Smart functional nucleic acid chimeras
Enabling tissue specific RNA targeting therapy
Aaldering, Lukas J.; Tayeb, H.; Krishnan, S.; Fletcher, S.; Wilton, S. D.; Veedu, R. N.

Published in:
R N A Biology

DOI:
10.1080/15476286.2015.1017234

Publication date:
2015

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Aaldering, L. J., Tayeb, H., Krishnan, S., Fletcher, S., Wilton, S. D., & Veedu, R. N. (2015). Smart functional nucleic acid chimeras: Enabling tissue specific RNA targeting therapy. R N A Biology, 12(4), 412-425. https://doi.org/10.1080/15476286.2015.1017234

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 27. Apr. 2019
Smart functional nucleic acid chimeras: Enabling tissue specific RNA targeting therapy

Lukas J Aaldering, Hossam Tayeb, Shilpa Krishnan, Susan Fletcher, Stephen D Wilton & Rakesh N Veedu

To cite this article: Lukas J Aaldering, Hossam Tayeb, Shilpa Krishnan, Susan Fletcher, Stephen D Wilton & Rakesh N Veedu (2015) Smart functional nucleic acid chimeras: Enabling tissue specific RNA targeting therapy, RNA Biology, 12:4, 412-425, DOI: 10.1080/15476286.2015.1017234

To link to this article: http://dx.doi.org/10.1080/15476286.2015.1017234
Smart functional nucleic acid chimeras: Enabling tissue specific RNA targeting therapy

Lukas J Aaldering1,2, Hossam Tayeb3, Shilpa Krishnan4, Susan Fletcher5,6, Stephen D Wilton5,6, and Rakesh N Veedu1,3,5,6,*

1Nucleic Acid Center; Department of Physics, Chemistry and Pharmacy; University of Southern Denmark; Odense, Denmark; 2Institute of Plant Biology and Biotechnology (IBBP); University of Munster; Munster, Germany; 3School of Chemistry and Molecular Biosciences; The University of Queensland; Brisbane, Australia; 4Department of Nuclear Medicine; Odense University Hospital; Odense, Denmark; 5Center for Comparative Genomics; Murdoch University; Perth, Australia; 6Western Australian Neuroscience Research Institute; Murdoch, Western Australia

Keywords: aptamers, siRNA delivery, Aptamer targeted delivery, aptamer chimera, modified nucleotides, miRNA delivery

Introduction

Technological advancement in targeting and delivery of therapies to the site of action within a patient could greatly improve both the standard of living for a patient, as well as decrease costs associated with wasted therapeutics. Toward this goal, nucleic acid aptamers are re-emerging as a prominent class of biomolecules capable of delivering target specific therapy and therapeutic monitoring by various molecular imaging modalities. This class of short oligonucleotide ligands with high affinity and specificity are selected from a large nucleic acid pool against a molecular target of choice. Poor cellular uptake of therapeutic oligonucleotides impedes gene-targeting efficacy in vitro and in vivo. In contrast, aptamer-oligonucleotide chimeras have shown the capacity to deliver siRNA, antiimRs, small molecule drugs etc. toward various targets and showed very promising results in various studies on different diseases models. However, to further improve the bio-stability of such chimeric conjugates, it is important to introduce chemically-modified nucleic acid analogs. In this review, we highlight the applications of nucleic acid aptamers for target specific delivery of therapeutic oligonucleotides.

A major obstacle for effective utilization of therapeutic oligonucleotides such as siRNA, antisense, antimiRs etc. is to deliver them specifically to the target tissues. Toward this goal, nucleic acid aptamers are re-emerging as a prominent class of biomolecules capable of delivering target specific therapy and therapeutic monitoring by various molecular imaging modalities. This class of short oligonucleotide ligands with high affinity and specificity are selected from a large nucleic acid pool against a molecular target of choice. Poor cellular uptake of therapeutic oligonucleotides impedes gene-targeting efficacy in vitro and in vivo. In contrast, aptamer-oligonucleotide chimeras have shown the capacity to deliver siRNA, antiimRs, small molecule drugs etc. toward various targets and showed very promising results in various studies on different diseases models. However, to further improve the bio-stability of such chimeric conjugates, it is important to introduce chemically-modified nucleic acid analogs. In this review, we highlight the applications of nucleic acid aptamers for target specific delivery of therapeutic oligonucleotides.

© Lukas J Aaldering, Hossam Tayeb, Shilpa Krishnan, Susan Fletcher, Stephen D Wilton, and Rakesh N Veedu

*Correspondence to: Rakesh N Veedu; Email: R.Veedu@murdoch.edu.au; rveedu@sdu.dk

Submitted: 10/30/2014; Revised: 01/22/2015; Accepted: 01/28/2015

http://dx.doi.org/10.1080/15476286.2015.1017234

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

large proteins and even whole cells. These characteristics make aptamers an attractive platform for applications relating to drug delivery, biosensing and theranostics. During the first decade after the discovery, aptamers gained their foothold in therapeutic development.1,2 In 2004, vascular endothelial growth factor (VEGF) targeting RNA aptamer (Mucagen or Pegaptanib sodium) was approved by the Food and Drug Administration (FDA) for age related macular degeneration.3

Aptamers are typically generated from a large oligonucleotide pool (~1015 members) via an in vitro reiterative combinatorial selection process called Systematic Evolution of Ligands by EXponential enrichments (SELEX, Fig. 1).4-9 Although this process generally takes around 2–6 months, there are few reports of single or limited step aptamer selection protocols.10-13 It is noteworthy mentioning that aptamer selection procedure may sound simple enough, however, it may not be as straightforward. In some cases, often there may not be any aptamers depending on the diversity of the starting nucleic acid pool, or sometimes the developed aptamers may not be as specific as necessary even with proper negative control selections. Aptamers may possess several advantages over conventional antibody-based therapeutic approaches. First of all, aptamers do not require live animals for production as these can easily be synthesized in a synthetic laboratory setting in very large scale.14 Aptamer synthesis is not vulnerable to bacterial or viral contaminations. They generally have longer shelf-lives and are non-immunogenic, because aptamers are small in size, can easily access protein epitopes and also show better internalization, which is more difficult for large molecules such as antibodies.15,16 Additionally, aptamers offer freedom to introduce chemical modifications for conjugating additional chemical functionalities and also for systematic truncations of the parent aptamer itself.

