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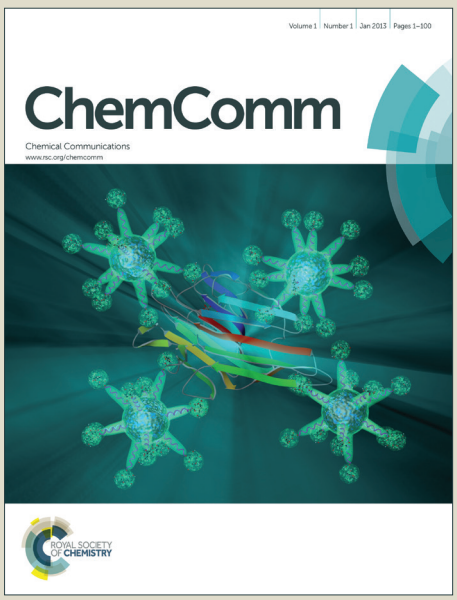

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Mononuclear Nonheme Iron(III) Complexes that Show Superoxide Dismutase-like Activity and Antioxidant Effects against Menadione-Mediated Oxidative Stress

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This paper describes the superoxide dismutase (SOD)-like activity of mononuclear iron(III) complexes with pentadentate monocarboxylamido ligands. The SOD activity can be controlled by the electronic nature of the substituent group on the ligand. The nitro-substituted complex showed clear cytoprotective activity against menadione-mediated oxidative stress in cultured cells.

The superoxide anion ($O_2^{\bullet-}$) is a by-product of the mitochondrial respiratory chain and play a role in a number of medical issues, including inflammation, ischemia-reperfusion injuries and carcinogenesis.[1] Superoxide dismutases (SODs) catalyse the disproportionation of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) and dioxygen (O_2) to protect cells from oxidative damage caused by $O_2^{\bullet-}$.[2] Therefore, malfunctioning SODs and the over-production of $O_2^{\bullet-}$ beyond the catalytic capacity of SODs are suspected to be related to certain neurological diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.[3]

Catalysts that can show SOD-like activity *in vivo* may have potential as pharmaceuticals for these neurological diseases or ischemia-reperfusion injuries. Natural SOD enzymes have manganese, iron, nickel or copper/zinc ions as catalytic centres. The search for metal-based SOD mimics has attracted great attention.[4] However, despite recent advances in the field, creating SOD mimics that possess high activity and bio-compatibility remains a great challenge. Most SOD mimics reported thus far are manganese complexes,[5] and studies on other metal-based SOD mimics are still limited.[6] In general, manganese-based SOD mimics show catalase activity; therefore, they are often named SOD/catalase mimics,[5b,7] and their antioxidant abilities have been successfully demonstrated in *in vivo* model systems. For example, one manganese-based SOD mimic, EUK-134 (Chart 1), has been shown to be capable of reducing oxidative stress in rat kidneys and limbic systems.[7b,8] In contrast, although several classes of iron-based SOD mimics have been reported, SOD mimics that show high SOD-like activity and can reduce oxidative stress against cultured cells are limited.[6a,9]

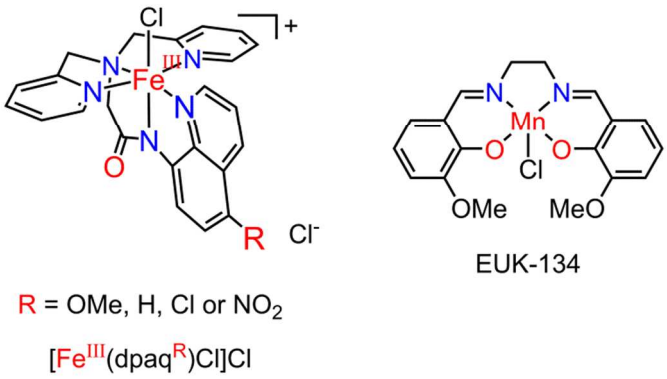


Chart 1 Chemical structure of $\mathbf{1}^{\mathbf{R}}$ and EUK-134.

