SUPPLEMENTARY MATERIAL
Evaluation of the Anti-Oxidant Properties of a SOD-mimic Mn-Complex in Activated Macrophages
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1. Chemicals
Murine recombinant interferon-γ (IFN-γ; specific activity, 2×10^7 U mg⁻¹) was provided by R&D Systems. Escherichia coli lipopolysaccharide O111:B4 (LPS), PMA (Phorbol myristate acetate), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), ferricytochrome c were purchased from Sigma. Locke 1X physiological medium (5.6 mM Glucose, 3.6 mM HCO₃⁻, 159.6 mM Cl⁻, 157.6 mM Na⁺, 5.6 mM K⁺, 5 mM HEPES-NaOH, 2.5 mM CaCl₂, 1.2 mM MgCl₂) was used in all amperometric experiments. Purified water from a Milli-Q system (resistance 18 MΩ cm⁻¹; Millipore) was used in the preparation of all solutions. DEANONOate (Diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate) and sodium peroxynitrite in alkaline solution were purchased from Cayman Chemical (USA). Complex 1(PF₆) was dissolved in DMSO and diluted in physiological Locke 1X solution for cells experiments (0.1 % DMSO).

2. Stability of the complex
As previously published, complex 1 shows a potential that ensures an efficient SOD-like activity. Mn(ClO₄)₂ in DMEM is not electroactive in the potential range tested (see figure S1-C). Complex 1 in HEPES (pH 7.4) or DMEM showed a very similar voltammogram, which indicates no decoordination, in the 100 µM range, in DMEM.

![Figure S1. Cyclic voltammograms of 1 180 µM in deoxygenated HEPES buffer (pH 7.4, 100mM) (A), 1 180µM in deoxygenated DMEM (B) and Mn(ClO₄)₂ 200 µM in deoxygenated DMEM (C); v=0.1 V s⁻¹, working electrode: glassy carbon; counter electrode: Pt wire; reference electrode: SCE.](image-url)
3. Cell culture
The murine macrophage RAW 264.7 (American Type Culture Collection) cell line was cultured at 37°C under a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g L⁻¹ D-glucose and sodium pyruvate (Invitrogen). The medium was supplemented with 5% fetal bovine serum (Invitrogen) and 20 μg mL⁻¹ gentamicin (Sigma). 18 to 24 h prior to electrochemical studies, confluent monolayers of RAW 264.7 cells were harvested mechanically and resuspended and plated in tissue culture Petri dishes (Nunc; 35 mm diameter).

4. Cell growth
Cell growth and viability was assessed by the mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan as previously reported. After 24 hours of incubation without or with 0, 2, 5, 10 or 50 µM complex, the macrophage cultures in the Petri dishes were incubated at 37°C with MTT (0.5 mg mL⁻¹) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in 0.04 M HCl in absolute ethanol. The extent of reduction of MTT to formazan by the cells was quantified by the measurement of OD₅₅₀. The results are arbitrarily normalized to control samples (Figure S2) and evidenced that incubations of 1 is cytotoxic for concentrations up to 10 µM.

![Figure S2](image_url)

**Figure S2.** Effect of concentration of 1 on the viability of RAW 264.7 macrophages (MTT assay). The values are normalized as a percentage with respect to untreated cells (control experiments) after 24 h of growth at 37°C under a 5% CO₂ atmosphere. The experiments were performed in triplicate and are presented as mean ± SEM.

5. SOD-activity evaluation in a cellular context using ferricytochrome c.

**Effect of intracellular 1**
Ferricytochrome c reduction was used to assess the superoxide ion production in RAW 264.7 cells. Cells were seeded in a six-well plate to reach 90% of confluency after 3 days. They were stimulated with culture medium containing 20 U mL⁻¹ IFN-γ, 50 ng mL⁻¹ LPS with or without antioxidant (10 µM 1, 10 µM MnCl₂ or 100 U mL⁻¹ SOD) for 24 h at 37°C (except for the control). After two washing with NaCl 0.9%, they were then
incubated 1h at 37°C with 1 mL of a medium (containing 14.6 mM glucose, 358 mM NaCl, 12.7 mM KCl, 3.1 mM KH₂PO₄, 6.1 mM MgSO₄, 3.1 mM CaCl₂, 13 mM NaHCO₃, 0.4 mM EDTA in 53 mM pH 7.4 HEPES buffer) with 100 µM ferricytochrome and 600 nM PMA. The absorbance of supernatants was read at 550 nm with a Cary 300 Bio Spectrophotometer. Contribution of ferricytochrome c at 100 µM was subtracted from the absorbance of each sample. Data are the mean ± SEM of six independent experiments (see figure 2 in the text).

