# Protamine-Fragment Peptides Fused to an SV40 Nuclear Localization Signal Deliver Oligonucleotides That Produce Antisense Effects in Prostate and Bladder Carcinoma Cells

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The development of antisense technology has focused on improving methods for oligonucleotide delivery into cells. In the present work, we describe a novel strategy for oligonucleotide delivery based on a bifunctional peptide composed of a C-terminal protamine-fragment that contains a DNA-binding domain and an N-terminal nuclear localization signal sequence derived from the SV40 large-T antigen (The sequences of two of the peptides are R6WGR6−PKKKRKV [s-protamine-NLS] and R4SR6FGR-6VWR4−PKKKRKV [l-protamine-NLS]). We demonstrated, by intrinsic fluorescence quenching, that peptides of this class form complexes with oligodeoxynucleotides. To evaluate delivery, we used a 20-mer phosphorothioate oligomer (Isis 3521) targeted to the 3′-untranslated region of the PKC-α mRNA and G3139, an 18-mer phosphorothioate targeted to the first six codons of the human bcl-2 open reading frame, and complexed them with either of two peptides (s- or l-protamine-NLS). These peptides bind to and deliver antisense oligonucleotides to the nucleus of T24 bladder and PC3 prostate cancer cells, as demonstrated by confocal microscopy. Furthermore, as shown by Western and Northern blotting, the peptide−oligonucleotide complexes produced excellent downregulation of the expression of the complementary mRNAs, which in turn resulted in downregulation of protein expression. However, under certain circumstances (predominately in PC3 cells), incubation of the cells with chloroquine was required to produce antisense activity. Using this strategy, PKC-α protein and mRNA expression in T24 and PC3 cells and bcl-2 expression in PC3 cells was reduced by approximately 75 ± 10% at a minimum concentration of oligomer of 0.25 μM, in combination with 12−15 μM peptide. On the basis of our results, we conclude that arginine-rich peptides of this class may be potentially useful delivery vehicles for the cellular delivery of antisense oligonucleotides. This new strategy may have several advantages over other methods of oligonucleotide delivery and may complement already existing lipid-based technologies.

## INTRODUCTION

Antisense oligonucleotides, especially those containing the phosphorothioate backbone, are currently in clinical trials as potential therapeutic agents and also may be valuable reagents for the validation of gene function. However, at least in tissue culture, antisense oligonucleotides almost invariably must be condensed with a delivery reagent to ensure adequate cellular uptake and release from sequestered sites in the endosomes/lysosomes. The most commonly employed delivery reagents are cationic lipids (e.g., Lipofectin, Lipofectamine), but these reagents may contribute their own cytotoxic effects, which would thus affect the phenotype produced after treatment of the cells with the antisense effector molecule.

Accordingly, a large number of peptide delivery vehicles for antisense oligonucleotides have been devised. Tung and Stein (*1*), in their recent review of covalent peptide−oligonucleotide conjugates, have listed approximately 20 peptides that increase the delivery of oligonucleotides to cells. These include pH sensitive fusogenic peptides (*2*−*5*), Antennapedia-type peptides (*6*, *7*), and the HIV tat C-terminus peptide (*8*, *9*). Other peptides that have been covalently conjugated to oligonucleotides include the ER-retaining peptide YKDEL (*10*) and various nuclear localization signal peptides (including the PKKKRKV sequence derived from the SV40 large-T antigen (*11*−*14*).

Noncovalent peptide oligonucleotide complexes have also been employed to increase cellular delivery (reviewed by (*15*)). Morris et al. (*16*) used a 27-mer peptide, called MPG, which was composed of the N-terminal domain of the HIV gp41 fusion sequence fused to the C-terminal domain derived from the nuclear localization signal derived from the SV40 large-T antigen. Nuclear localization of oligonucleotide in fibroblasts was observed. Pichon et al. (*3*) employed a permeabilizing peptide derived ultimately from an analogue of the N-terminal sequence of the HA2 subunit of the influenza virus hemagglutinin. Permeabilization was successful as judged by the nuclear localization of a fluoresceinated oligonucleotide. This

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same group (*17*) subsequently employed histidylated oligolysines to deliver antisense oligonucleotides targeted to ICAM-1, which demonstrated excellent antisense activity.

However, with this exception, in almost all the examples of peptide-mediated delivery given above, it is unclear if any sequence specific downregulation of the expression of a target mRNA was observed. In this work we have produced a bifunctional peptide comprising a C-terminal DNA-binding domain which is a protamine fragment, and an N-terminal nuclear localization signal derived from the SV40 large-T antigen. We demonstrate that peptides of this class both bind to and deliver antisense oligonucleotides to cells, and in addition, permit antisense activity by the oligonucleotide. However, in some cases, nuclear delivery, as well as antisense competency, only occur if the cells are pretreated with the lysosomotropic agent chloroquine, thus highlighting the difficulties that oligonucleotide/peptide complexes have in penetrating the endosomal barrier unassisted.

The antisense oligonucleotides we have employed are the 20-mer phosphorothioate antisense PKC-α, first developed by Dean and colleagues (*18*), which we have recently used to evaluate a novel type of porphyrin carrier (*19*). This oligomer, and G3139, the 18-mer antisense bcl-2 oligonucleotide (*20*) we have also employed in this study, are widely considered to be sequence-specific inhibitors of gene expression. Because of this property, they are both well suited for the evaluation of a novel delivery strategy.

## MATERIALS AND METHODS

**Peptide Synthesis.** The preparation of these peptide sequences required the difficult coupling of multiple positively charged amino acid monomers adjacent to each other. To prepare them in reasonable yields, we used the following monomers, reagents and conditions: Fmoc-Arg-(Pbf)-OH (pentamethyldihydrobenzofuransulfonyl), Fmoc-Ser(tBu)-Opfp (*tert*-butyl), Fmoc-Trp(tBoc)-Opfp (*tert*-butyloxycarbonyl), Fmoc-Lys(tBoc)-Opfp (*tert*-butyloxycarbonyl), Fmoc-Lys(Mtt)-OH (methoxytrityl), Fmoc-Phe-Opfp, Fmoc-Gly-Opfp, Fmoc-Val-Opfp and Fmoc-Val-OH. The resin used was PAC−PEG-PS, (PerSeptive Biosystems, UK). Amino acids, *N*,*N*-dimethylformamide (DMF), 2-(1*H*-benzotriazole-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DIPEA (diisopropylethylamine), and TFA (triethylsiliane) were purchased from Auspep Australia. HOBt (1-Hydroxybenzotriazole) was purchased from Fluka, TIS (triethylsilane) from Aldrich, and piperidine from Merck.

A 0.05 mmol scale peptide synthesis, using PAC−PEG-PS resin with a loading of 0.19 mmol/g, was carried out on a Rainin PS3 Automated Solid-Phase Peptide Synthesizer. Fmoc-amino acids were used in 5 mol equiv and activated with 5 mol equiv of HOBt or HBTU in 1 M DIPEA (in DMF) for 5 min, followed by a 35 min coupling. HBTU was used for the free alcohols and HOBt for the Opfp esters. Fmoc deprotection was carried out in 20% piperidine in DMF. Cleavage and side chain deprotection involved treating the peptide with a mixture containing 95% TFA, 2.5% TIS, and 2.5% $H_2O$, for 2 h at room temperature with constant stirring. The peptide was then isolated by ether extraction.

**Purification and Characterization of the Peptides.** Reverse phase HPLC was carried out using a Vydac 218TP C18 protein and peptide RP 5 μM column (4.6 × 250 mm) on a Waters 996 Photodiode Array Detector. Samples were monitored at 254 nm.

A mobile gradient buffer of 0.1% TFA and HPLC grade acetonitrile (0−60% in 30 min) were employed. The retention times for the peptides (not all of which were employed as delivery agents) are R6WGR6 (18 min), R6WGR6−PKKKRKV (19 min), R6WGR6−PKGKRKV (21min), R6WGR6−KPKRKVK (19 min), R4SR6FGRVWR4 (21.5 min), R4SR6FGR6VWR4−PKKKRKV (25.5 min), R4SR6FGR6VWR4−PKGKRKV (20 min), and R2FGR2VWR2 (19 min).

Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) was carried out on a Bruker Biflex spectrometer, and all data was collected and analyzed using Solaris software. Peptides were analyzed in positive linear mode as 50 ρM in 3:7 acetonitrile/water and 0.1% trifluoroacetic acid.

**Cell Culture.** Cells were obtained from American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852). T24 cells were grown in McCoy's 5A medium (Gibco BRL, Grand Island, NY), containing 10% (v/v) heat-inactivated (56 °C) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), supplemented with 25 mM Hepes, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. PC3 cells were grown in RPMI 1640 medium (Gibco BRL), containing 10% (v/v) FBS, to which were added 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Stock cultures were maintained at 37 °C in a humidified 5% $CO_2$ incubator.

**Reagents.** The anti-PKC-α mouse monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). The anti-bcl-2 mouse monoclonal antibody was purchased from Dako (Carpinteria,CA). The anti-mouse horseradish peroxidase conjugated secondary antibody was from Amersham (Arlington Heights, IL).

**Synthesis of Oligonucleotides.** Phosphorothioate oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) model 380B DNA. Following cleavage from controlled pore glass support, oligodeoxynucleotides were base-deblocked in ammonium hydroxide at 60 °C for 8 h and purified by reversed phase HPLC [0.1 M triethylammonium bicarbonate (TEAB)/acetonitrile, PRP-1 support]. Oligomers were detritylated in 3% acetic acid and precipitated with 2% lithium perchlorate/acetone, dissolved in sterile water, and reprecipitated as the sodium salt from 1 M NaCl/ethanol. Concentrations were determined by UV spectroscopy.

The sequences of the oligonucleotides used were Isis 3521 (targeted to the 3′ region of the PKC-α mRNA), 5′-GTTCTCGCTGGTGAGTTTCA-3′ (*18*); G3139 (targeted to the first six codons of the human bcl-2 open reading frame), 5′-TCTCCCAGCGTGCGCCAT-3′ (*20*). The sequence of the control, scrambled oligonucleotide (Isis 4559) is 5′-GGTTTTACCATCGGTTCTGG-3′ (*18*).

**Treatment of Cells with Oligonucleotide−Peptide Complexes.** Cells were grown in six-well plates until 65−75% confluent. The peptides, at the stated peptide/oligonucleotide molar ratios, were diluted in 200 μL of distilled water, and oligonucleotide was then added to the required concentration. The solution was mixed gently and incubated at room temperature for 30 min to allow oligonucleotide−peptide complexes to form. Then, 300 μL of Opti-MEM media was added, and the solution was mixed and overlaid onto the cells, which had been rinsed with Opti-MEM medium and pretreated with 500 μL 50 μM chloroquine for 15 min (the chloroquine with final concentration 25 μM was allowed to remain in solution during incubation with the complexes). The cells were then incubated at 37 °C for 5 h or refed with complete media containing 10% FBS and allowed to

incubate for an additional 19 h (for PKC-α) or 68 h (for bcl-2) before cell lysis and extract preparation.

**Fluorescence Titrations.** Fluorescence experiments were performed on an Aminco Bowman series 2 luminescence spectrometer (SLM Aminco, Urbana, IL). The intrinsic tryptophan fluorescence of peptide was excited at 290 nm, and the emission spectrum was recorded between 310 and 410 nm with a spectral band-pass of 4 nm. A fixed concentration of protein (0.5 μM) was titrated by increasing the concentration of Isis 3521 (from 0 to 0.35 μM) at room temperature in PBS buffer. Curve fitting was performed with the Grafit program (Excel Software).

**Confocal Microscopy.** T24 cells were seeded in glass bottom microwells (MatTec Corp., Ashland, MA) and treated with 5′-fluorescein-Isis 3521 (2 μM) complexed with 15 μM s-protamine-NLS in the presence of 25 μM chloroquine at 37 °C for 5 h. Cellular internalization was examined using an LSM 410 laser scanning confocal microscope (Zeiss, Thornwood, NY) equipped with a krypton/argon laser and attached to a Zeiss Axiovert 100 TV microscope. The 515−540 nm band-pass for fluorescein was used. Z-series were taken of a 1 to 2 micron optical section at 2 μm intervals. For measurements, a maximum projection of all sections was employed. Images were printed using Adobe Photoshop.

**Western Blotting.** Cells were treated with oligodeoxynucleotide−peptide complexes, scraped, washed with cold PBS, and then extracted in 40−50 μL of lysis buffer [50 mM Tris-HCl, pH 7.5; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 50 μg/mL Pefablock SC; 15 μg/mL aprotinin, leupeptin, chymostatin, and pepstatin A; 1 mM $Na_3VO_4$; 1 mM NaF] at 4 °C for 1 h. Cell debris was removed by centrifugation at 14000*g* for 20 min at 4 °C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA).

Aliquots of cell extracts containing 20−30 μg of protein were resolved by 10% or 12% SDS−polyacrylamide gel electrophoresis and transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL). Filters were incubated at room temperature for 1−2 h in Blotto A [5% nonfat milk powder in TBS-T: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (for PKC-α) and 5% BSA in PBS, 0.5% Tween (for Bcl-2)] and then probed overnight at 4 °C with 1:1000 dilution of anti-PKC-α or 1:500 dilution of anti-Bcl-2 in Blotto A. After washing in TBS-T or PBS-T buffer (3 × 7 min, room temperature), filters were incubated for 1 h at room temperature in 5% milk/TBS-T or 5% milk/PBS-T buffer containing a 1:3000 dilution of peroxidase-conjugated anti-mouse secondary antibody. The filters were then washed (3 × 10 min, room temperature), and ECL was performed according to the manufacturer's instructions.

**Northern Blot Analysis**. Total cellular RNA was isolated using TRIZOL Reagent (Gibco BRL). 30−40 μg was resolved on 1.2% agarose gel containing 1.1% formaldehyde and transferred to Hybond-N nylon membranes (Amersham). Human PKC-α and bcl-2 cDNA probes were $^{32}$P-radiolabeled with [α-$^{32}$P]dCTP by random primer labeling using a commercially available kit (Promega) according to the manufacturer's instructions. The blots were then hybridized with the cDNA probes in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 0.1 mg/mL of salmon sperm DNA overnight at 42 °C. The filters were washed at room temperature, twice for 15 min in 2X SSC and 0.1% SDS, once for 20 min in 1X SSC and 0.1% SDS, and finally twice for 15 min in 0.1X SSC and 0.1% SDS at 65 °C. The filters were exposed to Kodak X-ray film for 12−24 h with intensifying screens at −70 °C and then developed.

**Cellular Viability Assay.** Cells were seeded in 96-well tissue culture plates and treated the next day with oligonucleotide−peptide complexes in the presence of 25 μM chloroquine for 5 h at 37 °C. At the end of this time, the Opti-MEM media was then replaced with complete media containing various concentrations of paclitaxel. After 3 days treatment with paclitaxel (at the indicated concentrations as described in the Results), cellular viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, the cells were incubated for 4 h at 37 °C, 5% $CO_2$ with 0.5 mg/mL MTT in complete media. An equal volume of solubilization solution (10% SDS in 0.01 M HCl) was then added and allowed to incubate overnight at 37 °C. After the formazan crystals were dissolved, the plates were read on a Dynatech MR600 Microplate Reader at 540 nm.

