Catalytic Antioxidants

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Amphiphilic/Bipolar Metallocorroles That Catalyze the Decomposition of Reactive Oxygen and Nitrogen Species, Rescue Lipoproteins from Oxidative Damage, and Attenuate Atherosclerosis in Mice**

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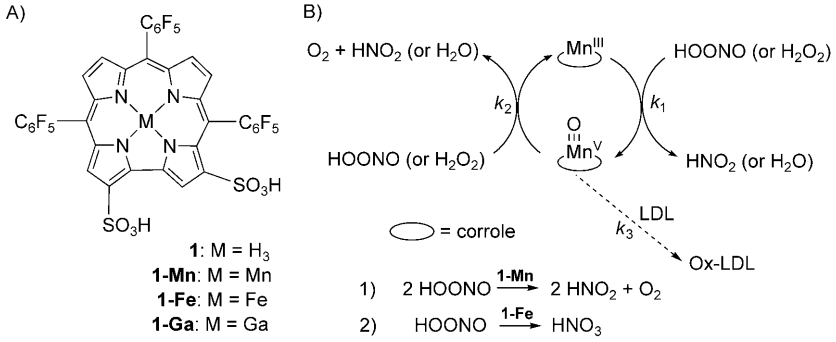
Accumulating evidence points towards the involvement of reactive oxygen and nitrogen species (ROS and RNS, respectively) in a very wide variety of diseases.[1] The relationship between atherosclerosis and oxidative/nitrosative stress—the imbalance between the desired production of ROS/RNS and the capability of biological systems to detoxify excess amounts thereof—is often analyzed in terms of damage to the cholesterol-transporting high- and low-density lipoproteins (HDL and LDL, respectively).[2] Oxidized LDL are taken up at an enhanced rate by arterial macrophages in a noncontrolled fashion and modified HDL are less effective in performing cellular cholesterol efflux, thus leading to the formation of cholesterol-loaded foam cells, the hallmark of early atherosclerosis. Although the atherosclerosis-preventing effect of certain natural antioxidants is in harmony with this hypothesis,[3] it is seriously challenged by the fact that many potent in vitro antioxidants fail to display beneficial in vivo effects.[4]

Recent focus on peroxynitrite (HOONO) revealed that this molecule is involved (directly and through $^{\bullet}OH$ and $^{\bullet}NO_2$ derived from it) in the damage of a wide variety of molecules,[5] including those that are of vital importance for proper cardiovascular function.[6] The problem unique to peroxynitrite is that, in contrast to other ROS and RNS and their precursors, there is no known biological defense system against it, and most natural antioxidants are very poor peroxynitrite scavengers.[7] This calls for the development of synthetic reagents that could act on and neutralize peroxynitrite by one or more of the following ways: 1) interfere with its formation by eliminating its precursors (superoxide anion and nitric oxide); 2) decompose it to biologically benign products;[8] and 3) repair the damage caused by it.

The motivation for the current investigations was the fact that the particular iron(III) and manganese(III) corroles shown in Scheme 1 A were disclosed as potent catalysts for decomposition of peroxynitrite in purely chemical systems, with the former acting very fast and the latter operating through the novel mechanism shown in Scheme 1 B.[9] The same metallocorroles (of unique amphiphilicity and bipolarity resulting from the positioning of sulfonic acid head groups on the otherwise lipophilic corrole) were also shown to have a large affinity to various proteins,[10] a very important factor that may be used for selective delivery purposes.



Scheme 1. A) Structures of the corroles used. B) The disproportionation mechanism for catalytic decomposition of hydrogen peroxide and peroxynitrite by **1-Mn** and the balanced equations for catalytic decomposition of peroxynitrite by **1-Mn** and **1-Fe**.

Herein, we show that the iron corrole **1-Fe** (Scheme 1 A) rescues small molecules from ROS-induced oxidation more efficiently than the manganese complex **1-Mn**, that the opposite holds for arresting RNS-induced nitration, and that **1-Fe** and **1-Mn** are anti- and pro-oxidants, respectively, with regard to their in vitro effects on oxidative damage to LDL/HDL. An important finding with regard to targeting is that both metallocorroles, as well as the non-transition-metal complex **1-Ga**, bind to lipoproteins more strongly than to all other serum components. All three corroles also affect cholesterol levels and distribution in plasma, and **1-Fe**, but

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not **1-Mn** or **1-Ga**, increases cellular cholesterol efflux from macrophages. The iron complex is more effective than natural antioxidants for attenuation of atherosclerosis development in mice, which is attributable to the synergetic effects that were deduced from the in vitro investigations.

