Published in final edited form as:

Free Radic Biol Med. 2015 June ; 83: 89–100. doi:10.1016/j.freeradbiomed.2015.01.031.

MnTE-2-PyP⁵⁺ acts as a pro-oxidant to inhibit electron transport chain proteins, modulate bioenergetics and enhance the response to chemotherapy in lymphoma cells

Melba C. Jaramillo¹, Margaret M. Briehl¹, Ines Batinic-Haberle², and Margaret E. Tome³

¹Department of Pathology, University of Arizona, Tucson, AZ 85724

²Department of Radiation Oncology, Duke University School of Medicine, Durham, NC 27710

³Department of Medical Pharmacology, University of Arizona, Tucson, AZ 85724

Abstract

The manganese porphyrin, manganese (III) *meso*-tetrakis *N*-ethylpyridinium-2-yl porphyrin (MnTE-2-PyP⁵⁺), acts as a pro-oxidant in the presence of intracellular H₂O₂. Mitochondria are the most prominent source of intracellular ROS and important regulators of the intrinsic apoptotic pathway. Due to the increased oxidants near and within the mitochondria, we hypothesized that the mitochondria are a target of the pro-oxidative activity of MnTE-2-PyP⁵⁺ and that we could exploit this effect to enhance the chemotherapeutic response in lymphoma. In this study, we demonstrate that MnTE-2-PyP⁵⁺ modulates the mitochondrial redox environment and sensitizes lymphoma cells to anti-lymphoma chemotherapeutics. MnTE-2-PyP⁵⁺ increased dexamethasone-induced mitochondrial ROS and oxidation of the mitochondrial glutathione pool in lymphoma cells. The combination treatment induced glutathionylation of Complexes I, III and IV in the electron transport chain, and decreased the activity of Complexes I and III, but not the activity of Complex IV. Treatment with the porphyrin and dexamethasone also decreased cellular ATP levels. Rho(0) malignant T-cells with impaired mitochondrial electron transport chain function were less sensitive to the combination treatment than wild-type cells. These findings suggest that mitochondria are important for the porphyrin's ability to enhance cell death. MnTE-2-PyP⁵⁺ also augmented the effects of 2-deoxy-D-glucose (2DG), an antiglycolytic agent. In combination with 2DG, MnTE-2-PyP⁵⁺ increased protein glutathionylation, decreased ATP levels more than 2DG treatment alone and enhanced 2DG-induced cell death in primary B-ALL cells. MnTE-2-PyP⁵⁺ did not enhance dexamethasone- or 2DG-induced cell death in normal cells. Our findings suggest that MnTE-2-PyP⁵⁺ has potential as an adjuvant for the treatment of hematologic malignancies.

© 2015 Published by Elsevier Inc.

Corresponding author: Margaret E. Tome, PhD. Department of Pharmacology. P.O. Box 245050. University of Arizona, Tucson, AZ 85724. Phone: 520-626-6771. mtome@email.arizona.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

lymphoma; MnTE-2-PyP⁵⁺ (AEOL10113); glutathionylation; mitochondria

Introduction

The redox environment has emerged as a promising target for anti-cancer drug discovery. Cancer cells have constitutively elevated levels of reactive oxygen species (ROS) compared to non-transformed normal cells [1]. The differential ROS between normal and cancer cells represents a specific vulnerability in cancer cells and provides a therapeutic window that can be targeted by redox modulating drugs [2, 3]. Use of an agent that increases ROS equally in cancer and normal cells is expected to induce cell death in the tumor cells to a greater degree than in the normal cells because the tumor cells are closer to the apoptotic threshold.

Several standard chemotherapeutics including anthracyclins, bleomycin, bortezomib and glucocorticoids increase intracellular ROS [2, 4, 5]. The increased ROS may or may not contribute to the chemotherapeutic efficacy. For example, in the treatment of lymphoid malignancies, the ROS generated by glucocorticoid treatment contribute to the therapeutic effect [4, 5]. Specifically, glucocorticoids increase the level of H₂O₂; the amplitude of the H₂O₂ signal determines the sensitivity of the cells to glucocorticoids [4]. On the other hand, the ROS produced by anthracyclins are not thought to contribute to the cell killing of lymphoma cells [6–8]. The amount (or species) of ROS produced may be insufficient to contribute to the therapeutic effect. These data suggest that redox cycling compounds could be combined with standard chemotherapeutics that generate ROS to enhance chemotherapeutic efficacy. By amplifying the ROS signal or altering the type and ratio of oxidants produced, redox cycling compounds could be effective adjuvants.

Previous work in our laboratory tested the possibility that combining a redox active compound with a standard chemotherapeutic that generated H₂O₂ could enhance chemotherapeutic efficacy. Specifically, we combined glucocorticoids with the manganese porphyrin, manganese (III) *meso*-tetrakis (*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP⁵⁺). Traditionally, cationic Mn(III) *N*-substituted pyridylporphyrins have been viewed as antioxidants and superoxide dismutase (SOD) mimetics, due to their ability to catalyze O₂^{•−} dismutation [9, 10]. However, in the presence of glucocorticoids, MnTE-2-PyP⁵⁺ exhibits a pro-oxidative activity that enhances glucocorticoid-induced apoptosis [11]. The pro-oxidative activity is not due to an increased H₂O₂ flux [11] because of increased SOD activity, a possibility indicated by the work of Buettner et al. [12]. Instead, our data indicate that the H₂O₂ produced by glucocorticoid treatment oxidizes the manganese in MnTE-2-PyP⁵⁺ which cycles back to a reduced state using reducing equivalents from glutathione (GSH) [9, 13]. The redox cycling of MnTE-2-PyP⁵⁺ promotes glutathionylation of intracellular proteins. Glutathionylation plays an important role in redox signaling by regulating protein function [14, 15]. The ability of MnTE-2-PyP⁵⁺ to enhance glucocorticoid-induced apoptosis depends on the presence of both H₂O₂ and GSH [11] indicating that MnTE-2-PyP⁵⁺ promotes glutathionylation of critical survival proteins. In lymphoma cells, MnTE-2-PyP⁵⁺ promotes glutathionylation of the p65 NF-κB subunit and

consequently inhibits NF- κ B activity [11]. Inhibition of NF- κ B enhances glucocorticoid-induced apoptosis in lymphoid cells that depend on NF- κ B [11].

There are likely other critical targets of MnTE-2-PyP⁵⁺, since it enhances glucocorticoid-induced apoptosis in lymphoma cells that are not dependent on NF- κ B [11]. The goal of this study was to identify additional MnTE-2-PyP⁵⁺ targets. Elucidating MnTE-2-PyP⁵⁺ targets will allow us to both define the mechanism by which MnTE-2-PyP⁵⁺ enhances glucocorticoid-induced apoptosis in lymphoma cells not dependent on NF- κ B and suggest other potential chemotherapeutic uses of MnTE-2-PyP⁵⁺ in lymphoma and other tumor types. We hypothesized that mitochondria are MnTE-2-PyP⁵⁺ targets. Our hypothesis was based on the observation that mitochondria are the major source of the increased H₂O₂ produced in response to glucocorticoid treatment [16, 17]. Previous studies have shown that MnTE-2-PyP⁵⁺ enters mitochondria at biologically relevant concentrations [18, 19]. Due to the central role of mitochondria in apoptosis, metabolic regulation and ROS production in tumor cells, defining the effects of MnTE-2-PyP⁵⁺ on mitochondria could enhance our ability to use MnTE-2-PyP⁵⁺ effectively in the clinic. In the current study, we tested whether: 1) mitochondrial proteins are a target of the pro-oxidative activity of MnTE-2-PyP⁵⁺; 2) the pro-oxidative activity of MnTE-2-PyP⁵⁺ in mitochondria contributes to its ability to enhance glucocorticoid-induced apoptosis; and 3) we could exploit the pro-oxidative activity of MnTE-2-PyP⁵⁺ to develop novel chemotherapeutic strategies for lymphoid malignancies.

Materials and Methods

Cell culture

Murine thymic lymphoma WEHI7.2 cells were maintained in suspension in Dulbecco's Modified Eagle Medium-low glucose (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT). Human leukemia Molt4 cells were obtained from Dr. Lisa Rimsza (University of Arizona, Tucson, AZ). They were maintained in suspension in RPMI 1640 medium (Mediatech, Inc., Manassas, VA), supplemented with 10% fetal bovine serum (ATCC, Manassas, VA); 2 mM L-glutamine (Invitrogen) and 50 U/ml of penicillin and streptomycin (Invitrogen). Rho(0) Molt4 cells were obtained from Dr. Lionel D. Lewis (Dartmouth Medical School, Lebanon, NH) [20]. They were maintained in suspension in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μ g/mL uridine, and 100 μ g/mL sodium pyruvate (Invitrogen). All cell lines were incubated at 37°C in a controlled 5% CO₂ humidified environment.

