Facilitation of the Cellular Uptake of a Triplex-Forming Oligonucleotide by Novel Polyamine Analogues: Structure−Activity Relationships†

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ABSTRACT: The inefficient uptake of oligodeoxynucleotides, including that of TFO, through the cell membrane is a limiting factor in developing gene therapy approaches for cancer and other diseases. To develop a new strategy for oligonucleotide delivery into the nucleus, we synthesized a series of novel polyamine analogues and examined their effects on the uptake of a 37-mer [^{32}P]-labeled TFO, targeted to the promoter region of c-*myc* oncogene. We used MCF-7 breast cancer cells to investigate the efficacy of polyamines on the internalization of the TFO. The uptake of TFO was enhanced by complexing it with several unsubstituted polyamine analogues at 0.1−5 μM concentrations, with up to 6-fold increase in TFO uptake in the presence of a hexamine, 1,21-diamino-4,9,13,18-tetraazahenicosane ($H_2N(CH_2)_3NH$-$(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ or 3−4−3−4−3). TFO uptake increased with the cationicity of the polyamines; however, bis(ethyl) substitution and structural features of the methylene bridging region had significant effects on TFO uptake. The majority of labeled TFO was recovered from the nuclear fraction containing genomic DNA. Electrophoretic mobility shift assay revealed enhanced binding of TFO to a target duplex containing promoter region sequence of c-*myc* oncogene. Treatment of MCF-7 cells with the TFO complexed with 0.5 μM 3−4−3−4−3 suppressed c-*myc* mRNA level by 65%, as determined by Northern blot analysis. These data indicate a novel approach to deliver oligodeoxynucleotides to the cell nucleus, and suppress the expression of target genes, and provide new insights into the mechanism of oligonucleotide transport in living cells.

The inactivation of gene expression at the transcriptional level using a short sequence of single-stranded oligodeoxynucleotides capable of forming a triplex DNA structure has been viewed as a potential strategy to specifically inhibit the transcription of disease-related genes[1] (*1*−*11*). The success of this strategy depends on the presence of triplex-forming homopurine−homopyrimidine sequences at the promoter regions of disease-related genes (*12*). This method differs from the antisense approach in which oligonucleotides are used to base pair with the complimentary sequences of mRNA, thereby suppressing the activity of targeted genes at the translational level (*12*−*13*). To exert their effects at transcriptional or translational level, oligonucleotides have to be taken up by cells and remain stable for a period of time sufficient for induction of triplex or DNA−RNA hybrid formation, leading to transcriptional inactivation or translational arrest. The slow rate of cellular uptake of administered oligonucleotides through the plasma membrane and a lack of understanding of the mechanism of oligonucleotide uptake impose serious limitations on the therapeutic usefulness of oligonucleotides in gene therapy (*14*, *15*).

As the DNA is a negatively charged macromolecule, it does not cross the membrane barrier very efficiently. Therefore, various methods have been developed to deliver

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[1] Abbreviations: ODN or oligonucleotide, oligodeoxyribonucleotide; TFO, triplex forming oligonucleotide; DAP or 3, 1,3-diaminopropane; BE, bis(ethyl); G3PDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; DCC, dextran coated charcoal; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; NSO, nonspecific oligonucleotide; 3−3, norspermidine; 4, 1,4-diaminobutane or putrescine; 4−4, homospermidine; 3−3−3, norspermine; BE−3−3−3, N^1,N^{11}-bis(ethyl)norspermine; BE−3−4−3, N^1,N^{12}-bis(ethyl)spermine; BE−3−7−3, 1,15-bis(ethyl)amino-4,11-diazapentadecane; 4−4−4, homospermine; BE−4−4−4, N^1,N^{14}-bis(ethyl)homospermine; 3−3−3−3, 1,15-diamino-4,8,12-triazapentadecane; BE−3−3−3−3, 1,15-bis(ethylamino)4,8,12- triazapentadecane; 4−4−4−4, 1,19-diamino-5,10,15-triazanonadecane; BE−4−4−4−4 1,19-bis(ethylamino-5,10,15-triazano-nadecane; 3−3−3−3−3, 1,19-diamino-4,8,12,16-tetraazanonadecane; 3−3−4−3−3, 1,20-diamino-4,8,13,17-tetraaza-icosane; 3−4−3−4−3, 1,21-diamino-4,9,13,18-tetraazahenicosane; PEI, polyethylenimine.

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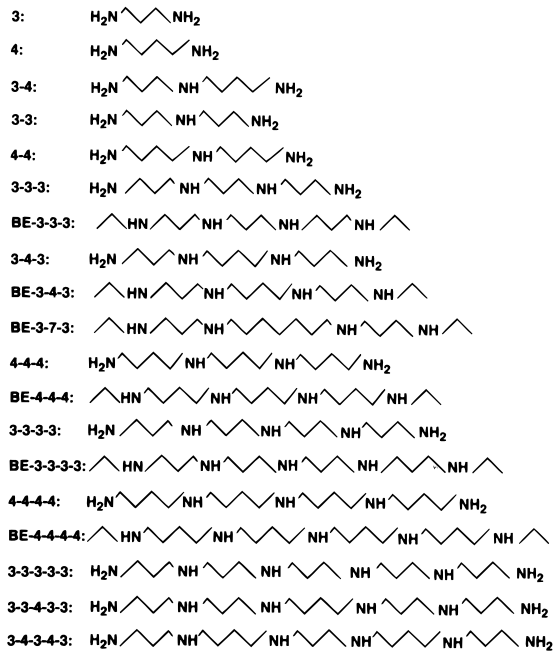

FIGURE 1: Chemical structure of polyamine analogues. For convenience, these molecules are abbreviated by a number system, which represents the number of methylene groups between the amino and imino groups of the polyamines.

oligonucleotides to the cellular compartments. These include conjugation of oligonucleotide with cross-linking or intercalating agents (*6*, *16*, *17*), substitution of 3′-propylamine group (*18*) or cholesteryl moiety at the end of the oligonucleotide (*5*), use of cationic lipids or polylysine (*19*, *20*), polyaminolipids (*21*), polyarginine (*22*), or complexing with metal ions (*23*). Boussif et al. (*24*) reported that polyethylenimine could be used as a versatile vector for gene and oligonucleotide transfer into cells. Although these methods are useful in enhancing the oligonucleotide uptake, in many cases, the final outcome of gene inactivation is not achieved due to interference of the carrier molecule, which may lower the hybridization potential of the oligonucleotide at the target site.

Another problem associated with the triplex DNA-mediated gene therapy approach is the stability of triplex DNA under physiologically compatible pH and salt concentrations (*12*, *13*, *25*). Previous studies from our laboratory have shown that triplex DNA stability can be significantly improved by the use of polyamines and their analogues (*7*, *26*−*28*). Thomas et al. (*7*) further reported that a triplex-forming oligonucleotide complexed with a polyamine analogue, DAP, inhibited the c-*myc* gene expression in MCF-7 breast cancer cells. Thus, complexing TFOs with polyamine analogues is a promising anti-gene strategy for treating different forms of cancer.

