 3.6. HYCO-3 affects macrophage inflammatory profile

We next narrowed our investigation to tissue macrophages and addressed whether changes in the expression of inflammatory markers by HYCO-3 were linked to macrophage phenotype. We first measured the expression of murine monocyte chemoattractant protein-1 (MCP-1/CCL2) and found that LPS increased its levels in all organs of C57BL/6 mice (Fig. 5A and C, Supplementary Fig. S5A and C). HYCO-3 did not modify this expression while DMF, CORM-401 and DMF + CORM-401 diminished MCP-1/CCL2 to control values. Similar results were obtained in Nrf2<sup>-/-</sup> mice challenged with LPS and LPS + HYCO-3 (Fig. 5B and D, Supplementary Fig. S5B and D). These results suggested that HYCO-3 did not affect the recruitment of macrophages elicited by LPS but most likely changed their phenotype. We then determined the percentage of macrophages in the brain (using Mac3+ immunostaining) and the liver (immunostaining of CD68+ cells) of C57BL/6 mice. We found that macrophages in the brain were slightly increased by LPS and further augmented by HYCO-3 (Fig. 6A and B). Macrophage levels were similar to control values in animals treated with LPS and DMF, CORM-401 or DMF + CORM-401, in accordance with MCP-1/CCL2 expression. In the liver we did not observe changes in macrophages with LPS or LPS + HYCO-3, although there was a tendency to increase the percentage in animals receiving LPS and DMF, CORM-401 or DMF + CORM-401 (Fig. 7A and B). Immune cells in the brain showed only limited iNOS staining (M1 phenotype) in LPS-treated animals as well as in mice challenged with LPS+HYCO-3, DMF or CORM-401 (Fig. 6C). Levels of iNOS were significantly increased only in the LPS + CORM-401 + DMF group. In addition, staining of the anti-



**Fig. 3.** HYCO-3 induces the expression of Nrf2-dependent genes in brain and liver of C57BL/6 mice challenged with LPS: comparison with Nrf2-deficient (Nrf2<sup>-/-</sup>) mice. Mice were treated following the protocol reported in Fig. 2A. A, B. Brain mRNA expression for heme oxygenase-1 (HMOX1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic subunit (GCLC) and gamma-glutamylcysteine synthetase (GCLM) in C57BL/6, Nrf2<sup>+/+</sup> (WT) and Nrf2<sup>-/-</sup> mice. C, D. Liver mRNA expression for HMOX1, NQO1, GCLC and GCLM in C57BL/6, Nrf2<sup>+/+</sup> (WT) and Nrf2<sup>-/-</sup> mice. Results are expressed as the mean ± S.E.M. of n = 5 animals per group. \*p < 0.01 versus LPS group.

