Inhibition of leukaemia cell proliferation by folic acid–polysine-mediated introduction of c-myb antisense oligodeoxynucleotides into HL-60 cells

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Summary. The inhibitory effect of c-myb antisense oligodeoxynucleotides (ODNs) conjugated to folic acid (FA) on HL-60 cell proliferation was examined. Folic acid was covalently linked to a polysine chain and purified by gel chromatography. Sterile FA-polysine was complexed with c-myb sense and antisense. Exposure of HL-60 cells to the FA–polysine–c-myb antisense ODN complex resulted in a down-regulation of c-myb expression and a greater inhibition of proliferation than that obtained using free ODNs. Moreover, FA–polysine conjugate alone or complexed to c-myb sense ODN was not toxic to cells. The antigenic properties and uptake of the vitamin were not affected by the polysine chain. These data suggest that this strategy is potentially useful for the selective delivery of anti-oncogene-targeted ODNs into cancer cells.

Antisense oligodeoxynucleotides (ODNs) have proven useful for selective inhibition of gene expression (Holt et al., 1988; Szczylık et al., 1991). However, their rate of cellular uptake appears to be quite slow, and consequently attempts have been made to enhance their stability and their delivery into cells. For instance, receptor-mediated endocytosis has been used to increase the uptake of synthetic ODNs and other foreign molecules such as proteins complexed to specific ligands (Wu & Wu, 1987, 1988; Cotten et al., 1990; Leamon & Low, 1991; Citro et al., 1992; Manfredini et al., 1993). Since the receptors for some growth factors, vitamins and hormones are overexpressed in rapidly dividing tumour cells (Rothenberg & Da Costa, 1971; Asok et al., 1981; Schub & Franklin, 1984; Lacey et al., 1989), the ligands of these receptors can be exploited to selectively introduce therapeutic compounds into the cells. The use of modified ligands for specific cell-surface receptors as carriers of oncogene-targeted antisense ODNs represents a potentially useful therapy to be used alone or in combination with antineoplastic drugs.

We have previously reported that a c-myb antisense–transferrin–polysine complex produces an enhanced uptake into HL-60 cells, resulting in an increased biological effect. Recently, we have also observed that a polysine chain covalently linked to compounds such as insulin, folic acid, retinoic acid, oestrone and testosterone can be used for specific interactions with nucleic acids in physiological ionic conditions (G. Citro, unpublished observation).

The presented study describes the efficacy of folic acid receptor-targeted c-myb antisense in the HL-60 cell line. The effect of the complexed phosphodiester (PO) ODNs was compared with that of phosphorothioate (PS) ODN antisense given alone.

With doses of 20 and 30 µg ml⁻¹, we found that PS c-myb antisense actively inhibited the rate of cell proliferation while free PO c-myb antisense had no effect. However, when free PO c-myb antisense ODNs were complexed to FA–polysine, their inhibitory effect on the cell proliferation was even greater than that obtained using the free PS oligos. Furthermore, whereas recent research has indicated there are some drawbacks to the use of PS oligos in systemic therapy (Stein & Cheng, 1993), PO oligos might prove useful since their metabolites are similar to physiological compounds, resulting in less aspecific toxicity.

Materials and methods

Folic acid–polysine and oligodeoxynucleotide conjugates

Folic acid (FA) was dissolved in 20 mM sodium phosphate buffer at pH 4.5 and incubated with a 6-fold molar excess of a water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce) for 1 h at room temperature. A 3 M excess of the modified vitamin was then added to the polysine solution (MW 21,000 in 20 mM sodium phosphate, pH 4.5) and incubated overnight at room temperature. The same procedure was performed to obtain the FA-fluorescein conjugate (Sigma).

The conjugate was purified by Sephadex G-25 gel chromatography (100 mM phosphate saline buffer pH 7.4) monitoring spectrophotometrically at 287 nm. The extent of FA conjugation to polysine was determined spectrophotometrically at 363 nm (folic acid Σ = 6,200 in PBS, pH 7.4). In addition, folate conjugate was identified by using a minimum amount of [3H]folic acid (Amersham) in the reaction mixture. In order to eliminate unbound or absorbed FA, the purified complex was extensively dialysed in 100 mM phosphate-buffered saline solution at pH 7.4 (1,000 ml day⁻¹ for 4 days) at 4°C. To verify that the unbound or absorbed FA was completely removed, gel filtration chromatography (Sephadex G-25) in the presence of high ionic strength (2 M sodium chloride in PBS, pH 7.4) was performed.

