# Thiol regulation by Mn porphyrins, commonly known as SOD mimics

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## ARTICLE INFO

## ABSTRACT

Superoxide dismutases play an important role in human health and disease. Three decades of effort have gone into synthesizing SOD mimics for clinical use. The result is the Mn porphyrins which have SOD-like activity. Several clinical trials are underway to test the efficacy of these compounds in patients, particularly as radioprotectors of normal tissue during cancer treatment. However, aqueous chemistry data indicate that the Mn porphyrins react equally well with multiple redox active species in cells including $H_2O_2$, $O_2^{\cdot-}$, $ONOO^-$, thiols, and ascorbate among others. The redox potential of the Mn porphyrins is midway between the potentials for the oxidation and reduction of $O_2^{\cdot-}$. This positions them to react equally well as oxidants and reductants in cells. The result of this unique chemistry is that: 1) the species the Mn porphyrins react with in vivo will depend on the relative concentrations of the reactive species and Mn porphyrins in the cell of interest, and 2) the Mn porphyrins will act as catalytic (redox cycling) agents in vivo. The ability of the Mn porphyrins to catalyze protein S-glutathionylation means that Mn porphyrins have the potential to globally modulate cellular redox regulatory signaling networks. The purpose of this review is to summarize the data that indicate the Mn porphyrins have diverse reactions in vivo that are the basis of the observed biological effects. The ability to catalyze multiple reactions in vivo expands the potential therapeutic use of the Mn porphyrins to disease models that are not SOD based.

## 1. Rationale for making SOD mimics

The quest to synthesize SOD mimics was initiated by the discovery of Irwin Fridovich and Joe McCord that the function of the previously identified copper porphyrin (now known as Cu,ZnSOD) was a superoxide dismutase [1]. This discovery, combined with the series of subsequent experiments establishing the key role of both Cu,ZnSOD and MnSOD in human health and survival [2,3], indicated the therapeutic potential of compounds with SOD-like activity. Several different classes of compounds have been developed that mimic SOD activity to varying degrees; most of them are metal complexes containing Mn. Fe complexes have also been explored [4]. While the Fe complexes are equal to the Mn analogs in their ability to act as SOD mimics, Fe complexes are inferior for use in vivo because they may lose Fe during redox cycling. Subsequently, free iron, $Fe^{2+}$, would impose Fenton-like toxicity due to hydroxyl radical formation in a reaction with hydrogen peroxide, $H_2O_2$.

Among Mn complexes, most frequently studied are the Mn(III) porphyrins (MnPs), Mn(II) cyclic polyamines and Mn(III) salen compounds (see reviews [5,6]). Mn(III) salen compounds are modest SOD mimics, while Mn(III) porphyrins and Mn(II) cyclic polyamines possess similarly high SOD-like activity [7]. Although they are similarly potent SOD mimics, Mn(III) porphyrins and Mn(II) cyclic polyamines have different physical and chemical properties which result in differing redox properties [5,8]. The properties associated with each class of compounds is expected to result in some overlap in clinical efficacy as well as clinical applications unique to each class of compounds. Currently, the Mn(III) salen, EUK134, is used only in cosmetic skin preparations by Estee Lauder; however, one Mn(II) cyclic polyamine and two Mn(III) porphyrins have reached the clinic and are presently being tested in several clinical trials.

Two Mn(III) porphyrin analogs are currently in clinical trials. The efficacy of MnTnBuOE-2-PyP$^{5+}$ (BMX-001) for the radioprotection of normal tissue during glioma (NCT02655601), head and neck (NCT02990468) and anal cancer treatment (NCT0338650) as well as radioprotection of normal brain in cancer patients who suffer from brain metastases has been explored (NCT03608020). It is important to note that BMX-001 does not protect cancer cells or cancerous tissue; moreover, it acts as a tumor radio- and chemosensitizer [9,10]. A second porphyrin analog, MnTE-2-PyP$^{5+}$ (AEOL10113, BMX-010), is being tested for a non-cancer application – atopical dermatitis and itch (NCT02457858).
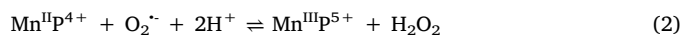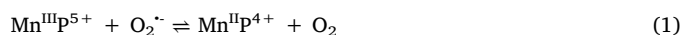
The Mn(II) cyclic polyamine, GC4419, is being tested as a radio-protector of salivary glands and mouth mucosa with head and neck cancer patients (NCT02508389) – the same application as BMX-001. In addition, GC4419 is being tested in combination with chemotherapy for locally advanced, non-metastatic squamous cell cancer of the head and neck, where, in addition to the radioprotective effect, the antitumor effect will be assessed (Phase I: NCT01921426). Two clinical trials, (phase 1/2b; PMID: 29174131 and phase 2 randomized clinical trials; http://www.galeratx.com/data-galera-therapeutics-223-patient-phase-2b-clinical-trial-gc4419-presented-masccisoo-2018-annual-meeting), have demonstrated the efficacy of GC4419 in suppressing oral mucositis in head and neck cancer patients. The success of these clinical trials has led to the first phase 3 clinical trial (NCT03689712) using an SOD mimic (GC4419); it is now open and accruing patients in the USA.

The progression of these compounds to clinical trials supports our rationale for the key therapeutic role of SOD mimics in disease and justifies over 3 decades of development of such compounds. Our continued studies on Mn(III) porphyrins provide substantial evidence that the more powerful the SOD mimic is, the better able it is to undergo diverse reactions in vivo with biological targets. The redox potential of these compounds, midway between the oxidation and reduction potential of $O_2^{\cdot-}$, uniquely positions them to act as reductants or oxidants depending on small changes in the cellular redox environment. In other words, the redox property of the Mn center, which allows the Mn porphyrin to be a powerful SOD mimic, allows it to easily undergo

reactions with other critical redox reactive species involved in cellular metabolism – simple thiols (glutathione) and protein thiols (cysteines), ascorbate, hydrogen peroxide ($H_2O_2$), peroxynitrite (ONOO⁻), nitric oxide ($^{\cdot}NO$), tetrahydrobiopterin, hypochlorite (ClO⁻) and sulfite ($SO_3^{2-}$). Given the richness of the redox chemistry of Mn porphyrins and the complex redox biology of a cell, the discovery of many more interactions with biological targets are anticipated. The diversity of reactions the Mn porphyrins undergo in the cell, suggests that some therapeutic actions occur via mechanisms other than mimicking SOD activity. The purpose of this review is to highlight the diverse reactions of the Mn porphyrins and summarize the evidence that the catalysis of the oxidation/S-glutathionylation of signaling proteins cysteines (thiols) may play a prominent role in their therapeutic effects.

## 2. Mimicking the thermodynamics (metal-centered reduction potential, $E_{1/2}$ for the Mn(III)/Mn(II) redox couple) and the kinetics of the dismutation process by SOD in the catalysis of superoxide dismutation by Mn porphyrins

Superoxide dismutation is a process that consists of two steps: oxidation and reduction of superoxide, $O_2^{\cdot-}$ [equations (1) and (2)]. SOD enzyme or its mimic (MnP) oxidizes $O_2^{\cdot-}$ in a first step [eq. (1)] and reduces it in a second step [eq. (2)]. The SOD enzyme undergoes both reactions with identical rate constants [11].

$$Mn^{III}P^{5+} + O_2^{\cdot-} \rightleftharpoons Mn^{II}P^{4+} + O_2 \tag{1}$$

$$Mn^{II}P^{4+} + O_2^{\cdot-} + 2H^+ \rightleftharpoons Mn^{III}P^{5+} + H_2O_2 \tag{2}$$

The properties of the SOD enzyme guided us and others in developing its mimics. Numerous compounds were synthesized and the structure-activity relationship evaluated to determine which compounds most closely approximated the activity of the enzyme [5]. The ability of a compound to undergo the catalysis of dismutation (thermodynamics) combined with the rate at which the process occurs (kinetics) were the critical properties required for a mimic to have therapeutic potential in biological systems. The thermodynamics of dismutation is described by the metal-centered reduction potential for the Mn(III)/Mn(II) redox couple, $E_{1/2}$, which is around $+300$ mV *vs* NHE (normal hydrogen electrode) for the enzyme regardless of the type of metal (Cu, Mn, Fe, Ni). This value of $E_{1/2}$ is essential for superoxide to efficiently exchange electrons with the metal center. The kinetics of $O_2^{\cdot-}$ dismutation is directly related to the electrostatic facilitation of the approach of the anionic superoxide towards the SOD metal site through the tunnel pulled by the cationic amino acid residues – in turn the catalysis of $O_2^{\cdot-}$ dismutation by SOD occurs with a diffusion-limited rate constant $k_{cat}(O_2^{\cdot-}) \sim 10^9$ $M^{-1}$ $s^{-1}$ [12].

We aimed to synthesize Mn porphyrins with this $E_{1/2}$ and appropriate electrostatics. Our goal was to have a molecule that has cationic charges to guide anionic $O_2^{\cdot-}$ towards the Mn (Fig. 1). Unsubstituted Mn pyridyl porphyrin, MnT-2-PyP$^+$, has a very negative $E_{1/2}$ of $-280$ mV *vs* NHE. This value of $E_{1/2}$ tells us that the compound is too electron-rich to be able to accept an electron from superoxide and undergo reduction in the 1st step of the dismutation process [eq. (1)]. The presence of *ortho* pyridyl groups in the *meso* positions of MnT-2-PyP$^+$ (which could be quaternized with alkyl substituents) allowed us to achieve both goals. Once quaternized, the pyridyl nitrogens become cationic and the whole molecule gets 5 positive charges: 4 on pyridyl nitrogens and a single charge on the Mn center. The *ortho*(2) positions of cationic pyridyl nitrogens are closest to the Mn site and thus simultaneously: **(i)** attract anionic $O_2^{\cdot-}$ [13] and **(ii)** withdraw electron density from the Mn, making it electron deficient and ready to accept an electron from $O_2^{\cdot-}$. This so-called "*ortho*" effect results in a large shift of $E_{1/2}$ (500 mV) from $-280$ mV (for MnT-2-PyP$^+$) to $+220$ mV for its quaternized methyl analog MnTM-2-PyP$^{5+}$.

