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Development of hammerhead ribozymes targeting miR-21

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Polish application
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Applicant: Institute of Bioorganic Chemistry PAS, Poznań, Poland
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Title of invention: *Rybozymy typu "hammerhead", kompozycja, środek terapeutyczny je obejmujące, ich zastosowania oraz sposób hydrolizy miR21 i prekursorów miR21*
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Applicant: Institute of Bioorganic Chemistry PAS, Poznań, Poland
Inventors: M.Z. Naskręt-Barciszewska, A. Belter, K.M. Rolle, M. Piwecka, P. Sosińska, A. Fedoruk-Wyszomirska
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Applicant: Institute of Bioorganic Chemistry PAS, Poznań, Poland
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Abstract

The object of the invention are hammerhead ribozymes directed against the sequence of miR-21 and/or miR-21 precursors, having the ability to specifically cleave miR-21 and/or miR- 21 precursors, and wherein they have acatalytic core with a sequence as shown in SEQ ID No 1. The invention also relates to a composition comprising such ribozymes, a therapeutic agent comprising them, a use of such ribozymes and a method of selective cleavage of miR-21 and/or 1 miR-21 precursors employing such ribozymes.

Introduction

Despite tremendous efforts worldwide, glioblastoma multiforme (GBM) remains a deadly disease for which no cure is available. Recently, miR-21 has emerged as a key omnipotent player in carcinogenesis, overexpressed in most human tumors including brain tumors. miR-21 is. It is recognized as an indicator of glioma prognosis and a prosperous target for anti-tumor therapy.

Here we show the idea, which is protected by law, that hammerhead ribozymes can be rationally designed to target miR-21 and/or its precursors. This approach enables specific hydrolysis of these RNAs resulting in a significant decrease of their levels in glioblastoma-derived cell lines. Our results demonstrate that anti-miRNA catalytic nucleic acids can be potentially used as a therapeutic agent.

Demand for the invention

Gliomas are the most common type among cancers of the central nervous system. Glioblastoma multiforme (GBM) represents more than 50% of all gliomas and is the most malignant (grade IV according to WHO malignancy grade) type from all the primary brain tumors. GBM is characterized by infiltrative growth pattern, abundant vascularization, rapid proliferation and aggressive clinical course. Moreover, because of its localization

and substantial resistance to conventional therapies, survival prognosis are very poor.

Invariably, for many years the standard treatment in gliomas remains surgical resection accompanied with radio-and chemotherapy. Maximum cytoreduction (> 98% of the tumor) prolongs survival for as long as 9-12 months. It also improves subject's response to radio- and chemotherapy. Very often due to tumor localization and its infiltrative character, the surgical intervention is not possible. Radiotherapy (RT) is usually implemented as the first adjuvant therapy after tumor resection. The standard treatment in chemotherapy is temozolomide (TMZ) and gliadel (Westphal et al., 2003; Stupp et al., 2005). In case of recurrence, faster and more aggressive growth of the tumor, as well as its enhanced resistance to treatment is observed among the patients treated with TMZ and RT.

In recent years, there was a significant progress in understanding the molecular basis of GBM. Many therapeutic targets and many potential therapeutic agents have been identified. Most of the new therapeutic approaches are focused on small-molecule inhibitors, monoclonal antibodies and peptides vaccines, used for the regulation of cellular pathways crucial for tumor development, angiogenesis and elimination of tumor cells drug resistance. Despite the good prognosis for these approaches, the majority of them have been rejected during clinical trials. Gliomas are one of the most difficult tumors to treat, with the worst prognosis and average survival time of less than one year.

In the absence of effective treatments for gliomas and their resistance to conventional therapies, the challenge is to study new therapeutic targets and new approaches to the treatment of GBM.

Downregulation of miR-21 with anti-miR-21 tools

Recently, miR-21, non-coding regulatory RNA molecule, has been identified as a new target for brain tumor therapy. The miR-21 content in the clinical specimens of brain glial tumors is significantly higher in comparison to the levels of this miRNA in healthy cells with cerebral origin and its expression level correlate well with the tumor's malignancy and it is significantly higher in patients with a grade III tumor, compared to the patients with a grade II tumor.

