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Best Minimally Modified Antisense Oligonucleotides According to Cell Nuclease Activity

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ABSTRACT

Minimally modified oligonucleotides belong to the second-generation antisense class. They are phosphodiester oligonucleotides with a minimum of phosphorothioate linkages in order to be protected against serum and cellular exonucleases and endonucleases. They activate RNase H, have weak interactions with proteins, and have thus a better antisense efficiency. Two of them have been designed from an all-phosphorothioate antisense oligonucleotide directed against *mdr1*-expressing cells. They are protected against serum and cellular enzymatic degradation by the self-forming hairpin d(GCGAAGC) at their 3′-end and by judiciously located phosphorothioate residues, depending on the cellular composition in exonucleases or endonucleases. Besides their already demonstrated ability to cleave pyrimidine sites, endonucleases show some specificity for CpG sites. Their activity is hindered if specific sites are involved in secondary structure as hairpin.

INTRODUCTION

MULTIPLE DRUG RESISTANCE (MDR) is a major problem in cancer therapeutics. One of its forms is based on overexpression of the *mdr1* gene encoding to the transmembrane P-glycoprotein (P-gp), which acts as a nonspecific efflux pump (Fojo et al., 1987; Ling, 1992; Roninson, 1992). A number of attempts at reversing the MDR phenotype through antisense strategy have been described (Alahari et al., 1996; Bertram et al., 1995; Cucco and Calabretta, 1996; Dassow et al., 2000; Liu et al., 1996). Antisense oligonucleotides have been designed against the *mdr1* gene, most of them complementary to the region of the initiation codon of the *mdr1* mRNA. Hybridization of antisense oligonucleotides to mRNA can block gene expression through several mechanisms, such as RNase H degradation of target mRNA (Walder and Walder, 1988), translation arrest (Boiziau et al., 1991), or interference with splicing of pre-mRNA (Kulka et al., 1989).

Because unmodified phosphodiester oligonucleotides are rapidly degraded by serum or intracellular nucleases, a number of analogs of oligonucleotides have been proposed. Among them, phosphorothioates, in which a nonbridging oxygen of the phosphodiester bond is replaced by a sulfur, have been extensively studied and tested in human clinical trials (Agrawal and Zhao, 1998). They have the characteristic of maintaining activation of RNase H (Agrawal and Iyer, 1997), which seems essential in the case of *mdr1* (Alahari et al., 1996). Nevertheless, phosphorothioate (PS) oligonucleotides cause nonantisense effects due to their nonspecific binding to proteins (Stein, 1996; Stein and Cheng, 1993). At high concentrations, they can even inhibit RNase H (Crooke, 1992). Second-generation antisense oligonucleotides have been designed. They have the desirable properties of PS oligonucleotides (resistance to nucleases, RNase H activation) but weak interactions with proteins. Mixed backbone oligonucleotides (MBO) were first proposed. They contain appropriately placed segments of PS oligodeoxynucleotides (ODN) and differently modified ODN (Agrawal and Zhao, 1998; Zhou and Agrawal, 1998) or oligoribonucleotides (Metelev et al., 1994) and have shown improvements over PS oligonucleotides even in terms of biologic activity. More recently, minimally modified antisense ODN have been described (Uhlmann et al., 2000). These are phosphodiester ODN protected (1) by two to five PS residues at their 3′-end, as the serum enzymes are mainly 3′-exonucleases (Shaw et al., 1991), (2) by two PS residues at their 5′-end against potential degradation by 5′-exonucleases, and (3) by PS linkages at internal pyrimidine nucleotides against degradation by endonucleases. Single-strand specific endonucleases in serum and inside the cells have been shown to cleave oligonucleotides preferentially at pyrimidine sites (Peyman and Uhlmann, 1996; Rait et al., 2000; Uhlmann et al., 2000), this cleavage becoming dominant in the case of several adjacent pyrimidines (Uhlmann et al., 2000). Minimally modified oligonucleotides have a higher cellular uptake than all-PS oligonucleotides (Peyman et al., 1997) and have biologic antisense effects with (Rait et al., 2000) and without (Tanaka et al., 1996) delivery systems. In other ap-

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proaches, the increased nuclease resistance of phosphodiester oligonucleotides having different secondary structures (hairpin, looped, snail-like) has been investigated (Maksimenko et al., 1999).

