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# Fluorimetric study of the pro-oxidant activity of EUK8 in the presence of hydrogen peroxide

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**Abstract** The catalase mimetic complex Mn(III)-salen chloride (EUK8) was found to be pro-oxidant under low hydrogen peroxide concentrations. The increase in the fluorescence rate of the probe 1,2,3-dihydrorhodamine (DHR) in solution, as well as the carbonyl content of human serum albumin were found to be maximum at $H_2O_2$:EUK8 molar ratios ranging from 0 to 2, supporting previous findings regarding the mechanism of EUK8 catalase activity and the formation of highly oxidative Mn(V)-$O^{2-}$ species. This pro-oxidant effect is precluded by the presence of glutathione. Cytotoxicity to HeLa cells, as probed by increased rate of oxidation of intracellular DHR, was not observed. Our findings suggest that the combination of $H_2O_2$ and EUK8 at specific molar ratios, in the absence of reductants/antioxidants, induces the oxidation of organic molecules. It is shown that the fluorimetric determination of pro-oxidant activity of metal complexes is more sensitive than the colorimetric quantification of protein carbonyl content. The implications of our findings with respect to the somewhat confusing results arising from in vivo studies of EUK8 and other Mn(III) antioxidant metal complexes are discussed.

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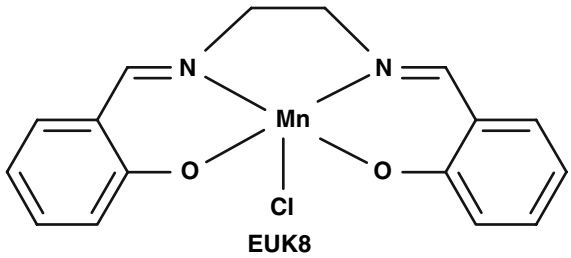
## Introduction

Several metal complex catalysts have been studied as superoxide dismutase (SOD) and/or catalase mimetics, aiming at the obtention of improved metallodrugs with antioxidant activity. Among these compounds, manganese(III) complexes such as Mn(III)-salen chloride (EUK8; Scheme 1 (Doctrow et al. 2002; Watanabe et al. 2006)) and manganese(III)-desferrioxamine B (MnDFB; (Hahn et al. 1991; Faulkner et al. 1994)) have been found to show promising activity.

In animal models, EUK8 has been found to reduce oxidative stress in the heart muscle (van Empel et al. 2006) and to improve postischemic cardiac (Xu et al. 2004) and brain (Baker et al. 1998) recovery following injury. It has also been reported that EUK8 can ameliorate renal dysfunction and hepatocellular injury in endotoxic shock with concomitant reduction of protein nytrosilation in the liver and kidney (McDonald et al. 2003). Aging retardation is one of the most intriguing possibilities of antioxidant therapy with EUK8. However, conflicting results have been obtained so far, as the effect seems to be species-specific and/or dependent upon the route of absorption (Jung et al. 2001; Bayne and Sohal 2002;




EUK8

**Scheme 1** Mn(III) salen chloride

Keaney and Gems 2003; Sampayo et al. 2003; Keaney et al. 2004). Curiously, the life span of flies treated with a relatively low concentration of EUK8 was significantly shortened under hyperoxic conditions (Bayne and Sohal 2002).

Electroanalytical experiments conducted with EUK8 have indicated that the species of the compound with SOD activity must be a Mn(II) complex. The same authors found that besides the antioxidant activity, this metal-reduced form may behave as a pro-oxidant by forming superoxide in the presence of relatively high concentrations of dissolved oxygen (O'Hare et al. 2003). Supporting evidence was provided by Vezin et al. who successfully synthesized superoxide-generating Mn(II)-salen complexes designed for high DNA cleaving activity (Vezin et al. 2002).

