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Human ribonuclease Dicer inhibition using RNA aptamers

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Abstract

The aim of this invention was to select RNA aptamers that specifically bind human ribonuclease Dicer, which is one of the main enzymes that take part in miRNA biogenesis and the biogenesis of other short regulatory RNAs, and then to find out if pre-selected molecules affect enzyme activity. Other aims of this solution include: 1) using RNA aptamers as ribonuclease Dicer competitive inhibitors; 2) using RNA aptamers that are allosteric ribonuclease Dicer inhibitors, 3) identifying RNA aptamers that are selective inhibitors of emergence of selected miRNAs.

Key words: Ribonuclease Dicer, SELEX, aptamers

State of the art

To read out the information that is stored in genomes of living organisms is one of the most important tasks faced by contemporary molecular biology and bioinformatics. It has been proven that a single gene can be a source of more than one type of transcript. Furthermore, many different mRNAs may arise from a single type of transcript. Only in bacteria and simple animal or plant organisms, most of the genome encodes proteins. In higher organisms, protein coding sequences constitute only a small portion of the genetic material (less than 5% in humans). A real turning point in research concerning mechanisms that govern the expression of genetic information has taken place due to an unexpected discovery of the phenomenon called RNA interference (RNAi) (Fire et al., 1998). It has been found that sequences that encode regulatory RNA molecules, the so-called miRNAs, are present in genomes of eukaryotic organisms. miRNA containing transcripts are called pri-miRNA. In their structures, there usually occur about 50-80 nucleotide long, not fully complementary double-strand regions, which, due to their shape, are referred to as the hairpin type structures. It was observed that miRNAs arise both in plants and animals, but their maturation is different in both types of organisms. In case of human cells, three basic stages of miRNA biogenesis can be distinguished (Bartel, 2004). At the first one, the enzyme called Drosha cuts a double-strand fragment (hairpin structure) out of the pri-miRNA. At the second stage, the newly emerged 50-80 nucleotide (nt) molecule – pre-miRNA is transported from nucleus to cytoplasm by exportin 5. At the third stage, pre-miRNA is recogni-

zed by ribonuclease Dicer, which cuts a 20-23 nt duplex out of it. Then, the duplex is conveyed into another protein complex RISC (RNA-induced silencing complex). At the next stage, RISC is activated. This process consists of removing one strand of the duplex, whereas the other one, called miRNA, remains within the complex, and serves as a probe that makes it possible to specifically recognize fully or partially complementary RNA molecules (Nykanen et al., 2001; Hammond et al., 2000). The active RISC complex can bind with mRNA. If mRNA is fully complementary to the miRNA that is present in the RISC complex, then it is cut (Haley and Zamore, 2004). As a consequence, within a relatively short time, the whole mRNA pool becomes degraded, and the so-called posttranscriptional gene silencing (PTGS) takes place. However, if mRNA is only partially complementary to the miRNA that is present in the RISC complex, then it is not degraded, but remains bound with the protein complex, and thus it cannot serve as a template for protein synthesis. As a result, the gene is silenced at the level of translation (TR – translational repression).

Ribonuclease Dicer is a member of the RNase III family, i.e. those endoribonucleases that specifically cut double-strand RNA molecules. All enzymes that belong to this family are characterized by the presence of one or two ribonuclease domains, called the RNase III domain. As a result of dsRNA digesting by RNases III, short duplexes are generated. They possess a phosphate group at the 5′ ends, and two non-paired nucleotides at the 3′ ends. In case of miRNA biogenesis, ribonuclease Dicer is responsible for cutting 20-23 nt miRNA – containing duplexes, out of precursor pre-miRNAs. Based on biochemical data, it has been found that human ribonuclease Dicer and bacterial RNase III contain only one active centre, in which both of the RNA strands are cut (Zhang and Nicholson, 1997; Macrae, 2006). It was also proved, however, that in case of human Dicer, two RNase III domains act independently from each other (i.e. each of them cuts only one of the strands) (Macrae 2006).

