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Inhibition of the Continuum of Radiation-Induced Normal Tissue Injury by a Redox-Active Mn Porphyrin

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Abstract

Normal tissue damage after head and neck radiotherapy involves a continuum of pathologic events to the mucosa, tongue and salivary glands. We examined the radioprotective effects of MnBuOE, a redox-active manganese porphyrin, at three stages of normal tissue damage: immediate (leukocyte endothelial cell [L/E] interactions), early (mucositis) and late (xerostomia and fibrosis) after treatment. In this study, mice received 0 or 9 Gy irradiation to the oral cavity and salivary glands $\pm$ MnBuOE treatment. Changes in leukocyte-endothelial cell interactions were measured 24 h postirradiation. At 11 days postirradiation, mucositis was assessed with a cathepsin-sensitive near-infrared optical probe. Stimulated saliva production was quantified at 11 weeks postirradiation. Finally, histological analyses were conducted to assess the extent of long-term effects in salivary glands at 12 weeks postirradiation. MnBuOE reduced oral mucositis, xerostomia and salivary gland fibrosis after irradiation. Additionally, although we have previously shown that MnBuOE does not interfere with tumor control at high doses when administered with radiation alone, most head and neck cancer patients will be treated with the combinations of radiotherapy and cisplatin. Therefore, we also evaluated whether MnBuOE would protect tumors against radiation and cisplatin using tumor growth delay as an endpoint. Using a range of radiation doses, we saw no evidence that MnBuOE protected tumors from radiation and cisplatin. We conclude that MnBuOE radioprotects normal tissue at both early and late time points, without compromising anti-tumor effects of radiation and cisplatin.

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INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) have an incidence of 62,000 new cases in the U.S. annually (accounting for 3% of all cancers) (1), radiotherapy with curative intent is an important treatment modality for these patients. Although radiotherapy is generally effective for HNSCC, >90% of patients develop side effects as a result of their treatment, including dermatitis, xerostomia (loss of saliva production) and oral mucositis (inflammation and ulceration of the oropharyngeal and/or esophageal mucosa). These side effects are classified as “severe” in at least two-thirds of radiation-treated HNSCC patients (2–4). Mucositis and xerostomia develop in a sequential fashion on the order of days/weeks to months after the initiation of radiotherapy, and pose a particular risk because they can interfere with oral food intake and dramatically reduce patient quality of life. There are no FDA approved therapies for the management of mucositis, and the only approved drug for treating xerostomia, amifostine, has its own adverse effects and provides incomplete protection (5–7). Consequently, the acute effects of radiation result in unplanned treatment breaks in roughly 30% of therapy courses for HNSCC (8, 9). However, while treatment breaks may reduce symptoms, they increase the risk of local tumor recurrence and are therefore an option of last resort (10). There exists an important and unmet need for methods that provide normal tissue radioprotection without decreasing the efficacy of radiation.

Our group has recently reported on the development of Mn(III) meso-tetrakis(N-n-butoxyethylpyridinium-2-yl)-porphyrin (MnBuOE), a redox-active manganese-porphyrin (11). MnBuOE shares a mechanism of action with superoxide dismutase (SOD), which regulates the oxidative environment of the cell by interacting with reactive oxygen (ROS) and reactive nitrogen (RNS) species (11–14). Depending on the redox environment of the cell, MnBuOE will yield differential effects after irradiation, sparing normal tissue while promoting tumor cell death (15). We have previously shown that MnBuOE radio-protects normal oral mucosa and salivary gland function without decreasing the efficacy of radiation at achieving xenograft tumor control/remission (13).

In prior work, the earliest time point that normal tissue damage was examined was at 11 days postirradiation. There are normal tissue effects that can occur earlier than this, including increased leukocyte-endothelial (L/E) interactions, upregulation in the expression of inflammatory cytokines, and changes in the intracellular oxidative state. Since our first report, pharmacokinetic analysis has revealed that although the plasma half-life of MnBuOE is on the order of hours, the drug persists in tissues for longer periods of time (16). These results suggested that maintenance doses taken every 2–3 days would be sufficient to maintain drug levels in tissues of interest. This schedule results in lower overall drug dosing, compared with our earlier study.

Therefore, the aims of this study were threefold: 1. To evaluate the potential protective effects of MnBuOE when combined with radiation and cisplatin treatments over the timeline for immediate, early and late effects; 2. To verify that MnBuOE does not protect HNSCC from the combined treatments of radiation and cisplatin; and 3. To apply recent pharmacokinetic findings to evaluate the efficacy of low doses of MnBuOE given 3 times

per week at preventing late injury while using the clinically relevant combined treatments of fractionated radiation with cisplatin.

MATERIALS AND METHODS

Animals

For normal tissue studies, we used six–eight-week-old female C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME). For tumor control studies, female nu/nu mice were obtained from the Duke University Breeding Facility. Mice were group housed in a barrier facility and allowed to acclimate to their new environment for at least one week before experiments. All work was approved by Duke University Institutional Animal Care and Use Committee.

