Modified Antisense Oligonucleotides Directed against Tumor Necrosis Factor Receptor Type I Inhibit Tumor Necrosis Factor α-Mediated Functions

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ABSTRACT: Tumor necrosis factor alpha (TNFα), a polypeptide produced by activated macrophages, is a highly pleiotropic cytokine which elicits inflammatory and immunological reactions. The binding of TNFα to tumor necrosis factor receptor type I (TNFRI) is considered the initial step responsible for some of the multiple biological functions mediated by TNFα. The role of TNFα as an inflammatory mediator through human TNFRI makes TNFRI an attractive target for intervention in both acute and chronic inflammatory diseases. In this study, we have identified partial phosphorothioate oligodeoxyribonucleotides (ODNs) containing C-5 propynyl or hexynyl derivatives of 2′-deoxyuridine which specifically inhibited TNFRI and subsequently inhibited the functions of TNFα mediated through TNFRI. The most active ODNs were directed against the 3′-poly adenylation signal site on the TNFRI mRNA, and in a cellular assay, gene-specific antisense inhibition occurred in a dose-dependent fashion at submicromolar concentrations, in the presence of Cellfectin. The inhibition of gene expression correlated with the binding affinity of the ODN for the target mRNA. The ODNs lowered TNFRI protein levels and TNFα-mediated functions by specifically reducing levels of TNFRI mRNA. These anti-TNFRI ODNs offer a novel approach for controlling biological functions of TNFα and may be useful as human therapeutic agents for treating diseases in which TNFα has been implicated.

Tumor necrosis factor α (TNFα)[1] is a mononuclear phagocytic cell-derived protein (Decker *et al*., 1987; Turner *et al*., 1987; Spriggs *et al*., 1988). There are two forms of TNFα, a type II transmembrane protein with a relative molecular mass of 26 kDa and a soluble, 17 kDa form generated from the cell-bound protein by proteolytic cleavage (Aggarwal & Vilcek, 1992). Some of the biological effects elicited by this highly pleiotropic cytokine include hemorrhagic necrosis of transplanted tumors, cytotoxicity, septic shock, multiple sclerosis, cachexia, inflammation, autoimmunity, and other immunological and pathological reactions (Aggarwal & Vilcek, 1992).

The mechanisms by which TNFα mediates its multiple activities are largely unknown, but as in the case for most polypeptide hormones, binding to specific cell surface receptors is an initial event. TNF receptors have been detected on a wide variety of normal tissues and cell lines that are sensitive or resistant to TNFα (Kull *et al*., 1985; Baglioni *et al.*, 1985). Two distinct TNF receptors (TNFRs) of approximately 55 kDa (TNFRI, p55) and 75 kDa (TNFRII, p70 or p75) have been identified (Tartaglia & Goeddel, 1992a; Wiegmann *et al*., 1992; Heller & Kronke, 1994; Tartaglia *et al*., 1993; Pfeffer *et al*., 1993). Currently, one of the key issues in TNFα biology is the respective role of these two TNFRs. The intracytoplasmic domains of the two TNFR chains do not share significant homology, suggesting that the two receptors trigger distinct intracellular events. Indeed, most of the known cellular TNF responses are attributed to TNFRI activation exclusively (Aggarwal & Vilcek, 1992). TNFRI-dependent responses include accumulation of *c-fos*, interleukin-6, and manganous superoxide dismutase mRNA, prostaglandin E2 synthesis, interleukin-2 receptor and MHC class I and II cell surface antigen expression, growth inhibition, and cytotoxicity (Engelmann *et al*., 1990; Espevik *et al*., 1990; Shalaby *et al*., 1990; Hohmann *et al*., 1990; Thoma *et al*., 1990; Naume *et al*., 1991; Tartaglia *et al*., 1991; Kruppa *et al*., 1992; Brakebusch *et al*., 1992).

TNFRI also triggers second messenger systems such as phospholipase A_2, protein kinase C, phosphatidylcholine-specific phospholipase C, and sphingomyelinase (Wiegmann *et al*., 1992; Schutze *et al*., 1992). Recently, TNFα induction of nuclear transcription factor κB (NF-κB) activity has been shown to be critically dependent on the generation of ceramides by an acidic sphingomyelinase, which in turn is secondary to the production of l,2-diacylglycerol by a TNFRI-responsive phosphatidylcholine-specific phospholipase C (Schutze *et al*., 1992). These signaling pathways most likely account for the aforementioned TNFRI-specific cellular responses. In contrast, information about the func-

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[1] Abbreviations: ODN, oligodeoxyribonucleotide; PT, total phosphorothioate; pPT, partial phosphorothioate; TNFα, tumor necrosis factor α; TNFRI and TNFRII, tumor necrosis factor receptor type I and II, respectively; T_m, melting temperature; IL-6, interleukin-6; IL-8, interleukin-8.

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tion of TNFRII is scarce. TNFRII triggering has been reported to induce cytotoxicity in fibroblasts and proliferation of thymocytes and CT6 cells (Tartaglia & Goeddel, 1992b; Heller *et al*., 1992). To date, the molecular mechanisms of TNFRII signaling remain unknown.

Since the plethora of data suggest that TNFRI rather than TNFRII is a key mediator of TNFα inflammatory action, we decided to target the function of this receptor by the antisense oligodeoxyribonucleotide (ODN) approach. The disruption of the TNFRI gene expression was expected to reduce the number of receptors available for TNFα binding which subsequently would result in diminished TNFα-mediated biological effects.

Antisense ODNs designed to specific mRNA sequences have been utilized to inhibit the expression of a number of cellular and viral proteins (Uhlmann & Peyman, 1990; Chiang *et al*., 1991; Peyman *et al*., 1996b; Peyman & Uhlmann, 1996a; Zon, 1988; Stein & Cohen, 1988; Dolnick, 1990; Helene & Toulme, 1990). The ODNs bind to the target mRNA or pre-mRNA using normal Watson−Crick base pairing. Therefore, knowing the DNA sequence of the target protein or gene, complementary ODNs can be designed to bind the target RNA and thereby inhibit gene expression or function. Antisense ODN technology has been used, for example, to define the role of oncogenes such as *c-myc* or *c-myb* in cell proliferation and maturation (Heikkila *et al*., 1987; Holt *et al*., 1988; Gewirtz & Calabretta, 1988; Venturelli *et al*., 1990) and the dependence of Th 1 helper T-cell proliferation on interleukin-2 (IL-2) and IL-4 synthesis (Harel-Bellan *et al*., 1988).

The optimal target site on mRNA for antisense ODNs has not been carefully examined; however, most studies target antisense ODNs to the AUG translation initiation (Zon, 1988; Stein & Cohen, 1988; Dolnick, 1990; Helene & Toulme, 1990; Heikkila *et al*., 1987; Holt *et al*., 1988; Gewirtz & Calabretta, 1988; Venturelli *et al*., 1990). Some studies, however, have achieved greater potency with antisense ODNs by targeting other loci on the mRNA. For example, it was determined that an ODN targeted to the 5′-cap site of *c-Ha-ras* mRNA was more active than the one directed against the AUG region (Daaka & Wickstrom, 1990). In addition, it has also been reported that an antisense ODN to the 5′-untranslated region of the IL-1β gene inhibited IL-1β expression (Manson *et al*., 1990). Hence, antisense ODN target sites on the mRNA other than the AUG codon may provide better activity for antisense effects.

In this report, we describe the design, synthesis, and evaluation of antisense ODNs directed against two sites on the TNFRI mRNA. These ODNs were tested for their ability to selectively inhibit the TNFRI gene expression and function in two different cell lines using multiple end points. Furthermore, we show that the optimum activity of these ODNs in culture assays is dependent on the pyrimidine base modifications and the presence of an uptake enhancer. We also demonstrate that the most active ODNs exert their effect by reducing the levels of TNFRI mRNA.