For initial appreciation regarding possible superiority of the synthetic catalysts relative to natural antioxidants, we determined the effect of excess (45–135 μM) punicalagin—the active polyphenolic hydrolyzable tannin ingredient of pomegranate juice[11]—on the decomposition of peroxynitrite (40 μM). This information, together with the previously determined rate constants for catalytic decomposition of peroxynitrite (385 μM) by **1-Fe** and **1-Mn** (5–20 μM; Scheme 1),[9a] were used for calculating the minimal concentrations required for reducing the half-life of peroxynitrite by 50%. The results revealed that 250 mol% punicalagin, 2.5 mol% **1-Mn**, and 0.05 mol% **1-Fe** are needed for that purpose, that is, the catalytically acting **1-Fe** and **1-Mn** are 5000 and 100 times, respectively, more effective than the most potent dietary antioxidant, pomegranate punicalagin, which acts in a sacrificial mode.

Another early investigation focused on the kind of peroxynitrite-induced damage that may be eliminated by the metallocorroles, by utilizing small molecules that represent targets for hydroxyl radicals and nitrogen dioxide (Table 1).[12,13] Catalytic amounts of **1-Fe** or **1-Mn** rescued dimethyl sulfoxide (DMSO) and deoxyribose from being oxidized by peroxynitrite-derived hydroxyl radicals, but only **1-Mn** completely eliminated $^{\bullet}NO_2$-accredited reactions (**1-Fe** had no effect on the nitration of tyrosine, and was only partially effective in inhibiting that of fluorescein). With $CuSO_4$/glutathione as the initiator of ROS (by the complex Fenton-type oxidation that produces the hydroxyl radical by the involvement of a superoxide anion radical and hydrogen peroxide),[14] **1-Fe** eliminated the oxidation of DMSO to formaldehyde completely, whereas **1-Mn** did it only partially (65% inhibition). These investigations clearly point towards the following conclusions: 1) both corrole metal complexes serve very well for preventing the formation of the hydroxyl radical from peroxynitrite; 2) the iron complex is more efficient in rescuing molecules from copper-induced formation of hydroxyl radicals; and 3) the manganese complex is more efficient in inhibiting the formation of RNS from peroxynitrite.

Table 1: The inhibitory effect of metallocorroles on oxidation/nitration of small molecules by peroxynitrite; pH 7.4, $T = 25\,^{\circ}C$.

Reactive species	Substrate	Product	Yield [%] relative to peroxynitrite		
			no additive	**1-Mn**	**1-Fe**
$^{\bullet}OH$	DMSO	formaldehyde	11.5	0	0
$^{\bullet}OH$	deoxyribose	malondialdehyde	1.7	0	0
$^{\bullet}NO_2$	fluorescein	nitrofluorescein	30[a]	0	4[a]
$^{\bullet}NO_2$	L-tyrosine	3-nitro-L-tyrosine	11	0	11

[a] Yield relative to fluorescein.

These findings are consistent with the differences determined so far in the modes of action of these complexes on ROS and RNS. No nitrating species are released in the catalytic cycle for decomposition of peroxynitrite by **1-Mn** [Scheme 1B, Eq. (1)],[9a] which is most likely not true for **1-Fe** [Scheme 1B, Eq. (2)].[15] On the other hand, the catalytic rate for decomposition of hydrogen peroxide (the precursor of $^{\bullet}OH$ in Cu-induced oxidations) by **1-Fe** appears to be larger than that of **1-Mn**,[16] thus explaining the better antioxidant properties of the former.

