Increased Manganese Superoxide Dismutase Expression or Treatment with Manganese Porphyrin Potentiates Dexamethasone-Induced Apoptosis in Lymphoma Cells

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Abstract

Glucocorticoid-induced apoptosis is exploited for the treatment of hematologic malignancies. Innate and acquired resistance limits treatment efficacy; however, resistance mechanisms are not well understood. Previously, using WEHI7.2 murine thymic lymphoma cells, we found that increasing the resistance to hydrogen peroxide (H2O2) by catalase transfection or selection for H2O2 resistance caused glucocorticoid resistance. This suggests the possibility that increasing H2O2 sensitivity could sensitize the cells to glucocorticoids. In other cell types, increasing manganese superoxide dismutase (MnSOD) can increase intracellular H2O2. The current study showed that increased expression of MnSOD sensitized WEHI7.2 cells to glucocorticoid-induced apoptosis and H2O2. Treatment of WEHI7.2 cells with the catalytic antioxidant Mn(III) meso-tetraakis(N-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP5+), a manganoporphyrin, mimicked the effects of increased MnSOD expression. MnTE-2-PyP5+ also sensitized WEHI7.2 cells to cyclophosphamide and inhibited cell growth; it had no effect on the WEHI7.2 cell response to doxorubicin or vincristine. In primary follicular lymphoma cells, MnTE-2-PyP5+ increased cell death due to dexamethasone. Treatment of H9c2 cardiomyocytes with MnTE-2-PyP5+ inhibited doxorubicin cytotoxicity. The profile of MnTE-2-PyP5+ effects suggests MnTE-2-PyP5+ has potential for use in hematologic malignancies that are treated with glucocorticoids, cyclophosphamide, and doxorubicin. [Cancer Res 2009;69(13):5450–7]

Introduction

Glucocorticoids are effective in the treatment of lymphoid malignancies because of their ability to induce apoptosis. Unfortunately, innate and acquired resistance limits their efficacy. Progress has been made in defining critical events for glucocorticoid-induced apoptosis (reviewed in refs. 1–3). Glucocorticoid-induced apoptosis requires binding of the steroid to a cytosolic receptor. The steroid-receptor complex translocates to the nucleus, where it acts as a transcriptional activator and repressor of gene networks. This results in a complex and poorly understood interaction of signals that decides the life or death of the cell. Apoptosis then proceeds through the release of cytochrome c from the mitochondria and activation of caspases.

Clinically, some cases of glucocorticoid resistance have been traced to nonfunctional (or decreased) glucocorticoid receptors (4, 5); however, this mechanism may be relatively rare (5). Glucocorticoid-induced release of cytochrome c from the mitochondria seems to be controlled by the relative amount of proapoptotic and antiapoptotic Bcl-2 family members (6–8). Inability to appropriately up-regulate the proapoptotic Bcl-2 family member, Bim (9), or up-regulation of antiapoptotic Bcl-2 family members (10, 11) are also correlated with treatment failure in the clinic. Given the complexity of the signaling pathways that determine glucocorticoid sensitivity, multiple resistance mechanisms clearly exist. Additional characterization of the signaling phase of dexamethasone-induced apoptosis in model systems is continuing to identify other potential sources of resistance (reviewed in refs. 2, 3).

Previous work in our laboratory using the WEHI7.2 murine thymic lymphoma cell model has shown that WEHI7.2 cells resistant to hydrogen peroxide (H2O2) via catalase transfection or selection for resistance to H2O2 are cross-resistant to dexamethasone (12, 13). The oxidative stress-resistant cells show a delay or lack of cytochrome c release in response to dexamethasone treatment proportional to their expression of catalase, an enzyme that metabolizes H2O2 (12, 13). This suggests that H2O2 is a key signal in dexamethasone-induced apoptosis.

