Effects of decoy molecules targeting NFKB transcription factors in cystic fibrosis IB3-1 cells

Recruitment of NFKB to the IL-8 gene promoter and transcription of the IL-8 gene

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Keywords: NFKB, transcription factor decoy, inflammation, peptide nucleic acids, PNA-DNA chimeras

Abbreviations: ODN, oligodeoxyribonucleotides; PNA, peptide nucleic acids; PDP, PNA-DNA-PNA chimeras; TFD, transcription factor decoy; NFKB, nuclear factor kappa B; IkB, IkappaB, inhibitor of NFKB; EMSA, electrophoretic mobility shift assay; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PAO-1, Pseudomonas aeruginosa, strain O1; IL-8, Interleukin 8; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; ChIP, chromatin immunoprecipitation

Introduction

Cystic fibrosis (CF) is a severe inherited disease caused by mutations of a gene encoding a chloride channel termed Cystic fibrosis transmembrane conductance regulator (CFTR).1 Although most of the CF patients are affected by multiple organ pathologies, lung disease is the major cause of morbidity and mortality in CF. In the lung of CF patients a hyper-inflammatory condition is established, which is characterized by predominant infiltrates of polymorphonuclear neutrophils (PMNs) in bronchial lumina and increased expression of pro-inflammatory cytokines and chemokines, in particular interleukin-8 (IL-8).2-4 Recently, it has been established that excessive IL-8 release from lung epithelial cells may also play a role in bacterial proliferation and adhesion.5 Accordingly, IL-8 is presently considered a critical pharmacological target to reduce the excessive inflammation in CF lungs.6 We recently described an analysis of the transcription machinery of the IL-8 gene in human bronchial epithelial cells, and found NFKB pathway very important.7,8 These results are in line with several reports indicating lung inflammation in CF related to an increased NFKB signaling causing the induction of IL-8 gene expression.9 One of the possible strategies to inhibit IL-8 gene transcription is the so-called “transcription factor decoy” (TFD) approach,10-13
with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices and are resistant to both nucleases and proteases. PNAs were found to be excellent candidates for antisense and antigene therapies. Among PNA-based molecules, we found that PNA-DNA chimeras are of great potential in gene therapy being active as decoy molecules against the NFκB and Sp1 transcription factors.

Following these considerations, we determined the effect of PNA-DNA-PNA (PDP) chimeras mimicking the binding sites of NFκB on the transcription of IL-8 gene in cystic fibrosis IB3-1 cells infected by Pseudomonas aeruginosa. We have described this system in several reports and reviews, pointing out that after Pseudomonas aeruginosa exposure, IB3-1 cells infected by PAO1 are induced to increase accumulation of IL-8 mRNA in respect to basal levels of uninfected cells. In addition to IL-8 mRNA, other sequences induced by PAO1 are GRO-γ, GRO-α, IL-6, IL-1β, ICAM-1. Therefore, IB3-1 cells infected by P. aeruginosa are an excellent system to verify (1) whether decoy molecules against NFκB inhibit IL-8 gene transcription and (2) whether this effect is restricted to the IL-8 gene, or affects

In this respect, in recent reports we presented the possible use of peptide nucleic acids (PNAs) as alternative reagents in experiments aimed at the control of gene expression involving the TFD approach. In PNAs, the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units. PNAs hybridize

