Targeted light-inactivation of the Ki-67 protein using theranostic liposomes leads to death of proliferating cells

*Ramtin Rahmanzadeh[1$], Prakash Rai[1$], Johannes Gerdes[2], Tayyaba Hasan[1]**

[1]*Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114*

[2]*Department of Immunology and Cell Biology, Research Center Borstel, D-23845 Borstel, Germany*

[$] *Authors contributed equally*

ABSTRACT

Nanomedicine is beginning to impact the treatment of several diseases and current research efforts include development of integrated nano-constructs (theranostics) which serve as probes for imaging and therapy in addition to delivering macromolecules intracellularly. In cancer, there is a vital unmet need for effective alternative treatments with high specificity and low systemic toxicity. This can be achieved by targeting key molecular markers associated with cancer cells with reduced effective drug doses. Here, we show an innovative proof-of-principle approach for efficient killing of proliferating ovarian cancer cells by inactivating a protein associated with cell proliferation namely, the nuclear Ki-67 protein (pKi-67), using nanotechnology-based photodynamic therapy (PDT). Antibodies against pKi-67 are widely used as prognostic tools for tumor diagnosis. In this work, anti pKi-67 antibodies were first conjugated to fluorescein isothiocyanate (FITC) and then encapsulated inside liposomes. After incubation of OVCAR-5 ovarian cancer cells with these liposomes, confocal microscopy confirmed the localization of the antibodies to the nucleoli of the cells. Irradiation with a 488 nm laser led to a significant loss of cell viability. The specificity of this approach for pKi-67 positive cells was demonstrated in confluent human lung fibroblasts (MRC-5) where only a small population of cells stain positive for pKi-67 and only minimal cell death was observed. Taken together, our findings suggest that pKi-67 targeted with nano-platform is an attractive therapeutic target in cancer therapy.

Keywords: Nanotechnology, Ovarian Cancer, Proliferative Index, Photodynamic Therapy, Antibody

1. INTRODUCTION

Cancer therapies are now in development which block or interrupt specific pathways or proteins that are intricately involved in the proliferation of cancer cells. One such protein that is involved in cell proliferation is pKi-67. Expression of the nuclear Ki-67 protein is strongly up regulated in proliferating cells[1,2]. For this reason, antibodies against this protein are widely used as prognostic tools for the assessment of cell proliferation in biopsies from cancer patients. Proliferating cells highly express the Ki-67 protein in all active phases of the cell cycle (G1, S and G2, as well as mitosis)[3]. Photodynamic therapy (PDT) is an approved treatment modality for various malignant and non-malignant diseases and is FDA approved as first line treatment for age-related macular degeneration. It has had considerable clinical success in treating various cancers and is routinely used for obstructive esophageal cancer and cases of advanced and early lung cancers. PDT involves light-based activation of a chemical called photosensitizer (PS) to produce cytotoxic free radicals[4-7].

*thasan@partners.org ; phone 1 617 726-6996; fax 1 617 726-8566; http://www.massgeneral.org/wellman/people/thasan.asp

Reporters, Markers, Dyes, Nanoparticles, and Molecular Probes for Biomedical Applications II, edited by Samuel Achilefu, Ramesh Raghavachari, Proc. of SPIE Vol. 7576, 757602
 · doi: 10.1117/12.843850

Nanoparticles, in particular liposomes have been used for intracellular delivery of macromolecules and provide a multifunctional platform for the incorporation of contrast agents for enhanced diagnostics, treatment and therapy monitoring[8-11]. Nanoparticle formulations of anti-cancer agents are in various stages of pre-clinical and clinical development. Several such formulations are already used in the clinic for treatment of various diseases including cancer.

In the present study we target the nuclear Ki-67 protein by combining nanotechnology and PDT. In this preliminary proof-of-principle study we demonstrate inactivation of pKi-67 using two antibodies, namely TuBB-9[12] and MIB-1[13] conjugated to a widely used chromophore, fluorescein 5(6)-isothiocyanate (FITC). The two antibodies recognize Ki-67 in two different states. We use a nanotechnology based intracellular delivery system where the antibody-FITC conjugates are encapsulated inside non-cationic liposomes. The treatment effects are demonstrated in monolayer ovarian cancer cells. The specificity of this approach for cells that are positive for pKi-67 is demonstrated in confluent human lung fibroblasts (MRC-5) where only a small population of cells expresses pKi-67. The results of the study suggest that pKi-67 is an attractive therapeutic target in cancer and this approach holds promise as an effective alternative therapy for cancer.

2. RESULTS

2.1 Liposomal constructs deliver antibody intracellularly

The liposomal constructs consisting of FITC-labeled TuBB-9 or MIB-1 antibody and a lipid bilayer were first characterized by DLS and found to be in a size range of ~180 nm. OVCAR-5 cells were then incubated with these constructs at a concentration of 20 nM FITC equivalent. After 24 hours FITC fluorescence could be detected inside the nucleoli of the cells by confocal laser scanning microscopy, indicating the localization of the FITC labeled antibodies at the site of the Ki-67 protein (Fig. 1a, b).

