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PAPER

Methoxy-derivatization of alkyl chains increases the *in vivo* efficacy of cationic Mn porphyrins. Synthesis, characterization, SOD-like activity, and SOD-deficient *E. coli* study of *meta* Mn(III) *N*-methoxyalkylpyridylporphyrins

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Cationic Mn(III) *N*-alkylpyridylporphyrins (MnPs) are potent SOD mimics and peroxynitrite scavengers and diminish oxidative stress in a variety of animal models of central nervous system (CNS) injuries, cancer, radiation, diabetes, *etc.* Recently, properties other than antioxidant potency, such as lipophilicity, size, shape, and bulkiness, which influence the bioavailability and the toxicity of MnPs, have been addressed as they affect their *in vivo* efficacy and therapeutic utility. Porphyrin bearing longer alkyl substituents at pyridyl ring, MnTnHex-2-PyP^{5+}, is more lipophilic, thus more efficacious *in vivo*, particularly in CNS injuries, than the shorter alkyl-chained analog, MnTE-2-PyP^{5+}. Its enhanced lipophilicity allows it to accumulate in mitochondria (relative to cytosol) and to cross the blood-brain barrier to a much higher extent than MnTE-2-PyP^{5+}. Mn(III) *N*-alkylpyridylporphyrins of longer alkyl chains, however, bear micellar character, and when used at higher levels, become toxic. Recently we showed that *meta* isomers are ~10-fold more lipophilic than *ortho* species, which enhances their cellular accumulation, and thus reportedly compensates for their somewhat inferior SOD-like activity. Herein, we modified the alkyl chains of the lipophilic *meta* compound, MnTnHex-3-PyP^{5+} *via* introduction of a methoxy group, to diminish its toxicity (and/or enhance its efficacy), while maintaining high SOD-like activity and lipophilicity. We compared the lipophilic Mn(III) *meso*-tetrakis(*N*-(6′-methoxyhexyl)-pyridinium-3-yl)porphyrin, MnTMOHex-3-PyP^{5+}, to a hydrophilic Mn(III) *meso*-tetrakis(*N*-(2′-methoxyethyl)pyridinium-3-yl)porphyrin, MnTMOE-3-PyP^{5+}. The compounds were characterized by uv-vis spectroscopy, mass spectrometry, elemental analysis, electrochemistry, and ability to dismute $O_2^{\bullet-}$. Also, the lipophilicity was characterized by thin-layer chromatographic retention factor, R_f. The SOD-like activities and metal-centered reduction potentials for the $Mn^{III}P/Mn^{II}P$ redox couple were similar-to-identical to those of *N*-alkylpyridyl analogs: log $k_{cat} = 6.78$, and $E_{1/2} = +68$ mV *vs.* NHE (MnTMOHex-3-PyP^{5+}), and log $k_{cat} = 6.72$, and $E_{1/2} = +64$ mV *vs.* NHE (MnTMOE-3-PyP^{5+}). The compounds were tested in a superoxide-specific *in vivo* model: aerobic growth of SOD-deficient *E. coli*, JI132. Both MnTMOHex-3-PyP^{5+} and MnTMOE-3-PyP^{5+} were more efficacious than their alkyl analogs. MnTMOE-3-PyP^{5+} is further significantly more efficacious than the most explored compound *in vivo*, MnTE-2-PyP^{5+}. Such a beneficial effect of MnTMOE-3-PyP^{5+} on diminished toxicity, improved efficacy and transport across the cell wall may originate from the favorable interplay of the size, length of pyridyl substituents, rotational flexibility (the *ortho* isomer, MnTE-2-PyP^{5+}, is more rigid, while MnTMOE-3-PyP^{5+} is a more flexible *meta* isomer), bulkiness and presence of oxygen.

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Introduction

Numerous diseases have oxidative stress in common[1] and strategies to reduce it have been aggressively sought. Natural and synthetic antioxidants have been explored. We have recently reviewed the biology, chemistry and medical effects of those synthetic antioxidants, whose at least one mode of action is mimicking superoxide dismutases and which have been explored as potential therapeutics, such as Mn porphyrins, Mn salen derivatives, Mn cyclic polyamines, Mn corroles, nitroxides, Mn complexes with simple ligands, different oxides such OsO_4 and CeO_2, fullerenes, *etc.*[2,3]

Mn porphyrins have been studied as SOD mimics and $ONOO^-$ scavengers for around two decades.[2–5] The cationic β-substituted Mn *N*-alkylpyridylporphyrins are the most potent SOD mimics. The β-octabrominated $MnBr_8TM\text{-}3\text{-}PyP^{4+}$ has k_{cat} close or identical to that of SOD enzymes, log $k_{cat}(O_2^{\bullet-}) \geq 8.85$[6] (log k_{cat}(SOD) 8.84–9.30).[2,4 and ref. therein] Based on the same thermodynamic and kinetic parameters that determine high k_{cat}, Mn(III) *N*-substituted pyridylporphyrins (MnPs) are among the most potent $ONOO^-$ scavengers with log $k_{red}(ONOO^-) > 7$. They are also able to scavenge/reduce $CO_3^{\bullet-}$ and are likely good in reducing HClO also.[4,7]

Due to the rich redox and protolytic equilibria of Mn complexes with electron-deficient porphyrins,[2,4,8] the Mn center can *in vivo* attain several oxidation states, which makes their biology complex and challenging to comprehend. Due to their easy reducibility, and high levels of endogenous reductants (in particular mM cellular concentrations of ascorbate and glutathione), cationic Mn(III) *N*-substituted pyridylporphyrins *in vivo* will likely be readily reduced to Mn(II)Ps.[9–12] Such coupling with reductants would allow them to remove $ONOO^-$ in a catalytic manner. Moreover MnPs can couple with reductants in a removal of $O_2^{\bullet-}$ also, and thus act as superoxide reductases instead of superoxide dismutases.[2–4] Thus, the Mn(III) porphyrin gets reduced to a Mn(II)P in a first step. In a subsequent one- or two-electron reaction, it will reduce either $O_2^{\bullet-}$ or $ONOO^-$, leading to formation of H_2O_2 or benign nitrite NO_2^-, respectively.[13] Importantly, upon reduction, Mn(II) *N*-alkylpyridylporphyrins gain up to 3 orders in lipophilicity,[14] which may account for their high accumulation in critical cellular compartments such as mitochondria and nucleus. Another *in vivo* scenario is the one-electron oxidation of Mn(III)P to a O=Mn(IV)P with $ONOO^-$, $CO_3^{\bullet-}$ or with other highly oxidizing species such as HClO, H_2O_2 or peroxide-derived species. In a subsequent step, O=Mn(IV)P, a highly oxidizing species in its own right, could be removed, *i.e.* reduced with glutathione, ascorbate or uric acid.[10,13,15–19] The nature of the reactive species and the Mn porphyrin redox couples involved would be determined by (1) the redox status of the *in vivo* environment; (2) the redox ability of MnPs; and (3) the extracellular, cellular, and subcellular localization of Mn complexes.

A reduced Mn(II)P was *in vivo* evidenced by uv-vis spectroscopy in the homogenates of *E. coli* treated with a variety of MnPs (both *ortho* and *meta* isomers).[14,20,21] Reduction happened in the absence of exogenous ascorbate, *via* endogenous reductants of *E. coli*. Generally speaking and as noted above, when formed *in vivo*, Mn(II)P species can subsequently scavenge $O_2^{\bullet-}$ or $ONOO^-$,[2,13] thereby producing Mn(III)P and O=Mn(IV)P, respectively. While the involvement of such a Mn(II)P/O=Mn(IV)P redox couple is more probable due to easy reducibility of cationic Mn(III) *N*-alkylpyridylporphyrins, the aqueous chemistry and *in vitro* data also support the possible *in vivo* involvement of a one-electron oxidation with a strong oxidant such as $ONOO^-$ *via* a Mn(III)P/O=Mn(IV)P redox couple.[5,9,10,13,15–17] Reactions of Mn porphyrins with other strong oxidizing species was addressed only marginally, except the reduction of $CO_3^{\bullet-}$ in an aqueous system.[17] HClO reduction by MnPs was studied in aqueous systems only; the porphyrins explored were cationic *para*-porphyrin, $MnTM\text{-}4\text{-}PyP^{5+}$ and anionic porphyrin $MnTBAP^{3-}$.[2,4,22] Based on such data and data on $ONOO^-$ reaction with $MnTM\text{-}4\text{-}PyP^{5+}$,[10] the high reactivity of *ortho* analogs towards HClO is very likely.[7] The chemistry of cationic Mn(III) *N*-alkylpyridylporphyrins with peroxide involving O=Mn(IV)P species, in particular catalase-like activity, has not been explored in detail.[2,4,23–25]

