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Targeting the Human Androgen Receptor Gene with Platinated Triplex-Forming Oligonucleotides

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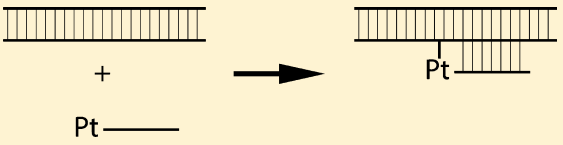
ABSTRACT: Platinum-derivatized homopyrimidine triplex-forming oligonucleotides (Pt-TFOs) consisting of 2′-*O*-methyl-5-methyluridine, 2′-*O*-methyl-5-methylcytidine, and a single 3′-N7-*trans*-chlorodiammine platinum(II)-2′-deoxyguanosine were designed to cross-link to the transcribed strand at four different sequences in the human androgen receptor (AR) gene. Fluorescence microscopy showed that a fluorescein-tagged Pt-TFO localizes in both the cytoplasm and nucleus when it is transfected into LAPC-4 cells, a human prostate cancer cell line, using Lipofectamine 2000. A capture assay employing streptavidin-coated magnetic beads followed by polymerase chain reaction (PCR) amplification was used to demonstrate that 5′-biotin-conjugated Pt-TFOs cross-link *in vitro* to their four designated AR gene targets in genomic DNA extracted from LAPC-4 cells. Similarly, the capture assay was used to examine cross-linking between the 5′-biotin-conjugated Pt-TFOs and the AR gene in LAPC-4 cells in culture. Three of the four Pt-TFOs cross-linked to their designated target, suggesting that different regions of the AR gene are not uniformly accessible to Pt-TFO cross-linking. LAPC-4 cells were transfected with fluorescein-tagged Pt-TFO or a control oligonucleotide that does not bind or cross-link to AR DNA. The levels of AR mRNA in highly fluorescent cells isolated by fluorescence-activated cell sorting were determined by RT-qPCR, and the levels of AR protein were monitored by immunofluorescence microscopy. Decreases in mRNA and protein levels of 40 and 30%, respectively, were observed for fluorescein-tagged Pt-TFO versus control treated cells. Although the levels of knockdown of AR mRNA and protein were modest, the results suggest that Pt-TFOs hold potential as agents for controlling gene expression by cross-linking to DNA and disrupting transcription.



Polypurine tracts in double-stranded DNA are potential binding sites for homopyrimidine or homopurine oligonucleotides, resulting in the formation of triple-stranded structures.[1,2] Homopyrimidine triplex-forming oligonucleotides (TFOs) bind in the major groove of DNA with a backbone orientation parallel to that of the purine tract through the formation of Hoogsteen hydrogen-bonded T-AT and C^+-GC triads. Homopurine TFOs, on the other hand, use reverse Hoogsteen hydrogen bonds to form A-AT and G-GC triads with the target purine tract and their sugar−phosphate backbones oriented antiparallel to this tract. There has been considerable interest in exploiting the ability of TFOs to bind to DNA as a means of controlling gene expression both in cell culture and in whole organisms.[3−9] In contrast to genome editing approaches such as the CRISPR/Cas9 system that knock out DNA sequences, TFOs modulate gene expression by inhibiting transcription. Derivatization of TFOs with psoralen, a photoreactive functional group, allows the TFO to cross-link to its DNA target.[10] Psoralen-derivatized TFOs have been shown to cross-link to their intended DNA targets in living cells despite potential interference from histones and other DNA binding proteins.[11−16] In addition, psoralen-derivatized TFOs have been shown to inhibit transcription[17] and to create mutations in specific genes[13,18−22] in TFO-treated mammalian cells in culture.

Derivatization of homopyrimidine TFOs with N7-*trans*-chlorodiammine platinum(II)-2′-deoxyguanosine (Pt-TFOs) provides an alternative approach to cross-link TFOs to their DNA targets. Unlike psoralen-derivatized TFOs, which require long wavelength UV light to activate cross-linking, the platinum group of the Pt-TFO can react directly with N7 of a suitably positioned guanine in the target DNA.[23,24] Cross-linking can occur when the platinated deoxyguanosine is located on either the 5′- or 3′-end of the TFO.[24] The target guanine can be located on the strand that contains the TFO binding site or on the complementary strand of the DNA duplex. We have shown that transcription of plasmid DNA that has been cross-linked to a Pt-TFO is inhibited significantly upon transfection of the cross-linked plasmid into Chinese hamster ovary cells.[25]

In this report, we demonstrate that Pt-TFOs targeted to purine tracts in the human androgen receptor (AR) gene cross-link with their intended genomic targets upon being transfected into human prostate cancer (LAPC-4) cells in culture. We also show that transfection of LAPC-4 cells with one of these Pt-TFOs results in reduced levels of AR mRNA and protein.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis. Triplex-forming oligonucleotides TFO X, TFO A, TFO B, TFO C, and NBC (Table 1) were synthesized on a 1 μmol scale on 500 Å controlled pore glass supports using an Applied Biosystems 3400 DNA

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Table 1. Sequences of TFOs and MALDI-TOF Mass Spectrometry Results

oligo	sequence (5′−3′)[a]	calculated mass	observed mass
TFO X	TTTCTTCTTTTCTCTCTTdG	6278.42	6277.34
Pt-TFO X	TTTCTTCTTTTCTCTCTTdG^{Pt}	6543.02	6544.40
NBC	TTTTTTTCCCCCTTTTTTdG	6278.42	6277.82
Pt-NBC	TTTTTTTCCCCCTTTTTTdG^{Pt}	6543.02	6544.60
fluoro-TFO X	fluorescein-TTTCTTCTTTTCTCTCTTdG	6815.42	6816.09
fluoro-Pt-TFO X	fluorescein-TTTCTTCTTTTCTCTCTTdG^{Pt} + Na^+	7103.01	7102.46
fluoro-NBC	fluorescein-TTTTTTTCCCCCTTTTTTdG	6815.42	6816.19
fluoro-Pt-NBC	fluorescein-TTTTTTTCCCCCTTTTTTdG^{Pt} + Na^+	7103.01	7105.22
biotin-TFO X	biotin-TTTCTTCTTTTCTCTCTTdG	6848.02	6850.63
biotin-TFO A	biotin-TTTTCTCTCTCTCTCTCTdG	6846.06	6845.75
biotin-TFO B	biotin-CTTTCCTTTCTCTCTCTCTdG	7179.30	7180.13
biotin-TFO C	biotin-CTTTTTCTTTCCTCTCTTTCTdG	7848.74	7848.74

[a]Oligonucleotides are composed of 2′-*O*-methyl-5-methyluridine, 2′-*O*-methyl-5-methylcytidine, and deoxyguanosine. Oligonucleotides were additionally modified with a 5′-fluorescein (Fluoro) or with a 5′-biotin. dG^{Pt} is N7-*trans*-chlorodiammineplatinum(II)-2′-deoxyguanosine.

Table 2. Sequences, Melting Temperatures (T_m), and Cross-Linking Levels of TFOs and Their Target Duplexes

oligo	sequence (5′−3′)[a]	T_m (°C) at pH 7.4	% cross-linking
AR duplex sense	GATTCTTCTCTCTTTTCTTCTTTAGTAG	64	N/A
AR duplex antisense	CTACTAAAGAAGAAAAGAGAGAAGAATC		
TFO X	2′-mr-TTTCTTCTTTTCTCTCTTdG	74	–
Pt-TFO X	2′-mr-TTTCTTCTTTTCTCTCTTdG^{Pt}	–	75
NBC duplex sense	GATTCTTTTTTCCCCCTTTTTTTAGTAG	66	N/A
NBC duplex antisense	CTACTAAAAAAAGGGGGAAAAAAGAATC		
NBC	2′-mr-TTTTTTTCCCCCTTTTTTdG	22	–
		39[b]	–
		54[c]	–
Pt-NBC	2′-mr-TTTTTTTCCCCCTTTTTTdG^{Pt}	–	0
		–	83[b]
		–	77[c]

[a]All oligonucleotides are composed of deoxynucleotides with the exception of TFO X and NBC, which are composed of 2′-*O*-methyl-5-methyluridine, 2′-*O*-methyl-5-methylcytidine, and deoxyguanosine. dG^{Pt} is N7-*trans*-chlorodiammineplatinum(II)-2′-deoxyguanosine. [b]At pH 6.5. [c]At pH 6.0.

synthesizer and commercially available 2′-*O*-methyl-5-methyluridine and 2′-*O*-methyl-5-methylcytidine phosphoramidites purchased from ChemGenes, Inc. The oligonucleotides were deprotected by treating the support overnight at 55 °C with a solution containing 100 μL of 95% ethanol and 300 μL of concentrated ammonium hydroxide. The oligonucleotides were purified on a DNAPac PA100 high-resolution anion exchange HPLC column using a linear 0 to 0.5 M sodium chloride gradient in a buffer containing 10% methanol and 100 mM Tris-HCl (pH 7.6). TFOs modified with a 5′-biotin-TEG or 5′-fluorescein group were prepared in a similar manner using commercially available biotin-TEG or 5′-fluorescein phosphoramidites purchased from Glen Research, Inc. The oligodeoxyribonucleotides (see Tables 2−4) used as polymerase chain reaction (PCR) primers and to form DNA duplexes were purchased from Sigma-Aldrich, Inc.