Figure 1. (A) Experimental design followed for the analysis of the effects of the transcription factor decoy (TFD) strategy employing TF decoy ODNs against NFκB and human bronchial epithelial cells. (#1) Human bronchial CF-derived respiratory epithelial IB3-1 cells were pre-incubated for 24 h with NFκB ODNs or PDP/PDP chimeras before infection with the laboratory strain of P. aeruginosa PAO1 (#2). After 4 h post-infection, total RNA was extracted, reverse-transcribed to cDNA and analyzed by RT-qPCR (#3). In parallel, chromatin was purified by the IB3-1 cells for chromatin immunoprecipitation assay (ChIP) (#4). In (B) representation of the genomic region located: 6 kb upstream of IL-8 gene. The location of primers used for IL-8 promoter amplification in the ChIP assay and the respective product length are indicated: PCR product obtained from IL-8 promoter amplification, containing NFκB binding site (301 bp, in blue); PCR product obtained using control primers flanking a genomic region: 5 kb upstream of IL-8 promoter (255 bp, in red).
The experimental system and preliminary assays on the specificity of the decoy molecules. Figure 1 shows the experimental strategy followed in our study. Complexes of cationic liposomes with NFkappaB ODNs or PDP/PDP chimeras have been pre-incubated with cystic fibrosis IB3-1 cells 24 h before exposure to the PAO1 laboratory strain of *P. aeruginosa* (100 CFU/cell) for a further four hour time period. A scrambled ODN was used as negative control (Table 1). After the treatment RNA was extracted and real-time quantitative RT-PCR performed; at the same time chromatin was purified from IB3-1 cells for chromatin immunoprecipitation assay (ChIP). In the first set of experiments, described in Figure 2, we provided evidences that the TF decoy approach interferes with the NFkappaB activity in vitro and in the IB3-1 cellular system. In order to verify the ability of this decoy ODNs to compete for the binding of NFkappaB to the sequences contained in the promoter of IL-8 gene, the NFkappaB decoy ODNs was incubated with nuclear extracts from IB3-1 cells in the presence of a radiolabeled probe 100% homologous with the NFkappaB binding sequences and performed electrophoretic mobility shift assays (EMSA). Complete inhibition of interaction of the 32P-labeled probes with specific transcription factor proteins (NFkappaB/DNA complexes) has been obtained, providing the proof of principle of the competition of this NFkappaB decoy ODNs for the DNA consensus sequence contained in the promoter of the IL-8 gene. On the contrary, other ODN containing the binding sites for other transcription factors are inactive. ODN for CREB, NF-IL6, AP-1 and CHOP were selected because these transcription factors play a crucial role in the control of transcription of the IL-8 gene.11

Effects of decoy ODNs targeting NFkappaB on gene expression of *Pseudomonas aeruginosa* infected IB3-1 cells. The results reported in Figure 3A show that treatment with 0.5 μg/ml of ODN *P. aeruginosa* induced IL-8 gene increased expression, as outlined in Figure 3B, showing that treatment of PAO1 infected IB3-1 CF cells with NF-IL6 decoy does not lead to significant reduction of IL-8 mRNA. In order to clarify the effects of decoy molecules on the NFkappaB intracellular dynamics, we studied by chromatin immunoprecipitation (ChIP) the possible effects of the treatment with decoy ODNs on the recruitment of NFkappaB to the IL-8 gene promoters. Chromatin was isolated from uninfected IB3-1 cells, from cells infected with PAO1, and from cells infected with PAO1 and treated with 2 μg/ml of ODN NFkappaB decoy. After immunoprecipitation (Fig. 1B), amplification of the IL-8 gene promoter segment containing the NFkappaB binding sites was performed; as a negative control a parallel PCR was also performed using primers amplifying an upstream sequence of the IL-8 gene (located at -5 Kb from the transcription start site) lacking NFkappaB consensus elements. Figure 3B shows one important feature of our experimental cell systems, i.e., that NFkappaB is recruited with high efficiency to the IL-8 gene promoter following infection with PAO1; second, the data clearly indicate that the NFkappaB decoy ODN interferes with this recruitment. No changes in occupancy by NFkappaB were detected when ChIP-DNA was amplified using PCR primers specific for the upstream sequence of the IL-8 gene lacking NFkappaB consensus elements.

