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TITLE: Targeting ATM-SMC1 Pathway to Sensitize Breast Cancer Cells to Therapeutic Interventions

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Targeting ATM-SMC1 Pathway to Sensitize Breast Cancer Cells to Therapeutic Interventions

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Previously we have shown that ATM phosphorylates SMC1 to regulate radiosensitivity. This project is to investigate whether interfering the ATM-SMC1 DNA damage signaling will increase the sensitivity of breast tumor cells to radiotherapy and chemotherapy. To achieve this goal, we have synthesized two small peptides containing the original amino acid sequence of SMC1 around Ser957. We have found that the peptide that has the wild-type SMC1 sequence possess the inhibitory ability on ATM kinase activity in vitro. We have also designed TAT-fusion peptides that can deliver the SMC1 peptide into cells. We have shown that the TAT-fusion peptides: 1) can be internalized into cells in a time- and dose-dependent manner; 2) can abrogate radiation-induced S-phase checkpoint; and 3) have minimal cytotoxicity to breast cancer cells in the absence of DNA damage. These insights are providing a basis for developing strategies for increasing selective tumor cell cytotoxicity after chemotherapy and radiotherapy. Future experiments will be focusing on testing the radio- and chemo-sensitization effect of the fusion peptides.
Introduction:
Recent studies have revealed that cellular sensitivity to DNA damage agents is controlled by the Ataxia-Telangiectasia-Mutated (ATM) protein kinase and its signal to the Structural Maintenance of Chromosome protein one (SMC1)(6,7,11). ATM phosphorylation of SMC1 at two serine sites is required for limiting the amount of radiation sensitivity. We hypothesized that targeting the ATM-SMC1 pathway may lead to a novel approach for developing sensitizers to therapeutic interventions for breast cancer. This hypothesis has been tested in this proposal by studying the effect of synthetic peptides that aim to block the in vivo phosphorylation events on breast cancer cellular response to DNA damage. The long term goal of this project is to develop the concept for utilizing novel approach to targeting DNA damage pathway in order to sensitize breast tumor to radiotherapy and chemotherapy.

Body:

A. Development of a small peptide to inhibit ATM phosphorylation of SMC1 in vitro. (SOW Task 1a and 1b)
Since ATM phosphorylation of SMC1 at two serine sites is required for limiting the amount of radiation sensitivity, we hypothesized that small molecule that can modulate ATM-SMC1 phosphorylation may function as radiosensitizers. Since a general consensus target motif has been defined for ATM—its phosphorylates a serine or threonine residue only if it is followed by a glutamine (the “SQ/TQ motif”)(5,8), these molecules could consist of the SQ sequence of SMC1 (at Ser957 and Ser966), presumably targeting ATM phosphorylation of SMC1. To test this hypothesis, we synthesized two small peptides to test the inhibitory effect on ATM kinase activity in an in vitro kinase assay (Fig 1A). One peptide contains the original amino acid sequence of SMC1 around Ser957 (peptide 2), and the other originates from the sequence except two serine and the glutamine residues were replaced (peptide 1). Flag-tagged ATM was immunoprecipitated, followed by an in vitro kinase assay using GST-SMC1 (amino acid 951-964) as a substrate in the presence of 32P-ATP. Our data shows that peptide 1 does not effect ATM phosphorylation on SMC1, while peptide 2 totally abrogates the phosphorylation signal (Fig 1B). This observation suggests that small peptides containing the amino acid sequence of ATM phosphorylation may have an inhibitory effect on ATM substrate recognition and phosphorylation; therefore they can be tested as in vivo inhibitors and radiosensitizers.

