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Self-Neutralizing oligonucleotides with enhanced cellular uptake

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Abstract

There is tremendous potential for oligonucleotide (ON) therapeutics, but low cellular penetration due to their polyanionic nature is a major obstacle. We addressed this problem by developing a new approach for ON charge neutralization in which multiple branched charge-neutralizing sleeves (BCNSs) are attached to the internucleoside phosphates of ON by A phosphotriester bonds. The BCNSs are terminated with positively charged amino groups, and are optimized to form ion pairs with the neighboring phosphate groups. The new modified ONs can be prepared by standard automated phosphoramidite chemistry in good yield and purity. They possess good solubility and hybridization properties, are not involved in non-standard intramolecular aggregation, have low cytotoxicity, adequate chemical stability, improved serum stability, and above all, display significantly enhanced cellular uptake. Thus, the new ON derivatives exhibit properties that make them promising candidates for the development of novel therapeutics or research tools for modulation of the expression of target genes.

INTRODUCTION

Several decades ago Drs. Paul Zamecnik and Mary Stephenson pioneered a new potential therapy approach. They foresaw that intracellular introduction of oligonucleotides (ONs) complementary to specific mRNA may serve as a tool to nullify deleterious gene products.^{1, 2} In subsequent years, this nucleic acid-based form of therapy became known as antisense therapy and has evolved to include RNA interference (siRNA, microRNA) therapy approaches.³ These techniques allow selective silencing of target genes, and, thus, are very attractive for the treatment of cancer, detrimental gene mutations, and even pathogenic microorganism-mediated diseases.

[†]Electronic supplementary information (ESI) available: NMR Spectra, 30 pages; differential melting curves of oligonucleotide duplexes, 14 curves; fluorescent high resolution images of incubation of oligonucleotides with A172 cells, 2 images.

The main obstacles in the realization of those therapeutic promises are poor pharmacokinetic (PK) properties and inefficient cellular uptake of the natural ONs. Natural ONs are highly charged molecules that are repelled by negatively-charged cellular membranes and are subject to inefficient intracellular transport. Inside the cells, ONs undergo endosome entrapment and lysosomal degradation resulting in very low cytoplasmic levels. In circulation, quick nuclease degradation and fast renal clearance result in very short half-life. This prompted the development of a large number of synthetic analogs of natural ONs, such as 2'-position modified ONs^{4, 5}, phosphorothioates⁶, methylphosphonates⁷, boranophosphonates⁸, benzylphosphonates⁹, locked nucleic acids^{10–12}, alkynyl phosphonates¹³, peptide nucleic acids (PNA)¹⁴, and morpholino oligonucleotides^{15, 16}, which have been recently reviewed extensively by Wan and Seth.¹⁷ While many of those modifications successfully solved the problem of nuclease degradation, they did little to improve the intracellular penetration.

In attempts to reduce or eliminate the ONs negative charge, and hence improve protein binding and cell membrane penetration various polyamines have been conjugated to a variety of ON derivatives.^{18–21} The polyamines have been attached either to the 5', or the 3' end of the ONs, (known as ZIP-ONs)^{22, 23} with few reports for attachment to the nucleobases.^{24, 25} In most cases, polyamine conjugation had beneficial effects on target hybridization,²⁶ nuclease resistance, and cellular uptake.^{18, 19} Unfortunately, attachment of a one or more polyamine molecule to either the 5' or the 3' end of the ON has certain drawbacks. Clustering of the positive charges at the end of the ONs is not the optimal arrangement for formation of tight ion pairs with the negative charges throughout the ON's backbone, and can often result in intermolecular aggregations and solubility problems. For significant charge reduction of larger ONs (20-mer or more), the size of the polyamine moiety becomes unacceptably large, often bringing cell toxicity. On the other side, attachment of polyamine molecules to the nucleobases has unpredictable effect on the biological activity.^{24, 25} Although the polyamine–ON conjugation represents a promising direction for further development of oligonucleotide-based therapeutics and tools, for their realization certain problems need to be resolved: (1) the polyamine moieties have to be optimized for forming intimate ion pairs with the phosphate groups of the same ON molecule without significant entropy penalty, thus reducing the possibility for aggregation and non-specific interactions; (2) chemical means of attachment of multiple polyamine moieties to the ON need to be developed, preferably without modification of the nucleobases.

One of the widely used modifications of ONs is the attachment of amino groups to oligonucleotides via linkers. The attached amino groups are mostly used as anchor groups for post-synthetic derivatization. As with the case of the polyamine conjugates, the linker is typically attached to the 5'-end of the ON upon the completion of the automated synthesis.^{27, 28} Attachment of amino linkers to the internucleoside phosphoroamidate groups has been performed using H-phosphonate chemistry,^{29, 30} but this approach limits the number of amino linkers that can be incorporated.

We have addressed the need for attachment of multiple amino-group terminated linkers in a sequence specific manner by developing a method for their attachment to the internucleoside

phosphate groups through phosphotriester bond, using phosphoramidite chemistry.^{31–34} The linkers were optimized not for charge neutralization, but for stability and efficiency of post-synthetic fluorescent reporter groups attachment: The linkers were based on triethylene glycol (9 atoms long), which moves the amino groups away from the phosphate backbone, increasing their steric accessibility, and reducing the propensity for linker scission through intramolecular attack of the amino group on the carbon atom attached to the phosphotriester group. We realized that, if the linker is optimized for formation of close ion pairs between the amino groups and the neighboring phosphates, and if the stability issues due to the above mentioned intramolecular linker scission are carefully addressed, this approach could be further developed for the attachment of charge neutralizing polyamine moieties. This led us to the development of new concept for modified ON with reduced or completely eliminated overall negative charge. Specifically, the modified ONs contain a defined number of short, branched, charge-neutralizing sleeves (BCNSs) with positively charged amino groups at their termini. The BCNSs are attached covalently to the internucleoside phosphates through phosphotriester bonds. The branch length is optimized in such a way that their terminal positive charges can reach the neighboring phosphate groups and neutralize them through ion-pairing (Fig. 1). Branching of the sleeves at the site of attachment reduces the possibility of sleeves scission during the alkaline deprotection by intramolecular amino groups attack on the attachment side. The chemical nature of the BCNS is designed to add sufficient hydrophobicity to the ONs without compromising their aqueous solubility. This modification approach is compatible with the currently used phosphoramidite chemistry for automated ON synthesis, and can be combined with other ON modifications or delivery systems. By varying the chemical nature of the BCNSs, the type of terminal positively-charged groups, the number of attachments, and the site of BCNS incorporations, our approach can be used to optimize the properties of the ONs.

In this report we present our studies aimed to validate this concept. We have synthesized four modified phosphoramidites comprising thymidine and 2'-OMe-uridine, which contain three different types of BCNSs (Scheme 1, compounds **12**, **14**, **16**, and **22**, and Fig. 3). Those phosphoramidites were used in conventional automated phosphoramidite chemistry to prepare a number of modified 2'-deoxy, or 2'-OMe ONs having different types and numbers of BCNSs attached to their phosphate or phosphorothioate backbones.

We have performed preliminary evaluation of the basic but essential biophysical and biochemical properties of the novel ONs and report that the BCNS modifications preserve (and in some cases improve) the hybridization properties and does not compromise aqueous solubility. Preliminary evaluation of these ONs also indicated that they have low cytotoxicity, enhanced serum stability, and, most importantly, significantly increased cellular uptake. More detailed evaluation of their biophysical and biochemical properties as well as the selective modulation of the activity of target genes will be the topics of future reports.

RESULTS AND DISCUSSION

BCNSs optimization by computer modeling

The chain lengths and structures of the BCNSs described in this report were initially designed and optimized for ion-pairing with the neighboring $n=\pm 1$ phosphate groups in the

single stranded ONs using Discovery Studio software. Three major criteria were considered during the computer modeling: 1) chain length optimization of both BCNS branches, 2) potential steric effects of terminal amino and dimethylamino groups of BCNS, and 3) potential impact of different stereo configurations of the chiral phosphotriester groups formed after covalent attachment of BCNS to the internucleoside phosphates. As it is demonstrated in Fig. 2, the optimal length of each branch is four atoms between the carbon attached to the phosphate oxygen and the terminal amino groups of BCNS. Formation of ion-pairs by protonated dimethylamino groups is sterically equal to that of protonated primary amino groups (Fig. 2, panels B, and C). Attachment of BCNS to the phosphate group renders the phosphorus atom asymmetric, i.e., the resulted phosphotriester group can have two stereo configurations. Our modeling shows that both configurations enable formation of ion-pairs at minimum energy level, and both diastereomers (A and B in Fig. 2) exhibit similar spacing between negatively charged phosphate oxygens and the nitrogen atoms on the BCNS, enabling ion-pairs formation (distances within 2.59 – 2.68 Å).

Selection of BCNS

The hydrophobicity of the BCNSs can have a significant impact on the properties of the modified ON. On the one hand, increased hydrophobicity can positively affect the ON interaction with the cellular membranes and improve the intracellular penetration, increase the circulation time and improve the pharmacokinetic properties. On the other hand, too high hydrophobicity can have significant negative effects. For instance, entirely hydrocarbon-based linkers, in order to minimize the surface that interacts with water molecules tend to adopt a globular conformation in aqueous environment. This may interfere with ion-pair formation. High hydrophobicity can also impair aqueous solubility, cause aggregation, undesired binding to receptors, other proteins, or tissues resulting in toxicity. Therefore, the degree of hydrophobicity of the BCNS needs to be carefully analyzed and optimized. For these purposes, we chose to prepare three types of BCNS. The first type, with primary amino groups at the ends and two oxygen atoms in the sleeve (BCNS 1 in Fig. 3) was selected as the most hydrophilic BCNS. Another one, having two dimethylamino groups at the ends and two oxygen atoms in the sleeve (BCNS 2, Fig. 3) was selected as a representative with intermediate hydrophobicity, and a third one, having two dimethylamino groups at the ends and an entirely hydrocarbon-based sleeve (BCNS 3, Fig. 3), represented the most hydrophobic BCNS.

