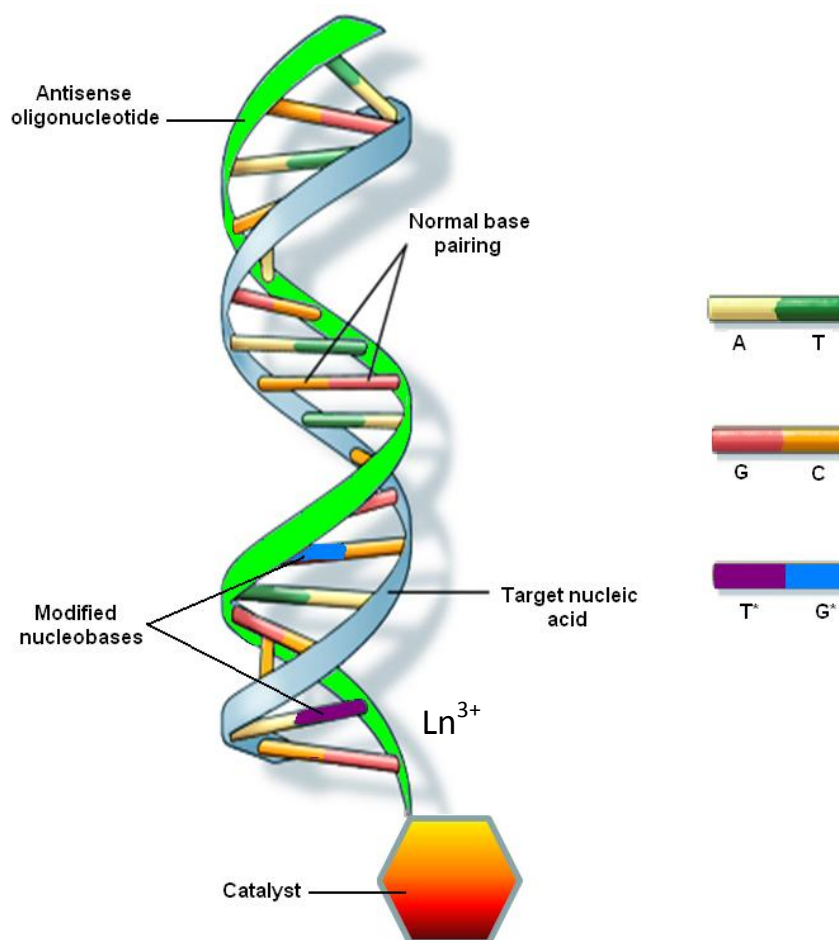


NEW ANTIVIRALS AGAINST HEPATITIS C BASED ON NOVEL GENE SILENCING PLATFORM

Novel gene silencing platform

BTD, Ltd. has introduced a new direction in the use of gene silencing agents in therapeutics. The 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 (Figure 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., Pilv, 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



