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Aptamer-SiRNA Technology Provides a Simple Platform for Cancer Combination Treatment

Hong Yan Liu*

Georgia Cancer Center, Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta University, Augusta, Georgia, USA

***Corresponding author:** Hong Yan Liu, Georgia Cnacer Center, Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta University, Augusta, USA. Tel: + 707-721-7149; Email: HOLIU@augusta.edu

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Cancer is inherently complex [1-3]. Cancer cells may adopt several mechanisms to escape a single-regimen therapy by switching signaling pathways, activation of alternative pathways and cross-talks among varied pathways [4,5]. To address tumor complexity/heterogeneity and effective blocking signaling compensation and cross-talks, the combination therapy is essential to co-target multiple oncogenic pathway simultaneously [6,7].

Combination of small chemical drugs and/ or kinase inhibitors usually shows some efficacy initially, but reaches plateau. Small chemical drugs and kinase inhibitors have no tumor targeting capability and tend to kill most dividing cells. Their combinations have shown the overlapping toxicities that have limited the extensive combination. Although antibody has tumor targeting capabilities, it has high immunogenicity, high cost, and inaccessibility to the targets locating in cytoplasm and nuclei. Antibody production is associated with growing animals, and purification and transportation suffer from high cost since protein-based antibody is sensitive to temperature and pH value and cannot be renatured after denature.

Small interfering RNA (siRNA) has emerged as a third class of therapeutics after antibody and chemical drugs and is promising to address "undruggable" targets [8,9]. In 2018, FDA approved first siRNA drug patisian which represents that siRNAs will fulfil the promise to be a new class of medicine. It only takes 12 years since Nobel Prize was awarded to Fire and Mello in 2006 for their discovery of RNA interference mechanism.

Recently, aptamers (synthetic DNA/RNA ligands) have proven to be a promising platform for delivering siRNA into cells [10-12]. Aptamers are developed through an iterative in vitro selection process called as SELEX (systematic evolution of ligands by exponential enrichment) [13,14]. Cell-based SELEX allows the selection of internalized aptamer which can induce cell-targeted cargo delivery through receptor-mediated endocytosis [15,16]. Like antibody, aptamer has high affinity and high specificity to the targets. In contrast to antibody, aptamer can be generated in vitro and is thermostable and can be easily renatured after denature [17].

Aptamer-siRNA chimera (AsiC), employing only RNA molecules, is a new targeting therapeutic [18,19] and has shown the promise of minimizing off-target effects that are usually associated with small molecule drugs, and immunogenicity associated with antibody-based therapeutic. As a single-component entity, AsiC also has advantages in ease of synthesis and high tissue penetrability. Importantly, AsiC-based drugs can utilize endogenous enzymes (e.g. dicer, argonaute) and enable cell type- and mRNA sequence-specific gene silencing, which can provide selective and effective inhibition of protein targets regardless their cellular localization. For examples, CD4 aptamer-tat/rev siRNA chimera has shown the efficacy in inhibition of HIV transmission, PSMA aptamer-PLK1siRNA enables the regression of prostate cancer [20]. CTLA4 aptamer-STAT3 siRNA inhibits tumor-associated Tregs and reduces tumor burden in multiple mouse tumor models [21]. EpCAM aptamer-survivin siRNA enables reversal of doxorubicin resistance and prolongs survival in mice bearing chemo-resistant tumors [19].

In our studies, we have developed a bivalent aptamer-dual siRNA chimera: PSMA aptamer-survivin siRNA-EGFR siRNA-PSMA aptamer chimera (PSEP) as shown in (Figure 1A). PSMA is a prototypical cell-surface marker of Prostate Cancer (PCa) [22,23]. We have advanced current aptamer-siRNA technology, one aptamer and one siRNA , and developed a bivalent aptamer-dual siRNA chimera PSEP targeting EGFR and survivin1. PSEP is able to suppress angiogenesis and tumor growth in PCa subcutaneous xenografts. Bivalent PSMA aptamer with antibody like structures offers significantly increased target specificity and enhanced cargo internalization compared with current single aptamer constructs. This platform technology enables delivery of multiple siRNAs into target cells efficiently. PSEP contains a bivalent PSMA aptamer

and two siRNAs specific to EGFR and survivin, thus PSEP is a triple targeting molecule by targeting prostate tumor and two oncogenes (EGFR and survivin). Although EGFR overexpression is associated with castration resistance and bone metastasis of PCa [24], and survivin has been correlated with recurrence and therapeutic resistance of PCa [25], single target antagonists against EGFR [26] or survivin [27] have limited effect on PCa treatment. Co-targeting EGFR and survivin with one drug has not been reported. From our studies, co-targeting EGFR and survivin with PSEP chimera can significantly suppress neo-angiogenesis and inhibit tumor growth, which is better than any single target antagonist alone. Survivin has been known as an "undruggable" target since only those molecules express on cell surface or having enzymatic activity are considered to be druggable. By virtue of siRNAs, survivin can be easily targeted which will fill the gap in the design of survivin inhibitors, and EGFR can be targeted without the issue of drug resistance, unlike EGFR chemical inhibitors which can be pumped out of cells by P-glycoprotein [28]. Prostate tumor- specific delivery will overcome off-target effect and reduce systemic toxicity. In contrast to combinations of chemical drugs and antibodies, combination of siRNAs driven by tumor specific aptamers have shown low toxicity, low immunogenicity, and high tumor targeting capability.