In contrast to the SOD-like mechanism, the mechanism for EUK8 catalase activity has been shown to proceed through the oxidation of the metal center to generate a Mn(V)-oxo complex as the first step of the dismutation reaction. This species is attacked by a second $H_2O_2$ molecule to regenerate the catalyst (Eqs. 1, 2) (Sharpe et al. 2002).

$$\mathrm{Mn(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Mn(V)O^{2-}} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{Mn(V)O^{2-}} + \mathrm{H_2O_2} \rightarrow \mathrm{Mn(III)} + \mathrm{H_2O} + \mathrm{O_2} \quad (2)$$

Mn(V)-oxo complexes and, to a lesser extent, Mn(III)-salen complexes are themselves powerful oxidants used in the catalytic oxidation of organic substrates (Katsuki 1995; Sharpe et al. 2002). Specifically, DNA seems to be a sensitive target for the pro-oxidant activity of Mn(III)-salen complexes, either in the presence (Gravert and Griffin 1996) or absence (Fucassi et al. 2001) of another oxidant species. The catalytic activity of EUK8 is sensitive to the presence of added chelators, but does not seem to be affected by the presence of plasma proteins (Baudry et al. 1993). Per-species other than peroxide (peroxinitrite, peracetate and persulphate) also lead to the formation of Mn(V)-oxo species (Sharpe et al. 2002). Taken together, these results suggest that low molecular weight metal complexes might not behave as antioxidants in all concentration ranges, in contrast to catalase, where the catalytic heme center is protected at the bottom of a hydrophobic cleft, being 2–3 Å wide and ~25 Å below the surface of the protein (Putnam et al. 2000).

The most common method for the assessment of catalase activity is the electrochemical detection of the oxygen produced in the reaction $H_2O_2 \rightarrow H_2O + ½O_2$ (Gonzalez et al. 1995). This method requires considerable sample manipulation, high sample volume (1.0 ml), careful handling of electrodes and, particularly, relatively high (1–10 mM) $H_2O_2$ concentration (Gonzalez et al. 1995; Baker et al. 1998; Doctrow et al. 2002) to obtain adequate readings. Usually, the $H_2O_2$:complex molar ratio in the in vitro evaluations is around 100, which precludes the study of the behavior of the enzyme mimetic at physiologically relevant peroxide concentrations and, most importantly, the assessment of any side-reactions catalyzed by the metal complex should the oxidant levels be depleted and thus unable to rapidly regenerate the catalyst. Substituted Mn(III) porphyrins also display catalase activity, however, again only high $H_2O_2$:EUK8 molar ratios (20–200) have been studied (Kachadourian et al. 2004). Also, the number of samples that can be studied at the same time in oxymeters/oxygraphs is limited to only a few, depending upon the instrument. A cheaper, high-throughput method which allows the rapid performance screening of candidate catalase-like compounds (or any metal complexes with purported antioxidant activity) at physiologically relevant hydrogen peroxide concentrations and, specifically, lower-than-unity $H_2O_2$:complex ratios, is clearly desirable for the medicinal inorganic chemistry practitioner.

Hydrogen peroxide is one of the reactive oxygen species (ROS) that can promote oxidation of the fluorescent probe 1,2,3-dihydrorhodamine (DHR) to its fluorescent form 1,2,3-rhodamine (RHA) (Hensley et al. 2003). However, other radical species may also induce DHR oxidation to RHA. DHR has been previously employed in the quantification of peroxides in cell lysates (Royall and Ischiropoulos 1993)

and in the high-throughput quantification of redox-active iron in clinical samples (Esposito et al. 2002, 2003).

In this paper we present the optimization of a fluorescence method to quickly evaluate catalase activity of candidate Mn(III) complexes in common microplate readers, based on the changes in the rate of DHR oxidation. Our data indicate that, at $H_2O_2$:EUK8 molar ratios lower than 2, EUK8 behaves as a pro-oxidant species, which is in accordance with the two-step model of EUK8 catalase activity described above (Eqs. 1, 2). This pro-oxidant effect is precluded by the presence of glutathione. The amount of carbonyls in human serum albumin (HSA) after the treatment with peroxide and EUK8 at different $H_2O_2$:EUK8 ratios (<2, 2 and >2) corroborated the fluorimetric data, although the method required both $H_2O_2$ and EUK8 in higher concentrations. The relevance of these findings is discussed in the light of fluorescence microscopy measurements performed on HeLa cells loaded with DHR, which indicated that this pro-oxidant effect of EUK8 may be effectively counteracted by cellular reductants. Our results contribute to an understanding of the somewhat confusing results regarding the safety of antioxidant Mn(III) complexes.