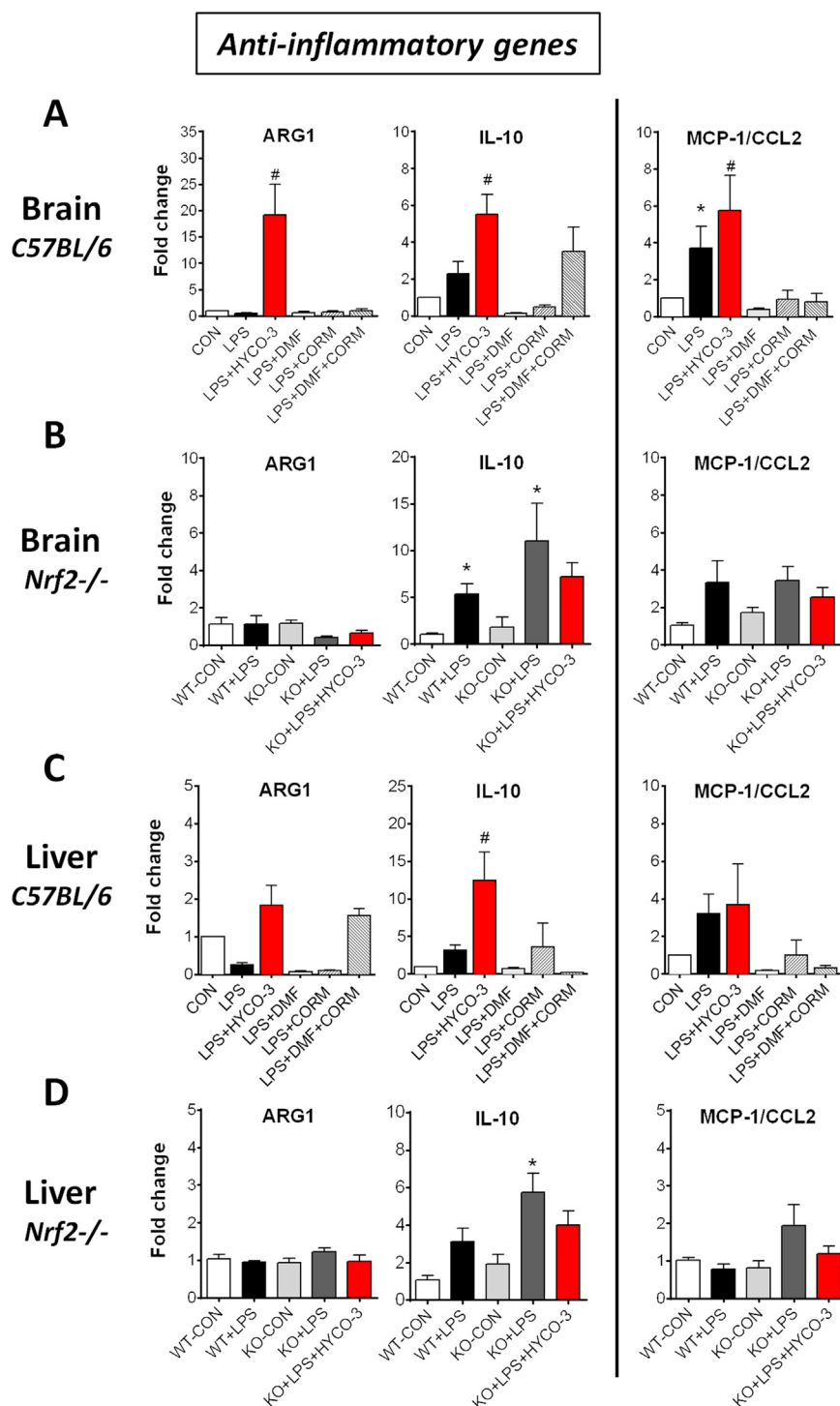




**Fig. 4.** HYCO-3 reduces the expression of pro-inflammatory genes in brain and liver of C57BL/6 mice challenged with LPS: comparison with *Nrf2*<sup>-/-</sup> mice. Mice were treated following the protocol reported in Fig. 2A. A, B. Brain mRNA expression for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) in C57BL/6, *Nrf2*<sup>+/+</sup> (WT) and *Nrf2*<sup>-/-</sup> mice. C, D. Liver mRNA expression for TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and iNOS in C57BL/6, *Nrf2*<sup>+/+</sup> (WT) and *Nrf2*<sup>-/-</sup> mice. Results are expressed as the mean  $\pm$  S.E.M. of  $n = 5$  animals per group. \* $p < 0.01$  versus CON group; # $p < 0.01$  versus LPS group.

inflammatory marker CD206 (M2 phenotype) was reduced in the LPS group and was significantly higher in LPS + HYCO-3 but was unchanged by the other compounds (Fig. 6D). In the liver iNOS staining was

significantly increased by LPS and slightly lowered by all the compounds (Fig. 7C). Levels of ARG1 (M2 phenotype) were lower than control in the LPS-treated group and slightly, but not significantly,



**Fig. 5.** HYCO-3 induces the expression of anti-inflammatory genes in brain and liver of C57BL/6 mice challenged with LPS: comparison with Nrf2-deficient (Nrf2<sup>-/-</sup>) mice. Mice were treated following the protocol reported in Fig. 2A. A, B. Brain mRNA expression for arginase 1 (ARG1), interleukin-10 (IL-10) and monocyte chemoattractant protein-1 (MCP-1/CCL2) in C57BL/6, Nrf2<sup>+/+</sup> (WT) and Nrf2<sup>-/-</sup> mice. C, D. Liver mRNA expression for ARG1, IL-10 and MCP-1/CCL2 in C57BL/6, Nrf2<sup>+/+</sup> (WT) and Nrf2<sup>-/-</sup> mice. Results are expressed as the mean  $\pm$  S.E.M. of n = 5 animals per group. \**p* < 0.01 versus CON group; #*p* < 0.01 versus LPS group.

