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# Amelioration of Radiation Esophagitis by Orally Administered p53/Mdm2/Mdm4 Inhibitor (BEB55) or GS-Nitroxide

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**Abstract.** *Background/Aim: Esophagitis is a significant toxicity of radiation therapy for lung cancer. In this study, reduction of irradiation esophagitis in mice, by orally administered p53/Mdm2/Mdm4 inhibitor, BEB55, or the GS-nitroxide, JP4-039, was evaluated. Materials and Methods: BEB55 or JP4-039 in F15 (liposomal) formulation was administered intraesophageally to C57BL/6 mice prior to thoracic irradiation of 29 Gy × 1 or 11.5 Gy × 4 thoracic irradiation. Progenitor cells were sorted from excised esophagus, and nitroxide was quantified, by electron paramagnetic resonance (EPR). Mice with Lewis lung carcinoma (3LL) orthotopic lung tumors were treated with BEB55 or JP4-039 prior to 20 Gy to determine if the drugs would protect the tumor cells from radiation. Results: Intraesophageal BEB55 and JP4-039 compared to formulation alone increased survival after single fraction (p=0.0209 and 0.0384, respectively) and four fraction thoracic irradiation (p=0.0241 and 0.0388, respectively). JP4-039 was detected in esophagus, liver, bone marrow, and orthotopic Lewis lung carcinoma (3LL) tumor. There was no significant radiation protection of lung tumors by BEB55 or JP4-039 compared to formulation only as assessed by survival (p=0.3021 and 0.3693, respectively). Thus, BEB55 and JP4-039 safely ameliorate radiation esophagitis in mice.*

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*Key Words:* Radiation protection, JP4-039, BEB55, esophagitis, p53, Mdm2, Mdm4.

Esophagitis is a significant complication of radiation therapy for thoracic malignancies, including non-small cell lung cancer (1). Radiation dose escalation to increase tumor control is limited by radiation esophagitis, and ulceration (2). Effective concurrent chemotherapy in many treatment protocols often exacerbates radiation esophagitis (1, 3). Therefore, effective amelioration of radiation esophagitis should improve radiotherapy treatment outcomes (3).

A recent approach to esophageal radiation protection is through the local (swallowed) administration of small-molecule radiation protector nitroxides (*e.g.* JP4-039) (2). Nitroxides are cell permeable, stable free radical scavenger compounds with superoxide dismutase-like activity capable of protecting mammalian cells from oxidative stress (4). Tempol is one hydrophylic nitroxide with radioprotective properties both *in vitro* and *in vivo* (5), and while the mechanism of protection includes scavenging of free radicals, oxidation of low-valency transition metals and superoxide dismutase activity (6), its use is limited by the requirement for high therapeutic levels that are associated with somnolence, hypotension, tachycardia and grand mal seizures (6).

Attaching gramicidin S (GS)-derived peptide isostere sequences to Tempol targets the radioprotector to the mitochondria (7). One of these products, JP4-039, provides radioprotection at much lower concentrations, and reduces radiation esophagitis in mice (2). Two populations of cells that are damaged in irradiation-induced esophagitis include the esophageal epithelium and esophageal progenitor cells (8). While JP4-039 was detected in whole esophagus after intraesophageal administration (2), it is not known whether drug reached esophageal stem cells and whether it reached other organs including tumors.

We recently described a p53/Mdm2/Mdm4 inhibitor, BEB55 (9), which is a radiation protector and mitigator that operates in a p53-dependent manner *in vitro* (9). The

proposed mechanism of action is that the imidazole-indole derivative prevents Mdm2 and Mdm4 from binding to p53 for ubiquitin-mediated degradation (9, 10). Increased p53 levels are hypothesized to slow cell cycle progression after irradiation, allowing better DNA repair (10-12). In the present studies, we delivered BEB55 or JP4-039 to the esophagus of mice by orally administered cationically charged multilamellar liposome formulation (F15) and tested the relative effect of each on irradiation-induced esophagitis.

## Materials and Methods

*Preparation of JP4-049 and BEB55 in F15 formulation*. The formulation of F15 has been described previously (2). F15 is a cationically charged multilamellar liposome that allows for enhanced esophageal surface coating and time-release of drug from liposome particles (2). It is composed of soy phosphatidyl choline (Avanti Polar Lipids, Alabaster, AL, USA), Tween-80 (Sigma-Aldrich, St. Louis, MO, USA) and *N,N*-di-oleylamine amido-L-glutamate at a 4:1:1 w/w ratio. The GS-nitroxide JP4-039 (7) and the p53/Mdm2/Mdm4 inhibitor BEB55 (9) were formulated at 8 mg/ml in F15 formulation as described previously (2). The final product was 1 mg of JP4-039 or BEB55 formulated in 125 μl volumes.

