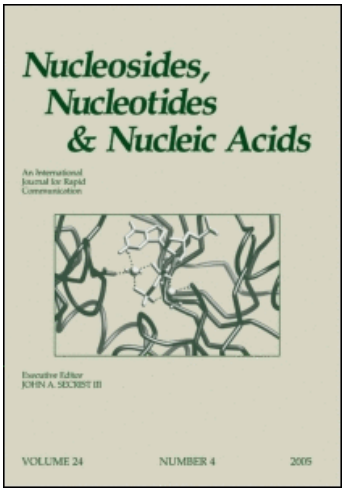

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ENHANCED CELLULAR UPTAKE OF G-RICH OLIGONUCLEOTIDES

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Abstract. Sequence-dependency of cellular uptake of oligonucleotides into Vero cells has been studied. Cellular uptake of 5'-[^{35}S]-labelled homopolymers decreased in the order $(dG)_{16}$ >> $(dT)_{16}$ > $(dA)_{16}$ > $(dC)_{16}$. The change of two base-pairs (dG→dA) in a dG-rich antisense oligonucleotide with good antiviral activity dramatically decreased cellular uptake and abolished antiviral activity.

Biological efficacy of antisense oligonucleotides is generally considered to depend on their stability against nucleases, their ability to penetrate cellular membranes and on their binding affinity to specific target sequences.[1-5] While binding affinity is known to be a function of base composition of antisense oligonucleotides, no systematic studies have been published regarding sequence-dependency of nuclease stability and cellular uptake. Indeed, it has been reported that oligonucleotides up to 30 bases in length are taken up by cells in a sequence-independent manner.[5] During our studies with antisense oligonucleotides as antiviral agents we observed some unusual properties of certain oligonucleotide sequences like strong aggregation in HPLC or PAGE which seemed to correlate in some way with their biological potency.

In order to explore the possibility that cellular uptake of oligonucleotides can be significantly influenced by their sequence, we synthesized all four homoplymers $(dN)_{16}$ and labelled them at their 5'-termini using γ-[^{35}S]-ATP. After purification of the 5'-labelled oligonucleotides by polyacrylamide gel electrophoresis (PAGE), they were incubated with Vero cells at 1 μM extracellular oligonucleotide concentration for 24 hours at 37°C in Dulbecco's modified Eagle medium (DMEM) in the absence of serum. Following incubation, cells were washed extensively with DMEM to remove surface-bound oligonucleotide, trypsinized, and then associated radioactivity was determined by means of a scintillation counter. Interestingly, cellular uptake increased in the order C<A<T<G, and $(dG)_{16}$ was taken up about 40-times better than $(dC)_{16}$. (TABLE 1). Additionally, the homopolymer of dG proved to be the most stable compound of all four dN's in Vero cell culture.

TABLE 1. Uptake of homopolymers at 1 μM extracellular concentration into Vero cells at 37°C.

Oligonucleotide	Cellular uptake [pmol / 10^5 cells]
$(dC)_{16}$	0.11
$(dA)_{16}$	0.15
$(dT)_{16}$	0.25
$(dG)_{16}$	4.3

During our studies with antisense oligonucleotides against HSV-1 we found that the G-rich antisense oligonucleotide AO2 (TABLE 2) has considerably better antiviral activity than AO1. While AO1 showed normal elution patterns on HPLC and a single band on PAGE, AO2 heavyly aggregated on HPLC. Aggregation could only be overcome by chromatography at pH 12. Even when loaded in 80% formamide onto a 7 M urea polyacrylamide gel, mainly bands of high molecular weight were observed provided that the sample has not been heated prior to loading (FIG. 1, lane 1). Heating of the sample before loading followed by slow cooling (lane 2), by quick cooling (lanes 3 and 5) or omitting cooling (lanes 4 and 6), was in favour of the non-aggregated form of the oligonucleotide. Higher oligonucleotide concentration seemed to promote aggregation (lanes 5 and 6: 3 OD's; lanes 1 to 4: 1 OD).

To show that there exists an equilibrium between the aggregated and non-aggregated form, we have isolated band I (aggregated) and band II (non-aggregated) from lane 5 and re-analyzed the recovered material by PAGE (FIG. 1, right panel). Aggregated oligonucleotide isolated from band I showed after heating in 80 % formamide mainly the non-aggregated form (lane 7), whereas the monomeric oligonucleotide isolated from band II revealed again high molecular weight aggregates when loaded without heating in 80 % formamide (lane 8).

Cellular uptake of AO2 turned out to be about 5-times higher as compared to AO1 in Vero cell culture (TABLE 2). This compares with a 3-fold higher anti-HSV-1 activity for the G-rich AO2 relative to AO1. Oligomer AO1 is directed against the coding region of DNA polymerase of HSV-1, whereas AO2 is directed against the translational start region of IE110. Introduction of a few internal phosphorothioate residues into AO2 results in derivative AO3 which was considerably more stable against nucleases while its cellular uptake was decreased.