The compositions of the oligonucleotides were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (see Table 1). Oligonucleotides (0.1 A_{260} unit; ∼0.5 nmol) were concentrated and purified using ZipTip Pipette Tips (Millipore). Oligonucleotides were resuspended in 5 μL of water. Samples were spotted on a plate containing 0.5 μL of oligonucleotide combined with 4 μL of 3-hydroxypicolinic acid (50 g/L in 50% aqueous acetonitrile) and 1 μL of 40 mM ammonium citrate.

Thermal Denaturation Experiments. Solutions that consisted of 0.5 μM DNA target duplex and TFO in triplex buffer containing 50 mM 4-morpholinopropanesulfonic acid (MOPS), 100 mM sodium chloride, and 5 mM magnesium chloride were prepared. The solutions were heated at 90 °C for 10 min and slowly cooled to room temperature. The solutions were degassed under vacuum in a Speed Vac for 1 min prior to recording the thermal denaturation curves. These curves were obtained by monitoring the absorbance at 260 nm versus temperature using a Carey 3E UV/vis spectrophotometer fitted with a thermostated cell compartment. The solutions were heated at a rate of 0.45 °C/min over a temperature range of 5−80 °C.

Synthesis of Platinated TFOs. TFOs X, A, B, and C and NBC (4 A_{260} units; ∼ 20 nmol) were each incubated in 500 μL of an aqueous solution that contained 1 mM *trans*-diamminedichloroplatinum(II) for 30−90 min at 37 °C. The Pt-TFOs were purified by strong anion exchange HPLC using a linear gradient of 0 to 0.5 M sodium chloride in a buffer containing 10% methanol and 100 mM Tris-HCl (pH 7.6). The Pt-TFOs were desalted on Sep Pak C18 reversed phase cartridges (Waters Inc.) and stored for up to 2 weeks in 50% aqueous acetonitrile at 4 °C.

Electrophoretic Mobility Shift Assays under Denaturing Conditions To Measure Cross-Linking. The 5′-^{32}P-labeled DNA target duplexes (1 μM) were incubated with a Pt-

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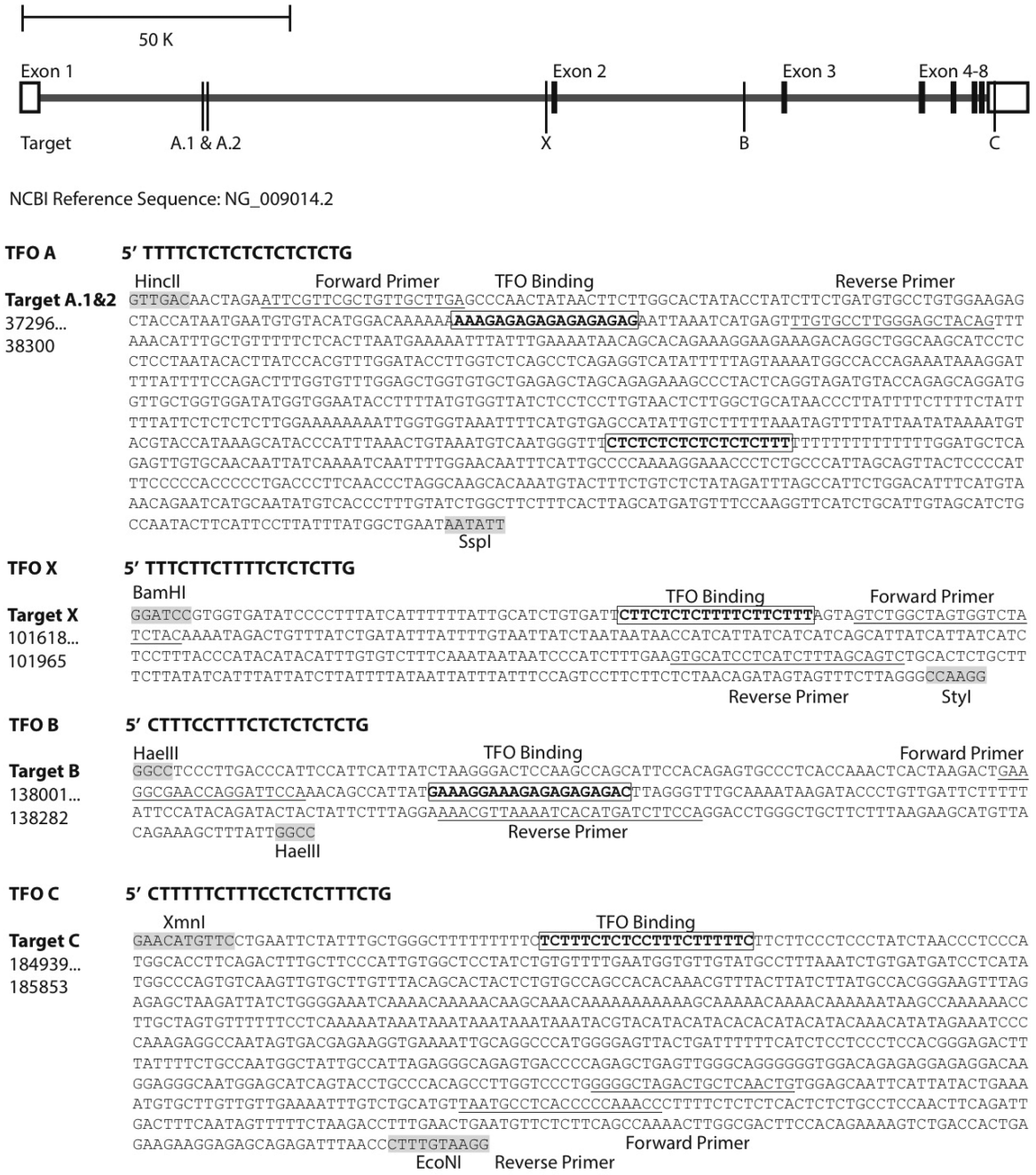

Figure 1. Sequences of Pt-TFO targets in the human AR gene. The locations of the target sequences are indicated on the leftmost side of the figure below the target name. Boxed sequences indicate TFO binding and cross-linking sites. Sequences highlighted in light gray indicate restriction enzyme recognition sites. Underlined sequences indicate forward and reverse PCR primer binding sites.

TFO (10 μM) in 50 μL of triplex buffer containing 50 mM MOPS, 100 mM sodium chloride, and 5 mM magnesium chloride, or triplex buffer supplemented with 0−1 M glutathione, overnight at 37 °C. The reactions were stopped by addition of an equal volume of 2× formamide loading buffer. Aliquots (15 μL) were electrophoresed at 600 V on 20% denaturing polyacrylamide gels. Radioactivity in the gels was detected by phosphorimage plates, which were scanned on a Fuji FLA-7000 phosphorimager, and the percent cross-linking quantified using ImageGauge.

Interaction of Pt-TFOs with LAPC-4 DNA *in Vitro*. Genomic DNA was isolated from LAPC-4 cells using a PerfectPure DNA Cultured Cell Kit (5 Prime, Inc.). The DNA was digested with restriction enzymes specific for the target of interest (see Figure 1). A solution containing 0 or 10 μM 5′-biotinylated Pt-TFO and 5 μg of digested LAPC-4 DNA in 50 μL of triplex buffer was incubated overnight at 37 °C. Excess unreacted Pt-TFO was removed using a PCRExtract Mini Kit (5 Prime Inc.). The DNA was concentrated under vacuum to 5 μL and added to a solution containing 25 μL of 2× wash/binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, and 0.01% Tween 20]. Sheared salmon sperm DNA (20 μL, 10 mg/μL) was added to minimize nonspecific binding of DNA to the streptavidin-coated magnetic beads. An 80 μL aliquot of Dynabeads MyOne C1 beads (Life Technologies) was washed three times with 500 μL aliquots of 1× wash/

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Table 3. Sequences and Annealing Temperatures of PCR Primers

primer	sequence (5′−3′)[a]	product size (bp)	PCR annealing temperature (°C)
plasmid X forward	ATTGCTAACGCAGTCAGTG	190	55
plasmid X reverse	CTTTATGTTTTTGGCGTCTTCCA		
TFO X forward	GTCTGGCTAGTGGTCTATCTAC	181	62
TFO X reverse	GACTGCTAAAGATGAGGATGCAC		
TFO A forward	ATTCGTTCGCTGTTGCTTGA	160	62
TFO A reverse	CTGTAGCTCCCAAGGCACAA		
TFO B forward	GAAGGCGAACCAGGATTCCA	167	68
TFO B reverse	TAAAGAAGCAGCCCAGGTCC		
TFO C forward	GGGGCTAGACTGCTCAACTG	95	68
TFO C reverse	GGTTTGGGGGTGAGGCATTA		

[a]All primers were designed using NCBI Primer-BLAST.

binding buffer. The beads were suspended in a solution containing 50 μL of sheared salmon sperm DNA (10 mg/mL) and 50 μL of 2× wash/binding buffer. A 25 μL aliquot of the beads was added to the DNA solution, which was gently rotated for 15 min at room temperature, and then incubated for an additional 15 min on ice. The beads were washed three times with 400 μL aliquots of cold 1× wash/binding buffer and resuspended in 50 μL of water. A 5 μL aliquot of the bead suspension was subjected to PCR using primers specific for the target of interest (see Table 3) and the following cycle conditions: initial denaturation at 94 °C for 60 s; 26−30 cycles at 94 °C for 15 s, 62 to 66 °C for 20 s, and 68 °C for 60 s; a final extension at 68 °C for 5 min; and a hold temperature of 10 °C. The optimal number of cycles was determined empirically for each target. The PCR amplicons from Pt-TFO-treated versus untreated DNA were subjected to electrophoresis on 2% agarose gels run in TBE buffer.