Synthesis of BCNS-containing phosphoramidites

First, we synthesized three diaminoalcohols, (compounds **7**, **8**, and **20** in Scheme 1), which served as starting materials for the synthesis of the four BCNS-bearing phosphoramidites (**12**, **14**, **16** and **22**). The synthesis of a key intermediate for **7** and **8**, the dibromo analog of **3** has been published.³⁵ Yet, this 4 step synthesis involves rather expensive starting materials - (2-*O*-benzyl-glycerol and *O*-(2-tetrahydropyranyl)-2-chloroethanol), and is difficult to scale up. Therefore, for the synthesis of **3** we adopted the method described for the synthesis of an analogous compound (WO2005/014049A2) from epibromohydrin and 2-(2-chloroethoxy)ethanol, replacing the latter with 2-chloroethanol (See Scheme 1). According to this scheme we prepared the dichloroalcohol **3** in 3 steps and in 49% overall yield. Ca.

10% of isomeric dichloroalcohol **4** was formed in step c due to reaction of the chloroethanol at the more hindered, secondary position of the oxirane **2**.

The diethylamino analog of **20** has been prepared from azelaic acid (5-oxononanedioic acid)³⁶ Since azelaic acid is prohibitively expensive for larger scale synthesis, we prepared compound **20** from the dichloroketone **18**. We prepared **18** by two alternative routes: in two steps from δ -valerolactone (**23**) or in one step from olean (1,7-dioxaspiro[5.5]undecane, **17**).

The four phosphoramidites, **12**, **14**, **16**, and **22** were prepared from the diaminoalcohols **7**, **8**, and **20** by reaction with bis-(diisopropylamino)chlorophosphine (**9**, prepared *in situ* from phosphorus trichloride and diisopropylamine), followed by reaction with the corresponding DMT-protected nucleosides in the presence of 5-ethylthiotetrazole. The phosphoramidites with dimethylamino-terminated sleeves (**16**, **22**) were purified by silica gel chromatography. The more polar phosphoramidites **12** and **14**, which contained trifluoroacetyl protected primary amino groups were co-eluted on silica gel with impurities, and had to be purified by preparative reversed phase chromatography, which required rigorously deoxygenated (with He) and ice chilled mobile phase in order to avoid oxidation to the corresponding phosphoramidates. The final phosphoramidites were prepared in 2.5 to 7 g scales, and yields of 57% (**12**), 44% (**14**), 71% (**16**), and 77% (**22**) with purity above 90%. Each impurity was characterized (LC-MS) and was found inert with respect to the oligonucleotide synthesis.

Synthesis of BCNS-containing ON

BCNS-containing phosphoramidites **12**, **14**, **16** and **22** were used to synthesize a number of 2'-deoxy-, and 2'-O-methyl ONs (from 19-mers to 23-mers) comprising different numbers of BCNSs in their sequence, and phosphate or phosphorothioate backbones as shown in Table 1. ON were synthesized on a 394 DNA/RNA synthesizer (Applied Biosystems) using standard phosphoramidite chemistry and UltraMILD[®] phosphoramidites (Glen Research). The coupling rates and efficiencies of the BCNS-containing phosphoramidites did not differ from those of standard phosphoramidites, and were above 98% (determined optically by the trityl deprotection method), and the coupling times didn't have to be optimized (the default coupling times of 10 min were used). The BCNS-containing phosphoramidites can be used for synthesis of both sugar-phosphate and sugar-thiophosphate segments. For the incorporation of phosphorothioate segments, oxidation was performed using Beaucage thiolation reagent⁵ or DDTT ((dimethylamino-methyldene)amino)-3H-1,2,4-dithiazaoline-3-thione, ChemGenes) under the manufacturer recommended conditions. For the incorporation of fluorescein (FAM) labels at the 5' end, 6-fluorescein phosphoramidite (Glen Research) was used. The removal from the solid phase and the bases deprotection were performed by 80 min incubation in concentrated ammonia/40% methylamine (1:1 v/v) mixture. Representative HPLC analysis of the deprotected mixtures (with DMT groups on) are shown in Fig. 4, A and B. Panel A represents the analysis of deprotected reaction mixture of control ON (without BCNS groups, ZT27 in Table 1) and panel B shows the analysis of deprotected reaction mixture of ON having the same sequence and 3 incorporated BCNSs (ZT25). There is no significant increase of the curtailed, capped sequences (peaks around 10 min), indicating similar coupling efficiency of standard and BCNS containing phosphoramidites.

After deblocking, the 5'-DMT protected ONs were purified by reversed phase HPLC, detritylated (80% acetic acid, 15 min at r.t.) and desalted (Glen-Pak™ cartridges). The final purity of the ONs was determined by analytical HPLC, and was above 94% (see Fig. 4 C for representative chromatogram). The characterization was done by negative mode electrospray MS analysis (Table 1).

Phosphoramidite **22**, which has an entirely hydrocarbon-based sleeve, showed excellent coupling properties during the automated synthesis of ONs (based on DMT deblocking values). However, after the alkaline deprotection, the yields of the final ONs, according to the HPLC analysis, were very low. Mass spectrometric analysis of the products showed significant loss of BCNSs during the deprotection. We have also detected some loss of BCNSs with phosphoramidites **12**, **14**, and **16** during the alkaline deprotection step in the synthesis of ON containing higher number of BCNSs when extended (more than 80 min) deprotection time was used. This problem was avoided by minimizing the deprotection time to 80 min, in which case no loss of BCNS groups was observed and the yields of BCNS-containing ONs was compatible with those of control unmodified ONs. Yet, even after optimization of the deprotection time, the yields of ONs, containing type 3 BCNS (made with phosphoramidite **22**) were unacceptably low.

We anticipated the cleavage of BCNS groups during the alkaline deprotection as a potential side reaction: an intramolecular nucleophilic attack of the terminal amino group of the sleeve on the carbon atom connected to the phosphate group would go through an entropically favorable 6 atoms transition state. Since the phosphodiester group is a relatively good leaving group, such an attack could result in cleavage of the sleeve. Indeed, the 4-(N-methyl-N-(trifluoroacetyl)amino)butyl group has been developed as an alternative to the cyanoethyl group for phosphate protection in ON synthesis.³⁷ The authors observed that in concentrated ammonia at 55°C, after trifluoroacetyl group removal, the N-methyl-4-aminobutyl group was cleaved instantaneously (5 carbon atom transition state), while 5-aminopentyl and the 2-(2-aminoethoxy)ethyl groups (6 atoms transition states) were cleaved after 2 and 10 h, respectively. While anticipating such a side reaction, we expected that the presence of a branching at the site of the phosphate attachment (increased steric hindrance), and utilization of "Ultramild®" deprotection chemistry, where the alkaline deprotection is carried out at r.t. would mitigate this problem. These expectations were realized only in the case of the more hydrophilic, oxygen-containing BCNSs. Our observations are in agreement with the earlier described work,³⁷ where the oxygen-containing 2-(2-aminoethoxy)ethyl sleeve was 5 times more stable than the 5-aminopentyl one.

One possible explanation of the different behavior of the ONs containing BCNS with oxygen atoms in the sleeve and the ONs carrying BCNSs with an entirely hydrocarbon sleeve may be the lower basicity of the amino groups in the former due to the effect of β -oxygen substitution.³⁸ Due to low chemical stability during the alkaline deprotection step of oligonucleotides having BCNS 3 (enabled by phosphoramidite **22**) further studies were performed only with ONs containing BCNS 1 and 2.

Thermal melting studies

We evaluated the ability of the BCNS-containing ONs to form duplexes with complementary ONs by measuring the annealed duplexes melting temperatures. Representative melting curves are shown in Fig. 5, and the melting temperatures are listed in Table 2. We found that the melting temperatures (T_m) of DNA duplexes formed with ONs containing BCNS with primary amino groups (Type 1, Fig. 3) did not differ from those of the control duplexes and remained unchanged within the sensitivity of the method regardless of the number of BCNSs incorporated (Table 2, rows 1 to 4, 0.5 μ M duplex concentrations in 10 mM $MgCl_2$, 15 mM KCl, 25 mM HEPES, pH 8.2 with heating rate 0.5 deg/min).

In lower ionic strength buffer (25 mM HEPES, pH 7.3), the 2'-OMe duplexes containing dimethylamino groups terminated BCNSs showed gradual increases in the T_m as the number of BCNSs increased (rows 6 to 10, Table 2). These data are in agreement with published reports describing that spermine, covalently attached to the termini or heterocycles of one of the ONs in the duplex, increases the T_m of the duplex.^{24, 39} As it is shown in rows 5 and 11, no melting was detected when oligonucleotides with BCNSs (both with primary and tertiary amino groups) were mixed with non-complementary ONs (see Supporting Information for the differential melting curves), thus indicating the absence of non-standard inter-molecular aggregation.

Solubility and chemical stability

Solubility in PBS was evaluated by dissolving 100 nmol of ONs in PBS (100 μ l) to obtain 1 mM solution. After sonication for 5 min and centrifugation, the UV absorption of the supernatant was measured and compared with the calculated.^{40–43} All compounds, including highly backbone-neutralized ones (ZT7 and ZT11 from Table 1), were completely soluble at 1mM concentration.

During the purification stage, compounds were incubated in concentrated ammonia for over 1 h and later in 80% acetic acid for 15 min. In other words, all compounds from Table 1 were stable at pH range from 3 to 12. Sterile PBS solutions of compounds ZT11 and ZT12 were stored at r.t. for 1 month without substantial degradation according to HPLC analysis. The only ONs that showed low stability during the 80 min r.t. alkaline deprotection were ONs containing BCNS 3.

Stability in serum

The stability of BCNS-bearing 2'-OMe ONs (ZT11, 6 BCNSs, and ZT12, 4 BCNSs) in serum was evaluated relatively to the stability of 2'-OMe ON with same sequence and backbones but without BCNS modifications (ZT15). For that purpose equimolar mixtures of ZT11 with ZT15, and ZT12 with ZT15 (5 μ M each) were incubated in PBS/bovine serum at 37 °C for 8 h and compared, after HPLC analysis with a control mixtures of the same oligonucleotides in serum, which were not subjected to incubation. The ratio of the peak areas of ZT11 and ZT12 to the peak area of ZT15 (no BCNS control, used as an internal standard) increased from 1:1 (no incubation) to 12:1 and 10:1, respectively, (8 h incubation) indicating that BCNS modifications not only do not compromise the stability relative to those of the 2'-OMe oligonucleotides, but, on the contrary, significantly increase their

stability in nuclease-containing biological fluids. These observations are in agreement with previous reports that multiple attachments of polyamines to the bases of ON have positive, and additive effects on the stability of ONs,^{24, 43} and these effects are independent of the positions of the polyamine-bearing bases within the sequence.^{43, 44}

Evaluation of key biological parameters

Cytotoxicity—The cytotoxicity of ONs containing different numbers of type 1 or type 2 BCNSs (Fig. 3) was evaluated in 4 different cell lines. ONs without BCNS groups served as controls. All ONs employed in this part of the evaluation did not have sequence-specific targets in the cell lines used. The list of ONs, their concentrations, and the cell lines are summarized in Table 3.