Reagents and drug treatments

MnTE-2-PyP⁵⁺ was provided by Dr. Ines Batinic-Haberle (Duke University School of Medicine, Durham, NC). All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

The concentrations of dexamethasone and 2-deoxy-D-glucose used in this study were selected based on the EC₅₀ values for each cell line. WEHI7.2 cells were treated with 1 μ M dexamethasone in an ethanol vehicle (0.01% final concentration) for 12 hours; wildtype

Molt4 and rho(0) Molt4 cells were treated with 250 and 10 μ M dexamethasone, respectively, for 48 hours. WEHI7.2 and Molt4 cells were also treated with 15 mM 2-deoxy-D-glucose for 12 or 24 hours, respectively. Treatment times and doses were chosen based on the drug sensitivity and time course of apoptotic events in each cell type. To test the effect of MnTE-2-PyP⁵⁺ on the dexamethasone and 2-deoxy-D-glucose response, WEHI7.2 cells were pretreated with 50 nM MnTE-2-PyP⁵⁺, and Molt4 and Rho(0) Molt4 cells were pretreated with 0.5 μ M MnTE-2-PyP⁵⁺, 2 hours prior to the addition of dexamethasone. To test the importance of glutathione in mediating the porphyrin effects, WEHI7.2 and Molt4 cells were pretreated with buthionine sulfoximine (BSO) for 4 hours at 1 and 5 μ M, respectively. These concentrations deplete cytosolic GSH by more than ninety percent [11].

Protein measurements

Total cellular or mitochondrial protein was measured in clarified lysates using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

MitoSOX measurements

Cells were incubated in a final concentration of 5 μ M MitoSOX (Molecular Probes, Eugene, OR) in DMEM with 10% calf serum at 37°C for 3 hours. The rate of increase in MitoSOX fluorescence (Ex: 530 nm/Em: 590 nm) was measured using a Synergy HT plate reader (BioTek Instruments, Inc. Winooski, VT). Rates were normalized to sample protein measured as described above.

Imaging mitochondrial redox changes with mitoroGFP2

We obtained the redox sensitive GFP plasmid with a mitochondrial localization signal, mitoroGFP2, from Dr. S. James Remington (Eugene, OR) in order to measure redox changes in the cells' mitochondria [21]. The plasmid was electroporated into WEHI7.2 cells using the Amaxa Nucleofactor™ II kit (Amaxa GmbH, Germany). Cells were then grown in phenol-red free DMEM (Invitrogen) supplemented with 10% calf serum for 24 hours, to allow the cells to recover from the electroporation. The cells were pretreated with 50 nM MnTE-2-PyP⁵⁺ for 2 hours, followed by 1 μ M dexamethasone or vehicle control for 12 hours. Cells were imaged using the DeltaVision Restoration Microscopy System (Applied Precision, Inc., Issaquah, WA) using excitation wavelengths at 407 nm and 488 nm and a 510/21 nm emission filter. Data were collected and processed using Scion Image (Scion, Frederick, MD). Images were corrected for background fluorescence by subtracting the intensity of a nearby cell-free region. Fluorescence excitation ratios were then calculated by dividing the integrated intensities of the cells at different excitation wavelengths using the mathematical formulas described in Hanson *et al.* [21]. Sixteen cells were analyzed per cell treatment.

Mitochondria isolation

Mitochondria were isolated using the Mitochondria Isolation Kit for Cultured Cells (Pierce, Rockford, IL) following the manufacturer's instructions. Briefly, cells were harvested by centrifugation, washed in PBS, and resuspended in mitochondrial isolation buffer. The cells were lysed and nuclei, large debris, and intact cells were removed by centrifugation at 750 x

g for 10 min. The resulting supernatant was centrifuged at 21,000 x g for 15 min. The resulting mitochondrial pellet was then resuspended in lysis buffer (0.25 M sucrose, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 1% Triton X100).

Mitochondrial protein glutathionylation

To measure mitochondrial protein glutathionylation, mitochondrial lysates were separated by SDS-PAGE. Blots were probed for glutathionylation using a 1:1,000 dilution of the anti-GSH antibody (Virogen, Watertown, MA) followed by a 1:1,000 dilution of horseradish peroxidase-linked goat anti-mouse Ig secondary antibody (Cell Signaling). Blots were also probed with a 1:5,000 dilution of anti-Hsp60 (AbCam, Cambridge, MA) to control for equal loading. To visualize multiple bands on the same blot, blots were stripped with Restore Western Blot Stripping Buffer (Pierce) before being probed with a new antibody. Proteins were normalized to Hsp60 to correct for loading differences. To determine the overall level of protein glutathionylation, bands were quantified using a ScanJet 4C scanner (Hewlett Packard, Palo Alto, CA) and Image J analysis software (National Institutes of Health, Bethesda, MA). To measure Complex III protein glutathionylation, mitochondrial cell lysates were immunoprecipitated for Complex III using a Complex III Immunocapture kit (AbCam), separated by SDS PAGE and immunoblotted with the anti-GSH antibody, as previously described [11].

2-D gel electrophoresis and proteomic analysis

Mitochondrial samples were isolated and resuspended in SDS Boiling Buffer using the MK-1 Kit provided by Kendrick Labs (Madison, WI). Beta-mercaptoethanol was added to reach a 5% final concentration and the sample was diluted 1:1 in Urea Buffer from the MK-1 Kit. Two-dimensional gel electrophoresis was performed by Kendrick Labs. The immunoblots were probed with the anti-GSH antibody (Virogen) at a 1:1,000 dilution to identify the glutathionylated proteins. Kendrick Labs then identified the glutathionylated proteins by nano-LC-MS/MS and SEQUEST database search.

Electron transport chain complex activity assays

The activities of Complexes I and III were measured using clarified whole cell lysates. To measure Complex IV activity, mitochondria were first isolated, as described above. Complex I activity was measured as described in Janssen et al. [22]; Complex III activity was measured as described in Kramer et al. [23]; and Complex IV was measured as described in Zhang et al. [24]. The activities were normalized to cellular or mitochondrial protein levels.

Cellular ATP measurements

ATP was measured using the Bioluminescent Somatic Cell Assay Kit (Sigma) as previously described [25]. Luminescence was quantitated using a Synergy HT plate reader (BioTek Instruments). To measure non-mitochondrial ATP production, cells were treated with 10 μ M carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) for 2 hours before cell harvest [25].

Cell viability and caspase-3 activity measurements

The number of viable cells was determined by dye exclusion as previously described [26]. Apoptosis was confirmed by measuring caspase-3 activity as previously described [11]. We also used the Nonradioactive Cell Proliferation Assay (MTS; Promega Corp) to measure the relative cell number after 2-deoxy-D-glucose treatment in WEHI7.2 and Molt4 cells. Plates were read at 490 nm using a Synergy HT plate reader (BioTek Instruments). The mathematical drug synergy model described in Chou et al. [27] was used to determine whether the interaction between the porphyrin and 2-deoxy-D-glucose was additive or synergistic.

Caspase-3 activity was measured using an assay dependent on the enzymatic cleavage of a synthetic caspase-3 specific substrate, Ac-DEVD-*p*-nitroanilide (Enzo Life Sciences, Inc. Farmingdale, NY), as described previously [28]. Caspase-3 activity was normalized to cellular protein and expressed as a percentage of control activity.

Primary B-ALL cells and normal human PMBC

Three primary human tumor samples with a diagnosis of acute lymphoblastic B cell leukemia (B-ALL) were obtained from the Arizona Lymphoma Tissue Repository in accordance with University of Arizona regulations for the use of primary human tissue. Normal human peripheral mononuclear blood cells (PMBC) were purchased from ReachBio LLC (Seattle, WA). The cells were thawed and resuspended in Iscove's modified Dulbecco's medium (Invitrogen) with 20% fetal bovine serum (Gemini Bio-Products, Woodland, CA) in the presence or absence of MnTE-2-PyP⁵⁺, dexamethasone and 2-deoxy-D-glucose. Viable B-cell content of the B-ALL samples was analyzed before the addition of drugs and after incubation at 37°C in a 5% CO₂ humidified environment for 24 hours in the presence or absence of drugs. Cells were labeled with phycoerythrin-labeled anti-CD20 (AbD Serotec, Raleigh, NC) to identify B-cells and 7.5 µg/mL 7-actinomycin D (7-AAD), which stains dead cells, for 20 minutes. CD20 positive/7-AAD negative cells were considered viable B-cells and measured by flow cytometry (EPICS XL-MCL, Beckman Coulter, Inc., Brea, CA). An isotype control was used for each run to gate out CD20-negative cells; debris was also gated out. At thaw, samples from patient 1, patient 2, and patient 3 contained 69%, 68% and 76% viable B-cells, respectively. A minimum of 5,000 events were analyzed per sample. For the data analysis, the percentage of viable B-cells in the sample containing no drug was set to 100%. Viable PMBC content was analyzed similarly except the samples were labeled only with 7.5 µg/mL 7-AAD. At thaw, samples 1 and 2 contained 95% and 98% PMBC, respectively.

Statistical analysis

Means were compared using student's t-tests with the algorithm in Excel (Microsoft Corp., Redmond, WA). Means were considered significantly different when $p < 0.05$.