Based on these results, we hypothesized that increasing the intracellular production of H2O2 is a potential strategy for sensitizing the cells to dexamethasone-induced apoptosis. For this purpose, we chose to overexpress manganese superoxide dismutase (MnSOD). MnSOD is a mitochondrial enzyme that dismutates superoxide (O2−) forming H2O2 in the process (14). In other cell types, increasing MnSOD elevated intracellular H2O2 (14, 15). There is evidence from IM-9 multiple myeloma cells that increasing MnSOD increases dexamethasone sensitivity (16); however, gene dose of MnSOD in thymocytes did not alter dexamethasone sensitivity (17). In the current study, we tested the hypothesis that increasing MnSOD could sensitize WEHI7.2 cells to dexamethasone. By testing this hypothesis in a lymphoma cell line, we can determine whether manipulating MnSOD to increase glucocorticoid sensitivity could be a unique characteristic of lymphoid tumor cells that can be exploited for chemotherapy. We further tested the extent to which we could replicate the MnSOD effects by pretreating the cells with a manganoporphyrin, Mn(III) meso-tetraakis(N-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP5+).
Materials and Methods

Reagents and drug treatments. MnTE-2-PyP\(^{5+}\) was provided by Aeolus Pharmaceuticals. All other chemicals and drugs were purchased from Sigma Chemical Co., unless otherwise stated.

For the tissue culture cells, sensitivity to dexamethasone was determined by incubating cells in a final concentration of 1 \(\mu\)mol/L dexamethasone in an ethanol vehicle (final concentration of ethanol, 0.01%) or vehicle alone. For MnTE-2-PyP\(^{5+}\) effects on drug or oxidant response, the cells were pretreated with the indicated concentrations of MnTE-2-PyP\(^{5+}\) 2 h before the addition of drugs or oxidants. For measurements of MnTE-2-PyP\(^{5+}\) effects on apoptosis and growth, the cells were treated with the indicated concentrations of MnTE-2-PyP\(^{5+}\) for 24 and 48 h, respectively.

Cell culture and transfections. The mouse thymic lymphoma WEHI7.2 parental cell line (18) was maintained as previously described (19). Stable WEHI7.2 clones overexpressing MnSOD were constructed by electroporation (18) with a pCDNA3.1 vector containing the human cDNA MnSOD sequence, a gift from Dr. Larry Oberley (University of Iowa; ref. 19); clones were isolated and maintained as previously described (13).

H9c2 cells, obtained from Dr. Qin Chen (University of Arizona), are an immortalized clonal cell line derived from BD1X rat embryonic heart tissue (20). They do not beat in culture but retain certain electrophysiologic and biochemical properties of cardiac cells and can form myotubes upon confluency (20, 21). H9c2 cells were chosen for this study due to their frequent use as a doxorubicin cardiotoxicity model (e.g., ref. 22). H9c2 cells were maintained in DMEM-high glucose (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products) and 2 mmol/L l-glutamine (Invitrogen), at 37°C in a 5% CO\(_2\) humidified environment until 60% to 85% confluence.

Primary follicular lymphoma cells. Two primary human tumor samples with the diagnosis of follicular lymphoma (FL) were obtained from the Arizona Lymphoid Tissue and Blood Repository in accordance with University of Arizona regulations for the use of primary human tissue. The cells were thawed and resuspended in Iscove’s modified Dulbecco’s medium (Invitrogen) with 20% fetal bovine serum (Gemini Bio-Products) in the presence or absence of MnTE-2-PyP\(^{5+}\) and 750 mmol/L dexamethasone.

Drug concentrations were chosen based on the response in a human lymphoma-derived cell line. Viable B-cell content was analyzed before the addition of drugs and after incubation at 37°C in a 5% CO\(_2\) humidified environment for 24 h in the presence or absence of drugs. Cells were labeled with phycoerythrin-labeled anti-CD20 to identify B-cells (AbD Serotec) and a FITC-labeled anti-CD27 to identify CD27+ cells (BD Pharmingen). An isotype control was used for each run to gate out CD20-negative cells; debris and events that did not pass forward and side light scatter were also gated out.

For MnTE-2-PyP\(^{5+}\) effects on drug or oxidant response, the cells were treated with MnTE-2-PyP\(^{5+}\) in addition of drugs or oxidants. For measurements of MnTE-2-PyP\(^{5+}\) effects on apoptosis and growth, the cells were treated with the indicated concentrations of MnTE-2-PyP\(^{5+}\) for 24 and 48 h, respectively.