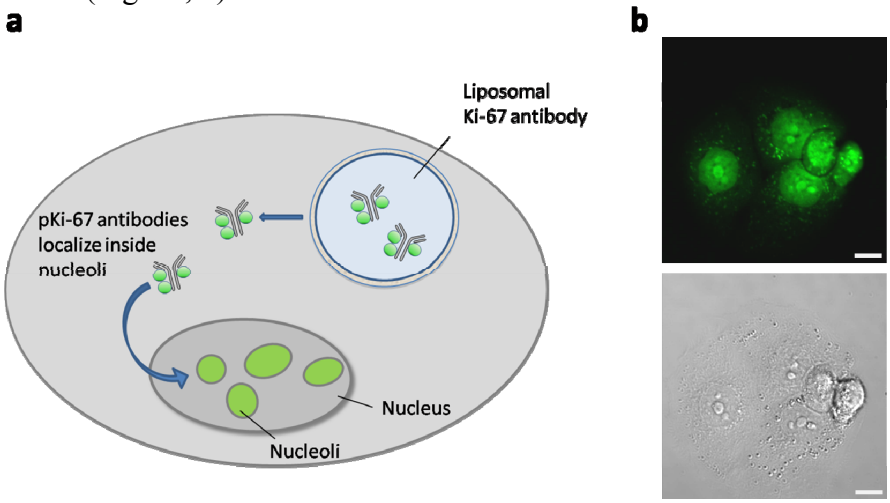

Fig. 1 (a) Schema showing the nanotechnology-based pKi-67 targeting strategy. (b) Confocal lasers can images showing delivery of TuBB-9-FITC to the cell nucleoli (scale bars, 10 μm).

2.2 Light irradiation leads to cell death in OVCAR-5 monolayer cultures

Next we investigated the effect of irradiation with an Argon laser at 488nm and 5J/cm^2 on cell viability of OVCAR-5 cells, which have been incubated with the liposomal constructs for 24 h. The MTT results showed a prominent decrease in viability of cells incubated with L-TuBB-9-

FITC and interestingly no significant effect on viability in cells incubated with L-MIB-1-FITC. Cell viability decreases 48 h after irradiation to 24.49% (Fig 2). The controls show that irradiation of the free TuBB-9-FITC antibody without liposomal envelope has no significant influence on cell viability. Also is cell viability unaffected by irradiation of cells without any conjugate or L-TuBB-9-FITC incubation without light.

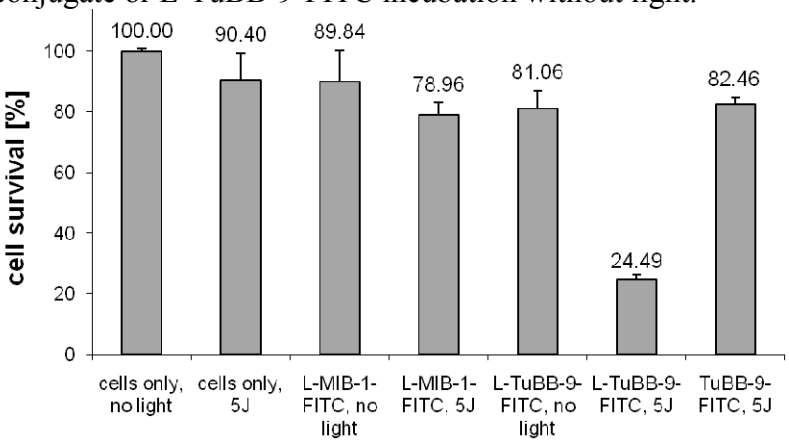

Fig. 2 Light irradiation leads to cell death in ovarian cancer cells, previously incubated with the liposomal TuBB-9 construct L-TuBB-9-FITC. Cell viability was assessed by MTT-assay 48 h after irradiation with a 488 nm Argon laser at 5 J/cm^2.

2.3 Irradiation of non-cancer human lung fibroblasts cells (MRC-5)

Flow cytometer stainings of pKi-67 in MRC-5 cells demonstrated that non-confluently grown cells were almost to 80% positive for pKi-67, while cells grown to confluency expressed pKi-67 in less than 20% of cells. To test the treatment efficacy on low Ki-67 expressing non-cancer cells we incubated MRC-5 cells with the constructs and determined cell viability after light irradiation. Interestingly the MRC-5 cells grown to confluency showed no significant effect on cell viability with neither of the constructs (Fig. 3). In contrast, non-confluently grown cells showed a decrease in cell viability to 22%, when incubated with the L-TuBB-9-FITC constructs.

