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Evaluation of the Anti-Oxidant Properties of a SOD-mimic Mn-Complex in Activated Macrophages

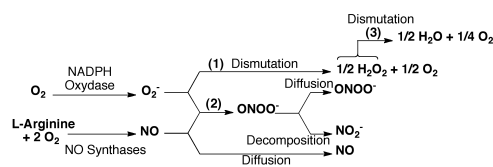
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SOD-mimic are small complexes that reproduce the activity of superoxide dismutases, natural proteins that catalytically
dismutate the superoxide anion. Activated macrophages, which produce ROS and RNS fluxes, constitute a relevant model to challenge antioxidant activity in a cellular context and were used to test a Mn-complex which was shown to efficiently alter the flow of O_2^- , $ONOO^-$ and H_2O_2 .

Reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are continuously produced as byproducts of normal cellular aerobic metabolism.^{1, 2} They are known to be part of a complex network involving also reactive nitrogen species (RNS) derived from nitric oxide (NO) as O_2^- and NO can react together leading to the harmful peroxynitrite ($ONOO^-$) (see scheme 1). Efficient protection pathways against damaging ROS have evolved and involve either low molecular weight antioxidants (E and C vitamin, GSH, etc.) or metalloenzymes (superoxide dismutases, catalases, peroxidases). When these protection pathways are overwhelmed, oxidative stress arises, which occurs in a wide range of pathophysiological processes,²⁻⁵ including diabetes, inflammatory and neurodegenerative diseases, reperfusion injury after ischemia, cancer, and also aging.

The fact that the removal of the superoxide anion has a beneficial outcome in oxidative stress is now well documented.^{2, 4} Superoxide dismutases are metalloenzymes involved in the control of the superoxide concentration inside bacteria or cells by catalytically dismutating this anion. However, major drawbacks limit the therapeutic use of the enzymes (low stability, limited cellular accessibility, immunogenicity, short half-lives, cost of production, proteolytic digestion, contamination risks),^{2, 4} therefore low molecular-weight molecular complexes have been developed as SOD-mimics, using metal ions such as copper, iron, manganese or, more rarely, cobalt.^{6, 7} In the last decade, special interest has been devoted to Mn-complexes^{2, 4, 5, 8, 9} as they do not lead to Fenton chemistry that induces pro-oxidant effects by the production of the reactive hydroxyl radical. Indeed, to date, a large variety of Mn-derivatives have been reported for their ability to react with superoxide in *non-cellular* conditions (labelled herein as *in vitro*), either as a true SOD mimic with catalytic activity or as a stoichiometric scavenger.⁹ Recently, some differences have been evidenced between intrinsic SOD-mimic Mn-complexes' activities evaluated *in vitro*, and their *in cellulo* or *in vivo* efficiency, that were assigned to a differential cell-location.¹⁰ This example emphasizes the importance of

testing the activity, not only *in vitro*, but also in biological models of oxidative stress. *In vivo* models range from radiation injuries,¹¹⁻¹³ chemotherapy or chemical stress,^{14, 15} ischemia-reperfusion injury,¹⁶⁻¹⁹ neuronal oxidative stress,^{20, 21} endotoxic shock,²² diabetes,²³ or inflammation.²⁴ However, there are much less data concerning investigations on cellular models. Most of the data concern the efficiency of SOD-mimics to rescue SOD deficient strain of *E. coli*.^{10, 23, 25, 26} Some other models involve other SOD deficient cells,^{10, 27} cells under irradiation,^{28, 29} chemical stress or infection,^{14, 15} or activated cells producing ROS species, such as macrophages³⁰⁻³³ or human leukemia cells.³⁴



Scheme 1: Reaction scheme describing the reactive oxygen and nitrogen species in the oxidative burst of RAW 264.7 macrophages.