Results

Construction and characterization of the MnSOD-transfected clones. To determine the effect of MnSOD on dexamethasone-induced lymphoid cell apoptosis, we first established WEHI7.2 cell clones with increased MnSOD by transfection. As shown in Fig. 1A, we established several WEHI7.2 cell clones that have increased MnSOD expression at the mRNA level. Two vector-only transfectants (Mneo1 and Mneo2) and three MnSOD-transfected clones (MnSOD5, MnSOD9, and MnSOD20) were selected for the current study. The MnSOD-transfected clones had significantly greater MnSOD protein levels than either of the two vector-only transfected clones (Fig. 1B). The MnSOD transfectants expressed 2.5-fold to 3-fold of the Mneo1 MnSOD protein and were not significantly different from each other. MnSOD protein in the Mneo2 vector-only transfected clone was also significantly higher than that in Mneo1 but still significantly lower than in the MnSOD-transfected clones. MnSOD activity increased with elevated MnSOD protein; the magnitude of the increase in activity was slightly less than that for the protein (Fig. 1C).

The enzymatic function of MnSOD is to metabolize O\(_2^-\), forming H\(_2\)O\(_2\) in the process (14). Cells that overexpress MnSOD may increase their ability to metabolize H\(_2\)O\(_2\), because elevated MnSOD can increase the cellular H\(_2\)O\(_2\) load (14, 28). Our previous studies indicate that resistance to H\(_2\)O\(_2\) causes cross-resistance to dexamethasone (12, 13). Therefore, to interpret the phenotype of the MnSOD-transfected clones, it was critical to understand their ability to metabolize H\(_2\)O\(_2\). To assess the ability of the clones to metabolize H\(_2\)O\(_2\), we measured catalase activity and the EC\(_{50}\) for H\(_2\)O\(_2\). Catalase is the primary enzyme in WEHI7.2 cells that metabolizes H\(_2\)O\(_2\). The EC\(_{50}\) for H\(_2\)O\(_2\) was used to indicate the overall H\(_2\)O\(_2\) sensitivity of the clones. Catalase activity in the MnSOD-transfected clones, vector-only transfectants, and the

bicinchoninic acid protein assay kit (Pierce) according to the manufacturer’s instructions. Enzyme activities were normalized to cellular protein.

**EC\(_{50}\)** measurements. Relative cell number was measured using the Cell Proliferation kit II (Roche Diagnostics; H\(_2\)O\(_2\) only) or the Nonradioactive Cell Proliferation Assay (MTS; Promega Corp.) according to the manufacturer’s protocol. The plates were read at 490 nm using a Microplate EL-311 or a Synergy HT plate reader (Bio-Tek Instruments). Fraction control absorbance was calculated as previously described (26). The EC\(_{50}\) was defined as the concentration at which the absorbance was 50% of the control.

Viable cell number and apoptosis measurements. Viable cell number was determined by dye exclusion as previously described (13). For the MnSOD-transfected clones, apoptosis was confirmed by morphologic examination using previously described criteria (13). The fraction of apoptotic cells, as measured by Annexin V staining, was determined using the apoptosis detection kit (R&D Systems, Inc.) as previously described (27).

Casparase-3 activity was measured using a colorimetric assay that depends on the cleavage of the synthetic caspase-3–specific substrate, Ac-DEVD-p-nitroanilide (pNA; BIMOL International LTD.). Briefly, cells were lysed in 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.01% Triton X-100 by sonication. The samples were clarified by centrifugation at 10,000 \(\times\) g for 10 min. An aliquot of the supernatant was first incubated in 10 mmol/L PIPES (pH 7.4), 2 mmol/L EDTA, 0.01% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate hydrate, 5 mmol/L DTT, 200 mmol/L Ac-DEVD-pNA for 2 h, and then the absorbance at 405 nm was measured using a SynergyHT plate reader (Bio Tek Instruments, Inc.). Activity was normalized for cellular protein measured as described above.

