Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 403–407
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Increased uptake of antisense oligonucleotides by delivery as double stranded complexes

Anna Astriab-Fisher[a], Michael H. Fisher[a], Rudy Juliano[a], Piet Herdewijn[b,*]

[a]*Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA*
[b]*Laboratory for Medicinal Chemistry, Katholieke Universiteit Leuven, Minderbroedersstraat 10, Leuven, Belgium*

Received 21 January 2004; accepted 19 March 2004

Abstract

Antisense oligonucleotides were potentially very powerful tools to modulate gene expression. Progress in chemical modification of oligonucleotides to enhance the strength and stability of interaction, without loosing specificity, has made the antisense strategy very attractive for therapeutic manipulation of the gene expression. However, pharmacological applications of oligonucleotides have been hindered by the inability to effectively deliver these compounds to their sites of action within cells. In this study we evaluated a new concept for antisense delivery in cellular systems. We have shown that formation of a duplex between the active oligonucleotide (with a chemically modified backbone) and an easily degradable complementary oligodeoxynucleotide in the presence of Lipofectamine 2000 leads to better intracellular uptake and more significant pharmacological effect of the active oligonucleotide. To evaluate our approach we targeted the MDR1 gene, which coded for *P*-glycoprotein, a membrane ATPase associated with multi-drug resistance in tumor cells. The 2′-*O*-methyl gapmer antisense RNA (active component of the duplex) was complementary to a site flanking the AUG of the MDR1 message. Effective inhibition of *P*-glycoprotein expression was attained with sub-micromolar concentrations of duplexes under serum-replete conditions and was much stronger than with traditional single stranded antisense delivery. The results obtained suggested that double stranded delivery could provide a simple and effective means for enhancing cell uptake of pharmacologically active oligonucleotides.

Keywords: Antisense; Drug resistant; Drug delivery; Duplex oligonucleotides; *P*-glycoprotein; Gapmers

1. Introduction

Antisense oligonucleotides have proven to be powerful tools for selective regulation of gene expression and are being evaluated intensively as potential therapeutics. Currently there are around 30 different oligonucleotides being tested in about 40 different clinical trials, mostly in Phase II [1,2]. However, so far, the FDA, has approved only one antisense-based drug [3]. There are several reasons why applying antisense strategy in the clinic seems to be very challenging task. Besides specificity and stability of oligonucleotides (both addressed by various chemical modifications) in second and third generation oligonucleotides [4,5], crucial for successful clinical application is efficient delivery of the compounds to their sites of action within cells. It has been demonstrated that, at the best, no more than 6–12% of oligonucleotides targeting an RNA sequence are efficient at forming the duplex necessary for the antisense effect [6]. Therefore a strategy that improves uptake and optimizes intracellular distribution to key targets could be very important for experimental and therapeutic applications. In the present study we evaluate a strategy of delivering chemically modified antisense sequences complexed with a shorter unmodified sense sequence as a duplex. The antisense sequence is chemically modified to increase stability against enzymatic degradation. A gapmer was used in order to maintain RNaseH activity involved in the antisense mechanism. The sense sequence is an unmodified oligodeoxynucleotide which is prone to degradation by phosphodiesterases. It was reasoned that, once taken up by cells, the sense oligodeoxynucleotide should be easily degraded and lib-

Abbreviations: MDR, multi-drug resistance; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; TAMRA, tetramethylrhodamine

* Corresponding author. Tel.: +32 16 337388; fax: +32 16 337340.
E-mail address: Piet.Herdewijn@rega.kuleuven.ac.be (P. Herdewijn).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.03.040

erate the antisense oligonucleotide for hybridization with its RNA target.

Using this approach, and targeting the *MDR1* gene, we demonstrated significantly stronger inhibition of *P*-glycoprotein expression compared to the effect with traditional delivery of a single strand oligonucleotide. *P*-glycoprotein is an ATP-driven *trans*-membrane pump that can expel a wide variety of drug molecules from cells [7,8]. Overexpression of *P*-glycoprotein in tumor cells confers a multi-drug resistance (MDR) phenotype that can impede the effectiveness of cancer chemotherapy [9,10]. Thus, a number of approaches have been devised to attempt to reverse the MDR phenotype, either through inhibition of *P*-glycoprotein function [11], or through reduction in its expression using molecular techniques [12,13]. We and others have previously sought to inhibit expression of *P*-glycoprotein message and protein using antisense oligonucleotides [14–18], but with only moderate success. The new strategy we present in this study significantly improves the delivery and pharmacological effects of antisense oligonucleotides targeted to MDR1 as compared to the traditional single stranded approach.

