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Beta-cyclodextrin derivatives as carriers to enhance the antiviral activity of an antisense oligonucleotide directed toward a coronavirus intergenic consensus sequence

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Summary. The ability of cyclodextrins to enhance the antiviral activity of a phosphodiester oligodeoxynucleotide has been investigated. A 18-mer oligodeoxynucleotide complementary to the initiation region of the mRNA coding for the spike protein and containing the intergenic consensus sequence of an enteric coronavirus has been tested for antiviral action against virus growth in human adenocarcinoma cells. The phosphodiester oligodeoxynucleotide only showed a limited effect on virus growth rate (from 12 to 34% viral inhibition in cells treated with 7.5 to 25 μM oligodeoxynucleotide, respectively, at a multiplicity of infection of 0.1 infectious particle per cell). In the same conditions, the phosphorothioate analogue exhibited stronger antiviral activity, the inhibition increased from 56 to 90%. The inhibitory effect of this analogue was antisense and sequence-specific. Northern blot analysis showed that the sequence-dependent mechanism of action appears to be the inhibition of mRNA transcription. We conclude that the coronavirus intergenic consensus sequence is a good target for an antisense oligonucleotide antiviral action. The properties of the phosphodiester oligonucleotide was improved after its complexation with cyclodextrins. The most important increase of the antiviral activity (90% inhibition) was obtained with only 7.5 μM oligonucleotide complexed to a cyclodextrin derivative, 6-deoxy-6-S-β-D-galactopyranosyl-6-thio-cyclomaltoheptaose in a molar ratio of 1:100. These studies suggest that the use of cyclodextrin derivatives as carrier for phosphodiester oligonucleotides delivery may be an effective method for increasing the therapeutic potential of these compounds in viral infections.

Introduction

The use of antisense oligonucleotides is an interesting approach to study cellular and viral gene function and to block gene expression [2, 3, 60]. Oligonucleotides have been considered as ideal agents for inhibiting viral

replication due in part to their high specificity for viral DNA and RNA sequences. These agents have been successfully employed to block the replication and cellular expression of several viruses, including herpes simplex virus [20], cytomegalovirus [43] and especially human immunodeficiency virus [33]. However, the vulnerability of oligonuleotides to nucleases' attack, combined with their intracellular distribution and uptake properties, have limited their therapeutic potential. To avoid the problem of nucleases' digestion and to improve the cellular uptake of oligonucleotides, either chemical modifications of the internucleoside phosphodiester linkages [35] or of the ribose sugar [25] and various oligonucleotide conjugates have been tested. One chemical analog that is widely employed for enhanced stability is the phosphorothioate oligonucleotide. But, although this analog has increased resistance to degradation in vivo and in vitro [1, 46, 59] it has exhibited sequence-specific and non sequence-specific activities [11, 54, 55]. The oligonucleotide conjugates include oligonucleotide adsorbed onto poly (isobutylcyanoacrylate) nanoparticles [40], encapsulated in liposomes [23] or conjugated to the cholesterol [18, 26]. These strategies have resulted in an increase of the cellular uptake of oligonucleotide and / or stability against nucleases. However, the inherent properties of the cholesterol, liposomes or nanoparticles carriers led to increase the immunogenicity of the oligonucleotide complex and the tendency of the complex to be phagocytosed by the reticuloendothelial system [44].

Investigations in cyclodextrin chemistry field have been increased since several decades. Cyclodextrins are molecular hosts acting as carriers. It frameworks describes a group of cyclic polysaccharides containing 6 to 8 glucose units in α-(1,4) linkage (α-CD, β-CD and γ-CD have respectively 6, 7 or 8 glucose units). They possess an hydrophobic internal cavity which can include various small molecules and a peripheral structure containing a large number of hydroxyl groups making the molecule water soluble. The cyclodextrins form complexes with numerous compounds and β-cyclodextrin is the most employed for numerous applications. A wide variety of cyclodextrins and derivatives has been studied to enhance the solubility and the availability of various water-soluble and water-insoluble pharmacological agents [6, 9, 21, 38, 64]. This approach using cyclodextrin derivatives has been recently studied to increase the cellular uptake of phosphorothioate oligonucleotides [65]. In this work, we have investigated the ability of native β- and γ-cyclodextrins and β-cyclodextrin derivatives as carriers to enhance the antiviral activity of a phosphodiester oligonucleotide directed toward the initiation region of the mRNA coding for the spike protein and containing the intergenic consensus sequence of an enteric coronavirus.

Materials and methods

Chemicals

The native cyclodextrins were purchased from Roquette-frères (Dijon, France) for β-cyclodextrin (β-CD) and from Wacker-chemie GmbH, (Lyon, France) for γ-cyclodextrin

(γ-CD). The chemically modified cyclodextrins were synthetised in our laboratory according to [17] for 6-deoxy-6-S-β-D-galactopyranosyl-6-thio-cyclomaltoheptaose (S-Gal-β-CD) and heptakis (6-deoxy-6-S-β-D-galactopyranosyl)-6-thio-cyclomaltoheptaose (7(S-Gal)-β-CD) or to [39] for heptakis [6-deoxy-6-(1-2-amino) ethylamino]-cyclomalto-heptaose (7(ethane-diamino)-β-CD). All the derivatives were soluble in distilled water and sterilized by filtration through a 0.22 μm membrane (Millipore, France) before use. Their chemical structures are presented in Fig. 1.

