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# Cell death in NF-κB-dependent tumour cell lines as a result of NF-κB trapping by linker-modified hairpin decoy oligonucleotide

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**Abstract** **The transcription factor NF-κB is frequently activated in cancer, and is therefore a valuable target for cancer therapy. Decoy oligodeoxynucleotides (ODNs) inhibit NF-κB by preventing its binding to the promoter region of target genes. Few studies have used NF-κB-targeting with ODNs in cancer. Using a hairpin NF-κB-decoy ODN we found that it induced growth inhibition and cell death in NF-κB-dependent tumour cell lines. The ODN colocalized with the p50 subunit of NF-κB in cells and directly interacted with it in nuclear extracts. In TNFα-treated cells the ODN and the p50 subunit were found in the cytoplasm suggesting that the complex did not translocate to the nucleus. Transcriptional activity of NF-κB was efficiently inhibited by the ODN, whereas a scrambled ODN was without effect on transcription. Thus, ODN-mediated inhibition of NF-κB can efficiently promote cell death in cancer cells providing a potentially powerful approach to tumour growth inhibition.**


*Keywords:* Decoy oligonucleotide; NF-κB; Tumour cell lines; Cell death; Linker-modification

## 1. Introduction

NF-κB is a transcription factor regulating the expression of a wide variety of genes that activate innate and adaptive immunity, angiogenesis, cell adhesion and inflammation. NF-κB is involved in many cancers: constitutively active NF-κB has been detected in human tumour cell lines and in tumour tissues derived from patients (reviewed in [1]), such as multiple myeloma, acute myeloblastic leukemia, acute lymphoblastic leukemia, prostate, breast cancers and colon cancers [2–4].

Activation of NF-κB makes it a valuable target for cancer therapy. Various pharmacological inhibitors of the NF-κB signaling pathway include inhibitors of the proteasome [5], inhibitors of the IKKs (aspirin, sulfasalazine, and sulindac) that result in trapping the NF-κB precursor subunits in the cytoplasm. Other anti-inflammatory agents such as corticosteroids or thalidomide, inhibit the NF-κB pathway through unidentified mechanisms [5]. Alternatively, the NF-κB pathway is inhibited by peptides designed to interact with the nuclear localization sequence of the p50 subunit of NF-κB. For example, cell-permeable peptide SN50 reduced NF-κB DNA binding and increased apoptosis in retinoblastoma and multiple myeloma cells [6]. Decoy oligodeoxynucleotides (ODNs) result in potentially highly specific NF-κB antagonism by preventing its binding to the promoter region of target genes. Indeed, inhibition of NF-κB by ODNs was achieved in a cystic fibrosis epithelial cell line [7], in allografts [8–10] and in animal models of cardiovascular disease and ischemia–reperfusion injury [11–15] and intimal hyperplasia [16]. The decoy therapy was also used for the treatment of asthma [17], rheumatoid arthritis [18] and atopic dermatitis [19]. In cancer, the inhibition of NF-κB by antisense ODNs interfered with the adhesion of cells and caused tumour regression in vitro and in vivo [20]. Previously, injections of phosphodiester NF-κB ODNs in the tumours of mice was shown to have no influence on the growth of the tumours [21], whereas a phosphorothioate NF-κB decoy ODN was found to sensitize MCF7 cells to anti-cancer drugs [22]. However, the relationship between the interaction of an ODN with NF-κB within cancer cells and the effect on growth has not been thoroughly studied. Previously, we showed that a hairpin NF-κB decoy ODN that could covalently bind the p50 subunit of NF-κB could induce cell death in cells in culture, however, substantial efficiency was difficult to achieve probably as the result of intracellular thioredoxin-based reducing systems [23]. The aim of this study was to evaluate the inhibition of NF-κB by a hairpin NF-κB decoy ODN and to analyse the interaction of the ODN with the transcription factor. In order to allow analysis of the cells, low concentrations of the hairpin decoy ODN were used. We tested the hairpin NF-κB decoy ODNs on two cell lines that are dependent on NF-κB for their growth (SW480 and MCF7) and on a cell line that is independent on NF-κB (2C4).

In this report, we show the ability of a hairpin NF-κB-decoy ODN to reduce the growth of NF-κB-dependent cancer cells, we show colocalization of the hairpin decoy ODN with NF-κB in these cells, and show the ability of the hairpin decoy ODN to block the transcriptional activity of NF-κB.

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## 2. Materials and methods

### 2.1. Cell culture

MCF7 (breast), SW480 (colon), 2C4 (fibrosarcoma) cell lines were grown in 10%FCS/ DMEM (GibcoBRL, Life technologies, Cergy-Pontoise, France), 100 U/mL penicillin, 10 μg/mL streptomycin (GibcoBRL), 1 mM sodium pyruvate (GibcoBRL), MEM vitamins 100× (GibcoBRL) and 5 μg/mL plasmocin (Cayla InvivoGen, Toulouse, France). Chemicals: the NF-κB inhibitor Bay 11 was from Bayer AG (France), curcumin was from Acros organics (Halluin, France).

