Int. J. Radiation Oncology Biol. Phys., Vol. 63, No. 2, pp. 545–552, 2005

0360-3016/05/$–see front matter

doi:10.1016/j.ijrobp.2005.05.026

**BIOLOGY CONTRIBUTION**

# A MANGANESE PORPHYRIN SUPEROXIDE DISMUTASE MIMETIC ENHANCES TUMOR RADIORESPONSIVENESS

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**Purpose: To determine the effect of the superoxide dismutase mimetic Mn(III) tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP$^{5+}$) on tumor radioresponsiveness.**
**Methods and Materials: Various rodent tumor (4T1, R3230, B16) and endothelial (SVEC) cell lines were exposed to MnTE-2-PyP$^{5+}$ and assayed for viability and radiosensitivity *in vitro*. Next, tumors were treated with radiation and MnTE-2-PyP$^{5+}$ *in vivo*, and the effects on tumor growth and vascularity were monitored.**
**Results: *In vitro*, MnTE-2-PyP$^{5+}$ was not significantly cytotoxic. However, at concentrations as low as 2 $\mu$mol/L it caused 100% inhibition of secretion by tumor cells of cytokines protective of irradiated endothelial cells. *In vivo*, combined treatment with radiation and MnTE-2-PyP$^{5+}$ achieved synergistic tumor devascularization, reducing vascular density by 78.7% within 72 h of radiotherapy ($p < 0.05$ vs. radiation or drug alone). Co-treatment of tumors also resulted in synergistic antitumor effects, extending tumor growth delay by 9 days ($p < 0.01$).**
**Conclusions: These studies support the conclusion that MnTE-2-PyP$^{5+}$, which has been shown to protect normal tissues from radiation injury, can also improve tumor control through augmenting radiation-induced damage to the tumor vasculature.** © 2005 Elsevier Inc.

**Manganese porphyrin, Superoxide dismutase, Radiotherapy.**

## INTRODUCTION

Normal tissue toxicity remains a major limiting factor for escalating radiation doses to better treat tumors. Chemical radioprotection offers the promise of minimizing dose-limiting normal tissue radiotoxicity without compromising tumor control. Classic radioprotectors are designed to interfere with the damaging free radical cascades initiated by radiation (1). They are often selectively beneficial for normal tissues, owing to differences in either uptake or target sensitivity between normal and malignant cells (2, 3). Many antioxidants, both naturally occurring and synthetic, have demonstrated capacity to protect normal tissues from radiation injury (4, 5).

As more has been learned about how oxidative stress promotes normal tissue radiotoxicity, new opportunities for radioprotection have arisen. It has long been recognized that free radicals initiate radiation-induced cellular damage (6). More recent studies have suggested that oxidative stress continues to promote radiation-induced damage long after normal tissues are irradiated (7, 8). Because these later free radical–mediated signals are components of natural physiologic/pathophysiologic processes, they might respond well to natural cellular antioxidants.

The superoxide dismutase (SOD) enzymes have been investigated as potential natural radioprotectors. The four known forms of SOD all serve to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen (9, 10). They are localized within and outside the cell, are widely expressed throughout the body, and are important in redox homeostasis (11–13). Preclinical models have demonstrated that treating irradiated rodents with exogenous SOD—delivered by injection of the enzyme or through liposome- or viral-mediated gene therapy—can ameliorate radiation-induced pneumonitis (14–19). The same principle has also been demonstrated in transgenic mice overexpressing SOD in type II pneumocytes (20). Although these studies generated promising results, the difficulties of delivering SOD to target tissues, with gene therapy or otherwise, have somewhat impeded translation into the clinic.

To exploit the benefits of SOD administration while

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Supported by Incara Pharmaceuticals, by National Institute of Health Grants CA40355 (M.W.D.) and P30CA14236 (Z.V.), and by U.S. Department of Defense Congressionally Directed Medical Research Programs Grant BC024326 (I.B.H.).

Received May 24, 2004, and in revised form May 3, 2005. Accepted for publication May 4, 2005.

bypassing delivery and antigenicity problems, small-molecular-weight mimetics of the enzyme have been synthesized (21, 22). Besides catalytically eliminating superoxide, these compounds effectively scavenge other reactive oxygen and nitrogen species (ROS/RNS), such as peroxynitrite, carbonate radical, and nitric oxide (21, 23–25). The MnTE-2-PyP$^{5+}$ [Mn(III) tetrakis(*N*-ethylpyridinium-2-yl)porphyrin] compound evaluated in this study also has various signaling effects: it blocks DNA binding by the transcription factors nuclear factor (NF)-$\kappa$B and activator protein (AP)-1 and inhibits downstream expression of important proteins, such as tumor necrosis factor and interleukin-1$\beta$ (26, 27). This mimetic has been shown to prevent pathology in a wide variety of preclinical models of diseases wherein oxidative stress plays a role, including stroke (28–30), diabetes (31), and sickle cell anemia (32). Relevant to the current study, systemic administration of MnTE-2-PyP$^{5+}$ has also been shown to protect against pathologic and functional radiation pneumonitis (33). Radioprotection has also been demonstrated preclinically with a methyl analog of the compound, MnTM-2-PyP$^{5+}$ (22, 34). These studies strongly support the concept that SOD-based therapeutics might serve as clinically useful radioprotectors.