The in vivo stability of the transfected oligonucleotide is also a commonly encountered problem because the unmodified phosphodiester oligonucleotides are highly susceptible to enzymatic cleavage by serum-derived and cellular nucleases (*29*). However, oligonucleotides can be protected from nuclease attack by structural modifications and changes in the DNA conformation by different chemical agents (*30*). Structural modifications of the backbone structure of TFO, by replacement of one of the phosphate oxygen atom with sulfur to form phosphorothioate or methyl residue to form methyl phosphonate, have also been used to enhance stability of TFOs (*29*). Alternately, polyamine-mediated condensation of DNA may protect the oligonucleotide against enzymatic degradation. For example, Baeza et al. (*31*) showed that polyamine-mediated DNA condensates are resistant to the action of DNase I using electron microscopy and biochemical techniques.

In an attempt to develop a new strategy for facilitating the cellular uptake and enhanced stability of triplex DNA, we synthesized a series of polyamine analogues and studied their effect on the cellular uptake of a TFO targeted to the promoter region of c-*myc* oncogene in MCF-7 breast cancer cells. Our results suggest that complexing TFO with specific polyamine analogues is an effective strategy for enhancing the oligonucleotide uptake and achieving a biological response in terms of suppressing the level of transcripts produced from the targeted gene.

MATERIALS AND METHODS

Polyamines and Chemicals. Putrescine·2HCl, 1,3-diaminopropane·2HCl, spermidine·3HCl, and spermine·4HCl were purchased from Sigma Chemical Co. (St. Louis, MO). The polyamine analogues, 3−3, 4−4, 3−3−3, BE−3−3−3, BE−3−4−3, BE−3−7−3, 4−4−4, BE−4−4−4, 3−3−3−3, BE−3−3−3−3, 4−4−4−4, BE−4−4−4−4, 3−3−3−3−3, 3−3−4−3−3, and 3−4−3−4−3 were synthesized by procedures described by Igarashi et al. (*32*). The chemical structure and purity of the synthesized compounds were determined by elemental analysis, NMR, HPLC, and mass spectrometry. The chemical structures of these polyamine analogues are given in Figure 1. Polyamine analogue stock solutions (20 mM) were prepared in sterile, double distilled water, and appropriate dilutions were made prior to use.

Fetal bovine serum, cell culture media, antibiotics, mevalonic acid, hydroxyurea, nocodazole, aminoguanidine, and other chemicals were purchased from Sigma. Lovastatin was a gift from Merck Pharmaceuticals (Rahway, N. J.). [^{32}P]-ATP was purchased from New England Nuclear (Boston, MA).

A cDNA probe for c-*myc* mRNA was purchased from Oxford Biomedical Research (Oxford, MI). A control probe for G3PDH was purchased from Clontech (Palo Alto, CA). These probes were [^{32}P]-labeled using the random primer method (Prime-a-Gene Labeling System, Promega, Madison, WI).

Oligodeoxynucleotides. We used the following oligonucleotides for this study: (i) a 37-mer TFO targeted to the promoter region of c-*myc* oncogene (ODN1); (ii) a 37-mer NSO (ODN2); (iii) a 37-mer pyrimidine rich strand corresponding to the promoter region of c-*myc* oncogene (ODN3); (iv) a complementary 37-mer purine rich strand corresponding to the promoter region of c-*myc* oncogene (ODN4). The nucleotide sequence of these oligonucleotides is given below:

TFO or
ODN1: 5′-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGGGT-3′

NSO or
ODN2: 5′-GGTGGTGGGTTGGGGTGGTGGTGGGGTGGTGGTGTTG-3′

ODN3: 5′-CTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCA-3′

ODN4 : 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGGAGGAG-3′

Oligonucleotides were purchased from Oligos, Etc. (Wilsonville, OR) and were HPLC purified. Oligonucleotides were 5′ end labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). The ODN's were purified and unincorporated label removed by electrophoresis through 8% polyacrylamide gel, followed by electroelution and ethanol precipitation.

Cell Culture. MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% FBS and supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, 40 μg/mL gentamicin, 2 μg/mL insulin, 0.5 mM sodium pyruvate, 10 mM nonessential amino acids and 2 mM L-glutamine (referred to as "regular medium"). Phenol red-free DMEM containing 10% fetal bovine serum (pretreated with DCC, consisting of 0.5% Norit A, and 0.05% Dextran T-70) was used for all uptake experiments in order to avoid the effects of serum-derived estrogenic compounds and phenol red (*7*).

Measurement of TFO Uptake by MCF-7 Cells. MCF-7 cells were seeded in 6 well plates at a density of 5×10^5 cells/well, using 4 mL of phenol red-free DMEM containing 4 nM estradiol. Cells were allowed to attach to the wells for 24 h. Prior to each experiment, the medium was removed and replaced with 0.5 mL (per well) of fresh phenol red-free medium, containing 10% DCC-treated FBS. Different amounts of [^{32}P]-labeled TFO ($2.5 \times 10^4 - 5 \times 10^5$ cpm or ~0.25−5 nM TFO) were added to the medium, and the cells incubated at 37 °C in a 5% CO_2 incubator. The cells were harvested at 0, 0.5, 1, 2, 4, 6, 12, and 24 h time points, the medium was immediately removed, and cells were washed 3 times with ice-cold PBS. Cells were lysed using 1 mL of 1 N NaOH, followed by heating at 60 °C for 30 min, and the lysate was neutralized with 1 mL of 1 N HCl. Aliquots of cell lysate (1 mL) along with 5 mL of Ready Safe Scintillation Cocktail (Beckman Instruments, Palo Alto, CA) were taken into scintillation vials and radioactivity quantified using a Beckman (LS 5000 TD) Scintillation Counter.

Since MCF-7 cells are estradiol-responsive, we included 4 nM estradiol in all TFO uptake experiments. This concentration was used because optimal cell growth stimulation of MCF-7 cells was observed at 4 nM estradiol in our previous studies (*33*).

To examine the uptake of TFO in the presence of polyamines, labeled TFO was added to different concentrations of polyamines/analogues, incubated for 15 min at 22 °C, and the mixture stored overnight at −70 °C. The polyamine/oligonucleotide mixture was thawed to 22 °C and incubated at this temperature for 1 h before adding to MCF-7 cells in culture (5×10^5 cells/well) in six well plates. (Similar uptake of TFO was observed when the polyamines/TFO mixture was used without the freezing step.) Each well contained 0.5 mL of medium with 4 nM estradiol. The final concentrations of polyamines/analogues ranged from 0.1 to 10 μM, and the level of probe used was 2.5×10^5 cpm (2.5 nM). The control group received 2.5×10^5 cpm level of probe alone. Aminoguanidine (1 mM) was added to the medium to inhibit the oxidative stress due to polyamine oxidase activity (*33*). Groups of cultures were harvested immediately after the addition of TFO (0 h) or at 4 h of treatment. The time-course of uptake of TFO was measured in the presence of 0.25, 0.5, 1 and 2.5 μM of hexamines, 3−4−3−4−3, 3−3−3−3−3, and 3−3−4−3−3 at 0, 4, 12, and 24 h time intervals by the same procedure.

