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Minimally modified phosphodiester antisense oligodeoxyribonucleotide directed against the multidrug resistance gene *mdr1*

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Abstract

In the perspective of reversing multidrug resistance through antisense strategy while avoiding non-antisense effects of all-phosphorothioate oligonucleotides which non-specifically bind to proteins, a minimally modified antisense phosphodiester oligodeoxyribonucleotide has been designed against *mdr1*, one of the multidrug resistance genes. Its stability in lysates prepared from NIH/3T3 cells transfected with the human *mdr1* gene has already been demonstrated. Confocal microspectrofluorometry using a fluorescence resonance energy transfer technique allowed its stability inside living cells to be proven. Its internalization into the cells was achieved with different delivery agents (addition of a cholesteryl group, Superfect® or an amphotericin B cationic derivative) and has been followed by fluorescence imaging. For each of the delivery systems, Western blotting allowed its antisense efficiency to be compared to that of an all-phosphorothioate antisense oligonucleotide. No antisense efficiency was demonstrated for the minimally modified ODN when internalized with Superfect®. In both other cases, the best extinction of the P-glycoprotein expression has always been achieved with the all-phosphorothioate antisense. While the difference was significant in the case the amphotericin B derivative was used as delivery agent (20% remaining protein expression with the all-phosphorothioate vs. 40% with the minimally modified antisense), it was negligible for the cholesterol conjugates (2% vs. 6%). It is of great interest to prove that an almost all-phosphodiester oligonucleotide can be an efficient antisense against an overexpressed gene. The reduction of non-antisense effects as non-specific binding to proteins are of importance in the case relatively high ODN concentrations are used, which can prove to be necessary in the case of overexpressed genes.

Keywords: Multidrug resistance; Minimally modified antisense oligonucleotides; Hairpin; Fluorescence imaging; Fluorescence resonance excitation transfer; Western blot

1. Introduction

While effective chemotherapeutic drugs are available, approximately 70% of patients do not respond to them because of innate or acquired MDR [1]. The reversal of this resistance is one of the present challenges in cancer research. This resistance has been correlated to transmembrane ''pumps'' which trigger drug efflux. The most well characterized of these transmembrane proteins is the P-gp [2–4] encoded by the *mdr1* gene. One cause of MDR is related to overexpression of this gene. Since the use of chemical inhibitors of the P-gp is restricted because of their toxicity, a lot of work has been achieved intending to modulate the *mdr1* gene expression. Several attempts at reversing the MDR phenotype through antisense strategy have been described [5–11]. Most of the antisense ODNs have been designed against the region of the initiation codon of the *mdr1* mRNA. Antisense ODN have to be protected against serum and cellular nucleases. Since antisense activity on *mdr1* very likely occurs through a mechanism involving RNase H degradation [5], most of the proposed antisenses have an all-phosphorothioate backbone, which is about the single modification with boranophosphates [12] which maintain RNase H activity [5–10]. A mixed-backbone oligonucleotide with five methoxyethoxy groups at both ends and interior residues with standard phosphorothioates has also been described [11]. However, phosphorothioates cause non-antisense effects due to their non-specific binding to proteins [13,14]. At

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; ODN, oligodeoxyribonucleotide; AMA, amphotericin B 3-dimethylamino propyl amide; FRET, fluorescence resonance energy transfer.

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high concentrations they can even inhibit RNase H [15]. They lead to reduction of the binding affinity to RNA (0.3–0.5° per phosphorothioate linkage) [16]. Therefore, the concept of minimally modified ODN has been proposed [16]. These ODNs are protected at their 3′-end against 3′-exonucleases which are the main serum enzymes [17] and at their 5′-end or at specific internal positions depending on whether the cells are rich in 5′-exonucleases or endonucleases. In a previous work [18] we determined which modifications should be introduced in a phosphodiester oligonucleotide devised from an efficient all-phosphorothioate anti-*mdr1* ODN [5]. We have shown that the self-forming d(GCGAAGC) hairpin at their 3′-end [19,20] efficiently protects the ODN against serum and cellular 3′-exonucleases. Two phosphorothioate at the 5′-end protect the ODN in lysates prepared from NIH-MDR-G185 cells which are rich in 5′-exonucleases whereas a few internal phosphorothioate linkages at specific locations protect them in lysates of K562 cells rich in endonucleases.

