

NOVEL GENE SILENCING TECHNOLOGY ANTISENSE-NUCLEASE CONJUGATES

Antisense technology

Antisense technology is one of the most straightforward approaches for suppressing unwanted gene expression. Antisense oligonucleotides bind to the target mRNA and block its translation and/or cause its degradation by attracting RNaseH activity. Unlike RNA interference, this approach does not depend on the host machinery required for housekeeping functions of the cell and organism. Therefore it can be applied as a treatment for the largest variety of disorders, from neurodegenerative diseases and cancer to various infectious diseases. Nevertheless, despite these promising properties, multiple trials involving the use of antisense drugs have failed to produce satisfactory results. The problematic issues of the existing technology, revealed in these trials, are low efficiency, bioavailability, inactivation of the target mRNA, and the intracellular localization of the antisense molecules and their stability.

Thus, still to date only one antisense compound, Fomivirsen or Vitravene, designed for the treatment of human cytomegalovirus retinitis, has been approved.

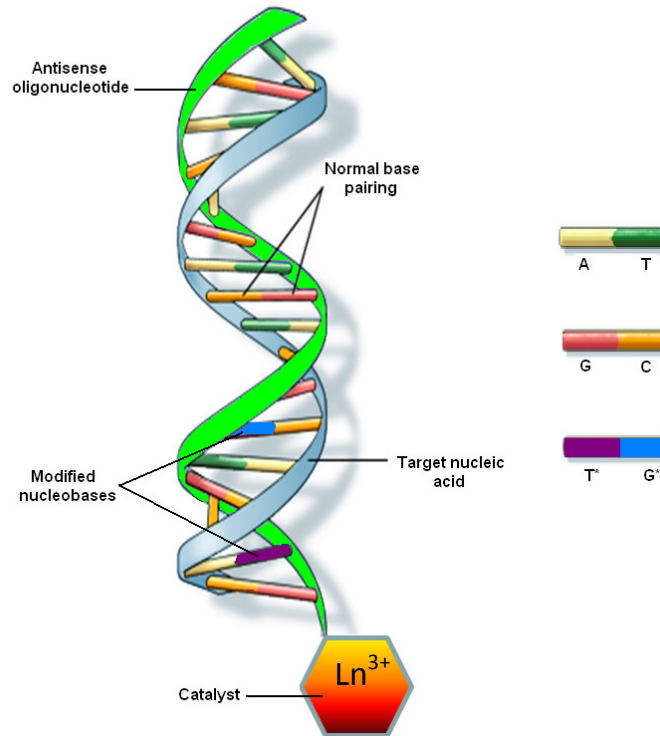
The major obstacles to the application of antisense therapy are inefficient delivery of oligonucleotides to cells and their poor bioavailability to intracellular targets. Therefore, much effort has been spent on building an effective carrier system. However, despite using various technologies like cell-penetrating peptides, morpholino oligos, programmable fusogenic vesicles, dendrimers, gold nanoparticles, magnetofection, receptor-mediated endocytosis etc., the resulting ineffective level of antisense oligonucleotides in targeted cells has remained the main obstacle for their wider use as therapeutic agents. The liver tissue is one of few known exceptions which is relatively easy to target with antisense oligonucleotides using currently existing technologies.

Novel gene silencing platform

BTD, Ltd. has introduced a new direction in the use of gene silencing agents in therapeutics that can lead to breakthroughs in the development of antisense drugs. As described in this summary, it has already resulted in the development of promising antiviral agents, as demonstrated by their significant *in vitro* effects on hepatitis C virus activity as well as on other virus types. We have also demonstrated that these novel drug candidates can efficiently suppress gene expression, including expression of viral genes, *in vivo*.

