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Evaluation of the compounds commonly known as superoxide dismutase and catalase mimics in cellular models

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ABSTRACT

Oxidative stress that results from an imbalance between the concentrations of reactive species (RS) and antioxidant defenses is associated with many pathologies. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase are among the key enzymes that maintain the low nanomolar physiological concentrations of superoxide and hydrogen peroxide. The increase in the levels of these species and their progeny could have deleterious effects. In this context, chemists have developed SOD and CAT mimics to supplement them when cells are overwhelmed with oxidative stress. However, the beneficial activity of such molecules in cells depends not only on their intrinsic catalytic activities but also on their stability in biological context, their cell penetration and their cellular localization. We have employed cellular assays to characterize several compounds that possess SOD and CAT activities and have been frequently used in cellular and animal models. We used cellular assays that address SOD and CAT activities of the compounds. Finally, we determined the effect of compounds on the suppression of the inflammation in HT29-MD2 cells challenged by lipopolysaccharide. When the assay requires penetration inside cells, the SOD mimics Mn(III) meso-tetrakis(N-(2′-n-butoxyethyl)pyridinium-2-yl)porphyrin (MnTnBuOE-2-PyP^{5+}) and Mn(II) dichloro[(4aR,13aR,17aR,21aR)-1,2,3,4,4a,5,6,12,13,13a,14,15,16,17,17a,18,19,20,21,21a-eicosahydro-11,7-nitrilo-7Hdibenzo[b,h] [1,4, 7,10] tetraazacycloheptadecine-κN5,κN13,κN18,κN21,κN22] (Imisopasem manganese, M40403, CG4419) were found efficacious at 10 μM, while Mn(II) chloro N-(phenolato)-*N*,*N*′-bis[2-(*N*-methyl-imidazolyl)methyl]-ethane-1,2-diamine (Mn1) requires an incubation at 100 μM. This study thus demonstrates that MnTnBuOE-2-PyP^{5+}, M40403 and Mn1 were efficacious in suppressing inflammatory response in HT29-MD2 cells and such action appears to be related to their ability to enter the cells and modulate reactive oxygen species (ROS) levels.

1. Introduction

Oxidative stress is associated with many physiological pathologies such as diabetes, inflammatory bowel diseases, cancers, and neurodegenerative diseases [1]. It results from an imbalance between the concentration of reactive species (RS) responsible for deleterious damages, and antioxidant defenses [2]. Among naturally occurring antioxidants, the metalloenzymes superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPxs) play crucial roles in redox homeostasis. SODs catalyze the dismutation of the superoxide anion $O_2^{\bullet-}$ into H_2O_2 and O_2, CAT catalyzes H_2O_2 dismutation into H_2O and O_2 and GPxs catalyze the reduction of H_2O_2 and others hydroperoxides into H_2O or alcohols. These enzymes are thus an extraordinary source of inspiration for the design of low molecular weight complexes possessing antioxidant properties that could be used as catalytic drugs to supplement these enzymes under pathological situations [3]. Complexes mimicking SOD have attracted much interest since superoxide is the first reactive species in the O_2 reduction cascade produced as byproduct during respiration in living aerobic organisms [4–9].

Among the metals used to develop SOD mimics, manganese is the

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most studied. If released, manganese does not induce Fenton chemistry which would have otherwise led to increased oxidative stress [6,10]. Indeed, to date, a huge diversity of manganese complexes has been reported for their ability to react catalytically with superoxide. They involved ligands such as salen derivatives [11–13], cyclic polyamine [14–19], tri- or dipodal nitrogen-centered ligands [20–24], 1,2-ethanediamine-centered ligands [25–27], desferrioxamine derivatives [15,28,29], polyaminocarboxylato- [30–32] or polycarboxylato ligands [33], peptides [34–36], porphyrins [37–42], phthalocyanines [43], texaphyrins [44,45], corroles [46–48] or biliverdin and its derivatives [49]. Some of these complexes have thus far been assayed on cellular [49–55] and in vivo models [9,53,56–65]. Importantly, Mn cyclic polyamine Mn(II) dichloro[(4aR,13aR,17aR,21aR)-1,2,3,4,4a,5,6,12,13,13a,14,15,16,17,17a,18,19,20,21,21a-eicosahydro-11,7-nitrilo-7Hdibenzo[b,h] [1,4,7,10] tetraazacycloheptadecine-κN5,κN13,κN18,κN21,κN22] (Imisopasem manganese, M40403, CG4419) and Mn porphyrins (Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin called MnTE-2-PyP^{5+} (BMX-010, AEOL10113) and Mn(III) meso-tetrakis(N-(2′-n-butoxyethyl)pyridinium-2-yl)porphyrin called MnTnBuOE-2-PyP^{5+} (BMX-001) have advanced to several clinical trials, which justifies further efforts in the field of SOD and catalase mimics [66].

The efficiency of the complexes was linked to their intrinsic SOD activity but also to parameters such as cellular uptake, localization inside cells and cellular fragments and their stability. It has been shown that SOD mimics can also exhibit catalase activity and their dual activities have been recently reviewed by Batinic-Haberle et al. and Signorella et al. [5,66–69]. We aimed here to undergo comparative study of several compounds which are frequently tested in cellular and animal models where they showed efficacy ultimately reducing oxidative stress. Small molecules such as those used here do not have protein tertiary structure that would allow specificity for certain reactive species, and thus react with variety of reactive species, both oxidizing or reducing them [5,7,66,68]. In particular, studies on Mn porphyrins have demonstrated that these compounds upregulate MnSOD, catalase, glutaredoxins (Grx), peroxiredoxins (Prxs) and other antioxidants via oxidizing cysteine of Kelch-like enoyl-coenzyme A hydratase-associated protein 1 (Keap1) of Nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor [66,68,70]. In that way, rather than acting as SOD and catalase mimics, they indirectly affect levels of $O_2^{\bullet-}$ and H_2O_2.