The NFkappaB decoy PNA-DNA-PNA chimeras are strong inhibitors of IL-8 gene expression and do not require transfection reagents. While it is firmly established that the entry of oligonucleotides into eukaryotic cells can occur through a nucleic acid channel,30 the complexation with lipofectamine (or other delivery systems) is required in vitro, since the degradation of ODNs exposed to fetal calf serum is an important drawback of these transfection approaches.31,32 On the contrary, we have elsewhere reported that, unlike ODN-based decoys, PDP/PDP chimeras are fully resistant to serum and cytoplasmic extracts.18,28 This information is of great impact, in our opinion, for the development of stable molecules to be used in non-viral gene therapy. Therefore, we compared the effects of ODN-based and PDP-based NFkappaB decoys on *P. aeruginosa* infected cells in the presence or in the absence of the lipofectamine transfection reagents. In the first set of experiments we confirmed that the NFkappaB decoy PNA-DNA-PNA chimera (see Fig. 4A for molecular structure) reproduces the activity of the NFkappaB decoy ODN on *P. aeruginosa* induced genes. In fact, the NFkappaB PDP/PDP chimera exhibits differential effects on expression of PAO1...
Recent studies have indicated that the transcription factor decoy (TFD) strategy targeting NFkappaB might be of interest to develop anti-inflammatory approaches for cystic fibrosis.12,13 The first set of data of the present study demonstrate that decoy ODNs against NFkappaB interfere with the NFkappaB pathway in cystic fibrosis cells infected with *Pseudomonas aeruginosa*. This was demonstrated by chromatin immunoprecipitation (ChIP) using as a model system cystic fibrosis IB3-1 cells infected with *Pseudomonas aeruginosa*. Treatment with ODN decoys against NFkappaB led to a decreased occupancy of the IL-8 gene promoter by NFkappaB factors.

In order to develop more stable therapeutic molecules, peptide nucleic acids (PNAs) based agents were considered. PNAs are DNA mimicking molecules in which the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units.19-21 PNAs activated genes (Fig. 4B), such as ICAM-1, GRO-γ, IL-1β, IL-6 and IL-8. The major inhibitory effect was found on IL-8 gene expression, confirming on one hand elsewhere reported studies on the role of NFkappaB for IL-8 gene transcription and, on the other hand, that decoy for NFkappaB might retain differential effects of genes regulated by promoters containing NFkappaB signal sequences, such as ICAM-1, GRO-γ, IL-1β and IL-6. This suggest that other transcription factors are the master regulators of these genes (such as Sp1 for IL-6 gene).33

Despite the fact that this specific issue should be object of future investigations, the effects of the NFkappaB PNA-DNA-PNA chimeras, as shown in the insert of Figure 4B, reproduce those obtained using NFkappaB ODNs. The results shown in Figure 5, while confirming that the NFkappaB decoy ODN exhibit lipofectamine-dependent effects, provide evidence that the NFkappaB decoy PDP interferes with the NFkappaB activity without the need of lipofectamine.

### Table 1. Sequence of synthetic oligonucleotides used in this study

| Double-stranded oligonucleotides used in gel shift assays and decoy transfections* | Sequences                      |
|--------------------------------------|--------------------------------|
| **NFkappaB**                         | 5'-AGA GGA ATT TCC ACG ATT-3' |
| **CREB**                             | 5'-AAA ACT TTC GTA ATA CTC-3' |
| **NF-IL6**                           | 5'-CAT CAG TTA CCA ATG GTC-3' |
| **AP-1**                             | 5'-TGT GAT GAC TCA GGT TTG-3' |
| **CHOP**                             | 5'-CGC TGG TGT GAT GCA CGG-3' |
| **SCRAMBLED**                        | 5'-CAC AAA GTG TAA CAG TCT-3' |

**ChIP Q-PCR primers**

| Amplified region | Sequences                      | Accession number |
|------------------|--------------------------------|-----------------|
| IL-8 ChIP f      | IL-8 promoter region           | 5'-TCA CCA ATG TGG GCT TCA GTA T-3' | AF385628.2 |
| IL-8 ChIP r      | IL-8 promoter region           | 5'-GGC TCT TGT CCT AGA ACG TTG TGT-3' |  |
| Neg ChIP f       | Negative control region a      | 5'-TCC CCA TCT CTT CTT CAA GGT GC-3' |  |
| Neg ChIP r       | Negative control region b      | 5'-CGT GCA TTT AAT TGT GTC TTG TGG-3' |  |

