

Discretionary Transduction of MMP-Sensitized Touselled in Head and Neck Cancer

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Oral radiotoxicity is often a limiting factor in cancer treatment. Previously, we demonstrated that transfer of cell-permeable, TAT-fusion Touselled-like kinase 1B (TLK1B) protein in salivary glands effectively mitigates radiation-induced salivary dysfunction. However, similar to most radioprotectors, TLK1B can carry the risk of limiting cancer treatment efficacy. The central goal of the study was, therefore, to reengineer TLK1B as a selective radioprotector of normal cells. Degradation of the extracellular matrix by proteases such as matrix metalloproteinases (MMPs) is a hallmark of aggressive tumors. Increased expression of membrane type 1-MMP (MT1-MMP; also called MMP14) is observed in a variety of cancers including head and neck squamous cell carcinoma (HNSCC). To limit TLK1B transduction to normal cells, we rendered the protein susceptible to MT1-MMP cleavage on the premise that high expression of MT1-MMP on the cell surface of HNSCC will suppress TLK1B internalization. Two optimal MT1-MMP-sensitive sequences (MS) were identified that when incorporated in TAT-TLK1B excluded its cellular entry in HNSCC, SCC40, but not immortalized salivary acinar cells, NS-SV-AC. Importantly, administration of MS-harboring TAT-TLK1B did not affect the sensitivity of tumors to radiation in a nude mouse xenograft tumor model. We conclude that a MMP-sensitive TLK1B can be an attractive therapeutic to allay salivary radiotoxicity without compromising cancer treatment efficacy.

INTRODUCTION

Radiotherapy is a common modality of treatment for head and neck cancer. A majority of head and neck cancer patients present with spread to regional lymph nodes at time of diagnosis,^{1,2} and radiation treatment fields invariably encompass either a part or whole of the major salivary glands. As a result, nearly all patients receiving radiotherapy to the head and neck region develop debilitating oropharyngeal complications. One of the major limiting factors in attaining a tumor-curative outcome is the radiation dose threshold of normal tissues. Oral mucositis and salivary hypofunction are the most common complications of head and neck radiotherapy, and the sequel of ulcerations, pain, and life-threatening dysphagia often interrupt cancer treatment.^{3–5} For optimal treatment outcome, the protection of vital normal tissues becomes critical. Palliative approaches to treat radiotoxicity have not been effective, and pre-emptive measures to prevent complications are being explored. The inclusion

of cytoprotective drugs or biological response modifiers can reduce radiation toxicity; however, most agents lack the ability to discriminate between healthy and cancer cells, and they inevitably risk limiting treatment efficacy. Therefore, there is a need for a selective radioprotector to reduce normal tissue toxicity without sacrificing tumor control.

Human Touselled-like kinase 1 (TLK1) is a serine/threonine kinase that is actively involved in chromatin assembly of nucleosomes on newly replicated DNA, as well as in DNA damage response.⁶ Expression of an alternate start variant, TLK1B, that is devoid of the N-terminal 236 amino acids, but otherwise identical to the full-length protein including the catalytic domain, improves cell survival against ionizing radiation (IR).^{7,8} Conversely, a kinase-defective mutant induces genomic instability and sensitizes the cells to radiation.^{7,8} Earlier reports demonstrated that preemptive expression of TLK1B in rat salivary glands through retroviral gene or protein transfer improves salivary gland survival and function against radiation.^{9–11} Protein therapy avoids the inherent risk of insertional mutagenesis that accompanies gene therapy, and in that regard, cell-permeable TAT-TLK1B holds clinical relevance. However, unwanted spread of protein to tumors can negate the effectiveness of radiotherapy, and a rational design that imparts tissue selectivity in protein transduction can further translate to patient care. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteases that are secreted or membrane tethered. They play a critical role during development and physiological remodeling of tissues, but are also involved in pathological states such as cancer.^{12–14} Many aggressive tumors, including those of the head and neck, express high levels of MMPs.¹⁵ Extracellular MMP proteolytic activity in the tumor microenvironment promotes growth, invasion, and metastatic spread of tumor cells. Membrane type 1 (MT1)-MMP is

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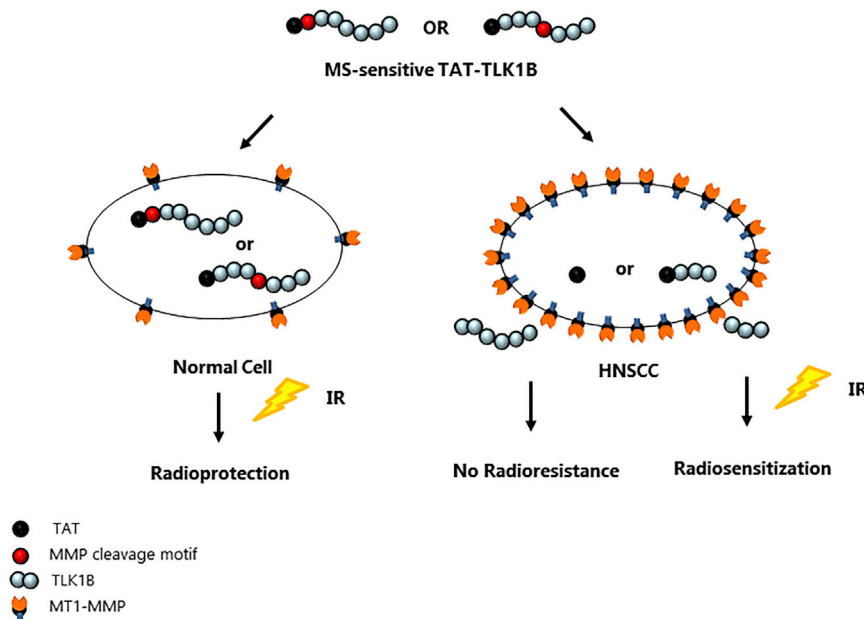


Figure 1. Strategies Designed to Limit Uptake of TLK1B in HNSCC.