We used here two assays that are specific for $O_2^{\bullet-}$ and H_2O_2 to see if we can correlate the SOD and CAT like activities of those compounds to their efficacy. For these assays, we have intentionally set up experiments lasting less than 1 h to focus on the mimics reactivity and to avoid upregulation of antioxidant enzymes. Then, we looked at their efficacy in suppressing the inflammation in intestine epithelial HT29-MD2 cells submitted to bacterial lipopolysaccharide (LPS) challenge. The three cellular assays have been combined with inductively coupled plasma mass spectrometry (ICP-MS) quantification of the Mn center of compounds to get insights into their cell penetration.

2. Experimental

2.1. Reagents and instruments

LPS (*Escherichia coli* O55:B5), bovine CuZnSOD, Nicotinamide adenine dinucleotide phosphate (NADH), ferricytochrome *c* and pyruvic acid were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Interleukine-8 (IL-8) detection by enzyme-linked immunosorbent assay (ELISA) was performed using a kit (Duoset) provided by R&D Systems (Minneapolis, Minnesota, USA). Bicinchoninic acid assay (BCA) and bovine serum albumin (BSA) were from Uptima-Interchim (Montluçon, France). Detection Enhanced Chemiluminescence (ECL) system and nitrocellulose membranes were from Amersham Biosciences (Piscataway, New Jersey, USA). Dulbecco's modified Eagle medium (DMEM), DMEM without $NaHCO_3$ (10 g.L^{-1}) and blasticidin, were from Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Fetal bovine serum (FBS) was from GE Healthcare Life Sciences (South Logan, Utah, USA). (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (1 M), Ethylenediaminetetraacetic acid (EDTA) solution (0.5 M), and Dulbecco's phosphate Buffered Saline (10×, DPBS) were from Gibco (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The protease inhibitor cocktail was from Roche Diagnostics (Meylan, France). Mn Porphyrins were obtained from I. Batinic-Haberle and prepared as detailed in: Rajic Z. et al. for MnTnBuOE-2-PyP [71], Reboucas J.S.R. et al. for Manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP) [72], Batinic-Haberle I. et al. for MnTE-2-PyP [73,74]. 2,2,6,6-Tetramethyl-4-[[5-(triphenylphosphonio)pentyl]oxy]-1-piperidinyloxy bromide (Mito-Tempol) was purchased from Sigma Aldrich, the enantiomer CG4419 of M40403 from Astatech and Mn(III) 2,2′-[1,2-Ethanediylbis(nitrilomethylidyne)]bis[6-methoxy-phenol) (EUK134) from Clinisciences. Mn1 ligand (N-(hydrobenzyl)-*N*,*N*′-bis[2-(*N*-methyl-imidazolyl)methyl]-etha ne-1,2-diamine) and complex were synthesized according to previously described protocols [75]. UV–visible spectra were recorded on a Varian Cary 300 Bio spectrophotometer and ICP-MS analysis were performed on an Agilent 7700 X.

2.2. Methods

2.2.1. Stock solutions

2.2.1.1. Mito-Tempol, M40403 and EUK-134. 5 mM stock solutions in H_2O were prepared and the compounds were then added to reach the expected incubation concentrations.

2.2.1.2. Mn1. A 10 mM stock solution of the 1:1 complex was prepared in HEPES (50 mM, pH 7.5). The complex was then added to reach the expected incubation concentrations.

2.2.1.3. Mn Porphyrins. The stock solutions in water were obtained from Ines Batinic-Haberle and the compounds were directly added to reach the expected incubation concentrations. [MnTE-2-PyP^{5+}] = 4.5 mM, [MnTnBuOE-2-PyP^{5+}] = 5.23 mM and [MnTBAP^{3-}] = 4.47 mM.

2.2.1.4. Assay on macrophages

2.2.1.4.1. Cell culture. Murine macrophage RAW 264.7 (American Type Culture Collection) cell line was cultured at 37 °C under a 5% CO_2 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).

2.2.1.4.2. Viability assay. Cell growth and viability of murine macrophages (RAW 264.7) was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as previously reported [55].

2.2.1.4.3. Superoxide measurement. Ferricytochrome c reduction was used to assess the superoxide ion production in RAW 264.7 cells. Cells were stimulated with culture medium containing 1 U mL^{-1} Interferon gamma (IFN-γ) and 1 ng mL^{-1} LPS for 24 h at 37 °C (except for the negative control). They were then incubated 1 h at 37 °C in a home-made medium with 100 μM ferricytochrome c (Sigma Aldrich, prepared without using trichloroacid (TCA)), with or without antioxidant and with 800 nM phorbol 12-myristate 13-acetate (PMA). A home-made medium is necessary to get rid of the colored pH indicator present in DMEM that absorbs in the same range of wavelength as ferricytochrome c. The medium is composed of 14.6 mM glucose, 358 mM NaCl, 12.7 mM KCl, 3.1 mM KH_2PO_4, 6.1 mM $MgSO_4$, 3.1 mM $CaCl_2$, 13 mM $NaHCO_3$ and 53 mM HEPES buffer. The absorbance of supernatants was measured at 550 nm, where ferrocytochrome *c* displays a peak. Contribution of ferricytochrome *c* at 100 μM was subtracted from the absorbance of each sample. This protocol leads to an integration over 1 h

of the production of superoxide in the extracellular medium. Note that this assay was shown to provide a positive result with antioxidants not able to enter cells, such as purified SOD in the extracellular medium [55], with its overall negative charge, as found again here in Fig. 2. For that reason, it was labeled "extracellular activity assay" [55], but it most probably provide an activity measurement composite between extracellular and the amount that penetrate in cells during the 1-h incubation.