Results

MnTE-2-PyP⁵⁺ enhances dexamethasone-induced mitochondrial ROS and oxidation of the mitochondrial redox environment

Recently, we reported that mitochondria are a major source of the ROS produced in response to dexamethasone treatment in WEHI7.2 cells [11]. To determine whether MnTE-2-PyP⁵⁺ enhances the levels of mitochondrial ROS produced by dexamethasone during the signaling phase of glucocorticoid-induced apoptosis, we measured mitochondrial ROS by quantifying the oxidation of MitoSOX [29] after 12 hours of treatment. We found that treatment with 50 nM MnTE-2-PyP⁵⁺ alone slightly increased mitochondrial ROS; although, the change was not statistically significant. As we reported previously, treatment with 1 μ M dexamethasone for 12 hours increases mitochondrial ROS. In combination with dexamethasone, MnTE-2-PyP⁵⁺ augmented the levels of mitochondrial ROS induced by dexamethasone approximately 2-fold (Figure 1A).

To determine whether the increase in ROS is sufficient to oxidize the mitochondrial redox environment we used the redox-sensitive green fluorescent protein (GFP) probe, mito-roGFP2, that localizes to the mitochondrial matrix [21]. The relative oxidation of mito-roGFP2 has been used as an indicator of the mitochondrial redox environment and most closely reflects the oxidation state of the GSH and glutathione disulfide (GSSG) redox pair; however, other factors can contribute [21]. Figure 1B shows the relative amounts of oxidized mitochondria-targeted GFP after a 12 hour treatment. In control cells treated with vehicle alone, $10.21 \pm 0.96\%$ of the mito-roGFP2 was oxidized. Treatment with 50 nM MnTE-2-PyP⁵⁺ alone did not change the percentage of oxidized mito-roGFP2. In the presence of 1 μ M dexamethasone, the amount of oxidized mito-roGFP2 increased to $16.97 \pm 1.03\%$. Combining 1 μ M dexamethasone with 50 nM MnTE-2-PyP⁵⁺ increased the relative amount of mito-roGFP2 in the oxidized state to $29.65 \pm 1.11\%$. Overall, these data indicate that in combination with dexamethasone, MnTE-2-PyP⁵⁺ increases mitochondrial oxidants and oxidizes the mitochondrial redox environment in lymphoma cells over that caused by dexamethasone treatment alone.

MnTE-2-PyP⁵⁺ induces mitochondrial protein glutathionylation

Previously, we showed that MnTE-2-PyP⁵⁺ increases glutathionylation of p65 NF- κ B, a cytosolic redox sensitive protein in WEHI7.2 cells [11]. Given that MnTE-2-PyP⁵⁺ increases dexamethasone-induced mitochondrial ROS and likely oxidizes the 2GSH:GSSG redox couple we investigated whether MnTE-2-PyP⁵⁺, in combination with dexamethasone, increased glutathionylated proteins in mitochondria. Treating WEHI7.2 cells with 50 nM MnTE-2-PyP⁵⁺, 1 μ M dexamethasone, or MnTE-2-PyP⁵⁺ in combination with dexamethasone for 12 hours induced protein glutathionylation in the mitochondria (Figure 2). We saw two prominent bands when we probed the total mitochondrial protein samples for glutathionylated proteins (Figure 2); longer exposures indicated that there were additional glutathionylated proteins (Supplementary Figure 1). On its own, MnTE-2-PyP⁵⁺ treatment increased glutathionylation of these mitochondrial proteins approximately 2-fold greater than the amount seen in cells treated with a vehicle (control). Dexamethasone also increased protein glutathionylation, but not to the same extent as MnTE-2-PyP⁵⁺ alone. We

measured the largest increase in mitochondrial protein glutathionylation when the cells were treated with MnTE-2-PyP⁵⁺ plus dexamethasone. The combination treatment enhanced the protein glutathionylation of these proteins four times the amount caused by dexamethasone treatment alone. Pretreatment with 1 μ M buthionine sulfoximine (BSO), a glutathione synthesis inhibitor, decreased the porphyrin's ability to induce protein glutathionylation or enhance dexamethasone-induced protein glutathionylation (Figure 2). We previously showed that 1 μ M BSO depletes the levels of GSH in WEHI7.2 cells by more than 90% [11]. Taken together, these findings suggest that MnTE-2-PyP⁵⁺ induces glutathionylation of redox sensitive proteins in the mitochondria.

We performed a proteomic analysis to identify the glutathionylated proteins in the mitochondria. We treated WEHI7.2 cells with 50 nM MnTE-2-PyP⁵⁺ and 1 μ M dexamethasone for 12 hours and used LC-MS/MS to identify mitochondrial proteins that were glutathionylated more than 2-fold the amount glutathionylated in the samples treated with dexamethasone alone. The identified proteins are listed in Table 1 and include: stress proteins; enzymes, and proteins in the electron transport chain (ETC), including NADH ubiquinone oxidoreductase (Complex I), and cytochrome c oxidase (Complex IV).

MnTE-2-PyP⁵⁺inhibits the activity of enzymes in the mitochondria electron transport chain

Given that glutathione is required for MnTE-2-PyP⁵⁺ to enhance glucocorticoid-induced apoptosis [11], we hypothesized that proteins with increased glutathionylation in the combined treatment compared to dexamethasone alone are most likely to contribute to the enhanced cell death. We focused on Complex I and Complex IV of the ETC for two reasons. First, both showed increased glutathionylation after the combined treatment. Second, dexamethasone treatment inhibits glucose uptake in lymphoid cells [25, 30] forcing the cells to generate ATP from other sources, particularly glutamine, via mitochondrial processes [25, 31]. Inhibition of mitochondrial ATP production could enhance the glucocorticoid response and suggest new therapeutic strategies using MnTE-2-PyP⁵⁺.

Previous studies in other cell systems have shown that Complexes I and IV are glutathionylated under conditions of oxidative stress [32]; however, the consequences of glutathionylation of these complexes is not well understood. To investigate how glutathionylation affects the function of the enzymes in the ETC in lymphoma cells, we measured their activity following treatment with MnTE-2-PyP⁵⁺ or dexamethasone alone, and MnTE-2-PyP⁵⁺ in combination with dexamethasone for 12 hours. As shown in Figure 3, MnTE-2-PyP⁵⁺ treatment alone decreased Complex I activity by 90%, but did not affect Complex IV activity. Dexamethasone treatment alone also decreased Complex I activity, but only by approximately 65%. Complex IV activity was not affected by dexamethasone treatment. Cotreatment with dexamethasone did not further decrease Complex I activity compared to what was seen with MnTE-2-PyP⁵⁺ alone and did not affect the activity of Complex IV.

Although the proteomic analysis did not identify Complex III as a protein with increased glutathionylation in the combination treatment, we hypothesized that the location of cysteine residues in Complex III would make them vulnerable to this modification [33]. We immunoprecipitated complex III and found that the Rieske iron sulfur protein in Complex III

is glutathionylated by MnTE-2-PyP⁵⁺ and dexamethasone (Figure 3C). Additionally, the activity of complex III decreased after treatment with MnTE-2-PyP⁵⁺ or the combination (MnTE-2-PyP⁵⁺/dexamethasone) by approximately 65% and 70%, respectively (Figure 3D). To verify that the ability of MnTE-2-PyP⁵⁺ to induce protein glutathionylation is responsible for the decrease in complex activity we pretreated the cells with 1 μ M BSO for 4 hours. In the presence of BSO, MnTE-2-PyP⁵⁺ and the combination treatment did not significantly decrease the activity of Complexes I or III (Figure 3A and D).

MnTE-2-PyP⁵⁺ accelerates the decrease in cellular ATP levels

We previously showed that a 24 hour dexamethasone treatment decreases cellular ATP in WEHI7.2 cells [25]. The ability of MnTE-2-PyP⁵⁺ and dexamethasone to inhibit the activity of several complexes in the ETC led us to hypothesize that the combination treatment would accelerate the decrease in cellular ATP levels. We measured the amount of ATP in WEHI7.2 cells after treatment with each drug alone, and the combination for 12 hours. As shown in Figure 4, the cellular ATP levels were not affected by MnTE-2-PyP⁵⁺ or dexamethasone treatment alone. The combination treatment, however, decreased the amount of ATP by $31.50 \pm 7.11\%$ in the WEHI7.2 cells. To determine whether the decrease in ATP was due to the glutathionylation of the complexes, we treated the cells with BSO plus MnTE-2-PyP⁵⁺ in combination with dexamethasone. We have previously shown that 1 μ M BSO does not cause cell death or enhance dexamethasone-induced apoptosis [11]. In the presence of BSO, the levels of ATP remained similar to control levels. Our findings suggest that: 1) the combination treatment is capable of decreasing cellular ATP levels; and 2) the glutathionylation of the complexes in the ETC contributes to the decrease in ATP levels after treating the cells with MnTE-2-PyP⁵⁺ and dexamethasone.