Extension of purely chemical findings into any in vivo system is very challenging,[18] and could be particularly severe for metal complexes of corrole **1** that were shown not to enter cells in protein-free medium and to form tightly bound noncovalent conjugates with serum proteins.[10] Accordingly, mutual interactions between the complexes and lipoproteins were checked to test the probability of the former reaching the place where the latter are oxidized—the arterial wall. Recording of the electronic spectra of aqueous (protein-free) solutions of metal complexes of corrole **1** before and after addition of LDL or HDL, as well as after dialysis, revealed the binding stoichiometry: 40 ± 5 and about 10 corrole molecules bind with high affinity to each LDL and HDL particle, respectively, which is significantly more than the number of natural antioxidants present therein.[2c] The interpretation of these data is, however, quite restricted because they do not provide information about the dissociation constants of the corroles from the lipoproteins, or about the affinity relative to other serum proteins. These concerns were addressed by density-gradient ultracentrifugation of whole serum preincubated with metallocorroles. Figure 1 clearly shows that these corroles preferably bind to lipoproteins at the expense of all serum proteins,[19] and spectral analysis of the separated fractions (see the Supporting Information) implies that 65–70% of the corroles are bound to HDL. These results suggest that the lipoproteins might carry the metallocorroles all the way to the arterial wall, where the antioxidant properties of the latter are needed.

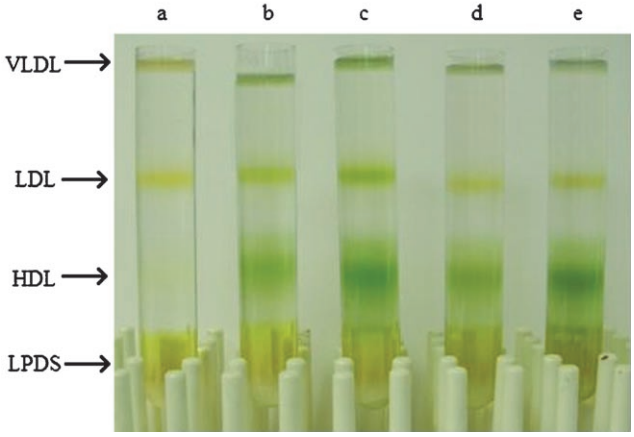

Figure 1. Density-gradient ultracentrifuged serum with a) no additive, b) 20 μM **1-Mn**, c) 40 μM **1-Mn**, d) 20 μM **1-Fe**, and e) 40 μM **1-Fe**. The yellow rings arise from different serum fractions and the green color from the associated corroles. VLDL = very-low-density lipoprotein; LPDS = lipoprotein-deficient serum.

Examination of the different fractions isolated from the above-mentioned experiments (Figure 1) further revealed that the amount of cholesterol in LDL from corrole-treated serum was lowered by about 20%, whereas it was increased in the HDL and LPDS fractions. This finding suggests that strong binding of the metallocorroles does affect cholesterol distribution. A further indication of a favorable alteration of cellular cholesterol transport by metallocorroles was obtained by studying cholesterol efflux from J-774 macrophages in a lipoprotein-free medium. The iron (but not the manganese or gallium) complex of corrole **1** increased the efflux by up to 20% in a dose-dependent fashion.

The effect of the metallocorroles on oxidative damage to LDL and HDL solutions, tested by established methods,[20,21] revealed the following results. The formation of conjugated dienes (the earliest intermediates of lipid peroxidation) by treatment of LDL with SIN-1, a reagent that slowly generates peroxynitrite,[22] was increased by **1-Mn** and completely eliminated by **1-Fe** (see the Supporting Information). Experiments performed according to the most commonly used copper-induced protocol revealed that in the presence of **1-Mn**, the delay time in formation of conjugated dienes (as a result of consumption of "free radicals" by endogenous antioxidants) was shortened from 30 to 15 min (Figure 2A), the concentration of hydroperoxides rapidly reached a maximum at 45 min (because of subsequent transformation to the final aldehyde products, Figure 2B), and aldehydes were formed faster and to a much larger extent than in the control experiment (Figure 2C). On the other hand, *practically no damage was observed in the presence of **1-Fe** by all the above three criteria* (Figure 2A–C), and even after 24 h no thiobarbituric acid reactive substances (TBARS) were detected. Similar results were obtained for copper-induced oxidation of HDL. Importantly, approved drugs, such as carvedilol, do not suppress the oxidation of isolated LDL induced by peroxyl radicals or cupric ions.[17]