Engineering of MMP-sensitive TAT-TLK1B such that MS, when placed between TAT and TLK1B, or between the N-terminal regulatory region and the kinase domain, will promote protein cleavage in tumor milieu. Protein cleavage will preclude cellular entry of full-length or functional TLK1B protein in HNSCC.

with synthetic PTD-tagged TLK1B (Figure S1). Because TAT-PTD was determined to be the most favorable, all subsequent studies were conducted using TAT-TLK1B.

Sensitivity of MS Sequences In Vitro

To assess the sensitivity of MT1-MMP recognition motifs to enzymatic cleavage, MT1-MMP-sensitive (MS) sequences were inserted between TAT-PTD and TLK1B (a schematic of all constructs is shown in Figure 2A). Cleavage assays using recombinant MT1-MMP revealed that

a plasma membrane-anchored protease that shows broad substrate specificity in cleaving pericellular proteins including collagen, fibronectin, laminin, and vimentin. A number of MMPs are initially expressed as inactive forms, and proteolytic cleavage of the pro-domain is essential to their enzymatic activation. Apart from its own proteolytic activity, MT1-MMP plays an important role in cleaving and activating pro-MMP2 to MMP2. High concentration of MT1-MMP on the cell surface of HNSCC and activated MMPs in the milieu play important roles in degrading target substrates, and proteolytic cleavage of MMP-sensitive TLK1B selectively in tumors forms the basis of the proposed complementary approach to minimize normal tissue toxicity without compromising tumor sensitivity to radiotherapy.

RESULTS

Two potential strategies were designed to achieve tumor-sparing TLK1B transduction by preventing entry of either: (1) full-length TLK1B, or (2) a functional TLK1B in HNSCC (Figure 1). Because TLK1 and TLK2 homo- or hetero-dimerize to autophosphorylate and attain full activity, we expected that the latter approach will have dual benefit in protecting normal cells and sensitizing tumor cells for a greater impact on the therapeutic index.

PTD and Transduction Efficiency

Synthetic peptides (protein transduction domain [PTD]1–3; Figure S1) that had shown improved transduction efficiency in Jurkat cells¹⁶ were examined against TAT-PTD in NS-SV-AC cells. Because results of immunofluorescence staining and immunoblotting showed no differences between groups (data not shown), the effectiveness of different PTD-TLK1B against IR was assessed based on the premise that improved protein transduction would promote cell survival. Results showed that cells transduced with TAT-TLK1B consistently displayed a trend toward increased survival compared

recognition motifs, VPLS-LRSG (MS1) or RPAH-LRDS (MS3), when incorporated within TAT-TLK1B rendered the protein sensitive to cleavage compared with the parent protein (% full-length protein at 30 min [mean \pm SD]: TAT-TLK1B, 76.15 \pm 2.7; TAT-MS1-TLK1B, 59.96 \pm 5.73; and TAT-MS3-TLK1B, 32.72 \pm 0.22; Figures 2C–2E). Although MS1 sequence aligned well with the collagen consensus motif, Pro-X-X-X_H, MS3 sequence was more sensitive to MT1-MMP cleavage (Figure 2B).

An optimal location to insert MS within TLK1B was determined based on mapping of the critical elements in the conserved serine/threonine kinase region. MS1 was inserted distal to the substrate binding regions, the ATP binding pocket, and the activation loop. The resultant protein, TAT-TLK-MS1, though sensitive to proteolytic cleavage was compromised in activity as observed by lack of phosphorylation of histone H3 (S10), a known TLK1 substrate,⁷ (Figure S2). Therefore, MS1 was then incorporated 5' to the kinase region. As expected, the insertion of MS1 between the regulatory and the kinase domains of TLK1B (TAT-TL-MS1-K) sensitized the protein to MT1-MMP cleavage. Interestingly, the MS1 location influenced sensitivity; TAT-TL-MS1-K was more sensitive to cleavage than TAT-MS1-TLK1B (% full-length protein at 60 min [mean \pm SD]: TAT-MS1-TLK1B, 22.48 \pm 10.66; and TAT-TL-MS1-K, 9.88 \pm 1.96; Figure 2E). Phosphorylation of H3 (S10) demonstrated that the insertion of MS1 between the regulatory and the catalytic domains of the protein does not negatively affect its activity (Figure 3B). Because kinase activity was unaffected, the possibility to further increase protein sensitivity to MT1-MMP was explored. Incorporating alternating MS3 and MS1 sequences for a total of five MS -highly sensitized the protein, TAT-TL-MS5-K, to cleavage (% full-length protein at 30 min [mean \pm SD]: TAT-TL-MS1-K, 32.27 \pm 15.8; and TAT-TL-MS5-K, 5.33 \pm 4.2; Figures 3A–3C), without conceding protein activity (Figure 3D).