MnBuOE Synthesis and Administration

MnBuOE was synthesized as previously described elsewhere (11). Each preparation of MnBuOE passed quality and purity tests (i.e., thin-layer chromatography, UV-vis, ESI-MS and fluorescence and elemental analyses). Mouse injections were administered subcutaneously, with MnBuOE dissolved in sterile saline. Mice received an initial dose of 0.2, 0.6 or 2.0 mg/kg, followed by three-times-per-week (TIW) maintenance dose of one-half of the loading dose (i.e., 0.1, 0.3 or 1.0 mg/kg, respectively) (termed 2/1, 0.6/0.3 and 0.2/0.1 dosing schedules) (FIG. 1A). Mice used for intravital microscopic analysis of leukocyte-endothelial cell interactions and the GSH/GSSG assay received a single dose of 0.2 mg/kg 24 h before irradiation.

Salivary Gland and Oral Mucosa Irradiation

Twenty-four hours after administration of a MnBuOE loading dose, mice were anesthetized with 1.5% isoflurane gas mixed with oxygen and placed in an X-RAD 225Cx (Precision X-ray Inc., North Branford, CT) small animal micro-CT irradiator. A collimating cone that produced a 15 × 40 mm radiation field was used to target the radiation beam to the salivary gland and oral cavity (FIG. 1B). The radiation field included all major and minor salivary glands (located primarily in the neck region of mice) and the oral mucosa, including glands of the cheeks, lips and tongue. The target tissues were localized within the radiation field with a source-to-subject distance of 30.76 cm, using fluoroscopy at 40 kVp and 2.5 mA with a 2-mm aluminum filter. QC dosimetry studies confirmed that the radiation dose administered during the fluoroscopy session was low (6–12 cGy). The target area was then irradiated using opposed lateral beams at a dose rate of 300 cGy/min at target depth with 225 kVp and 13 mA and a 0.3-mm copper filter for a total dose of 9 Gy. Control/sham mice were anesthetized but not imaged fluoroscopically or irradiated.

Mucositis Evaluation Using Fluorescence Molecular Tomography

As reported previously, we used ProSense 750EX (Perkin Elmer, Waltham, MA) to detect mucositis. ProSense 750EX is a near-infrared (NIR) imaging agent that is cleaved by cathepsins, a family of enzymes that are expressed during inflammation (17). Mice were injected intravenously with 100 µL ProSense 750EX. At 24 h after injection, mice were anesthetized via isoflurane and imaged using the Visen FMT 2500LX (Perkin Elmer). The

ProSense 750EX signal was quantified using TrueQuant software (Perkin Elmer Life and Analytical Sciences, Downers Grove, IL).

Imaging Leukocyte-Endothelial Cell Interactions Using Intravital Microscopy

Imaging was performed 24 h after irradiation or sham irradiation. Mice were anesthetized with 80/8 mg/kg ketamine/xylazine mixture. Acridine orange (Sigma-Aldrich, St. Louis, MO) was injected intravenously (100 μ L of 0.1% acridine orange in saline). The ventral surface of the tongue was placed on a glass cover slip before mounting the mouse onto a Zeiss Observer.Z1 (Carl Zeiss AG, Oberkochen, Germany) inverted microscope equipped with Zeiss Filter Set 38. Fluorescent excitation was performed at 470 nm, and video was obtained at 525 nm using a 40 $\times$ objective. Two separate regions with at least four veins in the 15–50 mm range were identified, and a 60–120 s long video at 5 frames/s was obtained using Zen Imaging Software (Carl Zeiss AG). Mice were kept warm while anesthetized using a heated stage.

Leukocyte-Endothelial Cell Interaction Analysis

All video analysis was performed in a blinded fashion. Rolling leukocytes were defined as cells that marginated along the vessel wall and were clearly dissociated from the bulk blood flow. Four vessels were analyzed per mouse. Leukocyte counting was repeated in triplicate for each vessel and averaged to obtain the flux of rolling leukocytes ($F_{rolling}$). Vessel diameter (D) and the velocity of at least one free-flowing leukocyte(s) (V_L) were also measured. A pseudo shear rate (g_s) was also calculated for each vessel as: $g_s = 8 V_L / D$. These methods have been previously described elsewhere (18). The observer was blinded to treatment groups for these analyses.