**Statistics.** Means were compared using ANOVA or Student’s \(t\) tests, where appropriate, with the algorithms in Excel (Microsoft Corp.). Means were considered significantly different when \(P \leq 0.05\).
regulating components that would confer H$_2$O$_2$ resistance. These
C, MnSOD activity in the selected clones.

**Figure 1.** Comparison of the MnSOD expression in the MnSOD-transfected and vector-transfected WEHI7.2 cells. A, Northern blot showing MnSOD mRNA and the fold mRNA increase after correction for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. B, representative immunoblots showing MnSOD and actin protein in selected clones. Bottom, average amount of MnSOD protein relative to that in Mneo1 quantitated from three independent blots. Representative full-length blots are presented in Supplementary Fig. S1. C, MnSOD activity in the selected clones. Columns, mean (n = 3); bars, SE. *, significantly different from Mneo1 values (P ≤ 0.05).

Increased MnSOD confers sensitivity to dexamethasone-induced apoptosis in WEHI7.2 cells. We hypothesized that increased MnSOD expression could sensitize WEHI7.2 cells to dexamethasone because of the ability of MnSOD to produce H$_2$O$_2$.

Our hypothesis was based largely on our previous work, showing that WEHI7.2 cells with increased catalase or selected for resistance to H$_2$O$_2$ are resistant to dexamethasone (12, 13). Recent data showing that increased MnSOD levels sensitize myeloma cells to dexamethasone (16) also suggested that lymphoma cells might have a similar response.

To test our hypothesis, we measured apoptosis in the MnSOD and vector-only transfectants in three ways. As shown in Fig. 2A, when we treated the MnSOD-transfected cells with dexamethasone, we saw a faster loss of cells from the culture than in the vector-only transfected cells. After 24 hours in dexamethasone, MnSOD5 and MnSOD9 cell cultures had significantly fewer viable cells than the Mneo1 cell cultures. By 32 hours after dexamethasone treatment, all three MnSOD transfectants had fewer viable cells than the control transfectants (P ≤ 0.05). The vector-only transfectants showed kinetics of cell loss similar to that previously reported for the WEHI7.2 cells (12).

Annexin V binding measurements showed that the MnSOD-transfected clone cultures had a greater amount of apoptosis 16 hours after dexamethasone treatment than the vector-only transfecteds (Fig. 2B). Increased apoptosis due to dexamethasone in the MnSOD-transfected clones was also confirmed by morphologic examination of culture aliquots (data not shown). These data suggest that increased MnSOD expression is accelerating dexamethasone-induced apoptosis.

**MnTE-2-PyP$^{5+}$ decreases growth in WEHI7.2 cells.** Our next step was to determine whether we could mimic the effects of MnSOD overexpression with a pharmacologic agent. For these studies, we used the manganoporphyrin, MnTE-2-PyP$^{5+}$. We chose MnTE-2-PyP$^{5+}$ because, in a cell-free assay, it dismutates O$_2$•$^-$ with a mechanism and thermodynamics similar to that of MnSOD and has a low affinity for H$_2$O$_2$ (29–31).

To provide an overview of the effects of MnTE-2-PyP$^{5+}$ alone and in combination with dexamethasone, we measured the number of WEHI7.2 cells in culture in the presence or absence of drugs by dye exclusion (Fig. 3A). In the cultures treated with either concentration of MnTE-2-PyP$^{5+}$ and vehicle, we saw lower cell numbers than in the WEHI7.2 cells treated with vehicle alone. WEHI7.2 cells treated with dexamethasone showed an initial increase in cell number followed by a loss of cells from the culture. In cultures of WEHI7.2 cells treated with the combination of dexamethasone and MnTE-2-PyP$^{5+}$, cell numbers were slightly lower at 16 hours than in the WEHI7.2 plus dexamethasone cultures. The rate of cell loss in the cultures from 16 to 32 hours was significantly faster in the dexamethasone plus 1.5 µmol/L MnTE-2-PyP$^{5+}$ compared with dexamethasone-only cultures (2.5 ± 0.2 × 10$^4$ versus 1.9 ± 0.3 × 10$^4$ cell/hours, respectively; P ≤ 0.05). We confirmed the cell number data at the 24-hour time point using the MTS assay, which measures the number of metabolizing cells. Using WEHI7.2 cells treated with vehicle alone as the control MTS absorbance set at 100 ± 0.6%, cultures treated with vehicle plus 0.75 or 1.5 µmol/L MnTE-2-PyP$^{5+}$ had significantly lower absorbances, 81.2 ± 3.0 or 64.3 ± 7.4%, respectively. WEHI7.2 cells treated with dexamethasone alone had 22.8 ± 1.4% control absorbance compared with 5.5 ± 1.4% or 2.7 ± 0.8% control absorbance for cells treated with dexamethasone plus 0.75 or 1.5 µmol/L MnTE-2-PyP$^{5+}$, respectively.