In the present work we will check whether a minimally modified ODN whose stability has been demonstrated in lysates of NIH-MDR-G185 cells still remains resistant to nucleases in living cells and keeps the antisense potential of its all-phosphorothioate model. Since the efficient uptake of ODNs to cells require the use of delivery agents, the impact of three of them on the antisense effectiveness has been assessed. It has been shown that, according to the chemical nature of the ODN and the chosen carrier, an antisense is effective or not [21]. We used as delivery agents a cholesterol conjugated at the 5′-end of the ODN [5], a commercial transfectant (Superfect® from Qiagen) or an Amphotericin B derivative, AMA [22].

In order to get comparable results, the amount of ODN, when expressed in concentration terms, was the same whatever the delivery system or the chemical nature of the ODN.

2. Materials and methods

2.1. ODN

The oligonucleotides listed in Table 1 have been synthesized by Eurogentec. AS-ODN and (*CH*)-AS-ODN are the all-phosphorothioate antisense ODN 5995 complementary to the region of the initiation codon of human *mdr1* mRNA [5]. CH is a cholesterol conjugated at the 5′-end. (*CH*)-S-ODN is the corresponding sense ODN; it carries a cholesteryl group at its 5′-end. ODN1 is a phosphodiester ODN designed from AS-ODN with minimal modifications. It is protected against serum nucleases by the self-forming hairpin d(GCGAAGC) at its 3′-end and against NIH-MDR-G185 enzymes by two phosphorothioate groups at its 5′-end. Since the last 3′-terminal bases cannot any more hybridize with the target mRNA [23], three bases complementary to the mRNA (GAT) are then added at the 5′-end of the ODN in order to restore the duplex stability [18]. (*CH*)-ODN1 carries a cholesteryl group at its 5′-end. (*F*,*R*)-ODN1 is labeled with fluorophores (fluorescein and rhodamine as indicated). (*F*,*R*)-ODN2 is the same except it lacks the protection of the two phosphorothioates at its 5′-end. (*CH*,*F*)-ODN1 carries a cholesteryl group and is labeled with fluorescein at its 5′-end. ODN3 is an antisense control of ODN1: it keeps the protections afforded by the two phosphorothioate groups at its 5′-end and the hairpin in 3′ but its stem part has the sense orientation. All oligonucleotides were solubilized in TE (10 mM Tris, 1 mM EDTA) buffer, pH 7.8, containing 20 mM NaCl in the case of hairpins which were then heated for 20 min at 80° then slowly cooled down in order to favor the hairpin formation [19].

2.2. Cells

NIH/3T3 cells were obtained from the American Type Culture Collection (ATCC). NIH-MDR-G185 cells transfected with a plasmid containing the human *mdr1* gene (pSK1 MDR) were a gift from M. Gottesman [24]. They were grown in DMEM medium supplemented with 10% decomplemented fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin in a 5% CO_2 atmosphere. In order to maintain P-glycoprotein expression, the medium of NIH-MDR-G185 cells was supplemented with 60 ng/mL colchicine. The day before transfection, 3×10^5 cells were seeded per 35 mm dish in order to get an exponential growth phase on the day of transfection.

2.3. ODN delivery

Cholesteryl ODN (1 μM) were incubated with NIH-MDR-G185 cells in DMEM medium without serum in order to minimize cell division and therefore optimize antisense effect.

Superfect® (3 mg/mL) was purchased from Qiagen. It is a dendrimeric structure presenting 60 positively charged NH_2 groups on its surface at pH 7. It was used according to the supplier transfection protocol, i.e. complex formation in a medium without serum and transfection in the presence of serum (700 μL final medium volume). ODN (5 μg) was complexed to 6 μL of Superfect® [25] for 3×10^5 cells seeded the day before transfection. This corresponds to a charge ratio(±) = 2. In concentration terms [ODN] = 1 μM. After a 4-hr incubation (1) 1 mL DMEM was added in order to reduce the serum concentration and minimize cell division or (2) transfection medium was removed and replaced by DMEM.