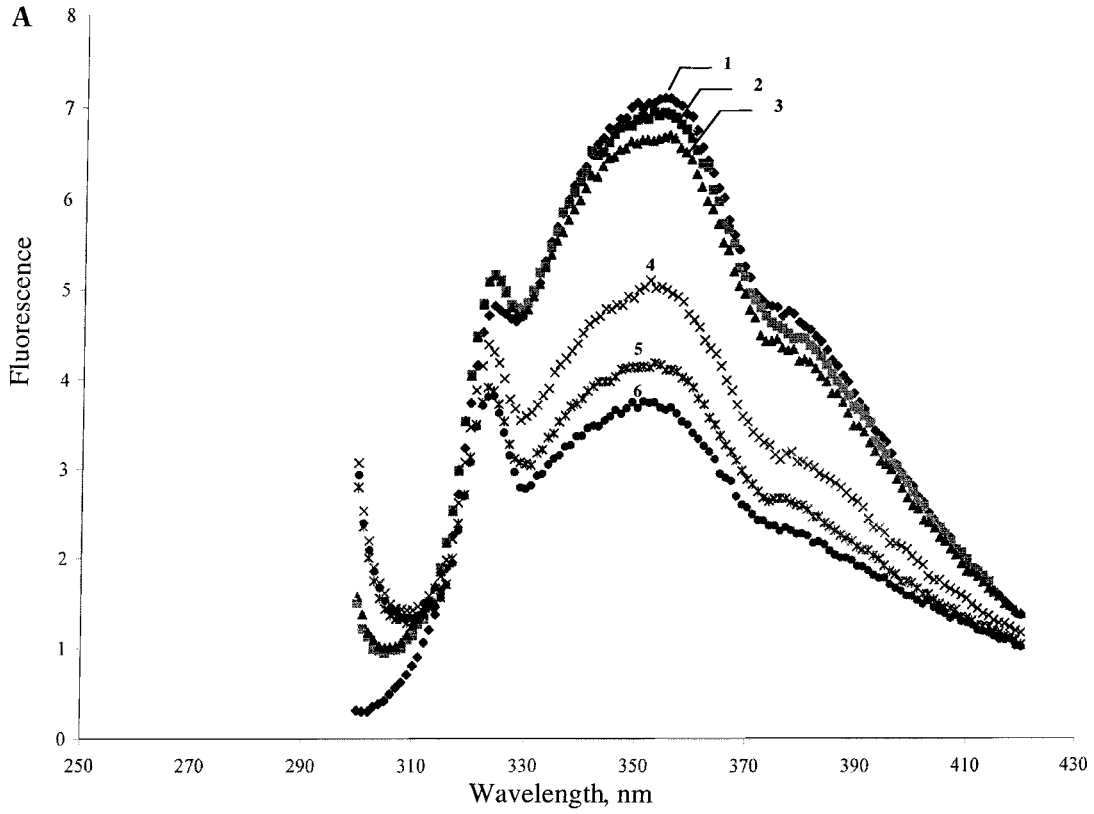
Experiments were performed in quadruplicate and data are expressed as the mean absorbance ± the SD. Statistical analysis of the results was performed using the Analysis ToolPack provided by Microsoft Excel. A Student's two-sample t-test, assuming unequal variances, was used to determine the equality of the means of two samples (* = $p < 0.05$).

## RESULTS

**Peptides Form Complexes with Oligodeoxynucleotides.** The interaction of Isis 3521, a 20-mer phosphorothioate antisense oligonucleotide, with protamine-NLS peptides was evaluated by fluorescence spectroscopy. The peptide contains a single Trp residue, which can be excited at 290 nm and emits fluorescent light at 330 nm. The ability of a bound oligonucleotide to quench this fluorescent emission constitutes a relatively sensitive probe for the monitoring of molecular interactions.

As shown in Figure 1A, the titration of a 0.5 μM solution of l-protamine-NLS with increasing concentrations of Isis 3521, both in PBS and in Opti-MEM (without serum, to mimic our transfection conditions), led to dramatic fluorescence quenching. At low oligonucleotide concentration (relative to l-protamine-NLS peptide) a plot of the decrease in fluorescence (due to quenching) vs oligonucleotide concentration fits an equation of the Michaelis−Menton type (Figure 1B). A Lineweaver−Burke (double reciprocal) plot gives $K_d$ = 80 nM ($R^2$ = 0.99). However, as oligonucleotide concentration is increased, Trp fluorescence continues to decline, but a similar plot no longer fits a Michaelis−Menton-type equation, and the binding becomes increasingly complex (not shown). Similarly, for the s-peptide-NLS, the binding at relatively low oligonucleotide concentration also fits a single-site model of the Michaelis−Menton type (Figure 1C). A Lineweaver−Burke plot of the data gives $K_d$ = 2 μM. At higher oligonucleotide concentration, the single site binding model also breaks down. For both peptides, these data are somewhat approximate because we could not obtain measurements at lower oligonucleotide concentrations than those indicated.

**Protamine-NLS Peptides Deliver Oligonucleotides to the Cell Nucleus.** We evaluated the ability of the protamine-NLS peptides to deliver the oligonucleotides into the cell nucleus by confocal microscopy. 5′-Fluorescein-labeled ISIS 3521 (2 μM) was complexed with s-protamine-NLS-peptide (15 μM) and incubated with T24 cells in the presence of chloroquine (25 μM). As shown in Figure 2, the peptide effectively delivered fluoresceinated oligonucleotide to the cell nucleus after


A
Fluorescence
Wavelength, nm
1
2
3
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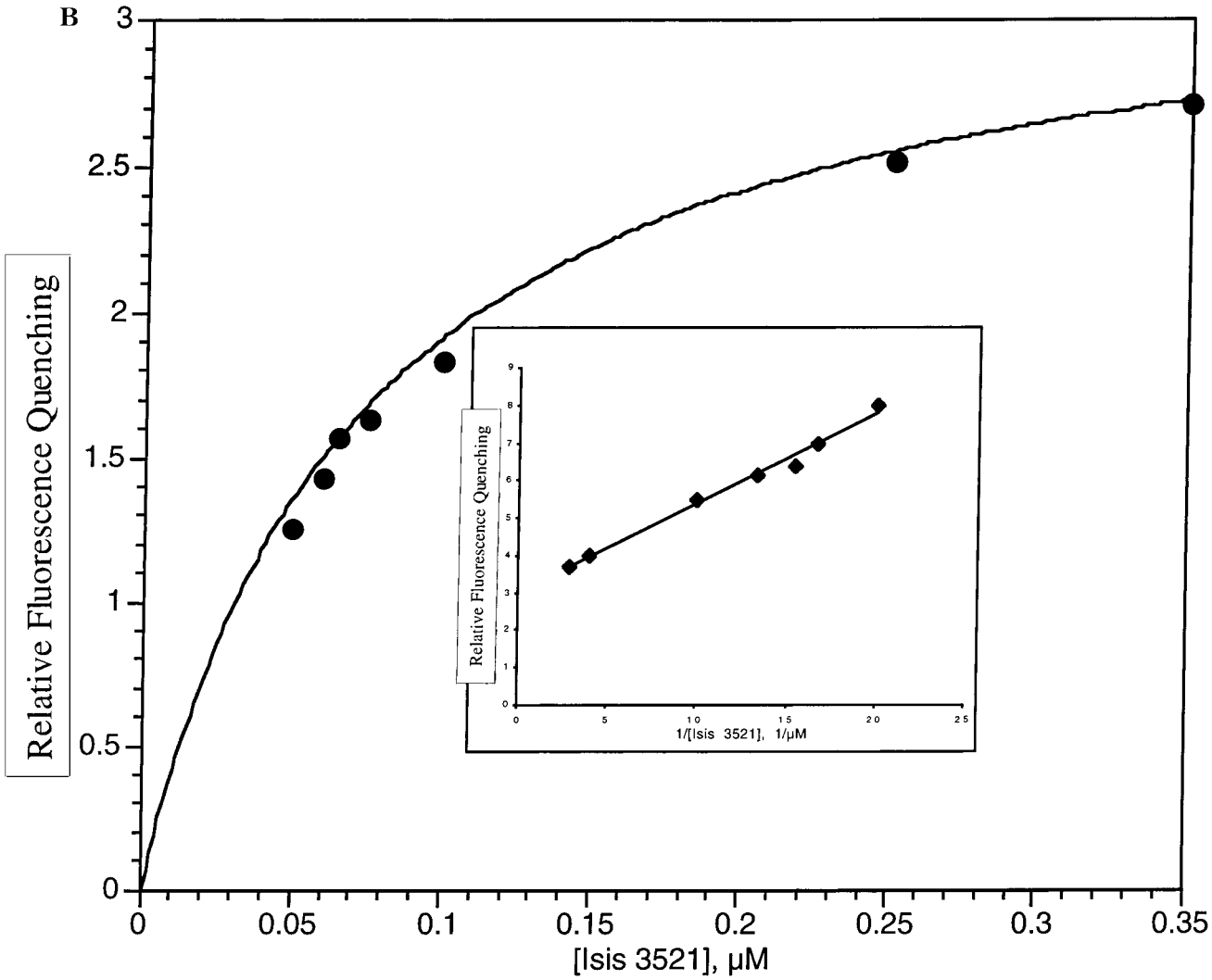
5
6


