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Selective inhibition of the cytopathic effect of type A influenza viruses by oligodeoxynucleotides covalently linked to an intercalating agent

Aurelio Zerial, Nguyen T.Thuong[1] and Claude Hélène[2]

Rhône-Poulenc Santé, Centre de Recherches de Vitry, 13 Quai Jules Guesde, 94400 Vitry sur Seine, [1]Centre de Biophysique Moléculaire, CNRS, 45071 Orléans Cedex 02 and [2]Laboratoire de Biophysique, INSERM U.201-CNRS UA.481, Muséum National d'Histoire Naturelle, 61 Rue Buffon, 75005 Paris, France

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ABSTRACT

Oligodeoxynucleotides covalently linked to an acridine derivative were targeted to part of the 3'-terminal sequence which is common to the eight RNAs of type A influenza viruses. The cytopathic effect of the virus on MDCK cells in culture was strongly decreased by a heptanucleotide covalently attached to the acridine ring. Control experiments using other oligonucleotide sequences showed that the effect was specific for the complementary sequence of the 3'-terminal region of the viral RNAs. The RNA transcriptase reaction of a type A virus was also selectively inhibited **in vitro** by the heptanucleotide-acridine conjugate. A type B influenza virus was used as a control. The common sequence at the 3' end of its eight viral RNAs is different from that of type A viruses. Three mismatches were expected with the heptanucleotide which was fully complementary to type A viral RNAs. This heptanucleotide had no effect on the cytopathic effect of a type B influenza virus. These results demonstrate that viral RNAs are specific targets for the oligonucleotide-acridine conjugate that inhibits the cytopathic effect of type A influenza viruses.

INTRODUCTION

Selective antiviral agents usually exploit differences existing between virus and host cell enzymes (1). Another strategy to achieve selectivity in antiviral chemotherapy would be to use oligonucleotides specifically designed to interact with only viral nucleic acid sequences. The possibility of using oligodeoxynucleotides to block gene expression has previously received experimental support (2-7). Oligonucleotides targeted to messenger RNAs can inhibit translation (3-5). When targeted to viral RNAs they may block viral development (2,3,6,7). Influenza viruses have a segmented genome consisting of eight single-stranded negative-sense RNA molecules which code for the different viral proteins (8,9). These eight RNAs have common sequences at their 3' and 5' ends. The 3'-terminal sequences of the eight viral RNAs of type A viruses are identical over twelve nucleotides except for a C/U degeneracy at the fourth position (8,10). This 3'-terminal sequence constitute a potential target for oligonucleotides which could be used as specific antiviral agents. This approach has been followed by Stridth **et al.** (11) in an

attempt to selectively inhibit the influenza virus RNA transcriptase. However negative results were reported.

We have recently described the synthesis of a new family of molecules in which an oligodeoxynucleotide is covalently linked via its 3'-phosphate to an intercalating agent, namely, 2-methoxy, 6-chloro, 9-amino-acridine [12-14]. The oligonucleotide retains its binding specificity towards the complementary sequence. The intercalating agent provides an additional binding energy due to its insertion between the base pairs formed by the oligonucleotide with its complementary sequence. These composite molecules can be used to inhibit messenger RNA translation in cell-free extracts or in microinjected **Xenopus** oocytes [15,16]. The acridine derivative facilitates uptake by living cells and protects the oligonucleotide against 3'-exonucleases (unpublished results). Because of these promising characteristics we attempted to use such oligonucleotide-acridine conjugates to inhibit the multiplication of influenza viruses in cell cultures. Here we show that an oligonucleotide covalently linked to an acridine derivative and targeted to part of the 3'-terminal common sequence of type A viruses inhibits the cytopathic effect of these viruses on MDCK cells in culture. The 3'-terminal sequence of type B influenza viruses differ from that of type A viruses at four positions. Due to the presence of mismatches the oligonucleotide that inhibits the cytopathic effect of type A viruses has no effect on a type B virus.

