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Variables That Influence Cellular Uptake and Cytotoxic/Cytoprotective Effects of Macrocyclic Iron Complexes

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Supporting Information

ABSTRACT: Determination of the cellular uptake of macrocyclic iron(III) complexes by a facile method, accompanied by cell viability tests under both basal and induced oxidative stress, demonstrates that protection against *intracellular* oxidative stress requires reasonably high internalization and favorable anti/prooxidant profiles. Of the four tested complexes, only amphipolar iron(III) corrole met these criteria.

Reactive oxygen and nitrogen species (ROS/RNS) are key players in the development of numerous diseases because of an activity decline of enzymes responsible for decomposition of the superoxide anion radical (O_2^{-}) and hydrogen peroxide (H_2O_2). The reactions of these moderately toxic molecules with improperly chelated ("free") iron induce the formation of the extremely damaging hydroxyl radical. One therapeutic approach is chelation therapy, used to arrest the ability of iron to catalyze undesired redox reactions.[1] On the other hand, catalytic antioxidant therapy relies on iron complexes that still participate in redox reactions but in a beneficial fashion.[2] This is commonly achieved by using macrocyclic molecules (mainly porphyrins and recently corroles as well), whose strongly chelated metal complexes may detoxify O_2^{-}, H_2O_2, and peroxynitrite (HOONO, formed by the reaction of O_2^{-} with NO) in a catalytic fashion.[2a,b,3] Iron is not released from these complexes under physiological conditions, and they have been shown to display beneficial effects in an impressively large number of disease model systems.[4] Two major concerns regarding that approach are that (a) such complexes may possess both *anti-* and *pro*oxidant activity and (b) the synthetic ROS/RNS decomposition catalysts must enter the cells in sufficient amounts for providing protection against *in cellulo* formed species.

The aim of this study was to examine the above variables by looking at cellular uptake and cytotoxic versus cytoprotective effects of four macrocycle-chelated iron(III) complexes—hemin, FeTPPS, FeTMPyP, and **1-Fe** (Figure 1)— selected for the following reasons: (a) natural hemin is considered toxic,[5] while the three synthetic complexes are under investigation in the context of catalytic antioxidant therapy;[2b,c,4] (b) FeTMPyP is positively charged, and the three other complexes carry negatively charged head groups; (c) FeTPPS and FeTMPyP may be considered hydrophilic because their charged substituents are symmetrically distributed, while **1-Fe** and hemin are amphipolar with charged groups on only one side of the macrocycle, structural differences that are likely to affect cellular internalization very much.[6] This study explored the intracellular accumulation of the various iron(III) macrocycles and the consequential effects on cell viabilities under both basal and induced oxidative stress.

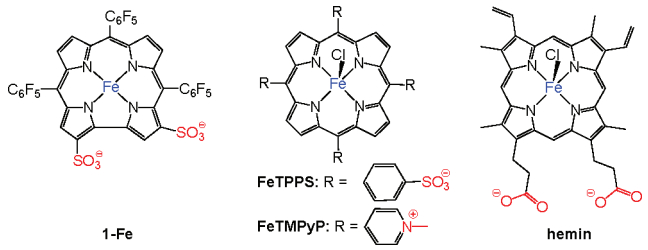

Figure 1. Structures of **1-Fe**, FeTPPS, FeTMPyP, and hemin.

The major methodology for *in cellulo* detection and quantification of drug candidates is optical imaging, which cannot, however, be used for nonemissive transition-metal complexes. Relatively simple methods reported for quantification of metalloporphyrin-based catalytic antioxidants at *submicromolar* concentrations include (a) atomic absorption, a method requiring a very large quantity of metal-containing cells, and (b) postreplacement of the porphyrin-chelated manganese(III) by zinc(II) and quantification of the zinc(II) porphyrin by fluorescence, an elegant but nonstraightforward method limited to cases in which the metal may indeed be substituted.[7] We now introduce a facile and sensitive detection method, based on a catalytic rather than an optical property. It relies on the emission of blue light in the luminol/H_2O_2 reaction, resembling routinely employed methods in biochemical research and forensic detection that are based on horseradish peroxidase and hemoglobin, respectively.[8] The prosthetic group responsible for catalysis in these biomolecules is iron(III) protoporphyrin IX, and synthetic iron(III) porphyrins have been used for the same purpose in purely chemical systems.[9] The potency of the iron(III) corrole **1-Fe** toward the luminol/H_2O_2 reaction is identified here for the first time.