Effect of Cell Cycle Phase on TFO Uptake. To determine the effects of cell cycle phase of MCF-7 cells on TFO uptake, we arrested these cells using chemical agents and measured TFO uptake. MCF-7 cells were plated in 60 mm culture dishes (2×10^6 cells/dish) using phenol red-free DMEM containing 4 nM estradiol and allowed to attach to the dishes for 24 h. The cell cycle was arrested at the G_1 phase by treating cells with 20 μM lovastatin for 36 h, as described by Keyomarsi et al. (*34*). The effect of lovastatin was inhibited by adding 2 mM of mevalonic acid. Cell cycle arrest in S phase was achieved by treating cells with 500 μM hydroxyurea for 24 h (*35*). G_2/M arrest was achieved by treating cells with 6.6 μM of nocodazole for 24 h (*36*). The drug containing medium was removed, fresh medium was added and TFO uptake was measured as described above.

Quantification of the percentage of cells in different phases of cell cycle was accomplished using flow cytometry, as described previously (*33*).

Cytoplasmic and Nuclear Uptake of TFO. MCF-7 cells (2×10^6 cells/dish) were plated in 60 mm dishes using 10 mL of phenol red-free medium, containing 4 nM of estradiol, and incubated at 37 °C for 24 h. The medium was removed, 4 mL of fresh medium added, and then cells were treated with 2×10^6 cpm of labeled TFO in the absence or presence of different concentrations (0.1−5 μM) of the polyamine analogue, 3−4−3−4−3. At the 0 and 4 h time points, the medium was removed, cells washed 3 times with ice-cold PBS, and then harvested by mild trypsinization, suspended in 1 mL ice-cold PBS, and concentrated by centrifugation at 500*g* for 5 min in a Beckman GS-6KR centrifuge. The cytoplasmic and nuclear extracts were prepared as described by Postel et al. (*1*), Greenberg and Ziff (*37*), and Wickstrom et al. (*38*). The cell pellet was lysed with 1 mL of NP-40 lysis buffer (10 mM Tris·HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 v/v). The lysate was centrifuged at 500*g* for 5 min to separate the cytoplasmic fraction. The resulting nuclear pellet was lysed with 0.2 mL of 1% SDS lysis buffer (10 mM Tris·HCl pH 7.4/150 mM NaCl and 1% Na dodecyl sulfate). The nuclear lysate was centrifuged at 500*g* for 5 min and the supernatant collected. The remaining pellet was also saved, and radioactivity associated with each fraction and nuclear pellet quantified by scintillation counting.

Electrophoretic Mobility Shift Assay. We conducted EMSA experiments to examine the effect of 3−4−3−4−3 on the stabilization of triplex DNA formed between the TFO and its target duplex, consisting of ODN3 and ODN4. For this experiment, ODN4 was labeled with [^{32}P] and mixed with ODN3 in 1:1 molar ratio to prepare the labeled duplex (*28*). The mixture was heated at 100 °C for 10 min, and allowed to cool to room temperature. The labeled duplex thus prepared was mixed with 10 nM of unlabeled ODN1 (TFO) and 0.25 or 0.5 μM 3−4−3−4−3 in a reaction buffer containing 20 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, and 10% sucrose and incubated at 37 °C for 1 h. The reaction products were analyzed on a 10% nondenaturing polyacrylamide gel containing 90 mM Tris·boric acid and 5 mM $MgCl_2$ (pH 7.8), with the same gel running buffer.

Control experiments were performed with [^{32}P]-labeled ODN3−ODN4 duplex and NSO under the same reaction conditions as used for TFO.

Northern Blot Analysis of c-myc mRNA in MCF-7 Cells Treated with TFO Complexed with 3−4−3−4−3. To examine the biological response of TFO uptake in the presence of polyamines, we examined the level of c-*myc* mRNA in MCF-7 cells treated with different concentrations of TFO and 3−4−3−4−3. For these experiments, MCF-7 cells were grown in phenol red-free media for two weeks and seeded in 60 mm dishes at a density of 2×10^6 cells/dish. These cells were synchronized at G1 phase of cell cycle by isoleucine starvation for 36 h, as described by Thomas et al. (*7*). (Eighty percent of cells were in G1 phase after this treatment, as assessed by flow cytometry.) Prior to the experiment, isoleucine-free medium was replaced with 2 mL of complete medium, and estrogenic effect was restored by adding 4 nM of estradiol. TFO was complexed with different concentrations of 3−4−3−4−3 and added to the cells. A control group was maintained without drug treatment. The cells were harvested at 1 and 2 h after the addition of TFO/3−4−3−4−3 complex, and total RNA was isolated by using 1 mL of Trizol reagent (Gibco-Life Technologies, Grand Island, NY), followed by chloroform extraction and precipitation with 2-propanol. The purity of the RNA preparation was monitored by recording the absorbance at 260 and 280 nm. The absorbance ratio (A_{260}/A_{280}) was ∼1.8. Equal amounts of RNA samples were separated on a 1% agarose gel under denaturing conditions. RNA was intact without any degradation as indicated by 18S and 28S bands of the gel, as visualized by ethidium bromide staining. The RNA was transferred to nylon membrane and probed with a [^{32}P]-labeled cDNA probe for c-*myc* oncogene (*7*). The level of c-*myc* mRNA was visualized by autoradiography and quantified by densitometry using a ScanJet 4c flatbed scanner (Hewlett-Packard) and NIH Image v1.6 software.

To normalize the level of RNA loading and transfer, we first removed c-*myc* cDNA by stripping the membrane in boiling SDS solution (0.5%) for 10 min, and then probed it with a control probe, G3PDH. We also performed another control experiment to examine the effect of the NSO complexed with 3−4−3−4−3 on the level of c-*myc* mRNA produced at 1 and 2 h of treatment of MCF-7 cells.

RESULTS

Time-Course of TFO Uptake. Figure 2 shows the time-dependent uptake of different concentrations of labeled TFO by MCF-7 cells. There was a steady increase in TFO uptake up to 12 h, followed by a leveling off of the radioactivity associated with TFO uptake by these cells except at the highest level of TFO used. A dose-dependent increase in TFO uptake was also observed. On the basis of these data, we used 2.5×10^5 cpm level (2.5 nM) of TFO and 4 h time point for subsequent experiments, unless specified otherwise. Although MCF-7 is an estradiol-responsive cell line, the presence of 4 nM estradiol had no significant effect on TFO uptake when control experiments were performed in its absence (results not shown).