*Intraesophageal drug administration*. Adult female C57BL/6HNsd mice (20-25 g) (Harlan Laboratories, Indianopolis, IN, USA) received 100 μl distilled water intraesophageally via feeding tube followed by 100 μl F15 vehicle, manganese superoxide dismutase-plasmid liposome (MnSOD-PL) (8), JP4-039 (2), or BEB55 (9) prior to irradiation and/or tissue excision (see below for n in each experiment). The stock solutions were diluted 1:1 with F15 so that the total amount of JP4-039 or BEB55 delivered with each administration was 400 μg (2).

*Esophageal progenitor cell isolation*. To determine if intraesophageal administration of JP4-039 resulted in drug uptake by esophageal multipotential cells (8) and/or differentiated epithelial cells, the stem cell-enriched side population (SP) compared to non-SP (NSP) cells were isolated according to published methods (13, 14). Ten minutes after intraesophageal administration of JP4-039, mouse esophagi were removed (n=24), minced, and then incubated in a solution of 0.2% Collagenase type II (Gibco, Carlsbad, CA, USA), 0.3% Dispase (Gibco) and 0.025% Trypsin (Cellgro, Manassas, VA, USA) for 45 min at 37˚C. The cell aggregates were disrupted by passing through sequentially smaller needles (to 23-gauge) and then filtered with a 40 μM cell strainer into Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Walkersville, MD, USA) supplemented with 40% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Single- cell suspensions were pelleted by centrifugation and resuspended at $10^6$ cells/ml in pre-warmed DMEM (2% FBS, 10 mM HEPES). Cells were incubated in 6 μg/ml Hoechst 33342 (Sigma Chemical, St. Louis, MO, USA) for 90 min to identify SP cells. Verapamil (Sigma Chemical), which inhibits the efflux of Hoechst, was used at a concentration of 50 μM for the purpose of cell gating. Cells were pelleted and resuspended in cold Hank's balanced salt solution (HBSS) (2% FBS, 10 mM HEPES) and incubated with anti-CD45-phycoerythrin (PE)-fluorescein isothiocyanate (FITC) and/or anti-Ter119-PE-Cy7 antibodies (BD Pharmingen, San Diego, CA, USA) at 1:200 dilutions to discriminate hematopoietic cells. Antibody-treated cells were incubated on ice for 20 min, washed in cold HBSS, filtered, pelleted and resuspended in cold HBSS. Propidium iodide was added at 2 μg/ml immediately prior to flow cytometry (14). SP and NSP cells were quantified, sorted into separate collection tubes containing cold HBSS (2% FBS, 10 mM HEPES) and pelleted. The supernatant was then aspirated and the cells snap-frozen in liquid nitrogen. JP4-039 content in sorted SP and NSP cells was quantified by electron paramagnetic resonance (EPR) analysis using a JEOL-RE1XEPR spectrometer (JEOL, USA, Inc., Peabody, MA, USA) as described previously (15).

Table I. *JP4-039 content in normal tissues and tumor after intraesophageal administration. Mouse esophagus, lung orthotopic tumor, liver, and peripheral blood samples were each removed at 10, 30 and 60 minutes after intraesophageal administration of JP4-039 in F15 liposome formulation (n=3/time point) (2). Tissue samples were snap-frozen on dry ice and JP4-039 content was quantified by EPR analysis, as described previously (15).*

| | JP4-049 (pmol/mg) | | |
|---|---|---|---|
| | 10 min | 30 min | 60 min |
| Esophagus (2) | 430.1±21.5 | 243.5±38.6 | 108.8±188.5 |
| Blood | 39.0±9.7 | 51.1±12.4 | 31.6±20.3 |
| Liver | 122.2±49.6 | 100.4±43.1 | 73.8±50.2 |
| Bone marrow | 10.0±0 | 10.0±0 | 10.0±0 |
| 3LL Orthotopic tumor | 169.5±67.0 | 276.0±189.1 | 134.4±68.5 |

*Measurement of JP4-039 uptake in cells*. To determine the uptake of JP4-039 by different tissues, esophagus, lung orthotopic tumor, liver, and peripheral blood samples were each removed at 10, 30 and 60 min after intraesophageal drug administration. Samples were snap-frozen on dry ice and JP4-039 content was quantified by EPR analysis as described previously (15).

*Cell cycle analysis*. To evaluate the effects of BEB55 on the cell cycle progression of mouse esophageal cells, mice received 400 μg BEB55 intraesophageally, alone or prior to 28 Gy upper-body irradiation and esophagi were excised 24, 48 or 72 h later. Single-cell suspensions were prepared as described above, washed in PBS, fixed in 70% ethanol and stored at −20˚C for at least 24 hours, then stained with 0.1 μg/ml propidium iodide and cell cycle analysis performed as described previously (16).