Extremely promising approaches that has evolved during the last decade are the use of RNA interference (RNAi)17,18 using short interfering RNA (siRNA),19 antisense oligo (ASO)20 for silencing gene expression, and targeting microRNAs (miRNA)19-21 that are responsible for several diseases including tumor development. However, while siRNA, antisense and miRNA targeting therapies provide alternatives to conventional chemotherapies, significant hurdles related to the delivery and efficacy of treatment must still be overcome before this technology can be fully utilized. Indeed, in an in vivo setting, the application of nucleic acid-based technologies have been complicated by poor serum stability (due to the presence of nucleases), off-target effects and inability to gain sufficient
concentration at the required target site. Thus, it is clear that innovative methods of both packaging, delivery and targeting oligonucleotide therapies are required to advance this technology that has shown such huge promise in vitro. One promising strategy would be to develop and use aptamers targeting cell-surface receptors for effective cellular uptake via receptor-mediated endocytosis. In this regard aptamer selection against particular cells in vitro (Cell–SELEX) and against particular tissues in vivo (in vivo Selection, Fig. 2) would be very advantageous.

**Aptamers as Tools for siRNA Delivery**

RNA interference (RNAi) is a biological process that occurs at the molecular level and mediates gene silencing among the post-transcriptional modification process. RNAi has been harnessed for several years to cease the function of several genes for therapeutic purposes toward various diseases. A major obstacle for developing siRNA as therapeutic agents is to deliver them specifically to particular tissues. Many scientists aimed to solve this problem by investigating different guidance systems for siRNA, ranging from small molecules, lipids, peptides and synthetic nanostructures. Aptamers, chemical (non-protein) antibodies, are emerging as a promising tool for delivering siRNA.

With the dawn of new millennium, the application of aptamers was further extended to target specific delivery of therapeutic compounds. Due to their low immunogenicity, ease of production, freedom for chemical alteration and high target specificity, the scientific community quickly accepted this concept. Since then, the application of aptamers for delivering siRNA has been widely explored. For example, in cancer therapy, aptamers have shown great potential to deliver siRNA specifically to tumor cells, minimizing the cytotoxicity to normal cells and harsh side effects of chemotherapeutic drugs. Functional aptamer-siRNA chimera toward a wide range of diseases have been developed in recent years, making aptamer-siRNA chimeras one of the most rapidly growing class of therapeutics (Fig. 3 describes a possible mechanism of aptamer-siRNA chimera mediated gene silencing).

Chu and colleagues were among the first to perform a functional delivery of siRNA using an aptamer in 2006. In this work, they used aptamers against prostate-specific membrane antigen (PSMA). The aptamers A9 and A10 were reported to be capable of transporting nanoparticle into the cells expressing PSMA. Streptavidin–biotin interaction was utilized to construct an aptamer-siRNA chimera in which 2 biotinylated anti-PSMA aptamers were connected to 2 biotinylated siRNAs. These conjugates were not only able to deliver the siRNA efficiently to PSMA-expressing LNCaP cells in vitro but also decreased the amount of target mRNA expression level. In the same year, McNamara and colleagues reported the delivery of siRNA.
targeting polo-like kinase 1 (PLK1) and BCL2 to PSMA-positive LNCaP prostate cancer cells by using PSMA binding RNA aptamer A10. This remarkable work clearly demonstrated that the aptamer-guided siRNA delivery system efficiently decreased the proliferation of prostate cancer cells and apoptosis.

In 2008, Zhou and colleagues developed an aptamer-siRNA delivery system with dual inhibitory function for HIV-1 therapy.41 The dual inhibitory function means that both the aptamer and the siRNA components have potent anti-HIV activities, making this capable of targeting the disease at 2 different levels. In this work, they used an anti-gp120 RNA aptamer, targeting the gp120 glycoprotein, a surface protein on the virion that largely determines the entry of HIV into cells, its cellular tropism as well as elements of its pathogenesis.41-44 The aptamer itself is able to bind this protein and neutralize the strains.45 The other part of the chimera contains an anti-tat/rev siRNA that inhibits HIV replication. Zhou et al. showed that the aptamer-siRNA chimera was able to utilize gp120 expressed on HIV infected cells for the delivery of its anti-HIV siRNA. This study demonstrates vast potential of aptamer-siRNA chimeras, because it uses the full capacity of an aptamer and leading the technology from just a target specific ligand to a full therapeutic tool to significantly increase the therapeutic efficacy.

For efficient endocytosis, it has been suggested that multiple ligands to receptor binding may be needed to meet the required free energy for complete wrapping of the membrane.46,47 In regard to this theory, Yoo et al. reported a rod-shaped comb-type aptamer-siRNA chimera.48 In this study, a mucin 1 (MUC1) targeting DNA aptamer was conjugated to the siRNA. MUC1 is a cell surface associated protein, highly over-expressed in malignant adenocarcinomas.49,50 The anti-MUC1-aptamer carrying sense strands of siRNA was hybridized complementary to the multimeric antisense strand to fabricate comb-like-aptamer-siRNA conjugate (Comb-Apt-siR). The intracellular uptake of Comb-Apt-siR in MUC1-positive MCF-7 cells was visually compared to conventional aptamer-siRNA and dimeric aptamer-siRNA conjugates using a red fluorescent dye, POPO-3. Comb-Apt-siR exhibiting the strongest fluorescence, and showed enhanced internalization compared to di- and monomeric aptamer-siRNA conjugates. The enhanced internalization of Comb-Apt-siR was explained by its ability to bind multiple receptors on the cell membrane initiating cluster formation leading to efficient endocytosis. The siRNA was designed to target the green fluorescent protein (GFP) gene expression. Despite an enhanced cell uptake, only Comp-Apt-siR inhibited the expression of the GFP gene efficiently, suggesting that the multivalent aptamer-siRNA conjugations might have improved the internalization capabilities compared to the monomers. The mechanism involved in the endosomal release of the chimera after cell entry is not fully understood.