Aptamers are relatively short RNA or DNA molecules, which bind with a strictly defined molecule with high affinity and specificity (Tuerk and Gold, 1990; Ellington and Szostak, 1990). Up till now, a substantial number of various aptamers has been obtained. They were selected as a ligands binding a wide spectrum of individuals, from small inorganic molecules to single-cell organisms (Blank and Blind, 2005; Nimjee et al., 2005; Yan et al., 2005; Proske et al., 2005). Specificity and high binding force (dissociation constant amounting to nano- or picomoles) result in aptamers being often compared to antibodies. Aptamer molecular mass is 10-15 kDa on average, i.e. about 10 times less than the mass of a single antibody (White et al. 2000), which makes it easier for them to be spread within the organism and to be promptly removed from the blood circulation system.

Aptamers are usually obtained by *in vitro* selection that is called the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method. The method makes it possible to identify the molecules that possess a desired property, e.g. strong binding with a selected molecule (target molecule – TM), from the input pool of many RNA or DNA molecules with an undefined, random sequence, called a combinatorial library. It is a set of all possible to synthesize oligonucleotides with definite length. Such oligonucleotides beside the centrally situated random sequence, also contain flanking regions with known nucleotide sequences. They are used to amplify and clone molecules within the library. The number of oligonucleotides that create the full combinatorial library (complete input pool) depends on random sequence's length since in each of its positions, one of the four nucleotides: A, G, C, or T/U can occur. Therefore, in case of n-nt random sequence, it is possible to synthesise 4^n of various oligonucleotides.

Typical RNA aptamer selection takes place according to the following scheme. Initially, a possibly high number of single-strand DNA molecules that contain n-nt random sequence is obtained by chemical synthesis. These molecules are then converted into dsDNA, which serves as a template to synthesise a combinatorial library of ssRNA molecules, by the *in vitro* transcription method. RNA is then incubated with a selected target molecule (TM). Then, all non-bound RNA molecules are removed and molecules bound with TM are recovered from the complex. RNAs thus obtained are converted into dsDNA by the RT-PCR using starters that are complementary to flanking sequences. The product obtained serves as a template to synthesise the narrowed RNA pool by *in vitro* transcription that then undergo the selection process again. Upon having conducted the planned number of selection cycles, dsDNA is ligated into the plasmid and cloned in bacterial cells. Finally, individual clones are sequenced. In this way, the sequences of RNA molecules that bind tightly with TM are identified.

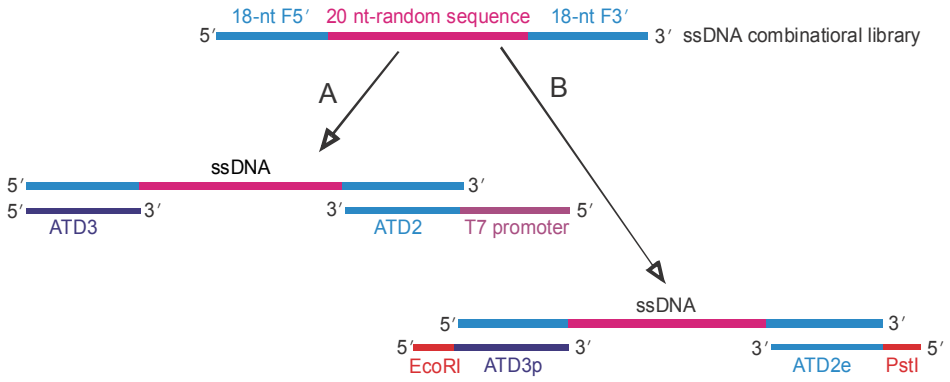

Fig. 1. Graph presents the ssDNA combinatorial library and starter sequences used in *in vitro* selections. ATD2 and ATD3 starters were used to obtain dsDNA that constituted a template for the *in vitro* transcription reaction. The ATD2 starter additionally contained RNA T7 polymerase promoter. ATD2e and ATD3p starters were used to obtain dsDNA, which was cloned to a plasmid, thus both of these starters contained sequences that were recognised by restrictive enzymes, EcoRI and PstI respectively