Another experiment has been conducted under the same conditions with an additional chaotropic shock (NaCl 1M) for 1 before the two washing with NaCl 0.9 %. The results are presented in figure S3.

**Figure S3.** Mean extracellular superoxide amount for n=3 experiments under several conditions: control (A), IFNγ/LPS/PMA (B), IFNγ/LPS/PMA + 1 10 µM (C), IFNγ/LPS/PMA + 1 10µM with chaotropic shock (D). Error bars represent SEM.

**Effect of extracellular 1**
Ferricytochrome c reduction was used to assess the superoxide ion production in RAW 264.7 cells. Cells were seeded in a six-well plate to reach 90% of confluency after 3 days. They were stimulated with culture medium containing 20 U mL⁻¹ IFN-γ and 50 ng mL⁻¹ LPS for 24 h at 37°C (except for the control). They were then incubated 1h at 37°C with 1 mL of a medium (containing 14.6 mM glucose, 358 mM NaCl, 12.7 mM KCl, 3.1 mM KH₂PO₄, 6.1 mM MgSO₄, 3.1 mM CaCl₂, 13 mM NaHCO₃, 0.4 mM EDTA in 53 mM pH 7.4 HEPES buffer) with 100 µM ferricytochrome c, with or without antioxidant (10 µM 1, 10 µM MnCl₂ or 100 U mL⁻¹ SOD) and with 600 nM PMA. The absorbance of supernatants was read at 550 nm with a Cary 300 Bio Spectrophotometer. Contribution of ferricytochrome c at 100 µM was subtracted from the absorbance of each sample. Data are a mean ± SEM of ten independent experiments.
Figure S4. Mean extracellular superoxide amount for n=10 experiments under several conditions: control (A), IFN γ/LPS/PMA (B), IFN γ/LPS/PMA + 10 µM (C), IFN γ/LPS/PMA + MnCl₂ 10 µM (D), IFN γ/LPS/PMA + SOD 100 U mL⁻¹ (E). Error bars represent SEM.

6. Microelectrodes fabrication
The preparation of microelectrodes has been previously described in detail.³, ⁴ Briefly, individual carbon fibers (10 µm diameter; Thornel P-55S, Cytec Engineered Materials) were sealed into pulled-glass capillaries (1 mm diameter; GC120F-10, Clark Electromedical Instruments) and the protruding carbon fibers were insulated by electrochemical deposition of poly-(oxyphenylene) following literature procedure. The tip of the insulated carbon fiber was then polished at an angle of 45° on a diamond particle whetstone microgrinder (Model EG-4, Narishige) to expose a clean, elliptical carbon surface. The polished carbon surface was then platinized by reducing hydrogen hexachloroplatinate (Sigma) in the presence of lead acetate (Sigma) at —60 mV versus SSCE. The platinization process, followed on a computer, was interrupted when the electrical charge of the signal reached 5 µC, which corresponds to the optimal activity and sensitivity of the electrode surface in this series of experiments.

7. Single-cell measurements
The macrophages were stimulated with culture medium supplemented with IFN-γ (20 U ml⁻¹) and LPS (50 ng mL⁻¹) to induce the production of ROS and RNS in the presence or absence of complex (1 or 10 µM). After 24h, the culture medium into the Petri dish is replaced by a Locke solution supplemented with 600 nM PMA. The cells are thus ready for the electrochemical detection of ROS/RNS release. Experiments were performed at controlled room temperature (22±1°C) on the stage of an inverted microscope (Axiovert 135, Zeiss) placed in a Faraday cage. The microelectrode was positioned about 30 µm above the cell of interest and polarized for 3 min at the start of each measurement. The microelectrode tip was then lowered slowly with a micromanipulator (MHW-103, Narishige) till it was just in contact with the cell. The release of ROS/RNS was detected in real time by amperometry (AMU130 amperometer, Radiometer Analytical) at a series of constant potential (E) versus a sodium-saturated calomel reference electrode. Values of E (+300, +450, +650, and +850 mV) were selected based on previous in vitro voltammetric studies of the oxidation of independent H₂O₂, ONOO⁻, NO, and NO₂⁻.⁵ The time course of the amperometric current was monitored and stored on a computer (Latitude D600, Dell) through a D/A converter (Powerlab 4SP, ADInstruments) and its software interface (Chart version 4.2 for Windows, ADInstruments). The exponential
decrease of the baseline current due to microelectrode polarization over the course of
the measurement was fitted and subtracted by using commercial software (Origin
version 7.0, OriginLab Corporation). Each resultant curve was then integrated over time
(1 h duration) to obtain the total charge (Q) that corresponds to the overall oxidation
process(es) occurring at each specific measurement potential.

