SUPPORTING INFORMATION

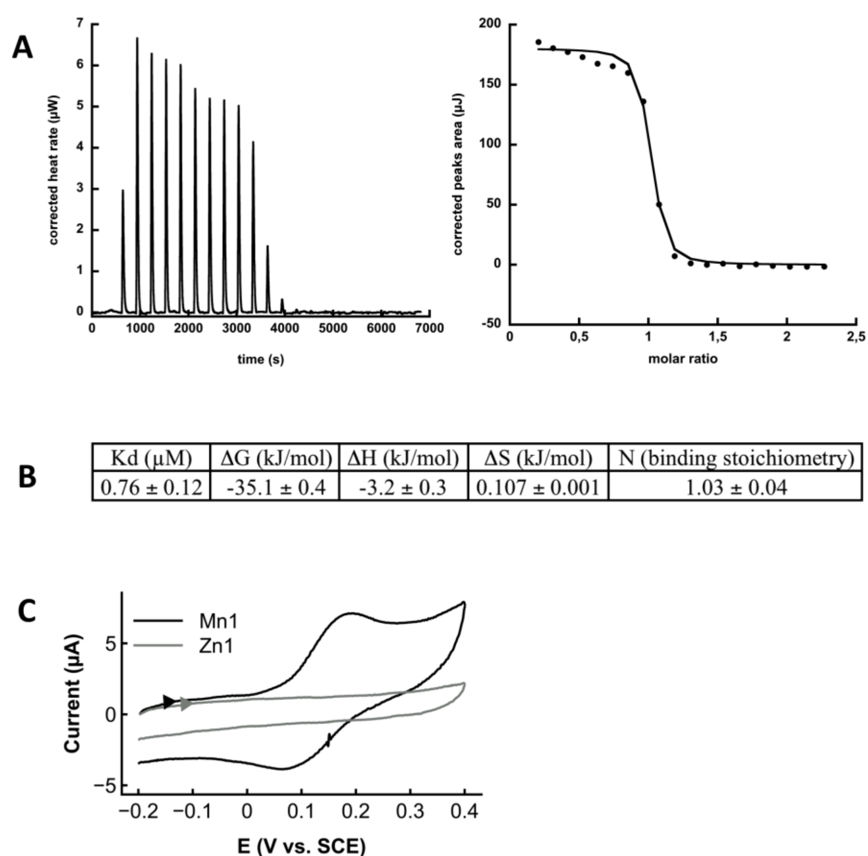
A cell penetrant manganese SOD-mimicisabletocomplementMnSODandexerts an anti-inflammatoryeffect on cellularandanimalmodelsofinflammatoryboweldiseases

Emilie Mathieu,[a,b]‡ Anne-Sophie Bernard,[a,b]‡ Nicolas Delsuc,[a,b]ElodieQuévrain,[a,b]GéraldineGazzah,[a,b] Barry Lai,[c] Florian Chain,[d] Philippe Langella,[d] Maria Bachelet,[d] Joelle Masliah,[a] Philippe Seksik,[d] and Clotilde Policar*[a,b]

Table of contents

1. Supplementary Figures
2. Supplementary Experimental Procedures
    a. Materials
    b. Intracellular quantification of Mn1by EPR in mitochondria-enriched fractions
    c. Mn1 anti-superoxide activity: Titration of superoxide in HT29-MD2 cells
    d. Cell assays
        Cytotoxicity
        Protein assay
3. SupplementaryReferences

1. Supplementary Figures



Kd (µM)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol)	N (binding stoichiometry)
0.76 ± 0.12	-35.1 ± 0.4	-3.2 ± 0.3	0.107 ± 0.001	1.03 ± 0.04

Figure S1 (related to Figure 1): Isothermal titration of ligand L with $MnCl_2$, and cyclic voltammogramm of Mn1 and Zn1 in DMEM. A: Isothermal titration of ligand L (500 µM) with $MnCl_2$ (5mM) in HEPES buffer (pH 7.4, 100 mM) containing NaCl (150 mM) at 25 °C. *Left*: Thermogramm: heat rate was corrected by the dilution heat rate of $MnCl_2$ in buffer. *Right*: Binding isotherm: dots represent the heat of reaction measured by peak integration as a function of $MnCl_2$ /L molar ratio. The solid line represents the best fit to experimental data. **B:**Thermodynamic parameters calculated from these experiments.**C:**Cyclicvoltammograms of Mn1 and after addition of 1 equivalent of $ZnBr_2$ (*in situ* formation of **Zn1**, the redox inactive analog of **Mn1**). Voltammogrammswererecorded in DMEM at 37 °C under argon atmosphereat a glassycarbonelectrode, scan rate 0.5 $V.s^{-1}$.