GSH/GSSG Sample Preparation

This procedure was modified from a previously published method analyzing glutathione in whole blood (19). After euthanasia, mouse tongues were resected and flash frozen in liquid nitrogen and stored at -80°C until use. Samples (~20 mg) were homogenized with: 4 parts (g/vol) of GSH-trapping solution [20 mM N-ethylmaleimide (NEM; Sigma), 2% SSA and 2 mM EDTA in 15% methanol], 10 parts (g/vol) of 1% formic acid (Fluka Inc., St. Louis, MO) and two 2.5 mm Zr-silica beads (Biospec Products Inc., Bartlesville, OK) in FastPrep (Thermo Fisher Scientific, Waltham, MA) homogenizer at speed 5 for 40 s. After 30 min incubation at room temperature, 500 μ L of chloroform was added to 150 μ L of the homogenate, mixed for 40 s, followed by centrifugation at 16,000g for 5 min. For GSH analysis, 5 μ L of supernatant, 1 mL of deionized water and 100 μ L of 1 μ M GSH-NEM-d3 (internal standard) was combined and placed into autosampler at 4°C . For GSSG analysis 20 μ L of supernatant and 20 μ L of 1 μ M GSSG-d6 in mobile phase A was combined and placed into an autosampler at 4°C .

LC-MS/MS Analysis

The measurement of GSH (as GSH-NEM derivative) and GSSG was performed on Shimadzu 20A series liquid chromatography (LC; Shimadzu Scientific Instruments, Columbia, MD) and Applied Biosystems/SCIEX API5500 QTrap tandem-mass

spectrometer (MS/MS; Applied Biosystems, Foster City, CA). LC conditions: Column Agilent ZORBAX Eclipse Plus, C18 4.6 × 50 mm 1.8 µm particle size (P/N 959941-902) analytical column (Agilent Technologies, Santa Clara, CA) and Phenomenex, C18 4 × 3 mm guard cartridge (P/N AJ0-4287) at 45°C (Phenomenex, Torrance, CA). Mobile phase solvents (all MS-grade); A: 0.1% formic acid in water, 2% acetonitrile; B: acetonitrile. Elution gradient at 1 mL/min: 0–3 min 0–50% B-solvent, 3–3.1 min 50–95% B-solvent, 3.13.5 min 95% B-solvent, 3.5–3.6 min 95–0% B-solvent. Run time: 7 min. MS/MS conditions: MRM transitions (*m/z*) followed for GSH-NEM, GSH-NEM-d3, GSSG and GSSG-d6 were 433-304, 436.2–307.1, 613.1–355 and 619.1–361, respectively. Calibration samples (n = 6) in mobile phase A solvent were prepared in the following appropriate ranges: 0.375– 6.00 mM for GSH and 1.88–30 µM for GSSG analysis and analyzed alongside the study samples. Quantification was performed by Analyst 1.6.2 software.

Xerostomia Quantification by Stimulating Saliva Production

Mice were injected intraperitoneally with 3 µg carbomylcholine chloride (Sigma) dissolved in sterile phosphate buffered saline. Salivation began 2 min after injection. Saliva was collected via pipette for 4 min. The volume was measured gravimetrically, assuming a density of 1 g/mL.

Salivary Gland and Tongue Histology and Fibrosis Quantification

Salivary tissues and tongues were excised from the euthanized mice, placed in formalin for 24 h and embedded in paraffin. Sections (4-µm thick) that passed through the submandibular and sublingual glands were stained with Masson's trichrome that results in bright turquoise staining of fibrotic tissue and red staining of healthy tissue. Images were obtained using a Zeiss Imager A.1, equipped with AxioVision 4.7 software and processed using MATLAB software (MathWorks, Natick, MA). Images from three different regions of each gland were collected and used for quantification. Whole tongue images were obtained, but blue staining connective tissue beneath the papillae was excluded from analysis for blue:red quantification to account for different tongue orientations during imaging.

Tumor Growth Delay Study

Human pharyngeal carcinoma (FaDu) cells were obtained from the Duke University Cell Culture facility and were confirmed to be free of mycoplasma and known rodent pathogens. Cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Mice were injected s.c. in the right flank with 3×10^6 cells suspended in 100-µl serum-free media. Seven days after implantation mice were randomized to treatment groups that received either MnBuOE treatment [0.6 mg/kg loading dose followed by TIW 0.3 mg/kg (loading/3× weekly maintenance)] or an equivalent volume of saline. Tumors were measured daily with calipers and the volume was calculated using the equation $V = (A^2 \times B \times \pi)/6$, where A is the shortest diameter and B is the longest diameter. As tumors reached 200 mm³, they randomized into radiation treatment groups consisting of three daily fractions of radiation (6, 9, 12, 15 or 17 Gy/fraction) administered on three consecutive days. Cisplatin (6 mg/kg; APP pharmaceuticals, Schaumburg, IL) was injected i.p. 4 h prior to the first radiation fraction. Sham-irradiated mice were anesthetized and did not receive cisplatin injections.

Statistics

Statistical analysis was conducted using GraphPad Prism 6 (La Jolla, CA). A log-rank test (Mantel-Cox) was used to compare saline vs. MnBuOE treatments at specific radiation doses. Exploratory analysis of treatment efficacy was based on comparison of tumor tripling times across treatment arms. However, formal hypothesis testing using tripling times proved difficult due to many animals not achieving the required volume threshold, particularly at higher radiation doses. To capture the inhibitory effect in these (and other) animals, we estimated animal specific time varying tumor inhibition curves using the dynamic treatment effect (DTE) methodology (20). We used the area under the curve (AUC) of animal specific inhibition curves as a summary measure for dose-response analysis. A linear dose-response curve was fit with the control arm as baseline with differential slopes and intercepts for the MnBuOE arm. The isodose effect of MnBuOE was quantified and tested in terms of the estimated differential slope.

