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Original article

Oligonucleotide–polyamine conjugates: Influence of length and position of 2′-attached polyamines on duplex stability and antisense effect

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Abstract

Tethering cationic ligands to oligonucleotides results in zwitterionic molecules with often improved target affinity and better cell membrane permeation. Due to the ideal distance between cationic groups, polyamines are perfect counter ions for oligonucleotides. Using an easy and versatile procedure for attaching ligands to the 2′-position, polyamines were conjugated to distinct terminal and internal positions of oligonucleotides. With polyamines attached to terminal nucleosides, the affinity to complementary DNA or RNA strands increased with growing number of cationic amines. Tethering polyamines to an internal nucleoside of wild type DNA oligonucleotides resulted in a considerable decrease in duplex stability, but in phosphorothioates, no significant decrease was detected. Conjugates exhibited progressively higher target downregulation ability with increasing polyamine chain length in a human melanoma cell culture assay.

Keywords: Antisense; Oligonucleotide conjugation; Polyamines; bcl-2

1. Introduction

Antisense oligonucleotides have been in the development as drug candidates since the discovery of their sequence specific gene downregulation activity more than 25 years ago. Despite persisting efforts, only one product, administered locally, has reached the market so far. Recently, several promising oligonucleotide drug candidates have failed in their respective phase III clinical trials. As an example, Genasense® (oblimersen) was tested in a large trial against malignant melanoma, but failed to significantly increase overall survival [1]. Still, several clinical trials against a number of cancers are ongoing [2,3], and the results of clinical trials against chronic lymphocytic leukemia (CLL) have been presented [4]. Treatment of patients with relapsed or refractory CLL with oblimersen in combination with fludarabine and cyclophosphamide chemotherapy resulted in a significant increase in major response. However, some doubts have been raised if oblimersen's main mechanism of action is antisense specific at all [5–8].

Meanwhile, the advent of siRNA has shifted much of the attention to double stranded RNA for gene knockdown. Attempts to exploit this nowadays widely used laboratory technique for therapeutical application are still in their infancy [9]. Early clinical trials, using local administration, have started only recently. Despite having a different molecular mode of action than antisense oligonucleotides, many challenges regarding siRNA based drug development remain the same.

The polyanionic character of antisense and siRNA oligonucleotides is a major cause of side effects like binding to serum proteins and insufficient cellular uptake. Nonionic modifications like PNA have shown improved counter strand binding, but have insufficient membrane permeating ability [10,11]. Zwitterionic oligonucleotides, possessing cationic functionalities in addition to the anionic phosphate backbone, have been shown to exhibit promising features [12–14].

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The 2′-position is particularly suited for tethering cationic groups, featuring minimal perturbance of base pairing abilities. However, due to their RNA-like character, 2′-*O*-modified oligonucleotides often lose the ability for RNAse H activation. RNAse H is a nuclease selectively recognizing duplexes consisting of one DNA and one RNA strand and cleaving the RNA strand only [15]. If an oligonucleotide activates RNAse H, it is itself released from the duplex with the targeted mRNA and thus provided with a catalytic effect. The ability to activate RNAse H is deemed as crucial for the development of efficient antisense therapeutics. Generally, only RNAse H activating oligonucleotides show an efficient antisense effect. So called gapmer oligonucleotides, consisting of an internal stretch of unmodified nucleotides, typically between seven and 12, flanked by several modified nucleotides have been developed. These gapmers are adequately protected against nuclease degradation, and the internal stretch of unmodified nucleotides suffices for RNAse H activation [16].

Aminoalkyl groups have been used primarily for the introduction of positively charged functionalities. Studies on the hybridization affinity of 2′-*O*-aminohexyl [17] and 2′-*O*-aminopropyl [18] modified oligonucleotides have been published. Although the overall counter strand affinity as determined by melting point measurements was slightly lower than for wild type DNA oligonucleotides, it was considerably higher when compared to 2′-*O*-alkyl modifications of similar length. It was concluded that the negative steric effect caused by the alkyl chain in the minor groove was partly abolished by the positive impact of the positively charged amino group that reduces the electrostatic repulsion of the polyanionic backbones.

