REVIEW

Advanced therapeutic platforms based on Non-Classical tripodal interfering RNA structural format

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The flexibility of human RNAi (RNA interference) machinery to accommodate unconventional structures has opened the way for the development of novel RNAi triggering formats, suitable for advanced therapeutic applications. Non-classical siRNA structural variants show improved functional features over the conventional siRNA formats including increased potency, reduced non-specific responses, and enhanced cellular delivery. These non-classical RNAi structures with enhanced structural and functional properties, in combination with appropriate delivery vehicles, can be an effective therapeutic module. Here we summarize our recent efforts on expanding the structural repertoire of classical siRNAs, using tripodal interfering RNA formats, to develop novel RNAi based therapeutics for advanced health-care applications.

Keywords: RNA interference; small interfering RNA; tripodal interfering RNA; nanoparticles; cationic polymers

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The salient feature of RNAi technology is their ability to utilize the cellular machinery mechanism responsible for the post-transcriptional gene silencing (PTGS). RNAi mechanism allows targeting of complementary transcripts, and this often results in potent down-regulation of gene expression in most eukaryotic cells [1]. Short duplex RNA units, referred to as small interfering RNAs (siRNAs), are regarded as the most powerful PTGS tool available for both functional genomics and therapeutic applications. siRNA typically featuring 19–21bp long double stranded RNA (dsRNA) structure, usually recognized by the RNAi machinery, are capable of executing sequence-dependent degradation of target mRNA [2]. Foremost advantage of RNAi technology is their ability to knock-out virtually any gene in the cells with high degree of specificity, providing a broader therapeutic window compared with conventional small-molecule drugs.

Ever since the significance of RNAi technology was established on a clinical setting, wide range of gene delivery systems have been evaluated for their systemic delivery [3].An ideal delivery technology, which can effectively regulate the therapeutic response of siRNAs, seems a key factor in developing clinically relevant therapeutic RNAi platforms. Even with their success in the pre-clinical and clinical stages, lipid-based platforms for siRNA delivery suffer limitations regarding their safety and target organs (mainly targeting liver). Synthetic cationic polymers appear to be the most eligible candidate for RNAi delivery, as they can be rationally designed to incorporate various functional domains capable of executing endosomal buffering and tissue targeting [4]. However, the structural incompetency of short dsRNAs to form stable and effective nano-complexes with cationic polymers remains a major obstacle.

A conventional siRNA features rigid structure and poor charge density, and this often limits its association with the polymeric delivery vehicle. Thus, development of