2.2.1.5. Assay on HeLa HyPer cells

2.2.1.5.1. Cell culture. Stable cell line HeLa HyPer1 was prepared using the HeLa Flp-In cell line, which was kindly provided by Stephen Taylor [76], and cultured at 37 °C under a 5% CO_2 atmosphere in DMEM containing 4.5 $g.L^{-1}$ D-glucose (Invitrogen) supplemented with 10% of heat-inactivated FBS (Invitrogen). HyPer1 expression in this stable cell line was controlled by doxycycline, added 24 h after seeding. The cells were cultured for an additional 24 h before being processed for analysis. 1 h before imaging, the medium was replaced by DMEM without $NaHCO_3$ containing 1.0 $g.L^{-1}$ D-glucose and 30 mM HEPES (pH 7.5). This cell is of interest in this context as they endogenously produce H_2O_2 a way to evaluate the catalase activity of antioxidants.

2.2.1.5.2. Pharmacological treatments. Cells were incubated with or without 100 μM of antioxidant during 1 h at 37 °C under a 5% CO_2 atmosphere.

2.2.1.5.3. Imaging. Imaging was performed with a CSU-W1 Yokogawa spinning disk coupled to a Zeiss Axio Observer Z1 inverted microscope equipped with a sCMOS Hamamatsu camera and a 63× objective (63×/1.4 oil WD: 0.17 mm) oil objective. DPSS 100 mW 405 nm and 150 mW 491 nm lasers and a 525/50 bandpass emission filter were used.

2.2.1.5.4. H_2O_2 levels quantification and statistical analysis. Images were processed with the Fiji software, to obtain the HyPer1 ratio of the emission at 530 nm (491/530)/(405/530). HyPer ratio was then measured for several cells, and normalized to the ratio value of the control condition. Data were analyzed using Kalaeidagraph and expressed as the mean ± standard error of the mean (SEM). Statistical significance was calculated using an ordinary one-way ANOVA followed by Tukey's multiple comparison test. Note that this assay was shown to provide a positive result with antioxidants not able to enter cells, such as purified catalase in the extracellular medium [77]. Indeed, H_2O_2 is able to cross membranes and its consumption in the extracellular medium shifts the equilibrium and, in fine, consumes also H_2O_2 inside cells, where it is measured by the Hyper probe. In this set-up, with a short incubation, the measure is most probably dominated by the extracellular effect, although a contribution of the amount of antioxidants that may have penetrated in cells in 1 h is not excluded.

2.2.1.6. Assay on HT29-MD2 cells

2.2.1.6.1. Cell culture. HT29 human colon adenocarcinoma were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, UK) and stably transfected to over-express MD2 as previously described [78]. Cells were cultured in DMEM supplemented with 10% of heat-inactivated FBS, and 0.1% of blasticidin (10 $\mu g\ mL^{-1}$) at 37 °C in a 5% CO_2/air atmosphere.

2.2.1.6.2. Cytotoxicity assay. Cytotoxicity of tested compounds and controls, with LPS, was assessed using lactate dehydrogenase (LDH) release assay, by following the release of the cytosolic lactate dehydrogenase (LDH) into the supernatant, indicating membrane damages [79]. Cytotoxicity was considered when LDH release was more than 10%.

Concentration of LDH in supernatant: 800 μL of a pyruvate/NADH solution (see below) was added into a 1 mL plastic cuvette, as well as 200 μL of supernatant, and the decrease in absorbance at 340 nm was immediately monitored for 1 min. The slope is proportional to LDH concentration in supernatant.

Concentration of LDH in cell lysate: 800 μL of a pyruvate/NADH solution (see below) was added into a 1 mL plastic cuvette, as well as 190 μL of 0.1 M PBS and 10 μL of cell lysate, and the decrease in absorbance at 340 nm was immediately measured for 1 min. The slope is proportional to LDH concentration in cell lysate.

The percentage of LDH released in the supernatant was calculated as follows:

$$\%LDH_{released} = \frac{Slope_{supernatant} \times V_{supernatant} \times 5}{Slope_{supernatant} \times V_{supernatant} \times 5 + Slope_{lysate} \times V_{lysate} \times 100} \times 100$$

Solution of pyruvate/NADH: 4.1 mg of pyruvic acid (0.62 mM), and 7.7 mg of NADH (0.18 mM) in 60 mL of 0.1 M PBS (pH 7.4).

2.2.1.6.3. Cell activation with LPS and incubation with the compounds. HT29-MD2 cells were seeded in 12 or 24 well-plates at 200000 cells/well to reach 90% confluence after 3 or 4 days. Cells were incubated with media only, or tested compounds at the desired concentration for 6 h in DMEM supplemented with 10% of heat-inactivated FBS and with LPS (0.1 $\mu g\ mL^{-1}$). Supernatants were collected, and stored at −20 °C before ELISA and LDH assay.

2.2.1.6.4. Determination of proteins concentrations in cell lysates. In order to normalize the IL-8 concentrations measured, protein content of each cell lysate was measured. They were determined using BCA protein assay reagents and BSA as standard according to the manufacturer's instructions. Briefly, a solution (98% BCA, 2% $CuSO_4$) was added to the protein solution in 96-wells plate. After 30 min at 37 °C, the 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.

2.2.1.6.5. IL8 quantification. Levels of the pro-inflammatory cytokine IL8 produced by cells were determined in cell supernatants using a commercially available ELISA kit according to the instructions of the manufacturer. IL8 levels were normalized by the protein content determined in the corresponding cell lysates.

