Single Polyplex Based Image-Guided Combined siRNA and Enzyme/Prodrug Cancer Therapy

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Introduction: We recently synthesized a prototype agent[1] consisting of a cancer therapeutic prodrug enzyme labeled with multimodal MR and optical imaging reporters. The prodrug enzyme, cytosine deaminase (CD), converts a non toxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). This prototype agent has been used to demonstrate the feasibility of image-guided prodrug enzyme therapy using MRI to minimize cytotoxic side-effects[2] (Figure 1). In the next stage of these studies we are using this prototype platform to combine prodrug enzyme delivery, and the delivery of siRNA to target choline kinase. Choline kinase downregulation with siRNA has been previously shown to amplify the effect of 5-FU in human breast cancer cells but not nonmalignant breast cells[3]. Downregulation of choline kinase can also be detected by spectroscopic imaging. A combined siRNA prodrug enzyme strategy can be used to target repair enzymes and sensitize cancer cells to therapy while minimizing damage to normal tissue.

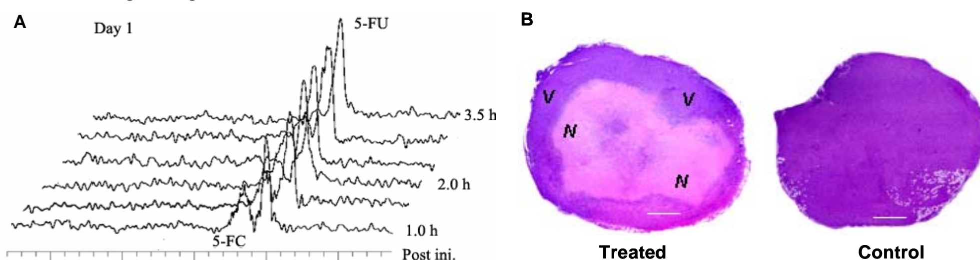

Figure 1. (A) *In vivo* ^{19}F MRS of a wild type MDA-MB-231 tumor when 5-FC (450 mg/kg *i.v.*) was injected 2 h after administration of bCD-PLL conjugate (1000 mg/kg *i.v.*). (B) Representative histological sections of MDA-MB-231 tumor 8 days after the treatment of bCD-PLL (250 mg/kg)/5FC (200 mg/kg) or PBS only. Regions ***V*** indicate viable tumor tissues, and the regions ***N*** indicates tumor necrosis (***N***). *Scale bar*, 1.0 mm.

Methods: Bacterial cytosine deaminase (bCD) was chosen as the therapeutic enzyme due to its high enzymatic and thermodynamic stability[4]. Linear polyethyleneimine (LPEI) (MW = 25 kDa) was selected as siRNA delivery vector due to its strong buffering capacity that helps the escape of siRNA from endosomes[5]. siRNA specific for choline kinase (siRNA-chk) with the sequence 5'-GCUGUUCCAGUGCUCC-3' was prepared a duplex[6]. The complexation between PEI and siRNA was achieved through electrostatic interactions. In this preliminary study, LPEI was PEGylated to improve its water-solubility and biocompatibility, and functionalized with Alexa Fluor-633 to dynamically monitor the intracellular delivery and biodistribution of targeting conjugate *in vitro* and *in vivo*. The fluorophore can also track the enzyme in excised tissue. The bCD protein and PEI moiety were conjugated through a cleavable disulfide bond to enable the transit of PEI-siRNA polyplexes from endosomes. The resulting bCD-PEI-siRNA-chk polyplex (MW > 300 kDa, Figure 2A) is expected to extravasate into the tumor interstitium, but not the normal tissues due to the high permeability of tumor vasculature.