**RT Q-PCR primers**

| Amplified region | Sequences                      | Accession number |
|------------------|--------------------------------|-----------------|
| IL-8 f           | IL-8 transcripts               | 5'-GAC CAC ACT GGC CCA ACA-3' | AF385628.2 |
| IL-8 r           | IL-8 transcripts               | 5'-GCT CTC TCT CAT CAG AAA GTT ACA TAA TTT-3' |  |
| ICAM-1 f         | ICAM-1 transcripts             | 5'-TAT GGC AAC GAC TCC TTC TCG-3' | NM_000201 |
| ICAM-1 r         | ICAM-1 transcripts             | 5'-CTC TGC GGT CAC ACT GAC TGA-3' |  |
| GRO-γβ f         | GRO-γβ transcripts             | 5'-CCG GAC CCC ACT GCG-3' | M36821 |
| GRO-γβ r         | GRO-γβ transcripts             | 5'-TTC CCA TCT TTG AGT GTG GCT A-3' |  |
| IL-1-β f         | IL-1-β transcripts             | 5'-CTC CAC CTC CAG GGA CAG-3' | BT07213.1 |
| IL-1-β r         | IL-1-β transcripts             | 5'-GGG CAT GGA GAA CAC CAC TTG TT-3' |  |
| IL-6 f           | IL-6 transcripts               | 5'-CCG TAC ATC CTC GAC GGC-3' | NM_000600 |
| IL-6 r           | IL-6 transcripts               | 5'-CTT GTT ACA TGT CTC TCT CTT CAG G-3' |  |
| GAPDH f          | GAPDH mRNA                     | 5'-GTG GAG TCC ACT GGC TTC TT-3' | NM_001404.3 |
| GAPDH r          | GAPDH mRNA                     | 5'-GCA AAT GAC CCC AGC CTG C-3' |  |

| aSequence of decoy ODNs based on IL-8 promoter regulatory elements; bRegion about 5 kb upstream of the IL-8 promoter, lacking NFkappaB binding sites.

Discussion

Recent studies have indicated that the transcription factor decoy (TFD) strategy targeting NFkappaB might be of interest to develop anti-inflammatory approaches for cystic fibrosis.12,13 The first set of data of the present study demonstrate that decoy ODNs against NFkappaB interfere with the NFkappaB pathway in cystic fibrosis cells infected with *Pseudomonas aeruginosa*. This was demonstrated by chromatin immunoprecipitation (ChIP) using as a model system cystic fibrosis IB3-1 cells infected with *Pseudomonas aeruginosa*. Treatment with ODN decoys against NFkappaB led to a decreased occupancy of the IL-8 gene promoter by NFkappaB factors.

In order to develop more stable therapeutic molecules, peptide nucleic acids (PNAs) based agents were considered. PNAs are DNA mimicking molecules in which the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units.19-21 PNAs...
are resistant to both nucleases and proteases\textsuperscript{22} and, more importantly, hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices.\textsuperscript{21} For these reasons, PNAs were found to be excellent candidates for anti-sense and antigen therapies.\textsuperscript{23-26} In recent studies, PNA-DNA chimeras have been described as reagents for the transcription factor decoy approach.\textsuperscript{27-29} PNA-DNA chimeras are PNA-DNA covalently bonded hybrids and were designed on one hand to improve the poor cellular uptake and solubility of PNAs, on the other hand to exhibit biological properties typical of DNA, such as the ability to stimulate RNaseH activity and to act as substrate for cellular enzymes (for instance DNA polymerases). The results published by Romanelli et al.\textsuperscript{29} Borgatti et al.\textsuperscript{27,28} and Moggio et al.\textsuperscript{27} firmly demonstrate that decoy molecules based on PNA-DNA chimeras are powerful decoy molecules. In respect to experiments aimed at pharmacological modification of gene expression, PNA-DNA-PNA chimeras are molecules of interest for several points of view: (1) unlike PNAs, they can be complexed with liposomes and microparticles;\textsuperscript{28} (2) unlike ODNs, they are resistant to DNases, serum and cytoplasmic extracts;\textsuperscript{22} (3) unlike PNA/PNA and PNA/DNA hybrids,\textsuperscript{35} they are potent decoy molecules.\textsuperscript{27-29}

