Biomaterials 122 (2017) 163–178

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

miRNases: Novel peptide-oligonucleotide bioconjugates that silence miR-21 in lymphosarcoma cells

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ARTICLE INFO

Article history:
Received 21 November 2016
Received in revised form
9 January 2017
Accepted 12 January 2017
Available online 13 January 2017

Keywords:
Peptide-oligonucleotide conjugates
RNA cleavage
Antisense oligonucleotides
Anti-miRNA therapy
Oncogenic miRNA
miR-21

ABSTRACT

MicroRNAs (miRNAs) are active regulators in malignant growth and constitute potential targets for anticancer therapy. Consequently, considerable effort has focused on identifying effective ways to modulate aberrant miRNA expression. Here we introduce and assess a novel type of chemically engineered biomaterial capable of cleaving specific miRNA sequences, *i.e.* miRNA-specific artificial ribonucleases (hereafter 'miRNase'). The miRNase template presented here consists of the catalytic peptide Acetyl-$[(LeuArg)_2Gly]_2$ covalently attached to a miRNA-targeting oligonucleotide, which can be linear or hairpin. The peptide C-terminus is conjugated to an aminohexyl linker located at either the 3′- or 5′-end of the oligonucleotide. The cleavage efficacy, structural aspects of cleavage and biological relevance of a set of these designed miRNases was assayed with respect to highly oncogenic miR-21. Several miRNases demonstrated effective site-selective cleavage of miR-21 exclusively at G-X bonds. One of the most efficient miRNase was shown to specifically inhibit miR-21 in lymphosarcoma cells and lead to a reduction in their proliferative activity. This report provides the first experimental evidence that metallo-independent peptide-oligonucleotide chemical ribonucleases are able to effectively and selectively down-regulate oncogenic miRNA in tumour cells, thus suggesting their potential in development of novel therapeutics aimed at overcoming overexpression of disease-related miRNAs.

1. Introduction

In the last decade, non-coding RNAs and in particular miRNAs have been the focus of considerable research due to their association with a range of pathological conditions, from cancer to neurodegenerative, cardiovascular and autoimmune diseases [1–3]. MiRNAs represent a new class of regulatory molecule; they are 18–25 nucleotides in length and, through binding to specific mRNAs, are capable of post-transcriptional repression of gene expression, either by the RNA interference pathway or by translational arrest [4–6]. A large amount of evidence suggests that miRNAs exert control over fundamental physiological processes, both within the cell and at the level of the whole body [7,8]. Disturbance in normal miRNA expression results in changes in the activity of target genes and is often associated with the initiation and progression of a wide spectrum of diseases, including oncology [9–12]. Tumour development is accompanied by an excess or deficiency of certain miRNAs compared to normal tissues. Increased expression of oncogenic miRNAs contributes to the development of neoplasia by suppression of tumour-suppressor genes, whereas a significant lack of tumour-suppressor miRNAs results in the overexpression of oncogenes [13,14].

Modulation of activity of tumour-associated miRNAs is therefore of great scientific, biomedical and clinical interest. To date, a number of approaches have been developed for microRNA-based and miRNA-targeted therapies [6,15]. Restoring the level of deficient miRNA can be achieved by using miRNA mimics, representing synthetically prepared miRNAs [16,17]; or viral constructs, encoding for miRNAs [18,19]. Suppression of miRNA activity can be achieved using small-molecule inhibitors, acting at the transcriptional level [20]; via miRNA sponges, representing transcripts that contain

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http://dx.doi.org/10.1016/j.biomaterials.2017.01.018
0142-9612/

multiple tandem-binding sites adsorbing deleterious miRNAs [21,22]; by miR-mask oligonucleotides or target protectors, that are fully complementary to predicted miRNA binding sites in the 3′-UTR of the target mRNA [23–25]; and using antisense oligonucleotides, complementary to the target miRNA and inducing either its degradation or steric blockage [26,27].

Numerous positive results have been achieved using strategies based on inhibition of oncogenic miRNAs. Indeed, the approaches based on miRNA suppression using miRNA-sponges, representing molecular traps, were shown to restore the activity of tumour-suppressor genes. For example, miRNA sponges designed for modulation of miR-10b, miR-21, miR-155 and miR-221/222 mediate an impact on the activity of many protein targets, such as HOXD-10, PDCD4, Smad4, SRC3, Bcl-2 Bim, FOXO3a, PTEN and RhoA. Thus they provide inhibition of proliferation, activation of apoptosis and increase in the sensitivity of tumour cells to chemotherapy [28–30]. Suppression of oncogenic activity of miR-522 in a non-small cell lung cancer model was successfully achieved by the use of miRNA masking [31]. Antisense technology also has proven successful in blocking miRNA activity: synthetic oligonucleotides of different chemistries targeted to known oncogenic miRNAs, such as miR-17, miR-21, miR-155, and miR-221/222 promoted not only induction of apoptosis and inhibition of proliferation, but also tumour regression and metastasis suppression *in vivo* [32–38]. The drugs Miravirsen and Regulus RG-101, aimed at suppressing miRNA-122 for the treatment of hepatitis C, have successfully progressed into clinical trials, and thus provide grounds to believe that, in the near future, effective antisense-based anti-miRNA therapies will be developed to combat oncopathology [39,40].