### 2.2. Synthesis of the hairpin NF-κB decoy ODN

The following oligonucleotides were used in this study: $RNH(CH_2)_6$-CTGGAAAGTCCCTCGAAGAGGGACTTTCCAG-$(CH_2)_3NHR$ (hairpin NF-κB decoy ODN) and $RHN(CH_2)_6$-TGCAGTCAC-TACGCGAAGCGTAGTGACTGCA-$(CH_2)_3NHR$ (hairpin scrambled ODN) where R is either H, Cy3 or biotin. Synthesis of decoy oligonucleotides with R = H and Cy3 was published elsewhere [24]. To synthesize oligonucleotides with R = biotin 7–10 nanomoles of the oligonucleotide bearing 3′- and 5′-aminoalkyl linkers were dissolved in 20 μL of 0.1 M $NaHCO_3$. EZ-Link NHS-Biotin (Pierce) (10 μL of a 65 mM solution in dimethyl sulfoxide) was added, and the mixture was incubated at room temperature for 6–16 h in the dark. Then 25 μL of water were added, and the modified oligonucleotide was separated from the excess of hydrolyzed reagent by two consecutive separations on Micro Bio-Spin 6 columns following the manufacturer's recommendations. After the second spin, the biotinylated oligonucleotide was precipitated with ethanol–sodium acetate.

### 2.3. Preparation of liposomes

Liposomes were formulated using a cationic lipid (3β-[*N*-(*N*′,*N*′,*N*′-triethylaminopropane)-carbamoyl] cholesterol) iodide (TEAPC-Chol) and neutral colipid dioleoyl phosphatidylethanolamine (DOPE), as previously described [25]. Briefly, TEAPC-Chol and colipid (DOPE) were mixed at a ratio of 1:1 (w/w) and dissolved in chloroform. The solution was dried in vacuum. Sterile water was then added and the mixture was sonicated to clarity for 1 h in cycles of 15 min. Using light scattering we found that the size distribution of the liposomes was unimodal. The concentration of cationic lipid was monitored by UV spectroscopy at 226 nm and the value was used to calculate the charge ratio assuming one positive charge for each cationic lipid molecule.

### 2.4. Transfection using liposomes

Cells were grown in 96-wells plates to a density of 1 million cells/mL. When the cells reached 50–60% confluence, they were transfected with the hairpin NF-κB decoy ODN or the hairpin scrambled ODN (0.5, 1 and 2 μg corresponding to 100, 200 and 400 nM, respectively) in 100 μL of DMEM medium (without SVF) combined to the liposomes (0.5, 1 or 2 μg of cationic lipid) thus yielding liposomes/ODN ratios of 0.5/0.5, 2/2, 1/0.5 and 1/1 (μg/μg). After 6 h at 37 °C in a humidified 5% $CO_2$ incubator, the cells were placed in fresh serum-containing medium. Expression was analysed after 24 h. In control experiments, the liposomes were used alone at the same lipid concentrations.

### 2.5. Flow cytometry

The uptake of FITC-labeled hairpin NF-κB decoy ODN was measured by flow cytometry, gating on FL1-positive signal on EPICS XL Beckman-Coulter counter (Beckman Coulter, Villepinte, France). To measure the rate of cell death, cells were resuspended in annexin V-binding buffer, incubated with 5 μL of FITC-labeled annexin V (BD Pharmingen, Morangis, France) and 5 μL of propidium iodide and analysed in a EPICS XL Beckman-Coulter counter.

### 2.6. Cell viability

Cell viability was determined using the Cell Titer-Glo kit (Promega-France, Charbonnières, France), following the manufacturer's procedure. The assay was performed by using a luminometer (Clarity Luminescence Microplate Reader, Fisher Bioblock Scientific, Illkirsch, France). In addition, cell viability was assessed using the trypan-blue exclusion method.

### 2.7. Luciferase activity

To measure the transcriptional activity of NF-κB, cells were cotransfected with the 0.4SK-luc plasmid (a generous gift of Dr. A. Israël, Institut Pasteur, France) containing the transcriptional regulatory element of the IκBα promoter with its 3 κB sites in front of the luciferase gene [26] and either the hairpin NF-κB decoy ODN or the hairpin scrambled ODN (0.5 μg, corresponding to 100 nM) combined to liposomes. Cells were lysed 48 h later for 30 min with a lysis buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% NP40 and 1 mM dithiothreitol. The lysates were clarified by centrifugation and assayed for luciferase activity using the Luciferase Assay System (Promega) and a luminometer. Protein concentrations were measured using the Bradford method. The experiments were done in triplicate.

