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14. ABSTRACT
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; 3) have minimal cytotoxicity to breast cancer cells in the absence of DNA damage, and more importantly 4) can significantly enhance radiation and chemotherapeutic effect. These insights have provided a basis for developing strategies for increasing selective tumor cell cytotoxicity after chemotherapy and radiotherapy.

15. SUBJECT TERMS
ATM, SMC1, DNA damage, radiotherapy, chemotherapy, peptide

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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)(Kim et al., 2002; Kitagawa et al., 2004; Yazdi et al., 2002). 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 was tested by studying the effect of synthetic peptides that aim to block the in vivo phosphorylation events on breast cancer cellular response to DNA damage, and the radiosensitization effect proven to be significant. 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--it phosphorylates a serine or threonine residue only if it is followed by a glutamine (the "SQ/TQ motif) (Kim et al., 1999; O'Neill et al., 2000), 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 ^{32}P-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.

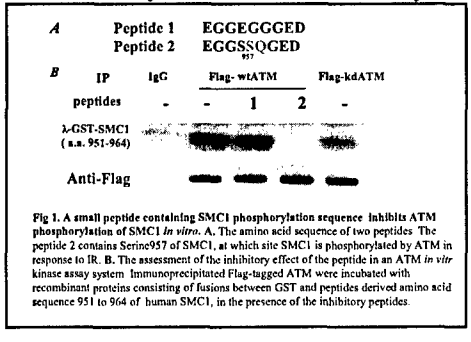

Fig 1. A small peptide containing SMC1 phosphorylation sequence inhibits ATM phosphorylation of SMC1 *in vitro*. A. The amino acid sequence of two peptides The peptide 2 contains Serine957 of SMC1, at which site SMC1 is phosphorylated by ATM in response to IR. B. The assessment of the inhibitory effect of the peptide in an ATM *in vitr* kinase assay system Immunoprecipitated Flag-tagged ATM were incubated with recombinant proteins consisting of fusions between GST and peptides derived amino acid sequence 951 to 964 of human SMC1, in the presence of the inhibitory peptides.

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 (Schwarze et al., 1999), 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 (Ford et al., 2000). TAT-mediated delivery requires a short 11 amino acid region of the TAT protein (Ezhevsky et al., 1997; Gius et al., 1997; Gius et al., 1999; Schwarze et al., 1999). 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 (Schwarze et al., 1999). 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.

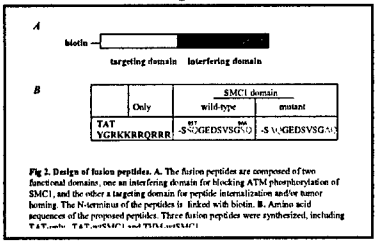

Fig 2. Design of fusion peptides. A. The fusion peptides are composed of two functional domains, one an interfering domain for blocking ATM phosphorylation of SMC1, and the other a targeting domain for peptide internalization and/or tumor homing. The N-terminus of the peptides is linked with biotin. B. Amino acid sequences of the proposed peptides. Three fusion peptides were synthesized, including

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 immunofluoresence 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.

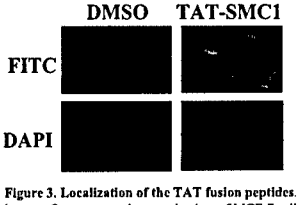

Figure 3. Localization of the TAT fusion peptides. Shown is the immunofluoresence microscopic view of MCF-7 cells treated with 10μM of TAT-wtSMC1 for 30 minutes.

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)-2, 5-diphenyltetrazolium bromide) 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

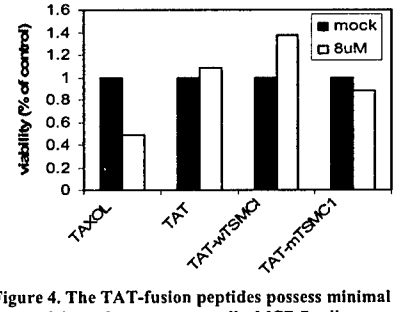

Figure 4. The TAT-fusion peptides possess minimal cytotoxicity to breast tumor cells. MCF-7 cells were treated with different doses of TAT-peptides or Taxol for three hours. After incubation, cell viability was quantified using a standard MTT assay. Shown is cell survival in the presence of 8uM TAT-peptides.

