

# Non-antisense cellular responses to oligonucleotides

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**Abstract** Oligonucleotides induce various cellular responses which are not due to the blockade of protein synthesis by an antisense mechanism. Oligonucleotides presenting double-stranded or G-quartet structures (ribo- or deoxyribonucleotides, phosphodiester or phosphorothioated) induce retraction of neurites and aggregation of chicken retinal cells within 10–20 h. This effect is reversible, non-toxic; it appears to require internalization and can be mimicked by treatment of the cells with an RGDS peptide. The oligonucleotides appear to trigger a cascade of intracellular events, affecting the adhesive properties of integrins. In addition, a subset of oligonucleotides induced platelet aggregation, probably through their interaction with membrane receptors. Recognition of these effects is important for the design and interpretation of antisense experiments. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Antisense oligonucleotide; Double strand; Cell aggregation; G-quartet; Neurite; Platelet

## 1. Introduction

Antisense oligonucleotides are extremely useful for exploring gene functions [1]. They can be applied to organotypic cultures and to cultures of primary or transformed cells, under defined physiological conditions. They can also be used in vivo, by intravenous injection or even by oral administration to rats or mice, and for therapeutic purposes in humans [2–4]. Their effect is limited by their metabolic instability, but this can be circumvented by using stabler derivatives. In addition to the expected decrease in protein synthesis, antisense oligonucleotides have also been shown to induce responses which are not related to the targeted gene. For example, it has been shown that oligonucleotides containing a cluster of guanines forming a G-quartet structure near their 3'-terminus could interact with integrins and thus modify the adhesiveness of cells [5,6]. G-quartet-containing oligonucleotides can also interfere with cell proliferation, probably by interacting with nucleolin [7,8].

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**Abbreviations:** AChE, acetylcholinesterase; BChE, butyrylcholinesterase; D.I.C., days in culture; DMEM, Dulbecco's modified Eagle's medium; DOTMA, *N*-[1-(2,3 dioleloxy)propyl]-*N,N,N*-trimethylammonium chloride

In the course of experiments in which we wished to block the expression of acetylcholinesterase (AChE) in neural cells with antisense oligonucleotides, we observed dramatic morphological changes. Although this effect was not produced by sense and scrambled oligonucleotides used as negative controls, we found that it was not related to AChE synthesis, because it could be elicited by totally unrelated oligonucleotides. This observation prompted us to investigate this phenomenon and to examine the effects of oligonucleotides on blood platelets, which do not synthesize proteins. We found that a subset of oligonucleotides that were active on neural cells induced a potent platelet aggregation. The effects observed on the two types of cells appear to result from entirely distinct mechanisms.

We thus report that oligonucleotides can elicit powerful cellular responses in neural cells and platelets, independently of any effect on protein synthesis.

## 2. Materials and methods

### 2.1. Materials

Human  $\alpha$ -thrombin was purchased from Enzyme Research Laboratories and collagen from Nycomed (Nycomed Arzneimittel, Germany). The protein kinase C inhibitor RO 31-8220 was from Calbiochem, the src kinase inhibitor pyrazolopyrimidine-type inhibitor 1 (PP-1) and the tyrosine kinase Syk inhibitor piceatannol were from Biomol (USA). Other reagents were from Sigma.

### 2.2. Cell cultures

Six-day-old chick embryo neural retinas were dissected and mechanically dissociated into a single cell suspension, after mild trypsinization. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2% chicken serum, 2 mM L-glutamine, 100 000 U/l penicillin/streptomycin, and 0.02 mg/ml gentamicin. Cells ( $3\text{--}5 \times 10^5$ ) were seeded in 35-mm tissue culture plastic dishes pre-coated with a poly-L-lysine solution (100 mg/ml), and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

### 2.3. Treatment with oligonucleotides

The sequences of oligonucleotides used are given in Table 1. Most oligonucleotides were custom-synthesized by Genset (Paris, France); the origins of others are indicated in the table. The oligonucleotides (0.5–1  $\mu$ M final concentration) were added 24 h after plating. Phosphodiester oligonucleotides were applied in serum-free DMEM with a four-fold higher quantity (2–4  $\mu$ M) of DOTMA (*N*-[1-(2,3-dioleloxy)propyl]-*N,N,N*-trimethylammonium chloride) (lipofectin, Gibco BRL Life Technologies). Phosphorothioated oligonucleotides were applied to the cells in aqueous solution.

