Manganeseoporphyrins Increase Ascorbate-Induced Cytotoxicity by Enhancing H₂O₂ Generation

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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 O₂ for generating H₂O₂. We hypothesized that catalytic manganeseoporphyrins (MnP) would increase AscH⁺ oxidation rates, thereby increasing H₂O₂ 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 H₂O₂. MnPs increased steady-state concentrations of Asc⁺ in vivo upon ex vivo addition to whole blood obtained either from mice infused with AscH⁺ or patients treated with pharmacologic AscH⁺. Finally, tumor growth in vivo was inhibited more effectively by combining MnT4MPyP with AscH⁺. We concluded that MnPs increase the rate of oxidation of AscH⁺ to leverage H₂O₂ flux and ascorbate-induced cytotoxicity. Cancer Res; 73(16); 5232–41. ©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 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 H₂O₂. The sequential one-electron oxidation of ascorbate can occur via the dianion: Asc– auto-oxidizes in the presence of dioxygen to produce the Asc–, dehydroascorbic acid, and H₂O₂ (12–14). Asc– 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 (pKₐ₁ = 4.1 and pKₐ₂ = 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– and the associated rate of AscH⁺ oxidation, leading to the production of O₂⁻ and H₂O₂ (15, 16). Superoxide can further react with AscH⁺ to form H₂O₂. Redox active metals can increase the flux of H₂O₂ by increasing the rate of oxidation of AscH⁺. The reduced metal can react with O₂ to form O₂⁻ and then with the aid of superoxide dismutase (SOD), H₂O₂ is formed. Although AscH⁺ can act as an electron donor to convert O₂⁻ to H₂O₂, the efficiency of SOD would make this a minor process in most settings.

Manganeseoporphyrins (MnP, manganese porphyrins) are being developed as SOD mimics (17). The Mn⁴⁺ is chelated by a substituted porphyrin ring; substituents on the porphyrin ring system affect the half-cell reduction potential (E₁/₂) of the
Manganese porphyrins (MnPs) to that of the Ascorbic Oxidants, Dionex Corp.) in PBS (GIBCO) or in (10% FBS) Dulbecco’s medium. 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-ethylpyridinium-2-yl) porphyrin pentachloride (MnTBAP) were purchased from Axxora Platform. Mn(III) tetrakis(N-methylpyridinium-4-yl) porphyrin pentachloride (MnTMPyP) and Mn(III) tetrakis(4- benzoic acid) porphyrin chloride (MnTBPAP) were purchased from Axioxa Platform. Mn(IV) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin pentachloride (AEOI 10113, MnTBPAP) 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 4°C. When dissolved in nanopure water, the solution was stored at 4°C in colored vials. Cytotoxicity, adenosine transfections, and Western blots were conducted as previously described (6).

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-nicotinyl-2-pyridine) porphyrin pentachloride (MnNTPP), and Mn(III) tetrakis(N-methylpyridinium-4-yl) porphyrin chloride (MnTBPAP) were purchased from Axxora Platform. Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin pentachloride (AEOI 10113, MnTBPAP) 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 Bistat multielectrode system (ESA Products, Dionex Corp.) in PBS (GIBCO) or in (10% FBS) Dulbecco’s Modified Eagle’s Medium (DMEM; ref. 27). The effect of different MnPs on the OCR of Ascorbate- was then determined. Accumulation of \(H_2O_2\) 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 \(Ascorbate^-\) (100 mmol/L), \(\alpha\)-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 × 10^3; modulation amplitude, 0.70 G; microwave frequency, 9.85 GHz; and nominal microwave power, 10.0 mW. To determine \([Ascorbate^-]\), 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 \(Ascorbate^-\) (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 ip, ascorbate 4 g/kg ip, MnTBPAP 0.2 mg/kg s.c., and ascorbate 4 g/kg + MnTBPAP 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 CO2 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 Ascorbate- 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 applied to compare different groups with a time-varying covariate (32).
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