Effective downregulation of miRNA levels in cells can be achieved by its selective, irreversible cleavage using agents that are capable of recognizing particular miRNA sequences. A direct approach to create such an artificial site-selective ribonuclease can be based on design of conjugates comprising of (i) antisense oligonucleotides (asON), which can form a complementary complex with a specific miRNA, and (ii) chemical moieties able to cleave phosphodiester bonds. These include metal complexes, imidazoles or cleaving peptides [41]. Over the last couple of decades, some progress has been achieved in the field of designing site-selective artificial ribonucleases [42–54]. It was shown that short peptides, containing either alternating leucine and arginine residues or imidazole-based catalytic groups, conjugated to antisense oligonucleotides targeting tRNA, were able to hydrolyze linkages adjacent to an oligonucleotide-binding site without involvement of exogenous species such as metal ions, enzymes or cofactors (*e.g.*, RISC, RNase H) [42,44,45,47,50,51]. Effective cleavage of complementary substrates was also demonstrated for tris(2-aminobenzimidazole) ribonuclease conjugated to PNA oligomers [49]. It is important to emphasize though that none of the above developments have been demonstrated against clinically relevant RNA sequences, and most of the studies in this area have been carried out so far using either short, linear RNA sequences or model RNAs (*e.g.* tRNAs).

Despite the fact that considerable success has been achieved in the area of development of site-selective, metal-free artificial ribonucleases, to date no studies have been reported demonstrating a successful downregulation of clinically significant miRNAs by such metallo-independent sequence-specific catalytic bioconjugates in eukaryotic cells. The key challenge of our research therefore was to assess whether such chemically engineered peptide-oligonucleotide conjugates (POCs) could potentially induce detectable downregulation of specific oncogenic miRNA in tumour cells, which is essential factor for future application in anticancer therapy. Here we report the development of miRNA-specific peptide-oligonucleotide conjugate ('miRNases') against highly oncogenic miR-21, and present the first experimental evidence of efficient, site-selective cleavage of this miRNA exclusively at G-X linkages by the most successful structural variants. Furthermore, we demonstrate here that one of the most efficient conjugates is capable of inducing specific inhibition of miR-21 in lymphosarcoma cells. This leads to reactivation of tumour-suppressor protein PDCD4, the direct target of miR-21, and subsequent reduction in proliferative activity of lymphosarcoma cells. To obtain structural insights into specific interactions between miRNases and miR-21 and to guide the future design of such conjugates, we carried out 1 μs molecular dynamics simulation of the hybridized complex between **5′-h-6/14** conjugate and miR-21.

2. Materials and methods

2.1. Instrumentation

The details of chemicals, reagents and facilities used in this research have already been provided in our previous publications [50,51]. The software Topspin 3.2 was used to analyse the NMR data obtained. After electrophoresis the gels were analysed using Molecular Imager FX (Bio-Rad, USA). PCR amplification was carried out using Bio-Rad iQ5 (Bio-Rad, USA).

2.2. Oligonucleotides

Oligodeoxyribonucleotides with an aminohexyl linker attached to either the 5′- or 3′-terminal phosphate of the oligonucleotide sequence were synthesized in the Laboratory of Medicinal Chemistry, ICBFM, Russia, by the standard phosphoramidite protocol on an ASM-800 synthesizer (Biosset, Russia) using solid support, nucleoside phosphoramidites and chemical phosphorylation reagent from Glenn Research (USA). Oligonucleotides were isolated by consecutive ion-exchange (Polysil SA-500 columns, Russia) and reverse-phase HPLC (LiChrosorb RP-18 columns, Merck, Germany) according to standard protocols.

2.3. Peptide synthesis and purification

Catalytic peptide Acetyl-(LeuArg)$_2$-Gly-(LeuArg)$_2$-Gly-COOH was synthesized by manual solid-phase methodology on Fmoc-Gly-Wang resin using the Fmoc/^{t}Bu strategy as described in Ref. [50]. Following completion of the peptide sequence, the N-terminus was acetylated by shaking with acetic anhydride (10 mmol) and DIPEA (10 mmol) in DMF (10 mL) for 30 min, followed by resin washing with DMF (2 × 10 mL), DCM (2 × 10 mL) and DMF (2 × 10 mL). After acetylation of the sequence, the peptide was cleaved from the resin using the same protocol reported in Ref. [50]. Crude lyophilized peptide was solubilized in 30% acetic acid and purified using RP-HPLC as described earlier [50].

2.3.1. Acetyl-(LeuArg)$_2$-Gly-(LeuArg)$_2$-Gly-COOH

Fractions collected at 33 min were combined and lyophilized to yield the TFA salt of the peptide as a fluffy white material. ESI-MS: m/z = 626.4 for $[M + H + H]^{2+}$ (MW = 1250.80 calcd. for $[C_{54}H_{102}N_{22}O_{12}]$). ^{1}H NMR ($D_2O$ with TSP (0.1 μM), 400 MHz): δ 0.83–0.97 (m, 24H, Leu-H$^{\delta}$), 1.49–1.94 (m, 28H, 8 × Arg-H$^{\beta}$, 8 × Arg-H$^{\gamma}$, 8 × Leu-H$^{\beta}$, 4 × Leu-H$^{\gamma}$), 2.04 (s, 3H, CH_3), 3.21 (m, 8H, Arg-H$^{\delta}$), 3.82–4.41 (m, 12H, 2 × Gly-H_2, 8 × Leu/Arg-H$^{\alpha}$) (see also Fig. S1).

2.4. Synthesis of the conjugates

Oligonucleotides containing an aminohexyl linker attached to either the 5′- or 3′-end of the oligonucleotide, (50 nmol) in H_2O