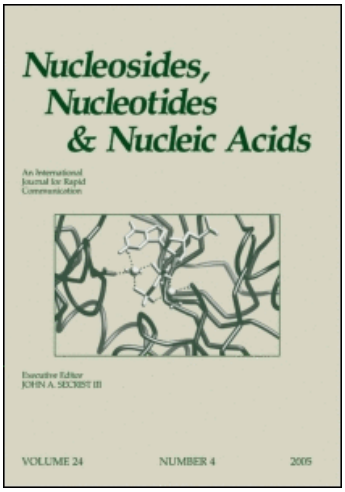

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DEVELOPMENT OF A RAPID SCREENING SYSTEM TO TEST ANTISENSE ODN MODIFICATIONS AND CARRIERS

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ABSTRACT : We developed a rapid screening system, using two reporter genes under the control of the same promoter, to identify the biological activity of modified or/and vectorized antisense oligodeoxynucleotides (ODNs). The ability of a dendrimeric structure and a monocationic cholesterol derivative to enhance ODN cellular uptake was previously investigated by fluorescence analysis. Then, the assay system was validated through investigating the effect of both vectors on antisense ODN efficiency.

INTRODUCTION

Antisense ODNs are useful tools for studying gene function and expression and they are potential therapeutic agents especially for the treatement of viral infections, malignant and inflammatory diseases[1,2,3]. But their use is limited by problems related to ODN stability in biological media, ODN cellular uptake and transport to RNA targets[4,5]. Furthermore, the search for chemical modifications and new ODN carriers able to enhance the biological activity of ODNs is often hampered by the absence of a rapid assay system. We developed a rapid screening system which measures the transient expression of two reporter genes, one used as target, the other one as control and vice versa. This system was validated by testing the effectiveness of two commercial transfecting reagents (a polyamidoamine dendrimer[6,7] and a monocationic cholesterol derivative) in enhancing the biological activity of antisense ODNs. A sequence-specific inhibition of 76% was obtained

for one reporter gene with a phosphodiester ODN containing two phosphorothioate groups at each end and complexed to the dendrimer vector. We also found that biological effects obtained were closely correlated with the observations done by fluorescence analysis with vectorized 3'- and 5'-FITC labelled phosphodiester ODN.

MATERIALS AND METHODS

Biological assay system : The day before cell transfection, HeLa cells were seeded on 6-well plates to obtain 60-80% of confluency. The reporter genes (pEGFP-N1 and pCMVß-gal (Clontech)) were co-transfected simultaneously into cells with a given transfecting reagent (SuperFect™ (Qiagen) or DAC-30™ (Eurogentec)). The ODNs (Eurogentec) complexed with the same transfecting reagent were added to cells, either at the same time or 2 h before the plasmids. ß-galactosidase (ß-gal) and green fluorescent protein (GFP) expression was measured in the cell lysate, 16 h after plasmid co-transfection. Cells were incubated with Reporter Lysis Buffer (Promega), scraped and centrifuged. For the ß-gal assay, the supernatant was mixed with a CPRG reaction buffer in a 96-well plate and incubated at 37°C for 20 minutes. ß-gal activity was then detected by reading the absorbance at 570 nm using an automatic reader spectrophotometer. The amount of GFP was directly measured on the remaining supernatant with a spectrofluorimeter using a 488 nm excitation wavelength and reading the emission at 507 nm.

Flow cytometry analysis : The day before FITC-ODN transfection, cells were seeded on 24-well plates. 3' and 5' labelled FITC-ODN complexed to the dendrimer vector (SuperFect™) or to the monocationic cholesterol derivative (DAC-30™) were incubated with cells. Mean cellular fluorescence intensities for 10,000 viable cells were determined on a Coulter EPICS Elite dual-laser flow cytometer. Dead cells were excluded by two means : through forward and side scatter gatings and by using propidium iodide.

Confocal microscopy analysis : The day before FITC-ODN transfection, cells were plated on Lab-Teck chambered coverglass (4 chambers). After incubation with vectorized 3' and 5' labelled FITC-ODN, cells were scanned with a Meridian ACAS-570 (Meridian Instruments).

RESULTS AND DISCUSSION

Flow cytometry and confocal microscopy analyses showed insignificant fluorescence when cells were incubated with the FITC-ODN alone (the FITC-ODN sequence is the

phosphodiester analog of IN-GFP antisense, see below). Results obtained by confocal microscopy showed that the fluorescence observed by flow cytometry corresponded to ODN uptake. Then, as shown in Fig. 1, both transfecting reagents promoted FITC-ODN uptake in HeLa cells. Furthermore, the number of fluorescent cells and the cellular fluorescence intensity were greater with the dendrimer-FITC-ODN complex. The fluorescence observed inside cells corresponds to intact the FITC-ODN because : (i) no fluorescence was detected inside cells with FITC, SuperFect™, DAC-30™ or FITC-ODN alone, (ii) FITC alone complexed to SuperFect™ or DAC-30™ penetrated into cells but the fluorescence distribution exhibited was completely different from the one observed with the FITC-ODN, (iii) no significant ODN degradation was observed when the FITC-ODN, complexed to SuperFect™ or DAC-30™, was extracted from HeLa cells after 16 h of incubation and analysed by PAGE.