So far, the possibility of employing molecular tools targeting miR-21 has been analyzed (i.e. small molecule inhibitors) (Gumireddy et al., 2008). However, the disadvantages of the proposed tools as therapeutics are: poorly understood mechanism of action, low specificity of the compounds, potential additional inhibition of other pre-miRNA and low therapeutic index. The abovementioned studies did not directly relate to glial tumors (Table 1).

Anti-miR-21 antisense oligonucleotides (AS-ON) have been tested in glial tumors (Kurreck, 2003). The AS-ONs used in the context of miR-21 and glioblastoma were used in basic research, to verify and identify potential target sequences for the miRNA and to investigate the effects of miR-21 inhibition. However AS-ONs have many disadvantages such as: short half-life in serum, susceptibility to degradation by endo- and exonucleases (Campbell et al., 1990), low specificity of the AS-ONs (so-called off-target effect), lower stability of AS-ON:RNA complexes, hampered transport of the AS-ONs through cell membranes and blood-brain barrier, the necessity of using a carrier, immunological response induction by the synthetic AS-ONs containing unmetylated CpG dinucleotides and dependency of the AS-ON:miRNA heteroduplex degradation on the endogenous protein machinery, mainly RNase H (Table 1).

A hammerhead ribozyme and its modified, nuclease-resistant variant, complementary to pre-miR-21 and miR-21 are known (Suryawanshi et al., 2010). Its catalytic activity was shown at *in vitro* conditions with respect to pre-miR-21, but not to mature miR-21 (Table 1).

The aim of treatment with hammerhead ribozymes

The object of the invention are the hammerhead ribozymes that specifically and efficiently cleave miR-21 RNA and/or its precursors (pri-miR21 and pre-miR-21), the composition and the therapeutic agent comprising them, their use for the downregulation of cellular miR-21 in therapy of brain tumors and the method of cleavage of miR-21 and/or its precursors.

The invention relates to hammerhead ribozymes targeting miR-21 and its precursors.

Hammerhead ribozymes are the most well-known and the smallest catalytic RNAs (Ferre-D'Amara et al., 2010). There were found in viroids and viral satellite RNAs. During viroid replication, hammerheads act in *cis*-, self-cleaving the RNA in which they are embedded through a single turnover mechanism. Their activity is due to the catalytic core, their specificity due to the ribo-

Table 1. Examples of molecular tools targeting miR-21 and their disadvantages

Anti-miR-21 molecular tool	Disadvantages
Small molecule inhibitors	Poorly understood mechanism of action, low specificity of the compounds, potential additional inhibition of other pre-miRNA and low therapeutic index. The above mentioned studies did not directly relate to glial tumors
Anti-miR-21 antisense oligonucleotides	Short half-life in serum, susceptibility to degradation by endo- and exonucleases, low specificity of the AS-ONs (so-called off-target effect), lower stability of AS-ON:RNA complexes, hampered transport of the AS-ONs through cell membranes and blood-brain barrier, the necessity of using a carrier, immunological response induction by the synthetic AS-ONs containing unmetylated CpG dinucleotides and dependency of the AS-ON:miRNA heteroduplex degradation on the endogenous protein machinery, mainly RNase H
Hammerhead ribozyme and its modified, nuclease-resistant variant	Rribozymes catalytic activity were shown at *in vitro* conditions with respect to pre-miR-21, but not to miR-21. The observed effects of the use of ribozymes in cell culture were the following: decrease in the pool of endogenous miR-21, increased expression of PDCD4 protein (miR-21 target protein) and reduced cell survival. It has not been demonstrated however that the known ribozymes cleave mature miR-21 as well as pre-miR-21. Immunological response in case of ribozyme containing modified dinucleotides is highly probable