### 2.8. Immunofluorescence

Cellular uptake and subcellular localization of the Cy3-labeled hairpin NF-κB decoy ODN were analysed on cells grown for 24 h on glass slides (Lab-Tek, Nunc, USA). Cells were washed twice in PBS, fixed in 3.7% formaldehyde in PBS for 15 min, permeabilized in 0.1% Triton X-100 for 15 min and blocked with 5% FCS, 0.1% Tween in PBS for 1 h. Cells were incubated with the primary antibody (anti-pl05/p50, dilution 1:100, Cell Signaling) for 1 h. The signal was amplified by the formation of the streptavidin–biotin complex (Universal LSAB kit, Dako France SAS, Trappes, France) using Alexa Fluor 488-labeled streptavidin (Invitrogen-Molecular Probes) diluted at 1:500 (90 min incubation). After counterstaining with DAPI, coverslips were mounted onto glass slides in Vectashield (Vectorlabs, Clinisciences, Montrouge, France). Fluorescent images were digitally acquired using a Zeiss Axioplan2 Deconvolution microscope (CarlZeiss, Le Pecq, France) and analysed with Metafer4 (Metasystems, Altlussheim, Germany).

### 2.9. Oligonucleotide pull-down and Western blotting

Nuclear protein extracts were obtained as follows: 20 million cells were resuspended in lysis buffer containing 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 10 mM KCl, 0.3% NP40, 0.5 mM dithiothreitol, 0.1 mM EDTA and protease inhibitors (Compete™, Boerhinger) at 4 °C for 5 min. The lysates were centrifuged at $12000 \times g$ for 20 min at 4 °C, and the supernatants containing the cytoplasmic proteins were discarded. The pellets were resuspended in the cell lysis buffer adjusted with 20% glycerol and 0.35 M NaCl for 30 min at 4 °C. After centrifugation at $20000 \times g$ for 5 min at 4 °C, the supernatants were stored at −80 °C. For pull-down assays, 100–200 μg of nuclear protein extracts were incubated for 30 min at 4 °C in binding buffer containing: 1% NP40 (Sigma), 50 mM HEPES, pH 7.6 (Euromedex), 140 mM NaCl (Sigma), salmon sperm DNA (1 μg/assay) and 1 μg of the biotinylated hairpin decoy ODN. The complexes were captured by incubating with 50 μl of avidin–sepharose beads (neutravidin, Pierce) for 2 h at 4 °C, washed three times with Tris-buffered saline (TBS) 20 mM NaCl (Sigma), 500 mM Tris–HCl, pH 8 (Euromedex), and once with TBS-0.1% Tween (TBS-T). After resuspension in sample buffer, complexes were separated on a SDS–polyacrylamide (10%) gel, and subjected to immunoblotting using anti-p50 NF-κB (Cell Signaling) followed by chemiluminescence (LumiGLO, Cell Signaling) and autoradiography (X-Omat R, Kodak).

## 3. Results

### 3.1. Efficient lipid-mediated delivery of NF-κB hairpin decoy to MCF7 and SW480 tumour cells

To determine lipids/decoy ODN ratios that result in optimal cellular uptake of the hairpin NF-κB decoy into SW480 cells, we prepared mixtures of cationic lipid/neutral colipid (DOPE) and decoy ODN at different weight ratios and transfected cells with increasing concentrations of FITC-labeled hairpin NF-κB decoy ODN. The intensity of FITC fluorescence was measured by flow cytometry: the uptake of the decoy in cells showed concentration dependence with no apparent saturation in the hairpin NF-κB decoy ODN concentration range studied (Fig. 1A). Similar results were obtained with MCF7 cells (not shown).