In the cellular environment, polyamines take over the function of phosphate counter ions. Apart from their role in cell growth and maintenance of cell viability, the natural polyamines putrescine, spermine and spermidine are involved in the regulation of gene function [19–23]. In vitro, they stabilize DNA and RNA duplexes [24,25], especially the ones with imperfect base pairing [26]. The distance between amino groups of three and four carbon atoms is practically the same as the distance between phosphate anions in DNA backbone, making polyamines the perfect compounds for creating zwitterionic oligonucleotides. Several possibilities for polyamine conjugation to oligonucleotides have been reported [27–31] Most of these reports describe a polyamine tethered to a nucleobase. Generally, a low destabilization effect was observed in the hybridization properties of these polyamine conjugates against DNA. Likewise, the attachment of propane-1,3-diamine or spermine to a 2′-carbamate via a short alkyl linker resulted in a decrease of around 4 °C in counter strand affinity of a test oligonucleotide [30]. Recently, a polycationic tail consisting of phosphate tethered spermine moieties was conjugated to an oligonucleotide, increasing its counter strand affinity as judged by the melting temperatures [32]. However, no biological data are available for polyamine oligonucleotide conjugates. Likewise, 2′-*O*-polyamine conjugated oligonucleotides have not been examined so far. Therefore, our aim was to use the previously described easy and versatile procedure for attaching polyamines at the 2′-position of oligonucleotides and evaluate the resulting conjugates for their binding affinity and their efficacy in a cell culture assay.

2. Chemistry

To examine the influence of polyamine length, the easily available, natural and ubiquitous polyamines putrescine (**3**), spermidine (**4**) and spermine (**5**) (Fig. 1) were chosen for attachment to the 2′-position of DNA oligonucleotides. In addition, an artificially prolonged polyamine with five amino groups was used. The attachment of a polyamine creates an amide bond, destroying one cationic amino group. The actual net charge is diminished by more than one, because the pK_as of the secondary amino functions may decrease slightly [33]. Spermine attachment thus leads to a functional equivalent of a spermidine oligonucleotide conjugate, bearing three amino functions with three and four carbons in between. For a conjugate with spermine properties, an artificially prolonged polyamine has to be used as a ligand. The synthesis of N^1,N^5,N^{10},N^{14}-tetra-*tert*-butoxycarbonyl-1,5,10,14,18-pentaazaoctadecane (**7**) was accomplished following a procedure from the literature [34] with slight changes (Fig. 2). The boc protection groups were finally removed with trifluoroacetic acid to give **6**.

The syntheses of polyamine conjugates were carried out as previously described for fluorescein and oligolysine conjugates [35,36]. The modified benzyl protected 2′-succinylamido-2′-desoxy uridine building block **1** was directly coupled to the solid phase for the preparation of 3′-terminal modified conjugates or converted to the corresponding 3′-*O*-phosphoramidite **2** for internal and 5′-terminal linker attachment (Fig. 3). The key preparation step consists of selectively deprotecting the carboxyl linker using a phase-transfer hydrogenolysis while the oligonucleotide remains attached to the resin. Subsequently, amines can be coupled to the free carboxylic acid functionality. For putrescine, no amine protection is necessary, since the molecule is symmetric and the two potential binding sites are identical. Regarding spermidine, spermine, and **6**, the secondary amines were left unprotected, because all amines are highly basic and consequently protonated at physiological pH [32]. Oligonucleotides were synthesized in DMT-on mode using standard procedures. The full-length product, still fully protected and bound to the solid phase, was subjected to the polyamine attachment reaction.

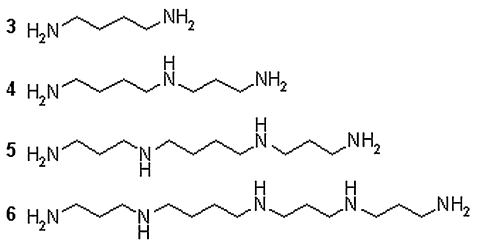

Fig. 1. Polyamine ligands used for attachment to oligonucleotides. **3**: putrescine, **4**: spermidine, **5**: spermine, **6**: synthetic pentaamine.

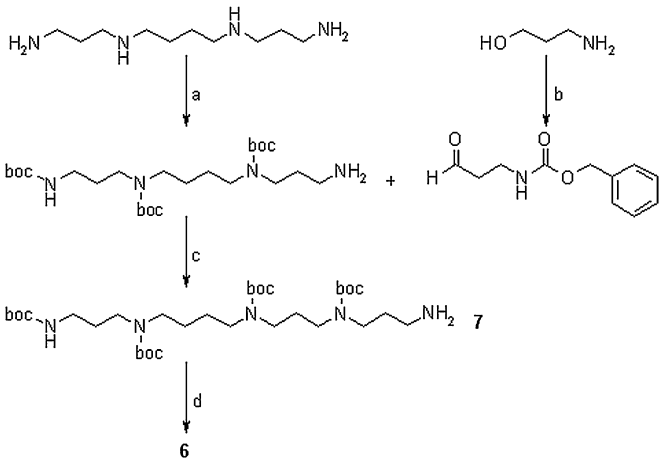

Fig. 2. Synthesis scheme for pentaazaoctadecane **7**. Reagents and conditions: (a) trifluoroacetate, −78 °C; di-*tert*-butyl dicarbonate, r.t.; conc. NH_4OH, r.t.; (b) benzyloxycarbonyl chloride, NaOH, 0 °C; oxalyl chloride, −78 °C; (c) $Na(CN)BH_3$, acetic acid, r.t.; Pd/C, H_2, r.t.; di-*tert*-butyl dicarbonate, r.t.; (d) trifluoroacetic acid, r.t.