MATERIALS AND METHODS

Antiviral studies

The antiviral effect of oligodeoxynucleotide-acridine conjugates was based on the inhibition of the cytopathic effect (CPE) of different strains of influenza virus on MDCK cells in culture. MDCK cells were grown in minimal Eagle's medium-Earle's salts (MEM) supplemented with 5 % fetal calf serum, penicillin and streptomycin sulfate at 40 I.U. and 40 µg/ml, respectively. To confluent cell monolayers in 24 well-Falcon plates were added 0.85 ml of MEM medium (without serum but supplemented with 5 µg/ml trypsin and antibiotics as above) and 50 µl of investigated compound (concentrated 20 X). Cultures were then infected with 0.1 ml of virus (containing 20 x TCID 50) and incubated for three days at 37°C in a 5 % CO_2 atmosphere. Cultures were then examined under the microscope to assess the degree of the cytopathic effect [17]. In some instances inhibition of the cytopathic effect was visualized by fixing and staining the remaining monolayer with formaldehyde (15 %) and crystal violet (0,1 %).

RNA transcriptase assays

Influenza virus A/Philippines/2/82 , grown in eggs, was kindly provided by Institut Merieux. The virus was purified on sucrose gradient as described [18], except that the RNAse step was omitted. Aliquots (180000 u HA/ml) were stored frozen at - 70°C. RNA transcriptase activity was measured at 34°C according to Plotch and Krug [19], using ^{3}H-UTP (400 cpm/pmole) as labelled precursor, 1 mM ApG as primer and 50 µg/ml virion proteins. Compounds dissolved in H_2O (or H_2O alone as a control) were added at the beginning of the reaction. 40 µl of the reaction mixtures were precipitated at different times with 10 % trichloroacetic acid (TCA) on Whatman 3MM filters and counted. No significant activity was observed in the absence of the ApG primer.

Synthesis of the oligonucleotide-acridine conjugates

Oligodeoxynucleotides were synthesized by the phosphotriester method. The 9-amino group of the intercalating agent 2-methoxy, 6-chloro, 9-aminoacridine was covalently attached to the 3'-phosphate of the oligodeoxynucleotide via a pentamethylene linker as previously described [20]. Oligonucleotide-acridine conjugates were purified by HPLC on ion exchange HR 5/5 Pharmacia columns. Their purity was checked by electrophoresis after 5'-end labelling with ATP-γ-^{32}P and polynucleotide kinase (Amersham).

RESULTS

Inhibition of the cytopathic effect of type A influenza viruses

Three oligonucleotides used in this study were complementary to part of the 12 nucleotide-long 3'-terminal sequence of influenza viruses of type A (Figure 1). Since influenza virus is a negative-strand virus, oligonucleotides complementary to messenger RNAs were also synthesised. They were directed against the 5'-end of mRNAs in the region which is complementary to the common 12-nucleotide sequence at the 3'-end of viral RNAs. Experiments were carried out using MDCK cells in culture. Three strains of type A influenza viruses were used to infect cells : A/Puerto Rico (A/PR/8/34), A/Victoria/3/75, A/Philippines/2/82. Oligonucleotides covalently linked to the acridine derivative were added at the time of infection. Their effect on virus multiplication was assayed after different times of incubation by scoring under the microscope the extent of the cytopathic effect (CPE).

As shown in Table 1, two undecanucleotide-acridine conjugates (compounds [1] and [2]), complementary to nucleotides 1-11 of viral RNAs were found inactive against A/Victoria/3/75 virus multiplication. These two compounds differed by one base to take into account the C/U degeneracy at the

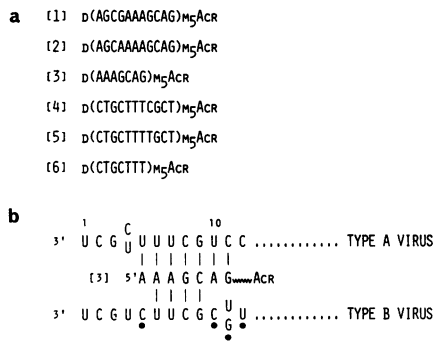

Figure 1 : a) Sequence (5' → 3') of oligodeoxynucleotides covalently linked to 2-methoxy, 6-chloro, 9-amino-acridine (Acr) through a pentamethylene chain (m_5). Compounds [1] [2] and [3] are complementary to viral RNAs. Compounds [4], [5] and [6] are complementary to the messenger RNAs.

b) Common nucleotide sequence at the 3' end of the eight viral RNAs in influenza viruses of type A and type B. The dots indicate the positions at which the common sequence differs in the two virus types. Oligonucleotide [3] shown in between the two viral sequences forms seven base pairs with the viral RNAs of type A but only four with viral RNAs of type B.

fourth position of the viral RNAs. A 1:1 mixture of them (final concentration up to 100 µM) was also inactive.