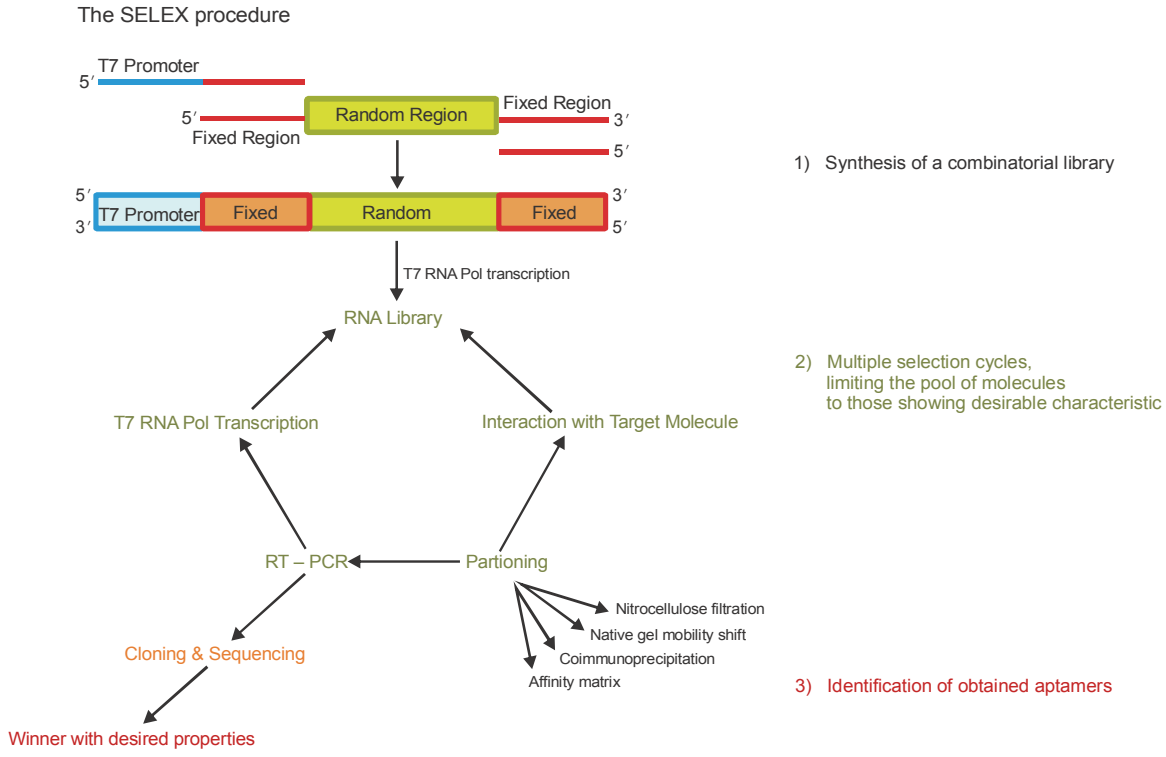

Fig. 2. In SELEX the initial random-sequence DNA pool can be easily manipulated and is typically generated by chemical DNA synthesis and contains a core of randomized nucleotides that are flanked by constant regions, which are required for enzymatic amplification. The constant regions may include promoter sequences, necessary for *in vitro* transcription of RNA, and specific restriction sites that aid cloning of selected species. Thus, the original single-stranded DNA pool can be readily converted into either a double-stranded DNA pool or a single-stranded RNA pool. There are three main steps of *in vitro* selection – SELEX: 1) synthesis of a combinatorial DNA library; 2) multiple cycles of selection that limit a pool of RNA molecules to those showing the desired characteristic; 3) identification of the obtained RNA aptamers

The subject of the presented invention is an *in vitro* method to inhibit ribonuclease Dicer, ribonuclease Dicer inhibitor, and use of RNA aptamers to inhibit ribonuclease Dicer. More specifically, this solution relates to using RNA aptamers as competitive ribonuclease Dicer inhibitors, use of RNA aptamers as allosteric ribonuclease Dicer inhibitors, and use of RNA aptamers as selective inhibitors of emergence of the selected miRNAs.