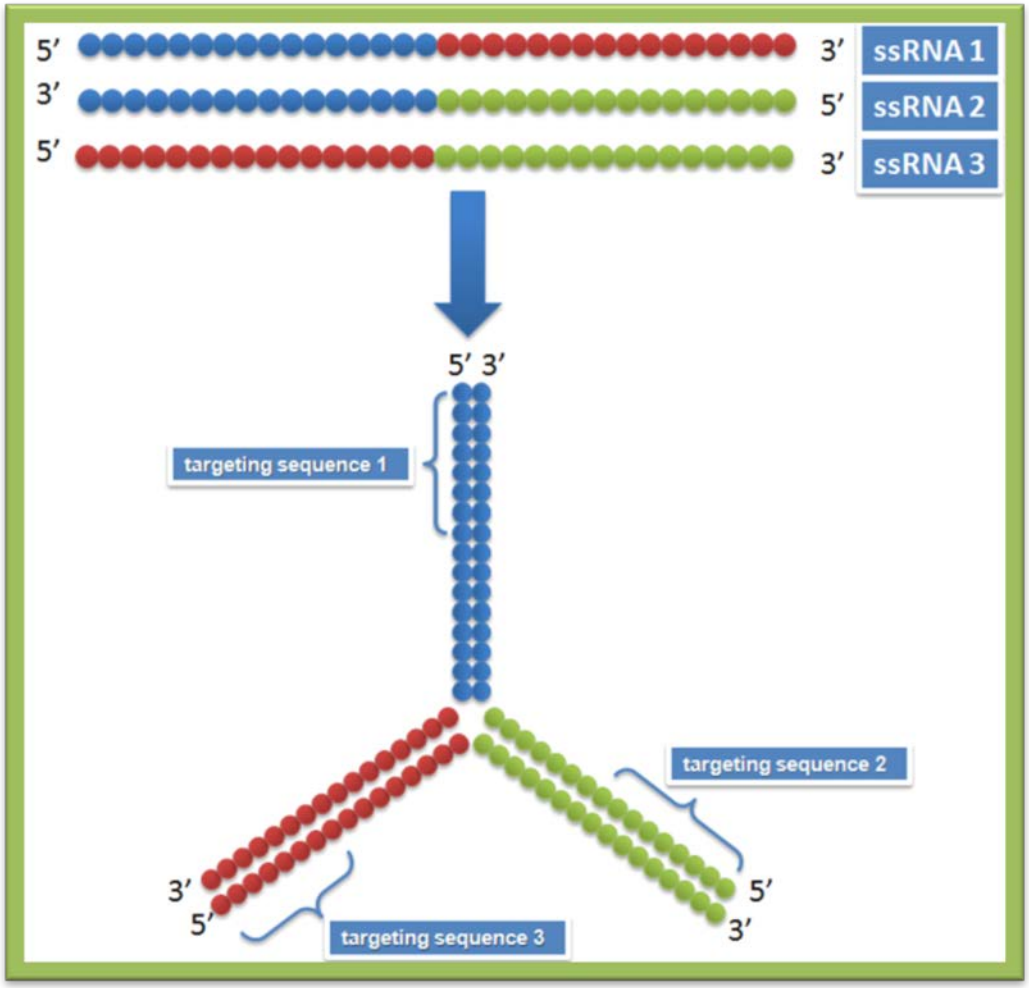

Figure 1. Schematic representation of tripodal interfering RNA (tiRNA) structure. Tripodal interfering RNA (tiRNA) was constructed via complementary base paring of three single stranded RNA (ssRNA) units. Each 5' terminus of the tiRNA structure encodes target sequence of interest and this structure can implement multi-target gene silencing. (Complementary sequences share same color coding in this scheme).

unconventional RNAi-triggering structures, with increased charge density and improved backbone flexibility, was undertaken to enhance the affinity between cationic polymers and inhibitory RNAs [5-10]. Chemical conjugation and/or complementary hybridization strategies have been employed to generate polymeric siRNAs resembling the feature of long plasmid DNA, and this allowed the formation of stable polyelectrolyte complexes upon interaction with the cationic carriers [5-7]. However, unlike conventional siRNAs, these polymeric siRNAs lacks well-defined structural format and their exact intra-cellular processing mechanism can be ambiguous. Therefore, our strategy has been focused mainly on developing well-defined RNAi triggering units, by taking advantage of facile complementary hybridization strategy [11-13].

In an effort to develop novel RNA interfering molecules with superior gene silencing and enhanced delivery efficacy over conventional siRNAs, we designed a tripodal-interfering RNA (tiRNA) format with multiple gene targeting capability [12]. This structure was designed via complementary base paring strategy, by paring three single stranded RNA (ssRNA) units. Combination of three ssRNAs resulted in the formation of Y-shaped dsRNA structures, with three different siRNA sequences embedded at each arm (Figure 1). For each arm, antisense sequence against target mRNA was paired with a complementary sense sequence from another ssRNA, similar to that of classical siRNAs. In other words, a tiRNA structure can be considered as a combination of three different siRNAs held in a tripodal structural format. Apart from their ability to knock-down multiple genes simultaneously, salient feature of tiRNA structures was their superior gene silencing efficacy compared with conventional siRNA structures, especially when combined with conventional cationic polymers.

Our original tiRNA structure was constructed by annealing three 38-nt ssRNAs, with each duplex region in the tiRNA composed of a 19 bp siRNA unit. We first constructed a tiRNA structure harboring three siRNA units intended for silencing Lamin A/C (siLamin), DBP (siDBP), and TIG3 (siTIG3) mRNA, respectively. This tiRNA structure was capable of executing multiple-target gene silencing with enhanced RNAi efficacy, over corresponding mixture of conventional siRNAs, when complexed with positively charged cationic polymers [12]. Physicochemical studies supported the concept that the efficacy of tiRNA structure was largely due to their enhanced interaction with cationic polymers, possibly due to the increased charge density and molecular flexibility of the RNA units. Compared with other non-conventional RNAi triggering formats, such as polymeric siRNAs, the tripodal structure retains a well-defined structure and can execute specific RNAi-mediated gene silencing without the induction of any innate immune response.