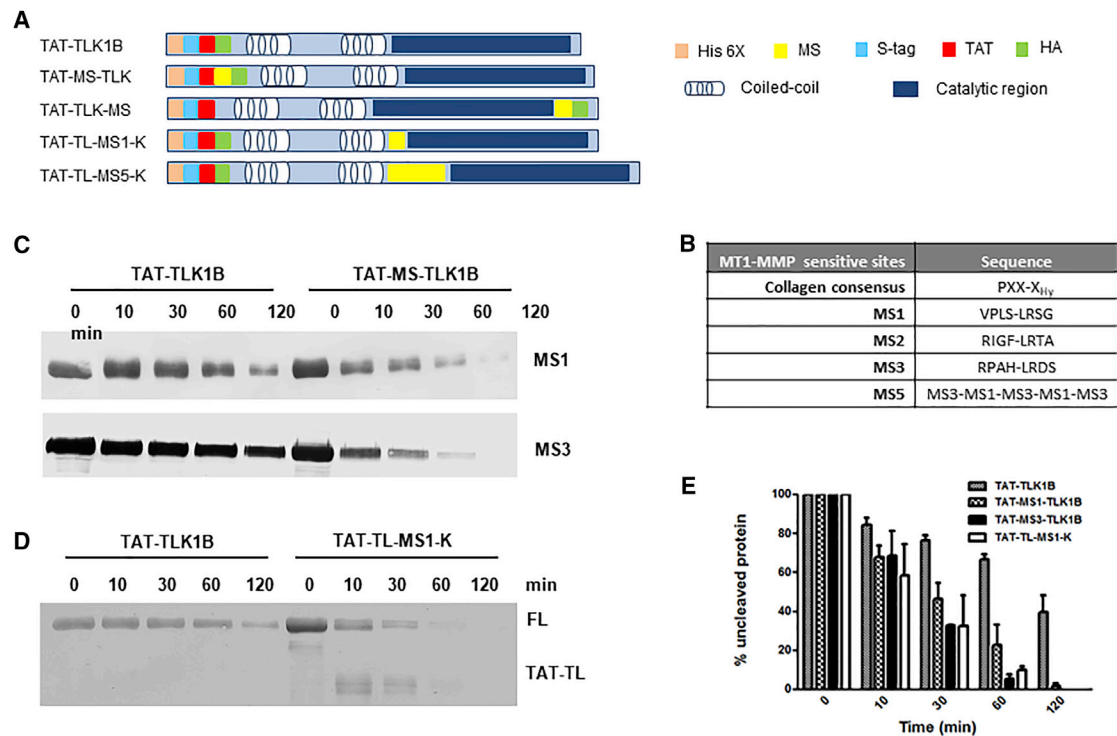


Figure 2. Sensitivity of Reengineered TAT-MS-TLK1B to Cleavage by Recombinant MT1-MMP *In Vitro*.

(A) Schematic of various TAT-TLK1B protein constructions. (B) MT1-MMP recognition sequences identified in previous studies. (C and D) *In vitro* cleavage assays with TAT-TLK1B, TAT-MS1-TLK1B, TAT-MS3-TLK1B, or TAT-TL-MS1-K. Proteins were reacted with recombinant catalytic domain of MT1-MMP (0.02 mU) at 22°C, and cleavage assessed by immunoblotting with anti-polyhistidine. (E) Quantification (mean ± SD) of full-length, uncleaved protein in three or more independent experiments. HA, hemagglutinin tag; His, Histidine 6×; MS, MT1-MMP-sensitive sequence; TAT, HIV TAT-PTD.

Selective TLK1B Transduction in Cancer Cells *In Vitro*

To directly demonstrate that MMP suppresses TLK1B transduction in HNSCC, protein transfer to SCC40 was assessed after seeding cells on collagen to activate expression of MT1-MMP.¹⁷ Three major forms of MT1-MMP have been described that conform to a proform at ~68 kDa (sometimes migrates as a doublet), an active form at ~65 kDa that migrates below pro-MT1-MMP, and the autocatalytic products at 55 kDa and 42 kDa.¹⁷ Expression of MT1-MMP in collagen-cultured SCC40 cells as determined by immunoblotting to MT1-MMP showed strong immunoreactive bands corresponding to auto-proteolytic products in TAT and TAT-TLK1B-treated cells. In contrast, autocatalytic cleavage products were greatly reduced in the presence of TAT-MS1-TLK1B, now a substrate of MT1-MMP (Figure 4A).

MT1-MMP degrades extracellular matrix components, and to determine whether the inclusion of MS accelerates extracellular degradation and suppresses entry of TLK1B protein in cancer cells, SCC40 were cultured on collagen in serum-depleted medium before the addition of the recombinant protein. Lysates analyzed by immunoblotting against His or S-tag at 6 h showed that TAT-TLK1B crosses the cell membranes unhindered, but the incorporation of MS1 5' to TLK1B reduced entry of full-length TLK1B protein (Figure 4B). However,

unlike a single motif, the attachment of an array of MS (TAT-TL-MS5-K) effectively constrained permeation of a functional TLK1B in SCC40 (Figure 4C). In contrast, proteins that harbor, or not, MS readily traversed cell membranes of immortalized salivary NS-SV-AC cells (Figure 4D).

Inactive TLK1B sensitizes cells to radiation,⁸ which raised the possibility that a kinase domain-deficient protein could radiosensitize cells too. Preliminary studies on a kinase domain-deleted TLK1B protein (TAT-TL) isolated from bacteria showed that pretreatment of NS-SV-AC cells with TAT-TL reduced the number of surviving colonies following radiation compared with TAT-peptide (survival fraction at 2 Gy [mean ± SEM]: TAT-TL, 50.64 ± 6.72; TAT, 65.73 ± 3.35; $p < 0.01$; Figure S3). To determine whether similar radiosensitization of SCC40 can be realized with transduction of merely the N-terminal regulatory region of TLK1B, cell survival against radiation was assessed. SCC40 cells cultured on collagen were treated with TAT, TAT-TLK1B, or MS-harboring proteins, TAT-TL-MS1-K or TAT-TL-MS5-K, prior to radiation. Results showed that, as anticipated, transduction of TAT-TLK1B in SCC40 improved cell survival compared with TAT-treated control cells (survival fraction at 4 Gy [mean ± SEM]: TAT-TLK1B, 63.78 ± 8.88; and TAT, 42.95 ± 3.61; Figure 5). The inclusion of MS1 sequence diminished cell survival