2. Material and methods

2.1. Cells

NIH 3T3 cells transfected with a plasmid containing the human *mdr1* gene (pSKl MDR) were gift from M.M. Gottesman [19]. The MDR-3T3 cells were grown in DMEM (Dulbecco minimal cultured media) medium containing 10% FCS (fetal calf serum).

2.2. Oligonucleotides

2′-*O*-methyl gapmer RNA (gap phosphorothioate DNA residues indicated in bold) 20-mer anti-MDRl 5′-d(CCA-UCC-**CGA-CCT-CGC**-GCU-CC)-3′ and mismatch 5′-d(CCA-UAC-**CAA-CAT-CAC**-GCU-CC)-3′ oligonucleotides with and without FITC marker on the 5′ end were synthesized. Regular, unmodified oligodeoxynucleotides complementary to the antisense sequence: including 14-mer-5′-d (G-CGC-GAG-GTC-GGG-A)-3′, 16-mer-5′-d(AG-CGC-GAG-GTC-GGG-AT)-3′, and 18-mer-5′-d(GAG-CGC-GAG-GTC-GGG-ATG)-3′, with and without TAMRA fluorescence marker were from Sigma Genosys and from the Oligonucleotide Facility of the UNC Linberger Cancer Center.

2.3. Treatment of cells with antisense oligonucleotides

The experimental protocols were similar to those previously described [15]. Briefly, MDR 3T3 cells were grown in 162 mm flasks to 95% confluency and then seeded into 100 mm dishes at 5×10^6 per dish in 10% FCS/DMEM and incubated 24 h. The cells were washed twice with PBS. Duplexes between sense and antisense strand were prepare in PBS by heating the solutions to 90 °C and then cooling to room temperature for 15 min. Complexation of the double stranded and single stranded antisense oligonucleotides with the cationic lipid Lipofectamine 2000 (Invitrogen) was done according to standard procedure. The compounds with cationic lipids were mixed in 10% FCS/DMEM and incubated with cells at 37 °C overnight. Oligonucleotide concentrations ranged from 0.025 to 0.1 μM in various experiments (see Legends).

2.4. Uptake and sub-cellular distribution of peptide–oligonucleotide conjugates

Cells treated with fluorescent oligonucleotides were harvested by trypsinization and then examined by fluorescence microscopy or by flow cytometry. For microscopy, cells resuspended in 1 ml 10% FCS/DMEM were incubated for 6 h on fibronectin-coated cover slips. The fluorescence patterns were analyzed on an Olympus Confocal FV300 fluorescent microscope with 60× oil-immersion objective. Images were captured with a slow scan charge-coupled device 3CCD Video Camera System interfaced to a PC using the Olympus Fluoview Software. For flow cytometry analysis cells were resuspended in 500 μl PBS and measured for the accumulation of TAMRA marker using a Becton Dickinson flow cytometer with Cicero software (Cytomation, Inc.).

2.5. Toxicity of antisense treatment

The cytotoxicity of the various treatments used in the oligonucleotide experiments was evaluated by cell enumeration. Briefly, the cells treated with antisense oligonucleotides in single or double stranded format were harvested by trypsinization at different time points after treatment and then analyzed by using an electronic particle counter (Elzone 80, Micro-meritics). Cell counts were normalized to untreated control.

2.6. Pharmacological effects of antisense treatment

Analysis of the pharmacological effects of oligonucleotides was based on a previously described assay [14] involving antisense inhibition of *P*-glycoprotein expression in mouse MDR 3T3 cells. The cell surface expression of *P*-glycoprotein was determined by immunostaining and quantitation by flow cytometry, as described [14,15]. Briefly, the MRK16 anti-*P*-glycoprotein antibody (Kamiya Biochemicals), which is directed against an external epitope, was employed as the primary antibody. After the incubation, the cells were washed twice in 10% FBS/PBS. The level of Phycoerythrin (PE) conjugated goat anti-mouse IgG (Sigma) was used as the second antibody. *P*-glycoprotein expression in viable cells (viability deter-

mined by light scatter) was quantitated using the Summit V3.0 software application (Cytomation Inc.) on a Becton Dickinson flow cytometer.

