Manganoporphyrins Increase Ascorbate-Induced Cytotoxicity by Enhancing H$_2$O$_2$ Generation

Malvika Rawal$^1$, Samuel R. Schroeder$^1$, Brett A. Wagner$^1$, Cameron M. Cushing$^1$, Jessemee L. Welsh$^2$, Anna M. Button$^3$, Juan Du$^1$, Zita A. Sibenaller$^1$, Garry R. Buettner$^{1,3}$, and Joseph J. Cullen$^{1,2,3,4}$

Abstract

Renewed interest in using pharmacological ascorbate (AscH$^-$) to treat cancer has prompted interest in leveraging its cytotoxic mechanism of action. A central feature of AscH$^-$ action in cancer cells is its ability to act as an electron donor to O$_2$ for generating H$_2$O$_2$. We hypothesized that catalytic manganoporphyrins (MnP) would increase AscH$^-$ oxidation rates, thereby increasing H$_2$O$_2$ fluxes and cytotoxicity. Three different MnPs were tested (MnTRAP, MnT2EPyP, and MnT4MPyP), exhibiting a range of physicochemical and thermodynamic properties. Of the MnPs tested, MnT4MPyP exerted the greatest effect on increasing the rate of AscH$^-$ oxidation as determined by the concentration of ascorbate radical [Asc$^-$] and the rate of oxygen consumption. At concentrations that had minimal effects alone, combining MnPs and AscH$^-$ synergized to decrease clonogenic survival in human pancreatic cancer cells. This cytotoxic effect was reversed by catalase, but not superoxide dismutase, consistent with a mechanism mediated by H$_2$O$_2$. We concluded that MnPs increase the rate of oxidation of AscH$^-$ to leverage H$_2$O$_2$ flux and ascorbate-induced cytotoxicity. Cancer Res; 73(16); 1–10. ©2013 AACR.

Introduction

Adenocarcinoma of the pancreas is the fourth leading cause of cancer death in the United States and is increasing in incidence (1). Current studies have investigated an entirely new approach, using pharmacological ascorbate as an adjuvant to radiotherapy, to treat pancreatic cancer. Intravenous ascorbate (i.e., ascorbic acid, vitamin C), but not oral ascorbate, produces high plasma concentrations (2), which are in the range cytotoxic to tumor cells (3–5). Ascorbate induces oxidative stress and cytotoxicity in pancreatic cancer cells, which seems to be greater in tumor versus normal cells (6). We have firmly established that pharmacological ascorbate is a prodrug for delivery of hydrogen peroxide (H$_2$O$_2$) in vitro and in vivo via its autoxidation (3, 6, 7). A recent phase I study has shown that pharmacological ascorbate is safe and well tolerated in oncology patients (8). In addition, phase I studies specifically in pancreatic cancer treatment have shown that pharmacologic ascorbate, combined with standard of care chemotherapy regimens, is safe and well tolerated and may lead to overall clinical benefit (9, 10). Ascorbate (AscH$^-$, vitamin C) is a classic donor antioxidant (11). AscH$^-$ scavenges oxidizing free radicals by donating an electron/hydrogen atom forming ascorbate radical (Asc$^-$), thereby “repairing” the oxidizing radical. In sequential one-electron oxidations, AscH$^-$ can donate 2 electrons to oxygen resulting in formation of dehydroascorbic acid and H$_2$O$_2$. The sequential one-electron oxidation of ascorbate can occur via the dianion; Asc$^{2-}$ auto-oxidizes in the presence of dioxygen to produce the Asc$^-$, dehydroascorbic acid, and H$_2$O$_2$ (12–14). Asc$^{2-}$ can dismute, reduce thermodynamically accessible metals, or be reduced by enzymes. At physiologic pH (pH 7.4), very little (0.01%) of the total AscH$^-$ is present as the dianion (p$K_{a1}$ = 4.1 and p$K_{a2}$ = 11.4; ref. 12). In the presence of redox active transition metals (e.g., iron, copper, or manganese), there is a substantial increase in the level of Asc$^{2-}$ and the associated rate of AscH$^-$ oxidation, leading to the production of O$_2$ and H$_2$O$_2$ (15, 16). Superoxide can further react with AscH$^-$ to form H$_2$O$_2$. Redox active metals can increase the flux of H$_2$O$_2$ by increasing the rate of oxidation of AscH$^-$. The reduced metal can react with O$_2$ to form O$_2$ and then with the aid of superoxide dismutase (SOD), H$_2$O$_2$ is formed. Although AscH$^-$ can act as an electron donor to convert O$_2$ to H$_2$O$_2$, the efficiency of SOD would make this a minor process in most settings.