In the present work, we have determined what could be the best minimally modified antisense oligonucleotide against *mdr1*. Our starting point was an all-PS antisense oligonucleotide that has proven its efficiency against this gene (Alahari et al., 1996). The stability against serum 3′-exonucleases of oligonucleotides protected at their 3′-end by the sequence d(GCGAAGC) has been used (Hirao et al., 1994; Réfrégiers et al., 1996). This sequence spontaneously forms a hairpin known for its extraordinary stability with regard to thermal denaturation or nuclease degradation (Hirao et al., 1994; Khan and Coulson, 1993). It is stabilized through only two G-C base pairs and a non-Watson-Crick G-A pair (Hirao et al., 1994; Yoshizawa et al., 1997). This hairpin does not prevent hybridization of the 5′-stem part of the oligonucleotide. When this occurs, it loses its stability and can itself contribute to hybridization (Jolles et al., 1997). Additional modifications preventing intracellular degradation have been proposed for the two following systems: multidrug-resistant K562 cells and NIH-MDR-G185 cells transfected with a plasmid containing the human *mdr1* gene. Contribution of the hairpin to protection against endonucleases has been demonstrated.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

The oligonucleotides listed in Table 1 were synthesized by Eurogentec (Seraing, Belgium). They are phosphodiester oligonucleotides with possibly some PS linkages. They are designed from the sequence of the antisense all-PS oligonucleotide 5995 complementary to the initiation codon of human *mdr1* mRNA (Alahari et al., 1996). ODN-H have the self-forming hairpin d(GCGAAGC) at their 3′-end. This is not the case for reference ODN-R, for which a G/C inversion prevents hairpin formation. ODN-H1 to ODN-H5 have PS linkages as indicated. H is a dodecamer that forms the hairpin; R is its linear reference. Both of them are labeled by rhodamine at their 5′-end and by fluorescein at their 3′-end. All oligonucleotides were solubilized in TE buffer, pH 7.8, containing 20 mM NaCl in the case of hairpins, which were then heated for 20 minutes at 80°C, then slowly cooled in order to favor the hairpin formation (Hirao et al., 1994).

Cells

Multidrug-resistant K562 cells were a gift from A. Garnier (Laigle et al., 1996), and NIH-MDR-G185 cells transfected with a plasmid containing the human *mdr1* gene (pSK1 MDR) were a gift from M. Gottesman (Bethesda, MD) (Kane et al., 1989). They were grown in RPMI (or DMEM) medium supplemented with 10% decomplemented newborn calf serum (or fetal bovine serum [FBS]), 50 U/ml penicillin, 50 μg/ml streptomycin in a 5% CO_2 atmosphere. To maintain P-gp expression, K562 cells were regularly treated with 0.4 μM doxorubicin, and the medium of NIH-MDR-G185 cells was supplemented with 60 ng/ml colchicine.

Cell lysates

K562 and NIH-MDR-G185 cell lysates were prepared as described (Maksimenko et al., 1999). Protein concentration was quantitated using Bio-Rad Protein Assay (Hercules, CA). Cell lysates were diluted in 10 mM sodium phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, and 1 mM DTT (Maksimenko et al., 1999) to achieve the chosen protein concentration.

PAGE analysis

All 23-mer ODN were 5′-end labeled with T4 polynucleotide kinase (Pharmacia, Uppsala Sweden) and γ-^{32}P-ATP (ICN Pharmaceuticals, Costa Mesa, CA). Oligonucleotide degradation was studied in cell culture medium (10% serum); respectively in cell lysates in the following way. Solution A contained 4.10^6 cpm of one of the labeled oligonucleotides plus 120 pmol of the same oligonucleotide unlabeled in a total volume of 10 μl cell culture medium without serum, respectively lysate dilution buffer. A 110-μl solution B contained cell culture medium (11% serum, respectively 63 μg K562 lysate proteins in dilution buffer or 146 μg NIH-MDR-G185 lysate proteins in dilution buffer). At time 0, solutions A and B were mixed. (Final protein concentrations were 10% serum, respectively 0.52 mg/ml K562 cell lysate proteins or 1.22 mg/ml proteins of NIH-MDR-G185 cell lysate). A 20-μl aliquot was immediately removed, and other aliquots were removed during a 24-hour incubation period at 37°C. The reactions were stopped by the addition of an equal volume of formamide containing 10 mM EDTA, pH 8, bromophenol blue, and xylene cyanol. Before migration, all samples were heated at 90°C for 4 minutes. Samples were analyzed on a 20% polyacrylamide/7M urea sequencing gel. Percentages of intact or cleaved oligonucleotides and

TABLE 1. OLIGONUCLEOTIDE SEQUENCES

ODN-H	5′ GATCCATCCCGACCTCGCGAAGC 3′
ODN-R	5′ GATCCATCCCGACCTCCGGAAGC 3′
ODN-H1	5′ GAT*C*C*AT*CCCG*ACCTCGCGAAGC 3′
ODN-H2	5′ GATC*C*A*TC*CCG*ACCTCGCGAAGC 3′
ODN-H3	5′ G*ATCCATCCCGACCTCGCGAAGC 3′
ODN-H4	5′ G*A*TCCATCCCGACCTCGCGAAGC 3′
ODN-H5	5′ G*A*T*CCATCCCGACCTCGCGAAGC 3′
H	5′ R-TTCTCGCGAAGC-F 3′
R	5′ R-TTCTCCGGAAGC-F 3′

*PS linkage.
R, rhodamine, F, fluorescein.

degradation $t_{1/2}$ were evaluated after densitometric scanning of the lanes and treatment with NIH image. The error is 5%.