RESULTS

Determination of Minimum Effective Dose of MnBuOE in Reducing Mucositis

We first performed a MnBuOE dose-response experiment to determine the minimum effective dose that still prevented mucositis (FIG. 2). Sham-irradiated mice that received either saline or 2/1 mg/kg MnBuOE did not show an appreciable NIR signal. Radiation (1×9 Gy) induced a significant ($P < 0.05$) increase in NIR signal compared with sham-irradiated control mice. Irradiated mice treated with MnBuOE demonstrated a decreased NIR signal with the greatest decrease occurring in mice receiving the lowest levels of MnBuOE [0.6/0.3 or 0.2/0.1 mg/kg MnBuOE ($P < 0.05$)].

Leukocyte-Endothelial Cell Interactions

Previous studies have utilized intravital microscopy to determine the magnitude of the early inflammatory response initiated by radiation by directly quantifying the number of rolling leukocytes in the terminal venules of irradiated tissues including the small bowel (21), skin (18) and ear pinna (manuscript in preparation). To assess leukocyte-endothelial interactions, we obtained real-time images of the sublingual vascular bed in mice treated with saline or MnBuOE $\pm 1 \times 9$ Gy irradiation (FIG. 3A). Images were used to quantify rolling leukocytes 24 h postirradiation (FIG. 3B, sub-panel i) To determine if differences in leukocyte rolling were masked by compensatory differences in shear stress, we measured the diameters and velocities of free-flowing leukocytes in vessels used for counting and calculated a shear rate for each vessel (FIG. 3B, sub-panels ii–iv). Mice treated with saline and 9 Gy had a significant increase in average velocity compared with mice treated with saline or MnBuOE alone ($P < 0.05$). MnBuOE treatment alone increased vessel diameters compared to saline control mice, but MnBuOE-treated mice that received 9 Gy irradiation had vessel diameters similar to those of the saline control mice ($P = 0.52$). The difference in vessel diameter contributed to significantly lower shear rates in the MnBuOE-treated mice compared to all other treatment groups [$P < 0.05$ (Supplementary Tables S1–S3; <http://dx.doi.org/10.1667/RR14757.1.S1>)].

GSH/GSSG Oxidative Stress Assay

The reduced to oxidized glutathione ratio (GSH/GSSG) has been used in multiple tissue types to measure the oxidative environment (19, 22, 23). To assess the oxidative stress at the time of intravital imaging, mice were administered a single 0.2 mg/kg dose of MnBuOE 24 h prior to irradiation (9 Gy) and euthanized 24 h postirradiation. We observed that radiation decreased the GSH/GSSG ratio (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14757.1.S1>) in tongue tissue, reflecting increased oxidative stress in the cells in response to radiation-induced ROS and RNS, but the 16% decrease was not statistically significant. The addition of MnBuOE alone slightly increased the GSH/GSSG ratio compared to the saline and saline + radiation groups, which may suggest an overall reduced oxidative stress in the tissue by MnBuOE alone. Mice receiving the combination of MnBuOE + radiation treatments had increased GSH/GSSG compared to either saline control arms, averaging only 4% lower than the 0 Gy MnBuOE tongue levels.

Xerostomia

Stimulated salivation was assessed at 11 weeks postirradiation (FIG. 4). In accordance with previous studies, radiation treatment significantly decreased salivation in saline-treated mice (13, 24–26). In this study, the lowest doses of MnBuOE (0.6/0.3 or 0.2/0.1 mg/kg) gave the highest level of protection against xerostomia. For both the 0.6/0.3 and 0.3/0.1 mg/kg doses of MnBuOE there was no significant difference in salivation compared to sham-irradiated-MnBuOE-treated mice.

Salivary Gland and Tongue Fibrosis

Fibrosis in the salivary glands and tongue were examined 12 weeks postirradiation (FIG. 5A). Radiation treatment resulted in a significant increase in fibrosis in both salivary glands and tongue while MnBuOE treatment protected against this fibrosis in both salivary gland and tongue, particularly at the lower MnBuOE doses. Saline + radiation treatments significantly increased blue:red pixel ratio in the salivary glands ($P < 0.05$) (FIG. 5B). The blue:red pixel ratio was not significantly different among mice treated with MnBuOE + radiation (at all levels of MnBuOE) compared to sham-irradiated control animals. In FIG. 5C, we show that in the tongue, radiation + 0.6/0.3 or 0.2/0.1 mg/kg MnBuOE treatments resulted in a significant decrease in fibrosis ($P < 0.05$).