This invention fulfils the aims described as above, and solves problems related with the possibility to use RNA aptamers as ribonuclease Dicer competitive inhibitors and as allosteric ribonuclease Dicer inhibitors, as well as selective inhibitors of emergence of the selected miRNAs.

Claims

1) The *in vitro* method to inhibit ribonuclease Dicer, wherein ribonuclease Dicer is inhibited by means of RNA aptamer, characterised in that it is selected from the SEQ ID. NO. 1 to 4 and it includes the conditions of the selection process of RNA aptamers that specifically bind ribonuclease Dicer, checking the influence of preselected aptamers upon enzyme activity, wherein flanking sequences of pre-selected RNA aptamers contain from 10 to 30 nucleotides, while the centrally situated random sequence contains from 10 to 30 nucleotides, and using pre-selected aptamers to inhibit Dicer ribonuclease activity, whereas each preselected RNA aptamer contains two flacking sequences and a centrally situated random sequence and wherein ribonuclease Dicer is inhibited by means of the aptamer that acts upon the competition basis or the RNA aptamer, which is an allosteric inhibitor or the aptamer that acts as a selective or non-selective inhibitor of emergence of the selected miRNAs.
2) The method according to claim 1, wherein pre-miRNA cutting efficiency decreases together with increase in RNA aptamer concentration, whereas the scope of the aptamer's molar excess as compared to Dicer is contained within the range from 1 to 100.
3) The method according to claim 1 or 2, wherein mammalian, preferably human ribonuclease Dicer, is inhibited.
4) The ribonuclease Dicer inhibitor, wherein the inhibitor is an RNA aptamer, which has been obtained in the way of *in vitro* selection, and which binds ribonuclease Dicer, and it contains two flanking sequences and a centrally situated random sequence, wherein flanking sequences contain from 10 to 30 nucleotides, while the central random sequence contains from 10 to 30 nucleotides and it is selected from the SEQ ID No. 1 to 4.
5) Inhibitor according to claim 4, wherein acts upon the competition basis.
6) Inhibitor according to claim 4, wherein the inhibitor is an allosteric inhibitor.
7) Inhibitor according to claim 4, wherein inhibitor selectively or non-selectively inhibits emergence of the selected miRNAs.
8) Inhibitor according to claim 4, wherein it is mammalian, preferably human ribonuclease Dicer inhibitor.
9) Use of RNA aptamers, in which RNA aptamers are selected from the SEQ ID. NO. 1 to 4 and arc obtained in the way of *in vitro* selection and contain two flanking sequences each and the centrally situated random sequence, in which flanking sequences of preselected RNA aptamers contain from 10 to 30 nucleotides, while the centrally situated random sequence contains from 10 to 30 nucleotides, to inhibit ribonuclease Dicer.
10) Use according to claim 9, in which pre-miRNA cutting efficiency decreases together with increase in aptamer concentration, whereas the scope of the aptamer's molar excess as compared to Dicer is contained within range from 1 to 100.
11) Use according to claim 9, in which ribonuclease Dicer is inhibited by means of the aptamer that acts upon the competition basis.
12) Use according to claim 9, in which ribonuclease Dicer is inhibited by means of the RNA aptamer that is an allosteric inhibitor.
13) Use according to claim 9, in which ribonuclease Dicer is inhibited by means of aptamer that acts as selective or non-selective inhibitor of emergence of the selected miRNAs.
14) Use according to claim 9, in which mammalian, preferably human ribonuclease Dicer, is inhibited.

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