The screening system consisted of two different reporter genes coding for readily measurable proteins (ß-galactosidase (ß-gal) and green fluorescent(GFP) proteins). These reporter genes were under the control of the same promoter to avoid possible bias resulting from ODN interaction with the promoter region. In our study, four 18-mer antisense ODNs were respectively targeted on the initiation codon (**AUG-βgal** : $T_{(S)}A_{(S)}A$ ACG ACA TGG TGA $C_{(S)}T_{(S)}T$; **AUG-GFP** : $T_{(S)}G_{(S)}C$ TCA CCA TGG TGG $C_{(S)}G_{(S)}A$) or an internal coding region (**IN-βgal** : $T_{(S)}G_{(S)}G$ TAG CGA CCG GCG $C_{(S)}T_{(S)}C$; **IN-GFP** : $G_{(S)}A_{(S)}G$ CTG CAC GCT GCC $G_{(S)}T_{(S)}C$) of each of the reporter genes. A double mismatched ($G_{(S)}A_{(S)}G$ CT<u>C</u> CAC GC<u>A</u> GCC $G_{(S)}T_{(S)}C$), a scrambled ($T_{(S)}C_{(S)}C$ GCC CTG AGC TGA $G_{(S)}C_{(S)}G$) and the reverse sequence of IN-GFP antisense ODN were tested as controls for antisense sequence-specificity. In order to enhance ODN resistance to nucleases, two phosphorothioate groups were introduced at both 3' and 5' ends. To evaluate the inhibitory effect of the ODNs, a first ratio between protein activities was measured in the presence of each ODN (R_{ODN}). In order to eliminate possible variations in gene expression due to a polyanionic effect caused by any short nucleic acid sequence, a second ratio between protein activities (R_C) was determined in the presence of a random ODN. The proportion between the above-described ratios (R_{ODN}/R_C) allowed us to determine the inhibitory activity of ODN. Thus, the non-targeted gene served as an internal control for cytotoxic effects and non-sequence-specificity of the ODNs. No inhibition was observed when the ODNs were not vectorized. Sequence-specific inhibition

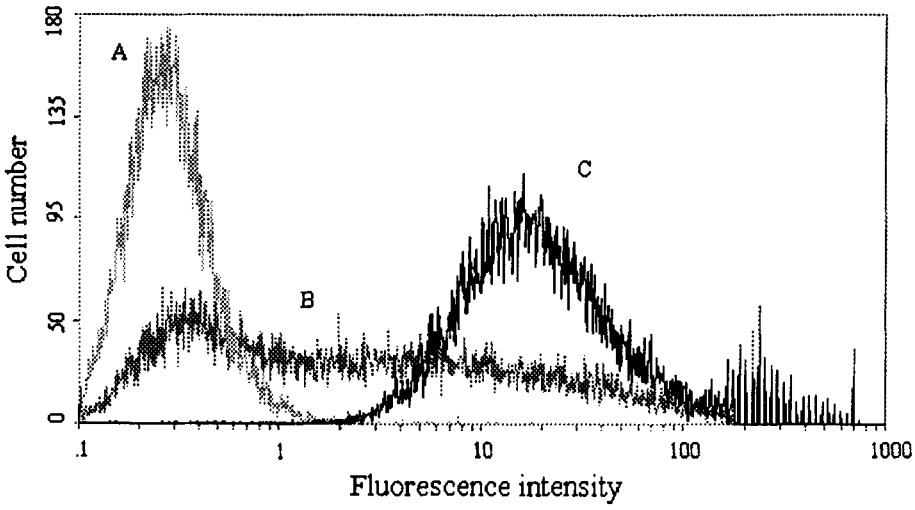

FIG. 1 : Comparison of cellular fluorescence intensity
Uptake of vectorized FITC-ODN was determined by flow cytometry after 16 h of incubation on HeLa cells. A : control without FITC-ODN ; B : FITC-ODN complexed to Dac 30™; C : FITC-ODN complexed to Superfect™.

