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Antisense activity of an anti-HIV oligonucleotide conjugated to linear and branched high molecular weight polyethylene glycols☆

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Abstract

An anti-HIV 12mer oligonucleotide (ODN) conjugated to two different high molecular weight monomethoxy polyethylene glycols (MPEGs) has been tested for its antisense activity. The capacity of these conjugates to protect the MT-4 cells against HIV infection has been compared to the unmodified, native ODN, and the effect of the different structures of the supporting polymer has been discussed. It was found that only the ODN conjugated to the linear MPEG shows an anti-HIV activity in the investigated conditions. The same 12mer, when conjugated to a branched $(MPEG)_2$, is fully inactive, as well as the native, unmodified ODN.

Keywords: Oligonucleotide; Antisense; HIV; Polyethylene glycol

1. Introduction

Since the pioneering work of Zamecnik and Stephenson [1], the discovery that synthetic oligonucleotides (ODNs) can inhibit gene expression had opened a new field of research aimed at the preparation of extremely powerful drugs [2]. Exogenous genetic material, as that derived from viral infections, is one of the most important targets of this antisense strategy, due to the difficulty in producing drugs able to specifically operate without damaging the activity of the hosting cell. In this regard, current therapies against HIV have been addressed toward different parts of the virus life cycle by using ODNs that interact with the genomic RNA reverse transcription and the viral mRNA transcription and translation [3].

Unfortunately, natural ODNs are subjected to the enzymatic degradation that hampers their in vivo application; moreover, difficulties in targeting and penetration into the specific cells had somewhat diminished the initial enthusiasm for antisense therapy [4]. Modification of ODNs by attachment of macromolecules to their ends or to the backbone had produced a series of conjugated ODNs that were more stable to nucleases and presented an increased binding affinity [5]. Among few polymeric units, high molecular mass polyethylene glycol (PEG) received much attention due to the absence of toxicity, the profitable amphiphilicity, and the demonstrated capability, at the protein level, to increase the stability and the body lifetime of its conjugates [6].

It has been demonstrated that a 20mer complementary to the sequence of the (+) DNA chain from HIV, and having 5′-terminal modification, exhibits a high anti-HIV activity [7]. Recently, the same sequence has been operated as the tandem of shorter sequences made by 3′- and 5′-terminal 8mers, and the central 4mer. The two octamers were modified at their extremities with a phenazinium (Phn) derivative, to take advantage of its intercalating aptitude that increases the duplex stability, once formed [8]. It has been demonstrated that the stability of the double helical complexes between the nucleic acid target and the tandem combination of the

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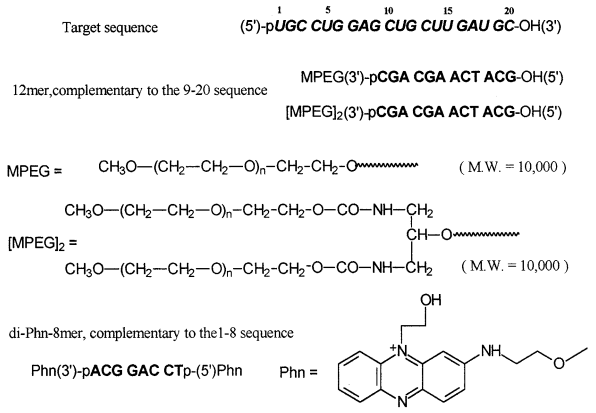

Fig. 1. Oligonucleotide sequences and structure of their conjugated molecules.

shorter sequences bearing the Phn intercalating unit was comparable with that given by the single, longer native sequence [9].

On this basis, we decided to test the antisense activity of a sample 12mer, part of the anti-HIV 20mer sequence previously recalled, conjugated to high molecular weight MPEGs. The study was addressed to a 12mer sequence, instead of a longer one, for synthetic convenience. The anti-HIV activity of this ODN, modified at its (3′) extremity with a cholesteryl or Phn unit, has been positively ascertained [10]. As a conjugating molecule a 10 000 Da monomethoxy polyethylene glycol has been utilized, both as a linear (MPEG) or branched $(MPEG)_2$ derivative, to investigate the different role of the supporting polymer structure on the biological activity of the ODN. The thermal denaturation and the enzymatic stability of these MPEG-12mers have been previously compared: they demonstrated the same duplex stability as the native unmodified sequence, whilst showing an almost identical, effective stabilization against the enzymatic attack [11]. The target sequence and the structure of the investigated compounds are reported in Fig. 1.

2. Results

The antisense activity of the conjugated ODNs was evaluated for the protection against HIV-1 infection on MT-4 cells. The synthesized 12mers have been used in the tandem combination with the 5′,3′-di-*N*-(2-hydroxyethyl)-phenazinium (Phn) 8mer representing the other part of the active 20mer sequence. Since this tandem system gave promising results in the first biological studies, it has been decided to apply this approach for the investigation of the MPEG-conjugated antisense activity with unusually short sequences, as those here reported, to simplify the synthetic effort. As control, the following derivatives have been used: native 12mer, MPEG, $(MPEG)_2$, tandem combinations with 3′,5′-diPhn-8mer non-complementary to the target DNA sequence, (3′) azido-thymidine (AZT).