Tumor Growth Control Studies

We have previously shown that higher doses of MnBuOE sensitized a head and neck tumor xenograft to radiation (13). However, most head and neck cancer patients will receive radiotherapy with chemotherapy, such as cisplatin. Therefore, it was prudent to investigate the effects of MnBuOE at a lower dose, to mimic what was performed for normal tissues, above, in conjunction with radiation + cisplatin. We found that there was a significant radiation effect on tumor growth ($P < 0.0001$), but MnBuOE (0.6 mg/kg loading dose followed by TIW 0.3 mg/kg maintenance dosing) did not significantly affect tumor growth, relative to radiation and cisplatin treatments. Kaplan-Meier analysis showed no significant differences at any radiation dose between MnBuOE- and saline-treated mice (FIG. 6). By day 90 postirradiation, the percentages of mice with complete tumor regression in the 3 ×

12, 3 × 15 and 3 × 17 Gy groups were 14%, 40% and 20%, respectively, for saline-treated mice, and 33, 40 and 50%, respectively, for the MnBuOE-treated mice (Supplementary Fig. S2A; <http://dx.doi.org/10.1667/RR14757.1.S1>). No mice were tumor-free at day 90 postirradiation in the sham-irradiated, 3 × 6 or 3 × 9 Gy irradiated groups.

As a deeper analysis, we compared the dose-response relationship of tumor inhibition, as quantified by the AUC of animal-specific dynamic-treatment effect curves, across the control and MnBuOE arms. There was no significant difference in the slope of the dose-response curve for the two arms [$P = 0.47$ (Supplementary Fig. S2B; <http://dx.doi.org/10.1667/RR14757.1.S1>)].

Normal Tissue Protection from Combined Radiation and Cisplatin Treatments

Finally, to confirm that normal tissue injury resulting from combination therapies of radiation and cisplatin would not be too great for MnBuOE to protect against, we conducted a study to compare mucositis and xerostomia in mice treated with 1 × 9 Gy + 6/mg/kg cisplatin. To answer the question of how long MnBuOE should be administered after irradiation, MnBuOE administration continued for 5 weeks (FIG. 7A). As expected, the combination of radiation + cisplatin treatments significantly increased NIR signaling compared to untreated, saline controls (FIG. 7B). Mice that were treated with 0.6 mg/kg loading dose followed by TIW 0.3 mg/kg maintenance dose of MnBuOE showed significantly less NIR signal than did mice treated with saline + radiation + cisplatin. The NIR signal for mice receiving the 0.6/0.3 dose of MnBuOE was not statistically different from sham-irradiated-saline-control mice.

We aimed to evaluate xerostomia at 12 weeks after radiation + cisplatin treatments; however, the saline control mice in the radiation + cisplatin group had deteriorated to the point where we were unable to collect saliva without causing the ulcerations in the mouth to bleed, thereby confounding our results. Notably, the radiation + cisplatin-treated mice failed to gain weight in the weeks postirradiation (FIG. 7C), and had severe moist desquamation of the skin in the irradiated region that failed to heal (FIG. 7D, see left-side panels). The mice that had received MnBuOE after radiation + cisplatin treatments did not have actively bleeding mouth ulcers, and only 1 out of 4 mice still had ulcers in the mouth and surrounding skin and had failed to gain weight after irradiation. An additional mouse in this group showed scarring of the skin, but had had weight gain similar to untreated controls (FIG. 7D, see right-side panels). The remaining two mice showed leukotrichia only, with normal weight gain and no scarring or ulcers.

DISCUSSION

MnBuOE Radioprotection Dose Response is Nonlinear

Our data show that in this model, MnBuOE TIW is effective at decreasing mucositis at very low doses (0.2/0.1 mg/kg, TIW). We have previously reported that higher levels of MnBuOE [1.5 mg/kg twice daily (bid)] are associated with protection against radiation-induced mucositis, xerostomia and salivary gland fibrosis. In addition, this higher MnBuOE dose enhanced local-control rates of the tumor compared with controls showing that MnBuOE

widens the therapeutic margin by decreasing the radiation dose required to control the tumor and at the same time increasing tumor resistance to radiation-mediated injury (13). The current study shows a substantial level of protection against the radiation-induced effects of mucositis, xerostomia and both salivary gland and tongue fibrosis using a maintenance dose that is approximately 60 times lower (0.1 mg/kg TIW compared to 1.5 mg/kg bid).

We previously reported that a single fraction of 9–15 Gy administered to the submandibular salivary gland significantly decreased salivation and increased fibrosis after irradiation. In this study the dosing schedule was modified based on additional pharmacokinetics data [see ref. (16)] that suggested that constant tissue levels can be maintained with less frequent dosing (2–3 doses/week of one-half the initial loading dose). This pattern of maintenance dosing is more practical than is a bid dosing schedule. We also identified a similar impact of protection against tongue fibrosis using the low-level MnBuOE dosing schedule. To our knowledge, this is the first preclinical study using tongue fibrosis as a normal tissue injury endpoint for a radioprotectant.