## Materials and methods

### Materials

The following reagents were purchased from commercial sources and used without further purification: DHR (Biotium); catalase from bovine liver, reduced glutathione, guanidine, 2,4-dinitrophenylhydrazine (2,4-DNP), Chelex®, Hepes, $MnCl_2 \cdot 4H_2O$, salicylaldehyde, ethylenediamine (Sigma); human serum albumin 20% (Hemope, Recife, Brazil); $H_2O_2$ 2.7 M, trichloroacetic acid (Cromoline, Diadema, Brazil). Hydrogen peroxide concentrations were determined photometrically by the method of Cotton (Cotton and Dunford 1973). EUK8 was synthesized according to literature methods (Boucher 1974). The DHR fluorogenic medium is described elsewhere (Esposito et al. 2003) and consists of a 50 μM solution of DHR in iron-free Hepes Saline Buffer (HBS; pH 7.4) prepared immediately before use from freezer stocks of DHR (100 mM in dimethyl sulfoxide). Iron-free HBS was prepared by washing the buffer solution with Chelex® (1 g/100 ml) as an important precaution against a parallel Fenton reaction catalyzed by trace amounts of Fe(III).

### Fluorescence measurements

Aliquots of 300 μl of the fluorogenic medium were transferred to a transparent 96-well microplate. The fluorescence microplate reader was a BMG FluoStar Optima operating at an incubation temperature of 37°C, $\lambda_{exc}/\lambda_{emis} = 485/515$ nm and gain of 600. Fluorescence intensity was measured at 1–2 min intervals for 60 min. All data were presented as slopes (fluorescence intensity/time) rather than as endpoints in order to minimize errors. All determinations were carried out in duplicate.

### Protein oxidation

Samples of HSA (20 μM in HBS; 1.0 ml) were incubated at 37°C for 2 h in the presence of EUK8, $H_2O_2$ and a mixture of the two at different concentrations. The carbonyl content of HSA was determined through the reaction with 2,4-DNP (Levine et al. 1990). UV–Visible spectra were recorded in a Shimadzu UV-2401-PC spectrophotometer in quartz cuvettes.

### Cell studies

HeLa cells were harvested in full DMEM medium (10% fetal calf serum) without phenol red under 5% $CO_2$ at 37°C. On the day before the microscopy measurements, cells were trypsinized, transferred ($1 \times 10^3$ cells/ml) to plastic Petri dishes with a final cell suspension volume of 2.0 ml and allowed to adhere to the plastic dish overnight in the incubator. The DHR-loading of the cells was carried out by a modification of Royall's protocol (Royall and Ischiropoulos et al. 1993). The cells were treated with 2.0 ml of 10 μM DHR in DMEM for 1 h in the incubator. The medium was then removed, the cells were washed once with PBS and new medium (without DHR) was added to the dishes. Whenever

necessary, $H_2O_2$ (incubation time: 1 h) or EUK8 were added to these DHR-loaded cells. The fluorescence microscope was a Carl Zeiss LSM 510 operating with Ar laser (excitation: 486 nm; emission: 522–544 nm) and a 40× objective.

## Results and discussion

Initially, the concentration-dependent effect of $H_2O_2$ oxidation over the fluorescence of DHR and the protective effect of catalase were confirmed in the experimental setup (Fig. 1). The $H_2O_2$ concentration-dependent oxidation of DHR is clearly visible, showing that the method is sensitive enough even at the small volumes and concentrations involved. EUK8 alone induced considerable oxidation of the fluorescent probe, however, at concentrations which are not expected to be found in a clinical setup.

EUK8 has been previously reported to be therapeutically active in experimental setups at concentrations of around 10 μM (Doctrow et al. 2002), that is, around 1,000 times higher than those of catalase. We therefore chose similar EUK8 concentrations and studied the effect of varying $H_2O_2$:EUK8 molar ratios on the fluorescence rate. This approach has previously proved to be useful in the study of the pro-oxidant effect of Fe(III) complexes when in the presence of low levels of ascorbate (Esposito et al. 2003). Interestingly, a peak of fluorescence activity was observed at $H_2O_2$:EUK8 $\sim$ 2.6 (Fig. 2), which is understood as increased oxidative damage to the fluorescence probe.

