Macrocyclic Ligands

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Striking Inflammation from Both Sides: Manganese(II) Pentaazamacrocyclic SOD Mimics Act Also as Nitric Oxide Dismutases: A Single-Cell Study**

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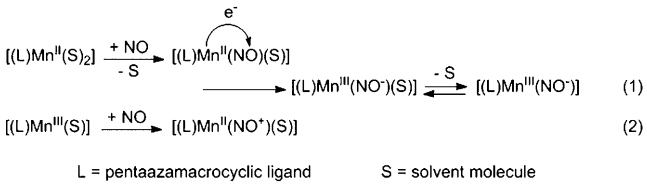
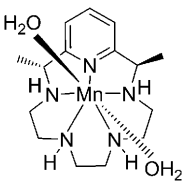
During the course of an inflammatory response, nitric oxide ($NO^{\bullet}$; formed by inducible NO synthase, iNOS) and superoxide ($O_2^{\bullet-}$; formed by NADPH oxidase or NOX2) are both generated in quantities that surpass physiological levels.[1] Consecutively, $NO^{\bullet}$ and $O_2^{\bullet-}$ react at a diffusion-controlled rate ($k \approx 10^{10}\,M^{-1}s^{-1}$) to form peroxynitrite ($ONOO^-$) and other reactive species (such as $NO_2^{\bullet}$ and $OH^{\bullet}$), which induce cytotoxic effects through DNA damage, low-density lipoprotein oxidation, protein nitration and oxidation, aconitase inactivation, and inhibition of respiration.[2] Several attempts have been made to either inhibit $NO^{\bullet}$ production, by designing selective iNOS inhibitors,[3a] or to mimic the activity of superoxide dismutase (SOD), by developing low-molecular-weight metal complexes,[3b] to treat diseases characterized by hyperinflammation. However, the pharmacological application of iNOS inhibitors[3c] or SOD mimics[3d] is restricted by a certain lack of selectivity, stability, and/or bioavailability of these compounds.

Mn^{II} pentaazamacrocyclic complexes feature the presently most potent synthetic SOD mimics. They were discovered just a decade ago[4a] and have since entered phase II clinical trials in the USA.[4b] Their main advantage is reported to be a strict selectivity towards superoxide.[4a–e] However, drawing an analogy with native enzyme,[5a] some of us have recently shown that such complexes do react with $NO^{\bullet}$, albeit at lower rates than with $O_2^{\bullet-}$ (the rate constant for aerobic reaction with NO was estimated to be $850\,M^{-1}s^{-1}$, whereas the catalytic rate constant for $O_2^{\bullet-}$ dismutation is $1\times10^7\,M^{-1}s^{-1}$).[5b] We proposed a new dismutation mechanism which involves the formation of labile metal–nitrosyl complexes and leads to the catalytic removal of large amounts of $NO^{\bullet}$ from solution[5b] [Eqs. (1) and (2)]. Therefore, this class of complexes might also act towards $NO^{\bullet}$ generation during an inflammatory response.



To clarify the pharmacological effects related to the chemistry of Mn^{II} pentaazamacrocyclic SOD mimics, we used $[Mn^{II}(pyane)Cl_2]$ (Scheme 1)[6] as a general representative of this class of complexes and studied its effect on the production of $O_2^{\bullet-}$ and $NO^{\bullet}$ by living cells.

Macrophages are the main actors in the production of reactive oxygen (ROS) and reactive nitrogen (RNS) species in inflammation,[7] so they were selected as cell models in this study. Moreover, some of us have shown that electrochemistry at ultramicroelectrodes offers the unique possibility of in situ, real-time, and direct measurements of ROS and RNS generated by cells, including species that are short-lived like $ONOO^-$.[8] Thus, the ROS/RNS production by single immu-



Scheme 1. Chemical structure of $[Mn^{II}(pyane)Cl_2]$ as a general representative of Mn^{II}–pentaazamacrocyclic SOD mimics.

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nostimulated (interferon-γ/lipopolysaccharide (IFN-γ/LPS)) macrophages was monitored by amperometry and quantified by using this established method to evaluate the in vivo reactivity of $[Mn^{II}(pyane)Cl_2]$.

