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ORIGINAL ARTICLE

Cytotoxic effects of Mn(III) *N*-alkylpyridylporphyrins in the presence of cellular reductant, ascorbate

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Abstract

Due to the ability to easily accept and donate electrons Mn(III)*N*-alkylpyridylporphyrins (MnPs) can dismute $O_2^{\cdot-}$, reduce peroxynitrite, but also generate reactive species and behave as pro-oxidants if conditions favour such action. Herein two *ortho* isomers, MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+}, and a *meta* isomer MnTnHex-3-PyP^{5+}, which differ greatly with regard to their metal-centered reduction potential, $E_{1/2}$ ($Mn^{III}P/Mn^{II}P$) and lipophilicity, were explored. Employing $Mn^{III}P/Mn^{II}P$ redox system for coupling with ascorbate, these MnPs catalyze ascorbate oxidation and thus peroxide production. Consequently, cancer oxidative burden may be enhanced, which in turn would suppress its growth. Cytotoxic effects on Caco-2, Hela, 4T1, HCT116 and SUM149 were studied. When combined with ascorbate, MnPs killed cancer cells *via* peroxide produced outside of the cell. MnTE-2-PyP^{5+} was the most efficacious catalyst for peroxide production, while MnTnHex-3-PyP^{5+} is most prone to oxidative degradation with H_2, and thus the least efficacious. A 4T1 breast cancer mouse study of limited scope and success was conducted. The tumour oxidative stress was enhanced and its microvessel density reduced when mice were treated either with ascorbate or MnP/ascorbate; the trend towards tumour growth suppression was detected.

Keywords: *Mn porphyrin, cancer cells, ascorbate, SOD mimics, oxidative stress, MnTE-2-PyP^{5+}*

Abbreviations: *Charges in the MnP abbreviations are omitted throughout descriptions of Figures; MnPs, Mn(III) N-alkylpyridylporphyrins; SOD, superoxide dismutase; ROS, reactive oxygen species; RNS, reactive nitrogen species; RS, reactive species; $ONOO^-$, peroxynitrite; $O_2^{\cdot-}$, superoxide; $^{\cdot}OH$, hydroxyl radical; Asc was used as a general term with no intention to account for its protonation or redox equilibria; H_2A, fully protonated ascorbic acid; A, dehydroascorbic acid; HA^-, monodeprotonated ascorbate; $HA^{\cdot}$, ascorbyl radical; $A^{\cdot-}$ deprotonated ascorbyl radical; $Mn^{III}P^{5+}$ and $Mn^{II}P^{4+}$, oxidized (Mn +3 oxidation state) and reduced Mn porphyrin (Mn +2 oxidation state), total charge 5 + and 4 + are omitted in text; MnTM-4-PyP^{5+}, Mn(III) meso-tetrakis(N-methylpyridinium-4-yl)porphyrin; MnTE-2-PyP^{5+}, AEOL10113, FBC-007, Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin; MnTE-3-PyP^{5+}, Mn(III) meso-tetrakis(N-ethylpyridinium-3-yl)porphyrin; MnTnHex-2-PyP^{5+}, Mn(III) meso-tetrakis(N-n-hexylpyridinium-2-yl)porphyrin; MnTnHex-3-PyP^{5+}, Mn(III) meso-tetrakis(N-n-hexylpyridinium-3-yl)porphyrin; DMF, N,N'-dimethylformamide; DMSO, dimethyl sulfoxide; TMRE, tetramethylrhodamine ethyl ester, perchlorate; Rf, thin-layer chromatographic retention factor that presents the ratio*

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between the solvent and compound path in 1:1:8=$KNO_{3(sat)}$:H_2O:CH_3CN solvent system; P_{OW} partition between n-octanol and water; $E_{1/2}$, half-wave reduction potential for $Mn^{III}P/Mn^{II}P$ redox couple; SOD, superoxide dismutase; NHE, normal hydrogen electrode; HIF-1α, hypoxia inducible factor-1, NF-κB, nuclear factor κB; AP-1, activator protein-1; SP-1, specificity protein 1; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; MCP-1, monocyte chemotactic protein-1; HER2, human epidermal growth factor receptor 2; IBC, inflammatory breast cancer; fL, femtoLiter; PBS, phosphate buffered saline; ip, intraperitoneal; sc, subcutaneous; DMEM, Dulbecco's Modified Fagle Medium; EBS, fetal bovine serum.

Introduction

Those Mn(III) *N*-alkylpyridylporphyrins (MnPs) (Figure 1) which have metal-centered reduction potential, $E_{1/2}$ for $Mn^{III}P/Mn^{II}P$ redox couple around + 300 mV, alike SOD enzymes, are equally able to efficiently reduce and oxidize superoxide, $O_2^{\cdot -}$. Thus, MnPs: (a) are powerful catalysts of $O_2^{\cdot -}$ dismutation, and mimic SOD enzymes which are the first-line of endogenous antioxidative defenses [1–5]; and (b) bear potential to be pro-oxidants. The ability of MnP to dismute $O_2^{\cdot -}$ parallels its ability to reduce $ONOO^-$, and to modulate cellular transcriptional activity [1–5]. MnPs can *in vivo* adopt 4 oxidation states, + 2, + 3, + 4 and + 5. The pro-oxidative action can arise directly or indirectly from all four oxidation states. During the facile reduction from $Mn^{III}P$ to $Mn^{II}P$, they could oxidize cellular redox-able low-molecular weight compounds and proteins as well as $O_2^{\cdot -}$. In a reduced state, $Mn^{II}P$ could reduce $O_2^{\cdot -}$, or bind oxygen and reduce it to $O_2^{\cdot -}$. Subsequently, H_2O_2 will be formed which could act as oxidant in its own right, but could also affect signalling pathways, perpetuating oxidative stress [6–8]. Under physiological conditions peroxide will be removed by abundant peroxide-removing enzymes such as catalases, glutathione peroxidases, glutathione reductases, glutathione transferases, thioredoxins, peroxyredoxins, etc. The $O=Mn^{IV}P$ and $O=Mn^{V}P$ are strong oxidants in their own right also, and were reportedly formed in the reaction of $Mn^{III}P$ and $Mn^{II}P$ with $ONOO^-$ [9–11].

We have already shown in an *E. coli* model that MnPs become pro-oxidants in the presence of cellular reductant ascorbate [12]. In that model MnPs alone are able to substitute for the lack of cytosolic SODs and allow SOD-deficient *E. coli* to grow aerobically as well or better than the wild type [1,3]. However, in the presence of ascorbate, MnPs catalyzed ascorbate-driven H_2O_2 formation. The increased peroxide levels forced *E. coli* to fight back/adapt *via* induction of *oxyR* regulon, and upregulation of peroxidases and catalases [12,13].

Our earlier understanding was that the inhibition of transcription factor activation is due to the removal of signalling reactive species by MnPs [1–5]. However, Tse et al [14] and Jaramillo et al [15,16] have shown that the ability of MnPs to prevent activation of the master transcription factor, NF-κB, could be pro-oxidative in nature also [14–16]. The oxidation of p50 subunit of NF-κB in the nucleus [14,17,18] and/or glutathionylation of p65 subunit in the cytosol in the presence of glutathione and H_2O_2 has been suggested to result from the pro-oxidative action of MnP (Figure 2). In the latter case, MnTE-2-PyP^{5+} enhances anticancer effects of glucocorticoids in a lymphoma study [15,16]. Growing number of studies have recognized the glutathionylation as a major modification of critical proteins [16,19–24]. In our earlier mouse studies on the inhibitory effect of MnTE-2-PyP^{5+} on transcription factors HIF-1α and AP-1, the removal of signalling reactive species was offered as an explanation [25,26]. It may well be that the protein cysteine modification, observed with NF-κB subunits, is operative with HIF-1α and AP-1 also. Importantly, the data on transcription factors warn us to distinguish between the mecha-

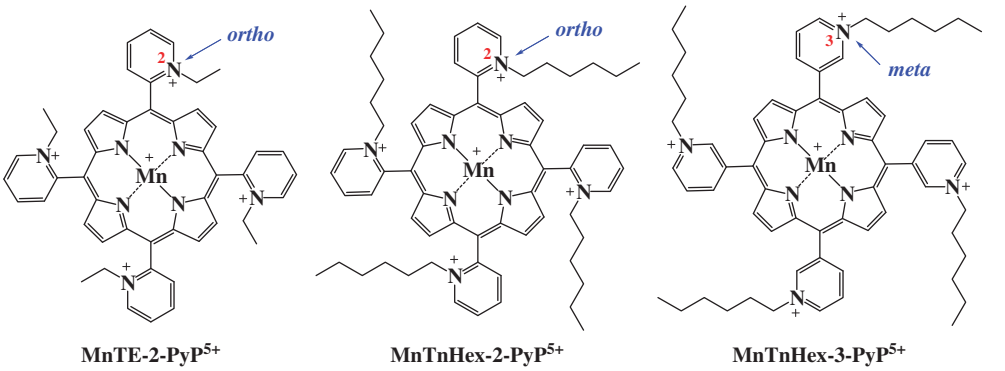

Figure 1. Structures of three manganese porphyrins (MnPs) studied: MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+}. *Ortho(2)* and *meta(3)* positions of nitrogen atoms in pyridyl rings are indicated.

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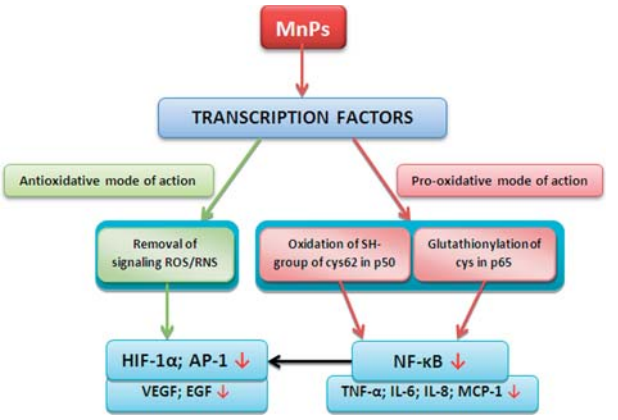

Figure 2. The mechanism of action of MnPs upon transcription factors was reported to be anti- and pro-oxidative. In a cellular study it was shown that treatment of 4T1 cells with H_2O_2 or ·NO donor activates HIF-1α while the activation was fully inhibited in the presence of MnTE-2-PyP^{5+} [25]. With AP-1 again, the inhibition of its activation was ascribed to the removal of ROS/RNS [26]. With NF-κB convincing evidence was provided to conclude that the NF-κB subunits, p50 (in the nucleus) [4,14,17,18,27] and/or p65 (in the cytosol, in the presence of GSH and H_2O_2), were oxidatively damaged by MnTE-2-PyP^{5+}; glutathionylation was suggested in the latter case [15,16,19,27]. Gluthationylation has been increasingly reported as a critical protein cysteine modification [16,19–24]. The same modification could have happened with HIF-1α and AP-1. HIF-1α is reportedly under the control of NF-κB also [28].

nism of MnP action and the effects observed. For example, in a diabetes model, MnP presumably acted *via* pro-oxidative mechanism, while the beneficial effects observed were clearly of antioxidative nature [27]. The type of MnP action, anti- or pro-oxidative (Figure 2), would depend upon the cellular levels of reactive species and antioxidants, cellular ratio of superoxide- to peroxide-removing systems, levels of oxygen, and cellular and subcellular distribution of MnP. It would also depend whether the normal cell (with abundant peroxide-removing enzymes) or the cancer cell (with compromised oxidative status and different transcription factor profile) is considered.