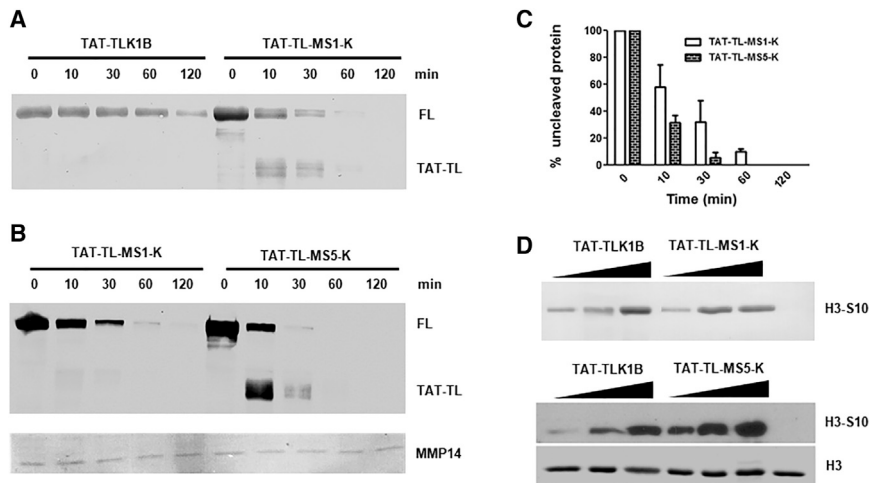


Figure 3. MT1-MMP Sensitivity of TAT-TL-MS-K Proteins.

(A and B) Recombinant proteins, TAT-TL-MS1-K and TAT-TL-MS5-K, were digested with MT1-MMP and reactions stopped at increasing times. Immunoblots were probed with polyhistidine antibody, and (C) band intensity of full-length protein (mean \pm SD) was quantified using ImageJ ($n = 3$). (D) Kinase activity was assessed after reacting increasing amounts of TAT-TL-MS-K proteins (0, 0.15, 0.3, 1.5 μ g) with histone H3 (1.5 μ g) at 30°C for 1 h, and phosphorylated substrate was detected with anti-H3-S10.

in relation to TAT-TLK1B, but not TAT (survival fraction at 4 Gy [mean \pm SEM]: TAT-TL-MS1-K, 53.03 ± 9.8 ; Figure 5A). Treatment with the highly sensitive protein with five MS reduced cell survival more than TAT (survival fraction at 4 Gy [mean \pm SEM]: TAT-TL-MS5-K, 38.09 ± 2.19 ; Figure 5B). On the other hand, treatment of NS-SV-AC with TAT-TLK1B or MS-harboring proteins significantly improved survival in relation to TAT (survival fraction at 4 Gy [mean \pm SEM]: TAT-TLK1B, 56.67 ± 2.58 ; TAT-MS1-TLK1, 65.82 ± 4.18 ; TAT-TL-MS5-K, 65.46 ± 0.06 ; and TAT, 49.17 ± 0.7 ; Figures 5C and 5D).

Mouse Xenograft Tumor Model

To assess the ability of reengineered TLK1B protein in sensitizing tumors *in vivo*, mice bearing SCC40 xenografts were pretreated with peri-tumoral protein administration before radiation. The results showed that 5 Gy, daily for 3 days, reduced tumor volume significantly in the TAT group (mean \pm SEM: TAT, 2.054 ± 0.233 ; and TAT+IR, 0.774 ± 0.243). Surprisingly, treatment with either protein also inhibited tumor growth (mean \pm SEM: TAT-TLK1B, 0.64 ± 0.18 ; and TAT-TL-MS5-K, 1.01 ± 0.241) similar to the TAT+IR group (Figure 6A). However, protein administration in conjunction with radiation further reduced tumor volume in TAT-TL-MS5-K-treated animals (mean \pm SEM, 0.585 ± 0.195), but not in the TAT-TLK1B group (mean \pm SEM, 1.005 ± 0.334). A two-way ANOVA analysis showed a statistically significant interaction between treatment and radiation groups ($p < 0.006$), and Bonferroni's multiple comparison test between TAT and TAT-TLK1B- or TAT and TAT-TL-MS5-K-treated groups was significant ($p < 0.05$). However, there was no statistically significant difference between non-irradiated and irradiated TAT-TLK1B or TAT-TL-MS5-K groups. Histopathology of tumors showed central coagulative necrosis that was more pronounced after radiation (Figure 6B).

DISCUSSION

Tumoricidal radiation used to ablate tumors also injures healthy tissues that lie within the radiation fields. A major limitation to attaining

a cancer-curative result is the dose threshold that normal critical tissues can withstand. The use of radioprotectors offers the opportunity to minimize normal tissue toxicity and attain better tumor control with high radiation doses. But, the caveat with their use is the possibility of tumor protection. To reduce salivary gland morbidity in patients treated with radiotherapy, we previously demonstrated that a complementary TLK1B gene or protein therapy improves survival and function of rat salivary glands against radiation.^{9–11}

Devoid of the risk of insertional mutagenesis, protein therapy is considered a safer alternative to gene therapy for expression of biological molecules in human cells. Numerous studies have shown that PTDs can act as inert transport vectors to transport biological cargoes across cell membranes.¹⁸ The HIV TAT-PTD is versatile in transducing a variety of cells, and a TAT-fused protein cargo can be effectively delivered across plasma membranes of salivary gland cells in culture and *in vivo*.¹⁰ Importantly, the transfer of biologically active TAT-TLK1B to the salivary glands suppressed the decline in salivary function following radiation.¹⁰ Augmentation of peptide delivery using rationally designed synthetic PTDs have shown improved transduction efficiencies in various cells.^{16,19} Three artificial PTDs optimized for α -helical content and arginine residues that had shown improved peptide transfer across the lipid bilayer previously were not more efficient than TAT in transducing TLK1B protein and improving cell survival against radiation. TAT-PTD can be internalized by receptor-dependent or -independent endocytosis. Small cargoes fused to TAT can easily permeate cells via micropinocytosis. However, cellular entry of larger cargoes such as proteins was shown to depend on anchorage of TAT to actin cytoskeleton and facilitated cytoskeletal remodeling for uptake via macropinocytosis.²⁰ We believe that the discrepancy in cellular uptake of synthetic PTDs between studies may, therefore, be due to the size difference of the attached cargo and, or, the strength of synthetic PTD-actin interaction for protein internalization.