### 2.4. Preparation of platelets

Human blood was collected from a forearm vein, and platelet suspensions were prepared as previously described [11]. Aggregation was

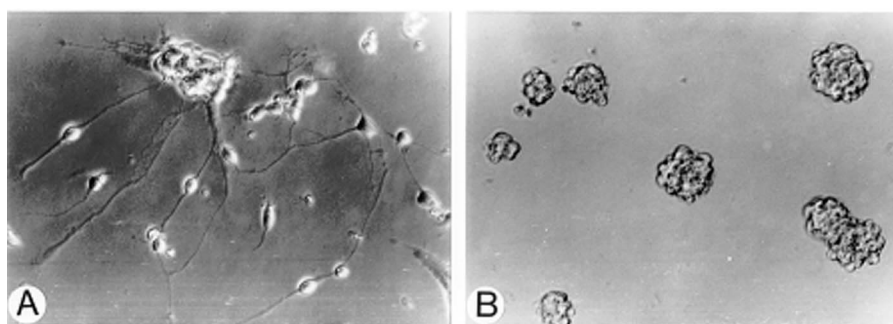


Fig. 1. Effect of oligonucleotides on chicken retinal cell cultures. A: Control cells, 48 h after plating on poly-L-lysine-coated dishes. B: Spherical aggregates induced by a mixture of AChE antisense oligonucleotides A\* and J\* (Table 1). We obtained similar results when each oligonucleotide was applied separately.

Table 1  
Oligonucleotides used in this study

Name	Sequence	Characteristics	Effect on retinal cells	Effect on platelets	
A	A*	AGAGGGCCCATCGGGGGGGCT	antisense against AChE (initiation codon)	+++	—
B	B*	GTACGGCGGAGCGCGAGTGCC	scrambled, derived from A	—	—
C	C*	GCATGGGGTACGCGGAGCGC	derived from A, scrambled	+++	n.d.
	D*	AGAGGTGTTACTGTGGGGGGTTC	derived from A, exchanging Ts and Cs	+++	—
	E*	GACCCCCACAGTAACACCTCT	complement of C	—	—
F	F*	AGAGGGCCCATCGCGGG	part of A	—	n.d.
G	G*	AAGAGAGGGCCCATCGCG	antisense, overlapping with A	—	n.d.
H	H*	AGCCCCCGCGATGGCGCCTCT	sense oligonucleotide, complement of A	—	n.d.
I	I*	CCCGCGATGGCGCCTCT	sense oligonucleotide, complement of F	—	n.d.
J	J*	GTCCTCCGGGGACCGTGGCGTTG	antisense against AChE (overlapping exon 2–exon T)	+++	—
	K*	CAACGCCACCGGTCCCCGGAGGAC	complement of J	—	—
	L*	CCTGGGGGAGTATTGCCAAGGAAGG	ATP-binding aptamer, containing G-quartets (DH25.43)	+++	++
	M*	CATTGGGGTCCCCCGTGGGGCGAAGT	antisense against AChE (overlapping exons 1–2)	+++	n.d.
	N*	GGGGTTGGGGTTGGGGTTGGGG	G-quartet forming sequence (G4T3)	+++	+++
	O*	GGGTTAGGGTTAGGGTTAGGG	human telomeric sequence (G3T2A)	+++	—
	P*	AGAGGGCCCATCGCTTATCGCGATGGCGCCTCT	hairpin-forming sequence, including part of A	+++	—
Q		CAATCGGATCGAATTCGATCCGATTG	palindromic sequence, double-stranded hairpin (ds26)	++	n.d.
	R*	GAATACGAACTCAATCTCGTAA	antisense oligonucleotide, corresponding to AChE (exon 1)	—	—
	S*	TTACGAGATTGAGTTTCGTATTC	sense oligonucleotide, corresponding to AChE (exon 1)	—	n.d.
	T*	GAATACGAACTCAATCTCGTAA	mixture of two complementary, inactive oligonucleotides	+++	n.d.
	U*	TTACGAGATTGAGTTTCGTATTC TCATCCCATTGGCCCAACCAT	antisense oligonucleotide, corresponding to BChE	—	n.d.
	V*	ATGGTTTGGGCAAATGGGATGA	sense oligonucleotide, corresponding to BChE	—	n.d.
	W*	TCATCCCATTGGCCCAACCAT	mixture of two complementary, inactive oligonucleotides	+++	n.d.
	X*	ATGGTTTGGGCAAATGGGATGA GTGGCTGTTTGAAGGGCAT	antisense oligonucleotide, corresponding to creatine kinase	+	n.d.
	Y*	ATGCCCTTCTCAAACAGCCAC	sense oligonucleotide, corresponding to creatine kinase	—	n.d.
Z	Z	poly[(I):poly(C <sub>12</sub> U) (Ampligene <sup>®</sup> )	imperfect ribonucleotide double-strand	+++	n.d.