*Design of MnTM-2-PyP$^{5+}$ was a breakthrough in the development of Mn porphyrins*. Its ethyl analog, MnTE-2-PyP$^{5+}$ (AEOL10113, BMX-010),

mV *vs* NHE

+400

III
stage

II
stage

SOD enzyme
$E_{1/2} = \sim +300$ mV
$\log k_{cat} (O_2^{\cdot-}) = \sim 9$

+200

MnTnHex-2-PyP$^{5+}$
$E_{1/2} = +314$ mV
$\log k_{cat} (O_2^{\cdot-}) = 7.48$

MnTnBuOE-2-PyP$^{5+}$
$E_{1/2} = +277$ mV
$\log k_{cat}(O_2^{\cdot-}) = 7.83$

MnTE-2-PyP$^{5+}$
$E_{1/2} = +228$ mV
$\log k_{cat} (O_2^{\cdot-}) = 7.76$

0

I
stage

Superoxide self-dismutation

$O_2^{\cdot-} + 2H^+ + e^- \Longleftrightarrow H_2O_2$    $E_{1/2} = +890$ mV vs NHE

$O_2^{\cdot-} \Longleftrightarrow O_2 + e^-$    $E_{1/2} = -180$ mV vs NHE

-200

*ortho* position of *N vs* Mn site

MnT-2-PyP$^+$
$E_{1/2} = -280$ mV
$\log k_{cat} (O_2^{\cdot-}) = 4.29$

-400
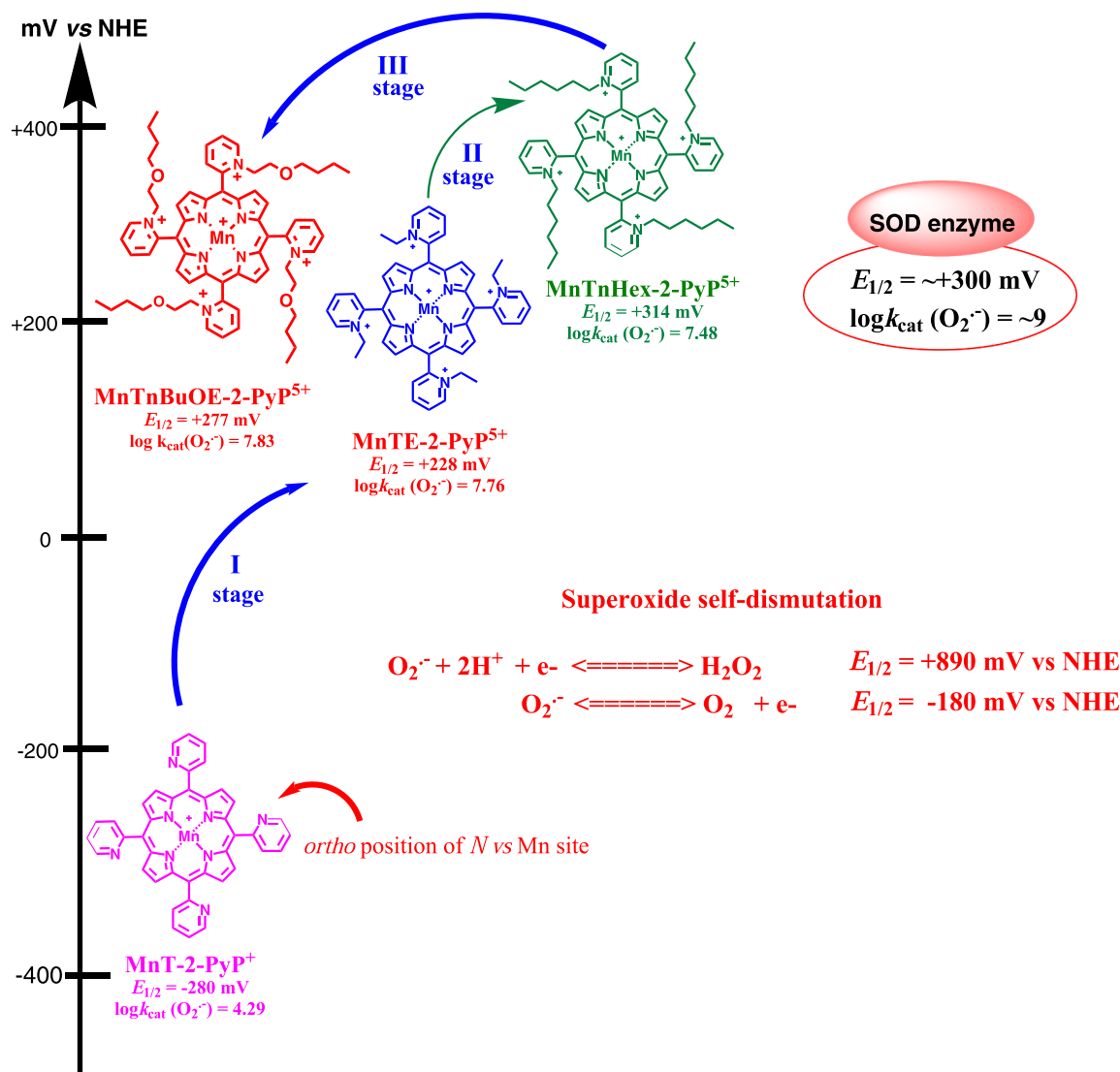
**Fig. 1. Design of the most powerful SOD mimics – *ortho*-substituted alkyl and alkoxyalkylpyridylporphyrins.** We aimed to mimic the catalysis of $O_2^{\cdot-}$ dismutation by the enzyme. The SOD enzyme catalyzes the $O_2^{\cdot-}$ dismutation at the potential that is right in between the potential for the reduction and oxidation of $O_2^{\cdot-}$, $\sim +300$ mV *vs* NHE. At that reduction potential there is an equal facilitation for the both steps of dismutation process, i.e. the enzyme reduces and oxidizes $O_2^{\cdot-}$ with identical rate constants [12]. The rate constants for the reduction and oxidation of $O_2^{\cdot-}$ were also identical in the case of a powerful SOD mimic, MnTE-2-PyP$^{5+}$ [15]. We started the development of SOD mimics from non-substituted Mn(III) *meso*-tetrakis (*N*-pyridinium-2-yl) porphyrin, MnT-2-PyP$^+$. The compound is overly stable in the +3 oxidation state and electron-rich. It does not readily accept an electron from $O_2^{\cdot-}$ which would have resulted in the reduction of the Mn to the +2 oxidation state. To increase the ability of the Mn center to accept an electron, the strategy was to reduce the electron density at the Mn site by substituting pyridyl nitrogens with alkyl groups (**stage I**). Once modified, the nitrogens become positively charged and in turn impose electron-withdrawing effects upon the Mn site, thereby reducing its electron richness. This modification introduces both a favorable thermodynamics (positive charges increase $E_{1/2}$ by 500 mV via the electron-withdrawing effect) and kinetics/electrostatics – positive charges attract negatively charged $O_2^{\cdot-}$. We first synthesized *N*-methylpyridylporphyrin, MnTM-2-PyP$^{5+}$, and subsequently its ethyl analog, MnTE-2-PyP$^{5+}$. The ethyl analog is a bulkier compound and, therefore, has decreased interactions with nucleic acids. The design of MnTE-2-PyP$^{5+}$ comprised a breakthrough in the development of Mn porphyrins and identified a critical role of the *ortho* positions of the nitrogens in the close vicinity of the Mn site. We were fortunate to have available another compound, MnBr$_8$T-2-PyP$^+$, that has no charges at the periphery and a single charge on the Mn site. The bromines on the pyrrolic rings exert an electron-withdrawing effect and adjust the $E_{1/2}$ to an essentially identical value ($E_{/12} = +219$ mV) as that of +228 for MnTE-2-PyP$^{5+}$. However, due to the lack of electrostatic facilitation for the approach of anionic $O_2^{\cdot-}$ towards the Mn site, MnBr$_8$T-2-PyP$^+$ has a very low SOD-like activity. The tetracationic charge at the periphery contributes to a 100-fold increase in $k_{cat}(O_2^{\cdot-})$ going from MnBr$_8$T-2-PyP$^+$ to MnTE-2-PyP$^{5+}$. The two other major stages in the development were the improvement in the lipophilicity of Mn porphyrins, giving rise to MnTnHex-2-PyP$^{5+}$ (**stage II**) and a decrease in the toxicity while maintaining the lipophilicity with MnTnBuOE-2-PyP$^{5+}$ (**stage III**).

was subsequently synthesized to increase the bulkiness of the molecule and suppress unfavorable interactions of MnP with the phosphate groups of nucleic acids. Interaction with nucleic acids is responsible for the reduced SOD-like activity of a planar *para*(4) analog (with pyridyl nitrogens farthest away from the Mn site), MnTM-4-PyP$^{5+}$ [14]. MnTE-2-PyP$^{5+}$ is still the most frequently studied compound (reviewed in [5]).

We have synthesized additional Mn porphyrin analogs to increase

bioavailability and decrease toxicity. MnTE-2-PyP$^{5+}$ is very hydrophilic and has a low ability to cross the blood brain barrier. We increased the lipophilicity of the molecule by quaternizing pyridyl nitrogens with longer alkyl chains while maintaining the *ortho* positions of the pyridyls – a property essential for appropriate $E_{1/2}$ and electrostatic facilitation for the approach of $O_2^{\cdot-}$. These analogs have an increased ability to cross the blood brain barrier; however, the increased lipophilicity was accompanied by increased toxicity [16]. Further

**Table 1**

**Oxidation and reduction reactions of Mn porphyrins with biological targets**. Reactions of MnPs with the following species were explored: superoxide [5], peroxynitrite [21,23], carbonate radical [21], hypochlorite [29], hydrogen peroxide [30], ascorbate [31], tetrahydrobiopterin [32], oxygen [32], nitric oxide [33], glutathione [26], sulfite [28], hydrogen sulfide (Olson, unpublished) and nitroxyl [27]. The ability to mimic glutathione peroxidase activity was also studied [5]. In the case of glutathione and cysteine, the spectrophotometric evidence demonstrated the one-electron reduction of $Mn^{III}P^{5+}$ into $Mn^{II}P^{4+}$ with GSH and formation of $GS^{\bullet}$ radical [5] and references therein. The reduction potential for the $O=Mn^{IV}P/Mn^{III}P$ redox couple, that would be involved in one-electron process, is 372 mV more positive than the reduction potential for the $O=Mn^{IV}TE\text{-}2\text{-}PyP^{4+}/Mn^{III}TE\text{-}2\text{-}PyP^{4+}$ redox couple that would be involved in a two-electron process (see Table 2). Thus, the $O=Mn^{IV}P/Mn^{III}P$ redox couple is more likely to be involved in GSH and protein cysteine oxidation. However, the involvement of the $O=Mn^{IV}TE\text{-}2\text{-}PyP^{4+}/Mn^{II}TE\text{-}2\text{-}PyP^{4+}$ redox couple cannot be excluded, so we have included the alternative two-electron processes in equations (23), (24), (30) and (31). Reactions in parentheses do not include the porphyrin, but support the porphyrin chemistry in the reactions above.