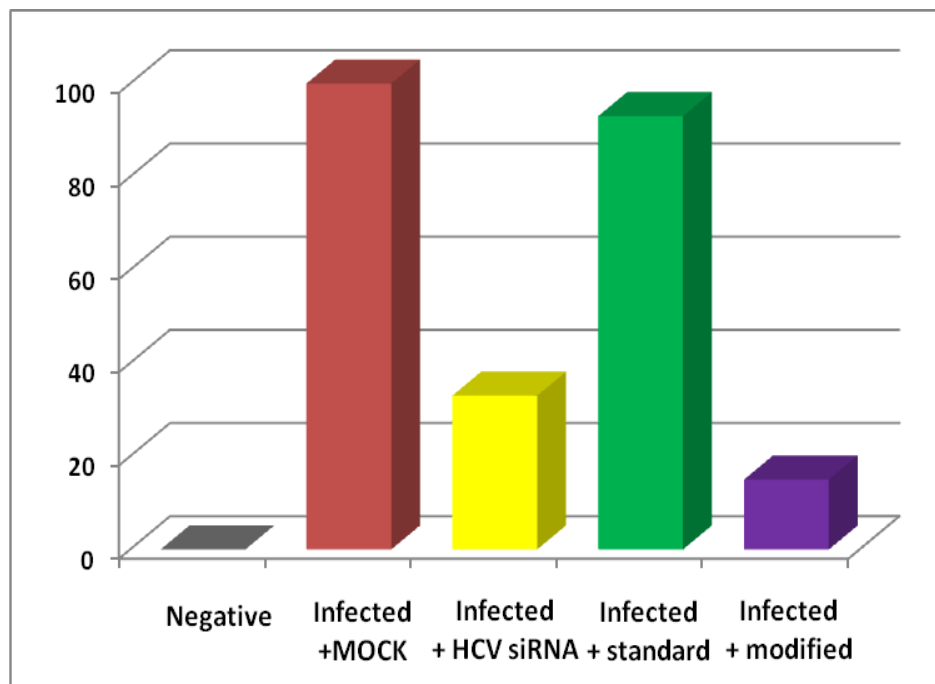
Novel agents against hepatitis C

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 efficiency (% of reduction of viral genomes) of modified antisense oligonucleotides against virus containing HCV target sequence through expression of *Renilla luciferase* reporter is depicted in Figure 2. The infected cells were treated with complexes containing antisense compounds or siRNA control at 30 nanomolar concentration, control cells were mock-treated 24 h prior infection. *Renilla luciferase* activity, measured at 24 h post infection, is proportional to the copy number of viral genomes. All results were normalized to activity in mock-treated cells, which is taken as 100%. siRNA indicates the effect of highly active anti-HCV control siRNA. Similar data was obtained if the antisense compounds were used to target HCV 1b replicons in stable cell line indicating that modified antisense compounds are effective against established (persistent) HCV infection as well.

Figure 2. Demonstration of the efficiency of modified antisense oligonucleotides against virus containing HCV target sequence and expressing *Renilla luciferase* reporter (% of reduction).



The additional effect to the antiviral efficiency of the artificial nuclease complex conjugated to modified antisense oligonucleotides against virus containing HCV target sequence and expressing *Renilla luciferase* reporter is presented in Figure 3. The conditions of the experiment were the same as described above. The dependence of antiviral effect from the concentration of inhibitor for both the modified oligonucleotides with and without conjugated artificial nuclease is given in Figure 4.

Figure 3. Demonstration of the additional effect of nuclease complex to the antiviral efficiency of modified antisense oligonucleotides against virus containing HCV target sequence and expressing *Renilla luciferase* reporter (conditions of the experiment and presentation of the data same as in Figure 2).

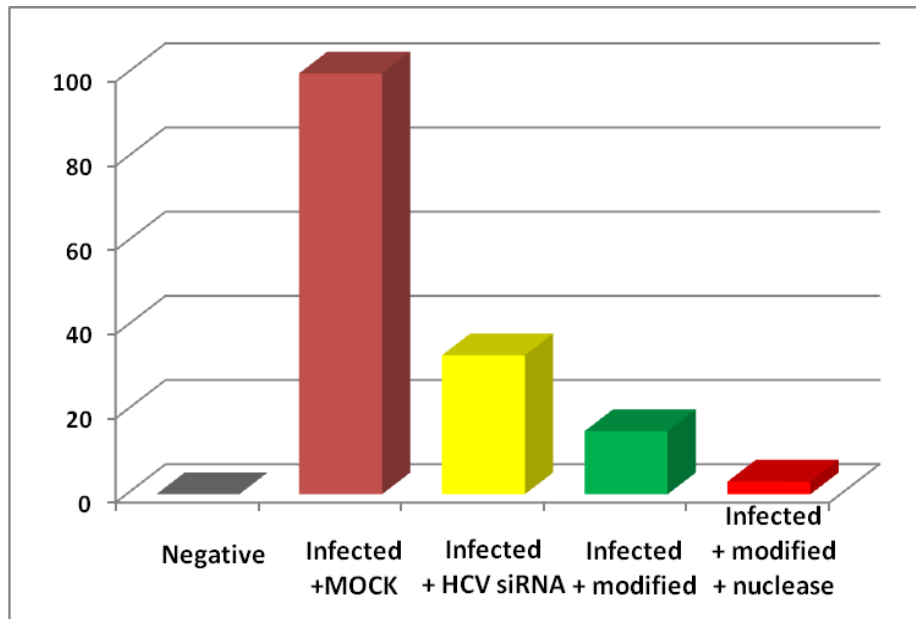
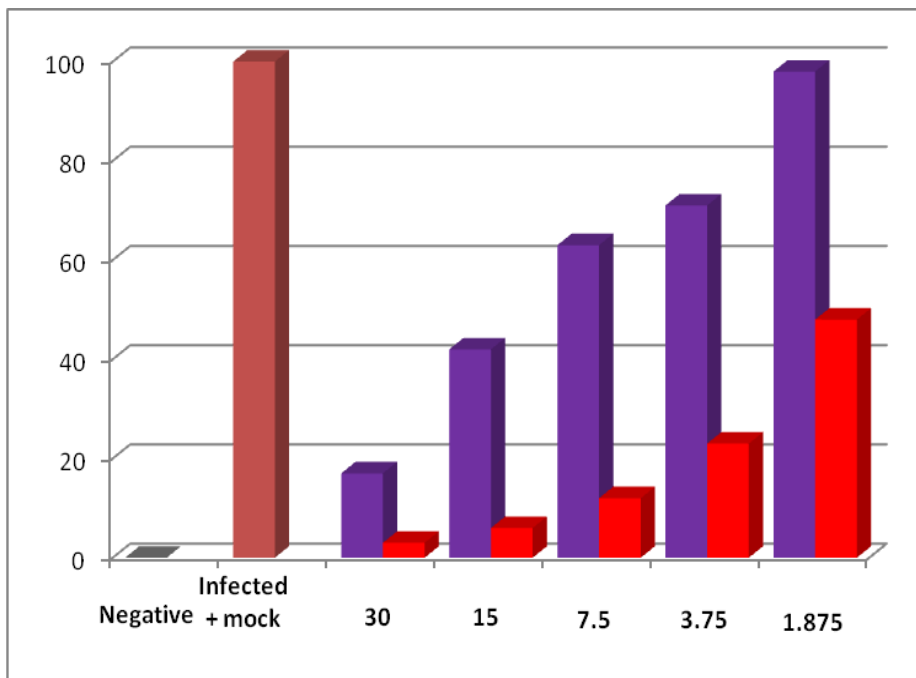