Only porphyrins with a positive $E_{1/2}$ for the Mn(III)P/Mn(II)P redox couple could be reduced easily from Mn(III)P to Mn(II)P with cellular reductants. For example, electron-rich $MnTBAP^{3-}$ ($E_{1/2}$ = –194 mV *vs.* NHE), and $MnTSPP^{3-}$ ($E_{1/2}$ = –160 mV *vs.* NHE), as well as electron-rich corroles whose Mn site is stabilized in the +4 oxidation state relative to porphyrins (due to corrole being a trianionic ligand) cannot be reduced with ascorbate or glutathione to Mn(II) species under physiological conditions. Therefore the scavenging of $ONOO^-$ involves Mn(III) and O=Mn(IV) species with $MnTBAP^{3-}$ and $MnTSPP^{3-}$ and Mn(III) and O=Mn(V) species with corroles.[26,27] Thus far we have evidence that Mn(III)$TBAP^{3-}$ can be reduced to Mn(II)$TBAP^{4-}$ with biologically irrelevant dithionite [Batinic-Haberle *et al.*, unpublished data]. Based on *E. coli* data *para* Mn(III) *N*-alkylpyridylporphyrins are *in vivo* associated/intercalated with nucleic acids, which makes them unavailable to the approach of $O_2^{\bullet-}$;[2,4] therefore it is unlikely that they are involved in any other redox reactions to a large extent.

By removing reactive species Mn porphyrins suppress primary oxidative damage and spare biological molecules. Reactive species are widely recognized as mediators of cellular signaling pathways.[28] Thus, by decreasing the levels of reactive species, MnPs also suppress excessive cellular transcriptional activity, and thus secondary and cycling oxidative stress.[2–5]

In addition to the high antioxidant potency of MnPs (dominated by the proximity of positive charges to the Mn site), lipophilicity, size, bulkiness, rotational flexibility and the shape of a molecule and the nature of substituents also account for the final effect of Mn porphyrins *in vivo*. We are lately exploring the means to improve the bioavailability and diminish the toxicity of MnPs. Both the increase in the length of the alkyl chain for each carbon atom, and the shift of alkyl chains of the same length from the *ortho* to the *meta* positions of the pyridyl groups increase the lipophilicity by 10-fold.[20,21] We reported that increased lipophilicity of the *meta* isomer, $MnTE\text{-}3\text{-}PyP^{5+}$, can compensate for its lower antioxidant potency relative to *ortho* $MnTE\text{-}2\text{-}PyP^{5+}$; in turn they were equally effective in protecting SOD-deficient *E. coli* when growing aerobically. Thus both *ortho* and *meta* isomers possess therapeutic potential. Importantly, the synthesis of *meta* compounds is more facile than of *ortho* analogs.

With very lipophilic longer-chained *N*-alkylpyridylporphyrins we targeted CNS injuries in particular, trying to improve their transport across the blood-brain barrier (BBB). Indeed $MnTnHex\text{-}2\text{-}PyP^{5+}$ is up to 120-fold more efficacious and crosses

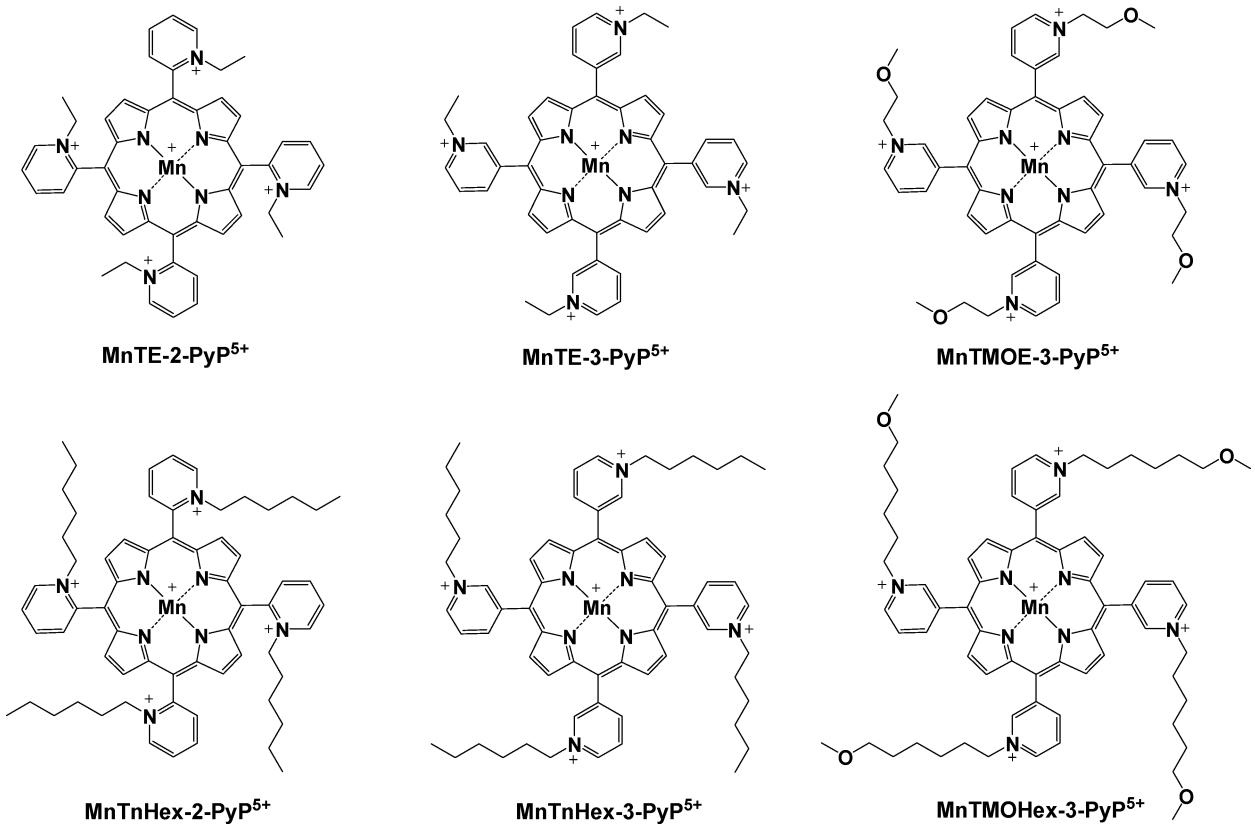

Fig. 1 Mn porphyrins studied: MnTE-2-PyP^{5+}, MnTE-3-PyP^{5+}, MnTMOE-3-PyP^{5+}, MnTnHex-2-PyP^{5+}, MnTHex-3-PyP^{5+}, and MnTMOHex-3-PyP^{5+}.

the BBB 12-fold more than MnTE-2-PyP^{5+}.[29] Importantly it also accumulates in mitochondria relative to cytosol, and at a much higher extent than MnTE-2-PyP^{5+}.[30–32] However, it possesses a surfactant nature and may damage cellular walls/membranes at higher concentrations. We thus sought to diminish its toxicity while maintaining high SOD-like activity. Any modification of the porphyrin structure should keep cationic charges preserved as they dominate the kinetics and thermodynamics of the reaction of MnPs with negatively charged reactive species, such as $O_2^{\bullet-}$, $ONOO^-$, ClO^-, $CO_3^{\bullet-}$ *etc.*[2,4] We herein synthesized the Mn(III) *N*-methoxyalkylpyridylporphyrins; cationic charges were preserved, and thus also SOD-like activity (Fig. 1). *Via* methoxy group insertion into the pyridyl substituents we aimed at diminishing the porphyrin toxicity, and therefore increasing its *in vivo* efficacy.

Experimental

Materials

H_2T-3-PyP was purchased from Frontier Scientific. *n*-Hexyl *p*-toluenesulfonate (>98%) and 2-methoxyethyl *p*-toluenesulfonate (>98%) were from TCI America. Pyridine (99%), tetrabutylammonium chloride hydrate (98%), Na_2SO_4 anhydrous (99%), heptafluorobutyric acid (98%) and 1,6-dibromohexane (96%) were from Aldrich. $MnCl_2{\cdot}4H_2O$ (99.7%) and hexane were supplied by J. T. Baker. NH_4PF_6 (99.99% pure) was from GFS chemicals. Diethyl ether anhydrous and acetone were from EMD chemicals, while absolute methanol, ethyl acetate, dichloromethane, chloroform, acetonitrile, EDTA and KNO_3 were purchased from Mallinckrodt. *N*,*N*-Dimethylformamide anhydrous of 99.8% purity (kept over 4 Å molecular sieves) and plastic-backed silica gel TLC plates (Z122777-25EA) were from Sigma-Aldrich. Silica (Siliaflash® G60, 70-230 mesh) was purchased from Silicycle (Canada). Xanthine and equine ferricytochrome *c* (Lot 7752) were from Sigma, whereas xanthine oxidase was prepared by R. Wiley.[33] All chemicals were used as received without further purification. (+)-Sodium L-ascorbate (>98%) was from Sigma.