The metalloporphyrins (exogenous and endogenous) or other redox-cycling agents were shown with a few other laboratories also, to catalyze the ascorbate-driven H_2O_2 production *in vivo* and *in vitro,* and in turn exert anticancer effects [6,29–34]. Such studies are highly relevant due to the high endogenous intracellular levels of ascorbate. Several groups are already exploring the clinical anticancer potential of ascorbate in the presence and absence of redox-cycling agent [35–38]. The use of ascorbate/menadione system is already in clinic [35,39].

Mn(III) *N*-alkylpyridylporphyrins differ vastly with respect to the metal-centered reduction potential for the $Mn^{III}P/Mn^{II}P$ redox couple, $E_{1/2}$, and lipophilicity (Table I). The $E_{1/2}$ shows which oxidation state of a porphyrin Mn site (+3 in $Mn^{III}P^{5+}$ or +2 in $Mn^{II}P^{4+}$) is a stable one, *i.e.* $E_{1/2}$ shows the affinity of $Mn^{III}P$ towards reduction to $Mn^{II}P$. Structure–activity relationship studies showed that $E_{1/2}$ is linearly related to log k_{cat} ($O_2^{\cdot -}$) and is thus also an indicator of SOD-like potency of MnP [1–5]. Finally, $E_{1/2}$ determines the MnP stability towards oxidative degradation with H_2O_2, whose ascorbate-driven production it catalyzes. The $E_{1/2}$ of *meta* isomers is up to 300 mV more negative than the $E_{1/2}$ of *ortho* isomers. Thus *meta* isomers, which favour Mn+3 oxidation state, are not as easily reduced as *ortho* analogs; upon reduction they readily reoxidize whereby producing H_2O_2 which in turn degrades them (Table I) [1,2,40–43]. Upon reduction of $Mn^{III}P$ to $Mn^{II}P$, the single charge from the metal site gets lost, which vastly enhances the MnP lipophilicity. The gain in lipophilicity upon reduction is higher with *ortho* than with *meta* species (Table I). With both isomers, the gain in lipophilicity depends in a bell-shape fashion upon the size of the alkyl chain length; it culminates with butyl porphyrins, and decreases towards ethyl and hexyl analogs [44]. MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+} would become ~100-fold more lipophilic upon reduction, while essentially not much gain has been observed with

Table I. Physicochemical properties of MnPs: the metal-centered reduction potential for $Mn^{III}P/Mn^{II}P$ redox couple ($E_{1/2}$), the SOD activity ($O_2^{\cdot -}$ dismuting catalytic rate constant, log k_{cat}), and the lipophilicity of $Mn^{III}P$ and $Mn^{II}P$ described in terms of chromatographic retention factor, R_f, n-octanol-water partition coefficient, log P_{ow}, and the gain in lipophilicity upon $Mn^{III}P$ reduction, Δ log P_{OW}.

Compound	$Mn^{III}P/Mn^{II}P$ redox couple $E_{1/2}$/mV *vs* NHE[a]	SOD activity log k_{cat} ($O_2^{\cdot -}$)[b]	Lipophilicity			
			R_f[c]	log P_{ow} ($Mn^{III}P$)	log P_{ow} ($Mn^{II}P$)	Δ log P_{ow} (upon reduction)
MnTM-4-PyP^{5+} [1,2]	+60	6.58	0.064			
MnTE-2-PyP^{5+} [45,46]	+228	7.76	0.085	–6.70[d]	–4.69[d]	2.01
MnTnHex-2-PyP^{5+} [47,48]	+314	7.48	0.394	–2.76[e]	–0.73[d]	2.03
MnTnHex-3-PyP^{5+} [46,47]	+64	6.64	0.489	–2.06[e]	–1.74[f]	0.32

[a]$E_{1/2}$ data measured in 0.05 M phosphate buffer, pH 7.8, 0.1 M NaCl. [b]SOD activity measured by the cyt *c* assay in 0.05 M phosphate buffer, pH 7.8, 25 ± 1°C. [c]data obtained in plastic-backed silica-gel thin-layer chromatography plates eluted with 1:1:8 = $KNO_{3(sat)}$:H_2O:CH_3CN. [d]Calculated according to equation: log P_{OW} = 12.18 · R_f – 7.43 [46]. [e]Determined in water/n-butanol (log P_{BW}) system and converted to water/n-octanol system using equation: log P_{OW} = 1.55 (log P_{BW}) – 0.54 [49]. [f]Calculated according to equation: log P_{OW} = 8.78 · R_f – 7.12 [46].

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MnTnHex-3-PyP^{5+} (Table I). In turn, the reduced Mn^{II}TnHex-2-PyP^{4+} is more lipophilic and may accumulate more within cell than the reduced Mn^{II}TnHex-3-PyP^{4+} (Table I). Such considerations are relevant to *in vivo* conditions due to the abundance of reductants; many of them (ascorbate, glutathione, tetrahydrobiopterin, flavoenzymes) could reduce Mn^{III}P [1–5].

This study was designed to elucidate which MnP may be the preferred candidate for further exploration of the therapeutic potential of MnP/ascorbate system. A *meta* and two *ortho* MnPs were compared. The *ortho* compounds used herein are the most studied cationic Mn porphyrins, MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+}. MnTE-2-PyP^{5+} is 13,500-fold more hydrophilic and has ~100 mV less positive $E_{1/2}$ than MnTnHex-2-PyP^{5+}. MnTE-2-PyP^{5+} has showed efficacy in numerous models of oxidative stress disorders [1–5]. Yet, a lipophilic MnTnHex-2-PyP^{5+} is preferred over MnTE-2-PyP^{5+} in central nervous system injuries, as well as brain tumours, where transport across the blood brain barrier is critical for its efficacy [18]. Moreover, this compound is advantageous over hydrophilic MnTE-2-PyP^{5+} due to its predominant accumulation in mitochondria [5,50–53].

The comparative *E. coli* study of *ortho vs meta* Mn(III) *N*-alkylpyridylporphyrins clearly showed that lipophilicity may compensate for somewhat inferior MnP redox-ability [47]. The *ortho* MnTE-2-PyP^{5+} is 10-fold more potent SOD mimic, but *meta* MnTE-3-PyP^{5+}, with 10-fold lower superoxide dismuting ability, is 10-fold more lipophilic and accumulates therefore 10-fold more within *E. coli* cell. Consequently both *ortho* and *meta* MnTE-2-(and 3)-PyP^{5+} are equally efficacious in protecting SOD-deficient *E. coli*. The study clearly showed that *meta* isomers bear therapeutic potential also [47]. They differ from *ortho* analogs predominantly with respect to lipophilicity and reducibility, but with regards to the shape and size of the molecule also; their comparison would allow us to study the impact of such factors upon the anticancer efficacy of MnPs. Therefore, we have chosen to compare the *meta* MnTnHex-3-PyP^{5+} to its *ortho* analog MnTnHex-2-PyP^{5+}.

We have explored 5 different cancer cell lines: human epithelial colorectal adenocarcinoma Caco-2, human epithelial cervical cancer Hela, mouse mammary cancer 4T1, human colon cancer HCT116 and human inflammatory breast cancer SUM149. Inflammatory breast cancer is an aggressive, highly invasive tumour affecting younger women with racial disparity and with one of the worst clinical outcomes among breast cancers [54,55], and is thus of particular interest for therapeutic screening.

Materials and methods

Chemicals

The H_2T-2-PyP and H_2T-3-PyP were purchased from Frontier Scientific. *n*-Hexyl *p*-toluenesulfonate (>98%) was from TCI America. The ethyl *p*-toluenesulfonate (>98%), and tetrabutylammonium chloride hydrate (98%) were from Aldrich. $MnCl_2 \cdot 4H_2O$ (99.7%) was 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, chloroform, acetonitrile, EDTA and KNO_3 were purchased from Mallinckrodt. *N,N*-dimethylformamide anhydrous (DMF) of 99.8% purity (kept over 4-Å molecular sieves) and plastic-backed silica gel TLC plates (Z122777-25EA) were from Sigma-Aldrich. All chemicals were used as received without further purification. (+)-Sodium L-ascorbate (>98%) and Hoechst 33342 were from Sigma. Due to the sensitivity of the ascorbate towards oxidation, its aqua solution was prepared daily, immediately before the start of an experiment. When the ascorbic acid is dissolved in water the aqueous solution becomes acidic, whereas pH does not change upon dissolving sodium ascorbate. At physiological pH 7.4, ascorbic acid is dissociated and is present as ascorbate. For both reasons, it is more convenient to use sodium ascorbate instead of ascorbic acid.

Tetramethylrhodamine ethyl ester, perchlorate, TMRE was from Molecular Probes. EF5, 2-nitroimidazole was from Cameron Koch, University of Pennsylvania, Philadelphia, PA. Premixed WST-1 cell proliferation reagent from Clontech Laboratories, Inc., was used as received and stored in aliquots at -20°C. The absorbance was measured in a microplate reader (Fluostar optima, BMG labtech).

Cell lines

Human epithelial colorectal adenocarcinoma Caco-2, human epithelial cervical cancer Hela, mouse mammary cancer 4T1, human colon cancer HCT116 were maintained in Alpha-MEM medium supplemented with 10% fetal bovine serum at 37°C. The presence of fetal bovine serum in the medium has not affected the results (data not shown). Human inflammatory breast cancer cell line (mammary epithelial cells) SUM149 was obtained from Asterand, Inc. (Detroit, MI) and was cultured in 6-well plates (Corning Incorporated, Corning, NY) in Ham's F12 supplemented with insulin, hydrocortisone, and 5% fetal bovine serum, FBS until they reached 70–80% confluence. SUM149 are triple negative basal-like cancer cells with activated EGFR and isolated from primary IBC tumour [56]. Previous studies have shown a correlation between ROS generation and sensitivity to a dual EGFR/HER2 kinase inhibitor approved for IBC therapy [55,56].

