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Research paper

Fragment-based solid-phase assembly of oligonucleotide conjugates with peptide and polyethylene glycol ligands

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ABSTRACT

Ligand conjugation to oligonucleotides is an attractive strategy for enhancing the therapeutic potential of antisense and siRNA agents by inferring properties such as improved cellular uptake or better pharmacokinetic properties. Disulfide linkages enable dissociation of ligands and oligonucleotides in reducing environments found in endosomal compartments after cellular uptake. Solution-phase fragment coupling procedures for producing oligonucleotide conjugates are often tedious, produce moderate yields and reaction byproducts are frequently difficult to remove. We have developed an improved method for solid-phase coupling of ligands to oligonucleotides via disulfides directly after solid-phase synthesis. A 2′-thiol introduced using a modified nucleotide building block was orthogonally deprotected on the controlled pore glass solid support with N-butylphosphine. Oligolysine peptides and a short monodisperse ethylene glycol chain were successfully coupled to the deprotected thiol. Cleavage from the resin and full removal of oligonucleotide protection groups were achieved using methanolic ammonia. After standard desalting, and without further purification, homogenous conjugates were obtained as demonstrated by HPLC, gel electrophoresis, and mass spectrometry. The attachment of both amphiphilic and cationic ligands proves the versatility of the conjugation procedure. An antisense oligonucleotide conjugate with hexalysine showed pronounced gene silencing in a cell culture tumor model in the absence of a transfection reagent and the corresponding ethylene glycol conjugate resulted in down regulation of the target gene to nearly 50% after naked application.

1. Introduction

Oligonucleotide gene silencing and splice-switching technologies have grown into indispensable tools in biomedical research as well as promising therapeutic modalities. However, their widespread therapeutic application is still significantly challenged by their poor pharmacokinetics, particularly insufficient cellular uptake [1–3]. Considering the overall moderate clinical success of oligonucleotides so far, it seems imperative to develop novel oligonucleotide derivatizations that properly shield the active agent up to its site of action without perturbing its pairing avidity.

One of the most promising approaches to improve the cellular uptake of the ONs is the development of bioconjugates with uptake enhancing ligands [4,5]. By attaching specific ligands, oligonucleotide distribution and cellular uptake can be rationally modulated to a certain extent. Depending on the ligand, efficient shielding from degrading enzymes, increased circulation times, receptor-specific cell binding and uptake, or enhanced membrane permeation can be conferred to oligonucleotides. Revusiran is the prime example of GalNAc-siRNA conjugates which make use of highly efficient receptor-mediated uptake in hepatocytes through the asialoglycoprotein receptor [6–8].

The generation of ligand-oligonucleotide conjugates requires efficient fragment-(or stepwise-) based synthesis schemes, which are ideally versatile to enable coupling of a variety of different ligands [9,10]. Precise and site-specific conjugation of small molecule ligands, or mono-disperse peptides and polymers with a defined chemical structure allows the generation of homogeneous ON conjugates for structure-activity relationship investigations and further preclinical development [11]. Arginine or lysine rich cell-penetrating peptides, glycoclusters, lipids, (linear or branched)

Abbreviations: ON, oligonucleotide; PEG, polyethylene glycol; CPG, controlled pore glass; LCAA, long chain amino alkyl; TCEP, tris(2-carboxyethyl)phosphin; TBP, tri-*n*-butylphosphine; HOBt, 1-hydroxybenzotriazole; DIPEA, N,N-diisopropylethylamine; BOP, benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate.

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polyamines, (linear or branched) poly- or oligo-ethylene-glycol chains and numerous small molecules have all received interest as possible oligonucleotide ligands [12].

Although conventional postsynthetic coupling reactions on the 3′- and 5′- positions of oligonucleotides in solution seem to be straightforward, there are some serious limitations in many cases, particularly for ligands with poor solubility in aqueous solutions. Often, multistep deprotection and activation procedures with subsequent purification steps are necessary, and electrostatic interactions of some ligands (particularly basic peptides) with oligonucleotides often hamper the efficiency of the coupling reactions [13]. These standard methods, typically making use of amino- or, less frequently, thiol-linkers introduced during oligonucleotide synthesis, are limited to attaching a single ligand at a terminal nucleotide position. Small molecules such as cholesterol or carbohydrates can also be tethered to the 5′-position of oligonucleotides by using respective phosphoroamidite building blocks [14,15]. Attachment on the 2′-position via linkers attached to the hydroxyl group of modified ribose nucleotides offer more versatile conjugation at any nucleotide within the sequence as well as multiple attachments within a single sequence [16]. Ligands are usually attached to the cleaved and purified oligonucleotide by solution-phase fragment coupling. In addition, stepwise solid-phase synthesis of peptide-oligonucleotide conjugates is possible, starting either with assembly of the ON followed by on-line ligand synthesis or vice versa [10,17]. The use of appropriately derivatized phosphoramidite building blocks carrying the desired ligand is another possibility to generate conjugates with small molecule ligands. In this strategy, ligands are tethered to the oligonucleotides via phosphate linkages, and it has been recently widely employed particularly for preparing single- and double stranded GalNAc conjugates for hepatocyte targeting [8,18–21].

There are a variety of chemical linkages used to join ligands with oligonucleotides, including amides, hydrazines, oximes, and many more [10,11,22]. Thiols afford an orthogonal reaction site to form ON-ligand conjugates through thioether, thioester or disulfide linkages [17,23]. Due to its ease of formation and their favorable stability profile for therapeutic applications, native ligation via a disulfide bond is perhaps the most extensively explored linkage in oligonucleotide peptide bioconjugations [22,24,25]. Disulfide linkages between ligands and oligonucleotides have been predominantly formed by employing post-synthetic approaches [25]. Although the exact extent and the kinetics of cleavage within endosomes are not fully elucidated, disulfide linkages are designed to at least partially release the delivered oligonucleotide from the ligand within endosomal compartments after successful cellular internalization [26]. Basic molecules are believed to contribute to subsequent endosomal escape through the proton sponge effect [27]. Linker cleavability is crucial for at least conjugates with large ligands such as PEGylated oligonucleotides and peptide- or protein-oliognucleotide conjugates, because bulky substituents impede interaction with the respective effector nucleases [28].

Solid-phase fragment coupling offers an alternative method for the stepwise synthesis on a single support. In this approach, the entire conjugate is assembled on a single support, either by conjugation of a prefabricated conjugate group or by assembling the conjugate moiety by a stepwise process prior to or after the ON synthesis [9]. The advantage of this method compared to solution fragment coupling is the application to a broad spectrum of ligands including lipophilic molecules and the ease of separating excess reagents and byproducts. We have recently communicated a facile post-synthetic disulfide formation method for solution based fragment coupling [29]. An S-sulfonate protected cysteine is utilized as the complementary ligand part to attach a peptide ligand onto the 2′-thioethyl arm of the ON in aqueous buffered solutions. In order to circumvent the problems associated with solution-phase approaches, i.e. the cumbersome purification and its limited applicability to lipophilic ligands, we evolved the method to allow on-bead coupling. Besides enabling facile purification, this method is also applicable to a wider range of ligands including those not easily soluble in aqueous buffers [10,30].

2. Results and discussion

To enable efficient formation of disulfide linked oligonucleotide conjugates and facilitate removal of ligand educts and reaction side product, we developed a feasible method for fragment coupling on the controlled pore glass (CPG) solid support. A resin-bound, still protected oligonucleotide with a free thiol group facilitates the coupling of lipophillic ligands in organic solvents. This is of particular importance considering the high hydrophilicity and anionic character of oligonucleotides which renders them unfavorable for conjugation reactions with other charged ligands due to aggregation [31]. In contrast, the resin-bound ON is practically neutrally charged since the internucleosidic oxygen atom is still protected as cyanoethyl ether. For the sake of achieving high coupling efficiencies, the ligand is usually used in considerable excess, which complicates the purification when the conjugation reaction is carried out in solution. In contrast, on a solid support, the unreacted conjugate group and the side products are easily removed by simple filtration, which markedly facilitates purification procedures.