For HEK 293 cell line, relative viability of cells in each test condition was determined as the ratio of apoptotic cells labeled with the nuclear stain/late-stage apoptosis label to total number of cells. As shown in Fig. 6, no statistically significant cytotoxicity toward HEK 293 cells was observed in the presence of 1 μ M and 10 μ M ONs. Average cell viability for HEK 293 was 88% – 98% with no notable differences in viability between the untreated cells and any of the ON treated cells (Fig. 6). Cytotoxicity was also determined visually by assessing basic cell/monolayer morphology. For 10 μ M concentrations of ONs, viability data for HEK 293 cells are presented in Fig. 7.

Average cell viability for A172, MCF7, and HeLa cells treated with ZT11 to ZT15 was over 95% with no significant differences in viability between the untreated cells and any of ON-treated (including unmodified control ZT15) cells (data not shown). Representative images of HeLa and MCF7 cells treated with ZT12 (ON with four BCNSs) and control ZT15 (ON with the same sequence but without BCNSs) are shown in Fig. 8. In conclusion, visual analysis of basic cell/monolayer morphology did not reveal any abnormalities or significant differences between cells treated with modified ONs and those treated with control.

Effect on cellular uptake—The uptake of BCNS-containing modified ONs by cultured anchorage-dependent cells was studied in three cell lines: A172, MCF7, and HeLa. For this part of the characterization, a series of ONs with identical sequence but with different number of incorporated BCNSs and with a fluorescent label (6-FAM, 6-carboxyfluorescein) at the 5' end were employed. In a preliminary range finding experiment, we incubated control ON ZD17 (without BCNS modifications) with A172 cells at concentration of 0, 0.1, 1 and 10 μ M. At 0.1 μ M, the fluorescence levels observed after cell washing and lysing were close to the background fluorescence (no oligonucleotide control). At concentration of 1 μ M, the fluorescence levels were 4 times above the background and all subsequent experiments were done at that concentration.

ONs at a final concentration of 1 μ M were added to near confluent cells in Dulbecco's Modified Eagle Medium/0.5% Fetal Bovine Serum without any additional formulation or cellular uptake enhancers. After incubation for 30, 60, 120, and 180 min, the cells were washed, lysed, and the cell-associated fluorescence was measured with a plate reader. Representative curves showing the changes in the amount of cell-associated fluorescence after the incubation of the cells with different ONs are presented in Fig. 9. The amount of

cell-associated fluorescence increased as the incubation progressed, and the rate of increase was proportional to the increase in the number of BCNSs in the ON. For instance, data presented in panel A indicate that the slopes of the curves for ZT18 (2 BCNSs), ZT19 (4 BCNSs) and ZT20 (6 BCNSs) are 2.1, 7.3, and 9.8 times higher than the slope of the curve for the control ON (ZT17, no BCNS). In other words, the rate of oligonucleotide uptake increased, on average, 1.5 times per each incorporated BCNS. In panel B, the uptake of two pair of control, and BCNS containing ONs by A172 cells is presented. The first pair consists of control 2'-OMe ON ZT8 (no BCNS) and the same sequence ON ZT30 containing BCNSs type 1 at positions 2, 8, 11, 14 and 17 from the 5' end. The second pair consists of the control 2'-OMe ON ZT31 (no BCNS) with sequence: FAM-CAC AAA AUC GGU TCU ACA GGG UA and the same sequence ON ZT32 bearing four type 2 BCNSs at positions 8, 12, 15, and 22 from the 5' end. From these data, it appears that the type 2 BCNS (dimethylamino groups at the termini) has higher effect on the cellular uptake than the type 1 BCNS (primary amino groups). The kinetics of the fluorescence increase suggests that this is not merely due to association of the oligonucleotides with the cell membrane or membrane surface proteins, but involve endocytosis or diffusion through the cell membrane.

For the visualization of cellular uptake, fluorescent images of A172 cells incubated with ZT17 and ZT20 for 150 min and counterstained with DAPI were taken. Fig. 10 depicts cell association/intracellular distribution of FAM-labeled oligonucleotides (green colored inclusions) in live 172 cells visualized by fluorescent microscopy. Blue color is derived from 4',6-diamidino-2-phenylindole (DAPI) DNA/nuclei counterstaining. Conventional oligonucleotide (ZT17) shows only a slight degree of cell-associated staining while BCNS-modified ON displays very high level of fluorescence associated with virtually 100% of captured cells.

Although these experiments clearly demonstrate the significant effect of the BCNS modifications on the cell penetration rates in the chosen cell lines, further research is necessary to elucidate the specific pathways of cell penetration of the new modified ONs and the exact role of BCNS in this process. Also, in future studies, the intracellular localization, as well as the cell type and cell cycle dependence of the cellular uptake of the BCNS-containing ONs needs to be addressed. Our initial experiments indicate that the new ONs can be effective gene expression modulators. For instance, ZT22 which has base pair complementarity to miR10b microRNA was effective in suppression of the growth of miR10b-dependent A172 glioblastoma cells. At least 80% growth inhibition was observed after incubation with 5 μ M for 48 h whereas only 15% inhibition was detected in the presence of no BCNSs-containing control ZT21 (data not shown).

CONCLUSIONS

In summary, we have designed and synthesized dT- and 2'-OMe-U-containing phosphoramidites bearing branched sleeves with charge neutralizing amino groups on their termini. Branching of the sleeves at the carbon atom attached to the phosphate group reduces the possibility of sleeves scission by intramolecular attack by the amino groups. The sleeves length was optimized in order to enable the formation of ion-pairs between the terminal amino groups and the neighboring n-1 and n+1 phosphate groups. We prepared

phosphoramidites with 3 types of BCNSs (Fig. 3) featuring primary amino or dimethylamino groups at their termini, and sleeves with different degree of hydrophobicity. We have demonstrated that phosphoramidites having BCNS 1 and 2 can successfully be used for automated chemical synthesis of oligonucleotides utilizing standard commercial reagents, equipment, synthetic protocols, and purification schemes. The phosphoramidites having BCNS 3 (with entirely hydrocarbon based sleeve, Fig. 3) were not sufficiently stable during the alkaline deprotection step. We have prepared a number of 2'-deoxy, and 2'-OMe oligonucleotides and phosphorothioate oligonucleotides (19-, to 23-mers) with different numbers of incorporated BCNS 1 and 2 and studied their base pairing properties, solubility, serum stability, cytotoxicity, and cell penetration rates.

The presence of BCNSs conserved and in some cases even enhanced the ability of modified ONs to base pair with their complementary ONs. The new ONs were not involved in non-specific associations with non-complementary ONs. They possessed good aqueous solubility and chemical stability, and enhanced serum stability. The new oligonucleotides were not cytotoxic at up to 10 μ M concentrations, and, most importantly, showed significantly enhanced cellular uptake. The ONs featuring BCNS 2 (dimethylamino groups at the termini) were clearly superior to the ONs with BCNS 1 (primary amino groups at the termini) in the cell penetration assay, and, therefore, BCNS 2 was selected for further development.

Based on these data, we conclude that the new modified ONs containing BCNSs on their internucleotide phosphates display a set of properties, which makes them promising candidates for the development of gene expression modulation therapeutics or research tools, and warrants further investigation and development.

EXPERIMENTAL

Chemistry

All solvents and reagents used were obtained commercially and used as such unless noted otherwise. ^1H and ^{31}P NMR spectra were recorded in CDCl_3 or DMSO-d_6 solutions at 298°K using a Bruker Avance 300 instrument. Chemical shifts are reported as parts per million (ppm) relative to tetramethylsilane (TMS) for ^1H , and phosphoric acid for ^{31}P NMR. All ^{31}P spectra were proton decoupled unless otherwise noted. Spin multiplicities are given as s (singlet), br s (broad singlet), ms (multiple singlets), d (doublet), t (triplet), q (quartet), or m (multiplet). All chemical reactions and the purity of the intermediates were analyzed using TLC (silica gel 60 F₂₅₄ plates, UV, I₂, or Hanessian's (cerium molybdate) stain) and LC-MS (ThermoFinnigan ICQ Advantage with Surveyor LC system) with a XBridge C18 3.5 μ m 2x50 mm column and a gradient from 0.1 to 100% acetonitrile in 0.1% formic acid (unless otherwise indicated in procedure) at a flow rate of 0.2 ml/min. Detection was at 210–420 nm for UV and electrospray ionization in the positive and the negative ionization modes (ESI+, ESI–) for MS. Preparative silica gel chromatography was performed using an ISCO Flash chromatography system and RediSep flash cartridges (particle size: 35–70 μ m, Teledyne ISCO) or PuriFlash cartridges (particle size: 50 μ m, spherical, Interchim). Preparative reversed phase chromatography was carried out using 25 x 10 cm column (Pharmacia) packed with Chromalite PCG C with a binary gradient from 50 mM ammonium acetate to methanol at a flow rate of 100 ml/min and UV detection. Triethylammonium

bicarbonate buffer (TEAB) was prepared by saturation of water/triethylamine mixture with carbon dioxide.

All procedures in the synthesis of the phosphoramidites were conducted under argon using syringe or cannula techniques. The glassware was flame-dried and cooled under argon. All solvents were absolute and septum sealed under argon or nitrogen.

The purity of the phosphoroamidites was determined by LCMS (LCQ Advantage with Surveyor LC system and XCalibur software, Thermo-Fisher, column, XBridge C18, 3 μ m, 2.1x50 mm, Waters, Inc., mobile phases, A, 10 mM ammonium acetate pH 9, B, acetonitrile, gradient, % B in A, from 0% to 35% for 1 min, then to 100% for 8 min, and then isocratic 100% B for 1 min at 0.2 ml/min), and was above 95% unless otherwise specified in the text. The purity of the ONs was determined by analytical HPLC (Waters 2695 Alliance system and C18 column, using a linear gradient from A, 5% acetonitrile in 0.1 M triethylammonium acetate (TEAA) to B, 40% A in acetonitrile for 27 min at flow rate of 1.5 mL/min and UV detection at 260 nm) and was above 95%.