To further improve the efficacy of aptamer-siRNA chimeras, endosome rupturing nanocarrier conjugation can be an alternative. However, Walter et al. showed that the positive net charge of nanomaterials could block the correct folding of an aptamer by triggering it to unfold on the surface.51 Such a conformational change will inhibit any interaction between the aptamer and its target, ultimately destroying its siRNA guiding property. To overcome this problem, Bagalkot and Gao developed a 2-step process using aptamer and siRNA separately to build a functional chimera.52 First, they applied siRNA molecules with a thiol-reactive terminal group to a polyethylene imine coated nanoparticle. This non-covalent interaction reduces some of the positive charge on the carrier. Next, the aptamer containing a single thiol–group was added to form a functional chimera.52 First, they applied siRNA molecules with a thiol-reactive terminal group to a polyethylene imine coated nanoparticle. This non-covalent interaction reduces some of the positive charge on the carrier. Next, the aptamer containing a single thiol–group was added to form a functional chimera with the nanocarrier bound siRNA. Their approach showed significantly increased gene silencing efficacy compared with conventional one-step assemblies. Recently, a new strategy using a simple protein tag was used to improve the endosome disruption.53 In comparison
with nanoparticles, this small protein tag consisted of 2 functional domains; a dsRNA binding domain and a polyhistidine. The dsRNA binding domain binds selectively to the siRNA part of the chimera, and depending on the pH, the polyhistidine induces endosomal membrane disruption. Table 1 summarizes recent efforts on aptamer mediated siRNA delivery for enhanced gene silencing efficacy.

Aptamer Targeted Delivery of shRNA

Similar to siRNA approach, shRNA (short hairpin RNA) can be used to initiate target gene silencing. shRNAs consist of 2 complementary RNA sequences linked by a short loop region and mimics the naturally-occurring miRNA precursor in miRNA biogenesis. A ribonuclease III family member called Dicer cleaves the shRNAs into small interfering RNA duplexes with symmetric 2-3 nucleotides 3' overhangs for creating conventional siRNAs. In order to trigger high gene silencing efficiency, shRNAs, like conventional siRNAs, are designed to match their target perfectly.

Aptamers can be utilized to further improve the target gene silencing efficacy and the major benefit of using shRNAs-aptamer chimeras is that the whole complex can be synthesized in one step, avoiding the annealing of 2 separated sense and antisense RNA strands, usually required for siRNA. Recently, Ni and colleagues used shRNA-aptamer chimeras to target the catalytic subunit of DNA-activated protein kinase, catalytic polypeptide (DNAPK). The aptamer-shRNA conjugate was designed as a single intact nuclease-stabilized 2'-fluoro-modified pyrimidine transcript. The treatments with the chimera lead to significant reductions in DNAPK mRNA levels. This report not only showed the enhanced RNAi capabilities of aptamer-shRNA chimera, but also the simplicity of the chimera synthesis.

Figure 3. Aptamer-siRNA mediated gene silencing approach.