MnTE-2-PyP⁵⁺ induces glutathionylation and inhibits the activity of proteins in the mitochondria of malignant human T-cells

In order to determine whether our data translated to a human model, we determined whether MnTE-2-PyP⁵⁺ glutathionylates mitochondrial redox sensitive proteins in human Molt4 T-cell leukemia cells. We treated these cells with 0.5 μ M MnTE-2-PyP⁵⁺ and 250 μ M dexamethasone for 24 hours; the concentrations are the EC₅₀ values for MnTE-2-PyP⁵⁺ and dexamethasone in this cell line. As shown in Figure 5A, we measured a slight increase in the levels of glutathionylated mitochondrial proteins after treatment with 0.5 μ M MnTE-2-PyP⁵⁺ for 24 hours; however, the change was not statistically significant. Similarly, treatment with 250 μ M dexamethasone for 24 hours also increased mitochondrial protein glutathionylation, but it was also not statistically different from vehicle-treated cells. The combination treatment (MnTE-2-PyP⁵⁺ and dexamethasone), increased mitochondrial protein glutathionylation 3-fold the amount in vehicle-treated Molt4 cells, and 2-fold the amount measured in cells treated with dexamethasone alone. In combination with 5 μ M BSO, a concentration we previously showed depletes GSH by more than 90% [11], the combination treatment did not induce protein glutathionylation. 5 μ M BSO treatment does not cause cell death or enhance apoptosis due to dexamethasone in Molt4 cells [11].

We next tested whether MnTE-2-PyP⁵⁺-induced protein glutathionylation inhibited the activity of Complexes I, III and IV in the Molt4 human cells. We treated the cells with 0.5

μM MnTE-2-PyP⁵⁺ and 250 μM dexamethasone for 24 hours. Our data (Figure 5B–D) show that on its own, MnTE-2-PyP⁵⁺, did not significantly decrease the activity of Complexes I, III or IV. Dexamethasone treatment decreased complex I and III activity by 40 and 30%, respectively. Dexamethasone treatment, however, did not affect Complex IV activity in these cells. In combination with dexamethasone, MnTE-2-PyP⁵⁺, inhibited Complex I and III activity more than dexamethasone treatment alone. The combination treatment inhibited Complex I activity more than 90%, and Complex III activity nearly 60% compared to vehicle-treated cells. Pretreatment with 5 μM BSO, however, blocked the ability of the MnTE-2-PyP⁵⁺/dexamethasone combination to decrease Complex I and III activity. The combination treatment did not affect Complex IV activity.

We also measured the amount of ATP in the Molt4 cells after treatment with MnTE-2-PyP⁵⁺ or dexamethasone alone and the combination (MnTE-2-PyP⁵⁺/dexamethasone) for 24 hours. As shown in Figure 5E, the cellular ATP levels were not affected by MnTE-2-PyP⁵⁺ or dexamethasone treatment. The combination treatment decreased the amount of ATP by $30.11 \pm 3.75\%$. To determine whether the decrease in ATP was due to the glutathionylation of the complexes we treated the cells with BSO plus MnTE-2-PyP⁵⁺ in combination with dexamethasone. In the presence of BSO, the levels of ATP remained at control levels. Taken together, these findings show that in combination with dexamethasone, MnTE-2-PyP⁵⁺: 1) promotes glutathionylation of mitochondrial redox sensitive proteins; 2) inhibits the activity of Complexes I and III by inducing glutathionylation; and 3) decreases cellular ATP levels in human malignant T-cells. Our findings suggest that MnTE-2-PyP⁵⁺ targets the mitochondria in murine and human lymphoma cells.

Mitochondria are important for the porphyrin's ability to enhance dexamethasone-induced apoptosis

To determine whether mitochondrial effects are important for the porphyrin's ability to enhance dexamethasone-induced apoptosis we tested the effect that MnTE-2-PyP⁵⁺ has on cell viability in rho(0) Molt4 cells treated with dexamethasone. Rho(0) Molt4 cells have depleted mitochondrial DNA and impaired ETC function compared to wild-type Molt4 cells [20]. We previously showed that MnTE-2-PyP⁵⁺ enhances dexamethasone-induced cell death in the wild-type Molt4 cells [11]. We compared the effect of the combination treatment in the wild-type cells to the rho(0) Molt4 cells to determine whether they were equally sensitive (Figure 6). In the wild-type cells, the percent viable cells after treatment with 0.5 μM MnTE-2-PyP⁵⁺ for 24 hours was $95.04 \pm 3.10\%$. Similarly, the number of viable rho(0) Molt4 cells in culture after treatment with 0.5 μM MnTE-2-PyP⁵⁺ for 24 hours was $97.63 \pm 1.98\%$. The percent viable cells after a 24 hour treatment with the dexamethasone EC₅₀ concentration was $53.89 \pm 2.16\%$ in the wild-type Molt4 cells and $52.51 \pm 2.17\%$ in the rho(0) Molt4 cells. In combination with dexamethasone, MnTE-2-PyP⁵⁺ decreased the percentage of viable cells more than when they were treated with dexamethasone alone in both cell types. However, the wild-type cells were more sensitive to MnTE-2-PyP⁵⁺/dexamethasone treatment than the rho(0) Molt4 cells. The percentage of viable cells remaining after treating the wild-type Molt4 cells with MnTE-2-PyP⁵⁺ and dexamethasone was $23.66 \pm 3.16\%$, whereas in the rho(0) Molt4 cells the percentage of viable cells was $43.24 \pm 3.16\%$. Caspase 3 activity measurements confirmed the induction of

apoptosis with treatments that decreased viable cell number (Supplementary Figure 2). These findings suggest that mitochondria are targets of MnTE-2-PyP⁵⁺ and that they contribute to the porphyrin's ability to enhance dexamethasone-induced cell death in lymphoma cells.

MnTE-2-PyP⁵⁺ synergizes with 2-deoxy-D-glucose to enhance cell death and decrease cellular ATP levels

Emerging studies show that dual targeting of mitochondrial and glycolytic pathways are a promising chemotherapeutic strategy for several different types of cancers [34]. One of the most frequently used anti-glycolytic agents is 2-deoxy-D-glucose (2DG). 2DG inhibits ATP generated via the glycolytic pathway [34]. The ability of MnTE-2-PyP⁵⁺ to inhibit the ETC suggested that MnTE-2-PyP⁵⁺ could be combined with 2DG to enhance cell death. We treated WEHI7.2 cells with 15 mM 2DG and 50 nM MnTE-2-PyP⁵⁺. In combination with 2DG, MnTE-2-PyP⁵⁺ decreased the cellular ATP levels, more than 2DG treatment alone (Figure 7A). As proof of principle, we tested whether the combination treatment could decrease mitochondrial ATP to the same extent as a 2 hour treatment with carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), a mitochondrial uncoupler; we found that the combination treatment decreased ATP levels more than CCCP treatment alone. The combination treatment decreased the ATP levels in the WEHI7.2 cells to the same extent as when the cells were treated with 2DG and CCCP. Taken together, these findings suggest that in combination with 2DG, MnTE-2-PyP⁵⁺ can decrease mitochondrial ATP to the same extent as CCCP and that the porphyrin targets the mitochondria. We also found that in combination with 2DG, MnTE-2-PyP⁵⁺ enhanced the ability of 2DG to induce cell death by $14.65 \pm 1.6\%$ (Figure 7B). Caspase 3 activity measurements confirmed the induction of apoptosis with treatments that decreased viable cell number (Supplementary Figure 3). Using a mathematical model to test for drug synergy [27], we determined that MnTE-2-PyP⁵⁺ synergizes with 2DG to enhance cell death. *MnTE*-

2-PyP⁵⁺ induces protein glutathionylation and augments 2-deoxy-D-glucose-induced cell death in primary B-ALL patient samples

We obtained three primary acute lymphoblastic B cell leukemia (B-ALL) samples to test the effectiveness of the porphyrin in combination with 2DG in primary tumor cells. We found that the overall expression of glutathionylated proteins increased approximately 2.5-fold over control levels after treatment with 0.5 μ M MnTE-2-PyP⁵⁺ for 24 hours (Figure 8A). As a single agent, MnTE-2-PyP⁵⁺ decreased cell viability between 10 and 35% in a dose dependent manner (Figure 8B). Treatment with 15 mM 2DG decreased the number of viable B-cells between 20 and 40%. In combination with 2DG, MnTE-2-PyP⁵⁺ enhanced 2DG-induced cell death in all three primary B-ALL samples. These data indicate that MnTE-2-PyP⁵⁺ accelerates 2DG-induced cell death in primary tumor cells, and suggests that MnTE-2-PyP⁵⁺ has clinical potential for the treatment of hematological malignancies combined with anti-glycolytic agents.