Uptake of TFO in the Presence of Polyamine Analogues. In the next set of experiments, we examined the effect of a series of polyamine analogues on TFO uptake by MCF-7 cells. For this purpose, cells were treated for 4 h with [^{32}P]-labeled TFO complexed with different concentrations (0.1 to 10 μM) of polyamines/analogues. The efficacy of each compound to alter TFO uptake was calculated as a percentage of the control uptake. Figure 3 shows the effects of a series of free and corresponding bisethylated tetramines and pentamines, and 3 free hexamines on TFO uptake. Compared

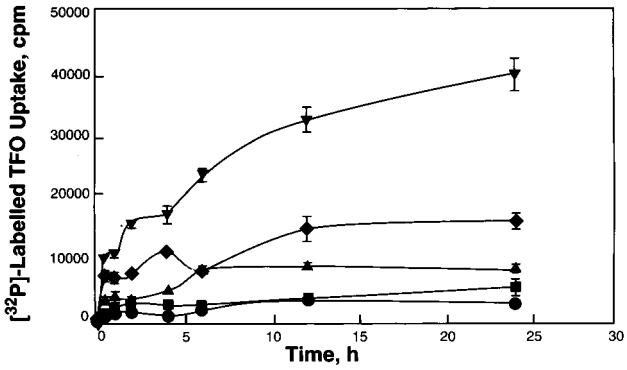

FIGURE 2: Time course of uptake of ^{32}P-labeled TFO by MCF-7 cells. Cells (5×10^5/well) were plated in 6 well plates and allowed to adhere for 24 h. Triplicate wells were treated with different concentrations of ^{32}P-labeled TFO in 0.5 mL of pre-warmed (37 °C) medium. Cells were harvested at the indicated time points. Cell lysate was prepared and radioactivity determined by scintillation counting. The cpm values shown are the mean from total cell lysate from each of the wells. The concentration of TFO added to different groups were: 2.5×10^4 (●); 5×10^4 (■); 1×10^5 (▲); 2.5×10^5 (◆); and 5×10^5 (▼) cpm. Values are mean ±SD from triplicate experiments.

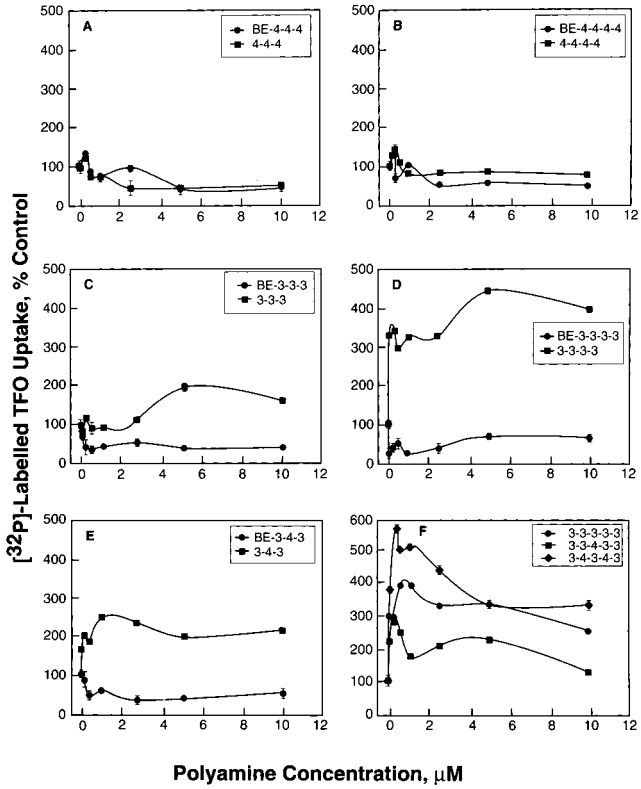

FIGURE 3: Effect of polyamine analogues on the uptake of ^{32}P-labeled TFO by MCF-7 cells. For these experiments, ^{32}P-labeled TFO (2.5×10^5 cpm ∼2.5 nM) was mixed with different concentrations of polyamine analogues, incubated at 22 °C, and used in TFO uptake experiments as described in legends to Figure 2. TFO uptake was quantified at the 4 h time point and expressed as percent control (in the absence of polyamines).

Table 1: EC_{50} Values for Polyamine Analogs[a]

compound[b]	EC_{50} (μM)
3	0.57
4	4.56
3−4	0.10
3−3	1.41
4−4	>10
BE−3−4−3	0.85
BE−3−3−3	3.52
BE−3−7−3	0.95
BE−4−4−4	3.33
BE−3−3−3−3	>10
BE−4−4−4−4	9.6

[a] EC_{50} values were calculated for compounds exerting an inhibitory effect on TFO uptake. [b] Polyamine analogues are designated by the abbreviated names as shown in Figure 1.

Table 2: Concentration of Polyamine Analogs that caused Maximum Uptake of ^{32}P-Labelled TFO by MCF-7 Cells

compound	concentration (μM)	% of TFO uptake[a]
3−4−3	1	245.4 ± 3.60
3−3−3	5	196.3 ± 9.30
4−4−4	0.25	120.3 ± 6.9
3−3−3−3	5	446.7 ± 4.3
4−4−4−4	0.25	137.1 ± 10.5
3−3−3−3−3	0.5	397 ± 5
3−3−4−3−3	0.25	283 ± 3
3−4−3−4−3	0.25	572 ± 8

[a] Data presented as a percentage of control. ± indicates S. D. (n = 3).

to control (100%), the BE-polyamine analogues suppressed TFO uptake. In contrast, the majority of polyamine analogues with free primary amine end groups facilitated TFO uptake. Hexamines were the most efficient analogues to enhance TFO uptake, and the maximal increase was observed at very low concentrations (0.1 to 5 μM). Among all the compounds tested, 3−4−3−4−3 (Figure 3F) produced maximum increase in TFO uptake (6-fold) at 0.25 μM concentration. There was a decrease in TFO uptake at >1 μM concentrations with certain polyamine analogues, indicating that these polyamines might compete for oligonucleotide uptake pathways at higher concentrations. Similar experiments were performed with all polyamine analogues listed in Figure 1 and the ability of each analogue depended on its chemical structure and cationicity.

To understand the structure−activity relationships of polyamines in modulating TFO uptake in MCF-7 cells, we calculated the EC_{50} (concentration of polyamine that inhibited 50% uptake compared to controls) values of those analogues that suppressed TFO uptake. Table 1 presents our results. Putrescine, spermidine, and related small compounds inhibited TFO uptake in the low μM concentrations. The EC_{50} values for bis(ethyl) polyamine analogues were in the range of 0.85 to 10 μM. In general, bis(ethyl) analogues inhibited TFO uptake, whereas unsubstituted polyamines facilitated TFO uptake. In the case of polyamines that facilitated TFO uptake, we determined the optimal concentration and percent uptake (Table 2). The facilitating effect increased with the cationicity of the polyamine analogue and decreased with increased charge separation (3−3 vs 4−4), as shown in two homologous series of the polyamine analogues (Figure 4).