2.2.1.7. Quantification of manganese in cell lysates by ICP-MS. Cells were cultured in a 75 cm^2 plastic flask to reach 90% confluency. They were incubated with medium only, or tested compounds at the desired concentration for one or twenty-four hours, at 37 °C. Cells were then washed twice with 0.9% NaCl. 700 μL of HEPES 0.1 M was then added to scratch cells. For HT29-MD2 cells, 50 μL of the cell suspension was added to an aqueous solution of HNO_3 2% (950 μL). The resulting solution was heated at 90 °C overnight to ensure decoordination of Mn from the porphyrins (Fig. S5a, SI). The solution was diluted with a solution of HNO_3 2% (1 mL), filtrated on 0.2 μm sized filters to get rid of cellular debris before analysis. A calibration curve was established using anhydrous $MnCl_2$. A range of concentration going from zero to 100 ppb was generally used for calibration.

For RAW cells, the same above-mentioned procedure was used but using 100 μL of the cell suspension in 0.9 mL of an aqueous solution of HNO_3 2%.

2.2.1.8. Quantification of porphyrins in HT29-MD2 cell lysates by UV–vis spectrometry. Cells were cultured in a 75 cm^2 flask to reach 90% confluency. They were incubated with medium only, or tested compounds at the desired concentration for one or twenty-four hours, at 37 °C. Cells were then washed twice with 0.9% NaCl. 700 μL of HEPES 0.1 M was then added to scratch cells. Cells were lysed by sonication. UV–vis spectra of the resulting solutions were recorded and the spectrum of a solution of non-incubated cell was subtracted to get rid of the cell absorption. The absorbance of the Soret band was compared with those of solutions (5 μM) of the pure porphyrins in water. To compare the concentrations found by UV–vis with the concentrations measured by ICP-MS, the concentrations in μM were converted into ppb considering the dilution used for samples preparation in ICP-MS experiments (dilution × 40).

3. Results and discussion

A nitroxide radical (Mito-TEMPOL) and several MnSOD mimics (Fig. 1) belonging to the above-mentioned SOD mimics families, covering a large range of SOD activity and/or catalase activity were selected (Table 1). Mito-TEMPOL was included for comparison, since it does not involve a metal cation to perform the redox catalysis, does not exhibit catalase activity but shows a weak SOD activity only in acidic region [8,67]. The catalysis of superoxide dismutation occurs via redox couple $RNO^{\bullet}/RNO^{+}$ [80]. In addition, Mito-TEMPOL, possesses a triphenylphosphonium group to increase its accumulation into mitochondria where reactive oxygen species (ROS) are mainly produced [81]. Among the Mn porphyrins studied, two positively charged and differently lipophilic (MnTE-2-PyP^{5+} and MnTnBuOE-2-PyP^{5+}) and one negatively charged ($MnTBAP^{3-}$) have been selected. The positively charged Mn porphyrins were shown to enter the cell, where they prefer nucleus and mitochondria being driven there by anionic phosphate groups and mitochondrial negative potential [5,70,82,83]. It is expected that the negatively charged porphyrin will be less reactive towards the negatively charged superoxide and less cell penetrant.

While not reported as not being a catalytic SOD mimic [94], $MnTBAP^{3-}$ can be interesting as it reacts with peroxynitrite and has reportedly shown efficacy in vitro and in vivo [8,95,96]. We refer herein as *intrinsic activities* to the kinetics of the reaction with superoxide or hydrogen peroxide outside of any cellular context, whether it is catalytic or not, typically reported in Table 1 [6].

In order to compare the antioxidants selected for their SOD activity in cells, we have first used an assay involving murine macrophages RAW 264.7 [55]. On this cell line, the production of RS including superoxide can be induced upon stimulation with bacterial lipopolysaccharide (LPS) and interferon γ (IFN-γ). The release of these RS in the surrounding medium is then triggered using phorbol 12-myristate 13-acetate (PMA) [54,55,97]. Quantification of superoxide concentration is made possible by using ferricytochrome *c* as superoxide UV–vis marker [55]. As ferricytochrome *c* is not able to penetrate cells, only the superoxide released extracellularly is measured. CuZnSOD was incubated at 100 U mL^{-1} and all of the other antioxidants were incubated, in presence of ferricytochrome *c*, for 1 h at 5 μM. At this concentration, no toxicity was observed (MTT assay, Fig. S1, SI). It is worth mentioning that 1 h-inccubation may be too short to lead to antioxidant proteins upregulation [98]. In presence of superoxide, the ferricytochrome *c* is reduced into ferrocytochrome *c*. A SOD mimic competes with ferricytochrome *c* for reaction with superoxide and leads to a lowering of the amount in ferrocytochrome *c*. The ferrocytochrome *c* concentrations measured in the cell supernatants were compared and the concentration found after activation and in absence of any antioxidant was set at 100%. Of note, even if the superoxide concentration is measured outside the cells, an

Table 1
Antioxidants intrinsic dismutation constants. k_{McCF} refers to the rate constant calculated from the McCord and Fridovich assay and k_{cat} corresponds to the catalytic constant determined by fast kinetics in presence of an excess of superoxide. [a] Ferricytochrome c, phosphate buffer (50 mM, pH 8). [b] Ferricytochrome *c*, phosphate buffer (50 mM, pH 7.8). [c] HEPES (60 mM, pH 7.4). [d] Phosphate buffer (50 mM, pH 7.4). [e] Phosphate buffer (50 mM, pH 7.8). [f] Unless specified, catalytic constants were measured by polarography using a Clark-type electrode in tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM, pH 7.8). [g] HEPES (100 mM, pH 7.4). [h] Value found for EUK-8 instead of EUK-134 that differs only by the two methoxy substituents. SF means stopped-flow and PR pulse radiolysis.