First of all, to assess a potent toxicity of $[Mn^{II}(pyane)Cl_2]$ and its optimal concentration to be used,[9] cultures of RAW 264.7 macrophages were incubated with either 5, 25, or 100 μM of the complex over three days.[10] Reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) (MTT), a common test leading to the purple formazan dye by the mitochondrial electron-chain process in living cells, was used to assay cell survival.[11]

As shown in Figure 1, incubation of macrophages with $[Mn^{II}(pyane)Cl_2]$ at a concentration of 25 μM for 18–24 h (duration of cell culture) was clearly nontoxic for the cells.

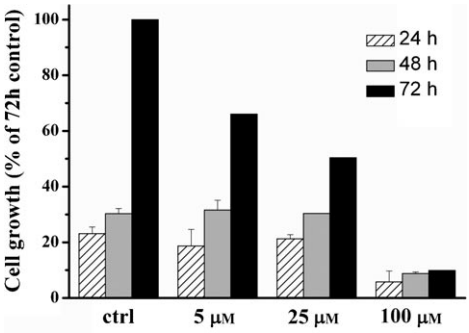

Figure 1. Effects of $[Mn^{II}(pyane)Cl_2]$ concentration on the viability of RAW 264.7 macrophages as determined by MTT assay. The values are normalized as a percentage with respect to control (untreated) cells after 72 h of growth at 37 °C under a 5% CO_2 atmosphere. The experiments were performed in triplicate and are presented as mean ± standard deviation.

Additionally, these conditions did not lead to any observable differences in cell morphologies prior to or following stimulation by immunological factors (see the Supporting Information, Figure S1).[12] Beyond these thresholds, for example, longer incubation times and/or higher complex concentrations, a significant decrease in cell viability was observed as well as a clear apoptotic morphology for activated macrophages (not shown). Consequently, the following studies were conducted on cells incubated with $[Mn^{II}(pyane)Cl_2]$ at 25 μM for 18–24 h, in comparison with solely IFN-γ/LPS-activated ones.

Amperometric analysis of the release of ROS/RNS from macrophages under both sets of conditions was performed as previously reported.[13] In each experiment, a platinized carbon-disk microelectrode was positioned in close proximity to the cell membrane of a single macrophage (see the Supporting Information, Figure S2a,b).[13a] The release of ROS/RNS was detected in real time by amperometry at a constant potential. Then, series of amperometric measurements at different potentials (+300, +450, +650, and +850 mV versus a sodium-saturated calomel reference electrode (SSCE)) were conducted to identify and quantify individually the electroactive species (H_2O_2, $ONOO^-$, $NO^\bullet$, or NO_2^-) released by the activated macrophages.[13] Typical amperometric responses of a single immunostimulated macrophage (see the Supporting Information, Figure S2c) and a detailed description of the method used to calculate the emission flux of each species (that is, the amount detected over a 1 h time period) are presented in the Supporting Information.

The production of endogenous ROS/RNS by immunostimulated macrophages that had, or had not, been co-treated with $[Mn^{II}(pyane)Cl_2]$ was evaluated. As shown in Table 1,

Table 1: Mean quantities of ROS and RNS released by single IFN-γ/LPS-stimulated RAW 264.7 macrophages over 1 h.

	H_2O_2 [fmol]	$ONOO^-$ [fmol]	$NO^\bullet$ [fmol]	NO_2^- [fmol]
control	8 ± 3	22 ± 7	44 ± 4	10 ± 1
25 μM $[Mn^{II}(pyane)Cl_2]$	21 ± 2	approx. 0	approx. 0	46 ± 2

treatment of immunostimulated cells with the complex for 18–24 h led to a significant increase (about a factor of 3) in H_2O_2. Since H_2O_2 is one of the two products of SOD-catalyzed $O_2^{\bullet-}$ disproportionation, this observation reflected the SOD-like activity of the complex, which was consistent with previous reports for this class of SOD mimics.[4]

Intriguingly, a complete attenuation of $ONOO^-$ and $NO^\bullet$ release from macrophages treated with the mimic was observed, while the amount of nitrite increased by about a factor of 4. This finding strongly suggested that the abrogation of $NO^\bullet$ detection did not result from the inhibition of its production, but from its efficient scavenging and transformation by $[Mn^{II}(pyane)Cl_2]$. To exclude a possible modulation by the complex of iNOS messenger RNA (mRNA) and/or iNOS protein expression, both levels were quantified by reverse transcription polymerase chain reaction (RT)-PCR and Western-blot analysis, respectively. Treatment for 24 h with the complex of nonstimulated macrophages did not change the basal levels of iNOS mRNA (Figure 2) and failed to induce expression of iNOS (Supporting Information, Figure S3). After IFN-γ/LPS stimulation of macrophages, a large increase of both iNOS mRNA and protein levels was observed, as previously reported.[14] No difference between complex-treated or untreated cells was detected, thereby confirming that the complex is only prone to remove endogenous $NO^\bullet$ and to transform it into more benign species without inhibiting the expression of iNOS.