We also examined the cellular uptake of ^{32}P-labeled ODN1 (2.5 nM) in the presence of increasing concentrations of

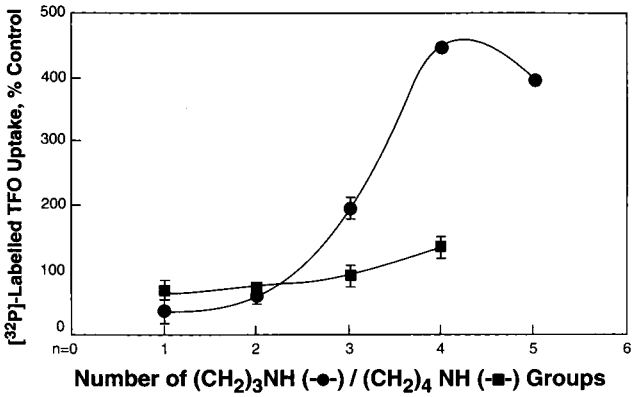

FIGURE 4: Effect of polyamine chain length on TFO uptake in two homologous series. Percent maximal uptake exerted by different polyamines is plotted against the number of trimethylene or tetramethylene bridging groups in the analogues. The concentrations at which the maximal uptake occurred varied with the compound, as presented in Table 2.

unlabeled ODN1 up to 10 μM concentration. There was a concentration-dependent decrease in the uptake of labeled ODN1 by unlabeled ODN1, and the cpm level associated with the cellular extract decreased to 10% of the control in the presence of 10 μM unlabeled ODN1. This result is consistent with reports from other laboratories also (*39*, *40*). This inhibitory effect of the unlabeled TFO was not modified by the presence of polyamines.

To determine the intracellular stability of the TFO in the presence of 3−4−3−4−3, we incubated MCF-7 cells with 1×10^7 cpm level (100 nM) of ^{32}P-labeled ODN1 complexed with 2.5 μM 3−4−3−4−3. ODN1 was isolated from the cellular lysate by phenol extraction and ethanol precipitation and electrophoresed on 12% polyacrylamide/7M urea gel (*21*). There was a significant degree of protection of the ODN1 up to 24 h in cells treated with ODN1 complexed with 3−4−3−4−3, whereas the ODN1 was mostly degraded in the absence of the polyamine analogue. This result is consistent with previous studies showing the persistence of intact ODN in cells in the presence of polyaminolipids (*21*), and the resistance of polyamine-condensed DNA against DNAse I (*31*).

We also studied the time-course of uptake of TFO complexed with the hexamines, 3−4−3−4−3, 3−3−3−3−3, and 3−3−4−3−3 at different concentrations (0.25−2.5 μM). There was a 2- to 5-fold increase in TFO uptake in the polyamine-treated groups compared to the control (Figure 5). The polyamine uptake increased in a time- and polyamine dose-dependent manner, and a leveling off of the internalized TFO concentration was found by 12 h of treatment, except for cells treated with 3−3−4−3−3. This result also shows that the TFO remained stable within the cell up to 24 h. This result is important because enzymatic cleavage of the oligonucleotides is a major concern in antigene and antisense applications of ODNs (*29*). Taken together, these results suggest the exciting possibility of using certain polyamine analogues as oligonucleotide delivery vehicles for gene therapy applications in breast cancer cells.

Effect of Cell Cycle Arrest on TFO Uptake. In the next set of experiments, we examined the effect of cell cycle phase on TFO uptake using synchronized MCF-7 cells. The cell cycle was arrested at the G_1, S, and G_2/M phases using lovastatin, hydroxyurea, and nocodazole, respectively (*33*−

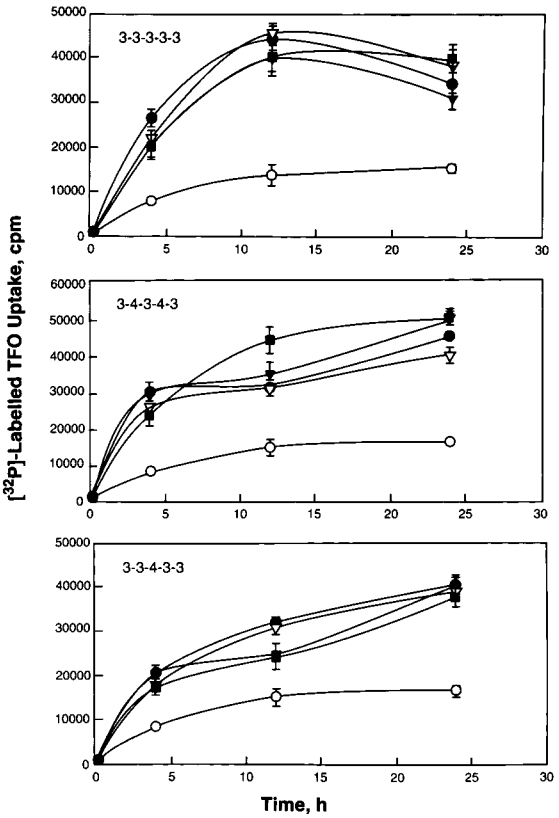

FIGURE 5: Time course of TFO uptake in the presence of 3−3−3−3−3, 3−4−3−4−3, and 3−3−4−3−3. TFO uptake experiment was performed as described in legends to Figures 2 and 4. The concentrations of hexamines used in these experiments were: 0 (○); 0.25 (▼); 0.5 (■); 1 (▽), and 2.5 (●) μM. Data shown are mean ±SD from triplicate experiments.

Table 3: Effect of Cell Cycle Phase on TFO Uptake

treatment	% cells in each phase			% of TFO uptake[a] (%)
	G_0/G_1	S	G_2-M	
none	59 ± 1	28 ± 3	13 ± 0.2	100
lovastatin (20 μM, 36 h)	81 ± 1	14 ± 1	5 ± 1.5	100 ± 10
hydroxyurea (500 μM 24 h)	14 ± 2.5	80 ± 1.5	5.4 ± 0.7	104 ± 1.00
nocodazole (6.6 μM, 24 h)	7.5 ± 0.1	14. ± 0.91	78.5 ± 2.7	101.8 ± 6.5

[a] Data presented as a percent of control. TFO uptake was determined in the presence of 0.25 μM 3−4−3−4−3 at 4 h after addition of TFO. ± indicates S.D. ($n = 3$).

36). The percentage of cells arrested by different treatments and the percentage of TFO uptake, as compared to controls, are presented in Table 3. Our results on cell cycle arrest by various treatments are consistent with that reported in the literature (*33*, *36*). Our results show that cell cycle arrest has no significant effect on TFO uptake by MCF-7 cells.