3. Results and discussion

The antisense strategy is a powerful approach to inhibiting gene expression and the method often works quite well in cell culture systems. Several chemical modifications are known to increase the thermal stability of the duplex between the antisense oligonucleotide and its target RNA, and to increase their resistance to enzymatic degradation. Therapeutic applications, however, are hampered by the low intracellular uptake of the antisense oligonucleotide and by the lack of methods to target the oligo to specific cells (or organs). Increased delivery can be obtained by using cationic lipids or by using oligonucleotide conjugates (with groups like cholesterol, cell-penetrating peptides, or ligands for cellular receptors) [20–22]. The disadvantage of the latter method is the covalent attachment of the conjugate group to the oligonucleotide, which may influence intracellular distribution, and hybridization to the target RNA.

Antisense oligonucleotides are usually delivered as single stranded molecules. Here we investigated the possibility of delivering a chemically modified antisense oligonucleotide together with its sense sequence in the DNA-form, using cationic lipids as an additional delivery agent. The sense-DNA is labile to phosphodiesterase degradation and should be hydrolyzed easily in the cell, so that the enzymatically stable antisense oligo can exert its function in the cytoplasm and the nucleus (Fig. 1). Neither part of the delivery system (sense DNA and cationic lipid) is covalently bond to the antisense oligo. The sense-DNAs have different lengths, as the stability of the duplex with the antisense oligo may influence the biological activity.

2′-*O*-methyl gapmer RNA and complementary unmodified oligodeoxynucleotides strand were hybridized together, complexed with Lipofectamine 2000 and introduced to the cells. As a control single stranded artisense 2′-*O*-methyl gapmer RNA with Lipofectamine 2000 was used. We used fluorescently labeled antisense, but not sense strand to evaluate uptake efficiency and compare single and double stranded delivery. We also evaluated the optimal length of sense strand, ranging from a 14-mer to a 18-mer. As presented in Fig. 2, the uptake of the double stranded oligonucleotide is significantly higher than for the single stranded. The sense strand 16- and 18-mers seem to be the best with regard to promoting uptake. The difference between single stranded and double stranded delivery was four fold after 3 h incubation and reached 6 times after 24 h incubation. There was no significant difference in uptake performance between 16- and 18-mer duplexes.In parallel, using the same time points used for the uptake analysis, we evaluated the toxicity of double stranded delivery. We counted the number of live cells after 3, 12 and 24 h and normalized the data to untreated control. As shown in Fig. 3, the proposed strategy does not have a negative impact on the number of living cells, rather some increase in the number of living cells for both 16- and 18-mer duplexes was observed.

To show actual cellular localization of antisense and sense strand after double stranded delivery we analyzed cells after 24 h of incubation of the 18-mer sense/20-mer antisense duplex (also with Lipofectamine 2000). We labeled the antisense strand with fluorescein and the sense strand with Texas Red and analyzed the cell images using a confocal microscope. As presented in Fig. 4 (and as expected) there is a strong green fluorescence from the antisense strand in the nucleus. The sense strand was not transported to the nucleus as evidenced by a lack of fluorescence in the nucleus. The red signal from the sense strand stays in the cytoplasm and it is most probably still in

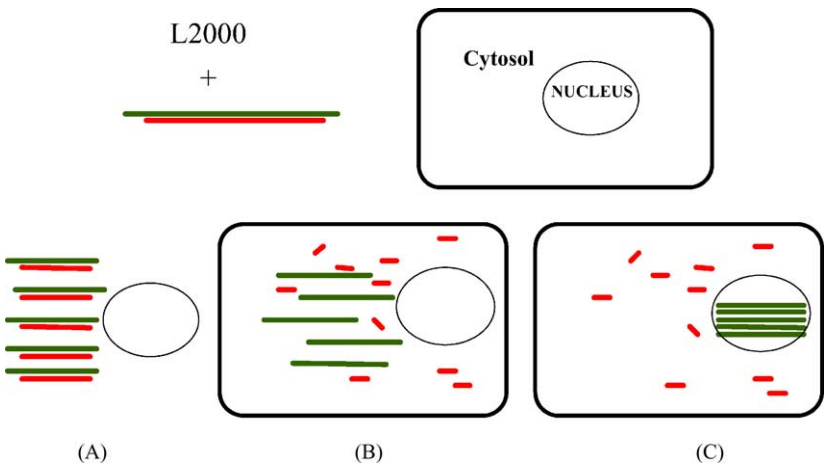

Fig. 1. The concept of double stranded delivery of antisense oligonucleotides. (A) Delivery as double stranded hybrids complexed with Lipofectamine 2000 into the cytoplasm (unmodified strand red, modified strand green). (B) Enzymatic degradation of the non modified strand. (C) Transport of the modified strand into the nucleus.