*Orthotopic Lewis lung carcinoma 3LL tumors*. 3LL carcinoma cells ($1\times10^6$) were intratracheally administered to mice according to published methods (17). Seven days later mice received intraesophageal JP4-039 (n=9), after which the tumor-filled lung was excised. JP4-039 uptake was calculated by comparing content per mass unit in tumor compared to that of normal lung.

*Irradiation*. Mice were immobilized for irradiation with intraperitoneal Nembutal anesthesia after intraesophageal drug administration. For single-fraction irradiation, mice received 29 Gy upper-body irradiation at 526 cGy/min on a Varian LINAC (6 MV photons) (Varian LINAC; Varian Medical Systems, Palo Alto, CA, USA). Mice were given the following treatments: i) F15 plus 29 Gy; ii) MnSOD-PL 24 h prior to 29 Gy; iii) BEB55 immediately before 29 Gy; or iv) JP4-039 immediately before 29 Gy (15 mice per group).

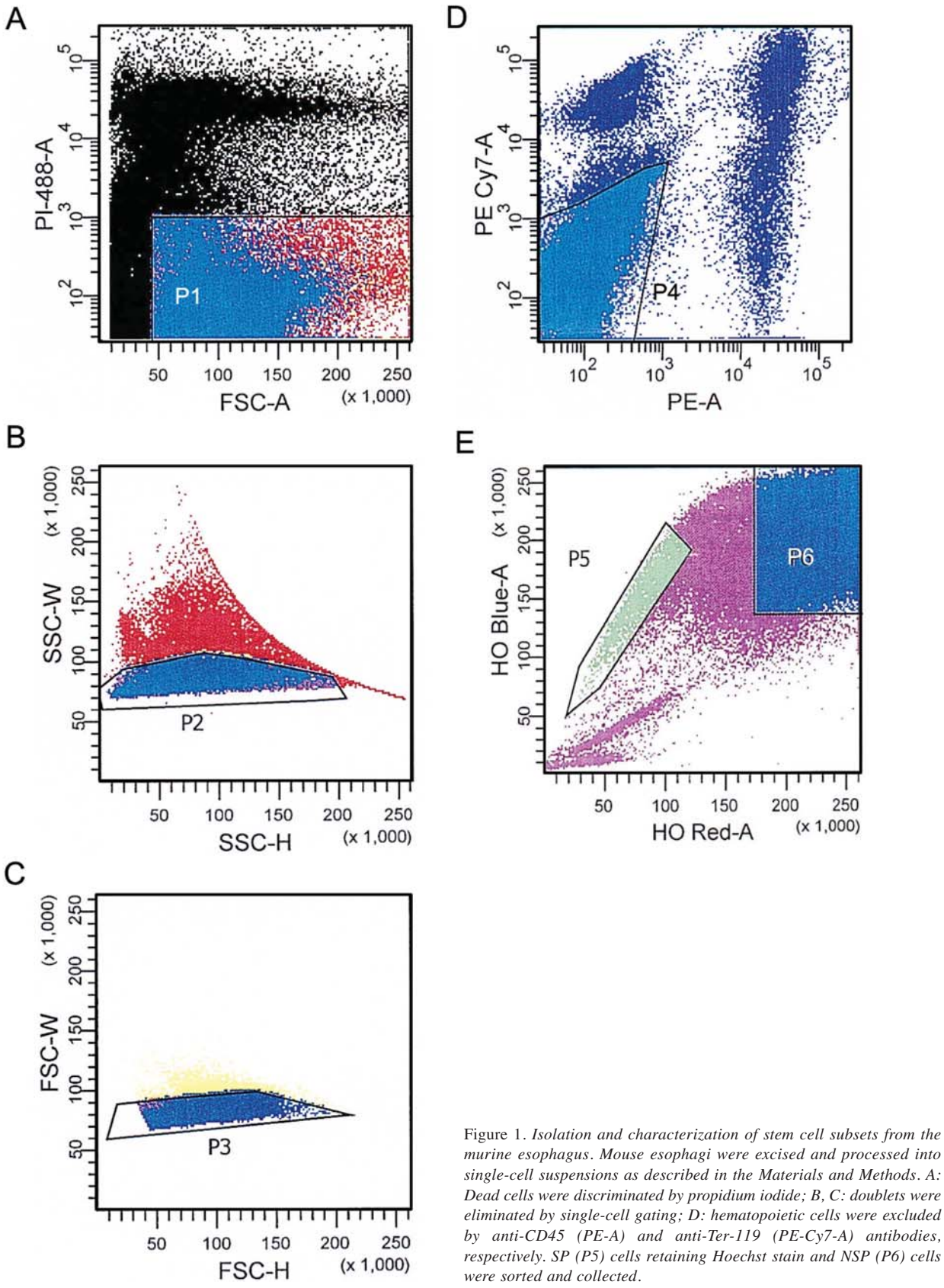


Figure 1. *Isolation and characterization of stem cell subsets from the murine esophagus. Mouse esophagi were excised and processed into single-cell suspensions as described in the Materials and Methods. A: Dead cells were discriminated by propidium iodide; B, C: doublets were eliminated by