After selective cleavage of the benzyl ester in a phase-transfer reaction catalyzed by palladium nanoparticles, the respective polyamines were coupled to the carboxylic linker using DIC/HOBt activation.

3. Pharmacology

For an assessment of the ability to mediate gene expression in vitro, these polyamines were attached to oligonucleotides with the sequence of oblimersen. Its sequence comprises four thymidine nucleotides that can potentially be substituted by our modified uridine building block. Two of those are located internally and two at the respective ends. To evaluate the influence of the polyamine length, putrescine (**3**), spermidine (**4**), spermine (**5**) and pentaazaoctadecane **6** were attached to the 3′-terminal nucleoside. To examine the influence of terminal and internal polyamine ligation, oligonucleotides with an increasing number of spermines attached were prepared. Both phosphodiester (DNA) and phosphorothioate (S-DNA) backbones were used. The structures of the oligonucleotides **8**–**19** can be found in Table 1.

The effect on the target protein bcl-2 was examined in the human melanoma cell line 607B. Antisense oligonucleotides were complexed to lipofectin and applied to the cells at a final concentration of 100 nM. After 48 h, the impact on the target protein was determined using western blotting.

4. Results and discussion

All polyamines were coupled using identical reaction conditions as described for oligolysine attachment. HPLC and MALDI mass spectrometry confirmed successful polyamine attachment. Due to the difference in pK_as and steric hindrance between the primary and secondary amines, attachment of the unprotected polyamines spermidine and spermine can be strongly assumed to take place at a primary amine. The fact that conjugates prepared with a protected (**7**) or unprotected (**6**) pentaamine are identical according to HPLC and CD, further substantiates this presumption.

Circular dichroism spectra were recorded to detect possible changes in secondary structure caused by the polyamine ligands. All single stranded oligonucleotides gave basically the same CD curve with slightly varying band intensities. After hybridization of the phosphodiesters **8**–**12** to their DNA complementary strand only subtle differences in their CD spectra were found (data not shown). Up to three 2′-modified nucleotides, which adopt neither a clear 2′-endo nor 3′-endo configuration [35], do not alter the helical properties of the oligonucleotide.

Duplexes of **8**–**12** with RNA counter strand presented a similar picture (Fig. 4). These DNA/RNA duplexes clearly show an A-type CD curve, featuring an intense positive band at 275 nm with only a negligible negative band at 248 nm. In addition, the negative band at 210 nm is typical for oligonucleotides in their A-form. Compounds **9** and **10**, possessing only terminal modifications, show less intensive bands than the wild type hybrid duplex **8**, indicating a more flexible secondary structure.

Like the phosphodiesters, spermine conjugated phosphorothioates **13**–**15** and **18** were hybridized to the respective DNA (data not shown) and RNA (Fig. 5) complementary strands for recording CD spectra. No differences in curve shapes and virtually no changes in band intensities were found. The same holds true for the duplexes with other polyamines bound to the 3′-end (**16**, **17**, **19**, data not shown). The CD curves of S-DNA/RNA hybrids are very similar to their DNA/RNA counterparts. Both phosphodiester and phosphorothioate

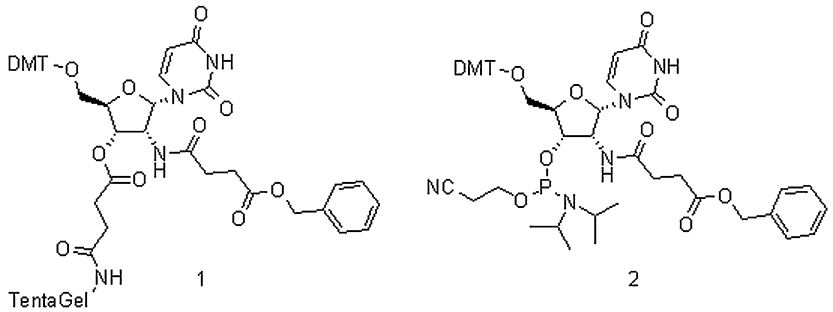

Fig. 3. Nucleoside building blocks used for oligonucleotide synthesis and as subsequent polyamine attachment sites.