AMA was kindly provided by Prof. E. Borowski (Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk). Its use as antisense delivery agent has already been demonstrated in our laboratory [22]. It is used in a medium without serum at the best charge ratio(±) = 0.5 [22]. The concentration of ODN is 1 μM.

Table 1

AS-ODN	5′C*C*A*T*C*C*C*G*A*C*C*T*C*G*C*G*C*T*C*C3′
(*CH*)-AS-ODN	5′(*CH*)-C*C*A*T*C*C*C*G*A*C*C*T*C*G*C*G*C*T*C*C3′
(*CH*)-S-ODN	5′(*CH*)-C*C*T*C*G*C*G*C*T*C*C*A*G*C*C*C*T*A*C*C3′
ODN1	5′G*A*TCCATCCCGACCTCGCGAAGC3′
(*CH*)-ODN1	5′(*CH*)-G*A*TCCATCCCGACCTCGCGAAGC3′
(*F*,*R*)-ODN1	5′G*A*TCCATCCCGACCTCGCGAAGC3′ (Fluorescein, Rhodamine)
(*F*,*R*)-ODN2	5′GATCCATCCCGACCTCGCGAAGC3′ (Fluorescein, Rhodamine)
(*CH*,*F*)-ODN1	5′(*CH*)-G*A*TCCATCCCGACCTCGCGAAGC3′ (Fluorescein)
ODN3	5′C*T*CCAGCCCTACCTAGGCGAAGC3′

(*CH*): cholesteryl group, (GCGAAGC): self-forming hairpin.
* Phosphorothioate linkage.

2.4. Measurement of ODN stability by confocal microspectrofluorometry

After a 4-hr incubation time with (*F*,*R*)-ODN1 or (*F*,*R*)-ODN2 internalized with Superfect® (see ODN delivery), NIH-MDR-G185 were washed with DMEM. FRET measurements inside the cells were recorded with a UV-Vis microspectrofluorometer prototype developed in our laboratory [26]. Like in the case of fluorescent imaging, washing the cells is essential to get rid of the fluorescence of non-incorporated ODNs. Despite confocality, about 10% of the signal recorded inside a cell results from external fluorescence. The excitation was the 488 nm wavelength of an Argon laser. The beam power was reduced to about 0.1 μW by the use of neutral density filters. The integration time of a spectrum was 1 s. A water immersion 63× objective was used.

2.5. Measurements of cellular ODN uptake by fluorescence imaging

After a short incubation time (4–5 hr) or a long incubation time (24 hr) with fluorescently labeled ODN ((*CH*,*F*)-ODN1 or (*F*,*R*)-ODN1 internalized with Superfect® or AMA), NIH-MDR-G185 were washed with DMEM and observed by fluorescence microscopy on a Nikon Optiphot-2 epifluorescence microscope. Images were detected with a cooled CCD camera (Micromax, Princeton Instruments) with a 12-bit detector (RTEA-1317 K, Kodack). Standard fluorescein and rhodamine filter sets have been used. Analysis and display were performed using IP Lab software (Scanalytics).

2.6. Western blotting measurement of P-glycoprotein expression

The day before transfection 3×10^5 NIH-MDR-G185 or NIH/3T3 cells were seeded onto 35 mm dishes and incubated for 24 hr in serum containing medium. The day of transfection NIH-MDR-G185 cells were treated with ODNs as described above and incubated for 48 hr in a medium without serum. Non-treated NIH-MDR-G185 and NIH/3T3 cells were used as references of resistant and sensitive cells. Cells were trypsinized, washed in PBS then resuspended in RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet-P40, 0.5% deoxycholate) at the ratio of 100 μL buffer for 2×10^6 cells. After 30 min on ice with some vortexing, the lysates were centrifuged at 10,000 *g* for 10 min at 4° and the resulting supernatants were checked for protein content. Equal amounts of proteins (∼20 μg) were mixed with concentrated SDS reducing buffer (final concentrations are 60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, bromophenol blue). Protein samples were separated on 7.5% SDS–PAGE then transferred onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was blocked with 5% non-fat dry milk in 0.1% Tween-PBS and treated with 0.5 μg/mL C219 anti-P-glycoprotein antibody (DAKO). Detection was carried out by enhanced chemiluminescence (ECL plus® kit with mouse IgG, HRP linked whole antibody from Amersham Pharmacia Biotech). The P-gp expression has been evaluated after densitometric scanning of the films and analysis with NIH Image software. The error has been evaluated to 5%.