Bromo-3-(2-chloroethoxy)propan-2-ol (1)—2-Chloroethanol (161 g, 134 ml, 2 mol) was dissolved in 60 ml anhydrous dichloromethane. Boron trifluoride etherate (1 ml) was added with stirring, and the resulting solution was cooled, protected from moisture, to -4°C . Epibromohydrin (54.8 g, 34.2 ml, 0.4 mol) was added dropwise during 35 min with stirring and cooling at -7 to -5°C . The clear solution was stirred for 3 h at 0°C and then left to warm up to r.t. The progress of the reaction was monitored by TLC (petroleum ether/ethyl acetate 2:1) and Hanessian's stain. Upon completion, the reaction mixture was evaporated under vacuum (12 mm Hg) in a 45°C bath to obtain 82.6 g (95%) of crude product which appeared as yellowish oil. According to ^1H NMR, this material contained 10% (w/w) 2-chloroethanol. This product was used in the next step without further purification. Analytically pure material was obtained by vacuum distillation. ^1H NMR (300 MHz, CDCl_3) δ : 3.99 (q, $J = 5.32$ Hz, 1H), 3.79 (m, 2H), 3.65 (m, 4H), 3.53 (m, 2H), 2.42 (bs, 1H); ^{13}C NMR (75MHz, CDCl_3) δ : 72.38, 71.43, 69.87, 42.85, 34.81.

2-((2-chloroethoxy)methyl)oxirane (2)—Crude 1-bromo-3-(2-chloroethoxy)propan-2-ol, **1** (82.6 g, 0.38 mol) was dissolved in dry THF (150 ml). The solution was cooled in an ice bath. Tertiary potassium butoxide (1M solution in THF, 360 ml, 0.36 mol) was added drop-wise during 30 min with stirring. The mixture was stirred for 1 h on ice, and then was filtered under vacuum. The precipitate was washed with petroleum ether (2x150 ml). The combined filtrate and washings were evaporated under vacuum to oil. This oil was dissolved in diethyl ether (300 ml), and the solution was extracted with water (2x200 ml), filtered through a cotton plug, and evaporated under vacuum to give 37 g of crude **2**. This material was further purified by vacuum distillation at 0.1 mm Hg. After a small (0.32 g) pre-run of 2-chloroethanol, the main fraction was distilled at 36 – 45°C to give 28.0 g (57%) of pure **2**. ^1H NMR (300 MHz, CDCl_3) δ : 3.90 – 3.73 (m, 3H), 3.65 (m, 2H), 3.46 (dd, $J_1 = 5.9$ Hz, $J_2 = 11.7$ Hz, 1H), 3.18 (m, 1H), 2.82 (dd, $J_1 = 4.2$ Hz, $J_2 = 5.0$ Hz, 1H), 2.64 (dd, $J_1 = 2.7$ Hz, $J_2 = 5.0$ Hz, 1H); ^{13}C NMR (75MHz, CDCl_3) δ : 71.79, 71.32, 50.69, 43.97, 42.80.

1,3-Bis(2-chloroethoxy)propan-2-ol (3)—Boron trifluoride etherate (0.42 ml) was added dropwise with stirring to cooled (–15 – –10 °C) 2-chloroethanol (79.6 g, 66.0 ml, 0.988 mol). 2-((2-Chloroethoxy)methyl)oxirane, **2** (27 g, 0.198 mol) was added dropwise during 30 min with stirring at the same temperature. The reaction mixture was kept at 0 – 5 °C for 3 h, and then concentrated under vacuum (12 mm Hg, 45 °C) to an oily liquid. This oil was dissolved in ethyl acetate (150 ml), and the solution was washed with 10% sodium carbonate (50 ml) and saturated brine (50 ml), and filtered through a cotton plug. The solvent was evaporated on a rotary evaporator (12 mm Hg, 45 °C). The residue was subjected to vacuum distillation to give 27.7 g (64.6%) of distillate at 115 – 121 °C (0.1 mm Hg). The ¹H and ¹³C NMRs of this distillate revealed that it contained ca. 10% of the isomeric 2,3-bis(2-chloroethoxy)propan-1-ol (**4**). The product was purified from this isomer by flash chromatography on silica gel. Distillate (5.3) g was loaded on a silica gel cartridge (Agela Silica-CS, 120 g) and eluted with a gradient of ethyl acetate/petroleum ether from 1:10 (2 L) to 2:1 (3 L). Fractions 5–32 (15 ml each) containing pure product were combined and evaporated to give 2.27 g of **3** as a colorless oil. Fractions 33–90 containing impure product were combined, evaporated and subjected to a second silica gel chromatography under the same conditions to give an additional 0.72 g of pure product. The yield of purified product was 36%. ¹H NMR (300 MHz, CDCl₃) δ: 3.98 (m, 1H), 3.76 (m, 4H), 3.68–3.51 (m, 8H), 2.82 (d, J = 4.3 Hz, 1H); ¹³C NMR (75MHz, CDCl₃) δ: 72.05, 71.38, 69.34, 42.86.

1,3-Bis(2-azidoethoxy)propan-2-ol (5)—1,3-Bis(2-chloroethoxy)propan-2-ol, **3** (8.76 g, 0.04 mol of crude distillate containing ca. 10% of isomeric **4**), sodium azide (9.97 g, 0.153 mol) and sodium iodide (7.2 g, 0.048 mol) were stirred in 100 ml DMF for 16 h at 95–100 °C. After cooling to rt, the mixture was filtered and the filtrate was evaporated under vacuum to oil. This oil was dissolved in dichloromethane (250 ml), washed with water (4x150 ml), and evaporated under vacuum to give 8.5 g, 92% of crude product as a slightly yellow oil, containing ca. 10% of isomeric 2,3-bis(2-azidoethoxy)propan-1-ol. This material was purified in two portions by silica gel chromatography on Agela Silica-CS 120 g cartridges. The elution was carried out with a gradient of ethyl acetate/petroleum ether from 1:10 to 1:2. The fractions containing pure material (TLC, Hanessian's stain) were combined and evaporated, to give, after drying under vacuum at rt, 5.2 g of pure **5** as a light oil. The mixed fractions were evaporated and subjected to a second purification under the same conditions as described above to give an additional 0.92 g of pure material. Total yield of pure product was 6.12 g (67%). ¹H NMR (300 MHz, CDCl₃) δ: 4.00 (m, 1H), 3.72 (m, 4H), 3.66–3.55 (m, 4H), 3.41 (m, 4H), 2.48 (bs, 1H); ¹³C NMR (75MHz, CDCl₃) δ: 72.05, 70.36, 69.31, 50.63.

1,3-Bis(2-aminoethoxy)propan-2-ol (6)—A solution of triphenylphosphine (8.68 g, 33 mmol) in THF (8 ml) was added to a solution of **5** (3.05 g, 13.2 mmol) in THF (4 ml) under argon with stirring. The flask was equipped with a bubbler and cooled slightly, to maintain the reaction temperature below 30 °C. After 5 h, the release of nitrogen ended. Water (0.65 ml) was added with stirring, and, again, the flask was cooled slightly to keep the temperature below 30 °C. After 24 h, the reaction mixture was partially concentrated under vacuum (strong foaming was observed), and diluted with water (100 ml). After 30 min stirring, the white precipitate of triphenylphosphine oxide was filtered under vacuum and washed with

water (3x20 ml). The combined filtrate and washings were evaporated under vacuum, to give, after drying on high vacuum at rt, 2.36 g (100%) of product as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ: 3.99 (m, 1H), 3.54 (m, 8H), 2.89 (t, J = 5.2 Hz, 4H), 1.96 (bs, 5H). MS (ESI+) m/z: observed, 179.10; calculated for C₇H₁₉N₂O₃⁺, 179.13.

1,3-Bis(2-((trifluoroacetyl)amino)ethoxy)propan-2-ol (7)—Methyl trifluoroacetate (6.77 g, 5.32 ml, 53 mmol) was added dropwise to **6** (2.36 g, 13.2 mmol) with stirring in an ice bath. The resulting solution was kept overnight at r.t. The volatiles were evaporated under vacuum, and the residue was purified on silica gel cartridge (Agela Silica-CS, 120 g) using ethyl acetate/petroleum ether 2:1. Fractions containing the product were evaporated. The residue was dissolved under argon in a mixture of anhydrous diethyl ether and toluene (30 ml each), and then treated under argon with 30 g molecular sieves (3 Å) in a septum sealed flask. After 3 h, the solution was removed by a syringe, and the molecular sieves were washed with 2x20 ml mixture of anhydrous diethyl ether/toluene 1:1. The washing was conducted under argon using the same syringe technique without unsealing the flask. The combined solution and washings were evaporated under vacuum to give, after drying under high vacuum at r.t., 3.18 g (65%) of product as a clear oil, which was stored under argon and protected from moisture. ¹H NMR (300 MHz, CDCl₃) δ: 7.03 (bs, 1H), 3.99 (m, 1H), 3.66 (m, 4H), 3.63-3.49 (m, 8H), 2.66 (bs, 1H); ¹³C NMR (75MHz, CDCl₃) δ: 157.42 (q, J = 37.3 Hz), 115.80 (q, J = 287.2 Hz), 71.95, 69.69, 69.20, 39.64.

1,3-Bis(2-(dimethylamino)ethoxy)propan-2-ol (8)—1,3-Bis(2-chloroethoxy)propan-2-ol, **3** (2.0 g, 9.2 mmol) was mixed with 24 ml of 40% (w/w) solution of dimethylamine in water, sealed, and stirred for 36 h at rt. The reaction mixture was passed through 40 ml of Biorad AG MP-1M anion exchange resin in the OH⁻ form. The resin was eluted with water until the eluate became neutral. The eluates were evaporated under vacuum to give **8** (2.3 g, 100% yield, contained 6% water) as a white semi-solid substance. Compound **8** was rendered anhydrous by dissolving in a mixture of dry toluene (60 ml), THF (40 ml), and acetonitrile (100 ml), and stirring for 3 h with molecular sieves (4 Å), filtering anaerobically, washing with 3x5 ml THF, followed by evaporation of the filtrate and washings under vacuum. ¹H NMR (300 MHz, D₂O) δ: 3.89 (m, 1H), 3.60 (m, 4H), 3.46 (m, 4H), 2.82 (m, 4H), 2.43 (s, 12H); ³¹P NMR (75MHz, D₂O, ¹H dec.) δ: 71.68, 68.82, 66.39, 56.94, 43.53.