EXPERIMENTAL PROCEDURES

*Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-5-(1-hexynyl)-2′-deoxyuridine (***2b***).* A mixture of 5-iodo-2′-deoxyuridine (**1**, 3.18 g, 9 mmol), dry DMF (45 mL), CuI (0.34 g, 1.8 mmol), triethylamine (2.52 mL, 18 mmol), 1-hexyne (3.09 mL, 27 mmol), and tetrakis(triphenylphosphine)palladium (1.04 g, 0.9 mmol) (Hobbs, 1989) was stirred at room temperature for 18 h under an argon atmosphere. DMF and volatile materials were evaporated, and the residue was coevaporated with toluene (25 mL). The residue was purified by chromatography on a silica gel column (2.5 × 25 cm), and the product was flash eluted using 0−5% MeOH in CH_2Cl_2 as the eluent. The homogeneous fractions containing C-5-(1-hexynyl)-2′-deoxyuridine (**2a**) were pooled and evaporated. Mp: 140−141 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.88 (t, 3 H, CH_3), 1.42 (m, 4 H, 2CH_2), 2.12 (m, 2 H, C$_{2'}H$ and C$_{2''}H$), 2.35 (t, 2 H, CH_2), 3.45 (m, 2 H, C$_{5'}H_2$), 3.77 (m, 1 H, C$_{4'}H$), 4.21 (br s, 1 H, C$_{3'}H$), 5.06 (t, 1 H, C$_{5'}$OH), 5.21 (d, 1 H, C$_{3'}$OH), 6.10 (t, 1 H, C$_{1'}H$), 8.09 (s, 1 H, C$_6H$), 11.53 (s, 1 H, N$_3H$).

To a solution of the above solid (**2a**, dried by coevaporation with dry pyridine) in pyridine (40 mL) was added 4,4′-dimethoxytrityl chloride (DMT-Cl, 2.7 g, 7.97 mmol), and the mixture was stirred at room temperature for 4 h. An additional 1.0 g of DMT-Cl was added. After 2 h of stirring, the reaction mixture was diluted with CH_2Cl_2 (200 mL), and the organic phase was washed with water (50 mL). The aqueous layer was extracted with CH_2Cl_2 (50 mL), and the combined organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by chromatography on a silica gel column (2.5 × 25 cm), and the product was flash eluted using 0−1.5% MeOH in CH_2Cl_2 as the eluent to yield 3.45 g (63%) of **2b**. Mp: 106−108 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.77 (t, 3 H, CH_3), 1.23 (m, 4 H, 2CH_2), 2.20 (m, 4 H, CH_2, C$_{2'}H$, C$_{2''}H$), 3.20 (m, 2 H, C$_{5'}H_2$), 3.73 (s, 6 H, 2OCH_3), 3.91 (br s, 1 H, C$_{4'}H$), 4.27 (s, 1 H, C$_{3'}H$), 5.30 (br s, 1 H, C$_{3'}$OH), 6.12 (t, 1 H, C$_{1'}H$), 6.87 (d, 4 H, *DMT*), 7.20−7.42 (m, 9 H, *DMT*), 7.87 (s, 1 H, C$_6H$), 11.57 (s, 1 H, N$_3H$). Anal. Calcd for $C_{36}H_{38}N_2O_7 \cdot 0.25H_2O$: C, 70.28; H, 6.31; N, 4.55. Found: C, 69.93; H, 6.23; N, 4.41.

*Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-5-(1-hexynyl)-2′-deoxyuridine 3′-O-(2-Cyanoethyl) N,N-Diisopropylphosphoramidite (***3***).* A mixture of **2b** (1.83 g, 3 mmol), CH_2Cl_2 (20 mL), *N*,*N*-diisopropylethylamine (2.08 mL, 12 mmol), and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.83 mL, 3.9 mmol) (Sinha *et al*., 1984) was stirred at room temperature for 30 min under an argon atmosphere. An additional 0.2 mL of phosphitylating agent was added. After 20 min of stirring, the reaction mixture was diluted with EtOAc (100 mL), and the organic phase was washed with saturated aqueous $NaHCO_3$ solution (50 mL). The organic layer was dried (Na_2SO_4) and evaporated, and the residue was purified by chromatography on a silica gel column (2.5 × 25 cm) packed in a mixture of CH_2Cl_2/EtOAc (1/1) containing 5% NEt_3. The product was flash eluted using the same solvent mixture. The homogeneous fractions containing the desired product were collected and the solvents evaporated. The residue was dissolved in CH_2Cl_2 (5 mL), and the CH_2Cl_2 solution was added to rapidly stirred cold (−50 °C) pentane (200 mL). The supernatant was decanted from the precipitated product, and the solid was dried under high vacuum to yield 2.29 g (94%) of **3**. ^{31}P NMR (CD_3CN): δ 149.65. Anal. Calcd for $C_{45}H_{55}N_4O_8P$: C, 66.65; H, 6.84; N, 6.91; P, 3.82. Found: C, 66.24; H, 6.80; N, 6.83; P, 3.67.

Reagents. Cellfectin (a liposomal preparation of the cationic lipid tetramethyltetrapalmitylspermine and the phospholipid dioleoylphosphatidylethanolamine) was obtained

from Life Technologies (Gaithersberg, MD). The enzyme-linked immunosorbent assay (ELISA) kits for the detection of interleukin-6 (IL-6), interleukin-8 (IL-8), TNFRI, and TNFRII were purchased from R&D Systems (Minneapolis, MN), as were recombinant human TNFα (rhTNFα) and recombinant human IL-1β (rhIL-1β).

ODN Synthesis. ODNs were synthesized on an PerSeptive Biosystems Expedite model 8909 automated synthesizer using modified pyrimidine phosphoramidites obtained from Glen Research (Sterling, VA). The nonmodified 5′-protected nucleoside phosphoramidite monomers were obtained from PerSeptive Biosystems, and other standard reagents were obtained from Perkin-Elmer, Applied Biosystems division (Foster City, CA), with the exception of acetonitrile, which was obtained from Baxter (McGraw Park, IL). RNA oligonucleotides were also synthesized and purified using standard procedures (PerSeptive Biosystems, Framingham, MA) with commercially available monomers (Glen Research). ODNs containing phosphorothioate were prepared using Beaucage reagent (Glen Research) (Iyer *et al*., 1990). Briefly, the synthesis proceeds through deblocking, coupling, capping, oxidizing, and capping cycles. After the deblocking step, the coupling step begins immediately after the monomer is added to the column. Following the completion of each synthesis, ODNs containing phosphodiester, phosphorothioate, and partial phosphorothioate backbones were cleaved and deprotected in ammonium hydroxide at 56 °C for 24 h.

Propynyl- and hexynyl-modified pyrimidine containing ODNs were synthesized using the same protocol except the coupling step was extended to 300 s after the propynyl or hexynyl monomer was added to the column and the resulting ODNs were cleaved and deprotected in ammonium hydroxide at room temperature for 48 h as opposed to 56 °C for 24 h.

Crude ODNs were purified using a Waters high-performance liquid chromatography (HPLC) system by anion-exchange chromatography on a Q-Sepharose column (1.5 × 10 cm). Standard sodium chloride (0.5−3 M)/sodium hydroxide (10−15 mM) mobile phases were used depending on the backbone. The purified ODNs were desalted on Sep-Pak Plus C18 cartridges purchased from Waters. The purity of the ODN was confirmed by analytical HPLC and by gel electrophoresis in a 20% polyacrylamide gel containing 7 M urea.

Cell Culture. Human lung embryonic fibroblast (MRC-5) or human newborn foreskin fibroblast (Hs68) cells were grown in minimal essential medium (MEM) supplemented with Earl's salts, L-glutamine (GIBCO BRL, Life Technologies, Inc.), 10% heat-inactivated fetal bovine serum (GIBCO BRL), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Measurement of ODN T_m. Absorbance *versus* temperature profiles were measured at a 1 μM concentration of each strand ODN in 20 mM NaCl and 2 mM sodium phosphate. Antisense ODNs containing total or partial phosphorothioate backbone with or without pyrimidine modifications were synthesized; the complementary DNA or RNA ODN strands were synthesized with phosphodiester backbones. ODN strands in the phosphate buffer were heated at 90 °C for 10 min and then cooled to 20 °C. After equilibration at 20 °C for 10 min, absorbance (at 260 nm) *versus* temperature (degrees Celsius) profiles were obtained at a heating ratio of 0.5 °C per 30 s from 20 to 80 °C using a Hewlett-Packard 8452 diode array spectrophotometer equipped with a temperature-controlled cell holder. T_m values (which serve as a measure of the strength of binding of ODN strands when forming duplexes or triplexes) and free energies of the duplex formation were obtained from fits of data to a two-state model with linear sloping base lines (Petersheim & Turner, 1983).