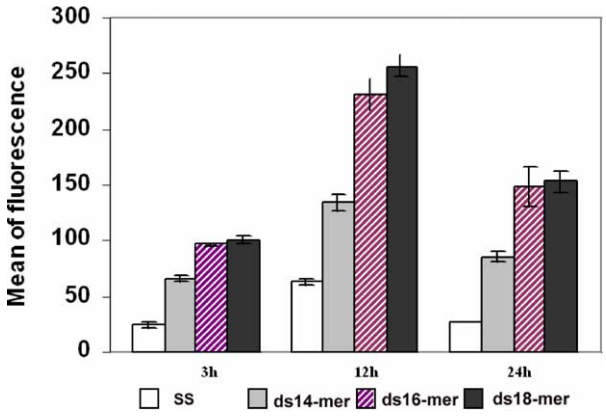

Fig. 2. Flow cytometry analysis of the cellular uptake of the antisense oligonucleotides in single and double stranded format (ss, single stranded 20-mer antisense; dsl4-mer, double stranded duplex antisense 20-mer/sense 14-mer DNA; dsl6-mer, double stranded duplex antisense 20-mer/sense 16-mer DNA; dsl8-mer, double stranded duplex antisense 20-mer/sense 18-mer DNA).

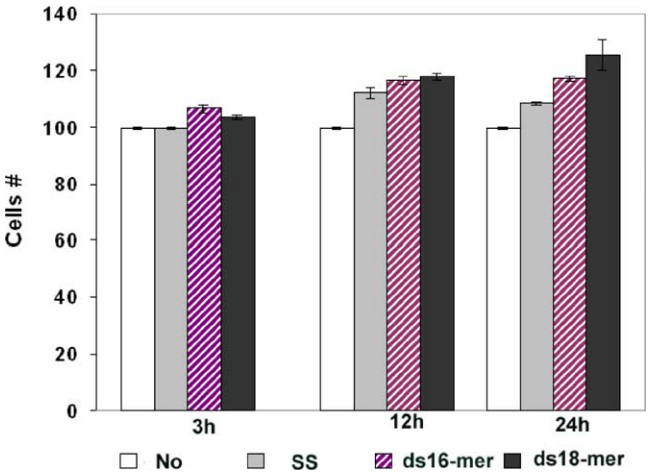

Fig. 3. Toxicity of antisense oligonucleotides in single and double stranded format (No, no treatment; ss, single stranded 20-mer antisense; ds 16-mer, double stranded duplex antisense 20-mer/sense 16-mer DNA strand; ds 18-mer, double stranded duplex antisense 20-mer/sense 18-mer DNA).

duplex form with antisense strand (however, this assumption needs to be evaluate in the future studies) as green and red spots are co-localized.

As final tests of double stranded delivery approach, we evaluated also the double stranded mismatch duplex and the inhibition of the *P*-glycoprotein expression by single and double stranded oligonucleotides. As shown in Fig. 5, the mismatch reference duplex has no activity at all on *P*-glycoprotein expression (compared to the correct double stranded antisense duplex). The normalized dose response inhibition of the *P*-glycoprotein expression is presented in Fig. 6. As shown, there is a significantly greater reduction of *P*-glycoprotein expression in the samples treated with the double-stranded complexes as opposed to the single stranded samples, particularly at low concentrations (0.025 and 0.05 μM). These results correlate well with uptake experiments and indicated that double stranded delivery leads to better uptake of the antisense oligonucleotides and as a result stronger biological response to antisense treatment.

From these experiments, we conclude that delivery of a stable antisense oligonucleotide together with its complementary DNA sequence in unstable form (and in the presence of Lipofectamine, 2000) increases the uptake and the biological activity of the antisense oligonucleotide. This approach may provide a simple and effective means for enhancing the delivery of pharmacologically active oligonucleotides in cells. At this point the mechanism underlying this effect is unknown. It is not clear if increased initial uptake, reduced release from the cell, or increased stability is the key factor for explaining the effect of this formulation; these issues will be pursued in subsequent studies together with its applicability in vivo. Also the role of the cationic lipid and the possibility of chemically modifying the sense strand with a reporter