Hence, there is a need for studies involving cellular models of oxidative stress to bridge the gap between *in vitro* and *in vivo* studies. We have chosen to investigate the properties of a 1,2-diaminoethane based Mn(II) complex (**1**, see Figure 1).³⁵ This complex, among the series of N-centered complexes developed by some of us,³⁵⁻⁴¹ is of interest as it displays a good anti-superoxide activity *in vitro*, which is in line with its redox potential being close to that of SODs, a key parameter for the SOD-activity.^{38, 42} Complex **1** has been shown to have a $E_{1/2}=0.2$ V/SCE (0.44 V/NHE),³⁵ close to the optimal value of 0.12 V/SCE (0.36 V/NHE)^{38, 42, 43} and with $k_{McCF} = 7.0 \pm 0.3$ M⁻¹s⁻¹.^{35, 44} In this communication the activity of **1** was investigated in macrophages immuno-activated by interferon- γ /lipopolysaccharide (IFN- γ /LPS) and phorbol-12-myristate-13-acetate (PMA), and compared with that of MnCl₂. As major actors in inflammation, macrophages constitute an interesting model for oxidative stress as they generate ROS and RNS high fluxes under immuno-stimulation.^{45, 46} The activities of **1** and MnCl₂ were first estimated on a collection of cells after incubation. To provide further *in situ* insights in the activity, experiments were conducted directly on single living cells using amperometry at ultramicroelectrodes to record real-time release of ROS and RNS,⁴⁷ as we and others have previously shown.⁴⁸

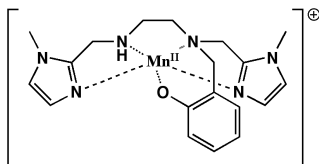


Figure 1: Schematic structure of complex **1**, used as hexafluorophosphate derivative.

Stability and cytotoxicity. The stability of the complex in cell-culture medium was monitored. Using its clear signature in cyclic voltammetry, **1** was found to be stable in DMEM-culture medium (Figure S1). The cytotoxicity of **1** was then evaluated on RAW 264.7 macrophages in order to determine an appropriate range of concentration to evaluate the activity of **1** on these cells. Cultures of macrophages were incubated with 2, 5, 10, 20 or 50 μM of complex **1** over 24 hours. The MTT reduction assay⁴⁹ showed that 1 and 10 μM concentrations were suitable (see Supp. Mat., Figure S2) for the 24h incubation required for the IFN γ /LPS activation.

Macrophages activation. Macrophages can be activated to produce ROS/RNS species that mostly derived from primary nitric oxide and superoxide anion. The oxidative burst is elicited through a successive IFN γ /LPS and PMA activation (see Supp. Mat.), the majority of ROS/RNS produced over IFN- γ /LPS stimulated macrophages being due to iNOS expression, while PMA is known as a NADPH oxidase activator.⁵⁰⁻⁵²

Evaluation of the SOD-activity in a cellular context using ferricytochrome *c*. As a first test, superoxide produced by a collection of macrophages was quantified using ferricytochrome *c* reduction in the extracellular medium.⁵³

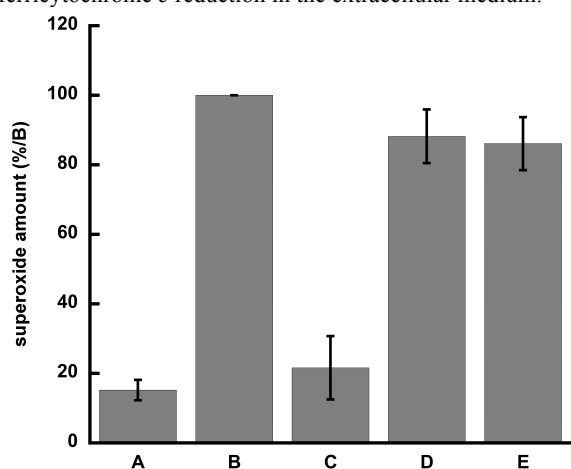


Figure 2. Mean extracellular superoxide amount for $n=6$ independent experiments under several conditions: control (A), IFN γ /LPS/PMA (B), IFN γ /LPS/PMA + **1** 10 μM (C), IFN γ /LPS/PMA + MnCl_2 10 μM (D), IFN γ /LPS/PMA + SOD 100 U/mL (E). Error bars represent SEM. This experiment reveals the intracellular activity of **1** (see text).