Figure 4. Dependence of antiviral effect of modified oligonucleotides without (colored columns) and with (open columns) nuclease from the concentration of inhibitor (shown below the columns, in picomol/ml). Conditions of the experiment and presentation of the data are the same as in previous examples.



The efficiency of the modified oligonucleotides has been also **compared with the existing locked nucleic acid (LNA) technology**. First, siRNAs, which have much higher anti-HCV effect than control siRNA used in above (Figure 2, 3) were selected; their target sites were used for design of modified

antisense oligonucleotides. The selection of the DNA oligonucleotide target site utilizing RNAi approach is described in Figure 5. The Hepatitis C Virus (HCV) RNA genome with viral genes encoded by it is given in Fig. 5A (upper panel) and bicistronic HCV ET RNA replicon, containing cell culture adaptive mutations (asterisks). The siRNA target site selection is further explained in Figure 5B. The Huh-luc/neo-ET1 cell line derived from human hepatocarcinoma cell line, which harbors HCV subgenomic bicistronic replicon (developed by Dr. Volker Lohmann and Dr. Ralf Bartenschlager, ReBlikon GmbH) was used for siRNA screening. siRNAs targeting HCV RNA were designed by using a specialized algorithm-driven software. The siRNAs (6.25 nM) were reverse-transfected using Lipofectamine™ RNAiMAX (Invitrogen) reagent and luciferase activity was measured after 48 hours. Dashed arrows schematically indicate target sites of siRNAs in the replicon RNA. Dashed box delimits HCV RNA-specific siRNAs. Ffluc(GL2+GL3), siRNA targeting firefly reporter gene (AM4629, Ambion). #4611, negative control non-targeting siRNA (AM4611, Ambion). Pos. Ctrl., positive control. Neg. Ctrl., negative control. Note the logarithmic scale of y-axis. siRNAs targeting sites #1 and site #2 were found to be the most potent HCV replication inhibitors in a cell culture experiment and hence were chosen as the primary sites for DNA oligonucleotides design.

Figure 5. siRNA target selection.