We recently reported that a mononuclear nonheme iron-monocarboxylamido complex, $[Fe^{III}(dpaq^{H})(H_2O)](ClO_4)_2$ ($dpaq^{H}$ = 2-[bis(2-pyridinylmethyl)-amino]-*N*-8-quinolinylacetylamido), was stable in buffers at pH values ranging from 3 to 9, but did not catalyse oxidation reactions of external substrates such as guaiacol (2-methoxyphenol), which use H_2O_2 as an oxidant.[10] Additional experiments to further explore the bio-related catalytic activity of $Fe^{III}(dpaq^{H})$ in aqueous solutions disclosed that it has a higher SOD-like activity than Mn-salen complexes. Furthermore, we found that the introduction of electron-withdrawing groups at the 5-position of the quinoline moiety of the pentadentate ligand enhances its SOD activity as well as its antioxidant activity, which protects cultured cells from menadione-mediated oxidative stress. Herein, we report the SOD and catalase activity and the antioxidant effect of a series of the formula $[Fe^{III}(dpaq^{R})Cl]Cl$ ($\mathbf{1}^{\mathbf{R}}$; The substituent R is located at the 5-

position of the quinoline moiety. R = OMe, H, Cl or NO_2. See Chart 1).[11]

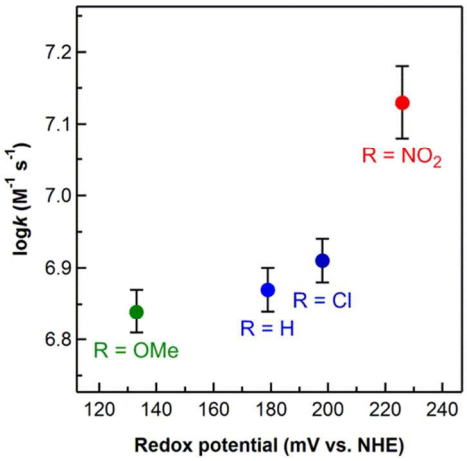
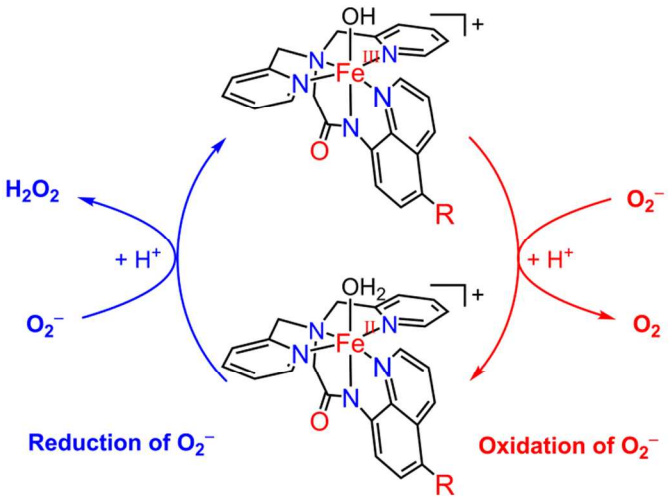


Fig. 1 Correlation between the SOD activity and the redox potential of $\mathbf{1}^{\mathbf{R}}$. The rate constants of the reaction between $\mathbf{1}^{\mathbf{R}}$ and $O_2^{\bullet-}$, k (M^{-1} s^{-1}) were recalculated form IC_{50} values obtained by following the inhibition of the reduction of a water soluble tetrazolium salt WST-1 in the presence of xanthine/xanthine oxidase, a superoxide-generating system. The rate constants of the reaction between $\mathbf{1}^{\mathbf{R}}$ and $O_2^{\bullet-}$, k_2 (M^{-1} s^{-1}) were recalculated by using the following equation: $k = k_{WST-1}\times[WST\text{-}1]/IC_{50}$,[14d] where k_{WST-1} denotes the formation rate constant of formazan from WST-1 in the absence of $\mathbf{1}^{\mathbf{R}}$, and was 4.6×10^4 M^{-1} s^{-1}. The data are the mean and standard deviations of quintuplicate experiments.



Scheme 1 Proposed $O_2^{\bullet-}$ disproportionation catalysed by iron complexes $\mathbf{1}^{\mathbf{R}}$.