B. Determination of peptide internalization (SOW Task 1a, c and d)
Since small peptides can transport through cellular membrane only when the molecules are very small—typically less than 600 Daltons (9), the proposed peptides need to link to a protein transduction domain. Recent reports have suggested that conjugation of peptides, proteins and antisense to short highly basic peptides, such as the human immunodeficiency virus (HIV) TAT, results in their rapid translocation into cells (2). TAT-mediated delivery requires a short 11 amino acid region of the TAT protein (1,3,4,9). This region corresponds to amino acid 45-57 of TAT (YGRKKRRQRRR) and
has a high net positive charge at physiological pH with nine out of 11 of its amino acids being either arginine or lysine. The 11-amino-acid TAT sequence carries full-length proteins or small peptides into cells in a rapid, concentration-dependent fashion that appears to be independent of receptors and transporters and instead is through targeting the lipid bi-layer component of cell membrane. Therefore, in principal, all mammalian cell type should be susceptible to the internalization (9). Based on above information, we synthesized three fusion peptides. These peptides are composed of two functional domains, one an interference domain and the other a targeting domain (Fig 2A). The N-terminus of the peptides was linked with biotin for visualization. For the interference domain, the SMC1 phosphorylation sequence (amino acid sequence around Serines 957 and 966) was included. Mutant sequence (serine to alanine substitution, which abrogates the phosphorylation) was generated as a negative control (Fig 2B). For the targeting domain, we utilized the TAT sequence.

Our first set of experiments to characterize the peptides was to test the internalization effect of the fusion peptides. MCF-7 cells were incubated with 10μM of the peptides for 30 minutes and the immunofluorescence microscopic assay was used to assess the localization of the peptides. Since the peptides are linked with biotin on their N-terminus, an FITC-conjugated anti-streptavidin antibody, which recognizes the tetrameric protein that can bind to biotin, was used to visualize the peptides (Figure 3). Our data confirmed that the fusion peptides localize within cytoplasmic and nuclear portions of the cell.

### C. Determine the cytotoxicity effect of the peptides (SOW Task 1e)

A promising radiosensitizer should be non-toxic to cells, but sensitize only tumor cells to radiation. To test the toxicity of the peptides, MCF-7, a well characterized breast cancer cell line on which we have studied the radiation sensitivity and cell cycle checkpoints was utilized. Cell viability was measured using the MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyldimazole) assay. We find that the TAT peptides have minimal cytotoxicity to MCF-7 cells as shown in Figure 4 that there is no decrease of cell survival after 24 hours treatment of the peptides. The effect of the peptides on cell cycle distribution was also investigated.

Cells treated with peptides were harvested, fixed in 70% of ethanol, and stained with propidium iodide, followed by a flow cytometric analysis. No change of basal cell cycle distribution is detected (data not shown).
D. Investigate the inhibitory effect of the peptides on IR-induced S-phase checkpoint activation (SOW Task 2b).

Since ATM phosphorylation of SMC1 regulates IR-induced S-phase arrest, we then investigated the inhibitory effect of the TAT-SMC1 peptides on IR-induced S arrest in MCF-7 cells. To measure the S-phase arrest, the RDS assay (10) was utilized. We find that in the presence of TAT-wtSMC1 peptide, MCF-7 cells display the Radio-Resistance DNA synthesis phenotype, indicative of abrogation of the S-phase checkpoint (Figure 5). However, the TAT only and TAT-mtSMC1 peptides have no effect on IR-induced S-phase checkpoint. These observations suggest that the wild-type SMC1 phosphorylation sequence specifically interferes with endogenous SMC1 phosphorylation after IR; therefore it is quite likely that this peptide will function as a powerful radiosensitizer.

**Key research accomplishments:**

1. We have found that a small peptide that contains the SMC1 phosphorylation sequence has an inhibitory effect on ATM activity; and
2. This peptide, when linked with the TAT sequence, can block radiation-induced S-phase checkpoint.

**Reportable outcomes:**

We will present our observations in major scientific meetings, such as the Annual Meeting of American Association of Cancer Research.

**Conclusions**

The major focus of this project is to develop the concept for utilizing novel approach to targeting DNA damage pathway in order to sensitize breast tumor to radiotherapy and chemotherapy. We have found that it is possible to use small peptides containing SMC1 phosphorylation sequence to interfere with ATM-mediated SMC1 phosphorylation. These peptides can then be tested as powerful sensitizers. This project may yield new therapeutic agents to improve the sensitivity of human breast cancers to radiotherapy and chemotherapy. This approach may also generate specific inhibitors to DNA damage pathways for basic breast cancer research.

**References**


Appendices

N/A