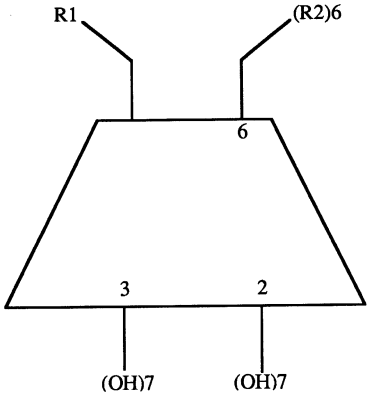
Oligonucleotides were purchased from Appligene (Strasbourg, France). An antisense sequence (3′ TGGTATTAGATTTGTACA 5′), synthetized as natural phosphodiester (O^-oligo) and phosphorothioate analog (S^-oligo), a sense sequence (5′ ACCATAATC-TAAACATGT 3′), synthetized as phosphorothioate analog (S^+oligo) and a random sequence (5′ GAATTGTGATATTTCATG 3′), synthetized as phosphorothioate analog (S^roligo) have been tested. All these oligonucleotides were purified by HPLC, lyophilized and the powder was dissolved in distilled water.

Virus and cells

The F15 strain of Bovine Enteric Coronavirus (BECV) was obtained from Laporte (INRA, Jouy-en-Josas, France) and propagated in the human rectal adenocarcinoma tumor cell line (HRT-18) grown in RPMI 1640 medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 2% foetal calf serum (FCS) (Life Technologies, Eragny, France), 10 μg/ml ofloxacin as antibiotic (Roussel Uclaf, Romainville, France) and incubated at 37 °C for 3 or 4 days in a humidified atmosphere containing 5 % CO_2 [30, 31].

Preparation of the [oligonucleotide-cyclodextrin]complexes

The complexes were prepared taking in account the β- and γ-cyclodextrins solubility data according to Higuchi and Connors [19].



β-CD	R1 = R2 = OH
S-Gal-β-CD	R1 = S-β-D-Galactopyranosyl R2 = OH
7(S-Gal)-β-CD	R1= R2 = S-β-D-Galactopyranosyl
7(ethane-diamino)-β-CD	R1 = R2 = -NH-(C_2H_4)-NH_2

Fig. 1. Structure of the native β-CD and β-CD derivatives. Cyclodextrins are regular cyclic oligosaccharidyl molecules exhibiting a tronconic ring which are for simplicity represented by a truncated cone with the wider side formed by the secondary 2- and 3-hydroxyl groups and the narrower side by the primary 6-hydroxyl

A volume of 7.5 μM phosphodiester oligonucleotide (O^- oligo) was added to 75 μM β-CD, 75 μM γ-CD, 7.5 to 750 μM S-Gal-β-CD, 75 μM 7(S-Gal)-β-CD or 75 μM 7(ethanediamino)-β-CD in a 0.5 ml total volume of RPMI 1640 medium. The solutions were mixed overnight at 25 °C by vortexing at 200 rpm with a Rotatest 74 401 apparatus (Bioblock-Scientific, Illkirch, France) and the resulting complexes were immediately tested [51]. Complex formation has been controlled by circular dichroïsm measurement and observation of oligonucleotide absorbance intensity at 260 nm (data not shown).

Cytotoxicity assays

The cellular viability was evaluated by the modified MTT assay as previously described [4]. HRT-18 cells were grown at confluence in 96-well flat bottom culture plates (Costar, Cambridge, MA, USA) at 4×10^4 cells per well and treated with varying concentrations of oligonucleotides (1 to 100 μM) or native cyclodextrins (1 to 8 mM) or CD derivatives (0.1 to 1 mM) for 16 h at 37 °C. At the end of the incubation period, the cytotoxic effect of the drug was evaluated by determination of the mitochondrial dehydrogenase activity using the MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (Sigma Chemical Co., St Louis, MO, USA). The absorbance of the solubilized material was determined using a Titertek Multiscan MCC/340 MK II apparatus (Labsystems, Helsinki, Finland). A cytotoxic effect corresponds to a decrease in purple formazan production. Results shown are the average from three experiments.

Antiviral assays

HRT-18 cells were exposed for 1 h at 37 °C to various concentrations of free oligonucleotides or oligonucleotides complexed with cyclodextrins and, at the same time, were inoculated with a BECV suspension at multiplicities of infection (MOI) of 0.1, 1 or 10 PFU / cell (Plaque Forming Units / cell). At 16 h post-infection, the virus titration was performed by the plaque forming assay using crystal violet for visualization, as previously described [30, 31, 61]. In interpreting viral titers, confidence intervals were determined and were taken into consideration [7].