Measurement of Glutathione Levels in LAPC-4 Cells. The measurement of glutathione levels in LAPC-4 cells was adapted from a procedure developed by Hissin and Hilf.[26] Cells were grown to confluence in a six-well (35 mm^2) plate. Culture medium was removed from the cells and replaced with 200 μL of 5% metaphosphoric acid. Cells were scraped from the dish, transferred to a microcentrifuge tube, and sonicated for 20 s. The cell lysate was centrifuged for 5 min at 13000*g*, and the supernatant was collected. To measure glutathione levels, 5 μL of cell lysate or 5 μL of diluted glutathione standard solution was combined with 185 μL of phosphate buffer [0.1 M sodium phosphate and 5 mM EDTA (pH 8.0)] and 10 μL of *o*-phthalaldehyde (OPA) in methanol and incubated for 15 min at room temperature. The fluorescence was measured on a VersaFluor fluorometer using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The cellular glutathione content was calculated from standard curves generated from concurrently run glutathione solutions that were freshly prepared in phosphate buffer at a concentration of 100 μg/mL. A diluted glutathione solution (5 μL) was combined with 185 μL of phosphate buffer and 10 μL of OPA in methanol and incubated for 15 min at room temperature prior to being measured.

Transfection of LAPC-4 Cells with Pt-TFOs. Human prostate cancer cells (LAPC-4) were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum; 1 nM androgen analogue, R1881; penicillin; and streptomycin. Six-well (35 mm^2) culture plates were seeded with 2 mL aliquots of a suspension containing 4×10^5 cells/mL and incubated for 24 h. The culture medium was removed and replaced with 1.5 mL of Opti-MEM. A solution containing 9 μL of Lipofectamine 2000 and 485 pmol of Pt-TFO in 500 μL of Opti-MEM was added dropwise to the cells and incubated for 4−6 h. The medium was then replaced with 2 mL of complete medium, and incubation was continued for 24 h. The cells were then treated with a second dose of a Pt-TFO/Lipofectamine 2000 complex (second transfection) as described above. The medium was replaced with 2 mL of complete medium and incubated for an additional 24 h before the cells were analyzed by immunofluorescence, RT-qPCR, fluorescence-activated cell sorting (FACS), or Western blotting.

For the live cell fluorescence microscopy studies, LAPC-4 cells were grown in glass bottom dishes (MatTek, Inc.). The cells were transfected with fluorescein-labeled oligonucleotides and observed 24 h after the second transfection.

For FACS analysis, LAPC-4 cells were grown in 60 mm dishes, transfected with fluorescein-labeled oligonucleotides, and prepared as single-cell suspensions 24 h after the second transfection. To make a single-cell suspension, the cells were treated with trypsin for 4 min. An equal volume of soybean trypsin inhibitor was added. The cells were centrifuged for 5 min at 400*g*, resuspended in FACS medium (HBSS without calcium and magnesium, 0.03% BSA), and made into a single-cell suspension by passing the cells through an 18-gauge syringe. Cells were analyzed and processed using a Beckman Coulter MoFlo cell sorter. Untreated cells were used as a negative control to assess the relative fluorescence intensity of cells treated with fluorescein-labeled oligonucleotides. Cells with a relative intensity of approximately 500 fluorescence units were considered positive for transfection and were selected for analysis of AR mRNA levels by RT-qPCR as described below.

Interaction of Pt-TFOs with Genomic DNA in LAPC-4 Cells. LAPC-4 cells were transfected with biotinylated Pt-TFOs as described above. Genomic DNA was isolated from cells using a PerfectPure DNA cultured cell kit. The DNA was digested with restriction enzymes specific to the target of interest (see Figure 1), and DNA fragments cross-linked to the Pt-TFO were captured on M280 Dynabeads as described above. Because of the low level of cross-linking in these experiments, 10 μg of DNA was digested and prepared for capture, and the beads were washed twice with 1× wash/binding buffer. Captured fragments were amplified by PCR and the amplicons analyzed by agarose gel electrophoresis as described above.

Effect of Pt-TFO X on the AR mRNA Level in LAPC-4 Cells. LAPC-4 cells were transfected with fluorescein-labeled Pt-TFO X, TFO X, or Pt-NBC and sorted using FACS as described above. RNA was extracted from the sorted cells using a PerfectPure RNA cultured cell kit (5 Prime Inc.) 24 or 48 h

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after the second transfection. An iScript cDNA synthesis kit (Bio-Rad) was used to convert 1 μg of RNA to cDNA in a 20 μL reaction mixture that was then diluted with nuclease-free water to a final volume of 100 μL. qPCR was conducted in 20 μL of solution containing 2 μL of the diluted cDNA and 10 μL of SsoAdvanced SYBR Green Supermix (Bio-Rad) supplemented with 375 nM target-specific primers (see Table 4).

Table 4. Sequences of PCR Primers Used for Androgen Receptor (AR) Expression Studies

primer	sequence (5′−3′)	product size (bp)
AR forward[a]	CCTGGCTTCCGCAACTTACAC	168
AR reverse	GGACTTGTGCATGCGGTACTCA	
TBP forward[b]	GAATATAATCCCAAGCGGTTTG	226
TBP reverse	ACTTCACATCACAGCTCCCC	

[a]AR was used as the target gene. [b]TBP was used as the reference gene.

Primer sequences for the androgen receptor (AR)[27] and TATA-box binding protein (TBP) were selected from the published literature. The qPCR cycle conditions were as follows: 95 °C for 30 s and 35 cycles at 95 °C for 5 s and 62 °C for 30 s. The results were analyzed using CFX Manager Software (Bio-Rad) to assess the relative gene expression levels of AR normalized to TBP.

Effect of Pt-TFO X on AR Protein Level in LAPC-4 Cells As Determined by an Immunofluorescence Assay. LAPC-4 cells were cultured in two-well Lab-Tek chamber slides, transfected with fluorescein-labeled Pt-TFO X, TFO X, or Pt-NBC as described above, and cultured in complete culture medium supplemented with R1881. Following a 24 h incubation after the second transfection, the transfected cells were fixed in 10% buffered formalin for 15 min, washed four times with PBS at 2 min intervals, permeabilized in 0.2% Triton X-100 diluted in PBS for 15 min, and washed with PBS. The fixed cells were blocked with an Image-iT FX signal enhancer (Life Technologies) for 30 min and then washed with PBS. AR primary goat antibody (Thermo Scientific Lab Vision, RB-9030-P1) diluted 1:250 in 2% BSA in PBS was added, and the cells were incubated overnight at 4 °C. The cells were then washed with PBS and incubated with anti-goat secondary antibody conjugated to a red fluorescent Alexa Fluor 594 dye (Life Technologies, A11072) diluted 1:500 with 2% BSA in PBS at room temperature for 2 h. The cells were washed and mounted with ProLong Gold with DAPI antifade mountant (Life Technologies). The fluorescence in the cells was visualized with a Zeiss Observer Z1 fluorescence microscope, and the images were processed using AxioVision Software Release 4.8.2. AR fluorescence was normalized to DAPI fluorescence to measure relative protein levels in the cell.

Effect of Pt-TFO X on AR Protein Level in LAPC-4 Cells As Determined by Western Blot Analysis. LAPC-4 cells were transfected with Pt-TFO X, TFO X, or Pt-NBC. Following the first and second transfection, the Opti-MEM culture medium was replaced with androgen-free complete culture medium (IMDM supplemented with 10% charcoal/dextran-treated fetal bovine serum, penicillin, and streptomycin). Transfected cells were treated with 100 μL of trypsin for 4 min, followed by further treatment with 400 μL of soybean trypsin inhibitor. The cell suspension was centrifuged for 5 min at 3100 rpm (~800*g*). The supernatant was removed, and the cell pellet was suspended in 100 μL of 1× lysis buffer [150 mM NaCl, 0.1% Triton X-100, 50 mM Tris-HCl (pH 8), 0.5% sodium deoxycholate, and protease inhibitor cocktail] followed by gentle agitation for 30 min at 4 °C. The resulting cell lysate was sonicated for 5 min, diluted with 35 μL of 4× NuPAGE LDS sample buffer containing 5% β-mercaptoethanol, incubated for 10 min at 95 °C, and centrifuged at 13000 rpm (16000*g*) for 10 min. Aliquots (10 μL) of the processed lysate were electrophoresed at 125 V for approximately 1 h on polyacrylamide gels comprised of a 4% stacking gel and a 10% resolving gel. The proteins in the gel were electroblotted to a nitrocellulose membrane at 100 V for 100 min. The membrane was blocked in Tris-buffered saline and Tween 20 (TBST) with 5% BSA for 1 h at room temperature, washed three times for 5 min in TBST, and incubated with primary antibody overnight at 4 °C. AR was detected using a 1:500 dilution of an anti-AR primary antibody (Thermo Scientific Lab Vision Polyclonal anti-Androgen Receptor, RB-9030-P1) in TBST. A 1:10000 dilution of anti-β-actin antibody (GeneTex, GTX110564) in TBST was used as a loading control. The membrane was then washed three times for 5 min in TBST and incubated for 4 h at room temperature with HRP-conjugated secondary antibody (Southern Biotech anti-rabbit 4030-05) diluted 1:10000 in TBST. The membrane was washed three times with TBST, treated with SuperSignal West Pico Chemiluminescent Substrate, and visualized with the FluorChem E System (ProteinSimple).