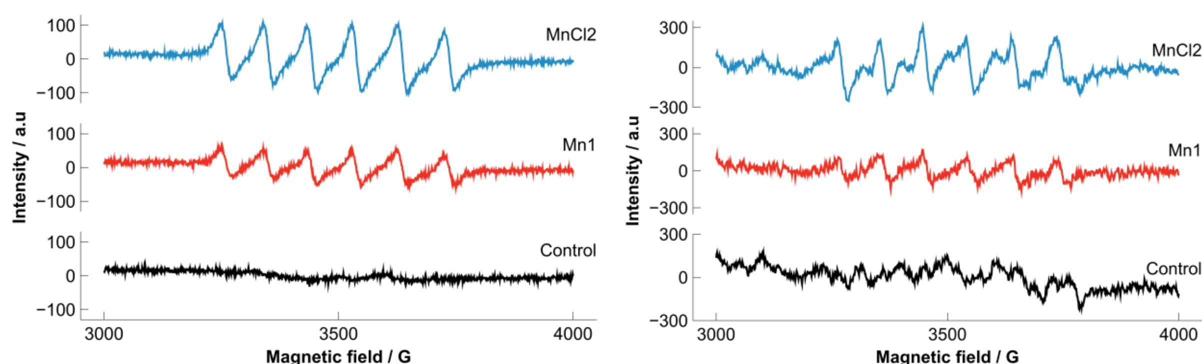


Figure S2 (related to Figure 2): Detection of Mn^{2+} in cell lysate (left) or mitochondria enriched fractions (right) by EPR. ***Left:***EPR spectra of acid-digested lysates of HT29-MD2 cells incubated for 3 h under several conditions: medium only (control), Mn1 (100 µM) or $MnCl_2$ (100 µM). EPR spectra were recorded on aElexsys 500 spectrometer from Bruker in a glass capillary tube: frequency: 9.82 GHz, microwave power: 32 mW, amplitude of the modulation: 2G, 9 scans, room temperature. ***Right*****:** EPR-spectra of acid-digested mitochondria enriched fraction of HT29-MD2 cells incubated for 6 h under several conditons: medium only (control), Mn1 (100 µM), or $MnCl_2$ (100 µM). EPR spectra were recorded on aElexsys 500 spectrometer from Bruker in a glass capillary tube: frequency: 9.82 GHz, microwave power: 80.5 mW, amplitude of the modulation: 18G, 9 scans, room temperature.The spectra were normalized by the protein content. Spectrum of the cavity was substracted.

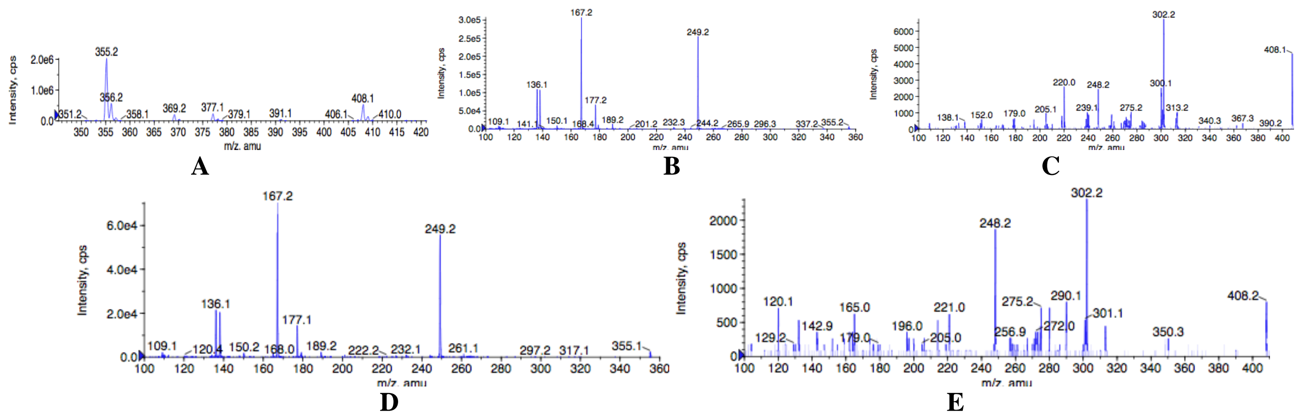


Figure S3: MS and MS/MS spectra for the pure complex Mn1 in water (A, B and C), and of cell-lysates incubated with Mn1 (100 µM, 3h, D and E). MS spectrum (**A**), MS-MS spectrum of the ion at m/z 355.2 corresponding to the free ligand (**B**) and MS-MS spectrum of the ion at m/z 408.1 corresponding to Mn1 (**C**) for 10 pmol of complex Mn1 in water injected in QTRAP LC-MS-MS system. MS-MS spectrum of the ion at m/z 355.2 detected in the lysate (**D**) and MS-MS spectrum of the ion detected at m/z 408.1 in the lysate (**E**) for 10 µL of filtrated lysate of cells incubated with complex Mn1 (100µM, 3h) injected in QTRAP LC-MS-MS system.