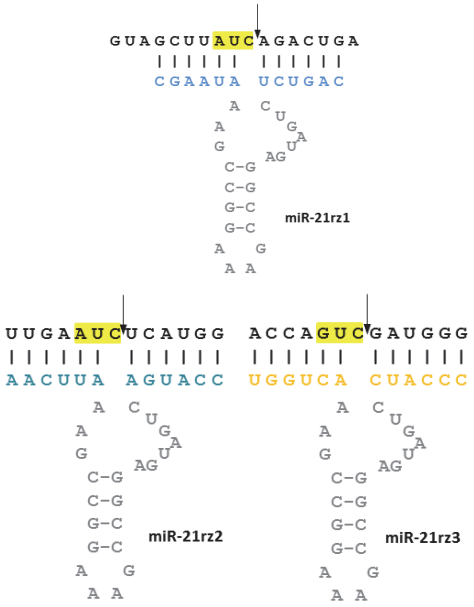
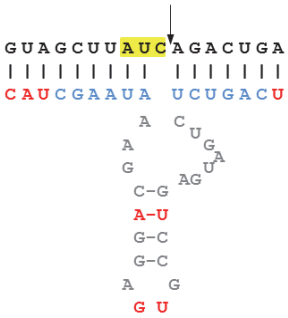
zyme arms complementarity to the sequences of the substrate flanking the cleavage site. Ribozymes catalyse a sequence-specific hydrolysis of RNAs containing a 5′-UH-3′ type trinucleotide, wherein N means any nucleotide, U means uridine and H means adenosine, cytosine or uridine, which is not linked by hydrogen bonds. The efficiency of the hydrolysis of the bonds depends mainly on the target RNA sequence (Sun et al., 2000). Other factors determining their activity, together with specificity, are the length and composition of the ribozyme arms surrounding the cleavage site. The arms ensure stable bonding of the ribozyme to the substrate and also facilitate its release from the cleavage products, such that the ribozyme becomes available for subsequent substrate molecules. Elongation of the arms may be associated with a decrease in hydrolysis efficiency; while their shortening may be associated with a decrease in ribozyme specificity.

The invention relates to H. Suryawanshi et al. hammerhead ribozymes targeting miR-21 and its precursors and its modified, nuclease-resistant variant, complementary to pre-miR-21 and miR-21 are known. Its catalytic activity was shown at *in vitro* conditions with respect to pre-miR-21, but not to miR-21. The observed effects of the use of ribozymes in cell culture were the following: decrease in the pool of endogenous miR-21, increased expression of PDCD4 protein (miR-21 target protein) and reduced cell survival. It has not been demonstrated however that the known ribozymes cleave mature miR-21 as well as pre-miR-21. Based on the complementarity of the arms sequence to miR-21 and on the activity against pre-miR-21of the ribozymes described by H. Suryawanshi et al. it can only be theorized that the ribozyme may be active against miR-21 as well. Also, the cell-based tests do not prove an activity of the proposed ribozymes against miR-21. The observed cellular effects may be a result of cleavage of pre-miR-21 only. Immunological response in case of ribozyme containing modified dinucleotides is high.

The aim of our invention is to overcome the indicated disadvantages and to provide new improved hammerhead ribozymes that specifically and efficiently cleave miR-21 and/or its precursors, while their properties enable their use in reducing the miR-21 pool in the treatment of brain tumors. The aim of the present invention is also to provide new uses of the improved ribozymes that efficiently cleave miR-21 and/or its precursors in the treatment of diseases with elevated cellular miRNA content for miR-21 and/or its precursors. The aim of the invention is also to provide a composition and therapeutic agents for the treatment of diseases with elevated cellular miRNA content for miR-21 and/or its precursors.

Moreover, the invention relates to a composition, comprising at least one ribozyme according to the invention or a mixture thereof. Such composition comprising ribozymes according to the invention, preferably con-

Table 2. Comparison of Ribozymes of polish and PCT applications and ribozymes being a state of art: Sequence of RNA substrate is marked in black, of ribozymes in green, nucleotides which differentiate A miR21rz1 of P.403341, PCT/IB2014/060188, EP 14719866,7 and ribozyme being a state of art in red, trinucleotide within substrate sequence recognized by ribozymes in yellow