Radiation induces both acute and chronic oxidative stress and inflammation. The question of how long to continue MnBuOE treatment after irradiation will need to be determined with an extensive dosing study, however, we were encouraged to find that cessation of MnBuOE at five weeks after radiation + cisplatin treatments conferred protection against late ulcerations and sustained weight loss. Although we were unable to satisfactorily quantify saliva production, due to the small number of mice and deteriorating oral mucosa, we did indeed see a trend towards reduced xerostomia at 10, 11 and 12 weeks in mice that received MnBuOE for the first five weeks after radiation + cisplatin treatments (data not shown.) Future studies are needed to determine the optimum length of time for which MnBuOE should be administered after irradiation.

Intravital Microscopy can be Used to Noninvasively Quantify Leukocyte-Endothelial Cell Interactions and Microvessel Characteristics in the Oral Cavity

It is well-established that radiation causes an increase in leukocyte-endothelial cell interaction (rolling and adhesion) in post-capillary venules (18, 21). Leukocytes that initiate an inflammatory response in the tissue first roll along the endothelium before adhering and ultimately migrating through the vessel wall and into the parenchyma (18, 21). Rolling and adhesion occur in response to changes in cytokine, selectin and integrin expression that begin within minutes of exposure to radiation. In model systems, such as the mouse small bowel, this effect persists in excess of 24 h, with a period of increased rolling generally preceding a period of increased adhesion before returning to normal (21).

To the best of our knowledge, we are the first to report this noninvasive method for directly measuring leukocyte-endothelial cell interactions in the sublingual vascular bed. Our findings are different from previous studies in two key respects: 1. there was no appreciable degree of leukocyte adhesion and 2. treatment with radiation alone did not lead to an increase in the quantity of rolling leukocytes 24 h postirradiation. These differences stress the importance of considering anatomical differences when assessing vascular radiation injury. MnBuOE treatment alone significantly increased vessel diameter, which translated into a significantly reduced shear rate (FIG. 3B, sub-panel iv). One would expect that a

lower shear rate would promote leukocyte rolling, as marginating leukocytes experience reduced forces parallel to the direction of blood flow (18). The observation that MnBuOE treatment alone decreased shear rate without increasing leukocyte rolling could suggest a decrease in adhesion molecule expression. Future studies should focus on elucidating the cytokine and proteomic basis of the observed anti-inflammatory effect of MnBuOE, potentially studying the drug's effect on expression of stimulants for rolling (including IL-1, H₂O₂, superoxide) and adhesion (including C5a, leukotriene B₄, platelet activating factor) of leukocytes.

It is surprising that we did not observe an increase in leukocyte rolling in response to radiation, because a single 9 Gy dose was sufficient to induce mucositis, xerostomia and salivary gland fibrosis. It is possible that the increase in rolling occurred and resolved completely before the time that imaging was performed, although it has been established that increased rolling persists through 24 h in other tissue models (21). Further trials with sequential imaging might be able to determine when maximum leukocyte rolling occurs in this model. Our studies were initiated with the anticipation that observed changes in leukocyte rolling could serve as an image-based biomarker for later radiation-mediated normal tissue injury. However, our results show that for the oral mucosa, late injury develops in the absence of measurable very early vascular changes.

Our GSH/GSSG analysis (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14757.1.S1>) did not show significant differences in oxidative stress in the tongues at 24 h postirradiation, which was likely due to large variability seen within samples that diminished the effects of the observed trends. A more comprehensive study with multiple time points will surely be informative. It is also possible that prolonged radiation-induced activation of NF- κ B will result in chronic effects on the cellular environment by inducing secondary oxidative stress (27). Thus, MnBuOE, which suppressed activation of NF- κ B, will exhibit its protective effect as long as secondary oxidative stress persists. Optimal protection against this mechanism of injury may require administration of MnBuOE for a prolonged period of time. The full effect of oxidative damage is therefore not necessarily apparent within the timescale we investigated with intravital microscopy, and as a result the efficacy of MnBuOE may not be fully apparent on this timescale in this model.

MnBuOE Decreases Radiation-Induced Fibrosis

Xerostomia progressively develops in the weeks/months after irradiation and is a reflection of damage to the salivary gland stem cells, which decreases the tissues' regenerative capacity (28). Xerostomia is often irreversible and has significant long-term consequences for a patient's oral health as well as overall quality of life (2, 28, 29). Our salivary gland fibrosis results demonstrate persistent damage after irradiation, which is prevented by MnBuOE treatment and may correlate with reduced risk of chronic xerostomia.