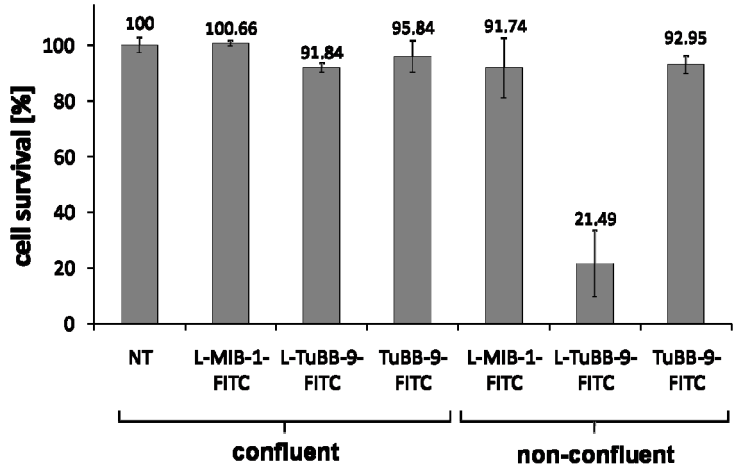

Fig. 3 Treatment of human lung fibroblasts cells (MRC-5) shows the specificity of the approach for pKi-67 positive proliferating cells. Only non-confluent cells (high pKi-67) show a significant reduction in viability after incubation with L-TuBB-9-FITC.

3. CONCLUSIONS

Here, we show the first antibody-targeted inactivation of a nucleolar protein in large cell populations and consequently the first evidence that inactivation of pKi-67 leads to cell death in proliferating cells. Most protein knock-down methods interfere with the synthesis pathway of the protein; however, here we show an innovative approach for targeting the active protein itself. Packaging of the antibodies inside liposomes made the delivery into the cytoplasma of cells

possible. In agreement with earlier findings, that the pKi-67 fraction recognized by TuBB-9 is involved in the synthesis of ribosomal RNA[12,14], light irradiation of ovarian cancer cells showed a significant decrease in viable cells when incubated with liposomal TuBB-9-FITC constructs, but not if incubated with liposomal MIB-1-FITC constructs. For the MIB-1 recognized fraction a function has not been addressed yet. It also demonstrates vividly the specificity of the photo inactivation, because photoactivatable FITC is delivered to the nucleoli of the cells with both antibodies, but only pKi-67 targeted with TuBB-9 constructs lead to cell death. Interestingly, low pKi-67 expressing human lung fibroblasts showed no significant effect on cell viability after treatment. This may make an additional targeting of the constructs for *in vivo* therapy obsolete. For *in vivo* studies a PS with higher absorbance in the red wavelength range should be used for higher tissue penetration of the light. Taken together, we show a novel approach for targeting and elimination of proliferating cancer cells. The results of the study suggest that targeting pKi-67 with nanotechnology-based PDT could be an attractive alternative cancer therapy.

ACKNOWLEDGEMENTS

We acknowledge support from the National Institute of Health (NIH) Grant R01 CA119388-03. R.R. acknowledges support from Deutsche Forschungsgemeinschaft (DFG) Grant Ra1771/1-1

REFERENCES

1. Gerdes, J., Schwab, U., Lemke, H. & Stein, H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int.J.Cancer* **31** (1), 13 (1983).
2. Gerdes, J. *et al.* Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J.Immunol.* **133** (4), 1710 (1984).
3. Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J.Cell Physiol.* **182** (3), 311 (2000).
4. Dougherty, T. J. *et al.* Photodynamic therapy. *J Natl Cancer Inst* **90** (12), 889 (1998).
5. Hasan, T., Ortel, B., Moor, A. C. E. & Pogue, B. W., in *Cancer Medicine* edited by H. Frei (BC Dekker, Inc, New York, NY USA, 2003), pp. 605.
6. Hasan, T., Ortel, B., Solban, N. & Pogue, B., in *Cancer Medicine*, edited by D. W. Kufe et al. (B.C. Decker, Inc., Hamilton, Ontario, 2006), pp. 537.
7. Price, M., Reiners, J. J., Santiago, A. M. & Kessel, D. Monitoring singlet oxygen and hydroxyl radical formation with fluorescent probes during photodynamic therapy. *Photochem Photobiol* **85** (5), 1177 (2009).
8. Sengupta, S. *et al.* Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system. *Nature* **436** (7050), 568 (2005).
9. Torchilin, V. P. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* **4** (2), 145 (2005).
10. Peer, D. *et al.* Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* **2** (12), 751 (2007).
11. Sengupta, S. & Sasisekharan, R. Exploiting nanotechnology to target cancer. *Br J Cancer* **96** (9), 1315 (2007).
12. Bullwinkel, J. *et al.* Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J.Cell.Physiol.* **206** (3), 624 (2006).

13. Key, G. *et al.* New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest.* **68** (6), 629 (1993).
14. Rahmanzadeh, R., Hüttmann, G., Gerdes, J. & Scholzen, T. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif.* **40** (3), 422 (2007).