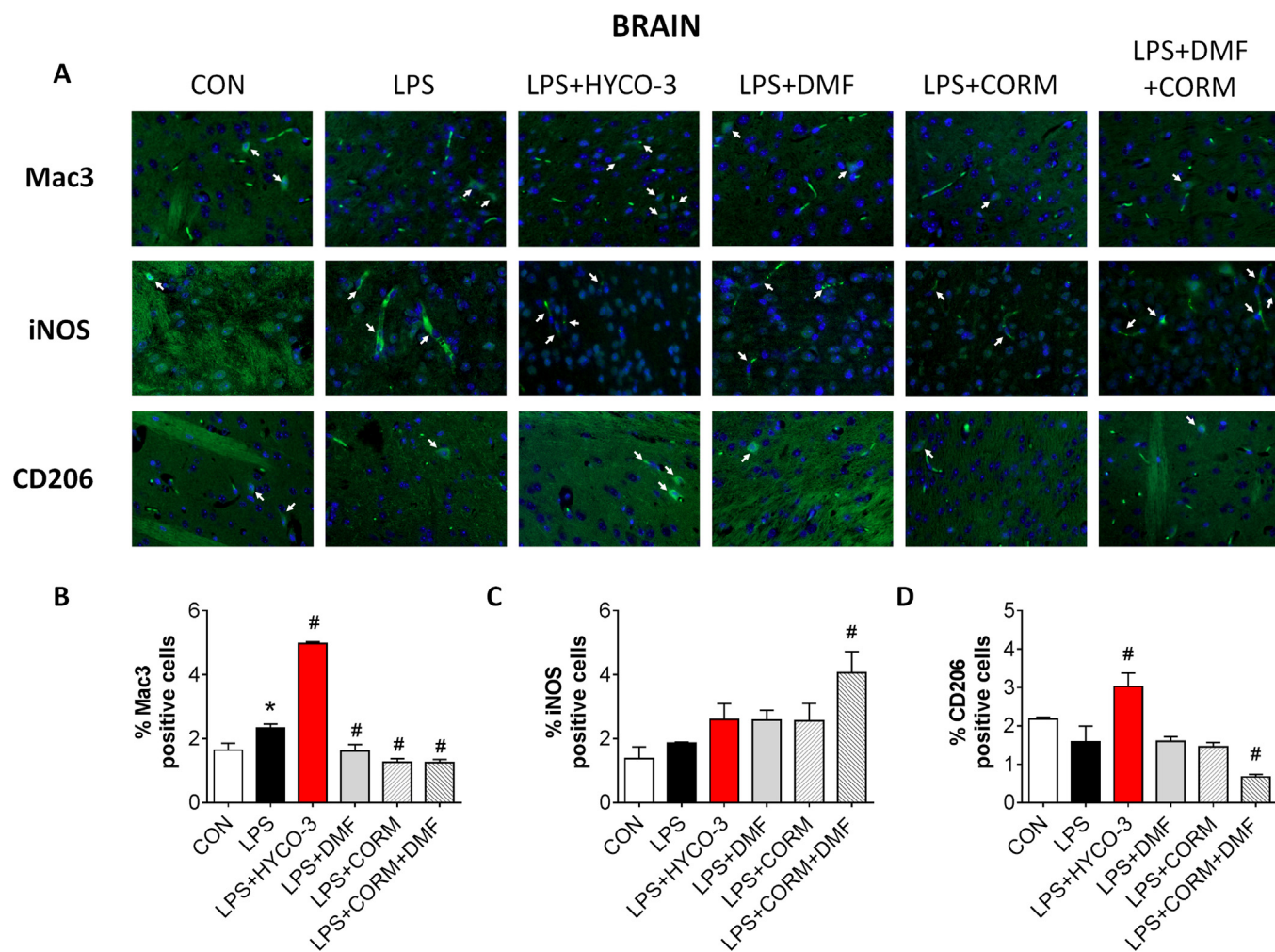
recovered by all treatments (Fig. 7D).