A crucial point is the necessity of orthogonal cleavage of the thiol protection group and the linkage compatibility with the final global deprotection and cleavage step which is usually achieved in concentrated aqueous ammonia. The stability of peptide amide bonds during standard deprotection/cleavage is well documented [32]. However, when preparing disulfide linked conjugates, the oligonucleotide deprotection/cleavage step has to be adapted due to cleavage of the disulfide bond under those basic conditions.

In order to enable on-resin coupling, an adequate orthogonal oligonucleotide protection/deprotection strategy had to be developed. Among the conventional sulfur protecting groups adapted to ON synthesis and post-synthetic in-solution coupling, the S-trityl group has found widespread use due to its stability towards phosphoramidite ON synthesis strategy and subsequent deprotection/cleavage and conjugation procedures, particularly with respect to RNA ligation reactions [33]. However, with respect to solid-phase conjugation approach, this strategy bears important shortcomings; the standard argentometric deprotection of the trityl group followed by regeneration produces insoluble side products which cannot be thoroughly washed off the resin. As a result, poor reagent diffusion results in drastic reduction of yields during the subsequent coupling steps [33,34]. We chose the *tert*-butyl sulfanyl group because it possesses favorable characteristics for post-synthetic on-bead deprotection as well as adequate stability for withstanding the conditions of phosphoramidite based oligonucleotide synthesis. In contrast to solid-phase coupling, the risk of the generation of homodimeric oligonucleotides is minimized.

By using S-sulfonate derivatized ligands [29], the required deprotection and activation steps of other disulfide-building procedures are avoided, as is the risk of homodimerization. We selected a highly cationic peptide and an amphiphilic ethylene glycol ligand for coupling to prove the use of the method for compounds with different solubility profile. In particular, basic peptides are difficult to attach to oligonucleotide because of their high charge density, which makes them prone to aggregation and hampers the use of aqueous solutions. The S-sulfonate group withstands the deprotection conditions of the Fmoc peptide synthesis strategy thus enabling peptide synthesis products to directly

undergo thiolysis for bioconjugations. The free deprotected thiol group of the resin-bound ON can thus directly react with the S-sulfonate protected thiol function of the ligand. For oligolysine peptides, we attached a cysteine at the N-terminus as a tether. For facilitating coupling, we utilized the more lipophilic S-methane sulfonate group for a short ethylene glycol ligand.

For synthesis of the nucleotide building block, the hydroxyl group of the 2′-side chain (**4**) [29] was transiently converted to a bromide to produce a key compound (**5**) capable of evolving into various building blocks. We performed conversion of the bromide **5** to the desired activated disulfide terminal methylsulfonyl-protected thiol (**6**), followed by replacement of the methyl-sulfonyl group with a more stable protecting group for oligonucleotide synthesis, the *tert*-butyl sulfanyl group (**7**).

The removal of the 3′- and 5′-silyl groups was quantitatively carried out with triethylamine trihydrofluoride (**8**), thereby minimizing the risk of partial exocyclic amine and S-*tert*-butyl-sulfanyl side chain cleavage. Subsequent 5'-dimethoxytritylation yielded the nucleoside building block **9**, which was purified by recrystallization. This key intermediate can be easily phosphitylated and thus allows incorporation of 2′-attachment sites at any position within an oligonucleotide chain.

We attached the nucleoside to a CPG solid support via a succinyl spacer (**10**) for use at the 3′-terminal site. The first adenosine of a model sequence directed against bcl-2 was exchanged for a modified 2′-*O*-thioethyl grafted adenosine. A long chain alkyl amine controlled pore glass (LCAA-CPG) solid phase loaded with the thioethylene modified adenosine **10** was successfully applied in automated phosphodiester oligonucleotide synthesis using standard phosphoramidite coupling protocols.

2.1. Preparation of peptide and ethylene glycol ligands for coupling

The ligands were chosen based on two main strategies applied for improving the pharmacokinetic properties of the ONs: charge-neutralizers with polycationic molecules such as basic peptides [12], and ethylene glycol chains as used in the Stealth™ technology [35]. It has been demonstrated that use of basic peptides not only partially neutralizes the multiple negative charges of the oligonucleotide backbone, but also protects the oligonucleotide in acidic environments of endosomal compartments and facilitates endosomal escape [36,37].

We prepared oligolysines $\mathbf{P_{1\text{-}4}}$ (Fig. 1) by conventional Fmoc/tBu strategy on a Rink amide resin [29]. To provide a thiol-terminated linker, sulfo-cysteine was sequentially incorporated at the N-terminus of the oligolysine amide chains (3–6 L-lysine residues, Fig. 1).

Polyethylene glycol (PEG) conjugation has been shown to prolong the half-life of the ON conjugates in plasma [28]. Long and short polyethylene glycol chains have been demonstrated to minimize the enzymatic degradability of the ONs and limit their unspecific interactions with serum proteins and non-targeted cells [38]. For antisense and siRNA applications, it has been shown that an increasing length of ethylene glycol chains reduces the gene silencing effect, likely by interfering with binding to the effector nucleases [39]. Shorter strands, on the other hand are well tolerated by the gene silencing machinery and do not reduce silencing efficiency [40,41]. Furthermore it has been shown that independently of the molecular weight, branched PEG was less efficient than linear PEG. Several studies have demonstrated that long chains are not necessarily a prerequisite for repellency towards proteins [42,43] In order to avoid clearance by the mononuclear phagocytic system, short chains are sufficient. Although only longer PEG chains can efficiently rescue oligonucleotide from rapid renal elimination in the absence of plasma protein binding, conjugates with shorter ethylene glycol chains have utility in inferring an increasing stability and a certain degree of enhanced intracellular uptake. Such conjugates can easily be used in combination with other chemical modifications or delivery systems that improve circulation times and biodistribution.

To evaluate on-bead coupling for amphilic and hydrophobic, positively charged ligands, we coupled a short polyethylene glycol chain and several oligolysine peptides [29] to the oligonucleotide. Therefore, a short ethylene glycol fragment consisting of five ethylene glycol units was derivatized with a terminal S-methanesulfonyl protected thiol (Fig. 2).

2.2. Deprotection of the thioalkyl tether and coupling of the ligands

Deprotection of the *tert*-butyl sulfanyl can be carried out either with a phosphorous-containing reagent, preferably tris(2-carboxyethyl)phosphin (TCEP) or tri-*n*-butylphosphine (TBP), or a sulfur-containing reducing agent such as dithiothreitol or ethanethiol [44,45]. Due to the high functionalization of the ONs and in order to decrease the risk of side reactions or cleavage from the resin, we opted for the phosphorous-based reduction. Hence, the 2′-thiol was deprotected after successful ON assembly using tributylphosphine (Fig. 3). After thorough washing to remove the reducing agent, the conjugation of the ethylene glycol chain was achieved by addition of a 4-fold excess of the corresponding ligand in anhydrous acetonitrile. The same reaction conditions produced only moderate yields for oligolysines, which were thus applied in a mixture of acetonitrile and aqueous Tris buffer. Triple repetitions of the coupling step with 10 equiv. oligolysines were necessary for complete conversion. The deprotection and disulfide formation steps were monitored using the colorimetric Ellman's test [46]. Although a rather large excess of the peptides needed to be employed, the unreacted surplus can easily be recovered from the reaction solution.

2.3. Cleavage and full deprotection of the conjugate

Due to the relatively high susceptibility of disulfide bond cleavage in aqueous alkaline conditions which carry the risk of a β-elimination reaction, the standard oligonucleotide deprotection and cleavage step was replaced with treatment with methanolic ammonia (Fig. 3). After cleavage, deprotection, and desalting, all products were analyzed by HPLC and gel electrophoresis (supporting information) for purity and their structures and integrity were verified by high-resolution mass spectrometry (Table 1). All conjugates were easily soluble in aqueous solution at stock concentrations of 100 μM. The conjugates produced using this method showed at least 90% purity without any additional purification after resin cleavage (supporting information). The oligolysine conjugates **10**–**13** are identical with the compounds generated using solution-phase fragment coupling and their analytical properties reported earlier [29].