Manganoporphyrins (MnP, manganese porphyrins) are being developed as SOD mimics (17). The Mn$^{3+}$ is chelated by a substituted porphyrin ring; substituents on the porphyrin ring system affect the half-cell reduction potential ($E_{1/2}$) of the Mn$^{3+}$.
central Mn$^{3+}$ to Mn$^{2+}$ (18). The reduction potential correlates with the ability of the Mn$^{3+}$ to enter into redox reactions with Asc$^{-}$ (19). Both Asc$^{-}$ and MnPs are well-known antioxidants and can protect cells against oxidizing species generated as a result of metabolism, disease, and ionizing stimuli like radiation (20–23). However, at pharmacologic concentrations, Asc$^{-}$ acts as a pro-oxidant. MnPs increase the flux of Asc$^{-}$-generated peroxide in vitro (18, 24, 25). To enhance Asc$^{-}$ oxidation, the MnPs must be able to redox cycle with Asc$^{-}$. Comparing the half-cell reduction potential ($E_{1/2}$) of MnPs to that of the Asc$^{-}$/Asc$^{+}$ couple (neutral pH), one can predict whether a MnP would efficiently redox cycle with Asc$^{-}$ and O$_2$ to produce H$_2$O$_2$.

We hypothesized that MnPs would enhance the rate of Asc$^{-}$ oxidation as a function of their reduction potential, thereby increasing the flux of H$_2$O$_2$. This increased flux of H$_2$O$_2$ will then potentiate Asc$^{-}$-induced cytotoxicity. Here we investigated the effects of MnPs on the [Asc$^{-}$/Asc$^{+}$]$_m$ in an Asc$^{-}$ solution and on oxygen consumption rate (OCR), both associated with an increased flux of H$_2$O$_2$, correlating the chemical findings with biological endpoints.

Materials and Methods

Cell culture and reagents

The human pancreatic cancer cell lines MIAPaCa-2, Panc-1, and AsPC-1 were purchased from the American Type Culture Collection and passaged for fewer than 6 months after receipt. No additional authentication was conducted. Mn(III) tetrakis(N-methylpyridinium-4-yl) porphyrin pentachloride (MnT4MPyP), and Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBPAP) were purchased from Axorora Platform. Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin pentachloride (AEOL 10113, MnT2EPyP) was from Dr. James D. Crapo (National Jewish Medical and Research Center, Denver, CO). Because MnPs undergo photooxidation (26), the solid was stored in colored vials at −20°C, or when dissolved in nanopure water, the solution was stored at 4°C in colored vials. Clonogenic survival, adenovirus transfections, and Western blots were conducted as previously described (6).

Oxygen consumption via Clark electrode

The rate of oxygen consumption (OCR, $-d[O_2]/dt$) was determined using a Clark electrode oxygen monitor (YSI Inc.) connected to an ESA Biostat multielectrode system (ESA Prod. Gioin Corp.) in PBS (GIBCO) or in (10% FBS) Dulbecco’s Modified Eagle Medium (DMEM; ref. 27). The effect of different MnPs on the OCR of Asc$^{-}$ was then determined. Accumulation of H$_2$O$_2$ was determined using catalase (Sigma).

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectra were obtained with a Bruker EMX ESR spectrometer (Bruker Biospin) using an ER 4119HS cavity. To make stable stock solutions of Asc$^{-}$ (100 mmol/L), L-ascorbic acid was dissolved in PBS (50 mmol/L, treated with Chelex resin, pH 7.4–7.6; ref. 28). Samples were contained in Pyrex capillary tubes (Fisher Scientific) with 1 mm outer diameter, supported in quartz sample tubes of 4 mm outer diameter (Wilmad-LabGlass). EPR instrument settings were: center field, 3507.62 G; sweep width, 10.00 G; receiver gain, 5.02×10$^5$; modulation amplitude, 0.70 G; microwave frequency, 9.85 GHz; and nominal microwave power, 10.0 mW. To determine [Asc$^{-}$], 3-carboxy-PROXYL (3-CP; CAS No. 2154-68-9; Sigma-Aldrich) radical was used as a standard, taking into account saturation effects (29).