In Table 1 the inhibition of HIV reproduction in MT-4 cells by the oligonucleotide derivatives, as indicated from the p24 antigene, is reported and compared with the effect of the MPEG polymers alone, as well as of a well-known effective drug (AZT).

In Table 2 the percentage of inhibition is reported, measured at the same concentration of the investigated compounds (10 μM).

Interestingly, all the analyzed compounds were non-toxic on uninfected MT-4 cells at 50 μM concentration, determined by the cell viability, as indicated in Table 3.

As observed, MPEG and $(MPEG)_2$ alone, as well as the unmodified 12mer (data not shown), were completely inactive in the reported conditions. Moreover, both the MPEG-supported 12mers, as well as the 3′,5′-diPhn 8mer, when separately observed, did not show any measurable activity. The tandem constituted by the MPEG-12mer and a 3′,5′-diPHN non-complementary 8mer, gave the same negative results. Thus, the study of the antisense activity of the conjugated ODNs proves that only the tandem made by the linear MPEG-conjugated 12mer and the complementary 3′,5′-diPhn 8mer inhibits the HIV reproduction, with an $ID_{50} \approx 10$ μM.

Table 1
Entity of HIV infection, as judged by the amount of p24 antigene released (ng/ml)

Sample	Concentration (μM)			
	2.5	5	10	15
MPEG	3950 ± 160	4395 ± 1000	4240 ± 220	3670 ± 930
$(MPEG)_2$	3820 ± 450	4050 ± 330	4105 ± 100	3870 ± 475
3′,5′-DiPhn-8mer	3860 ± 145	4340 ± 60	4065 ± 120	3815 ± 385
MPEG-12mer	3915 ± 140	4290 ± 240	3800 ± 520	3880 ± 270
$(MPEG)_2$-12mer	3605 ± 180	3620 ± 80	3370 ± 270	3980 ± 450
MPEG-12mer + diPhn-8mer	**4070 ± 250**	**3770 ± 45**	**1950 ± 300**	**855 ± 70**
MPEG-12mer + diPhn-8mer[a]	3830 ± 105	3775 ± 310	3550 ± 550	3980 ± 150
$(MPEG)_2$-12mer + diPhn-8mer	3450 ± 600	3790 ± 375	3880 ± 220	4050 ± 180
AZT		240 ± 0.5		

[a] Not complementary sequence = Phn–d(TGCCTGCA)–Phn; control: MT-4, HIV infected cells = 4370 ± 675 ng/ml.

Table 2
Percentage of inhibition of the investigated derivatives (as from the data in Table 1)

MPEG	$(MPEG)_2$	3′,5′-DiPhn-8mer	MPEG-12mer	$(MPEG)_2$-12mer	**MPEG-12mer + diPhn-8mer**	$(MPEG)_2$-12mer + diPhn-8mer[a]	$(MPEG)_2$-12mer +diPhn-8mer	AZT
5.77 ± 7.71	11.0 ± 2.25	-3.38 ± 2.93	5.64 ± 13.99	8.18 ± 9.75	**78.36 ± 0.51**	-2.60 ± 18.34	7.52 ± 5.27	96.36 ± 3.15

The same ODN conjugated to the branched $(MPEG)_2$ is, on the contrary, inactive.

3. Discussion

In a previous study [11] the thermal stability of the duplexes formed by the 12mers conjugated with the linear MPEG, as well as the branched $(MPEG)_2$, and the complementary, single strain nucleic acid sequence have been investigated and compared with that built with the unmodified ODN. Moreover, the effect of the stabilization given by the MPEGs against the enzymatic degradation was also tested. From these studies it has been ascertained that the high-molecular mass conjugated polymers do not hamper the interaction of the ODNs with the biological target, since the melting behaviors of all the formed duplex are strictly similar. On the other hand, they are responsible for an effective protection of the supported ODNs from biodegradation, as judged for their reduced susceptibility to the attack of nucleasic enzymes. These effects were almost identical for the two MPEGs employed.

From the presently reported data the following conclusions can be drawn:

1. the stabilizing effect of the MPEG on the ODN, as ascertained previously, seems to improve the activity of the molecule: the native sequence is inactive in the same conditions;
2. the structure of the supporting MPEG appears to play a role on the activity of the conjugated ODN: the branched one is inactive in the conditions where the linear polymer exhibits a measurable anti-HIV effect.