Synthesis

The general synthetic procedure for *meso*-tetra(*N*-methoxyalkylpyridinium-3-yl)porphyrins and their manganese complexes is shown in Fig. 2. The 2-methoxyethyl *p*-toluenesulfonate and 1-bromo-6-methoxyhexane were used to synthesize cationic *meso* *N*-substituted porphyrins.

H_2TMOE-3-PyPCl$_4$. To a solution of H_2T-3-PyP (100 mg, 0.147 mmol) in anhydrous DMF (10 mL, preheated at ~100 °C for 10 min), 2-methoxyethyl *p*-toluenesulfonate (13.96 mL, 0.073 mol) was added. *N*-Alkylation of H_2T-3-PyP proceeded smoothly and it was completed in 3.5 h. The completion of the reaction was determined by the observation of a single spot on silica gel TLC plates using 1 : 1 : 8 = $KNO_{3(sat)}$: H_2O : acetonitrile (AN) as a mobile phase. The reaction mixture was filtered into the separatory funnel containing H_2O and chloroform and extracted several times with chloroform. The water layer was then filtered over filter paper into the glass flask where it was precipitated as a PF_6^- salt by the addition of solid NH_4PF_6. The losses during the precipitation were insignificant. The precipitate was filtered off and washed

Table 1 The spectral properties of metal-free ligands and their Mn complexes

Porphyrin	λ_{max}/nm (log ε)[a]
H_2TMOE-3-PyPCl$_4$	263.0 (4.36), 418.0 (5.49), 515.0 (4.23), 582.0 (3.79)
MnTMOE-3-PyPCl$_5$	213.5 (4.71), 261.5 (4.56), 373.5 (4.69), 396.0 (4.67), 460.0 (5.16), 502.0 (3.82), 557.5 (4.13), 674.0 (3.20), 767.0 (3.36)
H_2TMOHex-3-PyPCl$_4$	263.0 (4.36), 417.5 (5.49), 514.0 (4.24), 581.0 (3.86), 634.0 (3.23)
MnTMOHex-3-PyPCl$_5$	214.0 (4.75), 261.0 (4.55), 373.0 (4.69), 395.5 (4.68), 460.0 (5.15), 500.0 (3.88), 557.0 (4.13), 675.5 (3.21), 767.0 (3.36)

[a] Spectra were taken in water at room temperature. Molar absorption coefficients (M^{-1} cm^{-1}) were determined within 5% errors. λ_{max} (nm) were determined with errors inside ± 0.5 nm.

Table 2 Electrospray ionization mass spectrometry (ESI-MS) data for *N*-methoxyalkylpyridylporphyrins and their Mn complexes

	m/*z* [found (calculated)]			
Species[a]	H_2TMOE-3-PyP^{4+}	MnTMOE-3-PyP^{5+}	H_2TMOHex-3-PyP^{4+}	MnTMOHex-3-PyP^{5+}
$[P^{n+} + HFBA^-]^{(n-1)+}/(n-1)$	355.9 (355.8)	280.3 (280.1)	430.9 (430.6)	336.4 (336.1)
$[P^{n+} + 2HFBA^-]^{(n-2)+}/(n-2)$	640.3 (640.2)	444.7 (444.4)	752.5 (752.3)	519.4 (519.2)
$[P^{n+} + 3HFBA^-]^{(n-3)+}/(n-3)$	—	773.1 (773.1)	—	885.2 (885.2)
$P^{4+}/4$	213.9 (213.6)	—	270.1 (269.7)	—
$[P^{4+} - H^+]^{3+}/3$	284.6 (284.5)	—	359.4 (359.2)	—

[a] ~1 μM solution of porphyrins and metalloporphyrins in 1 : 1 v/v acetonitrile: H_2O (containing 0.01% v/v heptafluorobutyric acid (HFBA)) mixture, 20 V cone voltage; n = 4 or 5 corresponding to ligands and MnPs, respectively. P stands for both MnPs and ligands.

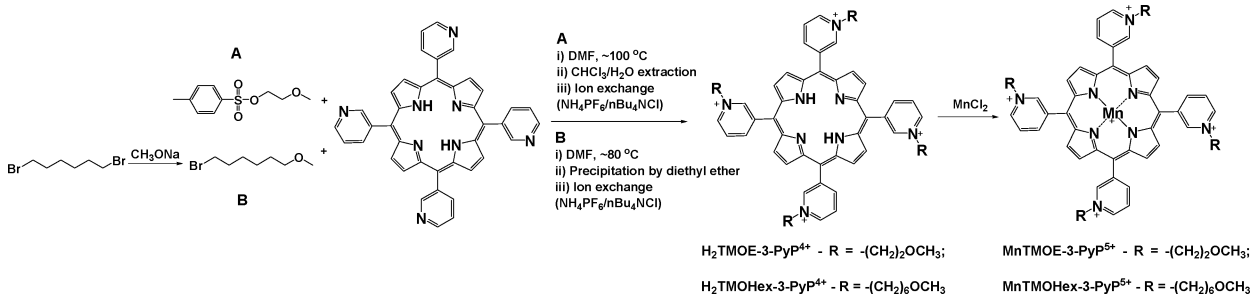

Fig. 2 Synthesis of *meta N*-methoxyalkylpyridylporphyrins (H_2TMOE-3-PyP^{4+} and H_2TMOHex-3-PyP^{4+}) and their Mn complexes (MnTMOE-3-PyP^{5+} and MnTMOHex-3-PyP^{5+}).

thoroughly with anhydrous diethyl ether (300 mL in total). The dried precipitate was dissolved in acetone and precipitated as a chloride salt by the addition of a saturated acetone solution of tetrabutylammonium chloride. The precipitate was washed thoroughly with acetone (500 mL in total). Yield (calculated as a chloride salt): 137 mg (93.8%). The uv-vis and ESI-MS data are summarized in Tables 1 and 2, respectively.

MnTMOE-3-PyPCl$_5$. The aqueous solution of H_2TMOE-3-PyPCl$_4$ (2.4 mM, 60 mL) was basified with 1 M NaOH to pH ~11. Then $MnCl_2 \times 4H_2O$ was added (20-fold excess, 2.752 mmol, 0.542 g), which resulted in the pH drop of the solution to ~8.4. The reaction mixture was stirred overnight at room temperature. The course of metallation was monitored by thin-layer chromatography on silica gel TLC plates using 1 : 1 : 8 = $KNO_{3(sat)}$: H_2O : ANacetonitrile as a mobile phase as well as by uv/vis spectroscopy and fluorescence measurements (on the presence of fluorescent metal-free ligand) employing long wavelength UV lamp (~350 nm). After completion of the metallation, the Mn oxo/hydroxo complexes were filtered over coarse and then smooth filter paper and porphyrin was precipitated as a PF_6^- salt by the addition of solid NH_4PF_6. Similar to the ligand, MnTMOE-3-PyP(PF$_6$)$_5$ was not soluble in water, so the losses were insignificant. The precipitate was filtered off and washed thoroughly with anhydrous diethyl ether (350 mL in total). The dried precipitate was dissolved in acetone (80 mL) and precipitated as a chloride salt by the addition of a saturated acetone solution of tetrabutylammonium chloride. The precipitate was washed thoroughly with acetone (500 mL in total) and dissolved in H_2O (30 mL) and the whole precipitation procedure was repeated. The product was dried *in vacuo*, at room temperature. Yield (calculated as a chloride salt): 0.133 g (89.1%). The uv-vis, ESI-MS, electrochemistry and $k_{cat}(O_2^{\bullet -})$ data are summarized in Tables 1, 2 and 3, respectively.