MnPs increase the generation of Asc−•, which is dependent on their reduction potential

Ascorbic acid exists predominantly (>99.9%) as the ascorbate monoanion (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, ≈120 nmol/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−• ([Asc−•]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 [Asc−•]ss in solution (pH 7.5–7.6) in a dose-dependent manner. The reduction potential (E°) of the Asc−•/AscH− couple is +282 mV (12) whereas the Mn5+ in MnT2EPyP, MnT4MPyP, and MnTBAP has reduction potentials of +228 mV (34), +60 mV (35), and −194 mV (36), respectively. The increase in the [Asc−•]ss upon introduction of MnT4MPyP and MnT2EPyP indicates that AscH− is able to effectively reduce both MnPs. Only a minimal increase in the [Asc−•]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.

MnPs increase the OCR of ascorbate and generation of H2O2

To determine if the increase in [Asc−•]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 H2O2. A 1.0 mmol/L solution of AscH− consumes oxygen at an initial rate of 2 to 5 nmol/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 H2O2. If H2O2 accumulates, then addition of catalase will result in the return of oxygen. If all of the oxygen consumed accumulates as H2O2, 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 nmol/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 heme proteins 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 nmol/L/s; addition of MnT4MPyP increased this rate two-fold to 90 nmol/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 H2O2. In these experiments, the accumulation of H2O2 was less than the loss of oxygen, indicating that some of the H2O2 was reduced to H2O. Some MnPs have a catalase activity, which would also remove H2O2. However, the relatively long-time frame of these experiments coupled with the clear accumulation of H2O2 indicates that the removal of H2O2 is a very slow process.

MnPs increase ascorbate-induced cytotoxicity

We hypothesized that the ability of MnPs to serve as catalysts for AscH− oxidation and associated generation of H2O2 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 [AsC−] 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 [AsC−]ss in solution only modestly and has little effect on the OCR of AsCH− in solution. It is, however, able to enhance AsCH− cytotoxicity to a lesser extent (Fig. 3E). 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 AsC− 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 AsC− is a function of the MnPs’ reduction potential and their ability to enhance AsC− 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\textsuperscript{−}−induced cytotoxicity in pancreatic cancer cell lines. A, treatment with AscH\textsuperscript{−} or MnT4MPyP alone does not alter clonogenic survival of MIA PaCa-2 cells. However, the combination of AscH\textsuperscript{−} (1 mmol/L) and MnT4MPyP (0.5 μmol/L) decreases plating efficiency to 5 ± 1%. When
The effects of MnP on AscH− cytotoxicity may be mediated by H2O2. The combination of AscH− (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 AscH− and MnT4MPyP cotreatment, suggesting indirectly that H2O2 is playing a significant role in the observed cytotoxicity.

**Ascorbate and MnPs synergize to enhance cytotoxicity in vitro**

To determine if MnPs were additive or synergistic to AscH−, 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 AscH− alone versus AscH− + 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 × 10^3 and 8 × 10^6. Ascorbate DRI calculations showed values in the 6 to 22 range (Supplementary Table S1), also suggesting synergy between AscH− and MnT4MPyP. The DRI values were less for AscH− as compared with MnT4MPyP in the same combination setting, suggesting that AscH− is the main cytotoxic agent in this combination whereas MnT4MPyP is an adjuvant, acting mainly as a catalyst to enhance AscH−-induced cytotoxicity.

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

To determine if the SOD-mimicking functionality of the MnPs was responsible for the enhanced toxicity of AscH−, MIA PaCa-2 cells were exposed to polyethylene glycol-superoxide dismutase (PEG-SOD; 100 U/mL), PEG alone (100 U/mL), AscH− (1 mmol/L), or the combination of PEG-SOD + AscH−. 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 AscH− 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). Transfection 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 AscH−. Treatment of MIA PaCa-2 cells transfected with AdEcSOD with AscH− (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 AscH−.