Most deoxy-oligonucleotides were synthesized and HPLC-purified by Genset (France). Phosphodiester oligonucleotides are designated A, B, C, etc., while the corresponding phosphorothioated oligonucleotides are designated A\*, B\*, C\*, etc. In Fig. 3, we used fluorescein-labelled analogs of oligonucleotides A\* and I\*. A mixture of complementary ribo-oligonucleotides (Z) was a generous gift from Dr. M.N. Thang. AChE antisense oligonucleotides were designed from the chicken cDNA sequence [9]. N\* and O\* were shown to form an intramolecular G-quartet [10]. The hairpin oligonucleotide Q (also called ds26) was a gift of Dr. J.-L. Mergny. Oligonucleotides were generally applied to the cells in aqueous solution. The same effect was obtained with 1  $\mu$ M phosphorothioated oligonucleotide or 3  $\mu$ M phosphodiester oligonucleotide, probably due to the lower stability of the latter in the culture medium and in the cells (note that this concentration is lower than generally used in antisense experiments). The concentration could be reduced in the presence of the cationic lipid DOTMA which forms unilamellar liposomes. Only a subset of oligonucleotides were tested on platelet aggregation (n.d.: not determined).

monitored using a Chrono-log dual channel aggregometer with stirring at 900 rpm at 37°C ( $5 \times 10^8$  platelets/ml) as described [12].

### 3. Results and discussion

#### 3.1. Oligonucleotides designed for antisense experiments induce reversible neurite retraction and aggregation of chick retinal cells

Dissociated chick retinal cells adhere to polylysine-coated dishes and extend neurites within 24 h. Upon addition of antisense phosphodiester oligonucleotides A and J, or phosphorothioated oligonucleotides A\* and J\* (Table 1), directed against AChE, the cells retract their neurites within 8–10 h and form spherical aggregates; these changes are complete after 18 h (Fig. 1). An identical effect is seen with neurons isolated from the brainstem of 8-day chick embryos (not shown). After 3–4 days, the cells recover their adherence to the dish and resume neurite extension (Fig. 2); a second addition of the same oligonucleotides again induces neurite retraction and cell aggregation, and the process can be repeated a third time (not shown).

Neurite retraction and aggregation appear to involve specific adhesion molecules, because they do not occur in fibronectin-coated dishes, even at a 10-fold higher concentration of phosphorothioated oligonucleotides (10  $\mu$ M) (not shown). It

is noteworthy that our cell cultures did not show any sign of toxic effects under these conditions [2,13].