Fluorescence resonance energy transfer (FRET) analysis

Fluorescently labeled R and H oligonucleotides were mixed with cell culture medium (10% serum) or cell lysates (0.6 mg/ml proteins for both K562 and NIH-MDR-G185 lysates). Immediately after mixing and all during the experiment, aliquots of 7 μl were directly laid down on an X63 Zeiss plan Neofluar objective (Zeiss, Oberkochen, Germany). Fluorescence measurements were made with the UV-visible microspectrofluorometer developed in our laboratory (Sureau et al., 1990). The excitation wavelength was the 488 nm line of an argon laser. The beam power was reduced to about 0.1 μW by the use of neutral density filters. Duration of a spectrum was 0.2–1 second.

RESULTS AND DISCUSSION

PAGE analysis

The sequence of the best all-PS antisense oligonucleotide against *mdr1* as determined by Alahari et al. (1996) has been chosen as the starting point of this study, d(CCA TCC CGA CCT CGC GCT CC). It recognizes a region flanking the AUG initiation codon of *mdr1* mRNA. To protect against serum 3′-exonucleases a minimally modified phosphodiester oligonucleotide of the same sequence, ODN-H ends on its 3′-side with the d(GCGAAGC) sequence that spontaneously forms a very stable hairpin (Hirao et al., 1994; Khan and Coulson, 1993). The four 3′-terminal bases cannot hybridize any more with the target mRNA (Chen et al., 1990). Three bases complementary to the mRNA (GAT) are then added on the 5′-side of the oligonucleotide to restore duplex stability. The nuclease resistance of this oligonucleotide and its improvement by different modifications have been checked in two cell lines expressing the human *mdr1* gene, resistant K562 cells and transfected NIH-MDR-G185 cells (see Materials and Methods).

The protection provided by the self-forming hairpin against serum 3′-exonucleases has been checked in culture medium of both cell lines. Figure 1 presents the degradation patterns of ODN-H and ODN-R (for which a G/C inversion prevents formation of the hairpin) in a medium containing 10% newborn calf serum. Longer exposures (E+) of ODN-R and one of its degradation lanes (>2 hours incubation) allows the oligonucleotide sequence to be read. Intact ODN-H migrates faster than ODN-R because of its more compact structure (Hirao et al., 1992). The increasing ladder of smaller products in the degra-

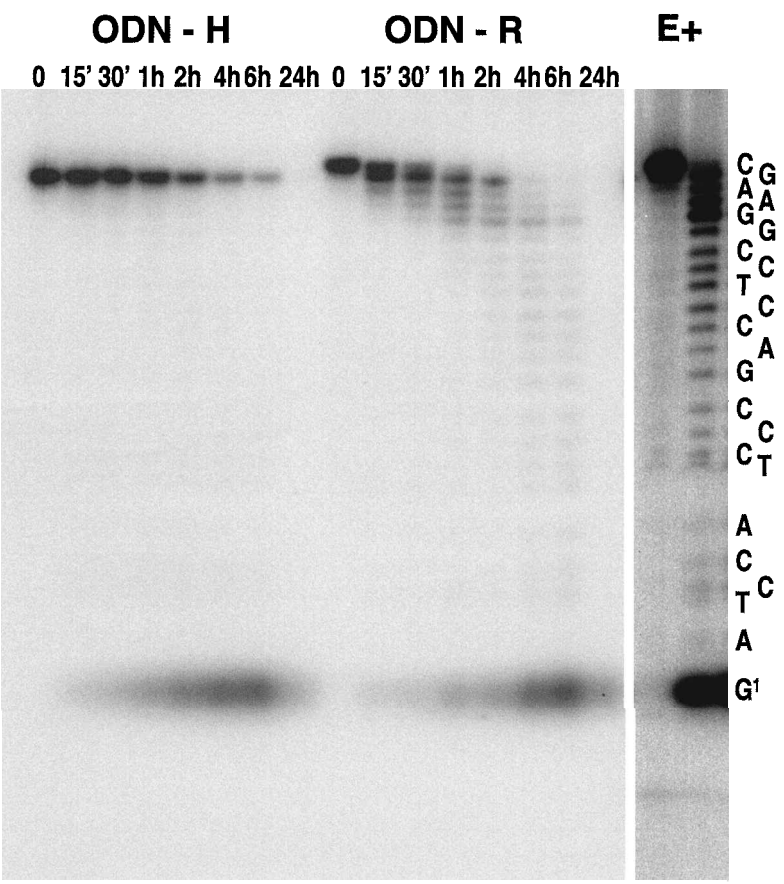

FIG. 1. Electrophoretic patterns of the degradation as a function of incubation time of 5′-γ^{32}P end-labeled ODN-H and ODN-R in culture medium (10% serum) (20% polyacrylamide/7 M urea gel). E+, longer exposure of lanes of ODN-R before and after limited degradation in 10% serum.