Ethylene glycol conjugate **14** was characterized by reversed-phase HPLC which showed a single product with a longer retention time than the oligonucleotide educt (supporting information). Polyacrylamide gel electrophoretic analysis showed a slightly lower migration caused by the increase of molecular weight. High-resolution mass spectrometry analyses proved the identity of all bioconjugates (Table 1). Circular dichroism spectroscopy of the conjugates indicated the retention of B-type DNA structure, showing that the attachment of the basic peptides caused no profound alteration of the spatial structure of the nucleic acids with only slight differences in the far UV region (supporting information).

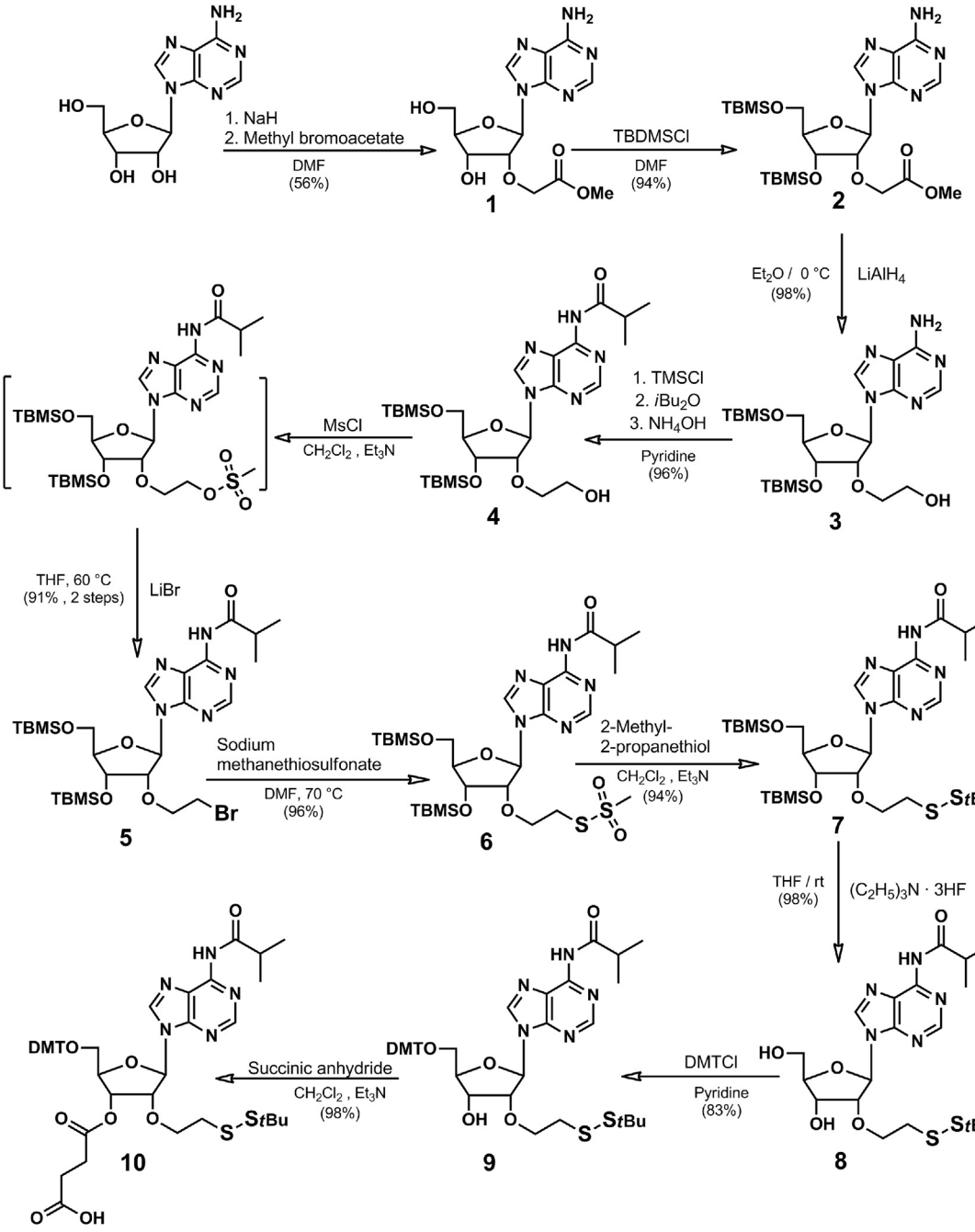

Fig. 1. Preparation of the nucleoside building block **10** for attachment to CPG and oligonucleotide synthesis. Optimized procedures resulted in a total yield of 32%.

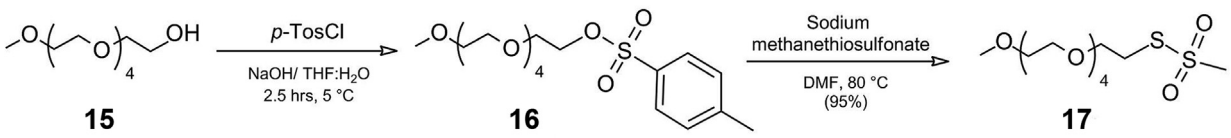

Fig. 2. Synthesis of the pentaethylene glycol ligand **17** for on-bead coupling to 2′-thiol-modified oligonucleotides.

2.4. Evaluation of gene-silencing properties

The gene-silencing activity of the conjugates was examined in the absence of any transfection enhancing agent in a melanoma tumor model with the antiapoptotic Bcl-2 protein as the target. Successful gene silencing was measured by western blotting and quantification of Bcl-2 protein bands (Fig. 4). All experiments were conducted with naked ONs with phosphodiester backbones and 1:1 stoichiometric mixtures of the corresponding isosequential ON and the ligand as controls to verify the conjugation-dependent