Bis(diisopropylamino)chlorophosphine (9)—Diisopropylamine (3.92 g, 5.47 ml, 39 mmol, dried for 3 days under molecular sieves 3 Å) and dry toluene (20 ml) were loaded into a round bottom flask containing a teflon stirring bar. The flask was cooled in ice, and phosphorus trichloride (1.22 g, 0.78 ml, 8.88 mmol) was added dropwise with stirring at a rate slow enough to keep the temperature of the mixture below 10 °C. The flask was equipped with a flame-dried and cooled (under argon) reflux condenser. The entire content was refluxed with stirring for 24 h. The reaction was controlled by ³¹P NMR in CDCl₃, which showed complete conversion of the PCl₃ singlet at 221.8 ppm to a multiplet at 142.9 ppm.

((1,3-Bis(2-((trifluoroacetyl)amino)ethoxy)-2-propyl)oxy)-N,N,N',N'-tetraisopropylphosphordiamidite (10)—1,3-Bis(2-

((trifluoroacetyl)amino)ethoxy)propan-2-ol, **7** (2.63 g, 7.10 mmol) was dissolved under argon in THF (10 ml) and added dropwise with stirring to the reaction mixture from the above step at -10 – -15 °C. After stirring for 2 h at -10 °C, the reaction mixture was left to return to rt. The reaction was controlled by ^{31}P NMR, which showed complete conversion of the multiplet at 142.9 ppm to a multiplet at 119.5 ppm. The reaction mixture was filtered by a cannula under argon pressure through a glass fiber filter (the filter was rendered dry by flushing with 50 ml of dry THF under argon). The reaction flask and the filtered solids were washed with dry THF (3x12 ml). The combined filtrate and washings were concentrated under vacuum in 25 °C bath to oil.

5'-O-(4,4'-dimethoxytrityl)2'-deoxythymidine-3'-O-(O-((1,3-bis(2-((trifluoroacetyl)amino)ethoxy)-2-propyl)oxy)-N,N'-diisopropylphosphoramidite) (12)—5'-O-(4,4'-Dimethoxytrityl)thymidine, **11 (2.22 g, 4.08 mmol) was dissolved in 6 ml of dry DMF, and evaporated under vacuum (35 °C). The residue was dissolved under argon in 6 ml of dry DMF, and added under argon with stirring to the oil from the above step. A solution of 5-ethylthiotetrazole in acetonitrile (0.45 M, 3 ml) was added with stirring. After 2 h at r.t. ^{31}P NMR of the reaction mixture showed conversion of the multiplet at 119.5 to two multiplets (representing the two diastereomers of product) at 150.8 and 149.7 ppm, a multiplet at 151.4 (a by-product di-((1,3-bis(2-((trifluoroacetyl)amino)ethoxy)-2-propyl)oxy)-N,N'-tetraisopropylphosphoramidite), and two doublets of multiplets at 17.1 and 7.7 ppm (H-phosphonate hydrolysis byproducts).**

Triethylamine (0.5 ml) was added to the reaction mixture, and the volatiles were evaporated under vacuum. The residue was dissolved in dichloromethane containing 1% triethylamine, loaded on a silica gel cartridge (Agela Silica-CS, 120 g), which was pre-equilibrated with 30% ethyl acetate and 1% triethylamine in petroleum ether, and eluted with a gradient from 30% ethyl acetate and 1% triethylamine in petroleum ether to 1% triethylamine in ethyl acetate. This resulted in partial purification of the product. The final purification was done on a preparative reversed phase resin column (100x250 mm). The fractions from the normal phase column containing the product were evaporated, dissolved in 1% triethylamine in methanol (15 ml), loaded on the column, and eluted with a gradient from 50% methanol and 1% triethylamine in water to 1% triethylamine in methanol for 50 min, and then isocratically with 1% triethylamine in methanol for 50 min at a flow rate of 100 ml/min. Fractions containing the product were pooled and evaporated under vacuum. LC-MS and ^{31}P NMR analysis of this product showed that it contained ca. 17% of the corresponding amidate, a byproduct resulting from oxidation of the product during the reversed phase chromatography. This material was repurified by the same chromatographic procedure with the following modifications: the mobile phases were chilled in ice and purged with helium for 3 h before and during the chromatography. Fractions containing **12** were pooled and evaporated under vacuum. The residue was evaporated from dry acetonitrile (2x150 ml) and finally from dry toluene to give 2.44 g (57%) of **12** as a white foam with 96% purity, and containing 4% of phosphoramidate byproduct. Compound **12** consisted of 2 diastereomers in ratio 4:1 (from ^1H , ^{31}P NMR and HPLC). ^1H NMR (300 MHz, C_6D_6) δ : 7.68, 7.59 (bs, 1H, 6-H), 7.58-6.73 (multiple m, 15H, DMT Ar-H, CONH), 6.65 (dd, $J_1 = 6.3$ Hz, $J_2 = 7.7$ Hz,

0.8H, Diast.1 1'), 6.61 (dd, $J_1 = 5.6$ Hz, $J_2 = 8.7$ Hz, 0.2H, Diast.2 1'), 4.71 (m, 1H, H-3'), 4.44, 4.26 (m, 1H, H-4'), 4.11, 4.03 (m, 1H, (OCH₂)₂CHOP), 3.61-3.29 (multiple m, 14H, NHCH₂CH₂OCH₂, 5', 5'), 3.334, 3.327 (s, 6H, OCH₃), 3.21, 3.10 (m, 2H, NCHMe₂), 2.35 (m, 2H, H-2', 2'), 1.54, 1.53, 1.49 (s, 3H, dT-CH₃), 1.16, 1.12, 1.01 (d, $J = 6.8$ Hz, 12H, NCHCH₃); ³¹P NMR (121 MHz, C₆D₆) δ: 150.35 (m, ¹H dec., s), 148.53 (m, ¹H dec., s). MS (ESI⁻) m/z: observed, 1042.18 (100.0%), 1043.19 (55.9%), 1044.18 (17.6%), 1045.16 (4.9%); calculated for C₄₈H₅₉F₆N₅O₁₂P⁻ [M-H]⁻, 1042.39 (100.0%), 1043.39 (54.9%), 1044.39 (18.2%), 1045.40 (3.6%). Retention times: Diastereomer 1, 6.44 min; Diastereomer 2, 6.67 min (Column, XBridge C18, 3 μm, 2.1x50 mm, mobile phases, A, 10 mM ammonium acetate pH 9, B, acetonitrile, gradient (% B in A) from 0% to 45% for 1 min, then to 100% for 5 min, and then isocratic 100% B for 1 min at 0.2 ml/min).

5'-O-(4,4'-dimethoxytrityl)-2'-O-methyluridine-3'-O-(O-(1,3-bis(2-((trifluoroacetyl)amino)ethoxy)-2-propyl)-N,N'-diisopropylphosphoramidite) (14)—Compound **14** was prepared using the procedure for **12**, with the following modifications: in step 1 (preparing of compound **9**), 5.39 g, 7.52 ml, 53 mmol diisopropylamine, 1.22 g, 0.78 ml, 8.88 mmol phosphorous trichloride, and 30 ml toluene were used; in step 2 (preparing of compound **10**), 2.63 g, 7.10 mmol of compound **7** were used; and in step 3, 3.28 g, 5.86 mmol of 5'-(4,4'-dimethoxytrityl)-2'-O-methyluridine (**13**) were used. The purification was carried out directly on a 100x250 mm reversed phase resin column using helium de-gassed and chilled in ice mobile phases. The product obtained after this purification step contained 17% of nucleoside (**13**), and was re-purified with the same column and procedure but with extended gradient step – from 50% methanol and 1% triethylamine in water to 1% triethylamine in methanol for 100 min. The product obtained after this re-purification was 2.76 g (44.4%). This material had 90% purity (¹H and ³¹P NMRs, LC-MS) and contained 5% nucleoside (**13**), and 5% of methylphosphite resulted from a reaction of compound **14** with methanol from the mobile phase (replacing the diisopropylamino group of compound **14** by a methoxy group). Compound **14** consisted of 2 diastereomers in ratio 2:1% (from ¹H, ³¹P NMR and HPLC). ¹H NMR (300 MHz, C₆D₆) δ: 8.10, 7.95 (d, $J = 8.2$ Hz, 1H, H-6), 7.62-6.95 (multiple m, 15H, DMT Ar-H, CONH), 6.28 (d, $J = 4.5$ Hz, 0.66H, Diast.1 1'), 6.13 (d, $J = 2.3$ Hz, 0.33H, Diast.2 1'), 5.39, 5.35 (d, $J = 8.1$ Hz, 1H, H-5), 4.74, 4.60 (m, 1H, H-2'), 4.50, 4.37 (m, 1H, H-3'), 4.25, 4.03 (m, 1H, (OCH₂)₂CHOP), 4.14 (m, 1H, H-4'), 3.70-3.27 (multiple m, 14H, NHCH₂CH₂OCH₂, 5', 5'), 3.55, 3.49 (s, 3H, 2'-OMe), 3.377, 3.366, 3.347, 3.343 (s, 6H, DTM-OCH₃), 3.19, 3.11 (m, 2H, NCHMe₂), 1.15, 1.02 (d, $J = 6.5$ Hz, 12H, NCHCH₃); ³¹P NMR (121 MHz, C₆D₆) δ: 152.98 (m, ¹H dec., s), 151.54 (m, ¹H dec., s). MS (ESI⁻) m/z: observed, 1058.12 (100%), 1059.08 (48.5%), 1060.07 (15.6%), 1061.04 (3.8%); calculated for C₄₈H₅₉F₆N₅O₁₃P⁻ [M-H]⁻, 1058.38 (100.0%), 1059.38 (53.1%), 1060.38 (17.5%), 1061.39 (4.1%). Retention times: Diastereomer 1, 4.94 min; Diastereomer 2, 5.23 min (Column, XBridge C18, 3 μm, 2.1x50 mm, mobile phases, A, 10 mM ammonium acetate pH 9, B, acetonitrile, gradient (% B in A) from 0% to 60% for 1 min, then to 100% for 4 min, and then isocratic 100% B for 1 min at 0.2 ml/min).