### Reduction of reactive species by MnPs

**Superoxide, $O_2^{\bullet-}$**

$$Mn^{II}P^{4+} + O_2^{\bullet-} + 2H^+ <===> Mn^{III}P^{5+} + H_2O_2 \qquad k_{red}(O_2^{\bullet-}) \qquad (2)$$

**Peroxynitrite, $ONOO^-$**

$$Mn^{III}P^{5+} + ONOO^- <===> O=Mn^{IV}P^{4+} + {}^{\bullet}NO_2 \qquad k_{red1}(ONOO^-) \qquad (3)$$
$$Mn^{II}P^{4+} + ONOO^- <===> O=Mn^{IV}P^{4+} + NO_2^- \qquad k_{red2}(ONOO^-) \qquad (4)$$

**Carbonate radical, $CO_3^{\bullet-}$**

$$Mn^{II}P^{4+} + CO_3^{\bullet-} <===> Mn^{III}P^{5+} + CO_3^{2-} \qquad k_{red}(CO_3^{\bullet-}) \qquad (5)$$
$$Mn^{III}P^{5+} + CO_3^{\bullet-} + H_2O <===> O=Mn^{IV}P^{4+} + CO_3^{2-} + 2\,H^+ \qquad k_{red}(CO_3^{\bullet-}) \qquad (6)$$

**Oxygen, $O_2$**

$$Mn^{II}P^{4+} + O_2 <===> Mn^{III}P^{5+} + O_2^{\bullet-} \qquad k_{red}(O_2) \qquad (7)$$

**Hydrogen peroxide, $H_2O_2$**

$$Mn^{III}P^{5+} + H_2O_2 <===> (O)_2Mn^{V}P^{3+} + 2H^+ \qquad k_{red}(H_2O_2) \qquad (8)$$
$$Mn^{II}P^{4+} + H_2O_2 <===> O=Mn^{IV}P^{4+} + H_2O \qquad k_{red}(H_2O_2) \qquad (9)$$

**Hypochlorite**

$$(H_2O)_2Mn^{III}P^{5+} + ClO^- <===> (O)_2Mn^{V}P^{3+} + Cl^- + H_2O + 2H^+ \quad k_{red}(ClO^-) \qquad (10)$$

### Oxidation of reactive species by MnPs

**Superoxide, $O_2^{\bullet-}$**

$$Mn^{III}P^{5+} + O_2^{\bullet-} <===> Mn^{II}P^{4+} + O_2 \qquad k_{ox}(O_2^{\bullet-}) \qquad (1)$$

**Ascorbate, $HA^-$**

$$O=Mn^{IV}P^{4+} + HA^- + 2H^+ <===> Mn^{III}P^{5+} + HA^{\bullet} (HA^{\bullet} ----> A^{\bullet-} + H^+) + H_2O \qquad k_{ox}(HA^-) \qquad (11)$$
$$Mn^{III}P^{5+} + HA^- <===> Mn^{II}P^{4+} + HA^{\bullet} (HA^{\bullet} ----> A^{\bullet-} + H^+) \qquad k_{ox}(HA^-) \qquad (12)$$

**Nitric oxide, $^{\bullet}NO$**

$$Mn^{III}P^{5+} + {}^{\bullet}NO <===> NO^+ + Mn^{II}P^{4+} \qquad k_{ox}({}^{\bullet}NO) \qquad (13)$$
$$Mn^{II}P^{4+} + {}^{\bullet}NO <===> ({}^{\bullet}NO)Mn^{II}P^{4+} \qquad k_{nitrosylation}(Mn^{II}P) \quad (14)$$
$$[({}^{\bullet}NO)Mn^{II}P]^{4+} <===> [(NO^+)Mn^{I}P^{3+}]^{4+}, \text{ reorganization of } ({}^{\bullet}NO)Mn^{II}P^{4+}$$

**Nitroxyl, $NO^-$**

$$Mn^{III}P^{5+} + HNO <===> ({}^{\bullet}NO)Mn^{II}P^{4+} + H^+ \qquad k_{ox}({}^{\bullet}NO), k_{nitroxylation}(Mn^{III}P) (15)$$

**Hydrogen peroxide, $H_2O_2$**

$$(O)_2Mn^{V}P^{3+} + H_2O_2 + 2H^+ <===> (H_2O)_2Mn^{III}P^{5+} + O_2 \qquad k_{ox}(H_2O_2) \qquad (16)$$
$$O=Mn^{IV}P^{4+} + H_2O_2 <===> Mn^{II}P^{4+} + O_2 + H_2O \qquad k_{ox}(H_2O_2) \qquad (17)$$

**Glutathione, GSH**

One-electron oxidation of GSH

$$Mn^{III}P^{5+} + GS^- <===> Mn^{II}P^{4+} + GS^{\bullet} \qquad k_{ox}(GS^-) \qquad (18)$$
$$O=Mn^{IV}P^{4+} + GS^- + 2H^+ <===> Mn^{III}P^{5+} + GS^{\bullet} + H_2O \qquad k_{ox}(GS^-) \qquad (19)$$
$$(O)_2Mn^{V}P^{3+} + GS^- + 2H^+ <===> O=Mn^{IV}P^{4+} + GS^{\bullet} + H_2O \qquad k_{ox}(GS^-) \qquad (20)$$

$$(GS^{\bullet} + GS^{\bullet} <===> GSSG)$$

Two-electron oxidation of GSH

$$O=Mn^{IV}P^{4+} + 2GS^- + 2H^+ \Longleftrightarrow Mn^{II}P^{4+} + GSSG + H_2O \qquad k_{ox}(GS^-) \qquad (21)$$
$$(O)_2Mn^VP^{3+} + 2GS^- + 4H^+ \Longleftrightarrow Mn^{III}P^{5+} + GSSG + 2H_2O \qquad k_{ox}(GS^-) \qquad (22)$$

Or

$$O=Mn^{IV}P^{4+} + GS^- + H^+ \Longleftrightarrow Mn^{II}P^{4+} + GSOH \qquad k_{ox}(GS^-) \qquad (23)$$
$$(O)_2Mn^VP^{3+} + GS^- + 3H^+ \Longleftrightarrow Mn^{III}P^{5+} + GSOH + H_2O \qquad k_{ox}(GS^-) \qquad (24)$$

$$(GSH + GSOH \Longleftrightarrow GSSG + H_2O)$$
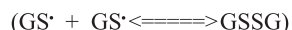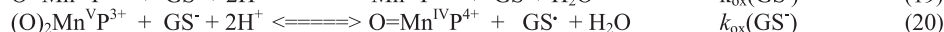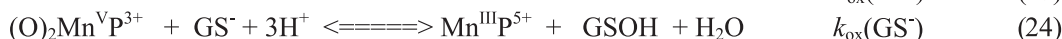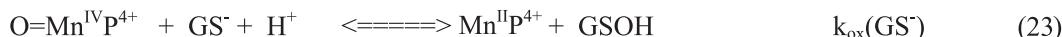
**Cysteine, Cys-SH**

One-electron oxidation of Cys-SH

$$Mn^{III}P^{5+} + Cys\text{-}S^- \Longleftrightarrow Mn^{II}P^{4+} + Cys\text{-}S^\cdot \qquad k_{ox}(Cys\text{-}S^-) \qquad (25)$$
$$O=Mn^{IV}P^{4+} + Cys\text{-}S^- + 2H^+ \Longleftrightarrow Mn^{III}P^{5+} + Cys\text{-}S^\cdot + H_2O \quad k_{ox}(Cys\text{-}S^-) \qquad (26)$$
$$(O)_2Mn^VP^{3+} + Cys\text{-}S^- + 2H^+ \Longleftrightarrow O=Mn^{IV}P^{4+} + Cys\text{-}S^\cdot + H_2O \quad k_{ox}(Cys\text{-}S^-) \qquad (27)$$

$$(Cys\text{-}S^\cdot + Cys\text{-}S^\cdot \Longleftrightarrow Cys\text{-}S\text{-}S\text{-}Cys)$$

Two-electron oxidation of Cys-SH

$$O=Mn^{IV}P^{4+} + 2Cys\text{-}S^- + 2H^+ \Longleftrightarrow Mn^{II}P^{4+} + Cys\text{-}S\text{-}S\text{-}Cys + H_2O \quad k_{ox}(Cys\text{-}S^-) \quad (28)$$
$$(O)_2Mn^VP^{3+} + 2Cys\text{-}S^- + 4H^+ \Longleftrightarrow Mn^{III}P^{5+} + Cys\text{-}S\text{-}S\text{-}Cys + 2H_2O \quad k_{ox}(Cys\text{-}S^-) \quad (29)$$

Or

$$O=Mn^{IV}P^{4+} + Cys\text{-}S^- + H^+ \Longleftrightarrow Mn^{II}P^{4+} + Cys\text{-}SOH \qquad k_{ox}(Cys\text{-}S^-) \qquad (30)$$
$$(O)_2Mn^VP^{3+} + Cys\text{-}S^- + 3H^+ \Longleftrightarrow Mn^{III}P^{5+} + Cys\text{-}SOH + H_2O \qquad k_{ox}(Cys\text{-}S^-) \qquad (31)$$

$$(Cys\text{-}SH + Cys\text{-}SOH \Longleftrightarrow Cys\text{-}S\text{-}S\text{-}Cys + H_2O)$$

**Sulfite, $SO_3^{2-}$**
$$Mn^{III}P^{5+} + SO_3^{2-} \Longleftrightarrow Mn^{II}P^{4+} + {}^\cdot SO_3^- \qquad k_{ox}(SO_3^{2-}) \qquad (32)$$
$$O=Mn^{IV}P^{4+} + SO_3^{2-} + 2H^+ \Longleftrightarrow Mn^{III}P^{5+} + {}^\cdot SO_3^- + H_2O \qquad k_{ox}(SO_3^{2-}) \qquad (33)$$

**Tetrahydrobioterin, $BH_4$**
$$Mn^{III}P^{5+} + BH_4 \Longleftrightarrow Mn^{II}P^{4+} + BH_4^{\cdot +} \qquad k_{ox}(BH_4) \qquad (34)$$

**Hydrogen sulfide, $H_2S$**
$$Mn^{III}P^{5+} + HS^- \Longleftrightarrow Mn^{II}P^{4+} + H_2S_2 \qquad k_{ox}(H_2S) \qquad (35)$$
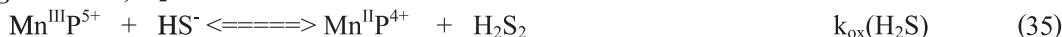
refinement of these analogs resulted in several Mn porphyrins that show a good balance between lipophilicity and toxicity in animal models. Of these, MnTnHex-2-PyP$^{5+}$ distributes at the highest levels to all organs and shows decreased toxicity induced by its micellar character compared to our initial lipophilic analogs. Due to its high bioavailability, MnTnHex-PyP$^{5+}$ has a better therapeutic window than MnTE-2-PyP$^{5+}$

[5]. We next inserted oxygen atoms into the hexyl chains and produced a compound, MnTnBuOE-2-PyP$^{5+}$, that is ~5-fold less toxic in an animal model [17]. Other properties of MnTnBuOE-2-PyP$^{5+}$, such as SOD-like potency and bioavailability, are similar to those of MnTnHex-2-PyP$^{5+}$. We recently modified Mn porphyrins by introducing fluorine atoms instead of hydrogen atoms into the alkyl chains. Two molecules,

monofluorinated ethyl-, MnTFE-2-PyP$^{5+}$, and the trifluorinated pentyl analog, MnTF$_3$Pen-2-PyP$^{5+}$, have been fully characterized and tested as radioprotectors and tumor radiosensitizers [18]. In efficacy studies where MnP analogs have been compared, those compounds that are the best performers with regards to all reactions thus far studied are exhibiting the best therapeutic potential in preclinical models [8,19,20].