Figure S5. Resulting difference curve obtained by subtraction of baseline and a typical
amperometric response from a single IFN-γ/LPS/PMA-stimulated and complex treated
macrophage (measured at +650 mV versus SSCE) in order to quantify the released species
amount.

The detected current at each potential, \( I_{\text{potential}} \), can be written as a linear
combination of the responses of each of the four species, with different weights. The
weights, dependent only on the detection potential and the type of electrode used, are
experimentally determined from in vitro steady state voltammograms of each respective
species:

\[
I_{850\text{mV}} = I_{H_2O_2} + I_{ONOO^-} + I_{NO} + I_{NO_2^-} \quad (1)
\]
\[
I_{650\text{mV}} = I_{H_2O_2} + I_{ONOO^-} + I_{NO} \quad (2)
\]
\[
I_{450\text{mV}} = 0.99 \times I_{H_2O_2} + 0.90 \times I_{ONOO^-} \quad (3)
\]
\[
I_{300\text{mV}} = 0.85 \times I_{H_2O_2} + 0.29 \times I_{ONOO^-} \quad (4)
\]

This system of linear equations can be extended to the coulometric charges detected at
each potential (Figure S5). Due to the definition of current (\( I = \frac{dQ}{dt} \)), those coulometric
charges directly correspond to the area of the amperogram integrated over one hour.
The amount \( (N_{\text{species}}) \) of each species released over one hour can then be calculated from
their respective charges obtained above through application of Faraday’s law (5):

\[
N_{\text{species}} = \frac{Q_{\text{total,species}}}{(n_{\text{species}} \times F)} \quad (5)
\]

where \( n_{\text{species}} \) is the number of electrons per molecule exchanged in the oxidation of a
given species \( (n_{\text{species}} = 2 \) for \( H_2O_2 \) and \( NO_2^- \); \( n_{\text{species}} = 1 \) for \( ONOO^- \) and \( NO \)) and \( F \) is the
Faraday constant. Therefore, amperometric measurements of statistically significant
numbers of single macrophages at four different potentials (+300, +450, +650, and +850
mV versus SSCE) enable a direct quantification with excellent precision of the release of
\( H_2O_2, ONOO^- \), \( NO \), and \( NO_2^- \). Furthermore, through the admitted scheme for evolution
of ROS/RNS species in macrophages (Scheme 1), primary (or initial) flux of superoxide
anion and nitric oxide can be back-calculated, or reconstructed:

\[
N_{O_2} = 2N_{H_2O_2} + N_{ONOO^-} + N_{NO_2^-}
\]
\[
N_{NO} = N_{ONOO^-} + N_{NO} + N_{NO_2^-}
\]
Results obtained in the case of complex 1 (incubation at 1 and 10 µM) or MnCl$_2$ (incubation at 10 µM) are displayed below (Figures S6 to S9 and figure 3 in text).

**Figure S6.** Mean charges measured per single cell over a 1 h period through electrochemical detection at four different potentials. Single cell measurements were performed on RAW 264.7 macrophages activated by IFN-γ/LPS - PMA and pre-treated with (gray bar) or without (white bar) 1 µM of 1. Error bars represent SEM (>30 independent measures at each potential).

**Figure S7.** Mean charges measured per single cell over a 1 h period through electrochemical detection at four different potentials. Single cell measurements were performed on RAW 264.7 macrophages activated by IFN-γ/LPS - PMA and pre-treated with (gray bar) or without (white bar) 10 µM of 1. Error bars represent SEM (>30 independent measures at each potential).