Ex vivo studies

Mice were treated with AscH$^-$ (4 g/kg) or NaCl (1.0 M) every day for 8 days. Blood was drawn 1 hour after the last intraperitoneal dose. Thirty-day-old athymic nude mice were obtained from Harlan Sprague-Dawley. For the human ex vivo experiments, the blood samples were from the phase I trial approved by The University of Iowa Human Institutional Review Board and the Protocol Review and Monitoring Committee of the Holden Comprehensive Cancer Center at The University of Iowa Hospitals and Clinics on May 22, 2008 (10). The trial was listed on www.clinicaltrials.gov under NCT01515046. Informed consent was documented by use of a written consent form approved by the Investigational Review Board and The University of Iowa.

In vivo studies

Thirty-day-old athymic nude mice were obtained from Harlan Sprague-Dawley. All of the nude mice protocols were reviewed and approved by the Animal Care and Use Committee of The University of Iowa. Each experimental group consisted of 12 to 16 mice. MIA PaCa-2 tumor cells (2×10$^6$) were delivered subcutaneously into the hind leg of nude mice. The tumors were allowed to grow until they reached between 3 and 4 mm in greatest dimension at 10 days, at which time treatment was initiated. Mice were divided into 4 treatment groups and treated daily for 22 days. The groups included controls that received 1 M NaCl i.p.; ascorbate 4 g/kg i.p.; MnT4MPyP 0.2 mg/kg s.c.; and ascorbate 4 g/kg + MnT4MPyP 0.2 mg/kg. Tumor size was measured every 3 to 4 days by means of a vernier caliper, and tumor volume was estimated according to the following formula: tumor volume = $\pi/6 \times L \times W^2$, where $L$ is the greatest dimension of the tumor, and $W$ is the dimension of the tumor in the perpendicular direction. Animals were sacrificed by CO$_2$ asphyxiation when the tumors reached 1,000 mm$^3$.

Statistical analysis

Statistical significance was determined using an upper tailed, one-sided Student $t$ test, with a value of $P<0.05$ considered significant. Combination index (CI) was calculated for AscH$^-$ and MnP to determine summation, antagonism, or synergy using the multiple drug interaction model (30). Dose reduction index (DRI) values were also calculated as described (30, 31). The 2 variables must be calculated from the same percent of cell killing (30, 31).

For the in vivo studies, the statistical analyses focused on the effects of different treatments on cancer progression. The primary outcome of interest was tumor growth over time. Tumor sizes (mm$^3$) were measured throughout the experiments, resulting in repeated measurements across time for each mouse. A generalized estimating equations model was
used to estimate and compare group-specific tumor growth curves. Pairwise comparisons were conducted to identify specific group differences in the growth curves. All tests were two-sided and carried out at the 5% level of significance. Analyses were conducted with SAS version 9.3 (SAS Institute Inc.).

Results

**MnP**s increase the generation of **Asc**−, which is dependent on their reduction potential

Ascorbic acid exists predominantly (>99.9%) as the ascorbate monooanion (AscH−) at physiological pH (7.36–7.44). One-electron oxidation of AscH− forms the ascorbate radical (Asc−). The ascorbate radical is a resonance stabilized tricarbonyl species that has a long half-life compared with most free radicals (32). Typically, it is detectable by direct EPR; some Asc− is always present in an aerated solution of AscH− (33).

Under our experimental conditions, \( \approx 120 \text{ mmol/L} \), Asc− was present in a 1.0 mmol/L AscH− solution (Chelex-treated PBS, pH 7.4, 25°C). The concentration of Asc− increases in AscH− solutions in proportion to the oxidative flux in the system (12).

We hypothesized that MnPs would increase the steady-state level of Asc− (\([\text{Asc}^-]_{\text{ss}}\)), consistent with an increased oxidative flux in the system. As seen in Fig. 1, the reduction potential of an individual MnP relates to the rate of AscH− oxidation. MnT4MPyP and MnT2EPyP increase the \([\text{Asc}^-]_{\text{ss}}\) in solution (pH 7.3–7.6) in a dose-dependent manner. The reduction potential (E\(^{\text{red}}\)) of the Asc−/AscH− couple is +282 mV (12) whereas the Mn\(^{3+}\) in MnT2EPyP, MnT4MPyP, and MnTBAP has reduction potentials of +228 mV (34), +60 mV (35), and −194 mV (36), respectively. The increase in the \([\text{Asc}^-]_{\text{ss}}\) upon introduction of MnT4MPyP and MnT2EPyP indicates that AscH− is able to effectively reduce both MnPs. Only a minimal increase in the \([\text{Asc}^-]_{\text{ss}}\) is observed upon introduction of MnTBAP, which is consistent with its much lower reduction potential. Thus, appropriate thermodynamics is an important consideration for redox active compounds that might accelerate AscH− oxidation.