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Mn porphyrins

Synthesis. The general synthetic procedure for *N*-alkylpyridylporphyrins (H_2TE-2-PyP^{4+}, H_2TnHex-2-PyP^{4+} and H_2TnHex-3-PyP^{4+}) and their Mn complexes (MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+}) were described earlier [3,48]. Briefly, the solution of H_2T-2-PyP (H_2T-3-PyP) in anhydrous DMF was stirred with ethyl *p*-toluenesulfonate or n-hexyl *p*-toluenesulfonate at ~105°C. After the reaction was completed (monitored by TLC plates using 1:1:8 = $KNO_{3(sat)}$:H_2O:CH_3CN as a mobile phase) the reaction mixture was worked up and counterion was replaced by chloride [3,48]. Porphyrins were subsequently metallated using 20-fold molar excess of $MnCl_2$ in basified aqueous medium. The uv/vis, thin-layer chromatography (TLC), ESI-MS and elemental analysis were performed to ensure the identity and purity of MnP preparations.

Stability of MnPs towards oxidative degradation. Stability of MnPs (6 μM) was previously determined in the presence of ascorbate (0.42 mM), and at pH 7.8 maintained by tris buffer on UV-2501 PC Shimadzu spectrophotometer with 0.5 nm resolution [43]. With hexyl analogs the oxidative degradation with 0.2 mM H_2O_2 in 0.1 M tris buffer was also followed.

In vitro *studies*

WST-1-based cytotoxicity assay. The assay is based on the reduction of tetrazolium salt WST-1 to a water-soluble colored formazan by mitochondrial dehydrogenases in a metabolically active cell. 10,000 viable cells were plated in each well of a 96-plate in Alpha-minimum essential medium (MEM) supplemented with 10% fetal bovine serum. After 24 h in 5% CO_2 at 37°C, the medium was replaced with 100 μL of fresh complete medium with different concentrations of drugs. A positive control contained the same number of cells in a complete medium with no drug added. A negative control without cells was used as a blank. Because the Caco-2 cells grow slowly, the plates were incubated for 96 h for Caco-2 cells and 48 h for other three cell lines. The doubling time for Caco-2 cells is ~62 h (ATCC catalog), while the doubling times for other cell lines are ~24 h (Hela) [57], ~23 h (4T1) [58] and ~16 h (HCT116) [59]. At the end of the incubation, the cells were washed twice with 1X PBS. Then 100 μL of Opti-Media with 10 μL of proliferation reagent WST-1 was added to each well and the plates were incubated at 37°C for another 2 h. The absorbance was measured in a microplate reader (Fluostar optima, BMG labtech) at 450/595 nm and the percent viability was calculated from the following equation: $[(A_{sample}\text{-}A_{blank})/(A_{control}\text{-}A_{blank})] \times 100$. The data were analyzed using the SPSS program version 11.5.0. Differences were considered significant with $p < 0.05$.

TMRE cytotoxicity assay. The viability of inflammatory breast cancer cells was assessed using the mitochondrial membrane potential marker tetramethylrhodamine ethyl ester, perchlorate, TMRE. SUM149 cells were cultured in 6-well plates until they reached 70–80% confluence. Cells were treated with either 30 μM MnTE-2-PyP^{5+} or 30 μM MnTnHex-2-PyP^{5+} in the presence and absence of 3 mM ascorbate for 24 h. After incubation, cells were harvested and incubated for 30 min with 500 nM TMRE. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson, Rockville, MD) and analyzed using Cellquest software (Beckton Dickinson). High fluorescence was calculated by setting a gate on the control cells where the peak reached a minimum, and all experimental samples were compared to this control gate.

Clonogenic assay. This assay measured the long-term effect of the MnPs alone or in combination with ascorbate on the overall survival and clonogenicity of the cells. 4T1 mouse breast carcinoma cells and HCT116 human colorectal carcinoma cells were plated in 6-well plates with 3 mL of DMEM complete growth media. Cells were plated at two densities (100 and 300 cells/well) and allowed to attach for 24 h under normal conditions (37°C, 5% CO_2). Then the cells were treated with deionized H_2O (vehicle control) or the indicated concentrations of sodium ascorbate (0.5 and 1 mM) and MnTnHex-2-PyP^{5+} (15 and 30 μM) alone or in combination for 24 h. After treatment, the media with drugs were removed and replaced with a fresh 3 mL of DMEM complete growth medium. The cells were then allowed to incubate under normal conditions for 7–10 days, or until the control cells grew colonies of ≥ 50 cells. Then the cells were rinsed with 1X PBS, fixed in 10% methanol-10% acetic acid and stained with a 0.4% crystal violet solution. Colonies with > 50 cells were counted. Plating efficiencies were determined and normalized to the control. Error bars represent standard error. Each experiment was repeated in a triplicate and a one-way ANOVA analysis with a Fisher's PLSD post-hoc test was used to determine significance values ($^{*}p < 0.05$).

MnTnHex-2-PyP^{5+} levels in 4T1 cells. Cells were plated at 1,440,000 cells per plate in 10 mL DMEM (high glucose + 10% FBS) and allowed 24 h to attach (80% confluence). Then the cells were treated with either 0.5 or 1 mM ascorbate or 5, 15 or 30 μM MnTnHex-2-PyP^{5+} or their combination. After 24 h, the media were poured off, cells were rinsed twice with PBS, and 500 μL of Ripa buffer (Sigma #9806, 10x) per plate was added. Cells were then scraped

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from plate and transferred to 1.5 mL tubes, incubated on ice for 10 min, and sonicated for 30 s. They were then centrifuged 5 min at 12,000 rpm. 10 μL of the supernatant was used to determine protein concentration using a Bradford protein assay [60]. The lysate was used for LCMS/MS analysis of MnTn Hex-2-PyP^{5+} as previously described in details for the analysis of tumour, cytosolic and mitochondrial homogenate [3,44]. The analyses were performed at Duke Comprehensive Cancer Center, Clinical Pharmacology Laboratory on Shimadzu 20A series (LC) and Applied Biosystems MDS Sciex 4000 Q Trap ESI-MS/MS.

Identification of the major species involved in cytotoxicity of MnPs/ascorbate system. In order to identify reactive species involved in cytotoxicity, under above described conditions Caco-2 cells were treated with MnPs/ascorbate system in the presence and absence of 1,500 units/mL of catalase (peroxide scavenger) and 5 mM mannitol (hydroxyl radical scavenger).

In vivo *study*

Mouse 4T1 breast cancer model. A total of seventy 6–8 week-old female Balb/c mice weighing an average of 22 g (Charles River Laboratories, Raleigh, NC) were used. Tumours were established from a 100 μL single cell suspension of 10^7 cells/mL (total of 10^6 cells) and were injected subcutaneously into the shaved right flank. Tumour volumes were measured daily with vernier digital caliper and calculated with the formula: $V = \text{length} \times \text{width} \times \text{width} \times \pi/6$. Sixty-eight mice were randomly enrolled into the study after the tumour of each mouse had reached a treatment volume of ~200 mm^3. The mice were divided into four treatment groups: (1) PBS (400 μL, intraperitoneally), (2) sodium ascorbate (2 g/kg, twice daily, intraperitoneally), (3) MnTnHex-2-PyP^{5+} (1 mg/kg, twice daily, subcutaneously), and (4) combination of MnTnHex-2-PyP^{5+} (1 mg/kg, twice daily, subcutaneously) and sodium ascorbate (2 g/kg, twice daily, intraperitoneally). In the 4th group, sodium ascorbate was delivered 1 h after the injection of MnTnHex-2-PyP^{5+}. The doses of MnP and ascorbate chosen were based on previously published *in vivo* experiments [42, 61]. Mice were treated for the whole duration of study (15 days). Once the tumour burden exceeded 2000 mm^3, 0.3 mL of EF5 (10 mM, obtained from Cameron Koch, University of Pennsylvania, Philadelphia, PA) was delivered intraperitoneally 2 h prior to sacrificing the animal, followed by intravenous administration of 100 μL of Hoechst 33342 (20 mg/mL, Sigma, in 0.9% saline) 2 min prior to sacrificing the animal. Tumours were excised and snap frozen using liquid nitrogen. All animal handlings and procedures were approved from Duke University Institutional Animal Care and Use Committee.

MnTnHex-2-PyP^{5+} levels in tumour and muscle. Tumours and muscles (from opposite legs) were homogenized, and proteins removed from tissue with 1% acetic acid in methanol, solvent evaporated and residue reconstituted in a LCMS/MS mobile phase. Analyses were performed on an Applied Biosystems MDS Sciex 3200 Q Trap and 4000 Q Trap LC/MS/MS spectrometer at Duke Comprehensive Cancer Center, Shared Resource PK Labs as described in details elsewhere [3,44].

Immunohistochemistry. The frozen tumours were stored at −80°C and cryosectioned at 10 μm thickness. These sections were stained using anti-CD31 mAB at a dilution of 1:100 (BD Bioscience) with Alexa Fluor 488 anti-rat IgG (Invitrogen), anti-CD68 Alexa Fluor 647 (Serotec) at a dilution of 1:100, anti-EF5 (obtained from Cameron Koch, University of Pennsylvania, Philadelphia, PA) at a dilution of 1:2 and Hoechst 33342 (Sigma) at a dilution of 1:1000.

Image analysis. Before staining, slides were dried at room temperature and imaged for Hoechst perfusion immediately. Whole tumour fluorescent images for CD31, CD68, EF5 and Hoechest 33342 staining were made using a fluorescence microscope (Axioskop 2plus; Zeiss) equipped with a cooled charge-coupled device digital camera (retiga 1300R; Q-Imaging) at × 50 magnification. Images acquired from stage scanning were stitched and overlaid for direct comparison of dual fluorescent images using Photoshop. ImageJ software was used to detect the stained cells area. Regions of interest were determined around the viable tumour area.