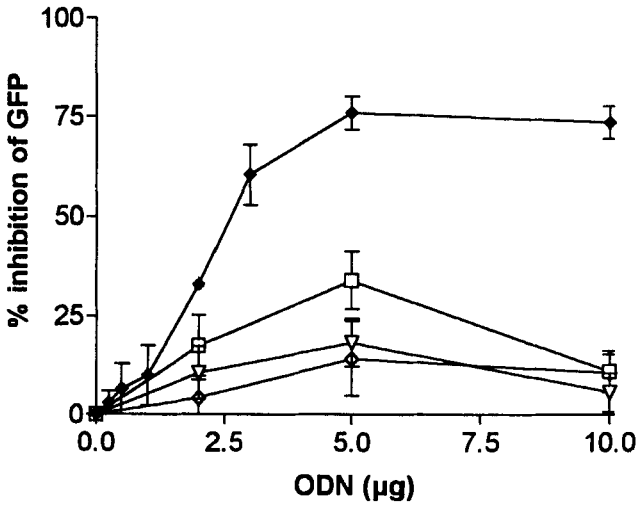

FIG. 2 : Inhibition of GFP expression by vectorized ODNs
Different quantities of IN-GFP ODNs (antisense and controls) were complexed to SuperFect™ and incubated 16 h with HeLa cells co-transfected with GFP and β-gal plasmids. The IN-GFP antisense (♦) and the IN-GFP controls (double mismatch (□), reverse (∇) and scramble (◊)) were added to cells 2 h before the plasmid co-transfection.

of the reporter genes was far greater when Superfect™ was used as the carrier than when Dac 30™ was used (76% versus 27% of GFP inhibition with 5µg of IN-GFP antisense) and these differences in biological effects were closely correlated with the differences observed in the uptake of the vectorized FITC-ODN. The ODNs targeting an internal coding region of each gene (IN-ODNs) were more efficient that those targeting the initiation codon (AUG-ODNs). Indeed, when 5 µg of ODNs were complexed to SuperFect™, the inhibition of their respective targets obtained with IN-ß-gal and IN-GFP ODNs was 54% and 76%, respectively, and only 16% and 49% with AUG-ß-gal and AUG-GFP ODNs (S.E. ≅ 8%). These results confirm that the initiation codon is not always the best target[8]. Furthermore, the IN-ODN against the GFP reporter gene seemed to be more active than the IN-ODN against the β-gal plasmid. This probably reflects different degrees of accessibility of targeted sites in mRNAs, a parameter which is very important, yet difficult to predict before cell experiments. Different quantities of the antisense ODN giving the best result (IN-GFP) were therefore complexed to SuperFect™ and tested (Fig. 2). The biological effect increased with the amount of ODN, and the dose-dependent curve reached a plateau at approximately 75% inhibition. The results obtained with the control ODNs (reverse and scramble) support the sequence-specificity of the observed antisense effects (Fig. 2). The inhibition measured with the mismatched ODN can be explained by the fact that its sequence differs in only two bases from the antisense one. Under the biological assay conditions, the cytotoxicity on HeLa cells, measured by a MTT assay, was not found superior to 10% with SuperFect™ and 15% with DAC-30™.

In conclusion, the screening system developed allows to study the biological activity and sequence-specificity of ODNs with various chemical modifications and/or complexed to different carriers. We found that a dendrimeric structure permits efficient vectorization of ODNs, giving sequence-specific inhibition in this assay system. Furthermore, flow cytometry well predicted biological effects obtained on cells. Consequently, fluorescence analysis appear to be powerful tools with which antisense ODN delivery and efficiency can be optimized.

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REFERENCES

1. Stein C.A. and Cheng Y.C. *Science*, **1993**, *261*, 1004-1012

2. Bennett C.F. *Biochem. Pharmacol.*, **1998**, *55*, 9-19.

3. Azad R.F., Brown-Driver V., Buckheit R.W. and Anderson K.P *Antiviral Res.*, **1995**,.*28* , 101-111.

4. Deshpande D., Toledo-Valasquez D., Thakkar D., Liang W.W. and Rojanasakul Y. *Pharmacol. Res.*, **1996**, *13*, 57-61.

5. Vlassov V.V., Balakireva L.A. and Yabukov L.A. *Biochim Biophys Acta*, **1994**, *1197*, 95-108.

6. Bielinska A., Kulowska-Latallo J.F., Johnson J., Tomalia D.A. and Baker J.R. *Nucleic Acids Res.*, **1996**, *24*, 2176-2182.

7. Delong R., Stephenson K., Loftus T., Fisher M., Alahari S., Nolting A. and Juliano R.L. *J. Pharm. Sci.*, **1997**, *86*, 762-764.

8. Monia P.B., Johnston J.F., Geiger T., Muller M. and Fabbro D *Nature Medecine*, **1996**,. *2*, 668-675.