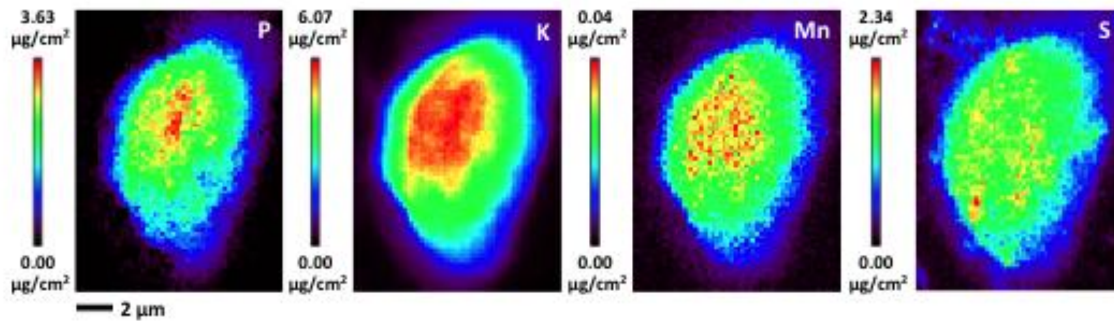


Figure S4 (related to Figure 3): Mappings of P, K, Mn, and S in intestinal epithelial cell incubated with Mn1. Phosphorus (P), potassium (K), manganese (Mn) and sulfur (S) detection by X fluorescence on cryofixed and freeze-dried HT29-MD2 cell incubated with Mn1 (100 µM, 2 h). Images recorded on 2-ID-D beamline of APS synchrotron, excitation at 6.8 keV, integration time 2 s / pixel, pixel size 200 nm. As can be seen in this series, the Mn-map does not match that of P, which is used in some studies to delineate the nucleus.[1] Mn is clearly distributed all over the cell as K, the map showing here a 2D-projection, with higher amounts where the cell is the thickest.

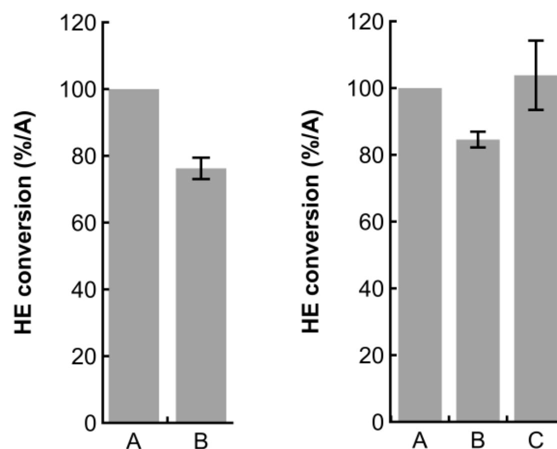


Figure S5: Oxidation of hydroethidine (HE) measured in HT29-MD2 cells incubated for 3 h under several conditions: (A) control; (B) Mn1 (100 µM); (C) $MnCl_2$ (100 µM). Data represent means ± SEM for 7 (*left*) or 3 (*right*) independent experiments.