**MnP**s increase the OCR of ascorbate and generation of H\(_2\)O\(_2\)

To determine if the increase in \([\text{Asc}^-]_{\text{ss}}\) in the presence of MnPs would lead to an increase in the production of reactive oxygen species, we measured the rate of oxygen consumption and changes in accumulation of H\(_2\)O\(_2\). A 1.0 mmol/L solution of AscH− consumes oxygen at an initial rate of 2 to 5 mmol/L/s in PBS. In the presence of MnT4MPyP (1 μmol/L), this rate increases 10-fold (Fig. 2A), whereas MnT2EPyP (1 μmol/L) increases this rate two-fold (Fig. 2B). MnTBAP (1 μmol/L) did not significantly alter the rate of oxygen consumption (Fig. 2C).

The oxidation of AscH− produces H\(_2\)O\(_2\). If H\(_2\)O\(_2\) accumulates, then addition of catalase will result in the return of oxygen. If all of the oxygen consumed accumulates as H\(_2\)O\(_2\), then 50% of the oxygen lost would be restored with the addition of catalase. Addition of catalase (500 U/mL) resulted in the return of 5% to 10% of the oxygen consumed in the AscH− + MnT4MPyP or AscH− + MnT2EPyP solutions (Fig. 2A and B). However, any return of oxygen in AscH−/MnTBAP solution was below the limit of detection by the Clark electrode (<2 μmol/L). These results are consistent with our observations with [Asc−]_{ss} and consistent with the reduction potentials of the MnPs.

As a surrogate for extracellular fluid, we determined the rate of oxidation of AscH− in DMEM containing 10% FBS. Because MnT4MPyP had the greatest effect on the rate of oxidation of AscH− in PBS, we examined MnT4MPyP as a catalyst in DMEM containing 10% FBS. In this media, AscH− consumes oxygen at a rate of 12 to 50 mmol/L/s; this variability is typical, probably due to small changes in the content of catalytic metals (28). This greater rate of oxygen consumption of AscH− in DMEM + FBS compared with PBS may be due to the presence of significant catalytic iron and other redox active metals in the DMEM and FBS; in addition, FBS contains hemeproteins that can act as promiscuous peroxidases (37). In a representative experiment with AscH− (1 mmol/L) alone in DMEM with 10% FBS, the OCR was 45 mmol/L/s; addition of MnT4MPyP increased this rate two-fold to 90 mmol/L/s (Fig. 2D). After the addition of catalase (500 U/mL), about 20% to 25% of the oxygen consumed was returned, indicating that about 45% of the oxygen consumed accumulated as H\(_2\)O\(_2\). In these experiments, the accumulation of H\(_2\)O\(_2\) was less than the loss of oxygen, indicating that some of the H\(_2\)O\(_2\) was reduced to H\(_2\)O. Some MnPs have a catalase activity, which would also remove H\(_2\)O\(_2\). However, the relatively long-time frame of these experiments coupled with the clear accumulation of H\(_2\)O\(_2\) indicates that the removal of H\(_2\)O\(_2\) is a very slow process.

**MnP**s increase ascorbate-induced cytotoxicity

We hypothesized that the ability of MnPs to serve as catalysts for AscH− oxidation and associated generation of H\(_2\)O\(_2\) would correlate with the ability of the MnPs to enhance AscH−-induced cytotoxicity in vitro. To determine this, we used
concentrations (0.5–2 μmol/L) of MnPs and AscH− (1 mmol/L) that alone did not affect clonogenic survival in any of the human pancreatic cancer cell lines examined. MnT4MPyP was able to potentiate AscH− cytotoxicity most significantly as compared with the other 2 MnPs (Fig. 3). The combination of AscH− (1 mmol/L) with MnT4MPyP (0.5 μmol/L) decreases plating efficiency of MIA PaCa-2 cells from 50 ± 2% in controls to 5 ± 1% (Fig. 3A). As the dose of MnT4MPyP is increased in combination with AscH−, the plating efficiency decreased. When MIA PaCa-2 cells are exposed to 2 μmol/L MnT4MPyP and 1 mmol/L AscH−, no clones survive (Fig. 3A). AsPC-1 cells were derived from ascites of a pancreatic cancer patient and are more resistant to gemcitabine (38) and AscH−-induced cytotoxicity compared with MIA PaCa-2 (6). However, their susceptibility to AscH− and MnP cotreatment was similar to that of MIA PaCa-2 cells (Fig. 3B). When cotreated with MnT4MPyP (1 μmol/L) and AscH− (1 mmol/L), no AsPC-1 clones survive. As seen in Fig. 3C, combined treatment with MnT4MPyP and AscH− resulted in the same effect with Panc-1 cells.