## 3. Mn porphyrins act as oxidants and reductants

During the process of $O_2^{\cdot-}$ dismutation, the cationic Mn(III) *N*-substituted pyridylporphyrin must act as a reductant and oxidant of $O_2^{\cdot-}$. Adjusting the $E_{1/2}$ of the MnPs to mimic SOD activity in aqueous chemistry experiments, resulted in compounds that are equally good as oxidants and reductants under physiological conditions. In cells, MnPs act as both reductants and oxidants of $O_2^{\cdot-}$ ($O_2^{\cdot-}$ dismutation). However, this property also suggests they can act as either a reductant or an oxidant in reactions with other biologically relevant molecules in a cellular milieu. The in vivo reactions of MnPs may involve 4 biologically accessible oxidation states of manganese and may occur through one- or two-electron processes. The lower oxidation states are Mn$^{II}$P and Mn$^{III}$P, while high-valent Mn(IV) and Mn(V) oxidation states are the O=Mn$^{IV}$P and (O)$_2$Mn$^V$P species. The reaction of Mn$^{II}$P with $^{\cdot}$NO may even involve the Mn(I) oxidation state. Since SOD and MnPs have identical thermodynamics, theoretically they should be able to undergo the same type of reactions. That indeed is true. Yet, the protein quaternary structure of SOD is constructed to allow the access of only very small molecules such as superoxide to the active site. Thus, reactions with larger molecules occur with very low rate constants in the case of SOD. The most obvious example is the reduction of ONOO$^-$ by MnPs that occurs with a rate constant of $> 10^7 \, M^{-1} \, s^{-1}$ [21]. The reaction of SOD with ONOO$^-$ is, however, more than 2 orders of magnitude slower with the $k_{red}(ONOO^-)$ of $2.5 \times 10^4 \, M^{-1} \, s^{-1}$ (monomer) [22].

In over 2 decades of research we demonstrated that MnPs can undergo the myriad of biologically relevant oxidation and reduction reactions depicted below with equations (1,35); charges are omitted in the text but provided in the equations (Table 1). Following closely on the characterization of the reaction of MnPs with superoxide, was the discovery that peroxynitrite is a target of Mn porphyrins. The one electron reduction of peroxynitrite into the $^{\cdot}$NO$_2$ radical employing O=Mn$^{IV}$P/Mn$^{III}$P, or two electron reduction of peroxynitrite into nitrite, NO$_2^-$, employing either O=Mn$^{IV}$P/Mn$^{II}$P or (O)$_2$Mn$^V$P/Mn$^{III}$P, seems to be as rapid as catalysis of superoxide dismutation with a rate constant $> 10^7 \, M^{-1} \, s^{-1}$ [11,21,23]. The two-electron reduction of hypochlorite, ClO$^-$, into chloride, Cl$^-$, by MnPs occurs rapidly as well [24]. While not very potent catalysts of H$_2$O$_2$ dismutation, Mn

porphyrins can reduce and oxidize it into O$_2$ and H$_2$O employing the same MnP redox couples that are used in the two-electron reduction of ONOO$^-$. Mn$^{II}$P rapidly binds/oxidizes $^{\cdot}$NO, or $^{\cdot}$NO is oxidized by Mn$^{III}$P in a slower reaction to NO$^+$. Mn$^{III}$P gets reduced, via a one-electron process, with available cellular electron donors such as glutathione, ascorbate and tetrahydrobiopterin. Given the abundance and high rate constant of the reaction, the reduction of Mn$^{III}$P with ascorbate resulting in the formation of ascorbyl radical, may be preferred in vivo. The oxidation of ascorbate may also happen with high-valent O=Mn$^{IV}$P [25]. While the oxidation of GSH [26] and/or protein cysteines is not fast with Mn$^{III}$P, it may happen more rapidly with high-valent O=Mn$^{IV}$P or (O)$_2$Mn$^V$P [25]. The one-electron reduction of carbonate radical, CO$_3^{\cdot-}$ to carbonate anion, CO$_3^{\cdot\cdot-}$, may occur via O=Mn$^{IV}$/Mn$^{III}$P or Mn$^{III}$P/Mn$^{II}$P. Oxidation of nitroxyl, NO$^-$ into $^{\cdot}$NO radical has also been reported [27].

More recent data suggest MnPs can react with additional biologically relevant sulfur species: sulfite and hydrogen sulfide. MnPs are able to oxidize sulfite, SO$_3^{2-}$ into sulfite radical, $^{\cdot}$SO$_3^-$ [28] and sulfide, H$_2$S, into H$_2$S$_2$ (Olson, unpublished); both reactions involve a one-electron processes and, thus, can possibly involve the same couples which are involved in oxidation of ascorbate: Mn$^{III}$P/Mn$^{II}$P or O=Mn$^{IV}$P/Mn$^{III}$P (Olson, unpublished). These reactions are new additions to the repertoire of MnP reactions with biological targets. Because of the rich chemistry of Mn porphyrins and rich redox biology there are likely other reactions of Mn porphyrins that have not yet been explored.

## 4. Mn porphyrins which are the most powerful SOD mimics are the most reactive with other redox active species

We have performed comprehensive studies on MnP reactions with several species including superoxide dismutation, peroxynitrite reduction, hydrogen peroxide dismutation, ascorbate oxidation, lipid peroxidation and glutathione peroxidase (GPx) mimicking to determine either the rate constant or the initial rate. The relation between the rate constants, or the initial rates, and metal-centered reduction potential for Mn$^{III}$P/Mn$^{II}$P redox couple has, in all cases, an identical bell shape. In other words, the compounds which are the best performers as SOD mimics are also the best catalase and GPx mimics, reductants of ONOO$^-$ and oxidants of ascorbate [5].

This concept is well illustrated by our data measuring the ability of the MnPs to mimic GPx activity in aqueous solutions. As shown in Fig. 2, SOD- and GPx-like activities follow the same pattern: the most efficacious SOD mimics are the most potent mimics of GPx. The SOD-like activity of MnPs is expressed in log values of the catalytic rate constant for $O_2^{\cdot-}$ dismutation, log $k_{cat}(O_2^{\cdot-})$ [6]. The GPx-like activities
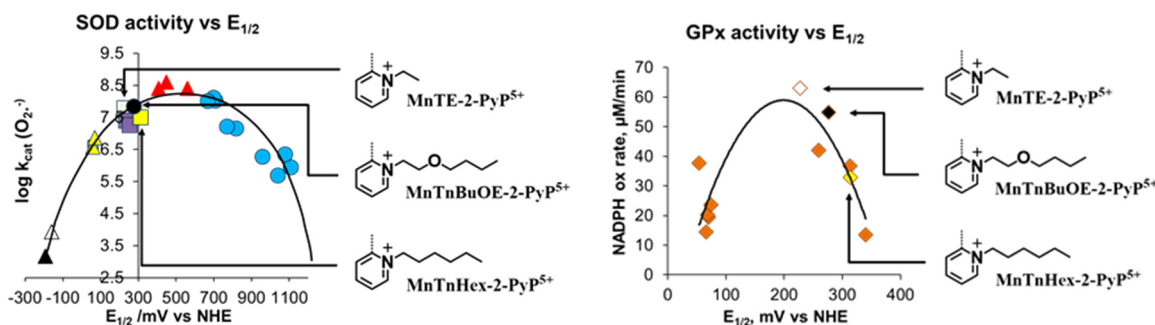


**Fig. 2.** Ability of cationic Mn(III) *N*-substituted pyridylporphyrins to mimic GPx activity is similar to their ability to mimic SOD activity. SOD-like activity is given by the log value of the catalytic rate constant for the $O_2^{\cdot-}$ dismutation, $k_{cat}(O_2^{\cdot-})$. GPx mimicking is described by the initial rates of NADPH oxidation [8]. Data are taken from refs [5,8] and relate to the following compounds with the $E_{1/2}$ values (in mV *vs* NHE) indicated in the parentheses. Such data allow the reader to locate these compounds on the plots: MnTBAP$^{3-}$ (-194), MnTSPP$^{3-}$ (-160), MnTE-2-PyPhP$^{5+}$ (-65), MnTM-3-PyP$^{5+}$ (+52), MnTE-3-PyP$^{5+}$ (+54), MnTM-4-PyP$^{5+}$ (+60), MnTnPr-3-PyP$^{5+}$ (+62), MnTnBu-3-PyP$^{5+}$ (+64), MnTnHex-3-PyP$^{5+}$ (+64), MnTMOE-3-PyP$^{5+}$ (+64), MnTnHex-4-PyP$^{5+}$ (+68), MnTMOHex-3-PyP$^{5+}$ (+68), MnTE-4-PyP$^{5+}$ (+70), MnTnOct-3-PyP$^{5+}$ (+74), MnTM-2-PyP$^{5+}$ (+220), MnTE-2-PyP$^{5+}$ (+228), MnTnPr-2-PyP$^{5+}$ (+238), MnTnBu-2-PyP$^{5+}$ (+254), MnTPhE-2-PyP$^{5+}$ (+259), MnTnBuOE-2-PyP$^{5+}$ (+277), MnTnHexOE-2-PyP$^{5+}$ (+313), MnTnHex-2-PyP$^{5+}$ (+314), MnCl$_3$TE-2-PyP$^{5+}$ (+408), MnCl$_4$TE-2-PyP$^{5+}$ (+448), MnCl$_5$TE-2-PyP$^{5+}$ (+560), Mn corroles (+670 to +1110).

**Table 2**
**Reduction potentials of different species of relevance to the discussion of the biological effects of MnPs** . Reduction potential values (mV vs NHE) and table design are from Buettner's 1993 article on the pecking order of free radicals and antioxidants [35] and references therein with the addition of the reduction potential values for the MnPs from [b]Weitner et al. [36] and from [c]Batinic-Haberle et al. [6].

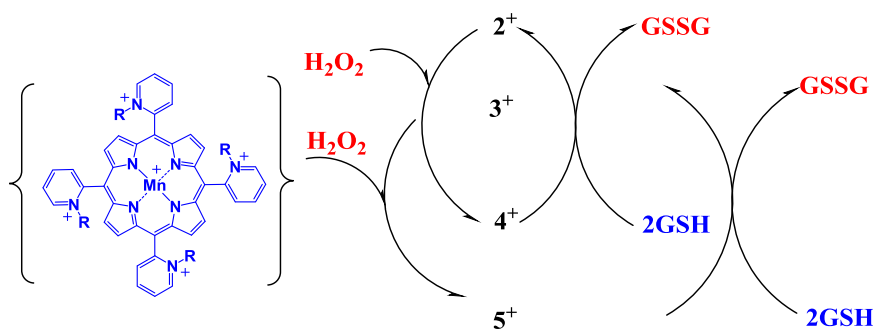| Couple | $E^{0'}$/mV vs NHE[a] |
|---|---|
| $HO^{\cdot}, H^{+}/H_2O$ | + 2310 |
| $O_2^{\cdot-}, 2H^{+}/H_2O_2$ | + 940 |
| $O=Mn^{IV}TE\text{-}2\text{-}PyP^{4+}/Mn^{III}TE\text{-}2\text{-}PyP^{5+,b}$ | + 889 |
| $O=Mn^{IV}TE\text{-}2\text{-}PyP^{4+}/Mn^{II}TE\text{-}2\text{-}PyP^{4+,b}$ | + 517 |
| $\alpha$-tocopheroxyl$^{\cdot}$, $H^{+}/\alpha$-tocopherol (TO$^{\cdot}$, $H^{+}$/TOH (Vitamin E) | + 500 |
| $H_2O_2, H^{+}/H_2O, {}^{\cdot}OH$ | + 320 |
| ascorbate$^{\cdot}$, $H^{+}$/ascorbate monoanion (vitamin C) | + 282 |
| ferricytochrome $c$/ferrocytochrome $c$ | + 260 |
| $Mn^{III}TE\text{-}2\text{-}PyP^{5+}/Mn^{II}TE\text{-}2\text{-}PyP^{4+,c}$ | + 228 |
| ubiquinone, $H^{+}$/semiubiquinone (CoQ/CoQ$^{\cdot}$) | − 36 |
| $O_2/O_2^{\cdot-}$ | − 330 |
| RSSR/RSSR$^{\cdot}$ (cystine or glutathione disulfide, GSSG) | − 1500 |

[a] pH = 7.0 for all measurements except those for the MnPs which were determined at pH = 7.4.

are expressed as initial rates of NADPH oxidation [8]. This property of the MnPs indicates that we can't assume that MnPs will act only as SOD mimics in vivo.