In contrast, the heptanucleotide-acridine conjugate [3] complementary to region 5-11 of the viral RNAs strongly reduced the cytopathic effect of all three strains of type A influenza virus (Table 1). Complete inhibition of virus multiplication was observed at concentrations higher than 50 µM. The same oligonucleotide without the covalently attached acridine had no effect (not shown). The results presented in Table 1 show that the acridine derivative including the linker ($Acrm_5OH$) had no antiviral activity. In contrast this compound exhibited a strong cytotoxic effect on MDCK cells when it was not covalently attached to an oligonucleotide. The concentration giving 50 % cell killing was approximately 2 µM. No cytotoxicity was observed with the oligonucleotide-acridine conjugates up to a concentration of 100 µM. These results demonstrate that free acridine was not released as this would have been expected if the oligonucleotide was completely hydrolysed by nucleases (13). Since the acridine derivative alone had no effect on the cytopathic effect of the virus at sub-toxic concentrations, it appears that the mere release of acridine from a small fraction of the oligonucleotide-acridine conjugate was not the source of the anti-cytopathic effect. This conclusion is also supported by the observation that no antiviral activity was detected with the oligonucleotide-acridine derivatives [4], [5], and [6] directed

TABLE 1

Effect of oligonucleotide-acridine conjugates and $Acrm_5OH$ on the inhibition of influenza virus multiplication in cell cultures.

Compound	Concentration (μM)	% inhibition of CPE			
		A/Victoria/3/75	A/Philippines/ 2/82	A/PR/8/34	B/Hong Kong/8/73
[1] d(AGCGAAAGCAG)m_5Acr or [2] d(AGCAAAAGCAG)m_5Acr or [1] + [2] added simultaneously	50	0	-	-	-
	100	0	-	-	-
[3] d(AAAGCAG)m_5Acr	50	50 ± 20 (a)	50	88 ± 12 (a)	0
	100	82 ± 18 (a)	80	85 ± 15 (a)	0
$Acrm_5OH$	0.7	0	-	0	-
	1.4	0 (b)	-	0 (b)	-
	3	0 (c)	-	0 (c)	-

(a) average inhibition ± SD on 3 different experiments each in duplicate ; (b) partial cytotoxicity detected on uninfected cells
(c) complete cytotoxicity

against the messenger RNA sequences. Because of the negative results obtained with A/Victoria and, in the case of compound [6], with both A/Victoria and A/PR/8/34 viruses, further studies against other strains and types were not performed with compounds [4], [5] and [6].

Selectivity of CPE inhibition

The inhibition observed with compound [3] (d(AAAGCAG)m_5Acr) could be due to an interaction of this molecule with a cellular or viral component essential for viral development rather than with viral RNAs. Changing the sequence of the oligonucleotide might alter this interaction and lead to a different behaviour. Therefore comparing two oligonucleotides with different sequences does not provide a good control in such systems. The best control would be to use a mutant with a point mutation in the target sequence of the oligonucleotide. No such mutant is available in the case of type A influenza viruses. However type B viruses, which have a similar infectious cycle, differ from type A viruses in the 3'-terminal sequence of their eight viral RNAs (figure 1) [10]. The heptanucleotide-acridine conjugate which is active against type A viruses has three mismatches with the type B common sequence (out of seven base pairs) (figure 1). This should strongly destabilize any complex involving this sequence. The heptanucleotide-acridine conjugate was therefore tested for its ability to inhibit the cytopathic effect of a type B virus (B/Hong Kong/8/73). As shown in Table 1, compound [3] was totally inactive against this type B influenza virus. Figure 2 compares the results obtained with compound [3] in the case of A/PR/8/34 (sensitive) and B/Hong Kong/8/73 (insensitive) influenza viruses. It can be seem that the heptanu-

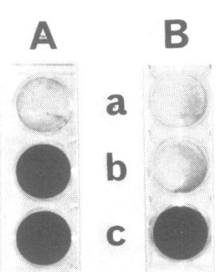

Figure 2 : Effect of oligodeoxynucleotide [3] on the cytopathic effect (CPE) of A/PR/8/34 and B/HK/8/73. Infected or mock-infected MDCK cells were cultured for 72 hours in the absence or in the presence of 50 µM of compound [3] and then stained with crystal violet. A : influenza virus A/PR/8/34 ; B : influenza virus B/HK/8/73. a : infected cells ; b: infected cells treated with 50 µM of oligonucleotide-acridine conjugate [3] ; c : mock-infected cells.

cleotide-acridine conjugate protects MDCK cells against type A virus infection whereas it has no effect on type B virus. These experiments led us to the conclusion that the oligonucleotide-acridine conjugate d(AAAGAAG)m_5Acr exerted its anti-cytopathic effect by hybridizing to the viral RNAs.