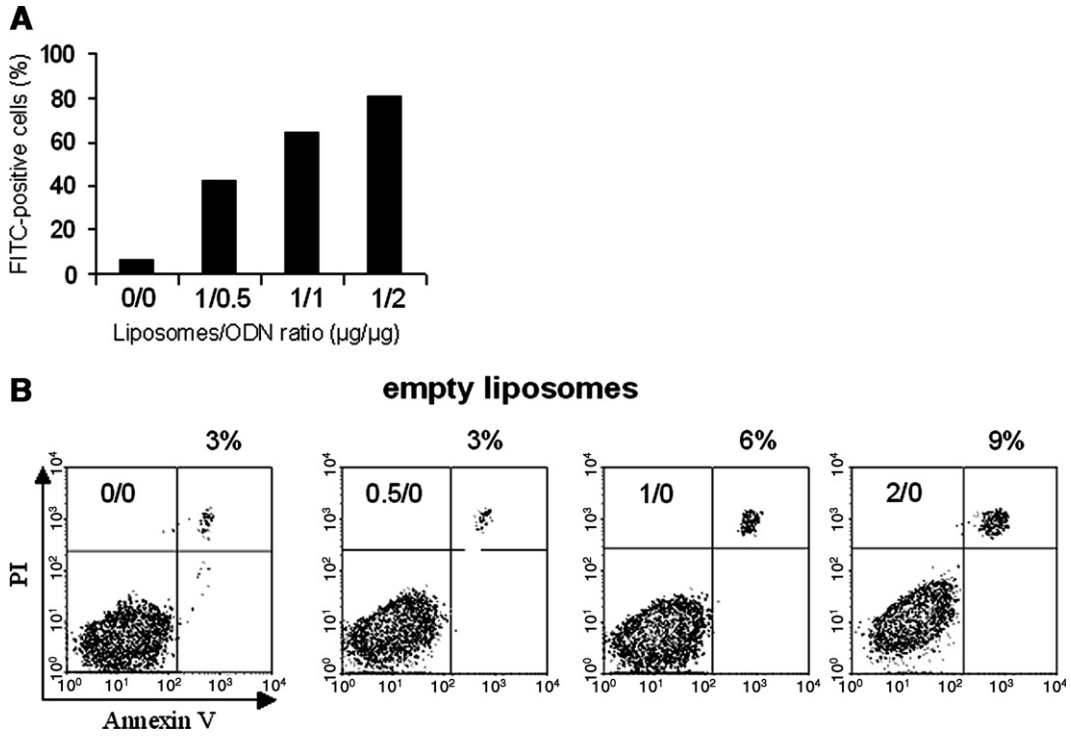


Fig. 1. Cellular uptake of the hairpin NF-κB decoy ODN. (A) Efficient uptake of the decoy ODN by SW480 cells using decoy/lipid complexes. The complexes were transfected using FITC-labeled NF-κB decoy/positively charged lipid/colipid complexes. After 6 h of incubation, the cells were placed in fresh culture medium containing 10% serum for 24 h. Fluorescence intensity was measured by flow cytometry after a treatment with lipids alone (liposomes/ODN ratio 1/0) or lipids combined to increasing concentrations of FITC-labeled hairpin NF-κB decoy ODN from 100 nM to 400 nM (liposomes/ODN ratio 1/0.5, 1/1, 1/2). (B) Effect of the treatment of MCF7 cells with lipids alone. Cells were treated with lipids for 6 h and analysed for annexin V binding and PI uptake after 24 h of culture: 0: no lipids: 3% dead cells, 0.5 µg of lipids: 3% dead cells, 1 µg of lipids: 6% dead cells, 2 µg of lipids: 9% dead cells.

### 3.2. Cell death in MCF7 and SW480 tumour cells treated with NF-κB hairpin decoy

MCF7 and SW480 cells were treated with the low molecular weight inhibitor of NF-κB (Bay-11) to verify that their growth was dependent on functional NF-κB. Concentrations in the micromolar range were sufficient to kill cells within hours (data not shown). Several lipid concentrations, ranging between 5 and 20 µg/ml were tested for their toxicity to MCF7 cells. The resultant content in dead cells, measured by annexin V binding and PI uptake, varied between 3% and 9% (Fig. 1B). After treatment of MCF7 cells with hairpin NF-κB decoy for 6 h, followed by a 24 h incubation, the proportion of dead cells increased over 2-fold when the ODN/lipid complexes ratio was 1/1, and reached 5-fold when the ODN/lipid complexes ratio was 2/2 (Fig. 2A, see histogram in 2B). In the case of SW480 cells, addition of lipids alone increased cell death by 10% (see Fig. 2C) and treatment with hairpin decoy ODN (lipid/ODN ratio equal to 1/0.5) for 30 h resulted in the highest rate of cell death (Fig. 2C). Using the trypan blue exclusion method we found a 2-fold increase in dead cells with a ratio of 1/0.5 and a 2.5 increase with a ratio of 1/1 (Fig. 2D). In the 2C4 fibrosarcoma cell line, the transfection of hairpin NF-κB decoy ODN did not induce cell death (Fig. 2E) although the hairpin NF-κB decoy ODN could efficiently enter the cells (Fig. 2F). On the other hand, curcumin, a chemical with multiple targets [27], could efficiently kill the 2C4 cells (Fig. 2E).

To further characterize the effect of hairpin NF-κB decoy ODN on cell viability, intracellular ATP was measured by luminometry. Twenty four hours after the MCF7 cells had been treated with the hairpin NF-κB decoy ODN (ratio 2/2), a 2-fold decrease in cell viability was observed (Fig. 3A). With the SW480 cells, a 3-fold decrease was observed after 24 h of treatment with the hairpin NF-κB decoy ODN (ratio 1/0.5) (Fig. 3B). In control experiments using the hairpin scrambled ODN (ratio 1/0.5) there was no effect on viability in neither cell line (Fig. 3C) nor did cell death increase (data not shown).

## 4. The hairpin NF-κB decoy ODN interacts with the p50 subunit of NF-κB

The decreased cell viability and the increased cell death caused by the treatment with the hairpin NF-κB decoy ODN could potentially be a consequence of events unrelated to NF-κB. To test whether the hairpin NF-κB decoy ODN interacts with NF-κB we performed pulldown assays using a biotinylated version of the hairpin NF-κB decoy ODN and nuclear protein extracts of SW480 cells. The results show that the hairpin NF-κB decoy ODN formed a complex with the p50 subunit (Fig. 4A), this complex was not detectable when nuclear protein extracts were first incubated with an excess of non-biotinylated hairpin NF-κB decoy ODN or when a biotinylated hairpin scrambled decoy ODN was used. These data demonstrate the ability of the hairpin NF-κB decoy ODN to interact with the p50 subunit of NF-κB in cellular extracts. To verify that this interaction also occurred within the cells, fluorescence