Table 1
Sequences and melting temperatures (°C) of oligonucleotides, **3**: putrescine, **4**: spermidine, **5**: spermine, **6**: synthetic pentaamine

	Sequence	Counter strand	
		DNA	RNA
Phosphodiester backbone			
8	TCTCCCAGCGTGCGCCAT	79.0	65.3
9	U_5CTCCCAGCGTGCGCCAT	80.0	63.0
10	U_5CTCCCAGCGTGCGCCAU_5	79.7	61.8
11	U_5CTCCCAGCGU_5GCGCCAT	74.5	57.8
12	$U_5C U_5$CCCAGCGU_5GCGCCAT	70.6	45.8
Phosphorothioate backbone			
13	TCTCCCAGCGTGCGCCAT	71.4	53.1
14	U_5CTCCCAGCGTGCGCCAT	74.3	53.2
15	U_5CTCCCAGCGU_5GCGCCAT	66.8	53.0
16	TCTCCCAGCGTGCGCCAU_3	67.6	50.1
17	TCTCCCAGCGTGCGCCAU_4	65.1	51.4
18	TCTCCCAGCGTGCGCCAU_5	68.5	53.5
19	TCTCCCAGCGTGCGCCAU_6	71.6	53.8

DNA usually adapt to the more rigid RNA secondary structure.

To evaluate the influence on duplex stability, melting experiments with both the RNA and the DNA counter parts were performed (Table 1). All melting curves showed cooperative shape. For the unmodified strands, the sequence in regard to duplex stability was RNA/RNA > DNA/DNA > S-DNA/DNA > DNA/RNA > S-DNA/RNA. Similar results have been demonstrated for other oligonucleotide sequences [24,37]. Despite the stepwise neutralization of the complementary strand by basic polyamines, a general decrease in melting temperature resulted. Phosphodiester oligonucleotides **9** and **10** showed an increase in the melting temperature when complexed to DNA, and only a small decrease in duplexes with RNA. The change of the nucleobase from thymidine to uridine is equal to the loss of a methyl group and was shown to lower the melting temperature by about 0.5 °C [38]. Thus, the effective duplex stabilization caused by the polyamine attachment can be assessed as more than 1.0 °C. In contrast to that, when the modified nucleoside was incorporated at internal positions in the sequence (**13**, **14**), a rather drastic reduction in duplex stability was observed. In particular, the affinity to RNA counter strand was reduced which is reflected by a melting temperature of **12** that is nearly 20 °C lower than that of unmodified **8**. To examine whether this effect was due to the ligand attachment at the minor groove or a result of an unfavourable conformation of building block **2**, we prepared an oligonucleotide with the sequence of **12**, but without any ligand attached to it. The same reduction in melting temperature was observed indicating that it is indeed caused by building block **2**. Judging from these data, the 2′-amido-2′-desoxy-ribonucleotide **2** adopts an adverse conformation that is tolerated in terminal positions, but it highly restricts duplex binding when present at an internal position. NMR data of the modified building block **1** suggest a sugar conformation unlike the north pucker adopted in an A-type helix. The coupling constant of protons at 1′ and 2′ is about 8.0 Hz, compared to 0–2 Hz of a north pucker, in which the dihedral angle of these protons is close to 90°. Consequently, this nucleoside is a well suited way to introduce ligands at either the 3′- or the 5′-end of oligonucleotides, but its sugar conformation lowers the RNA affinity when incorporated at internal sites.

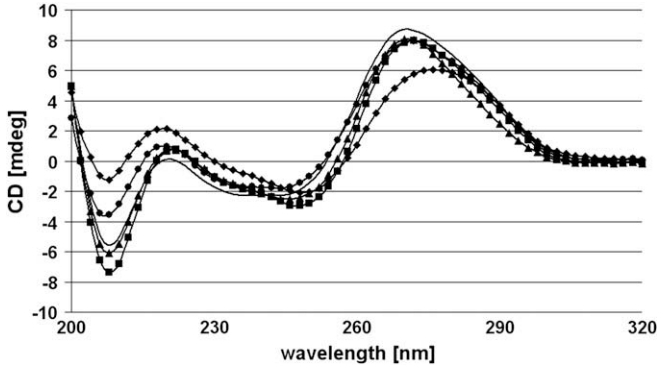

Fig. 4. CD spectra of phosphodiester spermine conjugates **8**–**12** with RNA counter strand; **8** straight line, **9** rhombuses, **10** circles, **11** squares, **12** triangles.