RESULTS

DNA Targets and Pt-TFOs. The TFOs described in this study target the human androgen receptor (AR) gene, which contains more than 70 homopurine tracts that are suitable for triplex formation. We used the following set of criteria to identify targets for the Pt-TFOs. (1) The target homopurine tracts are at least 18 nucleotides long and do not contain pyrimidine interruptions that could interfere with triplex formation. (2) Because three or more contiguous cytosines in the TFO can severely attenuate the thermal stability of the triplex at physiological pH, the target homopurine tracts contain no more than two adjacent guanines. (3) Cross-linking efficiency is significantly reduced if the platinated guanine of the Pt-TFO is adjacent to a cytosine.[25] Therefore, the guanine targeted by the Pt-TFO neighbors an adenine and not a guanine in the homopurine sequence. (4) We have found that Pt-TFOs that cross-link to the transcribed strand of a gene are more effective inhibitors of transcription regardless of whether the TFO binds to the coding strand or the transcribed strand.[25] Pt-TFOs that cross-link to the transcribed strand are more likely to block RNA polymerase, whereas Pt-TFOs that cross-link to the coding strand may reduce the rate of transcription only by stalling RNA polymerase. Therefore, the cross-linking sites for the Pt-TFOs in these studies lie in the transcribed strand of the AR gene. While bis-Pt-TFOs that can form interstrand cross-links with dsDNA targets are equally adept at inhibiting transcription as Pt-TFOs that form a single cross-link with the transcribed strand of DNA,[25] the AR sequences selected for this study were targeted by Pt-TFOs that contained a single 3′-platinated deoxyguanosine. This approach allowed the 5′-end of the Pt-TFOs to be further conjugated with biotin or a fluorescence marker for downstream biological assays.

Using these criteria, we employed a search engine[28] to identify four homopurine target sites, A, X, B, and C (see Figure 1), in the AR gene that are suitable for Pt-TFO cross-linking. Pt-TFO A, X, B, and C are composed of 2′-*O*-methyl-5-methyluridine and 2′-*O*-methyl-5-methylcytidine nucleosides

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and terminate at their 3′-ends with a 2′-deoxyguanosine. The 2′-*O*-methylribose backbone has been shown to enhance the thermal stability of triplexes formed by homopyrimidine TFOs.[29,30] Previous studies have shown that the apparent pK_a of 5-methylcytosine in homopyrimidine TFOs is higher than that of cytosine. This and the greater hydrophobicity of 5-methylcytosine versus cytosine most likely account for the ability of 5-methylcytosine-containing TFOs to form triplexes more stable than those of their cytosine-containing counterparts.[31,32] Pt-TFO A targets two homopurine tracts, A.1 and A.2, which lie within the coding strand and the transcribed strand, respectively, of intron 1. When bound to site A.1, Pt-TFO A can cross-link to a guanine in the coding strand, whereas cross-linking can occur on the transcribed strand when Pt-TFO A is bound to site A.2. Pt-TFO X and Pt-TFO C target homopurine tracts in the transcribed strand of intron 1 and exon 8, respectively, and can cross-link to guanines in that strand. We have previously shown that Pt-TOF X can inhibit transcription when it is cross-linked to a plasmid DNA that contains the same TFO binding site as target site X but a different sequence at the cross-linking site.[25] TFO B, on the other hand, targets a homopurine tract in the coding strand of intron 2 but can cross-link to a guanine in the transcribed strand.

The TFOs were synthesized using standard phosphoramidite chemistry and commercially available reagents. N7 of the 3′-terminal deoxyguanosine of the TFO was platinated by reaction with *trans*-diamminedichloroplatinum(II) as previously described.[23−25] Fluorescein-labeled and biotin-conjugated TFOs were prepared by coupling 6-(3′,6′-dipivaloylfluoresceinyl-6-carboxamido)hexyl-1-*O*-(2-cyanoethyl)(*N*,*N*-diisopropyl)-phosphoramidite and 1-dimethoxytrityloxy-3-*O*-(*N*-biotinyl-3-aminopropyl)triethylene glycolyl-glyceryl-2-*O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite, respectively, to the 5′-ends of the TFOs during synthesis. The TFOs were purified by strong anion exchange (SAX) HPLC and their compositions confirmed by MALD-TOF mass spectrometry (see Table 1).

In addition to Pt-TFOs A, X, B, and C, we designed a nonbinding control, Pt-NBC, whose base composition is identical to that of Pt-TFO X but whose sequence contains five contiguous cytosines (see Table 2). The difficulty of protonating successive, adjacent 5-metylcytosines in effect lowers their apparent pK_a[25,33] and was expected to prevent Pt-NBC from binding to its cognate homopurine tract at physiological pH.

We performed melting experiments to examine the ability of NBC to bind to its cognate DNA duplex (see Table 2). As expected, although NBC forms a stable triplex with the NBC duplex at pH 6.0, the melting temperature (T_m) of the triplex decreases dramatically when the pH is increased to 7.4. This sensitivity of binding to pH is reflected in the cross-linking activity of the platinum derivative, Pt-NBC (see Figure 2A). Significant cross-linking was observed at pH 6.0 and 6.5 (77 and 83%, respectively) when Pt-NBC was incubated with the NBC duplex at 37 °C, whereas no cross-linking was detected at pH 7.4.

We also confirmed that Pt-NBC does not cross-link to a DNA duplex (AR duplex) whose sequence is identical to that of the binding site for TFO X in the AR gene. TFO X forms a very stable triplex with the AR duplex at pH 7.4 (see Table 2), and Pt-TFO X cross-links with this duplex at a level of 75% under physiological conditions (see Figure 2B). In contrast, no cross-linking was observed when Pt-NBC was incubated with the AR duplex under the same conditions. These results rule out the possibility of indiscriminate cross-linking by the platinated guanine of the Pt-TFO and demonstrate that cross-linking depends upon the ability of the Pt-TFO to bind to its cognate homopurine target. Furthermore, examination of the AR gene shows it lacks a binding and cross-linking site for Pt-NBC.

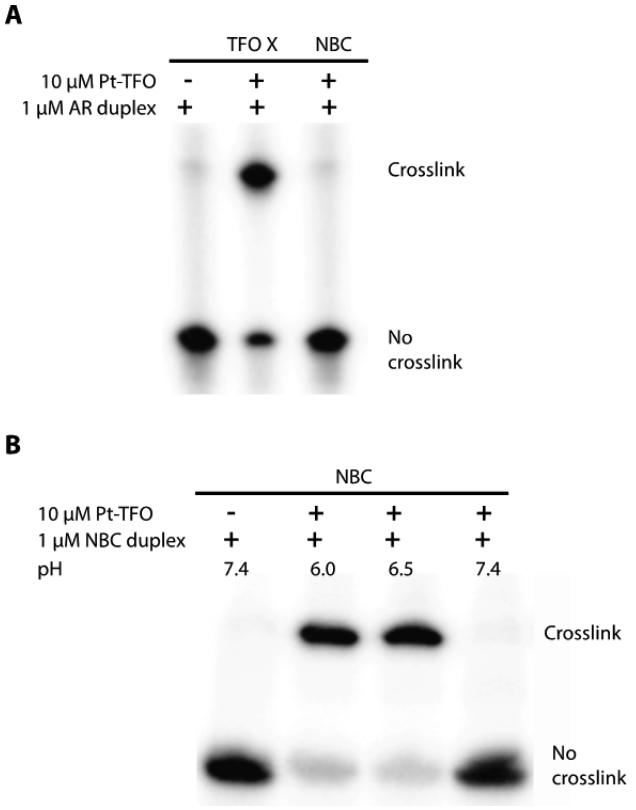

Figure 2. Interactions of Pt-TFO X and Pt-NBC with their target DNA duplexes. Solutions containing Pt-TFO and ^{32}P-labeled duplex in triplex buffer were incubated overnight at 37 °C, and the reaction mixtures were electrophoresed on denaturing 20% polyacrylamide gels. (A) Cross-linking between Pt-TFO X or Pt-NBC and AR duplex. Pt-TFOs and duplex were incubated at pH 7.4. (B) Cross-linking between Pt-NBC and NBC duplex. Pt-TFO and duplex were incubated at pH 6.0, 6.5, and 7.4.