Thus, it was possible to observe a positive effect of the linear, high-molecular mass MPEG molecule when conjugated to an antisense ODN. The stabilization against enzymatic degradation, without hampering the normal interaction with the biological target, improved the activity of the tested sequence; in fact, the native, non-conjugated 12mer, was fully inactive when subjected to the same experimental conditions. It is interesting to note that the same free 12mer has been reported to give an $ID_{50} \gg 5$ μM if bearing a fully modified thioate backbone [10], that is well known to induce a higher in vivo stability [12]. The absence of activity of the $(MPEG)_2$-conjugated ODN is not easily rationalized; eventually, the likely wider space occupied by this polymer along the final sequence could obstruct the correct interaction of the following 8mer in the tandem position, since this synthetic process affords 3′-conjugated ODNs. A further investigation will be addressed to the introduction of the same MPEG molecules in the 5′ position alone, or at both extremities, to fully delineate the effects of this macromolecular conjugation on the antisense properties of an ODN sequence.

4. Experimental

4.1. Oligonucleotides

MPEG-conjugated 12mer has been synthesized on a 0.1 mmol scale following the new liquid-phase procedure recently proposed (HELP) [6]. The detailed description of the synthesis and purification of these high-molecular mass conjugated ODNs has also been published on the Web, on a new online Journal [13].

Linear MPEG was a commercial product (Shearwater), while the new branched $(MPEG)_2$ was obtained, as previously reported, by reaction of activated MPEG molecules with 1,3-diamino-2-propanol [14]. The final polymers were of the same average molecular weight ($M_w = 10\,000$ Da).

Native 12mer was acquired from Genset (France) as HPLC purified product and used as such. 20mer RNA for Rnase H assay was kindly provided by Drs A.G. Venyaminova, and M.N. Repkova, (Novosibirsk Institute of Bioorganic Chemistry, Russia). All other ODNs were synthesized in Zarytova's laboratory following the standard procedures, and modified at their extremities as reported [8]. The concentrations of the ODNs in the experiments were determined spectrophotometrically,

Table 3
Toxicity of the tested compounds as determined from the cell viability

Tested compounds	Cell viability (%)
MPEG	94.41 ± 2.33
$(MPEG)_2$	94.65 ± 1.02
MPEG-12mer	97.69 ± 2.33
$(MPEG)_2$-12mer	96.45 ± 2.10
MPEG-12mer+3′,5′-diPhn-8mer	**95.50 ± 0.74**
None	98.11 ± 3.27

by using a molar extinction coefficient at 260 nm of 117 300 $M^{-1} \times 1$ cm for each 12mer and 185 600 for the target RNA.

4.2. Anti-HIV activity

The effect of the ODN derivatives on the inhibition of the HIV reproduction was analyzed in the widespread model of human lymphocytes (MT-4 cell line).

The MT-4 cells were infected by mixing the cell suspension (2×10^6 cell/ml) and the virus solution obtained from the supernatant of MT-4 cells infected by HIV-1/EVK strain (Institute of Virology, Moscow, Russia) and stored in liquid nitrogen until used. The multiplicity of infection (MOI) was 0.2–0.5. The mixture was incubated for 1 h at 37°C for the virus absorption. The infected cells were then diluted to 5×10^5 cell/ml by fresh complete medium made with RPMI-1640 medium supplemented with 10% heat-inactivated micoplasma free fetal bovine serum, 300 mg/ml L-glutamine, 100 mg/ml gentamicine. The cells were cultured in the presence of ODNs, from 0.25 to 50 μM, in 96-well microplate (Costar), in triplicate for each concentration at 37°C in a 5% CO_2 atmosphere. The control HIV-1 infected cells, without the ODNs, and the control uninfected cells were cultured under the same conditions. After 4 days cell viability, total virus antigen and p24 antigen were measured. Kits for p24 antigene detection were kindly provided by Dr N.V. Fedyuk (SRCVB Vector, Russia)

The inhibitory effect of the tested compounds was measured by determining the number of viral proteins by the ELISA assay. Tween 80 was added to the virus sample up to 0.1% final concentration, and a further HIV inactivation was performed at 45°C for no less than 24 h. Two-fold dilution of the infected material was prepared before being added to each well of the microplate containing immobilized human anti-HIV-1 IgG or monoclonal antibodies (MABs) to HIV-1 p24. After incubation at 37°C for 1 h, the microplate was washed four times with PBS (pH 7.4) containing 0.1% Tween 80, followed by the addition of IgG, or MABs to HIV-1 p24, conjugated to horseradish peroxidase. After 1.5 h at 37°C the wells were washed and a solution of *o*-phenylenediamine was added. After 20 min the reaction was stopped by the addition of 50 μl of 2 M sulfuric acid. The absorbance of each well was determined using a microplate reader at 492 nm. The positive control with known p24 antigen content was involved in each plate to standardize the results; the concentration of p24 antigen was calculated by interpolation from a standard curve considering the dilution of the test sample.

The viable cells were detected by the tripan blue exclusion staining method.

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