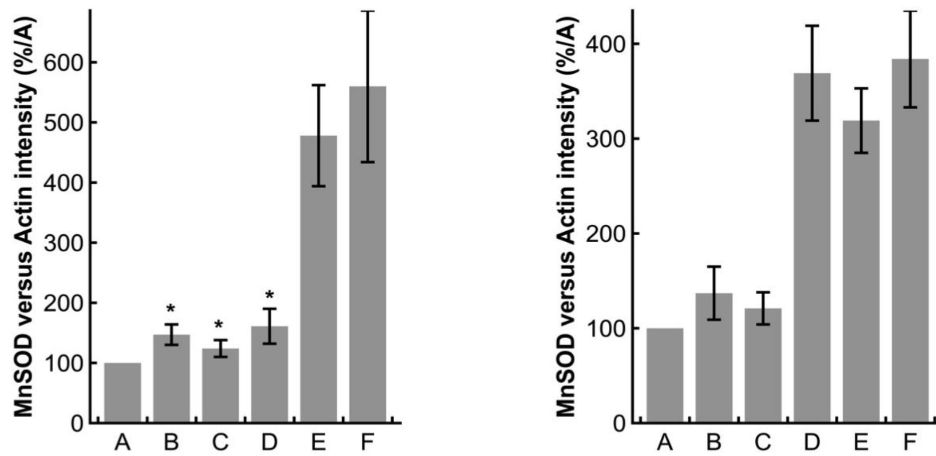
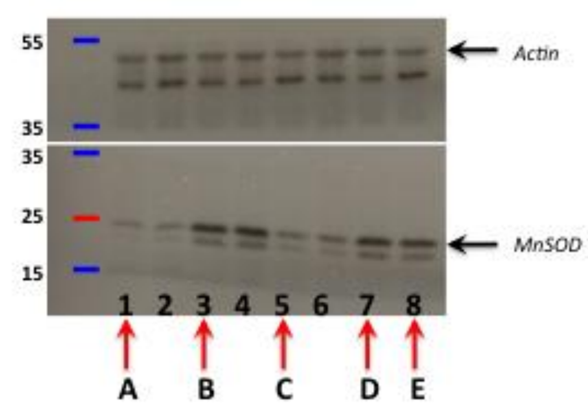
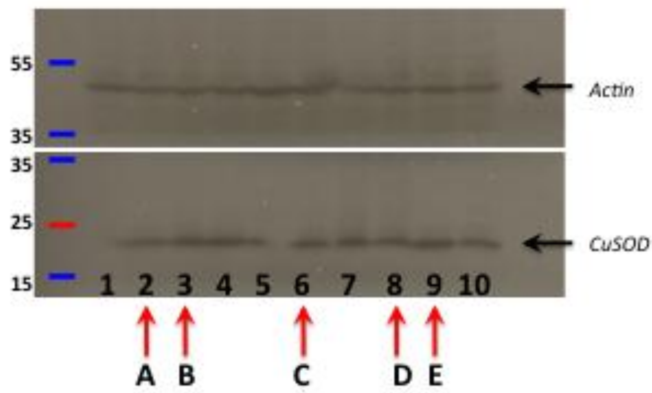


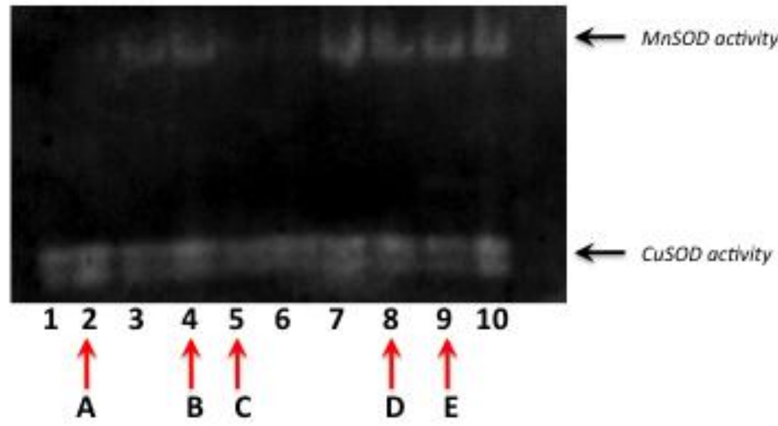
Figure S6 (related to Figure 4):Quantification of MnSOD expression in controls in intestinal epithelial cells.*Left:* Quantification of MnSOD versus actin expression obtained by western blot analysis. HT29-MD2 cells were incubated for 7 h under several conditions: (A) control (B) **Mn1** (100 μM), (C) $MnCl_2$ (100 μM), (D) HEPES, (E) LPS (F) HEPES + LPS. LPS (0.1 μg/mL) was added after 1 h of incubation. Data represent means ± SEM for 3 independent experiments. * $p< 0.05$ versus E. ***Right:*** Quantification of MnSOD versus actin expression obtained by western blot analysis. HT29-MD2 cells were incubated for 7 h under several conditions: (A) control (B) $ZnBr_2$ (100 μM), (C) **Zn1** (100 μM), (D) LPS, (E) $ZnBr_2$ (100 μM) + LPS, (F) **Zn1** (100 μM) + LPS. LPS (0.1 μg/mL) was added after 1 h of incubation. Data represent means ± SEM for 2 independent experiments.