	SOD activity		CAT activity	
	k_{McCF} (10^6 M^{-1} s^{-1})	k_{cat} (10^6 M^{-1} s^{-1})	$k_{cat}H_2O_2$ (M^{-1} s^{-1})[f]	References
Mito-TEMPOL	0.0297[a]		none	[84]
Mn1	7.0[b]	6.2[c] SF	2.3[g]	[55,75,85]
M40403	3.55[b]	1.9[d] SF	8.2	[7,67,86]
EUK-134	0.602[b]		13.0[h]	[52,67,87]
MnTE-2-PyP^{5+}	57[b]	54 PR	63.3	[67,73]
MnTnBuOE-2-PyP^{5+}	68[b]		88.5	[5,67]
$MnTBAP^{3-}$	0.00145[b]	none[d] SF	5.8	[67,72,86]
$MnCl_2$	1.3[b]	1.9[b] PR	none	[49,86,88]
$Mn(ClO_4)_2$	1.3[b]			[23,89]
CuZnSOD	870-2188[b]	2000[e] SF		[8,86,90–92]
MnSOD		ca. 3000 PR		[93]
Catalase from bovine liver			1.5 10^6	[67]

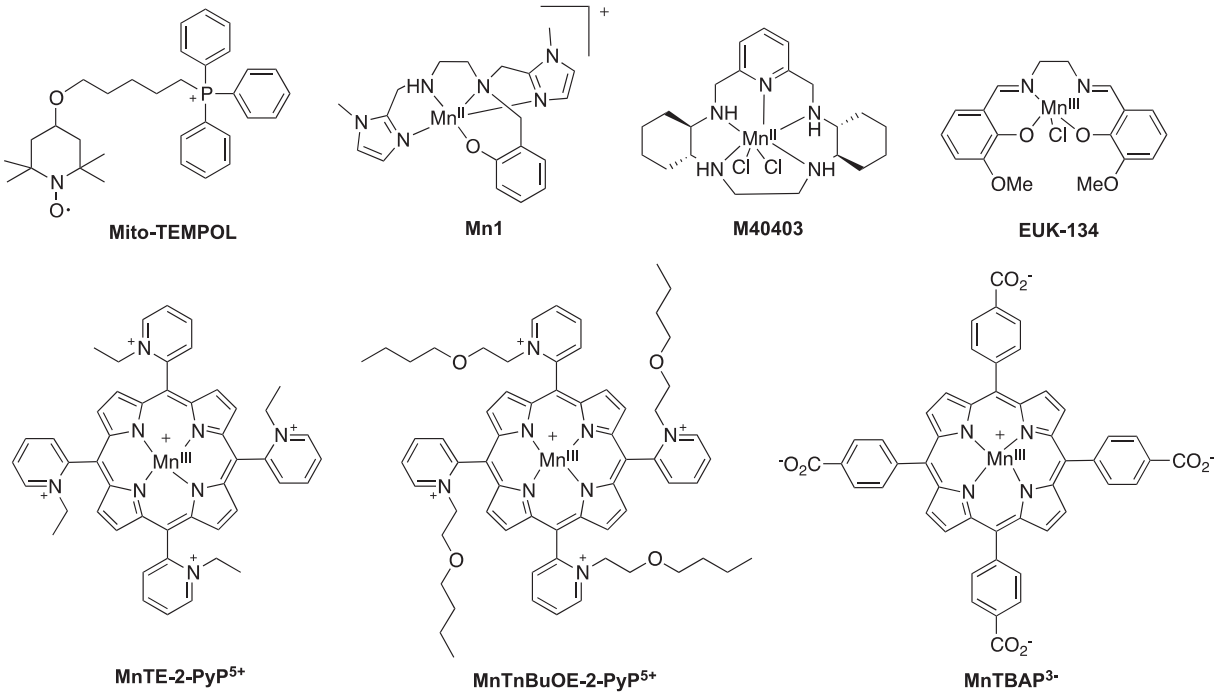

Fig. 1. Structure of the selected antioxidants studied in this article.

antioxidant that enters cells and decreases superoxide concentration inside cells would also result in lowering ferricytochrome *c* reduction [55]. As shown in Fig. 2, three main groups emerged: very efficient catalysts (% ferricytochrome *c* < 20% activated), efficient catalysts (20% < % ferricytochrome *c* < 80% activated), and inactive catalysts (% ferricytochrome *c* > 80% activated). In the first group, the recombinant CuZnSOD is very efficient at reducing the extracellular superoxide concentration and was even able to result in superoxide levels lower than that obtained for non-activated cells. In the second group, Mn1, M40403, EUK-134 and the two positively charged porphyrins MnTE-2-PyP^{5+} and MnTnBuOE-2-PyP^{5+} were able to decrease the extracellular superoxide concentrations to levels close to the non-activated situation. With exception of EUK-134, such data reflect their similar SOD-like activities. In the third group, Mito-TEMPOL and MnTBAP^{3-} were found inactive in the tested conditions. As expected, the obtained results are in line with the lack of their SOD activities reported in Table 1 (k_{McCF} or k_{cat}). Both compounds have SOD-like activities lower than the rate for $O_2^{\bullet-}$ self-dismutation of ~5×10^5 M^{-1} s^{-1} and are thus not true catalytic SOD mimics [7]

In a second assay, we wanted to compare the antioxidants according to their catalase activity. We have used HeLa cells expressing intracellularly the ratiometric fluorescent sensor of H_2O_2 called HyPer [99]. HyPer is a circularly permuted yellow fluorescent protein (cpYFP) emitting at 530 nm integrated into the regulatory domain of the bacterial H_2O_2 sensing protein OxyR (OxyR-RD). Upon oxidation of HyPer thiols by H_2O_2, disulfide bridges formation induces a modification of the spectral properties of the YFP. This leads to a ratiometric modification of the excitation spectrum of HyPer that possesses two excitation maxima at 420 and 488 nm. Thus, by measuring the normalized ratio of the intensity $I_{(491/530)}/I_{(405/530)}$, it is possible to monitor specifically H_2O_2 concentration in cells. It is worth mentioning that since H_2O_2 is able to passively diffuse across cytoplasmic membrane, the observed effect of the antioxidants may be composite: it may be due to dismutation of H_2O_2 inside or outside the cells. For instance, incubation of HeLa HyPer with the recombinant heme catalase, has shown to reduce H_2O_2 concentration inside cells whereas it does not cross cells membrane [77].