Based on Equations (1) and (2), the main products of NO dismutation through the complex activity are nitrosonium (NO^+) and nitroxyl (NO^-) species. The nitrosonium cation is highly unstable (half-life of about 3×10^{-10} s) in water,[15a] since it is rapidly hydrolyzed to form nitrite [Eq. (3)]. The other main product, nitroxyl, when loosely bound to the metal center of the complex may react with either oxygen, thus leading to $ONOO^-$ formation [Eq. (4)],[15b] or with $NO^\bullet$, which leads again to nitrite formation [Eqs. (5)–(7)).[15c] When protonated, $ONOO^-$ itself spontaneously decomposes rapidly into nitrate (approx. 70%) and nitrite (approx. 30%) [Eqs. (8)–(11)).[15d] While it is difficult to accurately predict the ultimate metabolic fate of nitroxyl, based on Equa-

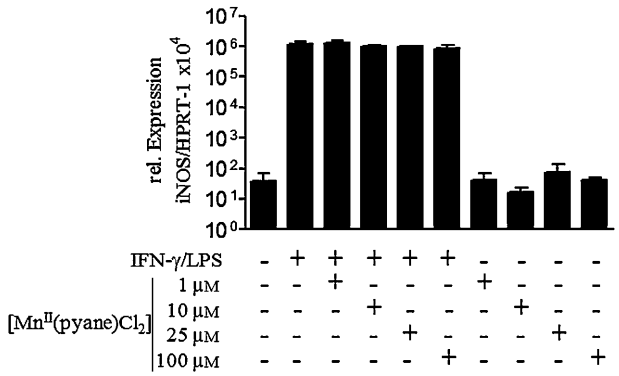

Figure 2. Real-time RT-PCR of iNOS mRNA expression in RAW 264.7 macrophages in the presence or absence of IFN-γ/LPS stimulation and treatment with $[Mn^{II}(pyane)Cl_2]$ (1, 10, 25, 100 μM) for 24 h. Mean ± standard error of the mean of two independent experiments is given. HPRT: hypoxanthine-guanine phosphoribosyl transferase.

tions (1)–(9) it seems plausible that more than 50% of the 44 fmol of dismutated NO• (Table 1) should be transformed into nitrite. Indeed, a significantly increased flux of NO_2^- (by approximately 36 fmol, compare Table 1) was released by stimulated macrophages treated with the SOD mimic (36 versus 22 fmol, assuming only 50% transformation). Residual NO_2^- may originate from either nitroxyl transformation and/or $ONOO^-$ decomposition.

$$NO^+ + H_2O \rightarrow H^+ + HNO_2 \rightarrow 2\,H^+ + NO_2^- \quad (3)$$

$$NO^- + O_2 \rightarrow ONOO^- \quad (4)$$

$$NO^- + NO^\bullet \rightarrow N_2O_2^- \quad (5)$$

$$N_2O_2^- + NO^\bullet \rightarrow N_3O_3^{\bullet -} \quad (6)$$

$$N_3O_3^{\bullet -} \rightarrow N_2O + NO_2^- \quad (7)$$

$$ONOOH \rightarrow {}^7/_{10}\,HNO_3 + {}^3/_{10}\,[{}^\bullet NO_2 + OH^\bullet] \quad (8)$$

$${}^\bullet NO_2 + OH^\bullet + ONOO^- \rightarrow {}^\bullet NO_2 + OH^- + O_2 + {}^\bullet NO \quad (9)$$

$${}^\bullet NO_2 + {}^\bullet NO \rightarrow N_2O_3 \quad (10)$$

$$N_2O_3 + H_2O \rightarrow 2\,H^+ + 2\,NO_2^- \quad (11)$$

We would like to acknowledge that reaction between CO_2 and $ONOO^-$ could take place leading to the formation of $CO_3^{\bullet -}$ and NO_3^- [Eqs. (12) and (13)].[15e] However, the contribution of this reaction on extracellular release of ROS and RNS is expected to be negligible in the studied case, since all our measurements were performed in phosphate-buffered saline (PBS; see the Supporting Information). Additionally, it has been recently shown that at distances < 5 μm, diffusion can outcompete the reaction with CO_2.[16]