MnTE-2-PyP⁵⁺ does not enhance dexamethasone or 2DG-induced cell death in normal PMBC

Our previous data shows that MnTE-2-PyP⁵⁺ enhances dexamethasone-induced cell death in primary follicular lymphoma patient samples [28]. In the current study, we found that MnTE-2-PyP⁵⁺ enhanced 2DG-induced cell death in B-ALL patient samples. However, the effect of these drug combinations on normal human blood cells is unknown. As shown in Table 2, MnTE-2-PyP⁵⁺ did not enhance either dexamethasone- or 2DG-induced cell death in normal PMBC, the normal human counterpart of lymphoma and leukemia cells. In one sample, MnTE-2-PyP⁵⁺ protected against 2DG-induced cell death of PMBC. These data suggest that the ability of MnTE-2-PyP⁵⁺ to enhance dexamethasone- and 2DG-induced cell death is specific to tumor cells.

Discussion

Using in vitro models and primary tumor samples we found a novel pro-oxidative activity of MnTE-2-PyP⁵⁺ at the mitochondria that results in glutathionylation of redox sensitive proteins. The pro-oxidative activity of MnTE-2-PyP⁵⁺ enhances therapeutic efficacy in these models. MnTE-2-PyP⁵⁺ does not enhance cell death in normal human PMBC due to the therapeutic agents suggesting MnTE-2-PyP⁵⁺ could act as an effective adjuvant for hematologic malignancies. MnTE-2-PyP⁵⁺ alone and/or in combination with dexamethasone induces glutathionylation of Complexes I, III and IV in the ETC. Glutathionylation of Complexes I and III inhibits their activity. Addition of MnTE-2-PyP⁵⁺ to dexamethasone augments dexamethasone-induced mitochondrial ROS, decreases cellular ATP levels and enhances dexamethasone-induced apoptosis in a synergistic manner. MnTE-2-PyP⁵⁺ is not able to enhance dexamethasone-induced apoptosis in cells lacking functional mitochondria to the same extent that it does in cells with intact mitochondria. These data indicate that mitochondrial effects are important for the ability of MnTE-2-PyP⁵⁺ to enhance glucocorticoid-induced apoptosis. Our data also show that the porphyrin can be combined with antiglycolytic agents such as 2DG. Use of the porphyrin to block oxidative phosphorylation enhances cell death due to 2DG in cell culture and in primary tumor samples. Our studies suggest that MnTE-2-PyP⁵⁺, due to its pro-oxidative activity at the mitochondria, can enhance the effect of existing chemotherapeutics and be used for the rational design of new therapeutic regimens. The model in Figure 9 summarizes the MnTE-2-PyP⁵⁺ effects we observed at the mitochondria.

The ability of MnTE-2-PyP⁵⁺ to induce protein glutathionylation is critical for its ability to enhance cell death due to the chemotherapeutics tested in the current study. For MnTE-2-PyP⁵⁺ to induce protein glutathionylation, redox cycling with an oxidant such as H₂O₂ is required. Dexamethasone treatment causes an increase in H₂O₂ [4]. If the H₂O₂ is removed by increasing catalase activity there is no increased protein glutathionylation in the presence of MnTE-2-PyP⁵⁺ or enhancement of apoptosis [11]. Addition of extracellular H₂O₂ can substitute for the dexamethasone treatment resulting in redox cycling of MnTE-2-PyP⁵⁺ and increased protein glutathionylation. Our data best fit a model whereby the manganese is oxidized from the +2 or +3 state to high-valent Mn oxo species then returns to the +2 or +3 state using reducing equivalents from glutathione or cysteine in the cell [11]. Aqueous

solution chemistry indicates this redox cycle can occur [35]. The oxidation of glutathione and cysteine in proteins results in protein glutathionylation. If this model is correct, the concentration and type of oxidants in the cell, which is determined by the generation and removal systems, would affect their vulnerability to MnTE-2-PyP⁵⁺. Some tumor cells with a high oxidant concentration would be susceptible to MnTE-2-PyP⁵⁺ as a single agent, while others with a lower concentration would only be susceptible when treated with a compound (chemotherapeutic) that causes the oxidant generation to outstrip the removal mechanisms. This feature could also provide a therapeutic window for treatment of tumors because of the relative oxidant load of normal and tumor cells. The inability of MnTE-2-PyP⁵⁺ to enhance dexamethasone- or 2DG-induced cell death of normal cells supports this concept.

To enhance cell death, MnTE-2-PyP⁵⁺ must promote glutathionylation of cellular targets critical for survival. In the current study we found that ETC proteins are MnTE-2-PyP⁵⁺ targets. S-glutathionylation is a post translational modification that can alter protein function and activity, usually by inhibiting it [36]. In lymphoma cells, similar to the observation in a Parkinson disease model [37], the activity of Complex I decreased when the complex was glutathionylated. Our studies also demonstrate that protein glutathionylation decreases the activity of Complex III in lymphoma cells. We found that the Rieske iron sulfur protein in Complex III is glutathionylated after treatment with MnTE-2-PyP⁵⁺ or MnTE-2-PyP⁵⁺ in combination with dexamethasone. Our findings are consistent with data from other groups showing that the cysteine on the Rieske protein is important for maintaining Complex III activity [33]; however, to the best of our knowledge, this is the first report that glutathionylation of this protein inhibits its activity. Although MnTE-2-PyP⁵⁺ induces glutathionylation of Complex IV, its activity did not change. Complex IV is comprised of 15 subunits, all of which contain cysteine residues. There are two cysteines in Complex IV located near the enzyme's active site (in the S2 subunit) that are essential for its activity [38]. It is possible that MnTE-2-PyP⁵⁺ did not promote glutathionylation of these cysteine residues, and as result the activity was not affected. On the other hand, several groups have reported that protein glutathionylation protects proteins from oxidative damage [32, 39]. In lung endothelial cells, the S2 cysteines are highly susceptible to nitrosylation, another redox-based post-translational modification [38]. S-nitrosylation of the cysteines inhibited Complex IV activity in these cells. MnTE-2-PyP⁵⁺ may therefore, glutathionylate the S2 cysteines in lymphoma cells, however, this event may be protecting Complex IV from nitrosylation that inhibits its activity.

Our findings suggest that the porphyrin promotes glutathionylation of the complexes on the outer surface of the inner mitochondrial membrane. Pretreatment with BSO decreased the ability of the porphyrin and the combination treatment to glutathionylate proteins in the mitochondria and restored the activity of the complexes. BSO is a GSH synthesis inhibitor; however, mitochondria do not have the cellular machinery to synthesize GSH [40]. GSH is produced in the cytosol and transported into the mitochondria [40]. Thus, with a short treatment time as in this study, BSO is thought to specifically decrease the amount of GSH in the cytosol [41]. The ability of BSO to decrease the level of protein glutathionylation induced by the combination treatment, therefore, suggests that the glutathionylation is occurring on the cytosolic side of the ETC proteins. Additionally, previous studies have shown that the reactive thiols (Cys-531 and Cys-704) on Complex I are located on the 75-

kDa subunit of Complex I [42]. These cysteines are located towards the outer surface of the mitochondrial inner membrane.

Glutathionylation of the complexes on the intermembrane space side is also consistent with our previous measurements of the MnTE-2-PyP⁵⁺ effect on the cellular glutathione pool. In WEHI7.2 cells, treatment with MnTE-2-PyP⁵⁺ alone decreases total cellular glutathione 42% compared to control cells [11]. Treatment with dexamethasone alone decreases glutathione 31% while cells treated with the MnTE-2-PyP⁵⁺/dexamethasone combination show a 61% decrease in glutathione. The combination treatment also increases glutathione disulfide 11-fold over that in control cells. Cellular glutathione is inversely proportional to the relative protein observed in the mitochondrial proteins (Figure 2). The observed pattern is what would be expected if the cytosolic glutathione pool is providing glutathione for protein modification on the cytosolic face of the ETC complexes. The relative mitochondrial protein glutathionylation does not fit the relative pattern of mitochondrial matrix ROS production and oxidation measured by mito-roGFP2 (Figure 1). If glutathionylation of the ETC complexes was occurring on the matrix side we would expect a glutathionylation pattern that was proportional to the mitochondrial matrix oxidation.

The results seen with the rho(0) Molt4 cells indicate that the mitochondria are a critical target of MnTE-2-PyP⁵⁺ and that they contribute to the porphyrin's ability to enhance dexamethasone-induced apoptosis. When combined with dexamethasone, the porphyrin augments the pro-oxidative effects of dexamethasone in the mitochondria. Previous studies have highlighted the therapeutic efficacy of combining ROS-inducing drugs and mitochondria targeted agents. Pelicano et al., recently demonstrated that partial inhibition of mitochondrial respiration with rotenone, a Complex I inhibitor, increased the levels of superoxide in the mitochondria and sensitized leukemia cells to anticancer agents whose action involve free radical generation [39]. Our data suggest that in combination with dexamethasone, MnTE-2-PyP⁵⁺ could work in a similar fashion. MnTE-2-PyP⁵⁺ induces glutathionylation and decreases the activity of the ETC complexes, which increases univalent reduction of oxygen to form superoxide [43, 44]. MnTE-2-PyP⁵⁺, in combination with dexamethasone, augments the oxidation of the mitochondrial glutathione pool which would alter the ability of the mitochondria to respond to oxidants. Inhibition of Complexes I and III by dexamethasone treatment could also make them more vulnerable to glutathionylation; recent data indicate mitochondrial respiratory state can influence mitochondrial protein glutathionylation susceptibility [45]. Alternatively, inhibition of ATP generation via glycolysis due to dexamethasone combined with inhibition of ETC function via MnTE-2-PyP⁵⁺ may combine to deplete ATP and enhance cell death.