1: Control
2: Control
3: LPS
4: LPS
5: LPS + Mn1 100 µM
6: LPS + Mn1 100 µM
7: LPS + $MnCl_2$ 100 µM
8: LPS + SOD 100 U/mL



1: Control
2: Control
3: LPS
4: LPS
5: LPS + Mn1 100 µM
6: LPS + Mn1 100 µM
7: LPS + $MnCl_2$ 100 µM
8: LPS + $MnCl_2$ 100 µM
9: LPS + SOD 100 U/mL
10: LPS + SOD 100 U/mL



1: Control
2: Control
3: LPS
4: LPS
5: LPS + Mn1 100 µM
6: LPS + Mn1 100 µM
7: LPS + $MnCl_2$ 100 µM
8: LPS + $MnCl_2$ 100 µM
9: LPS + SOD 100 U/mL
10: LPS + SOD 100 U/mL

Figure S7 (related to Figure 4):Representative experiment of CuSOD, MnSOD, and actin expression and activity in intestinal epithelial cells activated with LPS.HT29-MD2 cells were incubated for 7 h under several conditions: (A) control, (B) LPS, (C) Mn1 (100 µM) + LPS, (D) $MnCl_2$ (100 µM) + LPS, (E) bovine CuSOD (100 U/mL) + LPS. LPS (0.1 µg/mL) was added after 1 h of incubation. ***Top:*** Blot showing the expression of MnSOD in comparison with actin. ***Middle:*** Blot showing the expression of CuSOD in comparison with actin. ***Bottom***: NBT assay on non-denatured gel for the measurement of the SODs activities. On the left, main molecular weight markers (kDa). A,B,C,D,E: see figure 4.

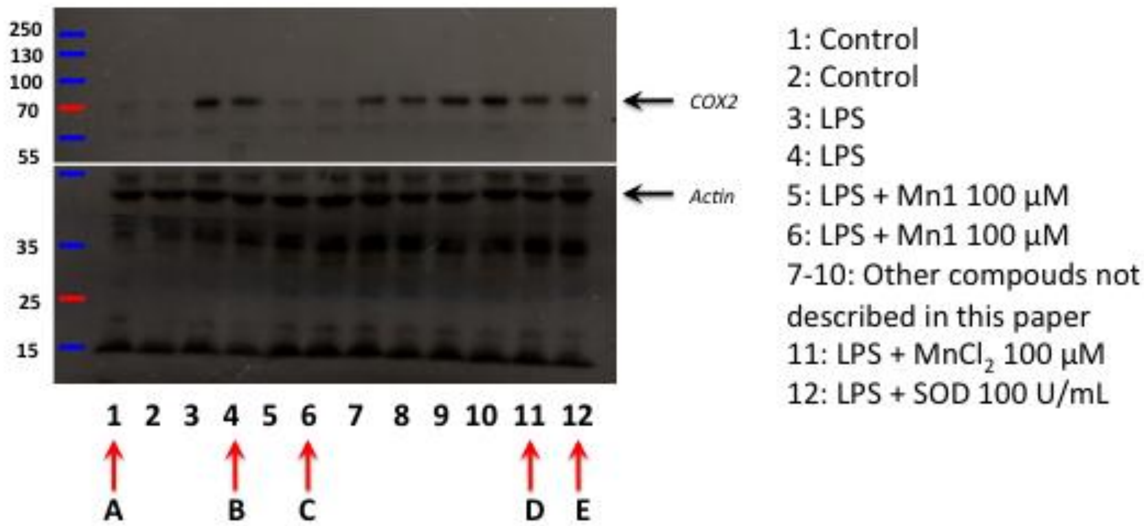
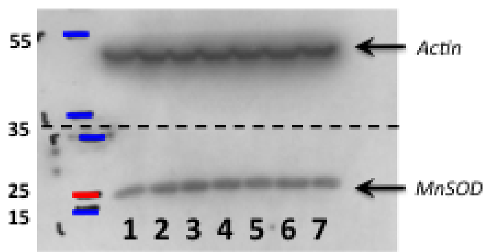
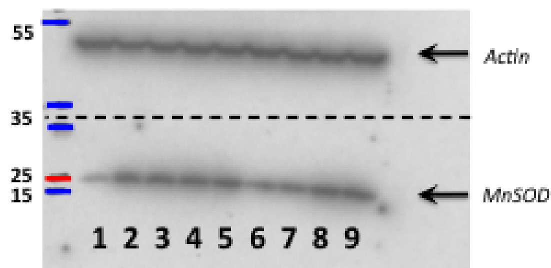


