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Growth inhibition and apoptosis induced by daunomycin-conjugated triplex-forming oligonucleotides targeting the *c-myc* gene in prostate cancer cells

Sara Napoli, Umberto Negri[1], Federico Arcamone[1], Massimo L. Capobianco[1], Giuseppina M. Carbone and Carlo V. Catapano*

Laboratory of Experimental Oncology, Oncology Institute of Southern Switzerland, Via Vela 6, Bellinzona, CH-6500 Switzerland and [1]ISOF-CNR, Via Gobetti 101, Bologna, 40129 Italy

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ABSTRACT

Covalent attachment of intercalating agents to triplex-forming oligonucleotides (TFOs) is a promising strategy to enhance triplex stability and biological activity. We have explored the possibility to use the anticancer drug daunomycin as triplex stabilizing agent. Daunomycin-conjugated TFOs (dauno-TFOs) bind with high affinity and maintain the sequence-specificity required for targeting individual genes in the human genome. Here, we examined the effects of two dauno-TFOs targeting the *c-myc* gene on gene expression, cell proliferation and survival. The dauno-TFOs were directed to sequences immediately upstream (dauno-GT11A) and downstream (dauno-GT11B) the major transcriptional start site in the *c-myc* gene. Both dauno-TFOs were able to down-regulate promoter activity and transcription of the endogenous gene. *Myc*-targeted dauno-TFOs inhibited growth and induced apoptosis of prostate cancer cells constitutively expressing the gene. Daunomycin-conjugated control oligonucleotides with similar sequences had only minimal effects, confirming that the activity of dauno-TFOs was sequence-specific and triplex-mediated. To test the selectivity of dauno-TFOs, we examined their effects on growth of normal human fibroblasts, which express low levels of *c-myc*. Despite their ability to inhibit *c-myc* transcription, both dauno-TFOs failed to inhibit growth of normal fibroblasts at concentrations that inhibited growth of prostate cancer cells. In contrast, daunomycin inhibited equally fibroblasts and prostate cancer cells. Thus, daunomycin *per se* did not contribute to the antiproliferative activity of dauno-TFOs, although it greatly enhanced their ability to form stable triplexes at the target sites and down-regulate *c-myc*. Our data indicate that dauno-TFOs are attractive gene-targeting agents for development of new cancer therapeutics.

INTRODUCTION

Purine-rich sequences representing potential target sites for triplex-forming oligonucleotides (TFOs) are scattered throughout the human genome and are particularly over-represented in gene regulatory regions (1). The high density of target sequences in genome coupled to their sequence-specificity makes TFOs attractive molecules to target individual genes and modulate their function (2,3). This approach can provide the means to design gene-targeted molecules that might be used to study a variety of DNA-associated processes and might have therapeutic applications for human diseases (3,4). TFOs acting as transcriptional repressors might down-regulate expression of over-active genes in cancer cells and be a valid alternative to current treatment modalities with the advantage of higher selectivity and lower toxicity. With this intent, we have investigated TFOs as tools to down-regulate expression of genes, like *c-myc*, which are frequently over-expressed in human cancers (5,6). M*yc*-targeted TFOs reduced *c-myc* expression and were able to induce growth arrest and death of cancer cells (5,6).

*To whom correspondence should be addressed at Oncology Institute of Southern Switzerland, 6500 Bellinzona, Switzerland. Tel: +41 91 820 0365; Fax: +41 91 820 0397; Email: carlo.catapano@irb.unisi.ch
Correspondence may also be addressed to Giuseppina M. Carbone. Tel: +41 91 820 0366; Fax: +41 91 820 0397; Email: pina.carbone@irb.unisi.ch

Although TFOs have been successfully used by others and us in cell-free and cellular systems, various factors limit their efficiency as gene-targeting agents and transcriptional repressors in cells (3,4). Efficient cellular and nuclear delivery is a major obstacle to overcome since sufficient amounts of TFOs need to reach the nucleus in order to drive triplex DNA formation. Another critical challenge is to improve the stability of triple helical complexes formed on chromatin-associated targets. Rapid dissociation of the complex would prevent any biological effect of TFOs. An approach to increase triplex stability is to attach DNA intercalating agents, like acridine and psoralen, to TFOs (2,3). TFO-intercalator conjugates have been shown to maintain sequence-specificity and induce triplex-mediated effects in different experimental contexts (2,3).

Our groups have recently explored the possibility to enhance triplex stability and biological efficacy of TFOs by attaching an anthracycline molecule like daunomycin (7–9). Anthracyclines are commonly used and very effective anti-cancer drugs (10). Unlike other DNA intercalators, anthracyclines intercalate into DNA with the anthraquinone moiety nearly perpendicular to the double helix (11). One end of the anthraquinone (ring D) reaches the major groove, while the other end (ring A), to which the aminosugar is attached, reaches the minor groove (11,12). TFOs were linked at their 5′ end to ring D of the anthraquinone, preserving both the orientation of the intercalating moiety and the alignment of the TFO in the major groove of the double helix (7–9). An initial study with a daunomycin-conjugated TFO (dauno-TFO) focused on an 11 bp purine-rich sequence immediately upstream the P2 promoter of the *c-myc* gene (9). The unmodified 11mer TFO formed a very unstable complex. The dauno-TFO, named dauno-GT11A, bound to the target sequence with much greater stability affording binding in near-physiological conditions (i.e. 37°C and neutral pH). The presence of the DNA intercalator did not affect sequence-specificity of dauno-GT11A as shown by electrophoretic mobility shift assay (EMSA) and footprinting experiments (9). Moreover, unlike the non-conjugated TFO, dauno-GT11A was active in cells, blocking promoter reporter activity and transcription of the endogenous gene (9).

In the present study we investigated the potential of dauno-TFOs for biological applications and evaluated the effects of dauno-GT11A and a new *myc*-targeted TFO, dauno-GT11B, on target gene expression, cell proliferation and survival in normal and cancer cells. Both dauno-TFOs were able to inhibit *c-myc* transcription. Furthermore, they inhibited growth and induced apoptosis of prostate cancer cells, while normal cells had minimal effects. The effects of *myc*-targeted dauno-TFOs were sequence- and target-specific, and clearly distinct from the non-specific cytotoxicity of free daunomycin. Our data indicate that attachment of daunomycin increases DNA binding and improves activity of TFOs in cells. Dauno-TFOs could be attractive gene-targeting and cancer therapeutic agents.

MATERIALS AND METHODS

Cell lines and oligonucleotides

Human prostate cancer cells DU145, PC3 and LNCaP cells were maintained in RPMI 1640, 22Rv1in T-Medium and primary cultures of normal human fibroblasts in DMEM, all supplemented with 10% heat-inactivated fetal bovine serum. Phosphodiester oligonucleotides conjugated at the 5′ end to daunomycin and with a propanediol tail at the 3′ end were synthesized and purified as described previously (7,9,13).

