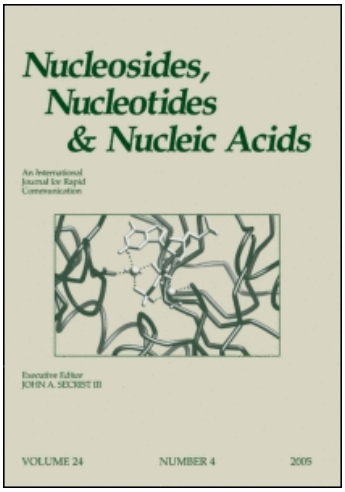

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Ojwang, Joshua O. , Rao, T. Sudhakar , Marshall, Hélène B. , Mustain, Shawn D. , Chaudhary, Nilabh , Walker, David A. , Peyman, Anusch , Uhlmann, Eugen , Revankar, Ganapathi R. and Rando, Robert F.(1997) 'Inhibition of Tumor Necrosis Factor Alpha (TNFα) Expression and Function *In Vitro* by Modified Antisense Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1703 — 1707
To link to this Article: DOI: 10.1080/07328319708006259
URL: http://dx.doi.org/10.1080/07328319708006259

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1703-1707 (1997)

INHIBITION OF TUMOR NECROSIS FACTOR ALPHA (TNFα) EXPRESSION AND FUNCTION *IN VITRO* BY MODIFIED ANTISENSE OLIGONUCLEOTIDES

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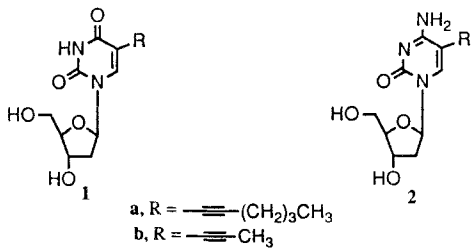
ABSTRACT: Antisense oligonucleotides containing C-5 hexynyl/propynyl modified pyrimidines were synthesized using solid phase phosphoramidite chemistry. These modified oligonucleotides were found to have significant inhibitory activity against TNFα production *in vitro*.

Tumor necrosis factor alpha (TNFα) is a mononuclear phagocytic cell derived protein which elicits inflammatory and immunological reactions.[1,2] The binding of TNFα to the p55 tumor necrosis factor receptor (type I receptor or TNFRI) is considered to be the initial step responsible for the multiple biological actions mediated by TNFα. The role of TNFα as an inflammatory mediator through human TNFRI makes both of these genes attractive targets for intervention in both acute and chronic inflammatory diseases.

Recently Wagner et al. reported[3] the antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. In the present study we have designed several antisense oligonucleotides containing C-5 hexynyl/propynyl dU/dC which specifically hybridize to the sequences of the human TNFα mRNA and inhibit its expression and function. The synthesis of C-5 hexynyl/propynyl-2'-deoxyuridine (**1a, b**) and 2'-deoxycytidine (**2a,b**) was accomplished starting from 5-iodo-2'-deoxyuridine utilizing the procedure of Hobbs.[4] The nucleosides were protected[5] and phosphitylated[6] by the conventional procedures and incorporated these monomers into oligonucleotides employing phosphoramidite methodology.[7]



Materials and Methods

Cell Culture Assay: THP-1 cells (3×10^6) were seeded in 48-well plates in 0.2 ml of Opti-MEM (GIBCO) medium. In a 96-well plate, varying concentrations of oligodeoxynucleotides (ODNs, 20 µl) were each added to 20 µl of Cellfectin (Life Technologies) in Opti-MEM (2.4 µl of 1 mg/ml Cellfectin added to 17.6 µl Opti-MEM) and incubated for 15 min at room temperature. The mixture was then added to the cells dropwise. The final concentration of the Cellfectin was 10 µg/ml in 0.24 ml of Opti-MEM. After 4 h at 37°C, 0.48 ml of 15%RPMI medium was added to each well to inactivate the Cellfectin. The final concentration of cells was 1×10^6 cells/ml in 10% RPMI. The cells were then stimulated with a combination of 100 ng/ml of PMA and 300 U/ml of IFNγ. To monitor the effects of ODNs on TNFα in culture medium 6 h post-stimulation the supernatants were collected and stored at -80°C until use. To monitor the effects on cell associated TNFα, the cells were centrifuged at 3500 rpm (~1000 × g) for 6 min, the supernatants were removed and the cell pellets were then resuspended in 200 µl of 0.25 mM Tris-HCl (pH 7.4). The cell associated protein was prepared by freezing the cell suspension in dry ice/ethanol and thawing at 37°C three times with vortexing in between freeze-thaw cycles. The cell lysates were stored at -80°C until used. The supernatants and cell lysates were analyzed for TNFα. The data were expressed as percent inhibition compared to control.