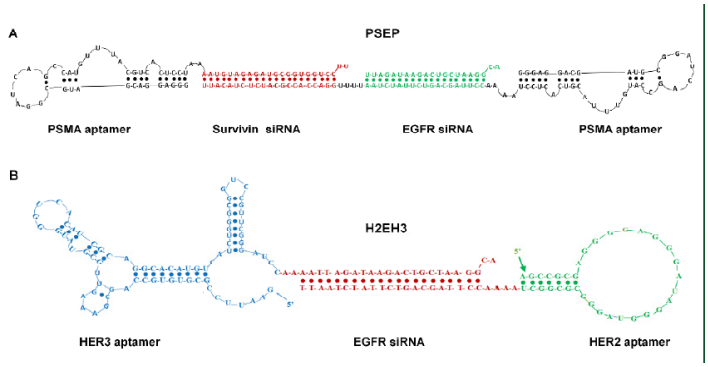

Figure 1: Combination treatments by aptamer-siRNA. **(A):** PSEP chimera containing bivalent PSMA aptamer and two siRNAs targeting EGFR and survivin [1]. PSEP is prostate cancer specific and enables silencing both EGFR and survivin genes; **(B):** Three-in-one H2EH3 chimera. H2EH3 is constructed by inserting EGFR siRNA between HER2 and HER3 aptamers. H2EH2 enables down modulation of EGFR/HER2/HER3 simultaneously [2].

In line with the notion of combination treatment, for HER-dependent cancers, co-targeting HER family is essential to over HER family member signaling cross-talk. The HER family consists of EGFR (HER1), HER2, HER3 and HER4. It is well known HER family is interdependent and displays functional redundancy in that the blockade of one HER receptor can often be compensated by another HER family member [29]. Through homo- and hetero-dimerization upon ligand binding, HER family members are well characterized to be capable of activation of PI3K/AKT survival pathway [30]. Extensive studies show that resistance to trastuzumab and lapatinib is associated with upregulation of EGFR and HER [31,32]. HER3 is a key node in many cancers and involved in the resistance against EGFR- and HER2- targeted therapies [33,34]. It has been demonstrated that resistance to lapatinb is due to increased EGFR-HER3 signaling. HER3-neuregulin signaling confers resistance to T-DM1. Since HER4 has generally been correlated with a favorable outcome in cancer patients [35,36], thus HER4 is not included as a therapeutic target in current HER family targeted studies. However, current HER2-targeted drugs do not have HER3 targeting agents. HER3 receptor can activate PI3K signaling in the absence of EGFR and HER2 function. Therefore, improved agents capable of inactivating EGFR, HER2, and HER3 in parallel are in need.

Antibody combination is the current strategy in targeting HER family. Pan-HER with six antibody mixture targeting EGFR, HER2 and HER3 has been developed and is able to overcome resistance to cetuximab [37] and trastuzumab [38]. However, most of antibodies are high cost in production and high immunogenicity. The six-antibody combination is expected to have significantly increased the toxicity and increased patient burden.

To address immunogenicity and high cost associated with Abs, in our current studies, we have engineered a multiple function AsiC with a siRNA and two RNA aptamers (Figure 1B). This chimera was constructed such that a single EGFR siRNA is positioned between HER2 and HER3 aptamers to create a HER2 aptamer-EGFR siRNA-HER3 aptamer chimera (H2EH3).The new three-in-one chimera is able to target EGFR/ HER2/ HER3 simultaneously [2].

In this design, EGFR siRNA was delivered to cells through two different aptamers (HER2 and HER3) which specifically target HER family. We have taken advantage of a well-known molecular mechanism that HER2/HER3 receptors upon ligand binding will form heterodimers, then internalize to cytoplasm. We propose that EGFR siRNA positioned between HER2 and HER3 aptamers can be delivered to HER2 expressing cells. We have proved this hypothesis and visualized EGFR silencing in cell lines and xenografts. In this three-in-one H2EH3 chimera, HER2 aptamer and HER3 aptamer play dual functions: 1) as agonists to block HER2 and HER3 signal pathways; 2) as delivery agents to drive EGFR siRNA to HER2- and HER3-expressing cells. EGFR siRNA situated between HER2 aptamer and HER3 aptamer also have two functions: 1) silencing EGFR and 2) as an adaptor to bridge HER2 aptamer and HER3 aptamer. Without three-in-one construction, single EGFR siRNA would not work since free siRNA cannot diffuse to cytoplasm. The new therapeutic is promising to replace complicated antibody combinations for treating trastuzumab resistant breast cancer. Three-in-one H2EH3 chimera with larger size than any single components will provide longer circulation time than single HER3/ HER2 aptamer alone, and render EGFR siRNA

function. H2EH3 chimera will have advantages over antibody in ease of production and modification, low immunogenicity, low cost, high thermos-stability, and high tissue penetration capability. In addition, generation of aptamer-siRNA will have less batch-to-batch variation. With three-in-one structure, new chimera will simplify the processes and cost in manufacturing, preclinical and clinical testing, and less complicated for patient administration. New three-in-one design will provide new therapeutic paradigm to address HER network and overcoming resistance to the therapies designed to target single HER family receptors.

In aforementioned studies, we have used chemical modification to increase nucleic acid chimera circulation stability by inclusion of 2′F-pyrimidines. 2′F- modification has been proved to confer ribonuclease resistance in vivo from our [39,40] and other laboratories [1,41-43] and is used in FDA proved siRNA drug Patisiran [45]. Extensive studies have shown that aptamer-siRNAs do not stimulate innate immunity and not elicit antibodies [21,44,45].

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In summary, we have proved the concept that AsiC can be constructed to simultaneously target multiple therapeutic targets with tumor specificity. The modularity of AsiC opens a way to design various combination treatment with a wide array of siRNAs and appearing tumor specific aptamers. The combination will increase overall size of AsiC that will prolong chimera circulation time. Owing to low immunogenicity, none toxicity, ease of generation and modification, AsiCs will play important roles to combat cancer complexity and block cancer cell signaling pathway switch.

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