We have also designed a well-defined T-tiRNA structure (T-tiSurvivin) that harbors three RNAi units of siSurvivin, with a trebler-DNA core [13]. In this structure, nucleotide extension was provided at the 3'-end of the antisense strand to facilitate interaction with the trebler-DNA core. T-tiRNA stucture was so designed that the 5' region of the antisense strand of each siRNA units was orientated towards the outside region, and this format retains the gene silencing activity of the individual siRNAs. When complexed with cationic delivery vehicles, quite similar to that with tiRNAs, T-tiRNAs showed enhanced gene knock-down efficacy over conventional siRNA mixture.

Next, we set out to investigate the optimal size limit of tripodal RNA structure for effective gene silencing activity. To address this issue, we constructed surrogate nucleic acid tripodal structures using complementary DNA units and their interaction with cationic polymers were studied systematically [14]. tiDNA of varying length were designed by changing the length of backbones DNA units and their complexation efficacy with cationic polymer was evaluated. By using ssDNA of size ranging from 16-40 nt, we observed that DNA units shorter than 26nt were unable to form the expected tripodal structure. In due course of experiments, we also observed that tiRNA composed of 32nt units was most appropriate of our experiments, and further experiments were performed with this structural format.

In an effort to choose the most appropriate delivery system for tiRNA delivery *in vivo*, we screened several potential candidates. Cationic polymers composed of polyethyleneimine units were polymers of our choice, owing to their inherent nucleic acid binding capability and the ability to facilitate endosomal escape pathways for the RNAi triggers[15]. However, the nucleic acid delivering ability of PEI is largely jeopardized by their toxicity effects; efforts have been made to develop biocompatible delivery system based on PEI units while retaining their nucleic acid delivering ability. In our studies, we choose a linear version of PEI, with 25 kDa, which is largely used in the delivery of pDNA. Linear PEI is not considered an effective reagent for siRNAs, largely due to their inability to deliver short RNA versions. With the introduction of tiRNA structure, we expected improved RNA-polymer association and this might further enhance their therapeutic potential. Physico-chemical characterizations clearly validated our expectations, as tiRNA structure formed stable nano-sized complex with linear PEI and showed much higher cellular uptake in our preliminary *in vitro* studies [14].

We then tested whether improved physico-chemical properties of tiRNA-PEI complex would be translated into an actual RNAi efficacy. This was validated with gene knock-down experiments using qRT-PCR, by checking the relative expression of endogenous targets in cancer cells with respect to house-keeping gene (GAPDH). The results clearly demonstrated the efficacy of tiRNA molecules over siRNA counterparts with PEI-based delivery system [14]. Next, efforts were made to include a targeting moiety to our tiRNA-PEI complex. We designed a liver-targeting delivery system based on galactose-modified PEI and this was further complexed with tiRNA structure. With a conclusive proof-of-concept in our *in vitro* experiments, we decided to perform an *in vivo* efficacy test for tiRNA.

We chose to use an Apolipoprotein B (ApoB)-targeting tiRNA sequences for *in vivo* investigations. Three different ApoB-targeting sequences were assembled into a single tiRNA format, and activity test was performed on *in vivo* animal models along with their siRNA counterparts. Results clearly validated the efficacy of tiRNA formats even under *in vivo* conditions. tiRNA assembled with cationic polymers were more effective in reducing ApoB mRNA level compared to a combination of siRNAs with similar carrier.

Considering the therapeutic potential, these functional RNA structures may offer researchers a novel tool for targeting multiple disease genes with enhanced therapeutic efficacy. However, the exact response of RNAi machinery towards these unconventional RNAi triggers is still unclear; further studies are required to elucidate the exact intracellular processing mechanism. Nevertheless, our studies indicate that the design of novel siRNA structural variants may provide alternative options to combat various diseases with improved safety and efficacy.

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