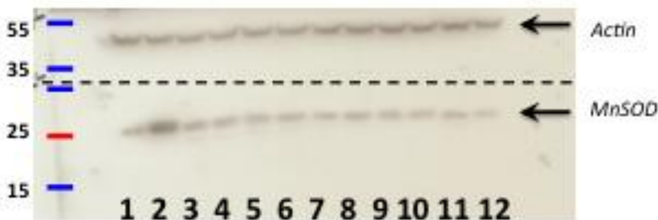
Figure S8 (related to Figure 5): Representative experiment of COX2, and actin expression in intestinal epithelial cells activated with LPS. HT29-MD2 cells were incubated for 7 h under several conditions: (A) control, (B) LPS, (C) Mn1 (100 µM) + LPS, (D) $MnCl_2$ (100 µM) + LPS, (E) bovine CuSOD (100 U/mL) + LPS. LPS (0.1 µg/mL) was added after 1 h of incubation. On the left, main molecular weight markers (kDa). A,B,C,D,E : see figure 5.



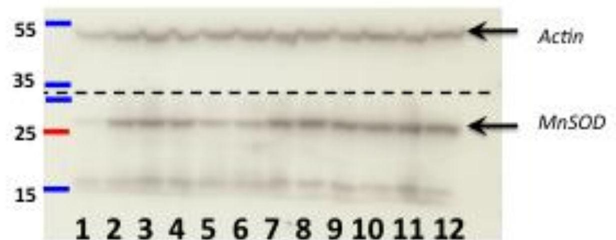
1: Control
2: $MnCl_2$ 100 µM
3: $MnCl_2$ 100 µM
4: Mn1 100 µM
5: Mn1 100 µM
6: HEPES
7: HEPES



1: Control
2: LPS
3: LPS
4: LPS + $MnCl_2$ 100 µM
5: LPS + $MnCl_2$ 100 µM
6: LPS + Mn1 100 µM
7: LPS + Mn1 100 µM
8: LPS + HEPES
9: LPS + HEPES



1: Control
2: LPS
3: $ZnBr_2$ 100 µM
4: $ZnBr_2$ 100 µM
5: Mn1 100 µM
6: Mn1 100 µM
7: Zn1 100 µM
8: Zn1 100 µM
9-12: Other compounds not described in this paper



1: Control
2: LPS
3: LPS + $ZnBr_2$ 100 µM
4: LPS + $ZnBr_2$ 100 µM
5: LPS + Mn1 100 µM
6: LPS + Mn1 100 µM
7: LPS + Zn1 100 µM
8: LPS + Zn1 100 µM
9-12: Other compounds not described in this paper

Figure S9 (related to Figure S6):Representative experiment of MnSOD, and actin expression in intestinal epithelial cells activated with LPS.HT29-MD2 cells were incubated for 7 h under several conditions. LPS (0.1 µg/mL) was added after 1 h of incubation. ***Top***: Blots related to Figure S6 *Left*. ***Bottom***: Blots related to Figure S6 *Right*.
On the left of each blot, main molecular weight markers (kDa).

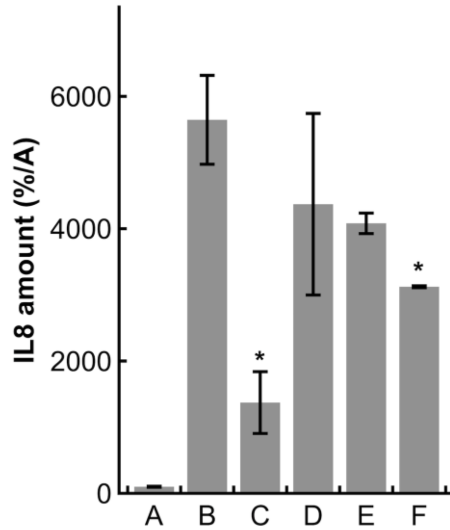


Figure S10: Comparisonof Mn1 and 5-ASA ant-inflammatoryactivity in intestinal epithelialcellsactivatedwith LPS. IL8 secretion was measuredby ELISA in supernatantsof HT29 MD2 incubated 7 h underseveralconditions: (A) control (B) LPS, (C) **Mn1** (100 µM) + LPS, (D) 5-ASA (100 µM) + LPS, (E) 5-ASA (1 mM) + LPS, (F) 5-ASA (10 mM) + LPS. LPS (0.1 µg/mL) was added after 1 h ofincubation. Data representmeans± SD for 2 replicates, * $p < 0.05$ versus B.