In vitro transcription of viral RNAs

Influenza viral RNAs are involved in two processes [8,9]. They are copied by the RNA transcriptase either into full length complementary plus-strands or into mRNAs utilizing the capped 5'-terminal region of an endogenous mRNA as primer. Influenza virions can be disrupted **in vitro** and release viral RNAs with the different subunits of RNA-dependent RNA polymerase bound to the 3'-end of viral RNAs [21]. When supplemented with a primer (such as ApG), the four ribonucleoside-triphosphates and Mg^{++}, virion lysates provide an **in vitro** system to assess the RNA transcriptase reaction [21].

As shown in Figure 3, compound [3] inhibited the enzymatic reaction by 70 % at 30 µM. The inhibition, however, did not appreciably increase at higher concentrations (100 µM). Acrm_5OH had no effect at 3 µM, a concentration which proved to be highly toxic to cells in culture (Table 1). At 30 µM Acrm_5OH inhibited the RNA transcriptase activity by 60 %. However the inhibitory effect of compound [3] was not mediated by the acridine moiety since the heptanucleotide-acridine conjugate [6], which has the same sequence as the viral template, did not inhibit the RNA transcriptase reaction. No effect was observed with the undecanucleotide-acridine conjugates [1] and [2] at 30 µM and 100 µM concentrations. These results suggest that the absence of antiviral activity of compounds [1] and [2] in cell cultures is not related to a lack of intracellular penetration but rather to the lack of accessibility of the very 3'-end of the viral RNAs resulting from binding of the transcriptase complex [21].

DISCUSSION

We have shown here that an heptanucleotide covalently linked to an acridine moiety (compound [3]) and targeted to part of the 3'-terminal sequence common to the eight viral RNAs of type A influenza viruses, inhibits the multiplication of these viruses in cell cultures. Although the antiviral activity was observed at high concentration ($\geq$ 50 µM), it appeared nevertheless to be selective. First, no cytotoxicity was observed at these concentrations. Second, only type A viruses were inhibited while a type B influenza virus was completely insensitive to the compound. In type B influenza viruses there is a common twelve nucleotide-long sequence at the 3'-termini with a

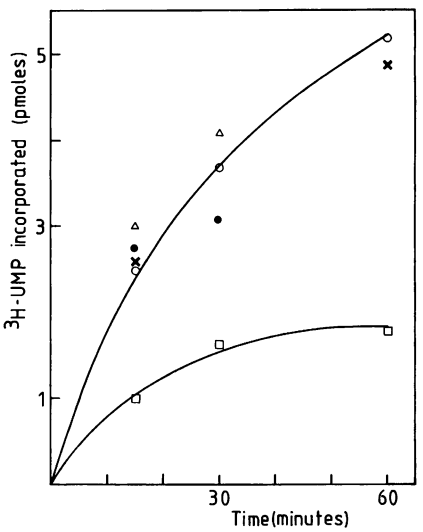

Figure 3 : Effect of oligodeoxynucleotide-acridine conjugates on the RNA transcriptase activity of A/Philippines/2/82 influenza virus. At the times indicated on the figure, 40 µl of the reaction mixture (prepared as described in Materials and Methods) were precipitated with 10 % TCA on Whatman 3 MM filters and counted. Incubation was carried out in the absence of any oligonucleotide (o) or in the presence of 30 µM of the oligonucleotide-acridine conjugates [3] (□), [1] (Δ) and [6] (●) or in the presence of 3 µM $Acrm_5OH$ (×).

G/U degeneracy at the eleventh position [10] but the 3'-terminal dodecameric sequence of type A and type B viruses differ at four positions (Figure 1b). Due to the presence of mismatches, the oligonucleotide which inhibits the cytopathic effect of type A viruses has no effect on type B viruses. This result strongly suggests that the 3'-region of the viral RNAs is the target for compound [3].