5'-O-(4,4'-dimethoxytrityl)-2'-O-methyluridine-3'-O-(O-(1,3-bis(2-(dimethylamino)ethoxy)-2-propyl)-N,N'-diisopropylphosphoramidite) (16)—

Compound **16** was prepared using the procedure described for **12**, with the following modifications: in step 1 (preparing of compound **9**), 6.48 g, 9.04 ml, 64 mmol diisopropylamine, 1.47 g, 0.93 ml, 10.7 mmol phosphorous trichloride, and 30 ml toluene were used; in step 2 (preparing of compound **15**), 2.30 g, 8.82 mmol of compound **8** were used; and in step 3, 3.28 g, 5.86 mmol of 5'-(4,4'-dimethoxytrityl)-2'-*O*-methyluridine (**13**) were used. The purification was carried out in two runs on a silica gel cartridge (Agela Silica-CS, 120 g) with a gradient of 3% triethylamine in ethyl acetate to 20% methanol and 3% triethylamine in ethyl acetate. Fractions containing the product were pooled, evaporated under vacuum (25 °C bath), and stripped from residual methanol by re-evaporation from dry acetonitrile containing 1% triethylamine to give, after drying on high vacuum, 3.50 g per run (7.0 g total, 71%) of compound **16** as a foam with 92% purity (¹H and ³¹P NMRs, LC-MS). It contained 4.5 % of the corresponding H-phosphonate (resulted from hydrolysis of the diisopropylamino group), 3% of the starting nucleoside (**13**), and 1% of the corresponding phosphoramidate (resulted from oxidation of compound **16**). Compound **16** was a mixture of 2 diastereomers with ratio 53:47 (from ¹H, ³¹P NMR and HPLC). ¹H NMR (300 MHz, C₆D₆) δ: 10.05 (bs, 2H, CONH), 8.16, 8.06 (d, J = 8.2 Hz, 1H, H-6), 7.69-6.76 (multiple m, 12H, DMT Ar-H), 6.20 (d, J = 1.8 Hz, 0.47H, Diast.1 1'), 6.17 (d, J = 1.2 Hz, 0.53H, Diast.2 1'), 5.44, 5.38 (d, J = 8.1 Hz, 1H, H-5), 4.89, 4.68 (m, 1H, H-2'), 4.51, 4.45 (m, 1H, H-3'), 4.37- 4.21 (m, 1H, (OCH₂)₂CHOP), 4.24, 4.16 (m, 1H, H-4'), 3.78-3.45 (multiple m, 14H, NHCH₂CH₂OCH₂, 5',5''), 3.65, 3.62 (s, 3H, 2'-OMe), 3.386, 3.369, 3.347, 3.340 (s, 6H, DTM-OCH₃), 2.67- 2.47 (m, 2H, NCHMe₂), 2.24, 2.22, 2.19, 2.11 (s, 12H, NMe₂), 1.22, 1.19, 1.10 (d, J = 6.7 Hz, 12H, NCHCH₃); ³¹P NMR (121 MHz, C₆D₆) δ: 153.29 (m, ¹H dec., s), 151.47 (m, ¹H dec., s). MS (ESI⁻) m/z: observed, 922.19 (100%), 923.17 (50.7%), 924.12 (16.7%), 925.24 (2.9%); calculated for C₄₈H₆₉N₅O₁₁P⁻ [M-H]⁻, 922.47 (100.0%), 923.48 (53.1%), 924.48 (16.1%), 925.48 (3.7%). Retention times: Diastereomer 1, 8.41 min; Diastereomer 2, 8.85 min (Column, XBridge C18, 3 μm, 2.1x50 mm, mobile phases, A, 10 mM ammonium acetate pH 9, B, acetonitrile, gradient (% B in A) from 0% to 35% for 1 min, then to 100% for 12 min, and then isocratic 100% B for 1 min at 0.2 ml/min).

1,9-Dichlorononan-5-one (18) (from olean) 1,7-Dioxaspiro[5.5]undecane (olean, **17**, 15 g, 97 mmol) was mixed with conc. HCl (38.7 ml) and the mixture was heated at 90 °C for 10 min under vigorous stirring. The mixture became quickly homogenous and darkened. After cooling to rt, the mixture was extracted with dichloromethane (300 ml), and the dichloromethane extract was washed with water (150 ml) and 8% sodium bicarbonate in water (150 ml), then dried overnight over anhydrous sodium sulfate, filtered, and evaporated under vacuum to an oil. This oil was subjected to vacuum distillation (0.1 mm Hg). Three fractions were collected: fraction 1, 56–113 °C, fraction 2, 113–116 °C, and fraction 3, above 116 °C. Fraction 2 consisted of pure product (2.3 g, 11.3%). ¹H NMR (300 MHz, CDCl₃) δ: 3.53 (t, J = 6.2 Hz, 4H, ClCH₂), 2.45 (t, J = 8.8, 4H, COCH₂), 1.83 – 1.66 (m, 8H); ¹³C NMR (75MHz, CDCl₃) δ: 209.85, 44.83, 41.89, 32.11, 21.20.

1,9-Dichlorononan-5-one (18) (from δ-valerolactone): δ-Valerolactone (4.00 g, 3.71 ml, 30 mmol) was loaded in a flask equipped with a reflux condenser, thermometer, and magnetic stirrer. Dry THF (6 ml) was added under argon. The mixture was cooled on ice and NaH (60% in mineral oil, 800 mg, 20 mmol) was added in portions within a couple of minutes.

The cooling bath was removed and the mixture was warmed carefully with a dryer – caution, exothermic reaction! The heating was turned on and the mixture was brought slowly to reflux. After reflux for 1.5 h, the mixture turned into a frothing semi-solid. Additional THF (6 ml) was added. Conc. HCl (8 ml) was added in portions causing much frothing at the beginning. The mixture was refluxed briefly, and then the reflux condenser was replaced by a straight one, and ca. 15 ml were distilled off. Conc. HCl (6 ml) was added again and the distillation was continued until the vapor temperature exceeded 100 °C. The mixture (two layers) was cooled and evaporated on a rotary evaporator until most of the HCl was evaporated and the salts crystallized. Conc. HCl (8 ml) was added once again, the mixture was refluxed vigorously for 20 min and cooled in ice. Salts crystallized again and a couple of milliliters of water were added to dissolve them. The mixture was extracted with 2x30 ml diethyl ether. The ether extract was dried over Na₂SO₄, filtered, and evaporated under vacuum. The resulting oil was subjected to vacuum distillation (0.1 mm Hg). Three fractions were collected: fraction 1 (45–86 °C), fraction 2 (86–160 °C), and fraction 3 (above 160 °C). Fraction 2 (1.4 g) contained the product along with other by-products. Silica gel chromatography of fraction 2 (Agela Silica-CS, 120 g, Ethylacetate/Petroleum ether, gradient from 1:50 to 1:10) gave 249 mg (5.9%) of pure **18**.

1,9-Dichloro-5-nonanol (19)—Sodium borohydride (358 mg, 9.5 mmol) was added to an ice cooled mixture of 1,9-Dichlorononan-5-one, **18** (20 g, 20 mmol), methanol (4 ml) and water (2 ml) with vigorous stirring. After 30 min at 0–4 °C the reaction mixture was extracted with ethyl acetate (3x50 ml). The extract was dried over Na₂SO₄, filtered, and evaporated under vacuum to give 3.92 g of **19** (92%) as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ: 3.63 (m, 1H, CHOH), 3.57 (t, J = 6.6 Hz, 4H, ClCH₂), 1.81 (m, 4H, CH₂CH₂Cl), 1.69 – 1.39 (m, 9H, CH₂, OH); ¹³C NMR (75MHz, CDCl₃) δ: 71.62, 45.16, 36.81, 32.74, 23.19.

1,9-Bis(dimethylamino)-5-nonanol (20)—1,9-Dichloro-5-nonanol, **19** (3.87 g, 18.8 mmol) was evaporated under vacuum from THF (50 ml) to remove any traces of ethyl acetate. The residue was mixed with dimethylamine (40% solution in water, 100 ml), sealed, and stirred at rt for 20 h. The mixture was diluted with water (200 ml), and extracted with diethyl ether (3x150 ml). The ether was removed under vacuum, and the resulting oil was rendered anhydrous by evaporation from toluene (2x100 ml) to give 3.3 g of **20** (76%) as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ: 3.67 (m, 1H, CHOH), 3.49 (bs, 1H, OH), 2.32 (m, 4H, CH₂NMe₂), 2.22 (s, 12H, NMe₂), 1.77 – 1.46 (m, 12H, CH₂); ¹³C NMR (75MHz, CDCl₃) δ: 71.07, 59.88, 45.50, 38.03, 28.10, 23.85.

5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(O-(bis-(4-(dimethylamino)butyl)methyl)-N,N'-diisopropylphosphoramidite) (22)—

Compound **22** was prepared using the procedure described for compound **12**, with the following modifications: in step 1 (preparation of **9**) 4.30 g, 5.99 ml, 42 mmol diisopropylamine, 0.972 g, 0.62 ml, 7.08 mmol phosphorous trichloride, and 20 ml toluene were used; in step 2 (preparation of **21**), 1.50 g, 6.51 mmol of **20** were used. Also, in step 2 after filtration, dimethylamine (2 ml) was added, and then **20** was added as neat oil, and the mixture reacted at r.t. for 2.5 h. In step 3, 3.85 g, 7.08 mmol of 5'-(4,4'-dimethoxytrityl)-

thymidine (**11**), and 3 mmol (6.6 ml of 0.45 M solution in acetonitrile) of ethylthiotetrazole were used. After 2 h and 15 min at r.t., ^{31}P NMR of the reaction mixture showed conversion of the multiplet at 107.5 to two multiplets (representing the two diastereomers of **22**) at 144.7 and 144.1 ppm. Triethylamine (1 ml) was added to the reaction mixture and the volatiles were evaporated under vacuum. The residue was dissolved in a minimal amount of ethyl acetate containing 3% triethylamine, loaded on a silica gel cartridge (Agela Silica-CS, 120 g), which was pre-equilibrated with the same solvent, and eluted with a gradient from 3% triethylamine in ethyl acetate to 3% triethylamine in ethyl acetate/methanol 4:1. Fractions containing the product were pooled and evaporated under vacuum. The residue was evaporated from toluene (2x150 ml) and finally from dry toluene (50 ml) to give, after drying for 3 h at high vacuum, 4.95 g (77%) of **22** as a white foam with 96% purity, and containing 2% of phosphoramidate byproduct. Compound **22** consisted of 2 diastereomers in ratio 1:1 (from ^1H , ^{31}P NMR and HPLC). ^1H NMR (300 MHz, C_6D_6) δ : 7.64-6.74 (mm, 14H, 6-H, DMT Ar-H), 6.65 (m, 1H, 1'), 4.77 (m, 1H, H-3'), 4.43, 4.29 (two m, 1H, H-4' of diast.1 and diast.2), 3.94, 3.80 (two m, 1H, $(\text{OCH}_2)_2\text{CHOP}$ of the two diastereomers), 3.68-3.29 (mm, 4H, 5', 5'', NCHMe_2), 3.35, 3.34 (s, 6H, OCH_3), 2.63-2.50 (mm, 2H, H-2', 2''), 2.40 (t, $J = 7.2$ Hz, 4H, NCH_2), 2.35 (s, 3H, dT- CH_3), 2.26, 2.23, 2.16, 2.11 (s, 12H, $\text{N}(\text{CH}_3)_2$), 1.76-1.39 (mm, 12H, CH_2), 1.22, 1.19, 1.15, 1.03 (d, $J = 6.9$ Hz, 12H, NCHCH_3); ^{31}P NMR (121 MHz, C_6D_6) δ : 145.15 (m, ^1H dec., s), 145.43 (m, ^1H dec., s). MS (ESI $^+$) m/z : observed, 903.94 (100.0%), 904.95 (40.9%), 905.90 (12.3%), 906.88 (2.6%); calculated for $\text{C}_{50}\text{H}_{75}\text{N}_5\text{O}_8\text{P}^+$ $[\text{M}+\text{H}]^+$, 904.54 (100.0%), 905.54 (55.2%), 906.54 (17.6%), 907.55 (2.7%). Retention times: Diastereomer 1, 6.34 min; Diastereomer 2, 6.72 min (Column, XBridge C18, 3 μm , 2.1x50 mm, mobile phases, A, 10 mM ammonium acetate pH 9, B, acetonitrile, gradient (% B in A) from 0% to 35% for 1 min, then to 100% for 8 min, and then isocratic 100% B for 1 min at 0.2 ml/min).