BTD's new technology platform is based on oligonucleotide analogs that contain specifically modified DNA bases and that are bound to organic complexes of lanthanides with highly selective artificial nuclease activity (Fig. 1). These compounds act as very efficient agents blocking gene expression and/or the replication of viruses belonging to different systematic groups. Depending upon the number of modified nucleobases in the oligonucleotide portion of the compounds, the binding ability to a complementary target nucleic acid can be increased many times compared to the ordinary antisense oligonucleotide, allowing for their much lower intracellular concentration. The catalytic activity of the new compounds conjugated with a chelating group binding a metal ion leads to further significant lowering of the effective concentration of the compounds. The modified nucleobases are tautomeric or ionic bases. The ligands of metal complexes (organic nuclease complexes) are various heterocyclic and macrocyclic compounds. The principles of the invention are described in Karelson, M., Saarma, M., Pily, M. Antisense Agents Combining Strongly Bound Base-Modified Oligonucleotide and Artificial Nuclease, U.S. Patent No. 7,786,292; August 31, 2010.

Figure 1. The principle of action of new gene silencing agents



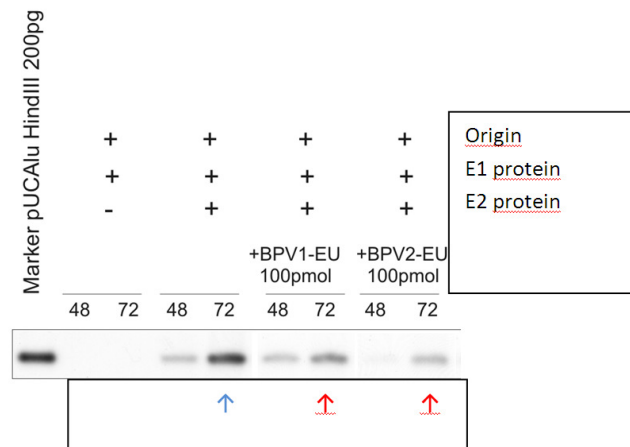
Novel efficient antiviral agents

The high efficiency of antiviral agents based on BTD's novel gene suppression technology is demonstrated for all types of viruses.

DNA viruses

The efficiency of the inhibition of DNA viruses has been examined using papillomavirus replication in transient replication assay. The model is bovine papillomavirus type 1 (BPV1).

Figure 2. The reduction of BPV1 expression by different agents.

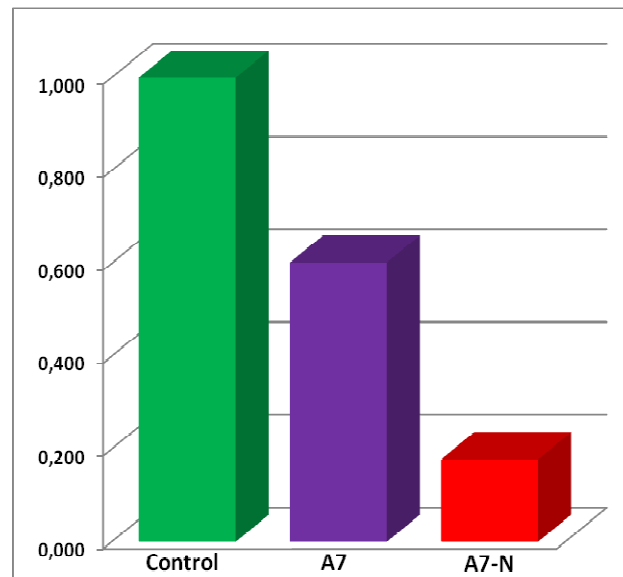


In Figure 2, the suppression of E2 dependent replication of BPV origin containing plasmids by asMOs targeted against E2 mRNAs is presented. Without oligonucleotides, the replication is activated by 48 h post transfection and occurs at high level at 72h (blue arrow). The presence of antisense oligonucleotides delays the start of replication and its level (red arrows). This may be also relevant to HPV induced cancers in which the activation of integrated papillomavirus origin may occur. Both oligonucleotides have same primary sequence and contain the artificial nuclease complex. The oligo BPV2-Eu contains in addition a modified nucleobase and has considerably stronger effect than the oligo having only native bases (BPV1-Eu).