single-cell gating; D: hematopoietic cells were excluded by anti-CD45 (PE-A) and anti-Ter-119 (PE-Cy7-A) antibodies, respectively. SP (P5) cells retaining Hoechst stain and NSP (P6) cells were sorted and collected.*

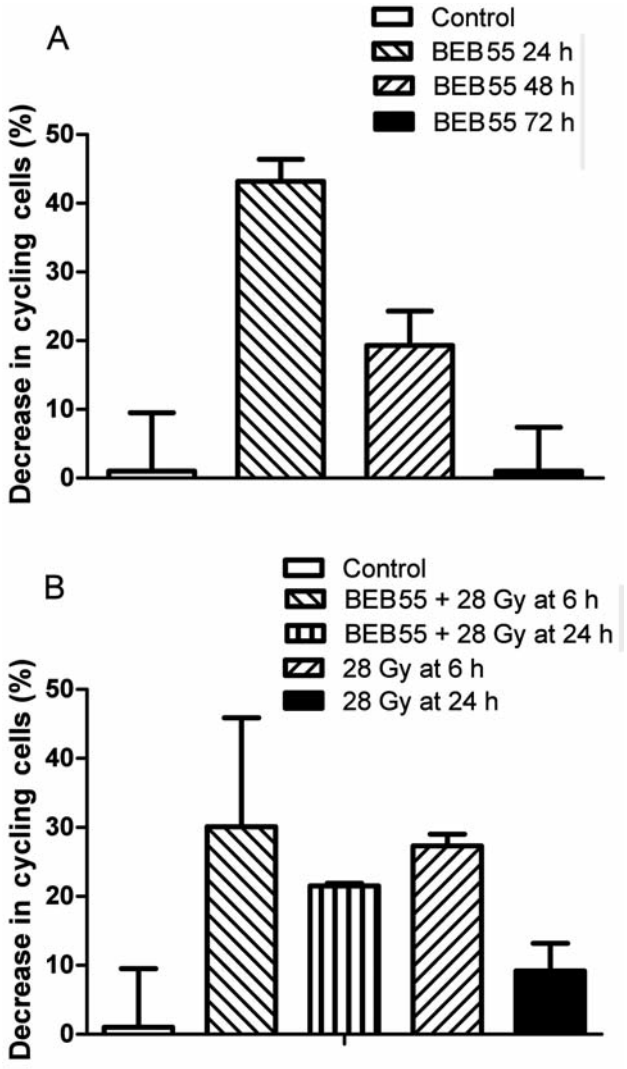


Figure 2. *Esophageal administration of BEB55 reduces esophageal cell cycle progression. Esophagi were excised after intraesophageal administration of BEB55 and cell cycle distribution assessed by flow cytometry. Comparisons of the number of cell in S and $G_2$/M phases of the cell cycle demonstrated changes in the number of cycling cells. A: BEB55 was administered intraesophageally with mice sacrificed at 24, 48 or 72 h later with the percentage of cells cycling determined. Treatment of cells with BEB55 resulted in a decrease in cycling cells at 24 hours compared to untreated cells (p=0.0091). B: BEB55 was administered intraesophageally 1 hour prior to 28 Gy upper-body irradiation and esophagi excised and processed for flow cytometry at 6 and 24 h after irradiation. There was a decrease in the percentage of cycling cells 24 h after treatment with BEB55 plus 28 Gy compared to untreated cells and cells that received 28 Gy only (p=0.0339 and 0.0269, respectively).*