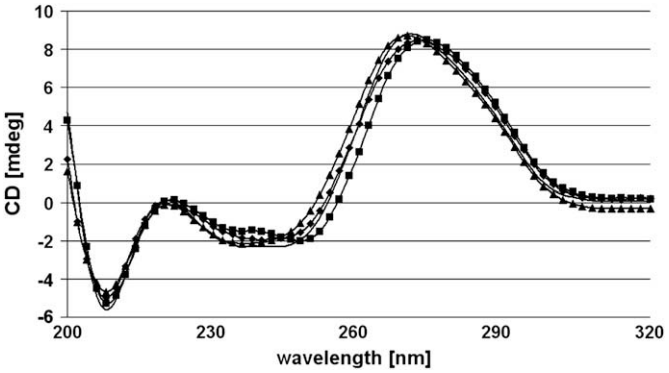

Fig. 5. CD spectra of phosphorothioate spermine conjugates **13**–**15** and **18** with RNA counter strand; **13** straight line, **14** rhombuses, **15** squares, **18** triangles.

Polyamines are known to stabilize oligonucleotide duplexes, as do other cations like Na^+ or Mg^{++}. Spermine and spermidine seem to be especially stabilizing when added to a duplex with imperfect base pairing such as base mismatches or bulged loops [26]. However, polyamines need to be added to the solution in a large excess up to 100-fold in order to see an influence on the duplex transition temperature. The simple addition of an equimolar amount of spermidine to the duplex of oblimersen and its DNA counter part had no influence on either the CD spectrum or the melting temperature. Melting profiles at pH 6.0–8.0 exhibit the same values for each conjugate indicating that duplex stability is largely independent of pH value. Given the pK_a values of spermine amino groups between 8 and 11 [32], all primary and secondary amines of the conjugates are expected to be protonated even at a pH of 8.

For the tested phosphorothioates, the decrease in duplex stability was lower. The affinity of spermine substituted oligonucleotides (**14**, **15**, and **18**) to the RNA counter strand was not significantly different than that of the unmodified phosphorothioate strand. Starting from the lower affinity level of

phosphorothioate oligonucleotides, the modified nucleoside seemingly has a less pronounced effect on the duplex stability. The smaller differences of the individual CD curves of the S-DNA series substantiate this assumption.

For the phosphorothioate conjugates **16**–**19**, melting temperatures of DNA/DNA and DNA/RNA duplexes basically increase with increasing polyamine chain length. An increasing alkyl chain length at the 2′-position progressively destabilizes duplexes [17] due to the increasing steric bulk in the minor groove. Aminoalkyl chains of the same length exhibit much higher duplex stability caused by the reduction of electrostatic repulsion due to the partial neutralization of negatively charged phosphate groups. The lower melting points of putrescine conjugate **16** and spermidine conjugate **17** may be explained by incomplete electrostatic hybridization. The two cationic groups of **17** are supposedly not enough to ensure a stable interaction with two adjacent phosphates of the counter strand. In this case, the destabilizing influence caused by the steric bulk overcomes the stabilizing electrostatic effect. In contrast, spermidine, spermine and pentaamine conjugates **17**–**19** exhibit a significant linear increase in duplex stability of about 3 °C per added amine with a DNA counter strand and about 1 °C with an RNA counter part. All duplexes of conjugates **16**–**19** have higher denaturation temperatures than the unconjugated oligonucleotide with the free succinyl tether [35], indicating that the modified nucleoside and the linkage type are responsible for lower target affinity. Pentaamine conjugate **19**, exhibiting four basic amines like spermine, showed practically the same melting point as the model phosphorothioate oblimersen (**13**). These conjugates are superior to 2′-*O*-aminoalkyl modified oligonucleotides in terms of melting behaviour [17,18].

Phosphorothioates with putrescine, spermidine, spermine and pentaamine (**16**–**19**) attached to the 3′-ends were tested in a cell culture assay [35,39]. Human melanoma cells 607B were transfected with the respective oligonucleotide, and after 48 h bcl-2 protein levels were examined using western blotting. Values reported in Fig. 6 are after actin standardization in relation to mock transfected cells and the result of triplicate