Results

In vitro *studies*

Mn porphyrins stability towards oxidative degradation. Among MnPs studied, MnTnHex-3-PyP^{5+} is the most unstable one and degrades significantly within 1.5 h in an aqueous system in the presence of 0.42 mM sodium ascorbate as observed by spectral change (Figure 3) [43]. Its *ortho* analog, MnTnHex-2-PyP^{5+}, is however the most stable towards oxidative degradation The affinity towards oxidative degradation is due to the fairly low $E_{1/2}$ of MnTnHex-3-PyP^{5+} (~250 and 150 mV more negative relative to MnTnHex-2-PyP^{5+} and MnTE-2-PyP^{5+}, respectively) (Table I), which favours the Mn + 3 oxidation state; when reduced, MnTnHex-3-PyP^{4+} readily reoxidizes, giving rise to H_2O_2. The same spectral change was observed when MnP

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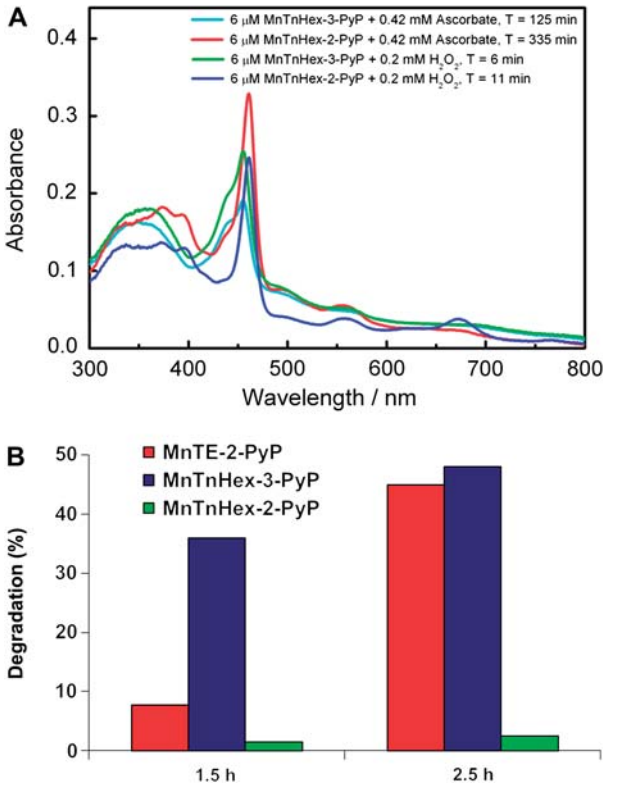

Figure 3. The stability of Mn porphyrins towards oxidative degradation induced by peroxide itself or by the MnP/ascorbate system. MnPs catalyze the ascorbate-induced hydrogen peroxide production. Accumulation of hydrogen peroxide results in the degradation of porphyrin macrocycle. The stability of MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+} were compared. **(A)** The uv/vis spectra of MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+} were taken at different time points in the presence of either ascorbate or H_2O_2. The conditions were: 6 μM MnPs, 0.42 mM ascorbate, or 0.2 mM H_2O_2, pH 7.8 maintained with 0.1 M tris buffer. The absorption spectra of MnPs with ascorbate mirror those with hydrogen peroxide. **(B)** The comparison of the stability of all three MnPs towards ascorbate-induced oxidative degradation [43]. The conditions were: 6 μM MnTnHex-2-PyP^{5+}, 0.42 mM ascorbate in 0.05 M tris buffer [43]. The degradation was assessed at 1.5 and 2.5 h after mixing MnP and ascorbate. The data show that the degradation rates of MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+} with ascorbate are the lowest and the highest, respectively, among MnPs explored.

was treated with either ascorbate or H_2O_2 (Figure 3A), undoubtedly indicating that with ascorbate, peroxide was the cause of porphyrin ring degradation (Figure 3) [40,62].

WST-1-based cytotoxicity study of MnP/ascorbate system. Data in Figures 4 and 5 were fitted to a four-parameter logistic equation to determine IC_{50} values and the results are summarized in Table II. Ascorbate itself was not cytotoxic up to ~3 mM concentration (Figure 5). MnPs alone were not toxic to Caco-2 cells at up to ~40 μM (Figure 4). Therefore, in subsequent studies we have chosen to work with 30 μM concentration of MnPs and 3.3 mM concentration of ascorbate with all cells.

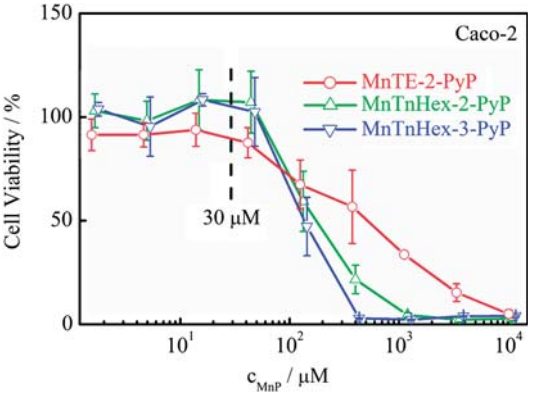

Figure 4. The toxicity of MnPs to Caco-2 cells. Cells were grown for 4 days at a cell density of 10,000 cells/well. Based on these data 30 μM MnPs was chosen for the subsequent studies. Cell viability was measured *via* reduction of tetrazolium salt WST-1 to a water-soluble colored formazan by metabolically active cell.

MnPs. In our earlier *E. coli* studies, in the absence of exogenous reductant, MnTnHex-3-PyP^{5+} accumulates several-fold more within the cell but is not accordingly more toxic than is MnTnHex-2-PyP^{5+} [47]. Herein, in the absence of ascorbate, despite being ~10-fold more lipophilic than MnTnHex-2-PyP^{5+}, MnTnHex-3-PyP^{5+} is not significantly toxic in its own right, while MnTnHex-2-PyP^{5+} is. That suggests that the difference in both their redox properties and bioavailibilities plays a role in their toxicity. Toxicity appears to be cell-type dependent also.

MnPs/ascorbate. Mn porphyrins enhanced cytotoxicity of ascorbate with all tumour cell lines, acting as catalysts of ascorbate-driven peroxide formation (Figures 5, 6 and 7). The combination of MnTnHex-3-PyP^{5+} with ascorbate was the least cytotoxic among MnPs studied (Figures 5 and 6), due to its fastest oxidative degradation (Figure 3, Table II).

In all our earlier comparative studies with *E. coli* and with mouse heart and yeast mitochondria [44,50,51], MnTnHex-2-PyP^{5+} accumulated more than MnTE-2-PyP^{5+} within cell and cellular compartments due to its higher lipophilicity [1–5]. We assumed therefore that it would accumulate more than other MnPs within cancer cells also, and thus exert higher toxicity (Figures 5 and 6). With Mn stabilized in + 3 oxidation state relative to MnTnHex-2-PyP^{5+} (described by lower $E_{1/2}$, Table I), the MnTE-2-PyP^{5+} reoxidizes more rapidly and is thus more efficacious in producing peroxide (Figure 12). Conversely, the MnTnHex-2-PyP^{5+} with nearly 100 mV more positive $E_{1/2}$ (Table I) is stabilized in |Mn + 2 oxidation state, and relative to MnTE-2-PyP^{5+} disfavours reoxidation which would have otherwise led to the peroxide production. Indeed, based on the slopes of the curves in Figure 5, in the presence of ascorbate, MnTE-2-PyP^{5+} appears equally (HCT116, Caco-2) or more cytotoxic (4T1, Hela)

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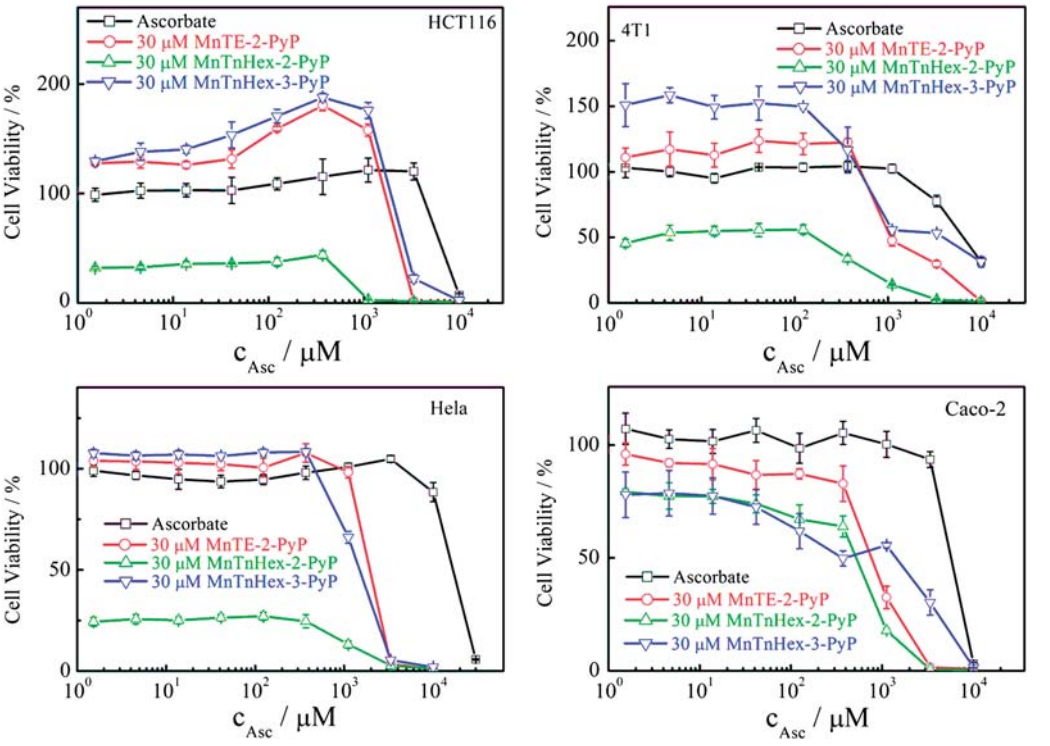

Figure 5. Dose-dependent cytotoxicity of sodium ascorbate in alpha-MEM medium to four tumour cell lines growing for 4 days (Caco-2) or 2 days (HCT116, 4T1 and Hela) at the density of 10,000 cells/well in 96-well cell culture plates in the absence and presence of 30 μM MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+}, or MnTnHex-3-PyP^{5+} under normoxic conditions. Sodium ascorbate starts to exert cytotoxicity at concentrations >1 mM. Addition of MnPs markedly potentiated the cytotoxicity of sodium ascorbate. Cell viability was measured *via* reduction of tetrazolium salt WST-1 to a water-soluble colored formazan by metabolically active cell.

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than MnTnHex-2-PyP^{5+}. The same was even more true with inflammatory breast cancer cell line (see Figure 7). Such data strongly suggest that the porphyrin bioavailability/lipophilicity has minor impact on the anticancer effects which are primarily due to its redox ability to cycle with ascorbate in extracellular space/medium. Consequently H_2O_2 is produced, which then enters the cell. The medium has no peroxide-removing systems unless tiny amount of H_2O_2-removing enzymes leaks from the cell [63].