3. Results

3.1. Cellular ODN stability

FRET allows nuclease degradation of ODNs inside the cells to be followed. ODN1 is a phosphodiester ODN designed from an efficient *mdr1* antisense [5]. It is protected by a hairpin at its 3′-end and two phosphorothioates at its 5′-end and its resistance in lysates prepared from NIH-MDR-G185 cells which are rich in 5′-exonucleases has already been demonstrated [18]. (*F*,*R*)-ODN1 is

labeled with fluorescein at its 5′-end (G^1) and with rhodamine at T^{15} inside the sequence. (*F*,*R*)-ODN2 is the same as (*F*,*R*)-ODN1 except it lacks the protection of the two phosphorothioates at its 5′-end. Since the fluorescence spectrum of fluorescein overlaps with the absorbance spectrum of rhodamine and since the distance between the two fluorophores is of the order of the Förster distance (about 50 Å for the fluorescein/rhodamine pair, ca. 15-mer) [27], the excitation of fluorescein induces fluorescence of rhodamine (577 nm) while its own fluorescence (520 nm) decreases: this is the FRET process. For a single ODN, FRET disappears at the first enzymatic cleavage between the two fluorophores. It is then possible to follow ODN degradation in cells by FRET disappearance, i.e. increase of the $I_{520\ nm}/I_{577\ nm}$ ratio [20].

Immediately after washing and along the time up to 25 hr, emission spectra of (*F*,*R*)-ODN1 and (*F*,*R*)-ODN2 were recorded inside the cells (Fig. 1(A)). The chosen delivery agent has been Superfect®. One must consider that the quantum yield of fluorescein is strong function of ionic strength, pH and the polarity of the environment surrounding the probe [28]. Its decrease inside the cell has been correlated to the location of the labeled ODN in an acidic environment as in endosomes [29]. Accordingly, spectra registered in the cytoplasm, the nucleus or vesicles (see next part) are very different. There is a strong quenching of fluorescein in the vesicles and the nucleus. The mean value of the $I_{520\ nm}/I_{577\ nm}$ ratio which is 0.4 in the cytoplasm just after washing of the cells is only 0.2 in the vesicles and the nucleus (not shown).This fluorescein quenching can be correlated to the pH decrease in the endocytosis vesicles and the hydrophobic environment due to DNA compaction in the nucleus. It is why the (*F*,*R*)-ODN1 and (*F*,*R*)-ODN2 degradation inside the cells was followed as the increase of the $I_{520\ nm}/I_{577\ nm}$ ratio recorded in cytoplasm at spots devoid of vesicles (Fig. 1(A)). Because of the dispersion of the results, a great number of spectra were registered for each incubation time. Fig. 1(B) shows in insert the fluorescence spectra of intact and fully digested (*F*,*R*)-ODN1 (after 48 hr in 50% serum at 37° [20]). The determined $t_{1/2}$ were, respectively, 130 hr for (*F*,*R*)-ODN1 which is protected by two phosphorothioate groups at its 5′-end and 21 hr in the case of (*F*,*R*)-ODN2 which lacks this protection. Therefore, the protections afforded by, on one hand, the hairpin against the 3′-exonucleases of serum and, on the other hand, two phosphorothioate residues against the 5′-exonucleases of the NIH-MDR-G185 lysates are still effective in living NIH-MDR-G185 cells. The two phosphorothioate at the 5′-end of (*F*,*R*)-ODN1 increase its protection of about a factor of six. The already high level of nuclease resistance of (*F*,*R*)-ODN2 is assigned to the location of fluorescein at its 5′-end.