Iron complexes $\mathbf{1}^{\mathbf{R}}$ were synthesized by treating the pentadentate ligand H-dpaq^R with $FeCl_3$ in methanol. The redox behaviour of the iron complexes was examined by cyclic voltammetry in piperazine-*N*,*N*'-bis(2-ethansulfonic acid) (PIPES) buffers at pH 7.5. Cyclic voltammograms showed pseudo-reversible behaviour at $E_{1/2}$ = 133 to 226 mV vs. the normal hydrogen electrode (NHE, ΔE = 76 to 166 mV, Fig. S1). These results support iron complexes $\mathbf{1}^{\mathbf{R}}$ having SOD-like activities, since the redox potential of SOD mimics should be between the reduction potential of $O_2^{\bullet-}$ at 891 mV vs. NHE ($O_2^{\bullet-}/H_2O_2$) and the oxidation potential of $O_2^{\bullet-}$ at −160 mV vs. NHE ($O_2/O_2^{\bullet-}$).[12] The redox potentials of iron complexes $\mathbf{1}^{\mathbf{R}}$ varied linearly with the Hammett substituent constants, giving a straight line graph with a slope of ρ = 84 ± 20, R = OMe, 133 mV; R = H, 179 mV; R = Cl, 198 mV and R = NO_2, 226 mV (Fig. S2), although the slope is slightly shallower than that observed for the related series $[Fe^{III}(dpaq^R)(MeCN)](ClO_4)_2$ in MeCN (ρ = 176).[11]

The SOD activity of iron complexes $\mathbf{1}^{\mathbf{R}}$, together with that of EUK-134 as a control, was assayed by following the inhibition of the reduction of a water soluble tetrazolium salt (WST-1, (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) in the presence of xanthine/xanthine oxidase, a superoxide-generating system (See the supporting information).[13] The half maximal inhibitory concentrations (IC_{50}) for iron complexes $\mathbf{1}^{\mathbf{R}}$ were found to range from 0.17 to 0.33 μM, which are much lower than that obtained for EUK-134 (IC_{50} = 3.4 ± 0.2 μM, Fig. S3). These results demonstrate that iron complexes $\mathbf{1}^{\mathbf{R}}$ are excellent iron-based SOD mimics. More importantly, the SOD-like activity of iron complexes $\mathbf{1}^{\mathbf{R}}$ increased as the redox potential became more positive (Fig. 1; R = OMe, 0.33 ± 0.02 μM; R = H, 0.31 ± 0.02 μM; R = Cl, 0.28 ± 0.02 μM and R = NO_2, 0.17 ± 0.01 μM). The correlation between the SOD activity and the redox potential has been reported with both iron and manganese SOD-mimics.[9b,9c,14] The present data indicate that the rate-determining step for the $O_2^{\bullet-}$ disproportionation by iron complexes $\mathbf{1}^{\mathbf{R}}$ is the reduction of the ferric complexes with $O_2^{\bullet-}$, but it is not the oxidation of the ferrous complexes with $O_2^{\bullet-}$ (Scheme 1).

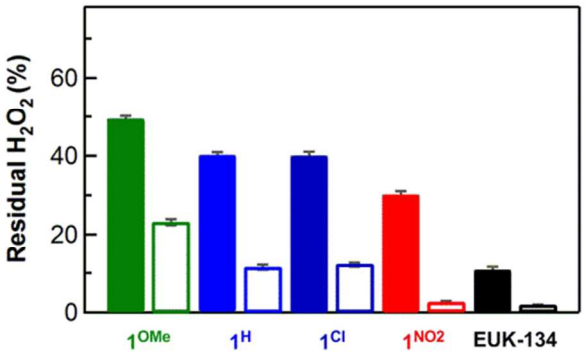


Fig. 2 Quantification of residual H_2O_2 after mixing $\mathbf{1}^{\mathbf{R}}$ (R = OMe, H, Cl and NO_2) and EUK-134 (10 μM) with H_2O_2 (100 μM) in a PIPES buffer (10 mM, pH 7.5) for 30 (filled bar) or 60 min (open bar). The reactions were started upon addition of ABTS (1 mM) and HRP (0.5 units/L). The data are the mean and standard deviations of quintuplicate experiments.

We previously reported that iron complex $\mathbf{1}^{\mathbf{H}}$ did not mediate one-electron oxidation of such substrates as ABTS (2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid)) and guaiacol by using H_2O_2 as an oxidant.[10] A lack of other iron complexes $\mathbf{1}^{\mathbf{R}}$ in peroxidase activity was confirmed using ABTS as a substrate in a pH 7.5 buffer solution, while EUK-134 exhibited significant peroxidase activity under the same conditions as the iron complexes $\mathbf{1}^{\mathbf{R}}$ series (Fig. S4).[15] The peroxidase activity of SOD mimics should be avoided if they are used as antioxidants, because the oxidation of biological molecules could potentially take place. We also found that iron complexes $\mathbf{1}^{\mathbf{R}}$ exhibited catalase activity (Fig. 2), although EUK-134 showed higher catalase activity. Thus, to the best of

our knowledge, iron complexes $\mathbf{1}^{\mathbf{R}}$ are the first unique SOD/catalase mimics that lack peroxidase activity.