The dependence of LDL oxidation on the corrole concentration was examined by analyses performed 2 h after the copper-initiated oxidation (Figure 2D). The non-redox-active gallium(III) complex **1-Ga**, examined to eliminate a possible effect of the corrole macrocycle itself, provided exactly the same results as those obtained without any corrole-based additive. In the presence of **1-Mn**, up to three times more TBARS than without any additive was obtained in a concentration-dependent manner. On the contrary, the iron complex **1-Fe** caused a minor increase in TBARS at a concentration of 0.5 μM, but complete inhibition was obtained at concentrations of 2.5 μM and higher (unmatched by any previously reported complex, vide infra). The dramatic differences between the two transition-metal complexes may be explained based on the mechanisms shown in Scheme 1: **1-Mn** efficiently traps hydrogen peroxide (the precursor of $^{\bullet}$OH in Cu-initiated oxidations), but the (oxo)-manganese(V) intermediate (k_1) reacts with lipid components (k_3) faster than with another hydrogen peroxide molecule (k_2) because of the very large differences in their local concentrations. On the other hand, the overall catalytic rate of the iron corrole is much faster and there is no evidence for the buildup of any oxidizing reaction intermediate.

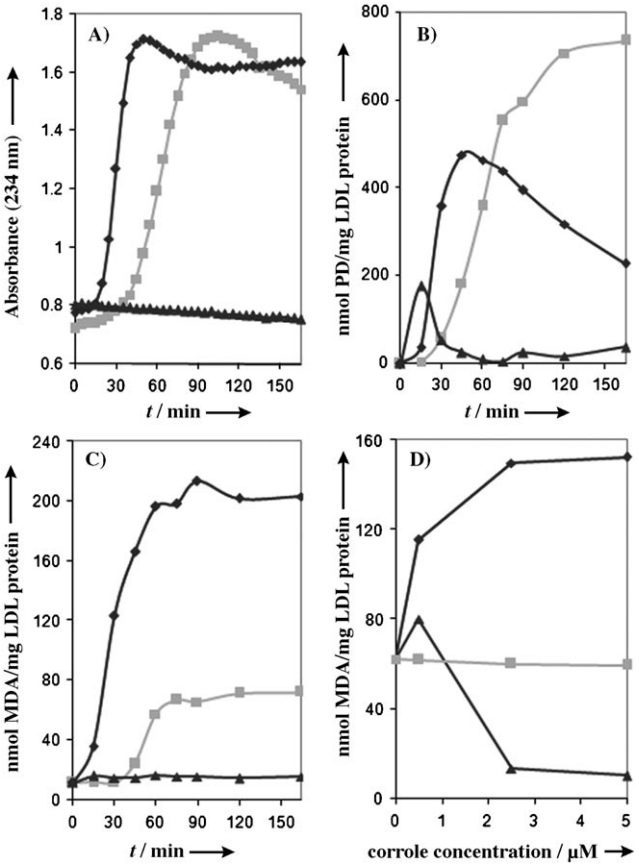

Figure 2. Effect of corrole (2.5 μM) on the kinetics of $CuSO_4$-induced (5 μM) oxidation of LDL (100 mg protein L^{-1}). A) Conjugated diene formation followed at 234 nm; B) lipid peroxide (PD) formation; C) aldehyde formation followed by TBARS measurement; and D) TBARS formation after 2 h for various corrole concentrations. ◆ with **1-Mn**; ▲ with **1-Fe**, and ■ without corrole for (A)–(C) and with **1-Ga** for (D). MDA = malondialdehyde.

The encouraging in vitro data with the metal complexes of corrole **1** were extended towards an in vivo investigation on apolipoprotein E-deficient (E^0) mice, the most common murine model used for atherosclerosis development studies. E^0 mice are hypercholesterolemic and develop spontaneous atherosclerotic lesions similar in development and morphology to those in humans.[23] The mice were divided into four groups that differed only in the type of supplied drinking water: no additive, or water containing either **1-Mn**, **1-Fe**, or **1-Ga** (0.2 mg per mouse per day). Histopathological development of lesions in the aorta obtained from mice sacrificed 10 weeks after treatment started revealed that the average lesion area was very high for untreated mice and for those that received **1-Ga** (Figure 3A). A small decrease in average lesion area (17% relative to the control group) was obtained for the **1-Mn** group, while the results obtained with **1-Fe** were truly dramatic. *Two of the mice receiving that treatment did not develop atherosclerotic lesions at all*, and the average lesion area for the other four mice was 60% smaller than in the control group. These results are much better than those obtained by other compounds, including E^0 mice that were treated by the most potent natural antioxidants (48 and 44%