The ability of MnTE-2-PyP⁵⁺ to inhibit the ETC and synergize with 2DG suggests this drug combination has potential in tumors that depend on glycolysis. Several studies have reported that cancer cells have increased glycolytic activity (Warburg effect) [46, 47], which represents a specific vulnerability of cancer cells. 2DG inhibits glucose metabolism in cell culture, increases intracellular oxidants and is preferentially taken up by tumor cells in vivo [48, 49]. In the clinic, 2DG has been tested for treatment of leukemia. It was well tolerated by patients and effectively decreases glucose metabolism in leukemia cells isolated from patients [50, 51]. However, as a single agent, 2DG does not alter the course of the disease or

improve patient survival [50, 51]. In the current study, we show that MnTE-2-PyP⁵⁺ augments cell death in murine, human and primary B-ALL cells treated with 2DG. When the ability of cells to generate ATP through mitochondrial oxidative phosphorylation (ETC) is compromised, cells increase their glycolytic activity to maintain their energy supply [52]. Thus, MnTE-2-PyP⁵⁺ may enhance the effects of 2DG by inhibiting the ETC, which would make the cells more dependent on glycolysis, and more sensitive to 2DG treatment.

Our studies demonstrate that the manganese porphyrin, MnTE-2-PyP⁵⁺, targets the mitochondria and acts as a mitochondrial pro-oxidant in lymphoma cells. The porphyrins have been used in a number of disease models where they are commonly regarded as SOD mimics and thus as antioxidants. The ability of the porphyrin to dismutate O₂^{•-} in an SOD-like fashion indicates that it can reduce and oxidize O₂^{•-} with similar efficiency [53]. This porphyrin chemistry indicates that it can also act as a pro-oxidant particularly in an environment where it can extract electrons from biological targets [54, 55]. Our findings suggest that it is the pro-oxidant activity of MnTE-2-PyP⁵⁺ that can be exploited for cancer therapy particularly in combination therapy. When used in combination with other drugs that produce oxidants, MnTE-2-PyP⁵⁺ promotes glutathionylation and inhibits proteins such as the ETC complexes and NF-κB [11] that are critical to the survival of tumor cells. The inability of MnTE-2-PyP⁵⁺ to enhance cell death due to established chemotherapeutics in normal cells suggests there is a therapeutic window for use of MnTE-2-PyP⁵⁺ as an adjuvant. Treatment regimens that utilize this unique activity of the porphyrins could be beneficial for treating a variety of tumor types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources: We thank: Dr. David Elliot for technical advice and assistance with the DeltaVision Restoration Microscopy System; Dr. James E. Remington for the mito-roGFP2 plasmid; and David Stringer and Dr. Eugene Gerner for use of critical equipment. Funding for this study comes from the National Cancer Institute grants CA-71768 (M.M.B) and CA-09213 (M.C.J), Arizona Cancer Support grant CA-023074, Lymphoma SPORE CA-130805 (M.E.T., M.M.B.), and Small Faculty Grant (M.E.T). IBH acknowledges her General Research Funds.

Abbreviations

7-AAD	7-actinomycin D
B-ALL	Acute lymphoblastic B-cell leukemia
BSO	Buthionine sulfoximine
CCCP	carbonyl cyanide <i>m</i> -chlorophenylhydrazine
2DG	2-deoxy-D-glucose
ETC	electron transport chain
GFP	green fluorescent protein
GSH	glutathione

GSSG	glutathione disulfide
MnTE-2-PyP⁵⁺	Manganese (III) <i>meso</i> -tetrakis <i>N</i> -ethylpyridinium-2-yl porphyrin
PMBC	peripheral mononuclear blood cells
SOD	superoxide dismutase

References

1. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* 1991; 51:794–8. [PubMed: 1846317]
2. Wondrak GT. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. *Antioxid Redox Signal.* 2009; 11:3013–69. [PubMed: 19496700]
3. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov.* 2009; 8:579–91. [PubMed: 19478820]
4. Tome ME, Jaramillo MC, Briehl MM. Hydrogen peroxide signaling is required for glucocorticoid-induced apoptosis in lymphoma cells. *Free Radic Biol Med.* 2011; 51:2048–59. [PubMed: 21964507]
5. Bera S, Greiner S, Choudhury A, Dispenzieri A, Spitz DR, Russell SJ, et al. Dexamethasone-induced oxidative stress enhances myeloma cell radiosensitization while sparing normal bone marrow hematopoiesis. *Neoplasia.* 2010; 12:980–92. [PubMed: 21170263]
6. Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. intermediacy of H(2)O(2)- and p53-dependent pathways. *J Biol Chem.* 2004; 279:25535–43. [PubMed: 15054096]
7. Trevino AV, Woynarowska BA, Herman TS, Priebe W, Woynarowski JM. Enhanced topoisomerase II targeting by annamycin and related 4-demethoxy anthracycline analogues. *Mol Cancer Ther.* 2004; 3:1403–10. [PubMed: 15542779]
8. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol.* 1999; 57:727–41. [PubMed: 10075079]
9. Batinic-Haberle I, Reboucas JS, Spasojevic I. Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential. *Antioxid Redox Signal.* 2010; 13:877–918. [PubMed: 20095865]
10. Miriyala S, Spasojevic I, Tovmasyan A, Salvemini D, Vujaskovic Z, St CD, et al. Manganese superoxide dismutase, MnSOD and its mimics. *Biochim Biophys Acta.* 2012; 1822:794–814. [PubMed: 22198225]
11. Jaramillo MC, Briehl MM, Crapo JD, Batinic-Haberle I, Tome ME. Manganese porphyrin, MnTE-2-PyP⁵⁺, Acts as a pro-oxidant to potentiate glucocorticoid-induced apoptosis in lymphoma cells. *Free Radic Biol Med.* 2012; 52:1272–84. [PubMed: 22330065]
12. Buettner GR, Ng CF, Wang M, Rodgers VG, Schafer FQ. A new paradigm: manganese superoxide dismutase influences the production of H₂O₂ in cells and thereby their biological state. *Free Radic Biol Med.* 2006; 41:1338–50. [PubMed: 17015180]
13. Spasojevic I, Colvin OM, Warshany KR, Batinic-Haberle I. New approach to the activation of anti-cancer pro-drugs by metalloporphyrin-based cytochrome P450 mimics in all-aqueous biologically relevant system. *J Inorg Biochem.* 2006; 100:1897–902. [PubMed: 16965820]
14. Anathy V, Roberson EC, Guala AS, Godburn KE, Budd RC, Janssen-Heininger YM. Redox-based regulation of apoptosis: S-glutathionylation as a regulatory mechanism to control cell death. *Antioxid Redox Signal.* 2012; 16:496–505. [PubMed: 21929356]
15. Janssen-Heininger YM, Aesif SW, van d V, Guala AS, Reiss JN, Roberson EC, et al. Regulation of apoptosis through cysteine oxidation: implications for fibrotic lung disease. *Ann N Y Acad Sci.* 2010; 1203:23–8. [PubMed: 20716279]