Cytotoxicity Analysis. The cytotoxicity of the ODNs or ODN/Cellfectin mixture was assayed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega, Madison, WI). For the ODNs alone, the viable cell number was determined by trypan blue staining and cells (MRC-5) were resuspended in MEM supplemented with 10% FBS (GIBCO). Eighty microliters of cell suspension (1.7×10^4 cells/well) was dispensed onto 96-well microtiter plates overnight. The following day, 20 μL of drug (or control) was added to appropriate wells. Each concentration was assayed in quadruplicate. The plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 days, and the MTS assay was performed according to the manufacturer's instructions (Promega). For the ODN/Cellfectin mixture, the cells were prepared as described above. Twenty microliters of various concentrations of the ODN/Cellfectin mixture (or control) was then added to appropriate wells. Each concentration was assayed in quadruplicate. After 4 h at 37 °C, the medium was removed and the wells were rinsed twice with MEM supplemented with 0.25% fetal bovine serum (FBS), and then 80 μL of fresh MEM supplemented with 0.25% FBS was added to each well. After 2 days of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the plates were assayed as above. The average absorbance obtained from both samples was plotted for each concentration.

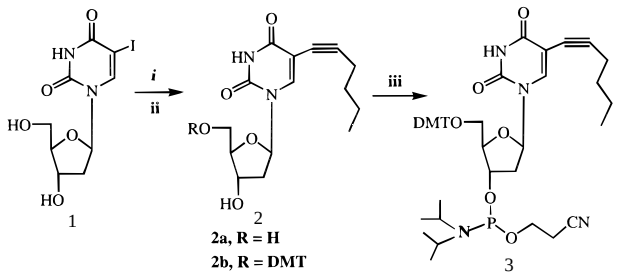
IL-6 or IL-8 Assay. Twenty five microliters of ODN at various concentrations was mixed with 2.5 μL of Cellfectin (1 mg/mL) in Opti-MEM. After a 10−15 min incubation at room temperature, 225 μL of Opti-MEM was added to each tube and then 100 μL of the ODN/Cellfectin mixture was added to MRC-5 cells. The MRC-5 cells were seeded the previous day at a cell density of 1×10^4 cells/well in 96-well plates. The cells were rinsed twice with 100 μL of Opti-MEM just before the addition of the ODN/Cellfectin mixtures. The final concentration of Cellfectin per well was 10 μg/mL. After 4 h at 37 °C, the medium was removed and the wells were rinsed twice with MEM supplemented with 0.25% FBS, and then 80 μL of fresh MEM supplemented with 0.25% FBS was added to each well. The cells were then stimulated by adding 20 μL of rhTNFα (5 ng/mL) (R&D) or rhIL-1β (50 ng/mL) (R&D). The final concentration of the TNFα or IL-1β in 100 μL was 1 or 10 ng/mL, respectively. Six or 18 h poststimulation, supernatants were collected and stored at −80 °C until used. The supernatants were assayed for IL-6 or IL-8 levels using the IL-6 or IL-8 ELISA kits, respectively, according to the manufacturer's instructions. The data were expressed as percent inhibition compared to the control.

TNFRI and TNFRII Assay. Twenty five microliters of ODN at various concentrations was mixed with 2.5 μL of Cellfectin (1 mg/mL) in Opti-MEM. After a 10−15 min incubation at room temperature, 225 μL of Opti-MEM was added to each tube and then 100 μL of the ODN/Cellfectin mixture was added to MRC-5 cells. The MRC-5 cells were seeded the previous day at a cell density of 1×10^4 cells/

well in 96-well plates and were rinsed twice with 100 μL of Opti-MEM just before the addition of the ODN/Cellfectin mixtures. The final concentration of Cellfectin per well was 10 μg/mL. After 4 h at 37 °C, the medium was removed and the wells were rinsed twice with MEM supplemented with 0.25% FBS, at which time 80 μL of fresh MEM supplemented with 0.25% FBS was added to each well. Six or 24 h postrinsing, aliquots were collected and stored at −80 °C until used. The cells were rinsed once with PBS and then treated with a buffer containing 40 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), and 150 mM NaCl for 10 min at room temperature. The rounded cells were then scraped, transferred into microfuge tubes, and centrifuged at 3500 rpm (∼1000*g*) for 6 min. The supernatants were discarded, and the cell pellets were resuspended in 200 μL of 0.25 mM Tris-HCl (pH 7.4). The cell-associated proteins were prepared by the freeze−thawing method, and the cell lysates were stored at −80 °C until used. Both supernatants and cell lysates were assayed for TNFRI or TNFRII levels using ELISA kits (R&D) according to the manufacturer's instructions. The data were expressed as percent inhibition compared to the control.

Isolation and Characterization of RNA. MRC-5 cells (5 × 10^5) were seeded in a 12-well plate, in 1 mL of medium, and allowed to reach ∼70−90% confluency. Cells were then treated with ODN at various concentrations in the presence of 10 μg/mL Cellfectin. Four hours post-ODN treatment, the cells were rinsed and incubated in fresh medium for an additional 3 h. The supernatants were saved for analysis of TNFRI, TNFRII, and IL-6 by ELISA. Cells were washed once with PBS (GIBCO, Life Technologies) prior to RNA extraction. Total cellular RNA was isolated using RNA-Zol^B (Tel-Test Inc., TX) according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometric absorbance at 260 nm, and the samples were stored at −20 °C until use.

Fifteen micrograms of extracted RNA was dissolved in 500 μL of an ice-cold solution containing 10 mM NaOH and 1 mM EDTA. The samples were then filtered through a S&S Nytran membrane (Schleicher and Schuell, Keene, NH) using a Bio-Rad Minifold slot-blot apparatus according to the supplier's instructions. The immobilized RNA was hybridized with the TNFRI cDNA probe or a β-actin cDNA probe labeled with [α-^{32}P]dCTP (New England Nuclear) using the Rediprime random primer labeling kit (Amersham, Arlington Heights, IL) to a specific activity of 1 × 10^7 cpm/μg. The partial human TNFRI cDNA probe was generated from total RNA isolated from MRC-5 cells using reverse transcriptase−polymerase chain reaction (RT-PCR). The PCR was performed using human TNFRI primer pairs (Clonetech, San Francisco, CA) designed to hybridize to the human TNFRI mRNA at positions 350−380 (5′-att tgc tgt acc aag tgc cac aaa gga acc-3′) and positions 936−906 (5′-gtc gat ttc cca caa aca atg gag tag agc-3′). The amplified cDNA fragment (587 bp) was purified using agarose gel electrophoresis and GENECLEAN (BIO 101, Vista, CA). The prehybridization, hybridization, and washes were performed according to the Rapid-hyb kit (Amersham) instructions. Autoradiography of the filters was performed at −80 °C with X-ray film (Eastman Kodak, Rochester, NY). The filters were also exposed to a phosphoroimaging plate and quantified using a Fujix Bioimaging Analyzer System BAS 1000 instrument. The values were expressed as percent inhibition of TNFRI mRNA compared to the control.



(i) 1-hexyne/CuI/tetrakis(triphenylphosphine)palladium, (ii) 4,4′-dimethoxytrityl-chloride (DMT-Cl), (iii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite

FIGURE 1: Reaction scheme utilized to synthesize the hexynyl-modified dU pyrimidine monomer.

Fluorescence Uptake Experiments. MRC-5 cells were seeded at a density of 2.5 × 10^5 in 60 mm dishes containing six glass coverslips. After 24 h, the medium in each dish was replaced with fresh medium containing 0.1 μM fluorescein-tagged ODN (T30964) in the presence or absence of 5 μg/mL Cellfectin. At 2 h after addition of ODN, the cells were washed four times with D-PBS containing 0.1% azide, fixed with methanol (−20 °C) for 6 min, and washed four more times with D-PBS. Coverslips were mounted on glass slides using Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA), sealed with clear nail polish, and observed by phase contrast or fluorescence microscopy on a Nikon Laborphot 2 system, using a 100× objective (120 s).

RESULTS

Monomers. ODNs containing C-5-hexynyl-modified dU monomers have been reported by Sagi *et al.* (1993); however, the report did not describe the synthesis of the monomers. Therefore, the C-5-hexynyl-modified dU monomers used in this study were synthesized according the reaction scheme outlined in Figure 1, while C-5-propynyl dU and dC monomers were purchased from Glen Research.