EMSA

Binding assays were performed as described (9). The pyrimidine-rich strands of the *c-myc* target sequences were 5′ end labeled with [γ-^{32}P]ATP and annealed to the complementary strand (9). TFOs were incubated with the radiolabeled targets for 18 h at 37°C in 90 mM Tris-borate (pH 8.0) and 10 mM $MgCl_2$ (TBM buffer). Binding was determined by gel electrophoresis under non-denaturing conditions using TBM as running buffer and maintaining the gel temperature at 20°C (9). The upper strand of target A was 5′-TGGCGGGAAAAAGAACGGAGGGAGGGATCGC-3′ and that of target B was 5′-AGAGCTGCGCTGCGGGCG-TCCTGGGAAGGGAGATCCGGAG-3′. (Underlined bases indicate TFO binding sites.)

Luciferase reporter gene constructs

The p262-Myc reporter vector has been described previously (9). Cells (2×10^4/well) were plated in 48-well plates and grown for 24 h prior to transfection with p262-Myc (100 ng), pRL-SV40 (10 ng) and 1 μM of oligonucleotides using DOTAP (Roche Diagnostics GmbH, Mannehim, Germany) as described (9). Cells were harvested 24 h later to measure Firefly and Renilla luciferase activity using Dual-luciferase assay system (Promega Corporation, Madison WI, USA). The pRL-SV40 vector was used to monitor transfection efficiency. Data were expressed as percentage of luciferase activity in TFO-treated cells compared to cells incubated with an equal concentration of control oligonucleotide.

RNA and protein analysis

Cells (1.5×10^5/well) were seeded in 6-well plates and transfected 24 h later with oligonucleotides using DOTAP as described (9). Myc RNA and protein levels were determined using semi-quantitative RT–PCR (Invitrogen, Carlsbad, CA, USA) and immunoblotting as described (9). To look at the effects of dauno-TFOs on exogenous *c-myc* expression, cells were transfected with a *c-myc* expression vector (PMT-2T-Myc) or an empty vector (PMT-2T) along with the oligonucleotides. After 24 h, cells were harvested and cell lysates prepared for immunoblotting. Antibodies against *c-myc* (clone 9E10, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-tubulin (Oncogene Research Products, San Diego, CA, USA) and peroxidase conjugated secondary antibodies (Amersham, Biosciences, Little Chalfont, Buckingamshire, UK) were used for immunoblotting. To examine *c-myc* expression by FACS, cells were washed and incubated in 100 μl of fixation medium (Caltag Laboratories, Burlingame, CA, USA) for 15 min at 37°C followed by 1 min on ice. Cells were recovered by centrifugation at 900 *g* for 5 min and incubated in ice-cold 90% Methanol for 30 min. Cells were then incubated with the anti-c-myc antibody for 45 min at room temperature followed by 30 min incubation with fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody (BD Biosciences, Heidelberg, Germany) and examined

by FACS (FACSCalibur, BD Biosciences, Heidelberg, Germany). The percentage of *c-myc* expressing cells was determined using Cell Quest software (BD Biosciences, Heidelberg, Germany).

Uptake of daunomycin-conjugated TFOs

Cells (1.5×10^5/well) were seeded in 6-well plates and transfected 24 h later with dauno-TFOs using DOTAP (9). After 24 h, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) containing 1% fetal bovine serum and then examined by FACS (9). The percentage of daunomycin positive cells and mean fluorescent intensity were calculated using the Cell Quest software. For fluorescence microscopy, cells were grown on glass coverslips and transfected with 1 μM of dauno-TFO using DOTAP or treated with DOTAP alone. After 24 h, cells were washed twice with PBS, fixed with 4% formaldehyde and counterstained with DAPI. Coverslips were transferred to microscopy slides and cells were examined using a fluorescence microscope equipped with a DAPI and Texas red filter sets.

Cell proliferation

Cells (1×10^3/well) were seeded in 96-well plates and transfected with oligonucleotides using DOTAP (14). Free daunomycin (Sigma Aldrich, Steinheim, Germany) was dissolved in sterile water, diluted and added directly to the culture medium at the desired concentrations. The number of viable cells was measured after 96 h by a colorimetric assay with MTT tetrazolium salt (Sigma Aldrich, Steinheim, Germany) as described previously (14). To assess clonogenic potential, cells transfected with oligonucleotides were plated at a low density (1×10^3/well) in 6-well plates (14). Colonies were stained with crystal violet and counted after 8–10 days. All the experiments were repeated at least three times and Student's *t*-tests were performed to assess statistical significance of the differences among treatment groups.

Apoptosis

To measure apoptotic cell death, cells transfected with oligonucleotides were stained with FITC-Annexin-V (Bender MedSystem GmbH, Vienna, Austria) for 10 min at room temperature and then analyzed by FACS without PI staining. A 488 nm excitation was used with a 515 nm bandpass filter for detection of FITC-Annexin-V and a 610 nm bandpass filter for detection of intracellular dauno-TFO. The percentages of Annexin-V and daunomycin positive cells were determined using Cell Quest software. Cells not stained with Annexin-V or daunomycin were used as negative controls.

RESULTS

Target sites of daunomycin-conjugated TFOs

Figure 1 shows the position of the two TFO target sites relative to the major transcriptional start site (P2 promoter) in the *c-myc* gene (15). Dauno-GT11A is directed to a purine-rich sequence (5′-GGAGGGAGGGA-3′) overlapping binding sites of transcription factors (e.g. Sp1, MAZ, Ets and E2F) known to regulate the activity of the P2 promoter (9). The second TFO, dauno-GT11B, is directed to a purine-rich sequence

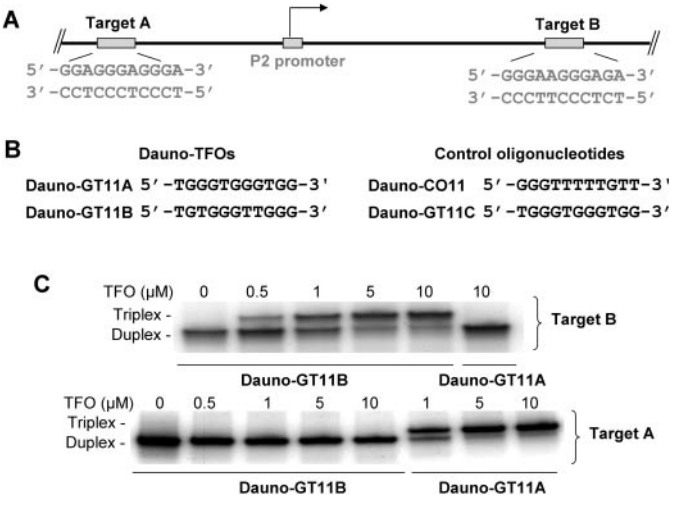

Figure 1. Triplex DNA formation by daunomycin-conjugated TFOs in the c-myc gene promoter. (**A**) Target sites in the *c-myc* promoter. Target A is an 11 bp sequence located 40 bp upstream of the P2 promoter. The 11 bp target B sequence is about 100 bp downstream the P2 promoter. (**B**) Sequence of dauno-TFOs and control oligonucleotides. Both dauno-GT11A and dauno-GT11B were designed to bind in antiparallel orientation to the purine-rich strand of the respective targets. Dauno-CO11 and dauno-GT11C are control oligonucleotides unable to form triplex DNA with sequences in the *c-myc* promoter. (**C**) EMSA. Oligonucleotides corresponding to the pyrimidine-rich strands of target A (lower panel) and target B (upper panel) were 5′ end labeled with [γ-^{32}P]ATP and annealed to the complementary strand. Duplex DNA (1 nM) was incubated for 18 h at 37°C with the indicated concentrations of either dauno-GT11A or dauno-GT11B. Gel electrophoresis was carried out under non-denaturating conditions. Positions of duplex and triplex DNA are indicated.