This observation indicates that for $H_2O_2$:EUK8 $<$ 2 the Mn(III) complex actually behaves as a pro-oxidant rather than an anti-oxidant, contrary to what is observed for catalase (Fig. 1) which behaves as an antioxidant in all concentration ranges. Our data support the postulated two-step mechanism for Mn(III)-salen catalase-like activity described above (Eqs. 1, 2), which implies that a low stoichiometric proportion of $H_2O_2$ would increase the concentration of the highly active oxidant Mn(V)-$O^{2-}$ species. If there is not enough peroxide present to reduce this species to the Mn(III) derivative and thus regenerate the complex, the assumed antioxidant may actually induce the formation of a powerful oxidant. The implications for the therapeutic use of this complex would be that unless the precise locale peroxide concentration is known, EUK8 treatment might be deleterious, which may explain some of the confusing results regarding the benefits of its administration (Bayne and Sohal 2002). In this regard, it is curious to observe that deleterious effects of EUK8 and other Mn(III) antioxidant species on the growth of Cu,

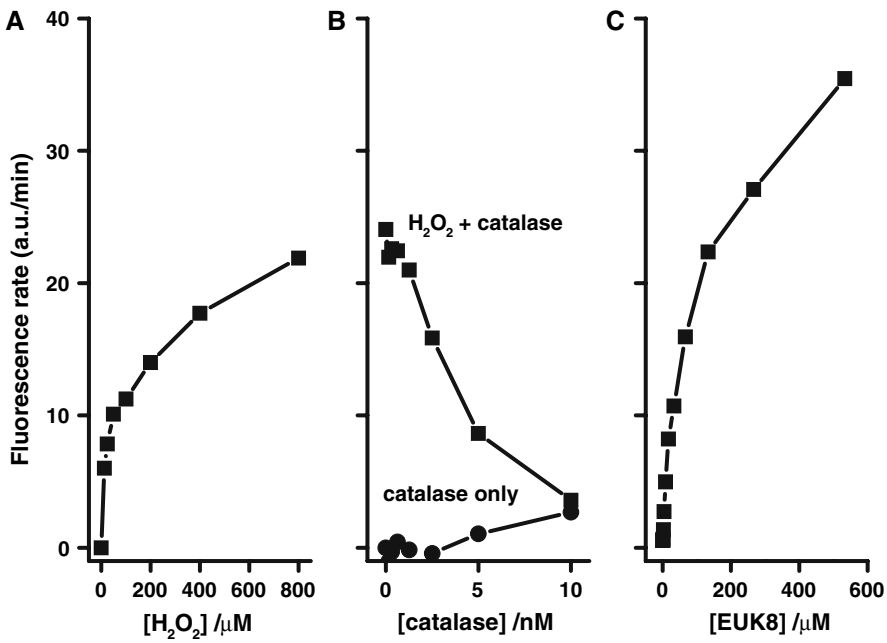



**Fig. 1** Effects of (**a**) $H_2O_2$; (**b**) catalase and 200 μM $H_2O_2$; (**c**) EUK8 on the fluorescence rate of DHR oxidation

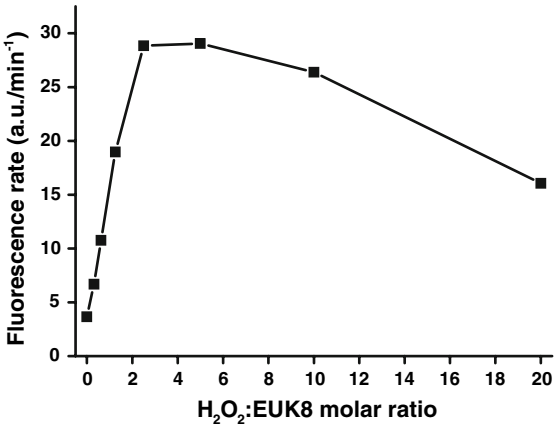



**Fig. 2** Fluorescence rate of DHR oxidation under the influence of different molar ratios of $H_2O_2$ ([EUK8] = 10 μM)

Zn-SOD knockout yeast have been detected, possibly due to imbalances in the levels of ROS and interference with cell signaling processes. Only free $Mn^{2+}$, which incidentally is not redox-active in our experimental setup (data not shown), was able to revert the phenotype of the knockout yeast (Sanchez et al. 2005). The pro-oxidant activity of low concentrations of EUK8 cannot be determined through the traditional oximetric determinations since they require relatively high (millimolar range) concentrations of $H_2O_2$ (Doctrow et al. 2002).