3.2. Cellular ODN uptake

The impact of the delivery agents on the distribution of ODNs inside the NIH-MDR-G185 cells has been checked. Uptake of (*CH*,*F*)-ODN1 (the same as ODN1 conjugated to a cholesteryl group and a fluorescein at its 5′-end), and of (*F*,*R*)-ODN1 complexed with Superfect® or AMA has been observed by fluorescence imaging. In the first phase of internalization, i.e. after 5 hr incubation, (*CH*,*F*)-ODN1 is localized in membranes and in cytoplasm with a diffuse fluorescence pattern with perinuclear accumulation (Fig. 2(A1)). After a longer incubation time (24 hr), membranes are no longer labeled and fluorescent vesicles are observable besides the diffuse fluorescence of

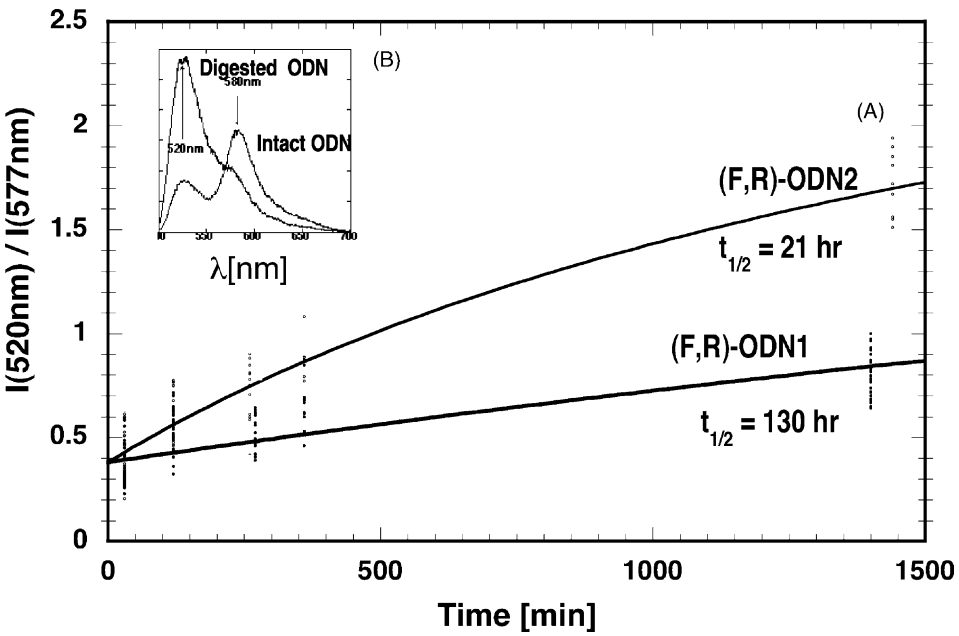

Fig. 1. (A) Kinetics of degradation of (*F*,*R*)-ODN1 and (*F*,*R*)-ODN2 in NIH-MDR-G185 cells. (B) Fluorescence spectra of intact and fully digested (*F*,*R*)-ODN1. $\lambda_{exc} = 488$ nm.