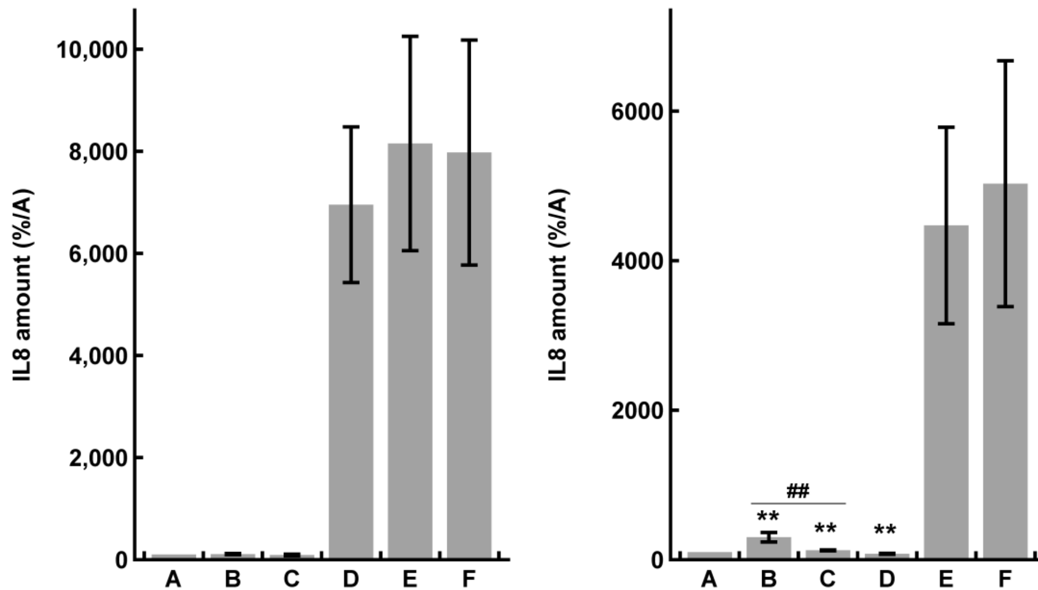


Figure S11 (related to Figure 5): IL8 secretion in controls in intestinal epithelial cells.*Left:*IL8 secretion in HT29-MD2 cells incubated for 7 h under several conditions: (A) control (medium only) (B) $ZnBr_2$ (100 µM), (C) **Zn1** (100 µM), (D) LPS, (E) $ZnBr_2$ (100 µM) + LPS, (F) **Zn1** (100 µM) + LPS. LPS (0.1 µg/mL) was added after 1 h of incubation. Data represent means ± SEM for at least 2 independent experiments. ***Right:***IL8 secretion in HT29-MD2 cells incubated for 7 h under several conditions: (A) control (medium only) (B) **Mn1** (100 µM), (C) $MnCl_2$ (100 µM), (D) HEPES, (E) LPS (F) HEPES + LPS. LPS (0.1 µg/mL) was added after 1 h of incubation. Data represent means ± SEM for 4 independent experiments. ** $p< 0.01$ versus E, and $^{\#\#}$ $p < 0.01$.

2. Supplemental Experimental Procedures

a. Materials

LPS (Escherichia coli O55:B5), bovine purified CuSOD, NADH, NBT, TEMED, riboflavin and pyruvic acid were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). IL8 detection ELISA kit (Duoset) was provided by R&D Systems (Minneapolis, Minnesota, USA). Goat polyclonal anti-human actin, mouse monoclonal anti-human COX2, rabbit polyclonal anti-human SOD2 (MnSOD), rabbit polyclonal anti-human SOD1 (CuSOD) were from Santa Cruz Biotechnology (Dallas, Texas, USA). Horseradish peroxidase-conjugated antibodies were from Rockland (Limerick, Pennsylvania, USA). BCA and BSA were from Uptima-Interchim (Montluçon, France). Detection ECL system and nitrocellulose membranes were from Amersham Biosciences (Piscataway, New Jersey, USA). Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, blasticidin, were from Invitrogen (Thermo Fisher Scientific, Waltham, Massachussetts, USA). Fetal bovine serum was from GE Healthcare LLife Sciences (South Logan, Utah, USA). HEPES buffer solution (1M), and Dulbecco's Phosphate Buffered Saline (10X, DPBS) was from Gibco (Thermo Fisher Scientific, Waltham, Massachussetts, USA). The protease inhibitor cocktail was from Roche Diagnostics (Meylan, France). Acrylamide-bis-acrylamide was from Q-Biogene (Carlsbad, California, USA). All other reagents not mentioned were from Sigma-Aldrich. Silicon nitride windows were purchased from Agar Scientific.