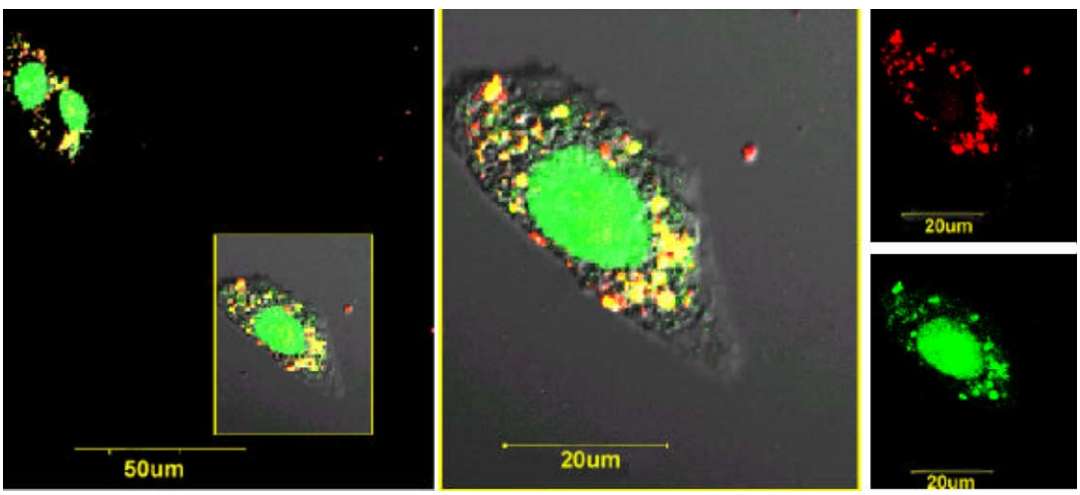

Fig. 4. Confocal Microscopy analysis of cellular distribution of the antisense 20-mer/sense 18-mer duplex 24 h after treatment (sense strand—red, antisense strand—green).

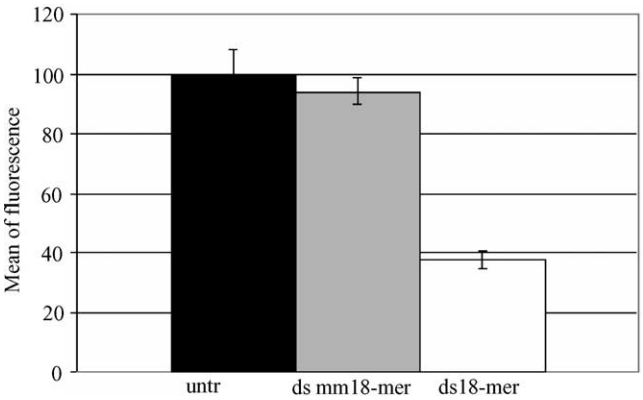

Fig. 5. Flowcytometry analysis of specificity of inhibition of the *P*-glycoprotein expression. Double stranded delivery approach at 0.1 μM concentration (untr, untreated; ds mm 18-mer, double stranded duplex, antisense mismatch 20-mer with sense 18-mer DNA; ds 18-mer, double stranded duplex antisense 20-mer with sense 18-mer DNA).

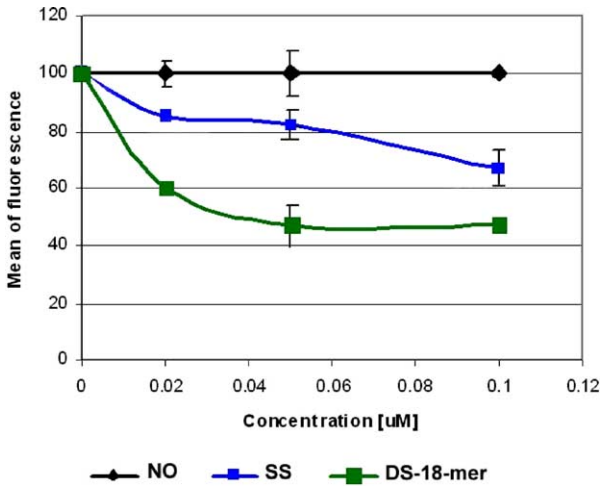

Fig. 6. Concentration-dependent inhibition of *P*-glycoprotein expression using antisense oligonucleotide (No, no treatment; ss, single stranded 20-mer antisense; ds 18-mer, double stranded duplex antisense 20-mer/sense 18-mer DNA).

group will be envisaged. In view of current results, a question may be asked as to whether the somewhat higher biological activity of siRNA (as compared to antisense) may be partly due to a better delivery process, since siRNA is also delivered as a duplex. An interesting question coming out of this research deals with the explanation of the biological activity of siRNA when the antisense strand is chemically modified. Such molecules may also be considered as antisense oligonucleotides, delivered with their biological degradable sense-RNA, and questions should be asked about the mode of action of such duplexes.