Cells were incubated for 24h with IFN γ /LPS and without or with **1**, MnCl_2 (10 μM) or bovine CuZnSOD (100 U/mL). It should be noted that this protein is not able to cross membranes.² The supernatants were then removed and a solution containing ferricytochrome *c* (100 μM) and PMA was added. After 1h, absorbance of the supernatants was measured at 550 nm.³¹ As shown in figure 2, the amount of extracellular

superoxide detected by reduction of the ferricytochrome *c* was significantly reduced for the cells pre-treated with **1** and activated (C) in comparison with activated cells (B), the level of superoxide in C being close to that of unactivated cells (A). No effect was recorded in the case of cells pre-treated with MnCl_2 (D) or CuZnSOD (E). The absence of effect of CuZnSOD is in line with the absence of any cell-penetration for the enzyme. To check that the reduction in the flux of superoxide anion was not due to **1** in interaction with the outer leaflet of the plasmic membrane, but to intracellular **1**, cells were washed using a chaotropic agent (NaCl 1M) before the addition of the ferricytochrome *c*. As shown in figure S3, no difference was observed with and without this chaotropic shock. Overall, these results indicate unambiguously that intracellular **1** is able to reduce efficiently the flux in superoxide.

To quantify the activity of **1** in comparison with CuZnSOD, the activity of extracellular **1** was estimated and compared with that of CuZnSOD. As previously, cells were activated (IFN γ /LPS) and then incubated 1 h with PMA, ferricytochrome *c* and with **1** (10 μM), MnCl_2 (10 μM) or CuZnSOD (100 U/mL). The activity of 10 μM of extracellular **1** was found close to that of 100 U/mL CuZnSOD, significantly higher than the weak activity of MnCl_2 (see Supp. Mat. and figure S4).

ROS and RNS fluxes at the single-cell level. Amperometric analyses of ROS and RNS release from macrophages cells using ultramicroelectrodes were performed as previously described.⁵⁵⁻⁵⁷ Experiments consist of positioning a platinized carbon fiber ultramicroelectrode in the close vicinity (~ 100 nm) of a single isolated living cell using micromanipulators ("artificial synapse" configuration). In the present experiments, macrophages were pre-incubated for 24 h with IFN γ /LPS and Mn derivatives (**1** or MnCl_2) at 10 μM or without. The pre-incubation medium was then removed, and a medium supplemented with PMA was added. The ROS/RNS release was then monitored at the single-cell level by amperometry at a constant potential as a function of time by applying a series of amperometric measurements at different potentials (+300 mV, +450 mV, +650 mV, and +850 mV versus sodium-saturated calomel reference electrode (SSCE)) and integrated during one hour (see Figure S5). Averaging these individual responses over 30 cells allowed a statistically significant quantification of the four electroactive species (H_2O_2 , ONOO^- , NO, or NO_2^-) fluxes (see Figure 3 and Supp. Mat., Table S1 and Figure S7). Cells pre-treated with **1** at 1 μM or MnCl_2 at 10 μM did not exhibit any alteration of ROS/RNS release in comparison with conditions without any Mn-salt (see Supp. Mat., Figures S6, S8 and S9.).

Extractions of the mean amount of H_2O_2 , ONOO^- , NO, and NO_2^- released over one hour by a single macrophage are summarized in Figure 3. The initial fluxes of O_2^- and NO can be back-calculated (see Supp. Mat. and Table S1) from the concentrations in H_2O_2 , ONOO^- , NO, and NO_2^- in Figure 3 and Table S1.⁵⁵⁻⁵⁷ The weak differences, if any, in the initial fluxes recorded with and without preincubation with **1** indicate that incubated **1** does not inhibit the production of superoxide nor that of NO. However, the peroxyxynitrite amount released during the oxidative burst was drastically reduced (about one fourth) in the case of cells pre-incubated with **1** (10 μM) in