Phosphorothioate and phosphodiester ODNs corresponding to c-myb codons 2–7 (18-mer) were supplied by Applied Biosystems (CA, USA). The sense and antisense c-myb sequences were 5'-GCC CCG CAC CCG AGA TGG-3' and 5'-GGG CGG GGG TCT TCG GGC-3' respectively. Sterile FA-polysine (30 ng µl⁻¹) was mixed with c-myb antisense or sense ODNs and left for 1 h at room temperature.

Immunoslot blot

Purified FA–polysine samples (20 µl) containing various amounts of FA were immobilised on nitrocellulose filters (Bio-Rad) using a Bio-Dot SF Microfiltration apparatus (Bio-Rad) following the manufacturer's suggestions.

Slots were incubated first with anti-FA monoclonal antibody (clone VP 52; mouse IgG2b; Sigma), then with goat anti-mouse horseradish peroxidase (HRP) conjugate, and developed using the HRP substrate 4-chloro-nitrophenol. The polysine not complexed to folic acid was used as a control to verify the absence of aspecific immunoreactivity.

Fluorescence microscopy

To ensure the same amount of fluorescein (FITC) in both compounds used in cell treatments, FITC-polylysine (Sigma) was coupled to FA or left unconjugated as control. HL-60 cells (10⁶ ml⁻¹) were incubated for different lengths of time (from 5 to 300 min) at 37°C with FITC–polysine–folic acid conjugate (final concentration of folic acid 10⁻⁷ M). Cells were then washed five times with cold PBS, cytocentrifuged (Shandon) and fixed at 4°C in absolute acetone for 15 min. Cells were photographed through a Leitz microscope with a 40 x phase-contrast/fluorescence objective.
Formation of the folic acid–polylysine–c-myb oligodeoxynucleotide complexes
Sterile FA–polylysine (30 ng μl⁻¹) was mixed with various amounts of c-myb antisense or sense ODN in sterile aqueous solution. Complexes were allowed to form for 1 h at room temperature before being added to the cells.

Cells and culture conditions
Human promyelocytic leukaemia cells (HL-60) were grown in suspension in a humidified atmosphere of 95% (v/v) air and 5% (v/v) carbon dioxide at 37°C in RPMI-1640 and 10% heat-inactivated fetal calf serum supplemented with 102 μg ml⁻¹ penicillin G, 102 μg ml⁻¹ streptomycin and 120 μg ml⁻¹ L-glutamine. The cells were grown to densities of 1 × 10⁶ cells before harvesting (Collins et al., 1977; Koehler, 1983). For all the experiments, cells were cultured in 24 well Costar plates at an initial concentration of 1 × 10⁴ in RPMI-1640 folate-deficient medium prepared according to Barton and Capdevila (1986). Doses of 10 or 20 μg ml⁻¹ ODNs were added to cells, followed by two subsequent doses of 5 μg at 24 and 48 h. The control cells were treated with the same doses of FA–polylysine conjugate (10⁻⁷ M) used in the oligo complex preparation.

Cell number and viability were determined using an electronic particle counter and trypsin blue exclusion assay every 2 days.

c-myb mRNA levels in HL-60 cells
Reverse transcription–polymerase chain reaction (RT–PCR) for detection of c-myb mRNA transcripts was carried out as previously described (Chomczynski & Sacchi, 1987; Venturelli et al., 1990). A 3' ODN primer c-myb corresponding to nucleotides 2,466–2,487 and a 5' ODN primer c-myb corresponding to nucleotides 2,258–2,279 of the published cDNA sequence were utilised (Majello et al., 1986). After 30 cycles, 10 μl of amplified product was electrophoresed on a 4% agarose gel and then transferred to a nylon filter. Filters were prehybridised and then probed with a 32P-end-labelled oligonucleotide probe (Sambrook et al., 1989) corresponding to a 50 base c-myb oligomer sequence contained within the amplified region from nucleotides 2,351 to 2,400. As control, β-actin mRNA was amplified with β-actin-specific primers and detected with a specific probe, as described by Nicolaides et al. (1991). Hybridisation was detected by autoradiography.

Results

Purification of the folic acid–polylysine conjugate
The elution profile of the polylysine and FA mixed in the absence of the coupling agent is shown in Figure 1 (top). Two separated peaks were observed under physiological ionic conditions (100 mM phosphate buffer saline, pH 7.4). fluoresceinated polylysine was recovered in fractions 4–8, while free folic acid was collected from fractions 23–35. The fluoresceinated polylysine–FA conjugate eluted in the excluded volume shows (Figure 1, bottom) as a single sharp peak with a strong UV absorption at 287 nm. The conjugate rechromatographed at high ionic strength (2 M sodium chloride in PBS, pH 7.4) showed a similar elution profile, demonstrating that the compounds were covalently bound (data not shown). The average conjugation ratio of FA–polylysine was 0.5.