Alphaviruses

These viruses cause mainly acute infection. The model studied was the Semliki Forest virus (SFV), close relative of Chikungunya virus. There is no vaccine or antiviral for any alphavirus. Several anti-SFV agents were used in the experiment, the results are shown in Fig. 3. In all experiments the control agent was standard antisense oligonucleotide while agents A7 and A7-N contained three modified residues. The agent A-7N was additionally modified by the inclusion of artificial nuclease complex. Both experimental and control substances were used at 15 nmol (final concentration).

Figure 3. The reduction of the SFV virus expressed Rluc activity in cells treated with indicated agent with respect to cells treated with control agent (standard antisense oligonucleotide).



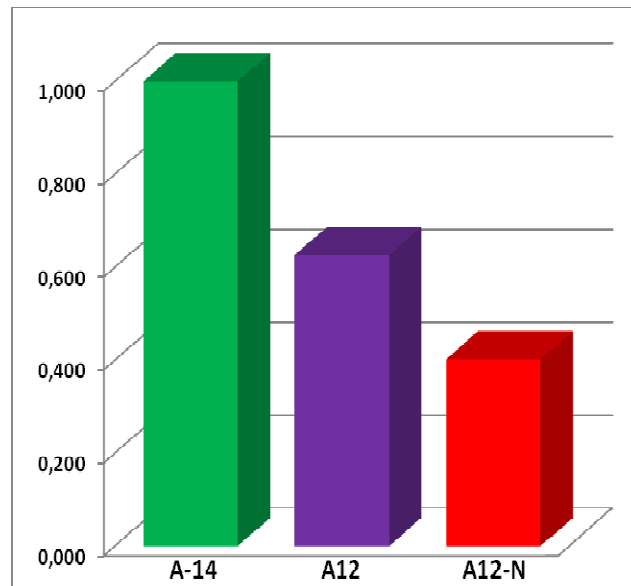
Retroviruses

HIV Tat-expression and hence Tat-mediated LTR promoter activation was targeted to model the novel antisense agent efficiency against retroviruses. The antisense oligonucleotides were taken in concentration 50 nmol (final concentration). LTR-promoter activation was measured by the expression of luciferase marker.

The virus reduction is presented through the fall of the luciferase activity in cells treated with indicated agents compared to activities measured from untreated cells (Figure 4).

The compounds A12, A12-N and A14 had identical sequences, with A12 and A12-N containing also two modified base residues. The compound A12-N was additionally modified by the inclusion of artificial nuclease complex. While control compound was totally inactive at used concentrations, both experimental compounds displayed clear ability to suppress Tat-mediated activation from HIV promoter.

Figure 4. The reduction of HIV Tat-expression by different agents (A14 – standard antisense oligonucleotide).



Hepatitis C virus

The effectiveness of BTD's new gene silencing platform is demonstrated by their application as antiviral agents against the Hepatitis C virus (HCV, genus *Hepacivirus*, family *Flaviviridae*).

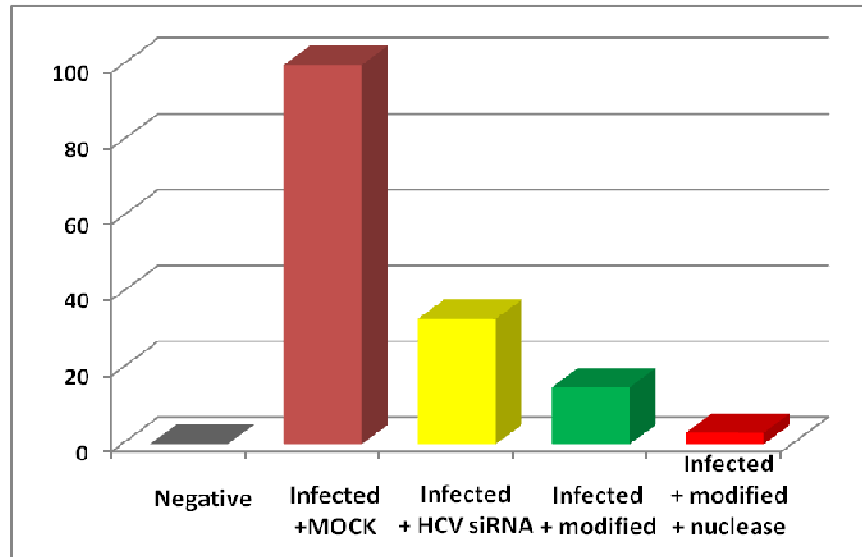
The new technology directly targets the virus replication system. In contrast to most anti-HCV antisense compounds the target sites have been selected in the coding region of HCV genome which offers much bigger selection of potential targets compared to typically targeted and highly structured IRES element. In most applications, this technology includes the use of oligonucleotides with lengths about 15...25 residues and containing 10-100% modified bases that can be linked to an organic nuclease complex..