Retroductal delivery of TAT-fusion protein to the submandibular glands showed marginal protein spread to the ipsilateral regional lymph nodes.¹⁰ Biospread of TLK1B especially to tumor cells can

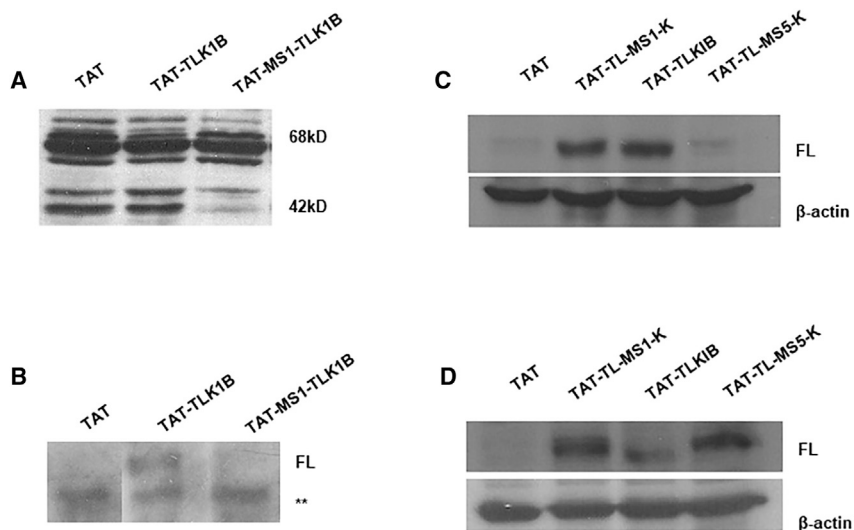


Figure 4. MT1-MMP in Discriminatory Protein Transduction of MS-Harboring TAT-TLK1B.

(A and B) MT1-MMP expression was induced by culturing HNSCC, SCC40 cells on collagen and cells were treated with TAT, TAT-TLK1B, or TAT-MS1-TLK. Cells were lysed at 6 h, and equal protein was loaded for immunoblotting using (A) anti-MT1-MMP or (B) HRP-conjugated anti-poly-histidine. Pro-MT1-MMP (68 kDa); autocatalytic cleavage products (55 kDa and 42 kDa). (C and D) SCC40 (C) or immortalized salivary acinar cells, NS-SV-AC (D), were transduced with TAT, TAT-TLK1B, TAT-TL-MS1-K, or TAT-TL-MS5-K, and cell lysates probed with anti-S-tag. Asterisks (**) represent non-specific band. FL, full-length protein.

hinder its successful application to patient care, and the ability of cell-permeable TLK1B to differentiate normal from tumor cells becomes critical to minimizing radiotoxicity without compromising tumor control. To this end, our approach exploited the signature overexpression of MT1-MMP in HNSCC to prevent TLK1B transduction in tumor cells. The digestion of extracellular matrix proteins by proteolytic enzymes such as MMPs has been implicated in growth and spread of tumors. Previous studies have favorably used proteases expressed in cancer to activate cytotoxic pro-drugs or pro-toxins selectively in the tumor milieu, thereby diminishing their off-target cytotoxicities.^{21–24} Other than possessing its own collagenolytic and fibrinolytic activities, MT1-MMP also activates MMP2,²⁵ and the MT1-MMP > MMP2 axis is responsible for activation of other MMPs such as MMP9.²⁶ As a result, upregulation of MT1-MMP can correlate with higher MMP2 and MMP9 activity in tumors. Similarities in substrate cleavage motifs have been observed, and the collagen consensus motif, Pro-X-X-[↓]-X_{H₂}, is recognized by multiple collagenases and gelatinases including MT1-MMP and MMP2.²⁷ Profiling substrate specificity of MMPs identified additional amino acid preferences around the cleavage site.^{28–31} Substrate sequence selectivity of VPLS[↓]LR (MS1) was common to MT1-MMP and MMP2, whereas RIGF[↓]LR (MS2) and RPAH[↓]LR (MS3) were deemed highly selective for MT1-MMP.^{27,30,32} Although a comparative analysis of sensitivity to MT1-MMP cleavage *in vitro* was MS3 > MS1 > MS2, the incorporation of MS1 motifs in combination with MS3 in the construction of MS5 was adopted to increase sensitivity of the fusion protein *in vivo* to active MMP2 as well.