1-Bromo-6-methoxyhexane. A solution of sodium methanolate (13.5 g, 0.25 mol) in anhydrous methanol (75 mL) was added over a five-hour period to a stirred solution of 1,6-dibromohexane (61 g, 0.25 mol) in anhydrous diethyl ether (25 mL), and refluxed for 2 days (the solution of sodium methanolate was prepared by a dropwise addition of a 10-fold molar excess of methanol over sodium filings).[34] The reaction was monitored by TLC (dichloromethane as a mobile phase). Precipitated NaBr was filtered off and the solution poured into the water (200 ml). The organic layer was separated and aqueous layer was washed with diethyl ether (3 × 100 ml). The organic

Table 3 Lipophilicity (R_f) of MnPs and their ligands, and metal-centered reduction potential $E_{1/2}$ *vs.* NHE (for $Mn^{III}P/Mn^{II}P$ redox couple), and log k_{cat} for $O_2^{\bullet-}$ dismutation of MnPs

Porphyrin	R_f[a]	$E_{1/2}$[b]/mV *vs.* NHE	log k_{cat}[c]
H_2TE-2-PyP^{4+}	0.12		
MnTE-2-PyP^{5+}	0.06	+228[38]	7.76[20]
H_2TE-3-PyP^{4+}	0.22		
MnTE-3-PyP^{5+}	0.09	+54[20,21]	6.65[20,21]
H_2TMOE-3-PyP^{4+}	0.27		
MnTMOE-3-PyP^{5+}	0.12	+64	6.72
H_2TnHex-2-PyP^{4+}	0.45		
MnTnHex-2-PyP^{5+}	0.43	+314[35]	7.48[20]
H_2TnHex-3-PyP^{4+}	0.54		
MnTnHex-3-PyP^{5+}	0.48	+64[20,21]	6.64[20,21]
H_2TMOHex-3-PyP^{4+}	0.42		
MnTMOHex-3-PyP^{5+}	0.39	+68	6.78
MnTnBu-3-PyP^{5+}	0.34	+64[21]	6.69[21]
SOD enzymes		~+300[49]	8.84–9.30[2,4,50]

[a] R_f (compound path/solvent path) on the silica gel TLC plates (1 : 1 : 8 = $KNO_{3(sat)}$: H_2O : AN as a mobile phase). [b] $E_{1/2}$ is determined in 0.05 M phosphate buffer (pH 7.8, 0.1 M NaCl). [c] k_{cat} is determined by cytochrome *c* assay in 0.05 M potassium phosphate buffer (pH 7.8, at 25 ± 1 °C).

layers were combined and washed with water (3 × 50 ml), dried (Na_2SO_4 anhydrous) and filtered. The solvent was removed under reduced pressure. The obtained oil was purified by column chromatography on silica gel using CH_2Cl_2 as eluent. Obtained pure product was a colorless liquid. Yield: 23 g (47.2%). ^{1}H-NMR ($CDCl_3$): δ = 1.40, 1.48, 1.59, 1.87 (4m; 8H, Br–CH_2–(CH_2)$_4$–), 3.33 (s; 3H, –O–CH_3), 3.37 (t; 2H, –O–CH_2–), 3.41 (t; 2H, –CH_2–Br). ^{13}C-NMR ($CDCl_3$): δ = 25.472 (Br–(CH_2)$_3$–CH_2–), 28.104 (Br–(CH_2)$_2$–CH_2–), 29.58 (O–CH_2–CH_2–), 32.88 (Br–CH_2–CH_2–), 33.715 (–CH_2–Br), 58.508 (–O–CH_3), 72.635 (–O–CH_2–).

H_2TMOHex-3-PyPCl$_4$. To the solution of H_2T-3-PyP (50 mg, 0.080 mmol) in anhydrous DMF (10 mL, preheated for 10 min at ~80 °C), 1-bromo-6-methoxyhexane (1.08 g, 5.54 mmol) was added. After 5 h, again 1.08 g of 1-bromo-6-methoxyhexane was added. The completion of the reaction (5 days overall) was followed by thin-layer chromatography, until one spot (tetra-quaternized product) appeared on silica gel TLC plates using 1 : 1 : 8 $KNO_{3(sat)}$: H_2O : AN as a mobile phase. The porphyrin was then precipitated by diethyl ether, filtered and washed with diethyl ether. Obtained precipitate was dissolved in water. The purification and isolation of H_2TMOHex-3-PyPCl$_4$ was done as described for H_2TMOE-3-PyPCl$_4$. Yield (calculated as a chloride salt): 92 mg (93.2%). The uv-vis and ESI-MS data are summarized in Tables 1 and 2, respectively.

MnTMOHex-3-PyPCl$_5$. To the aqueous solution of H_2TMOHex-3-PyPCl$_4$ (50 mg, 2.7 mM) 20-fold excess of $MnCl_2$·$4H_2O$ (243 mg, 1.23 mmol) was added. The solution was stirred for 24 h at ~100 °C. The metallation under basic conditions (at pH ~11) was avoided as the hydrolysis leads to the formation of species with 6-hydroxyhexyl chains. The course of metallation was monitored by thin-layer chromatography, as well as by uv/vis spectroscopy and fluorescence measurements. After completion of the metallation, the Mn oxo/hydroxo complexes were filtrated over the smooth filter paper. The purification and isolation of MnTMOHex-3-PyPCl$_5$ was done as described for MnTMOE-3-PyPCl$_5$. Yield (calculated as a chloride salt): 50 mg (93.3%). The uv-vis, ESI-MS, electrochemistry and $k_{cat}(O_2^{\bullet-})$ data are summarized in Tables 1, 2 and 3 respectively.

H_2TE-2-PyPCl$_4$, H_2TE-3-PyPCl$_4$, H_2TnHex-2-PyPCl$_4$, H_2TnHex-3-PyPCl$_4$. The synthesis and characterization of these porphyrins and their corresponding Mn complexes (MnTE-2-PyPCl$_5$, MnTE-3-PyPCl$_5$, MnTnHex-2-PyPCl$_5$, MnTnHex-3-PyPCl$_5$) were described earlier.[20,21,35]

Elemental analysis

Elemental analyses of the porphyrins and their Mn complexes were done by Atlantic MicroLab (Norcross, GA, USA).

H_2TMOE-3-PyPCl$_4$·7H_2O: Anal. Calcd for $C_{52}H_{68}Cl_4N_8O_{11}$: C, 55.62; H, 6.1; N, 9.98; Cl, 12.63%. Found: C, 55.2; H, 5.75; N, 10.14; Cl, 12.54%. **MnTMOE-3-PyPCl$_5$·12H_2O·0.3NH_4Cl**: Anal. Calcd for $C_{52}H_{77.2}Cl_{5.3}N_{8.3}O_{16}Mn$: C, 47.41; H, 5.91; N, 8.82; Cl, 14.26%. Found: C, 47.00; H, 5.24; N, 8.76; Cl, 14.01%. **H_2TMOHex-3-PyPCl$_4$·10.5H_2O·0.25NH_4Cl**: Anal. Calcd for $C_{68}H_{112}Cl_{4.75}N_{8.75}O_{15.5}$: C, 57.36; H, 7.65; N, 8.12; Cl, 10.58%. Found: C, 57.47; H, 7.55; N, 8.12; Cl, 10.08%. **MnTMOHex-3-PyPCl$_5$·12H_2O·0.5NH_4Cl**: Anal. Calcd for $C_{68}H_{110}Cl_{5.5}N_{8.5}O_{16}Mn$: C, 52.60; H, 7.14; N, 7.67; Cl, 12.56%. Found: C, 52.44; H, 7.03; N, 7.68; Cl, 12.52%.

Uv-vis spectroscopy

Uv-vis spectra were recorded in H_2O at room temperature on UV-2501PC Shimadzu spectrophotometer with 0.5 nm resolution.

Stability of MnPs towards oxidative degradation

In the presence of ascorbate (0.42 mM), and at pH 7.8 maintained by potassium phosphate buffer, MnPs (6 μM) get reduced and ascorbyl radical formed. During the oxidation of the ascorbyl radical to dehydroascorbate, $O_2^{\bullet-}$ and eventually H_2O_2 are formed also. In a subsequent step, MnPs reduce oxygen (bound or unbound on the metal site) whereby $O_2^{\bullet-}$ is formed. $O_2^{\bullet-}$ then dismutes to H_2O_2, which eventually oxidatively destroys porphyrin ring.[36,37] The same spectral change observed with ascorbate or H_2O_2 was discussed previously,[23,38,39] undoubtedly indicating the peroxide as a cause of porphyrin oxidative degradation.