The lower cell numbers in cultures treated with MnTE-2-PyP$^{5+}$ and vehicle alone (Fig. 3A) suggest that MnTE-2-PyP$^{5+}$ alone has an effect on the WEHI7.2 cells. One possibility is that MnTE-2-PyP$^{5+}$ itself causes apoptosis. To determine whether MnTE-2-PyP$^{5+}$
possible explanation of the lower-than-predicted values is that the drug combination causes increased apoptosis compared with dexamethasone alone. In WEHI7.2 cells, treatment with 1 μmol/L dexamethasone saturates the glucocorticoid receptors and causes apoptosis with reproducible kinetics (13). In these cultures, we see increases in Annexin V–positive cells at 16 hours, caspase-3 activation, and release of cytochrome c at 24 hours, followed by loss of viable cells from the culture beginning at 24 hours after the addition of dexamethasone (13, 27).

To determine whether MnTE-2-PyP5+ sensitizes cells to dexamethasone-induced apoptosis, we tested for the appearance of increased amounts of apoptotic markers at earlier time points. We measured both the activation of caspase-3 and the percentage of Annexin V–positive cells. As shown in Fig. 3C, 8 hours after dexamethasone addition, caspase-3 activity was minimal in the cells treated with dexamethasone alone. The MnTE-2-PyP5+ pretreated cells had significantly increased caspase-3 activity compared with the dexamethasone-treated cells. The MnTE-2-PyP5+ pretreated cultures also showed a significant increase in the percentage of Annexin V–positive cells in the presence of dexamethasone compared with the cells treated with dexamethasone alone (Fig. 3C). MnTE-2-PyP5+ pretreatment increased the Annexin V–positive cells at earlier time points; however, this was not significant. These measurements are corrected for the appropriate values in the vehicle-treated cells and, thus, reflect only the amount due to dexamethasone. Taken together, the data indicate that pretreatment with MnTE-2-PyP5+ increases in Annexin V–positive cells at 16 hours, caspase-3 activity compared with the dexamethasone-treated cells. The MnTE-2-PyP5+ pretreated cultures also showed a significant increase in Annexin V–positive cells at earlier time points; however, this was not significant. These measurements are corrected for the appropriate values in the vehicle-treated cells and, thus, reflect only the amount due to dexamethasone. Taken together, the data indicate that pretreatment with MnTE-2-PyP5+ accelerates apoptosis due to dexamethasone in the WEHI7.2 cells; increased apoptosis likely contributes to the decreased cell numbers in the cultures treated with MnTE-2-PyP5+ and dexamethasone combined.

MnTE-2-PyP5+ sensitizes primary FL cells to dexamethasone-induced death. To determine whether MnTE-2-PyP5+ has a similar effect in primary tumor cells, we measured the effect of dexamethasone and MnTE-2-PyP5+ individually and combined on B-cell death in primary FL cells from two different patients (Fig. 3D). As is typical of FL cells, the primary cells did not grow in culture, so the effect we measured was only on cell death. Treatment with MnTE-2-PyP5+ alone had no effect in one sample; however, the higher concentration decreased viable B cells in patient 2. In both patient samples, dexamethasone alone decreased the viable B cells. Adding MnTE-2-PyP5+ to dexamethasone increased B-cell death in both patient samples. These data indicate that MnTE-2-PyP5+ accelerates dexamethasone-induced cell death.