Estimation of TFO in the Cytoplasmic and Nuclear Fractions. From the previous experiments, we found that the complexing of TFO with 3−4−3−4−3 facilitated the uptake of the TFO by MCF-7 cells. Therefore, we used this system to determine the distribution of labeled TFO in the cytoplasm, nuclear extract, and nuclear pellet of MCF-7 cells. Cells were treated with labeled TFO complexed with different concentrations (0.1−2.5 μM) of 3−4−3−4−3 and harvested after 4 h of incubation. The radioactivity associated with cytoplasmic and nuclear extracts and nuclear pellet was

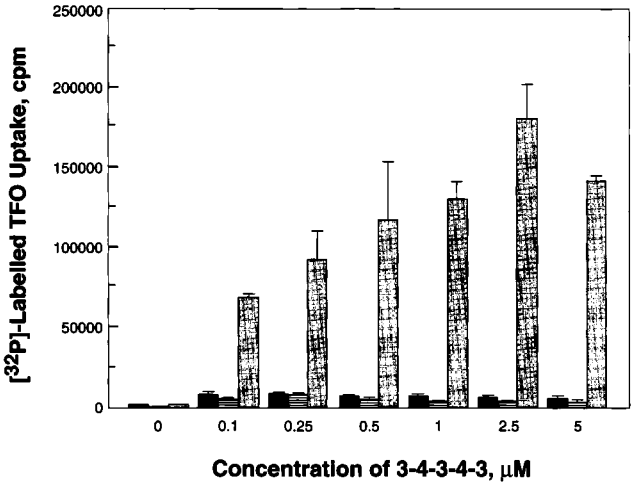

FIGURE 6: Nuclear uptake of ^{32}P-labeled TFO in the presence of 3−4−3−4−3. Cells (2×10^6) were plated in 60 mm dishes and allowed to adhere for 24 h. TFO (2×10^6 cpm) was added along with different concentrations of polyamine analogue 3−4−3−4−3. After the cells were washing three times with PBS, they were harvested with trypsin. The cytoplasmic and nuclear fractions were separated as described in Materials and Methods. Bars represent cytoplasmic fraction (solid), nuclear extract (striped), and nuclear pellet (shaded).

quantified by scintillation counting. The cytoplasmic fraction retained comparatively more radioactivity than the nuclear extract. In the absence of polyamines, the nuclear accumulation of labeled TFO was negligible (350 ± 50 cpm). In the presence of the hexamine, the nuclear pellet, consisting of DNA and tightly bound proteins, retained the majority of the radioactivity. The nuclear pellet showed a dose-dependent increase in the [^{32}P] label associated with it. There was up to 150-fold increase in radioactivity counts in nuclear pellet in the presence of polyamines compared to that of controls (Figure 6). Thus, 3−4−3−4−3 not only increased the uptake of TFO, but also enhanced its affinity for nuclear material, including DNA. Similar results were obtained with 3−3−3−3−3 and 3−3−4−3−3, although the nuclear uptake was only 70% of that observed with 3−4−3−4−3. Thus, the hexamine analogues are capable of delivering the TFO into the nucleus of the cell.

We also examined whether the radioactivity was associated with nuclear proteins or DNA. For this purpose, we carefully isolated genomic DNA from TFO/3−4−3−4−3-treated cells (3×10^7 cells) using protease K digestion, followed by phenol/chloroform extraction, and ethanol precipitation. The precipitate was dissolved in Tris−EDTA buffer and the radioactivity of the DNA was determined by scintillation counting. After this extraction procedure, we found 19 490 ± 2150 cpm of radioactivity associated with DNA isolated from samples treated with a combination of TFO and 0.5 μM 3−4−3−4−3. In contrast, the radioactivity associated with DNA isolated from the control sample was only 2020 ± 250 cpm. These results suggest that the hexamine facilitated high affinity binding of the labeled TFO with nuclear DNA.

Effect of 3−4−3−4−3 on Triplex DNA Stabilization. We next conducted a series of EMSA experiments to determine whether 3−4−3−4−3 could enhance the stability of triplex DNA formed between TFO and its target duplex strands, consisting of ODN3 and ODN4. Figure 7 shows our results

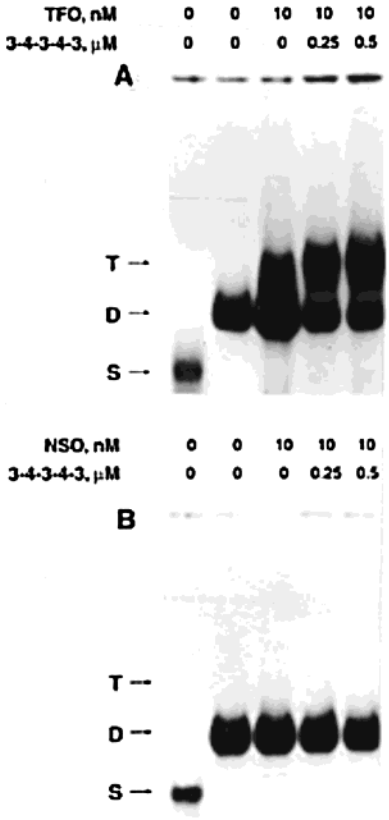

FIGURE 7: Autoradiogram of EMSA showing the effects of 3−4−3−4−3 on stabilizing triplex DNA formed between TFO and its target duplex, consisting of the c-*myc* promoter region sequences, ODN3 and ODN4 (Panel A). Labeled duplex (10 nM) was prepared first and incubated with unlabeled TFO at 37 °C for 1 h in a buffer containing 20 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$ and 10% sucrose. Lanes 1 and 2 were loaded with [^{32}P]-labeled ODN4 and ODN3−ODN4 duplex, respectively. Panel B shows that NSO cannot form triplex DNA in the presence of 3−4−3−4−3. Arrows in the margin indicate triplex (T), duplex (D), and single-stranded (S) DNA bands.

with TFO (A) and NSO (B) in the presence of 0.25 and 0.5 μM concentrations of 3−4−3−4−3. Triplex DNA formation was assessed by the presence of a species having lower mobility than duplex and single stranded DNA (*27*). In the absence of 3−4−3−4−3, there was a low level of triplex DNA formation as found in lane 3 of Panel A; however, triplex DNA formation was enhanced by as little as 0.25 and 0.5 μM concentrations of 3−4−3−4−3. Our control experiments with NSO (Figure 7B) showed that this oligonucleotide is incapable of forming triplex DNA. The addition of 3−4−3−4−3 could not induce triplex DNA formation in this case. Control experiments also showed that the mobility of the duplex band was not affected by complexing it with 3−4−3−4−3 or a combination of 3−4−3−4−3 and NSO (panel B).