MnBuOE Tumor Radiosensitization is Dose Dependent

Due to the intrinsic differences in redox environments of tumor vs. normal tissue (30, 31), Mn porphyrin exhibited differential effects in those tissues: healing of normal while suppressing growth of tumor. In our previous work, we showed that higher doses of

MnBuOE (1.5 mg/kg bid) radiosensitized FaDu tumors, shifting the TCD₅₀ curve to the left with a dose-modifying effect of 1.3 (13). We now report a null effect of MnBuOE on FaDu tumors in this study. There are significant differences in these experiments that likely explain the different responses of MnBuOE treatment on FaDu tumors.

First, we previously used five fractions of radiation (5–10 Gy/fraction), as opposed to the three fractions (6–17 Gy/fraction) described herein. It should be noted that these radiation doses were not chosen capriciously. Assuming a tumor, $\alpha/\beta = 10$, the BEDs chosen for this study ranged from 28.8–137.7, which provided adequate coverage of the previous study's BED range (37.5–100). Though the BED was constant, reducing our fractions from five to three could have decreased the radiosensitizing benefits of fractionation that come with reoxygenation and redistribution. A comprehensive evaluation of different dose fractionation schemes was beyond the scope of this study, but future experiments could investigate MnBuOE efficacy using different radiation approaches at a fixed MnBuOE dose.

Second, the mechanisms by which MnBuOE is predicted to sensitize tumor cells will differ as the drug dose decreases. With bid dosing of 1.5 mg/kg, MnBuOE accumulates in the tumor (eventually reaching ~1.5–2 μM in a FaDu flank xenograft; data not shown). The results found in this article were based on updated pharmacokinetic data that used recently clarified tissue half-life information and would have resulted in maintenance of a lower tumor MnBuOE concentration. Our recent study suggested that the major *in vivo* action of MnBuOE is pro-oxidative in nature, due to apoptosis-promoting protein thiol S-glutathionylation (32). The magnitude of such oxidative modification is directly related to the levels of major reactive species involved in the process: H₂O₂ and MnBuOE. In comparison to our earlier study, this study's lower MnBuOE dose likely precluded such apoptotic events.

Third, and finally, a difference between our studies was the addition of cisplatin, which was included to increase the study's clinical relevance. Importantly, cisplatin increases mitochondrial oxidative stress (33), which is associated with increased normal tissue injury (34, 35) that can be ameliorated by reducing oxidative stress (36). We sought to determine whether tumor cell killing and/or normal tissue injury by cisplatin were decreased or abrogated by the addition of MnBuOE. We demonstrate that treatment with low levels of MnBuOE protects against radiation-induced damage to normal tissues, without inhibiting tumor control achieved by the combined radiation + cisplatin treatments.

A Clinical Trial to Test MnBuOE with Daily Fractionated Irradiation and Cisplatin is Imminent

An open question is whether MnBuOE will be protective when administered with fractionated irradiation, which is the current standard. The studies described herein and earlier (13) have led to the design and approval of a Phase 1a–b clinical trial (NCT02990468) to examine MnBuOE (alternately referred to as BMX-001) in conjunction with daily fractionated intensity-modulated radiation therapy (IMRT; 2.0–2.1 Gy per fraction, to a cumulative radiation dose of 60–70 Gy) and cisplatin in head and neck squamous cell carcinoma patients.

CONCLUSION

This study demonstrates that MnBuOE is able to provide radioprotection in normal head and neck tissues at lower doses than previously reported. Radioprotection persists from 24 h to 12 weeks postirradiation, as measured by mucositis, saliva production and tissue fibrosis. Finally, MnBuOE did not affect tumor sensitivity to fractionated radiation + cisplatin treatments. Taken together, these data support the use of MnBuOE as a normal tissue radio-protecting agent during treatment of head and neck cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Schematic of study methods. Panel A: Comprehensive timeline of treatment course for radioprotection studies. Mice received a loading dose of MnBuOE or saline on day 1, followed by TIW maintenance doses up through 12 weeks. Radiation was given as a single 9 Gy fraction on day 0. Panel B: Radiation field was targeted to include the oral cavity and salivary glands, found in the neck. Care was taken to exclude the central nervous tissue from the treatment field.

FIG. 2. Mucositis signal on day 11 postirradiation. Inflammatory tissue (pink) was identified using ProSense 750EX, which is cleaved to become activated in the presence of cathepsins to yield a near infrared signal. A 9 Gy dose significantly increased in NIR signal in mice treated with both saline and 2/1 mg/kg MnBuOE. NIR signal was decreased in mice treated with either 0.6/0.3 mg/kg or 0.2/0.1 mg/kg MnBuOE. Representative FMT images are shown for all groups. N = 4–6 mice/group. Data is reported as mean with SEM. *Significant difference ($P < 0.05$) vs. saline/0 Gy control **Significant difference ($P, 0.05$) vs. 2/1 mg/kg MnBuOE/0 Gy control.

FIG. 3.