B
Relative Fluorescence Quenching
[Isis 3521], μM
Relative Fluorescence Quenching
1/[Isis 3521], 1/μM

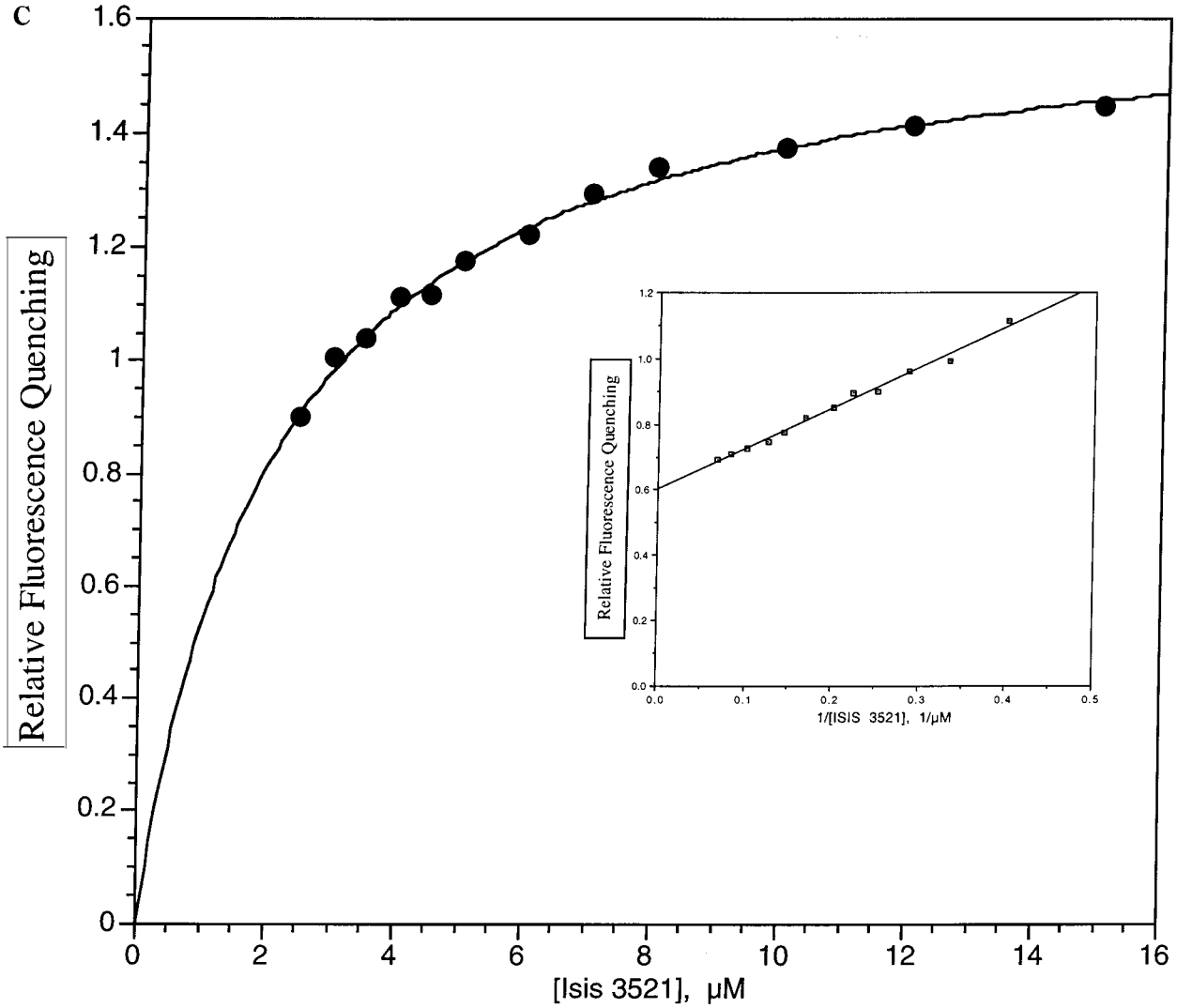



**Figure 1.** (A) Binding of l-protamine-NLS (0.5 $\mu$M) to increasing concentrations of Isis 3521 as monitored by intrinsic fluorescence quenching. The concentrations of Isis 3521 are (traces 1−6, respectively) 0, 0.06, 0.075, 0.1, 0.25, and 0.35 $\mu$M. The fluorescence of l-protamine-NLS was excited at 290 nm, and the emission spectrum was recorded between 310 and 410 nm. (B) Relative fluorescence of l-protamine-NLS at 350 nm plotted as a function of the concentration of Isis 3521, with extrapolation to zero concentration. The solid line represents the data plotted as per the Michaelis−Menton equation. The insert depicts a Lineweaver−Burke (double reciprocal) plot of these data. (C) Relative fluorescence of s-protamine-NLS at 350 nm plotted as a function of the concentration of Isis 3521, with extrapolation to zero concentration. The solid line represents the data plotted as per the Michaelis−Menton equation. The insert depicts the Lineweaver−Burke (double reciprocal) plot of these data.

an incubation time of 5 h. In the absence of peptide, oligonucleotides were internalized poorly by the cells, with most of the internalized material accumulating in a punctate pattern, signifying localization in vesicular structures (endosomes/lysosomes; not shown).

Surprisingly, under the same conditions and reagent concentrations, the s-protamine-(scrambled)NLS also promotes the delivery of the oligonucleotide to the nucleus (not shown). However, as described below, when delivered in this fashion, the oligonucleotide does not downregulate the expression of PKC-α protein as determined by Western blotting.

**Peptide−Oligodeoxynucleotide Complexes Demonstrate Antisense Activity.** *1. s-Protamine-NLS Complexes with Oligonucleotides.* Experiments using the protamine-NLS peptides to deliver antisense oligonucleotides were performed in T24 bladder carcinoma and PC3 prostate carcinoma cells. In addition to the 20-mer ISIS 3521, we also employed the 18-mer phosphorothioate oligonucleotide G3139, which is targeted to the first six codons of the human bcl-2 open reading frame. The

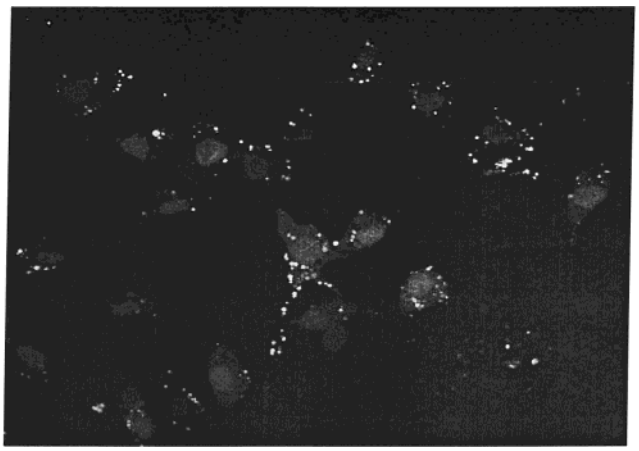


**Figure 2.** s-Protamine-NLS-mediated delivery of Isis 3521 in T24 bladder carcinoma cells. Cells were treated for 5 h at 37 °C with 5′-fluorescein-labeled Isis 3521 (2 $\mu$M) complexed to s-protamine-NLS (15 $\mu$M) in the presence of 25 $\mu$M chloroquine. Cellular localization was monitored by confocal microscopy, as described in the text.