<table>
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<tr>
<th>Table 1. Response to H2O2</th>
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<td>Cell variant</td>
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<td>Mneo2</td>
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NOTE: Values represent the mean ± SE (n = 3–6).
*Significantly different from WEHI7.2 and Mneo1 values (P ≤ 0.05).
in primary tumor cells and suggests MnTE-2-PyP$^{5+}$ has clinical potential for the treatment of lymphoma.

MnTE-2-PyP$^{5+}$ pretreatment alters the oxidant and chemotherapeutic drug response in WEHI7.2 cells. To better understand the redox properties of MnTE-2-PyP$^{5+}$ in the WEHI7.2 cells and interpret the effect of MnTE-2-PyP$^{5+}$ on the chemotherapeutic drug response, we measured the effect of MnTE-2-PyP$^{5+}$ pretreatment on the response to H$_2$O$_2$ and paraquat. In cell-free assays, MnTE-2-PyP$^{5+}$ has a low level of catalase activity (31). To determine whether MnTE-2-PyP$^{5+}$ is contributing to H$_2$O$_2$ removal in the WEHI7.2 cells, we measured the effect of MnTE-2-PyP$^{5+}$ pretreatment on the EC$_{50}$ for H$_2$O$_2$. As shown in Table 2A, pretreatment at the lower MnTE-2-PyP$^{5+}$ dose did not alter H$_2$O$_2$ sensitivity; however, at the higher dose, the cells were more sensitive to H$_2$O$_2$. This indicates that MnTE-2-PyP$^{5+}$ is not acting as a catalase mimic. It is more consistent with MnTE-2-PyP$^{5+}$ acting as an oxidant under these conditions to sensitize WEHI7.2 cells to H$_2$O$_2$.

Paraquat is a compound that redox cycles with a variety of oxidoreductases in cells to produce O$_2^·$ (34, 35). Paraquat toxicity has been linked to its ability to produce superoxide because both increased SOD and treatment with superoxide dismutase mimetics reduce toxicity (36, 37). In WEHI7.2 cells, MnTE-2-PyP$^{5+}$ pretreatment increased the EC$_{50}$ for paraquat in a dose-dependent manner, indicating that MnTE-2-PyP$^{5+}$ protected the WEHI7.2 cells from paraquat toxicity.

The data above suggest that MnTE-2-PyP$^{5+}$ is a candidate chemotherapeutic agent in lymphoma treatment because it sensitizes the lymphoid cells to dexamethasone. To determine whether MnTE-2-PyP$^{5+}$ would increase resistance to other commonly used lymphoma drugs, we tested MnTE-2-PyP$^{5+}$ in combination with cyclophosphamide, doxorubicin, and vincristine.
These three drugs plus prednisone, a glucocorticoid, comprise the CHOP regimen, a standard protocol for treating diffuse large B-cell lymphoma (DLBCL; ref. 38). These drugs are also used to treat other types of lymphoma (39). Testing MnTE-2-PyP5+ in combination with doxorubicin is particularly crucial because (a) doxorubicin can redox cycle, similar to paraquat, to produce $\text{O}_2^-/\text{C}1/\text{C}0$ (40–42) and (b) doxorubicin is a critical drug in lymphoma chemotherapy (43). As shown in Table 2B, MnTE-2-PyP5+ pretreatment increased the sensitivity of the cells to cyclophosphamide but had no effect on doxorubicin or vincristine sensitivity.

**MnTE-2-PyP5+ pretreatment protects H9c2 cardiomyocytes from doxorubicin-induced toxicity.** The clinical use of doxorubicin in lymphoma therapeutics is often limited by acute and chronic cardiotoxicity (44). A number of studies suggest that reactive oxygen species (ROS) produced by doxorubicin treatment play a key role in cardiotoxicity in cardiomyocyte cell culture and mouse models (e.g., refs. 41, 42, 45, among others). Both increased MnSOD expression and treatment with other MnSOD mimetics are protective (41, 45). Given the redox properties of MnTE-2-PyP5+, we tested whether the EC50 for doxorubicin was altered by MnTE-2-PyP5+ pretreatment in H9c2 rat heart myocardiocytes. As shown in Fig. 4, pretreatment with MnTE-2-PyP5+ protected H9c2 cells from doxorubicin-induced toxicity in a dose-dependent manner.