Aptamers as Tools for Delivering microRNAs

The discovery of microRNA (miRNA), short endogenous-initiated non-coding RNA molecules, is considered an important breakthrough in the molecular genetics field. It was initially revealed as regulator of the larval developmental stages of Caenorhabditis elegans. Studies on miRNA received great attention and this area is growing rapidly. The reason for that is the involvement of miRNAs in the regulation of various important gene networks that play a role in the development of various diseases. miRNAs function as gene modulators inducing either degradation or translational repression of a target mRNA (mRNA). Depending on the degree of complementarity of the miRNA to the target mRNA, negative regulation occurs via the cleavage or by translational biogenesis and regulated repression of the target mRNA. The perfect or almost perfect binding of the miRNA to the target site induces the cleavage of mRNA. This way of interfering is most common in plants, but it was also reported for animals. The major regulation pathway in animals as well as in humans, is the translational repression induced by imperfect binding of the miRNA to complementary sites within the 3' untranslated regions of mRNA blocking the translation into a protein. As imperfect target binding (compromised Watson-Crick base pairing rules) can block translation, one miRNA is able to regulate multiple
| Aptamer target                                      | Component                  | siRNA-Target                  | In vivo/in vitro target | Aptamer-siRNA linkage                          | Reference                  | Further Information                  |
|-----------------------------------------------------|----------------------------|-------------------------------|-------------------------|-----------------------------------------------|----------------------------|--------------------------------------|
| prostate-specific membrane antigen (PSMA)           | 2'-Fluoro RNA              | Lamin A/C or GAPDH            | LNCaP cells             | Biotinylated siRNA/aptamer linked by streptavidin | Chu et al., 2006 (38)       |                                      |
|                                                     | 2'-Fluoro RNA              | Polo-like kinase1 (PLK1) or   | LNCaP cells             | Conjugated via combined transcription         | McNamara et al., 2006 (40) |                                      |
|                                                     |                            | BCL2 mRNA                     |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | Polo-like kinase1 (PLK1) or   | athymic nude mice       | Multiple linking methods                      | Dassie et al., 2009 (54)   |                                      |
|                                                     |                            | BCL2 mRNA                     |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | Eukaryotic Elongation Factor 2 (EEF2) mRNA | LNCaP cells | Conjugated via combined transcription         | Wullner et al., 2008 (55) |                                      |
|                                                     |                            |                               |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | shRNA: Bcl-xL (anti-apoptotic | LNCAP & PC3 cells      | Branched polyethyleneimine (PEI) and polyethylene glycol bridge | Kim et al., 2010 (56)     |                                      |
|                                                     |                            | gene)                         |                         |                                               |                            |                                      |
|                                                     | DNA                        | Smg1 and Up2 (factors of     | B16/F10 & CT26 tumor cells & Balb/c or Nude cells | Conjugated via combined transcription         | Pastor et al., 2010 (57) |                                      |
|                                                     |                            | nonsense-mediated mRNA)       |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | shRNA: DNA-activated protein kinetic (DNAPK); mitotic spindle assembly checkpoint protein MAD2B (MAD2L2); and breast cancer type 2 susceptibility protein (BRCA2) | LNCAp cells |                                               | Ni et al., 2011 (58) | radio sensitization                   |
|                                                     | RNA                        | Enhanced green fluorescent   | Human prostate cancer cell line C4-2B | SPDP crosslinker                           | Bagalkot et al., 2011 (52) |                                      |
|                                                     |                            | protein (EGFP)                |                         |                                               |                            |                                      |
| Human epidermal growth factor receptor 2 (HER2) CD4 | 2'-Fluoro RNA              | Anti-apoptotic gene Bcl2      | N202.1A cells           | Conjugated via combined transcription         | Thiel et al., 2012 (59)   |                                      |
|                                                     |                            |                              |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | Firefly luciferase mRNA       | CD4 overexpressing T-cells | Dimerization using phi29 Motor pRNA           | Guo et al., 2005 (60)     |                                      |
|                                                     |                            |                              |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | Survivin & firefly luciferase mRNA | CD4 overexpressing T-cells | Dimerization using phi29 Motor pRNA           | Khaled et al., 2005 (61)  |                                      |
|                                                     |                            |                              |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | gag and vif or host CCR5      | CD4 overexpressing T-cells | Conjugated via combined transcription         | Wheeler et al., 2011 (62) |                                      |
|                                                     |                            |                              |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | gag and vif or host CCR5      | NOD/SCID/IL2Rγ−/− (NSG) mice | Conjugated via combined transcription         | Wheeler et al., 2013 (63) |                                      |
|                                                     | DNA                        | HIV-PR                        | CD4 overexpressing T-cells | Commercial synthesis                         | Zhu et al., 2012 (64)    |                                      |
|                                                     |                            | Asthma STAT5b gen             | CD4 overexpressing T-cells | Dimerization using phi29 Motor pRNA           | Qiu et al., 2012 (65)    |                                      |
| HIV-1 gp120                                         | 2'-Fluoro RNA              | HIV-1 tat/rev common exon     | HIV-1-infected CEM cells & HIV-1 infected Rag-Hu mouse | 4-nucleotide linker (CUCU) | Zhou et al., 2008 (41) |                                      |
|                                                     |                            | sequence                      |                         |                                               |                            |                                      |
|                                                     | 2'-OMe modified A and G and 2'-F modified U and C | HIV-1 tat/rev common exon     | CEM T-cells & primary blood mononuclear cells (PBMCs) | Non-covalent via sticky bridge               | Zhou et al., 2009 (66) |                                      |
|                                                     | 2'-Fluoro RNA              | HIV-1 tat/rev common exon     | CD4+ T & Humanized BALB/c RAG2−/− γc−/− mice | 2-nucleotide linker (UU) | Neff et al., 2011 (67) |                                      |
|                                                     |                            | sequence                      |                         |                                               |                            |                                      |
|                                                     | 2'-Fluoro RNA              | HIV-1 tat/rev common exon     | CHO-WT and CHO-EE cells & PBMCs |                                               | Zhou et al., 2011 (68) |                                      |
|                                                     |                            | sequence                      |                         |                                               |                            |                                      |
| Aptamer target | Component | shRNA-Target | in vivo/in vitro target | Aptamer-siRNA linkage method | Reference | Further Information |
|---------------|-----------|--------------|-------------------------|----------------------------|-----------|---------------------|
| CD8 DNA      | GNLY mRNA | CD8 overexpressing T-cells | Non-covalent via sticky bridge | Wang et al., 2013 (70) |          |                     |
| CD30 2'-O-methyl modified RNA | Anaplastic lymphoma kinase mRNA | human anaplastic large cell lymphoma | Non-covalent charge forces to carrier | Zhao et al., 2011 (71) |          | ALK siRNA and a RNA-based CD30 aptamer probe onto nano-sized polyethyleneimine-citrate carriers |
| Theophylline RNA | shRNA: albumin mRNA | hepatic (HepG2) cells | Theophylline aptamer inserted in the loop region of shRNA | Tuleuova et al., 2008 (72) |          |                     |
| Xanthine 5'-radiolabeled RNA | shRNA: enhanced green fluorescent protein (EGFP) | HEK293T cells | Theophylline aptamer inserted in the loop region of shRNA | Beisel et al., 2008 (73) |          | ligand-regulated RNAi |
|             | RNA   | shRNA: enhanced green fluorescent protein (EGFP) | HEK293 cells | An et al., 2006 (74) |          | ligand-regulated RNAi |
|             | RNA   | shRNA: firefly luciferase mRNA | HEK293T cells | Noguchi et al., 2011 (75) |          |                     |
| Xanthine 5'-radiolabeled RNA | shRNA: enhanced green fluorescent protein (EGFP) | HEK293T cells | Xanthine aptamer inserted in the loop region of shRNA | Beisel et al., 2008 (73) |          | ligand-regulated RNAi |
| Malachite green (MG) 2'-Fluoro RNA | Firefly luciferase mRNA | Human nasopharyngeal carcinoma KB cells | phi29 packaging RNA (pRNA) 3-way junction | Reif et al., 2012 (76) |          | Fluorogenic RNA NP for Monitoring RNA Folding & Degradation in Real Time |
| Transferrin receptor, CD71 (TfR) 2'-Fluoro RNA | Enhanced green fluorescent protein (EGFP) | HeLa-EGFP cells | Aptamers conjugated to liposomes | Wilner et al., 2012 (77) |          | aptamer-targeted siRNA-laden liposomes |
| murine 4-1BB 2'-Fluoro RNA | Diverse | HEK293T & HEPA1-6 cells | Conjugated via combined transcription | Berezhnoy et al., 2012 (78) |          | Paper focuses on thermal stability effects on inhibition |
| RNA | raptor mRNA | CD8 overexpressing T-cells | Conjugated via combined transcription | Berezhnoy et al., 2014 (79) |          |                     |
| 2'-Fluoro RNA | STAT3 mRNA | 4-nucleotide linker (CUCU) | Zhou et al., 2013b (80) |          |          |                     |