(5′-GGGAAGGGAGA-3′) downstream of the transcription start site. This sequence does not overlap known transcription factor binding sites. Binding to this site, dauno-GT11B could block transcription elongation or interfere indirectly with the assembly an active transcription complex. Two dauno-oligonucleotides, dauno-CO11 and dauno-GT11C, were used as controls in the study (Figure 1B). Dauno-CO11 matched in parallel orientation a sequence adjacent to the dauno-GT11A binding sequence (9,16). Dauno-GT11C was identical in sequence to dauno-GT11A but it was linked to daunomycin via the amino sugar (9). Both control dauno-oligonucleotides were unable to form triplex DNA within the *c-myc* promoter and transcription of the gene (9).

Binding of daunomycin-conjugated GT11B to the target site

Binding of dauno-GT11A was extensively characterized by EMSA and footprinting in a previous report (9). Dauno-GT11A bound with high affinity and specificity to DNA containing the target sequence, while binding was completely abolished on a mutated target (9). Binding of dauno-GT11B was assessed by EMSA under similar conditions. Incubation of dauno-GT11B with its target (target B) resulted in the formation of triplex DNA (Figure 1C, upper panel). Dauno-GT11B did not bind to the non-complementary target A at concentrations up to 10 μM (Figure 1C, lower panel). Similarly, dauno-GT11A bound to the complementary target A (Figure 1C, lower panel), but did not bind to the non-complementary target B (Figure 1C, upper panel). Affinity of the two dauno-TFOs

for the respective targets was similar, while both TFOs did not bind to the non-complementary targets even though they had very similar sequences. Binding of dauno-TFOs was clearly driven by the oligonucleotide sequence and required perfect matching with the target. Thus, the presence of daunomycin did not affect the ability of dauno-TFOs to discriminate between sites with high sequence similarity.

Inhibition of promoter activity and transcription of the *c-myc* gene by dauno-GT11B

To determine whether the new myc-targeted TFO, dauno-GT11B, was able to affect *c-myc* transcription in cells, we investigated its effects on promoter activity using luciferase reporter assays. The p262-Myc reporter, which contained the target sequences for both dauno-GT11A and dauno-GT11B, was transfected in normal fibroblasts along with oligonucleotides and a control vector. After 24 h, luciferase activity was measured in cell extracts. As shown in Figure 2A, incubation of cells with either dauno-GT11A or dauno-GT11B resulted in reduced reporter activity. Similar results were obtained with dauno-GT11A and dauno-GT11B in prostate cancer cell lines [ref. (9) and data not shown]. Control dauno-oligonucleotides were inactive in these assays [Figure 2A and ref. (9)].

Next, we evaluated the effects of dauno-GT11B on transcription of the endogenous *c-myc* gene. DU145 cells were transfected with oligonucleotides and *c-myc* RNA was measured 24 h later. The level of *c-myc* RNA was reduced in cells treated with dauno-GT11B compared to mock-transfected cells (Figure 2B). A similar level of inhibition was seen with dauno-GT11A, while the control dauno-GT11C had no effect on *c-myc* expression (Figure 2B). Incubation of cells with dauno-GT11B reduced *c-myc* protein level as shown by FACS (Figure 2C) and western blotting (Figure 2D). Mean fluorescence intensity in TFO-treated cells was reduced ~2-fold compared to untreated control cells (Figure 2C). Moreover, the fraction of cells with high *c-myc* level in the untreated cell population was considerably reduced upon incubation with dauno-GT11B. Dauno-CO11 had no effect on *c-myc* expression (Figure 2C). Thus, both dauno-TFOs reduced endogenous *c-myc* RNA and protein levels in