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

AscH−-induced cytotoxicity may be mediated by H2O2 (6). With the addition of a redox active metal like the Mn3+/Mn2+ 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 + AscH−-induced cytotoxicity, cells were treated with SOD or catalase. Although AscH−, MnT4MPyP, PEG-SOD, and PEG-catalase alone were not cytotoxic (Fig. 5), few clones survived when cells were treated with both AscH− and MnT4MPyP (Fig. 5). Addition of SOD did not reverse the AscH− + MnT4MPyP cytotoxicity (Fig. 5A). However, PEG-catalase (120 U/mL) reversed the combined cytotoxicity of AscH− + MnT4MPyP (Fig. 5B). These data suggest that although O2− may not play a significant role, accumulation of H2O2 mediates the cytotoxicity of AscH− combined with the MnPs in our experimental setting.

**MnT4MPyP increases [AscH−] ex vivo**

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

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MnT4MPyP (2 μmol/L) was added to AscH−, no clones survived; n = 3. *P < 0.001 vs. controls. B, MnT4MPyP (1 μmol/L) or AscH− (1 mmol/L) did not affect AsPC-1 clonogenic survival, whereas the combination led to no surviving clones; n = 3. *, P < 0.001 vs. control. C, MnT4MPyP (0.5–2 μmol/L) or AscH− (1 mmol/L) did not affect Panc-1 clonogenic survival. However, addition of MnT4MPyP (0.5 μmol/L) to AscH− (1 mmol/L) decreased survival to 3 ± 0.2%. Only 1 ± 0.1% clones survived exposure to 2 μmol/L MnT4MPyP in the presence of AscH−; n = 3. *, P < 0.001 vs. control. D, MnT4MPyP (0.5, 1, or 2 μmol/L) or AscH− (1 mmol/L) does not alter MIA PaCa-2 survival. MnT2EPyP (0.5 μmol/L) combined with AscH− (1 mmol/L) decreases plating efficiency to 10 ± 0.5%. When increased concentrations of MnT2EPyP are used, plating efficiency was reduced to <5%; n = 3. *, P < 0.001 vs. controls. E, MnTBAP (0.5, 1, or 2 μmol/L) does not alter MIA PaCa-2 plating efficiency. However, when MnTBAP was combined with AscH−, MIA PaCa-2 plating efficiency was decreased to 3±3%; n = 3. *, P < 0.001 vs. control. F, the OCR of AscH− when combined with MnPs correlates with log (plating efficiency) in pancreatic cancer cell lines (solid line, R² = 0.9, P < 0.05). In solution, [AscH−] in the presence of MnPs (from Fig. 1) strongly correlated with (log(plating efficiency)) in MIA PaCa-2, AsPC-1, and Panc-1 cells (hatched line, R² = 0.9, P < 0.05). The (2) below specific points on the graph indicate that 2 identical points at that value.
We then obtained whole blood from human pancreatic cancer patients receiving pharmacologic AscH\(^{\ast}\) (50–125 g AscH\(^{\ast}\) twice a week) as part of a phase I trial. Similar to trends seen in mice, Asc\(^{\ast}\) was not detected in whole blood drawn from patients prior to being infused with AscH\(^{\ast}\) (Fig. 6B). When MnT4MPyP (1.0 \(\mu\)mol/L) was added to pre-infusion blood, EPR detectable Asc\(^{\ast}\) increased to 82 nmol/L. Following intravenous infusion of pharmacologic AscH\(^{\ast}\) (100 g), EPR detectable Asc\(^{\ast}\) was increased to 120 nmol/L. Addition of MnT4MPyP to post-infusion blood increased Asc\(^{\ast}\) in the whole blood to 360 nmol/L. Thus, MnT4MPyP increased Asc\(^{\ast}\) in whole blood \textit{ex vivo} from mice and from patients. This increased Asc\(^{\ast}\) in \textit{ex vivo} whole blood indicates increased AscH\(^{\ast}\) oxidation, similar to our \textit{in vitro} observations; an increased AscH\(^{\ast}\) oxidation will lead to an increased flux of \(\text{H}_2\text{O}_2\).