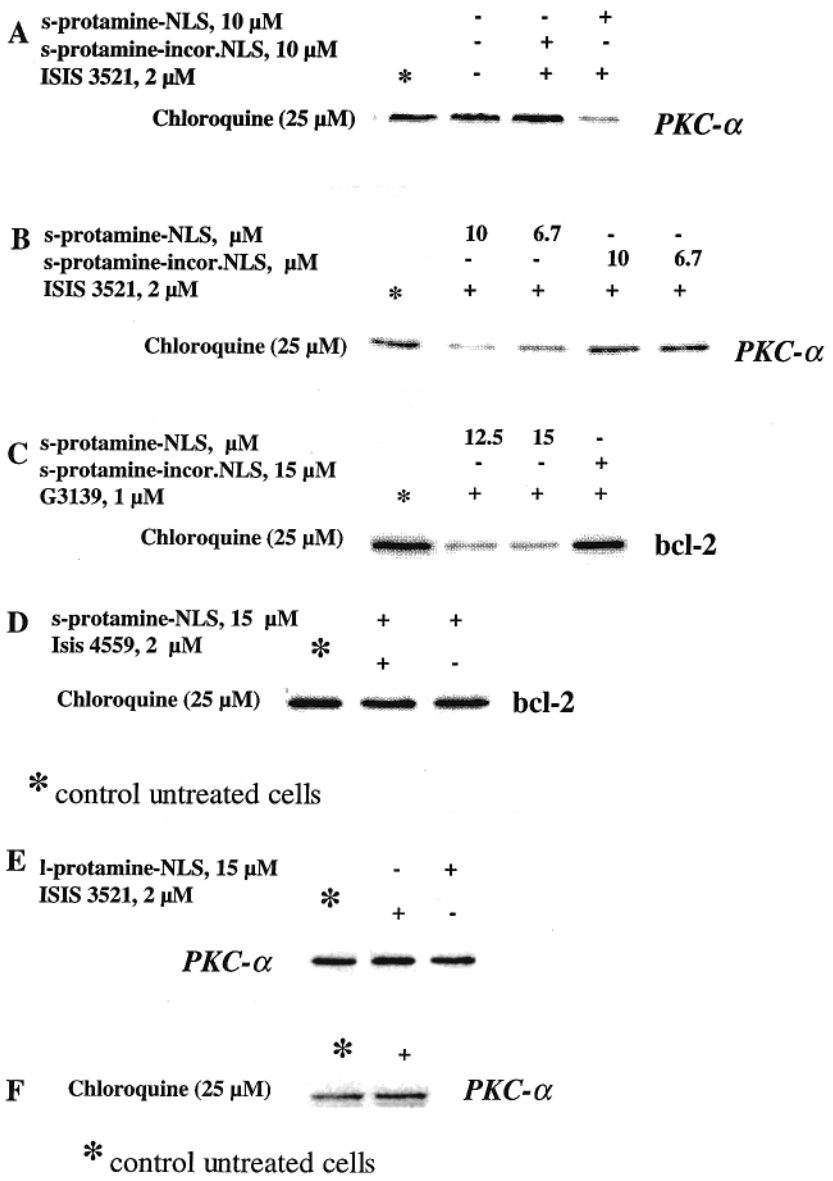



**Figure 3.** Inhibition of PKC-α protein expression by Isis 3521 (2 μM) (A, B) complexed with s-protamine-NLS (at the stated concentrations) in T24 (A) and PC3 (B) cells. (C) Inhibition of bcl-2 protein expression by G3139 (2 μM) complexed with s-protamine-NLS (15 μM) in PC3 cells. (D) Control oligonucleotide Isis 4559 complexed with s-protamine-NLS has no effect on bcl-2 expression in PC3 cells. (E) Uncomplexed l-protamine-NLS (15 μM) and Isis 3521 oligonucleotide in the presence of 25 μM chloroquine have no effect on PKC-α protein expression in PC3 cells. (F) Chloroquine (25 μM) alone has no effect on PKC-α protein expression in T24 cells. Cells were treated with complexes of Isis 3521 or G3139 oligonucleotides/s-protamine-NLS or control s-protamine-(incorrect)-NLS in the presence of 25 μM chloroquine for 5 h at 37 °C. 19 h (for PKC-α) or 67 h (for bcl-2) later, total protein was harvested, and Western blot analysis (25−30 μg of protein/lane) performed with anti-PKC-α and anti-bcl-2 antibodies as described in the Materials and Methods.

optimum incubation time of the cells with the peptide/oligonucleotide complexes was 5 h for Isis 3521 and G3139, followed by incubation periods of 24 h and 3 days, respectively.

To generate antisense effects, cells treated with s-protamine-NLS complexes with oligonucleotides were also pretreated with 50 μM chloroquine for 15 min. The chloroquine (final concentration = 25 μM) was allowed to remain in solution during incubation with the complexes (i.e., for 5 h). s-Protamine-NLS, when complexed to ISIS 3521 (2 μM), yielded significant antisense activity in both T24 (Figure 3A) and PC-3 cells (Figure 3B). By Western blotting and scanning densitometry, the expression of the PKC-α (T24 and PC3 cells) and Bcl-2 (Figure 3C; PC3 cells only) proteins were all reduced by approximately 70−80%. Naked oligomer (i.e., not complexed with peptide), at the identical concentration (2 μM) + 50 μM chloroquine preincubation and then 25 μM during the incubation period, was completely ineffective (Figure 3E), most likely because of the sequestration of the oligonucleotides in endosomes/lysosomes, as demonstated by confocal microscopy. A control, scrambled phosphorothioate oligonucleotide (Isis 4559; 2 μM)) complexed with the s-protamine-NLS-peptide, and ISIS 3521 complexed with s-protamine-(incorrect)-NLS-peptide under identical conditions (+ 50 μM chloroquine preincubation and then 25 μM during the incubation period), did not produce any reduction in target PKC-α protein levels in either T24 or PC3 cells (Figure 3A and 3B). Furthermore, 25 μM chloroquine alone did not downregulate PKC-α expression (Figure 3F), and neither the l-protamine-NLS alone (15 μM) nor Isis 3521 alone (Figure 3E) were active.

In addition, G3139 when complexed to s-protamine-NLS-peptide was "antisense" active in PC3 cells (Figure