These results indicate that the effects of HYCO-3 on pro- and anti-inflammatory gene profile in whole brain and liver tissue are broadly reproduced in markers of M1 and M2 phenotype in immune cells.

### 3.7. Preliminary studies on metabolic stability and pharmacokinetic of HYCOs

We finally conducted some preliminary experiments to assess the metabolic stability and pharmacokinetic of HYCOs *in vitro* and *in vivo*. This was assessed by first incubating HYCO-3 and HYCO-6 in the presence of mouse plasma or liver microsomes and then measuring by LC-

MS/MS technique the residual fraction of each compound still present in the suspensions at different time points. As shown in Supplementary Fig. S6, we found that HYCO-3 was significantly much more stable both in plasma and microsomes compared to HYCO-6 as indicated by the higher area under the curve (AUC) detected at times 0, 15 and 30 min. In fact, while HYCO-3 gradually disappeared over time, HYCO-6 was virtually absent already at time 0. These data are in agreement with the faster ability of HYCO-6 to release CO to Hb compared to HYCO-3 (see Supplementary Fig. S2). When administered orally to mice at a dose of 40 mg/kg, we found however that both compounds were undetectable in plasma at all time points considered (data not shown). Interestingly, oral administration of HYCO-3 and CORM-401 at the same dose



**Fig. 6.** Effect of HYCO-3 on the recruitment of macrophages and their inflammatory profile in the brain of C57BL/6 mice challenged with LPS. Mice were treated following the protocol reported in Fig. 2A. At the end of the experiments, brain tissue was collected for immunostaining analysis and macrophages identified by the expression of MAC3, iNOS and CD206. Representative pictures (A) and quantitative analysis (B, C and D) of the three markers are shown. See Material and Methods for details of the analysis. \* $p < 0.01$  versus CON group; # $p < 0.01$  versus LPS group.

resulted after 6 h in a significant accumulation of CO in liver and to a lesser extent in the heart, indicating that CO can be efficiently delivered in tissues by these compounds despite the apparent low pharmacokinetic profile *in vivo*.