ODN Design. Many of the cellular responses mediated by TNFα occur when TNFα binds to TNFRI (Aggarwal & Vilcek, 1992); therefore, we synthesized antisense ODNs with total phosphorothioate (PT) internucleoside linkages designed to hybridize to the human TNFRI mRNA (Nophar *et al.*, 1990) at various regions, including the translation initiation AUG codon (T30410) and the poly(A) signal site (T30411). One of the TNFRI-dependent responses to TNFα is the induction of interleukin-6 (IL-6) (Aggarwal & Vilcek, 1992). On the basis of this observation, we used an assay system which monitors the level of IL-6 in the culture medium of cells treated with rhTNFα to evaluate the biological activity *in vitro* of T30410 and T30411. In this assay, MRC-5 cells were treated with ODNs in the presence or absence of Cellfectin, a cationic lipid used to enhance the uptake and intracellular distribution of ODNs into cells, and the cells were then stimulated with rhTNFα. The IL-6 levels in the culture media or in the cell lysates were monitored 6 or 18 h poststimulation. No significant inhibitory activity was observed with any of the ODNs tested in the absence of Cellfectin (data not shown). In the presence of Cellfectin, T30411 was able to inhibit IL-6 production at submicromolar concentrations; however, no effect was observed with T30410 (Figure 2A). Therefore, for subse-

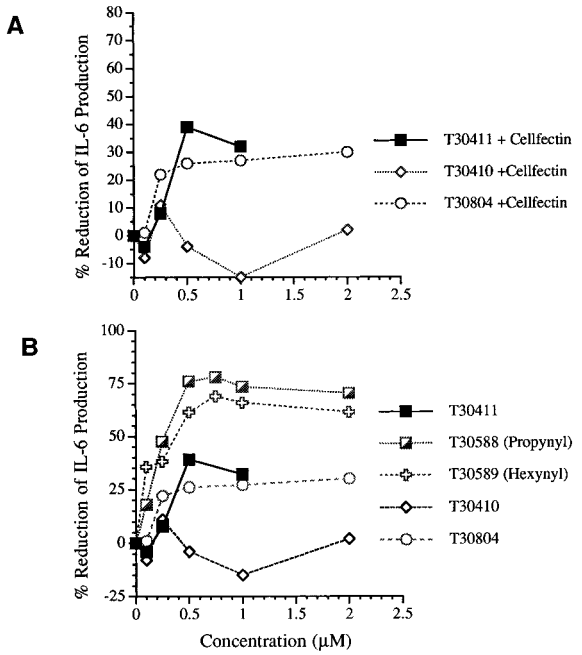


FIGURE 2: Effect of antisense ODNs on IL-6 production in MRC-5 cells. (A) Effects of T30804, T30410, and T30411 on IL-6 production when administered to cells in the presence of Cellfectin 6 h poststimulation with rhTNFα. (B) Inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with ODNs containing natural bases and hexynyl- or propynyl-modified pyrimidines in the presence of 10 μg/mL Cellfectin.

quent experiments, we synthesized several derivatives of the T30411 containing modified bases, different phosphorothioate patterns, or length and control ODNs (Table 1).

T30411 was first modified by reducing the number of PT linkages in the ODN [partial phosphorothioate (pPT) backbone], generating T30804. We have found previously that ODNs having a pPT backbone are reasonably stable in serum (Peyman & Uhlmann, 1996a) and show less nonspecific dependent side effects as compared to uniformly PT-modified ODNs (Fennewald & Rando, 1995). In this scheme, the rationale for the removal of the sulfur atom resided in the subsequent addition of modifed pyrimidines to the sequence motif. It was surprising to find that in the IL-6 induction assay T30804 was just as active as T30411 (Figure 2A).

Wagner *et al.*, (1993) recently reported on the enhanced efficacy of C-5-propynyl-modified pyrimidine in an antisense sequence. For this reason, ODN T30804 was modified by replacing some thymidines with C-5-propynyl (T30588) or C-5-hexynyl (T30589) derivatives of 2′-deoxyuridine (Table 1). Various concentrations of these ODNs were tested in the IL-6 assay system with Cellfectin (10 μg/mL, final concentration) to compare their biological activity with that of T30411 and T30804. The C-5-propynyl or -hexynyl-substituted ODNs inhibited IL-6 production in a dose-dependent fashion at submicromolar levels and were more potent than their parent unmodified counterparts (Figure 2B). No inhibition was observed with T30589 at these concentrations in the absence of Cellfectin (Figure 3A).

Optimization of Cationic Lipid Formulation. The preceding data implied that, in addition to C-5-pyrimindine modification, uptake enhancer was also required for optimal activity of ODNs *in vitro*. On the basis of these observations, we next determined the optimum formulation of ODN and cationic lipid. In this experiment, ODN (T30589) and Cellfectin (up to 10 μg/mL) at various concentrations were added to MRC-5 cells and the level of IL-6 produced after stimulation with rhTNFα was monitored. The results of this study indicated that the ODN activity increased with increasing Cellfectin concentration and that the best inhibition was achieved with 10 μg/mL of Cellfectin (Figure 3A). Cellfectin by itself had no effect on IL-6 induction by rhTNFα (Figure 3A).

Fluorescent Uptake Experiments. To determine whether the profound reduction in IL-6 by antisense ODN in the presence of Cellfectin is due to the efficient delivery and intracellular distribution of an ODN into cells, a fluorescein-conjugated ODN (T30964, Table 1) was added to MRC-5 cells in the presence or absence of Cellfectin (5 μg/mL) as described in Experimental Procedures. In the absence of Cellfectin, a very weak fluorescence signal was observed inside the cell, suggesting poor uptake of ODN (Figure 3B, panel I). In contrast, coadministration of Cellfectin with ODN resulted in brighter cytoplasmic and nuclear fluorescence, suggesting that the ODN is efficiently delivered inside the cells in the presence of Cellfectin (Figure 3B, panel II).

Cytotoxicity. To determine the toxicity of Cellfectin and modified ODNs, we performed growth inhibition assays in MRC-5 cells treated with C-5-modified pyrimidine-containing ODNs and the ODN/Cellfectin formulations. The cytotoxic effect of the ODNs was monitored using a standard 4 day growth inhibition study, while the effect of ODN/Cellfectin formulations was studied using a 2 day growth inhibition designed to mimic the drug regimen in the efficacy protocol. In these experiments, ODNs containing C-5-propynyl-modified bases (T30588) did not exhibit observable cytotoxic effects when concentrations up to 50 μM, the highest concentration tested, were used (Figure 4A). When the ODNs were formulated with Cellfectin, a clear pattern of toxicity was observed when concentrations above 10 μg/mL Cellfectin were used in MRC-5 cell cultures (Figure 4B). These data suggest that the biological activity of the antisense ODNs formulated in 10 μg/mL Cellfectin was not influenced by cytotoxicity of either the ODN or Cellfectin. Similar results were obtained with the C-5-hexynyl-modified ODN, T30589 (Table 1). For these reasons, the highest dose of Cellfectin used in all subsequent experiments was 10 μg/mL.

Specific Inhibition of TNFα-Mediated Events. To demonstrate the specificity of the activity observed with the propynyl- or hexynyl-modified ODNs in the presence of Cellfectin, the control ODNs T30691, T30779, and T30786 were tested in the IL-6 assay. T30779 is a scrambled sequence version of T30589; T30786 is the sense counterpart of T30588, and T30691 is a random control.

As shown in Figure 5A for propynyl-containing ODNs and Figure 5C for the hexynyl-containing molecules in the presence of Cellfectin, the control ODNs had no inhibitory effect on IL-6, suggesting that the activity observed with the anti-TNFRI antisense ODNs was sequence-specific.