HeLa HyPer cells were incubated with the antioxidants for 1 h at 100 μM in HEPES buffer and imaged at 530 nm by fluorescence microscopy upon excitation at two wavelengths 405 and 491 nm respectively (Figs. S2 and S3) [100]. This incubation time is long enough to detect an effect on H_2O_2 concentration and short enough to exclude a possible feedback of the cell expressing anti-oxidant enzymes [98]. Hence, this assay is meant to evidence a direct effect of the compounds to reduce the level of H_2O_2. The ratios of the intensity $I_{(491/530)}/I_{(405/530)}$ normalized against the control (NFR for normalized fluorescence ratio) was determined for several cells (typically 50–150 cells) and were reported in the Fig. 3. In this assay, cells were not stressed, and, consequently, the effects reported are those on basal H_2O_2 levels.

As shown in Fig. 3, again, three groups of antioxidants were observed: efficacious catalyst (0.5 < NFR < 0.75), moderately efficacious catalysts (0.75 < NFR < 1) and prooxidant compounds (NFR > 1). In the first group, the two more efficacious CAT mimics from aqueous chemistry studies were MnTE-2-PyP^{5+} ($k_{cat}H_2O_2$ = 63.3 M^{-1} s^{-1}) [67] and MnTnBuOE-2-PyP^{5+} ($k_{cat}H_2O_2$ = 88.5 M^{-1} s^{-1}) [67] and were indeed found with NFR of 0.68 and 0.60 respectively. The effect of MnTnBuOE-2-PyP^{5+} may be higher as it distributes to a higher level to cells than does MnTE-2-PyP^{5+} [68]. In the second group, Mn1 and EUK-134 reduced similarly the H_2O_2 concentration in HeLa cells which correlates with their similar moderate intrinsic CAT activities (2.3 and 13 M^{-1} s^{-1} respectively) [55,67,87]. Surprisingly, Mito-TEMPOL was found active in this assay whereas no CAT activity is reported for this compound. In contrast, although M40403 exhibits a CAT activity (8.2 M^{-1} s^{-1}) similar to Mn1 and EUK-134 in aqueous studies, in cells, M40403 induced increased levels of H_2O_2 [67]. In eukaryotic organisms, the steady state concentration of superoxide and H_2O_2 were estimated to be 10^{-10} and 50 10^{-10} M respectively, attesting the higher tolerance of eukaryotic cells for H_2O_2 than for $O_2^{\bullet-}$ [101]. Owing to this ratio, it is very unlikely that by catalyzing superoxide dismutation the antioxidants lead to a measurable increase of H_2O_2 concentration and thus to an increased fluorescence ratio. The increased level of H_2O_2 observed with M40403 may instead be due to a low stability of the complexes which may partly dissociate and lead to manganese release as reported recently [102,103]; there may be a ligand chemistry or toxicity we are still not aware of and would require further studies. Increased levels of H_2O_2 were also observed after incubation with the redox silent analogue of Mn1 called Zn1 and the manganese salt $MnCl_2$.The negatively charged porphyrin MnTBAP^{3-} did not show a significant effect on H_2O_2 concentration as it has no CAT-like activity. Overall, as H_2O_2 is mainly produced at the plasma membrane and can easily diffuse, and as the results are in line with the catalytic constants measured in test tubes with the positively charged porphyrins being the most efficacious [104], these results suggest that the measured activities are mainly due to the portion of antioxidants that remain outside the cells.

To go further, we have assayed the selected antioxidants on HT29-MD2 cells. These intestine epithelial cells have been stably transfected to overexpress MD2 protein in the extracellular environment and that promotes an enhanced inflammatory response in presence of bacterial lipopolysaccharide [78]. Besides, it has been shown that such inflammatory reaction is mediated by oxidative stress [105,106]. HT29-MD2 cells were thus incubated in presence of LPS and the antioxidants at