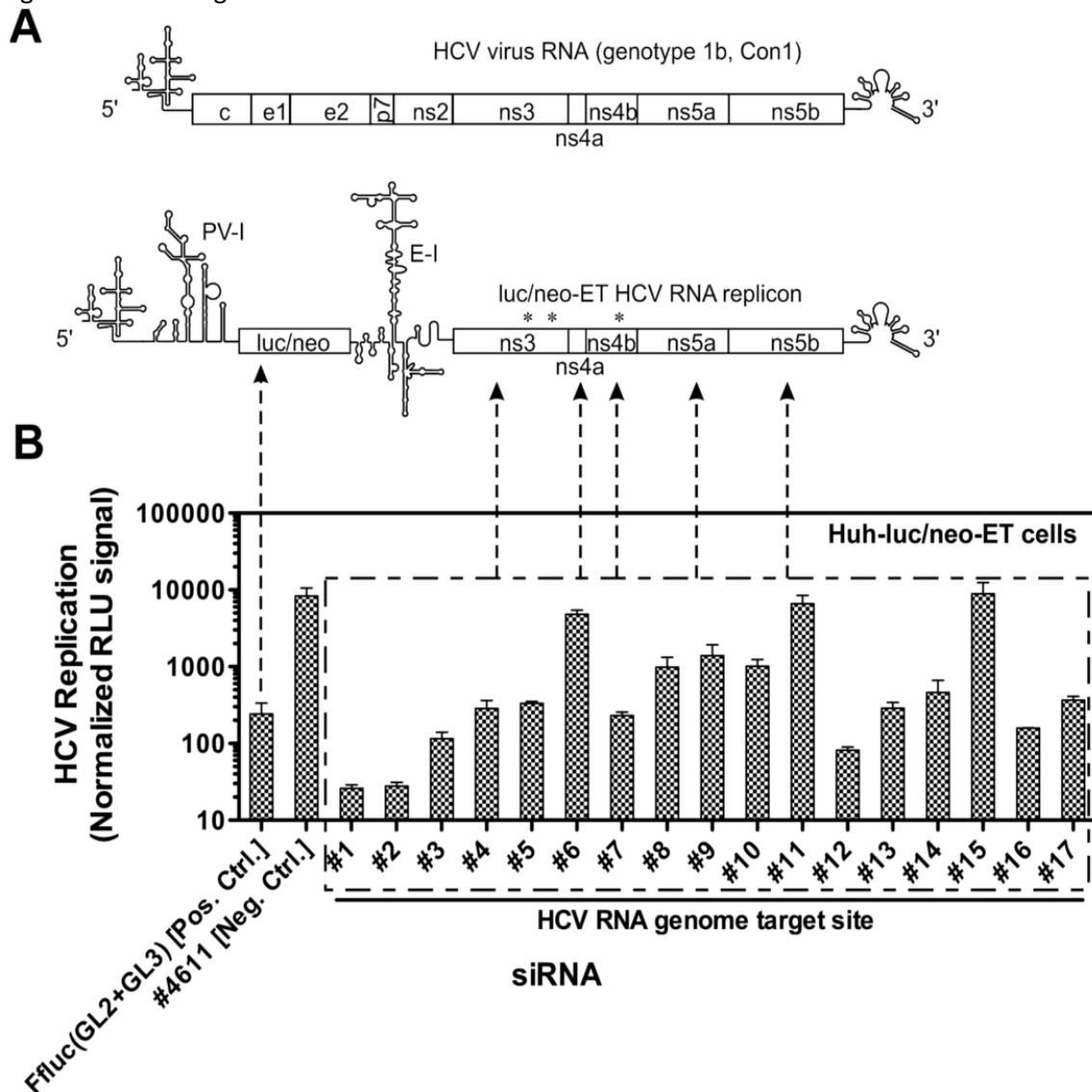


Figure 6. Comparison of antisense effects of DNA, modified DNA, modified DNA/LNA, and DNA/LNA oligonucleotides on siRNA target site #1.

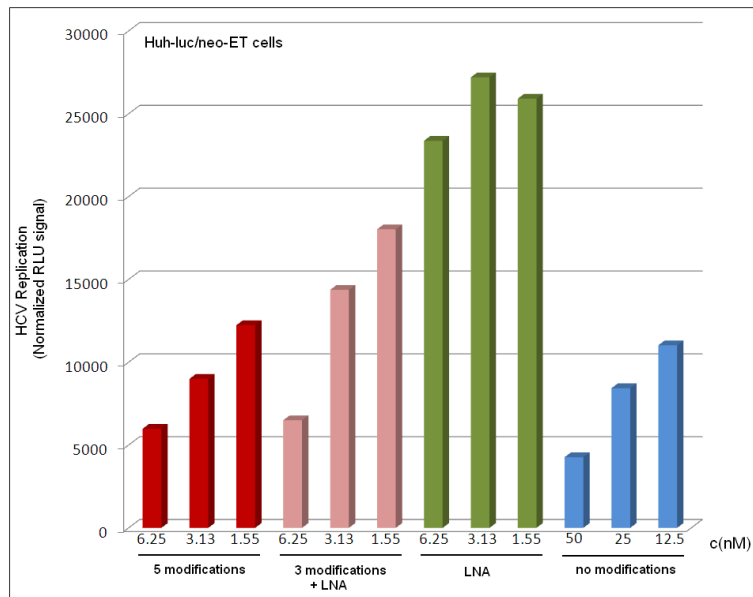
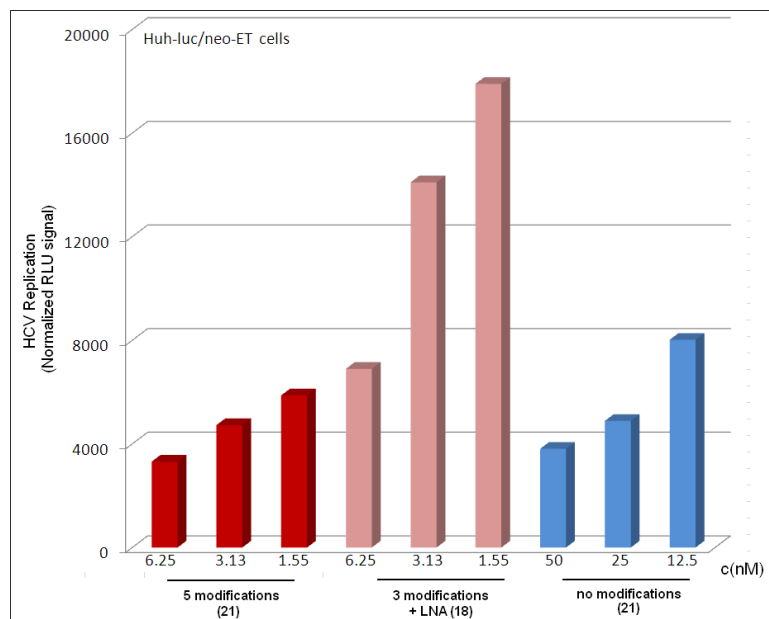