#### 3.2. The effect is not caused by an antisense mechanism

In experiments done to inactivate AChE expression, the level of mRNA is reduced to about 30% but AChE activity remains 70% of the control and the proportions of the different oligomeric forms of the enzyme [14] are not modified after 18 h (not shown), a time at which the morphological change is complete. All types of cells are affected, despite their heterogeneity [15]; only about 20% of the cells express AChE (not shown). Consequently, it does not appear possible to attribute the morphological changes to suppression of AChE expression, although they are obtained with antisense oligonucleotides but not with sense or scrambled oligonucleotides. In fact, a number of oligonucleotides unrelated to AChE also produce these effects (Table 1).

#### 3.3. Importance of the secondary structure of oligonucleotides

Although antisense and sense oligonucleotides directed against butyrylcholinesterase (BChE) have no effect when administered separately, they induce morphological changes when applied together (Table 1). This suggests that a double-stranded structure may be important. This was confirmed by the fact that self-complementary oligonucleotides (P\*, Q)

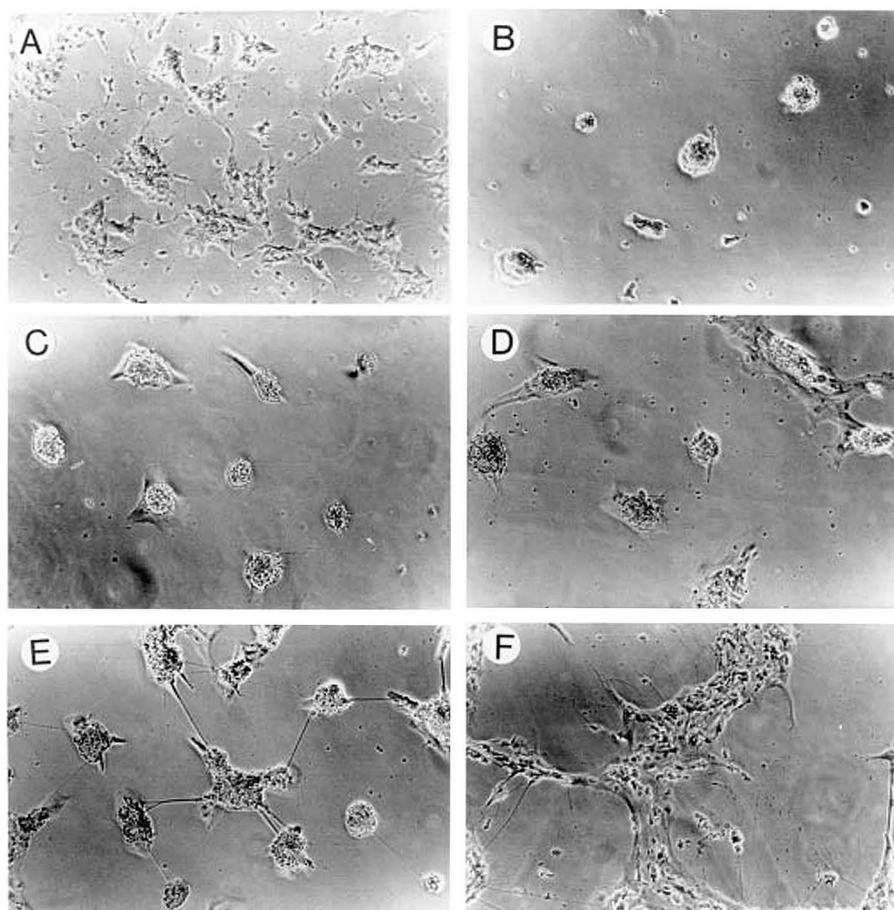


Fig. 2. Reversibility of morphological changes. A: Control cells, after 2 days in culture (2 D.I.C.). B: Twenty-four hours after plating, the cells were treated for 24 h with the oligonucleotides (as in Fig. 1). C: Two days after addition of the oligonucleotides (4 D.I.C.). D: At 6 D.I.C., the aggregates are flattening. E: At 8 D.I.C., some cells re-extend neurites. F: At 12 D.I.C., most cells are individualized and present extended neurites. In these experiments, the culture medium was changed every 2–3 days.