To determine the minimum length of the ODN required for maximum activity, we synthesized size variants of T30588 or T30589 (21-mers, Table 1). T30782 and T30783 are 18-nucleotide (nt) long derivatives of T30588 with 3 bases deleted from their 5′ and 3′ ends, respectively. T30776 and T30777 are the hexynyl-substituted counterparts of T30782 and T30783, respectively. T30784 and T30778 are 15-nt long derivatives of T30588 and T30589, respectively,

Table 1: Tumor Necrosis Factor Receptor I (TNFRI) ODN List

ID	Sequence[a]	Location[b]	Modification
T30410	5'-c*a*c*g*g*t*g*g*a*g*a*g*g*c*c*c*a*t*g*c*c -3'	273-253	
T30411	5'-a*g*a*a*t*t*t*t*a*g*t*g*t*a*t*g*t*a*c*a*a -3'	2155-2135	
T30804	5'-a*g*a*a*t t t t a*g*t*g*t*a t*g t a*c*a*a -3'	2155-2135	
T30837	5'-a*g*a*a*u u u u a*g*u*g*u*a u*g u a*c*a*a -3'	2155-2135	2′OMe
Propynyl-substituted ODNs			
T30588	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	2155-2135	prop
T30782	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	2152-2135	prop
T30783	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T*a -3'	2155-2137	prop
T30784	5'- a*t T T T a*g*t*g*t*a T g T*a -3'	2152-2137	prop
T30964	5'**F**-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	2155-2135	prop
Hexynyl-substituted ODNs			
T30589	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	2155-2135	hex
T30776	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	2152-2135	hex
T30777	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T*a -3'	2155-2137	hex
T30778	5'- a*t T T T a*g*t*g*t*a T g T*a -3'	2152-2137	hex
Control ODNs			
T30779	5'-g*a*g*g*t T T T g*a*t*a*t*g T a T*g*c*g*g -3'		hex, Scrambled
T30786	5'-t*t*g T a*c*a T a*c*a*c T a*a*a*a T T g*t -3'		prop, Sense
T30691	5'-C*a*T*g*g*a*T*g*T*C*T*a*T*C*a*g*C*g*c -3'		prop, Random

[a] Modifications to oligonucleotides include phosphorothioate internucleoside linkages (*). The lowercase letters (a, c, g, t, and u) indicate the presence of the natural pyrimidine or purine base at the corresponding position. Uppercase T = a C-5 propynyl (prop)- or hexynyl (hex)-modified dU at the indicated positions, while C = propynyl (prop)- or hexynyl (hex)-dC. T30837 contains 2′-*O*-methyl-modified nucleosides. F = fluorescein moiety. [b] The start site of the human TNFRI gene according to the sequences published by Nophar *et al.* (1990).

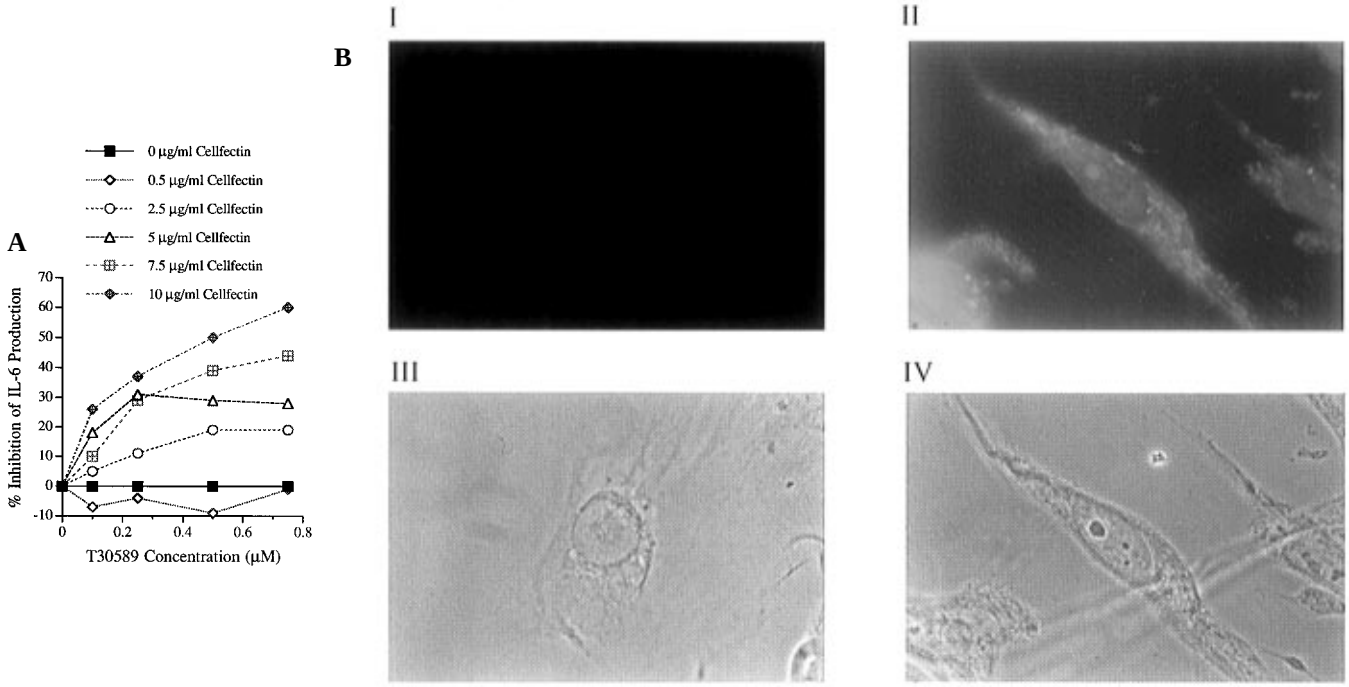


FIGURE 3: (A, left) Effect of the antisense ODN/Cellfectin ratio on IL-6 production. The left panel shows the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with ODNs containing hexynyl-modified pyrimidines (T30589, Table 1) in the presence of various concentrations of Cellfectin plotted against the varying ODN concentration. (B, right) Effect of Cellfectin on ODN Uptake. Panel I is a representation of the ODN delivery and distribution after 2 h in the absensce of Cellfectin. Panel II is a representation of the ODN delivery and distribution after 2 h in the presence of Cellfectin (5 μg/mL). Panels III and IV are phase contrasts of panels I and II, respectively.

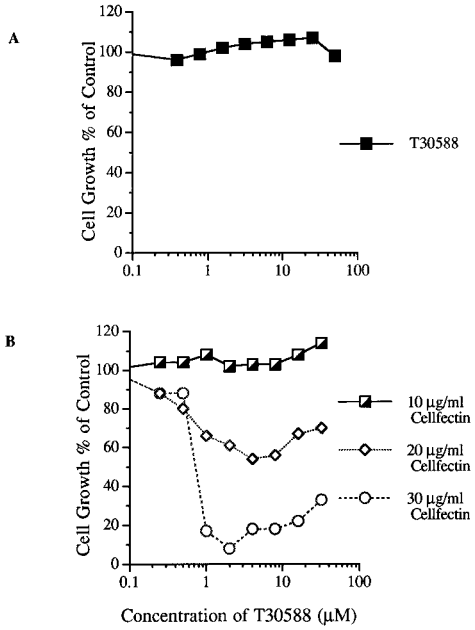


FIGURE 4: Cellular cytotoxicity of the ODNs and Cellfectin. Panel A representation of the percent inhibition of cell growth (MRC-5) of cells treated for 4 days with T30588 (propynyl-modified pyrimidines containing ODN) in the absence of Cellfectin. Panel B shows the percent inhibition of MRC-5 cell growth of cells treated for 2 days with T30588 (propynyl-modified pyrimidines containing ODN) in the presence of 10 (half-shaded boxes), 20 (◇), and 30 (○) μg/mL Cellfectin.

with 6 bases deleted, 3 bases from each end. These variants were tested for their ability to inhibit the induction of IL-6 by rhTNFα. The results show that all the 18-mer propynyl (Figure 5B) or hexynyl (Figure 5D) variants were as active as the full-length ODN while the 15-mer variants showed reduced activity compared to the parental sequence, suggesting that the minimum length for T30589 and T30588 may be between 15 and 18 nucleotides (panels B and D of Figure 5).

The ability of T30588 or T30589 to inhibit TNFα-mediated functions in multiple cell lines was evaluated by monitoring the effects of these ODNs on IL-6 production in human newborn foreskin fibroblast (Hs68, ATCC) cells. Hs68 cells, untreated or treated with ODNs, were stimulated with rhTNFα, and the IL-6 levels were monitored in the culture media collected at 6 or 18 h poststimulation. The inhibition of TNFα-mediated function(s) in this cell line was similar to the results obtained with MRC-5 cells (data not shown). The results obtained with both MRC-5 and Hs68 cells suggest that the C-5-propynyl or -hexynyl-modified ODNs can control TNFα-mediated functions in multiple cell lines.