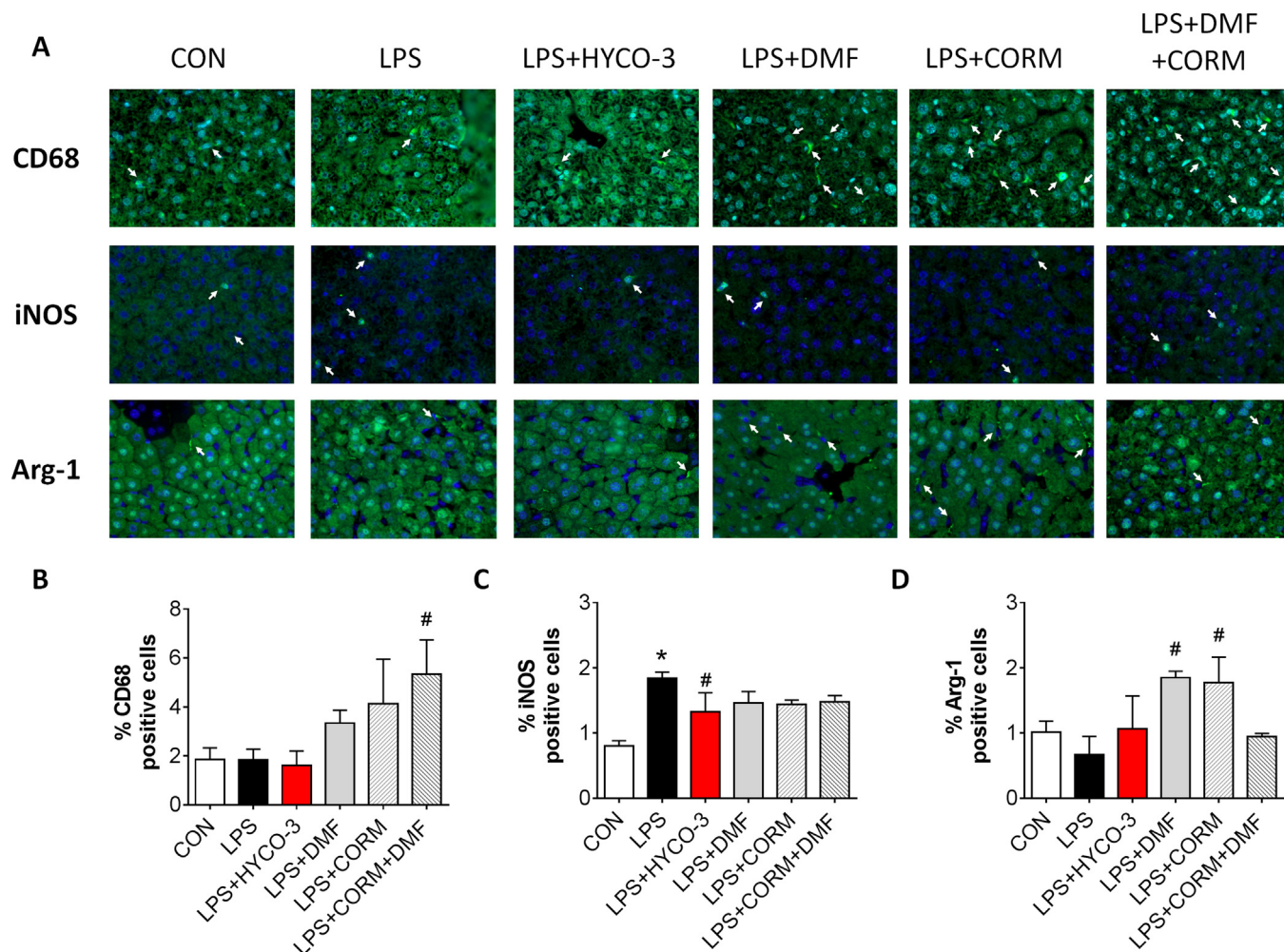
#### 4. Discussion

We report herein our findings on HYCO-3, a new hybrid compound obtained from the conjugation of a fumaric ester with a manganese-based carbonyl complex. We show that HYCO-3 activates Nrf2/HO-1 and delivers CO efficiently to cells *in vitro* and tissues *in vivo*, confirming that the two expected pharmacological actions of this novel group of agents are achieved. In mice challenged with LPS, orally administered HYCO-3 reduced the mRNA levels of the pro-inflammatory markers TNF- $\alpha$ , IL-1 $\beta$  and IL-6 while increasing the expression of the anti-inflammatory genes ARG1 and IL-10 in brain, liver, lung and heart. This effect was in contrast to that elicited by either DMF or CORM-401 alone or by the combination of DMF + CORM-401 given as separate components, as all these treatments decreased the expression of pro-inflammatory genes but had limited influence on anti-inflammatory markers. Furthermore, in Nrf2<sup>-/-</sup> mice, HYCO-3 diminished TNF- $\alpha$  and IL-1 $\beta$  in brain and liver but not in lung and heart, indicating that the CO-releasing part of this hybrid contributes to the reduction of pro-inflammation and that this effect is organ-specific. The anti-

inflammatory response of HYCO-3 was also less prominent in Nrf2<sup>-/-</sup> mice, implicating again Nrf2 activation and CO release as the main mechanisms mediating the effect on ARG1 and IL-10. Overall, these data highlight how the dual activity of HYCO-3 results in enhanced anti-inflammatory action compared to the parent compounds in a systemic model of inflammation; the fact that the molecule similarly affects the response of major body organs suggests a broad pharmacological activity during inflammatory conditions.

The first aim of this study was to design HYCOs with an improved pharmacological profile compared to hybrid compounds previously reported by our group [26,27] and to define their properties *in vitro* and *in vivo*. Since ruthenium (CORM-3) and manganese (CORM-401) carbonyls are the most active and well characterized CO-RMs in our laboratory [13,43] and DMF is an Nrf2 activator used in the clinic for the treatment of multiple sclerosis [19,20,44], we generated HYCO-6 and HYCO-3 by conjugating analogues of these two CO releasers to a fumaric ester. Although the two new hybrid compounds promoted the conversion of deoxyhemoglobin to COHb when tested in cuvette, only HYCO-3 caused significant accumulation of CO in cultured microglia cells. This could indicate that either HYCO-6 cannot enter the cell and deliver CO or that there is a different chemical reactivity of HYCO-3 and HYCO-6 towards COP-1, the probe we used to detect intracellular CO. Notably, we observed similar results using hemoCD, a unique CO scavenger that binds CO with high affinity and that allows the