The feasibility of using the luminol reaction for quantifying iron(III) macrocycles originating from cell cultures was first tested on cellular debris. Macrophages were selected in the present study because of their major contribution to the

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development of atherosclerosis, a disease against which **1-Fe** has been proven particularly efficient.[10] J774.A1 macrophage cells were grown in the luminescence examination plate and then ruptured by suspension in distilled water, followed by a freeze−thaw cycle. In nontreated cell debris, the addition of luminol and H_2O_2 produced a weak chemiluminescent reaction. Upon the addition of **1-Fe** to already ruptured cells,[11a] the emission was strongly enhanced in a dose-dependent manner

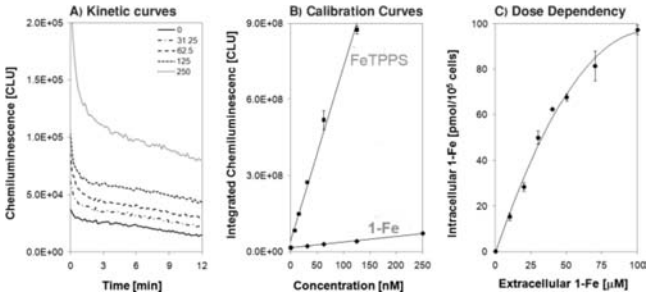

Figure 2. Quantitative analysis of cell-derived **1-Fe** and FeTPPS. (A) Luminol chemiluminescence kinetic curves in the presence of increasing concentrations of **1-Fe**. (B) Calibration curves for **1-Fe** and FeTPPS in the presence of cell debris. (C) Cellular concentrations of **1-Fe** following 2 h of incubation with J774.A1 macrophages. The results are mean (n = 3) ± SD.

(Figure 2A). Integration of the kinetic curves allowed for the construction of a calibration curve (Figure 2B), enabling quantification of the cellular-derived **1-Fe** down to a 30 nM concentration. The same procedure was applied for the iron(III) porphyrins FeTPPS, FeTMPyP, and hemin, which all displayed dose-dependent responses. Chemiluminescence was more intense for reactions catalyzed by iron porphyrins, with FeTPPS being so efficient that even 7 nM was easily detected (Figure 2B). The lower efficiency of **1-Fe** relative to porphyrins for catalyzing the luminol reaction is consistent with its much higher potency for the catalytic decomposition of H_2O_2,[3c] the reaction competing with the oxidation of luminol.

The dependence of the intracellular accumulation of **1-Fe** on its extracellular concentration was examined by incubation of the compound with living macrophage cells for 2 h, followed by removal of the extracellular fraction, cell rupture, and chemiluminescence quantification relative to a standard curve. The chemiluminescence intensity increased as a function of the initial concentration of **1-Fe** (Figure 2C), signaling an increase in the level of intracellular corrole, and reached saturation at high extracellular corrole concentrations.[11b] The calculated amount of intracellular **1-Fe** at saturation was 1 fmol/cell, or 6 × 10^8 molecules/cell. This is 2−3 orders of magnitude larger than the cellular amount of the most important and extremely fast acting (k_{cat} = 2 × 10^9 M^{-1} s^{-1}) enzymatic antioxidant, superoxide dismutase: 2.5 × 10^5 and 1 × 10^6 molecules/cell in erythrocytes and lymphocytes, respectively.[12]

The cellular uptake of the various iron(III) chelates was compared using the new method (Figure 3A). Following 24 h of incubation, FeTPPS accumulated to only about 8 pmol/well, 40−50 pmol/well was obtained for **1-Fe** and FeTMPyP, and 140 pmol/well for hemin. Meaningful analysis of this data must, however, take into account the effects that these compounds had on the cell viability (Figure 3B) because FeTMPyP and hemin greatly reduced the cell survival to 45−60%. The high intracellular amounts of these compounds are, hence, misleading because cell death is accompanied with the loss of