(Continued on next page)


| Aptamer target | Component | siRNA-Target | In vivo/In vitro target | Aptamer-siRNA linkage | Reference | Further Information |
|----------------|-----------|--------------|-------------------------|----------------------|-----------|---------------------|
| B-cell–activating factor receptor (BAFF-R) | Z'-Fluoro RNA | STAT3 mRNA | Jeko-1, Z138, Rec-1 & Granta-519 cells | Non-covalent via sticky bridge | Zhou et al., 2013b (80) |  |
| αvβ3 integrin | RNA | Eukaryotic Elongation Factor 2 (EEF2) mRNA | U-87 MG, SiHa & PC-3 cells | Conjugated via combined transcription | Hussain et al., 2013 (81) |  |
| Nucleolin | Oligodeoxynucleotides | snail family zinc finger 2 (SLUG) neuropilin 1 (NRPI) | CL1-5 cells | Hetero-bifunctional crosslinker, sulfo-SMPB | Lai et al., 2014 (82) |  |
| | Oligodeoxynucleotides | BRAF gene | A375 cells & Balb/c or Nude mice | Hetero-bifunctional crosslinker, sulfo-SMPB | Lai et al., 2014 (82) |  |
| | Oligodeoxynucleotides | MUC-1 DNA Green fluorescent protein (GFP) gene | MCF-7 & A549 cells | sRNA linear linked via crosslinker dithio-bismaleimidodithiane; aptamer to siRNA linking non-covalent via complementary base paring | Yoo et al., 2014 (48) |  |
| | | | | Unspecified linker | Hermann et al., 2014 (84) |  |
| Cytotoxic T lymphocyte–associated antigen 4 (CTLA4) U87-EGFRvIII cells | RNA | STAT3 mRNA | CD8 overexpressing T-cells & immunodeficient mice bearing human T cell lymphomas | Unspecified linker | Zhang et al., 2014 (85) |  |
mRNAs, making miRNAs an interesting tool for multi-target inhibition.

In comparison with normal cells, tumor cell lines often show a broad deregulation of miRNA expression. In most cancer type, miRNA down-regulation correlates with a lack of tumor suppressing functions, indicating their role as tumor suppressors. On the other hand some cancer types exhibit an increased expression of specific miRNAs that target tumor suppressor genes. Therefore, manipulating miRNAs would be a rational therapy considering their diverse roles in tumorigenesis and inducing tumor formation. An increasing number of studies have revealed that depending on the cellular context, one miRNA can act as tumor suppressor as well as an oncomir. One example for this 2-faced activity is miR-221. While being up regulated in most cases of epithelial tumors, miR-221 also play tumor suppressor role in erythroleukemic cells. Such examples will further complicate the use of miRNAs as therapeutic agents and demonstrates the requirement for cell specific delivery, further justifying the use of aptamers as a delivery tool.

The miRNAs miR-15a and miR-16-1 are known to act as tumor suppressors in prostate cancer. In 2011, Wu and colleagues used this tumor suppressing character to create a polya-midoamine (PAMAM)-based aptamer conjugation as a target-specific intracellular delivery carrier of miR-15a and miR-16-1 to treat prostate cancer. PAMAM was conjugated to the aptamer using a polyethyleneglycol (PEG) linker. ATP-PEG-PAMAM-miRNA complexes were created by an electrostatic interaction between miRNA and PAMAM. By utilizing the aptamer A10-3.2 targeting prostate-specific membrane antigen (PSMA), they were able to deliver the miRNAs specifically to PSMA expressing LNCaP cells and induce cancer cell death.

Another example of utilizing aptamers to deliver miRNA was performed by Dai and colleagues. They conjugated MUC1-aptamers (anti-MUC1 protein) to miRNA-29b to generate the chi-29b chimera for the purpose of re-expressing the tumor-suppressor gene, PTEN. The chi-29b chimera was delivered specifically to OVCAR-3 cells, which express MUC1 protein guided by the PTEN gene in the OVCAR-3 cells. chi-29b chimera successfully showed anti-tumor effects in ovarian cancer xeno-graft mice models. In another study, MUC1 aptamer was used for target specific delivery of let-7i miRNAs to reverse the paclitaxel-induced chemoresistance of OVCAR-3 cells in the ovarian carcinomas. The paclitaxel-induced chemoresistance has been successfully reversed by the MUC1/let-7i chimera, which has down-regulated the expressions of Dicer1, cyclin D1, cyclin D2 and PGRMC.

Aptamers as Tools for Delivering antimiRs

AntimiRs, short piece of single-stranded nucleic acids targeting miRNA are a recent tool for inhibiting miRNA activity. AntimiRs are mostly modified oligonucleotides binding complementary to the target miRNA preventing from binding to its biological target. For example, Elmen et al. demonstrated the function of LNA-modified antimiRs in vivo, demonstrating antimiRs as an important therapeutic tool.

In 2012, Kim et al. have developed an AS1411 aptamer-targeted theranostic platform composed of miRNA-221 targeting molecular beacon fused to a magnetic fluorescent nanoparticle. The beacon consisted of a perfect reverse complement sequence to mature miRNA-221. Aptamer and the miRNA beacon were covalently linked to the nanoparticle using the coupling reagent, N-(3-dimethylaminopropyl)-N’-ethyl-carbodiimide hydrochloride. While the aptamer conducts cell specific delivery of the antimiR beacon, the nanoparticle enables tracking and visualization of the complex. They successfully demonstrated a functional system for simultaneous targeting of cancer cells, imaging and oncomir inhibition.

Very recently, Pofahl et al. reported the first successful aptamer based antimiR delivery to the deregulated miRNA target miR-21 in breast cancer cells. The antimiR sequence should in principle be specifically delivered to the cancer cells and strongly bind to the target miRNA sequence to inhibit its function. In their study, nucleolin targeting aptamer AS1411 was used to deliver the antimiR sequence. The antimiR sequence was chemically modified by using phosphorothioate linkages and also by incorporating locked nucleic acid (LNA) nucleotides to enhance the antimiR-miR-21 interaction and to improve the stability. To test antimiR interference, an enhanced green fluorescent protein (EGFP)-expressing MCF-7 cell line was generated. In those cells, the EGFP expression was inhibited by miR-21. The study revealed that the chimera was successfully internalized in MCF-7 cells and exhibited antiproliferative properties while preventing miR-21 dependent EGFP inhibition. They coined the term AptamiR for this type of chimeras for combining aptamer and antimiR.
synthetic methods do not use large biological molecules like streptavidin, and thus can be less immunogenic.