dation pattern of the reference ODN-R is characteristic of a 3′-exonuclease attack. The $t_{1/2}$ of ODN-R is <15 minutes, as the amounts of intact 23-mer and 22-mer oligonucleotide that has lost the 3′-terminal base are about the same after a 15-minute incubation. There is no more intact ODN-R after 2 hours incubation. In contrast, the $t_{1/2}$ of ODN-H is of the order of 2 hours (60% intact ODN-H after 1 hour incubation, 45% after 2 hours). An amount of 25% intact ODN-H is still present after 6 hours incubation. The same amount of remaining ODN-R is observable as soon as after 30 minutes incubation. A faint band corresponding to the 5′-G^1 fragment is observable, particularly after 4 hours incubation. G^1 is the ultimate product of degradation by the main serum enzyme, 3′-exonuclease. The lack of 3′-protection in the case of ODN-R allows accumulation of intermediary fragments to be observed as an increasing ladder of smaller products. Small amounts of endonucleases, 5′-exonuclease, or phosphatase may contribute to oligonucleotide degradation. The amount of fragment G^1 decreases after 24 hours incubation, and a new fragment appears below on the gel (not shown), which is assigned to inorganic phosphate resulting from phosphatase activity in the serum. Degradation rates were similar in a medium containing 10% FBS (results not shown).

In view of these results and considering that delivery systems are used in most of the antisense technology protocols, the hairpin will be considered as satisfactory protection against enzymatic degradation of serum.

Is this protection sufficient inside the cell? To answer this question, oligonucleotide degradation was checked in lysates prepared from resistant K562 or NIH-MDR-G185 cells. As K562 lysate was more active, the total protein concentrations were, respectively, 0.52 mg/ml and 1.22 mg/ml for experiments in K562 and NIH-MDR-G185 lysates. Figure 2 compares degradation of ODN-R and ODN-H in K562 lysate. They were similar, with degradation patterns characteristic of an endonuclease attack. This attack was moderate compared with the 3′-exonuclease degradation in the culture medium. There was still about 10% intact ODN-R after 6 hours incubation ($t_{1/2}$ = 2 hours), whereas there was no ODN-R after 2 hours incubation in 10% serum. The hairpin was not expected to protect oligonucleotides against endonucleases. There was only 5% intact ODN-H after 6 hours incubation ($t_{1/2}$ = 1 hour 20 minutes). Degradation patterns showed several cleavage sites, which were assigned according to a lane of limited degradation of ODN-R in 10% serum run at the same time and exposed for a