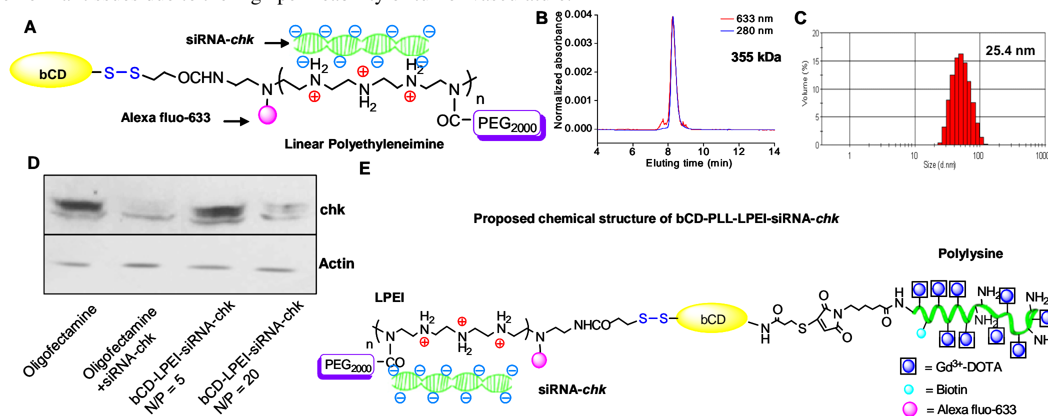

Figure 2. (A) Schematic of bCD-LPEI-siRNA-chk polyplex. (B) GPC of bCD-LPEI conjugate monitored at 280 and 633 nm. The overlapping of peaks monitored at 280 and 635 nm confirmed the conjugation between bCD and LPEI. (C) Hydrodynamic radius and size distribution of bCD-LPEI conjugate measured by dynamic light scattering (DLS). (D) Choline kinase Western blots of controls and bCD-LPEI-siRNA-chk polyplex (N/P ratio = 5 and 20) treated MDA-MB-231 cells at the 48-hour time point. Actin levels were probed as loading controls. (E) Schematic of bCD-PLL-LPEI-siRNA polyplex with Gd^{3+} chelators.

Results and Discussion: The molecular weight of the bCD-LPEI conjugate was determined as 355 kDa by size-exclusive chromatography, and its hydrodynamic radius were measured as 15.4 nm by dynamic light scattering (Figure 2B–C). Kinetic study demonstrated only slight enzymatic activity loss (< 7%) of the conjugate to both substrates cytosine and 5-FC compared with that of free bCD protein. The knockdown efficiency of bCD-PEI-siRNA-chk was studied in human breast MDA-MB-231 cell cultures and oligofectamine was used as a positive control. The corresponding choline kinase levels at different N/P ratios were determined by immunobolt using anti-choline kinase antibody as shown in Figure 2D. The highest choline kinase knockdown efficiency of this conjugate was observed with N/P ratio of 20 when siRNA concentration was 100 nM. We are evaluating intracellular delivery and subcellular location of bCD-PEI-siRNA-chk by confocal fluorescence microscopy. Cytotoxicity of bCD-PEI-siRNA-chk polyplex and its therapeutic efficiency induced by the combined enzyme/prodrug chemotherapy and siRNA interference will be evaluated by MTT assay in MDA-MB-231 breast cancer cells.

Conclusion: In summary, we have developed a novel prototype of prodrug-activating enzyme conjugate. This conjugate not only demonstrated high enzymatic activity, but also has the ability to deliver siRNA into the cytoplasm of cancer cells with a substantial down-regulation of choline kinase. The conjugate combines the promise of two cancer therapeutic strategies, chemotherapy and RNAi at the same time. The visualization of this conjugate by non-invasive diagnostic modalities *in vivo* can minimize the systemic toxicity by optimizing the timing of the prodrug injection. After verifying the combined siRNA and enzyme/prodrug therapeutic effectiveness from the prototype agent bCD-LPEI-siRNA, we are currently preparing another polyplex bCD-PLL-LPEI-siRNA polyplex (Figure 2E), in which multiple Gd^{3+}-DOTA chelates are labeled on a PLL moiety that is conjugated with bCD. This will achieve a single polyplex based multimodal image-guided combined siRNA and enzyme/prodrug cancer strategy.

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