## LIVER



**Fig. 7.** Effect of HYCO-3 on the recruitment of macrophages and their inflammatory profile in the liver of C57BL/6 mice challenged with LPS. Mice were treated following the protocol reported in Fig. 2A. At the end of the experiments, liver tissue was collected for immunostaining analysis and macrophages identified by the expression of the specific markers CD68, iNOS and ARG1. Representative pictures (A) and quantitative analysis (B, C and D) of the three markers are shown. See Material and Methods for details of the analysis. <sup>\*</sup> $p < 0.01$  versus CON group; <sup>#</sup> $p < 0.01$  versus LPS group.

determination of intracellular CO levels [45,46], confirming that only the manganese-containing HYCO-3, unlike its ruthenium-based counterpart (HYCO-6), delivers CO to cells at the concentrations used (data not shown, manuscript in preparation). This difference may also be explained by the increased bioavailability of HYCO-3, since we found that HYCO-3 is significantly more stable than HYCO-6 in plasma and microsomes. In addition, intracellular CO content after HYCO-3 exposure was equal or superior to that measured after CORM-401 treatment, indicating that the carbonyl part of HYCO-3 maintains its CO-releasing capacity towards cells even when coordinated to a fumaric ester. Focusing on HYCO-3 in the continuation of our study, we measured increased blood COHb levels when the compound was orally administered in mice in the presence or absence of LPS, thus confirming a consistent CO releasing ability of this molecule as shown in cuvette and in cells *in vitro*. In the absence of LPS, a dose of 40 mg/kg HYCO-3 caused a peak COHb of ~2% at 1 h and these levels remained unchanged at 6 and 24 h. This was in contrast to the effect of CORM-401 or DMF + CORM-401, which resulted in increased COHb to ~5% after 1 h followed by a decrease to 2% or less at 6 and 24 h. Since all compounds were prepared in the same solvent, we hypothesize that this difference is due to a delayed and prolonged absorption of HYCO-3 *in vivo*, possibly because of the heavier molecular weight of this hybrid compared to the parent compounds. Interestingly, 6 h after HYCO-3

administration, COHb levels were nearly 5% in LPS-challenged C57BL/6 mice. These data suggest that the further increase in COHb compared to untreated mice is a consequence of endogenously generated CO since a significant rise in HO-1 expression by HYCO-3 was observed in the organs of LPS-treated C57BL/6 mice. This idea is also supported by a much lower increase in COHb levels (~1.5%) in *Nrf2*<sup>-/-</sup> mice challenged with LPS, which accordingly displayed lower HO-1 expression. We cannot exclude that additional sources of endogenous CO contribute to the increase in COHb levels as it has been shown that CO can also be produced by lipid peroxidation under oxidative stress conditions [47,48]. We note that the expression of the other Nrf2-dependent genes investigated in this study was also more pronounced in C57BL/6 compared to *Nrf2*<sup>-/-</sup> mice, confirming that Nrf2 is a real target for activation by HYCO-3 *in vivo*. Similarly, previous studies demonstrated that DMF did not induce expression of several Nrf2-dependent genes in *Nrf2*<sup>-/-</sup> mice, with the exception of HO-1, which was partially attenuated in the absence of Nrf2 [20].