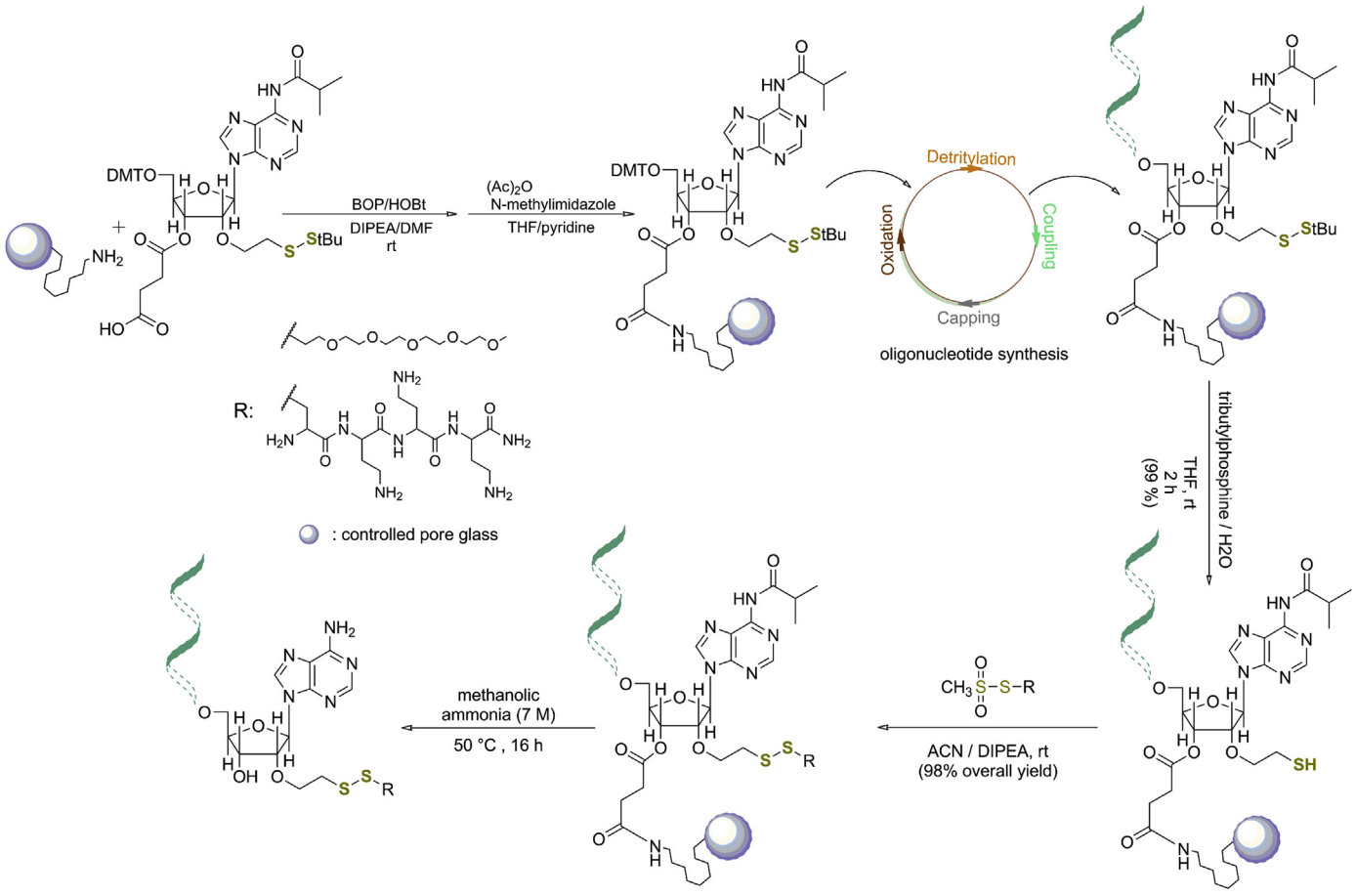

Fig. 3. Oligonucleotide synthesis and solid-phase coupling to PEG_5 and oligolysine peptides.

Table 1
Sequences and analytical data of the conjugates **10**–**14**. Oligonucleotide sequence is TTCTCCCAGCGTGCGCCA (no modifications apart from the 3′-nucleotide), and the indicated ligands were attached to the 2′-linker at the 3′-terminal nucleotide. High-resolution mass spectra were acquired in negative-ion mode after desalting in 1 M ammonium acetate solution. (A: methanol; B: 50 mM triethylammonium acetate buffer, pH 7).[1]: 28–33% solvent A in 30 min; flow rate = 1 mL min^{-1} at 25 °C; column: Thermo Scientific BDS Hypersil C18 (150 × 4 mm).[2]: 15–70% solvent A in 35 min; flow rate = 1 mL min^{-1} at 25 °C; column: Phenomenex Synergi 4u Fusion-RP 80A (150 × 4.60 mm).

Compound	Sequence	HPLC retention time (min)	Mass found/calculated
10	Oligonucleotide-K_3	6.11[1]	$[M-9H]^{9-}$: 664.5683 (664.5871)
11	Oligonucleotide-K_4	6.26[1]	$[M-9H]^{9-}$: 678.8310 (678.8285)
12	Oligonucleotide-K_5	6.48[1]	$[M-9H]^{9-}$: 693.0599 (693.0699)
13	Oligonucleotide-K_6	6.95[1]	$[M-10H]^{10-}$: 636.6681 (636.4793)
14	Oligonucleotide-PEG_5	24.42[2]	$[M-7H]^{7-}$: 820.8690 (820.7092); $[M-8H]^{8-}$: 718.1360 (717.9946); $[M-9H]^{9-}$: 638.2386 (638.1054); $[M-10H]^{10-}$: 574.3033 (574.1941)

silencing activity. We observed a concentration-dependent effect, which for the oligolysine conjugates was in direct relation with increasing peptide chain length as reported earlier [29]. Among the conjugates **10**–**14**, the hexalysine conjugate **13** showed the strongest effect (9-fold reduction) even at a 100 nM concentration (Fig. 4A). Neither the wild-type ON nor a complex of the corresponding oligolysine and ON had any significant effect on bcl-2 levels, underlining the importance of covalent attachment of the basic peptide to the phosphodiester ON for these conjugates.

Treatment of cells with conjugate **14** (PEG_5 ligand) resulted in statistically significant down regulation of Bcl-2 expression (Fig. 4B) at both 100 and 1000 nM concentrations. Co-administration of uncoupled PEG_5 ligand and the ON at resulted in less efficient, but also significant down-regulation. This effect might be explained by a certain destabilizing impact of the amphiphilic ethylene-glycol chain on the cell membrane, which is also present when co-administrating the two components.

For toxicity determination, 607B cells treated with the conjugates were assayed for their impact on cell proliferation and in a caspase cleavage assay (Fig. 5). No reduction in cellular viability or apoptosis induction was detected for any of the compounds **10**–**14** in concentrations as high as 1 μM. In contrast, an isosequential

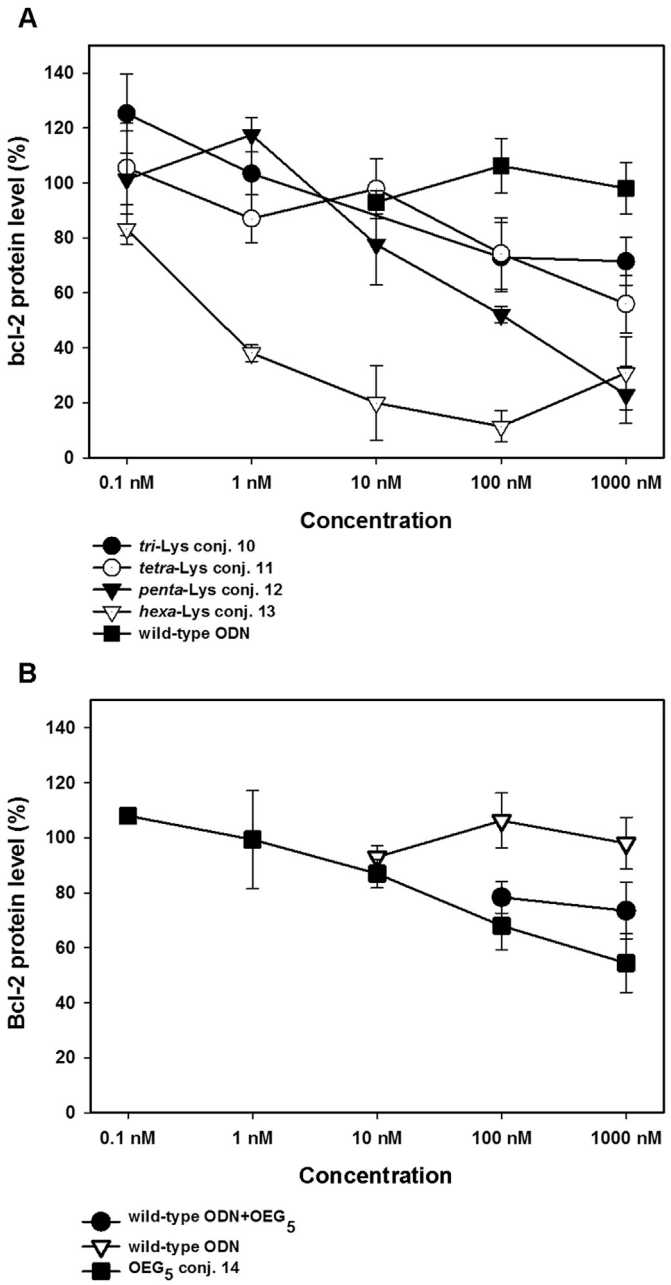

Fig. 4. Bcl-2 expression level of oligolysine-oligonucleotide disulfide conjugates **10**–**13** (**A**) [29] and PEG_5-oligonucleotide conjugate **14** (**B**), after naked application on 607B cell line after 72 h incubation followed by protein extraction and western blotting. Results are reported as mean values with n = 3 ± s.d.