As is the case for all radioprotectors, before considering MnTE-2-PyP$^{5+}$ as a clinical tool it is important to study what effects it might have on tumor radiosensitivity. There is convincing evidence in the literature to support the hypothesis that ROS/RNS are capable of promoting vascular angiogenesis (35) and that antioxidants have antiangiogenic activity (36–41). Additional data suggest that antiangiogenic compounds act synergistically with radiotherapy by increasing radiation damage to the vasculature, leading to secondary tumor cell kill (42), raising the possibility that MnTE-2-PyP$^{5+}$ might improve radiotherapeutic control of tumors through an antivascular mechanism.

On the basis of the aforementioned data, we hypothesized that the normal tissue radioprotector MnTE-2-PyP$^{5+}$ might enhance tumor radiosensitivity by augmenting destruction of the tumor vasculature after radiotherapy. We used a combination of *in vitro* and *in vivo* assays to test the feasibility of combining MnTE-2-PyP$^{5+}$ with radiotherapy. These studies might validate the potential utility of MnTE-2-PyP$^{5+}$ in the setting of tumor radiotherapy.

## METHODS AND MATERIALS

### *Cell lines and cell culture*

4T1 (mouse mammary carcinoma), B16 (mouse melanoma), R3230 (rat mammary carcinoma), and SVEC (SV40 large T antigen–transformed murine endothelial cell) lines were acquired from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (Gibco-BRL) and penicillin–streptomycin (Gibco-BRL) at 5% $CO_2$ and 21% $O_2$.

### *Viability assay*

Cells were plated into 96-well dishes at a density of $10^3$ cells per well in growth medium. Twelve hours later, MnTE-2-PyP$^{5+}$ was added at the desired concentration. After predetermined incubation periods, methylthiazolyldiphenyl-tetrazolium (MTT; Sigma Chemical, St. Louis, MO) was added to each well to a final concentration of 1 mg/mL. Four hours later, the medium was removed, MTT crystals were dissolved in 100% dimethyl sulfoxide, and the optical density was read on a spectrophotometer ($OD_{570}$).

### *Clonogenic assay*

Cells were harvested and incubated in a single-cell suspension with 5 $\mu$mol/L MnTE-2-PyP$^{5+}$ for 2 h at 37°C. Cells were then placed in a Mark IV Cesium Irradiator (dose rate = 7 Gy/min; JL Shepherd, San Fernando, CA) on a rotating platform and irradiated with predetermined doses. Immediately after irradiation, serial dilutions of the treated cells were plated in six-well plates, each in triplicate. After incubating for 9 days, colonies ($\geq$50 cells) were fixed in methanol, stained with crystal violet, and counted.

### *Endothelial radioprotection assay*

Subconfluent 4T1 cells were incubated at 0.5% $O_2$ for 12 h with or without MnTE-2-PyP$^{5+}$ (0–5 $\mu$mol/L) or amifostine (WR-2721) (0–5 mmol/L) to generate conditioned medium. Conditioned medium was then collected, and the appropriate drug was added to the previously drug-free samples. Endothelial cells (ECs) were plated as for viability assays. Twelve hours later, the medium was replaced with drug-containing conditioned medium. Two hours later, the cells were irradiated (10 Gy). Ninety-six hours after treatment, cells underwent a viability assay, as described above. Endothelial cell viability was determined from MTT results, according to methodology described previously (43). Briefly, spectrophotometric results were normalized according to the following equation:

$$V_{RxDr} = (A_{RxDr} - A_0)/(A_{Dr} - A_0)$$

where $V_{RxDr}$ is the relative viability of the cells treated with predetermined doses of radiation (Rx) and drug (Dr), $A_{RxDr}$ is the MTT absorbance of those cells, $A_{Dr}$ is the MTT absorbance of the unirradiated controls treated with the same dose of drug, and $A_0$ is the MTT absorbance of the cells before treatment.

### *Animals*

Female Fisher-344 rats, C57/Bl6 mice, and Balb/C mice were housed and treated in accordance with approved guidelines from the Duke University Institutional Animal Care and Use Committee.

### *Tumors*

4T1 tumors were grown in the flank of Balb/C mice by injecting a single cell suspension of tumor cells ($10^5$). Tumor volumes were measured with calipers and calculated according to two diameters with the formula: $v = (a^2 \times b)/2$, where v is the volume, a is the short diameter, and b is the long diameter. Animals were sacrificed once tumors reached five times their initial treatment volumes.

### *Skinfold window chambers*

Window chambers were implanted as described previously (44). Briefly, mice were anesthetized with sodium pentobarbitol (80 mg/kg, i.p.), and a 1-cm diameter circular incision was made in the dorsal skin flap, over which a titanium chamber was surgically implanted. A 10-$\mu$L suspension of tumor cells ($5 \times 10^3$ cells) was