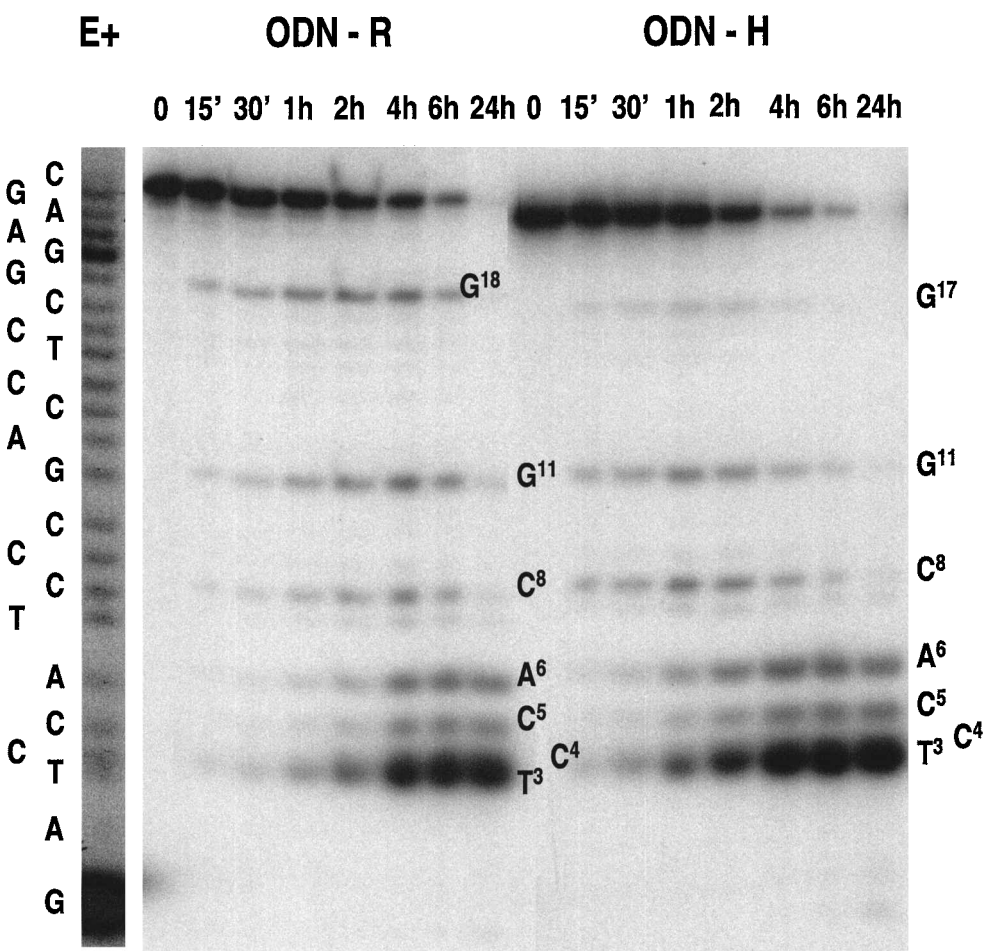

FIG. 2. Electrophoretic patterns of the degradation as a function of incubation time of ODN-R and ODN-H in K562 lysate (0.52 mg/ml protein). E+, longer exposure of a lane of ODN-R after limited degradation in 10% serum.

long time. All bases of the TCCA sequence located near the 5′-end of the oligonucleotides were cleaved, the main cleavage being on the 3′-side of T^3. Other cleavage sites were on the 3′-side of C^8, G^{11}, and G^{18} in the case of ODN-R, and G^{17} in the case of ODN-H. Although endonuclease activity has been described as occurring preferentially at pyrimidine sites, especially with two or more adjacent pyrimidines (Peyman and Uhlmann, 1996; Uhlmann et al., 2000), it is clear that this was not the case for endonuclease activity of K562 lysate. Besides cleavage at T^3, C^4, C^5, and C^8, three of the cleavage sites were purines: A^6, G^{11}, and G^{18} (or G^{17}). The shift of the cleavage from G^{18} (ODN-R) to G^{17} (ODN-H) was remarkable and shows the specificity of K562 endonucleases for the guanine site. It can be seen that all cleaved purines have a cytosine on their 5′-side: CA^6, CG^{11}, and CG^{18} (or CG^{17}). It should be emphasized that the patterns showed a protective effect of the hairpin against K562 endonuclease activity. The amount of ODN-H cleaved at the CG^{17} site was less than that of ODN-R at the CG^{18} site (2% instead of 9% after 2 hours incubation). G^{17} is the 5′-terminal base of the self-forming hairpin (Hirao et al., 1994; Yoshizawa et al., 1997). This protection does not lead to an increase in the degradation $t_{1/2}$ of ODN-H, as the decrease in the cleavage rate at this site is compensated by an increase in other cleavage rates (e.g., 1.5 times more cleavage at the T^3 site). If CpG is a specific site for K562 endonucleases, the absence of cleaving at the CG^{19} site of ODN-H could be explained by the fact that this site belongs to the center of the hairpin and is, thus, inaccessible to enzymes. This new protective effect of the d(GCGAAGC) hairpin will be further demonstrated.

Two minimally modified ODN-H having PS linkages as indicated in Table 1 (ODN-H1 and ODN-H2) have been checked in K562 lysate. The protective effect of the hairpin at the site G^{17} was used without further modification of the linkages around this site. A sequence of three successive PS residues is located in the region of the T^3, C^4, C^5, and A^6 cleavage sites, which perfectly protects the TCCA sequence, even if the main cleavage site at T^3 is not directly involved in the modification (ODN-H2) (Fig. 3). We suggest that considering the 5′-side location of T^3, wherever the modification in this region, it will prevent interaction with endonuclease and, therefore, cleavage. When oligonucleotides were protected by PS bridges at their pyrimidine sites, no differences in stability was found for PS linkages in the 5′ or 3′ position of the internal pyrimidines (Uhlmann et al., 2000). Such is not the case in the present work. When the PS linkage was on the 5′-side of C^8 (ODN-H1), the site was protected, but the cleavage shifted to C^9. On the other hand, when the PS linkage was on the 3′-side of C^8 (ODN-H2), the whole region was protected. Therefore, 5′ and 3′ protection of pyrimidines is not equivalent. This was probably not observed in previous works because adjacent pyrimidines are generally protected by several PS linkages. A PS linkage on the 3′-side of G^{11} protects this site against cleavage (ODN-H1 and