Data in Figures 5 and 6 also show that the low levels of each of MnTnHex-3-PyP^{5+}, MnTE-2-PyP^{5+} (but not MnTnHex-2-PyP^{5+}) and ascorbate are beneficial to the cell. Such data agree with our earlier observations from *E. coli* studies where some MnPs increase the growth of wild type presumably *via* easing the cell physiological oxidative burden [43,47]. The pro- *vs* anti-oxidative nature of ascorbate has been discussed in details [64–66]. The production of peroxide *via* metal-catalyzed ascorbate oxidation may be beneficial to cancer cell to some extent allowing it to proliferate, but detrimental if excessive. Such contemplations also point to the complex and not yet fully understood *in vivo* interactions of reactive species and redox-active compounds.

Cytotoxicity of ortho Mn(III) N-alkylpyridylporphyrins/ascorbate in inflammatory breast cancer cell model. Based on the data in 4 other cancer cell lines, the more

Table II. The IC_{50} values of sodium ascorbate with and without 30 μM MnPs in four cancer cells.

Cancer cell line	IC_{50}, mM[a]			
	Asc	Asc + MnTE-2-PyP^{5+}	Asc + MnTnHex-2-PyP^{5+}	Asc + MnTnHex-3-PyP^{5+}
HCT116	4.1 ± 1.0	1.5 ± 0.1	–	2.9 ± 0.1
4T1	6.5 ± 0.3	1.3 ± 0.2	–	2.7 ± 0.3
Hela	16.4 ± 1.1	1.9 ± 0.1	–	1.3 ± 0.1
Caco-2	5.3 ± 0.5	0.84 ± 0.14 (0.47 ± 0.05)*	0.54 ± 0.08 (0.17 ± 0.02)*	0.87 ± 0.21 (0.14 ± 0.02)*

[a]The IC_{50} values were calculated relative to the cells growing in medium with neither ascorbate nor MnP added (100% viability). MnTnHex-2-PyP^{5+} itself, with no ascorbate added, reduced the cell viability by more than 50% with all cells except Caco-2 (Figure 4).
*The IC_{50} values for MnPs in the absence of ascorbate.

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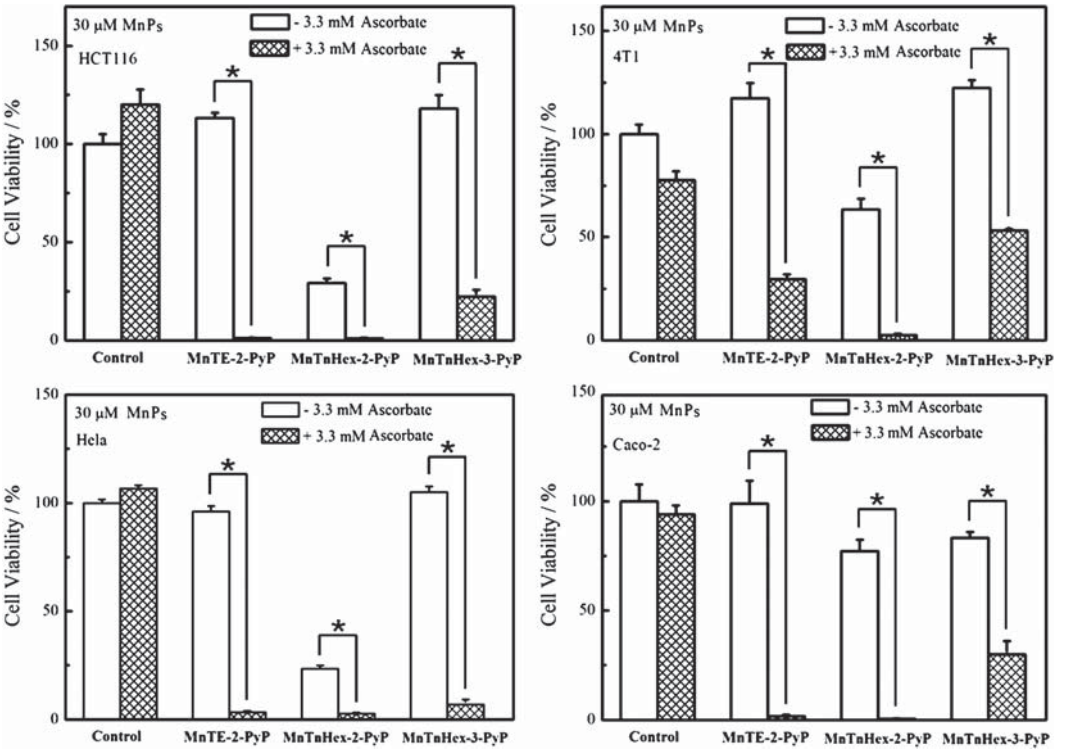

Figure 6. Cytotoxicity of 3.3 mM sodium ascorbate to 4 different cancer cell lines. Cells grew in alpha-MEM medium for 4 days (Caco-2) or 2 days (HCT116, 4T1 and Hela) at the density of 10,000 cells/well in 96-well cell culture plates in the absence and presence of 30 μM MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+}, or MnTnHex-3-PyP^{5+} under normoxic conditions. Cell viability was measured *via* reduction of tetrazolium salt WST-1 to a water-soluble colored formazan by metabolically active cell and the experiments were performed in triplicate. With Caco-2 cells, MnPs alone did not exert cytotoxicity. With other cell lines 30 μM MnTnHex-2-PyP^{5+} itself exerted toxicity. The viability was greatly or fully suppressed when ascorbate was added to the medium. The MnTnHex-3-PyP^{5+} was the least cytotoxic when combined with ascorbate. Differences were considered significant with $^{*}p < 0.05$.

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efficacious *ortho* isomers were forwarded to the inflammatory breast cancer cell model. MnTE-2-PyP^{5+}, but not MnTnHex-2-PyP^{5+}, appeared efficacious (Figure 7) in decreasing cell integrity and viability as measured by mitochondrial membrane potential marker, TMRE. While we do see efficacy of MnTnHex-2-PyP^{5+}/ascorbate in other cancer cells (see Results, Figure 6), with SUM149 there is none, which suggests that there may be differential response of cancer cell lines to MnPs. Neither of two MnPs was toxic in their own rights. As seen before with Caco-2 cells, ascorbate itself has beneficial effect on the viability of SUM149.

Clonogenicity assessment of the cytotoxicity of MnP/ascorbate system. Ascorbate and MnTnHex-2-PyP^{5+} alone did not affect the ability of two different cancer cell lines, 4T1 and HCT 116 cells, to produce colonies (Figure 8). The HCT116 cells seem to be more sensitive than 4T1 cells to the treatment. However, when combined, the treatment significantly decreased cell survival in both cell lines ($^{*}p < 0.0001$). In comparison with WST-1 assay, the clonogenic data show that 30 μM of MnTnHex-2-PyP^{5+} is not toxic to 4T1 and HCT116 cells, suggesting that the cells might have adapted and/or recovered from the stress induced by MnP.

MnTnHex-2-PyP^{5+} levels in 4T1 cells. Under given conditions, depending upon the total concentrations of ascorbate and porphyrin and their ratio, up to the 29% more of the MnP was found within the cells when they were treated with MnP/ascorbate compared to MnP treatment alone (Table III). This is somewhat lower than the effect of ascorbate on the MnP accumulation in tumour and muscle (Figure 10B) [44]. This is in agreement with the increased lipophilicity of the reduced Mn^{II}TnHex-2-PyP^{4+} relative to oxidized Mn^{III}TnHex-2-PyP^{5+} [44]. However, the accumulation of MnP within the cell appears not to be relevant for the efficacy of MnPs in our *in vitro* experiments, because our data indicate that the peroxide produced outside of the cell, in medium, is the main cause of cytotoxicity which agrees with Chen et al conclusions [6] (see Discussion also).

Assuming a cellular volume of 2.19 μL/mg protein [67], and 0.27 nmole MnP/mg protein determined (Table III), we calculated that when 1.44×10^6 cells in 10 mL plate were treated with 30 μM MnP, the intracellular MnP concentration was 123 μM (0.27×10^{-9} mole/2.19×10^{-6} L).

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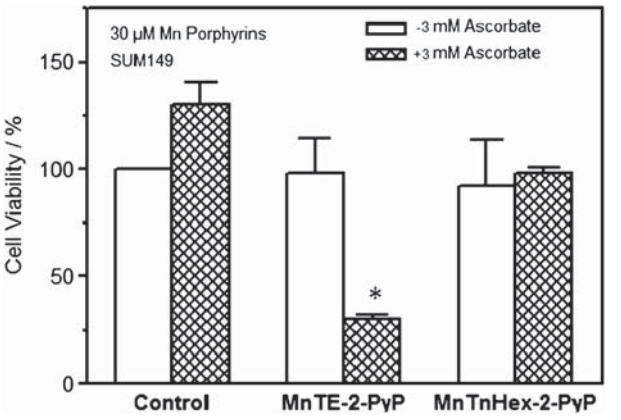

Figure 7. The cytotoxic effects of 30 μM MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+} on the inflammatory breast cancer line SUM149 in the presence of 3 mM ascorbate. Cell viability was assessed *via* measurements of mitochondrial membrane potential marker tetramethylrhodamine ethyl ester perchlorate, TMRE. Cells were incubated with MnPs ± ascorbate for 24 h. Bars represent mean ± SEM normalized to DMSO. The student's two-tailed t test was used to calculate p values. Compared to control, the $^{*}p < 0.0001$ for MnTE-2-PyP^{5+}/ascorbate.

Assuming a volume of a cell of 2000 fL (from the data listed for mouse lymphoblastoid cells, [68]), the total volume occupied by 1.44×10^6 cells was 1.44×10^6 cells $\times 2000 \times 10^{-15}$ L cell^{-1} = 2.88×10^{-6} L. Then the amount of MnP in 2.88×10^{-6} L is 3.44×10^{-10} mole (123×10^{-6} mole $\times$ L$^{-1} \times 2.88 \times 10^{-6}$ L). Total number of moles of porphyrin in 10 mL medium was 3×10^{-7} (30×10^{-6} mole $\times$ L$^{-1} \times 10^{-2}$ L). Although the MnP concentration in the cells is ~4 times higher than in the medium, the total volume of cells is only tiny fraction of the medium volume. Therefore, the final concentration of MnP in the medium remained essentially the same as initial, 29.97 μM {(3×10^{-7} –3.44×10^{-10}) mole/10^{-2} L}.