Increased expression of MT1-MMP has been observed in HNSCC tumor cells.^{33–36} Expression of MT1-MMP in tumors can promote extracellular degradation of a MS-harboring protein to prevent its entry in HNSCC cells. Because expression of a kinase-mutant sensitizes mouse mammary epithelial cells to radiation, kinase activity was regarded as vital to cell fitness, especially in response to genotoxic stress.⁸ Based on the crystal structure of the conserved serine/

threonine kinase domain of CDK2, MS was incorporated distal to the critical elements within the TLK1B kinase domain to least disrupt activity while maximally retaining the ability of the protein to homo/hetero-dimerize, a feature essential to its catalytic activation through autophosphorylation.^{37,38} Due to the unexpected loss in TAT-TLK-MS activity, placement of MS upstream of the C-terminal catalytic region was considered. The coiled-coil domains suggested to participate in oligomerization reside immediately upstream of the catalytic region,^{37,38} and MS inserted between the coiled-coil region and the kinase domain should transduce a kinase-devoid protein harboring the oligomerization motifs. The dominant-negative effect of TAT-TL was reproducible in SCC40 cells treated with TAT-TL-MS5-K, which ratified the strategy.

Radiation was highly effective at reducing tumor volume compared with the non-irradiated control group. Unexpectedly, treatment with either protein significantly suppressed tumor growth too. Human recombinant proteins can be antigenic, but minimal host immune response to TAT-TLK1B instilled in salivary glands suggested its unlikely impact.¹⁰ Breast cancer cells when co-cultured with fibroblasts depleted of TLK1 or TLK2 showed increased proliferation,³⁹ which suggested a pivotal role of TLKs in stromal signaling. Additionally, in murine embryonic stem cells, TLK1 downregulated expression of core pluripotency factors and promoted cell differentiation.⁴⁰ Therefore, it is possible that tumor control in response to TAT-TLK1B treatment is a result of its effects on surrounding fibroblasts and, or, cancer stem cells. Although details about TLK1/2 mutations in head and neck tumors remain to be investigated, this work reveals that the reengineered TLK1B protein therapy does not compromise radiation efficacy on tumor control.

Evidence of efficacy, lack of tumor protection, and limited toxicity are important factors to be considered before transitioning a promising radioprotector from preclinical phase to clinical care. Few radioprotectors have the ability to prevent radiotoxicity without untoward tumor protection. Previously, we demonstrated that TAT-TLK1B

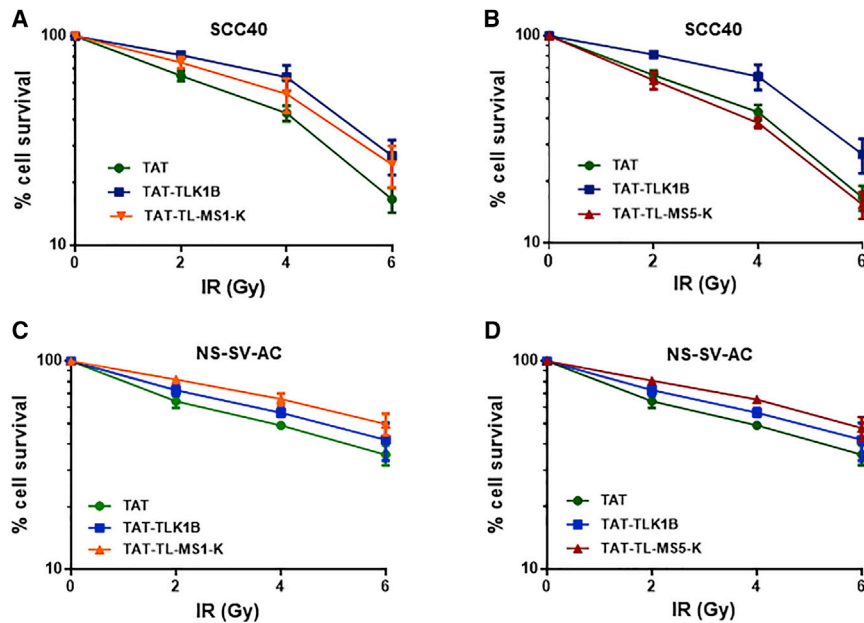


Figure 5. Cell Survival against Radiation in Cells Pretreated with MT1-MMP-Sensitive TAT-TLK1B.

SCC40 cells (A and B) or NS-SV-AC cells (C and D) were treated with TAT peptide, TAT-TLK1B, TAT-TL-MS1-K, or TAT-TL-MS5-K (100 μ g) before exposure to increasing doses (0, 2, 4, 6 Gy) of radiation. Colonies were stained with crystal violet on day 10 for SCC40 cells and day 14 for NS-SV-AC cells ($n = 3$). Data are shown as mean \pm SEM.

effectively radioprotects rat salivary gland function, and because activity that is vital to cellular recovery from radiation-induced checkpoint arrest is unaffected in TAT-TL-MS5-K, we anticipate that it will function similar to TAT-TLK1B in salivary glands *in vivo* as observed in culture *in vitro*. Future studies in an animal model of salivary gland radiation toxicity should validate the assumption. Nevertheless, by imparting the proficiency to transfer a kinase-devoid TLK1B in cancer cells, but a full-length active protein in healthy cells, the proof-of-concept study suggests the benefit of reengineered TLK1B in safely increasing the therapeutic ratio.

MATERIALS AND METHODS

Plasmid Constructions

To assess efficiency of three different PTDs, the TAT-TLK1B sequence was subcloned into pSP-NF fusion vector (Promega), and TAT-PTD was substituted with synthetic PTDs at HindIII and BamHI using 5' phosphorylated and hybridized oligonucleotides: PTD1 (5'-AAGCTTCTAGAGGCTACGCTCGCAAGGCTCGCCGCCAGGCTCGCCGCGGTGGATCC-3'), PTD2 (5'-AAGCTTCTAGAGGCTACGCTCGCGCTGCTCGCCGCGCTGCTCGCCGCGG TGGATCC-3'), or PTD3 (5'-AAGCTTCTAGAGGCTACGCTCGCGCTGCTGCTCGCCAGGCTC GCGCTGGTGGATCC-3'). Presence of insert was confirmed by digestion with XbaI, and PTD-TLK1B cassette was excised using HindIII and XhoI and inserted in similarly cut pET30b(+) vector. The coding cassette includes N-terminal 6 \times Histidine followed by S-tag, HIV TAT-PTD or a synthetic PTD, hemagglutinin (HA) tag, and TLK1B sequence.