We further investigated the validity of our findings through the quantification of the protein carbonyl content, an established method for assessing metal-catalyzed oxidation, based on the formation of a colored product after the reaction of carbonylated protein and 2,4-DNP (Levine et al. 1990). The results for some $H_2O_2$:EUK8 molar ratios are given in Table 1.

**Table 1** Carbonyl content of HSA (20 μM) incubated with EUK8 and $H_2O_2$

| Treatment | Carbonyl content/μM | $H_2O_2$:EUK8 molar ratio |
|---|---|---|
| None | 3.3 | – |
| $H_2O_2$ 200 μM | 3.2 | – |
| EUK8 20 μM | 2.8 | – |
| EUK8 100 μM | 4.3 | – |
| EUK8 200 μM | 4.1 | – |
| EUK8 20 μM + $H_2O_2$ 200 μM | 3.2 | 10 |
| EUK8 100 μM + $H_2O_2$ 200 μM | 3.6 | 2 |
| EUK8 200 μM + $H_2O_2$ 200 μM | 3.7 | 1 |

Again, it was possible to observe the oxidant effect of EUK8 alone at high concentrations, as well as its pro-oxidant effect when in the presence of less-than-ideal concentrations of hydrogen peroxide. When $H_2O_2$:EUK8 $< 2$ we observed an increase in the carbonyl content, which decreased at higher $H_2O_2$ concentrations. Since the photometric determination of the 2,4-DNP-tagged protein is observed only for high concentrations of EUK8, it could be argued either that the complex is intrinsically safe for therapeutic purposes or that the method is not capable of detecting the fine details of pro-oxidant activity of EUK8 in the presence of $H_2O_2$. In the light of the results for the fluorescence rate reported above, we believe that the latter scenario should be considered as an important possibility and, in spite of the greater resemblance to biological systems, protein carbonyl quantification might be unable to account for the phenomena which influence the results found at higher levels (animal and cell models).

The fact that most reports attest to the beneficial effects of EUK8 in a plethora of animal models prompted us to study the effect of the natural antioxidant glutathione in a mixture of EUK8 and $H_2O_2$ in a 1:2 molar ratio (therefore in the top of the pro-oxidant range of Fig. 2) (Fig. 3). As expected, cellular levels of glutathione were effective in counteracting the rate of DHR oxidation. This result

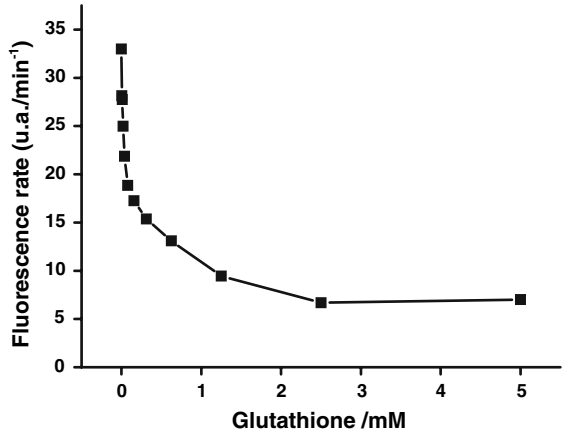



**Fig. 3** Effect of glutathione on the fluorescence rate of DHR oxidation catalyzed by a mixture of EUK8 10 μM and $H_2O_2$ 20 μM

further reinforces the need to assess accurately the antioxidant status of a given organism before drawing conclusions on the effects of a redox-active compound. It also indicates that these compounds should be used carefully if this status is unknown in a clinical setup.