Figure 7. Comparison of antisense effects of DNA, modified DNA, modified DNA/LNA, and DNA/LNA oligonucleotides on siRNA target site #2.

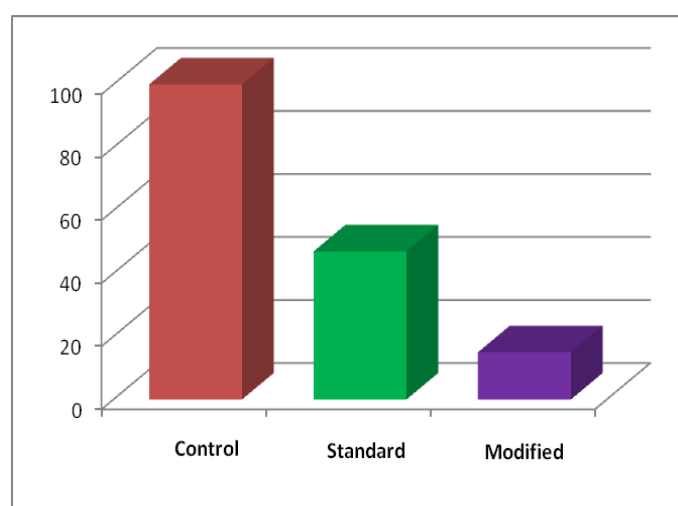


DNA oligonucleotides complementary to target sites #1 and #2 selected by RNAi approach (Figure 5B) were therefore synthesized and reverse-transfected into Huh-luc/neo-ET cell line. The comparison of the activity of the base-modified oligonucleotides with the normal and LNA-modified oligonucleotides (having the length of either 18 or 21 nucleotides) is given in Figures 6 and 7. As it is evident from this data selected standard antisense oligonucleotides are efficient inhibitors at concentration 12,5 nM which is very good efficiency for this class of compounds and demonstrates efficiency of used target-site selection procedure. The modified oligonucleotides, containing five modified bases, were, however, active at much lower concentrations showing the additive effect of

nucleobase modifications. LNAs of any design (including LNA/DNA gapmers) were inactive at these concentrations; the addition of three modified nucleobases considerably activated their ability to inhibit replication of HCV replicon.

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. All *in vivo* experiments were performed as follows: mice (each group consisting from 10 animals) were transfected using hydrodynamic shock method with reporter plasmid expressing Renilla luciferase (Rluc) marker fused to HCV fragment containing target site for antisense oligonucleotide. Another plasmid expressing Luciferase reporter and containing no target site was used as control. Oligonucleotides (750 picomol per 22-25 gram mice) and control siRNA against Rluc region (Ambion, 150 picomols per 22-25 gram mice) were co-delivered with plasmid reporters. Mice were sacrificed 24 post-transfection, livers were extracted, homogenized and the reporter activities measured. Positive control siRNA caused over 10-fold reduction of reporter expression indicating that the system was relevant for the analysis. The results for antisense oligonucleotides presented on Figure 8 are normalized to the signal produced by control plasmid in absence of antisense inhibitors. The presented data indicate that as little as five modified nucleobases per oligonucleotide increase ability to suppress expression from targeted mRNA more than twice (single delivery, in mice liver).

Figure 8. Demonstration of the *in vivo* efficiency of modified oligonucleotides (% of virus reduction).



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

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