Immunodetection of folic acid in the conjugate
As the specific MAb used was able to recognise both FA and its active metabolite, the conjugation of FA with polylysine chain did not alter the active site of the FA molecules. The specific MAb showed a dose-dependent reaction with the vitamin in the conjugate. Figure 2 shows the results of a slot blot assay (in duplicate) obtained using an increasing concent-

![Figure 1](https://example.com/figure1.png)

**Figure 1** Sephadex G-25 chromatography of the FA–polylysine conjugate. Purification of FA–polylysine conjugate was performed by gel chromatography on Sephadex G-25 in 10 mM sodium phosphate pH 7.4 (bottom). [3H]FA (25 × 10⁶ c.p.m.) was added to the reaction mixture as radioactive tracer. FITC–polylysine (MW 11,000; Sigma) was used as marker to identify the fractions where the free polylysine was eluted (top).

**Figure 2** Immunoblots. Amounts of FA–polylysine conjugate containing FA (from top to bottom: 300, 60, 30 and 3 ng) were blotted in duplicate onto nitrocellulose filter. After incubation with specific anti-folic acid MAb, the bound MAb molecules were then reacted with a goat anti-mouse IgG horseradish peroxidase conjugate. Enzymatic activity was detected via colour development as described in Materials and methods. Free polylysine 1 mg ml⁻¹ was used as negative control.
brane. The complex bound to the cell surface in 5–10 min (Figure 3d) and then gradually entered the cell cytoplasm over a period of 2–5 h with the fluorescence distributed in a somewhat patchy pattern (Figure 3d and e). In contrast, cells treated with FITC–polylysine lacking FA showed no fluorescence (Figure 3f). The presence of a 100-fold molar excess of free folate in the medium resulted in a significant decrease in the fluorescence intensity indicating that the uptake of FA–polylysine complex (as with FA) is mediated via the FA receptor mechanisms (data not shown).

Figure 3 Uptake of FA–FITC–polylysine conjugate. Phase-contrast a, b and fluorescence d, e micrographs of HL-60 cells incubated with FITC–polylysine conjugate. The micrographs shown refer to the incubation time of 15 and 120 min respectively. Phase-contrast e and fluorescence f micrographs refer to HL-60 cells treated with FITC–polylysine lacking FA (incubation time = 120 min).

Formation of folic acid–polylysine/c-myb oligodeoxynucleotide complexes
Complexes of FA–polylysine with c-myb ODNs were obtained as described in Materials and methods. Oligo binding to the FA–polylysine complex was demonstrated by gel mobility-shift assay (Figure 4). It is evident that ODN mixed with FA or alone migrated to the positive charged pole (Figure 4a and c). On the other hand the negative charge of the ODN when complexed to the FA–polylysine conjugate was completely neutralised by the polylysine chains (Figure 4b).

Effect of folic acid–polylysine–oligodeoxynucleotide complex on the proliferation of HL-60 cells
HL-60 cell proliferation is inhibited by exposure to c-myb antisense ODNs in excess of 10 μM (Anfossi et al., 1989; Ferrari et al., 1990; Nicolaides et al., 1991). In agreement with our previous results (Citro et al., 1992), 20 and 30 μg ml⁻¹ doses of free phosphodiester (PO) c-myb antisense ODNs had no effect on the HL-60 cell proliferation. Indeed, after 6 days the cell number of all the treated cells was similar to that of the control: PO sense 20 μg ml⁻¹ = 540 ± 7 × 10²; PO sense 30 μg ml⁻¹ = 515 ± 15 × 10²; PO antisense 20 μg ml⁻¹ = 490 ± 10 × 10²; PO antisense 30 μg ml⁻¹ = 495 ± 20 × 10²; control = 520 ± 10 × 10². However, doses of 20 and 30 μg ml⁻¹ phosphorothioate (PS) c-myb antisense ODNs clearly impaired HL-60 cell proliferation (Figure 5a). The same doses of ODNs phosphodiester complexed to the FA–polylysine conjugate induced a dose-dependent inhibition of HL-60 cell proliferation (Figure 5b) which was much greater than the inhibition induced by free phosphorothioate ODNs (Figure 5a). Moreover, the proliferation rate of HL-60 cells exposed to the FA–polylysine–sense ODN complex was unaffected (Figure 5b). To determine whether the marked