Total fluorescence measurements show that viable HeLa cells display some DHR oxidation capacity (Fig. 4a). Upon oxidation, DHR is converted to the fluorescent cationic form RHA. Resting levels of cellular oxidants ($H_2O_2$, labile $Fe^{2+}$) account for this oxidation, which can be further enhanced, for instance, by the addition of exogenous $H_2O_2$ (Fig. 4c). However, contrary to what is observed in buffered solutions (Fig. 1c), EUK8 does not substantially enhance DHR oxidation rate in viable HeLa cells (Fig. 4b). Fucassi et al. have recently observed that EUK8 damages free DNA but the complex is not cytotoxic to primary fibroblasts, probably because natural levels of other cell antioxidants, such as glutathione, are effective in counteracting any EUK8 pro-oxidant activity (Fucassi et al. 2007). As demonstrated above, we suggest that previous reports on the absence of beneficial effects of EUK8 might be better understood if the actual concentrations of natural antioxidants/reductants (e.g., glutathione) were known. Also, it is important to bear in mind that different organisms may possess different rates and routes for the metabolism of Mn(III) complexes.

## Conclusions

The increased rate of oxidation of the fluorescent probe DHR in solution indicates that therapeutically relevant levels of EUK8 may possess pro-oxidant action when sub-stoichiometric levels of $H_2O_2$ are present. Pro-oxidant activity increases for $H_2O_2$:EUK8 molar ratio $\leq 2$ and is precluded by glutathione. This suggests the accumulation of the highly oxidant Mn(V)-$O^{2-}$ species and the possibility of in vivo damage when local levels of EUK8 are relatively higher than those of hydrogen peroxide and glutathione. At EUK8 levels which are too high to be therapeutically relevant, the carbonyl content (a marker of metal-catalyzed oxidation) of HSA increases. It appears that this colorimetric method is not as effective as the fluorescence method to evaluate the generation of oxidant species in vitro. HeLa cells treated with EUK8 did not display increased pro-oxidant activity, as reported by the fluorescent probe DHR, indicating that intracellular antioxidants may be effective to counteract EUK8 activity. Our findings suggest that the combination of $H_2O_2$ and EUK8 at specific molar ratios, in the

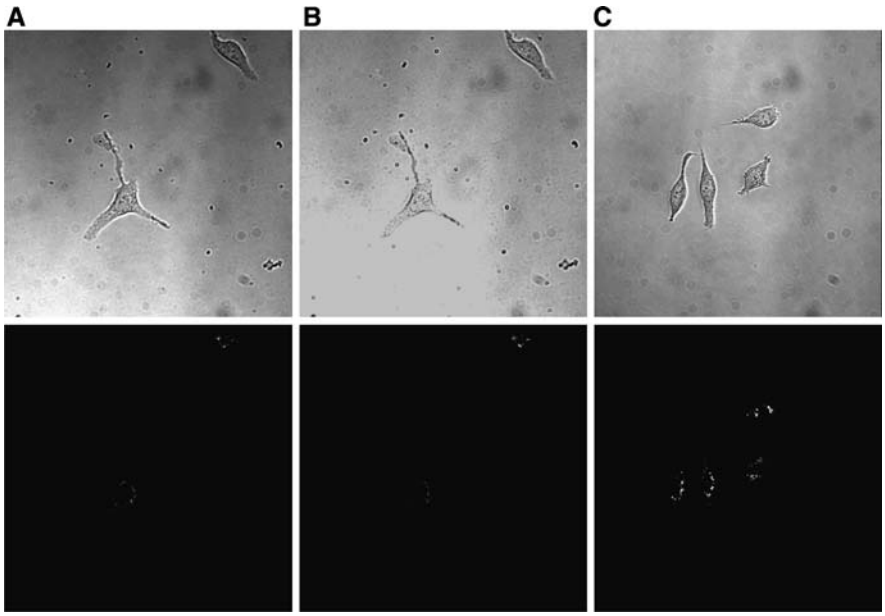



**Fig. 4** Fluorescence microscopy images of HeLa cells loaded with DHR and buffer (**a**), EUK8 100 μM for 1 h (**b**) and another set incubated with $H_2O_2$ 100 μM for 1 h (**c**). Upper panels: light field; lower panels: fluorescence field

absence of reductants/antioxidants, induces the oxidation of organic molecules, which may contribute to a better understanding of the problems associated with catalase-like mimetics in vivo.

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