16. Tome ME, Lee K, Jaramillo MC, Briehl MM. Mitochondria are the primary source of the H₂O₂ signal for glucocorticoid-induced apoptosis of lymphoma cells. *Exp Ther Med*. 2012; 4:237–42. [PubMed: 22844350]
17. Tonomura N, McLaughlin K, Grimm L, Goldsby RA, Osborne BA. Glucocorticoid-induced apoptosis of thymocytes: requirement of proteasome-dependent mitochondrial activity. *J Immunol*. 2003; 170:2469–78. [PubMed: 12594272]
18. Ferrer-Sueta G, Hannibal L, Batinic-Haberle I, Radi R. Reduction of manganese porphyrins by flavoenzymes and submitochondrial particles: a catalytic cycle for the reduction of peroxynitrite. *Free Radic Biol Med*. 2006; 41:503–12. [PubMed: 16843831]
19. Spasojevic I, Chen Y, Noel TJ, Yu Y, Cole MP, Zhang L, et al. Mn porphyrin-based superoxide dismutase (SOD) mimic, MnIIITE-2-PyP5+, targets mouse heart mitochondria. *Free Radic Biol Med*. 2007; 42:1193–200. [PubMed: 17382200]
20. Armand R, Channon JY, Kintner J, White KA, Miselis KA, Perez RP, et al. The effects of ethidium bromide induced loss of mitochondrial DNA on mitochondrial phenotype and ultrastructure in a human leukemia T-cell line (MOLT-4 cells). *Toxicol Appl Pharmacol*. 2004; 196:68–79. [PubMed: 15050409]
21. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, et al. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem*. 2004; 279:13044–53. [PubMed: 14722062]
22. Janssen AJ, Trijbels FJ, Sengers RC, Smeitink JA, van den Heuvel LP, Wintjes LT, et al. Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clin Chem*. 2007; 53:729–34. [PubMed: 17332151]
23. Kramer KA, Oglesbee D, Hartman SJ, Huey J, Anderson B, Magera MJ, et al. Automated spectrophotometric analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts. *Clin Chem*. 2005; 51:2110–6. [PubMed: 16141288]
24. Zhang S, Ding JH, Zhou F, Wang ZY, Zhou XQ, Hu G. Iptakalim ameliorates MPP⁺-induced astrocyte mitochondrial dysfunction by increasing mitochondrial complex activity besides opening mitoK(ATP) channels. *J Neurosci Res*. 2009; 87:1230–9. [PubMed: 19006086]
25. Tome ME, Lutz NW, Briehl MM. Overexpression of catalase or Bcl-2 alters glucose and energy metabolism concomitant with dexamethasone resistance. *Biochim Biophys Acta*. 2004; 1693:57–72. [PubMed: 15276325]
26. Tome ME, Baker AF, Powis G, Payne CM, Briehl MM. Catalase-overexpressing thymocytes are resistant to glucocorticoid-induced apoptosis and exhibit increased net tumor growth. *Cancer Res*. 2001; 61:2766–73. [PubMed: 11289160]
27. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984; 22:27–55. [PubMed: 6382953]
28. Jaramillo MC, Frye JB, Crapo JD, Briehl MM, Tome ME. Increased manganese superoxide dismutase expression or treatment with manganese porphyrin potentiates dexamethasone-induced apoptosis in lymphoma cells. *Cancer Res*. 2009; 69:5450–7. [PubMed: 19549914]
29. Mukhopadhyay P, Rajesh M, Yoshihiro K, Hasko G, Pacher P. Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochem Biophys Res Commun*. 2007; 358:203–8. [PubMed: 17475217]
30. Hallahan C, Young DA, Munck A. Time course of early events in the action of glucocorticoids on rat thymus cells in vitro. Synthesis and turnover of a hypothetical cortisol-induced protein inhibition of glucose metabolism and of a presumed ribonucleic acid. *J Biol Chem*. 1973; 248:2922–7. [PubMed: 4697402]
31. Dang CV, Semenza GL. Oncogenic alterations of metabolism. *Trends Biochem Sci*. 1999; 24:68–72. [PubMed: 10098401]
32. Hurd TR, Costa NJ, Dahm CC, Beer SM, Brown SE, Filipovska A, et al. Glutathionylation of mitochondrial proteins. *Antioxid Redox Signal*. 2005; 7:999–1010. [PubMed: 15998254]
33. Lhee S, Kolling DR, Nair SK, Dikanov SA, Crofts AR. Modifications of protein environment of the [2Fe-2S] cluster of the bc1 complex: effects on the biophysical properties of the rieske iron-sulfur protein and on the kinetics of the complex. *J Biol Chem*. 2010; 285:9233–48. [PubMed: 20023300]

34. Cheng G, Zielonka J, Dranka BP, McAllister D, Mackinnon AC Jr, Joseph J, et al. Mitochondria-targeted drugs synergize with 2-deoxyglucose to trigger breast cancer cell death. *Cancer Res.* 2012; 72:2634–44. [PubMed: 22431711]
35. Tovmasyan A, Weitner T, Jaramillo MC, Wedmann R, Roberts E, Leong KW, et al. We have come a long way with Mn porphyrins: from superoxide dismutation to H₂O₂ driven pathways. *Free Radic Biol Med.* 2013; 16:S133.
36. Mieyal JJ, Chock PB. Posttranslational modification of cysteine in redox signaling and oxidative stress: Focus on s-glutathionylation. *Antioxid Redox Signal.* 2012; 16:471–5. [PubMed: 22136616]
37. Chinta SJ, Andersen JK. Reversible inhibition of mitochondrial complex I activity following chronic dopaminergic glutathione depletion in vitro: implications for Parkinson's disease. *Free Radic Biol Med.* 2006; 41:1442–8. [PubMed: 17023271]
38. Zhang J, Jin B, Li L, Block ER, Patel JM. Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells. *Am J Physiol Cell Physiol.* 2005; 288:C840–C849. [PubMed: 15561762]
39. Pelicano H, Feng L, Zhou Y, Carew JS, Hileman EO, Plunkett W, et al. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *J Biol Chem.* 2003; 278:37832–9. [PubMed: 12853461]
40. Griffith OW, Meister A. Origin and turnover of mitochondrial glutathione. *Proc Natl Acad Sci U S A.* 1985; 82:4668–72. [PubMed: 3860816]
41. Griffith OW, Anderson ME, Meister A. Inhibition of glutathione biosynthesis by prothionine sulfoximine (S-n-propyl homocysteine sulfoximine), a selective inhibitor of gamma-glutamylcysteine synthetase. *J Biol Chem.* 1979; 254:1205–10. [PubMed: 33187]
42. Hurd TR, Requejo R, Filipovska A, Brown S, Prime TA, Robinson AJ, et al. Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: potential role of CYS residues in decreasing oxidative damage. *J Biol Chem.* 2008; 283:24801–15. [PubMed: 18611857]
43. Stowe DF, Camara AK. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. *Antioxid Redox Signal.* 2009; 11:1373–414. [PubMed: 19187004]
44. Taylor ER, Hurrell F, Shannon RJ, Lin TK, Hirst J, Murphy MP. Reversible glutathionylation of complex I increases mitochondrial superoxide formation. *J Biol Chem.* 2003; 278:19603–10. [PubMed: 12649289]
45. Garcia J, Han D, Sancheti H, Yap LP, Kaplowitz N, Cadenas E. Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates. *J Biol Chem.* 2010; 285:39646–54. [PubMed: 20937819]
46. Smith TA. Mammalian hexokinases and their abnormal expression in cancer. *Br J Biomed Sci.* 2000; 57:170–8. [PubMed: 10912295]
47. Warburg O. On the origin of cancer cells. *Science.* 1956; 123:309–14. [PubMed: 13298683]
48. Fath MA, Diers AR, Aykin-Burns N, Simons AL, Hua L, Spitz DR. Mitochondrial electron transport chain blockers enhance 2-deoxy-D-glucose induced oxidative stress and cell killing in human colon carcinoma cells. *Cancer Biol Ther.* 2009; 8:1228–36. [PubMed: 19411865]
49. Ahmad IM, Abdalla MY, Aykin-Burns N, Simons AL, Oberley LW, Domann FE, et al. 2-Deoxyglucose combined with wild-type p53 overexpression enhances cytotoxicity in human prostate cancer cells via oxidative stress. *Free Radic Biol Med.* 2008; 44:826–34. [PubMed: 18155176]
50. Laszlo J, Landau B, Wight K, Burk D. The effect of glucose analogues on the metabolism of human leukemic cells. *J Natl Cancer Inst.* 1958; 21:475–83. [PubMed: 13576101]
51. Laszlo J, Humphrys SR, Goldin A. Effects of glucose analogues (2-deoxy-D-glucose, 2-deoxy-D-galactose) on experimental tumors. *J Natl Cancer Inst.* 1960; 24:267–81. [PubMed: 14414406]
52. Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, et al. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. *Cancer Res.* 2005; 65:613–21. [PubMed: 15695406]

53. Batinic-Haberle I, Spasojevic I, Stevens RD, Hambright P, Neta P, Okado-Matsumoto A, et al. New class of potent catalysts of O₂.-dismutation. Mn(III) ortho-methoxyethylpyridyl- and di-ortho-methoxyethylimidazolylporphyrins. Dalton Trans. 2004:1696–702. [PubMed: 15252564]
54. Batinic-Haberle I, Spasojevic I. Complex chemistry and biology of redox-active compounds, commonly known as SOD mimics, affect their therapeutic effects. Antioxid Redox Signal. 2014; 20:2323–5. [PubMed: 24650329]
55. Batinic-Haberle I, Tovmasyan A, Roberts ER, Vujaskovic Z, Leong KW, Spasojevic I. SOD therapeutics: latest insights into their structure-activity relationships and impact on the cellular redox-based signaling pathways. Antioxid Redox Signal. 2014; 20:2372–415. [PubMed: 23875805]

Highlights

MnTE-2-PyP⁵⁺ induces S-glutathionylation of ETC Complexes I, III and IV

S-glutathionylation of Complexes I and III inhibits their activity

MnTE-2-PyP⁵⁺ enhances lymphoma cell death due to glucocorticoids or 2-deoxyglucose

MnTE-2-PyP⁵⁺ does not enhance normal cell death due to glucocorticoids or 2DG

Induction of S-glutathionylation is required for MnTE-2-PyP⁵⁺ to enhance cytotoxicity

Figure 1. MnTE-2-PyP⁵⁺ augments dexamethasone-induced mitochondrial ROS and oxidation of the mito-roGFP2

Panel A. Rate of the increase in MitoSOX fluorescence after 12 hours of treatment with MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), compared to cells treated with dexamethasone alone (D), MnTE-2-PyP⁵⁺ alone (Mn) or the vehicle-control (C). Values are the mean + S.E.M. (n = 3). *Panel B.* Percentage of mito-roGFP2 oxidized after treatment of cells as described in panel A, with the exception that cells were treated for 8 hours. Values are the mean + S.E.M. (n=16). * denotes significantly different from control values. ** denotes significantly different from dexamethasone-treated cells (p < 0.05).