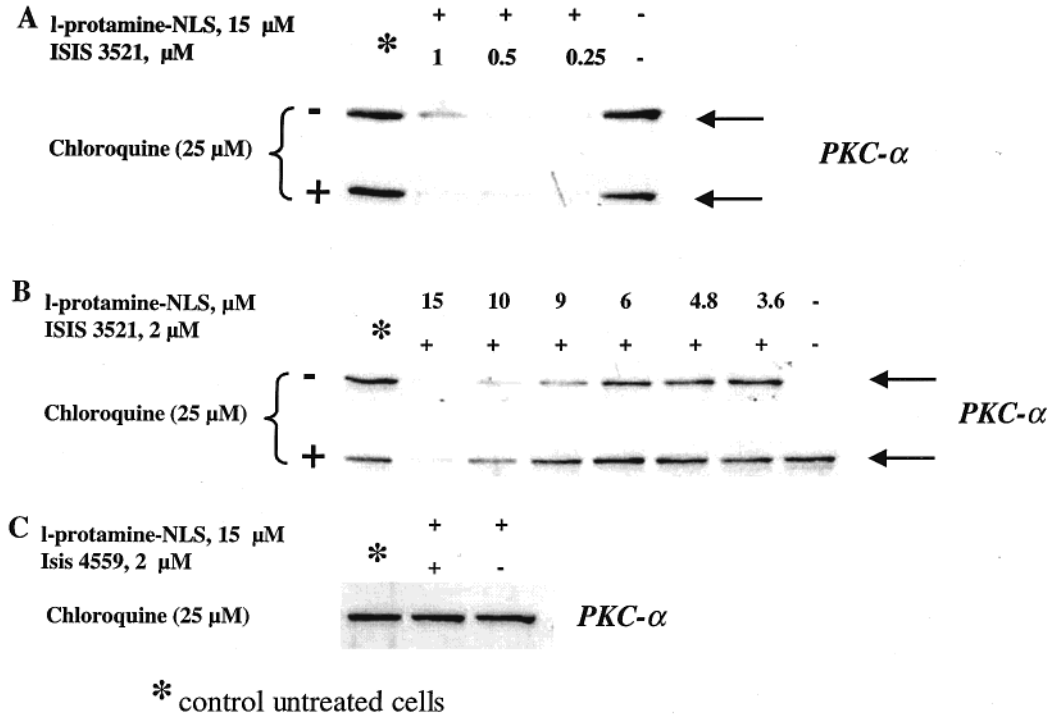



**Figure 4.** Inhibition of PKC-α protein expression in T24 cells by Isis 3521 (at the stated concentrations) complexed with l-protamine-NLS (15 μM) (A) and Isis 3521 (2 μM)/l-protamine-NLS (at the stated concentrations) (B) in the presence or absence of chloroquine. (C) Control oligonucleotide Isis 4559 (2 μM) complexed with l-protamine-NLS (15 μM), and uncomplexed l-protamine-NLS (15 μM), in the presence of 25 μM chloroquine, have no effect on PKC-α protein expression in T24 cells. Cells were treated with complexes of Isis 3521 or Isis 4559/l-protamine-NLS in the presence or absence of chloroquine for 5 h at 37 °C. 19 h later, total protein was harvested, and Western blot analysis (25−30 μg of protein/lane) was performed as described in the text.

3C). Similar controls as employed above (scrambled oligonucleotide Isis 4559, incorrect NLS sequence (15 μM), chloroquine alone (50 μM pretreatment then 25 μM during incubation; Figure 5C) were all totally inactive. However, we were not able to inhibit the expression of bcl-2 protein expression in T24 cells at any concentration of peptide/oligonucleotide complex.

*2. l-Protamine-NLS Complexes with Oligonucleotides.* We also evaluated the ability of a longer protamine peptide to diminish PKC-α and Bcl-2 protein expression in T24 and PC3 cells. Complexes of this peptide with the ISIS 3521 or the G3139 oligonucleotides were active over approximately the same concentration range in both cell lines. In T24 cells, as shown in the Western blots in Figure 4A and 4B, pretreatment by 50 μM chloroquine, and then treatment with 25 μM chloroquine during the incubation time was not necessary for the induction of an antisense effect. Instead, at a constant 2 μM concentration of Isis 3521 (Figure 4B), the concentration of peptide required for equivalent downregulation of PKC-α protein expression was slightly, but not dramatically, increased in the absence of chloroquine. At constant l-protamine-NLS concentration (15 μM; Figure 4A), the oligonucleotide concentrations required for maximal downregulation of PKC-α activity were also virtually identical in the presence and absence of chloroquine. However, the l-protamine-NLS (15 μM) complex with a control oligomer, Isis 4559 (2 μM; Figure 4C), did not produce any downregulation of PKC-α expression under the same experimental conditions. Congruent with these observations, the 8.5 kb and 4.0 kb PKC-α mRNA transcripts, as demonstrated by Northern blotting, were downregulated in the absence of chloroquine (Figure 6A).

In contrast, in PC3 prostate cancer cells, chloroquine (50 μM pretreatment + 25 μM during the incubation time) was still required for antisense activity. As shown in Figure 5A, a molar ratio of l-protamine-NLS/Isis 3521 of 7.5 provided maximal downregulation of PKC-α protein expression until a concentration of 1.9/0.25 μM was achieved. However, at a constant concentration of l-protamine-NLS (15 μM), Isis 3521 concentrations as low as 0.25 μM were still active (Figure 5B). Nevertheless, a decrease in peptide concentration to 10 μM virtually eliminated the antisense effect of Isis 3521. Similar to what was observed in the T24 cells, the control oligomer Isis 4559 (2 μM) complexed with 15 μM l-protamine-NLS was equally ineffective in the PC3 cells (Figure 5D). Furthermore, an l-protamine-NLS peptide in which the NLS sequence was mutated from PKKKRKV to PKGK-RKV did downregulate PKC-α protein expression (Figure 5D), but not quite to the same extent as the NLS peptide with the correct sequence.

G3139 (2 μM-0.25 μM) in complex with l-protamine-NLS (15−10 μM; Figure 5C) could effectively downregulate the expression of both bcl-2 protein (after 3 days) and mRNA (after 1 day; Figure 6B), but again only after pretreatment of the PC3 cells with 50 μM chloroquine and then treatment with 25 μM chloroquine during the incubation time. However, at lower peptide concentrations, antisense inhibition was lost (Figure 5C).

**Downregulation of PKC-α Increases Sensitivity of T24 Bladder Carcinoma Cells to Paclitaxel.** Reductions in cell proliferation was determined by MTT assay. Data are shown in Figure 7A and 7B, as determined after 3 days of drug treatment. This followed 24 h treatment of the T24 cells by complexes of s-protamine-NLS (15 μM)/Isis 3521 (2 μM) complexes and pretreatment of the cells with 50 μM chloroquine and then treatment with 25 μM during the incubation period. Isis 3521, s-protamine-NLS peptide, and 50 μM and then 25 μM chloroquine alone had no effect on cell viability, as assessed by MTT assay. Complexes of s-protamine-NLS