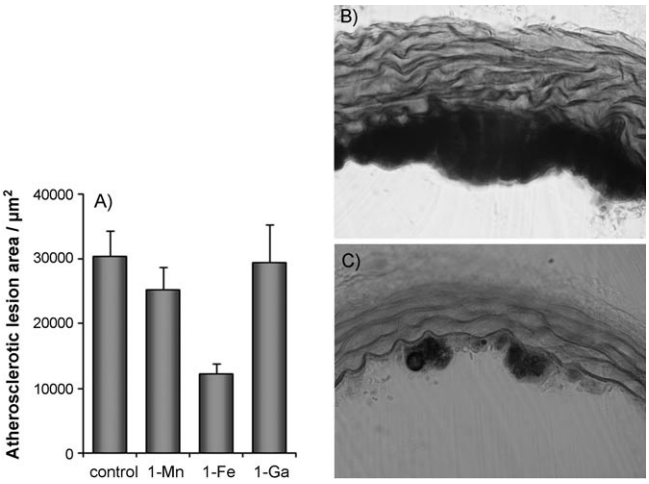

Figure 3. Effect of corrole consumption on the formation of lesions in E^0 mice. A) Size of atherosclerotic lesions in the different groups. Values are means ± standard error of the mean, $n = 6$. For the **1-Fe** group the mean is for $n = 4$ because two of the mice in the group did not show formation of a lesion at all. B) Cross-section from the aortic arch of a control mouse. C) Cross-section from the aortic arch of a mouse that consumed **1-Fe**. The lipid components are stained a brown-black color, thus highlighting the foam cells.

reduction for consumption of red wine and pomegranate juice, respectively).[3]

Examination of the sacrificed mice revealed another important result: the total blood cholesterol levels in mice that received the metallocorroles were significantly reduced (**1-Fe**: 40%, **1-Mn**: 26%, **1-Ga**: 20%) relative to the control animals. These ex vivo observations are nicely correlated with the in vitro results described earlier, which revealed that the amount of cholesterol in the LDL fractions of corrole-treated serum was lowered by about 20% and that **1-Fe** even affected cellular efflux of cholesterol.

All together, the observations obtained in the mouse model of atherosclerosis appear to be very much consistent with the in vitro results, which disclosed that the iron complex **1-Fe** is a very potent catalytic antioxidant that also affects cholesterol efflux from macrophages and lowers LDL cholesterol levels. The unique bipolarity of corrole **1** is apparently responsible for the selective binding to lipoproteins, which is very important for their protection from oxidative damage and may serve as a mechanism for delivering the complexes to the arterial wall. This finding, together with the very fast *catalytic* rates of **1-Fe**, is of particular relevance in the context of the comparison with dietary antioxidants: the noncatalytic activity of polyphenols was shown to occur at concentrations at least one order of magnitude higher than their bioavailability.[24]

The structure of corrole **1** and its metal complexes is also distinctly different from that of all metalloporphyrin-based decomposition catalysts of peroxynitrite reported to date,[25] wherein the symmetrical distribution of (either negative or positive) charges is likely to preclude their association with lipoproteins.[26] In fact, such synthetic iron porphyrins do not display potency for inhibiting LDL oxidation, and manganese porphyrins protect LDL from peroxynitrite only in the presence of reductants, such as ascorbate[27] or uric acid, at concentrations that are 40 times larger than those needed for full inhibition by **1-Fe**.[28] On the other extreme, hemin and structurally related iron porphyrins that associate very strongly with lipoproteins are pro- rather than antioxidants.[29] Differences in the mechanism and the rate of decomposition of ROS and RNS are apparently responsible for the anti- and pro-oxidant properties of **1-Fe** and **1-Mn**, respectively, as well as for **1-Mn** being more efficient in eliminating RNS. In fact, the somewhat positive in vivo effect of **1-Mn** may possibly be traced to its effect on nitrosative stress. Future studies will be devoted to tuning of the catalyst's ability regarding efficient decomposition of other ROS/RNS and better suppression of nitration, as well as to additional biochemical investigations. One example of the last aspect is that the preliminary results are indicative of increased macrophage lactonase activity (probably related to the cellular antioxidant enzyme, paraoxonase 2)[2a] in mice that consumed the metallocorroles. Apparently, the combination of several favorable effects is responsible for the quite dramatic effect displayed by the specific iron corrole described herein as regards the rescue of LDL/HDL from oxidative damage and the attenuation of atherosclerosis in mice.

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