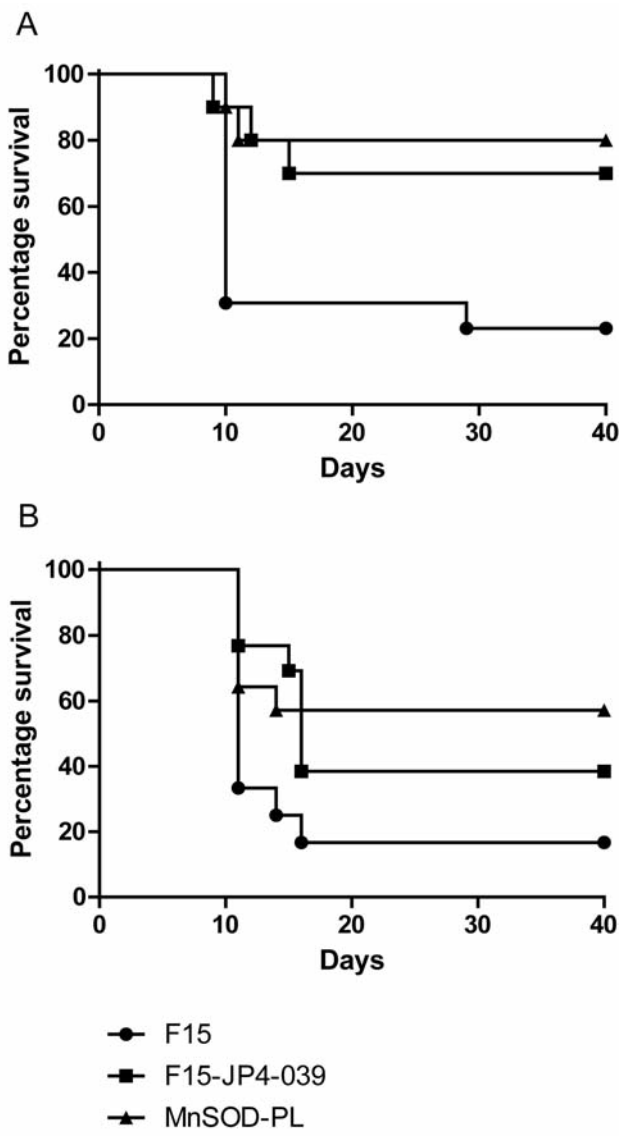


Figure 3. *Improved survival after thoracic irradiation by orally administered JP4-039. Mice were injected with JP4-039 IP prior to A: 29 Gy thoracic irradiation, or B: each of 4 daily fractions of 11.5 Gy thoracic irradiation. Mice that received JP4-039 prior to irradiation demonstrated increased survival compared to the vehicle control group in both single- and multiple-fraction administration (p=0.0384 and 0.0388, respectively). Mice treated with MnSOD-PL as a positive control (8) had increased survival compared to F15 plus irradiation in both single- and multiple-fraction administrations (p=0.0070 and 0.0343, respectively).*

For fractionated irradiation, mice received four daily fractions of 11.5 Gy upper-body irradiation. Subgroups of mice received i) F15 plus 11.5 Gy × 4; ii) MnSOD-PL 24 hours prior to the first and third fractions; iii) BEB55 before each fraction; or iv) JP4-039 prior to each fraction (15 mice per group).

To determine whether JP4-039 or BEB55 in tumor cells was radioprotective, mice with 3LL tumors were treated as follows: i) no additional treatment; ii) F15 plus 20 Gy × 1 thoracic irradiation (single fraction, 526 cGy/min); iii) BEB55 plus 20 Gy; iv) JP4-039 plus 20 Gy; or v) MnSOD-PL, 24 hours prior to 20 Gy.

*Animal assurance and welfare*. Animal protocols were approved by the University of Pittsburgh, Institutional Animal Care and Use