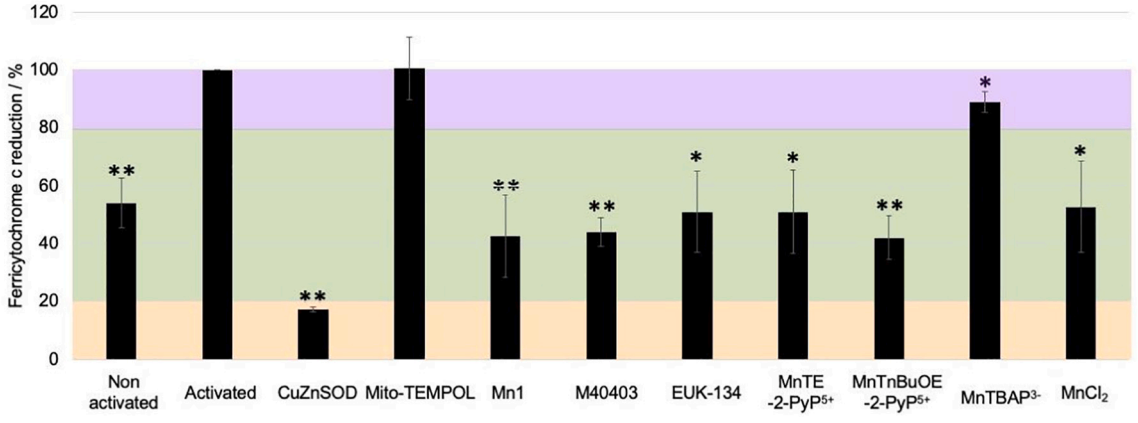

Fig. 2. Evaluation of antioxidants on murine macrophages RAW 264.7. Reported values are related to the values observed for activated cells and arbitrarily set at 100%. Macrophages were incubated with IFN-γ (1 U mL^{-1}) and LPS (1 ng mL^{-1}) for 24 h at 37 °C in DMEM followed by an incubation with ferricytochrome *c* (100 μM), PMA (800 nM) and antioxidants (100 μM) at 37 °C for 1 h in a homemade medium (see experimental section). Data are mean ± SEM of three independent experiments with (**) $p < 0.01$ and (*) $p < 0.05$ versus activated cells.

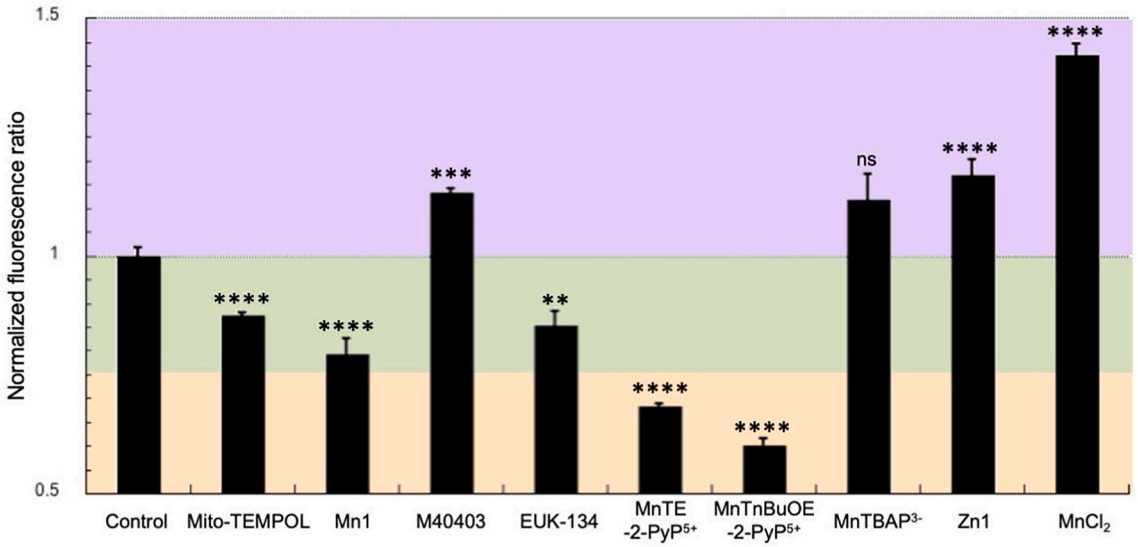

Fig. 3. H_2O_2 levels measured in HyPer HeLa cells. The cells were incubated 1 h at 37 °C in HEPES (30 mM pH 7.5) under 5% CO_2 without (control) or with antioxidants at 100 μM. Fluorescence images were recorded after excitation at 405 nm and 491 nm and the ratio of the emission at 530 nm $I_{(491/530)}/I_{(405/530)}$ was measured for several cells. The ratio was arbitrarily set at 1 for control cells i.e. cells incubated with HEPES only. Data are mean ± SEM of several cells (from 48 to 163 cells) with **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. control cells. ns means non-significant.

non-toxic concentrations: 10 and 100 μM (Fig. S4, SI) [9,103,107]. In this assay, 6 h incubations were required to observe a significant inflammatory response. Consequently, the observed anti-inflammatory effect of the antioxidants may be related to either their direct scavenging of $O_2^{\bullet-}$ or H_2O_2, or mediated metabolic processes that would result in reducing levels of those species and oxidative stress in general. The concentration of an inflammatory marker, the interleukin 8 (IL8) has been measured by ELISA to compare the effects of the antioxidants. As expected, upon LPS challenge, the IL8 concentration of non-incubated cells increased significantly. Thus, the decreased level of IL8 in the presence of an antioxidant was indicative of anti-inflammatory activity (Fig. 4). After incubation of the antioxidants at 10 μM, only M40403, and MnTnBuOE-2-PyP^{5+} to a lesser extent, showed a moderate anti-inflammatory effect. The other compounds were found inactive or even pro-inflammatory in the case of Mn1. Taking into account Mn1 dissociation constant in solution that has been estimated to be $6.5 \pm 1.7\ 10^{-7}$ M [85], at a concentration of 10 μM, it is expected that at least 23% of the ligand is not coordinated to Mn and this value may increase in a competitive ligand biological environment. Because the ligand is toxic for the cells [9], the pro-inflammatory response observed may be due to the toxicity of the non-coordinated ligand. Note that in this model, $MnCl_2$ was previously assayed and found to be not active or pro-inflammatory [9,103,107]. After incubation at a higher concentration (100 μM), as expected, M40403 and MnTnBuOE-2-PyP^{5+} were found