$$ONOO^- + CO_2 \rightarrow {}^7/_{10}\,NO_3^- + {}^7/_{10}\,CO_2 + {}^3/_{10}\,[NO_2^\bullet + CO_3^{\bullet -}] \quad (12)$$

$${}^\bullet NO_2 + CO_3^{\bullet -} \rightarrow NO_3^- + CO_2 \quad (13)$$

Previous reports have emphasized the selectivity of this class of Mn^{II} pentaazamacrocyclic SOD mimics in favoring $O_2^{\bullet -}$ disproportionation, specifically postulating that NO• and $ONOO^-$ did not react with this type of complex.[4] However, in these earlier studies it was noted that peroxynitrite production by pleural macrophages collected from rats pretreated with M40403 decreased in a dose-dependent manner.[4e] This was attributed to the fast removal of $O_2^{\bullet -}$, thereby preventing the formation of $ONOO^-$.

In our study, any possible direct interaction between $[Mn^{II}(pyane)X_2]$ and $ONOO^-$ was also checked in vitro by amperometric studies of mixtures of these two species. The principle of these analyses was to examine whether or not the presence of the manganese complex led to a faster consumption of peroxynitrite compared to its own decomposition rate. This was detected at a constant potential of +450 mV versus SSCE, which corresponded to the plateau potential for peroxynitrite oxidation at platinized carbon microelectrodes.[17,18] Figure 3 shows that the presence of increasing

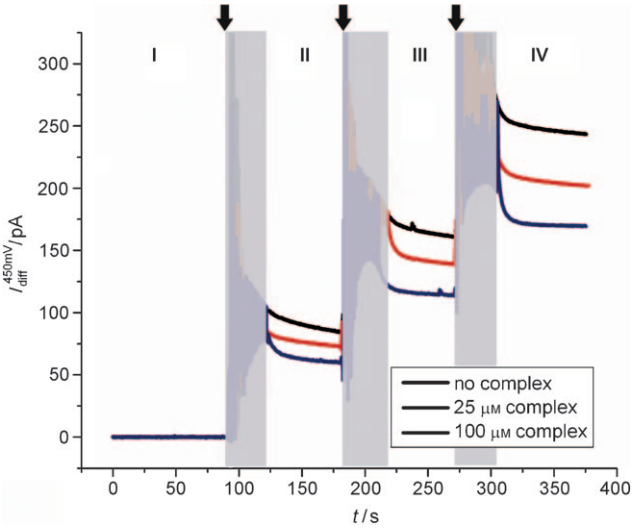

Figure 3. Subtracted amperometric responses at +450 mV (I_{diff}^{450mV}) versus SSCE in PBS/NaOH (pH ≈ 10.5) using the same platinized carbon microelectrode. The dotted curves were obtained in the absence of $[Mn^{II}(pyane)Cl_2]$, whereas the dashed and solid curves were obtained in the presence of 25 and 100 μM of the complex, respectively. Region I corresponds to the period prior to $ONOO^-$ addition, while regions II–IV correspond to initial $ONOO^-$ concentrations of 12.8, 25.0, and 36.6 μM, respectively. The arrows and shaded areas correspond to injections of $ONOO^-$ and mixing of solution.

concentrations of $[Mn^{II}(pyane)Cl_2]$ led to faster-decreasing rates in amperometric currents, thus showing that the SOD mimic exhibits a direct reactivity towards $ONOO^-$. In addition, this was confirmed by voltammetric studies which established that the oxidation wave of $ONOO^-$ decreased in amplitude in the presence of the complex (see the Supporting Information, Figure S4a).[19] Collectively, these electrochemical results obtained in vitro validated the above assumptions raised by experiments on cells.

Based on the estimated amounts of each ROS/RNS (Table 1) and according to the reaction Scheme S5 (Supporting Information), primary NO• and $O_2^{\bullet -}$ fluxes endogenously produced could be calculated, as we have shown previous-

ly.[13b] The effects of $[Mn^{II}(pyane)Cl_2]$ on the fluxes of both $NO^{\bullet}$ and $O_2^{\bullet-}$ were rationalized through the scheme depicted in Figure 4. Notably, this shows that on average (76 ± 8) and (47 ± 8) fmol of $NO^{\bullet}$ and $O_2^{\bullet-}$ (over an hour), respectively,