Cytotoxicity Assay: The cytotoxicity of the oligonucleotides or oligonucleotide/ Cellfectin mixture was assayed using the CellTiter 96TM Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega). For the oligonucleotide alone, viable cell number was determined by trypan blue staining and cells (THP-1) were resuspended in RPMI supplemented with 10% FBS (GIBCO). Eighty microliters of cell suspension (1.7×10^3 cells/well) was dispensed onto a 96-well microtiter plates. At this time 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 MTS assay was performed according to the manufacturer's instructions. For the ODN/Cellfectin mixture, the cells were prepared as described above. Twenty microliters of varying concentrations of the ODN/Cellfectin mixture (or control) were

TABLE 1

ID	Sequence	Location[8,9]
Test oligonucleotide and its shorter derivatives from the AUG site.		
T30797	5′-T*C*a*T g g T g T C C*T T*T g*C*a*g-3′	156-139
T30798	5′-T*C*a*T g g T g T C C*T T*T*g*C- 3′	156-141
T30799	5′-T*C*a*T g g T g T C C*T T*T*g- 3′	156-132
T30800	5′- a*T g g T g T C C*T T*T g*C*a*g-3′	154-139
T30801	5′- T*g*g T g T C C*T T*T g*C*a*g-3′	153-139
Control Oligos		
T30802	5′-T*C*g*T a g C g T C T*T C*T g*C*a*g-3′	18mer
T30803	5′-C*T*g C a a a g g a*C*a C*C a T*G*a-3′	156-139

*indicates PT linkage, T = C-5 propynyl dU; C = C-5 propynyl dC

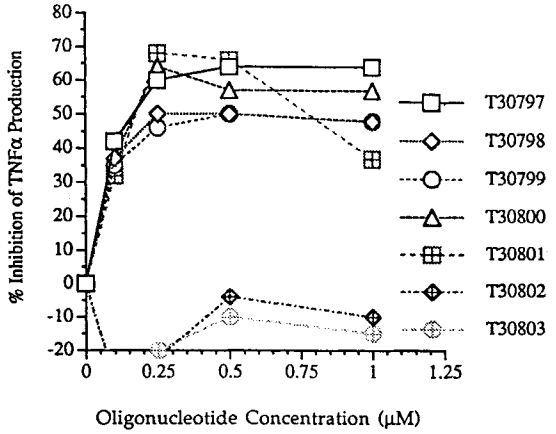


FIGURE 1

then added to appropriate wells. Each concentration was assayed in quadruplicate. After 4 h at 37°C, the medium was removed, the wells rinsed twice with 10% RPMI and then 80 μl of fresh 10% RPMI 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.

Results: The modified oligonucleotides containing C-5 propynyl pyrimidines synthesized for the present study are presented in Table 1. These oligonucleotides were evaluated *in vitro* for their ability to inhibit TNFα production in the presence of the uptake enhancer Cellfectin. The data obtained from these experiments (Figure 1) clearly show that the base modified partial phosphorothioate oligonucleotdes (T30797-T30801) have the ability of selective

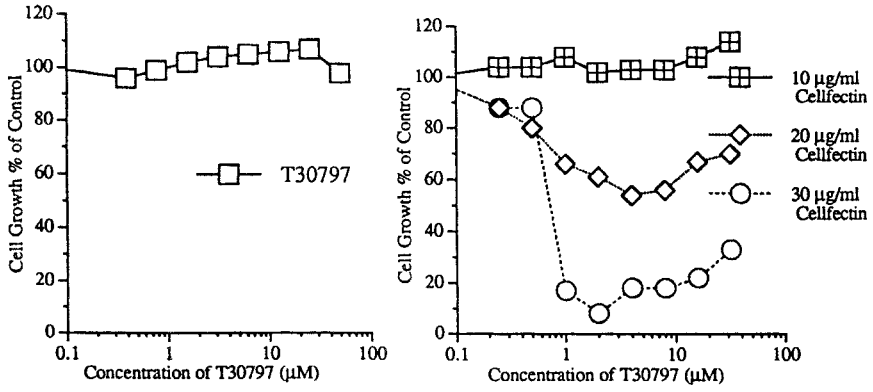


FIGURE 2

inhibition of TNFα production in a dose dependent fashion at submicromolar concentrations. Similar results were obtained with C-5 hexynyl pyrimidine containing oligonucleotides. The control phosphorothioate oligo-nucleotides (T30802, a scrambled 18 mer and T30803, sense oligonucleotide) with base modifications did not show any inhibition of TNFα production.

The cytotoxicity of the modified oligonucleotide T30797 or oligo-nucleotide/Cellfectin mixture was also analyzed using the CellTiter 96 TM Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega). In these experiments oligonucleotide itself did not exhibit any toxicity to the cells (Figure 2). In the case of oligonucleotide/Cellfectin mixture no toxicity was observed upto 10 μg/ml of Cellfectin, however toxicity was noticed with increasing concentrations of Cellfectin.

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