## 5. Cellular environment determines which species react with MnPs

The electrostatic properties of the MnPs, combined with the lack of steric constraints that are characteristic of the SOD enzyme, increases the likelihood that the observed biological effects of MnPs can be due to non-SOD reactions. Favorable electrostatics contribute vastly to the reactivity of cationic MnPs with anionic reactive species. In the case of superoxide, electrostatics contributes two orders of magnitude to the increase in the rate constant with pentacationic MnTE-2-PyP$^{5+}$ relative to monocationic MnBr$_8$T-2-PyP$^+$ [13]. Many, if not all, reactive species that MnP encounters are predominantly anionic at a physiological pH of 7.8, e.g., peroxynitrite, ascorbate, sulfite and carbonate. For some reactive species, even if the concentration of the protonated form is high, the portion of that species that is deprotonated will still react with MnPs. Reactive species with this characteristic include thiols and H$_2$O$_2$. In addition, while superoxide dismutation occurs mostly via an outer-sphere reaction [13], reactions with other molecules such as ONOO$^-$ and $^{\cdot}$NO involve binding to the Mn site. The binding is highly preferred because the Mn center is electron-deficient and readily binds an electron-donating ligand such as ONOO$^-$ [34]. These properties increase the complexity of the potential reactions in vivo.

The aqueous chemistry data in Table 1 indicate that the MnPs have favorable rate constants for reactions with a myriad of biologically relevant redox active molecules. The aqueous chemistry reactions summarized in Table 1 measured reactions when only a few reactants
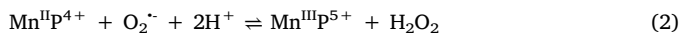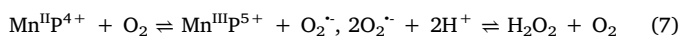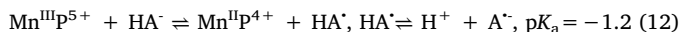
were present. It indicates which species *can* react with MnPs. However, in the cell, things are much more complex; there are multiple reactive species at varying concentrations in the milieu. This means that the species most likely to react with the MnPs will depend not only on the chemical properties of the reaction, but also on the cellular environment where those species will interact. For example, if there is more H$_2$O$_2$ or ONOO$^-$ than superoxide, the MnP will be more likely to react with them than superoxide. The relative amount of reactant species and the rate of the reaction with that particular species will determine which reactions occur. In cells, the key question is thus, given a certain milieu, which species *do* react with MnPs? For therapeutic purposes, the answer to this question will indicate pathologies where MnPs can be effective. It will also allow us to identify biomarkers that indicate treatment efficacy.

## 6. Mn porphyrins act as catalytic redox cycling agents in biological systems

The second property of the MnPs that it is critical to consider when they are used in vivo is that the $E_{1/2}$ values that make the MnPs equally able to act as oxidants and reductants in biological systems position them well to act as catalytic redox cycling agents in cells. In Gary Buettner's "pecking order" of redox active molecules in cells [35], the redox potential of the Mn(III)/Mn(II) redox couple [36] sits close to that of cytochrome $c$ (Table 2). When MnPs are added to cells, the Mn is in the Mn(III) state, but readily gets reduced to Mn(II). In Buettner's "pecking order," molecules higher in the order are stronger oxidants, i.e. likely to take electrons from molecules listed lower in the order. The reaction of MnPs with H$_2$O$_2$, as shown in Fig. 3, is an example of what occurs at the Mn center as the MnPs react with biological oxidants in cells. H$_2$O$_2$ is reduced by MnP while the Mn in the porphyrin is oxidized via a two-electron process to either O=Mn(IV) [starting from Mn(II)] or (O)$_2$Mn(V) [starting from Mn(III)]. In this state the MnPs are now strong oxidants; they move up in the "pecking order" (Table 2) so there are a variety of abundant biological reductants from which they can take electrons. They will oxidize biological reducing agents, particularly glutathione. This results in regeneration of the MnP in either the Mn(II) or Mn(III) state and oxidation of two molecules of glutathione. The MnPs are now available to perform another cycle. Redox cycling in the cellular milieu causes the removal of oxidants concomitant with the consumption of reducing equivalents. Consumption of reducing equivalents, particularly oxidation of the glutathione pool will have biological effects because it taps into the regulatory mechanisms affected by the cellular redox environment.

The example shown in Fig. 3, is the redox cycling with H$_2$O$_2$ and glutathione. This process works with other oxidants including peroxynitrite and superoxide and other reductants including protein cysteines and ascorbate. This redox cycling is the basis of the therapeutic potential of the combination of ascorbate/MnPs for the treatment of tumors. In this case, H$_2$O$_2$ is produced via cycling of MnP with ascorbate, HA$^-$(equations (12), (7) and (2)). Production of H$_2$O$_2$ is required to increase tumor cell death; the addition of extracellular catalase



**Fig. 3. Redox cycling of MnP with H$_2$O$_2$**. MnP gets oxidized with H$_2$O$_2$ when it is either in the + 2 or + 3 oxidation state to Mn + 4 or Mn + 5 oxidation state in a 2-electron process. MnP can get reduced back to a lower oxidation state with GSH where GSH is oxidized to GSSG. R relates to either alkyl or alkoxyalkyl groups on Mn porphyrin.

abolished the effect [31,37].

$$Mn^{III}P^{5+} + HA^- \rightleftharpoons Mn^{II}P^{4+} + HA^\bullet, HA^\bullet \rightleftharpoons H^+ + A^{\bullet-}, pK_a = -1.2 \quad (12)$$

$$Mn^{II}P^{4+} + O_2 \rightleftharpoons Mn^{III}P^{5+} + O_2^{\bullet-}, 2O_2^{\bullet-} + 2H^+ \rightleftharpoons H_2O_2 + O_2 \quad (7)$$

$$Mn^{II}P^{4+} + O_2^{\bullet-} + 2H^+ \rightleftharpoons Mn^{III}P^{5+} + H_2O_2 \quad (2)$$

The ability of the porphyrins to redox cycle in this way means that the reactions with cellular targets are amplified, i.e. they are catalytic. In other words, a low concentration of MnP is capable of performing a large number of reactions. For therapeutic purposes, the ability to act as a catalyst means that the small amounts remaining as the drug is cleared (from the plasma and tissues) can still have an effect.

## 7. Therapeutic effects of the most powerful Mn porphyrin-based SOD mimics

We have summarized most of the therapeutic effects of Mn(III) *N*-alkyl- or alkoxyalkypyridylporphyrins in several recent reviews [5,19,20]. In Fig. 4, we have listed the studies that directly promoted progress of MnPs into 5 clinical trials. Mn porphyrins were very efficacious in numerous ischemia/reperfusion injuries of organs such as liver, kidney, stroke, subarachnoid hemorrhage, cerebral palsy, and etc. Recent data indicates MnPs have therapeutic potential in cardiac disorders which may be relevant as such disorders occur as side effects of cancer chemotherapy. Ferrari's team showed MnPs act as a suppressor of sclerotic events in aortic valves [38]. Another cardiac application of

MnPs, demonstrated by Gomes's team, is based on their ability to suppress arrhythmias thereby preserving heart contractile function [39]. MnPs are excellent suppressors of tumor growth particularly in the presence of chemotherapy, radiotherapy and ascorbate. The data also indicate another attribute of the MnPs during cancer treatment. During cancer radio- and chemotherapy normal tissue gets damaged. As of now there is no efficacious clinically available radioprotectant of normal tissue. MnPs showed an impressive ability as agents for radio- and chemoprotection in various normal tissues. Such efficacy enabled their progression into 4 clinical trials where MnTnBuOE-2-PyP$^{5+}$ is being tested for its ability to radioprotect the brain, lower pelvic region, salivary glands and mouth mucosa. Ability to protect pancreatic cells during transplant enabled MnTE-2-PyP$^{5+}$ to progress into a clinical trial for the protection of β cells. MnTE-2-PyP$^{5+}$ suppressed oxidative stress but has not improved the success of the transplant [40]. The pre-clinical data on MnTnBuOE-2-PyP$^{5+}$ for the same application looks promising due to its enhanced ability to protect β cells and improve transplant success in an animal model [41]. Based on its efficacy in animal models [5,42], MnTE-2-PyP$^{5+}$ is now being tested in a clinical trial for atopical dermatitis and itch [43,44].