Acknowledgments

This work was supported by grants of the Katholieke Universiteit Leuven (IDO 02/014 and the GOA 2002/13). The authors thank Chantal Biernaux for editorial help.

References

[1] Opalinska J, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. Nature Rev Drug Discov 2002;1:503–14.
[2] Agrawal S, Kandimalla ER. Antisense therapeutics: is it as simple as complementary base recognition? Mol Med Today 2000;6:72–81.
[3] Roehr B. Fomivirsen approved for CMV retinitis. J Int Assoc Physicians AIDS Care 1998;4:14–6.
[4] Stein CA. Keeping the biotechnology of antisense in context. Nat Biotechnol 1999;17:209.
[5] Kutteck J. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 2003;270:1624–8.
[6] Herdewijn P. Heterocyclic modifications of oligonucleotides and antisense technology. Antisense Nucleic Acids Drug Dev 2000;10:297–310.
[7] Licht T, Pastan I, Gottesman M, Herrmann F. *P*-glycoprotein-mediated multidrugresistance in normal and neoplastic hematopoietic cells. Ann Hematol 1994;69:159–71.
[8] Bradley G, Ling V. *P*-glycoprotein, multidrug resistance and tumor progression. Cancer Metastasis Rev 1994;13:223–33.
[9] Chabner BA, Wilson W. Reversal of multidrug resistance. J Clin Oncol 1991;9:4–6.
[10] Dalton WS. Is *P*-glycoprotein a potential target for reversing clinical drug resistance? Curr Opin Oncol 1994;6:595–600.
[11] Dalton WS, Crowley JJ, Salmmon SS, Grogan TM, Laufman LR, Weiss GR, et al. A phase III randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma. Cancer 1995;75:815–20.
[12] Scanlon KJ, Ishida H, Kashani-Sabet M. Ribozyme-mediated reversal of the multidrug resistant phenotype. Proc Natl Acad Sci: USA 1994;91:11123–7.
[13] Kobayashi H, Dorai T, Holland JF, Ohnuma T. Reversal of drug sensitivity in multidrug-resistant tumor cells by an MDR1 (PGY1) ribozyme. Cancer Res 1994;54:1271–5.
[14] Alahari SK, Dean NM, Fisher MH, DeLong R, Manoharan M, Tivel KL, et al. Inhibition of expression of the multidrug resistance-associated *P*-glycoprotein by phosphorothioate and 5′ cholesterotconjugated phosphorothioate antisense oligonucleotides. Mol Pharmacol 1996;50:808–19.
[15] Alahari SK, DeLong R, Fisher MH, Dean NM, Viliet P, Juliano RL. Novel chemically modified oligonucleotides provide potent inhibition of *P*-glycoprotein expression. J Pharmacol Exp Ther 1998;286:419–28.
[16] Corrias MV, Tonini GP. An oligomer complementary to the 5′ end region of MDR1 gene decreases resistance to doxorubicin of human adenocarcinoma-resistant cells. Nucleic Acids Res 1992;12:1431–8.
[17] Efferth T, Volm M. Modulation of *P*-glycoprotein-mediated multidrug resistance by monoclonal antibodies, immunotoxins or antisense oligodeoxynucleotides in kidney carcinoma and normal kidney cells. Oncology 1993;50:303–8.
[18] Cucco C, Calabretta R. In vitro and in vivo reversal of multidrug resistance in a human leukemia-resistant cell line by MDR1 antisense oligodeoxynucleotides. Cancer Res 1996;56:4332–7.
[19] Kane SE, Reinhard DH, Fordis CM, Pastan I, Gottesman MM. A new vector using the human multidrug resistance gene as a selectable marker enables overexpression of foreign genes in eukaryotic cells. Gene 1989;84:439–46.
[20] Gait MT. Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues. Cell Mol Life Sci 2003;60:844–53.
[21] Hope MJ, Mui B, Ansell S, Ahkong Q-F. Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs. Mol Membr Biol 1998;15:1–14.
[22] Manoharan M. Oligonucleotide conjugates as potential antisense drugs and improved uptake, biodistribution, targeted delivery and mechanism of action. Antisense Nucleic Acid Drug Dev 2002;12:103–28.