Isolation and northern blot analysis of viral mRNAs

Intracellular RNAs were extracted from infected HRT-18 cells by the acid guanidinium thiocyanate-phenol-chloroform method [12] using a commercial ''Message systems''kit (Bioprobe Systems, Montreuil-sous-Bois, France). The RNA pellets were dissolved in nuclease free water and adjusted to 2.2 M formaldehyde – 50% formamide in MOPS (morpholinepropane sulfonic acid) buffer (20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA). Samples were denatured at 65 °C for 10 min and analyzed by electrophoresis in 1 % agarose gel and 2.2 M formaldehyde – MOPS buffer as previously described [37]. After electrophoresis, the gel was treated with 50 mM NaOH for 20 min, soaked in DEPC (diethylpyrocarbonate) treated water, then in 20 × SSPE (1 × SSPE is 3.6 M NaCl, 0.2 M sodium phosphate pH 7.7, 0.02 M EDTA) for 45 min. RNAs were transfered overnight on a hybond-N nylon membrane (Amersham, Buckinghamshire, England) in 20 × SSPE, then baked at 80 °C for 2 h. Nylon membrane was prehybridized for 2 h at 42 °C in plastic bags containing a solution of 50% formamide – 5 × Denhardt's solution (1 × is 0.02% Ficoll, 0.02% polyvinylpyrrolidine, 0.02% bovine serum albumine) – 0.5% SDS – 5 × SSPE and 200 μg/ml of sonicated calf thymus DNA. Hybridization was done overnight in the same solution at 42 °C using a [^{32}P] DNA probe prepared by random-primed labelling of gel

purified BECV 3′ end specific cDNA fragment (1.8 Kb) as previously described [13, 47]. The membrane was washed twice at room temperature for 10 min in 2 × SSPE, 0.1% SDS then once at 65 °C for 15 min in 1 × SSPE, 0.1% SDS and then once at 65 °C for 10 min in 0.1 SSPE, 0.1% SDS. The membrane was air-dried and autoradiographed on X-ray film (Kodak X Omat AR-5, N.Y, USA).

Results

Antiviral activity of the oligonucleotides

Influence of oligonucleotides on the cell viability

These effects were evaluated by the MTT assay after 16 h of treatment of the HRT-18 cells with increasing concentrations (1 to 100 μM) of each oligonucleotide. Figure 2 shows that S^r, S^- and O^-oligonucleotides exhibited no inhibitory effect on the viability of the cells at any concentration tested.

Antiviral activity of oligonucleotides in function of concentration

The antiviral activities of the antisense phosphodiester (O^-oligo) and its phosphorothioate analog (S^-oligo) were evaluated as described in Materials and methods. HRT-18 cells were exposed to various oligonucleotide concentrations (2.5 μM to 25 μM) and, at the same time, were infected by a BECV suspension at a MOI of 0.1 PFU/cell for 1 h at 37 °C. At 16 h post-infection the virus titers of the treated infected cells were determined comparatively to those obtained with control infected cells. We observed

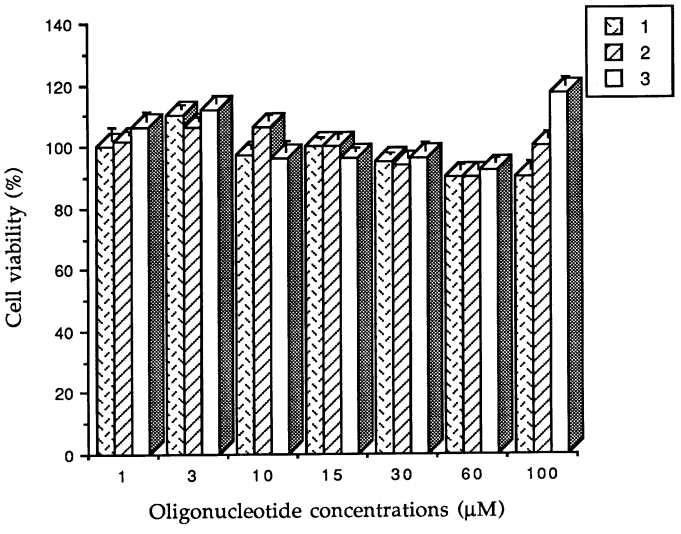


Fig. 2. Influence of oligonucleotides on cell viability. Cells grown to confluence (4.10^4 cells/well) were treated for 16 h with S^r (*1*), S^- (*2*) and O^- (*3*) oligonucleotides at concentrations ranging from 1 to 100 μM. Cell viability was evaluated by their dehydrogenase activity (MTT assay). Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

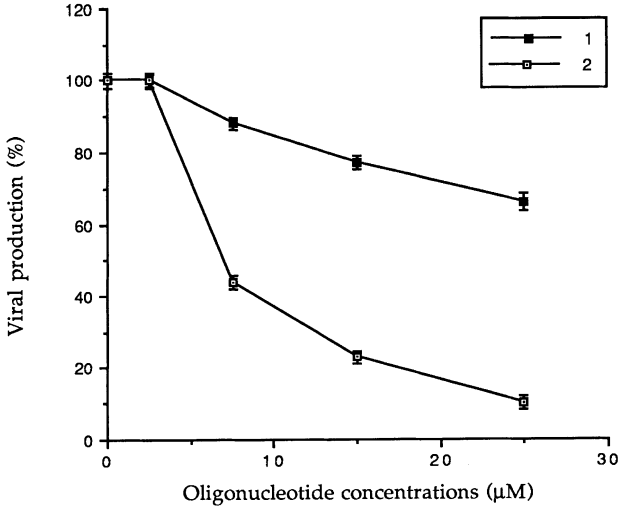


Fig. 3. Antiviral activity of the antisense oligonucleotides in function of their concentrations. Infected cells (MOI = 0.1 PFU/cell) were incubated for 16 h with various concentrations (2.5 to 25 μM) of O^-oligo (*1*) or S^-oligo (*2*). The virus titers were determined by the plaque forming assay. Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

(Fig. 3) about 88 to 66% of viral production when cells were treated with 7.5 to 25 μM of O^-oligo. In contrast the S^-oligo significantly reduced the ability of BECV to form plaques on HRT-18 cells. When cells were treated with 7.5 to 25 μM of the S^-oligo, from 44 to 10% of viral growth were observed comparatively to control infected cells. These results confirm that the inhibition is dose-dependent and that the phosphorothioate analogue exhibited a more pronounced antiviral activity than the phosphodiester oligodeoxynucleotide.