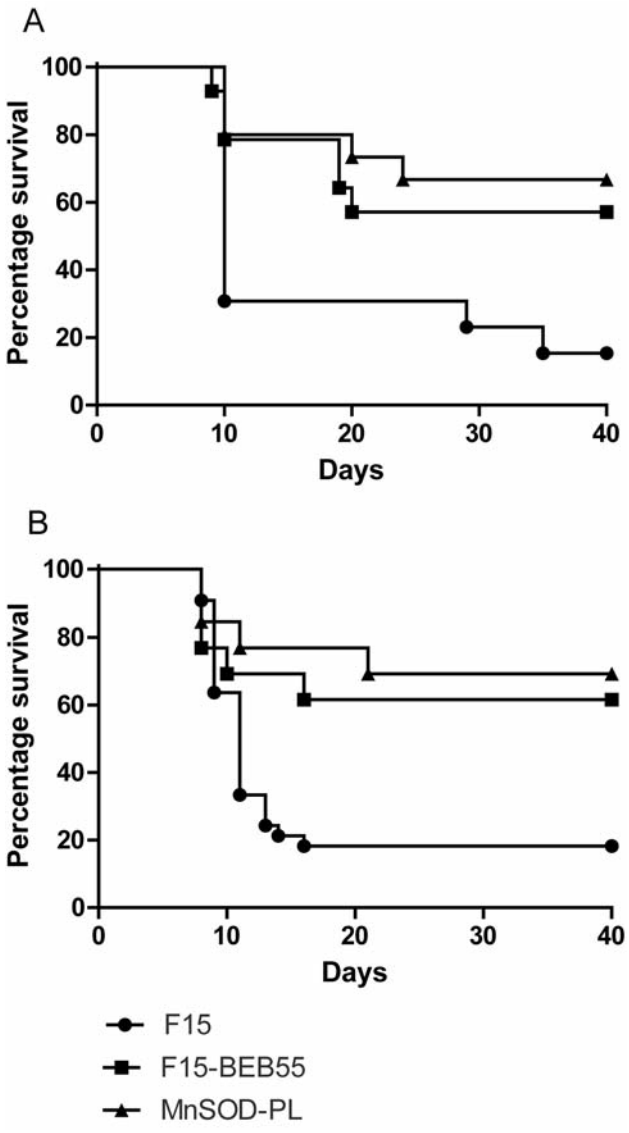


Figure 4. *Improved survival after thoracic irradiation by orally administered BEB55. Mice were injected with BEB55 IP prior to A: 29 Gy thoracic irradiation, or B: each of 4 daily fractions of 11.5 Gy thoracic irradiation. Mice that received BEB55 prior to irradiation demonstrated increased survival compared to the vehicle control group in both single- and multiple-fraction administration (p=0.0209 and 0.0241, respectively). Mice treated with MnSOD-PL as a positive control (8) had increased survival compared to F15 plus irradiation in both single- and multiple-fraction administrations (p=0.0014 and 0.0034, respectively).*

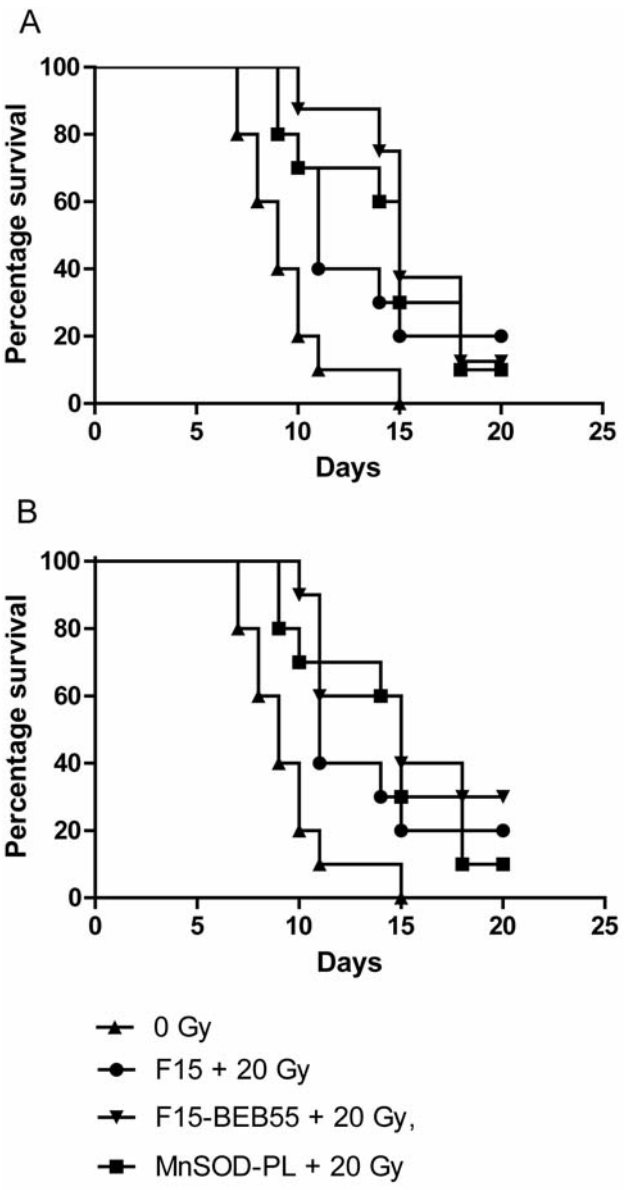


Figure 5. *Esophageal radioprotection by JP4-049 or BEB55 does not reduce therapeutic gain. Mice were administered 3LL cells intratracheally 1 week prior to receiving thoracic irradiation as described (17). Mice with orthotopic tumors received orally administered JP4-039 (A), or BEB55 (B) prior to 20 Gy thoracic irradiation. There was no significant difference in survival between groups of mice that received JP4-039 or BEB55 vs. those receiving F15 vehicle alone (p=0.3693 and 0.3021, respectively). Mice that received 20 Gy irradiation survived longer than mice that did not receive irradiation (p=0.0218, 0.0011 and 0.0008 for mice treated with F15 plus 20 Gy, JP4-039 plus 20 Gy and BEB55 plus 20 Gy, respectively). MnSOD-PL is the positive control.*

Committee (IACUC). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh.