Interaction of Pt-TFOs with Genomic DNA *in Vitro*. We used an approach (see Scheme 1) similar to that described by Besch and co-workers[14] to assess whether the Pt-TFOs shown in Figure 1 and Table 1 can cross-link to their designated targets in DNA isolated from LAPC-4 cells, a human prostate cancer cell line. In this method, the Pt-TFOs are conjugated with a 5′-biotin, a modification that was expected not to interfere with cross-linking by the platinated deoxyguanosine located at the 3′-end of the Pt-TFO. The biotin serves as a molecular handle and allows DNA cross-linked to the Pt-TFO to be captured by streptavidin-coated magnetic beads. Genomic DNA is reacted with the biotinylated Pt-TFO and then digested with restriction enzymes selected to produce <1 kb fragments, which is the maximal size that can be captured by the beads. Excess unreacted Pt-TFO is removed using a PCR extraction column that selectively enriches with DNA >40 bases in length. Under these conditions, Pt-TFOs are not expected to form stable triplexes through the PCR extraction procedure. As a result, only Pt-TFOs cross-linked to DNA are enriched. The DNA fragments are then mixed with the streptavidin-coated

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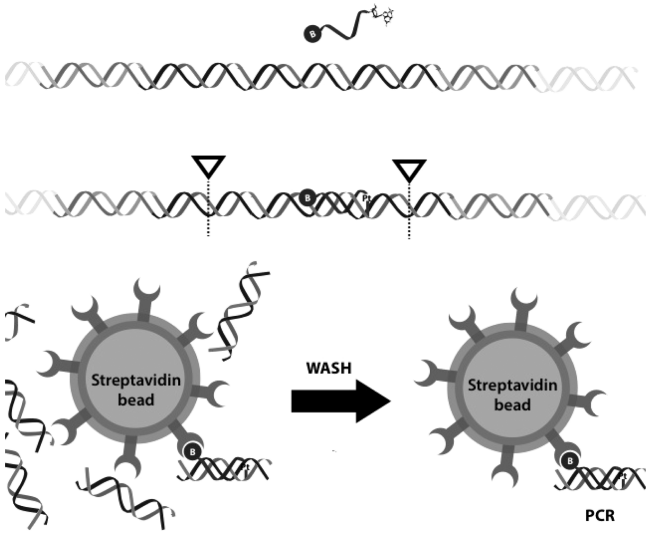
Scheme 1. Biotinylated Pt-TFO Cross-Linking Assay[a]



[a]After cross-linking, the DNA is digested with restriction enzymes, and the digest is incubated with streptavidin-coated magnetic beads. The beads are washed, and the captured DNA is amplified via PCR with sequence-specific primers.

magnetic beads that had been preincubated with sheared salmon sperm DNA to reduce the level of nonspecific binding. Unbound DNA is removed by washing the beads with cold buffer consisting of 1 M NaCl, 0.5 mM EDTA, 5 mM Tris-HCl (pH 7.5), and 0.005% Tween 20. We found that addition of the detergent prevents the beads from sticking to microcentrifuge tubes. Following these washes, an aliquot of the beads is subjected to PCR amplification using target-specific primers. The PCR product is electrophoresed on an agarose gel containing SYBR green dye to qualitatively assess whether the Pt-TFO has cross-linked to its designated DNA target.

To test the feasibility of this approach, we first conducted a capture experiment using biotinylated Pt-TFO X and plasmid X, a 5.7 kb circular DNA that contains a binding site and cross-linking site for this Pt-TFO. The cross-linking site is part of a recognition sequence for the restriction enzyme, BstEII (see Figure 3A). Biotinylated Pt-TFO X was incubated with plasmid X. Cross-linking was confirmed by the inability of BstEII to cleave the plasmid, whereas incubation with SacI, whose recognition site is upstream of the TFO binding site, did cleave the plasmid (see Figure 3B). The cross-linked plasmid was treated with SacI and EcoNI to excise a 781 bp fragment containing the Pt-TFO X binding site and cross-linking site. The DNA fragments from the digest were incubated with streptavidin-coated magnetic beads and, after being washed, subjected to PCR and analysis by agarose gel electrophoresis. A single band whose size corresponded to the expected size of the PCR amplicon was observed from the plasmid treated with biotinylated Pt-TFO X, but not from the untreated plasmid, thus indicating that biotinylated Pt-TFO X had cross-linked to plasmid X (see Figure 3C).

The capture procedure was repeated to determine if Pt-TFOs A, X, B, and C (see Table 1) cross-linked to targeted homopurine tracts A, X, B, and C, respectively, of the AR gene (Figure 1) in genomic DNA extracted from LAPC-4 cells. In these experiments, LAPC-4 DNA was first digested with a unique set of restriction enzymes to create <1 kb fragments for each targeted homopurine tract. The DNA in each digest was

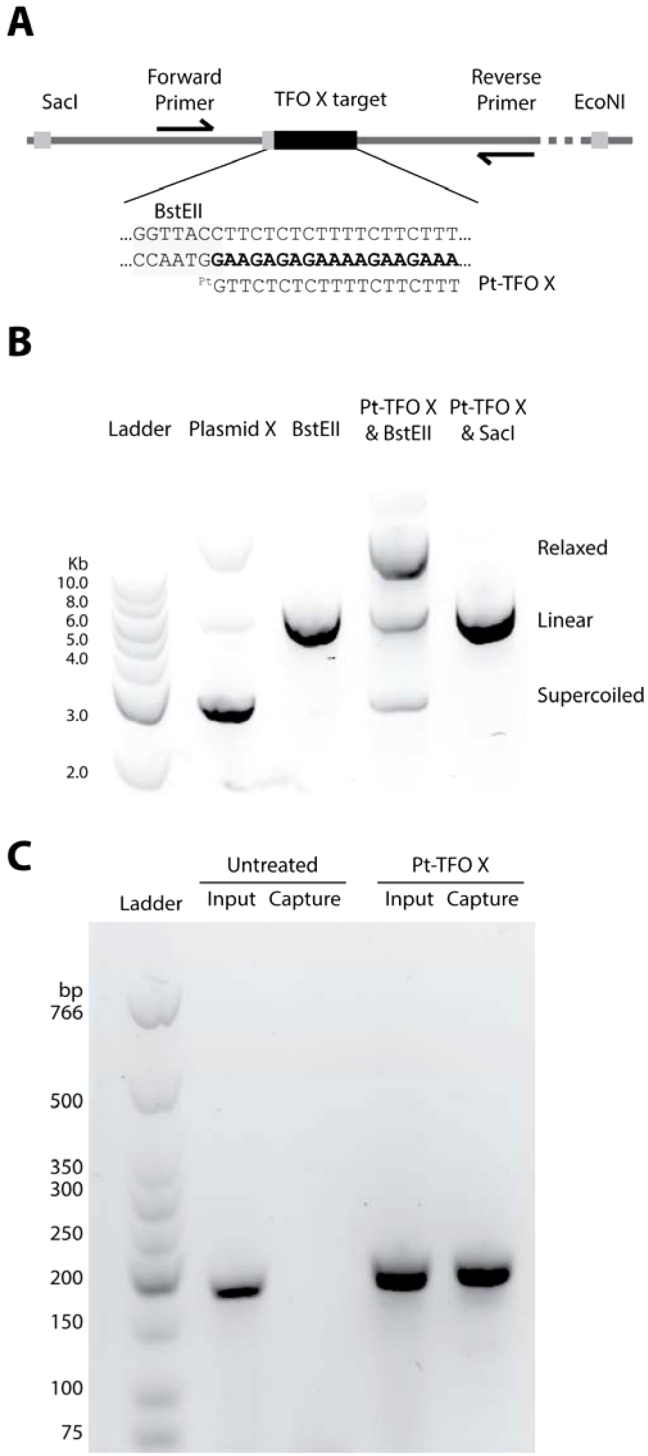


Figure 3. Cross-linking of biotinylated Pt-TFO X to plasmid X DNA. (A) Pt-TFO binding and cross-linking sites in 5.7 kb plasmid X. (B) Restriction enzyme digests of plasmid X DNA cross-linked to Pt-TFO X. (C) Capture of plasmid X DNA fragments cross-linked to biotinylated Pt-TFO X. Lanes marked "input" show the PCR amplicon obtained prior to mixing DNA fragments with streptavidin-coated beads.

then reacted with its corresponding Pt-TFO, and the cross-linked DNA fragments were captured as described above. The captured fragments were amplified by PCR using the appropriate primers (see Table 3), and the amplicons were analyzed by agarose gel electrophoresis. Amplicons of the

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expected size were observed from DNA treated *in vitro* with each of the Pt-TFOs, whereas essentially no amplicons were observed from DNA that had not been treated with a Pt-TFO (see Figure 4A, left). These results are consistent with cross-linking of the Pt-TFO to its designated LAPC-4 DNA target site.