**Discussion**

These data suggest that increasing MnSOD or treating with a pharmacologic agent that mimics MnSOD effects has the potential to improve outcome in hematologic malignancies that are treated with glucocorticoids. In addition to accelerating dexamethasone-induced apoptosis in the WEHI7.2 and primary FL cells, MnTE-2-PyP5+ potentiated cyclophosphamide toxicity while inhibiting lymphoma cell growth and attenuating doxorubicin toxicity in H9c2 cardiomyocytes. This combination of responses suggests that MnTE-2-PyP5+ could benefit lymphoma patients who receive combined therapy, which includes glucocorticoids, doxorubicin, and cyclophosphamide.

Increasing MnSOD protein by genetic manipulation sensitizes the WEHI7.2 cells to dexamethasone. This effect in lymphoma cells is similar to the sensitization of multiple myeloma cells to dexamethasone when MnSOD is increased by transfection or treatment with demethylating agents (16). However, in thymocytes, the gene dose of MnSOD (and resulting alterations in activity) did not track with dexamethasone sensitivity (17). This suggests the possibility that lymphoid tumor cells may respond differently from thymocytes in this regard, an attribute that could be exploited therapeutically. In DLBCL patient tumors, we found that decreased MnSOD expression correlates with a poorer outcome (46). The loss of MnSOD may contribute to chemoresistance in these patients because standard therapy for DLBCL includes the glucocorticoid prednisone.

The ability of increased MnSOD protein to sensitize WEHI7.2 cells to dexamethasone-induced apoptosis fits a model whereby

![Figure 4. MnTE-2-PyP5+ protected H9c2 cardiomyocytes from doxorubicin toxicity. Doxorubicin EC50 measurements of H9c2 cells pretreated with 2.5 or 5 μmol/L MnTE-2-PyP5+ for 2 h, followed by a 48-h doxorubicin treatment. Columns, mean (n = 3); bars, SE. *, significantly different from control (no MnTE-2-PyP5+ cells; P < 0.05).](image-url)
expected to track with glucocorticoid sensitivity. In our modelsystem, resistance to H2O2 results in carcinogenesis. MnTE-2-PyP5+ inhibits proliferation via cells are more sensitive to H2O2. This supports the hypothesis that, oxidized milieu, MnTE-2-PyP5+ can also be redox cycling and causing further oxidative stress. The H2O2 EC50 values in the presence of MnTE-2-PyP5+ can act as an oxidant, directly oxidizing proteins, such as nuclear factor-κB (NF-κB; ref. 50) or contributing to oxidative stress by depleting small molecule reductants.

MnTE-2-PyP5+ may potentiate dexamethasone-induced apoptosis in WEHI7.2 cells via several mechanisms. Dexamethasone treatment increases ROS and causes a more oxidized redox environment (51). MnTE-2-PyP5+ may act, in part, as an MnSOD mimic further increasing intracellular H2O2 and accelerating apoptosis. In a more oxidized milieu, MnTE-2-PyP5+ can also be redox cycling and causing further oxidative stress. The H2O2 EC50 values in the presence of MnTE-2-PyP5+ indicate that MnTE-2-PyP5+ is not detoxifying H2O2; in the cultures with the higher MnTE-2-PyP5+ concentration, the cells are more sensitive to H2O2. This supports the hypothesis that, in an oxidized environment, MnTE-2-PyP5+ can enhance cell death in the WEHI7.2 cells. MnTE-2-PyP5+ may also be oxidizing NF-κB. Oxidizing NF-κB inactivates it by inhibiting its binding to DNA (50). Dexamethasone treatment alone inhibits NF-κB activity in lymphoid cells, in part by inhibiting NF-κB binding to DNA (52). Loss of NF-κB survival signals likely contributes to glucocorticoid-induced lymphocyte apoptosis (52). MnTE-2-PyP5+ may potentiate dexamethasone-induced apoptosis by accelerating NF-κB inhibition. Additional mechanisms are likely because of the complex signaling required for dexamethasone-induced apoptosis.