<table>
<tr><th></th><th>Ribozymes designed by Inventors as claimed in of PCT/IB2014/060188</th><th>Ribozymes being a state of art</th></tr>
<tr><td>Ribozymes sequence</td><td> 
</td><td> 
</td></tr>
<tr><td>In vitro activity toward pre-miR-21 and miR-21</td><td>ribozymes miR21rz1 and miR21rz3 hydrolyze both miR-21 and pre-miR-21 at the physiological concentration (1 mM) of $MgCl_2$, preferably up to 5 mM, more preferably up to 10 mM, the most preferably at 25 mM Mg^{2+} concentration</td><td>ribozymes hydrolyze pre-miR-21 at 25 mM $MgCl_2$ (nonphysiological concentrations); the activity toward miR-21 has not been determined</td></tr>
<tr><td>Activity toward pre-miR-21 in EGFP reporter system</td><td>All ribozymes efficiently hydrolyze pre-miR-21 sequence. IC_{50} for each ribozymes in T98G and HeLa cells were determined
<table>
<tr><th></th><th>miR21rz1</th><th>miR21rz2</th><th>miR21rz3</th></tr>
<tr><td>IC_{50} (T98G)</td><td>115.5 nM</td><td>91.2 nM</td><td>99.2 nM</td></tr>
<tr><td>IC_{50} (HeLa)</td><td>60.2 nM</td><td>53.0 nM</td><td>69.2 nM</td></tr>
</table></td><td>not determined</td></tr>
<tr><td>In vivo activity on endogenous pool of miR-21</td><td>ribozymes miR21rz1, miR21rz2, miR21rz3 at 100 nM (ten times lower than in D1) concentration reduce the levels of endogenous miR-21 by respectively 80, 50 and 20%</td><td>modified and wild ribozymes at 1 μM concentration reduce the levels of endogenous miR21 levels respectively by 60 and 40%</td></tr>
<tr><td>In vivo activity on miR-21 targets (mRNA level)</td><td>mRNA of each analyzed miR-21 targets including PTEN, PDCD4, RECK and TIMP3 increase in T98G cells after transfection with ribozymes; miR21rz3: over 200% increase in PTEN and TIMP3 mRNA levels and around 100% increase in RECK and PDCD4 mRNA levels; miR21rz2: over 100% increase in PDCD4, TIMP3 and RECK mRNA levels, no effect on PTEN mRNA level miR21rz1: ~30%, ~80%, ~50% and ~90% increase in respectively PTEN, PDCD4, RECK and TIMP3 mRNA levels</td><td>not determined</td></tr>
<tr><td>In vivo activity on miR-21 targets (protein level)</td><td>miR21rz1, miR21rz2 and miR21rz3 ribozymes at 500 nM and 100 nM (in much lower concentration that in D1) concentration increase the level of PTEN protein by respectively 300, 200 and 500% and 100, 100 and 200%.</td><td>wild and modified ribozymes at 1 μM concentration increase the level of PDCD4 protein respectively by 80 and 60%</td></tr>
</table>

tains a carrier improving stability of nucleic acids or facilitating the transport of ribozymes through cell membranes. The composition preferably contains a carrier, which is Lipofectamine, for example Lipofectamine 2000.

The invented ribozymes in comparison with natural ribozymes:

- have higher activity against pre-miR-21 and miR-21,
- exhibit of the activity at physiological concentrations of magnesium ions (i.e. about 1 mM),
- consist of natural nucleotides only, without any chemical modifications, which allows to exclude the possibility of cellular response to modified nucleotides,
- efficiently reduce the levels of endogenous miR-21,
- increase mRNA levels of each analyzed miR-21 targets including PTEN, PDCD4, RECK and TIMP3 in T98G cells
- increase the level of PTEN protein (Table 2).

Perspectives

The invention is the object of patent applications (P.403341, PCT/IB2014/060188, EP 14719866,7) and has been proven recently. Preclinical pharmacological/pharmacokinetic testing, dosage formulation, safety testing of formulation, and clinical trials have to be completed before commercialization. Keeping in mind a high cost of these stages, the investors participation to the new drug approval is essential. The license granting is also considered at this stage.

The invention is the first stage to a new drug of a global range. Biotechnology and pharma companies, especially these specialized in RNAi therapeutics, such as may be the potential targetof the invention.

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