Reactive Oxygen Species (ROS)-responsive Protein Modification and Its Intracellular Delivery for Targeted Cancer Therapy

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Introduction: Although protein-based drugs have shown success, they have been limited mostly to cytokines, growth factors, enzymes and monoclonal antibodies, all of which function primarily extracellularly. There are a number of diseases, including genetic diseases and cancers, that have the potential to be treated through proteins with an intracellular target. However, proteins alone are not usually able to cross the cell membrane in order to reach their intracellular targets. An efficient and safe tool to deliver active proteins into the cytosol of targeted cells is highly desirable to advance protein-based therapeutics.

Mounting evidence suggests that, compared with their normal counterparts, many types of tumor cells and tissues have increased levels of reactive oxygen species (ROS). These increased levels of ROS provide a biochemical basis for developing novel therapeutic strategies exploiting ROS for targeted cancer treatment.

We report herein a convenient chemical approach to reversibly modulate protein (RNase A) function and develop reactive oxygen species (ROS)-responsive protein for targeted cancer therapy. The conjugation of RNase A with 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl carbonate (**NBC**) blocks protein lysine and temporarily deactivates the protein. However, the treatment of RNase A-NBC with hydrogen peroxide (one major intracellular ROS) efficiently cleaves NBC conjugation, restoring RNase A activity. Thus, RNase A-NBC can be reactivated inside tumor cells by high levels of intracellular ROS, thereby restoring the cytotoxicity of RNase A for cancer therapy. Due to higher ROS levels inside tumor cells than non-tumor cells, and resulting different levels of RNase A-NBC reactivation, RNase A-NBC has significantly specific cytotoxicity against tumor cells.

Methods:

ROS-responsive Modification of RNase A: RNase A was modified with *NBC* in 0.1 M $NaHCO_3$ buffer solution (pH = 9.5). The crude products were purified with Amicon Ultra for three times.

Lipid-protein nanoparticle formulation. Lipid EC16-80 was dissolved in a NaOAc buffered solution (25 mM, pH = 5.2) at a concentration of 1 mg/mL. EC16-80 and RNase-NBC were mixed in phosphate buffer (25 mM, pH = 7.5) at a weight/weight ratio (protein: lipid) of 1:2 for DLS measurements.

In Vitro Protein delivery. After the addition of the lipidoid/protein complexes to the B16F10, cells were incubated at 37 ºC for an additional 48 h. The cell viability was determined by MTT assay after 48 h of incubation. All transfection experiments were performed in quadruplicate.

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Results and Discussion: The capability of EC16-80/RNase A-NBC nanoparticles to selectively inhibit tumor cell proliferation was studied by testing a panel of non-cancerous and cancer cell lines. In this study, three non-cancer cell lines, including NIH-3T3 (embryonic fibroblast cells), Raw 264.7 (murine macrophages), and NCF (neonatal rat cardiac fibroblast) were exposed to EC16-80/RNase A-NBC nanoparticles under the same conditions as a panel of cancer cell lines (HeLa cervical cancer cells, B16F10 melanoma cells, PC-3 prostate cancer cells, and MDA-MB-231 breast cancer cells). The cytotoxicity of EC16-80/RNaseA-NBC complex against the proliferation of all seven cell lines was compared using an MTT assay. As shown in Figure 1, the four types of cancer cells decreased in viability to less than 40% after EC16-80/RNase A-NBC exposure (1.3 μg/mL of protein) and 24 h of treatment, however, all non-cancerous cells retained over 90% viability with the same treatments. The growth inhibition of non-cancer Raw264.7 cells was only observed when the cells were exposed to higher concentration of protein (up to 5.2 μg/mL) and longer treatments. These results suggest that RNase A-NBC treatment is highly selective in inhibiting tumor cell proliferation.

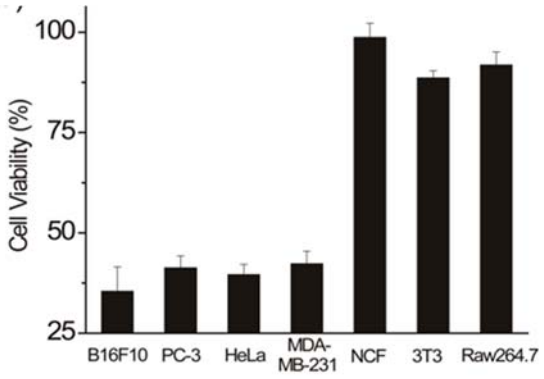

Figure 1. (a) Viability of EC16-80/RNase A-NBC pre-treated B16F10 cells with and without the second PMA (200 nM) treatment. (b) EC16-80/RNase A-NBC delivery selectively prohibited tumor cell proliferation.

Conclusion: we report the design of a ROS-responsive protein and its intracellular delivery as a tool for targeted cancer therapy. The chemical conjugation of lysine residues with aryl boronic acid (NBC) temporally de-activated proteins, and ROS-triggered NBC cleavage restored protein function in a highly selective manner. The EC16-80/RNase A-NBC nanoparticles prohibited tumor cell growth in both protein-concentration and intracellular-ROS-level-dependent manners.