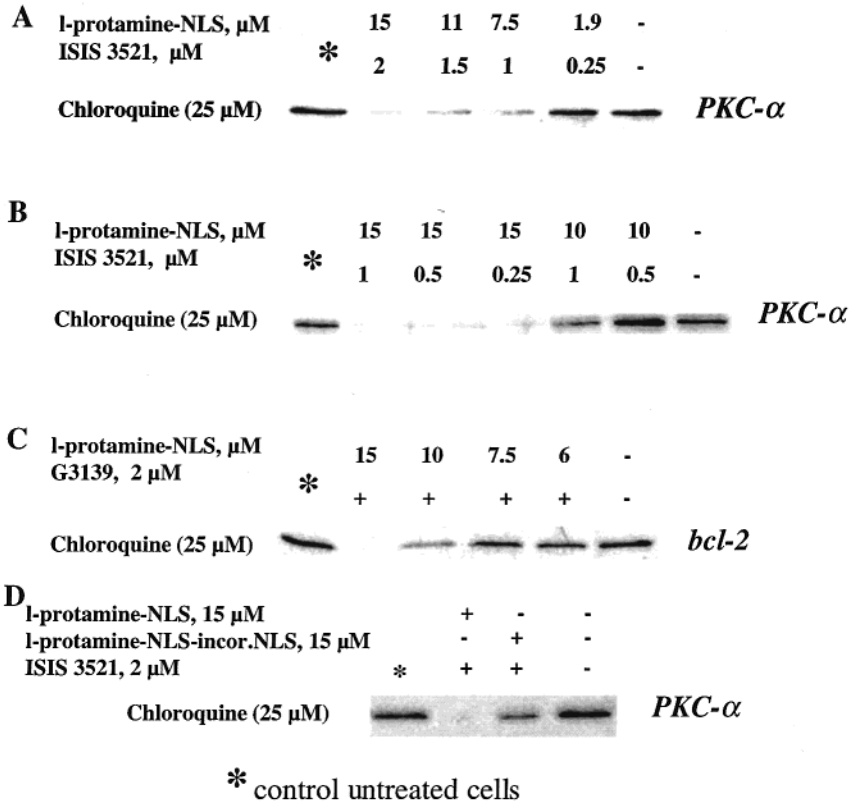



**Figure 5.** Inhibition of PKC-α protein expression by Isis 3521 (at the stated concentrations) complexed with l-protamine-NLS (at the stated concentrations) in PC3 cells (A, B). (C) Inhibition of bcl-2 protein expression by G3139 (2 $\mu$M) complexed with l-protamine-NLS (at the stated concentrations) in PC3 cells. (D) Complexes of Isis 3521/l-protamine-(incorrect)-NLS in the presence of 25 $\mu$M chloroquine have a diminished effect on PKC-α protein expression in PC3 cells, while chloroquine alone (25 $\mu$M) has no effect. Cells were treated with complexes of Isis 3521 or Isis 4559/l-protamine-NLS or complexes of Isis 3521/l-protamine-(incorrect)-NLS in the presence of 25 $\mu$M chloroquine for 5 h at 37 °C. 19 h later, total protein was harvested, and Western blot analysis (25−30 $\mu$g of protein/lane) was performed, as described in the text.

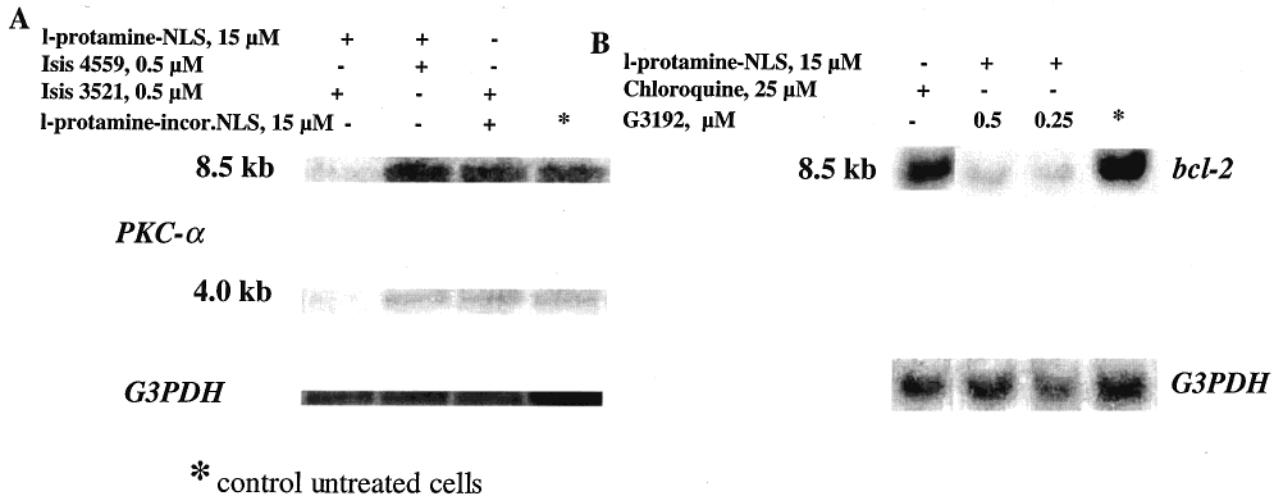



**Figure 6.** Inhibition of PKC-α mRNA expression by Isis 3521 (0.5 $\mu$M) complexed with l-protamine-NLS (15 $\mu$M) in T24 cells (A) and bcl-2 mRNA expression by G3139 (0.25 and 0.5 $\mu$M) complexed with l-protamine-NLS (15 $\mu$M) in PC3 cells (B). Cells were treated with complexes of Isis 3521, G3139 or control oligonucleotide Isis 4559/l-protamine-NLS in the presence of 25 $\mu$M chloroquine for 5 h at 37 °C. 19 h later, total mRNA was isolated, and Northern blot analysis was performed as described in the Materials and Methods. 30−40 $\mu$g of mRNA was loaded in each lane of the 1.2% denaturing agarose gel, transferred to Hybond-N nylon membranes, and probed with either a PKC-α, bcl-2, or control G3PDH cDNA probe.

(scrambled)-peptide/Isis 3521 reduced cellular viability to a much lesser extent. Only treatment of the cells with s-protamine-NLS/Isis 3521 was able to reduce cellular viability.

## DISCUSSION

The peptides in this study were designed in an attempt to satisfy the major requirements for efficient oligonucleotide delivery to the cell nucleus, and, what is of course the ultimate goal, the retention of antisense activity. We believe that these requirements for peptide sequence include (1) the ability to bind to oligodeoxynucleotides, (2) the ability of the peptide−oligonucleotide complexes to penetrate the endosomal/lysosomal membrane, and (3) the ability of the peptide to promote nuclear localization.