Electrochemistry

Cyclic voltammetry was performed on CH Instruments model 600 voltammetric analyzer using three-electrode system: glassy carbon button working electrode (3 mm diameter, Bioanalytical Systems), Ag | AgCl reference electrode and a Pt wire (0.5 mm) as an auxiliary electrode.[38,40] All measurements were done in small volume cell (3 mL) containing solutions of ~0.5 mM MnP, 0.1 M NaCl, 0.05 M potassium phosphate buffer, pH 7.8. The scan rates were 0.01 and 0.1 V s^{-1}. We originally used ferrocyanide/ferricyanide couple as well as ferrocenemethanol and ferrocene systems for calibration in order to determine the $E_{1/2}$ for MnTE-2-PyP^{5+}. Regardless of the system used for calibration we obtained the same $E_{1/2}$.[38,40,41] Thus we are now routinely using MnTE-2-PyP^{5+} as standard for all our electrochemical determinations. On any given day the electrode system is calibrated with MnTE-2-PyP^{5+} to assure the reliability and reproducibility of our measurements.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometric (ESI-MS) analysis was performed as described elsewhere[42] on Applied Biosystems MDS Sciex 3200 Q Trap LC/MS/MS spectrometer at Duke Comprehensive Cancer Center, Clinical Pharmacology Laboratory. All samples of 1 μM concentrations were prepared in acetonitrile–H_2O (1 : 1, v/v; containing 0.01% v/v HFBA) mixture and infused for 1 min at 10 μl min^{-1} into the spectrometer (curtain gas 20 V, ion spray voltage 3500 V, ion source 30 V, t = 300 °C, declustering potential 20 V, entrance potential 1 V, collision energy 5 V, gas N_2).[20] In the presence of soft anion, heptafluorobutyric acid, which pairs with cationic porphyrin, no fragmentation of the porphyrin has been detected. Thus any partially quaternized porphyrin found in the mass spectrum is the consequence of partial alkylation or methoxyalkylation, but not of fragmentation during spectral analysis.[42]

Catalysis of $O_2^{\bullet-}$ dismutation (cytochrome *c* assay)

The ability of newly synthesized MnPs to dismute $O_2^{\bullet-}$ was evaluated *via* cytochrome *c* assay which was proven to be equally effective as pulse radiolysis and stopped-flow methodology.[2,4,43,44–46] The cyt *c* assay is based on $O_2^{\bullet-}$ production *via* xanthine/xanthine oxidase reaction and MnPs ability to compete with ferricytochrome *c* in scavenging $O_2^{\bullet-}$. The experiments were conducted at room temperature (25 ± 1 °C) in 0.05 M potassium phosphate buffer, pH 7.8, 0.1 mM EDTA as previously described in detail.[44] The reduction of cytochrome *c* was followed at 550 nm. As described above with voltammetric measurements, similar care is taken with the cyt *c* assay, where we always measure the SOD activity of the compound of interest side-by-side with that of MnTE-2-PyP^{5+} (using same buffers, cuvettes, *etc.*). This double checking of the data has been routinely done (often exhaustively) in our laboratories for more than 15 years, to avoid any variability associated with the operator or the quality of the reagents used in the assay; obviously no doubtful values are reported.

Thin-layer chromatography

The chromatographic retention factor, R_f (compound path/solvent path), was determined on silica gel plates using 1 : 1 : 8 = $KNO_{3(sat)}$: H_2O : AN as a mobile phase for the porphyrins and their respective Mn complexes (Table 3) and compared with those of *N*-ethyl and *N*-hexyl analogs. It is extremely difficult-to-impossible to reproduce the R_f values from one experiment to another. Thus, for unambiguity of conclusions the best approach is to compare all the compounds of interest in a single experiment.

E. coli study

E. coli study was performed as previously described in detail.[4,20] *Escherichia coli* strains used in this study were AB1157, wild type (*F-thr-1*; *leuB6*; *proA2*; *his-4*; *thi-1*; *argE2*; *lacY1*; *galK2*; *rpsL*; *supE44*; *ara-14*; *xyl-15*; *mtl-1*; *tsx-33*), and JI132, SOD-deficient, $sodA^-sodB^-$ (same as AB1157 plus (*sodA*::*mudPR13*)*25* (*sodB-kan*)*1*-Δ*2*). 5 independent experiments were performed. In each experiment all compounds were tested in triplicate. The cultures were grown aerobically in minimal 5 amino acid (L-leucine, L-threonine, L-proline, L-arginine, L-histidine) medium in 96-well plates in a shaking thermostat at 37 ± 0.1 °C and 200 rpm. The effect of MnPs on the growth of the SOD-deficient strains was followed turbidimetrically at 600 nm and compared to the growth curves of both strains in the absence of MnPs (controls). Deionized water was used throughout the study.

Results and Discussion

Synthesis

In this study, *meta* Mn(III) *N*-methoxyalkylpyridylporphyrins (alkyl being ethyl or hexyl) were synthesized, and their thermodynamic and kinetic parameters determined and compared to those of Mn(III) *N*-alkylpyridylporphyrins. Mn(III) porphyrins were synthesized starting from the commercially available non-quaternized porphyrins, employing either commercially available 2-methoxyethyl *p*-toluenesulfonate (MnTMOE-3-PyP^{5+}) or 1-bromo-6-methoxyhexane (MnTMOHex-3-PyP^{5+}); the latter compound was prepared by a modified method[34] (Fig. 2). 1-Bromo-6-methoxyhexane was used as a quaternizing agent because its synthesis is more facile (one step less, thus easier purification), than is the synthesis of 6-methoxyhexyl *p*-toluenesulfonate. The preparation of 6-methoxyhexyl *p*-toluenesulfonate includes the synthesis of a respective alcohol, 6-methoxyhexane-1-ol and its subsequent reaction with *p*-toluenesulfonyl chloride. The 1–5% of the impurity (side product) obtained in a final quaternized product, when 1-bromo-6-methoxyhexane was used, and identified by mass spectrometry to be a species with three methoxyalkyl groups and one methyl group attached to the pyridyl nitrogens, is present at much higher level in *ortho* compounds, H_2TMOE-2-PyP^{4+} and H_2TMOHex-2-PyP^{4+}. To minimize the formation of such side products in H_2TMOHex-3-PyP^{4+}, the quaternization with 1-bromo-6-methoxyhexane was performed at 80 °C rather than at 100 °C. The mechanistic aspects of the quaternization of methoxy-derivatized porphyrins will be discussed in a subsequent publication (Rajic *et al.*, in preparation). The *meta* Mn(III) *N*-alkylpyridylporhyrins are 10-fold more lipophilic than analogous *ortho* isomers. We already showed that the higher lipophilicity of *meta* MnTE-3-PyP^{5+} than of *ortho* MnTE-2-PyP^{5+} compensates for its somewhat inferior SOD-like activity.[20,21] In turn both isomers appear equally efficacious *in vivo*. For such reasons, and because of more facile synthesis leading to a purer product, *meta* rather than *ortho* *N*-methoxyalkylpyridylporphyrins were explored herein.

Characterization

The compounds were characterized in terms of uv/vis spectral properties (Table 1), elemental analysis, electrochemistry (metal-centered reduction potential $E_{1/2}$ for $Mn^{III}P/Mn^{II}P$ redox couple) (Table 3), ability to catalyze $O_2^{\bullet-}$ dismutation, k_{cat} (Table 3) and ESI-MS (Table 2). All properties are dominated by cationic charges on pyridyl nitrogens; thus the data for *N*-methoxyalkyl- and corresponding *N*-alkylpyridylporphyrins are very similar.

Stability of MnPs towards oxidative degradation by ascorbate

The study aimed to compare *ortho* to *meta* MnPs, and MnPs with and without oxygen-bearing alkyl chains, as well as those MnPs of different reducibility ($E_{1/2}$) with respect to their stability

towards oxidative degradation by biologically relevant reductant, ascorbate. Time-dependent decrease in the concentration of six MnPs (from Fig. 1) in the presence of ascorbate at pH 7.8 is shown in the Fig. 3. Three trends were observed. (1) The *ortho* isomers (MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+}) are initially (within 1.5 h) more resistant to degradation than the corresponding *meta* analogs (MnTE-3-PyP^{5+}, MnTnHex-3-PyP^{5+}, MnTMOE-3-PyP^{5+}, MnTMOHex-3-PyP^{5+}). Once an excessive amount of H_2O_2 gets produced, the degradation of most resistant *ortho* MnPs accelerates. *Meta* porphyrins, having less positive $E_{1/2}$, are more stable in the +3 state relative to *ortho* analogs. Thus they are more readily oxidized back from +2 to +3 oxidation state with oxygen bound or unbound to Mn site, while producing $O_2^{\bullet-}$ and H_2O_2 which in turn oxidizes porphyrin.[23,25,47] (2) The longer the pyridyl chains of *ortho* analogs (but not of *meta*), the more stable the Mn porphyrin is. Herein, MnTnHex-2-PyP^{5+} was the most stable compound towards oxidative degradation (Fig. 3B). With ~100 mV more positive $E_{1/2}$ relative to *ortho* ethyl species, MnTnHex-2-PyP^{5+} is stabilized in +2 oxidation state; additionally the longer hexyl chains may afford steric protection of Mn site towards oxidation. (3) The introduction of oxygen into the pyridyl chains has no effect on the stability of MnPs. Spectral changes during oxidative degradation of *ortho* isomers have been reported.[23,25,38] The reaction mechanism for MnP/ascorbate system is complicated involving at least several ascorbate and MnP species, and gets more complex *in vivo*.[48] Our data along with those already published[23–25] imply that under such conditions at least one possible scenario *in vivo* involves the ascorbate-driven oxygen consumption catalyzed by MnPs, leading to superoxide and peroxide formation, which in turn degrade MnPs; the spectral changes are the same when MnPs were exposed to either H_2O_2 or to ascorbate; ascorbate/O_2 system yields H_2O_2 *in situ*.[23–25]