Polynucleotide linkage might be the easiest way to link aptamer and therapeutic oligonucleotides. In this case, a special phosphoramidite that may affect the total synthetic yield is not required. It is noteworthy mentioning that polynucleotide linkers are able to engage in base paring with other nucleotides within the sequence or other sequences. Therefore, the linker has to be chosen carefully and also to avoid its influence on the secondary structure of the aptamer. In addition, the polynucleotide linker can make the chimera less flexible compared to other chemistries.

A polyethylene glycol (PEG) based phosphoramidite can be used to establish a PEG linkage between aptamer and oligonucleotides. PEG is hydrophilic, which decreases aggregation and increases solubility of the complex, non-toxic, non-immunogenic and a usual approach for increasing the bioavailability in vivo. Furthermore, a PEG linkage is highly flexible and thus it could be a useful method for conjugation. Disulphide linkages are commonly found in bacterial protein toxins. These toxins utilize the cleavage of covalently linked disulphide bond by reducing it to thiol groups. The disulphide bond is mostly stable in serum, due to the oxidizing character of the extracellular space, but if exposed to the reducing intracellular space, the disulphide bond is cleaved. This will facilitate the cleavage of the aptamer-oligonucleotide complex and release of the interfering oligonucleotide upon cell entry. Using this approach, coagulation of aptamer and siRNA/miRNA can be avoided and the efficacy of the interfering oligonucleotide can be improved.

**Chemically Modified Aptamer-Oligonucleotide Chimera**

Stability of oligonucleotides is key for successful therapeutic efficacy in vivo. Virtually every organism possesses various enzymes to synthesize, modify or hydrolyze nucleic acids. Nucleases are important for nucleic acid turnover and as a defense mechanism against pathogens, such as bacteria and viruses. Consequently, aptamer-therapeutic oligonucleotide chimera composed of naturally occurring DNA or RNA nucleotide monomers have serious limitations toward therapeutic development, as they exhibit shorter half-life in vivo because of their poor nuclease resistance and bio-availability. To tackle these limitations, a number of modified nucleotides have been developed in recent years (Fig. 5).

Some of the most prominent examples are 2′-fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-methoxymethyl (2′-MOE), 2′-fluoroarabinose (2′-FANA), locked nucleic acid (LNA), unlocked nucleic acid (UNA), cyclohexenyl (CeNA) nucleic acid, peptide nucleic acid (PNA), phosphorimidate morpholino (PMO) etc. Although many of the modified nucleotides have been successfully utilized in various nucleic acid-based therapeutic technologies, their relatively poor or no enzymatic recognition properties often pose a major challenge toward the development of biostable aptamers.

In principle 2 different approaches are used to incorporate modified nucleotides into aptamers. First, fabrication of a preselected aptamer introducing modified nucleotides at various places
positions during solid phase oligonucleotide chemical synthesis ('post-SELEX'). In this approach, incorporation of a modified nucleotide can result in unfavorable shift or even in total loss of the binding affinity which highlight the importance of a systematic incorporation and analysis. A post-SELEX approach has been used during the development of the first aptamer drug Macugen® (Pegaptanib).\(^3\) Pegaptanib is a human vascular endothelial growth factor (VEGF)-binding RNA aptamer containing 2'-F pyrimidine and 2'-OMe purine nucleotides. While the aptamer origins from a 2'-F pyrimidine-containing library via conventional SELEX, the 2'-OMe modifications were introduced post-SELEX by substituting purines to enhance nuclease resistance and serum stability. Kuwahara et al. recently reviewed various successful post-SELEX modified aptamers.\(^1,2^1\)

The second approach is by conventional aptamer selection via SELEX approaches whereby a new aptamer is developed from an oligonucleotide library containing modified nucleotides (in-SELEX approach). The 2'-OH group is a suitable location for introducing chemical modifications, since the modification can be introduced equally in purines and pyrimidines. Furthermore, 2'-modifications is known to increase the stability against chemical and enzymatic degradation.\(^1,2^2-1,2^5\) Very recently, Lauridsen et al. reported a review article describing the enzymatic recognition capabilities of various 2'-modified nucleotides.\(^1,2^6\) Stemming from their initial enzymatic recognition studies, 2'-amino pyrimidines, 2'-fluoro pyrimidines and 2'-O-Methyl nucleotides have been successfully applied in aptamer development by conventional SELEX-based methodologies.\(^1,2^7-1,3^4\) LNA is one of the successful nucleotide analogs extensively utilized in various fields because of their remarkable properties.\(^1,3^5,1,1^4\) In LNA the sugar ring is conformationally locked by an O2'-C4'-methylene linkage to adopt N-type sugar puckering.\(^1,3^5-1,3^7\) Toward developing LNA-modified aptamers, Veedu et al. reported the enzymatic recognition capabilities of LNA nucleotides using DNA and RNA polymerases.\(^1,3^8-1,4^4\) In 2013, Kuwahara and co-workers
reported an LNA (BNA) aptamer against thrombin using capillary electrophoresis-based SELEX (CE-SELEX) method. 145,146

Summary and Outlook

Since their invention, aptamers have been applied to various applications including therapy, diagnosis, imaging and delivery. Aptamer selection is normally performed with a goal of generating a candidate sequence with very high target binding affinity (low nanomolar level) and specificity to a given molecular target. High affinity would be desirable for most applications, however for aptamers targeting proteins that are overexpressed in a particular disease condition (both intra-cellular and extra-cellular including cell-surface receptors), highest target binding affinity might not be necessary as it could increase the probability of binding to the same proteins needed for normal cellular functions. Aptamers are conventionally selected with a nucleic acid library with primer binding regions flanked to the randomized region. Secondary structures responsible for target binding may usually be expected from the random region; however, it is important to use the full-length oligonucleotide aptamer sequences (with primer flanks) for initial target binding analysis. Systematic truncation of the successful binding aptamer can then be performed using secondary structure prediction algorithms (e.g., mfold). 147