The second aim of this study was to determine whether HYCO-3 exhibited anti-inflammatory effects in a systemic model of inflammation. The concept underlying the synthesis of HYCOs is to bring together in one molecular entity the pharmacological properties of CORMs and Nrf2 activators, with the aim of enhancing the protective action of the individual parent compounds. Among the various CO-RMs to

be hybridized with fumaric esters, we decided to use CORM-401 because it can deliver CO with high efficiency *in vitro* and *in vivo* [30,35,49]. CO-RMs and fumaric esters, including DMF, are known modulators of inflammation [41,50–55], which justifies the choice of our model. For example, CORM-401 exerts anti-inflammatory effects by suppressing NF $\kappa$ B signaling in a model of porcine kidney transplantation [49], in line with previous reports showing that CO-RMs are very effective in reducing systemic inflammation in murine polymicrobial sepsis [50,56]. In the present study, we decided to administer the compounds after the stimulation of the inflammatory challenge, as this protocol has more relevance for potential therapeutic applications. Our findings reveal that HYCO-3 effectively reduces the expression of pro-inflammatory markers in C57BL/6 mice and this effect, although less pronounced, was still present in Nrf2<sup>-/-</sup> mice. Altogether, these results indicate that both the Nrf2-inducing and CO-RM moieties of HYCO-3 contribute to the modulation of inflammation. In particular, TNF- $\alpha$  and IL-1 $\beta$  were clearly diminished in brain and liver of Nrf2<sup>-/-</sup> mice, while these and other pro-inflammatory factors were unchanged in the heart and the lung. Thus, the CO liberated by HYCO-3 affords protective activity in specific organs even in the absence of an Nrf2 response. It could be argued that the inhibition of inflammation derives from Nrf2 and CO-independent mechanisms, as HYCO-3 may possess off-target effects. However, the fact that CORM-401 alone also decreased the expression of pro-inflammatory mediators in C57BL/6 mice supports a direct role of CO in this beneficial outcome. The reason for the heart and lung of Nrf2<sup>-/-</sup> mice being less responsive to HYCO-3 is currently unknown and we speculate that it may be due to tissue distribution and clearance of the hybrid. There is evidence that DMF also induces Nrf2-dependent genes in a tissue-specific manner, which was ascribed to differential Nrf2 tissue regulation and exposure to the drug [20]. Of special note are the data showing that HYCO-3 increased the anti-inflammatory mediators IL-10 and ARG1, in contrast to all the other treatments investigated. Since IL-10 and ARG1 are classical indicators of an alternative, pro-reparative phenotype [4,57], we propose that an important therapeutic advantage conferred by the hybrid compound is the simultaneous depression of pro-inflammation and rise in anti-inflammatory markers. It is likely that this profile extends to several cell types of the organs tested and we confirmed that immune cells, including monocytes/macrophages in the liver and macrophages/microglia in the brain, are similarly polarized. Interestingly, the ability of HYCO-3 to stimulate anti-inflammatory factors was attenuated in Nrf2<sup>-/-</sup> mice, emphasizing again that the dual activity of HYCO-3 is necessary to achieve the full pharmacological potential of the hybrid. Nevertheless, the maintenance of some protection in Nrf2<sup>-/-</sup> mice suggests that HYCO-3 can exert positive action even when the Nrf2 response is impaired, as reported in premature aging in Hutchinson-Gilford progeria syndrome [58] and skin disorders [59].

The third aim of our study was to compare the effect of HYCO-3 with that of the parent compounds. As DMF is an approved immunomodulatory drug [41,55], its comparison with HYCO-3 is particularly relevant for therapeutic perspectives. It is evident that none of the individual compounds or the combination of DMF + CORM-401 are efficient in up-regulation of Nrf2-dependent genes in major organs of C57BL/6 mice at the doses used in this study. This result is especially intriguing for the DMF group and the treatment with DMF + CORM-401, which mostly resembles HYCO-3 and would be expected to closely reproduce its pharmacological effects. Thus, HYCO-3 appears more potent than DMF and fumaric derivatives in activating Nrf2, a finding that we also obtained in microglia *in vitro*. In this respect, previous pharmacodynamic studies in mice tested DMF at 50, 100, 200, 400 and 600 mg/kg and showed induction of Nrf2-regulated genes in a dose-dependent manner [20], suggesting that the inability of DMF to increase HMOX1, NQO1, GCLC and GCLM expression in our investigation is likely due to the weak dose employed. In addition, a dose of 100 mg/kg by oral gavage was used to assess the immunomodulatory activity of DMF in experimental autoimmune encephalomyelitis, a model of