To determine the ability of T30588 to inhibit a second gene product induced by TNFα, we stimulated treated or untreated MRC-5 cells with rhTNFα and monitored the levels of IL-8 in the culture media. T30588 inhibited IL-8 production in a dose-dependent fashion at submicromolar concentrations in the presence of 10 μg/mL Cellfectin (data not shown). T30588 was also tested for its ability to reduce rhIL-1β-induced IL-6 expression. In this experiment, the treatment of MRC-5 cells with T30588 did not result in the inhibition of IL-6 production induced by rhIL-1β, suggesting that the ODN specifically inhibited TNFα-induced events (Figure 6).

Further evidence that the antisense ODN T30588 inhibited TNFα function through an antisense mechanism was obtained when T30588 was preincubated with the sense ODN T30786. In this experiment, the concentration of one ODN was kept constant at 0.5 μM while the concentration of the second ODN was varied. The antisense and the sense ODNs

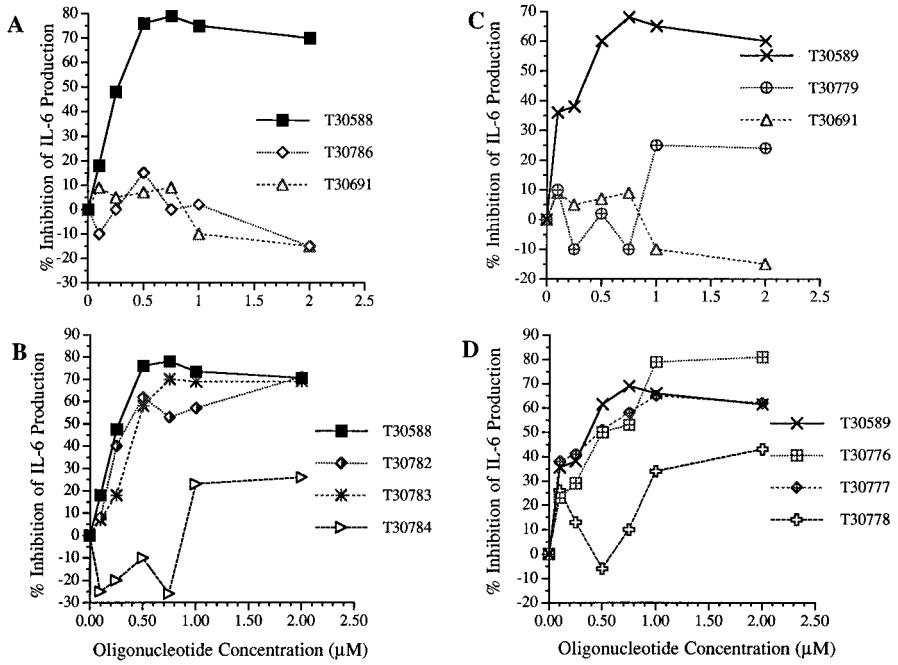


FIGURE 5: Effect of sequence and length which is dependent on TNFα-induced IL-6 production in the presence of a cellular uptake-enhancing agent. Panel A is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with T30588 (propynyl-modified pyrimidines containing ODN) and control ODNs in the presence of 10 μg/mL Cellfectin. Panel B is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with T30588 (propynyl-modified pyrimidines containing ODN) and short variants of T30588 in the presence of 10 μg/mL Cellfectin. Panel C is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with T30589 (hexynyl-modified pyrimidines containing ODN) and control ODNs in the presence of 10 μg/mL Cellfectin. Panel D is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with T30589 (hexynyl-modified pyrimidines containing ODN) and short variants of T30589 in the presence of 10 μg/mL Cellfectin.

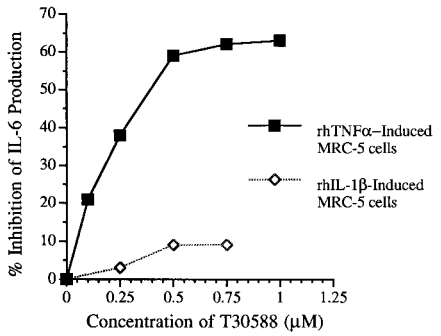


FIGURE 6: Effect of ODN on IL-6 production using rhIL-1β as an inducer compared to that with rhTNFα. The figure is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhIL-1β or rhTNFα and treated with T30588 (propynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin.

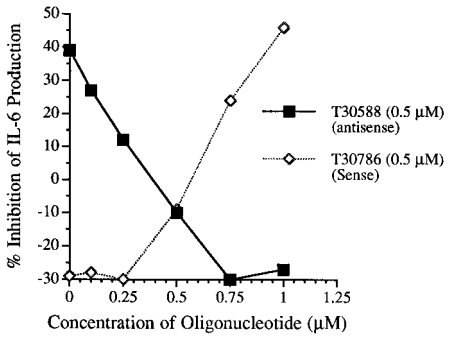


FIGURE 7: Effect of the antisense ODN/sense ODN ratio on IL-6 production. The figure is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 h poststimulation with rhTNFα and treated with either 0.5 μM antisense ODN (T30588) and various concentrations of sense ODN (T30786) or *vice versa* in the presence of 10 μg/mL Cellfectin.

were annealed at 37 °C for 10 min before addition to the MRC-5 cell cultures. Six or 18 h after rhTNFα induction, the level of IL-6 in the culture medium was monitored by ELISA. Prehybridization of antisense (T30588) to sense (T30786) ODN blocked the inhibition of rhTNFα-induced IL-6 expression in a dose-dependent fashion (Figure 7). The uptake of the annealed and linear ODNs was similar in the presence of Cellfectin (data not shown).

Inhibition of TNFRI Protein Expression. To determine if the observed inhibition of IL-6 expression was due to reduction of the target gene, the level of TNFRI was monitored. MRC-5 cells were treated with T30588 or T30589, and 6 or 24 h after drug treatment, the culture medium and cell lysates were collected and the level of TNFRI and TNFRII was determined. The protein levels found in the medium and the corresponding cell lysates were analyzed and plotted as extracellular and cell-associated TNFR levels, respectively. Analysis of the protein levels showed that the ODNs directed against TNFRI mRNA inhibited extracellular and cell-associated TNFRI protein in a dose-dependent fashion at submicromolar concentrations (panels A and B of Figure 8). In contrast, the analysis of the TNFRII protein expression in these cells revealed that the level of this protein was unchanged or slightly stimulated by treatment with either T30588 or T30589 (panels C and D of Figure 8). These results strongly suggest that the activity of these ODNs was both sequences and target-specific. This also implies that the inhibition of IL-6 production is due to the reduction in TNFRI and that TNFα induction of IL-6 is dependent upon TNFα binding to TNFRI and not to TNFRII.