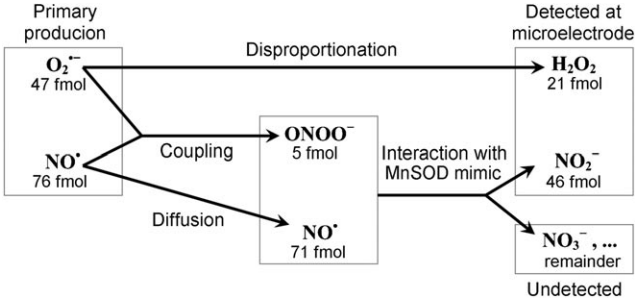

Figure 4. Proposed scheme accounting for the detection of 21 fmol H_2O_2 and 46 fmol NO_2^- from the primary production of 76 fmol $NO^{\bullet}$ and 47 fmol $O_2^{\bullet-}$.

were primarily released by each immunostimulated macrophage. Since our results showed that there is no change in iNOS expression and activity under the effect of $[Mn^{II}(pyane)Cl_2]$, it is inferred that a single mimic-treated immunostimulated macrophage produced the same amounts of primary $NO^{\bullet}$ and $O_2^{\bullet-}$. Hence, based on the disproportionation reaction stoichiometry and considering Equations (3)–(9), the 21 fmol of H_2O_2 detected resulted from 42 fmol of primary $O_2^{\bullet-}$, while the remaining 5 fmol of $O_2^{\bullet-}$ would react with 5 fmol of $NO^{\bullet}$ to yield $ONOO^-$. The corresponding excess of $NO^{\bullet}$ (71 fmol) would be disproportionated by $[Mn^{II}(pyane)Cl_2]$. The complex would also react with $ONOO^-$ (5 fmol). Overall, this accounts for the final release of 46 fmol of NO_2^- per single macrophage as detected at the microelectrode. The remaining products would either be undetected or undetectable,[18] such as NO_3^- which is not electroactive at our platinized microelectrodes though it is a major end species of RNS.

Contrary to generally accepted opinion that Mn^{II} pentaazamacrocyclic SOD mimics possess a high selectivity for superoxide, $[Mn^{II}(pyane)Cl_2]$ has been shown herein to interact also with $NO^{\bullet}$ and $ONOO^-$ produced by immune cells. Indeed, in addition to its "SOD mimic" activity that converts $O_2^{\bullet-}$ into H_2O_2, this complex also induces a near complete scavenging of $NO^{\bullet}$ and $ONOO^-$ releases. This study thus demonstrates that the previously established in vitro chemistry of the SOD mimics used here[5b] is valid in vivo as well, thus leading to a rare example where in vitro chemistry explains very well in vivo effects.

To the best of our knowledge, this is the first time that a chemical compound with a promising pharmacological effect has been reported to efficiently remove both $O_2^{\bullet-}$ and $NO^{\bullet}$, by transforming them into more benign species and without altering the normal enzymatic pathways for their production. This additional protective effect of this class of SOD mimics,[4] by comparison with other SOD mimics, might be relevant for the treatment of inflammation and inflammation-related diseases. The rational design of compounds that can remove more than one reactive species derived from $O_2^{\bullet-}$ or $NO^{\bullet}$ seems to be challenging from both the chemical and pharmacological points of view and warrants further consideration.

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[9] The complex should be used at sufficiently low concentrations to present low toxicity to RAW 264.7 macrophages and exclude the detection of ROS/RNS released by apoptotic cells. Conversely, a sufficiently high concentration was needed for the effects from the Mn SOD mimic to be more apparent. These boundaries defined the experimental concentration range used in this study.
[10] Experimental details, as well as supporting text and references, are given in the Supporting Information.
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significant morphological differences in comparison with cells that had been activated in the presence of IFN-γ/LPS only.
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[18] The resulting exponential current decay was followed for several minutes, so that the baseline current could be ascertained and subsequently subtracted from the measured response to extract the relevant Faradaic data. A stock solution of peroxynitrite was then rapidly injected with fast mixing into the Petri dish, to yield a diluted solution of known $ONOO^-$ concentration.
[19] As some of us have reported previously (C. Amatore, S. Arbault, C. Ducrocq, S. Hu, I. Tapsoba, *ChemMedChem* **2007**, *2*, 898 – 903), HNO can be oxidized at platinized carbon microelectrodes, with an apparent half-wave potential at about + 200 mV versus SSCE. In other words, some of the produced HNO might be oxidized at all four of the potentials that were used herein. Since its in vivo formation by macrophages has not been reported, HNO/NO^- oxidation was not considered here.