Most frequently, MnPs have been administered subcutaneously. This method of delivery of MnTE-2-PyP$^{5+}$, MnTnHex-2-PyP$^{5+}$ and MnTnBuOE-2-PyP$^{5+}$ results in the distribution to all organs [5,45]. The more lipophilic MnTnBuOE-2-PyP$^{5+}$ distributes to much higher levels in all organs than does MnTE-2-PyP$^{5+}$; the difference is the largest with regards to their accumulation in the brain [45]. Such an increase in

## Therapeutic effects drive progress of Mn porphyrins into clinical trials
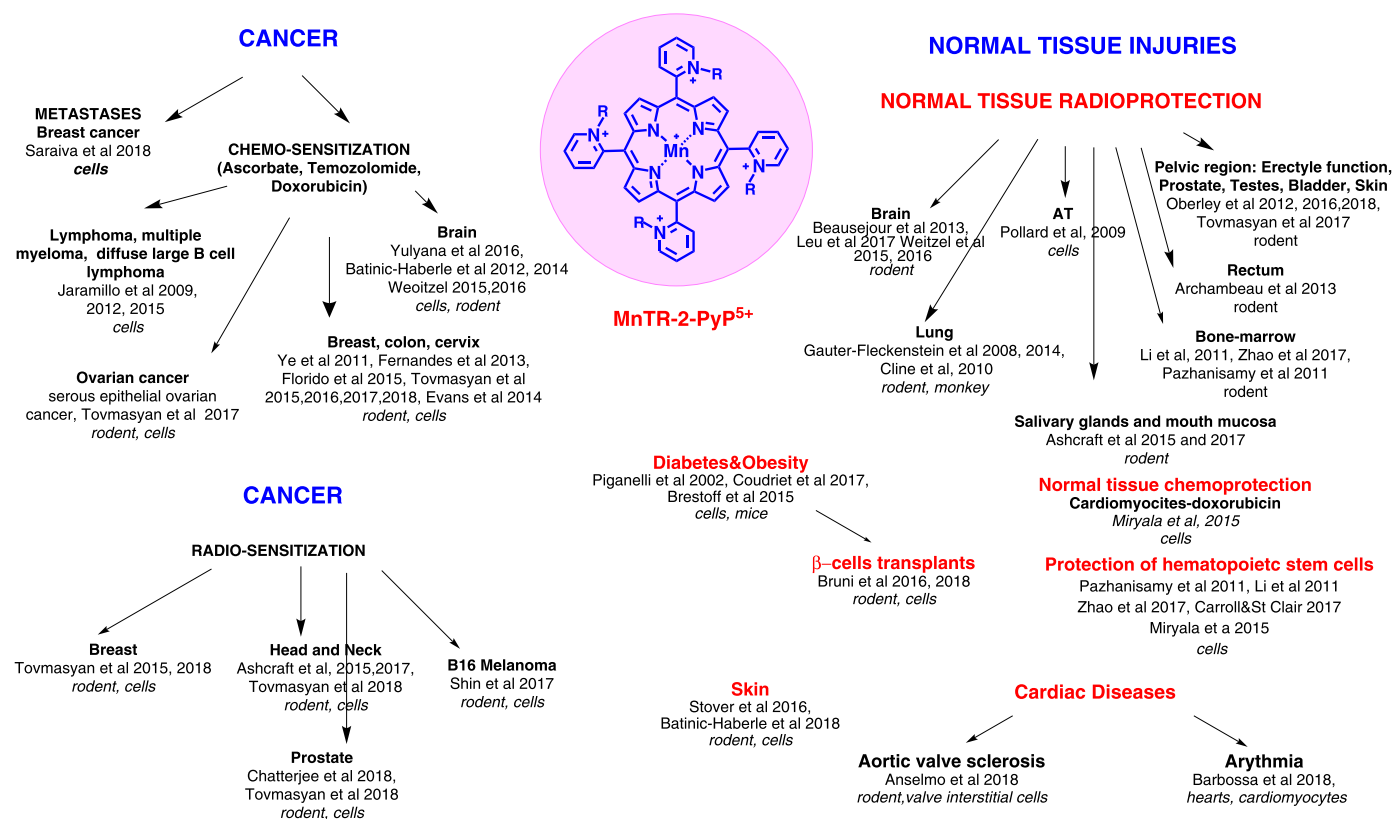


**Fig. 4. Therapeutic effects of Mn(III) *N*-substituted alkyl- and alkoxyalkylpyridylporphyrins which were the major force for driving MnPs into clinical trials**. The figure summarizes those effects of Mn porphyrins on healing of the normal cells/tissues while suppressing tumor growth which contribute to their progress into the clinic. Four clinical trials test the ability of MnTnBuOE-2-PyP$^{5+}$ to radioprotect normal tissue with cancer patients. One trial is testing MnTE-2-PyP$^{5+}$ on atopical dermatitis and itch [42,44]. R stands for the *N*-pyridyl substituent. It is either an alkyl substituent with carbon atoms ranging from 2 in ethyl (MnTE-2-PyP$^{5+}$, AEOL10113, BMX-010), to 6 in hexyl (MnTnHex-2-PyP$^{5+}$), or an alkoxyalkyl (butoxyethyl) substituent with 6 carbons and 1 oxygen atom in MnTnBuOE-2-PyP$^{5+}$ (BMX-001) (see Fig. 1). For other therapeutic effects and for a detailed description of those effects listed in Fig. 4 see our reviews [5,19,20] and references therein.

bioavailability manifests itself in up to a 50-fold difference in the dosing of the butoxyethyl *vs* the ethyl compound. Importantly, the data provided by Warner and Sheng in a middle cerebral artery occlusion stroke model show that if ethyl, hexyl and butoxyethyl porphyrins reach the targeted site (given intracerebroventricularly), the therapeutic efficacies are nearly identical due to their similar redox properties [5]. Favorable pharmacokinetics and pharmacodynamics have also been seen for delivery of the MnPs intravenously, intraperitoneally, orally and via lavage in animal models [43,46–48].

## 8. Oxidant effect of MnPs in vivo

The initial studies where MnPs were added to mammalian cells made the assumption that these compounds were acting as SOD mimics in the cellular milieu. However, several studies indicate that, although treatment with MnPs has the expected outcome, the major effect of MnPs is not as mimics of SOD in vivo. In retrospect this is not surprising given the multitude of biological redox active compounds that can react with the MnPs in aqueous solution. What has emerged from these studies is the indication that the pro-oxidant/redox cycling activity plays a critical role in the biological effects, particularly in the therapeutic potential the MnPs show in cancer.

The very first data that suggests mimicking SOD may not be the major action of MnPs came from a study from Piganelli's team. Their study, using a diabetes model, suggested that MnP may not act as a reductant but as an oxidant. The authors assigned the effect of MnP to the oxidation of a cysteine in the p50 subunit of NF-κB that prevented NF-κB binding to nuclear DNA [49]. Other evidence comes from use of MnTnHex-2-PyP$^{5+}$ in a rat kidney ischemia/reperfusion injury model. Dorai's team showed that MnSOD and EC-SOD were upregulated in addition to numerous other endogenous antioxidant defenses [50] (Fig. 5). These data raise the question: *why would MnP upregulate MnSOD if it acts as an SOD enzyme in its own right?*

Our work in several lymphoma models suggests that the ability of the MnPs to enhance the chemotherapeutic response is due to the redox cycling (oxidant and reductant) activity of the MnPs [51–53]. Treatment of the cells with MnPs in the presence $H_2O_2$ generated from chemotherapeutic agents results in oxidation of the cytosolic and mitochondrial redox environments, oxidation of the glutathione pool, and an increase in MnSOD protein. The data demonstrate that the major in vivo action of Mn porphyrins is most likely the oxidation and/or S-glutathionylation of cysteines of proteins in a GPx-like manner in the presence of $H_2O_2$ and glutathione. We found no evidence of an increase in SOD-like activity in the intact cells treated with MnPs [52]. In addition to S-glutathionylation and inhibition of the p65 subunit of NF-κB, we showed that MnP catalyzes S-glutathionylation of mitochondrial respiratory complexes I, III and IV. Some proteins of glycolysis were also S-glutathionylated [53] (Fig. 6). The initial studies looked at the effects of MnTE-2-PyP$^{5+}$, but similar effects were subsequently seen with MnTnBuOE-2-PyP$^{5+}$ in lymphoma and multiple myeloma cell culture models [54] (Tome, unpublished data).

Subsequently, a redox proteomics study was performed using the mouse breast cancer 4T1 cells which were exposed to ascorbate/MnP cycling (MnP = MnTE-2-PyP$^{5+}$) to produce $H_2O_2$. Numerous proteins showed peptidyl cysteine oxidation, suggesting that S-glutathionylation of protein cysteine may be indeed a major in vivo action of Mn porphyrins. This treatment affected 3605 peptidyl cysteines (Cys) in total, out of which 1577 were oxidized 1.3-fold or higher compared with control, untreated samples. Distribution analyses of MnP/ascorbate-induced oxidation of peptidyl Cys show that > 50% of oxidized peptidyl Cys bear a 1.3–2.0-fold increase in oxidation level. The MnP/ascorbate system affected 1762 proteins in total, among which 942 proteins were associated with a > 1.3-fold oxidized peptidyl Cys. Analysis of potential functional pathways revealed that MnP/ascorbate-driven oxidation of peptides is associated with regulation of cytoskeleton rearrangement, transcription/mRNA processing, translation, protein folding, cell cycle and adhesion. The data show that the NF-κB pathway was the major one affected. A number of endogenous antioxidant defense proteins had oxidized peptidyl cysteines as well as MAPK p38, p38α MAPK, protein kinase C (σ and τ) and Keap1 (Fig. 7) [55].

Most recently, a cellular and mouse 4T1 breast cancer study was conducted. Mice were treated with MnP in combination with ascorbate and radiation – a powerful source of $H_2O_2$. The redox status of the cell, the GSSG/2GSH ratio, was analyzed in cells from both tumor and non-tumor tissue. The increase in the GSSG/2GSH ratio was found only under triple treatment (MnP/ascorbate/radiation) and was accompanied by the increase in total S-glutathionylated proteins in tumor tissue (which received triple treatment) but not in non-irradiated tissues [8]. Huang's team also found an increase in total S-glutathionylated proteins in mouse cortex treated with radiation (5 Gy) and MnTnBuOE-2-PyP$^{5+}$, but not in non-irradiated liver [45].

Evidence continues to mount that an oxidant effect of the MnPs is responsible for the observed in vivo outcome in multiple model systems. Most recently, St. Clair's group reported that MnTnBuOE-2-PyP$^{5+}$ treatment activated the Nrf2 pathway in hematopoietic stem cells which resulted in the upregulation of MnSOD, catalase, glutathione S-
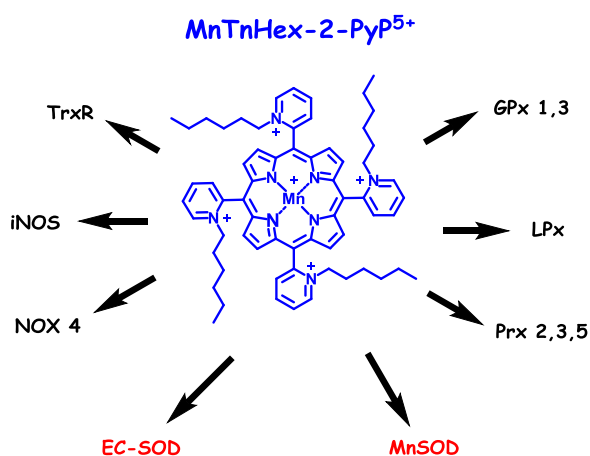


**Fig. 5. MnTnHex-2-PyP$^{5+}$ upregulates numerous endogenous antioxidants in a kidney ischemia/reperfusion, model**. Left renal artery and vein were clamped for 40 min which was followed by 48 h of reperfusion. The renoprotective cocktail, that contained MnTnHex-2-PyP$^{5+}$ (0.1 mg/kg/day) in addition to growth hormones (50 ng/kg/day) and mitochondria protecting compounds (125 μmol/kg/day), was given at −24, 0 and +24 h after surgery [50]. PCR analysis for oxidative stress genes was performed.
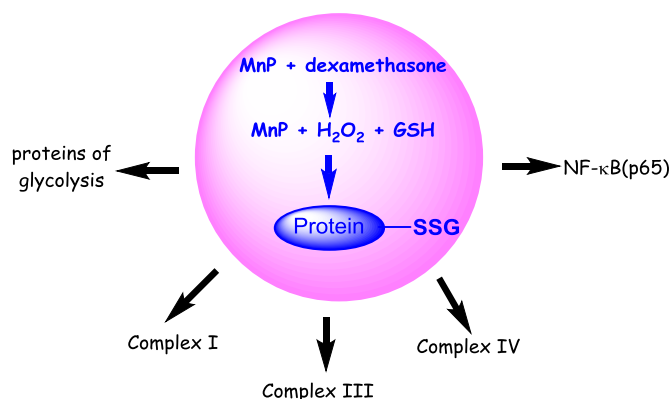