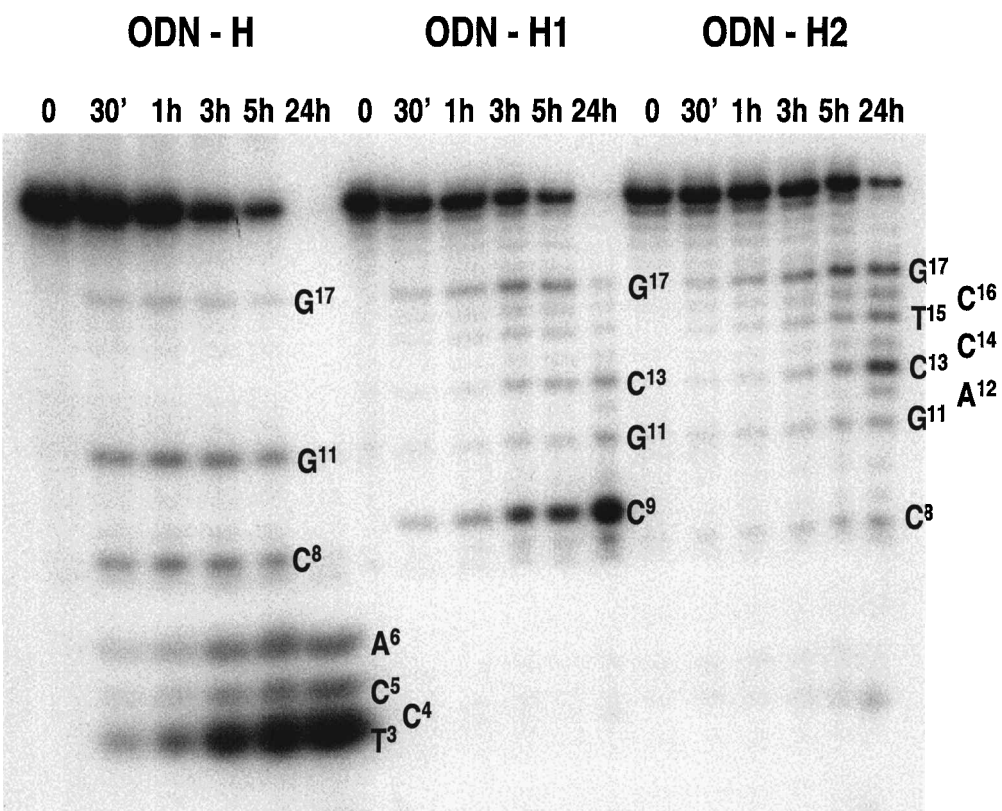

FIG. 3. Electrophoretic patterns of the degradation as a function of incubation time of ODN-H, ODN-H1, and ODN-H2 in K562 lysate (0.52 mg/ml protein).