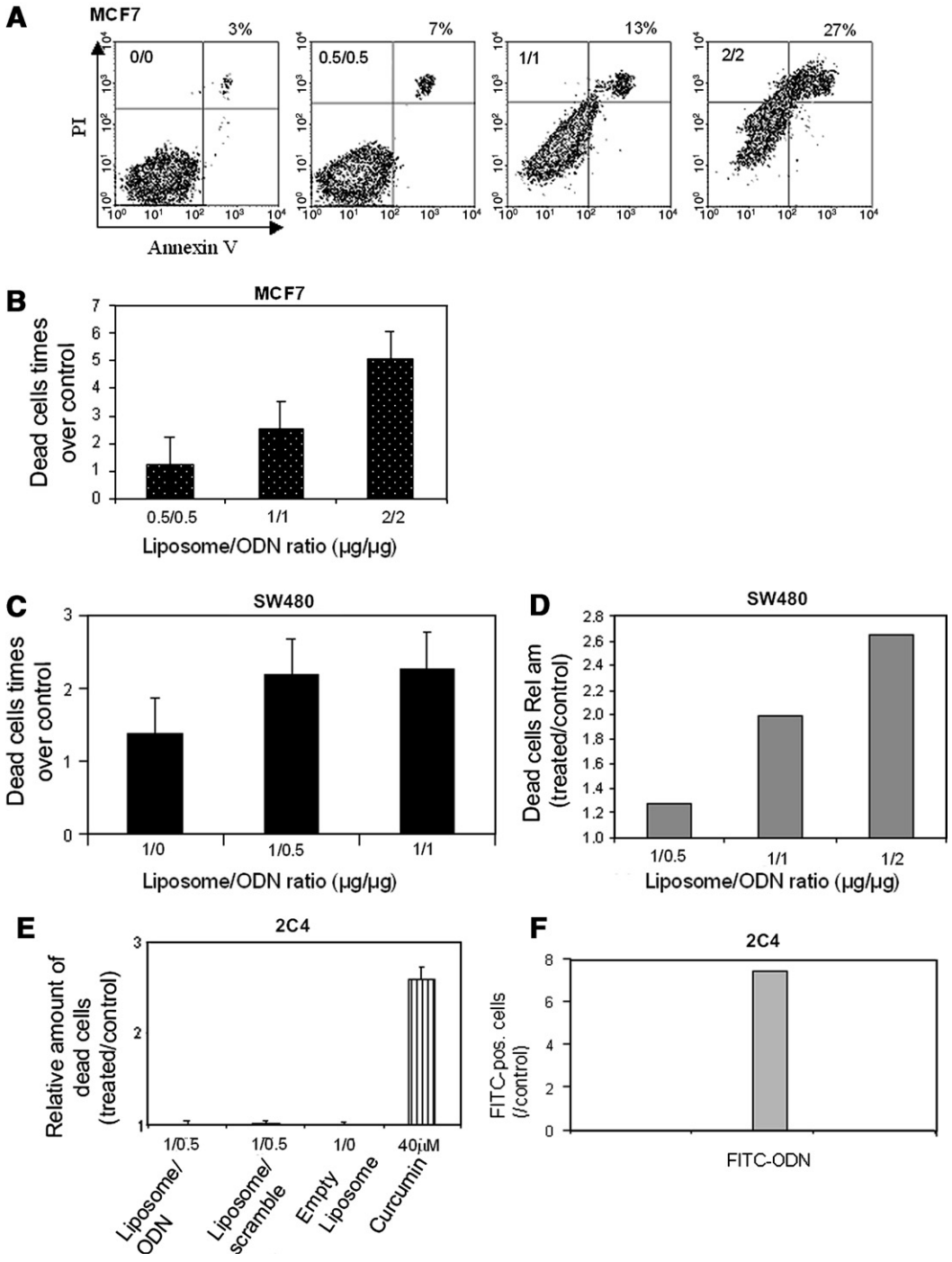


Fig. 2. Increased cell death of MCF7 and SW480 cells treated with hairpin NF-κB decoy ODN. (A) MCF7 cells were treated with empty lipid complexes (liposomes/ODN ratio 0/0), or lipid complexes combined with hairpin NF-κB decoy ODN at the liposomes/ODN ratios of 0.5/0.5, 1/1 or 2/2 for 6 h and analysed by flow cytometry for annexin V binding and PI uptake after 24 h of culture; a typical experiment is shown. (B) Histograms of several experiments of cell death induction by the hairpin NF-κB decoy ODN. Results are expressed as fold of dead cells over control (lipids alone). (C) Cell death induced by the ODN in SW480 cells as measured by annexin V binding. Cells were treated for 6 h with lipid complexes combined with hairpin NF-κB decoy ODN at the following liposomes/ODN ratios (µg/µg): 0/0, 1/0; 1/0.5 and 1/1, and analysed for annexin V binding by flow cytometry, after 24 h of culture; The combined results from several experiments are expressed as fraction of control (untreated cells). (D) Cell death induced by the ODN in SW480 cells as determined by the trypan blue exclusion assay. Results are expressed as fold of dead cells over control. A typical result is shown. (E) The 2C4 cells, which do not depend on the activity of NF-κB for their survival, were treated with empty liposomes (1/0), liposomes either combined to hairpin NF-κB decoy ODN 1/0.5 or to a hairpin scrambled ODN at the same concentrations or with curcumin at 40 µM. Viable cells were counted using the trypan blue exclusion assay. The relative amount of dead cells (treated cells/control cells) is shown, the experiment was repeated three times. (F) The 2C4 cells were transfected with the FITC-labeled hairpin NF-κB decoy ODN 1/0.5 or liposomes alone (1/0) and fluorescence intensity measured as in Fig. 1. The result is expressed as a fraction of the control.