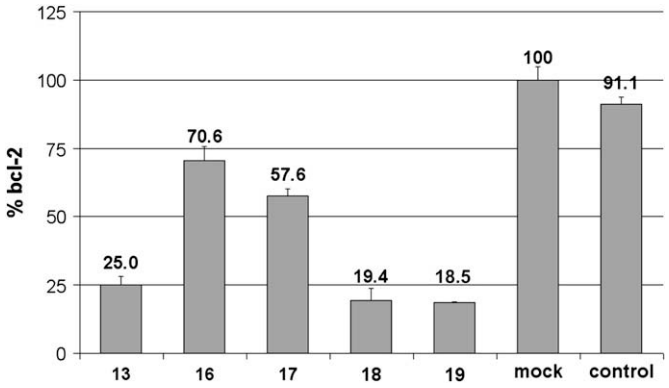

Fig. 6. bcl-2 levels of oligonucleotide conjugates (100 nM) **13** (no ligand), **16** (putrescine), **17** (spermidine), **18** (spermine), and **19** (pentaamine) in human melanoma cells 48 h after transfection in relation to mock transfected cells. A scrambled sequence was used as control.

tests. A scrambled control oligonucleotide resulted in no significant target downregulation. Compounds **16** and **17** both exhibit notable target downregulating ability, but the extent was clearly less than that of the unmodified control oblimersen (**13**). Compounds **18** and **19** on the other hand were highly efficient in downregulating the target bcl-2, even surpassing the effect of oblimersen. In addition to the rather small impact on target affinity, other factors like higher nuclease stability, better cellular uptake, differences in lipoplex formation, or higher release from the endosome seem to be responsible for the different in vitro efficiencies of **16** and **17** compared to **18** and **19**. In the in vitro experiment, polyamine length is highly important for the effect. It seems that at least three cationic amines are necessary for a beneficial effect, as a lower number is apparently not enough for a stable charge interaction. The nucleoside modification brings in a considerable steric bulk and leads to a lower activity. In addition to the slightly higher affinity to their RNA target, the longer polyamine tail of **18** and **19** may have an effect on the membrane permeation rate as well as on the complexation to the cationic transfection reagent. Still, even for pentaamine conjugate **19** less than a quarter of 17 phosphate charges is neutralized. Consequently, lipid-free cellular uptake is negligible, as only fully neutralized or positively charged oligonucleotide molecules are able to enter cells without the aid of a transfection reagent. CD spectra and melting behaviour indicate that the attachment via the modified nucleoside **2** is not feasible for the conjugation of multiple internally positioned polyamines. An RNA based nucleoside with a 2′-*O*-alkyl linker [13] appears to be the better option for internal attachment as well as for conjugating polyamines to RNA oligonucleotides for use as modified siRNA agents.

5. Conclusions

In conclusion, biophysical and in vitro data support the potentially auspicious properties assumed for conjugates of oligonucleotides and polyamines. The modified 2′-desoxy-2′-succinylamido-uridine building block led to substantial decrease in counter strand affinity when incorporated internally in DNA, but barely influenced the melting behaviour in S-DNA. The secondary structure of a duplex with complementary DNA or RNA is not dependent on the length of the attached polyamine, but melting point determinations showed increasing duplex stability with increasing polyamine length at a terminal position. A gradual increase both in duplex stability and in in vitro antisense effect was observed with increasing polyamine chain length when ligands were attached to a terminal nucleotide.

6. Experimental protocols

Reagents were used in standard quality for synthesis. Dichloromethane was dried over phosphorous pentoxide and distilled. NMR spectra were recorded on a Bruker Avance 200 MHz or on a Varian Unity 300 MHz machine. Shifts are reported relative to the solvent peak ($CHCl_3$ in $CDCl_3$:

δ 7.26 and 77.00), coupling constants are in Hz. Spin multiplicities are given with the following abbreviations: s singlet, d doublet, t triplet, q quartet, m multiplet. Thin layer chromatography (TLC) was performed using silica gel 60-F_{254} precoated aluminium plates by Merck. The acidic silica gel was neutralized by adding a 1% solution of triethylamine in dichloromethane. Spots were visualized by UV.

6.1. 2′-(Benzylsuccinyl)amino-3′-O-[(2-cyanoethoxy)-(diisopropylamino)phosphanyl]-2′-desoxy-5′-(4,4′-dimethoxytriphenyl)methyluridine (**2**)

2′-(Benzylsuccinyl)amino-2′-desoxy-5′-(4,4′-dimethoxytriphenyl)methyluridine [35] (650 mg, 0.88 mmol) and 1*H*-tetrazole (62 mg, 0.89 mmol) were dissolved in 10 ml dried dichloromethane and stirred under an argon atmosphere. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite (250 mg, 0.83 mmol) was added to the mixture. The reaction was monitored by TLC (dichloromethane/methanol 95:5, starting material R_f 0.45, product R_f 0.80) on triethylamine treated silica gel. After 2 h, another portion of the phosphordiamidite (80 mg, 0.26 mmol) was added and stirring continued for an hour. At that time TLC indicated nearly complete reaction. The mixture was distributed between dichloromethane and 5% $NaHCO_3$ and the organic phase was evaporated to dryness.