To generate MS-harboring, TAT-MS-TLK, MS sequences (MS1: 5'-GATCCGTGCCA CTGTCCCTGAGATCTGGTACCTATCCAT ATGACGTC CAGACTATGCTTC-3'; MS2: 5'-GAT CCCGATT GGCTTCTGCGTACCGCGCTAGCTATCCATATGACGTCCCA

GACTATGCTTC-3'; MS3: 5'-GATCCCGTCCGGCGCATCTGCGTGATTCGCGTAGCTATCCATATGAC GTCCAGACTATGCTTC-3') were generated in conjunction with HA sequence, and oligonucleotides were 5' phosphorylated and hybridized for subcloning in-frame at BamHI and NcoI before the start of the TLK1B sequence. TAT-TLK-MS were generated through gene block cloning using Gibson Assembly (NEB, Ipswich, MA, USA), whereas gene construction was used for place-

ment of MS sequence(s) before the catalytic region of TLK1B to derive TAT-TL-MS-K plasmids (GenScript, Piscataway, NJ, USA). All plasmids were sequenced to confirm in-frame insertion and sequence fidelity.

Protein Expression and Purification

Expression plasmids were transformed into BL21(DE3)pLysS competent cells (Novagen/Millipore, MA, USA), and transformants were grown in Luria broth with 30 μ g/mL kanamycin (USB/Thermo Fisher, Waltham, MA, USA) at 37°C until density of 0.6 at λ 600 was reached. The culture was then shifted to 20°C, and protein expression was induced by addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (Gold Biotech, St. Louis, MO, USA). After 16 h, bacterial cells were pelleted, washed in PBS, and re-suspended in lysis buffer (50 mM HEPES [pH 8.0], 300 mM NaCl, 20 mM imidazole) containing 1 mg/mL lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and incubated on ice for 30 min. PMSF (Thermo Fisher, Waltham, MA, USA) to a final concentration of 1 mM was added before sonication on ice at 45% amplitude, seven cycles of 30 s with 1-min intervals in between. Lysate was centrifuged twice at 17,000 rpm, 4°C, 60 min each, and the supernatant incubated with His60 Ni Superflow resin (Clontech/Takara, Mountain View, CA, USA). Recombinant proteins were eluted in elution buffer (50 mM HEPES [pH 8.0], 100 mM NaCl, 250 mM imidazole) before passing them over a cation exchange column. Isolated proteins were buffer exchanged, and glycerol was added to a final concentration of 10% before freezing the aliquots.

Unlike other recombinant TAT-TLK1B proteins, the kinase domain-deleted TLK1B (TAT-TL) was predominantly in the membrane-associated fraction. The protein was isolated after solubilizing it in urea followed by dialysis of the denaturant.

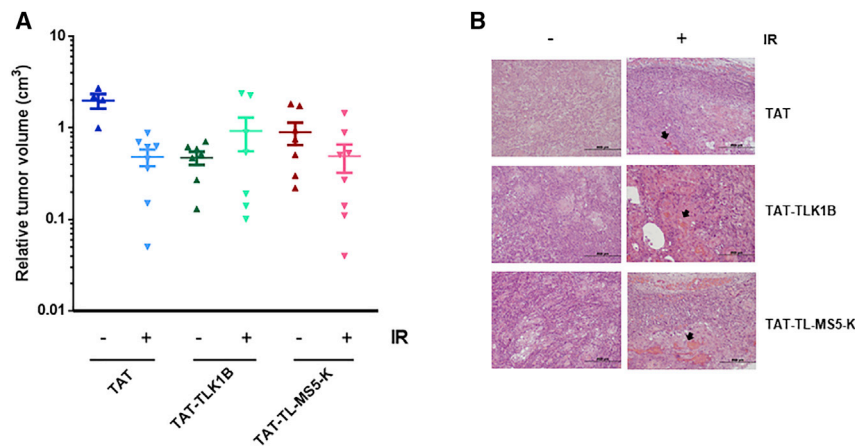


Figure 6. Treatment Effect on Xenograft Tumor Volume.

SCC40 cells were implanted in the lateral aspect of the neck of nude mice. After 2 weeks, tumor-bearing animals were treated with peritumoral administration of TAT, TAT-TLK1B, or TAT-TL-MS5-K (10 μ g) and irradiated (5 Gy) 6 h later. Protein treatment and radiation were conducted on 3 consecutive days (days 1–3); animals were euthanized on day 10. (A) Relative tumor volume was derived and plotted for each treatment group ($n = 8$; mean \pm SEM). (B) H&E-stained tissue sections. Arrows point to coagulative necrosis.

MMP Sensitivity Assay

TAT-TLK1B proteins (1.5 μ g) harboring, or not, MS recognition sequences were reacted with MMP14 catalytic domain (EMD Millipore, Burlington, MA, USA) at 22°C. At indicated times, the reaction was stopped by the addition of Laemmle loading dye, and samples were heated at 95°C before electrophoresis on 10% SDS polyacrylamide gels. Immunoblotting was performed using mouse monoclonal anti-polyhistidine (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) followed by corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Dako, Denmark) before reacting the blots to chemiluminescent reagents. The intensity of protein bands was measured using ImageJ software.