The antiviral effects our antisense compounds, demonstrated with a series of anti-HCV compounds, show significant effect with modified compounds (Figure 5). The inhibition is also efficient against viral genomes which have acquired mutations, providing resistance against low molecular weight inhibitors such as protease inhibitors.

These and other data, available from our detailed studies, confirm the extraordinary potential of the new technology. Compared to the standard antisense oligonucleotides the effective EC50 concentrations were hugely reduced (at least 100 times) to the low nanomolar range or, in case of some oligonucleotides with bound organic nuclease, even lower. It was also shown in parallel experiments that standard antisense oligonucleotides failed to show any significant antiviral activities at concentrations in the low micromolar range, which is consistent with reports from other research groups. The EC50 of all novel antisense oligonucleotides was at least two orders of magnitude lower than the concentration causing considerable cytotoxic effects (cytotoxic conc. 50).

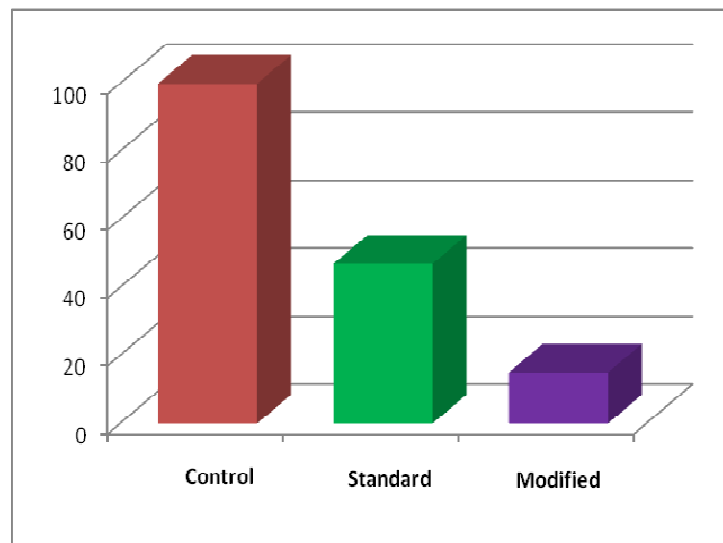
In addition, our data indicates that some previously developed modifications, such as introduction of PTO bonds or LNA nucleotides into antisense oligonucleotides targeting the HCV genome, has on its own no or even negative effect on the anti-HCV activity of the oligonucleotides. We have demonstrated that these problems can be overcome and high antiviral efficiencies obtained by incorporation of modified nucleobases and/or artificial nuclease groups into these modified oligonucleotides.

Figure 5. Demonstration of the efficiency of modified antisense oligonucleotides against virus containing HCV target sequence and expressing *Renilla luciferase* reporter (% of reduction).



The **animal tests** targeting the hepatitis C virus have been carried out with mice. When concentrations of compounds are adjusted to the body-weight of animals (ca 30 grams) the obtained results are in good correlation with results from *in vitro* systems (Figure 6.)