Effect of TFO-Complexed with 3−4−3−4−3 on c-myc Oncogene Expression. To determine the biological response of complexing TFO with 3−4−3−4−3, we examined the effect of this combination on c-*myc* expression in MCF-7 cells, using Northern blot analysis. Since c-*myc* mRNA expression is estradiol-sensitive and peaks at 1−2 h time points after initiation of cell cycle from G_1 arrest of MCF-7 cells (*7*), we performed these experiments with G_1-synchronized cells in the presence of 4 nM estradiol. Figure 8A shows the effects of TFO and 3−4−3−4−3 as individual

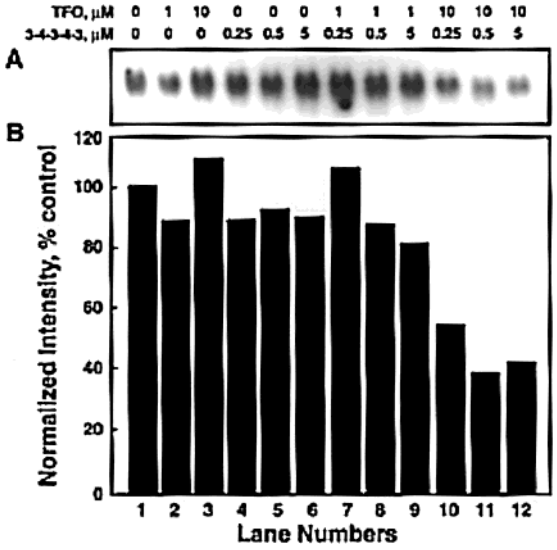

FIGURE 8: Autoradiogram showing the effects of TFO and 3−4−3−4−3 as single agents and in combination on the expression of c-*myc* oncogene at the mRNA level (Panel A). Panel B shows the intensities of different lanes, normalized to the intensities of the G3PDH control gene probed from the same membrane after boiling and removing the c-*myc* cDNA probe. Similar results were obtained in a separate experiment examining c-*myc*.

agents and in combination on c-*myc* mRNA signal at 1 h after initiation of cell cycle. The intensity of mRNA levels in different lanes of the same membrane probed with a control probe, G3PDH was comparable and was used for normalization of the intensity readings (Figure 8B). Treatment of cells with 1 and 10 μM TFO or 0.25, 0.5 and 5 μM 3−4−3−4−3 had no major effect on c-*myc* mRNA levels. A combination of 1 μM TFO with 3−4−3−4−3 produced a 20% decrease in c-*myc* mRNA signal intensity at the highest polyamine concentration only as compared to the control signal intensity (lanes 1). This level of decrease is comparable to that produced by TFO and 3−4−3−4−3 as single agents. In contrast, combinations of 10 μM TFO and 0.25 to 5 μM of 3−4−3−4−3 decreased c-*myc* mRNA level by 50−65% (lanes 10, 11, 12). (Similar results were obtained for the 2 h time period also.). Our control experiments with NSO/3−4−3−4−3 complex showed only a 10−20% decrease in c-*myc* mRNA level (results not shown), suggesting that the TFO/3−4−3−4−3 complex has a specific gene inhibitory effect.

DISCUSSION

The main objective of the present study was to design a new strategy to deliver oligonucleotides to MCF-7 breast cancer cells in order to develop a triplex DNA-mediated antigene strategy for breast cancer. We used a 37-mer single-stranded oligonucleotide, capable of forming a triplex DNA structure at the promoter region of the c-*myc* oncogene for this study. On the basis of our previous results of a structure-dependent stabilizing effect of polyamines on triplex DNA stabilization (*26*−*28*), we examined the use of these polyamine analogues as delivery vehicles for the internalization of the TFO. Our results on the time-course of TFO uptake showed that the TFO was internalized by MCF-7 cells in a concentration- and time-dependent manner (Figure 2). The uptake plateaued after 12 h, a result consistent with the findings of Shoji et al. (*41*). Exocytosis of the transported

oligonucleotides might have played a role in this process. Our results also showed that TFO uptake was not influenced by arresting of cells in any phase of the cell cycle. Estradiol also did not exert any significant effect on TFO uptake.

Our results demonstrate that TFO uptake can be enhanced severalfold by complexing it with appropriate concentrations of certain polyamine analogues. In this instance, cationicity and chemical structural features appear to play a major role. Among the nonbisethylated polyamine analogues, the hexamine 3−4−3−4−3 had the highest efficiency in facilitating TFO uptake. This molecule can be considered as two spermine molecules fused together, and hence, a possible pathway of its action involves the utilization of the polyamine transporter(s) (*42*) for the uptake of polyamine/TFO complex, in addition to the possible utilization of cell surface receptors. Several cell surface proteins have been shown to bind to oligonucleotides with high affinity (*40*, *43*, *44*). For example, Hawley et al. (*45*) recently reported the binding of a c-*myc* antisense oligonucleotide to cell surface proteins of 28−30, 46, 67, and 70−90 kDa, using fibroblast and epithelial cell lines. Polyamine-complexed oligonucleotide might be actively transported across the plasma membrane consisting of polyamine structure-specific interactions with these cell surface receptors. However, Stein et al. (*46*) reported that bulk internalization of phosphodiester oligonucleotide in HL60 cells occurs predominately by pinocytosis.

Another possible mechanism of polyamine action in enhanced oligonucleotide uptake efficiency of the hexamines might be related to their efficacy in condensing DNA. Monomolecular condensation of large DNA molecules and multimolecular condensation of plasmid size or smaller DNA fragments have been observed in dilute solutions using laser light scattering and electron microscopic investigations (*47*−*49*). Liquid crystalline packing of mononucleosomal size DNA has also been reported recently, with an interesting phenomenon associated with the precipitation and resolubilization of DNA in the presence of polyamines (*50*, *51*). We recently found that the 37-mer TFO used in this investigation is capable of undergoing this phenomenon (*52*). Among the various polyamine analogues used in this study, 3−4−3−4−3 was the most efficacious agent in collapsing of the TFO (Saminathan, M., Thomas, T., Shirahata, A., and Thomas, T. J., manuscript in preparation). Therefore, its ability to deliver the TFO to the cell nucleus might be related to this property. Recent studies have shown that the compaction of plasmid DNA into spherical particles, rather than the ionic charge of cDNA complexes, improved nuclear trafficking by mechanisms that may involve permeability through nuclear pores of about 9 nm diameter, or signal mediated transport requiring shuttle molecules (e.g., nuclear localization signal peptide) (*53*, *54*). A recent confocal microscopic investigation tracking the intracellular path of PEI/DNA complexes using fluorescence tagged PEI and fluorescent DNA also provided strong evidence for the nuclear entry of both the delivery vehicle (PEI) and DNA (*55*). A similar situation is conceivable in the case of 3−4−3−4−3/TFO complex because of the strong affinity of the hexamine for DNA.