**Fig. 6. S-glutathionylation of proteins in a lymphoma study by MnP/dexamethasone**. A combined treatment of MnTE-2-PyP$^{5+}$/dexamethasone produces larger amounts of $H_2O_2$ than either individually. MnP then uses $H_2O_2$ and GSH to S-glutathionylate cysteines of key proteins via process depicted in equations (8),(9), (36–42). When either GSH synthesis is inhibited, or catalase overexpressed the extent of S-glutathionylation was insignificant [52,53].
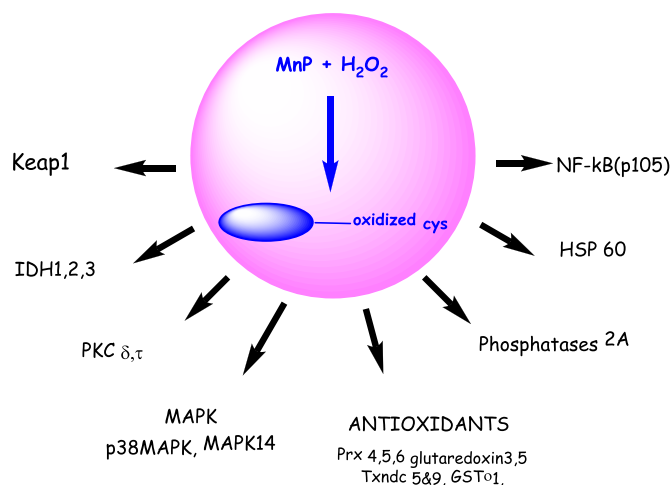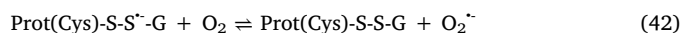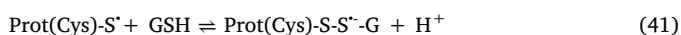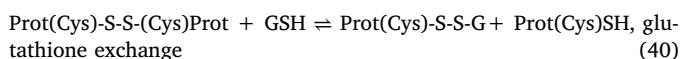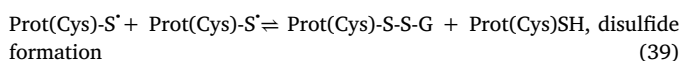
Fig. 7. Redox proteomics indicate numerous proteins in 4T1 breast cancer cells had oxidized peptidyl cysteines after MnP/ascorbate treatment. Only *some* of the oxidized proteins are listed here. Cells were treated for 4 h in a growth medium with 5 μM MnP (MnTE-2-PyP$^{5+}$) and 1 mM ascorbate [55] (Tovmasyan et al., in preparation). MnP/ascorbate is a $H_2O_2$ producing system; see equations (2), (7) and (12) [5,8,31].

transferase pi 1 and mitochondrial uncoupling protein 3 [56]. The studies summarized in this section have used different MnP analogs, yet they have observed an oxidant effect. Given the similar redox properties and bioavailabilities as well as numerous in vitro and in vivo data published using multiple Mn(III) *N*-substituted alkyl- and alkoxyalkylpyridylporphyrins analogs [5,19], it is highly likely that these analogs have similar reactions. The ability of the MnP analogs to act as oxidants should prove advantageous for some clinical applications, particularly the treatment of cancer.

## 9. S-glutathionylation of protein cysteines catalyzed by MnPs – chemistry

One of the biological effects of MnP treatment is a combination of protein cysteine (Prot(Cys)-SH) oxidation (Prot(Cys)-S-S-(Cys)Prot) and protein S-glutathionylation (Prot(Cys)-S-S-G). S-glutathionylation of proteins occurs when glutathione is linked to exposed protein cysteines. For the linkage to occur either protein cysteine or glutathione needs to be oxidized [57] (Fig. 8). This is a reversible reaction. In cells, addition of the glutathione group occurs through spontaneous or en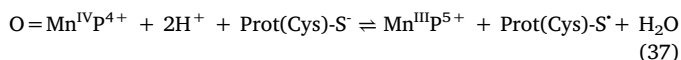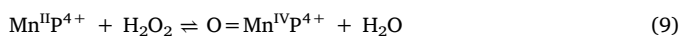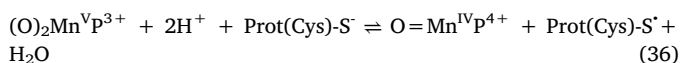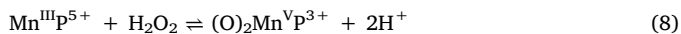zyme catalyzed reactions [58]. Removal of the glutathione group from the protein is enzymatic via glutaredoxin, thioredoxin or sulfiredoxin [59]. The MnPs can also catalyze the addition of glutathione to protein cysteines via reactions (8), (9), and (36–42).

$$Mn^{III}P^{5+} + H_2O_2 \rightleftharpoons (O)_2Mn^VP^{3+} + 2H^+ \qquad (8)$$

$$(O)_2Mn^VP^{3+} + 2H^+ + Prot(Cys)\text{-}S^- \rightleftharpoons O{=}Mn^{IV}P^{4+} + Prot(Cys)\text{-}S^{\bullet} + H_2O \qquad (36)$$

$$Mn^{II}P^{4+} + H_2O_2 \rightleftharpoons O{=}Mn^{IV}P^{4+} + H_2O \qquad (9)$$

$$O{=}Mn^{IV}P^{4+} + 2H^+ + Prot(Cys)\text{-}S^- \rightleftharpoons Mn^{III}P^{5+} + Prot(Cys)\text{-}S^{\bullet} + H_2O \qquad (37)$$

$$Mn^{III}P^{5+} + Prot(Cys)\text{-}S^- \rightleftharpoons Mn^{II}P^{4+} + Prot(Cys)\text{-}S^{\bullet} \qquad (38)$$

Prot(Cys)-S$^{\bullet}$ + Prot(Cys)-S$^{\bullet}$ ⇌ Prot(Cys)-S-S-G + Prot(Cys)SH, disulfide formation (39)

Prot(Cys)-S-S-(Cys)Prot + GSH ⇌ Prot(Cys)-S-S-G + Prot(Cys)SH, glutathione exchange (40)

$$Prot(Cys)\text{-}S^{\bullet} + GSH \rightleftharpoons Prot(Cys)\text{-}S\text{-}S^{\bullet-}\text{-}G + H^+ \qquad (41)$$

$$Prot(Cys)\text{-}S\text{-}S^{\bullet-}\text{-}G + O_2 \rightleftharpoons Prot(Cys)\text{-}S\text{-}S\text{-}G + O_2^{\bullet-} \qquad (42)$$

The MnPs, with the ability to catalyze this reaction, can alter the balance between the modified and unmodified proteins. This results in an ability to modify redox signaling pathways throughout the cell.

## 10. Biological effects of protein cysteine oxidation/protein S-glutathionylation

The cysteine oxidation/protein S-glutathionylation cycle has recently received a lot of attention for its ability to act as a redox switch in cells. There are a number of excellent recent reviews that discuss redox regulation via thiol switches and S-glutathionylation in depth [57,59–67] among others. The purpose of this review is not to extensively review this field. Here we are focusing on several concepts from this field that could pertain to the observed biological effects of MnPs. In general, protein cysteine oxidation/S-glutathionylation of proteins inhibits their activity. It provides redox regulatory mechanisms at several levels. In a microenvironment it can provide an on/off switch for an individual protein as depicted in Fig. 8. By altering activities of specific proteins, it can modify specific signaling pathways. Examples of this type of regulation include cysteine oxidation of the YVH1 phosphatase [68], S-glutationylation of MEKK1 to regulate the MAPK pathways [69] and cysteine oxidation and S-glutathionylation of NF-κB to inhibit DNA binding [49,52] and references therein. The growing list of proteins that are regulated by S-glutathionylation suggests that an agent that causes widespread S-glutathionylation, as the cycling MnPs have to potential to do, can tap into the global network of redox sensitive pathways to regulate overall biological processes in the cell. Dean Jones has hypothesized that global redox regulation occurs when the thiol redox cycles are disrupted [66]. In addition, he suggested that
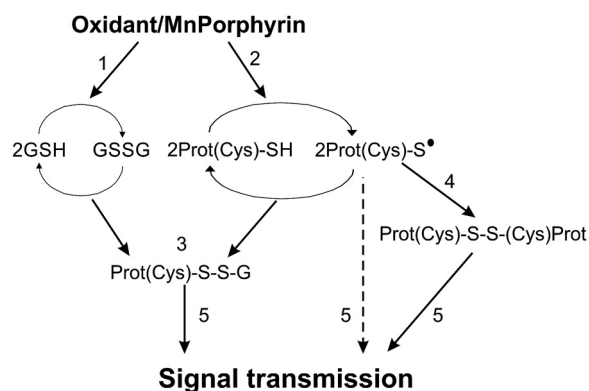