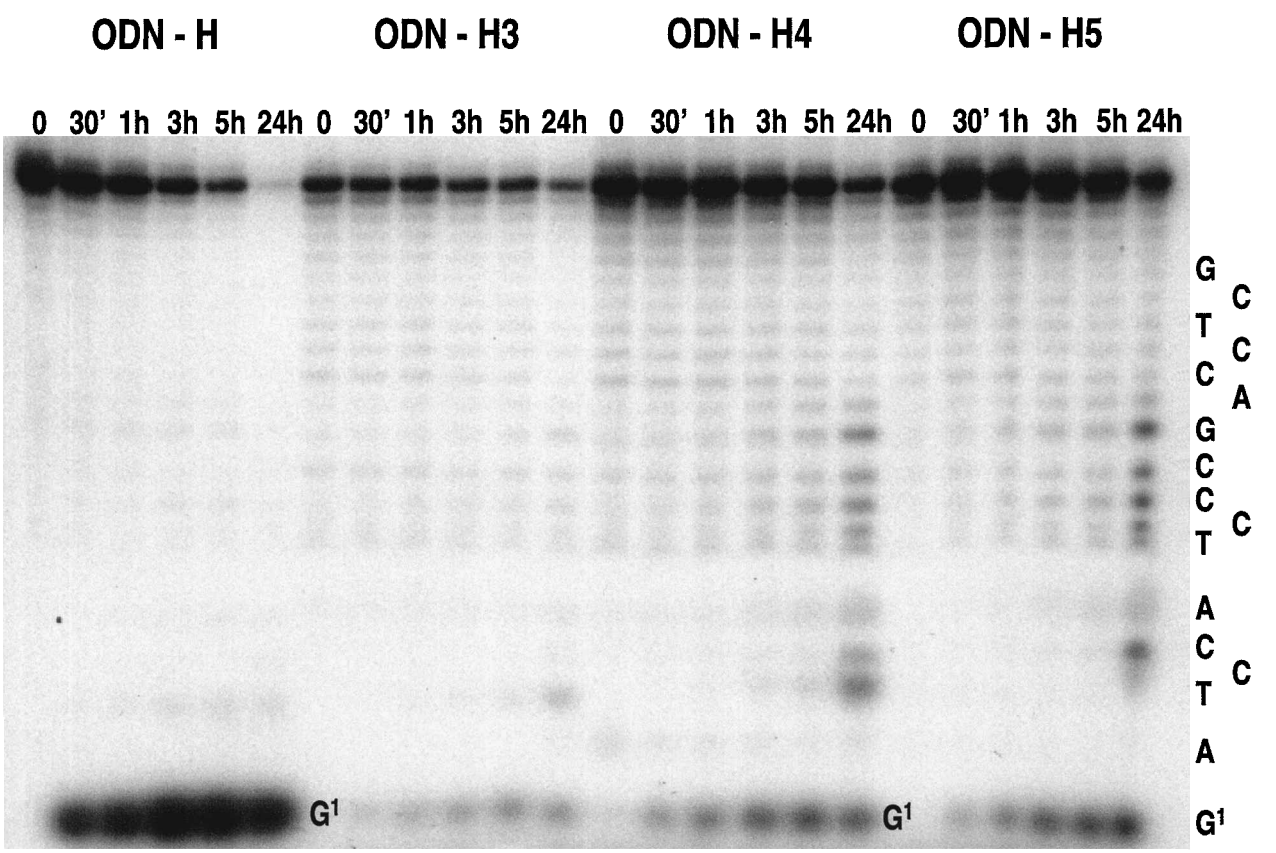

FIG. 4. Electrophoretic patterns of the degradation as a function of incubation time of ODN-H, ODN-H3, ODN-H4, and ODN-H5 in NIH-MDR-G185 lysate (1.22 mg/ml protein).

ODN-H2). When the initial cleavage sites are protected, more cleavage appears at pyrimidine sites. This occurs immediately (cleavage in C^9 of ODN-H1) or after long incubation time (C^{13}, T^{15}, C^{16} for ODN-H2 after 24 hours incubation). Therefore, we can consider that in the absence of specific sites (perhaps 5′-CpG sites), oligonucleotides are actually preferentially cleaved at adjacent pyrimidines. For use as an antisense oligonucleotide, ODN-H2 is a better choice than ODN-H1, which is itself preferable to ODN-H. There is still 1% ODN-H1 after 24 hours incubation. Its degradation $t_{1/2}$ is 4 hours instead of about 1 hour 45 minutes for ODN-H. There is 20% intact ODN-H2 after 24 hours incubation ($t_{1/2} \approx$ 10 hours). In conclusion, ODN-H2 is the best anti-*mdr1* oligonucleotide in resistant K562 cells. It is likely possible to reduce the number of PS linkages on the 5′-side.

Results obtained in NIH-MDR-G185 cell lysate are very different. ODN-H and ODN-R (not shown) are degraded in a similar way, with a pattern corresponding to a 5′-exonuclease attack (Fig. 4). A similar degradation pattern of nontransfected NIH/3T3 has been observed (Maksimenko et al., 1999). This is a moderate attack, and there is still 15% ODN-H after 5 hours incubation and 5% after 24 hours. It is, of course, possible to improve resistance by one (ODN-H3), two (ODN-H4), or three (ODN-H5) PS residues at the 5′-end of ODN-H (Fig. 4). In all cases, about 70% intact oligonucleotide is observed after 5 hours incubation and about 40% after 24 hours. ODN-H3, ODN-H4, and ODN-H5 are good anti-*mdr1* oligonucleotides in NIH-MDR-G185 cells.

FRET analysis

We have shown (Réfrégiers et al., 1996) that FRET (Förster, 1948) allows serum degradation of short oligonucleotides to be followed. Two dodecamer oligonucleotides that have (H) or have not (R) the self-forming hairpin at their 3′-end (Table 1) are labeled with rhodamine on their 5′-end and with fluorescein

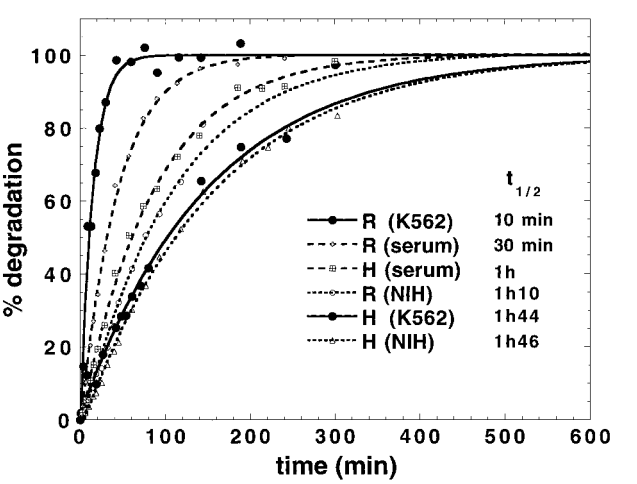

FIG. 5. Kinetics of degradation of R and H in 10% serum, K562, or NIH-MDR-G185 lysate (0.6 mg/ml protein).

on their 3′-end. Except for the two thymines on their 5′-end, H and R are similar to the 3′-moiety of ODN-H or ODN-R (from the C^{14} nucleotide). Because the fluorescence spectrum of fluorescein overlaps with the absorbance spectrum of rhodamine and the distance between the two fluorophores is of the order of the Förster distance (about 50Å for the fluorescein/rhodamine pair) (Parkhurst and Parkhurst, 1995), the excitation of fluorescein induces the fluorescence of rhodamine (577 nm) while its own fluorescence (520 nm) decreases. This is the FRET process. At the first enzymatic cleavage, FRET of a single oligonucleotide disappears. It is then possible to follow oligonucleotide degradation in solution by FRET disappearance, that is, increase in the I_{520nm}/I_{577nm} ratio (Réfrégiers et al., 1996).