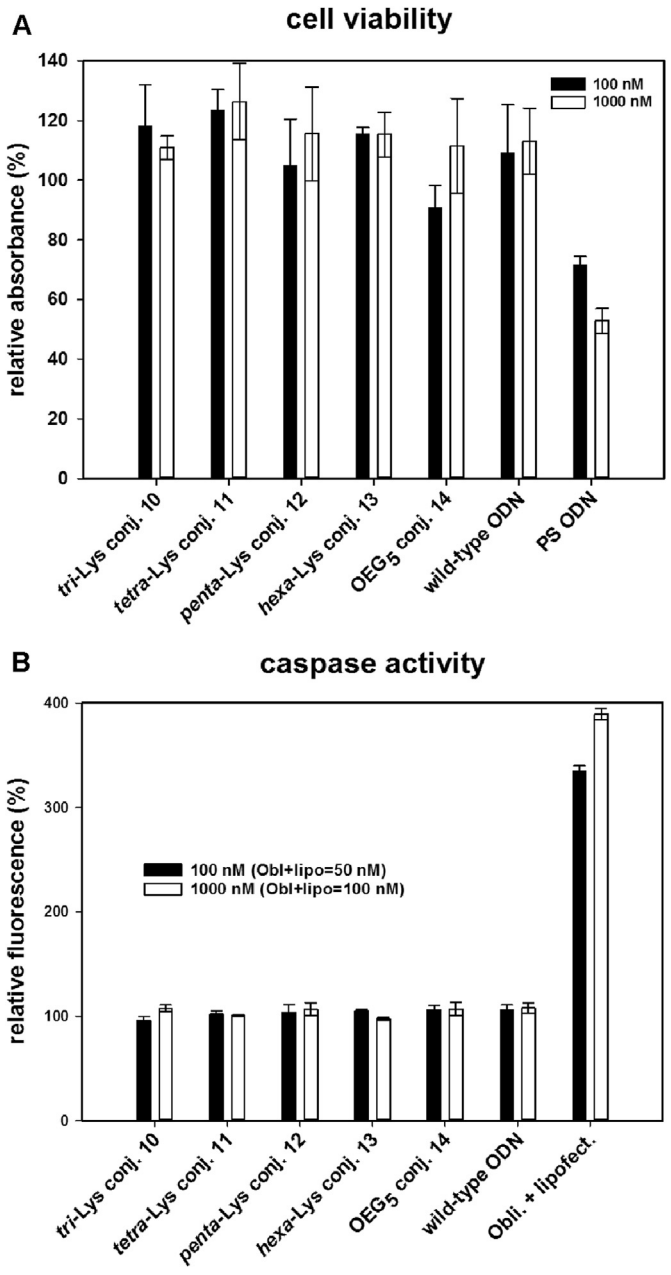

Fig. 5. Cell viability (**A**) and caspase activity (**B**) of oligolysine-oligonucleotide disulfide conjugates **10**–**14** (**A**) and oligoethylene-oligonucleotide conjugate **14** (**B**), after transfection-free application on 607B cell line after 24 h incubation. Results are reported as mean values with n = 3 ± s.d.

phosphorothioate oligonucleotide resulted in concentration-dependent inhibition of viability and an increase in caspase activation at both 100 nM and 1 μM concentrations. The documented intrinsic toxicity of phosphorothioate oligonucleotides results in inhibition of cellular proliferation which is independent on down regulation of the anti-apoptotic bcl-2 protein [47,48]. Although bcl-2 plays a pivotal role in apoptosis regulation, its depletion by gene silencing is not sufficient for increased apoptosis rates without an additional trigger through chemo- or radiotherapy [49,50].

3. Conclusion

The reported on-bead method for assembly of disulfide linked bioconjugates enables the coupling of a large range of ligands to oligonucleotides with quick and facile purification. The representative conjugates that were synthesized demonstrate the feasibility of conjugation of both highly charged and amphiphillic ligands and prove the sufficient stability during the modified cleavage and deprotection procedure. The prepared phosphodiester oligonucleotide conjugates show promising *in vitro* target down regulation in the absence of any transfection enhancing agent while being non-toxic.

4. Materials and methods

Synthesis reagents and solvents were obtained from

Sigma–Aldrich (St. Louis, MO, USA); Bachem (Bubendorf, Switzerland), or Fisher Scientific (Waltham, MA, USA) and were used without further purification. Dimethylformamide (Fisher) was dried by refluxing over calcium hydride followed by vacuum distillation. Peptide synthesis reagents and amino acids were purchased from Bachem and Sigma–Aldrich. Oligonucleotide synthesis reagents, solvents and nucleoside building blocks were supplied by Proligo (SAFC, Sigma–Aldrich). Thin-layer chromatography was carried out on Merck 60 F254 aluminum-coated silica gel plates. Product spots were visualized by UV shadowing (254 nm).

For column chromatography, Acros 0.035–0.070 mm, 60 Å silica gel was used. NMR spectra were recorded on a Varian 500 MHz or on a Bruker 200 MHz spectrometer. High-resolution mass spectra were collected on a Bruker Daltonics microTOF-Q II mass spectrometer with an ESI source with an accuracy of ±5 ppm. HPLC was performed on a Shimadzu LC-20 AD system equipped with a UV/VIS detector (260 nm) or a Merck Hitachi D-7000 with the UV/VIS detector set at 220 nm. CD spectra were recorded on a Jasco J-810 spectropolarimeter. Gel electrophoresis and blotting reagents and buffers were supplied by Serva (Heidelberg, Germany), and blots were digitalized using a Molecular Imager ChemiDoc™ XRS System and processed with Quantity One Software (both Bio-Rad Laboratories, Carlsbad, CA, USA). Absorbance and fluorescence measurements of apoptosis and viability assays were measured on a TECAN Infinite M200 Pro (Tecan, Grödig, Austria).

4.1. Synthesis of the modified nucleoside

4.1.1. 3′-,5′-Bis(tert-butyldimethylsilyl)-2′-O-(2-bromo-ethyl)-N^6-isobutyryl-adenosine (**5**)

A stirred solution of **4** (2.10 g, 3.44 mmol) [29] in 11 mL anhydrous dichloromethane and 4 mL triethylamine was cooled to 0 °C in an argon atmosphere. Methanesulfonyl chloride (0.43 g, 3.80 mmol) was diluted in 4 mL dichloromethane and added dropwise via a syringe. The reaction mixture was stirred at room temperature for 2 h and subsequently evaporated to dryness. The orange oily residue was taken up in ethyl acetate and washed with 1 M sodium bicarbonate solution, water and brine and dried over sodium sulphate followed by evaporation of the solvent under vacuum.

The residue was then dissolved in 15 mL anhydrous tetrahydrofuran and mixed with a solution of ultra-dry lithium bromide (0.90 g, 10.32 mmol) in anhydrous THF in an argon atmosphere. After heating the reaction mixture at 60 °C for 6 h, thin-layer chromatography showed complete conversion of the starting material. Afterwards, the solvent was removed and the pale yellow residue was redissolved in ethyl acetate.

The organic phase was washed with water and brine, dried over sodium sulphate and chromatographed on a silica gel column eluted with a gradient of 0–70% *tert*-butyl methyl ether in petroleum ether. The corresponding fractions were pooled and evaporated under vacuum to produce 2.11 g (91%) of a pale yellow powder as the product.

4.1.2. R_f 0.35 (1:20 MeOH/CH_2Cl_2)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.72 (s, 1H, H-2), 8.60 (br s, 1H, NH), 8.36 (s, 1H, H-8), 6.19 (d, *J* = 3.8 Hz,1H, H-1′), 4.51 (dd, *J* = 5.35 and 3.8, 1H, H-3′), 4.41 (dd, *J* = 4.9, 3.8 Hz, 1H, H-2′), 4.14 (dt, *J* = 5.7, 2.8 Hz, 1H, H-4′), 4.00 (dd, *J* = 11.7, 3.5 Hz, 1H, H-5′/1), 3.97–3.87 (m, 2H, OCH_2), 3.78 (dd, *J* = 11.7, 2.9 Hz, 1H, H-5′/2), 3.44 (t, *J* = 6.3 Hz, 1H, $BrCH_2$), 3.19 (septet, *J* = 6.0 Hz, 1H, *i*-prCH), 1.30 (d, *J* = 6.9 Hz, 6H, *i*-prCH$_3$), 0.92 (s, 18H, *tert*-BuCH$_3$), 0.11 (s, 3H, SiCH$_3$), 0.10 (s, 9H, SiCH$_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.0 (CON), 152.6 (C-2), 150.8 (C-4), 149.3 (C-6), 141.5 (C-8), 122.5 (C-5), 87.2 (C-1′), 85.0 (C-4′), 82.4 (C-2′), 70.7 (2′-OCH_2), 69.9 (C-3′), 61.6 (C-5′), 36.2 (*i*-prCH), 29.8 ($BrCH_2$), 26.0 (*tert*-BuCH$_3$), 25.7 (*tert*-BuCH$_3$), 19.2 (*i*-prCH$_3$), 18.4 (*tert*-BuC), 18.1 (*tert*-BuC), - 4.9 (SiCH$_3$), - 5.4 (SiCH$_3$).