Figure 6. Demonstration of the *in vivo* efficiency of modified oligonucleotides (% of virus reduction).

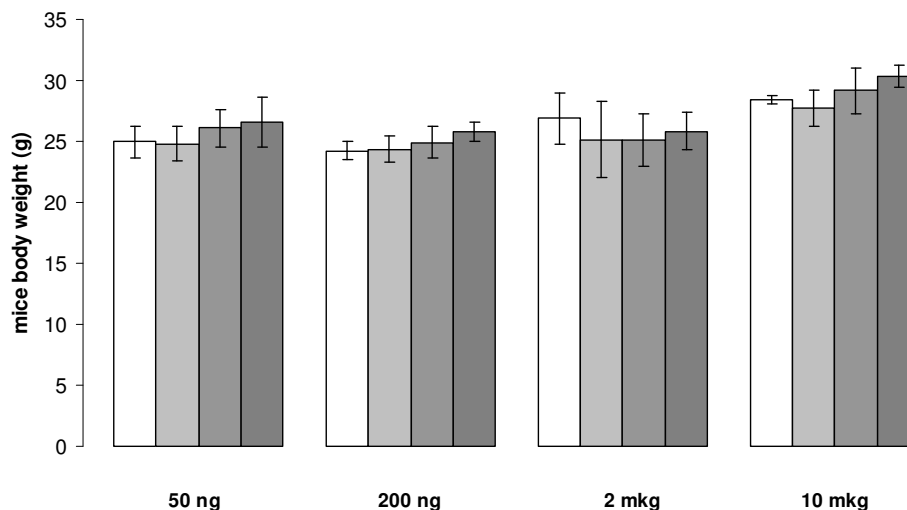


Modified oligonucleotides are non-toxic *in vivo*

Mice (approximately 25 g in bodyweight, 5 mice per group) were injected (hydrodynamic injection through tail vein) with increasing amounts of oligonucleotides containing five modified residues and antisense target in HCV. The body weight of animals was measured before (white bar in Figure 7) injection and 1, 5 and 14 days after injection (darker bars). No weight losses were documented at any

concentration of the agent. Results obtained for mice injected with oligonucleotide–nuclease complexes of with oligonucleotides containing eleven modified residues were essentially identical to those presented below. No abnormality (aside the effects caused by transfection procedure) was detected for animals in this or any other experiment.

Figure 7. Effect of injected modified oligonucleotides on the body weight of mice.



Conclusions

The results clearly demonstrate that the oligonucleotides with modifications and their conjugates with artificial nucleases are much more potent inhibitors than oligonucleotides without such modifications both *in vitro* and *in vivo*. At the same time, all compounds tested were well tolerated by animals.

Therefore, using innovative methods, BTD has been successful in creating the prototypes for a new generation of gene silencing drugs. Our approach has all the advantages of general antisense technology, namely the possibility of targeting almost any RNA (viral or cellular), the relatively easy optimization of the antisense drug structure to new targets, including those from drug-resistant virus genotypes (compared with optimization of drugs against proteins, encoded by these RNAs), and the possibility of constructing and simultaneously using a number of different antisense oligonucleotides without those interfering with each other and/or with essential cellular housekeeping systems. We have also shown that use of our modified nucleobases is compatible with other nucleic acid modifications used to improve the efficiency and stability of antisense compounds.

BTD, Ltd. is licensing the above described gene silencing technology for specified targets as well as delivers the necessary modified oligonucleotides and/or oligonucleotide-nuclease conjugates.

Contact:

Prof. Mati Karelson
 Baltic Technology Development, Ltd.
 Ahtri 8, Tallinn 10151
 Estonia
 Phone: +372 6155 099
 Fax: +372 6202 819
 E-mail: info@genecode.com

Prof. Andres Merits
 Institute of Technology
 University of Tartu
 Nooruse 1 50411, Tartu, Estonia
 Phone: +372 7375 007
 Fax: +372 6202 819
 E-mail: andres.merits@ut.ee