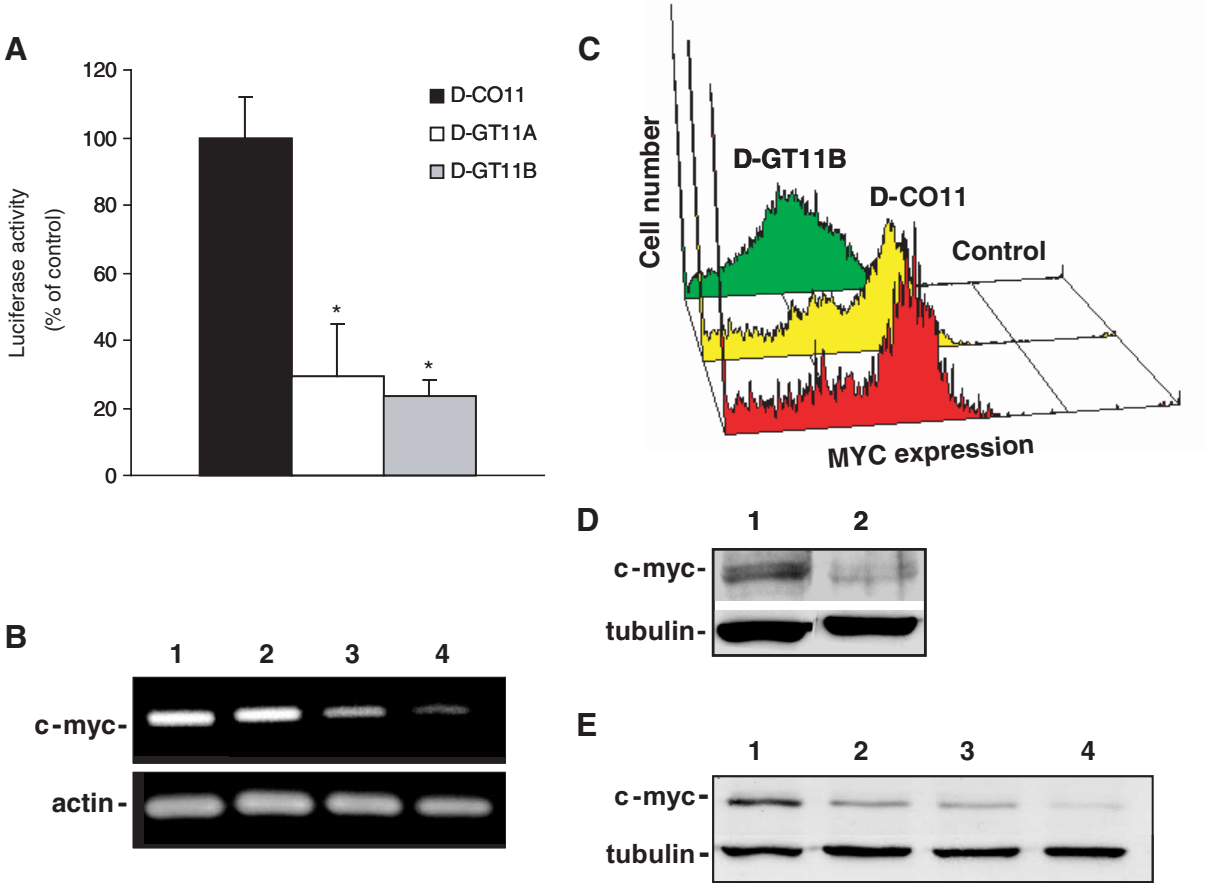

Figure 2. Inhibition of c-myc transcription by daunomycin-conjugated TFOs. (**A**) Luciferase reporter assay. Normal fibroblasts were transfected for 4 h with the p262-Myc reporter, pRL-SV40 and 1 μM of dauno-CO11, dauno-GT11A or dauno-GT11B. Luciferase activity was measured after 24 h. Data are presented as percent of luciferase activity compared to cells transfected with control oligonucleotide. *$P < 0.05$ compared to control trasfected cells. (**B**) RT–PCR. DU145 cells were left untreated (lane 1) or transfected with 1 μM of dauno-GT11C (lane 2), dauno-GT11A (lane 3) and dauno-GT11B (lane 4). Total RNA was extracted after 24 h. *c-myc* and β-actin RNA were determined by RT–PCR. (**C**) DU145 cells were left untreated (Control) or transfected with 1 μM of dauno-CO11 or dauno-GT11B. Cells were harvested after 24 h and *c-myc* protein level was examined by FACS. (**D**) DU145 cells were transfected with 1 μM of dauno-CO11 (lane 1) or dauno-GT11B (lane 2). Cell lysates were prepared after 24 h and *c-myc* protein level examined by western blot. (**E**) DU145 cells were transfected with 1 μM of the oligonucleotides along with either PMT-2T-Myc or PMT-2T. *c-myc* protein level was determined 24 h later by western blot. Lane 1, PMT-2T-Myc and dauno-CO11; lane 2, PMT-2T and dauno-CO11; lane 3, PMT-2T-Myc and dauno-GT11A; lane 4, PMT-2T and dauno-GT11A.

DU145 cells. On the other hand, dauno-TFOs did not affect *c-myc* expression in transiently transfected cells expressing the gene from a heterologous promoter, while still able to reduce expression of the endogenous gene (Figure 2E). The level of non-target proteins, like Ets1, which has similar sequences in its promoter region, was not affected by dauno-TFOs, suggesting a certain degree of target-selectivity [data not shown and ref. (9)].

Antiproliferative effects of daunomycin-conjugated TFOs in prostate cancer cells

The *c-myc* gene is over-expressed in many cancers, including prostate cancer (15,17,18). Studies with transgenic mice indicate that *c-myc* has an important role in development of prostatic intraepithelial neoplasia and invasive adenocarcinomas (19). Elevated expression of *c-myc* contributes also to the androgen-independent phenotype of prostate cancer cells (20). Reducing *c-myc* levels is sufficient to cause growth arrest and death of cancer cells in culture and tumor regression in mice (18,21,22). Thus, dauno-TFOs that reduce *c-myc* expression might be able to inhibit proliferation of prostate cancer cells. To test this hypothesis, we evaluated the effects of the *myc*-targeting dauno-TFOs on growth of prostate cancer cells that express the gene constitutively (20). Cells were transfected with the oligonucleotides for 4 h and then incubated for additional 96 h before measuring viable cell number by a colorimetric assay. As shown in Figure 3, both dauno-TFOs inhibited growth of DU145 and PC3 prostate cancer cells with IC_{50} of ~0.5 μM ($P < 0.01$ at 0.5 and 1 μM compared to both untreated and control treated cells). Similar results were obtained in LNCaP and 22Rv1 prostate cancer cells that were inhibited ~60–70% by 0.5 μM of dauno-TFOs. The control dauno-CO11 and dauno-GT11C had minimal effects on cell growth (≤20% inhibition) without statistically significant differences with respect to untreated control cells (Figure 3). The lack of activity of control oligonucleotides that were conjugated to daunomycin but lacked triplex-forming ability indicated that the effects of dauno-TFOs were not related to toxicity of daunomycin and likely triplex-dependent.

Studies were also done to determine the ability of dauno-TFOs to inhibit colony forming ability of prostate cancer cells. DU145 cells were transfected with oligonucleotides and then plated in tissue culture dishes at a low cell density to assess colony formation in anchorage-dependent conditions (Figure 4A). Cells treated with dauno-TFOs gave rise to a number of colonies significantly lower than cells treated either with DOTAP alone or control oligonucleotide (Dauno-GT11A, 66 ± 2%; Dauno-GT11B, 58 ± 1%; Dauno-CO11, 91 ± 5%; $P < 0.05$). Colonies formed by cells transfected with dauno-GT11A and dauno-GT11B were also considerably smaller than those formed by mock- and control-transfected cells (Figure 4B).

Daunomycin-conjugated TFOs induced apoptosis in prostate cancer cells

To investigate the mechanisms underlying the effects of dauno-TFOs on prostate cancer cells, we examined their