The second set of results presented in this paper (Figs. 4 and 5) demonstrated that PDP/PDP chimeras targeting NFκB are strong inhibitors of IL-8 gene expression even in the absence of protection with lipofectamine (Fig. 5) and mimic the biological activity of ODN-based lipofectamine-delivered decoys (insert of Fig. 4B). The extent of inhibition of IL-8 gene expression using naked NFκB decoy ODN (Fig. 5C) approaches that found in lipofectamine-delivered NFκB decoy ODN (Fig. 5B). This is the first research article from our group reporting this information, which is of great impact, in our opinion, for the development of stable molecules to be used in non-viral gene therapy. Despite the fact that these results should be considered as a proof-of-principle that PNA-DNA chimeras targeting NFκB retain selective biological functions, we like to remark that no translation to clinical settings can be proposed based on data reported in the present paper. On the other hand, we like to underline that IL-8 is one of the master genes in pro-inflammatory processes affecting cystic fibrosis. Accordingly, inhibition of its functions might have a clinical relevance and research efforts on this issue deserve great attention.

**Materials and Methods**

**Synthetic oligonucleotides and peptide nucleic acids.** The synthetic oligonucleotides used in this study were purchased from Pharmacia.

**PNA-DNA-PNA oligomer assembly.** Chimeras were assembled on solid phase, by sequential elongation of the PNA fragment, to whom DNA first and PNA were attached. The chimeras were obtained using Mmt(Bz)PNA monomers, as reported in previously published papers.\textsuperscript{29,36} ESI-MS for gcg-ACC CCT GAA AGG T-gcc: [M + 4 H]\textsuperscript{4+}: 1,438.2, [M + 5 H]\textsuperscript{5+}: 1,150.5, calculated for C\textsubscript{194}H\textsubscript{247}N\textsubscript{86}O\textsubscript{99}P\textsubscript{13}: 5,770.26. ESI-MS for gcg-TGG GGA CTT TCC A-cgg: [M + 4 H]\textsuperscript{4+}: 1,438.2, [M + 5 H]\textsuperscript{5+}: 1,150.5, calculated for C\textsubscript{194}H\textsubscript{247}N\textsubscript{86}O\textsubscript{99}P\textsubscript{13}: 5,770.26.

**Cell cultures and bacteria.** IB3-1 cells (LGC Promochem) are human bronchial epithelial cells immortalized with adenov12/SV40, derived from a CF patient with a mutant F508del/W1282X genotype.\textsuperscript{37} Cells were grown in LHC-8 basal medium (Biofluids) supplemented with 5% fetal bovine serum (FBS). All culture flasks and plates were coated with a solution containing 35 μg/ml bovine collagen (Becton-Dickinson), 1 μg/ml BSA (Sigma-Aldrich) and 1 μg/ml human fibronectin (Becton-Dickinson). \textit{P. aeruginosa}, PAO1 laboratory strain, was kindly provided by A. Prince (Columbia University). Bacteria were grown in trypticase soy broth (TSB) or agar (TSA) (Difco).

**Electrophoretic mobility shift assays.** The double-stranded oligonucleotides (ODN) used in the EMSA are reported in the present paper.

![Figure 3.](image-url)
Electrophoresis was performed at 200 V. Gels were vacuum heat-
dried and subjected to autoradiography.