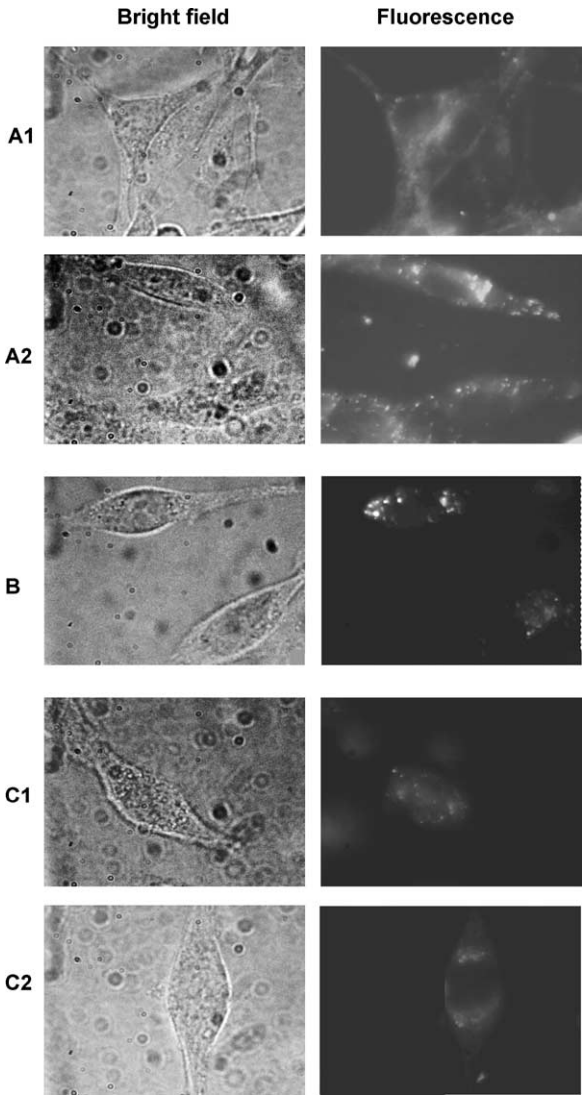

Fig. 2. Fluorescence imaging of (*CH*,*F*)-ODN1 after 5-hr incubation (A1) and 24-hr incubation (A2), (B) (*F*,*R*)-ODN1 internalized with Superfect® after 4-hr incubation; identical distribution was observed after 24-hr incubation, (C) (*F*,*R*)-ODN1 internalized with AMA after 4-hr incubation (C1) and 24-hr incubation (C2).

the cytoplasm and the perinuclear fluorescence accumulation (Fig. 2(A2)). Conjugation of ODN with lipophilic substituents as cholesterol enhances their ability to permeate the cell membrane [5]. The presence of fluorescent vesicles suggests that ODNs enter the cells not only by diffusion but also by endocytosis unless sequestration occurs after ODN uptake and these vesicules result from an exocytosis process.

In the case of internalization of (*F*,*R*)-ODN1 through Superfect® complexation, most of the cells have a punctuated perinuclear fluorescence beside diffuse cytoplasmic fluorescence (target for the FRET experiments) but some of them demonstrate intranuclear fluorescence. This is observed for both incubation times (4 and 24 hr) (Fig. 2(B)). This is consistent with previous observations [25] according which the intracellular distribution of ODNs vectorized by Superfect® is not time-dependent but function of the cell cycle. The ODNs would be rather localized in endocytosis vesicles during S and G1 phase. Alterations in the nuclear membrane during mitosis would allow ODN penetration into the nucleus.

After its vectorization with AMA, (*F*,*R*)-ODN1 led to a diffuse distribution mainly localized in the nucleus with some more intense speckles (Fig. 2(C1)). For a 24-hr incubation time, this fluorescence shifted towards the outside of cells (Fig. 2(C2)). Since AMA is a derivative of amphotericin B, it is expected to work in a similar way by creating transient membrane pores and favoring ODN uptake into cells [22]. Free ODNs fast diffuse into the nucleus [30]; the fluorescence of this one after a 4-hr incubation is thus not surprising. An exocytosis process could generate the fluorescent speckles and the ODN shift outside the nucleus.

3.3. P-glycoprotein expression

For each of the delivery systems, the antisense effect of ODN1 on the P-gp expression has been compared to that of the all-phosphorothioate antisense AS-ODN. In that purpose, Western blot analysis was carried out after a 48-hr treatment. This time has been shown to be sufficient to readily detect modifications in the level of the protein expression [5].

In the case a 5′-conjugated cholesteryl group favors the ODN internalization, the level of P-gp expression of NIH-MDR-G185 cells treated with (*CH*)-ODN1 has been compared to that of untreated resistant NIH-MDR-G185 cells (R), untreated sensitive NIH/3T3 cells (S), NIH-MDR-G185 cells treated with the full-phosphorothioate positive (antisense (*CH*)-AS-ODN) and negative (sense (*CH*)-S-ODN) controls (Fig. 3(A)). Sense (*CH*)-S-ODN control did not reduce P-gp expression which was the same as in resistant (R) cells. This expression was not detectable in sensitive (S) cells. It was drastically reduced after treatment with the positive control (*CH*)-AS-ODN (only 2% P-gp remaining) and with the minimally modified phosphodiester ODN (*CH*)-ODN1 (6%).