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>$H_2O_2$ (fmol)</th>
<th>ONOO$^-$ (fmol)</th>
<th>NO (fmol)</th>
<th>NO$_2^-$ (fmol)</th>
<th>$O_2^-_{initial}$ (fmol)</th>
<th>NO$_{initial}$ (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0.5 ± 0.1</td>
<td>2.6 ± 0.3</td>
<td>2.0 ± 0.6</td>
<td>1.2 ± 0.4</td>
<td>4.7 ± 0.8</td>
<td>5.8 ± 1.3</td>
</tr>
<tr>
<td>1 10 µM</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.3</td>
<td>3.9 ± 0.6</td>
<td>4.6 ± 1.1</td>
</tr>
</tbody>
</table>

**Table S1.** Mean amounts (fmol) of ROS/RNS species released by single IFN-γ/LPS - PMA stimulated RAW 264.7 macrophages for 1 hour with and without the complex at 10 µM (see figure 2 in the text).
Figure S8. Mean charges measured per single cell over a 1 h period through electrochemical detection at four different potentials. Single cell measurements were performed on RAW 264.7 macrophages activated by IFN-γ/LPS - PMA and pre-treated with (gray bar) or without (white bar) 10 μM of MnCl₂. Error bars represent SEM (>30 independent measures at each potential).

Figure S9. Mean amounts (fmol) of ROS/RNS species measured per single cell over a 1 h period through electrochemical detection at four different potentials and back-calculated (ini) superoxide and nitric oxide. Single cell measurements were performed on RAW 264.7 macrophages activated by IFN-γ/LPS - PMA and pre-treated with (gray bar) or without (white bar) 10 μM of MnCl₂ during 24h. The results correspond to 30 independent amperometric measurements per potential. Error bars represent SEM.

8. In vitro activity of 1 with NO
NO solution was obtained through a common NO donor, DEANONOate. Diethylamine-NONOate or DEANONOate [Diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate] dissociates into the free diethylamine and NO through a pH-dependent first order kinetic. Crystalline solid DEA-NONOate was stored under nitrogen at -20°C to avoid its decomposition. To initiate the release of NO, a solution was prepared by rapidly mixing DEANONOate with Locke buffer (at pH 7.4) to provide a 10 mM DEANONOate solution, immediately injected into the cell chamber for absorbance monitoring with or without 1 (1 eq.).
Figure S10. Decomposition of NO (formed through NO donor DEANONOate) with (dashed line with triangles) and without (solid line with circles) 1 monitored by UV-Vis spectroscopy (250 nm, initial concentration of 10 mM). $A_0$ (resp. $A_{\text{inf}}$) is initial (resp. final) absorbance.

9. In vitro activity of 1 with ONOO$^-$

The ONOO$^-$ aliquots (aqueous solution in NaOH 0.3 M at 41.2 mM) can be stored at -80°C without any decomposition over 6 months. UV investigations of peroxynitrite reactivity were achieved by rapidly mixing ONOO$^-$ with basified HEPES buffer 100 mM (at pH 10.6) to provide a 412 µM ONOO$^-$ solution, immediately injected into the cell chamber for absorbance monitoring at 302 nm with or without 1 (0.1 eq.).
Figure S11. A: Intrinsic decomposition of ONOO$^-$ monitored by UV-Vis spectroscopy in basified HEPES buffer 100 mM (pH 10.6). B: Intrinsic decomposition of ONOO$^-$ at 302 nm in basified HEPES buffer 100 mM (pH 10.6). C: Decomposition of ONOO$^-$ at 302 nm in presence of 0.1 eq of 1 in basified HEPES buffer 100 mM (pH 10.6).

The absorbance decrease observed in Figure S11C could be used to extract the rate constant of the reaction between ONOO$^-$ and 1. Unfortunately, a rigorous treatment requires to consider two competitive pathways, that are the intrinsic decomposition of ONOO$^-$ (first order) and the reaction between ONOO$^-$ and 1 (second order). In absence of basic simplifications (Ostwald isolation method, reaction in stoichiometric amounts), the corresponding kinetic equation could be only solved through a numeric treatment that is beyond the scope of the present work. Nevertheless, we can roughly estimate the rate constant by using initial rate method:

$$v_0 = k[\text{ONOO}^-]_0[1]_0 = -\left(\frac{d[\text{ONOO}^-]}{dt}\right)_0$$

Using Beer-Lambert’s law, we obtain:

$$v_0 = k[\text{ONOO}^-]_0[1]_0 = -\frac{1}{\varepsilon l} \left(\frac{dA}{dt}\right)_0$$