**Figure 4.** (A) Sequences of the double stranded NFkappaB DNA-
PNA chimeras. DNA sequences are within white boxes; PNA sequences are within black boxes. (B) Effects of PDP/PDP NFkappaB decoy molecules on induction of different pro-inflammatory mRNAs. The PDP/ PDP NFkappaB decoy chimera was tested on transcription of ICAM-1, IL-8, GRO-γβ, IL-1β and IL-6 genes in IB3-1 bronchial cells after infection with *P. aeruginosa*. Total RNA was extracted and processed for quantifi-
cation of transcripts as described in Materials and Methods. White bars: PAO-1 infected IB3-1 cells; black bars: IB3-1 cells infected with PAO-1 and treated with NFkappaB decoy PDP/PDP chimeras. Values are mean ± SEM of four separate experiments. Significance was determined using the Student’s t test between each scrambled and decoy ODNs: **p < 0.01. Insert. Relationship between the effects of PDP based and ODN-based NFkappaB decoys on accumulation of IL-8 (●), GRO-γβ (▲), ICAM-1 (○), IL-1β (◇) and IL-6 (■) in PAO-1 infected IB3-1 cells. Data represent mRNA content in respect to control decoy-untreated PAO-1 infected cells.

**Table 1.** ODN (3 pmol each) were 32P-labeled using 10 U T4 polinucleotide kinase (MBI Fermentas) annealed to an excess of complementary ODN and purified from [γ-32P]ATP (Perkin Elmer). Binding reactions were performed by incubating 2.5 μg of nuclear extract, obtained from IB3 cells as previously described,12 and 16 fmol of 32P-labeled double-stranded ODN, with or without competitor in a final volume of 20 μL of bind-
ing buffer (20 mM TRIS-HCl, pH 7.5, 50 mM KCl, 1 mM MgCl2, 0.2 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.01% TritonX100, 0.05 μg·μL−1 of poly dl-dC, 0.05 μg·μL−1 of a single-stranded ODN). Competitor (100-fold excess of unlabeled ODNs) and nuclear extract mixture were incubated for 15 min and then probe was added to the reaction. After a further incubation of 30 min at room temperature samples were immediately loaded onto a 6% nondenaturing polyacryl-
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overnight at 4°C and continued an additional 1 h after the addition of 60 μl protein A-agarose slurry. Thereafter the agarose pellets were washed consecutively with low salt, high salt and LiCl buffers. DNA/protein complexes were recovered from the pellets with elution buffer (0.1 M NaHCO₃ with 1% SDS), and cross-links were reversed by incubating overnight at 65°C with 0.2 M NaCl. The samples were treated with RNase A and proteinase K, extracted with phenol/chloroform and ethanol-precipitated. The pelletted DNAs were washed with 70% ethanol and dissolved in 40 μl of Tris/EDTA. Two μl aliquots were used for each real-time PCR reaction to quantitate immunoprecipitated promoter fragments. For quantitative real-time PCR reaction, a pair of primers that amplify a 301 bp region on the IL-8 promoter, containing the NFκB binding site, was designed (IL-8 ChIP f, IL-8 ChIP r, Table 1).

PCR reactions were also performed using negative control primers that amplify a 255 bp genomic region about 5 kb upstream of the IL-8 promoter, lacking NFκB binding sites (Neg ChIP f, Neg ChIP r, Table 1). Each Real-time PCR reactions were performed in 25 μl of final volume, using 2 μl of template DNA (from chromatin immunoprecipitations), 10 pmol of primers and 1 × iQ™ SYBR® Green Supermix (Bio-Rad) for a total of 45 cycles (96°C for 15 sec, 66°C for 30 sec and 72°C for 20 sec) using an iCycler IQ® (Bio-Rad). The relative proportions of immunoprecipitated promoter fragments were determined based on the threshold cycle (Tc) value for each PCR reaction. Real time PCR data analysis were obtained using the comparative cycle threshold method. The data represent the ratios between the IL-8 promoter Q-PCR performed on NFκB specific ChIP and negative IgG (preimmune rabbit serum) ChIP. Each value was derived employing chromatin isolated from untreated IB3-1 cells or cells infected with PAO-1 in the absence or in the presence of pre-treatment with the NFκB decoy ODN. Each sample was performed in duplicate on at least three separate experiments.

**Statistics.** Results are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made by using paired Student’s t test and a one-way analysis of variance (ANOVA). Statistical significance was defined with p < 0.05 (*significant) and p < 0.01 (**highly significant).

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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