Identification of the species involved in the cytotoxicity of MnPs/ascorbate system. In order to identify the reactive oxygen species involved in MnPs/ascorbate cytotoxicity, Caco-2 cells were treated with either MnPs or ascorbate alone, or their combination in the presence and absence of catalase (Figure 9A) and mannitol (Figure 9B). Both mannitol and catalase diminished the cytotoxic effects of MnP/ascorbate systems. The effect was somewhat larger with catalase indicating that the peroxide is the major species involved in cytotoxicity. In the presence of metals, H_2O_2 would lead to the production of $^{\cdot}OH$ radicals (Figure 12). The effect of mannitol on cell viability was only substantial with MnTnHex-3-PyP^{5+}. The results are in agreement with data reported by Tian et al on *ortho* methyl analog, MnTM-2-PyP^{5+}. These data also indicate that both lipophilic MnPs, MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+}, exert fair toxicity to Caco-2 cells in their own rights and presumably *via* production of H_2O_2 and $^{\cdot}OH$ radical also. In both experiments (Figure 9) MnTnHex-3-PyP^{5+} by itself is consistently less toxic than MnTnHex-2-PyP^{5+} which is in agreement with data shown in Figures 5 and 6.

In vivo *study*

Mouse 4T1 breast cancer study of the MnP/ascorbate system. The lipophilic MnTnHex-2-PyP^{5+}, was shown to accumulate more in cell and in particular in critical cellular compartments – mitochondria than MnTE-2-PyP^{5+}, and was therefore chosen for the *in vivo* study [5,41,51]. The dose of MnTnHex-2-PyP^{5+} used was taken from our previous *in vivo* brain tumour study [69], while the dose chosen for ascorbate (2 g/kg twice daily ip) was taken from the Levine et al study [42]. Levine group has shown clearly that only ascorbate given iv or ip, but

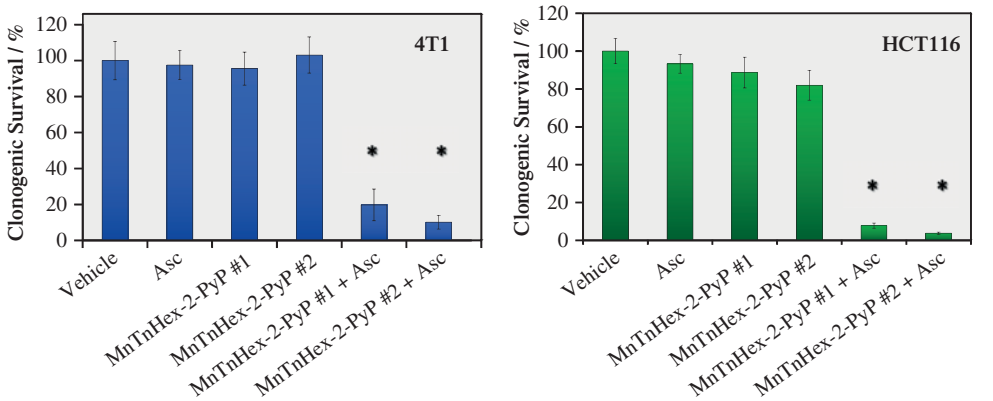

Figure 8. 4T1 mouse mammary cancer cells and HCT116 human colon cancer cells were treated with ascorbate (Asc) or MnTnHex-2-PyP^{5+} alone or in combination for 24 h. Clonogenic assays were conducted to assess the effect of MnP/ascorbate on cell survival and proliferation. The combination treatment in both cell lines is significantly more toxic than either of the individual treatments ($^{*}p < 0.001$). HCT116 cells were treated with ascorbate (0.5 mM), MnTnHex-2-PyP^{5+} #1 (15 μM) or MnTnHex-2-PyP^{5+} #2 (30 μM) alone or in combination for 24 h. 4T1 cells were treated with ascorbate (1 mM), MnTnHex-2-PyP^{5+} #1 (15 μM) or MnTnHex-2-PyP^{5+} #2 (30 μM) alone or in combination for 24 h.

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Table III. LCMS/MS analysis of the MnTnHex-2-PyP^{5+} levels in the 4T1 tumour cells cultured in the presence or absence of ascorbate.

Exp #	Concentration of MnTnHex-2-PyP (μM) in medium	Concentration of ascorbate (mM) in medium	MnP concentration in tumour cells (nmole/mg protein)	% increase in concentration
1	0	0.5	0.00	
2	0	1	0.00	
3	5	0	0.14	
4	5	0.5	0.15	7.1
5	5	1	0.18	28.6
6	15	0	0.21	
7	15	1	0.27	28.6
8	30	0	0.27	
9	30	1	0.25	none

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not per os, achieves therapeutic levels in plasma [42]. The *in vivo* study shows that there is a trend towards tumour growth delay with MnP/ascorbate system, although statistical significance was not reached under our experimental conditions (Figure 10).

The tissue analysis of MnTnHex-2-PyP^{5+} shows its higher accumulation in tumour relative to muscle from the opposite leg [44]. Also, ascorbate enhanced MnP accumulation in both tissues by 33% (tumour) and 54% (in muscle), which is due to the increased lipophilicity of the reduced Mn^{II}TnHex-2-PyP^{4+} relative to oxidized Mn^{III}TnHex-2-PyP^{5+} (Table I) [44]. The effect of ascorbate on MnP accumulation in tumour and muscle is of similar magnitude as the effect of ascorbate on MnP accumulation in 4T1 cells.

Immunohistochemical analysis of tissue sections (stained for microvessel density, perfusion, hypoxia and macrophage). In anti-CD31 staining for microvessels, all treatment groups showed significant reduction of microvessels compared with PBS group (Figure 11). The microvascular reduction was the highest in MnTnHex-2-PyP^{5+}/ascorbate group (Figure 11). No differences in EF5 staining, a marker of hypoxia, were detected, suggesting that these treatments did not change the overall hypoxic fraction of these tumours. However, increased perfusion (which suggests favorable drug delivery conditions) as indicated with increased Hoechst staining was observed for the treatment groups compared to the PBS control without reaching statistical significance (Figure 11). The CD68 staining showed that there was increased macrophage infiltration, which implied the increased tumour inflammation when the mice were treated with either ascorbate or MnTnHex-2-PyP^{5+}/ascorbate (Figure 11). The difference between the control group and MnTnHex-2PyP^{5+}/ascorbate group was of marginal significance ($p = 0.0849$).

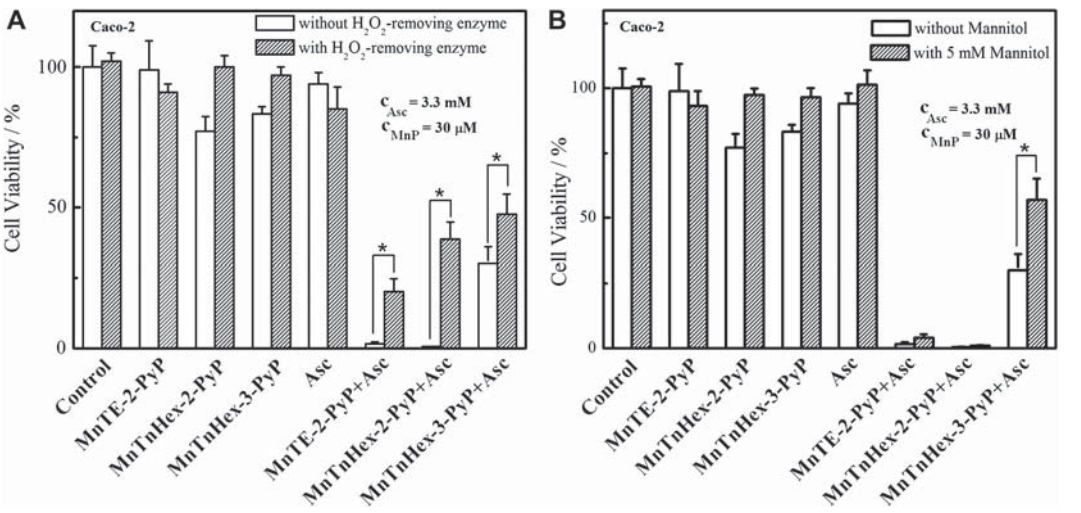

Figure 9. The cytotoxicity of MnP/ascorbate system to Caco-2 cells is due to the production of H_2O_2 and $^{\cdot}OH$. The 5 mM mannitol ($^{\cdot}OH$ radical scavenger) blocked significantly the cytotoxicity of MnTnHex-3-PyP^{5+} only. The experimental conditions are as stated for Figures 5 and 6. The addition of 1500 U/mL catalase (peroxide scavenger) significantly reduced the cytotoxic effect in all cases, suggesting peroxide as a major damaging species. The Caco-2 cell line was cultured in alpha-MEM supplemented with 10% heat-inactivated FBS, with or without 1500 U/mL catalase or 5 mM mannitol, respectively for 4 days at 37°C. WST-1 assay was used to measure the cell viability. Differences were considered significant with $^{*}p < 0.05$.

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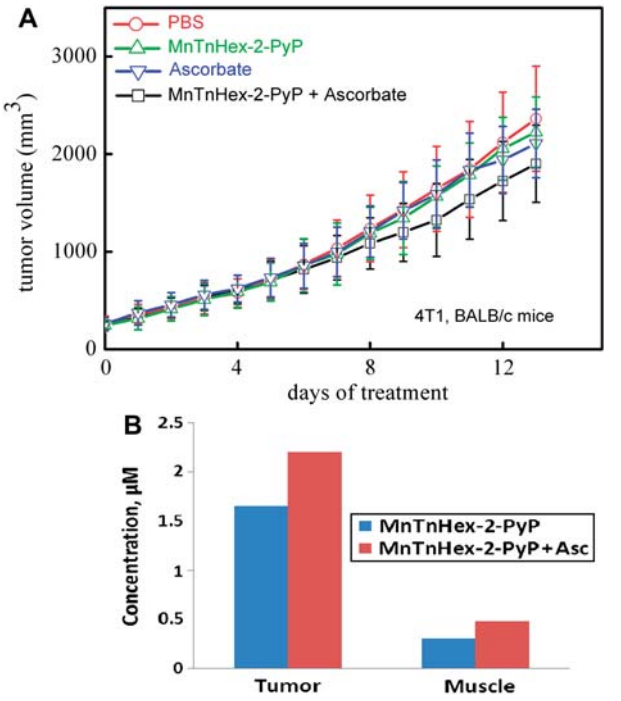

Figure 10. (A) The *in vivo* study of the effect of MnP/ascorbate treatment on the tumour growth delay in 4T1 mammary cancer Balb/c mouse study. Balb/c mice ($n = 17$ for each group) were implanted with 4T1 cells, as described in Materials and Methods. Treatments were started when tumour volume was ~200 mm^3. Four groups received: (1) PBS (400 μL, ip) as control; (2) ascorbate (2 g/kg, twice daily, ip); (3) MnTnHex-2-PyP^{5+} (1 mg/kg, twice daily, sc); and (4) combination of MnTnHex-2-PyP^{5+} (1 mg/kg, twice daily, sc) and ascorbate (2 g/kg, twice daily, ip), respectively. Tumour volumes were measured every day with vernier digital calipers and calculated with the formula $V = \text{length} \times \text{width} \times \text{width} \times \pi/6$ until they reach size of ~2000 mm^3. Under experimental conditions employed, the hypoxia was somewhat potentiated due to the ascorbate-driven oxygen consumption, which presumably counterbalanced the increased tumour oxidative stress and reduction in tumour microvessels and therefore diminished the overall beneficial anticancer effect of MnP/ascorbate. (B) The levels of MnTnHex-2-PyP^{5+} in a mouse tumour and muscle (when mice were treated with either MnP or MnP/ascorbate) expressed in μM in tissue homogenates determined by LCMS/MS method [3,44]. Ascorbate enhanced the MnP accumulation in tumour and muscle by 33% and 54%, respectively.