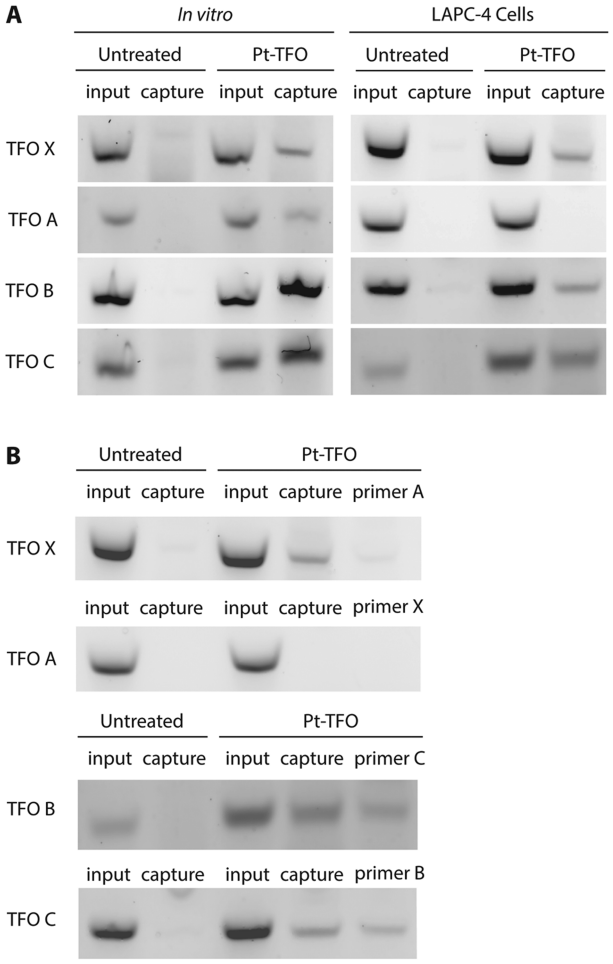

Figure 4. Cross-linking of biotinylated Pt-TFOs to LAPC-4 genomic DNA. (A) Biotinylated Pt-TFOs cross-linked to genomic DNA *in vitro* (left). Biotinylated Pt-TFOs B, X, and C each cross-linked to chromosomal DNA in LAPC-4 cells (right). (B) Cross-linking of biotinylated Pt-TFOs X and A to chromosomal DNA targets X and A in LAPC-4 cells (top). Cross-linking of biotinylated Pt-TFOs B and C to chromosomal DNA targets B and C in LAPC-4 cells (bottom). Lanes marked "input" show the PCR amplicon obtained prior to mixing DNA fragments with streptavidin-coated beads.

Effect of Glutathione on Cross-Linking. In their original studies on Pt-TFOs, Colombier and co-workers suggested that reaction of the platinum group of the TFO with sulfhydryl-containing biomolecules such as glutathione could attenuate cross-linking of Pt-TFOs to genomic DNA.[23] Using a fluorometric assay,[26] we determined that the LAPC-4 cells have an intracellular glutathione concentration of 0.5 mM. Therefore, before examining the binding of Pt-TFOs to genomic DNA in cultured LAPC-4 cells, we investigated the effect of incubating ^{32}P-labeled AR duplex with Pt-TFO X in the presence of increasing concentrations of glutathione ranging from 0 to 1 M. Cross-linking was monitored by denaturing polyacrylamide gel electrophoresis (see Figure 5A). The extent of cross-linking began to diminish at glutathione

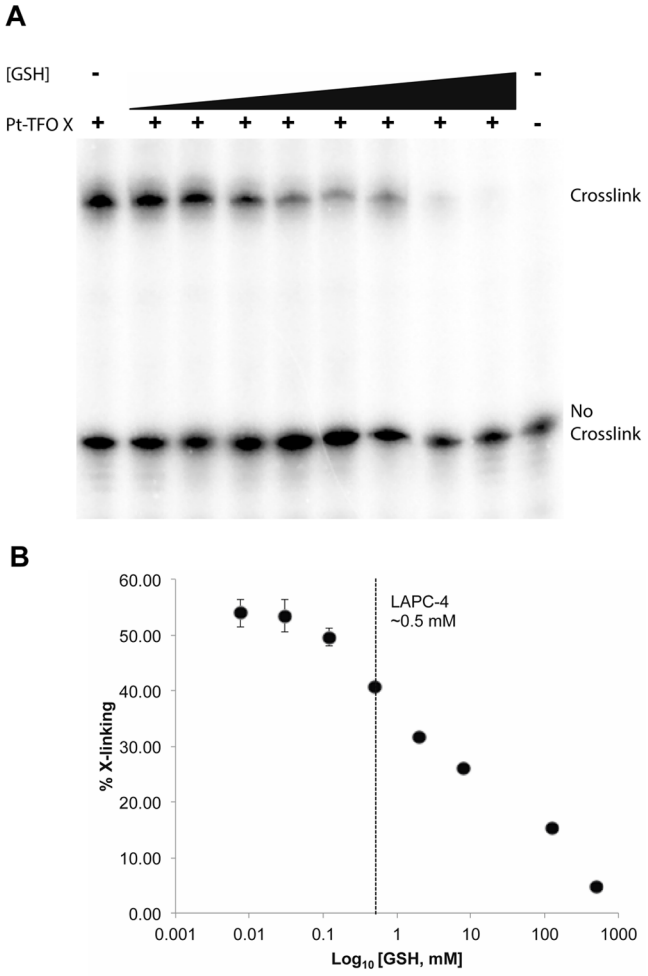

Figure 5. Effect of glutathione on Pt-TFO cross-linking. (A) EMSA, under denaturing conditions, of Pt-TFO X (10 μM) cross-linking with ^{32}P-labeled target duplex (1 μM) in the presence of increasing concentrations of glutathione (GSH). (B) Percent cross-linking in the presence of varying concentrations of glutathione. The bars represent the standard deviation of three experiments. The GSH concentration in LAPC-4 cells, indicated by the dashed line, is 0.5 mM.

concentrations above 0.1 mM (see Figure 5B). On the basis of our *in vitro* results, the concentration of glutathione within LAPC-4 cells would be expected to decrease the level of cross-linking by approximately 15%.

Interaction of Pt-TFOs with Chromosomal DNA in LAPC-4 Cells. We first examined the uptake of Pt-TFO X by LAPC-4 cells in culture to ensure that the cells could be transfected with the platinated TFO. LAPC-4 cells were transfected with fluorescein-labeled Pt-TFO X complexed with the polycationic lipid, Lipofectamine 2000. The cells were treated with the complex for 4–6 h in serum-free Opti-MEM, followed by overnight incubation in complete medium. This treatment (second transfection) was repeated 24 h later to increase the rate of Pt-TFO uptake. The cells were analyzed using live cell fluorescence microscopy 24 h after the second transfection. The majority of the cells appeared to efficiently take up the fluorescently labeled oligonucleotide (see Figure 6A). Although Pt-TFO X was distributed throughout the cell, a

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significant amount appeared to localize in the nucleus (see Figure 6B).

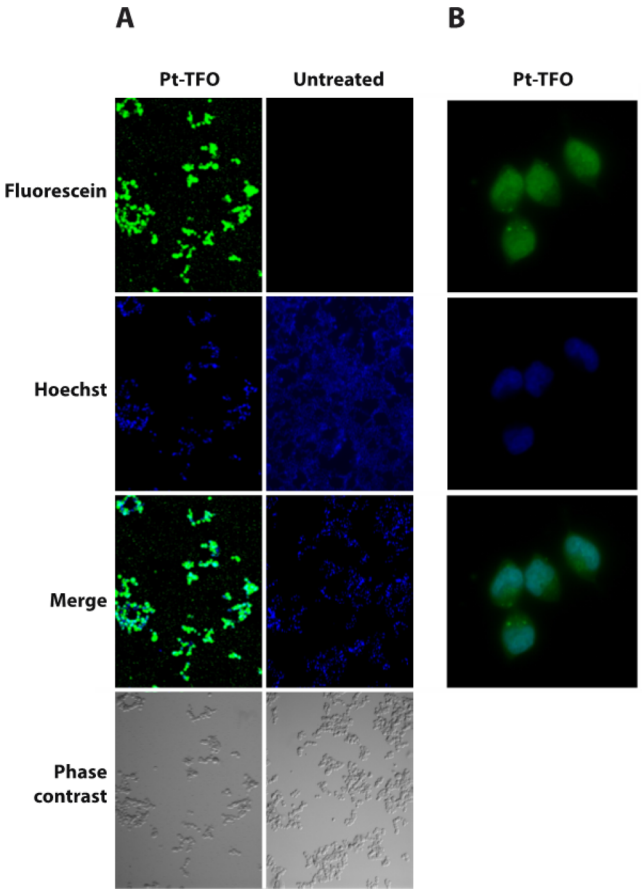

Figure 6. Live cell microscopy of LAPC-4 cells transfected with fluorescein-labeled Pt-TFO X. DNA is stained with Hoechst dye: (A) 100× magnification and (B) 400× magnification showing Pt-TFO localized in nuclei.

Uptake was also examined by FACS. In these experiments, LAPC-4 cells were transfected with two doses of fluorescein-labeled nonplatinated TFO X, Pt-TFO X, or Pt-NBC as described above. The transfected cells were treated with trypsin, prepared as a single-cell suspension in FACS buffer, and analyzed using FACS. The relative fluorescence levels of the transfected cells were normalized against cells that were not transfected. Each of the oligonucleotides appeared to be taken up to approximately the same extent. Furthermore, the platinum modification did not affect transfection efficiency (see Figure 7).