*Statistics*. Log-rank test was used to compare survival between mouse treatment groups. *P*-values less than 0.05 were regarded as significant.

## Results

*JP4-039 uptake in normal tissues and orthotopic tumors*. To determine if the orally administered JP4-039 also reached other tissues and orthotopic tumors (17), we tested the effect

of swallowed drug. Esophagus, peripheral blood, bone marrow, liver and 3LL orthotopic tumors were harvested at 10, 30 and 60 min after intraesophageal administration of JP4-039. JP4-039 content was quantified by EPR according to published methods (15). GS-nitroxide in liver peaked after 10 min at 122.2 pmol/mg protein and gradually decreased over time (Table I). JP4-039 levels in peripheral blood and orthotopic tumor peaked at 30 min at 51.1 and 276.0 pmol/mg protein, respectively (Table I). These data demonstrate that intraesophageal delivery of JP4-039 in F15 liposome formulation allows nitroxide uptake by both normal and tumor tissue. Lower levels of JP4-039 were detected in bone marrow up to 60 min after drug swallow compared to levels in liver and tumor tissue.

*JP4-039 is detected in esophageal SP and NSP cells*. The above data confirm and extend a prior report of detection of JP4-039 in esophagus by ESR (2). We next tested whether the drug had reached esophageal stem cells. Twenty mouse esophagi were excised 10 min after intraesophageal delivery of JP4-039 with subsequent isolation of SP and NSP cells (13, 14). The sorting results for esophageal SP and NSP cells are shown in Figure 1. JP4-039 uptake was then quantified by EPR as published previously (15). The 101,000 SP cell pellet contained 275.1 fmole JP4-039 (2.7 pmol per 106 cells). The 3,387,000 sorted NSP cells, these contained 221.3 fmole JP4-039 (65.3 fmol per $10^6$ cells). The data establish that swallowed JP4-039 in F15 formulation reaches and is detectable in both excised and isolated SP and NSP cells.

*BEB55 reduces esophageal cell cycle progression in vivo*. We next tested the effect of swallowed BEB55 as an esophageal radioprotector. We first evaluated whether BEB55 altered cell cycle progression after irradiation. Esophagi were excised at different time points after intraesophageal administration of BEB55 in F15. Single-cell suspensions were then assessed for cell cycling by flow cytometry. Actively cycling cells, or cells in the S and $G_2$/M phases, were identified by increased DNA content. By comparing the number of cells in the S and $G_2$/M phases of the cell cycle, changes in the number of cells which were actively cycling were determined. Twenty-four hours after intraesophageal administration of BEB55 there was a 43% decrease in the percentage of cells cycling (*i.e.* cells in the S, $G_2$ or M phase) ($p$=0.0091) (Figure 2). BEB55 administration prior to upper-body irradiation resulted in a 20% decrease in cycling cells at 24 h ($p$=0.0269). Thus, the F15 formulation allowed sufficient esophageal uptake of the p53/Mdm2/Mdm4 complex inhibitor BEB55, resulting in more free p53. Higher p53 values would theoretically result in more cells in the $G_1$ phase (12). There was detectable delay in cell cycle progression, with an increase in the number of cells in $G_1$ (Figure 3), a more radioresistant phase than $G_2$/M (18) and a phase important for DNA repair (12). The data demonstrate that intraesophageal BEB55 reduces the percentage of cycling esophageal cells *in vivo*.

*BEB55 and JP4-039 are radioprotective in single-fraction upper-body-irradiated mice*. To determine whether intraesophageal administration of BEB55 or JP4-039 in F15 would ameliorate irradiation-induced esophagitis in mice, mice were treated with F15 only, JP4-039 or BEB55, immediately prior to a single fraction of 29 Gy thoracic irradiation. As a positive control, MnSOD-PL was administered 24 h prior to the irradiation. Mice that were treated with JP4-039 or BEB55 prior to 29 Gy thoracic irradiation demonstrated increased survival compared to the F15 vehicle only group ($p$=0.0384 and 0.0209, respectively) (Figure 3A and 4A). The data indicate that intraesophageal administration of JP4-039 or BEB55 in F15 formulation ameliorates single-fraction irradiation-induced death from esophagitis.