b. Intracellular quantification of Mn1by EPR in mitochondria-enriched fractions

Cells were cultured in a 75 cm^2 flask to reach 90 % confluence. They were incubated with medium only (control), Mn1 or $MnCl_2$ (100 µM) for 0.5 to 7 hours, at 37 °C. After washing with NaCl 0,9 %, a chaotropic shock was performed by adding a solution of NaCl(1 M). Cells were further washed once with EDTA 50 mM, and twice with NaCl 0.9%. They were then harvested by scraping and centrifuged at 4°C during 10 min at 900 rpm. Protein content was determined for each sample (see Cell assays). Enriched mitochondria fraction was obtained as follow: cells were centrifuged at 4°C during 5 min at 800 rpm, supernatant was removed, a Mitochondria Isolation Kit for cultured cells (Thermo Fisher Scientific, Waltham, Massachussetts, USA) was used, and three freezing/thawing cycles in liquid nitrogen were performed on isolated fraction. Mn^{2+} spectra were recorded for total cell lysates and enriched mitochondria fraction after acidification with $HClO_4$ (10% v/v final concentration), which freed manganese (II) ion from coordination.[2] EPR spectra were recorded on an Elexsys 500 spectrometer from Bruker in a glass capillary tube. The spectra recorded were normalized by the protein content. A calibration curve was established using $MnCl_2$ in water acidified with $HClO_4$ and the quantification of the Mn-content was performed using the two first lines in the six-line Mn(II) X-band EPR-spectrum and reported *per* mg of proteins.

Cell lysates parameter (Figure S2,*left*):

EPR spectra were recorded on aElexsys 500 spectrometer from Bruker in a glass capillary tube: frequency: 9.82 GHz, microwave power: 32 mW, amplitude of the modulation: 2G, 9 scans, room temperature.

Mitochondria enriched fraction parameter(Figure S2, *right*):

EPR spectra were recorded on aElexsys 500 spectrometer from Bruker in a glass capillary tube: frequency: 9.82 GHz, microwave power: 80.5 mW, amplitude of the modulation: 18G, 9 scans, time constant: 40,9 ms, room temperature.

c. Mn1 anti-superoxide activity: Titration of superoxide in HT29-MD2 cells

The amount of intracellular superoxide ion was measured usinghydroethidine (HE) for detection. Cells were seeded in 96-wells black plate (10^5 cells/well). After 24 h, they were incubated for 1 h with Mn1 (100 µM) or $MnCl_2$ (100 µM), then HE (20 µM) was added for two more hours. Cells were then washed with NaCl 0.9 % and fluorescence intensity was measured at excitation wavelength of 480 nm and emission wavelength of 615 nm using a SpectraMax M5e fluorescent microplate reader from Molecular Devices.

d. Cell assays

Supernatants and cell lysates collected after the experiments were stored at -20 °C before analysis.

Cytotoxicity

Cytotoxicity of the compounds was tested systematically for each experiment by following the release of the cytosolic lactate dehydrogenase (LDH) into the supernatant, indicating membrane damages. Cytotoxicity was considered when LDH release was more than 10%. No cytotoxicity was observed in HT29-MD2 cells upon incubation, with or without LPS.

The assay is based on the ability of LDH contained in cell lysates or supernatants to catalyze the reduction of pyruvate (0.6 mM) into lactate in the presence of NADH (0.18 mM), which is oxidized to form NAD^+, in PBS. The level of LDH was proportional to amount of pyruvate consumed measured by monitoring the decrease in absorbance (340 nm) due to oxidationof NADH during 1 min. The percentage of LDH release in supernatants was calculated by dividing this LDH activity found in supernatant, by the sum of activity in supernatants and cell lysates.

Protein assay

Protein concentrations were determined in cell lysates using BCA protein assay reagents and bovine serum albumin (BSA) as standard according to the manufacturer's instructions. Briefly, a disclosing solution (98% BCA, 2% $CuSO_4$) was added to

the protein solution in 96-wells plate. After 30 min at 37°C, absorbance was monitored at 560 nm in a SpectraMax M5e microplate reader from Molecular Devices. Absorbance was linked to protein mass thanks to a calibration curve with BSA.

3. SupplementaryReferences:

[1] Aitken, J. B., Shearer, E. L., Giles, N. M., Lai, B., Vogt, S., Reboucas, J. S., Batinic-Haberle, I., Lay, P. A., and Giles, G. I. (2013) Intracellular targeting and pharmacological activity of the SOD mimics MnTE-2-PyP(5+) and MnTnHex-2-PyP(5+) regulated by their porphyrin ring substituents, *Inorg. Chem.52*, 4121-4123.

[2] Ash, D. E., and Schramm, V. L. (1982) Determination of free and bound manganese(II) in hepatocytes from fed and fasted rats, *J. Biol. Chem. 257*, 9261-9264.