microscopy was used. Cells were transfected with a Cy3-labeled version of the hairpin NF-κB decoy ODN under conditions similar to those described for the induction of cell death. After transfection, the p50 subunit was mainly detected in the

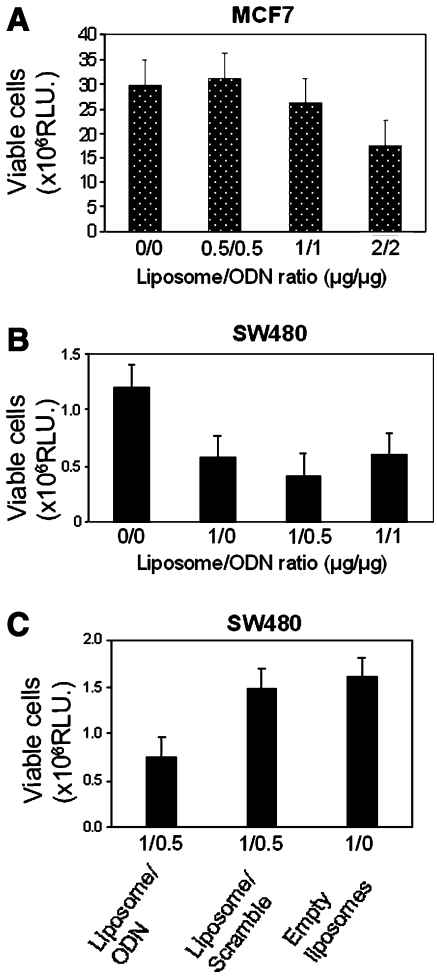


Fig. 3. The hairpin NF-κB decoy ODN reduces the viability of MCF7 and SW480 cells. MCF7 (A) and SW480 cells (B) were treated with hairpin NF-κB decoy ODN under the conditions used in Fig. 2 and cell viability determined by measuring the intracellular concentration of ATP on a luminometer. A control experiment included SW480 cells treated with either lipid complexes combined with the hairpin NF-κB decoy ODN or the hairpin scrambled ODN, at 1/0.5, or free lipid complexes (1/0). After 24 h of culture, ODN concentration-dependent decreased intracellular ATP was observed reflecting reduced viability of the cells. The effect was maximal between 0.5 µg (100 nM) and 1 µg (200 nM) of ODN (C). The experiment was performed in triplicate.

cytoplasm of the MCF7 cells and the Cy3-labeled hairpin NF-κB decoy ODN was found to be homogeneously distributed within the cytoplasm of most of the cells. Superimposition of the p50 (green fluorescence) and the Cy3 -labeled hairpin NF-κB decoy ODN (red fluorescence) images yielded areas of yellow color, suggesting that p50 and hairpin NF-κB decoy ODN colocalized in the cytoplasm (Fig. 4B). In the SW480 cells, the p50 subunit and the Cy3-labeled hairpin NF-κB decoy ODN were also detected in the cytoplasm (Fig. 4C, panels a and c). And after a treatment of the cells with TNFα, the p50 subunit fluorescence was detected in the nucleus (Fig. 4C, panel b), however, in cells that had also been treated with the Cy3-labeled hairpin NF-κB decoy ODN, the p50 subunit was detected in the cytoplasm, but not in the nucleus (Fig. 4C, panel d, arrow). These data support the notion that the hairpin NF-κB decoy ODN is engaged in direct binding with the p50 within the cell.

## 5. The hairpin NF-κB decoy ODN inhibits NF-κB transcriptional activity

To determine whether the hairpin NF-κB decoy ODN could inhibit the transcriptional activity of NF-κB, the cells were transfected with a luciferase reporter cDNA under the control of a promoter containing 3 NF-κB recognition sites and the intensity of the luminescence was measured. The results showed a 70% decrease of the luminescence in cells that were transfected with the hairpin NF-κB decoy ODN, whereas there was no inhibition when cells were transfected with the hairpin scrambled ODN (Fig. 5).

## 6. Discussion

The main goal of this work was to examine the ability of a hairpin NF-κB decoy ODN to induce cell death in cancer cells and to determine whether this effect correlated with the interaction of the hairpin NF-κB decoy ODN with NF-κB. The results obtained demonstrated that the decoy induced cell death in the cell lines tested and that it formed complexes with the p50 subunit of NF-κB, suggesting that it functions by directly blocking it in the cells. Inhibition of gene expression by targeting different transcription factors by ODNs has been shown to efficiently inhibit the growth of cancer cells [28–31]. The study attempted to investigate in more detail the mechanism of the anti-proliferative and the effects on cell death of NF-κB decoy ODN at the cellular level in NF-κB-dependent tumour cells. The decoy ODNs had aliphatic linker chains attached at both 3′ and 5′ termini to increase their resistance to nucleases within the cells, allowing to use the lowest possible concentrations. Indeed, in experiments conducted with the same hairpin decoy ODN without any end-substitutions there was no detectable effect on cell death (not shown).