Encouraged by these promising results, we then investigated the utility of iron complexes $\mathbf{1}^{\mathbf{R}}$ to reduce oxidative stress on living cells caused by menadione (2-methyl-1,4-naphthoquinone, vitamin K3). Menadione is commonly used as an oxidative stress-inducing reagent to generate $O_2^{\bullet-}$ through redox cycling inside cells.[16] $O_2^{\bullet-}$ is the first reactive oxygen species (ROS) generated from O_2, and it is then converted to H_2O_2 and more harmful ROS such as hydroxyl radicals and peroxynitrite. Therefore, the rapid disproportionation of $O_2^{\bullet-}$ is a key reaction to reduce oxidative stress caused by menadione.

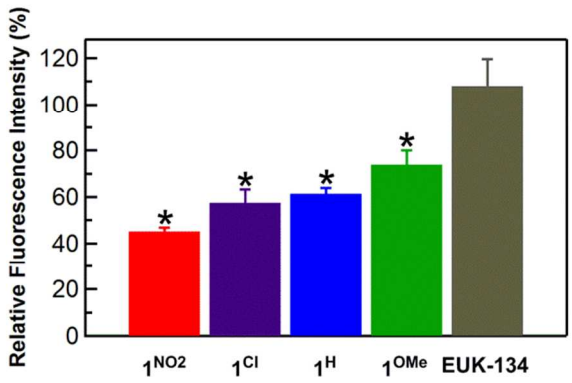


Fig. 3 Fluorescence intensity of ethidium cation formed inside cells incubated with varied concentrations of $\mathbf{1}^{\mathbf{NO2}}$ (red), $\mathbf{1}^{\mathbf{Cl}}$ (purple), $\mathbf{1}^{\mathbf{H}}$ (blue), $\mathbf{1}^{\mathbf{OMe}}$ (green) or EUK-134 (black), together with 10 μM 2-hydroethidium. The data are the mean and standard deviations of quintuplicate experiments. *P < 0.005 compared with the control.

Prior to assessing the antioxidant activity of iron complexes $\mathbf{1}^{\mathbf{R}}$, HeLa cells were first incubated with iron complexes $\mathbf{1}^{\mathbf{R}}$ at various concentrations for 3 h, and the cell viability was evaluated by MTT assay to examine the cytotoxicity of iron complexes $\mathbf{1}^{\mathbf{R}}$. Iron complexes $\mathbf{1}^{\mathbf{R}}$ were not cytotoxic at concentrations of up to 100 μM (Fig. S5). Therefore, in the experiments on the antioxidant activity, iron complexes $\mathbf{1}^{\mathbf{R}}$ at a concentration of 10 μM were incubated with HeLa cells under anaerobic conditions for 3 h and then exposed to 10 μM menadione. In this experiment, 2-hydroethidium, a highly specific fluorescent marker for $O_2^{\bullet-}$,[17] was used to quantify the amount of generated $O_2^{\bullet-}$. The fluorescence intensity from the generated ethidium was reduced significantly when iron complexes $\mathbf{1}^{\mathbf{R}}$ were co-incubated. Conversely, EUK-134 showed negligible effects under identical conditions. Interestingly, the fluorescence intensity was reduced to 73 ± 6, 61 ± 3, 58 ± 6 and 45 ± 2% of the control with no metal complex for iron complexes having the substituent group R = OMe, H, Cl and NO_2, respectively. Thus, the antioxidant effect of iron complexes $\mathbf{1}^{\mathbf{R}}$ followed the order of the SOD activity determined in vitro.

In conclusion, we found that a series of mononuclear iron complexes, $[Fe^{III}(dpaq^{R})Cl]Cl$, are novel iron-based antioxidants that protect cultured cells from oxidative stress caused by menadione. They are unique SOD/catalase mimics that lack peroxidase activity. It is interesting that they show catalase activity but lack peroxidase activity, and the reason for this has yet to be determined. Further work should be directed at understanding the reaction mechanisms of the catalase cycle. Finally, we anticipate these complexes will serve as pure SOD mimics in studies of oxidative stress as well as in clinical treatments of neurological diseases.

Notes and references

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† Electronic Supplementary Information (ESI) available: Figs. S1 to S5. Synthesis of $\mathbf{1}^{\mathbf{R}}$ and experimental details. See DOI: 10.1039/c000000x/

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