Synthesis of ON containing BCNS

ON in Table 1 were synthesized on a 394 DNA/RNA synthesizer (Applied Biosystems) using standard phosphoramidite chemistry and UltraMILD $^{\text{®}}$ deprotection phosphoramidite monomers (Cat. # 10-1601-xx, 10-1015-xx, 10-1621-xx Glen Research, Sterling, VA or ChemGenes, Wilmington, MA) in combination with the phosphoramidite monomers **12**, **14**, **16**, and **22**. Another difference from the standard phosphoramidite chemistry was using different CAP A (5% phenoxyacetic anhydride in THF/Pyridine, Cat. # 40-4210-57, Glen Research). ON were synthesized on Glen UnySupport $^{\text{TM}}$ 500 (Cat. # 20-5040-xx, Glen Research) solid support on 0.1 μM scale columns. Coupling duration for the dT (**12** and **22**) and 2'-OMe (**14** and **16**) phosphoramidites during the synthesis was as in default protocols for the synthesis with standard deoxy- and 2'-OMe phosphoramidite monomers. For the incorporation of phosphorothioate segments, oxidation was performed using Beaucage thiolation reagent 5 or DDTT ((dimethylamino-methylidene)amino)-3H-1,2,4-dithiazoine-3-thione, ChemGenes, Wilmington, MA) under the manufacturer recommended conditions. For the incorporation of FAM labeling groups at the 5'-positions of ON, 6-fluorescein phosphoramidite (Glen Research, Sterling, VA) was used. The removal from the carrier control pore glass and the bases deprotection were performed by 80 min incubation of CPG with ON in a mixture of concentrated ammonia:40% methylvamine (1:1 v/v).

Purification and purity analysis of the partially (DMT groups still on) and fully deprotected ONs were performed on the Waters 2960 (Waters, Corporation, Milford, MA) system equipped with 2487 Dual Wavelength Absorbance Detector (Waters Corporation). Purification of DMT protected ONs from other capped ONs was performed on 4.7 x 250 mm PartiSphere RP-C18 column (Wattman) with mobile phases, A: 0.1 M TEAA 5% acetonitrile, and B: 20% A in acetonitrile, and linear gradient from 100% A to 100% B for 27 min at flow rate of 1.5 mL/min and UV detection at 260 nm. Fractions containing product were evaporated on a Speed-vac®, and DMT groups were removed by incubation in 80% acetic acid at rt for 15 min. Reaction mixtures were evaporated, re-dissolved in 2 mL deionized water, and, after addition of 10 mL 70% ethanol, were evaporated to dryness to remove trace amounts of acetic acid. Final products were dissolved in 2 mL 0.1 M TEAA buffer pH 7 and desalted on Glen-Pak™ purification cartridges (Glen Research, Cat # 60-5200-10) as recommended by the manufacturer. The final purity of the ONs was determined by analytical RP HPLC. The purity of BCNS-containing ONs was similar to that of the control ONs, and was over 94%. The molecular weights were determined by negative mode electrospray MS analysis (direct infusion of 50 µM solutions in 20% methanol in water at 10 µL/min flow rate), and were determined after deconvolution of the primary spectra to be within 1.3 m/z from the theoretical values.

Thermal (UV) melting studies

The melting studies were performed with Cary 4000 (Agilent) spectrophotometer. All duplexes were annealed before measurement at 75, 65, 55, 40 and 18 °C, each step lasting 5 min. The heating rate was 0.5 deg/min. The measurements were done at concentration of 0.5 µM in 10 mM MgCl₂, 15 mM KCl, 25 mM HEPES, pH 8.2 for duplexes Nos. 1–5, and at 1.2 µM in 25 mM HEPES, pH 7.3 for duplexes Nos. 6–11 (Table 2).

Stability in serum

Solution of ZT11, or ZT12 (5 µM, 100 µl) in PBS was mixed with ZT15 (5 µM, 100 µl). Both ON mixtures were diluted with 9 volumes of bovine serum and incubated at +37 °C. Samples, 250 µL, were removed at 0, and 8 hrs, and precipitated by addition of 10 volumes of sodium perchlorate in acetone. After centrifugation and decanting, the pellet was dissolved in 2 mL of 0.1 M triethylammonium acetate pH 7 and filtrated through 10k size exclusion centrifugation filter (NanoSep® Omega, Pall Corp.). Further purification was done on solid phase extraction C18 cartridges (Glen-Pak® Cat. No. 60-5200-10, Glen Research), which were prepared according to the manufacturer recommendation. The solution was loaded on the cartridges, cartridges were washed with 2x1 ml water, and the captured ONs were eluted with 20% acetonitrile in water. The eluates were speed-vac evaporated, and the residues were re-dissolved in 500 µl of 0.1 M triethylammonium acetate buffer. These solutions were analyzed on C18 HPLC column on Waters Alliance HPLC system (Waters Corporation) using a liner gradient from A, 5% acetonitrile in 0.1 M TEAA to B, 40% A in acetonitrile for 27 min at flow rate of 1.5 mL/min and UV detection at 260 nm.

Cytotoxicity

Cell culture—HEK293 Null-1 cells (InvivoGen) were grown in complete DMEM supplemented with 10% FBS, 2mM L-Glutamine, and Penicillin/Streptomycin/Normicin. A172, MCF7, and HeLa cells were cultured in DMEM/10% FBS with no antibiotic/antimycotic agents added. All cell lines were routinely maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Cytotoxicity Assay—For HEK293 cytotoxicity, clean 18 mm² glass coverslips were coated with poly-L-lysine. HEK293 cells were seeded at a density of 3x10⁵ cells/ml in complete medium into 6-well tissue culture plates (3ml/well) containing coated coverslips. After overnight incubation, growth medium was removed, medium supplemented with 1μM or 10μM of test compounds was added, and cells were incubated for an additional 24 h.

For A172, MCF7, and HeLa cytotoxicity, cells were grown in 6-well plates containing glass coverslips or in 24-well plates without coverslips. All cells were incubated until early stage monolayers were formed. Again, growth medium was removed, DMEM/5% FBS supplemented with test compounds (10 μM) was added, and cells were incubated for the indicated time.

Toxicity was evaluated visually using phase-contrast microscopy.

Cell Viability Assay—To assess the viability of HEK293 cell incubated in the presence of test compounds, cells were stained with 50 μM propidium iodide (PI). Cells were then fixed in 4% formaldehyde and, to label all nuclei, cells were stained with 1 μg/ml Hoechst 33342. Following the staining, cells were washed in PBS and mounted cell-side down onto microscope slides in Vectashield Mounting Media. As a negative control for cell viability, cells treated with 0.5% Tween-20 which resulted in 100% of cells being PI-positive were used.

The effect of BCNS-containing ONs on viability and growth of A172, MCF7, and HeLa cells was estimated using cells grown in 96-well plates. Cells were seeded in DMEM/10% FBS (5x10² cell/well) and, after overnight incubation, growth medium was replaced with DMEM/5% FBS containing 10 μM test ONs. With no medium change, cells were incubated for 96 h and cell number in each well was determined using trypan blue exclusion. Triple wells were used for each treatment. Results were expressed as a ratio between mean numbers of cells in treated wells versus control wells.

Cell Uptake of ONs

Microscopic Observation—A172, MCF7, and HeLa cells were seeded in growth medium in 6-well plates (5x10⁴ cells/well) with glass coverslips and incubated until they covered 80–90% of the surface. Growth medium was removed and DMEM/0.5% FBS containing 1 μM of test FAM-labeled ON was added. After incubation at 37 °C for 2.5 h, all wells were washed with DMEM/0.5% FBS and complete Hanks' Balanced Salts Solution (HBSS). Cells were fixed with 4% formaldehyde in HBSS for 15 min at r.t.. Coverslips were mounted on microscopic slides using mounting solution containing DAPI and analyzed with

a Zeiss AX10 epi-fluorescent microscope equipped with computer running Zen-Blue 2011 program.

Kinetics of Cell Uptake—Kinetics of intracellular penetration of FAM-labeled ONs were studied on A172 and MCF7 cells. Cells were seeded into 96-well plates (10^4 cells/well) in DMEM/10% FBS and incubated overnight at 37 °C/5% CO₂. Two plates were prepared for each cell line: one for cell uptake and second for cell count. At defined time points, designated wells were washed and filled with DMEM/0.5% FBS containing 1 μM of labeled ON. Triplicate wells were used for each time point for each compound. At the end of incubation, all wells were washed with DMEM and HBSS, and cells were lysed with 0.5% lauroyl sarcosine (pH 9.0). The amount of fluorescence in each well was measured using Plate Reader TECAN Infinite F200. The Plate Reader data were normalized to corresponding cell numbers. All experiments were repeated three times.

Statistical Analysis

Unless otherwise stated, quantitative results are expressed as the mean ± SD of at least three independent measurements. Statistical significance was evaluated by using student's *t*-tests and *p* values 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dinucleotide segment of the modified ON comprising BCNS with positively-charged terminal amino groups. B^1 is Thymine or Uracil base; B^2 is any naturally occurring DNA or RNA base; X is H or OMe ; Z is O or S ; Y is O or CH_2 ; and R is H or CH_3 .