comparison with control cells, while the amount of hydrogen peroxide was significantly but only slightly increased (about one and a half). In addition, the NO and NO₂[−] amounts detected directly were not significantly modified. Considering the decomposition scheme of superoxide/nitric oxide occurring for macrophages activation (scheme 1), these observations are consistent with the SOD-activity of **1**, previously evaluated *in vitro*³⁵, in an extracellular environment (see figure S4) or inside macrophages (see figure 2). Indeed, the SOD-mimic **1** accelerates the superoxide conversion into hydrogen peroxide (scheme 1 reaction (1)), which is a pathway in competition with the reaction between NO and O₂[−] (reaction (2)), and thus decreases the peroxynitrite amount detected at the electrode. Since the peroxynitrite anion arises only from superoxide/nitric oxide reaction with a stoichiometry of 1 peroxynitrite *per* superoxide, and superoxide disproportionation provides ½ H₂O₂ *per* superoxide, the overall peroxynitrite decrease should be twice that of H₂O₂ increase. In these experiments, the average decrease in peroxynitrite was evaluated to 2 fmol whereas the average increase in H₂O₂ was evaluated to 0.3 fmol, which is below the expected value (*ca.* 1 fmol). This result points at a possible direct reaction of **1** with the peroxynitrite anion and/or with H₂O₂ (reaction (3)).

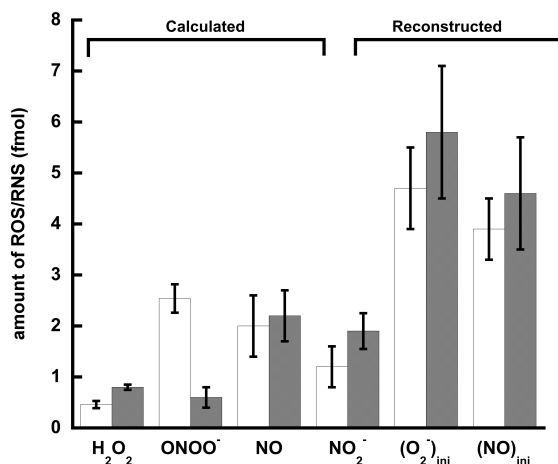


Figure 3. Mean amounts (fmol) of ROS/RNS species (H₂O₂, ONOO[−], NO and NO₂[−]) measured *per* single cell over a 1 h period through electrochemical detection at four different potentials. Original fluxes (labelled “ini”) can be reconstructed for superoxide and nitric oxide (see Supp. Mat. for details). 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** during 24h. The results correspond to 30 independent amperometric measurements *per* potential. Error bars represent SEM (n=30cells). See Table S1 and figure S9 for fluxes in the case of MnCl₂.

Additional *in vitro* reactivity of **1.** To rationalize these observations, reactivity of **1** with NO, ONOO[−], and H₂O₂ were investigated *in vitro*. First, the reactivity of the Mn-complex with NO was questioned using an NO-donor, namely, DEANONATE (see Supp. Mat.). Evolution of the expected NO absorbance (λ_{max} at 250 nm) decrease was monitored with or without **1** (1 eq.), showing that, under such experimental conditions, the intrinsic slow decomposition of NO was not affected by the presence of **1** (see figure S10). This result

establishes the absence of reactivity of **1** with NO, as expected from the single-cell fluxes determination. The reactivity of **1** with ONOO[−] was investigated *in vitro* at pH = 10.6. Indeed, ONOO[−] is intrinsically very unstable at acidic or physiological pH (7.4) (t_{1/2} ~ 0.5 s),⁵⁸ forbidding *in vitro* investigations at such a pH. Conversely, the reactivity with **1** could be tested in very basic conditions, in which its decomposition in absence of target molecules is slowed down.⁵⁹ As shown in figure S11, the peroxynitrite decomposition, monitored at 302 nm, was found to be faster in the presence of **1** (0.1 eq.), thus indicating a catalytic effect of **1**, close that of a previously published NO-dismutase Mn-complex (see supp. Mat.).³⁰ Although these strongly basic conditions are far from being physiological, this qualitative experiment indicates a possible reaction with peroxynitrite and **1**, whenever both species are present simultaneously in a same cellular compartment. Furthermore, the catalase activity, consisting of the catalysis of hydrogen peroxide dismutation, of **1** (20 μM) was assayed by monitoring the conversion of hydrogen peroxide (50 mM) into dioxygen using a Clark-type electrode (see Supp. Mat.).^{16, 60-62} The initial catalase rate for **1** was 69 ± 17 μM O₂ produced/min, while the maximal concentration in O₂ was 67 ± 10 μM, which is in the range of previously reported salen (EUK) complexes, known catalytic scavengers of H₂O₂.^{16, 60, 62} The present results, with disproportionation of 6.7 moles of H₂O₂ *per* mole of metal complex, is consistent with catalase activity.^{16, 60, 62}