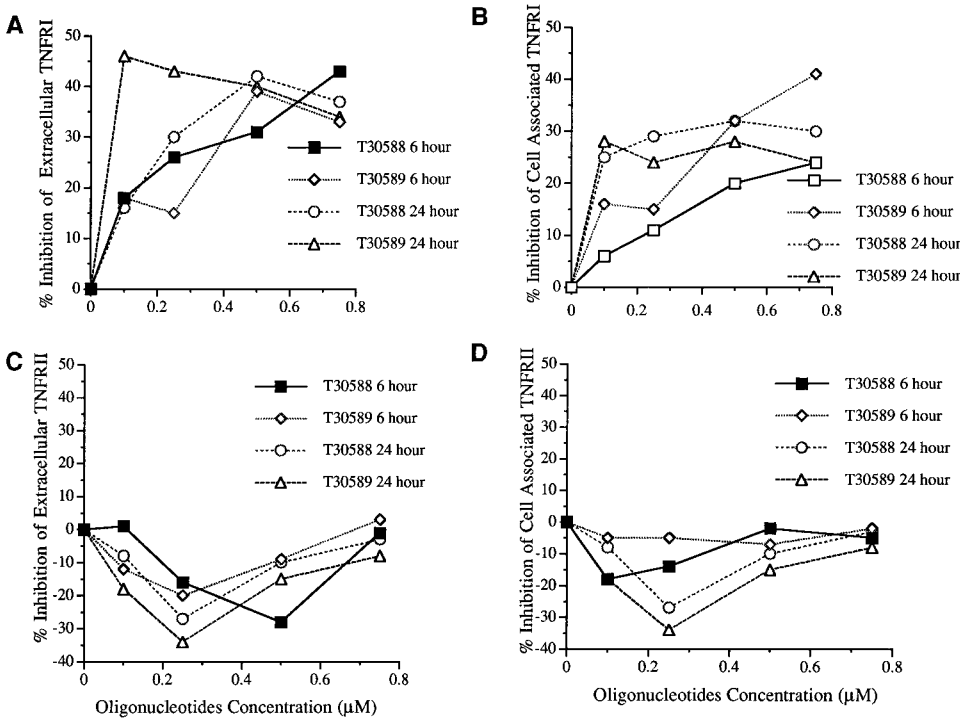


FIGURE 8: Effect of antisense ODNs on TNFRI and TNFRII expression. Panel A is a representation of the percent inhibition of TNFRI levels in MRC-5 cell supernatants (extracellular) collected 6 or 24 h after drug removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) or T30589 (hexynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin. Panel B is a representation of the percent inhibition of TNFRI levels in MRC-5 cell lysates (cell-associated) collected 6 or 24 h after drug removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) or T30589 (hexynyl-modified pyrimidines containing ODN) in the presence of 10 μg/ml Cellfectin. Panel C is a representation of the percent inhibition of TNFRII levels in MRC-5 cell supernatants (extracellular) collected 6 or 24 h after drug removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) or T30589 (hexynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin. Panel D is a representation of the percent inhibition of TNFRII levels in MRC-5 cell lysates (cell-associated) collected 6 or 24 h after drug removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) or T30589 (hexynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin.

The long-term effects of ODN treatment on TNFRI expression were also investigated. The experiment was performed using both log phase growing and confluent MRC-5 cells. The MRC-5 cells were treated with 1 μM T30588 for 4 h in the presence of Cellfectin, rinsed, and incubated in fresh media. Every 2−3 days, up to 24 days after drug removal, the culture supernatants were collected and stored at −80 °C until assayed for the presence of TNFRI. At the same time, the cells were trypsinized and re-seeded in fresh medium at a cell density of 1×10^4 cells/mL (for growing cell cultures) or a 1/2 dilution (for confluent cell cultures). In this experiment, the level of extracellular TNFRI expression was reduced for at least 3 days in growing MRC-5 cells and 7 days in semiconfluent MRC-5 cells, compared to the control untreated cells (Figure 9). Similar results were obtained for the levels of cell-associated TNFRI expression (data not shown).

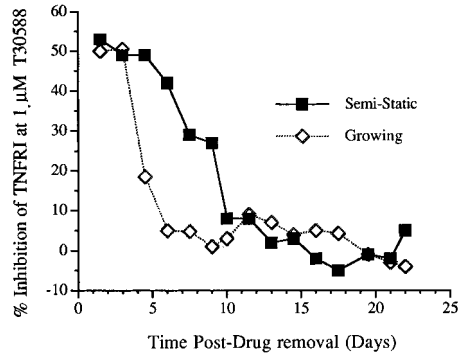


FIGURE 9: Long term effect of antisense ODN on TNFRI expression. The figure is a representation of the percent inhibition of TNFRI levels in MRC-5 cell supernatants (extracellular) collected from log phase growing cells (◇) and semiconfluent cells (■) as a function of time (days) after ODN removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin.

To determine if the observed reduction of TNFRI could be achieved in the presence of rhTNFα, MRC-5 cells were treated with ODNs in the presence of 10 μg/mL Cellfectin for 4 h and then rinsed and stimulated with rhTNFα. Six or 24 h poststimulation, culture supernatants and corresponding cell lysates were collected and assayed for TNFRI protein levels. This analysis showed that the antisense ODNs inhibited extracellular and cell-associated TNFRI protein in a dose-dependent fashion in the presence of rhTNFα (data not shown).

Mechanism of Anti-TNFRI ODN Action. To determine the effect of anti-TNFRI ODNs on TNFRI mRNA, total cellular RNA isolated from cells treated with test or control ODNs was analyzed for the presence of TNFRI mRNA by slot-blot hybridization. The level of TNFRI mRNA was quantitated and compared to the level of β-actin mRNA in the cells (Figure 10). In this experiment, T30588 formulated with Cellfectin significantly reduced the expression of the TNFRI mRNA relative to β-actin (Figure 10).

RNase H is an enzyme that specifically degrades the RNA strand of RNA−DNA hybrids. ODNs containing propynyl-modified pyrimidines and ODNs having pPT backbones have been reported to serve as substrates of RNase H (Wagner *et*

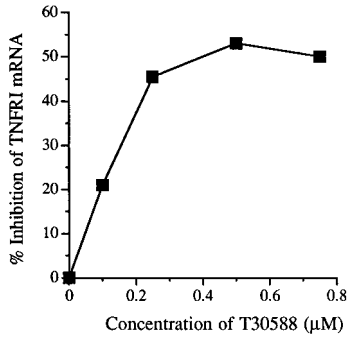


FIGURE 10: Effect of antisense ODNs on TNFRI mRNA. The figure is a representation of the percent inhibition of TNFRI mRNA levels in MRC-5 cells 3 h after drug removal when treated with T30588 (propynyl-modified pyrimidines containing ODN) in the presence of 10 μg/mL Cellfectin.

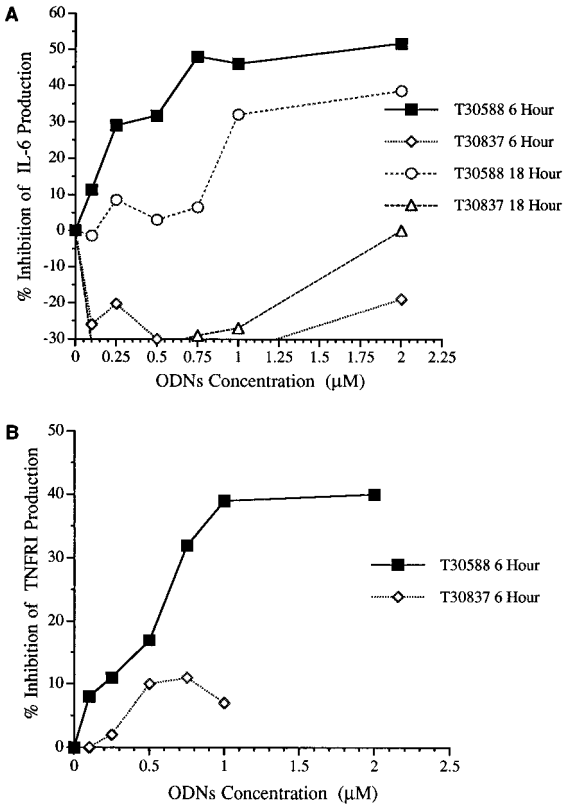


FIGURE 11: Effect of 2′-*O*-methyl modified ODN (T30837) on both IL-6 and TNFRI production. Panel A is a representation of the percent inhibition of IL-6 levels in MRC-5 cell supernatants 6 or 18 h poststimulation with rhTNFα and treated with T30588 or T30837 in the presence of 10 μg/mL Cellfectin. Panel B is a representation of the percent inhibition of TNFRI levels in MRC-5 cell supernatants (extracellular) collected 6 h after drug removal when treated with T30588 or in the presence of 10 μg/mL Cellfectin.

al., 1993; Walder & Walder, 1988), while 2′-*O*-methyl-containing ODNs do not (Inoue *et al*., 1987). To address the role of RNase H in the reduction of TNFRI mRNA mediated by the treatment of cells with T30588 or T30589, the 2′-*O*-methyl analog (T30837) of T30804 was tested in both IL-6 and TNFRI assay systems using MRC-5 and Hs68 cells. In these experiments, T30837 failed to inhibit IL-6 production (Figure 11A) or TNFRI expression (Figure 11B) in either cell line. The lack of activity of T30837 was not due to a decreased hybridization affinity as the T_m of T30837 complexed to an RNA target was superior to that of any of the other ODNs used in this study (Table 2). These data taken together suggest that the reduction in TNFRI mRNA observed when cells are treated with propynyl- or hexynyl-modified pyrimidine derivatives of T30804 was due to RNAse H-induced cleavage.