The *in vivo* systems are complex, as is the chemistry, biology and tissue and cellular distribution of MnPs. We are still at the infancy of understanding the role of all reaction species that may be involved and their interplay *in vivo* and as of now may only speculate on the nature of a wide range of possible reactions of MnPs. The mere fact that MnTE-2-PyP^{5+} and similar compounds oxidize and reduce $O_2^{\bullet-}$ with similar rate constants[9] makes them prone to remove and produce reactive species (as by-products, such as H_2O_2), which makes the studies of their *in vivo* effects even more challenging. We detailed such thoughts in recent reviews and papers.[2,14,24,48]

Lipophilicity of Mn(III) *N*-methoxyalkyl- *vs.* *N*-alkylpyridylporphyrins

For years we had no means to access log P_{OW} in order to provide the tool to compare the lipophilicity of MnPs to lipophilicity of other drugs of similar targets. We then developed a methodology to determine the log P_{OW} values, and showed that there is a linear relationship between R_f and log P_{OW}.[20] Such relationship justified the use of R_f values which could be more conveniently determined experimentally than log P_{OW}.[20] The small differences in R_f values translate into large differences in log P_{OW} values.[20] To assure the accuracy and account for the variability of silica gel plates and solvent saturation of the TLC chambers, we routinely compare R_f of new compounds to old compounds of relevance for the present study. We recently discussed the lipophilicity of reduced Mn(II)Ps in terms of both log P_{OW} and R_f values.[14]

The lipophilicity of compounds studied are shown in Fig. 3 in three different ways: (1) to indicate the differences between ligands and their Mn complexes; (2) to indicate the change in lipophilicity

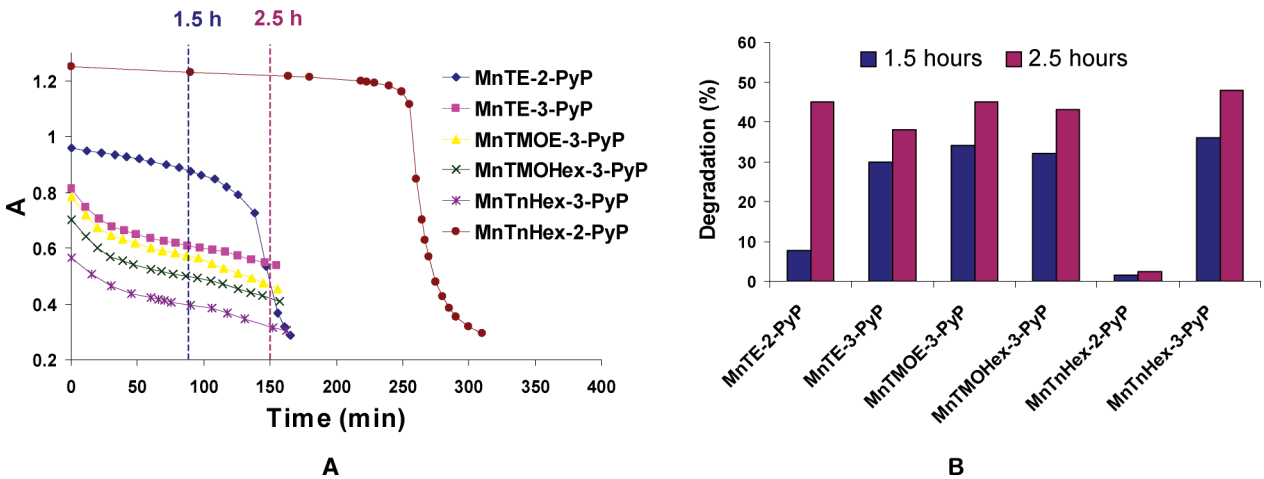

Fig. 3 Comparison of the stability of MnPs towards oxidative degradation with H_2O_2. Peroxide was produced in the interaction of MnPs with ascorbate.[23,25,47] The conditions were: 6 μM MnPs, 0.42 mM sodium ascorbate, pH 7.8 (Fig. 3A). The reduction of *ortho* MnPs, *i.e.* shift in Soret band, was observed upon the addition of ascorbate. The absorbance, *A*, of reduced Mn^{III}TE-2-PyP^{4+}and Mn^{III}TnHex-2-PyP^{4+} was measured at 437 and 439 nm, respectively. With *meta* isomers, at the time scale of our spectrophotometer, only species with Mn in +3 oxidation state were observed, and their disappearance followed at 460 nm. At higher concentrations, *i.e.* 30 μM MnP and 3.3 mM ascorbate, the reduced species and its disappearance was visible (not shown). Fig. 3B clearly indicates the enhanced stability of MnTnHex-2-PyP^{5+} relative to its *meta* analog, MnTnHex-3-PyP^{5+} and shorter-chained ethyl species, MnTE-2-PyP^{5+}, and much more so within a 2.5 h time frame and under the conditions indicated. A sharp drop in absorbance likely coincides with accumulated H_2O_2 needed to degrade more resistant *ortho* porphyrins. As seen from a shorter time scale of 1.5 h (Fig. 3A), *meta* porphyrins are more prone to oxidative degradation than *ortho*. Charges on porphyrins are omitted for simplicity.