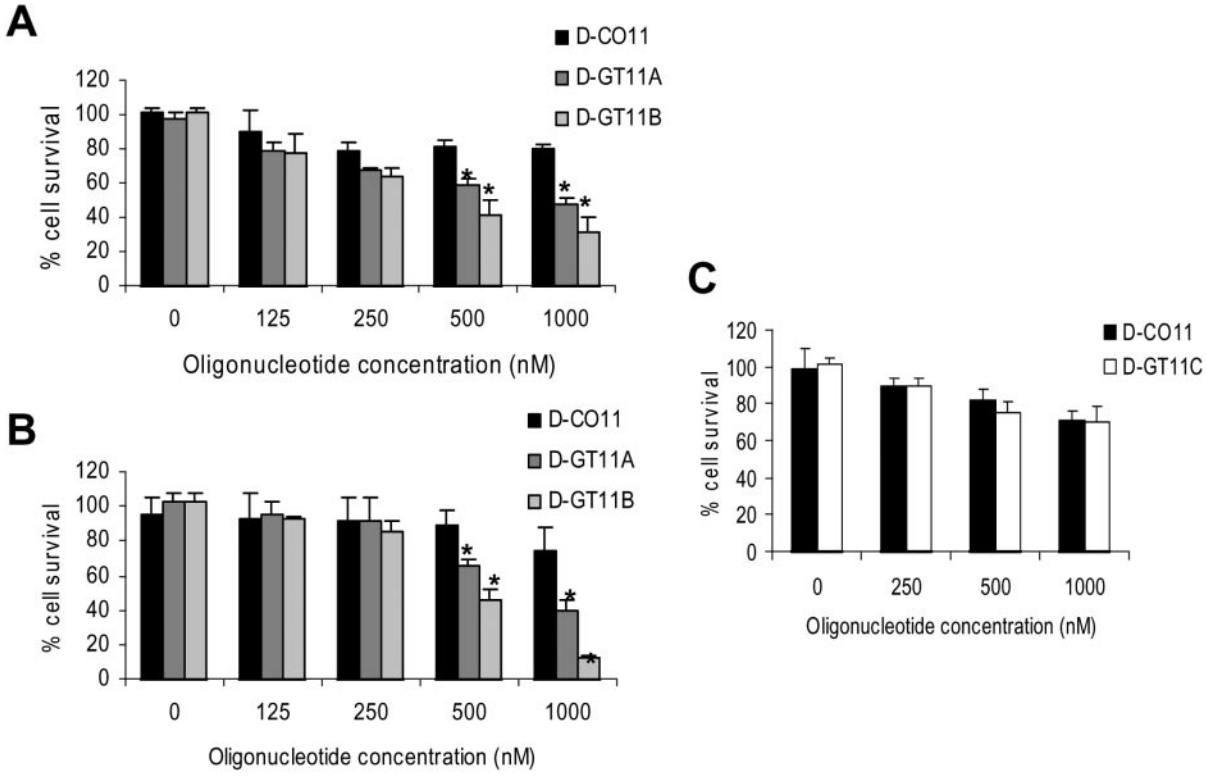

Figure 3. Inhibition of prostate cancer cell growth by daunomycin-conjugated TFOs. Prostate cancer cells DU145 (**A** and **C**) and PC3 (**B**) were transfected for 4 h with the indicated oligonucleotides using DOTAP. Viable cell number was determined after 96 h using MTT assays. Data are presented as percentage of viable cells compared to untreated control cells and are mean ± SD of triplicate samples from representative experiments. *$P < 0.01$ compared with untreated and control-transfected cells.

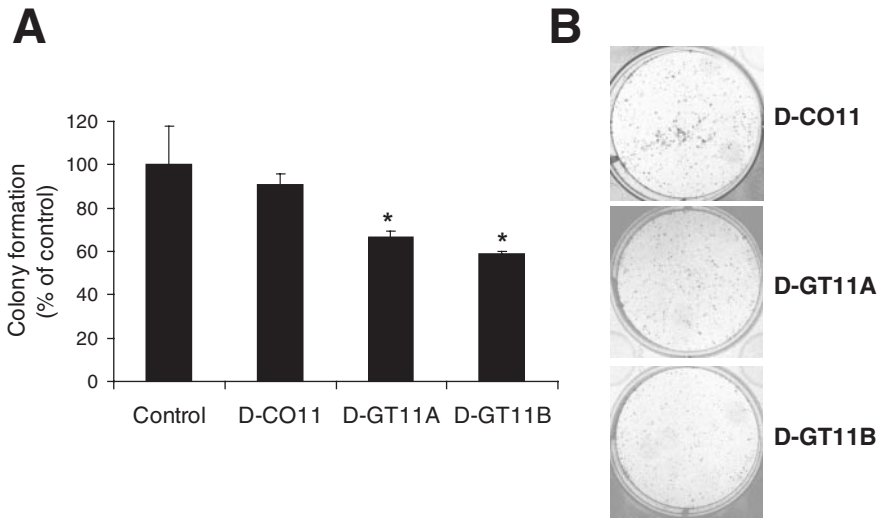

Figure 4. Reduced colony forming ability of prostate cancer cells treated with daunomycin-conjugated TFOs. DU145 cells were transfected with DOTAP alone or 1 μM dauno-CO11, dauno-GT11A or dauno-GT11B. Cells were counted and plated to determine colony forming ability in anchorage-dependent conditions. Colonies were stained with crystal violet after 8–10 days and counted. (**A**) Percentage of colonies relative to mock-transfected cells. Data are mean ± SD of triplicate samples from a representative experiment. $*P < 0.05$ compared to untreated and control-transfected cells. (**B**) Colonies formed by control and dauno-TFO-treated cells from a representative experiment.

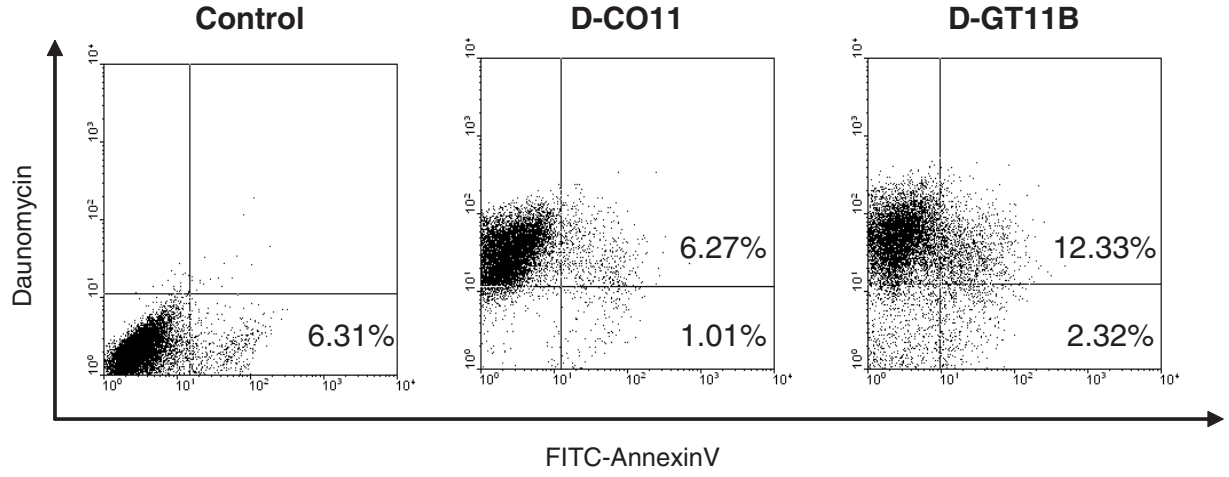

Figure 5. Induction of apoptotic cell death by daunomycin-conjugated TFOs in prostate cancer cells. DU145 cells were left untransfected (control) or transfected with 1 μM of dauno-CO11 and dauno-GT11B. After 24 h cells were harvested, stained with FITC-Annexin-V and analyzed by FACS to detect FITC and daunomycin positive cells.

ability to induce cell death. Because of the presence of the anthraquinone chromophore, we could examine simultaneously the amount of intracellular dauno-TFOs and percentage of apoptotic cells (Annexin-V positive cells) by flow cytometry and correlate cellular uptake with the induction of cell death. Figure 5 shows Annexin-V staining and daunomycin positivity in mock-transfected, dauno-CO11 and dauno-GT11B transfected cells. Only few cells were Annexin-V positive (∼6%) in the population of mock-transfected cells. The fraction of Annexin-V positive cells among cells transfected with dauno-CO11 was similar (∼7%) to that of mock-transfected cells. The percentage of Annexin-V positive cells was more than double (∼15%) in cells transfected with dauno-GT11B (Figure 5). A similar percentage (∼16%) was seen with an identical dose of dauno-GT11A (Figure 6). In all cases, ∼80% of cells had taken up the oligonucleotides. It should be noted that the Annexin-V staining probably underestimates the number of cells undergoing apoptosis because it detects preferentially cells at the early stages of the process, while cells that have progressed to later stages might be missed. In addition, other non-apoptotic mechanisms might contribute to cell growth inhibition and cell death.