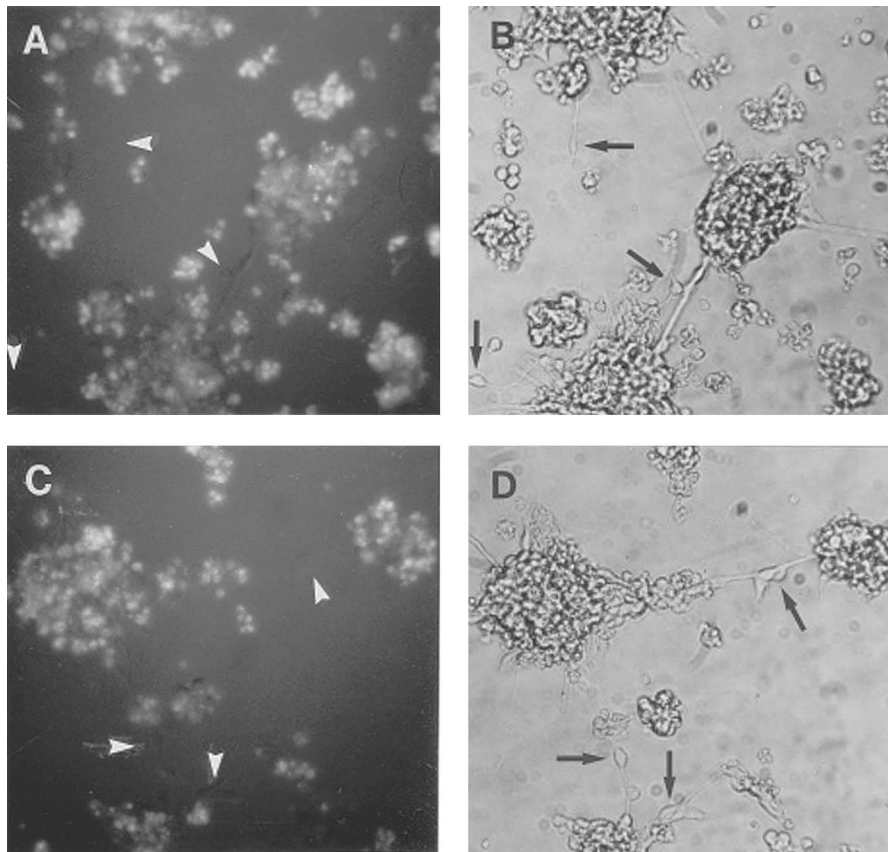


Fig. 3. Fluorescent labelling of transfected retinal cells, 3 D.I.C. Only unlabelled cells possess long fibers 70 h after oligonucleotide treatment. A: Fluorescence view of cells transfected with 1  $\mu$ M fluorescein-labelled oligonucleotide A\* without DOTMA (see Section 2). B: Phase contrast view of the same cells as in A. C: Fluorescence view of cells transfected with 1  $\mu$ M fluorescein-labelled oligonucleotide A\* (Table 1) with lipofectin. D: Phase contrast view of the same cells as in C. Arrows indicate cells which are not fluorescent and extend neurites.

and a mixture of complementary oligonucleotides (T\*, W\*) or of synthetic polyribonucleotides, poly(I) and poly(C<sub>12</sub>U) (Z), are also effective: thus, the effect appears to be linked to the secondary structure, but not to the chemical nature of the oligonucleotides (Table 1).

Some of the AChE antisense oligonucleotides that induced the effect contained four or five guanines, suggesting the formation of G-quartet structures [6,16], like the ATP-aptamer DH25.43 (L\*) [17]. It has been reported that a stretch of guanines at the 3' end of an oligonucleotide can interfere with cell adhesion [5], but guanines were located at other positions in some of our most effective oligonucleotides.