The dependence of the two cancer cell lines, MCF7 and SW480, on the activity of NF-κB for their growth [3,32,33] was verified using Bay 11 [5]. In order to be able to study the cells we chose to use the hairpin decoy ODNs at low concentrations. The two cell lines showed a considerable increase of cell death levels after transfection with the hairpin NF-κB decoy ODN; their sensitivity to hairpin decoy ODN were somewhat similar: with MCF7 cells, an increase was still noted at 2/2 but the toxicity of lipid itself did not allow to use higher amounts, whereas with SW480 the ratios of 0.5/1 and 1/1 gave identical results, as well as 1/2 (not shown), indicating that optimal conditions were reached. In contrast, in the 2C4 fibrosarcoma cell line, which shows no dependence on NF-κB transcriptional activity [34] NF-κB decoy ODN had no effect. Altogether, these results show that NF-κB-dependent cells are sensitive to a treatment by a hairpin NF-κB decoy ODN. The use of lipids was necessary to enable the incorporation of the decoy ODN into the cells, and when added alone the lipids showed low toxicity to the cells. The lipid complexes enabled homogeneous distribution of the Cy3-labeled ODN in the cytoplasm of most of the cells. The observation of a concentration-dependent inhibition of NF-κB, together with the absence of effect of a hairpin scrambled ODN suggests that direct interaction of the hairpin NF-κB decoy ODN with the DNA-binding region of NF-κB was required for inhibition. Indeed, the Cy3-labeled version of the hairpin NF-κB decoy ODN was found to colocalize with the p50 subunit of NF-κB

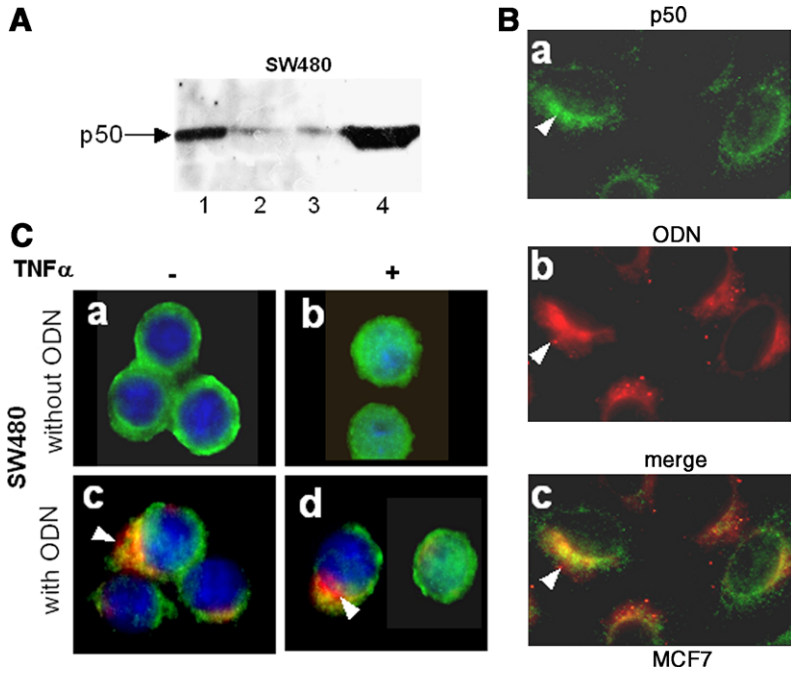


Fig. 4. Demonstration of the interaction of the hairpin NF-κB decoy ODN with the p50 subunit. (A) Pull-down assay with the hairpin NF-κB decoy ODN. Nuclear extracts (100 μg) were incubated with a biotinylated version of the hairpin NF-κB decoy ODN (1 μg) (lane 1), or a biotinylated hairpin scrambled ODN (1 μg) (lane 2). In one assay, an excess of non-biotinylated-NF-κB decoy ODN was added before the biotinylated ODN (lane 3). The complexes were analysed by Western blotting using anti-p50 NF-κB antibody. The amount of NF-κB p50 subunit in the nuclear extract is shown (input) (lane 4). Cellular uptake of the hairpin NF-κB decoy ODN and colocalization with the p50 subunit of NF-κB: (B), in MCF7 cells; the subcellular location of the p50 subunit NF-κB was analysed by fluorescence microscopy after fluorescent immunostaining (Alexa 488 green) and was mostly cytoplasmic (a, arrow). The distribution of the Cy3-labeled hairpin NF-κB decoy ODN, analysed under conditions identical to those described in Fig. 1, was homogenous and cytoplasmic (b, arrow). The merge (c) shows cytoplasmic colocalization of the p50 subunit and the Cy3-labeled NF-κB decoy ODN (arrow). (C) In SW480 cells; the p50 subunit of NF-κB was cytoplasmic in non-treated cells (panel a) and nuclear in TNFα-treated cells (panel b). The Cy3-labeled NF-κB decoy ODN was in the cytoplasm (c, arrow). The merge shows the colocalization of the labeled hairpin NF-κB decoy ODN and the NF-κB p50 subunit (d, arrow), and suggests inhibition of the TNFα-induced nuclear translocation of the p50 subunit by the hairpin NF-κB decoy ODN. Magnification: 100×.