Figure 4 Gel shift of oligodeoxynucleotide with FA–polylysine complexes. a, Three nmoles of native FA incubated with 10 nmol of c-myb antisense ODNs. b, Three nmoles of FA–polylysine incubated with 10 nmol of c-myb antisense ODNs. c, c-myb ODNs in distilled water. Samples were separated by electrophoresis on 1% agarose gel at 100 V with 1 x TAE (40 mM Tris-acetate/1 mM EDTA, pH 8) running buffer.
inhibition of HL-60 cell proliferation with the FA–polylysine/ c-myb antisense ODN complex correlated with c-myb transcript levels, total mRNA was extracted from cells treated with the FA–polylysine conjugate (Figure 6c) or the FA–polylysine–c-myb antisense ODN complex (Figure 6, lane s) or the FA–polylysine–c-myb antisense ODN complex (Figure 6, lane as), and c-myb expression was measured by RT-PCR. c-myb mRNA was barely detectable in cells treated with the FA–polylysine–c-myb antisense ODN complex, while it was highly expressed in sense-treated and control cells (Figure 6). Densitometric measurement of the c-myb hybridizing band in sense-vs-antisense oligodeoxynucleotide-treated samples indicated that the signal from the antisense-treated samples was <5% of that from the sense-treated samples.

Discussion

In this study we present a protocol for the synthesis and purification of a covalent conjugate of FA and polylysine chain (Figure 1) to be employed as vehicle for ODNs.

As shown in Figure 2 the covalent coupling of a polylysine chain to FA did not prevent the ligand being recognised by specific monoclonal antibodies, suggesting that FA maintained its biological activity. Other authors also provide evidence to support this hypothesis (Leamon & Low, 1991). Indeed, they found that FA covalently linked to proteins of different sizes was still recognised by specific monoclonal antibodies as well as by FA cell-surface receptors. Moreover, the immunoblot presented here can be used to recognise the FA–polylysine complex in the medium as well as in biological fluids in vivo. The addition of the polycationic peptide to FA resulted in a conjugate capable of binding to ODNs in physiological conditions. Owing to the cationic properties of the polypeptide chain, the FA–polylysine conjugate was able to avidly bind negatively charged ODNs, as shown in the band-shift experiments (Figure 3). The fluorescence microscopy results (Figure 4) clearly indicate that the conjugate interacts with the cell membrane after a few minutes and then enters the cells. Therefore, as this process can be competitively blocked by free folate, it would appear that the cells are capable of internalising folate conjugates through a folate receptor-mediated mechanism (Barton & Capdevila, 1986). The non-lysosomal pathway internalisation of folate into the cells (Rothemberg et al., 1990; Asok, 1992; Weitman et al., 1992) allows the ODNs–FA conjugate to enter directly into the cytoplasmic compartment. Because of Watson–Crick base pairing specificity, the ODNs can react with the complementary c-myb mRNA inside the cells, thus inhibiting cell proliferation. The high inhibitory effect on cell proliferation displayed by complexed ODNs can be ascribed to both their stability outside the cells and the increased uptake obtained by the receptor-mediated event. The FA–polylysine chain can form a complex with an ODN in the medium, thereby shielding it from nuclease attack. These observations are in agreement with data of other authors (Farber et al., 1975; Stein & Cheng, 1993). As with the delivery system based on the use of a transferrin–polylysine complex (Citro et al., 1992; Manfredini et al., 1993), the system described here represents a useful means of targeting and of intracellular uptake of ODNs into tumour cells. Furthermore, this study also shows that the FA–polylysine c-myb antisense complex, unlike FA–polylysine c-myb sense, specifically reduces the c-myb mRNA level in treated cells (Figure 6).

Taking into account the receptor expression on the tumour cell membranes, two or more vehicles carrying antisense
ODNs directed against the encoded mRNAs can be made. The ideal surface receptors to exploit for a selective delivery of antisense ODNs would undoubtedly be those exclusively expressed by tumour cells. Alternatively, receptors which are overexpressed in some neoplastic cells, such as the EGF, the transferrin and the FFA receptors (Klausner et al., 1983; Kamen et al., 1988; Hopkins et al., 1990; Asok, 1992; Simons et al., 1992; Weitman et al., 1992; Berczi et al., 1993), could also be used. Recent results (Ratajczak et al., 1993; T. Skorski et al., 1994) indicate that anti-oncogene-targeted phosphorothioate ODNs administered in vivo possess antitumoural activity against tumour cells, resulting in an increase in animal survival. Yet a relative paucity of phosphorothioate vs phosphodiester successes in tissue culture when targeted to mammalian mRNAs has also been reported (Stein & Cheng, 1993). Consequently, complexed phosphodiester ODNs should perhaps be considered as their metabolites are less toxic to the cells. However, as with any other drug in the developmental process, further studies are required to assess the potential role of antisense oligodeoxynucleotides in therapeutic applications.

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