Influence of the viral multiplicity of infection

The antiviral activity of 25 μM of S^-oligo in presence of various viral MOI (0.1, 1 or 10 PFU/cell) was also evaluated. The results are presented on Fig. 4. Respectively 10% or 34% of virus production were observed in cultures infected with 0.1 or 1 PFU/cell. In contrast in presence of an MOI of 10 PFU/cell, the use of 25 μM of S^-oligo inhibit weakly the virus infectivity, we obtained 66% of viral growth.

Sequence specific effect of the phosphorothioate oligonucleotide on the viral growth

In order to study the specificity of the antiviral activity of the phosphorothioate analogue, sense (S^+oligo), antisense (S^-oligo), and random (S^roligo) oligonucleotides were comparatively tested. Using oligonucleotide concentrations of 15 or 25 μM and a MOI of 0.1 PFU/cell we showed (Fig. 5) that in presence of S^roligo and S^+oligo, no significant antiviral effect was observed, whereas 25 μM S^-oligo allowed only 10% of viral production. These results confirm the antisense sequence specificity of the S^-oligo antiviral activity.

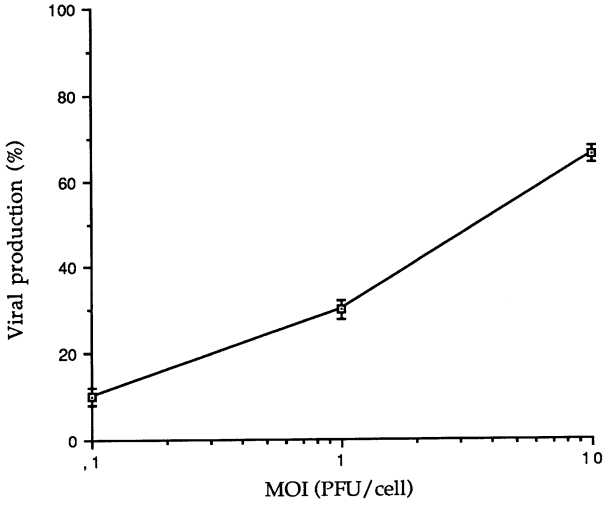


Fig. 4. Influence of the viral multiplicity of infection on the antiviral effect of the phosphorothioate oligonucleotide. Infected cells (MOI = 0.1, 1 or 10 PFU/cell) were incubated for 16 h with 25 μM of S^-oligo. The virus titers were determined by the plaque forming assay. Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

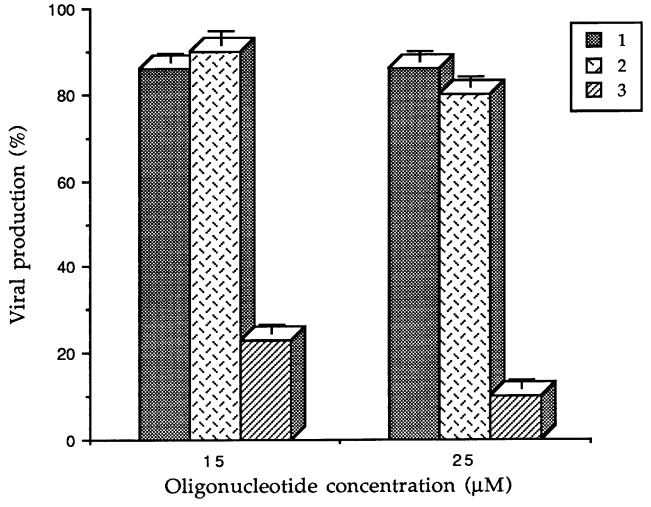


Fig. 5. Sequence specificity of the antiviral effect of the phosphorothioate oligonucleotide. Experiments were performed in presence of antisense, S^-oligo (*3*), random, S^roligo (*1*) and sense, S^+ oligo (*2*). Infected cells (MOI = 0.1 PFU/cell) were incubated for 16 h with 15 or 25 μM of oligonucleotides. The virus titers were determined by the plaque forming assay. Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

Effect of the oligonucleotides on the viral mRNA synthesis

Taking into account data in which viral mRNA synthesis reached its maximal rate between 6 and 8 h post-infection [24], HRT-18 cells infected with BECV

(MOI of 0.1 PFU/cell) or mock-infected (medium containing 2% FCS) were exposed to a 25 μM either S^-oligo, S^roligo or S^+oligo. Each oligonucleotide and the viral suspension were added together at the same time of infection. After 7 h post-infection intracellular RNA extracts were separated by electrophoresis, transfered to a membrane and hybridized to a cDNA probe representing the 3′ end of the BECV genome (including almost all the N and a part of the M gene) [13]