Colony Formation Assay

The SV40 T-Ag immortalized human salivary acinar cells, NS-SV-AC, were a kind gift from Dr. Masayuki Azuma, University of Tokushima School of Dentistry, Tokushima, Japan,⁴¹ and cells were grown in complete KGM2 (Lonza, Switzerland) containing 1 \times antibiotic-antimycotic (GIBCO/Thermo Fisher, Waltham, MA, USA). To assess effectiveness of synthetic PTD-fused TLK1B, exponentially growing human salivary gland acinar cells, NS-SV-AC, in 60-mm plates were treated with equimolar amounts of TAT-TLK1B or PTD-TLK1B protein (500 μ g) or TAT peptide (1 μ g; GenScript, Piscataway, NJ, USA). Following a 6 h incubation, cells were resuspended (1×10^5 cells/mL) and exposed to radiation (0, 2, 4, and 8 Gy) using the 6 MeV Elekta Precise linear accelerator (Ochsner LSU Health-Radiation Oncology). Cells were plated in triplicates at different densities (500–4,000 cells/well) in six-well plates, and colonies were counted after staining with crystal violet on day 14. Results were normalized to non-irradiated controls and expressed as percent cell survival. Data were averaged across three independent experiments and expressed as mean \pm SEM.

Induction of MT1-MMP Expression, Protein Transduction, and Colony Survival Assay

The HNSCC cell line, SCC40, was obtained from ATCC, and they were cultured in DMEM supplemented with 10% bovine growth

serum (Hyclone/GE Life Sciences, Marlborough, MA, USA), 1 \times minimum essential amino acids, and 1 \times antibiotic-antimycotic (GIBCO/Thermo Fisher, Waltham, MA, USA). To activate expression of MT1-MMP, a previously described procedure was followed.¹⁷ SCC40 were seeded at a density of 9×10^5 cells/well in rat tail type I collagen-coated six-well plates (BD Biocoat, Franklin Lakes, NJ, USA) and allowed to adhere overnight. The next day, medium was replaced with serum-free medium containing 0.1% BSA, and cells were incubated for 48 h. Floating cell debris was removed by brief centrifugation, and recombinant TAT-TLK1B protein harboring, or not, MS sequence (100 μ g diluted in PBS) was layered on added back conditioned medium. After 6 h, the cells were washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, and 1 mM PMSF). Lysate supernatant (30 μ g) was separated on 10% SDS-PAGE and immunoblotted with a HRP-conjugated anti-HA (1:1,000; Sigma-Aldrich, St. Louis, MO, USA), anti-S tag (1:2,000; Abcam, Cambridge, MA, USA), or anti-MMP14 (1:500; Millipore, MA, USA), followed by corresponding HRP-conjugated secondary antibody (Vector Labs, Burlingame, CA, USA).

The effect of recombinant protein treatment on radiation response was assessed using colony formation assays. SCC40 cells were grown on collagen in serum-free DMEM/0.1% BSA as before and treated with recombinant protein for 6 h. NS-SV-AC cells were grown in KGM2 and similarly treated with protein. Cells were trypsinized and resuspended at a density of 1×10^5 cells/mL before 0, 2, 4, or 6 Gy irradiation. Cells were then plated in triplicates at increasing density with increasing radiation dose, and colonies were stained with crystal violet on day 10 for SSC40 and on day 14 for NS-SV-AC cells. Experiments were repeated thrice, and results were expressed as the fraction of surviving colonies in comparison with the non-irradiated samples.

In Vitro Kinase Assays

In vitro kinase assays were performed in kinase buffer (15 mM HEPES [pH 7.4], 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, and

100 mM MgCl₂) with 0.2 mM ATP. Increasing amounts of recombinant TLK1B protein (0, 0.15, 0.3, or 1.5 µg) were incubated with 1.5 µg histone H3 (NEB, Ipswich, MA, USA) at 30°C for 1 h. Reactions were electrophoresed and immunoblotted with anti-phospho-S10 H3 antibody (Millipore) and corresponding HRP-conjugated secondary antibody.

In Vivo Experiments

Male and female Hsd:ATHymic nude-Foxn1^{nu} mice (null/knockout forkhead box N1; 6–7 weeks old; Envigo, East Millstone, NJ, USA) were anesthetized by intramuscular administration of ketamine (149 mg/kg) and xylazine (5 mg/kg), and 1 × 10⁶ SCC40 cells resuspended in 50% matrigel were injected subcutaneously in the lateral aspect of the neck in a 50 µL vol. After 2 weeks of tumor growth, animals were randomly divided into groups, and 10 µg of recombinant protein or 0.15 µg of TAT peptide in 50 µL vol was peritumorally administered. Because protein precipitation was observed at high concentrations, it prevented its intra-tumoral administration in a small volume. Six hours following protein injection, a tissue-equivalent bolus was placed over the head and neck region of anesthetized animals, and animals were irradiated (5 Gy) using a 6 MeV Elekta Precise linear accelerator. After recovery from anesthesia, the animals were housed at the vivarium with free access to food and water. Protein injection and animal irradiation were conducted on 3 consecutive days (total = 15 Gy), and on day 10, animals were euthanized, and tumors were excised, measured, and fixed in cold 4% paraformaldehyde/PBS before paraffin processing and H&E staining of tissue sections. Tumor volume was derived from caliper measurements as: Tumor volume = 1/2 × L × (D)². All procedures were approved by the institution's Animal Care and Use Committee and were conducted in accordance with NIH guidelines. A few animals in the non-irradiated TAT group were prematurely euthanized and excluded from the study as tumor size exceeded institutional Animal Care and Use Committee guidelines.

Statistical Analysis

Data analyses were done using GraphPad Prism software. Differences were considered significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omto.2019.02.003>.

AUTHOR CONTRIBUTIONS

R.P.N. conducted all experiments with TAT-TL-MS-K; P.S.T.S. conducted experiments with TAT-MS-TLK; and G.S.D. designed the study and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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