The residue was purified on triethylamine neutralized silica gel (Merck) using a dichloromethane/methanol mixture (98:2). The respective fractions were evaporated to give diastereomeric uridine phosphoramidite **2** (300 mg, 36%).

^{1}H NMR ($CDCl_3$, 200 MHz): $\delta = 7.84$ (d, $J_{56} = 8.2$, 1H, H-6); 7.45–7.21 (m, 14H, Ar-H); 6.80 (d, $J = 8.8$, 4H, Ar-H); 6.32 (d, $J_{2'1'} = 8.6$, 1H, H-1′); 5.38 (d, $J_{65} = 8.1$, 1H, H-5); 5.03 (s, 2H, $PhCH_2$); 4.80–3.30 (m, 11H, H-2′, H-3′, H-4′, OCH_3, H-5′); 2.92–2.44 (m, 4H, CH_2–CH_2).

^{13}C NMR ($CDCl_3$, 50 MHz): $\delta = 173.24/172.17$ (COOR/CONH); 163.39 (C-4); 158.94 (DMT); 151.48 (C-2); 144.51/144.32 (DMT); 139.82 (C-6); 135.52/135.30/135.00 (DMT/benzyl); 130.16–127.00 (DMT/benzyl); 117.82/116.90 (CN); 113.26 (DMT); 104.02 (C-5); 87.98 (CPh_3); 86.15 (C-1′); 84.90 (C-4′); 72.02 (C-3′); 66.88 ($PhCH_2$); 62.90 (C-5′); 58.90 (C-2″); 55.30 (OCH_3); 45.44/45.20 (CH_2OP); 43.34/43.25 ($CH(CH_3)_2$); 30.80/29.75 (CH_2–CH_2); 23.02/22.90 (CH_3); 20.40 (CH_2CN). ^{31}P NMR ($CDCl_3$): 150.98, 150.60. $C_{50}H_{58}N_5O_{11}P$: Calcd: C, 64.16; H, 6.25; N, 7.48. Found: C, 64.44; H, 6.10; N, 7.21.

6.2. Oligonucleotide synthesis

Acetonitrile was heated over potassium carbonate and distilled, followed by heating over calcium hydride and distillation. Tetrahydrofurane was heated over sodium and benzophenone until the indicator turned blue, and then distilled. Standard nucleotide phosphoramidites and tetrazole solution were obtained from Carl Roth GmbH & Co. KG. Other reagents used for DNA synthesis were obtained in analytical quality, purified and dissolved in dried acetonitrile or THF, respectively. Oligonucleotide syntheses were carried out on an ABI 392 DNA/RNA-Synthesizer in standard 1 μmol scale and DMT-on mode. When using the solid phase loaded with the modified building block **1**, the coupling of the first nucleotide was prolonged to 2 min. The coupling time of the modified phosphoramidite **2** was set to 15 min. Sulfurization was done with tetraethylthiuram disulfide, which was purchased from Sigma and recrystallized from ethanol before use. For the synthesis of phosphorothioates, the sulfurization step was performed before capping. 1*H*-Tetrazole was used as activation reagent.

6.3. Preparation of oligonucleotide–polyamine conjugates

The synthesis column was removed from the synthesizer and opened and the resin-bound oligonucleotide was transferred into a 2-ml vial. Cleavage of the benzyl protecting group was achieved by phase-transfer hydrogenation with PVP-stabilized palladium nanoparticles (0.7 ml) [35] and 1,4-cyclohexadiene (0.05 ml) as hydrogen donor. Cyclohexadiene was added to the PVP-stabilized suspension of palladium and the resulting solution was microfiltered (0.22 μm) to ensure that no precipitated palladium was applied to the resin. The reaction mixture was left standing at room temperature for 3 h.

The resin was washed three times with methanol and three times with DMF. Prior to coupling, the carboxyl group was activated by a solution of 1-hydroxy-1*H*-benzotriazole (0.4 mg, 3.0 μmol) and *N*,*N*′-diisopropylcarbodiimide (1.1 mg, 8.7 μmol) in 1 ml DMF. The mixture was left for 3 h and the solution was again filtered off. Coupling was done by adding a solution of 1-hydroxy-1*H*-benzotriazole (0.4 mg, 3.0 μmol), *N*,*N*′-diisopropylcarbodiimide (0.4 mg, 8.7 μmol) and polyamine (6.0 μmol) in 1 ml DMF and standing for 18 h. The resin was washed three times with DMF and three times with water. For cleavage and deprotection of oligonucleotides 2 ml concentrated ammonia was added and the mixture was heated to 55 °C for 18 h. The solution was filtered and the solvent was evaporated in vacuo.