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 2a. b).

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 (Xu et al., 2001) 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. Western blot analyses were also performed to investigate whether the SMC1 peptide could block radiation induced SMC1 phosphorylation. Our data demonstrate that SMC1 phosphorylation at Ser 966 after irradiation was abolished in the presence of wild-type SMC1 peptide, but not the mutant SMC1 peptide. This suggests that the SMC1 peptide indeed affects in vivo SMC1 phosphorylation.

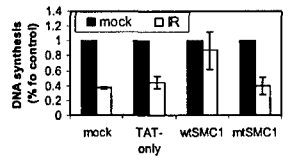

Fig 5. Exposure to the TAT-wtSMC1 peptide abrogates IR-induced S-phase checkpoint. To assess the S-phase checkpoint, MCF-7 cells were treated with 10μM of either TAT only, TAT-wtSMC1, or TAT-mtSMC1. 30 min after exposure to 10 Gy of ionizing radiation, replicative DNA synthesis was measured. *Columns*, averages of at least triplicate samples; *bars*, SE.

E. The radio- and chemo- sensitization effects of the SMC1 peptide (SOW 2c and d). The major goal of this project was to test whether the TAT-SMC1 peptides can be used as radio- and/or chemo-sensitizers. To reach this goal, we performed colony formation assays using a breast cancer cell line MCF-7 after radiation and chemotherapy. The chemotherapeutic compounds used include cyclophosphamide, methotrexate, 5-fluorouracil and paclitaxel. Before treatment of these DNA damage agents, cells were treated with the TAT only, TAT-wtSMC1 and TAT-mtSMC1 compounds for 30 minutes. Ten days after treatment, the number of colonies (cell numbers greater than 50 = one surviving colony) were counted and the surviving fraction were calculated. As shown in **Figure 6**, the TAT-wtSMC1 peptide significantly increases the sensitivity of MCF-7 cells to radiation and chemotherapy. The sensitization effect is at least two fold. Meanwhile, the control peptide (TAT-only) and the TAT-mtSMC1 peptides had limited effects on survival after DNA damage. These observations strongly suggest that the wtSMC1 peptide is a

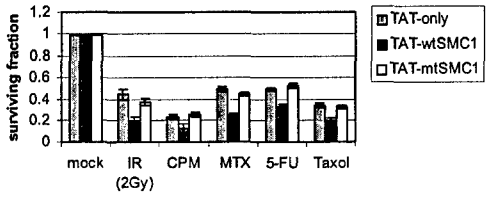

Figure 6. Radio- and chemo-sensitization of the TAT fusion peptides. MCF-7 cells were seeded into 6-well plates and treated with 10uM of the peptides. 30 minutes after the treatment, the cells were exposed to ionizing irradiation or indicated chemotherapeutic drugs. Colony formation assays were then processed and the surviving fractions were calculated.

powerful radio and chemo- sensitizer *in vitro*. Our next step is to text the *in vivo* radiosensitizing effect using breast cancer xenograft models in mice.

Key research accomplishments:

We have presented our observations in two scientific meetings, including the 3rd International Symposium on Targeted Anticancer Therapies in March 2005, Amsterdam, the Netherlands, and the Era of Hope 2005 in Philadelphia, PA , June 11, 2005. We are also preparing a manuscript focusing on our findings and we will submit the paper to a major scientific journal, including *Nature Medicine* or *Cancer Research.*

Reportable outcomes:

1. We presented our research in two scientific meetings, including the 3rd International Symposium on Targeted Anticancer Therapies in March 2005, Amsterdam, the Netherlands, and the Era of Hope 2005 in Philadelphia, PA , June 11, 2005.
2. The sequence we identified from our study will be submitted for an US patent;
3. The data we obtained were used as preliminary data to apply for major federal grants, including a NIH grant and a DoD grant.

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 were then tested whether they function as radio- and chemo- sensitizers. The presented data have suggested a significant increase of cellular sensitivity to DNA damage compounds if cells were treated with wild-type SMC1 peptides. Our study has provided a strong rationale for the wild-type SMC1 peptide sequence be explored as a powerful radio- and chemo- sensitizing compounds *in vivo*. 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.

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Appendices

N/A