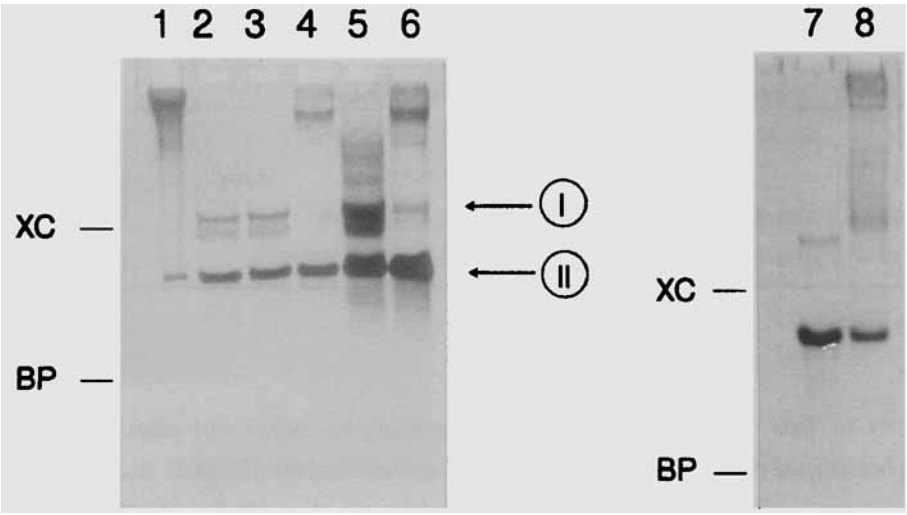

FIG.1. Aggregation of G-rich oligonucleotide AO2 as studied by PAGE (see text).

TABLE 2. Cellular uptake of 5'-[^{35}S]-oligonucleotides (1 μM) by Vero cells at 37 °C.

AO#	Oligonucleotide Sequence (* means phosphorothioate)	Cell uptake [pmol/10^5 cells]
AO1	G*C*A G G A G G A T G C T G A G G A*G*G	0.7
AO2	G*C*G G G G C T C C A T G G G G G T*C*G	3.5
AO3	G*C*G G G G C*T C*C*A*T G G G G G T*C*G	1.5
AO4	G*C*G G G G C*T C*C*A*T G G **A A** G T*C*G	0.6
AO5	G*C*G G **A** G C*T C*C*A*T G G **A** G G T*C*G	0.07

By changing two G's into A's we have disrupted one of the two G stretches resulting in a 3-times lower uptake for AO4. However, if both G-stretches were disrupted through one A substitution at each side (AO5), cellular uptake dropped by a factor of 20. Correspondingly, AO3 was about two orders of magnitude more active than AO4 in the HSV-1 cytopathic effect assay, whereas AO5 was completely inactive. We believe that the decrease in activity is not merely due to the two base-pair mismatches, but moreover reflects the dramatic decrease in cellular uptake caused by the A → G mutations.

In view of these results we thought to deliberately enhance the antiviral activity of antisense oligonucleotides with low intrinsic activity by adding putative non-base-pairing G

TABLE 3. Antiviral activity of G-rich oligonucleotides in a HSV-1 cytopathic effect assay.

AO#	Oligonucleotide Sequence (* means phosphorothioate)	MIC [μM]
AO6	G*C*A G G A G G A T*G C*T*G A G G A*G*G	27
AO7	G*G*A G G A T*G C*T G A*G*G "core"	> 80
AO8	G*G*G G G A G G A T*G C*T G A G G G*G*G	3
AO9	G*G*A G **A** **G** G **A** **G** T*G C*T **A** **G** G G **G***G*G	> 80

residues to their termini. Thus, AO6 corresponding to AO1 with additional internal phosphorothioate residues showed only moderate antiviral activity (TABLE 3). Shortening of this oligomer by three nucleotides at both ends results in a complete loss in antiviral activity (AO7). However, by adding three G units to either end of AO7, high antiviral activity was obtained for AO8. Notably, the G-rich oligomer AO8, in which three out of six flanking G residues do not match the target sequence, was about 10-times more active than the perfectly matching AO6. A further oligonucleotide (AO9) of almost identical base composition, but with 6 mismatches in the core region (plus two mismatches in the flanking region), turned out to be inactive against HSV-1.

Although our data do not prove an antisense oligonucleotide mechanism at this point, we were able to rationally modulate anti-HSV-1 activity of our oligonucleotides in a sequence-dependent manner. This was only possible by using partially phosphorothioate-modified oligomers while all-phosphorothioate oligonucleotides did not give any sequence-specific antiviral activities in our assay systems. Cellular uptake of our oligonucleotides did not only depend on the number of G residues, but was decisively influenced by the nucleotide sequence. Good antiviral activity of individual antisense oligonucleotides was connected to high cellular uptake which in turn is favoured by aggregation of the oligonucleotides to high molecular structures. These structures may arise from inter-strand G-tetrad formation rather than from intra-strand G-tetrad formation. Since there is an equilibrium between the aggregated and non-aggregated form of our oligonucleotides, the observed enhanced cellular uptake is likely to be responsible for the improved biological activity of these compounds.

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