The experiment shown in Figure 6 examines further the relationship between TFO uptake and cell death at increasing doses of dauno-TFO. Cellular uptake of dauno-TFO increased as function of the dose of dauno-TFO as indicated by the increased mean cell fluorescence intensity and number of daunomycin positive cells (Figure 6A and B). Moreover, there was a direct relationship between mean cell fluorescence

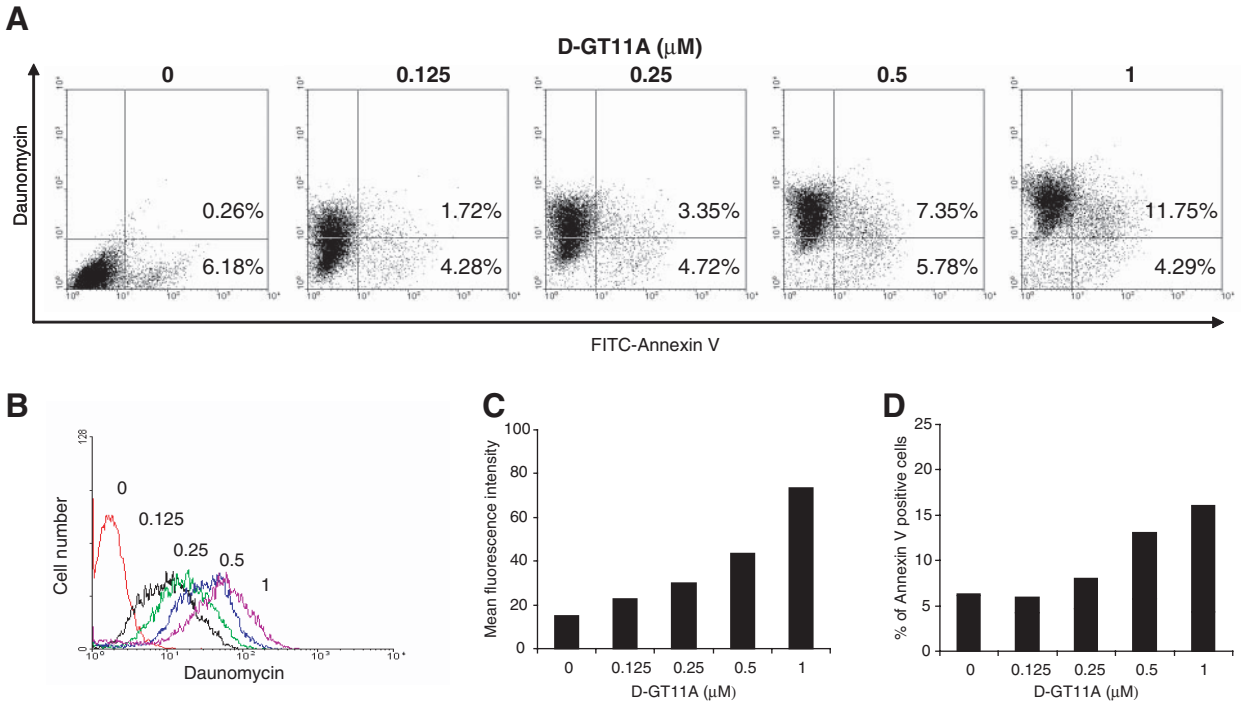

Figure 6. Cellular uptake and apoptosis in daunomycin-conjugated TFO-treated prostate cancer cells. DU145 cells were transfected with increasing concentrations of dauno-GT11A. FITC-Annexin-V staining and daunomycin uptake were measured by FACS. (**A**) Scatter plots of Annexin-V and daunomycin staining distribution. (**B**) Fluorescence intensity distribution in control and dauno-TFO-treated cells. (**C**) Plot of mean cell fluorescent intensity as function of dauno-TFO concentration. (**D**) Percentages of Annexin-V positive cells at increasing doses of dauno-TFO.

intensity (i.e. amounts of intracellular dauno-TFO) and the number of cells undergoing apoptosis (Figure 6C and D). At each dose, a larger fraction of Annexin-V positive cells were also positive for daunomycin, suggesting that cells undergoing apoptosis were predominantly those that had taken up the oligonucleotide.

Daunomycin-conjugated TFOs are selective toward cancer cells

Dauno-TFOs inhibited growth of prostate cancer cells constitutively expressing high levels of *c-myc*. Selective down-regulation of *c-myc* should be minimally toxic to cells that express the gene at low levels. On the contrary, if the antiproliferative effects of dauno-TFOs were due to non-specific effects of either the oligonucleotide or daunomycin, even low expressing cells would likely be affected. To address this point, primary cultures of normal human fibroblasts, which express low levels of *c-myc* (23), were transfected with dauno-TFOs or control oligonucleotides using DOTAP and growth was measured by a colorimetric assay. Under these conditions, normal fibroblasts took up dauno-TFO with an intracellular distribution similar to that seen in prostate cancer cells (Figure 7). In both cell types dauno-TFO accumulated in the cytoplasm as intensely fluorescent perinuclear foci and in nucleus with a more diffuse staining (Figure 7). The total amount of intracellular dauno-TFOs determined by FACS was also similar in normal fibroblasts and prostate cancer cells (Figure 8A). Furthermore, promoter reporter assays showed that dauno-TFOs were able to bind and block the target sequences in the *c-myc* promoter (Figure 2A) and immuno-blot analysis confirmed their ability to reduce endogenous *c-myc* expression in normal fibroblasts (Figure 8B). Despite their ability to inhibit *c-myc* transcription, dauno-TFOs did not have any effect on the growth of normal fibroblasts (Figure 8C). This was in striking contrast with daunomycin that was similarly toxic to prostate cancer cells and normal fibroblasts (Figure 8D). Thus, unlike daunomycin, *myc*-targeted dauno-TFOs were selective toward cancer cells expressing *c-myc* constitutively and less effective against normal cells. These data also indicated that the antiproliferative activity of dauno-TFOs was related to their ability to target the *c-myc* gene and not to non-specific toxicity of the conjugates.