Effect of Oligonucleotide T_m. It has been reported by Wagner *et al*. (1993) that the potency of antisense ODNs containing C-5-propynyl-modified pyrimidines correlated with enhanced binding affinity with the cognate target site. We have now completed a relatively thorough analysis of the effect of propynyl- and hexynyl-modified pyrimidine substitution on the antisense ODNs used in this study. In these experiments, the target strands were synthesized with phosphodiester backbones. Data were obtained by optical detection of the thermal denaturation. The data clearly show that propynyl- and hexynyl-modified pyrimidine substitution leads to a net stabilization of duplex formation when binding to either DNA or RNA sense strands (Table 2). In order to investigate the origin of the duplex stabilization by substituting with the propynyl group, we have analyzed the melting data according to the method described by Petersheim and Turner (1983). The data summarized in Table 2 suggest that the observed stabilization is associated with stabilization in the enthalpy term of the duplex formation. Even though the structure is different, Colocci and Dervan (1994) proposed that the stacking interaction extended by the propynyl group might explain the stabilization of triple helix, which contained propynyl-substituted pyrimidines in the third strand. However, the similar analysis of T_m data in other duplexes bearing propynyl-substituted pyrimidines we have carried out have suggested the entropy-driven stabilization of duplex (data not shown), which is probably due to the hydrophobic effect of the propynyl group. Since the extent of the stacking could be dependent on the sequence of duplex, it is most likely that the balance of two effects (extended stacking and hydrophobic effect) might determine the magnitude of total stabilization by propynyl substitution.

DISCUSSION

We have identified antisense ODNs targeted to the human TNFRI mRNA which specifically inhibit expression of TNFRI and subsequent functions of the gene product in two cell lines, human lung embryonic fibroblast (MRC-5) and human newborn foreskin fibroblast (Hs68). The data presented strongly imply an antisense mechanism of action for the test ODNs since the control ODNs failed to inhibit the TNFRI expression or TNFα-mediated functions, the test ODNs did not inhibit the internal control target, TNFRII, hybridization of the test ODNs to their complementary strands reversed the activity of the antisense ODNs, the effect of the ODNs on protein synthesis or protein function was specific, and the antisense ODNs specifically reduced the TNFRI transcript.

Several investigators have reported antisense activity with phosphorothioate ODNs containing unmodified bases (Wickstrom, 1991); however, the results reported in this study show that optimum activity at submicromolar concentrations was achieved with ODNs containing C-5-hexynyl- or C-5-propynyl-substituted 2′-deoxyuridines and a reduced number of PT linkages. The antisense ODN activity was dependent on the length and base modification used and correlated well with T_m and ΔG values obtained. These observations suggest that the pyrimidine modification was necessary to promote

Table 2: Thermodynamic Measurements of Anti-TNFRI ODNs[a]

ID	Sequence	Tm (°C)	ΔH (kcal/mol)	ΔS (eu)	ΔG (37°C) (kcal/mol)	Ka
DNA-DNA Duplex						
T30804	5'-a*g*a*a*t t t t a*g*t*g*t*a t*g t a*c*a*a -3'	36.6	-154	-469	-8.7	1.4×10^6
T30837	5'-a*g*a*a*u u u u a*g*u*g*u*a u*g u a*c*a*a -3'	36.2	-122	-366	-8.6	1.2×10^6
T30588	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	40.2	-168	-506	-10.6	3.3×10^7
T30782	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	34.3	-133	-405	-7.7	2.9×10^5
T30589	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	39.4	-171	-518	-10.2	1.7×10^7
T30776	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	31.6	- 91	-270	-7.3	1.4×10^5
DNA-RNA Duplex						
T30804	5'-a*g*a*a*t t t t a*g*t*g*t*a t*g t a*c*a*a -3'	31.1	-108	-327	-6.8	6.6×10^4
T30837	5'-a*g*a*a*u u u u a*g*u*g*u*a u*g u a*c*a*a -3'	48.3	-124	-356	-13.3	2.4×10^9
T30588	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	38.6	-142	-427	-9.7	6.7×10^6
T30782	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	36.5	-102	-302	-8.7	1.5×10^6
T30589	5'-a*g*a*a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	35.5	-105	-310	-8.4	9.0×10^5
T30776	5'- a*t T T T a*g*t*g*t*a T g T a*c*a*a -3'	32.3	-104	-313	-7.3	1.4×10^5

[a] Conditions: 2 mM sodium phosphate buffer (pH 7.0), 20 mM NaCl; strand concentration, 2×10^{-6} M (1×10^{-6} M each); and heating rate, 0.5 °C per 30 s.

enhanced hybridization affinity between the antisense ODNs and the target RNA to promote efficient antisense activity.

We were unable to detect significant activity with total or partial phosphorothioate ODNs with or without C-5-hexynyl- or C-5-propynyl-substituted pyrimidines in the absence of an uptake enhancer. The lack of activity of TNFRI antisense ODNs in the absence of uptake enhancer suggests that Cellfectin facilitates the efficient delivery of these ODNs to the target sites within these cells (Figure 3B). Cellfectin is a cationic lipid formulation initially reported as a delivery vehicle for transfection of DNA into cells (Macdonald *et al*., 1996). In addition, the use of other cationic lipids such as DOTMA as delivery agents for ODNs into mammalian cells has been reported (Chiang *et al*., 1991). Like DOTMA, Cellfectin may also enhance cell association of ODNs and significantly change the intracellular distribution of ODN, allowing higher cytoplasmic and nuclear concentrations of free ODN, resulting in increased biological activity. The use of cationic lipids, therefore, helps deliver and distribute ODN within a large number of cells, allowing the biochemical analysis of ODN functions to be performed.

Several mechanisms by which antisense ODNs may inhibit gene expression have been proposed. These include inhibition of new protein synthesis by translational arrest, promotion of mRNA degradation by RNase H, inhibition of mRNA maturation by masking sequences required for formation of spliceosomes, prevention of mRNA transport out of the nucleus, inhibition of gene transcription by forming a triple helix structure, or other unidentified mechanisms (Zon, 1988; Stein & Cohen, 1988; Dolnick, 1990; Helene & Toulme, 1990). The mechanism by which antisense ODNs modulate gene expression may depend on several factors, such as target cell or target mRNA and the chemical nature of the test ODNs. In this report, the active antisense ODN targeted the 3′-untranslated region of the TNFRI mRNA. T30588 and T30589 hybridize to the TNFRI mRNA at the poly(A) site; therefore, these ODNs should have no direct effect on translation of the protein. The analysis of steady-state mRNA levels from the ODN-treated MRC-5 cells showed that these ODNs caused a specific reduction in the TNFRI mRNA levels. The reduction of mRNA after treatment with the ODNs was due to mRNA destabilization either by an RNase H-dependent mechanism or by modulating natural processes that help to stabilize the TNFRI mRNA. It is important to note that the maximum of 50−70% reduction was observed for both TNFRI protein and mRNA production. This may mean that either only 50−70% of the cells were inhibited or all the cells were inhibited by 50−70%. The fact that IL-6 or IL-8 was also inhibited at the same levels suggests the former, because one would expect a certain threshold of TNFRI expression that would be sufficient for a maximal TNFα effect.

Several clinical evaluations have indicated an elevation of TNFα expression in a variety of systemic inflammatory disorders, including rheumatoid arthritis and other immunological reactions (Aggarwal & Vilcek, 1992). Although there has been no direct link between high levels of TNFα and inflammatory responses, it is believed that transient high levels of TNFα at these inflamed regions may contribute directly or indirectly to the disorder. Recently, monoclonal antibodies specific for TNFα have been used with promising results to treat rheumatoid arthritis in humans (Keffer *et al*., 1991; Williams *et al*., 1992). In another study, Pampfer *et al*. (1995) reported the protection of rat blastocysts from TNFα by blocking rat p60 expression. These studies provide strong evidence that inhibitors of TNFα or its specific receptor (TNFRI) could have beneficial effects in the treatment of inflammatory or immunological responses elicited by this cytokine.

Thus, we have shown that antisense ODNs with proper base and backbone modification and a good delivery agent are capable of specifically reducing the expression of TNFRI in a cell culture system. In addition, inhibition of TNFRI expression with antisense ODNs correlated with inhibition of TNFα-mediated biological functions. Therefore, inhibition of TNFRI expression with antisense ODNs offers a novel approach for controlling biological functions of TNFα and these antisense ODNs may be useful as human therapeutic agents in treating diseases in which TNFRI is the primary receptor.

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