Figure 2.

Computer model of tetranucleotide segments of the modified single stranded ON containing one BCNS. Panels A and B show BCNS terminated with two primary protonated amino groups and phosphorous atoms in opposite steric configuration. Panel C shows BCNS terminated with protonated dimethylamino groups. During the modeling the ON, segment had fixed position and BCNS allowed formation of ion-pairing with the lowest energy levels. Both diastereomers (A and B) exhibit similar spacing between the negatively charged phosphate oxygen atom and the positively charged nitrogen atom (2.60–2.68 Å). This distance remains in the same range when the protonated primary amino groups are replaced by protonated dimethylamino groups (panel C).

Figure 3.
Structures of the three BCNSs.

Figure 4. HPLC analysis of ONs after alkaline deblocking (A and B) and after HPLC purification (C). Panel A, ZT27 (control, no BCNS groups), Panel B, ZT25 (same sequence as ZT27 with 3 BCNS groups). Panel C, ZT25 after HPLC purification and DMT group removal. Peaks **1**, curtailed, capped (no-DMT) sequences; Peaks **2**, DMT-on sequences; Peak **3**, DMS-off final ON.

Figure 5.

Representative melting curves. All three duplexes are with the same sequence and are annealed before measurements (see Tables 1 and 2 for sequences and experimental part for details). ZT21 and ZT29 are control ONs (no BCNS), ZT1 has one, and ZT3 has three BCNSs

Figure 6.

Percent viability of HEK 293 cells treated with 1 μ M (A), or 10 μ M (B) ON. Viable cells were counted using ImageJ and defined as cells that were positive for Hoechst 33342, but negative for PI. Cell counts were normalized to the total number of cells captured in the microscope field (at 40X). No treatment (NT) cells served as a positive control for viability and cells treated with 0.5% Tween 20 in PBS were used as negative viability control. Error bars represent standard deviation. For group NT $n=5$, for all other groups $n=3$.

Figure 7. Morphology of HEK 293 cells after 24 h incubation with 10 μ M of test ONs. Representative phase-contrast images of control cells (NT) and cells treated with ZT4, ZT5, and ZT6 (40X magnification) are shown.

Figure 8. Morphology of monolayers formed by HeLa and MCF7 cells treated for 96 h with 10 μ M of control ON (ZT15, no BCNS), and ZT12 (ON with the same sequence as ZT15 but containing 4 type 2 BCNSs). Representative phase-contrast images at 10x magnification are shown.

Figure 9.

Cellular uptake of 5'-fluorescein-labeled BCNS-bearing ONs. Panel A, uptake of control 2'-OMe ON ZT17 (no BCNS) and the same sequence ONs containing type 2 BCNS: ZT18 (2 BCNSs), ZT19 (4 BCNSs), and ZT20 (6 BCNSs) by MCF7 cells. Panel B, uptake of control 2'-OMe ON ZT8 (no BCNS), and the same sequence ON ZT30, containing BCNSs type 1 at positions 2, 8, 11, 14 and 17 from the 5' end; control 2'-OMe ON ZT31 (no BCNS) with sequence: FAM-CAC AAA AUC GGU TCU ACA GGG UA, and ZT32, the same sequence ON bearing four type 2 BCNSs at positions 8, 12, 15, and 22 from the 5' end by A172 cells. The amount of fluorescence was calculated as relative to the control untreated cells and normalized to the corresponding cell numbers. Error bars represent standard deviation from 3 measurements.

Figure 10.

Fluorescent images of A172 cells incubated with ZT17 (control, no BCNS, bottom panel) and ZT20 (same sequence, 6 type 2 BCNSs, top panel) for 150 min and counterstained with DAPI (light blue fluorescence). Bright green fluorescence corresponds to localization of ONs with FAM labels inside the cells.

Scheme 1.

Synthesis of thymidine and 2'-OMe-uridine phosphoramidite monomers bearing BCNS groups.

a) 2-chloroethanol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, DCM, 3 h, 0°C , 95% crude; b) $t\text{-BuOK}$, THF, 1 h, 0°C , 57%; c) 2-chloroethanol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, 3 h, 0°C , 95% crude mixture (90% **3**, 10% **4**), 37% pure **3**; d) NaN_3 , NaI, DMF, 16 h, 100°C , 67%; e) Ph_3P , THF, water, 30 h, rt, 100%; f) $\text{CF}_3\text{CO}_2\text{CH}_3$, 12 h, rt, 65%; g) $(\text{CH}_3)_2\text{NH}$, water, 36 h, rt, 100%; h) reflux for 24 h in toluene; i) $i\text{-Pr}_2\text{NH}$ /THF, 2 h -10°C ; j) 5-ethylthiotetrazole, DMF, 2 h at rt; k) conc. HCl, 90°C , 10 min, 11.3%. TFA – trifluoroacetyl, DMT – 4,4'-dimethoxytrityl.

Table 1

BCNS-containing and reference ONs.

ID	Sequence ^a , 5'>3'	Phosphoramidite ^b	BCNS type ^c	Ribose type	Phosphate type ^d	MW by Mass Spectroscopy	
						Calc.	Det.
ZT1	AtA GTA GTA GTC CTA GTC T	12	1	2'-deoxy	O	5975.2	5974.7
ZT2	AtA GTA GTA GTC CTA GtC T					6135.3	6135.6
ZT3	AtA GTA GtA GtC CTA GTC T					6295.4	6295.9
ZT4	UUC GUA GUU GUC UUA GUC C	NA	NA	6217.1		6217.6	
ZT5	UUC GuA GUU GUC UUA GUC C			6377.7		6377.3	
ZT6	UUC GuA GUu GUC UUA GuC C			6698.1		6697.3	
ZT7	UuC GuA GuU GuC UuA GuC C	14	1	7177.8		7178.9	
ZT8	FAM-UUC GUA GUU GUC UUA GUC C			6754.2		6754.6	
ZT9	FAM-UUC GuA GUU GUC UUA GUC C			6914.3		6914.6	
ZT10	FAM-UuC GuA GuU GuC UuA GuC C	16	1	7714.9		7715.7	
ZT11	uCG uAC uUA uCU uAA uCC UAC			8104.3		8106.0	
ZT12	UCG uAC uUA UCU uAA uCC UAC			7671.9		7672.8	
ZT13	UCG uAC UUA UCU UAA uCC UAC	NA	2	7239.5		7240.6	
ZT14	UCG UAC UUA UCU UAA uCC UAC			7023.3		7024.2	
ZT15	UCG UAC UUA UCU UAA UCC UAC			6807.2		6807.9	
ZT16	GCG UAG GAU UAA GAU AAG UAC	NA	NA	7075.3		7076.0	
ZT17	FAM-UCG UAC UUA UCU UAA UCC UAC			7374.3		7375.4	
ZT18	FAM-UCG uAC UUA UCU UAA uCC UAC			7806.7		7807.8	
ZT19	FAM-UCG uAC uUA UCU uAA uCC UAC	16	2	8239.0		8240.7	
ZT20	FAM-uCG uAC uUA uCU uAA uCC UAC			8671.4		8673.5	
ZT21	CAC AAA AUC GGU UCU ACA GGG UA			8023.9		8026.8	
ZT22	CAC AAA AuC GG <u>u</u> UC <u>u</u> ACA GGG <u>uA</u>	16	2	S	8889.6	8891.5	
ZT23	CUG uGG AAG uCU A				4945.9	4946.7	
ZT24	CUG UGG AAG UCU A				4512.5	4513.6	
ZT25 ^e	ATA CC <u>u</u> CA <u>u</u> GGT AAT CC <u>u</u> GTT	6896.4	6897.3				

ID	Sequence ^a , 5'→3'	Phosphoramidite ^b	BCNS type ^c	Ribose type	Phosphate type ^d	MW by Mass Spectroscopy	
						Calc.	Det.
ZT26 ^e	A <u>u</u> A CC <u>u</u> CA <u>u</u> GGT AA <u>u</u> CCT GuT					7248.7	7250.0
ZT27	ATA CCT CAT GGT AAT CCT GTT	NA	NA			6368.1	6368.9

^a **t** and **u** in bold indicate locations of BCNS enabled by phosphoramidites **12** and **14**, respectively; *u* in bold and italics indicates locations of BCNS enabled by phosphoramidite **16**; FAM indicates a fluorescein label.

^b BCNS-bearing phosphoramidite (Scheme 1).

^c See Fig. 3 for structures.

^d O indicates phosphate backbone; S indicates phosphorothioate backbone.

^e These are hybrid ONs, containing 2'-deoxy conventional nucleotides, and 2'-OMe-U BCNS containing nucleotides.

Table 2

T_m of the duplexes containing different numbers and types of BCNS

#	Duplexes	# of BCNS	Type of BCNS	Types of ONs	T _m (°C)
1	ZT28 ^a :ZT29 ^b	0	NA	2'-deoxy/2'-deoxy	55.2
2	ZT11:ZT29 ^b	1	1		55.9
3	ZT2:ZT29 ^b	2			55.4
4	ZT3:ZT29 ^b	3			56.4
5	ZT3:ZT16 ^c	3		2'-deoxy/2'-OMe	No melting
6	ZT15:ZT16 ^d	0	NA	2'-OMe/ 2'-OMe	56.2
7	ZT14:ZT16	1	2		56.5
8	ZT13:ZT16	2			57.5
9	ZT12:ZT16	4			61.2
10	ZT11:ZT16	6		67.2	
11	ZT11:ZT29 ^e	6		2'-deoxy/2'-OMe	No melting

^aThe control 2'-deoxy ON ZT28 has sequence ATA GTA GTA GTC CTA GTC T.

^bThe control 2'-deoxy ON ZT29 has sequence AGA CTA GGA CTA CTA TT.

^cThis non-complementary nucleotide (Table 1) was used as non-Watson-Crick association control.

^dThe complementary region is from nucleotide 3 to nucleotide 21 from the 5' end.

^eThis non-complementary nucleotide was used as non-Watson-Crick association control.

Table 3

ONs and cell lines used for cytotoxicity study.

Cell Line	ON tested	Concentrations (μM)	Observation time (h)
HEK 293	ZT4, ZT5, ZT6	1, 10	Up to 24
A172	from ZT11 to ZT15	1, 5, 10	Up to 96
MCF7			
HeLa			