We next determined whether biotinylated Pt-TFOs could cross-link to chromosomal DNA in LAPC-4 cells. LAPC-4 cells were treated over the course of 48 h with two doses of a Lipofectamine complex of biotinylated Pt-TFO A, X, B, or C as described in the preceding section. The cells were incubated for an additional 24 h after the second treatment. Genomic DNA was extracted from the cells and digested with restriction enzymes specific to the target of interest (see Figure 1), and the biotinylated Pt-TFO cross-linked DNA fragments were captured on streptavidin-coated beads. The beads were then subjected to PCR using target-specific primers, and the products of these reactions were analyzed by agarose gel electrophoresis. DNA from cells treated with Pt-TFO X, B, or

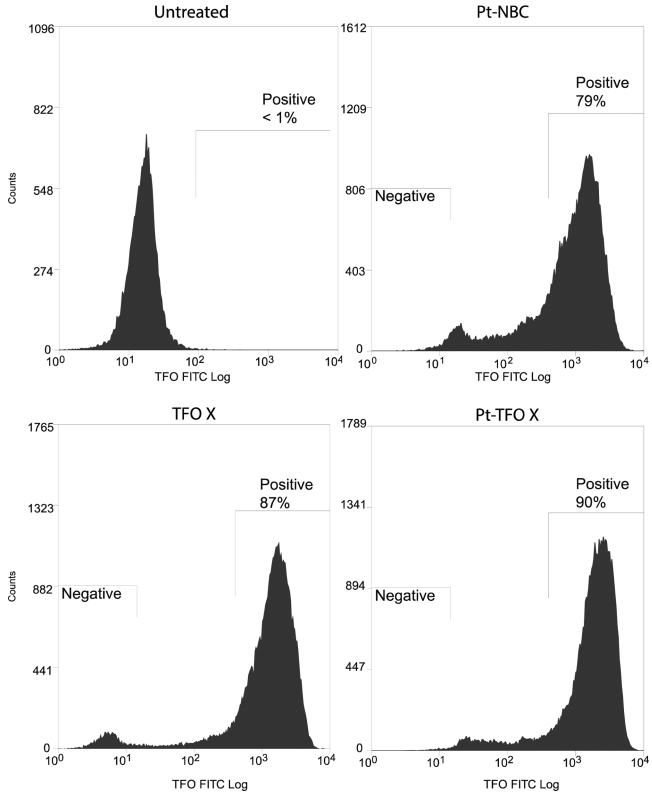

Figure 7. Fluorescence-activated cell sorting (FACS) of LAPC-4 cells transfected with fluorescein-labeled TFOs.

C each produced amplicons of the expected size, whereas an amplicon was not observed from cells treated with Pt-TFO A or from cells not treated with the biotinylated TFOs (see Figure 4A, right). These results demonstrate that three of the biotinylated Pt-TFOs were able to cross-link to their designated chromosomal DNA targets in the LAPC-4 cells. The specificity of DNA fragment capture and PCR amplification was demonstrated by the absence of an amplicon when DNA fragments captured from cells treated with Pt-TFO X were amplified using primers for target A (see Figure 4B). Similarly, no amplicon was observed when captured DNA fragments from cells treated with Pt-TFO A were amplified using primers for target X. However, cross reactivity with targets B and C was evident from the appearance of amplicons in Figure 4B with reciprocal primers for both Pt-TFO B and Pt-TFO C, which share significant sequence similarity, particularly at their 3′-ends adjacent to the platinated deoxyguanosine (see Table 1 and Figure 1).

Effects of Pt-TFO X on AR mRNA and Protein Levels in LAPC-4 Cells. Our previous studies demonstrated that Pt-TFO X, when cross-linked to the transcribed strand of a plasmid DNA, inhibited DNA transcription in Chinese hamster ovary cells.[25] We therefore conducted experiments to determine if Pt-TFO X could attenuate AR mRNA and protein levels in LAPC-4 cells. When LAPC-4 cells were transfected with an equimolar mixture of Pt-TFOs A, X, B, and C, there was no significant effect on the expression of the AR gene (data not shown). Because the transfection efficiency was not uniform, cells with lower intracellular concentrations of Pt-TFOs likely had unperturbed AR expression. To enrich highly transfected cells, LAPC-4 cells were transfected with two doses of fluorescein-labeled Pt-NBC, TFO X, or Pt-TFO X and single-

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cell suspensions in cell sorting buffer were prepared 24 h after the second transfection. Cells having the highest relative fluorescence were selected using FACS (see Figure 7B–D). Messenger RNA was isolated from the sorted cells and reverse transcribed into cDNA. The relative AR mRNA levels were determined using RT-qPCR and normalized to that from the housekeeping gene, TBP. The normalized AR mRNA levels were compared with those from untreated LAPC-4 cells (see Figure 7A). Cells treated with TFO X or Pt-TFO X had 46 and 42% knockdown of AR mRNA, respectively (see Figure 8). In

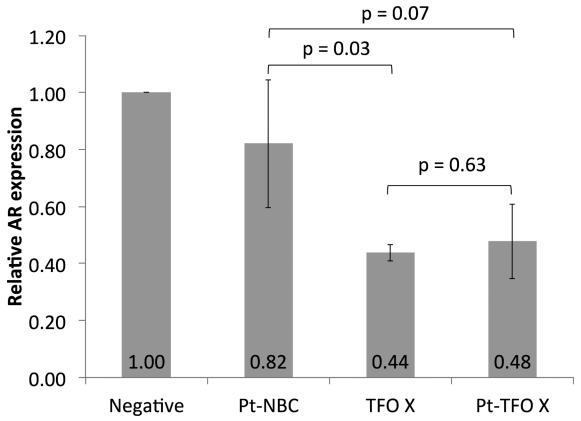

Figure 8. Effects of fluorescein-labeled TFOs on AR mRNA levels in FACS-sorted LAPC-4 cells as measured by RT-qPCR. AR mRNA levels normalized to that of TBP are compared in TFO-treated vs untreated cells. The bars represent the standard deviation of three separate experiments, each measured in triplicate. *P* values were determined with a Student's *t* test (paired and two-tailed).

contrast to these results, no significant knockdown of AR mRNA was observed when LAPC-4 cells transfected with fluorescein-labeled Pt-NBC, TFO X, or Pt-TFO X were not sorted by FACS prior to RT-qPCR (data not shown).

The effect of Pt-TFO X on AR protein levels was examined by immunofluorescence microscopy in LAPC-4 cells transfected with fluorescein-labeled Pt-NBC, TFO X, or Pt-TFO X (Figure 9A). The AR fluorescence was normalized to DAPI DNA fluorescence from images of cells that exhibited significant fluorescence following transfection with the fluorescein-labeled TFOs compared to untreated cells (see Figure 9 B). The level of AR protein in LAPC-4 cells treated with Pt-TFO X was reduced by 32%, whereas no significant reduction was observed in cells treated with nonplatinated TFO X.

We also used Western blots to examine the effect of the TFOs on AR protein levels in LAPC-4 cells. Protein lysates were prepared from LAPC-4 cells 24 h after transfection with two doses of Pt-NBC, TFO X, or Pt-TFO X. The TFOs were not labeled with fluorescein, and therefore, these cells were not sorted prior to cell lysis. The AR protein levels were quantified from immunoblot band densities normalized to β-actin and compared to those from untreated cells (see Figure 10). The amount of AR protein was reduced by 18% in cells treated with Pt-TFO X compared to that in cells treated with Pt-NBC. In contrast, there was no apparent reduction in the level of AR protein in cells treated with TFO X.

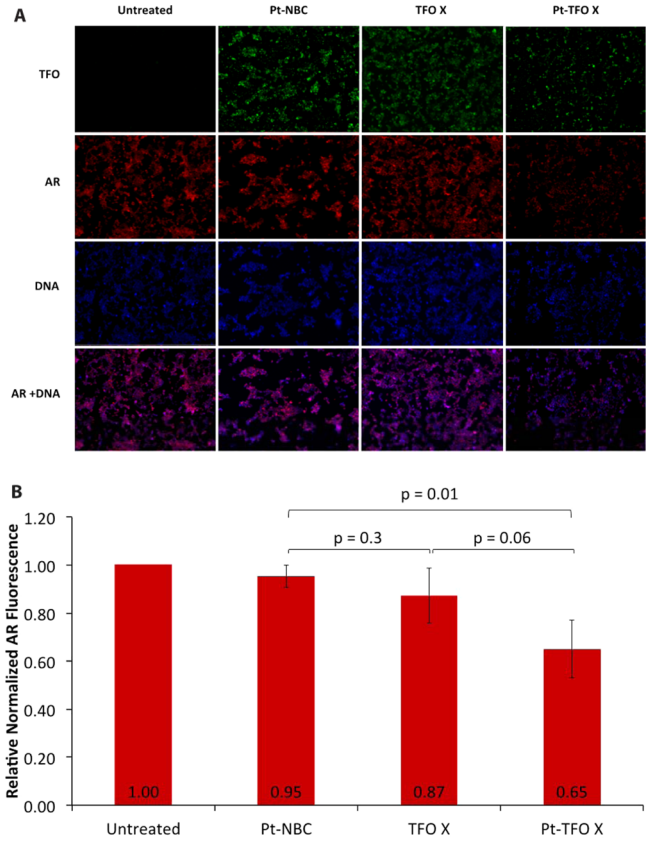

Figure 9. Effects of Pt-TFOs on AR protein levels in LAPC-4 cells. (A) Immunofluorescence images of LAPC-4 cells transfected with fluorescein-labeled TFOs at a 100× magnification. (B) AR protein fluorescence normalized to that of DNA in TFO-treated vs untreated cells. The bars represent the standard deviation of three experiments, each measured in triplicate. *P* values were determined with a Student's *t* test (paired and two-tailed) comparing Pt-NBC with TFO X or Pt-TFO X.