MnT2EPyP increases [AscH−] in solution but produces less H2O2 as compared with MnT4MPyP (Fig. 2). The potentiating effect of MnT2EPyP on AscH− cytotoxicity in MIA PaCa-2 cells (Fig. 3D) and in AsPC-1 (Supplementary Fig. S1A) and Panc-1 cells (Supplementary Fig. S1B), although significant, is not as marked as that of MnT4MPyP combined with AscH−. MnTBAP increases [AscH−] in solution only modestly and has little effect on the OCR of AscH− in solution. This effect was also showed in AsPC-1 (Supplementary Fig. S1C) and Panc-1 cells (Supplementary Fig. S1D). The decrease in plating efficiency of various pancreatic cancer lines by the addition of MnPs to AscH− correlates with the OCR (R² = 0.62, P = 0.01; Fig. 3F). Also, the plating efficiency correlates with the concentration of AscH− present in solution after the addition of MnPs (R² = 0.82, P = 0.001; Fig. 3F). Together, these data show that the enhanced cytotoxicity of AscH− is a function of the MnP’s reduction potential and their ability to enhance AscH− oxidation in solution.

Figure 2. MnPs increase the rate of oxygen consumption, leading to generation of H2O2. A. AscH− consumes oxygen at the rate of 2 to 5 nmol/L/s in PBS. Addition of MnT4MPyP (1.0 μM) increases the rate of oxygen consumption to 22–25 nmol/L/s. Addition of catalase (500 U/mL) leads to a return of O2 with 16 to 20 μmol/L H2O2 accumulating in solution; n = 3. Inset, MnT4MPyP molecular structure. B. addition of MnT2EPyP (1.0 μmol/L) to AscH− (1 mmol/L) solution increases the OCR from 5 to 10 nmol/L/s. Addition of catalase indicates 3 to 5 μmol/L of H2O2 has accumulated in solution after 60 minutes; n = 3. Inset, MnT2EPyP molecular structure. C. MnTBAP (1.0 μmol/L) addition to AscH− (1.0 mmol/L) solution does not alter the OCR. Addition of catalase does not return detectable amounts of oxygen, showing minimal H2O2 accumulation; n = 3. Inset, MnTBAP molecular structure. D. in DMEM with 10% FBS, oxygen consumption for AscH− (1 mmol/L) is 20 to 45 nmol/L/s. Addition of MnT4MPyP increases oxygen consumption to 80 to 100 nmol/L/s. Addition of catalase 30 minutes after MnT4MPyP leads to a return of oxygen, indicating that 50 to 60 μmol/L H2O2 has accumulated in solution; n = 3.
Figure 3. MnPs enhance Asch\(^{-}\)-induced cytotoxicity in pancreatic cancer cell lines. A. treatment with Asch\(^{-}\) or MnT4MPyP alone does not alter clonogenic survival of MIA PaCa-2 cells. However, the combination of Asch\(^{-}\) (1 mmol/L) and MnT4MPyP (0.5 \(\mu\)mol/L) decreases plating efficiency to 5\% ± 1%. When
The effects of MnP on Ascl⁻ cytotoxicity may be mediated by H₂O₂. The combination of Ascl⁻ (1 mmol/L) and MnT4MPyP (1 µmol/L) in DMEM + 10% FBS, resulted in the accumulation of 55–60 µmol/L H₂O₂ at 30 minutes. In addition, exposure of MIA PaCa-2 (Supplementary Fig. S2A) and AsPC-1 cells (Supplementary Fig. S2B) to a bolus dose of 60 µmol/L H₂O₂ reduced their survival to levels similar to those seen with Ascl⁻ and MnT4MPyP cotreatment, suggesting indirectly that H₂O₂ is playing a significant role in the observed cytotoxicity.