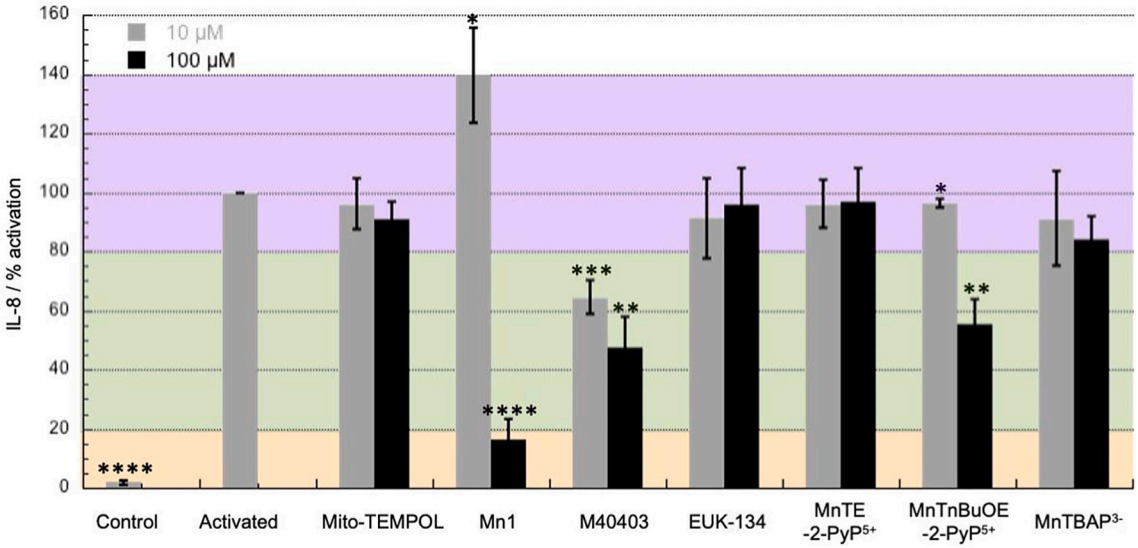

Fig. 4. Quantification of the inflammatory marker IL8 by ELISA after incubation of HT29-MD2 cells with the antioxidants at 10 or 100 μM. Antioxidants were incubated for 6 h at 37 °C in supplemented DMEM in presence of LPS (0.1 μg mL^{-1}) except for the control where no antioxidants and LPS were added. The absorbance measured for activated cells was arbitrary set at 100%. Data represent mean ± SEM for two independent experiments for MnTnBuOE-2-PyP^{5+} and M40403 and three independent experiments for Mito-TEMPOL, Mn1, EUK-134, MnTE-2-PyP^{5+} and $MnTBAP^{3-}$ with **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. activated cells.

more active than at 10 μM. Mn1 also exhibited high anti-inflammatory effect as already reported [9]. Surprisingly, MnTE-2-PyP^{5+} did not lead to a significant decrease of IL8 level whereas it possesses similar catalytic constants for superoxide and H_2O_2 dismutation as MnTnBuOE-2-PyP^{5+} [68].

The bioavailability of cationic Mn-porphyrins in cellular fragments, cells and tissue has been comprehensively studied and reported [70]. In this cellular assay, we observed that both the more hydrophilic MnTE-2-PyP^{5+} and MnTnBuOE-2-PyP^{5+} penetrate similarly, as shown by quantification of the Mn content by ICP-MS in cell lysates incubated with these porphyrins that lead to similar values (Fig. S6, SI). A similar trend was observed when the porphyrins were quantified in cell lysates using their specific UV–Vis signature (Fig. S5 b-c and Fig. S6, SI). To note, very surprisingly, quantification experiments showed that the negatively charged MnTBAP^{3-} was more internalized than the positively charged porphyrins (Fig. S5 b-c and Fig. S6, SI), which was not expected. It was though reported that MnTBAP^{3-}accumulated within 4T1 breast cancer cells, tumor, muscle and liver, but did not exhibit anticancer efficacy [68]. It should also be mentioned that MnTBAP^{3--} was suggested to display an intracellular and intramitochondrial bio-activity [108]. Although it penetrates in this model, no activity was observed for this negatively charged porphyrin. Interestingly too, this study suggests that M40403 and EUK134 penetrate into cells and confirmed the previous results reported for Mn1 [9,103,107]. The different activities observed here may be related to different cellular localizations. In this assay, antioxidants that are poor SOD mimics (MITO-TEMPOL, EUK-134, MnTBAP^{3-}) are not efficient whatever their catalase-like activity, suggesting that superoxide and/or its progeny may be the main reactive species that had to be controlled in this model and played an important role in the inflammatory responses. While inferior at lower 10 μM concentration, due to its low metal-ligand stability and/or inertia, at 100 μM, though, Mn1 showed the best anti-inflammatory activity in this cellular model, likely due to its high SOD activity and high bioavailability.

MnTnBuOE-2-PyP^{5+}, that is now in clinical trial, shows efficacy on all of the models. Its effects may be due to direct scavenging of H_2O_2 and $O_2^{\bullet-}$ (for the two first cellular models, with short incubation times) and/or to the Mn porphyrin-driven upregulation of MnSOD and catalase that would then take care of H_2O_2 and $O_2^{\bullet-}$ (inflammation model requiring a longer 6 h incubation, compatible with protein up-regulation). These data support its progress into clinical [66,109].

4. Conclusion

In conclusion, we have shown that the assays on macrophages (for SOD activity) and on HeLa HyPer cells (for CAT activity) are useful to validate the activity of antioxidants in cellular contexts. They allow verifying that the compounds are efficient under biological conditions which data justify their further evaluation under more complex conditions. The assay on HT29-MD2 cells affords more relevant information, in particular for SOD mimics, since in this assay, among the ROS produced, it seems that superoxide or its progeny plays an important role in the inflammatory response of the cells. The bioactivities measured were related not only to the intrinsic catalytic activities of the antioxidants but also with their penetration inside the cells. Evidences suggested that their cellular localization is also an important factor; this assay gained an insight into their bioavailabilities. This three-cell-model assay thus provides a comparison of different families of SOD mimics on cellular models relevant for the development of such compounds, and a broader overview of the potential of antioxidants for their use as catalytic drugs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2021.111431.

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