The differential effects of bisethylated and nonbisethylated polyamine analogues on TFO uptake indicate that the terminal ethyl groups may interfere with cellular oligonucleotide uptake. It has been reported that the bisethylated polyamine analogues were less efficacious in condensing/aggregating the DNA than their nonsubstituted parent compounds (*28*). Thomas et al. (*56*) further found that the relative binding constants of bisethylated polyamines for duplex and triplex DNA were ∼2-fold lower compared to that of nonbisethylated compounds. Basu et al. (*57*) also reported that the association constants of bis(ethyl)polyamines for double helical calf thymus DNA were 10- to 50-fold lower than the corresponding nonbisethylated analogues. A methyl substitution at one of the pendant amino groups of spermidine also caused a lowering of the association constants of polyamines with duplex DNA, when compared to spermidine (*58*). The reason for this lowered affinity of substituted polyamines for different forms of DNA is not clear at present; however, steric effects and hydrophobicity of the alkyl substituents might play an important role. The decreased cellular uptake of TFO/bisethylated polyamine complex could be attributed to the lowered affinity of these compounds to DNA, resulting in their inability to condense DNA in a form suitable for membrane uptake. The same explanation may hold for the inhibitory effects of some of the non-BE substituted di- and trivalent polyamines (*3*, *4*, *3*−*4*, *3*−*3*, *4*−*4*). The divalent polyamines could not provoke the condensation of even high molecular weight bacteriophage DNA, and this inability was explained in terms of a less than critical level (∼89%) of DNA phosphate charge neutralization by these ions in aqueous solution (*59*). Our ongoing investigations show that even trivalent polyamine analogues, such as 3−4, 3−3, and 4−4 are unable to cause the collapse of the 37-mer TFO (Saminathan, M., Thomas, T., Shirahata, A., and Thomas, T. J., manuscript in preparation). However, the reason for the different inhibitory efficacy among these polyamines to inhibit TFO uptake to different levels is not clear at present. Our future studies will address the interplay between the transport mechanisms of polyamines and TFO in breast cancer cells in order to understand the mechanism of this differential effect.

In the case of the two homologous series of nonbisethylated polyamines, we examined the chemical structural and cationicity effects of the polyamine analogues on TFO uptake (Figure 4). The ability of the polyamine analogues to facilitate TFO uptake increased with the cationicity only in the case of compounds separated by a trimethylene bridge between the amino and imino groups. With a tetramethylene bridge, the facilitating effect was much less, suggesting that the hydrophobic bridging region might interfere with the TFO uptake mechanism. Alternately, these chemical structural features contributed to charge density differences of the polyamines, with the polyamine having higher charge density acting as efficacious DNA condensing agents.

The mechanism of oligonucleotide internalization is not well-defined at present. It is believed that multiple pathways, such as receptor-mediated endocytosis with the involvement of a specific receptor, absorptivity-mediated endocytosis in which the oligonucleotide binds to the membrane through weak interactions and/or fluid phase endocytosis, in which plasma membrane folds in to bring in substances, are involved in cellular uptake of oligonucleotides (*43*−*46*, *60*, *61*). It has also been reported that the mechanism of ODN uptake is dependent upon the concentration of the ODN (*40*). Below 1 μM, uptake is thought to occur by receptor-mediated endocytosis, but at higher concentrations, fluid phase en-

docytosis may become the predominant mechanism. A disadvantage of many of these pathways is that oligonucleotides might reach endosomes or lysosomes from which they have to be released to cytoplasmic or nuclear site of action. The cationic delivery vehicles, such as lipopolyamines, facilitate increased nuclear localization of the ODN (*21*). In our studies, we found that most of the internalized TFO remained attached to the nuclear fraction, including the nuclear pellet containing genomic DNA, when 3−4−3−4−3 was used as an oligonucleotide delivery vehicle (Figure 7).

Our studies also provide evidence for a triplex DNA stabilizing effect of 3−4−3−4−3 using in vitro experiments with ^{32}P-labeled duplex target sequences and the TFO (Figure 8). This finding is in agreement with our previous studies using both purine- and pyrimidine-motif triplex DNA model systems and tri-, tetra-, and pentavalent polyamines (*26*−*28*, *56*, *62*). A general mechanism of polyamine effect on triplex DNA is to neutralize the high negative charge density imposed by the association of three nucleic acid chains. For example, the negative charge separation in triplex DNA is only 1.1 Å compared to that of 1.7 and 4.3 Å, respectively, in duplex and single stranded DNA (*63*). However, this explanation is inadequate to account for the remarkable structural effects of polyamines observed in our previous studies (*26*, *28*, *56*, *62*). Several X-ray crystallographic studies (*64*−*67*) have provided evidence for site-specific interactions between spermine and DNA, indicating the geometric fit of spermine to interact with the bases and/or phosphate groups of DNA. Molecular modeling (*68*) and solution structural studies (*69*) also support such interactions, especially those involving the binding of spermine in the major or minor groove of DNA. Taken together, the most plausible explanation for the ability of 3−4−3−4−3 to stabilize triplex DNA is the high affinity binding of this molecule with DNA because of its structural similarity with spermine (*3*−*4*−*3*).

The ability of the internalized TFO/3−4−3−4−3 complex to exert a biological response is revealed by the suppression of c-*myc* mRNA signal by 50−65% in cells treated with this combination. As individual agents, TFO and 3−4−3−4−3 had no significant effect on c-*myc* gene expression under the conditions of our experiment; however, certain combinations of these agents repressed gene transcription. The concentration of TFO necessary to exert this gene inhibitory effect is much higher than that used to demonstrate in vitro triplex DNA formation (Figure 8), suggesting that only a fraction of the TFO is actually taken up by the cells even in the presence of 3−4−3−4−3. The inhibitory effects of the TFO/3−4−3−4−3 combination observed here is comparable to the recent findings of Kim et al. (*70*) using the liposome vehicle, DOTAP [1,2-dioleoyloxy-3-(trimethylammonio)-propane]/DOPE (dioleoylphosphatidylethanolamine) for transfection. Inhibition of c-*myc* gene transcription appears to involve triplex DNA formation at the promoter region of this gene.

In summary, our results show that multivalent hexamine analogues of spermine are highly efficient in delivering the TFO to the cell nucleus. For the intracellular delivery of oligonucleotides, various kinds of lipid preparations are widely used (*71*); however, the interference of the lipid carrier on triplex DNA stability is not fully elucidated. According to Bennet et al. (*19*) many available cationic lipid preparations are toxic to cells at concentrations close to their effective doses if exposure time is prolonged. The concentrations of hexamine used for effective transport of oligonucleotides in this study were below the toxic range. For example, the growth inhibitory effects of these hexamines were not manifested even at 10 μM concentrations in MCF-7 cells, as determined by [^{3}H]-thymidine incorporation assays (Antony, T., Thomas, T. J., and Thomas, T., unpublished results.) In contrast, the maximal efficacy of these compounds in facilitating TFO uptake occurred at 0.25 μM concentration.

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