**Ascorbate and MnPs synergize to enhance cytotoxicity**

To determine if MnPs were additive or synergistic to Ascl⁻, the CI and DRI for the 3 different cell lines were calculated (30, 31). The CI allows for quantitative determination of drug interactions with CI < 1, = 1, and >1, indicating synergism, additive effects, or antagonism, respectively. The DRI was calculated as the molar ratio of Ascl⁻ alone versus Ascl⁻ + MnT4MPyP required to produce the same level of cytotoxicity. For all combinations, the CI was <0.1, indicating synergy (Supplementary Table S1). DRI indices for MnT4MPyP were between 4 × 10⁻⁵ and 8 × 10⁻⁶. Ascorbate DRI calculations showed values in the 6 to 22 range (Supplementary Table S1), also suggesting synergy between Ascl⁻ and MnT4MPyP. The DRI values were less for Ascl⁻ as compared with MnT4MPyP in the same combination setting, suggesting that Ascl⁻ is the main cytotoxic agent in this combination whereas MnT4MPyP is an adjuvant, acting mainly as a catalyst to enhance Ascl⁻-induced cytotoxicity.

**SOD activity does not alter Ascl⁻-induced cytotoxicity**

To determine if the SOD-mimicking functionality of the MnPs was responsible for the enhanced toxicity of Ascl⁻, MIA PaCa-2 cells were exposed to polyethylene glycol-superoxide dismutase (PEG-SOD; 100 U/mL), PEG alone (100 U/mL), Ascl⁻ (1 mmol/L), or the combination of PEG-SOD + Ascl⁻. No changes in clonogenic survival were observed, indicating that superoxide is not a major factor in ascorbate-induced cytotoxicity (Fig. 4A). To further investigate the role of SOD activity, cells were transfected with the AdEcSOD to determine if overexpression of EcSOD would enhance Ascl⁻ toxicity. Western blots confirmed that EcSOD immunoreactive protein was absent in control and AdEmpty transfected cells, whereas abundant in the AdEcSOD transfected cells (Fig. 4B). Transduction of cells with adenovirus vectors can decrease survival, as seen in Fig. 4C. There were no differences in clonogenic survival in cells treated with the combination of AdEmpty and Ascl⁻. Treatment of MIA PaCa-2 cells transfected with AdEcSOD with Ascl⁻ (1 mmol/L) had little effect on clonogenic survival (Fig. 4C). These data indicate that the SOD activity of MnPs is not responsible for the MnP-enhanced toxicity of Ascl⁻.

**Catalase reverses MnP + ascorbate-induced cytotoxicity**

Ascl⁻-induced cytotoxicity may be mediated by H₂O₂ (6). With the addition of a redox active metal like the Mn⁵⁺/Mn³⁺ couple of MnPs, the flux of O₂⁻ may be altered, which could enhance toxicity in addition to the accumulation of H₂O₂. To probe for the free radical species involved in the MnP + Ascl⁻-induced cytotoxicity, cells were treated with SOD or catalase. Although Ascl⁻, MnT4MPyP, PEG-SOD, and PEG-catalase alone were not cytotoxic (Fig. 5), few clones survived when cells were treated with both Ascl⁻ and MnT4MPyP (Fig. 5). Addition of SOD did not reverse the Ascl⁻ + MnT4MPyP cytotoxicity (Fig. 5A). However, PEG-catalase (120 U/mL) reversed the combined cytotoxicity of Ascl⁻ + MnT4MPyP (Fig. 5B). These data suggest that although O₂⁻ may not play a significant role, accumulation of H₂O₂ mediates the cytotoxicity of Ascl⁻ combined with the MnPs in our experimental setting.