**MnT4MPyP enhances ascorbate-induced cytotoxicity \textit{in vivo}**

To determine if MnPs could enhance ascorbate-induced cytotoxicity \textit{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\(^{\ast}\) 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\(^{\ast}\) increased to 22 \(\pm\) 5 nmol/L. Following i.p. administration of AscH\(^{\ast}\) (4 g/kg), EPR detectable Asc\(^{\ast}\) was increased to 120 \(\pm\) 40 nmol/L. In mice treated with both MnT4MPyP and AscH\(^{\ast}\), Asc\(^{\ast}\) increased to 310 \(\pm\) 66 nmol/L. Thus, MnT4MPyP enhanced AscH\(^{\ast}\)-induced Asc\(^{\ast}\) in treated mice showing increased AscH\(^{\ast}\) 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 \((^\circ, P < 0.01)\) or ascorbate alone \((^\circ, P < 0.05); \text{Supplementary Table S2}\). 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 mm\(^3\), whereas the ascorbate alone group had a mean tumor volume of 384 mm\(^3\). In mice treated with the combination of MnT4MPyP and ascorbate, mean tumor volume was 174 mm\(^3\).
Manganoporphyrins Enhance Ascorbate-Induced Cytotoxicity

**Discussion**

Pharmacologic \textit{AscH} induces tumor cell cytotoxicity \textit{in vitro} and \textit{in vivo} (3–6); this toxicity is mediated by the generation of \textit{H}_2\textit{O}_2 (3–6, 39). With our goal to increase the flux of \textit{H}_2\textit{O}_2 from \textit{AscH}, we hypothesized that MnPs combined with pharmacologic ascorbate would increase the rate of oxidation of \textit{AscH}, leading to an increased flux of \textit{H}_2\textit{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 \textit{in vitro} and \textit{in vivo} experiments (18, 19, 34, 36). Our study shows that MnPs can increase [\textit{Asc}^\cdot] 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 \textit{AscH} is an indicator of its rate of oxidation. This oxidation leads to a flux of \textit{H}_2\textit{O}_2. MnPs increased the OCR of \textit{AscH} in solution and lead to the

**Figure 5.** \textit{H}_2\textit{O}_2 mediates MnP-enhanced \textit{AscH}–induced cytotoxicity. A, PEG-SOD (100 U/mL) did not reverse MnT4MpyP-enhanced \textit{AscH}–induced cytotoxicity in MiaPaCa-2 cells, suggesting that superoxide is not directly responsible for the observed cytotoxicity; \textit{n} = 3. * \textit{P} < 0.001 vs. controls. # \textit{P} > 0.05 vs. \textit{AscH} + MnP. B, treatment with PEG-CAT (120 U/mL) reverses MnT4MpyP-enhanced \textit{AscH}–induced cytotoxicity, suggesting that \textit{H}_2\textit{O}_2 mediates the observed cytotoxicity; \textit{n} = 3. * \textit{P} < 0.001 vs. control. ** \textit{P} < 0.001 vs. \textit{AscH} + MnT4MpyP-treated cells.

**Figure 6.** MnT4MpyP enhances ascorbate radical concentration in whole blood as seen by EPR spectroscopy. A, in whole blood from mice, \textit{Asc}^\cdot is below the limit of detection. MnT4MpyP (1.0 \textit{m}mol/L) increased [\textit{Asc}^\cdot] by 97 nmol/L. When mice were treated with \textit{AscH} (4 g/kg), resulting in a plasma level of [\textit{AscH}] of 29 nmol/L, [\textit{Asc}^\cdot] was increased to 350 nmol/L. Upon addition of MnT4MpyP (1.0 \textit{m}mol/L), [\textit{Asc}^\cdot] increased more than 3-fold to 1,200 nmol/L; \textit{n} = 3. (Hyperfine splitting of \textit{Asc}^\cdot, \textit{a} = 1.76 G). B, in whole blood from humans, \textit{Asc}^\cdot is also below the limit of detection. [\textit{Asc}^\cdot] increases to 82 nmol/L upon addition of MnT4MpyP (1.0 \textit{m}mol/L) to human whole blood. After infusion of pharmacological \textit{AscH} (100 g), [\textit{AscH}] increased to 22 nmol/L and [\textit{Asc}^\cdot] increased to 120 nmol/L. Upon addition of MnT4MpyP (1.0 \textit{m}mol/L), [\textit{Asc}^\cdot] increased to 360 nmol/L; \textit{n} = 3.
expression of SOD (either PEG-SOD or Ad