*BEB55 and JP4-039 are radioprotective in multiple-fraction upper-body-irradiated mice*. To evaluate radioprotection by BEB55 and JP4-039 in multiple-fraction upper-body irradiation, mice were treated with intraesophageal BEB55 or JP4-039 prior to each of four fractions of 11.5 Gy thoracic irradiation. MnSOD-PL was administered as a positive control 24 h prior to the first and third fractions. Mice treated with JP4-039 or BEB55 prior to irradiation had increased survival compared to the F15 only control group ($p$=0.0388 and 0.0241, respectively) (Figure 3B and 4B). The data indicate that both BEB55 and JP4-039 are protectors against fractionated irradiation of the esophagus and are effective when given in multiple administrations.

*BEB55 and JP4-039 do not protect orthotopic tumors from radiation*. The above data indicate that BEB55 and JP4-039 were taken up by an orthotopic tumor after drug swallow (Table I). To determine whether the drugs also protected tumors from irradiation damage, we used an orthotopic lung tumor model. Mice received intratracheal injection of 3LL cells 1 week prior to exposure to 20 Gy thoracic irradiation (17). This dose of irradiation was chosen to reduce tumor growth but was below the level required for lethal esophagitis (17). Irradiated mice were divided into treatment groups of F15, BEB55 plus F15, JP4-039 plus F15 and MnSOD-PL. Control tumor-bearing mice received no irradiation. Non-irradiated mice died rapidly of progressive tumor within 15 days; irradiated mice survived significantly longer due to reduction in tumor growth (Figure 5). Irradiated mice that received orally administered drugs JP4-039 or BEB55, as well as those receiving positive control of MnSOD-PL, prior to 20 Gy did not survive significantly differently compared to mice given F15 alone ($p$=0.3693 and 0.3021, respectively). The data show that neither BEB55 nor JP4-039 protected tumors from irradiation.

## Discussion

Esophagitis is a significant complication of concurrent chemotherapy and radiotherapy for thoracic malignancies, with an incidence up to 80% of patients in some treatment protocols (19). Analgesics are used to manage pain (19). Drugs including sucralfate and amifostine have shown limited effectiveness in radiation esophagitis (20). We now present evidence that intraesophageal delivery of the p53/Mdm2/Mdm4 inhibitor BEB55, and the GS-nitroxide JP4-039, in F15 formulation, ameliorate radiation esophagitis in single-fraction and fractionated irradiated mice.

Two populations of cells could be targeted for preventing irradiation-induced esophagitis: quiescent stem cells and rapidly dividing populations (8, 14). We demonstrated that the GS-nitroxide JP4-039 is taken up by both the stem cell enriched SP and differentiated NSP cells. Successful detection in SP cells after drug delivery to esophageal cells may have been due to the slow esophageal transit of the F15 formulation and the extended release of JP4-039 containing liposomes (2). SP cells had 41-fold greater uptake of JP4-039 per cell compared to the NSP population, contrary to the expected equal uptake by SP and NSP cells (21). One possible explanation for the relative increase in SP cells may have been that the metabolically quiescent progenitor cells did not clear nitroxide as quickly as the more rapidly proliferating NSP cells.

We evaluated whether radioprotection of the esophagus by BEB55 was associated with alteration of cell cycle progression (18). The data with BEB55 indicate that intraesophageal administration allowed irradiated esophageal cells to remain in $G_0$ or $G_1$, which are more radioresistant phases of the cell cycle (18). As an inhibitor of p53/Mdm2/Mdm4 complex formation, BEB55 should increase available p53, slowing cell cycle progression and increasing the checkpoint time for DNA repair (12). This mechanism of protection may have been more effective in rapidly proliferating cells than in quiescent cells.

*In vivo*, both BEB55 and JP4-039 were effective radioprotectors against single- and multiple-fraction upper-body radiation. Since clinical radiation therapy protocols require multiple radiation doses administered over many weeks, the effectiveness of JP4-039 and its detection in several mouse organs after intraesophageal administration indicates that this drug may have utility in the clinic. Neither BEB55 nor JP4-039 was radioprotective for tumors, further supporting a potential clinical application for selectively protecting normal tissue during radiation therapy.

While BEB55 and JP4-039 confer radioprotection by different mechanisms, previous work did not detect an additive effect when combining the two drugs *in vitro* compared to each alone (9). This may be due to competitive uptake of the drugs and perhaps could be overcome by spacing the times of administration (21). Further investigation may reveal that BEB55 increases the uptake of JP4-039, or a synergistic radioprotective effect may be found when the two compounds are spaced appropriately.

These results are significant in highlighting a potential advantage of the small molecule protectors JP4-039 and BEB55 as esophageal radioprotectors over MnSOD-PL gene therapy (22). The small molecule protectors are relatively inexpensive to produce and do not require 24 h administration to show efficacy. Instead, they can be given immediately prior to radiation therapy (2), and are quickly cleared from tissues. The potential utility of BEB55 and JP4-039 as radiation protectors in the clinic justifies further investigation.

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