**MnT4MPyP increases [Asc⁻] ex vivo**

Infusions of pharmacologic doses of Ascl⁻ (0.6–1.5 g/kg body weight) over an extended period of time result in peak plasma Ascl⁻ concentrations of 15 to 25 mmol/L (8). Achieving this plasma [Ascl⁻] is central to cytotoxic Ascl⁻ therapy (4, 8, 10). Oxidation of Ascl⁻ in extracellular fluid results in increased generation of H₂O₂ leading to cytotoxicity (3). Enhanced oxidation of Ascl⁻ and increased steady-state levels of Asc⁻ are indicators of Ascl⁻ oxidation and resultant H₂O₂ production and cytotoxicity. MnT4MPyP increases [Asc⁻] and the OCR of Ascl⁻ in solution, synergizes with Ascl⁻ *in vitro*, and increases Ascl⁻-induced cytotoxicity in pancreatic cancer cell lines. To determine if MnT4MPyP increases the rate of Ascl⁻ oxidation ex vivo, whole blood [Asc⁻] was measured by EPR from mice infused with pharmacologic Ascl⁻. In untreated mouse whole blood Asc⁻ was below detection limits (<10 nmol/L; Fig. 6A). Addition of MnT4MPyP (1.0 µmol/L) increased Asc⁻ to 97 nmol/L. Mice were infused with Ascl⁻ (4 g/kg) resulting in plasma levels of 29 mmol/L Asc⁻; EPR detectable [Asc⁻] increased to 350 nmol/L in whole blood. However, addition of MnT4MPyP (1.0 µmol/L) to this mouse whole blood ex vivo led to an increase in [Asc⁻] to 1200 nmol/L.
Manganese porphyrins enhance ascorbate-induced cytotoxicity in vivo

To determine if MnPs could enhance ascorbate-induced cytotoxicity in vivo, we treated mice with preestablished MIA PaCa-2 human pancreatic tumors. There were no differences in weight changes among the 4 treatment groups and none of the animals during the study had to be sacrificed for continued weight loss or cachexia. We also obtained whole blood from separate groups of mice treated with the same combinations. Asc•− was not detected in whole blood drawn from mice treated with saline (Fig. 6A). When MnT4MPyP (1.0 μmol/L) was added to pre-infusion blood, EPR detectable Asc•− increased to 82 nmol/L. Following intravenous infusion of pharmacologic Asc•− (100 μmol/L), EPR detectable Asc•− was increased to 120 nmol/L. Addition of MnT4MPyP to post-infusion blood increased Asc•− in the whole blood to 360 nmol/L. Thus, MnT4MPyP increased Asc•− in whole blood ex vivo from mice and from patients. This increased Asc•− in ex vivo whole blood indicates increased Ascorbate oxidation, similar to our in vitro observations; an increased Ascorbate oxidation will lead to an increased flux of H2O2.

MnT4MPyP enhances ascorbate-induced cytotoxicity in vivo

We then obtained whole blood from human pancreatic cancer patients receiving pharmacologic Asc•− (50–125 g Asc•− twice a week) as part of a phase I trial. Similar to trends seen in mice, Asc•− was not detected in whole blood drawn from patients prior to being infused with Asc•− (Fig. 6B). When MnT4MPyP (1.0 μmol/L) was added to whole blood, EPR detectable Asc•− increased to 82 nmol/L. Following intravenous infusion of pharmacologic Asc•− (100 μmol/L), EPR detectable Asc•− was increased to 120 nmol/L. Addition of MnT4MPyP to post-infusion blood increased Asc•− in whole blood drawn from mice treated with Asc•− and none of the animals during the study had to be sacrificed for continued weight loss or cachexia. We also obtained whole blood from separate groups of mice treated with the same combinations. Asc•− was not detected in whole blood drawn from mice treated with saline (Fig. 7A). When MnT4MPyP (0.2 mg/kg) was given i.p., EPR detectable Asc•− increased to 22 ± 5 nmol/L. Following i.p. administration of Asc•− (4 g/kg), EPR detectable Asc•− was increased to 120 ± 40 nmol/L. In mice treated with both MnT4MPyP and Asc•−, Asc•− increased to 310 ± 66 nmol/L. Thus, MnT4MPyP enhanced Asc•−-induced Asc•− in treated mice showing increased Asc•− oxidation.

MnT4MPyP enhanced ascorbate-induced cytotoxicity as animals treated with the combination of ascorbate + MnT4MPyP had a slower rate of growth in tumors when compared with saline (controls) treatment (*, P < 0.01) or ascorbate alone (*, P < 0.05; Supplementary Table S2). The estimated tumor growth curve is displayed in Fig. 7B. On day 25 of the study, the control group had a mean tumor volume of 491 mm3, whereas the ascorbate alone group had a mean tumor volume of 384 mm3. In mice treated with the combination of MnT4MPyP and ascorbate, mean tumor volume was 174 mm3.
Discussion