#### 3.4. The cellular response requires internalization of the oligonucleotides and does not involve protein synthesis

Double-stranded RNA complexes elicit a number of cellular responses, for example during viral infection, including interferon induction [18]. However, the effect that we observed in this study could not be transmitted by conditioned medium (not shown), therefore cannot be ascribed to production of interferons or other diffusible factors. Furthermore, they could not be transmitted by cell-to-cell contacts, since cells which did not incorporate fluorescent oligonucleotides retained their extended neurites, even though they were in contact with aggregated cells (Fig. 3). Thus, only cells which internalized oligonucleotides showed the morphological response. Two other results support this view: (a) the effect was enhanced by the lipid compound DOTMA, and (b) bio-

tinylated oligonucleotides were active when added to the culture in soluble form, but not when they were immobilized on streptavidin-coated dishes prior to addition of the cells (not shown).

The effect was not suppressed by  $\alpha$ -amanitin (0.5  $\mu$ M) or cycloheximide (10  $\mu$ M), applied 2 h before the oligonucleotides (not shown), indicating that it was not related to either mRNA or protein synthesis.

We cannot entirely exclude the possibility that breakdown products of the oligonucleotides might participate in the observed effects. However, this appears very unlikely, because the effect was critically dependent on the sequence, but not on the phosphodiester or phosphorothioated nature of the oligonucleotides. The fact that lipofectin facilitated the effect also indicates that it does not result from an uptake of breakdown products. In addition, the effect was much more rapidly reversible with oligoribonucleotides (Z), suggesting that the products of intracellular degradation were inactive.

#### 3.5. Involvement of intracellular calcium stores; integrin-mediated effects

The oligonucleotide effect was blocked by 1  $\mu$ M of 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate, which prevents release of intracellular Ca<sup>2+</sup> stores, by 20  $\mu$ M thapsigargin, which inhibits endoplasmic reticulum Ca<sup>2+</sup>-ATPase and depletes Ins(1,4,5)P<sub>3</sub>-sensitive Ca<sup>2+</sup> pools, and by preincubation of the cells with 0.1 mM EGTA for 2 h before addition of the oligonucleotides (not shown). This suggests that intracellular

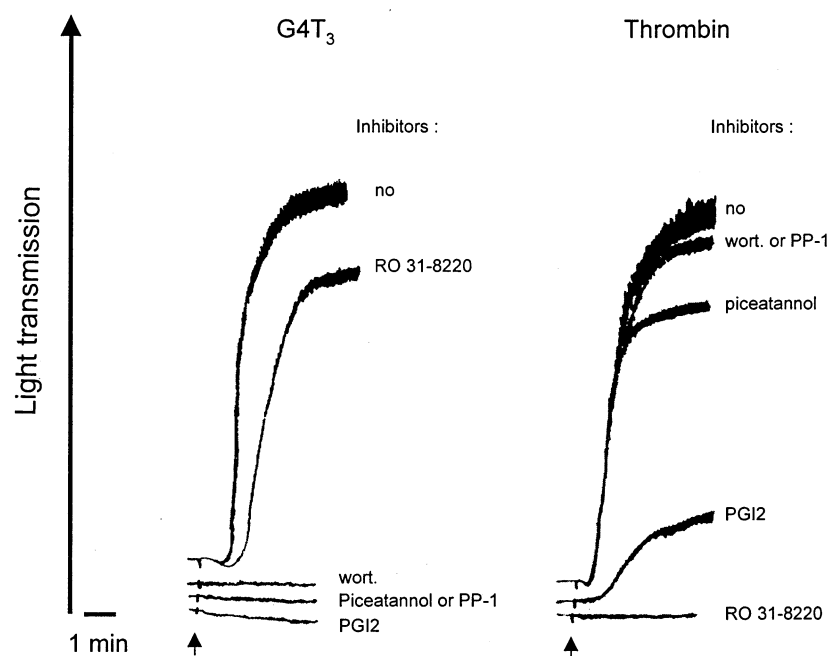


Fig. 4. Aggregation of human blood platelets produced by oligonucleotide N\*. Human platelets were preincubated for 10 min either without or with RO 31-8220 (5  $\mu$ M), wortmannin (wort., 25 nM), PP-1 (10  $\mu$ M) or piceatannol (50  $\mu$ M) and stimulated with oligonucleotide N\* (10  $\mu$ g/ml) or thrombin (1 IU/ml). Prostaglandin I<sub>2</sub> (PGI<sub>2</sub>, 10 ng/ml) was preincubated for 2 min before stimulation. The arrows indicate addition of the agonist. Aggregation was assessed using a Chrono-log dual channel aggregometer with stirring at 900 rpm ( $5 \times 10^8$  cells/ml). The tracings are representative of two experiments performed with platelets from different individuals, which yielded very similar results.