Autoradiograms (Fig. 6) showed that in infected cells, 7 RNA species could be detected at 7 h post-infection. These mRNAs were transcribed in non-equimolar amounts. They were named mRNA 2 to 7 (2, 2a, 3, 4, 5, 6, 7) according to decreasing order of size coding for viral proteins ns2, HE, S, ns3, ns4, M and N respectively, as already described [24]. Although the oligonucleotide had a sequence complementary to mRNA3, the synthesis of all mRNAs was significantly reduced when cells were treated with S^-oligo. In the same conditions, mRNA profiles or production were not affected when cells were treated with sense oligo (S^+oligo) and random oligo (S^r oligo) comparatively to untreated infected cells.

Antiviral activity of [O^-oligo-CD] complexes

Influence of cyclodextrins on the cell viability

The effect of native CD and cyclodextrin derivatives on cell viability have been tested. Native β-CD and γ-CD (Fig. 7) did not inhibit the cellular viability at concentrations varying from 1 to 8 mM. On the contrary, the β-CD stimulated the cell dehydrogenase activity, we obtained an increase of cell viability with concentrations ranging from 1 to 4 mM. Concerning the CD derivatives, it can be observed from Fig. 8 that S-Gal-β-CD and 7(S-Gal)-β-CD exhibited no

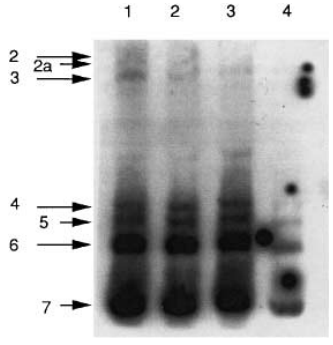


Fig. 6. Northern blot analysis of subgenomic RNAs. Intracellular RNAs were extracted at 7 h post-infection, separated by formaldehyde-1% agarose gel electrophoresis and transfered to a nylon membrane. The probe was prepared by random primed [^{32}P] labelling of enteric coronavirus-specific cDNA fragment corresponding to the 3′ end of genomic RNA. Autoradiograms were exposed overnight at −80 °C with an intensifying screen. Infected cells (MOI = 0.1 PFU/cell) (*1*), infected cells treated with S^roligo (*2*) or S^+oligo (*3*) or S^-oligo (*4*). Each oligonucleotide was used at a concentration of 25 μM

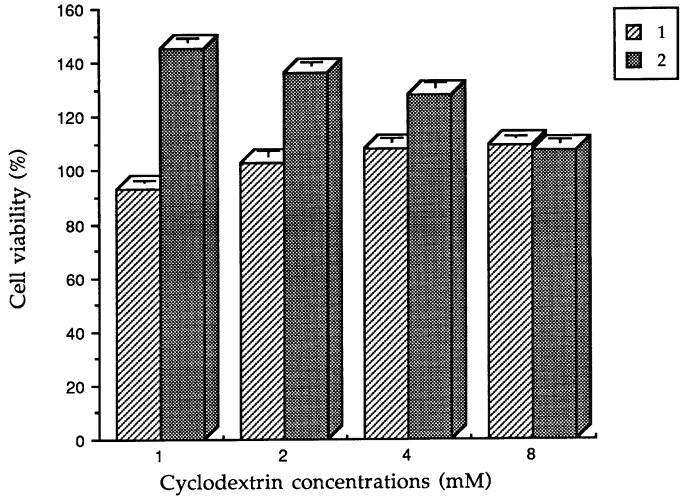


Fig. 7. Influence of native cyclodextrins on the cell viability. Cells grown to confluence (4.10^4 cells/well) were treated for 16 h with native cyclodextrins β-CD (*1*) and γ-CD (*2*) at concentrations ranging from 1 to 8 mM. Cell viability was evaluated by their dehydrogenase activity (MTT assay). Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

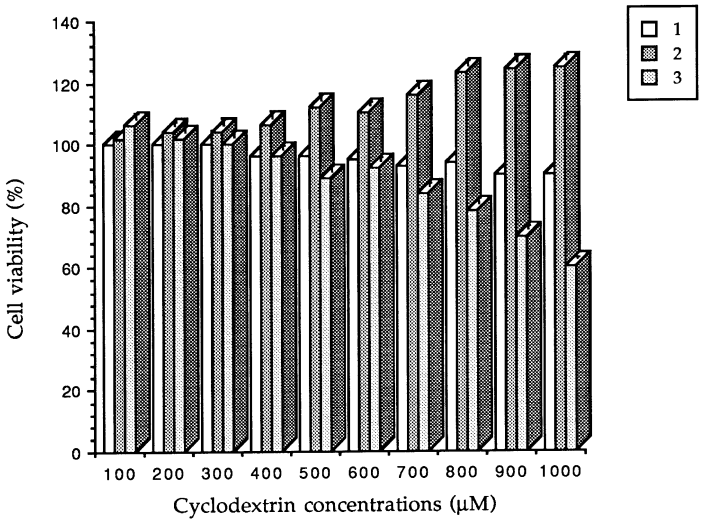


Fig. 8. Influence of cyclodextrin derivatives on the cell viability. Cells grown to confluence (4.10^4 cells/well) were treated for 16 h with S-Gal-β-CD (*1*), 7(S-Gal)-β-CD (*2*) and 7(ethane-diamino)-β-CD (*3*) at concentrations ranging from 100 to 1000 μM. Cell viability was evaluated by their dehydrogenase activity (MTT assay). Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

cytotoxic effect at concentrations varying from 0.1 to 1 mM. However, a decrease of cell viability to 40 % was observed in presence of 1 mM 7(ethane-diamino)-β-CD.