DISCUSSION

Previous studies by Colombier et al. demonstrated that deoxyribopyrimidine TFOs modified with a 5′-N7-*trans*-chlorodiammineplatinum(II)-2′-deoxygaunosine were capable of cross-linking to a homopurine tract in a DNA duplex.[23] Subsequent studies in our laboratory showed that platinum-modified 2′-*O*-methylribopyrimidine TFOs that cross-link to DNA under physiological pH and temperature conditions could be designed.[24] These latter results suggested that 2′-*O*-methylribopyrimidine Pt-TFOs could be targeted to cross-link to genomic DNA in living cells. To test this idea, we designed and synthesized four Pt-TFOs, Pt-TFO A, X, B, and C, that target different homopurine tracts within the human AR gene and examined their interactions with genomic DNA in LAPC-4 cells, a human prostate cancer cell line.

To do this, we used a procedure similar to that described by Besch et al. to detect cross-linking between psoralen-derivatized TFOs and genomic DNA.[14] In their approach, isolated nuclei or intact mammalian cells were transfected with biotin-conjugated psoralen TFOs and irradiated with long wavelength UV light. The genomic DNA was then isolated and digested with restriction enzymes. The digest was incubated with streptavidin-coated beads that were then washed with redistilled water at 60 °C to remove unbound DNA. The captured cross-linked fragments were amplified by PCR using primers specific

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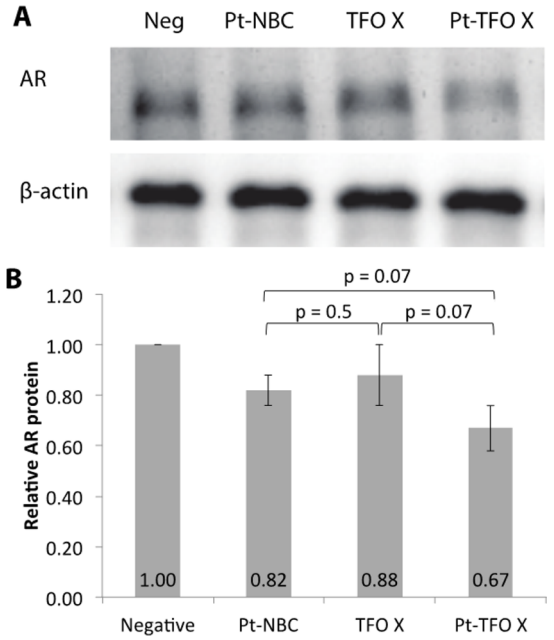

Figure 10. Western blot analysis of AR protein levels in LAPC-4 cells transfected with TFOs. (A) Blots were probed with AR- or *β*-actin-specific antibodies. (B) Relative AR protein levels in LAPC-4 cells transfected with TFOs vs untreated cells. Proteins were quantified from immunoblot band densities. AR protein levels were normalized to *β*-actin. The bars represent the standard deviation of three experiments. *P* values were determined with a Student's *t* test (paired and two-tailed).

to the region of the genome where cross-linking was expected to occur and the resulting amplicons analyzed by agarose gel electrophoresis. In our studies, intact LAPC-4 cells were transfected with biotin-conjugated Pt-TFOs. The restriction digest of the isolated genomic DNA was incubated with streptavidin-coated beads in the presence of salmon sperm DNA to reduce the level of nonspecific binding to the beads. Instead of washing the beads with redistilled water, we washed the beads with a cold wash buffer that contained 1 M NaCl. This latter modification was introduced on the basis of studies by Holmberg et al., who showed that incubating streptavidin-coated beads in deionized water at 60 °C for ≥1 min was sufficient to disrupt the biotin−streptavidin interaction.[34] Prior to conducting experiments in living cells, we used this capture procedure to verify that Pt-TFOs A, X, B, and C did indeed cross-link *in vitro* to their designated AR gene target sites in genomic DNA extracted from LAPC-4 cells.

Colombier et al. pointed out that sulfhydryl-containing biomolecules, particularly glutathione, are potential impediments to reaction of Pt-TFOs with genomic DNA in the cell.[23] Platinum(II) compounds are known to react with the sulfhydryl group of glutathione,[35] which is the most abundant thiol in the cell with an intracellular concentration ranging from 0.2 to 10 mM.[36] Several reports have implicated this ubiquitously expressed tripeptide as a major contributor to cisplatin resistance.[37,38] Like cisplatin, the transplatin group of a Pt-TFO is potentially vulnerable to reaction with glutathione, which could prevent the Pt-TFO from cross-linking with its DNA target. To address this issue, cross-linking reactions between Pt-TFO X and a target DNA duplex were conducted *in vitro* in the presence of increasing concentrations of glutathione. As suspected, cross-linking levels decreased with increasing concentrations of glutathione until essentially no cross-linking was detected at 1 M glutathione. However, our measurements in LAPC-4 cells suggested that the glutathione concentration is not sufficiently high to significantly interfere with Pt-TFO cross-linking in these cells.

Biotinylated Pt-TFOs were transfected into LAPC-4 cells as a complex with the widely used polycationic lipid, Lipofectamine 2000. Preliminary experiments employing 5′-fluorescein-labeled Pt-TFO X showed that it was distributed throughout the cytoplasm of the cell and, most importantly, localized in the nucleus. These results showed that the positively charged platinum(II) of the Pt-TFOs does not interfere with cationic lipid-mediated transfection and that the Pt-TFOs can potentially reach their chromosomal target in the nucleus.

Cross-linking to genomic AR DNA in the LAPC-4 cells was observed with three of the Pt-TFOs, X, B, and C, as assessed by the streptavidin−bead capture procedure. This behavior is in contrast to that seen *in vitro* where all four Pt-TFOs were found to cross-link to AR DNA. A number of studies have reported cross-linking of psoralen-derivatized TFOs to genomic DNA in mammalian cells.[11−16] With the exception of one study,[11] active gene transcription appeared to promote cross-linking.[12,15] The AR gene is actively transcribed in LAPC-4 cells,[39] and in analogy to psoralen-TFOs, Pt-TFOs would be expected to have access to genomic AR DNA. However, the lack of cross-linking by Pt-TFO A suggests that different regions of the AR gene, possibly because of the chromatin structure, are not uniformly accessible to binding and cross-linking by Pt-TFOs.

The observation that Pt-TFOs cross-link to AR genomic DNA in LAPC-4 cells coupled with our previous observation that Pt-TFO X was an effective barrier to transcription when cross-linked to a plasmid reporter gene[25] suggested they could attenuate AR gene transcription in these cells. We initially found no significant knockdown of AR mRNA when cells were transfected with Pt-TFO X alone or with a cocktail of Pt-TFOs A, X, B, and C. However, approximately 40% knockdown of AR mRNA was observed in the subpopulation of LAPC-4 cells that exhibited high levels of fluorescence following transfection with fluorescein-labeled TFO X or Pt-TFO X and hence could be isolated by FACS prior to RT-qPCR analysis. These results show that effective knockdown occurs only in cells that have taken up relatively high concentrations of TFO. Similar results were obtained by Besch et al. in their studies of psoralen-TFO cross-linking to genomic DNA.[14] They observed approximately 10-fold less cross-linking in whole cells than in isolated nuclei, which are able to take up much higher concentrations of the psoralen-TFO.

Both the nonplatinated and platinated forms of TFO X were capable of knocking down AR mRNA in the LAPC-4 cells. TFO X forms an unusually stable triplex (T_m = 74 °C) with its AR DNA target sequence under essentially physiological conditions. It appears that this strong binding is sufficient to attenuate transcription.

Although the level of AR mRNA knockdown in cells transfected with nonplatinated TFO X was apparently not sufficient to cause a significant decrease in the level of AR protein, cells transfected with Pt-TFO X did display a decreased amount of AR protein. This difference may be a consequence of the unique ability of Pt-TFO X to cross-link to its DNA target. Young et al. have shown that TFOs cross-linked to DNA serve as absolute blocks to transcription and, as a result, give rise to truncated transcripts.[40] In contrast, non-cross-linking TFOs, which interact exclusively with DNA through hydrogen bonds,

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can stall RNA polymerase and consequently reduce the rate of transcription but are eventually displaced, resulting in the production of full length transcripts. These full length transcripts would be expected to undergo normal translation, whereas the truncated or altered transcripts generated by Pt-TFO inhibition may actively interfere with processes downstream of transcription, resulting in a decreased level of protein synthesis.

Transfection of LAPC-4 cells with fluorescein-labeled TFO allowed us to focus on cells that had taken up high concentrations of TFO. Under these conditions, transfection with Pt-TFO X resulted in an approximately 30% reduction in the level of AR protein compared to that of cells treated with control Pt-NBC. Significantly less reduction (~18%) was seen when AR protein levels were determined by Western blot analysis, which monitors all cells, including those that have taken up little or no TFO along with those in which high concentrations of TFO have accumulated.

The results of our experiments are consistent with a mechanism by which Pt-TFO X binds and cross-links to its designated target site in the AR gene and, as a consequence, inhibits its transcription and subsequent AR protein synthesis. Although the levels of knockdown of AR mRNA and protein are modest, the results suggest that Pt-TFOs have potential as agents to control gene expression at the DNA level. Modifications to the platinum(II) group that increase stability and cross-linking activity and improvements in delivery methods could enhance the efficacy of these triplex-forming oligonucleotides.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AR, androgen receptor; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; mr, 2′-*O*-methylribo; PAGE, polyacrylamide gel electrophoresis; G^{Pt}, 3′-N7-*trans*-chlorodiammineplatinum(II)-2′-deoxyguanosine; Pt-TFO, platinated TFO; SAX, strong anion exchange; TFO, triplex-forming oligonucleotide; T_m, melting temperature.

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