Intravital quantification of L/E interactions and physical properties of blood vessels 24 h postirradiation. Panel A: Example image obtained from intravital microscopy of the inferior surface of a mouse tongue after i.v. administration of 0.1% acridine orange solution 24 h postirradiation. The three white arrows indicate rolling leukocytes visible along the venule. Panel B: Sub-panel i: Mean number of rolling leukocytes decreases in response to a combination of 0.2/0.1 MnBuOE and radiation. Sub-panel ii: Radiation increased the mean velocity of free-flowing leukocytes in the blood (V_L) in mice treated with saline. This effect was not observed in mice treated with MnBuOE. Sub-panel iii: Radiation or MnBuOE treatment alone (2/1) led to an increase in the average diameter (D) of blood vessels. Combined radiation + MnBuOE treatment (0.2/0.1) did not lead to an increase in diameter vs. the saline control. Sub-panel iv: Shear rates (g_s) were calculated with the formula $g_s = 8 V_L / D$. Only mice treated with MnBuOE alone (2/1) experienced a decrease in shear rate, primarily driven by increased vessel diameter. Data points are presented for each vessel, along with mean and SEM. N = 8–13 mice/group *Significant difference ($P < 0.05$) vs. saline/0 Gy control.

FIG. 4. Radiation-induced xerostomia at 11 weeks postirradiation (post-RT). To reduce variability, all mice were normalized to salivation levels for their appropriate control. Mice receiving saline or 2/1 mg/kg MnBuOE treatment experienced significant ($P < 0.05$) decreases in salivation in response to radiation, but mice treated with either 0.6/0.3 or 0.2/0.1 mg/kg MnBuOE experienced smaller, nonsignificant decreases in saliva production compared with unirradiated controls. N = 5–6 mice/group *Significant difference ($P < 0.05$) vs. saline/0 Gy control **Significant difference ($P < 0.05$) vs. 2/1 mg/kg MnBuOE/0 Gy control.

FIG. 5. Analysis of fibrosis in salivary glands and tongues at 12 weeks postirradiation. Panel A: Masson's trichrome staining in the salivary glands and tongue results in bright turquoise staining of fibrotic tissue and red staining of healthy tissue. Panel B: Plot of fibrosis in the salivary glands normalized to the control value of either saline or 2/1 mg/kg MnBuOE control. A 9 Gy dose increases fibrosis in mice that received saline only. MnBuOE attenuates fibrosis in irradiated mice, with all doses of MnBuOE equally effective at reducing fibrosis. N = 4–6 mice/group. *Significant difference ($P < 0.05$) vs. saline/0 Gy control. Panel C: Plot of fibrosis in the tongue normalized to the control value of either saline or 2/1 mg/kg MnBuOE control. As in the salivary glands, the 9 Gy dose led to an increase in fibrosis. Both 0.6/0.3 and 0.2/0.1 mg/kg MnBuOE treatment reduce fibrosis to a

greater degree than 2/1 mg/kg. N = 4–6 mice/group. *Significant difference ($P < 0.05$) vs. saline/0 Gy control.

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FIG. 6.

MnBuOE did not interfere with radiation/cisplatin-mediated tumor growth delay in a murine xenograft model. The human pharyngeal carcinoma tumor cell line (FaDu) was transplanted to flanks of nude mice randomized to receive fractionated radiation + cisplatin along with MnBuOE treatment or saline control injections. MnBuOE was started one week after transplanted and continued TIW for the duration of the experiment. Radiation + cisplatin treatments were initiated as tumors reached 200 mm³. Differences in tumor growth rate were compared by assessing time to tripling of tumor volume, using the initial treatment volume of each mouse as baseline.

FIG. 7.

MnBuOE protects mice against normal tissue injury after radiation + cisplatin treatments. Panel A: Mice were treated with 1×9 Gy + 6 mg/kg cisplatin. Saline or MnBuOE treatment (0.6 mg/kg loading dose) began 24 h prior to radiation + cisplatin treatments and maintenance dosing of MnBuOE (0.3 mg/kg) continued TIW for five weeks (post-RT). Panel B: Radiation + cisplatin treatments increased NIR signaling in saline-treated mice compared to unirradiated controls ($P < 0.01$) and to MnBuOE-treated mice that received radiation + cisplatin ($P < 0.01$). Mice treated with MnBuOE and/or radiation + cisplatin did not show significantly greater NIR signal than unirradiated controls [$P = 0.002$, across all groups (ANOVA), $N = 2-4$ /group]. Panel C: All radiation + cisplatin-treated mice showed initial weight loss 8–14 days postirradiation. At 10 weeks postirradiation + cisplatin, the body weight of the MnBuOE-treated mice were not significantly different from unirradiated control mice. Radiation + cisplatin-treated saline mice had significantly lower body weight compared to sham-irradiated mice ($P < 0.0001$), and radiation + cisplatin-MnBuOE-treated ($P < 0.01$) mice. Two-way ANOVA showed a significant interaction between time and treatment group ($P = 0.018$). Panel D: MnBuOE lessened long-term ulcerations and moist desquamation in the irradiated area (right side panels) compared to mice that received radiation + cisplatin with saline (left side panels).