Effect of [O⁻oligo-β-CD or γ-CD] or [O⁻oligo-β-CD derivatives] complexes on antiviral activity

To determine if cyclodextrins could improve the activity of the phosphodiester oligonucleotide, a series of native cyclodextrins and cyclodextrin derivatives was tested. This includes β-CD, γ-CD, S-Gal-β-CD, 7(S-Gal)-β-CD and 7(ethane-diamino)-β-CD. The cells were infected (0.1 PFU/cell) and at the same time, the various [oligonucleotide-cyclodextrin] complexes at a molar ratio of [1:10] were added. The final concentration of O⁻oligo was 7.5 μM. The viral titers were determined after 16 h post-infection. The results showed (Table 1) that with O⁻oligo the viral production was 75% which was similar to previously obtained (88%). After complexation with cyclodextrins, the inhibition was improved, 50% of viral production was obtained with [O⁻oligo-β-CD], [O⁻oligo-γ-CD] and [O⁻oligo-7(ethane-diamino)-β-CD] complexes and only 25% with [O⁻oligo-S-Gal-β-CD] and [O⁻oligo-7(S-Gal)-β-CD].

Influence of the molar ratio between oligonucleotide and S-Gal-β-CD on the improvement of the antiviral activity

We have evaluated the influence of the molar ratio between the O⁻oligo and the S-Gal-β-CD on the antiviral activity of the phosphodiester oligonucleotide. O⁻oligo was used at a concentration of 7.5 μM and S-Gal-β-CD at concentrations ranging from 7.5 μM to 750 μM. We showed (Fig. 9) that at molar ratio

Table 1. Antiviral activity of the phosphodiester oligonucleotide after its complexation with native cyclodextrin and β-cyclodextrin derivatives

Compounds	PFU/ml ($\times 10^8$)	L_l ($\times 10^8$)	L_u ($\times 10^8$)	Viral production (%)
no oligo	40	28.4	54.2	100
O⁻ oligo	30	20.8	43.8	75
[O⁻ oligo-β-CD]	20	12.8	31.9	50
[O⁻ oligo-γ-CD]	20	12.8	31.8	50
[O⁻ oligo-S-Gal-β-CD]	10	4.8	18.4	25
[O⁻ oligo-7(S-Gal)-β-CD]	10	4.8	18.4	25
[O⁻ oligo-7 (ethane-diamino)-β-CD]	20	12.8	31.8	50

HRT-18 cells were inoculated with viral suspension (MOI = 0.1 PFU/cell) in presence of different complexes at molar ratio of [1:10]. O⁻ oligo concentration = 7.5 μM; CD concentration = 75 μM. After 16 h post-infection, the virus titers were determined by the plaque forming assay. L_l Lower 95% limit of confidence interval, L_u upper 95% limit of confidence interval

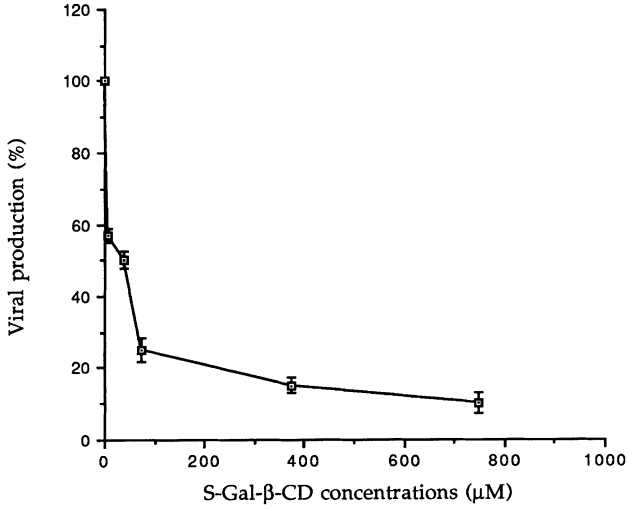


Fig. 9. Influence of the molar ratio between oligonucleotide and S-Gal-β-CD on the antiviral activity of (O^-oligo). Infected cells (MOI = 0.1 PFU/cell) were incubated for 16 h with [O^-oligo-S-Gal-β-CD] solutions at various molar ratio. O^-oligo = 7.5 μM; S-Gal-β-CD = 7.5 μM (molar ratio = 1:1) or 37.5 μM (molar ratio = 1:5) or 75 μM (molar ratio = 1:10) or 375 μM (molar ratio = 1:50) or 750 μM (molar ratio = 1:100). The virus titers were determined by the plaque forming assay. Each point is the mean of three independent experiments performed in triplicate and error bars represent standard error of mean

of [1:1] or [1:5], no significant antiviral activity of the O^-oligo was observed (viral production equal 57 and 50%, respectively). On the contrary the viral multiplication decreased to 25%, 15% and 10% when the molar ratio of O^-oligo and S-Gal-β-CD was [1:10], [1:50] and [1:100], respectively.