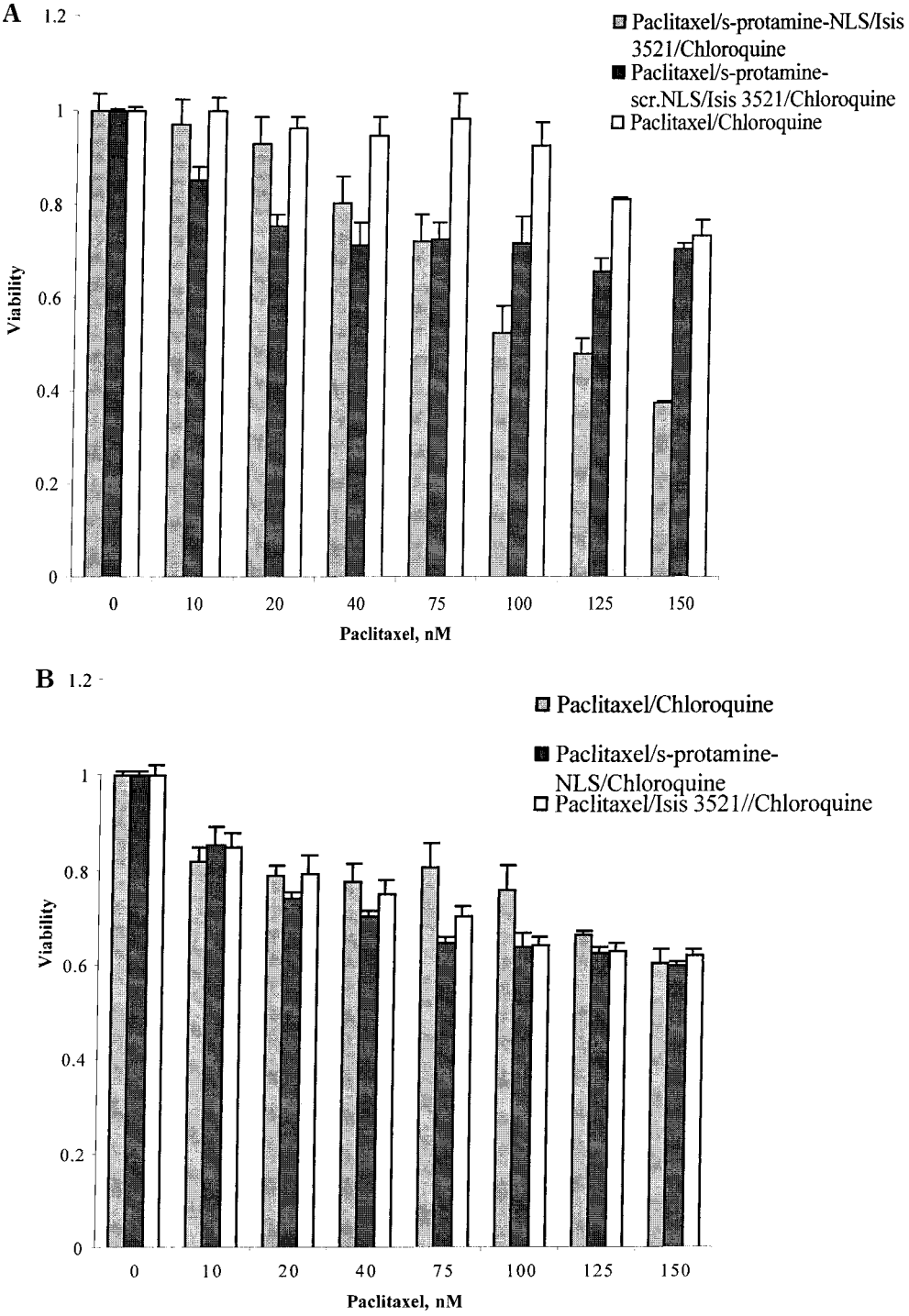



**Figure 7.** (A) Diminution in T24 cellular viability induced by the Isis 3521/s-protamine-NLS complex in response to increasing concentrations of paclitaxel. Neither control s-protamine-(scrambled)-NLS complexed to Isis 3521 (A), nor s-protamine-NLS or Isis 3521 alone (B) have any significant effect on T24 cellular viability in response to increasing concentrations of paclitaxel. Cells were treated with Isis 3521 complexed to s-protamine-NLS or s-protamine-(scrambled)-NLS or either s-protamine-NLS or Isis 3521 alone in the presence of 25 $\mu$M chloroquine for 5 h at 37 °C. The Opti-MEM media was then replaced with complete media containing various concentrations of paclitaxel. After 3 days of incubation, the MTT assay was performed. The absorbance at 540 nm was normalized to the controls to determine cellular viability. Experiments were performed in quaduplicate, and data are expressed as the mean absorbance ± the SD. A Student's two-sample t-test assuming unequal variances was used to determine the equality of the means of two samples (* = $p$ <0.05).

The peptides described in this work satisfy criteria 1 and 3, and with the addition of chloroquine, all of them.

Bull protamine is a 50 amino acid peptide with an arginine rich central domain (ARYRCCLTHSGSRCR-RRRRRRC **RRRRRRFGRRRRRR** VCCRRYTVI.RC-TRQ). The short (s) sequence that we have tested is the central region highlighted in bold, with the exception that we have replaced the phe (F) with a trp (W) amino acid in order to provide an intrinsic fluorescent probe. This region was chosen because it provides the required arginine-rich sequence for oligonucleotide binding, but lacks cysteines which could promote dimerization. The long (l) protamine sequence contains the region in bold above, but the F is retained. Instead, the l-protamine sequence deletes the C and extends the R residues on both the N-and C-termini, as shown in Table 1.

**Table 1. Peptides Used in This Work**

| peptide | sequence | mw | $m/z^{+1}$ |
|---|---|---|---|
| s-protamine | R6WGR6 | 2134.88 | 2136.88 |
| s-protamine-NLS | R6WGR6−PKKKRKV | 2998.97 | 3000 |
| s-protamine-(incorrect)-NLS | R6WGR6−PK**G**KRKV | 2927.97 | 2931 |
| s-protamine-(scrambled)-NLS | R6WGR6−KPKRKVK | 2998.97 | 2999.14 |
| l-protamine | R4SR6FGR6VWR4 | 3716.30 | 3717.03 |
| l-protamine-NLS | R4SR6FGR6VWR4−PKKKRKV | 4580.90 | 4580.9 |
| l-protamine-(incorrect)NLS | R4SR6FGR6VWR4−PK**G**KRKV | 4509.93 | 4509.9 |

It is known that the positively charged guanidinium functional groups (p$K_a$ = 12.5) electrostatically interact with the negatively charged phosphate backbone of DNA. These charge based interactions result in an overall charge neutralization leading to condensation of DNA.

In general, studies of protamine interactions have been carried out for double-stranded DNA. These have involved X-ray diffraction, Raman spectra, chemical reaction, and optical detection of magnetic resonance (*21*−*24*). Some of the reports are conflicting, but regardless it is now thought that the interaction of protamine and double-stranded DNA involves binding to the major groove with a $\beta$-form DNA and $\beta$-conformation for the protamine (*21*, *23*). Unfortunately, the interaction of protamine with single-stranded DNA remains poorly understood. Nevertheless, protamine is known to be an efficient condensing peptide and has been shown to enhance transfection (*25*) and lipid-mediated gene transfer (*26*). In these experiments, the binding of increasing concentrations of oligonucleotide to a much larger concentration of s-protamine-NLS fits a single-site binding model of the Michaelis−Menton type, with $K_d$ as derived from the Lineweaver−Burke analysis equaling 75 nM. The binding of additional molecules of s-protamine-NLS to oligonucleotide is mathematically complex, but on the basis of the spectroscopic measurements, Trp fluorescence is 100% quenched at a peptide/oligomer molar ratio of 4:1 (charge ratio of approximately 3.15:1).

Arginine-rich peptides (e.g., Tat-(48−60) appear to be able to be internalized even at 4 °C by an endocytosis-independent pathway (*27*). Despite this, it seems unlikely that an arginine-rich peptide, when complexed with negatively charged oligonucleotides, will undergo endocytosis-dependent internalization. Experiments with the lysosomotropic agent chloroquine support this idea.

Chloroquine is a weak base (p$K_a$ 10.2 and 8.1) that accumulates in endosomes/lysosomes in a time and concentration-dependent manner. It has been used extensively to improve nuclear delivery of plasmids, but to the best of our knowledge only rarely, if at all, for similar purposes for oligonucleotides (*28*). Intralysosomal concentrations of chloroquine may reach 9 mM, and the compound acts as a buffer to prevent the continuous endosomal acidification process (*29*). This causes a rise in intraendosomal osmolarity and its eventual lysis, releasing peptide/oligonucleotide complexes into the cytoplasm (the "proton sponge" hypothesis (*30*)). It is also possible that, in addition to this mechanism, chloroquine causes some degree of intraendosomal dissociation of the peptide/oligonucleotide complex (*29*). This dissociation may to some extent contribute to the retention of antisense activity (see below), but is probably overshowed in extent by peptide-mediated nuclear delivery.

A large family of nuclear localization signals exists, but the simian virus 40 (SV40 T) large T antigen nuclear localization sequence was chosen because it has resulted in efficient DNA transfer into the nucleus of various cell types, and has enhanced transgene expression (*31*−*34*). The peptide we employed ($^{126}$PKKKRKV$^{132}$) consists of a basic stretch of amino acids containing five consecutive positively charged residues, which are necessary for binding to the nuclear import protein karyopherin $\alpha$ (also known as importin $\alpha$) (*35*). This complex then binds to importin $\beta$ and, with the assistance of the GTPase Ran, is carried through the nuclear pore by mechanisms that are still poorly defined. Nevertheless, particles as large as 25 nm have been shown to be transported into the nucleus in this fashion (*35*). Since in preliminary experiments the particle size of the complexes is nearly 300 nm, significant intracellular decomposition must be occurring.

Lys-128 appears to be crucial for nuclear import (*36*). In this work, we have mutated Lys-128 to Gly and were unable to detect antisense downregulation of PKC-$\alpha$ protein expression. While these data strongly argue for the importance of the NLS in the generation of an antisense effect, other data suggest its role may be complex. For example, an s-protamine-NLS, in which the NLS has been scrambled, also promoted delivery of oligonucleotides to the nucleus, but did not downregulate the expression of PKC-$\alpha$ protein. This observation may suggest that the binding of the NLS to nuclear elements promotes dissociation of the oligonucleotide/peptide complex that results from particle degradation, thereby increasing the level of free nuclear oligonucleotide. However, this is speculative and other explanations may also be possible.

In summary, we have found a novel, efficient, arginine-peptide (and frequently, chloroquine) dependent way to transfer oligonucleotides into the nucleus of T24 and PC3 cells, where they produce downregulation of protein expression that is consistent with antisense effects. Provided that these substances can be manufactured at reasonable cost, they may be important complements to traditional cationic lipids as transfection vehicles.

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