Conclusion

Macrophages are considered as relevant models for oxidative stress and for testing the antioxidant properties of SOD-mimics in a cellular context.³⁰ Amperometric measurements of ROS and RNS provide a complete picture of the reactivity of the SOD-mimic in biological conditions, with a direct evaluation of extracellular fluxes in H₂O₂, ONOO[−], NO and NO₂[−], and a possible back calculation of initial fluxes/production of O₂[−] and NO. Complementary information was obtained from the ferriicytochrome *c* test, which measures the flux in superoxide in the extracellular medium. “Artificial synapse” amperometric measurements established that cells pre-treated with **1** showed a significantly reduced flux in superoxide without any modification in the superoxide initial production. In addition, these measurements indicated that intracellular **1** did not modify the fluxes in NO nor in NO₂[−], but significantly reduced the flux in ONOO[−], with an increase in H₂O₂ weaker than what could have been expected in the case of a pure SOD activity. These results suggested that, beyond its SOD-mimic activity, complex **1** can react with ONOO[−] and H₂O₂, that was evaluated *in vitro*.

Interestingly, intracellular **1** was found to be an efficient SOD-mimic, as expected from its design,^{32,35} and it was found to display attractive additional reactivity with the harmful ROS and RNS H₂O₂ and ONOO[−], without any reactivity with the beneficial NO. **1** is thus an interesting derivative for further test in cellular models of oxidative stress.

Abbreviations

DEANONOate = diethylammonium (Z)-1-(*N,N*-diethylamino)diazene-1-ium-1,2-diolate; DMEM = Dubelcco’s modified Eagle medium;

DMSO = dimethylsulfoxide; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid IFN- γ = interferon- γ ; GSH = glutathione; LPS = *Escherichia coli* lipopolysaccharide O111:B4; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; iNOS = inducible NO-synthase; PMA = Phorbol myristate acetate; RNS = reactive nitrogen species; ROS = reactive oxygen species; SEM = standard error of mean; SCE = KCl saturated calomel electrode (0.242V/NHE); SSCE = NaCl saturated calomel electrode (0.236V/NHE); SOD = superoxide dismutase

Notes and references

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ASB and CG have equally contributed to the work (first authors)

[†] Electronic Supplementary Information (ESI) available: 1. Chemicals, 2. Stability of the complex, 3. Cell-culture, 4. Cell-growth, 5. SOD-activity evaluation in a cellular context using ferricytochrome *c*, effect of intracellular and extracellular **1**, 6. Microelectrodes fabrication, 7. Single-cells measurements, 8. *In vitro* activity of **1** with NO, 9. *In vitro* activity of **1** with ONOO⁻, 10. *In vitro* activity of **1** with H₂O₂: catalase effect. See DOI: 10.1039/b000000x/

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47. After being stimulated as described here, macrophages act through a process named phagocytosis. Briefly, the macrophage first extends its membrane towards the target and subsequently traps it within a membrane-bound vacuole (phagosome). The macrophage digests and destroys the internalized target by releasing in situ a mixture of ROS/RNS species. During this process, significant ROS/RNS amounts are fastly produced by the enzymes NADPH oxidase and iNOS. Once degraded, the phagosome contents are then emptied into the extracellular medium. By comparison, since the ultramicroelectrode is localized in the close vicinity of the macrophage, the typically weak and broad peaks recorded through amperometry are due to the detection of one or more types of ROS/RNS, that are quasi-continuously generated by phagosomes fusion with the cell external membrane. In that way, the ROS/RNS species, though endogenously produced, are eventually detected after their release in the extracellular fluid. On the other hand 1 is removed from the cells environments during measurements so any effect detected due to cells pre-incubation with 1 cannot be ascribed to the release event but must necessarily be related to its action within the phagosomes.
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