In recent years, a number of studies showed the potential of aptamers to improve the efficacy of therapeutic oligonucleotide candidates for target specific gene silencing and generate a better clinical outcome. Endosomal release of aptamer-therapeutic oligonucleotide chimera could be another problem in addition to cellular uptake, with high amounts of chimeras required to produce relevant changes in gene expression. Attaching endosome disrupting molecules such as a nanoparticle or a protein/peptide tag to the aptamer-oligonucleotide chimera may prove useful to circumvent this limitation. In previous years, the main focus was on aptamer-targeted delivery of siRNA. But, the scope of miRNA targeting and antisense therapy continues to rise and this will surely broaden the applications of aptamers based delivery systems. A classical approach for targeting mRNA is to use antisense oligonucleotides (ASOs), 148 short pieces of single-stranded DNA sequence that anneal to the target mRNA. This RNA:DNA hetero-duplex then recruits the enzyme RNAse H, which specifically cleaves the target mRNA and block translation. Chemically-modified nucleotide-based ASOs are also widely applied for enhanced targeting efficacy and stability, and in this case a steric-block mechanism is also applied for preventing translation. Most importantly, the first therapeutic oligonucleotide entered the clinic is Vitavene (Formiviren), an ASO for the treatment of cytomagaloviral (CMV) retinitis in patients with HIV infection. 149 This approach has been widely explored for its applicability as therapeutics in various disease conditions both in vitro and in vivo. Target specific delivery is very important for high efficiency aptamers and efﬁcacy can be a vital tool for more efﬁcient delivery of ASOs. However, to the best of our knowledge so far, there are no reports on aptamer-mediated delivery of ASOs.

To summarize, the relatively new field of aptamer-therapeutic oligonucleotide chimera is currently advancing its potential for various therapeutic applications. Aptamer-guided delivery of therapeutic oligonucleotides could be one of the most exciting approaches toward the treatment of diseases and its broad applicability is limited by our knowledge and imagination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Keefe AD, Pai S, Ellington A. Aptamers as therapeutics. Nat Rev Drug Discov 2010; 9:337-50; PMID:20592747; http://dx.doi.org/10.1038/nrd3141
2. Famulok M, Hargir JS, Mayer G. Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. Chem Rev 2007; 107:3715-43; PMID:17715981; http://dx.doi.org/10.1021/cr0607643
3. Ng EW, Shima DT, Calais P, Cunningham ETJ, Jr., Gayer DR, Adams AP, Pegapatan, a targeted anti-VEGF aptamer for ocular vascular disease. Nat Rev Drug Discov 2006; 5:123-32; PMID:16518379; http://dx.doi.org/10.1038/nrd1955
4. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990; 249:505-10; PMID:2200121; http://dx.doi.org/10.1126/science.2290121
5. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind speciﬁcally to DNA. Nature 1990; 346:818-22; PMID:1967402; http://dx.doi.org/10.1038/346818a0
6. Brown D, Gold L. Template recognition by an RNA-dependent RNA polymerase: characterization and identiﬁcation of two RNA binding sites on Q beta replicase. Biochemistry 1995; 34:14765-74; PMID:7578085; http://dx.doi.org/10.1021/bi0004501
7. Klug SJ, Famulok M. All you wanted to know about SELEX. Mol Biol Rep 1994; 20:97-107; PMID:7536299; http://dx.doi.org/10.1007/BF00990358
8. Gopinath SC. Methods developed for SELEX. Anal Bioanal Chem 2007; 387:171-82; PMID:17072603
9. Stolzenburg R, Reinemann C, Strehlitz B. SELEX-a (r)evolutionary method to generate high-afﬁnity nucleic acid ligands. Biomol Eng 2007; 24:381-403; PMID:17627883; http://dx.doi.org/10.1016/j.biomeng.2007.06.001
10. Lauweryns JH, Shamilov HA, Edwards SL, Taran E, Veedu RN. Rapid one-step selection method for generating nucleic acid aptamers: development of a DNA aptamer against alpha-bungarotoxin. Plos One 2012; 7:e41782; PMID:22860007; http://dx.doi.org/10.1371/journal.pone.0041702
11. Nitsche A, Kurth A, Dunkhorst A, Panke O, Sielaff H, Junge W, Muth D, Scheller F, Stöcklein W, Dahmen C, et al. One-step selection of Vaccinia virus- binding DNA aptamers by MonoLEX. BMC Biotechnol 2007; 7:48; PMID:17607578; http://dx.doi.org/10.1186/1472-6750-7-48
12. Peng L, Stephens BJ, Bonin K, Cubicciotti R, Guthold M. A combined atomic force/fluorescence microscopy technique to select aptamers in a single cycle from a small pool of random oligonucleotides. Microsc Res Tech 2007; 70:372-81; PMID:17262788; http://dx.doi.org/10.1002/jemt.20421
13. Fan M, McMurrant SR, Andrews CJ, Allman AM, Bruno JG, Kiel JL. Aptamer selection express: a novel method for rapid single-step selection and sensing of binding DNA aptamers by MonoLEX. BMC Biotechnol 2007; 7:52; PMID:17672571; http://dx.doi.org/10.1186/1472-6896-7-48
14. Dua P, Kim S, Lee DK. Nucleic acid aptamers targeting cell-surface proteins. Methods 2011; 53:21-25; PMID:21300154; http://dx.doi.org/10.1016/j.ymeth.2011.02.002
15. Liu K, Lin B, Lan X. Aptamers: a promising tool for cancer imaging, diagnosis, and therapy. J Cell Biochem 2013; 114:250-5; PMID:22949372; http://dx.doi.org/10.1002/jcb.24337
16. Liu M, Petersen B, Wolf H, Prohaska E. An aptamer-based quartz crystal protein biosensor. Anal Chem 2007; 79:4488-95.D; PMID:12336360; http://dx.doi.org/10.1021/ac061294p
17. Bumcroft D, Moharan M, Koteliansky V, Sah DW. RNA therapeutic: a potential new class of pharmaceutical drugs. Nat Chem Biol 2006; 2:711-9; PMID:17108989; http://dx.doi.org/10.1038/nchembio839
18. Wilson RC, Doudna JA. Molecular mechanisms of RNA interference. Annu Rev Biophys 2013; 42:217-39; PMID:23654304; http://dx.doi.org/10.1146/annurev-biophysics-083012-150404
19. Cardwell RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell 2009; 136:642-55; PMID:19298866; http://dx.doi.org/10.1016/j.cell.2009.01.035
20. Dias N, Stein CA. Antisense oligonucleotides: basic concepts and mechanisms. Mol Cancer Ther 2002; 1:347-55; PMID:12489851; http://dx.doi.org/10.1158/1535-7163.MCT-01-0144
21. He L, Hanson GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5:522-31; PMID:15211354; http://dx.doi.org/10.1038/nrg1379
22. Ray P, White RR. Aptamers for targeted drug delivery. Pharmaceuticals 2010; 3:1761-1778; http://dx.doi.org/10.3390/ph3061761
40. Hussain AF, Tur MK, Barth S. An aptamer-siRNA