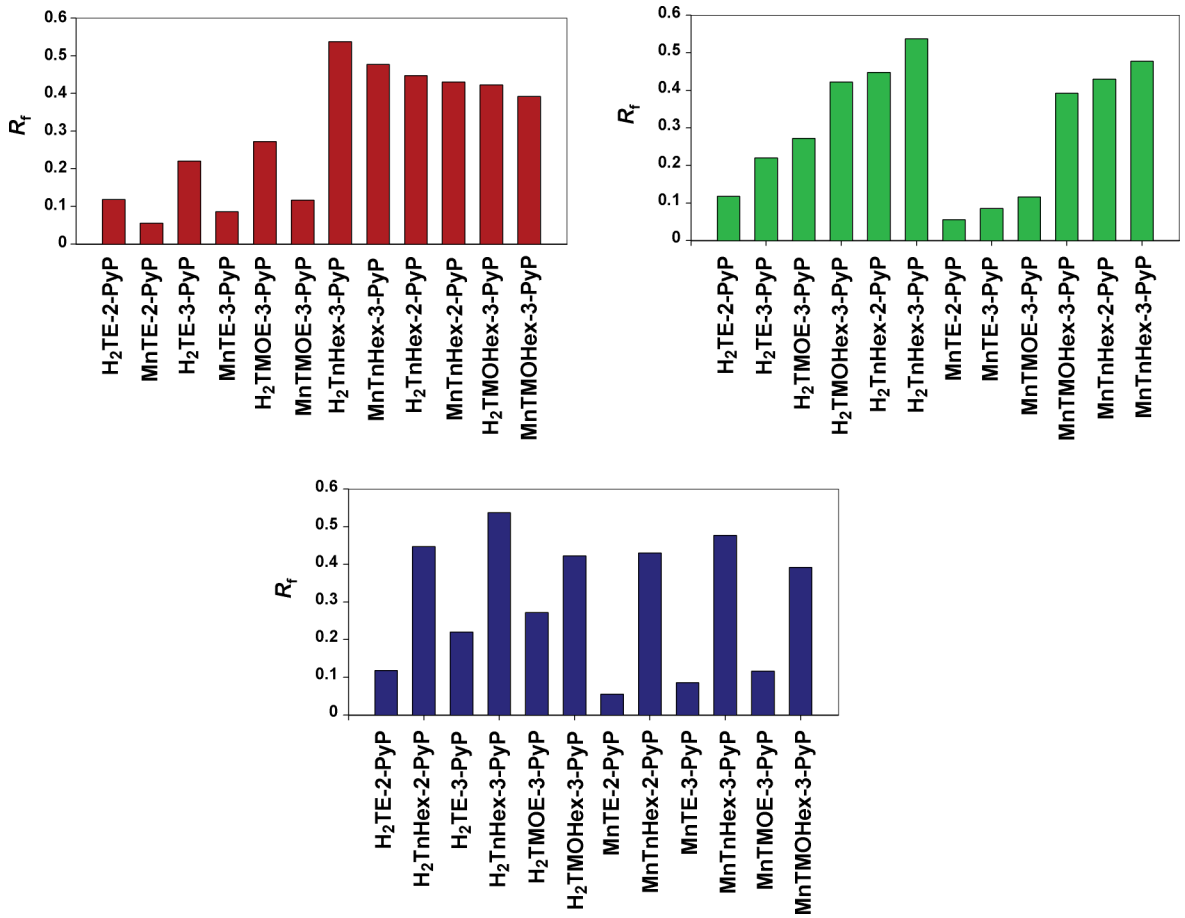

Fig. 4 The lipophilicity of metal-free porphyrins and their ligands expressed here in terms of chromatographic retention factor, R_f. The R_f values are linearly related to log P_{OW} values.[20] The small differences in R_f values translate into large differences in log P_{OW} values.[20] Lipophilicity of Mn complexes is lower than of their metal-free ligands due to higher solvation of the metal site. The effect is more drastic with porphyrins bearing shorter substituents. Longer-chained analogs, alkyl and methoxyalkyl are more lipophilic than their shorter-chained analogs. Introduction of a methoxy group reduces lipophilicity of longer alkyl analog, H_2TnHex-3-PyP^{4+} and its Mn complex. Charges on porphyrins are omitted for simplicity.

of alkyl- *vs.* methoxyalkylpyridylporphyrins; and (3) to indicate the changes going from shorter to longer chains. The numerical data are shown in Table 3.

As expected and indicated in Fig. 4, the introduction of a methoxy group into the hexyl alkyl chains reduces the lipophilicity of MnTnHex-3-PyP^{5+}. However, MnTMOE-3-PyP^{5+} was slightly more lipophilic than its ethyl analog, MnTE-3-PyP^{5+}; the proximity of oxygen electron pairs to pyridyl nitrogens and steric hindrance provided by longer methoxyethyl *vs.* shorter ethyl chains might have diminished/hindered the cationic charges on nitrogens.

A huge drop in lipophilicity was seen upon metallation of porphyrins bearing shorter chains (ethyl and methoxyethyl analogs). A smaller change was observed with porphyrins having longer chains (hexyl and methoxyhexyl porphyrins) where solvent molecules no longer distinguish between the species having protonated (metal-free ligands) and metallated inner pyrrolic nitrogens (Fig. 4). A similar observation has been made with the lipophilicity of Mn(III) and reduced Mn(II) *meso*-tetrakis(*N*-alkylpyridylporphyrins).[14] As the length of the alkyl chain increases beyond butyl, the difference between Mn(III)Ps and Mn(II)P decreases; the impact of a single charge residing on a Mn site of Mn(III)Ps becomes insignificant.[14]

E. coli study

The *meta* Mn(III) *N*-alkylpyridylporphyrins and their methoxyalkyl analogs were studied in *E. coli* model and compared to analogous *ortho* compounds, MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+}. The growth was followed in restricted five amino acid medium where the ability of MnPs to substitute for SOD deficiency is more critical than in M9 casamino acid medium. Thus, the SOD-deficient mutant grows poorly in five amino acid medium, containing only arginine, histidine, leucine, proline and threonine, since synthesis of sulfur-containing and branched amino acids requires the action of $O_2^{\bullet-}$-sensitive enzymes.[4] We have modified *meta* Mn(III) *N*-alkylpyridylporphyrins introducing methoxy groups into pyridyl chains. Such modifications led to more efficacious compounds MnTMOE-3-PyP^{5+} and MnTMOHex-3-PyP^{5+} relative to their alkyl analogs. With MnTMOHex-3-PyP^{5+} we were able to reduce toxicity of MnTnHex-3-PyP^{5+}, which is at least in part related to its micellar character. At concentrations

below 1 μM, MnTnHex-3-PyP^{5+} (not shown) is still not protective, at 1 μM it becomes toxic, and at 5 μM prevents JI from growing (Fig. 5). At 5 μM concentrations, where MnTnHex-3-PyP^{5+} and its *ortho* analog, MnTnHex-2-PyP^{5+}, are fully toxic, the methoxy *meta* analog, MnTMOHex-3-PyP^{5+} is similarly protective as MnTE-2(or 3)-PyP^{5+} (Fig. 5). Thus it appears that introduction of oxygen diminished its toxicity by ~10-fold. However, and possibly due to the higher cellular accumulation and/or partial micellar character, MnTMOHex-3-PyP^{5+} becomes toxic at higher concentrations ≥10 μM where the shorter-chain analog, MnTMOE-3-PyP^{5+} is still fully protective (Fig. 5, inset).

Thus far, it is not quite clear if introduction of methoxy groups into lipophilic MnTnHex-3-PyP^{5+} modifies its toxicity only *via* suppression of porphyrin lipophilicity, therefore its cellular accumulation and in turn toxicity, or the presence of oxygen in its own right has some impact. For reasons not fully understood, the shorter analog, MnTMOE-3-PyP^{5+}, is much more efficacious than its *meta* alkyl analogs, MnTE-3-PyP^{5+} and MnTnBu-3-PyP^{5+}, and *ortho* MnTE-2-PyP^{5+}. We discuss here MnTnBu-3-PyP^{5+} as the compound has chains of the same length as MnTMOE-3-PyP^{5+}. With MnTMOE-3-PyP^{5+} there may be a favorable balance of the size, length of alkyl chains, ratio of oxygen to the chain length, lipophilicity, rotational flexibility, hindrance of cationic charges, *etc.* Relative to MnTE-3-PyP^{5+} and MnTnBu-3-PyP^{5+}, MnTMOE-3-PyP^{5+} exerts less toxicity and/or is more efficacious, likely because of two reasons: (1) moderate, and thus favorable increase in lipophilicity, which increases its accumulation within the cell when compared to insufficiently lipophilic MnTE-3-PyP^{5+} and excessively lipophilic MnTnBu-3-PyP^{5+} (the later becomes toxic already at >10 μM[21]); and (2) hindrance of cationic pyridyl charges by longer pyridyl substituents (relative to MnTE-3-PyP^{5+}) whereby unfavorable interactions of porphyrin with biological molecules, such as phosphates of nucleic acids, are suppressed. When compared to our most studied Mn porphyrin, there may be a sufficient gain in the lipophilicity of MnTMOE-3-PyP^{5+} relative to MnTE-2-PyP^{5+}, which allows it to accumulate within the cell at