The initial slope can be easily extracted from the A = f(t) curve as a value of $-0.288 \text{ min}^{-1} = -4.8.10^{-3} \text{ s}^{-1}$. Knowing initial concentrations,$^b$ l and $\varepsilon$ values (respectively 1 cm and 1670 mol$^{-1}$ L cm$^{-1}$) allowed obtaining the rate constant k as:

$$k = \frac{-4.8.10^{-3}}{1670\times1\times354.10^{-6}\times0.1\times412.10^{-6}} \Rightarrow k = 197 \text{ mol}^{-1}\cdot\text{L}\cdot\text{s}^{-1}$$

$^a$ These two methods are precluded in the present case. Indeed, they need to work with at least one equivalent of 1. As such, its high molar extinction coefficient (4476 mol$^{-1}$ L cm$^{-1}$) by comparison with peroxynitrite (1670 mol$^{-1}$ L cm$^{-1}$) and a close wavelength for maximum absorption (286 nm) would prevent ONOO$^-$ monitoring.

$^b$ The initial peroxynitrite concentration is 412 µM. Nevertheless, the peroxynitrite solution was remained two minutes before 1 was added and was slightly altered. By extrapolation of Figure S11B, the peroxynitrite concentration was then estimated at 354 µM. The initial proportion 1:ONOO$^-$ was 1:10, thus leading to $[1]_0 = 41.2 \text{ µM}$. 

Furthermore, some of us recently reported a possible reactivity between peroxynitrite anion ONOO\(^-\) and a similar complex to that investigated here, [Mn(pyane)(H\(_2\)O)]\(^2+\) (a SOD-mimic that also acts as a NO dismutase).\(^6\) While this reactivity was qualitatively measured through electrochemical measurements, an estimation by the same methodology\(^c\) used here can be achieved and gives a rate constant value of 325 mol\(^-1\) L s\(^-1\), that is in the same range as the reactivity between 1 and ONOO\(^-\).

10. In vitro activity of 1 with H\(_2\)O\(_2\): catalase effect

The catalase activity of 1 was assayed similar conditions as described in the literature for Mn-salen complexes (EUK), which are known catalytic scavengers of H\(_2\)O\(_2\).\(^8\)-\(^11\) Catalase activity was assayed by using a Gilson Oxygraph equipped with a Clark-type electrode to monitor the conversion of H\(_2\)O\(_2\) to O\(_2\). In a typical experiment (n=5), a reaction mixture containing HEPES (100 mM, pH 7.4) and H\(_2\)O\(_2\) (50 mM) was stirred at room temperature and when the baseline was linear, 1 (20 \(\mu\)M) was added. The complex was solubilised using a minimal amount of DMSO which was less than 0.2 % v/v overall. Oxygen concentration was monitored at 2 s intervals and the baseline was calculated by linear regression using readings obtained 60 s before the addition of the metal complex and was subtracted from all values. The initial rate was calculated by linear regression using data from the first 6 s of the reaction. The maximum O\(_2\) concentration was calculated using data 20 s after the reaction endpoint. Blank experiments showed that the complex alone (without H\(_2\)O\(_2\)) or DMSO had no influence on the O\(_2\) level of the system. Data are a mean ± SEM of 5 independent experiments. The reaction was complete before all the substrate was consumed, suggesting the inactivation of the catalysis under the assay conditions, which has been previously observed for the related EUK complexes.\(^8\),\(^9\),\(^11\)

![Figure S12](https://example.com/figure12.png)

**Figure S12.** Dioxygen evolution from a 50 mM H\(_2\)O\(_2\) solution in HEPES 100 mM (pH 7.0) in the presence of 20 \(\mu\)M of 1 (corrected from natural disproportionation of H\(_2\)O\(_2\); baseline). Mean values ± SEM from 5 independent experiments.

\(^{c}\) The oxidation current of a peroxynitrite solution was monitored at a constant potential (450 mV vs SSCE) in presence of the Mn-complex. Knowing the electrode sensitivity (33 pA \(\mu\)M\(^-1\), calculated from standard solutions,\(^7\) the initial concentrations ([ONOO\(^-\)]\(_0\) = [Mn-cx]\(_0\) = 25 \(\mu\)M), and the initial slope of the I = f(t) curve (6.7 pA s\(^-1\)), the rate constant can be calculated with the method described above.