41. Zhou J, Tiemann K, Chomchan P, Alluin J, Swiderski P,

42. Noguchi K, Ishitu Y, Takaku H. Evaluating target

43. An CI, Trinh VB, Yokobayashi Y. Artificial con-

44. Tuleuova N, An CI, Ramanculov E, Revzin A, Yoko-

45. Zhou J, Shu Y, Guo P, Smith DD, Rossi JJ. Dual

46. Peck K. Synergistic inhibition of lung cancer cell inva-

47. Burnett J, Zhang X, Forman S, Chen R, Rossi J. Dual

48. Calin GA, Croce CM. MicroRNA signatures in

49. Garofalo M, Contel CW, Croce CM, Condorelli G. MicroRNAs as regulators of death receptors signaling. Cell Death Differ 2010; 17:200-9; PMID:19645940; http://dx.doi.org/10.1038/cdd.2009.105

50. Bartel DP. MicroRNAs: genomics, biogenesis, mecha-

51. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6:857-66; PMID:16994266; http://dx.doi.org/10.1038/nrc1997

52. Kernan A, Bredemeyer BL, Bressler PA, Hockenbery DM, Eckert GP. Characterization of human nucleolin as a target for nucleolin-directed specific gene silencing; Nucleic Acids Research 2010; 38:965-74; PMID:20032596; http://dx.doi.org/10.1093/nar/gkq125

53. Liu N, Zhou C, Zhao J, Chen Y. Reversal of paclitaxel resistance in epithelial ovarian carcinoma cells by a MUC1 aptamer-let-7i RNA level and inhibits 2012; 30:577-82; PMID:22882695; http://dx.doi.org/10.1097/01.jnum.0000375977.2012.072765

54. Eifler J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedjazian M, Hansten HF, Berger S, et al. RNA-modified microRNA silencing in non-human primates. Nature 2008; 452:896-9; PMID:18368051; http://dx.doi.org/10.1038/nature06783

55. Kim JK, Choi KJ, Lee M, Jo MH, Kim S. Molecular imaging of a cancer-targeting therapeutic using a nucleolin aptamer- and microRNA-221 molecular beacon-conjugated nanoparticle. Biomaterials 2012; 33:207-17; PMID:21944470; http://dx.doi.org/10.1016/j.biomaterials.2012.01.023

56. Potzial M, Wengel J. Multifunctional nucleic acids for tumor cell treatment. Nucleic Acid Ther 2014; 24:171-7; PMID:24494617; http://dx.doi.org/10.1098/rsat.2013.0472

57. Burns P, Lasser DA, Miller DM, Thomas SD, Trent JO. Discovery and development of the G-rich oligo-

58. Falnes PO, Sandvig K. Penetration of protein toxins into cells. Curr Opin Cell Biol 2000; 12:407-13; PMID:10873820; http://dx.doi.org/10.1016/S0955-0674(00)00019-5

59. Kawasuki AM, Casper MD, Freier SM, Lesnik EA, Zornes MC, Cummins LL, Gonzalez C, Cook PD. Uniformly modified 2'-deoxy-2'-fluoro-phosphorothioate-oligo-

60. Majlessi M, Nelson NC, Becker MM. Advantages of 2'-O-methyl oligoribonucleotides probes for detecting RNA targets. Nucleic Acids Res 1998; 26:2224-9; PMID:9547284; http://dx.doi.org/10.1093/nar/26.11.2224

61. Baker BF, Lot SS, Condon TP, Chench-Flournoy S, Lesnik EA, Sasmor HM, Bennett CF. 2'-O-(2-Methoxy-ethyl)-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA and ICAM-1 expression of he ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 1997; 272:11994-2000; PMID:9115264; http://dx.doi.org/10.1074/jbc.272.18.11994

62. Geary RS, Watanabe TA, Truong L, Freier S, Lesnik EA, Soult NB, Sasmor H, Manoharan M, Levin AA. Pharmacokinetic properties of 2'-O-(2-methoxy-ethyl)-modified oligonucleotides in rats. J Pharmacol Exp Ther 2001; 296:890-7; PMID:11413820; http://dx.doi.org/10.1124/jpet.296.2.890

63. Wilds CJ, Damha MJ. 2'-Deoxy-2'-fluoro-D-arabinonucleotides and oligonucleotides (2F-ANA): synthesis and physicochemical studies. Nucleic Acids Res 2000; 28:3625-35; PMID:10988285; http://dx.doi.org/10.1093/nar/28.19.3625

64. Veedu RN, Wengel J. Locked nucleic acid as a novel class of therapeutic agents. RNA Biol 2009; 6: 321-23; PMID:19345849; http://dx.doi.org/10.4161/rna.6.3.18070

65. Veedu RN, Wengel J. Locked nucleic acids: promising nucleic acid analogs for therapeutic applications. Chem Biodivers 2010; 7: 536-42; PMID:20232525; http://dx.doi.org/10.1002/cbdv.200900343

66. Nielsen P, Drevet LH, Wengel J. Synthesis and evalua-

67. Nielson P, Drei