ESI-HRMS *m/z* $[M+H]^+$: calculated for $C_{28}H_{51}BrN_5O_5Si_2$ 672.2606, found 672.2632.

4.1.3. 3′-,5′-Bis(tert-butyldimethylsilyl)-N^6-isobutyryl-adenosine-2′-O-(ethyl)-methanesulfonothioate (**6**)

To a solution of **5** (600 mg, 0.89 mmol) in 15 mL anhydrous dimethylformamide sodium methanethiosulfonate (0.131 g, 0.98 mmol) was added in an argon atmosphere. The reaction mixture was heated at 70 °C until thin-layer chromatography revealed completion of the reaction (16 h).

The solvent was then removed under vacuum and the residue was taken up in ethyl acetate, washed with water and brine and dried over sodium sulphate. The organic phase was evaporated to obtain 0.60 g (96%) of pale yellow crystals as the desired product which was used without further purification.

4.1.4. R_f 0.29 (tert-butyl methyl ether)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.70 (s, 1H, H-2), 8.53 (br s, 1H, NH), 8.37 (s, 1H, H-8), 6.18 (d, J = 3.2 Hz, 1H, H-1′), 4.52 (t, J = 5.1 Hz, 1H, H-3′), 4.32 (dd, J = 3.2, 2.9 Hz, 1H, H-2′), 4.14 (dt, J = 2.9, 2.5 Hz, 1H, H-4′), 4.01 (dd, J = 11.7, 2.8 Hz, 1H, H-5′/1), 3.95 (t, J = 5.7 Hz, 2H, OCH_2), 3.78 (dd, J = 11.7, 2.8 Hz, 1H, H-5′/2), 3.37 (dd, J = 9.5, 5.4 Hz, 2H, SCH_2), 3.34 (s, 3H, -SO_2CH_3), 3.21 (septet, J = 6.5 Hz, 1H, i-prCH), 1.31 (d, J = 7.0 Hz, 6H, i-prCH$_3$), 0.93 (s, 9H, tert-BuCH$_3$), 0.92 (s, 9H, tert-BuCH$_3$), 0.11 (s, 12H, SiCH$_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.9 (CON), 152.6 (C-2), 150.7 (C-4), 149.3 (C-6), 141.3 (C-8), 122.4 (C-5), 87.0 (C-1′), 84.8 (C-4′), 82.8 (C-2′), 69.6 (C-3′), 69.5 (2′-OCH_2), 61.5 (C-5′), 50.6 (-SO_2CH_3), 36.2 (SCH_2), 36.2 (i-prCH), 26.0 (tert-BuCH$_3$), 25.7 (tert-BuCH$_3$), 19.2 (i-prCH$_3$), 18.5 (tert-BuC), 18.1 (tert-BuC), - 4.5 (SiCH$_3$), - 5.4 (SiCH$_3$).

ESI-HRMS *m/z* $[M+H]^+$: calculated for $C_{29}H_{54}N_5O_7S_2Si_2$ 704.2998, found 704.2935.

4.1.5. 3′-,5′-Bis(tert-butyldimethylsilyl)-2′-O-(2-tert-butyldisulfanyl-ethyl)-N^6-isobutyryl-adenosine (**7**)

2-Methyl-2-propanethiol (0.092 g, 1.02 mmol) was added dropwise to a cooled (ice bath) solution of **6** (0.60 g, 0.85 mmol) in 5 mL dichloromethane containing 2 equiv of (0.24 mL) triethylamine. After stirring for 6 h (TLC) at room temperature, the solvents were removed under vacuum. The residue was taken up in ethyl acetate and washed with water and brine followed by drying over sodium sulphate. The solution was thereafter evaporated and the residue was chromatographed on a silica gel column. The title compound was eluted with a 0–50% gradient of *tert*-butyl methyl ether in petroleum ether to obtain 0.57 g (94%) of a white powder.

4.1.6. R_f 0.35 (1:1 tert-butyl methyl ether/petroleum ether)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.72 (s, 1H, H-2), 8.51 (br s, 1H, NH), 8.34 (s, 1H, H-8), 6.17 (d, *J* = 4.1 Hz,1H, H-1′), 4.50 (dd, *J* = 5.1, 4.7 Hz, 1H, H-3′), 4.39 (t, *J* = 4.4 Hz, 1H, H-2′), 4.13 (ddd, *J* = 7.9, 6.9, 3.2 Hz, 1H, H-4′), 4.00 (dd, *J* = 11.4, 3.8 Hz, 1H, H-5′/1), 3.84–3.76 (m, 3H, OCH_2 and H-5′/2), 3.21 (septet, *J* = 6.5 Hz, 1H, *i*-prCH), 3.37 (dd, *J* = 7.0, 6.6 Hz, 2H, SCH_2), 1.31 (d, *J* = 6.6 Hz, 6H, *i*-prCH$_3$), 1.28 (s, 9H, S-*tert*BuCH$_3$), 0.93 (s, 9H, Si-*tert*-BuCH$_3$), 0.92 (s, 9H, Si-*tert*-BuCH$_3$), 0.11 (s, 12H, SiCH$_3$), 0.12 (s, 3H, SiCH$_3$), 0.10 (s, 9H, SiCH$_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.9 (CON), 152.6 (C-2), 150.9 (C-4), 149.2 (C-6), 141.6 (C-8), 122.5 (C-5), 87.1 (C-1′), 84.2 (C-4′), 82.2 (C-2′), 69.9 (C-3′), 69.5 (2′-OCH_2), 61.8 (C-5′), 47.9 (S-*tert*BuC), 39.7 (SCH_2), 36.2 (*i*-prCH), 29.8 (S-*tert*BuCH$_3$), 26.0 (Si-*tert*-BuCH$_3$), 25.7 (Si-*tert*-BuCH$_3$), 19.2 (*i*-prCH$_3$), 18.4 (Si-*tert*-BuC), 18.1 (Si-*tert*-BuC), -4.5 (SiCH$_3$), -4.8 (SiCH$_3$), -5.40 (SiCH$_3$), -5.42 (SiCH$_3$).

ESI-HRMS *m/z* $[M+H]^+$: calculated for $C_{32}H_{60}N_5O_5S_2Si_2$ 714.3569, found 714.3601.

4.1.7. 2′-O-(2-Tert-butyldisulfanyl-ethyl)-N^6-isobutyryl-adenosine (**8**)

To a solution of **7** (0.25 g, 0.35 mmol) in 2 mL tetrahydrofuran triethylamine trihydrofluoride 97% (0.23 g, 1.40 mmol) was added dropwise and the mixture was stirred at room temperature for 4 h until thin-layer chromatography showed complete deprotection of the starting material.

The solvent was then evaporated and the residue was taken up in ethyl acetate, washed with water and brine and dried over sodium sulphate. The organic phase was removed to produce 0.170 g (98%) of the desired product as a pale yellow foam which was used without further purification.