Discussion

Three Mn porphyrins, MnTE-2-PyP^{5+}, MnTnHex-2-PyP^{5+} and MnTnHex-3-PyP^{5+} (Figure 1) with different superoxide dismuting ability (log k_{cat}), metal-centered reduction potential ($E_{1/2}$), stability towards oxidative degradation and lipophilicity (n-octanol/water partition coefficient, log P_{ow}) (Table I) were selected for the present study to explore the importance of these parameters in the ascorbate-mediated cancer cell toxicity.

In vitro *studies*

Ortho vs meta MnPs. Ortho isomers, with up to 300 mV more positive $E_{1/2}$ (Table I), are stabilized in Mn + 2 oxidation state and are thus more readily reducible from $Mn^{III}P$ to $Mn^{II}P$ than *meta* isomers. However, once reduced (with ascorbate or $O_2^{\cdot -}$), the *meta* isomers undergo more rapid reoxidation to $Mn^{III}P$ than *ortho* porphyrins, with either $O_2^{\cdot -}$ or O_2 giving rise to H_2O_2 or $O_2^{\cdot -}$. $O_2^{\cdot -}$ would subsequently self-dismute or be enzymatically reduced to H_2O_2. H_2O_2 produced would in turn degrade MnP (Figure 12). Thus, MnTnHex-3-PyP^{5+} appeared the least stable compound among three MnPs explored, and for the same reason the least efficacious catalyst of ascorbate oxidation (Figure 3). Ascorbyl radical, produced in the first step of $Mn^{III}P$ reduction to $Mn^{II}P$, would react with either O_2 or $O_2^{\cdot -}$; that would again lead to increased peroxide levels (Figure 12). Finally, in a radical-radical reaction, ascorbyl and superoxide would produce H_2O_2. Due to the large excess of ascorbate over MnP, the major peroxide production would arise from MnP catalysis of ascorbate oxidation.

Ortho MnPs. Hydrophilic MnTE-2-PyP^{5+} *vs lipophilic* MnTnHex-2-PyP^{5+}. The Mn site of MnTnHex-2-PyP^{5+}, with ~100 mV more positive $E_{1/2}$ relative to MnTE-2-PyP^{5+}, is more reducible and thus is more stable in reduced Mn + 2 oxidation state [48] (Table I). Further, longer alkyl chains obviously hinder the approach of H_2O_2 towards metal site and therefore this porphyrin is by far the most stable against oxidative degradation (Figure 3). Such sterical conformation may hinder approach of ascorbate as well. By disfavoring reoxidation, MnTnHex-2-PyP^{5+} produces less peroxide than MnTE-2-PyP^{5+}, and is thus less cytotoxic, *i.e.* less efficacious in killing cancer cell. Our earlier *E. coli* data [47,81] showed that MnTnHex-2-PyP^{5+} accumulates to a much higher degree within cell than MnTE-2-PyP^{5+}, thereby exerting more toxicity in its own right (Figures 5 and 6) for at least two reasons. Firstly, it has the higher micellar character and could damage cellular membranes [47]. Secondly, the data shown in Figure 9 indicate that, at least in part, MnTnHex-2-PyP^{5+} toxicity is also due to the production of peroxide and hydroxyl radical as it is reversed upon the addition of catalase and mannitol. Taken together the data imply that lipophilicity/bioavailability has only minor impact on the anticancer effects of an *in vitro* MnP/ascorbate system, and that it is the peroxide produced in the medium (extracellular space) that enters the cell and exerts there the cytotoxic effects [42,66,82]. The conclusion is strengthened with the data on inflammatory breast cancer cell line (Figure 7), where none of the two Mn porphyrins alone are toxic, and only MnTE-2-PyP^{5+}/ascorbate was efficacious.

As production of peroxide is related to the $E_{1/2}$ of MnP, one may conclude that the optimal $E_{1/2}$ of an ideal compound, being somewhere around + 200 mV *vs* NHE, would allow it to undergo numerous cycles

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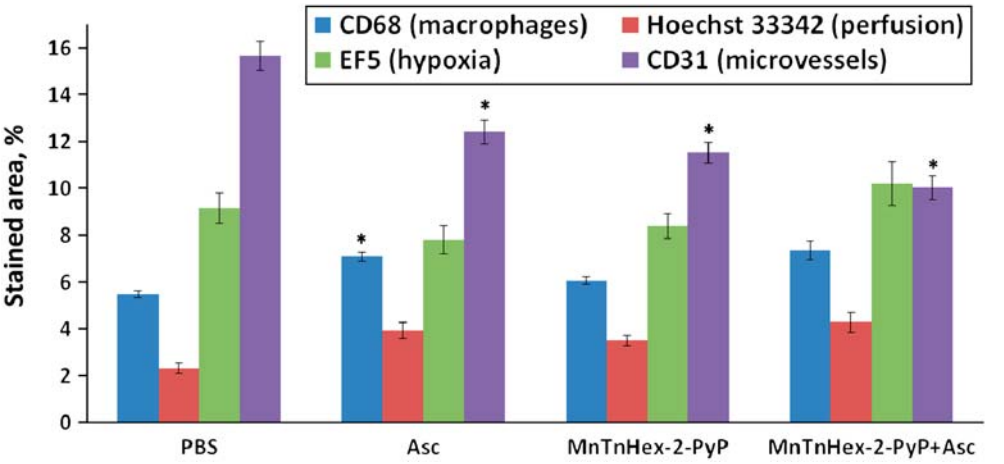

Figure 11. Histograms of stained areas of microvessels (CD31), hypoxia (EF5), perfusion (Hoechst 33342) and macrophage infiltration (CD68). The differences for staining fractions between each treatment groups were determined by the Mann–Whitney test. Differences were considered to be statistically significant if $^{*}p < 0.05$. The difference between the control group and MnTnHex-2-PyP^{5+}/ascorbate group was of marginal significance ($P = 0.0849$).

between + 3 and + 2 Mn oxidation states before being oxidatively destroyed. Our data support the notion (already elaborated in Introduction) that the same redox property that makes MnP an ideal SOD mimic, would likely make it an ideal catalyst of ascorbate-driven peroxide production, *i.e.* an ideal anticancer agent.

In vivo *study*

The *in vivo* system, though, is different than *in vitro* and is hard to predict to what degree the cytotoxic action of MnP/ascorbate may happen in extra- relative to intracellular space. Bioavailability may play a larger role in an *in vivo* setting. Not much is known about the transport of cationic MnPs, and the inhibition of their uptake which would both affect their bioavailability. *The effects in vivo would arise from the interplay of: (1) stability; (2) reducibility; (3) lipophilicity; (4) cellular distribution; (5) toxicity; and other factors that may affect bioavailability such as solvation, size, size and bulkiness of MnPs.*

Immunohistochemistry data from a 4T1 breast cancer mouse study, in particular those on the macrophage infiltration, undoubtedly show that there is a trend towards increased tumour oxidative stress with the MnP/ascorbate system, which along with decreased microvessel density resulted in a marginal tumour growth suppression (Figure 11). However, hypoxia if any was potentiated due to the ascorbate-driven oxygen consumption, and thus the ability of tumour to progress was maintained [83–85]. In light of the anticancer effects observed earlier with MnTE-2-PyP^{5+} in a 4T1 mouse study [61], and with MnTnHex-2-PyP^{5+} in a brain tumour study [69], as well as with Chen et al on ascorbate [6,42], the optimization of the MnP/ascorbate system may lead to the more pronounced anticancer effects and strengthen its clinical relevance.

The effects observed *in vitro* and *in vivo* may be best summarized in Figure 13. This and other studies by Espey et al [89], Tian et al [30], Chen et al [42] as well as our *E. coli* studies [12] confirm that peroxide is a major cytotoxic species arising from the catalysis of ascorbate-driven peroxide formation and oxygen consumption (Figure 12). In a healthy cell, the increased peroxide levels may not present a problem due to the abundance of peroxide-removing enzymes (Figure 12). However, the already compromised antioxidative status of the cancer cell [85,90,91] (depicted in Figure 13 as the narrower bell-shape curve relative to normal cell), along with additional oxidative burden (depicted in Figure 13 as the shift of the maximum of bell-shape curve relative to normal cell), would force it to undergo death. Alternatively, the cancer cell may try to fight back and upregulate catalases and peroxidases as did *E. coli* in our recent study when challenged by MnP/ascorbate [27]. The response may be cell type related. Under pathological conditions in non-cancer cells, such pro-oxidative action of MnPs alone (with no exogenously added ascorbate) may be operative also and exert beneficial effects by forcing cell to adapt *via* upregulation of endogenous antioxidative defences as shown by us [12] and others [92]. Note, that in such scenario, mechanism of action would be pro-oxidative while effects would be antioxidative. Finally, the most recent work by Dorai et al on rat kidney ischemia/reperfusion injury [93] showed clearly that, when rats were treated with renoprotective mixture containing MnTnHex-2-PyP^{5+}, growth factors, and mitochondrial amino acids, the whole plethora of endogenous antioxidant systems is upregulated. Why the upregulation of antioxidative defenses would happen if porphyrin itself is an oxidant: such data greatly support the pro-oxidative type of MnP action which forces cell to undergo adaptive response alike *E. coli* did [12].