multiple sclerosis [55], pointing to the fact that efficacious treatment of DMF in inflammatory diseases is usually performed with relatively high doses. Concerning the stimulation of pro-inflammation, we observed that all compounds significantly inhibited the expression of pro-inflammatory genes induced by LPS in all organs of C57BL/6 mice and HYCO-3 did not appear more powerful than the other compounds. Thus, it emerges from these findings that: 1) DMF at 40 mg/kg exerts immunomodulatory function despite its inability to induce classical Nrf2-dependent genes that mediate the salutary effects of Nrf2 activation; this is in line with data showing that DMF equally protected wild type and Nrf2<sup>-/-</sup> mice from the development of acute inflammatory autoimmune encephalomyelitis [55] and suppressed inflammatory activation of microglia in Nrf2-dependent and independent manner [60]; 2) CORM-401, which has been evaluated for the first time in our model of systemic inflammation *in vivo*, potently inhibits the pro-inflammatory response, possibly also by modulating the recruitment of macrophages in certain organs, such as the brain; 3) the combination of DMF + CORM-401 as separate entities or conjugated in the form of a hybrid (HYCO-3) does not enhance the effect of DMF or CORM-401 alone, which is not surprising since the two compounds already nearly abolished the up-regulation of pro-inflammatory genes. Importantly, and in contrast to the majority of reports where DMF or CO-RMs are given prior to or in correspondence with the initiation of inflammation [55,60], this study demonstrates that administration of DMF or CORM-401 after the initiation of inflammation is still very useful in alleviating the tissue inflammatory responses, broadening the therapeutic applicability of these agents and advancing our understanding of their pharmacological characteristics.

One aspect that needs to be addressed for the characterization of HYCOs and CO-RMs *in vitro* and *in vivo* relates to the development of appropriate assays to establish their metabolic stability and pharmacokinetic profiles. Our experience in this study is that classical assays that are commonly used for new compounds are not always adequate, since they measure the residual presence of the original substance in biological fluids over time. In our analysis HYCO-3 was more stable than HYCO-6 in plasma and microsomes preparations *in vitro* but when evaluated *in vivo* both compounds were untraceable in plasma at any time point considered (up to 120 min). However, increased COHb levels in blood were reproducibly measured in our setting 1 h after oral gavage of HYCO-3 and CORM-401, indicating the absorption and decomposition of the compounds. In fact, both HYCOs and CO-RMs can be considered as drug carriers that undergo rapid biotransformation in order to release one of the active components, CO, thus immediately modifying their chemical structure and preventing their detection with LC-MS/MS. In addition, in the case of HYCOs there is the possibility of hydrolysis between the fumaric ester and the CO-RM moiety, generating a variety of metabolites that have to be identified. Likewise, after oral administration, DMF is hydrolyzed to its metabolite monomethyl fumarate, which can be detected in the circulation and in tissues and is responsible for the activation of gene expression by Nrf2 [20]. Consequently, we recommend that assessments of the stability of these compounds must be coupled with measurements of specific parameters including: blood COHb levels, accumulation of CO in different tissues as well as the ability of the compounds to induce Nrf2-dependent genes in the case of hybrid Nrf2 activators coordinated to carbonyl groups.

To conclude, our findings reveal an anti-inflammatory action of HYCO-3 *in vivo* which is mediated by activation of the Nrf2 system and the CO-releasing capacity of the compound, emphasizing the dual activity character of this new hybrid and the proof-of-principle of an additive/ synergistic effect. Our hybrids have been conceived to potentiate two interrelated biochemical activities, that is, the activation of Nrf2 that leads to HO-1 induction and subsequent CO production. However, CO possesses pharmacological properties that are independent of Nrf2 and that may add to the therapeutic value of these novel compounds. In this respect, it would be very interesting to compare the therapeutic efficacy of HYCO-3 to that of DMF in multiple

sclerosis, since CO gas and CO-RMs counteracted neuroinflammation in experimental autoimmune encephalomyelitis [61,62]. The idea of combining Nrf2 inducers with other small active molecules is being currently explored by others, as exemplified by a recently described dual AMPK/Nrf2 activator shown to exert potent neuroprotective action in mice and rats [63]. Considering the multitude of cellular pathways controlling the inflammatory response, this dual approach may offer an exciting new avenue for the development of more robust and effective anti-inflammatory therapies.

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## 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 RF, RM, MR and TM as inventors.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.10.020

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