AscH

MnT4MPyP and ascorbate-treated animals had significantly slower tumor growth when compared with the control and ascorbate groups (*, P < 0.05, n = 12–16/group, means ± SEM). MIA PaCa-2 tumor cells (2 × 10^6) were delivered subcutaneously into the hind leg of nude mice. On day 25, there was nearly a three-fold decrease in tumor growth compared with the control and ascorbate groups.

Figure 7. MnT4MPyP enhances ascorbate-induced cytotoxicity and ascorbate radical concentration in vivo. A, in whole blood from a separate group of mice, Asc^−/− is below the limit of detection. [Asc]_{ss} increased to 22 nmol/L upon treatment with MnT4MPyP (0.2 mg/kg i.p.). After i.p. injection of pharmacologic AscH^− (4 g/kg), [AscH^−] increased to 30 mmol/L and [Asc]_{ss} increased to 120 nmol/L. Combining both treatments increased [Asc]_{ss} to 310 nmol/L. n = 3 for each determination. AscH^− vs. MnT4MPyP, P < 0.05. AscH^− vs. AscH^− + MnT4MPyP, P < 0.01. B, MnT4MPyP combined with pharmacologic ascorbate decreased MIA PaCa-2 tumor growth in nude mice. The MnT4MPyP and ascorbate-treated animals had significantly slower tumor growth when compared with the control and ascorbate groups (*, P < 0.05, n = 12–16/group, means ± SEM). MIA PaCa-2 tumor cells (2 × 10^6) were delivered subcutaneously into the hind leg of nude mice. On day 25, there was nearly a three-fold decrease in tumor growth in animals receiving the combination when compared with controls.

O_2^- does not ameliorate MnP-enhanced AscH^- cytotoxicity. Accumulation of H_2O_2 does play a significant role in the cytotoxicity because scavenging H_2O_2 with catalase ameliorates the combined cytotoxicity of AscH^- and MnPs. We also show a 300% increase in the steady-state [Asc^-] with the addition of MnPs from mice infused with AscH^- and in patients treated with pharmacologic AscH^-.

Although our results are consistent with other studies showing decreases in clonogenic survival with MnPs and AscH^- (24, 25), our current study extends these previous observations by showing the chemistry behind the observed biological effects. Most importantly, we show the effects of MnPs on AscH^- oxidation in vitro, ex vivo, and in vivo. Studies previously carried out with MnPs have used AscH^- as a reducing agent (25). However, we use AscH^- as the main cytotoxic agent and MnPs as catalysts to increase the flux of H_2O_2.

In summary, MnPs increased the rate of AscH^- oxidation, the steady-state level of [Asc^-], and the rate of oxygen consumption, with MnT4MPyP having the greatest effect. MnPs synergistically enhanced AscH^- -induced cytotoxicity in all pancreatic cancer cell lines studied. Catalase, but not SOD, reversed the cytotoxicity of the AscH^- and MnPs combination, suggesting an H_2O_2-mediated mechanism. In addition, there was a marked increase in [Asc^-]_{ss} in whole blood from mice upon the addition of MnPs and as well as in the blood from patients treated with pharmacologic AscH^-.

We conclude that MnPs can increase the rate of oxidation of AscH^- leading to an increased flux of H_2O_2 resulting in increased AscH^- -induced cytotoxicity. MnPs have the potential as adjuvants to pharmacologic AscH^- therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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