The anticancer effects shown here in an *in vitro* cancer cell model pointed to the extracellular action

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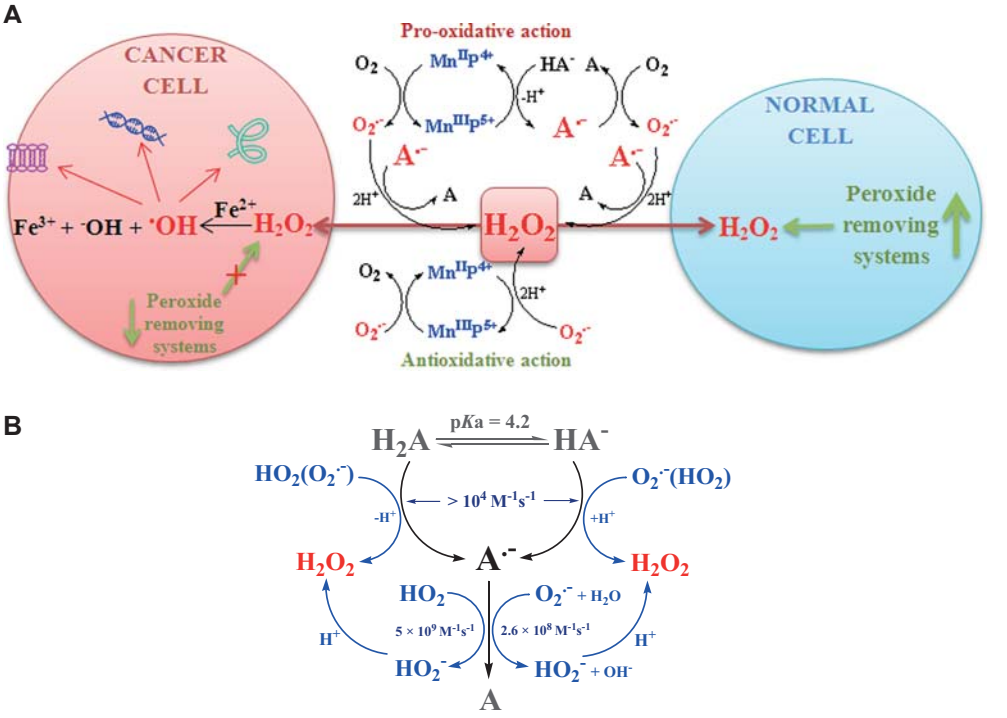

Figure 12. The proposed mechanism of the peroxide production in a MnP-catalyzed ascorbate oxidation. (A) Direct oxidation of ascorbate by oxygen, leading to a hydrogen peroxide formation is kinetically disfavoured, due to spin restriction [70], even though hydrogen peroxide production by ascorbate and oxygen is a thermodynamically favorable process [71,72]. The ascorbate oxidation is efficiently catalyzed by transition metals ions, such as iron and copper [42,71,73] as well as with metalloporphyrins [12,30,42]. Metal site of the porphyrin cycles between Mn + 3 and + 2 oxidation states while transferring electron to ascorbate and producing ascorbyl radical. The reoxidation of $Mn^{II}P$ to $Mn^{III}P$ may happen with O_2 and $O_2^{\cdot -}$. The rate constant for the oxidation of $Mn^{II}TM\text{-}4\text{-}PyP^{4+}$ with oxygen is 1.1×10^6 $M^{-1}s^{-1}$ [74]. Based on that value and our structure-activity relationship between MnP redox ability, $E_{1/2}$ and SOD-like activity [1–5,44], the rate constant for the reaction of *ortho* $Mn^{II}TE\text{-}2\text{-}PyP^{4+}$ with O_2 is estimated to be 8×10^4 $M^{-1}s^{-1}$, while the oxidation of $Mn^{II}TE\text{-}2\text{-}PyP^{4+}$ with $O_2^{\cdot -}$ is ~ 5×10^7 – ~ 10^8 M^{-1} s^{-1} [75]. Given the orders of magnitude higher availability of oxygen than superoxide, reduction of O_2 by MnP may still be preferred over $O_2^{\cdot -}$ reduction. (B) Both ascorbyl and superoxide anion radicals self dismute with a rate constant of ~10^5 $M^{-1}s^{-1}$ [66,70,76,77]. Interaction between these two radicals could happen under *in vitro* experimental conditions with a rate constant of > 10^8 $M^{-1}s^{-1}$. All ascorbate species present in the solution may react with superoxide anion and produce hydrogen peroxide [78–80]. Ascorbyl radical may react with oxygen to generate superoxide with a rate constant of ~ 10^2 $M^{-1}s^{-1}$ which is ~3 log unit lower than $A^{\cdot -}$ self-dismutation. Again, given the much higher availibility of oxygen in the medium than that of superoxide, this interaction could happen as well [70]. It is also 6 to 7 orders of magnitude smaller than the rate constant for the reaction of ascorbyl and superoxide radicals. The reduction of superoxide anion by either $Mn^{II}P$ or by ascorbyl radical to hydrogen peroxide happens at about the same rate [75,78]. However, due to the ~70 times higher ascorbate concentration, the reduction of superoxide to H_2O_2 likely happens at the expense of ascorbate species (Figure 12A).

of MnPs. The data are relevant for the *in vivo* studies, where the effects may occur both (i) extracellularly [6] when an organism is treated with exogenous ascorbate either alone [42] or in combination with MnP or any other redox cycling agent [35]; and (ii) intracellularly due to the excess of endogenous ascorbate over MnP catalyst and tumour MnP accumulation (Figure 10). For further discussion of the relevance of such system, consisting of ascorbate and redox-cycling agent, see refs [8,12,35,44,87,88].

Conclusions

Most cancer cells differ from normal cells due to their lower antioxidant defence capacity and thus increased oxidative stress, which is a driving force for their proliferation [94]. In particular, cancer cells have a perturbed ratio of H_2O_2- to $O_2^{\cdot -}$-removing enzymes [66]. Under such conditions any additional oxidative burden may force the cancer cell to undergo death. The study here shows that in the presence of ascorbate, MnP exerted pronounced pro-oxidative action. The action is due to the MnP catalysis of ascorbate-driven peroxide production and oxygen consumption, which greatly suppressed cell viability and its ability to grow colonies. In our *in vitro* setting the effects are primarily dependent upon the redox-ability of Mn porphyrin to cycle between oxidized $Mn^{III}P$ and reduced $Mn^{II}P$, and its stability towards oxidative degradation. The *meta* isomer, $MnTnHex\text{-}3\text{-}PyP^{5+}$ is the least efficient among MnPs explored, due to its lowest stability towards H_2O_2. The *ortho* isomers appeared more prospective than the *meta* analog. The 13,500-fold more hydrophilic $MnTE\text{-}2\text{-}PyP^{5+}$ is of similar or slightly

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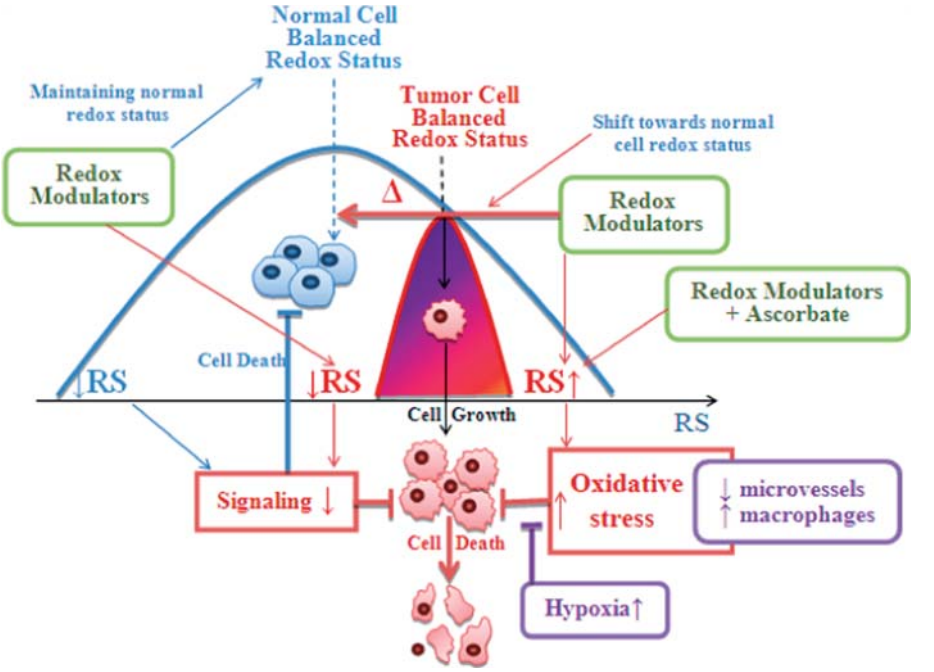

Figure 13. Tumour growth/suppression by MnP as a function of the oxidative stress/levels of reactive species (RS). Depending upon the levels of RS, different cellular events happen: at lower, physiological levels, signalling events predominate, while at very high levels oxidative events prevail. Such scenarii are depicted with two bell shape curves for the normal and cancer cell. Tumour is frequently under oxidative stress; such 'physiological' status is visualized here with the shift of the maximum of its bell shape curve towards higher levels of RS. The increased level of RS is a signal for the upregulation of the genes needed to support tumour angiogenesis and progression [84,85]. Tumour is however vulnerable and any further increase in oxidative burden would force tumour cells to undergo death. The strategy to treat tumours may thus be either to remove RS or to vastly increase their levels. In both cases redox-cycling agents would be efficacious as they can both remove and produce them, acting as anti- or pro-oxidants [5,12]. The latter strategy has already been used in clinic; one example is the combination of ascorbate and redox cycling agent menadione [35,86–88]. HIF-1α inhibition and VEGF downregulation and tumour angiogenesis suppression is a part of the first strategy where MnP scavenges RS, whereby affecting cell signalling [25,61]. In this work we aimed to show that indeed a redox-able MnP, when combined with ascorbate, exerted anticancer effects *via* increased oxidative burden, acting as a catalyst for ascorbate-driven H_2O_2 production [12]. Further work is needed to comprehend under which conditions MnP would act as antioxidant (removing signalling species) and under which it would kill cells *via* increasing RS levels.

better efficacy relative to MnTnHex-2-PyP^{5+}; the latter was found more bioavailable in our earlier *in vitro* and *in vivo* studies. Such finding indicates that the bioavailability in this *in vitro* setting has not played a major role in porphyrin efficacy, and suggests that it is the peroxide produced outside of cell that crosses the cell membrane and acts within the cell. Further work is needed to fully comprehend the mechanism/s of action/s of redox-able drugs.

In an *in vivo* 4T1 breast cancer Balb/c mouse study, we observed a trend towards tumour growth suppression by MnP/ascorbate-mediated increased oxidative stress/inflammation, which is at least in part due to the increased macrophages infiltration and reduced microvessel density. The study suggests that further optimization of MnP/ascorbate system for clinical development is warranted.

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Declaration of interest

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