Influence of the S-Gal-β-CD on the viral multiplication

The effect of the S-Gal-β-CD on the viral multiplication was evaluated. The results (Table 2) showed that no inhibitory effect in viral production was

Table 2. Effect of S-Gal-β-cyclodextrin derivative on the viral multiplication

S-Gal-β-CD (μM)	PFU/ml ($\times 10^8$)	L_1 ($\times 10^8$)	L_u ($\times 10^8$)	Viral production (%)
0	20	12.0	30.6	100
75	20	12.0	30.6	100
375	25	16.0	36.7	125
750	36	25.0	49.6	180

HRT-18 cells were inoculated with viral suspension (MOI = 0.1 PFU/cell) in presence of S-Gal-β-cyclodextrin at various concentrations. After 16 h post-infection the virus titers were determined by the plaque forming assay. L_1 Lower 95% limit of confidence interval, L_u upper 95% limit of confidence interval

observed. Nevertheless with 750 μM, there was a significant stimulation of about 180% in viral growth.

Discussion

Coronaviruses which have an original replication procedure can be candidates for antisense strategy. These viruses cause acute and/or persistent infections and might be etiological agents for autoimmune diseases [16, 41, 45]. They are enveloped viruses which contain a single-stranded, positive-sensed polyadenylated genome. They possess three or four major structural proteins. All coronaviruses have spike (S), membrane (M) and nucleocapsid (N) proteins and some have a haemagglutinin-esterase (HE) protein. In addition, there are at least four genes that encode nonstructural proteins (ns1 to 4) [29, 58]. During the replication in infected cells, genomic RNA is transcribed into genome-sized, negative-stranded RNA which serves as template for the synthesis of a 3′-coterminal nested set of 7 to 8 subgenomic mRNAs [52, 56] and only the 5′-proximal region which is not present in the next smaller RNA of the set is used for translation [48]. Another unique feature of coronavirus mRNAs is that the 5′ ends of genomic RNA and of all mRNAs contain a 72–77 nucleotide-long leader sequence [27, 28]. Studies made on the murine coronavirus mouse hepatitis [10, 50] have shown that the 3′ end of the leader sequence contains a repeated UCUAA pentanucleotide sequence included in the unique UCUAAAC consensus sequence (or similar sequence) observed in all intergenic regions of genomic RNA. Thus the mRNA transcriptional start is the result of the interaction between the leader sequence of the positive-stranded RNA and the complementary antileader sequence of the negative-stranded RNA [49, 50].

This intergenic consensus sequence, common to the transcription initiation sites of mRNAs could be an interesting target for an antisense oligonucleotide. We have targeted it by a 18-mer antisense oligodeoxynucleotide complementary to the initiation region of mRNA coding for S protein and which contains the unique UCUAAAC intergenic consensus sequence which is the promoter of genomic RNA and subgenomic mRNAs transcription [36].

We have shown that 25 μM phosphorothioate antisense (S^-oligo) oligonucleotide is able to inhibit 90% of enteric coronavirus growth in HRT-18 cells at a MOI of 0.1. The intensity of the inhibitory effect of S^-oligo is a function of its concentration and of the multiplicity of viral infection. In previous studies, efficient inhibition of gene expression by antisense sequence in cells requiring oligonucleotide concentrations in the range of 1 to 100 μM have been reported. The great varieties in the biological models investigated render comparisons difficult [3, 8, 20, 32, 33, 43, 57].

In the same experimental conditions, only 34% of the viral production is inhibited by the unmodified phosphodiester antisense (O^-oligo) oligonucleotide. The absence of a more significant inhibitory effect of this oligonucleotide may be related to its rapid degradation by nucleases and to its reduced cellular uptake. Our observations about the cellular uptake of fluorescein-conjugated

oligonucleotides have revealed (data not shown) that only 1 μM of S^-oligo is quickly (< 1 h) internalized by HRT-18 cells and that fluorescence is present both in the cytoplasm and in the nucleus of cells. In contrast, intracellular localization of even 20 μM of O^-oligo can not be noticed. Studies have shown [8, 22, 62] that the uptake of oligonucleotides is a complex process which depends on many factors including cell type, structure and chemical modifications of oligonucleotides, temperature and time of incubation. This uptake is a saturable process that probably occurs through an 80 kD protein but the true role of this protein is still unknown [5, 34]. The stronger inhibitory effect observed with S^-oligo could be related to an increase in stability and in lipophilicity resulting from the replacement of the oxygen atom by sulfur [15]. It has thus been reported that phosphorothioate oligonucleotides are more strongly associated with cell membranes than the corresponding phosphodiester [8].

As previously observed [3, 20, 32, 33, 43] the antiviral activity of S^-oligo is not due to an oligonucleotide induced cytotoxicity. Assays to measure host-cell viability reveal that the oligonucleotides tested do not affect the level of cellular metabolism even at high concentration (100 μM) which is 4-fold in excess of inhibitory concentration (25 μM).