Binding of compound [3] to the viral RNAs is expected to affect early events of influenza virus replication. As shown in Figure 3, the RNA transcriptase reaction is inhibited by this compound at concentrations which exert antiviral activity in cell cultures. The observation that inhibition of RNA transcriptase activity plateaued around 70 % when concentration increased above 30 µM might be related to a difference in accessibility of the target sequence in the eight viral RNAs. Since all eight RNAs have to be transcribed **in vivo** to observe virus production, a 70 % inhibition of the RNA transcriptase is certainly sufficient to block the cytopathic effect of the virus.

As compared to compound [3], the lack of antiviral activity of the

undecanucleotide-acridine conjugates (compounds [1] and [2]) was somehow unexpected. Since these compounds were also inactive **in vitro** in the RNA transcriptase reaction, the absence of inhibition of the cytopathic effect in cell cultures was not likely due to a decreased cell uptake as compared with compound [3]. It is known that the viral transcriptase complex is bound to the 3'-end of the viral RNAs (21). It seems therefore plausible to assume that the undecanucleotides which are directed against sequence 1-11 cannot hybridize to the very 3'-end of the viral RNAs. A dangling end might be destabilizing the complex (as a result, e.g., of repulsive interactions with the transcriptase complex) as compared to the heptanucleotide which covers sequence 5-11. Our results agree with a previously reported observation that a dodecanucleotide complementary to the 3'-end of the viral genome was without effect on the influenza virus RNA transcriptase **in vitro** (11).

Oligonucleotides specifically designed to block gene expression provide a basis for a rational approach in chemotherapy. As shown in the present study, not only the sequence but also the length of the oligonucleotide play an important role. Longer molecules which are expected to be more active on the basis of a stronger binding to the target sequence may not always be the most effective ones. In order to efficiently interact at physiological temperatures with targets dispersed inside intracellular compartments, one must increase the binding affinity of these molecules for the target sequences without loosing base pairing specificity. We have shown previously that this is possible by coupling the oligonucleotide to an intercalating agent, such as the acridine derivative used in the present study which was attached via its 9 amino group and a polymethylene linker to the 3'-phosphate group of the heptanucleotide (compound [3]) (12-16).

The results presented above show that an oligodeoxynucleotide covalently linked to an intercalating agent can behave as an antiviral agent provided the oligonucleotide sequence is complementary to a crucial region of the viral genome. Influenza virus provides an interesting example where a single oligonucleotide sequence can be targeted to all viral RNA segments. A similar situation is found in other organisms, e.g., in trypanosomes where all mRNAs possess a 35 nucleotide-long common sequence at their 5'-end. Oligonucleotides complementary to part of this sequence block mRNA translation **in vitro** (22,23). Shorter oligonucleotides can be made active provided they are covalently linked to an intercalating agent (24). In all these systems the intercalating agent appears to play several roles :

i) it stabilizes the complex formed by the oligonucleotide with its complementary sequence[12-16] ;

ii) it facilitates the uptake of these composite molecules inside living cells. The fluorescent acridine derivative provides a good probe to monitor by fluorescence microscopy the penetration of oligonucleotide-acridine conjugates inside living cells (unpublished data). The acridine appears to drag the oligonucleotide inside cells ;

iii) acridine covalently linked to the 3'-phosphate protects the oligonucleotide against 3'-exonucleases [13,25].

The antiviral activity of the oligonucleotide-acridine conjugate [3] is observed only at high concentrations. This arises mainly because oligodeoxynucleotides are rapidly degraded by nucleases present both in the culture medium and inside cells [25] and also because their penetration into living cells is limited despite the fact that the acridine facilitates uptake as compared to unsubstituted oligonucleotides.These two problems must be overcome before such antiviral agents can be considered in a practical context : the penetration through cell membranes must be improved and the oligonucleotide should be made resistant against nucleases. Both problems can be solved. For example, attaching oligonucleotides to positively-charged polymeric carriers such as poly-lysine strongly enhances their uptake by living cells in culture [26]. Modifying the phosphodiester backbone e.g., under the form of phosphonates, makes these molecules resistant to nucleases [3,7]. It was recently shown that oligodeoxynucleotides synthesized with the [α]-anomers of nucleosides instead of the natural [β]-anomers are resistant to nucleases [27,28]. Moreover they bind more strongly to ribonucleic acids than to deoxyribonucleic acids in their single-stranded form [27]. Their evaluation as specific blockers of messenger and viral RNAs is presently under way.

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