Ca<sup>2+</sup> stores are involved. Thus, the oligonucleotides appear to trigger a cascade of intracellular events involving intracellular Ca<sup>2+</sup> stores.

Similar morphological changes were induced by peptides RGDS and GRGDS but not by RGES, suggesting that they involve integrin-mediated cell adhesion, probably to vitronectin secreted by the retinal cells themselves. However, the peptides induced more rapidly and more completely reversible changes than most oligonucleotides and were also effective on fibronectin-coated dishes, leading to a reduction of integrin affinity for the substrate [19,20]. Although the morphological changes appear identical, the peptides and oligonucleotides may act through different pathways.

### 3.6. Effect of oligonucleotides on platelet aggregation

To confirm that oligonucleotides can induce a strong cellular effect independently of silencing of a specific gene, we checked their potential effect on human platelets. These anucleated cells respond rapidly to extracellular stimuli through ligation of specific membrane receptors, leading to morphological changes, secretion and aggregation, independently of protein synthesis. Oligonucleotides D\*, J, J\*, O\* and P\*, which are active on retinal cells, have no effect, but L\* and N\* stimulate aggregation of platelets. Aggregation with oligonucleotide N\* is rapid, comparable to that obtained with 1 IU/ml of thrombin, a potent physiological agonist (Fig. 4).

However, platelet activation induced by oligonucleotide N\* is totally blocked by wortmannin, piceatannol or PP-1, in contrast to activation induced by thrombin, indicating that they act via distinct intracellular signalling pathways. Activation by oligonucleotide N\* therefore requires phosphoinositide 3-kinase, a non-receptor tyrosine kinase of the Src family, as well as tyrosine kinase Syk. This resembles the activation

triggered by membrane receptors such as Fc $\gamma$ RIIa [12] and GpVI [21] (Bernard Payrastré, unpublished observation). The other oligonucleotides tested do not activate platelets, except L, which induces a modest aggregation.

## 4. Conclusion

We show here that half of 26 oligonucleotides tested induce neurite retraction and formation of globular cell aggregates of chick retinal cells, and that a subset of these oligonucleotides trigger aggregation of human platelets. These effects are not produced via an antisense mechanism, and do not involve protein synthesis.

Taken together, our observations suggest that the secondary structure of the oligonucleotides is important, either as double-stranded helices or as G-quartets, for inducing cellular responses. In contrast, the chemical nature of the oligonucleotides appeared irrelevant, since phosphodiester and phosphorothioated oligodeoxyribonucleotides, as well as oligoribonucleotides induce the morphological changes. These effects are independent of chemical toxicity. The effects observed on retinal cells and platelets are probably due to different mechanisms: the retinal cell response seems to require internalization, while platelet activation resembles that induced by membrane receptors. Further work will be needed to elucidate the detailed pathways involved in these effects.

However, the immediate importance of the present findings is to call attention to the fact that some antisense oligonucleotides, either alone or in combination with their mRNA target, may induce cellular responses which are not related to suppression of the transcript, at concentrations which are usually used in antisense experiments. These effects are therefore entirely different from those obtained with double-stranded

small interfering RNAs [22,23]. Because of effects such as those reported here, and in previous studies [24], the design of antisense experiments and of adequate controls may be more difficult than is currently realized. On the other hand, effects such as those illustrated here on neural cells and on platelets may exist *in vivo*, upon release of nucleic acid fragments from lytic or apoptotic cells, and could also be of major clinical relevance.

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