It is noteworthy that non-sequence-specific oligonucleotides can inhibit HIV replication especially at high concentrations [53–55]. So, a random unrelated phosphorothioate oligonucleotide (S^roligo) with the same base composition as S^-oligo but without homology with any known sequence in genomic RNA shows no activity. Therefore, the inhibition of virus growth does not seem to be due to a non-sequence-specific effect. In addition a sense oligonucleotide (S^+oligo) at any concentration employed does not inhibit virus growth showing that the antiviral mechanism of action of S^-oligo is antisense. These results are confirmed by northern blotting analysis in which all transcripts are significantly reduced in infected cells when 25 μM of S^-oligo is used. In contrast RNAs levels are unaffected by non-specific control (S^roligo) or by sense (S^+oligo) phosphorothioate oligonucleotides. In addition, northern blotting shows that the antisense phosphorothioate S^-oligo acts through a transcription inhibition mechanism in which the duplex made by hybridization of positive-stranded genomic RNA and S^-oligo stops the synthesis of the full length negative stranded-RNA preventing so a subgenomic and genomic RNA synthesis. The non-inhibitory effect of the sense oligonucleotide could be explained by considering the viral replication mechanism. The transcription of the mRNA is initiated after the hybridization between the leader and anti-leader sequences. The anti-leader sequence contains the anti-consensus sequence which is the target of the sense oligonucleotide. Consequently, we assume that a competition occurs between the leader sequence and the sense oligonucleotide for hybridization to their target. This competition might be in favour of the leader sequence mainly due to its total complementarity to its target and due also to a highest affinity. Our results provide evidence that the coronavirus intergenic consensus sequence of the positive-stranded genomic RNA is a good

target for an antisense oligonucleotide antiviral action. The inhibiting effect on virus growth is mediated by the ability of the oligonucleotide to interfere with mRNA transcription.

However, some potential disadvantages may be inherent to the use of phosphorothioate analogs. There is no assurance that substitution of a phosphorothioate for a phosphodiester in an oligonucleotide would preserve its ability to bind with similar high affinity to its target. In addition studies have shown [14] that the sulfur on phosphorothioate may provide a novel reactive center on a DNA molecule for drug interactions and this interaction may affect the efficacy of the antisense compound. It may also act by viral polymerase inhibition or by interference with viral adsorption or penetration or by inhibition of cellular enzymes or growth factors [55]. Moreover commercially phosphorothioates tend to be more expensive than the phosphodiester oligonucleotides. To avoid these problems, we attempted to improve the properties of the phosphodiester oligonucleotide by complexation with cyclodextrin supermolecules. We compared the biological activity of the phosphodiester antisense oligonucleotide (O^-oligo) either free or complexed with native β-CD and γ-CD or β-cyclodextrin derivatives. When the O^-oligo is complexed either with native β-, γ-CD, or with a 7(ethane-diamino)-β-CD derivative, 50% decrease in viral multiplication is observed. The inhibition is higher (75%) after its complexation with S-Gal-β-CD or 7(S-Gal)-β-CD. Moreover, the antiviral activity of O^-oligo complexed with S-Gal-β-CD is improved to 90% if the complexation is performed in the [O^-oligo-CD] molar ratio of [1:100; 7.5 μM: 750 μM]. The same level of inhibition is obtained in presence of 25 μM of S^-oligo, a concentration three fold up to that used in the complex.

It has been demonstrated in the last year that substituted cyclodextrins were no longer biological amorphous compounds, but could exhibit biological activities such as antiviral activity [42, 63]. We checked that the chemical modification of β-CD does not induce any cyclodextrin antiviral nor anticellular effect. S-Gal-β-CD induces no inhibitory effect on the viral multiplication at the concentrations used for the preparation of the complexes. Moreover, assays to measure cell viability show no cytotoxic action of the cyclodextrins. These observations exclude the possibility that the antiviral activity obtained with the [O^-oligo-S-Gal-β-CD] complexes is due to an effect of the S-Gal-β-CD itself.

From these results it might be concluded that the complexation of the oligonucleotide with the cyclodextrins protect the O^-oligo from its degradation by the nucleases perhaps by protecting the phosphodiester group. Cellular uptake studies recently reported that the fluorescent intensity of (FITC)-conjugated phosphorothioate oligonucleotides was greatly increased when the oligonucleotide was complexed with cyclodextrin derivatives, indicating that complexation increases cellular uptake [65]. In our case it might be expected that the chemical modification of the native β-CD by substitution of the hydroxyl of the primary alcohol of one glucose unit on the ring with a thio-galactose residue improves the cellular uptake of the O^-oligo by a ligand-

carbohydrate cell receptors interaction. As the surface of numerous normal and malignant eukaryotic cells are covered with galactose-specific lectins, galactose-substituted β-CD might be used as drug carriers for targeting oligonucleotides in vivo. Another possibility which may also contribute to the enhancement of the antiviral activity of O^-oligo complexed to cyclodextrin is that the complexation could modify the intracellular distribution of the antisense oligonucleotide. Probably oligonucleotide-cyclodextrin complexes enter the cells by a process different from endocytosis pathway in which the oligonucleotides may be trapped within endosomal or lysosomal vesicles. Further studies have to be achieved to study the intracellular distribution of oligonucleotide after treatment with [oligonucleotide-cyclodextrin] complexes and to elucidate the mechanism of the interaction between the oligodeoxynucleotide and cyclodextrin.

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