Superfect® has been used following two protocols. With protocol (1), the cells were incubated for 48 hr (like for both other internalization systems) with the complex ODN-Superfect®. This led to a strong decrease of the P-gp expression whatever the ODN (results not shown). For instance this expression was only 30% with the control ODN3 (minimally protected with sense orientation). This was attributed to Superfect® cytotoxicity for long incubation times. With protocol (2), Superfect® was eliminated after a 4-hr incubation. In that case, the all-phosphorothioate AS-ODN had a strong antisense effect (15% P-gp remaining) whereas the minimally modified ODN1 was without effect (Fig. 3(B)).

When AMA allows the ODN internalization, the sense control ODN3 has no effect whereas AS-ODN and ODN1 both lead to decrease of the P-gp expression (Fig. 3(C)).

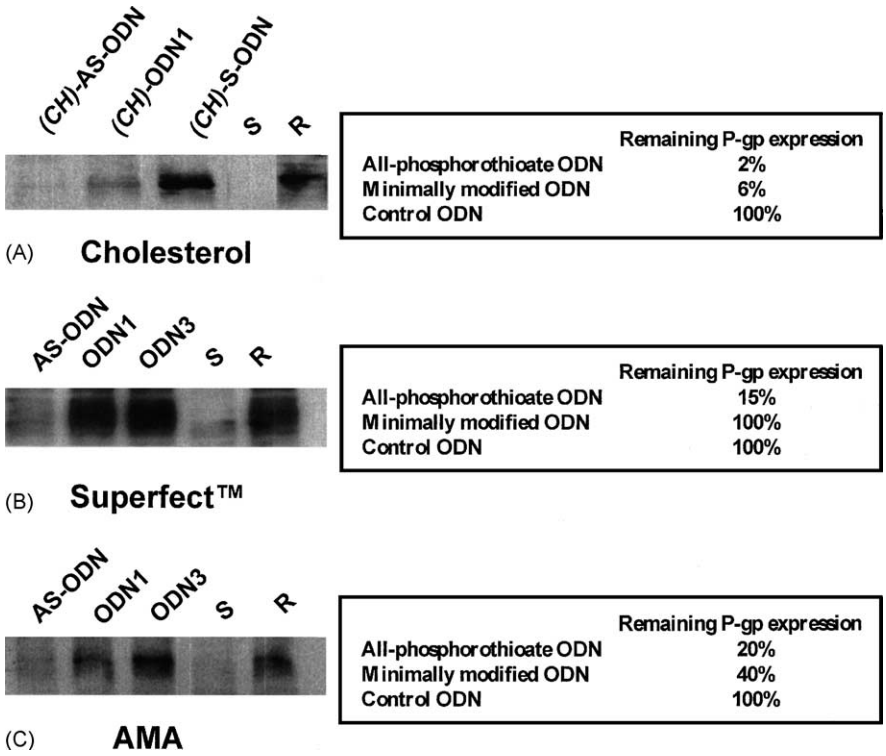

Fig. 3. Detection of total cellular P-gp by Western blotting. Equal amounts of cell lysate protein (20 μg) were run on 7.5% SDS–PAGE gels, transferred on PVDF membranes and detected with c219 monoclonal antibody and enhanced chemiluminescence. Cells had been treated with different ODN internalized with (A) a cholesterol conjugate, (B) Superfect®, (C) AMA. Lane R: lysate from untreated resistant NIH-MDR-G185 cells, lane S: lysate from untreated sensitive NIH/3T3 cells, other lanes: lysate from resistant NIH-MDR-G185 cells treated with the specified ODN.

The residual amounts are, respectively, 20 and 40%. There is antisense effect even if it is smaller than with (*CH*)-AS-ODN and (*CH*)-ODN1.