Fig. 8. Porphyrins impact signal transmission via cycling with thiols. This figure is modified from Fig. 1 in Winterbourn and Hampton [57] to illustrate how MnPs can alter protein cysteine oxidation/protein S-glutathionylation signaling networks. MnP redox cycles with oxidants to **1)** increase glutathione oxidation from GSH to GSSG (equations (18–24)), and **2)** protein cysteine oxidation from Prot(Cys)-SH to Prot(Cys)-S$^{\bullet}$ radical (equations (25) to (31)). Oxidation of glutathione and/or protein cysteines results in **3)** protein S-glutathionylation (equations (8), (9), and (36–42)). Oxidation of protein cysteines can also result in **4)** disulfide bond formation (equation (39)). Proteins that are S-glutathionylated, have oxidized cysteines or altered disulfide bond structure can have altered activities or abilities to bind other biomolecules. The changes in activity/binding **5)** alter signal transmission which taps into global redox signaling networks. We have assumed here that MnP oxidizes GSH and protein cysteine via a one-electron process. The justification for this assumption is the spectrophotometric evidence we provided where reduction of Mn$^{III}$P$^{5+}$ with GSH gave rise to the one-electron product, Mn$^{II}$P$^{4+}$, accompanied by the formation of GS$^{\bullet}$ radical [5] and references therein. With highly oxidizing Mn(IV) and Mn(V) oxo species (O$=$Mn$^{IV}$P$^{4+}$ and (O)$_2$Mn$^V$P$^{3+}$) the formation of two-electron products, GSOH or cysteine sulfenic acid, Cys-SOH, may occur (equations (23, 24), and (30, 31)). This also applies to the formation of protein cysteine sulfenic acid Prot(Cys)-SOH.
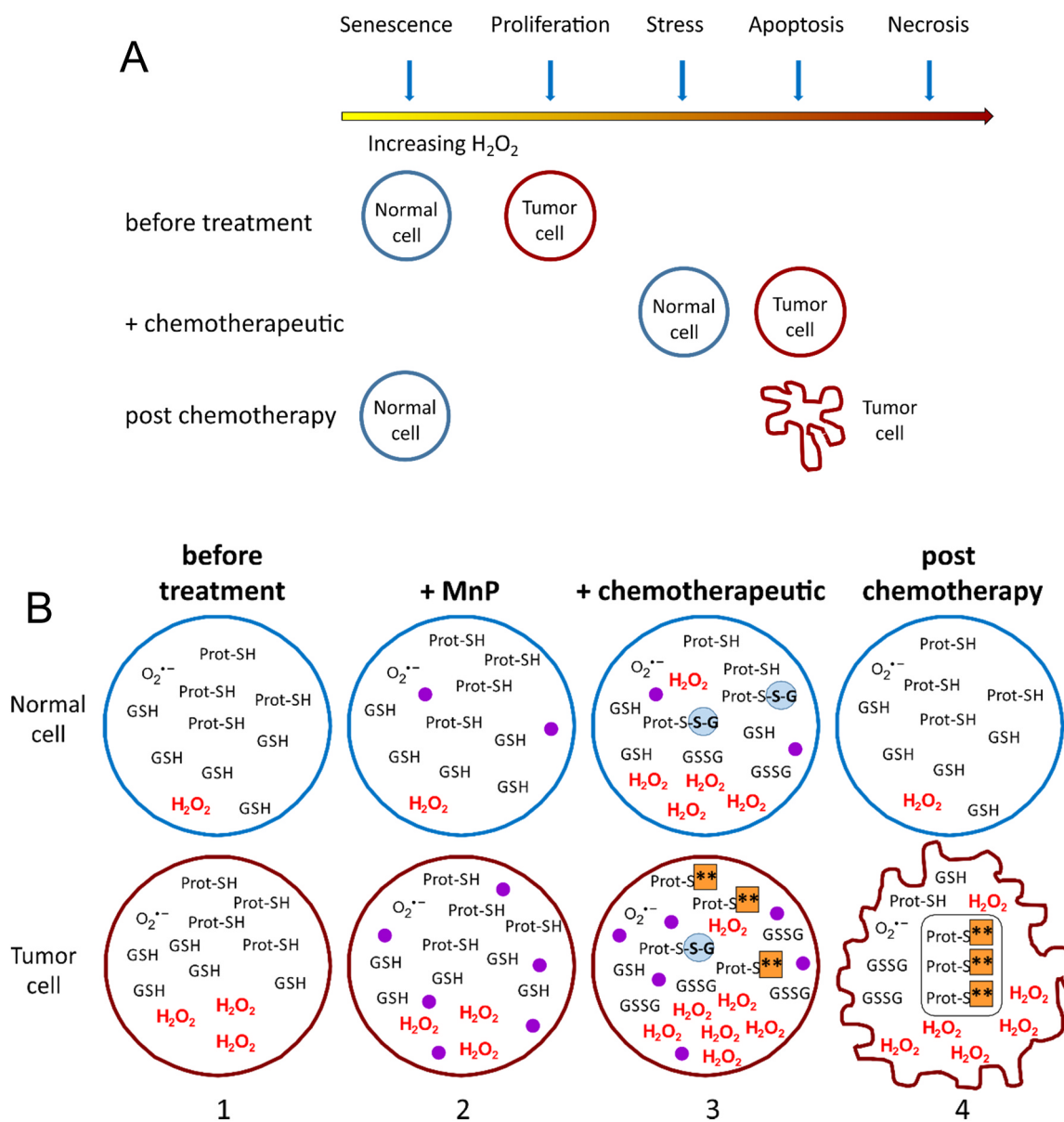
**Fig. 9. Model of oxidant/MnP differential effects in normal and tumor cells. A.** Model illustrating the general hypothesis of the mechanism for the differential effect of oxidant-based chemotherapeutics on normal and tumor cells. **B.** Model of the intracellular milieu when MnPs are combined with oxidant-producing therapeutics to enhance tumor cell death. **1.** Tumor cells have a higher baseline concentration of $H_2O_2$• **2.** Tumor cells take up more MnPs (purple dots) from the extracellular milieu than normal cells. **3.** Treatment with a chemotherapeutic that increases intracellular $H_2O_2$ results in redox cycling with the MnPs and protein cysteine S-glutathionylation (Prot-S-S-G) in both normal and tumor cells. However, with more molecules of both $H_2O_2$ and MnPs in the tumor cell there will be more oxidation of glutathione and protein cysteines than in normal cells. There will be increased irreversible oxidation of protein cysteines (Prot-S**). **4.** Normal cells will be able to remove the *S*-glutathionyl moiety from undamaged proteins so they will again be active. In the tumor cells the irreversibly damaged proteins can aggregate and/or be degraded (black box). The combination of the irreversibly damaged proteins and increased oxidative stress is expected to cause increased apoptosis in the tumor cells. The combination of the increased oxidants, increased MnP uptake and the ability of the MnPs to act as catalysts potentiates the oxidative stress in the tumor cells to increase tumor cell death. In panel B, Prot indicates Prot(Cys).

protein cysteines that are redox sensitive, but not involved in signaling pathways per se, act as redox-sensors [67]. In this capacity they serve to coordinate biological processes such as cell cycle without directly acting in specific signaling pathways. The biological effects of the MnPs could be due to these more global effects.

We have found that the MnPs are increasing protein cysteine oxidation/protein S-glutathionylation in every model in which we have looked for this process [8,52,53,55]. Whether this occurs in other models has not been tested. Until we test for this activity we will not know how prevalent this activity is in biological systems or whether it is key to the observed effects.

## 11. Differential effects of MnPs in normal tissue *vs* tumor cells

MnPs have the potential to widen the therapeutic window for cancer treatment by acting as a chemo- and radioprotectant of normal tissue while sensitizing the tumor cells to these agents. This differential effect of MnPs on normal and cancerous tissue could be one of its most powerful clinical attributes. Numerous studies have contributed to our knowledge of both protective and sensitizing effects of the MnPs. Fig. 4 shows a partial list of studies indicating the pre-clinical disease models where the MnPs were tested. The mechanisms by which the MnPs cause the differential effect are still being elucidated, however, the data thus far suggest some possibilities.

As shown by the aqueous chemistry data, the reactions the MnPs will undergo in the cell depends on the redox environment of the cell. Tumor cells have a more oxidized redox environment than normal cells [70,71]. In particular, they have a higher $H_2O_2$ load than normal cells through some combination of increased production and/or decreased removal. One hypothesis for the use of chemotherapeutics that cause oxidative stress to increase cell death in tumor cells is that they exploit the difference in oxidant load between normal and cancer cells [70,71]. The theory states that if an agent causes the same increase in oxidative stress in normal and cancer cells, the oxidative stress will be enough to induce apoptosis in the cancer cells, but not in the normal cells (Fig. 9**A**). This is because the cancer cells already have some oxidative stress and it would take less to push them over to apoptosis. The data suggest that MnPs cycle with $H_2O_2$ and cause oxidative stress in cells. Due to the increased $H_2O_2$ in the cancer cells, the MnPs would cycle more frequently with $H_2O_2$ in those cells and amplify the oxidative stress more in the cancer cells than in the normal cells.

This model could explain how the MnPs act as chemo/radio sensitizers in tumor cells relative to normal cells, but MnPs are also chemo/radioprotectants of normal cells. How could this work? The studies listed in Fig. 4 indicate that the MnPs protect normal cells from radiation and doxorubicin, an anthracycline. The data suggest that it could be due to the relative amount of redox active molecules and MnPs in tumor *vs* normal tissue. Radiation initially produces hydroxyl radical, superoxide and organic radicals [72] while anthracyclines initially produce superoxide [73]; $H_2O_2$ can be produced in subsequent reactions. MnPs react with hydroxyl radical, superoxide and $H_2O_2$. The major differences are that MnPs accumulate to a 3–10 fold greater degree in tumor tissue [8] and the tumor cells start with a greater amount of $H_2O_2$ than the normal cells (Fig. 9B). This combination suggests that the MnP catalyzed reactions would occur to a greater extent in the tumor cells and could result in more damage than in the normal cells.

The recent data indicating that MnPs cause protein cysteine oxidation/protein S-glutathionylation suggests another possibility for the differential effect of MnPs on tumor cells and normal tissue. Protein cysteine oxidation can be a mild reversible modification that regulates protein activity and can result in S-glutathionylation [57,68]. S-glutathionylation is a reversible modification that is usually inhibitory and protective so the cysteine isn't further oxidized [59,63]. More highly oxidized cysteines (e.g., sulfinic and sulfonic acids) are irreversible modifications that result in damaged and permanently inactivated proteins [59,63]. The increased $H_2O_2$ in the tumor cells cycling with the MnPs may cause more irreversibly oxidized cysteines and damaged proteins than in normal cells (Fig. 9B). In normal cells there may be more mildly oxidized cysteines/S-glutathionylated proteins that would recover upon removal of the agent (Fig. 9B). It is also possible that the increased S-glutathionylation in the tumor cells inhibits a key protein required for survival. The identification of NF-κB as a glutathionylation target of the MnP/$H_2O_2$ cycling is an example of such a target [52]. NF-κB is a required survival protein in one type of diffuse large B-cell lymphoma [74]. Normal tissues, which are often senescent, may not respond to widespread protein S-glutathionylation in the same way.

During chemo/radiation therapy, the dose-limiting effect is usually damage caused to normal tissues. This is the case for many commonly used agents, e.g. radiation, anthracyclines. Use of the MnPs to limit this damage, while improving tumor cell response to therapy, would improve patient outcome and decrease medical care for pathologies caused by damaged normal tissues. The MnPs, due to their redox properties, are uniquely positioned for clinical efficacy as an adjuvant in cancer treatment.

## 12. Conclusion

The progression of the MnPs to clinical trials justifies the effort that was required to synthesize these compounds. Although synthesized to mimic SOD-like activity, these compounds are highly reactive with a variety of redox active molecules. In many cases, their principal effect may not be due to SOD-like activity in vivo. The data to date indicate that these compounds have several critical properties that will dictate their biological effects. First, the reactions that will occur in vivo depend on the specific redox environment of the cell. Second, MnPs redox cycle in cells because their redox potential makes them equally likely to act as oxidants and reductants in the cellular environment. Third, they can act as widespread mild oxidants and catalysts of protein S-glutathionylation. These properties are a critical strength of these compounds and suggests new avenues to explore to determine potential therapeutic applications.

The parallel testing of two compounds (BMX-001 and GC4419), which are similarly potent SOD mimics yet have very different chemistry and thus biology, for the same clinical application is exciting. It is the very first testing ever of drugs that target the cellular redox environment in this way. The knowledge gained in these trials will help us to understand the biology and therapeutic effects of these two compounds as well as redox compounds in general.

The results from the clinical trials combined with continued use of the compounds in pre-clinical disease models will give us insight into the cellular therapeutic targets of these compounds and suggest other clinical uses for the SOD mimics.

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