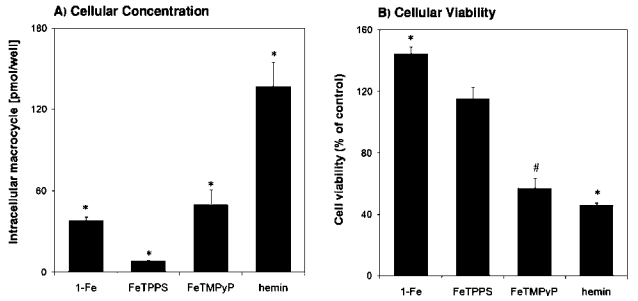

Figure 3. Intracellular concentrations (A) and cell viability (B) following incubation of J774.A1 macrophages with 20 μM macrocyclic iron(III) complexes for 24 h. Results are mean (n = 3) ± SD. (*) p < 0.001 and (#) p < 0.05 relative to nontreated cells.

membrane integrity. This cytotoxicity apparently reflects the prooxidant properties of hemin and the DNA-cleaving ability of FeTMPyP (on top of its antioxidant activity).[5a,13] The ≥5 times larger cellular concentration of **1-Fe** relative to FeTPPS may be attributed to differences in their interactions with serum proteins, needed for transporting such negatively charged molecules across cell membranes. This hypothesis is consistent with the demonstration that the amphipolar **1-Fe** (and hemin) conjugates spontaneously and extremely strong to lipoproteins, while the symmetrically substituted FeTPPS does not.[10b]

The results of Figure 3B also reveal a significant increase in cell viability (150%) following **1-Fe** treatment, which might reflect the effect of this catalytic antioxidant on the attenuation of damage induced by the high oxidative stress that is naturally present in macrophages. The importance of the redox-active iron may be appreciated by a comparison between **1-Fe** and its gallium(III) analogue: the latter is highly cytotoxic (<5% cell viability under the conditions of Figure 3), consistent with its use as an anticancer agent that promotes the formation of ROS.[14] **1-Fe** and FeTPPS, the two complexes that did not reduce cell viability, were, hence, further examined for their cytoprotection capability. Cell cultures pretreated as above (i.e., 24 h of incubation with one of the iron(III) complexes followed by removal of the extracellular fraction) were exposed to diverse agents that initiate oxidative stress-induced cell death: (a) the primary oxidants H_2O_2 and HOONO [progressively formed from the applied 3-morpholinosydnonimine (SIN-1)],[15] which the antioxidants may directly neutralize in a catalytic fashion; (b) the secondary oxidant oxLDL (oxidized low-density lipoproteins);[16] and (c) the combination of lipopolysaccharide (LPS) and interferon-γ (INF-γ),[17] which causes immunoactivation of cells for the production of ROS and RNS.[18] FeTPPS had no significant effect in any of the examined cases, but the 40 pmol/10^5 cells intracellular **1-Fe** increased cell survival relative to control for all applied toxins (Figure 4): from 25% to 55% for H_2O_2, from 35% to 50% for SIN-1, from 55% to 90% for oxLDL, and from 40% to 80% for LPS + INF-γ.

The superiority of **1-Fe** relative to FeTPPS is in line with its higher intracellular concentration, as well as with the larger catalytic rates that it has for decomposition of all major ROS/RNS, especially H_2O_2 (Table 1). Although most metalloporphyrins are bleached by H_2O_2, they still serve well for eliminating O_2^- and HOONO[2b,13b,19] and have been shown to be efficient against the addition of these ROS/RNS when present in culture media.[20] They are, however, quite ineffective against oxidants formed *in cellulo* by cellular activation.[2c,17a,21]

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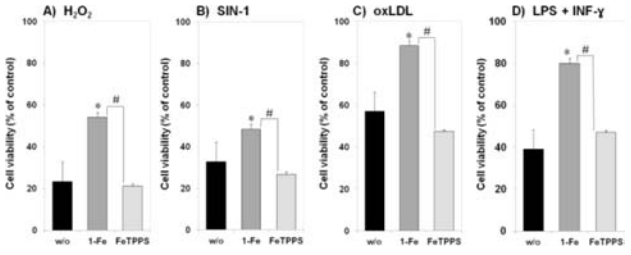

Figure 4. Cell viability following incubation of J774.A1 macrophages with or without 20 μM **1-Fe** or FeTPPS for 24 h, followed by medium replacement and exposure to several toxins. Results are mean (n = 3) ± SD. (*) $p < 0.001$ relative to w/o. (#) $p < 0.001$ relative to **1-Fe**.