DISCUSSION

Various factors affect the activity of TFOs in biological systems, including limited stability and rapid dissociation of triple helical complexes formed on chromatin-associated targets (2,24,25). Modifications of oligonucleotide chemistry and attachment of intercalating agents can be used to enhance formation and stability of triplex DNA in cells. We have recently explored the possibility of enhancing triplex stability by linking an anthracycline molecule to TFOs (7–9). Anthracyclines, like daunomycin, are potent DNA intercalators (10–12). Attachment of daunomycin to the 5′ end of a TFO led to a considerable increase in triplex stability *in vitro* under near-physiological conditions and activity in cells (9). Unlike the unmodified TFO, the 11mer dauno-TFO directed to a sequence immediately upstream of the *c-myc* P2 promoter bound with high affinity to the target

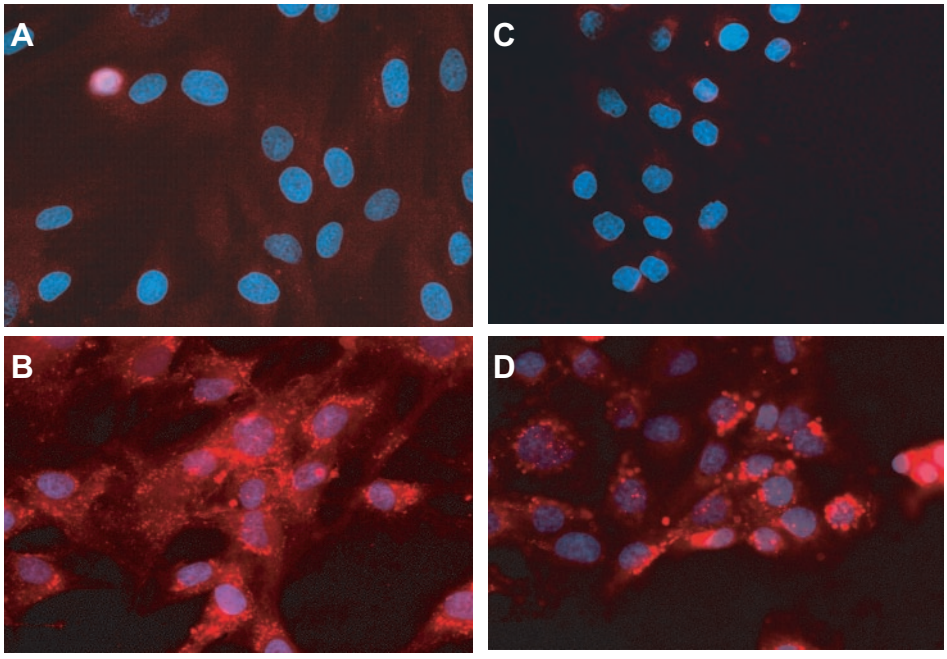

Figure 7. Uptake and intracellular distribution of daunomycin-conjugated TFOs in normal fibroblasts and prostate cancer cells. Fibroblasts (**A** and **B**) and DU145 cells (**C** and **D**) were treated with DOTAP alone (upper panels) or transfected with 1 μM of dauno-TFO using DOTAP (lower panels). After staining with DAPI, cells were examined on fluorescence microscope and images collected using DAPI and Texas red filter sets. Merged images of control and TFO-treated cells are shown.

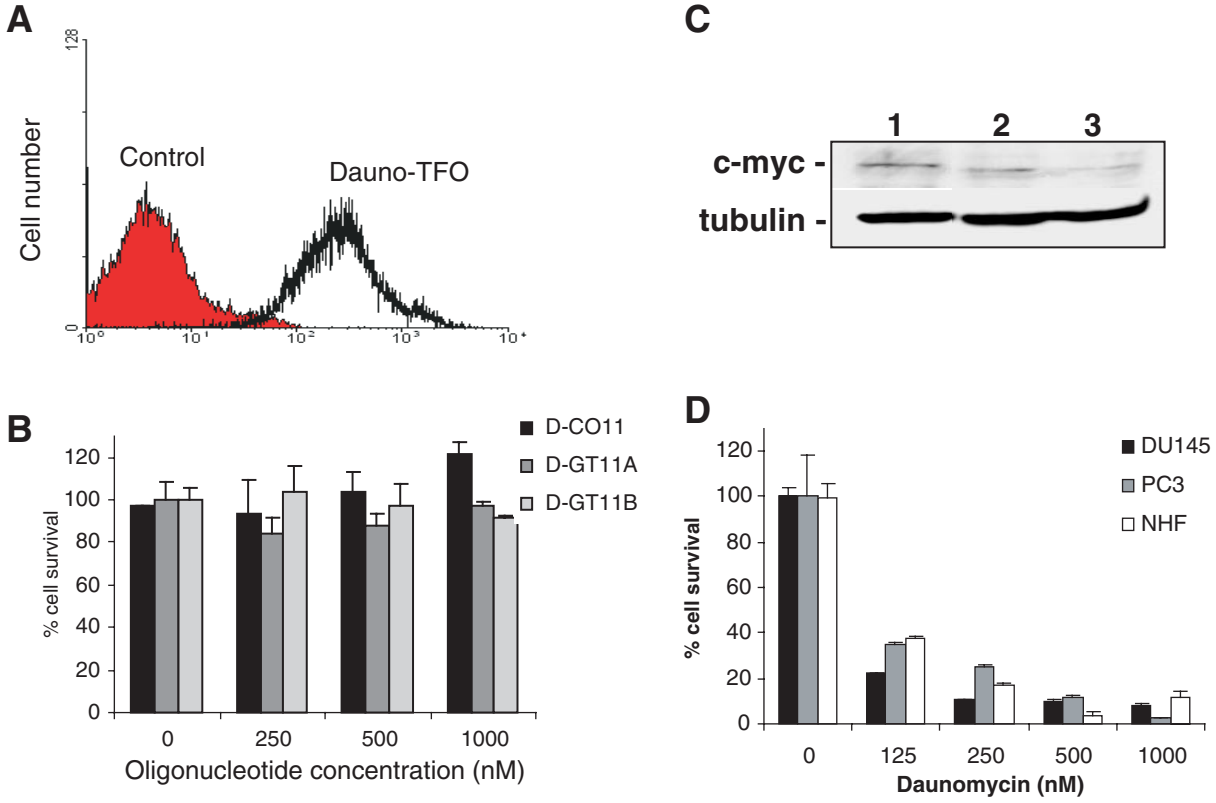

Figure 8. Activity of daunomycin-conjugated TFOs in normal human fibroblasts. (**A**) Fibroblasts were mock-transfected (Control) or transfected with 1 μM of dauno-TFO (Dauno-TFO) using DOTAP and cellular uptake determined by FACS. (**B**) Cells were transfected with dauno-GT11A, dauno-GT11B or dauno-CO11 and viable cell numbers determined by MTT assays after 96 h. (**C**) Fibroblasts were left untreated (lane 1) or transfected with 1 μM of dauno-GT11A (lane 2) and dauno-GT11B (lane 3). After 24 h, *c-myc* protein level was determined by immunoblotting. (**D**) Fibroblasts, DU145 and PC3 prostate cancer cells were incubated with daunomycin. Viable cell numbers were determined after 96 h by MTT assays.

DNA and inhibited transcription of the gene (9). A similar effect of daunomycin on triplex stability was seen in cell-free systems with TFOs designed both in antiparallel and parallel orientation (7,8,13). These data, along with recent improvements in synthesis of daunomycin-conjugated oligonucleotides (8,13), indicate that attachment of an antracycline moiety is a feasible and effective approach to enhance binding and efficacy of TFOs. In the present study, we evaluated the biological activity of two *myc*-targeted dauno-TFOs and their potential for *in vivo* applications as gene-targeting and anticancer agents. Our study shows that *myc*-targeted dauno-TFOs were effective transcriptional repressors and exhibited strikingly selective antiproliferative and pro-apoptotic activity toward cancer cells. Both dauno-GT11A and dauno-GT11B caused growth inhibition, reduced clonogenic potential and apoptotic cell death in prostate cancer cells with minimal effects on normal human fibroblasts. Dauno-TFOs directed to genes over-expressed in cancer cells, therefore, might be developed as gene-targeted cancer therapeutics.