Inhibition of proliferation and potentiation of cyclophosphamide toxicity are two additional characteristics of MnTE-2-PyP5+ that would contribute to chemotherapeutic efficacy. In a mouse skin carcinogenesis model, MnTE-2-PyP5+ inhibits proliferation via inhibition of activator protein-1 (AP-1) (33). Inhibition of AP-1 or NF-κB (50) could certainly contribute to the antiproliferative effects of MnTE-2-PyP5+ in WEHI7.2 cells; however, the actual source of proliferation inhibition is unknown. Cyclophosphamide normally is bioactivated through hydroxylation by the cytochrome P450 system in the liver (53). The hydroxylated cyclophosphamide released by the liver is thought to be responsible for the cytotoxic effects of cyclophosphamide. In a cell-free system, MnTE-2-PyP5+ acts as a cytochrome P450 reductase mimetic and hydroxylates cyclophosphamide (53). The potentiation of cyclophosphamide toxicity is consistent with MnTE-2-PyP5+ acting as a cytochrome P450 reductase mimetic in the WEHI7.2 cells.

The ability of MnTE-2-PyP5+ to protect cardiomyocytes from doxorubicin toxicity could provide an added chemotherapeutic benefit because cardiotoxicity is a significant problem in the clinical use of doxorubicin (44). The effect of MnTE-2-PyP5+ on doxorubicin toxicity is cell-type specific: MnTE-2-PyP5+ is protective in the H9c2 cardiomyocytes but not in the WEHI7.2 cells. However, MnTE-2-PyP5+ protects WEHI7.2 cells from paraquat. Both paraquat and doxorubicin redox cycle with similar enzymes to produce O2•− (34, 35, 42). This suggests that doxorubicin produces ROS in both cell types, but ROS are only involved in cardiomyocyte toxicity (40, 41, 45). There are at least two scenarios by which MnTE-2-PyP5+ pretreatment can produce these results: (a) MnTE-2-PyP5+ could be scavenging the O2•− produced by paraquat in the WEHI7.2 cells and doxorubicin in the H9c2 cells or (b) because MnTE-2-PyP5+ redox cycles using the same enzymes as paraquat and doxorubicin, MnTE-2-PyP5+ may be competing with both compounds for reducing equivalents (47). By decreasing the redox cycling of paraquat and doxorubicin, MnTE-2-PyP5+ can prevent O2•− generation. Inactivation of NF-κB by MnTE-2-PyP5+ could also play a role in cardiomyocyte protection because activation of NF-κB during doxorubicin treatment is proapoptotic in cardiomyocytes (54).

The profile of MnTE-2-PyP5+ effects: (a) sensitizing lymphoid cells to dexamethasone and cyclophosphamide, (b) not affecting the doxorubicin toxicity in the WEHI7.2 cells while (c) protecting cardiomyocytes from doxorubicin and (d) inhibiting proliferation suggests that MnTE-2-PyP5+ could be effective in DLBCL combined with CHOP. MnTE-2-PyP5+ also has potential for use in other hematologic malignancies that use glucocorticoids, especially multiple myeloma where there is decreased MnSOD and dexamethasone is standard treatment (16). These data further suggest that MnTE-2-PyP5+ could be investigated as a cardioprotectant for cancers treated with doxorubicin. The known chemistry of MnTE-2-PyP5+ suggests potential mechanisms by which some of the observed effects could occur. However, additional work is necessary to determine the molecular requirements for the MnTE-2-PyP5+ effect. In lymphoma, the heterogeneity of treatment response is a significant barrier to successful treatment. By further characterizing the mechanism of MnTE-2-PyP5+ action, it will be possible to identify the molecular profile of patients likely to benefit from treatment with MnTE-2-PyP5+.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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