Table 1. Catalytic Rates (M^{-1} s^{-1}) for ROS/RNS Decomposition and Cellular Concentrations (fmol/cell) of 1-Fe and FeTPPS (following 24 h of Incubation of 20 μM)

	O_2^-	H_2O_2	HOONO	fmol/cell
1-Fe	3×10^6 [3a]	6400[3c]	3×10^6 [3b]	0.4
FeTPPS	6×10^5 [13c]	none[19c]	9×10^5 [13b]	0.08

Our results suggest that this phenomenon likely reflects the low intracellular concentrations of these catalysts. The effectiveness of **1-Fe** against *in cellulo* formed oxidants not only is quite novel but also confirms that iron corrole identified by the chemiluminescence method is, in fact, intracellular and not just cell-associated.

We have demonstrated significant differences between the cellular accumulation of iron(III) macrocycles of different polarity and shown that both cytotoxicity and cytoprotection properties vary very much within the examined series. Determination of cell accumulation was achieved using a very simple, rapid, and sensitive detection method, which may, in principle, be applied for any cell type and for other compounds that catalyze the oxidation of luminol.[9] Of the four examined iron complexes, only the amphipolar corrole displays all desired features that are essential for utilization as a cell-protecting antioxidant: sufficiently large cellular uptake, nontoxicity toward the cells, and intracellular catalytic activity against the most important ROS and RNS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Kelm, M.; Dahmann, R.; Wink, D.; Feelisch, M. *J. Biol. Chem.* **1997**, *272*, 9922.

(2) (a) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 7493. (b) Batinić-Haberle, I.; Rebouças, J. S.; Spasojević, I. *Antioxid. Redox Signaling* **2010**, *13*, 877. (c) Salvemini, D.; Wang, Z.-Q.; Stern, M. K.; Currie, M. G.; Misko, T. P. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2659.

(3) (a) Eckshtain, M.; Zilbermann, I.; Mahammed, A.; Saltsman, I.; Okun, Z.; Maimon, E.; Cohen, H.; Meyerstein, D.; Gross, Z. *Dalton Trans.* **2009**, 7879. (b) Mahammed, A.; Gross, Z. *Angew. Chem., Int. Ed.* **2006**, *45*, 6544. (c) Mahammed, A.; Gross, Z. *Chem. Commun.* **2010**, *46*, 7040.

(4) Day, B. J. *Drug Discovery Today* **2004**, *9*, 557.

(5) (a) Camejo, G.; Halberg, C.; Manschik-Lundin, A.; Hurt-Camejo, E.; Rosengren, B.; Olsson, H.; Hansson, G. I.; Forsberg, G.-B.; Ylhen, B. *J. Lipid Res.* **1998**, *39*, 755. (b) Carlsen, C. U.; Moller, J. K. S.; Skibsted, L. H. *Coord. Chem. Rev.* **2005**, *249*, 485.

(6) Okado-Matsumoto, A.; Batinic-Haberle, I.; Fridovich, I. *Free Radical Biol. Med.* **2004**, *37*, 401.

(7) (a) Kasugai, N.; Murase, T.; Ohse, T.; Nagaoka, S.; Kawakami, H.; Kubota, S. *J. Inorg. Biochem.* **2002**, *91*, 349. (b) Spasojevic, I.; Chen, Y.; Noel, T. J.; Fan, P.; Zhang, L.; Rebouras, J. S.; St. Clair, D. K.; Batinic-Haberle, I. *Free Radical Biol. Med.* **2008**, *45*, 943. (c) Mandoj, F.; Nardis, S.; Pomarico, G.; Paolesse, R. *J. Porphyrins Phthalocyanines* **2008**, *12*, 19.

(8) Barni, F.; Lewis, S. W.; Berti, A.; Miskelly, G. M.; Lago, G. *Talanta* **2007**, *72*, 896.

(9) (a) Li, Y.; Zhu, C.; Wang, L.; Li, D.; Xu, J. *Microchim. Acta* **2003**, *143*, 19. (b) Liu, X.; Huang, L.; Baeyens, W. R. G.; Ouyang, J.; He, D.; Wan, G.; Zhang, L. *Electrophoresis* **2009**, *30*, 3034.