4. Discussion

Three delivery systems have been checked in this work: a 5′-conjugated cholesteryl group, a commercial dendrimer Superfect® and an amphotericin B derivative, AMA. In order to get comparable results, the amount of ODN, when expressed in concentration terms, was the same whatever the delivery system or the chemical nature of the ODN. One micromolar has been shown to be a good compromise between effective antisense effect and non-specific results [5] in the case of (*CH*)-AS-ODN. Satisfactory antisense effects have already been described with Superfect® [25] or AMA [22] used in these conditions.

The ODN delivery efficiency has been shown by fluorescence imaging. Differences in the ODN distribution inside the cells have been evidenced. For long incubation times, diffuse and punctuated fluorescence are observed in each case. Diffuse fluorescence is mainly located in the cytoplasm for cholesteryl-ODN and in the nucleus when Superfect® or AMA are used. Punctuated fluorescence has been assessed to endocytosis vesicles (Superfect®) or exocytosis vesicles (AMA). Both possibilities have been suggested for (*CH*)-ODNs.

There is not straightforward correlation between ODN distribution and antisense activity. The positive control, i.e. the all-phosphorothioate AS-ODN is effective for all the delivery systems. Most of the P-gp expression is extinct when using a conjugated cholesteryl group (2% remaining expression); only 15 and 20%, respectively, remain with Superfect® or AMA. The antisense activity of AS-ODN had already been demonstrated for its cholesterol conjugate (*CH*)-AS-ODN and to a lesser extent by using Lipofectin as uptake enhancer agent [5] as well as for a peptide-AS-ODN conjugate [31]. In all cases, the negative control ((*CH*)-S-ODN or CH3) is without effect on P-gp. The antisense activity of the minimally modified ODN1 has been demonstrated in this work for only two internalization systems: a 5′-conjugated cholesteryl group and AMA. It is ineffective when internalized with Superfect®. With (*CH*)-ODN1, only 6% P-gp expression remains, to be compared to the 2% measured in the case of the starting antisense (*CH*)-AS-ODN. With AMA, 40% P-gp expression remains after treatment with ODN1, only 20% with AS-ODN. In both cases, the antisense effect is better after treatment with the all-phosphorothioate AS-ODN. While this difference is significant in the case AMA is used, it is negligible for the cholesterol conjugates. Satisfactory antisense effects have already been obtained with ODNs stabilized with a minimum amount of phosphorothioate linkages and internalized with lipocationic uptake enhancers [16] or the addition of an hydrophobic tail at the 3′-end [32]. However,

ODN1 is the less modified phosphodiester antisense ODN since (i) it is protected at its 3′-end by the self-forming hairpin, (ii) it has only two phosphorothioate linkages at its 5′-end, which is made acceptable by the fact that the NIH/3T3 cells have a negligible endonuclease activity. The best antisense effect obtained with AS-ODN as compared to ODN1 may result from the shift of three bases on the target *mdr1* mRNA (ODN1 extended on its 5′-side to compensate for the loss of hybridization at the 3′-end of the hairpin, see Section 2). It is well known that the shift of some bases on the mRNA target can modify the antisense efficiency in a negative as well as in a positive way [33].

ODN1 is ineffective when internalized with Superfect®. Imaging experiments (Fig. 2) prove it is not the result of lower internalization of ODN, as compared to internalization with the other delivery systems. This is on no account attributable to fast nuclease degradation of ODN1 in these conditions. Indeed, the stability inside the cells of the minimally modified ODN1 had been controlled by using Superfect® as delivering agent (see cellular ODN stability in Section 3). We find once more that a chosen carrier leads or not to antisense effect, depending on the chemical nature of the ODN [21].

Even if it is not effective with all delivering systems, to get an almost all-phosphodiester antisense oligonucleotide efficient against an overexpressed gene as *mdr1* was an important challenge. The enhancement of the binding affinity to RNA of such an ODN and especially the reduction of non-antisense effects as non-specific binding to proteins are of importance in the case relatively high ODN concentrations are used, which can prove to be necessary in the case of overexpressed genes.

Acknowledgments

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