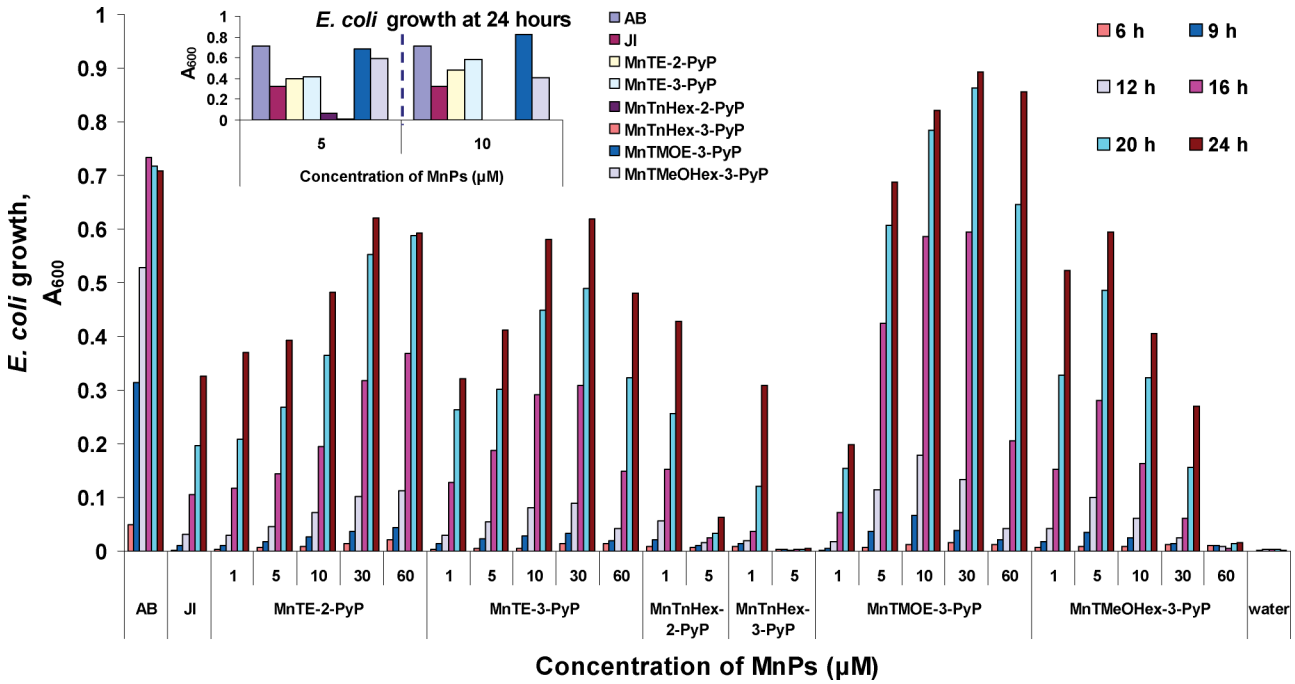

Fig. 5 The differential effect of the MnPs on the aerobic growth of SOD-deficient *E. coli*, JI in restricted five amino acid medium: impact of oxygen. The turbidity of medium was measured by absorbance at 600 nm, and is proportional to the *E. coli* growth. MnPs were given at 1–60 μM. The growth of JI and AB alone are shown also. Without cytosolic SOD enzymes or their mimics, SOD-deficient *E. coli* grows very poorly in a restricted medium. An SOD mimic substitutes for the lack of cytosolic SODs in the SOD-deficient strain JI. The magnitude of its effect on the *E. coli* growth depends upon its efficacy and ability to enter the *E. coli* cell. The *ortho* Mn porphyrin, MnTE-2-PyP^{5+}, is routinely used as an internal control in all our studies to account for the differences in the growth of *E. coli*. In numerous experiments MnTE-2-PyP^{5+} at 20 μM allowed the full growth of SOD-deficient *E. coli*.[4] Since the *meta* isomeric *N*-methoxyalkylpyridylporphyrins are explored in this study, to witness their advantage over *meta* alkylpyridylporphyrins, *meta* MnTE-3-PyP^{5+} and *meta* MnTnHex-3-PyP^{5+} were tested also. When compounds enter the cell and are potent SOD mimics also, they exert efficacy at very low 1 and 5 μM concentrations. Such was the case with both MnTMOE-3-PyP^{5+} and MnTMOHex-3-PyP^{5+}. Yet, when used at higher concentrations, MnTMOHex-3PyP^{5+} starts exerting toxicity, while MnTMOE-3-PyP^{5+} was fully efficacious up to 60 μM levels. Analogous hexylpyridylporphyrin, MnTnHex-3-PyP^{5+}, is much more toxic: no improvement in the growth of JI was seen at 1 μM concentration, and full suppression of its growth was observed at 5 μM. The *ortho* analogue, MnTnHex-2-PyP^{5+} is slightly less toxic than *meta* MnTnHex-3-PyP^{5+} as it is bulkier and less lipophilic. In five independent experiments, the methoxyethyl analog MnTMOE-3-PyP^{5+} was consistently the most efficacious compound: it allows SOD-deficient *E coli* to grow better than wild type AB, and better than both *ortho* and *meta* ethyl analogues, MnTE-2(or 3)-PyP^{5+}. Such a remarkable effect of MnTMOE-3-PyP^{5+} may be the consequence of the favorable interplay of lipophilicity, size, shape, rotational flexibility, and presence of oxygen. The inset shows the effect of MnPs at two concentrations and at 24 h. At 5 μM both methoxyalkylpyridylporphyrins are superior to the alkylpyridyl analogs tested; *i.e.* they help SOD-deficient *E. coli* (JI) to grow to a similar extent as does wild type strain (AB). Taken together, the data indicate that introduction of oxygens into the chains makes alkylpyridylporphyrins less toxic and more efficacious. See the Results and discussion section for possible explanation of such effects. The charges on Mn porphyrins are omitted for simplicity.

a high enough level to be efficacious despite it somewhat inferior $k_{cat}(O_2^{\bullet -})$. Finally, relative to the *ortho* MnTE-2-PyP^{5+},[51] the more flexible structure of the *meta* isomer, where substituted pyridyls can freely rotate while a molecule travels across the cell wall, could enhance the cellular accumulation of MnTMOE-3-PyP^{5+}. Again, the presence of oxygens may by itself also facilitate transport across the cell wall.

Lately, we are learning that *E. coli* is much more sensitive to excessive lipophilicity of Mn porphyrins than are the eukaryotic systems such as yeast and mammalian endothelial and cancer cells.[25] The reason probably lies in the difference between the structures of the *E. coli* cell wall *vs.* yeast cell membrane. Under the same concentration conditions, the porphyrins which exert significant toxicity to SOD-deficient *E. coli* are nontoxic and fully protective to SOD-deficient yeast.[52] MnTnHex-2-PyP^{5+} is the most obvious case. It is already toxic to *E. coli* at 1 μM, but not at all toxic to yeast and to five different cancer lines even at 30 μM.[25,52,53]

To fully understand the complexity of porphyrin efficacy and impact of oxygen within alkyl chains, we would need to compare compounds of identical lipophilicity, metal-centered reduction potential (which would assure the presence of reduced lipophilic species at the same level[14]) and antioxidant potency, differing only in the presence of oxygens in pyridyl substituents. Work is in progress to explore factors that affect cellular accumulation (bioavailability), toxicity and efficacy of potent cationic Mn(III) *N*-substituted pyridylporphyrins. Comparative studies are also in progress to evaluate SOD-deficient *E. coli* and SOD-deficient yeast *Saccharomyces cerevisiae* assays in justifying the therapeutic potential of Mn porphyrins.

Abbreviations

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
$ONOO^-$	Peroxynitrite
$O_2^{\bullet -}$	Superoxide
$^{\bullet}NO$	Nitric oxide
$CO_3^{\bullet -}$	Carbonate radical
BBB	Blood brain barrier
CNS	Central nervous system
H_2TE-2-PyP^{4+}	*meso*-tetrakis(*N*-ethylpyridinum-2-yl)porphyrin
H_2TE-3-PyP^{4+}	*meso*-tetrakis(*N*-ethylpyridinum-3-yl)porphyrin
H_2TnHex-2-PyP^{4+}	*meso*-tetrakis(*N*-n-hexylpyridinum-2-yl)porphyrin
H_2TnHex-3-PyP^{4+}	*meso*-tetrakis(*N*-n-hexylpyridinum-3-yl)porphyrin
H_2TMOE-3-PyP^{4+}	*meso*-tetrakis(*N*-(2′-methoxyethyl)pyridinum-3-yl)porphyrin
H_2TMOHex-3-PyP^{4+}	*meso*-tetrakis(*N*-(6′-methoxyhexyl)pyridinum-3-yl)porphyrin
MnPs	Mn(III) *N*-alkyl- and *N*-methoxyalkylpyridylporphyrins
Mn(II)P, Mn(III)P, O═Mn(IV)P	Mn porphyrins with Mn in its +2, +3 and +4 oxidation states
MnTE-2-PyP^{5+} (AEOL10113)	Mn(III) *meso*-tetrakis(*N*-ethylpyridinum-2-yl)porphyrin
MnTE-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-ethylpyridinum-3-yl)porphyrin
MnTnBu-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-n-butylpyridinum-2-yl)porphyrin
MnTnHex-2-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinum-2-yl)porphyrin
MnTnHex-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinum-3-yl)porphyrin
MnTMOE-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-(2′-methoxyethyl)pyridinum-3-yl)porphyrin
MnTMOHex-3-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-(6′-methoxyhexyl)pyridinum-3-yl)porphyrin
MnTM-4-PyP^{5+}	Mn(III) *meso*-tetrakis(*N*-methylpyridinum-4-yl)porphyrin
MnTDE-2-ImP^{5+} (AEOL10150)	Mn(III) *meso*-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin
MnTBAP^{3-} (MnTCPP^{3-}; AEOL10201)	Mn(III) *meso*-tetrakis(4-carboxylatophenyl)porphyrin
MnTSPP^{3-}	Mn(III) *meso*-tetrakis(4-sulfonatophenyl)porphyrin
R_f	Thin-layer chromatographic retention factor that presents the ratio between the solvent and compound path in 1 : 1 : 8 = $KNO_{3(sat)}$: H_2O : AN solvent system
$E_{1/2}$	Half-wave reduction potential
SOD	Superoxide dismutase
NHE	Normal hydrogen electrode
EDTA	Ethylenediaminetetraacetic acid.
HFBA	Heptafluorobutyric acid
AN	Acetonitrile, CH_3CN

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