4.1.8. R_f 0.31 (1:15 MeOH/CH_2Cl_2)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.70 (s, 1H, H-2), 8.61 (br s, 1H, NH), 8.05 (s, 1H, H-8), 6.13 (d, J = 11.7 Hz, 1H, OH-5′), 5.94 (d, J = 7.6 Hz, 1H, H-1′), 4.83 (dd, J = 7.6, 4.4 Hz, 1H, H-2′), 4.58 (d, J = 4.4, 1H, H-3′), 4.38 (s, 1H, H-4′), 3.97 (d, J = 13.00 Hz, 1H, H-5′/1), 3.79 (d, J = 12.00 Hz, 1H, H-5′/2), 3.77–3.67 (m, 2H, OCH_2), 3.24 (septet, J = 6.00 Hz, 1H, i-prCH), 3.14 (s, 1H, OH-5′), 2.76–2.68 (m, 2H, SCH_2), 1.31 (d, J = 7.00 Hz, 6H, i-prCH_3), 1.28 (s, 9H, S-tertBuCH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.0 (CON), 152.0 (C-2), 150.0 (C-4), 150.1 (C-6), 143.1 (C-8), 123.7 (C-5), 89.4 (C-1′), 88.0 (C-4′), 81.3 (C-2′), 70.9 (C-3′), 68.7 (2′-OCH_2), 63.3 (C-5′), 48.3 (S-tertBuC), 39.8 (SCH_2), 36.2 (i-prCH), 29.8 (S-tertBuCH_3), 19.2 (i-prCH_3), 19.1 (i-prCH_3).

ESI-HRMS *m/z* $[M+H]^+$: calculated for $C_{20}H_{32}N_5O_5S_2$ 486.1839, found 486.1843.

4.1.9. 2′-O-(2-Tert-butyldisulfanyl-ethyl)-5′-O-dimethoxytrityl-N^6-isobutyryl-adenosine (**9**)

4,4′-Dimethoxytrityl chloride (0.058 g, 0.17 mmol) was added to a cooled solution of **8** (0.075 g, 0.15 mmol) in 1 mL of anhydrous dichloromethane containing 3 equiv triethylamine (0.065 mL). The reaction mixture was allowed to warm up to room temperature and stirred for 16 h until thin-layer chromatography showed completion of the reaction.

After quenching the reaction for 0.5 h in the presence of 0.5 mL of methanol, the solvents were removed under vacuum and the residue was redissolved in ethyl acetate, washed with saturated sodium bicarbonate solution, water, brine and dried over sodium sulphate. The organic phase was then evaporated and the resulting yellow foam was recrystallized from *tert*-butyl methyl ether/*n*-hexane to give 0.098 g (83%) of the title compound as a pale yellow foam.

4.1.10. R_f 0.27(1:40 MeOH/CH_2Cl_2)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.65 (s, 1H, H-2), 8.46 (br s, 1H, NH), 8.20 (s, 1H, H-8), 7.43 (d, *J* = 7.60 Hz, 2H, Ph-2,6), 7.32 (dd, *J* = 8.9, 1.6 Hz, 4H, Ar-2,6), 7.29–7.22 (m, 3H, Ph-3,4,5), 6.81 (d, *J* = 8.60 Hz, 4H, Ar-3,5), 6.18 (d, *J* = 3.8 Hz,1H, H-1′), 4.63 (dd, *J* = 4.7, 4.1 Hz, 1H, H-2′), 4.52 (dd, *J* = 11.1, 5.4, 1H, H-3′), 4.24 (dt, *J* = 8.5, 4.1 Hz, 1H, H-4′), 4.05 (ddd, *J* = 10.4, 7.3, 5.4 Hz, 1H, OCH_2/1), 3.89–3.87 (m, 1H, OCH_2/1), 3.79 (s, 3H, OMe), 3.53 (dd, *J* = 10.70, 3.2 Hz, 1H, H-5′/1), 3.43 (dd, *J* = 10.8, 3.20 Hz, 1H, H-5′/2), 3.17 (septet, *J* = 6.81 Hz, 1H, *i*-prCH), 2.90–2.86 (m, 2H, SCH_2), 1.32 (d, *J* = 7.00 Hz, 6H, *i*-prCH_3), 1.28 (s, 9H, S-*tert*BuCH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 175.8 (CON), 158.6 (Ar-4), 152.6 (C-2), 150.9 (C-4), 149.3 (C-6), 144.4 (Ph-1), 141.4 (C-8), 135.6 (Ar-1), 135.6 (Ar-1), 130.1 (Ar-2,6), 128.2 (Ph-2,6), 127.9 (Ph-3,5), 127.0 (Ph-4), 122.6 (C-5), 113.2 (Ar-3,5), 87.1 (C-1′), 86.6 (trityl-C), 84.1 (C-4′), 81.9 (C-2′), 69.8 (C-3′), 69.3 (2′-OCH_2), 62.8 (C-5′), 55.2 (OMe), 48.2 (S-*tert*BuC), 39.9 (SCH_2), 36.2 (*i*-prCH), 29.8 (S-*tert*BuCH_3), 19.2 (*i*-prCH_3).

ESI-HRMS *m/z* $[M+Na]^+$: calculated for $C_{41}H_{49}N_5NaO_7S_2$ 810.2966, found 810.2970.

4.1.11. 2′-O-(2-Tert-butyldisulfanyl-ethyl)-3′-succinyl-5′-O-dimethoxytrityl-N^6-isobutyryl-adenosine (**10**)

Compound **9** (0.060 g, 0.076 mmol) was dissolved in 2 mL anhydrous dichloromethane containing 3 equiv (0.032 mL) triethylamine in an argon atmosphere. The reaction mixture was cooled in an ice bath before succinic anhydride (0.015 g, 0.15 mmol) was added.

The reaction mixture was stirred for 1 h at 0 °C and allowed to come slowly to room temperature. After 2 h, thin-layer chromatography revealed complete conversion of the starting material. Solvents were removed under vacuum, the residue redissolved in ethyl acetate and washed with saturated bicarbonate solution, water, brine and dried over sodium sulphate. The solution was then evaporated to dryness under vacuum and the residue was recrystallized from *tert*-butyl methyl ether/*n*-hexane to produce 0.066 g (98%) of the title compound as a pale yellow foam.

4.1.12. R_f 0.17(1:20 MeOH/CH_2Cl_2)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.90 (br s, 1H, NH), 8.63 (s, 1H, H-2), 8.16 (s, 1H, H-8), 7.40 (d, *J* = 7.60 Hz, 2H, Ph-2,6), 7.30 (d, *J* = 8.8 Hz, 4H, Ar-2,6), 7.27–7.16 (m, 3H, Ph-3,4,5), 6.81 (d, *J* = 7.60 Hz, 4H, Ar-3,5), 6.12 (d, *J* = 6 Hz,1H, H-1′), 5.47 (dd, *J* = 4.8, 3.5, 1H, H-3′), 4.93 (t, *J* = 5.7 Hz, 1H, H-2′), 4.36 (dt, *J* = 6.9, 3.5 Hz, 1H, H-4′), 3.78 (s, 3H, OMe), 3.84–3.67 (m, 2H, OCH_2), 3.53 (dd, *J* = 10.7, 3.5 Hz, 1H, H-5′/1), 3.41 (dd, *J* = 10.8, 3.8 Hz, 1H, H-5′/2), 3.14 (septet, *J* = 6.7 Hz, 1H, *i*-prCH), 2.81–2.66 (m, 6H, succin-CH_2 and SCH_2), 1.299 (d, *J* = 7.0 Hz, 3H, *i*-prCH_3), 1.295 (d, *J* = 6.9 Hz, 3H, *i*-prCH_3), 1.23 (s, 9H, S-*tert*BuCH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.0 (CON), 171.3 (COO), 158.6 (Ar-4), 152.7 (C-2), 151.3 (C-4), 149.5 (C-6), 144.2 (Ph-1), 141.2 (C-8), 135.4 (Ar-1), 135.4 (Ar-1), 130.1 (Ar-2,6), 128.1 (Ph-2,6), 128.0 (Ph-3,5), 127.1 (Ph-4), 122.4 (C-5), 113.3 (Ar-3,5), 86.9 (trityl-C), 86.7 (C-1′), 82.2 (C-4′), 80.1 (C-2′), 71.5 (C-3′), 69.7 (2′-OCH_2), 62.9 (C-5′), 55.2 (OMe), 48.9 (S-*tert*BuC), 39.8 (SCH_2), 36.1 (*i*-prCH), 29.8 (S-*tert*BuCH_3), 29.1 (succin-CH2), 19.2 (*i*-prCH_3).