The dauno-TFOs tested in this study were directed to distinct sites in the *c-myc* gene. Dauno-GT11A could interfere with transcription factors binding to sequences overlapping or adjacent to the TFO binding site (16), while dauno-GT11B, which is directed to a site downstream of the transcription start site, could interfere with the assembly of the initiation complex or block the elongation of *c-myc* transcripts. Both dauno-TFOs formed very stable triplexes *in vitro* and inhibited promoter activity and expression of *c-myc* to similar extents. Although it is difficult to rule out completely alternative mechanisms of action, like an aptameric effect, multiple lines of evidence support the conclusion that the activity of dauno-TFOs was sequence-specific and consistent with a triplex-mediated mechanism. The interaction of dauno-TFOs with the respective targets *in vitro* was strictly sequence-dependent as shown by the inability to bind to non-complementary targets despite close sequence homology [Figure 2A and ref. (9)]. Control oligonucleotides, which were unable to form triplex DNA with sequences in the *c-myc* promoter, did not inhibit *c-myc* transcription and did not have any effect on cell growth and survival. Their inability to induce any relevant biological effect excluded non-sequence-specific activity of dauno-TFOs. Oligonucleotides can also induce non-target specific effects via sequence-dependent mechanisms, e.g. acting as aptamers (26–28). The control dauno-GT11C had a sequence identical to dauno-GT11A and very similar to dauno-GT11B. However, it was unable to bind to DNA because of the different mode of attachment to the daunomycin (9). Consistent with its inability to form triplex DNA, dauno-GT11C did not have any activity on promoter reporter, transcription, cell growth and viability. Thus, these results ruled out both sequence and non-sequence dependent mechanisms that might lead to non-specific activity of dauno-TFOs. In conclusion, the activity of dauno-TFOs appeared to be consistent with a triplex-mediated mechanism and dependent upon down-regulation of the target gene. We cannot rule out that other mechanisms—e.g. topoisomerase II-mediated DNA cleavage (29) or secondary DNA damage induced by the DNA intercalator (30)—might contribute to the activity of dauno-TFOs in addition to transcription inhibition. However, based on the present evidence, any additional effect would still be dependent on the primary mechanism, i.e. triplex-directed binding at the target site in the *c-myc* promoter.

Since daunomycin is a potent cytotoxic drug, one must be particularly careful while considering the basis of the antiproliferative activity of dauno-TFOs. However, our data argue against the possibility that the antiproliferative and pro-apoptotic effects of dauno-TFOs were due to toxicity of the daunomycin moiety. When daunomycin was conjugated to oligonucleotides unable to form triplex DNA either because of the nucleotide sequence (dauno-CO11) or mode of attachment (dauno-GT11C), we did not observe cytotoxicity. This was consistent with the fact that the conjugated oligonucleotide modified the biophysical and biochemical properties of daunomycin affecting, e.g. cellular uptake and intracellular trafficking of daunomycin (9). Here, we show that the cellular activities of dauno-TFOs were also different from that of free daunomycin and strictly dependent on the oligonucleotide component and not the anthracycline moiety. This might be a direct consequence of the different DNA binding properties of daunomycin and dauno-TFOs. The presence of the oligonucleotide is likely to prevent random intercalation of daunomycin into DNA as it has been shown with other intercalating agents (31,32). Unlike free daunomycin, dauno-TFOs bound *in vitro* only at sites where the oligonucleotide found a perfect match with the target duplex [Figure 2 and ref. (9)]. Repulsion between the oligonucleotide and the duplex apparently prevents binding and intercalation of daunomycin at sites with non-matching sequences.

Another point of concern is that short oligonucleotides, like those used in this study, might not bind to unique sites in the genome. The shorter the oligonucleotide sequence, the higher is the probability to find similar targets at other sites in the genome (1). Furthermore, the presence of daunomycin with its triplex stabilizing effect could reduce the ability of dauno-TFOs to discriminate between matching and non-matching sequences, increasing the probability of binding to multiple sites in the genome. The latter possibility seems less likely since *in vitro* binding experiments showed that dauno-TFOs maintained a high discriminating power, similar to that of unmodified TFOs, and argue against a reduced sequence-selectivity of the intercalator-TFO conjugates. However, sequences identical to the 11mer *c-myc* targets are present in regulatory regions of a number of other genes and may represent additional sites of triplex formation by dauno-TFOs according to searches of public databases (e.g. DBTSS; http://dbtss.hgc.jp/). Looking for sequences in positions similar to the *c-myc* targets (i.e. −100 to + 200 bp from the transcriptional start site) 276 and 58 hits were found for target A and target B, respectively. Considering a wider region likely to contain additional regulatory elements (i.e. −1000 to + 200 bp from the transcriptional start site) there were 815 hits for target A and 243 for target B. Binding to multiple sites throughout the genome could induce non-target specific effects, partially or completely independent of *c-myc* down-regulation, which might contribute significantly to the activity of dauno-TFOs. To address these concerns in a cellular context, we examined the effects of dauno-TFOs in cells, like normal human fibroblasts, expressing low levels of *c-myc*. One would expect that, if dauno-TFOs bind to multiple sites scattered throughout the genome, they would probably down-regulate many genes or damage DNA at

multiple sites, reducing target-selectivity and resulting in cytotoxicity irrespective of target gene expression. We did not observe toxicity in normal fibroblasts at concentrations of dauno-TFOs that were effective in prostate cancer cells. In contrast, daunomycin, which is a non-sequence selective DNA damaging agent, was equally toxic to normal fibroblasts and prostate cancer cells. Thus, although the possibility of multiple gene-targeting exists, dauno-TFOs seemed rather selective toward cells expressing the intended target. Indeed, additional TFO binding sites might be located in regions not directly relevant for transcription or the putative target genes might not be transcribed or might not be critical for cell growth and survival, thus favoring the apparent target-selectivity of dauno-TFOs. The inability of dauno-TFOs to affect growth of normal cells is also relevant for potential therapeutic applications of this approach. Dauno-TFOs might have limited non-target specific toxicity and cells in which the target gene is not expressed or expressed only at low levels might not be affected significantly.

Collectively, our study provides evidence of the activity of dauno-TFOs as transcriptional repressors in cells and may open new avenues for design of gene-targeted therapeutics. The activity of dauno-TFOs was consistent with a triplex-mediated mechanism and was clearly different from the non-selective cytotoxic activity of daunomycin, supporting the idea that daunomycin and dauno-TFOs have distinct modes of action. In fact, both DNA binding and biological activity of dauno-TFOs were dictated exclusively by the oligonucletide sequence. Our study also indicates that triplex-mediated targeting of relatively short homopurine sequences in genomic DNA is possible with the addition of a strong intercalating and triplex stabilizing agent, like daunomycin. The number of potential TFO target sites as well as the range of applications of the triplex-mediated gene-targeting strategy could be considerably increased by this approach.

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