Figure 2. MnTE-2-PyP⁵⁺ induces glutathionylation of mitochondrial proteins

Panel A. Representative immunoblot probed with an anti-GSH antibody to measure the level of glutathionylated proteins in the mitochondria of WEHI7.2 cells treated with a vehicle control (C), MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), buthionine sulfoximine (B), or buthionine sulfoximine in combination with MnTE-2-PyP⁵⁺ and dexamethasone (BMnD) for 12 hours. Hsp60 was used as a control for equal loading of mitochondrial proteins. *Panel B.* Quantification of the relative amount of glutathionylated proteins in the mitochondria. Values are the mean + S.E.M. (n=3) * denotes significantly different from control and dexamethasone values (p 0.05).

Figure 3. MnTE-2-PyP⁵⁺ inhibits the activity of Complex I and III in the electron transport chain

Panels A, B and D. Activity of complexes I, III, and IV in the presence of MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), buthionine sulfoximine (B), and buthionine sulfoximine in combination with the porphyrin and dexamethasone (BMnD) for 12 hours in WEHI7.2 cells. Values have been corrected for protein and normalized to control values. Values are the mean + S.E.M (n=3). * denotes significantly different from control; ** denotes significantly different from control and dexamethasone treated cells (p < 0.05). *Panel C.* Representative immunoblot comparing the glutathionylation of complex III, specifically the Rieske iron sulfur complex, after treating cells with MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), or cells pretreated with BSO and then treated with the combination treatment (BMnD). Mitochondrial cell lysates were immunoprecipitated for complex III and then immunoblotted using an anti-GSH antibody. * denotes significantly different from

control; ** denotes significantly different from control and dexamethasone-treated cells ($p < 0.05$) ($n = 3$).

Figure 4. In combination with dexamethasone, MnTE-2-PyP⁵⁺ decreases cellular ATP levels in WEHI7.2 cells

The levels of ATP in WEHI7.2 cells treated with a vehicle-control (C), MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), buthionine sulfoximine (B), and buthionine sulfoximine in combination with the porphyrin and dexamethasone (BMnD) for 12 hours. Values have been corrected for protein and normalized to control values. Values are the mean + S.E.M. (n=3). * denotes significantly different from control treated cells ($p < 0.05$).

Figure 5. In combination with dexamethasone, MnTE-2-PyP⁵⁺ induces glutathionylation of mitochondrial proteins, inhibits the activity of proteins in the mitochondrial ETC, and depletes cellular ATP in malignant human T-cells

Panel A. Quantification of three immunoblots probed with an anti-GSH antibody to measure the protein expression of glutathionylated proteins in the mitochondria of Molt4 cells treated with a vehicle control (C), MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD), buthionine sulfoximine (B), or buthionine sulfoximine in combination with MnTE-2-PyP⁵⁺ and dexamethasone (BMnD) for 24 hours. Values are the mean + S.E.M. * denotes significantly different from control and dexamethasone values ($p < 0.05$). *Panels B-D.* Activity of complexes I, III, and IV in Molt4

cells treated as described in panel A. Values have been corrected for protein and normalized to control values. Values are the mean + S.E.M (n=3). * denotes significantly different from control treated cells. ** denotes significantly different from control and dexamethasone treated cells (p < 0.05). *Panel E.* ATP levels in Molt4 cells treated as in panel A. Values have been corrected for protein and normalized to control values. Values are the mean + S.E.M. (n=3). * denotes significantly different from control and dexamethasone treated cells (p < 0.05)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Mitochondria are important for the porphyrin's ability to enhance dexamethasone-induced apoptosis

Comparison of the percentage of viable cells after treatment with MnTE-2-PyP⁵⁺ (Mn), dexamethasone (D), and MnTE-2-PyP⁵⁺ in combination with dexamethasone (MnD) for 24 hours to control (C) Molt4 cells and Molt4 cells with depleted mitochondrial DNA and impaired mitochondrial function (rho(0) Molt4). Values are the mean + S.E.M. (n=3)

*denotes significantly different from wildtype Molt4 cells. (p < 0.05).

Figure 7. MnTE-2-PyP⁵⁺ synergizes with 2-deoxy-D-glucose to enhance cell death in WEHI7.2 and Molt4 cells and to decrease cellular ATP levels

Panel A. ATP levels in WEHI7.2 cells treated with a vehicle control (C), 2DG for 24 hours, MnTE-2-PyP⁵⁺ in combination with 2DG (Mn2DG) for 24 hours, 10 μ M CCCP 2 hours before harvest or CCCP in combination with a 24 hour treatment with 2DG. Values have been corrected for protein and normalized to control values. Values are the mean + S.E.M. (n=3). * denotes statistically different from control treated cells; ** denotes statistically different from 2DG treated cells; and # denotes statistically similar to each other (p > 0.05).

Panel B. Cell viability after treating WEHI7.2 cells with a vehicle control (C), MnTE-2-PyP⁵⁺ (Mn), 2-deoxy-D-glucose (2DG), and MnTE-2-PyP⁵⁺ in combination with 2-deoxy-D-glucose (Mn2DG) for 24 hours. Values are the mean + S.E.M. (n=3) *denotes significantly different from control treated cells; ** denotes significantly different from cells treated with 2DG (p > 0.05).

Figure 8. MnTE-2-PyP⁵⁺ induces protein glutathionylation and augments 2-deoxy-D-glucose-induced cell death in primary B-ALL patient samples

Panel A. Representative immunoblot of three different immunoblots showing the protein expression of glutathionylated proteins in primary B-ALL patient samples treated with a vehicle control (C) or MnTE-2-PyP⁵⁺ (Mn) for 24 hours. Values represent the mean + S.E.M. *denotes significantly different from control treated cells ($p < 0.05$). *Panel B.* Relative percentage of live B-cells remaining in culture 24 hours after treatment with MnTE-2-PyP⁵⁺, 2-deoxy-D-glucose (2DG), and MnTE-2-PyP⁵⁺ in combination with 2DG (Mn2DG). CD20 positive/7AAD negative cells were considered live B-cells. The value for the sample with no drugs for each patient was set to 100%.

Figure 9. Model showing the effect of MnTE-2-PyP⁵⁺ on the mitochondria in the presence of dexamethasone or 2-deoxyglucose

Panel A. Proposed reactions of the MnTE-2-PyP⁵⁺ in the cell. *Panel B.* Model of MnTE-2-PyP⁵⁺ targets within the mitochondria.

Table 1

Summary of identified mitochondrial glutathionylated proteins.

Protein	Dexamethasone	MnTE-2-PyP ⁵⁺ /Dexamethasone
<i>Stress Proteins</i>		
HSP70	X	X
HSP60	X	X
Stress-induced phosphoprotein 1	X	X
<i>Enzymes</i>		
Aldolase	X	X
6-Phosphogluconolactonase		X
Adenylate kinase 2	X	X
Cytochrome c oxidase		X
NADH ubiquinone oxidoreductase		X
<i>Other</i>		
T complex protein	X	X
Lymphocyte specific protein 1	X	X

Table 2

Viability of normal human PMBCs after DEX or 2-deoxy-D-glucose treatment in the presence of MnTE2-PyP⁵⁺

Treatment	Sample 1 (% viable cells)	Sample 2 (% viable cells)
Control	100.0*	100.0
50 nM MnTE-2-PyP ⁵⁺	97.8	106.4
100 nM MnTE-2-PyP ⁵⁺	101.4	101.3
250 nM Mn TE-2-PyP ⁵⁺	91.2	104.7
1 μ M DEX	77.7	102.3
1 μ M DEX + 50 nM MnTE-2-PyP ⁵⁺	75.1	102.3
1 μ M DEX + 100 nM MnTE-2-PyP ⁵⁺	77.1	101.4
1 μ M DEX + 250 nM Mn TE-2-PyP ⁵⁺	76.0	101.4
15 mM 2-deoxy -D-glucose	85.9	101.4
15 mM 2-deoxy -D-glucose + 50 nM MnTE-2-PyP ⁵⁺	86.6	106.6
15 mM 2-deoxy -D-glucose + 100 nM MnTE-2-PyP ⁵⁺	91.7	101.6
15 mM 2-deoxy -D-glucose + 250 nM Mn TE-2-PyP ⁵⁺	101.0	98.2

* Values are the viable peripheral mononuclear blood cell (PMBC) number relative to control after a 24 hour treatment with the indicated compounds.