Figure 5 presents the degradation percentages of H and R as a function of time in culture medium or NIH-MDR-G185 or K562 lysates (0.6 mg/ml protein in both cases). These percentages have been determined from the increase in the I_{520nm}/I_{577nm} ratio. Calculated $t_{1/2}$ are indicated. In the case where oligonucleotide degradation occurs through an exonuclease attack, except for R in culture medium, degradation is faster in FRET experiments compared with PAGE experiments. Thus, in the case of the 5′-exonuclease attack in NIH-MDR-G185 lysate, degradation $t_{1/2}$ are about 1 hour 10 minutes for R and 1 hour 45 minutes for H in FRET experiments and several hours for ODN-R and ODN-H in PAGE experiments (5% intact oligonucleotide at 24 hours). In PAGE experiments, the NIH-MDR-G185 protein concentration was nevertheless twice as much as in FRET experiments. In the case of the 3′-exonuclease attack in culture medium, degradation $t_{1/2}$ of H is 1 hour in FRET experiments vs. 2 hours for ODN-H in PAGE experiments. These results can be explained by the shorter size of the oligonucleotides in FRET experiments (12-mer instead of 23-mer). In the case of R in culture medium, the degradation $t_{1/2}$ is longer in FRET experiments, 30 minutes instead of 15 minutes. This could be due to the protection provided by fluorescein itself on the 3′-end of R (Réfrégiers et al., 1996). In all cases, the $t_{1/2}$ of H degradation is longer than that of R degradation. This difference is moderate: 1 hour for H in culture medium (2 hours for ODN-H in PAGE experiment) vs. 30 minutes for R (15 minutes for ODN-R in PAGE), 1 hour 45 minutes for H in NIH-MDR-G185 lysate vs. 1 hour 10 minutes for R (no difference for ODN-H and ODN-R in PAGE). The protection provided by the hairpin against 3′-exonuclease attack has been well demonstrated (Hirao et al., 1994; Réfrégiers et al., 1996). Advance of 5′-exonuclease along the oligonucleotide is likely hindered by the hairpin.

When oligonucleotide degradation occurs through endonuclease attack, that is, in K562 lysate, the results achieved in FRET experiments are drastically different from those obtained in PAGE experiments. The electrophoresis degradation patterns of ODN-H and ODN-R were very similar, with assessed $t_{1/2}$ this time in favor of a faster degradation of ODN-H ($t_{1/2}$ = 1 hour 20 minutes vs. 2 hours for ODN-R). In FRET experiments, the calculated degradation rate of R is nine times as high ($t_{1/2}$ = 10 minutes) as that of H ($t_{1/2}$ = 1 hour 45 minutes). Only one of the specific cleavage sites of the K562 lysate endonucleases remains in the short oligonucleotides: CG^{6} for H (which corresponds to CG^{17} of ODN-H) or CG^{7} of R (which corresponds to CG^{18} of ODN-R). Apart from the favorable cleavage at adjacent pyrimidines (Uhlmann et al., 2000) on the 5′-side of both H and R, CG^{6} of H (or CG^{7} of R) is thus the other main possibility of cleavage. PAGE experiments revealed a strong protection by the hairpin, 2% cleavage at the CG^{17} site of ODN-H vs. 9% cleavage at the CG^{18} site of ODN-R. This protection was counterbalanced by more cleavage at other possible sites of ODN-R. Such sites are missing in the case of the short oligonucleotides, explaining the great protection of the hairpin during K562 endonuclease attack.

In conclusion, when minimally modified oligonucleotides are used in antisense strategy, they are generally capped at both ends by PS linkages for protection against 3′-exonuclease degradation (mainly in serum) and 5′-exonuclease degradation (mainly inside the cells). Additional PS linkages are placed at internal pyrimidine nucleotides to ensure protection against cellular endonucleases (Peyman and Uhlmann, 1996; Rait et al., 2000; Uhlmann et al., 2000). We showed in this work that this last modification is not always the best solution. Guanines in CpG sites seem to be significant cleavage sites for K562 endonucleases. Their protection considerably reinforces the stability of the oligonucleotide. We also demonstrated that when cleavage occurs at a pyrimidine site, protection by PS linkage is better when it is at the 3′-side of the internal pyrimidine. In this work, oligonucleotides were not protected on their 3′-end by PS linkages but by the self-forming hairpin d(GCGAAGC). This was shown as sufficient protection against 3′-exonuclease degradation in serum and even as good protection against endonucleases-specific sites involved in hairpin base pairing.

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