Pharmacologic AscH\(^{-}\) induces tumor cell cytotoxicity in vitro and in vivo (3–6); this toxicity is mediated by the generation of H\(_2\)O\(_2\) (3–6, 39). With our goal to increase the flux of H\(_2\)O\(_2\) from AscH\(^{-}\)/C\(_0\), we hypothesized that MnPs combined with pharmacologic ascorbate would increase the rate of oxidation of AscH\(^{-}\)/C\(_0\), leading to an increased flux of H\(_2\)O\(_2\) enhancing cytotoxicity. MnPs are redox-active metal chelates that are being developed as SOD mimics (18). The catalytic and thermodynamic properties of MnPs have been extensively investigated resulting in MnPs with a range of reduction potentials and have been used in both in vitro and in vivo experiments (18, 19, 34, 36). Our study shows that MnPs can increase [Asc\(^{-}\)]\(_{ss}\) in solution, dependent on the [MnP] as well as the reduction potential of the individual MnP. The rate of oxygen consumption in a solution of AscH\(^{-}\) is an indicator of its rate of oxidation. This oxidation leads to a flux of H\(_2\)O\(_2\). MnPs increased the OCR of AscH\(^{-}\) in solution and lead to the

Figure 6. MnT4MPyP enhances ascorbate radical concentration in whole blood as seen by EPR spectroscopy. A, in whole blood from mice, Asc\(^{-}\)/C\(_0\) is below the limit of detection. MnT4MPyP (1.0 \(\mu\)mol/L) increased [Asc\(^{-}\)]\(_{ss}\) to 97 nmol/L. When mice were treated with AscH\(^{-}\)/C\(_0\) (4 g/kg), resulting in a plasma level of [AscH\(^{-}\)] of 29 mmol/L, [Asc\(^{-}\)]\(_{ss}\) was increased to 350 nmol/L. Upon addition of MnT4MPyP (1.0 \(\mu\)mol/L) to ascorbate-treated mice, [Asc\(^{-}\)]\(_{ss}\) increased more than 3-fold to 1,200 nmol/L; \(n = 3\).

Hyperfine splitting of Asc\(^{-}\), \(a_{hh} = 1.76 \) G. B, in whole blood from humans, Asc\(^{-}\) is also below the limit of detection. [Asc\(^{-}\)]\(_{ss}\) increases to 82 nmol/L upon addition of MnT4MPyP (1.0 \(\mu\)mol/L). With addition of MnT4MPyP (1.0 \(\mu\)mol/L) to ascorbate-treated mice, [Asc\(^{-}\)]\(_{ss}\) increased more than 3-fold to 1,200 nmol/L; \(n = 3\).
ascorbate decreased MIA PaCa-2 tumor growth in nude mice. The MnT4MPyP, a reducing agent (25), however, we use AscH as the main cytotoxic agent and MnPs as catalysts to increase the flux of $\text{H}_2\text{O}_2$.

In summary, MnPs increased the rate of AscH oxidation, the steady-state level of [Asc$^\cdot$], and the rate of oxygen consumption, with MnT4MPyP having the greatest effect. MnPs synergistically enhanced AscH$^\cdot$-induced cytotoxicity in all pancreatic cancer cell lines studied. Catalase, but not SOD, reversed the cytotoxicity of the AscH$^\cdot$ and MnPs combination, suggesting an H$_2$O$_2$-mediated mechanism. In addition, there was a marked increase in [Asc$^\cdot$]$_{ss}$ in whole blood from mice upon the addition of MnPs and as well as in the blood from patients treated with pharmacologic AscH$^\cdot$. We conclude that MnPs can increase the rate of oxidation of AscH$^\cdot$ leading to an increased flux of H$_2$O$_2$ resulting in increased AscH$^\cdot$-induced cytotoxicity. MnPs have the potential as adjuvants to pharmacologic AscH$^\cdot$ therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: M. Rawal, S.R. Schroeder, B. Wagner, G.R. Buettner, J.J. Cullen


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.R. Schroeder, B. Wagner, J.L. Welsh, J. Du, Z.A. Sibenaller, J.J. Cullen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Rawal, S.R. Schroeder, C.M. Cushing, A.M. Button, G.R. Buettner, J.J. Cullen

Writing, review, and/or revision of the manuscript: M. Rawal, S.R. Schroeder, B. Wagner, C.M. Cushing, Z.A. Sibenaller, G.R. Buettner, J.J. Cullen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Rawal, C.M. Cushing, G.R. Buettner, J.J. Cullen

Study supervision: J.J. Cullen

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References


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Malvika Rawal, Samuel R. Schroeder, Brett A. Wagner, et al.

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