ESI-HRMS *m/z* $[M+Na]^+$: calculated for $C_{45}H_{53}N_5NaO_{10}S_2$ 910.3126, found 910.3126.

4.2. Loading of the 2′-modified nucleoside **10** onto the LCAA-CPG

To a solution of **10** (20.90 mg, 26.76 μmol) in anhydrous dimethylformamide (0.5 mL) 1-hydroxybenzotriazole (HOBt, 4.05 mg, 29.96 μmol) and N,N-diisopropylethylamine (DIPEA, 11.1 mg, 85.6 μmol) were added. The mixture was then transferred to a reaction vessel containing LCAA-CPG (200 mg) followed by addition of benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 13.25 mg, 29.96 μmol). The slurry was shaken gently at room temperature in an argon atmosphere. After 16 h, the resin was filtered off and washed three times with dimethylformamide, methanol and dichloromethane, successively, and was air-dried.

The free amino groups were subsequently capped by addition of a mixture of acetic anhydride/pyridine/1-methylimidazole/anhydrous THF (1/1/1.5/15) the resin under an argon atmosphere. After 2 h, the resin was washed three times with acetonitrile, methanol and dichloromethane and air-dried for a short time before being completely dried in an exsiccator overnight. Loading was determined by adding 3% trichloroacetic acid in dichloromethane and determination of the absorption at 498 nm of the supernatant and

was estimated to be 51.6 μmol g^{-1}.

4.3. Oligonucleotide synthesis, bioconjugation, and analysis

Oligodeoxynucleotides were synthesized with phosphodiester bonds on a Polygen 10 column DNA synthesizer using standard phosphoramidite chemistry protocols using the DMT-off mode. After completed synthesis, the desired amount of the resin-oligonucleotide was transferred to a micro test tube for the consecutive deprotection and coupling steps.

The loaded oligonucleotide-LCCA-CPG (10 mg) was slurried in tetrahydrofuran (200 μL) followed by addition of tri-*N*-butylphosphine (4 equiv). After 5 min, one μL doubly-distilled water was added and the reaction mixture was agitated gently for 2 h at 25 °C. Subsequently, the resin was filtered off and washed three times with tetrahydrofuran, methanol and dichloromethane and dried in an evacuated exsiccator overnight. The assessment of deprotection and the optimization of the reaction were carried out employing the Ellman test.

The LCAA-CPG from the previous step (deprotection) was suspended in a mixture of PEG_5 (3 equiv) in 200 μL anhydrous acetonitrile followed by the addition of DIPEA (6 equiv). The reaction mixture was agitated gently at room temperature for 4 h and the resin was filtered off, washed three times with acetonitrile and dichloromethane and air-dried. Optimization of reaction conditions and reaction time was carried out by performing the Ellman test.

The LCAA-CPG-oligonucleotide-SH was suspended in a mixture of 2:1 ACN/Tris–HCl (pH 7.5) buffer followed by addition of the peptides [29]. The reaction mixture was agitated gently at room temperature overnight and the resin was filtered off, washed three times with 2:1 ACN/H_2O, ACN and dichloromethane and air-dried. Optimization of reaction conditions and reaction time was carried out by the Ellman test.

The resin was suspended in a screw-capped vial in concentrated methanolic ammonia solution (7 N) and heated for 16 h at 55 °C to afford cleavage from the solid support and removal of the protecting groups. After cooling down to room temperature and removing the excess of ammonia, the solvent was evaporated on a SpeedVac. The residue was then redissolved in a small volume (50–100 μL) of doubly distilled water and precipitated from isopropanol (1 mL). After overnight incubation at −20 °C, the suspension was centrifuged at 16,000 *g* for 15 min at 4 °C. The supernatant was removed and the pellet was air-dried. After redissolving in water and dilution to 100 μM stock solutions, the purity of the oligonucleotides was checked by HPLC. If required, the raw mixture was passed through a column of Sephadex™ G-25 for desalting. Oligonucleotide conjugates with a purity of at least 90% (as integrated from HPLC traces) were used without further purifications.

The overall efficiency of the process was assessed by HPL chromatography. Analytical HPLC of the oligolysine conjugates was carried out according to **method A** using a Thermo Scientific BDS Hypersil C18 column (150 × 4 mm) and 260 nm UV detection on a Shimadzu (LC-20 AD) HPLC instrument. A gradient of 28–33% solvent A (A: methanol; B: 50 mM triethylammonium acetate buffer, pH 7) was run in 30 min at a flow rate of 1 mL min^{-1} at 25 °C.

For the OEG_5-conjugate **method B** was applied on a Shimadzu (LC-20 AD) HPLC instrument equipped with a UV/VIS detector (260 nm) using a Phenomenex Synergi 4u Fusion-RP 80A (150 × 4.60 mm; 4 μ) column running a gradient of 15–70% solvent A (A: methanol; B: 50 mM *tri*ethylammonium acetate, pH 7) in 35 min at a flow rate of 1 mL min^{-1} at 25 °C.

For gel electrophoresis, a urea polyacrylamide gel (20% acrylamide, 1x Tris-borate buffer TBE) was prepared and run in a Bio-Rad Mini Protean II apparatus. Samples were mixed 1:10 in formamide loading buffer (95% formamide). As tracer, a mixture of bromophenol blue and xylene cyanol was used, and the gel was run in 1x TBE buffer until the dyes reached about one third and two thirds, respectively, of the migration distance of the gel. The gel was stained in methylene blue solution (0.002% in TBE), destained in water, and digitalized in a GS-710 densitometric scanner (Bio-Rad).

4.4. In vitro gene silencing activity

Gene silencing assays were performed as described previously [29]. In short, 607B cells were seeded in 24-well tissue culture plates (Greiner, bio-one, Kremsmünster, Austria). After 24 h, the cells were incubated with the indicated concentrations of oligonucleotides or conjugates, and after 72 h incubation, the culture medium was then removed and the cells were lysed at 4 °C. Aliquots of cell lysates containing 20 μg of total protein were separated on a 15% SDS-polyacrylamide gel and blotted onto a PVDF membrane. The blots were developed with the primary antibodies directed either against actin (Sigma–Aldrich, 1:5000) and Bcl-2 (Zymed, 1:500) and with HRP-coupled secondary antibodies in TBST (anti-rabbit for actin, anti-mouse for Bcl-2, 1:100,000 dilution). Bands were detected by chemiluminescence using the ImmunStar Western C Kit (Bio-Rad) and a Chemi-Doc gel imager (Bio-Rad). The protein bands were then analyzed utilizing the Quantity One™ (version 4.6.3) software package (Bio-Rad).

4.5. Cell viability assay

Cell viability and caspase activites were tested as described [29]. In short, 607B cells (2×10^4 cells per well) were seeded in transparent (viability) or black (caspase activity) 96-well microplates (Greiner bio-one). After 24 h, oligonucleotides and conjugates were added at the indicated concentrations and the plate was incubated for another 24 h. Subsequently, the EZ4U proliferation assay kit (Biomedica) was used according to the manufacturer's instructions to quantify cell viability/proliferation. The absorbance at 465 nm was recorded with 620 nm as reference wavelength. For the caspase activity readout, lysis buffer was added and the plates were incubated 20 min on ice. Assay buffer containing the peptide substrate Ac-DEVD-AMC was added and fluorescence was recorded at room temperature (excitation: 360 nm; emission: 460 nm; slit width: 5 nm) until optimal signal to noise ratios were reached. Cell viability and apoptosis rates are presented in relation to untreated cells.

4.6. Statistical analysis

Statistical significance of differences was determined by using the student's *t*-test (SigmaPlot 12.5). P values < 0.05 were considered to be significant.

Disclosure

A patent application comprising some of the described methodology and structures has been filed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2016.05.001.

These data include MOL files and InChiKeys of the most important compounds described in this article.

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