(10) (a) Haber, A.; Mahammed, A.; Fuhrman, B.; Volkova, N.; Coleman, R.; Hayek, T.; Aviram, M.; Gross, Z. *Angew. Chem., Int. Ed.* **2008**, *47*, 7896. (b) Haber, A.; Aviram, M.; Gross, Z. *Chem. Sci.* **2011**, *2*, 295.

(11) (a) Reaction volume was 100 μL, so 1000 nM in the calibration curve is translated to 100 pmol per well containing debris of 10^5 cells. (b) 100 pmol/10^5 cells corresponds to 1% of the 100 μM **1-Fe** added to the medium, indicating a specific uptake mechanism that reaches saturation even when excess corrole is in the medium.

(12) Nakano, M.; Kimura, H.; Hara, M.; Kuroiwa, M.; Kato, M.; Totsune, K.; Yoshikawa, T. *Anal. Biochem.* **1990**, *187*, 277.

(13) (a) Sari, M. A.; Battioni, J. P.; Mansuy, D.; Le Pecq, J. B. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 643. (b) Jensen, M. P.; Riley, D. P. *Inorg. Chem.* **2002**, *41*, 4788. (c) Pasternack, R. F.; Skowronek, W. R. *J. Inorg. Biochem.* **1979**, *11*, 261. (d) Rodriguez, M.; Kodadek, T.; Torres, M.; Bard, A. J. *Bioconjug. Chem.* **1990**, *1*, 123.

(14) Fernandez, G.; Spatz, E. S.; Jablecki, C.; Phillips, P. S. *Cleveland Clin. J. Med.* **2011**, *78*, 393.

(15) Martin-Romero, F. J.; Gutiérrez-Martin, Y.; Henao, F.; Gutiérrez-Merino, C. *J. Fluoresc.* **2004**, *14*, 17.

(16) Gieseg, S. P.; Leake, D. S.; Flavall, E. M.; Amit, Z.; Reid, L.; Yang, Y. T. *Front Biosci.* **2009**, *14*, 1230.

(17) (a) Szabó, C.; Day, B. J.; Salzman, A. L. *FEBS Lett.* **1996**, *381*, 82. (b) Assreuy, J.; Cunha, F. Q.; Epperlein, M.; Noronha-Dutra, A.; O'Donnell, C. A.; Liew, F. Y.; Moncada, S. *Eur. J. Immunol.* **1994**, *24*, 672.

(18) In both cases NADPH is activated, forming a high flux of O_2^-, that may dismutate to H_2O_2. In the latter system nitric oxide synthase (NOS) is also activated producing NO, which together with the O_2^- forms peroxynitrite.

(19) (a) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 6053. (b) Schwalbe, M.; Dogutan, D. K.; Stoian, S. A.; Teets, T. S.; Nocera, D. G. *Inorg. Chem.* **2011**, *50*, 1368. (c) Pasternack, R. F.; Halliwell, B. *J. Am. Chem. Soc.* **1979**, *101*, 1026.

(20) (a) Gardner, P. R.; Nguyen, D.-D. H.; White, C. W. *Arch. Biochem. Biophys.* **1996**, *325*, 20. (b) Dwyer, B. E.; Lu, S.-Y.; Laitinen, J. T.; Nishimura, R. N. *J. Neurochem.* **1998**, *71*, 2497. (c) Naunyn–Schmiedeberg's Arch: Klassen, S.; Rabkin, S. *Pharmacology* **2009**, *379*, 149.

(21) (a) Choi, I. Y.; Lee, S. J.; Ju, C.; Nam, W.; Kim, H.-C.; Ko, K. H.; Kim, W.-K. *Glia* **2000**, *31*, 155. (b) Zingarelli, B.; Day, B. J.; Crapo, J. D.; Salzman, A. L.; Szabó, C. *Br. J. Pharmacol.* **1997**, *120*, 259. (c) Trackey, J. L.; Uliasz, T. F.; Hewett, S. J. *J. Neurochem.* **2001**, *79*, 445. (d) Misko, T. P.; Highkin, M. K.; Veenhuizen, A. W.; Manning, P. T.; Stern, M. K.; Currie, M. G.; Salvemini, D. *J. Biol. Chem.* **1998**, *273*, 15646.