6.4. Purification and analyses of oligonucleotides

Oligonucleotide concentrations were determined by measuring OD_{260} in a Hitachi U3000 spectrophotometer. Molar extinction coefficients were calculated as the sum of nucleotides (A: 15 400, G: 11 700, C: 7300, T: 8800, U: 9950).

Initial purification of DMT-on oligonucleotides was done with Poly-Pak® columns obtained from Carl Roth GmbH & Co. KG according to manufacturer's instruction except for elution, which was done with 20% acetonitrile to ensure complete elution. Analytical HPLC was performed on a Nucleosil® CC 250/4 100-5 C18 column with the following gradient system: A: 0.1 M triethylammonium acetate in water, B: 0.1 M triethylammonium acetate in 80% acetonitrile, linear gradient 10–40% B in 0–30 min, flow rate 1 ml/min. If the purity after Poly-Pak®-purification was deemed insufficient, preparative

HPLC was performed on a LiChrosphere® 100 RP-18 using the same gradient as for analytical HPLC.

Mass spectrometric analysis was done on a Kratos seq MALDI mass spectrometer. Oligonucleotide solution (0.5 μl, 0.1 mM) was spotted on a target, followed by 0.5 μl of a mixture of 3-hydroxypicolinic acid (5%) and diammonium hydrogen citrate (1%). After careful mixing, the solution was air-dried. The mass spectrometer was run in the negative ion and reflectron mode and spectra were obtained by summing up 100 single laser pulses. Compounds **9**: calcd 5680.9, found 5681.3; **10**: calcd 5950.3, found 5951.4; **11**: calcd 5950.3, found 5951.3; **12**: calcd 6219.7, found 6219.4; **14**: calcd 5954.1, found 5953.4; **15**: calcd 6223.5, found 6224.6; **16**: calcd 5839.9, found 5839.7; **17**: calcd 5897.0, found 5897.4; **18**: calcd 5954.1, found 5954.9; and **19**: calcd 6011.1, found 6011.2.

Circular dichroism spectrometry was performed on a Jasco J-810 spectropolarimeter equipped with a Neslab RTE 7 thermostatic unit. Oligonucleotides were diluted to a 9 μM solution in 0.15 M NaCl and 0.01 M Tris–HCl (pH 7.0) buffer. CD spectra were collected from 320 to 210 nm using a quartz cuvette (Hellma 100-QS) with a path length of 1 mm. Duplexes were measured after heating the equimolar mixture of complementary strands to 50 °C for 10 min and subsequent slow cooling to room temperature.

Melting temperatures (T_m) of the duplexes were determined in the Jasco J-810 by slowly heating a 9 μM equimolar mixture of the complementary strands in 150 mM NaCl/10 mM Tris–HCl (pH 7.0) from 30 to 90 °C (50 °C/h) and recording CD at 248 nm or 260 nm and OD at 260 nm as a function of temperature. Repeated cooling and heating was performed to ensure the presence of stable hybridization. Melting curves showed cooperative form and T_m was obtained from the maxima of the first derivative plots. Temperatures given are that of the cuvette holder. All experiments were performed at least in triplicate; the standard deviation was lower than 1.0 °C in all cases.

6.5. Cell culture experiments

Oligonucleotides were transfected in a concentration of 100 nM for 4 h using lipofectin (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, transfection was repeated. Cells were lysed 48 h after the first transfection period. After work-up and protein quantitation by the Bradford assay, cell lysates were electrophoresed and blotted on a PVDF membrane. Western blot detection was performed using a monoclonal mouse bcl-2 antibody targeted at amino acids 41–54 (Santa Cruz, CA, USA, sc-509). Actin immunostaining using a polyclonal rabbit antibody (Sigma, St. Louis, USA, A2066) was used for loading control. After incubation with respective HRP-conjugated secondary antibodies (Santa Cruz, CA, USA, sc-2005, sc-2004), bands were visualized with ECL plus reagent (GE Healthcare, Chalfont St. Giles, Great Britain). X-ray films were exposed to the membranes and subsequently scanned with a GS-710 calibrated imaging densitometer (Bio-Rad Laboratories, Hercules, CA). For the densitometric analysis, bcl-2 bands of mock transfected cell lysates were set to 100%, and all bcl-2 bands were set in relation to the respective actin band to adjust for different loadings.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.05.012.

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