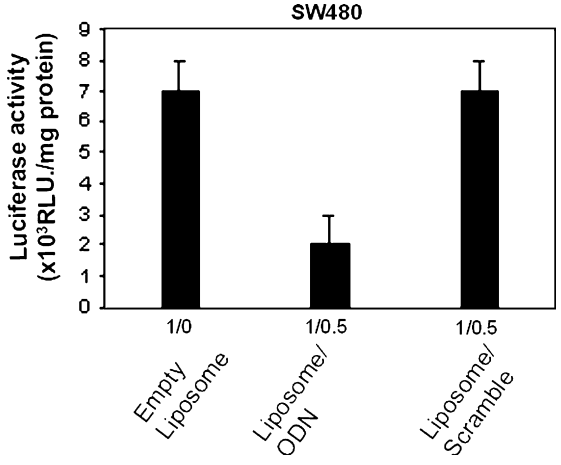


Fig. 5. Inhibition of the transcriptional activity of NF-κB by the hairpin NF-κB decoy ODN in SW480 cells. SW480 cells were cotransfected with a 0.4SK-luc plasmid and either hairpin NF-κB decoy ODN or a hairpin scrambled ODN, and the luciferase activity after 24 h incubation with the decoy ODN measured. Relative NF-κB transcriptional activity in transfected cells is shown. Each transfection experiment was done in triplicate.

within the cytoplasm. Furthermore, the hairpin NF-κB decoy ODN was apparently able to prevent the TNFα-induced translocation of the p50 subunit to the nucleus. As suggested by the fluorescence microscopy results, the treatment with hairpin NF-κB decoy ODN resulted in trapping the p50 subunit exclusively in the perinuclear area in the cytoplasm. The direct interaction of the ODN and NF-κB was also demonstrated by the efficient pulldown of the p50 subunit of NF-κB from nuclear extracts with a biotin-tagged version of the hairpin NF-κB decoy ODN. The observed reduced activity of the luciferase reporter construct containing an NF-κB response element in cells treated with the hairpin NF-κB decoy ODN suggested that p50-hairpin decoy ODN interaction was responsible for the inhibition of the transcriptional activity of NF-κB.

Overall, our results indicate that trapping of p50 in the cytoplasm could be sufficient to efficiently inhibit NF-κB. Previous reports using NF-κB decoy drew somewhat different conclusions: in some reports the trapping of the hairpin NF-κB decoy ODN to the cytoplasm is considered to be responsible for its absence of inhibitory efficiency [35,36]; in another report the efficient inhibition of NF-κB, measured by gel retardation assay is thought to be independent from the nuclear translocation of the transcription factor [37]. These observed differences may be due in part to variations in the experimental conditions. Nevertheless, the mechanism of activation of NF-κB involves its interaction with target DNA mostly as a heterodimeric complex comprising the p50 and p65 subunits, an interaction which is thought to take place in the cytoplasm once the complex has been freed from the I-κB inhibitory subunit. Crystallographic studies have also shown that the I-κB subunit

masks the nuclear localization signal (NLS) of the p65 subunit but not that of the p50 subunit [38], thus it is unclear why the hairpin decoy ODN used in this study is so efficient. It is possible that the ODN's high affinity for the p50 subunit interferes with the cytoplasm/nucleus equilibrium shuttling [39,40] of the NF-κB complex. Because of the differences between relative affinities of anti-p50 and anti-p65 antibodies it was problematic to faithfully assess the content of the hairpin decoy ODN/NF-κB subunit complex obtained in pull-down assays: in our hands the p65 subunit was present in these complexes but in lower amounts (not shown). Nevertheless, the most abundant forms of NF-κB complexes are the p50/p65 hetero- and the p50/p50 homodimers, the p50/p65 heterodimer being the most transcriptionally active form [41].

The mechanism of cell death induced by the hairpin NF-κB decoy ODN remains unclear. While trypan blue exclusion, PI and ATP viability tests showed clear effects of the hairpin NF-κB decoy ODN, the Annexin V binding and the detection of apoptotic bodies by DAPI were negative. Recent studies showed that several forms of programmed cell death exist apart from apoptosis [42,43]. The absence of PARP cleavage in the hairpin decoy treated cells suggests the involvement of a form of cell death different from apoptosis (data not shown).

In conclusion, we find that the efficient induction of cell death by a hairpin NF-κB decoy ODN involves its direct interaction with the transcription factor and its subsequent cytoplasmic trapping. This provides a basis for the modelling of high affinity inhibitory decoys interacting with a transcription factor's DNA-binding region.

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