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Inhibition of Potentially Anti-Apoptotic Proteins by Antisense Protein Kinase C-α (Isis 3521) and Antisense bcl-2 (G3139) Phosphorothioate Oligodeoxynucleotides: Relationship to the Decreased Viability of T24 Bladder and PC3 Prostate Cancer Cells

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ABSTRACT

Isis 3521 and G3139 are 20- and 18-mer phosphorothioate oligonucleotides, respectively, targeted to the protein kinase C (PKC)-α and bcl-2 mRNAs. Treatment of T24 bladder and PC3 prostate carcinoma cells with full-length and 3′-truncation mutants of Isis 3521 causes down-regulation of PKC-α protein and mRNA. However, at the level of a 15-mer and shorter, down-regulation of mRNA expression is no longer observed. Further, no diminution in cellular viability, as measured by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide assay, in response to increasing concentrations of paclitaxel, can be observed for these shorter oligomers. These observations not only indicate that PKC-α protein expression can be down-regulated by both RNase H-dependent and -independent mechanisms but also that down-regulation of PKC-α is insufficient by itself to "chemosensitize" cells. G3139, which down-regulates bcl-2 protein and mRNA expression, also down-regulates PKC-α protein and mRNA expression but not that of PKC-βI, -ε, or -ζ. However, the down-regulation of PKC-α and bcl-2 are not linked. When the carrier Eufectin 5 is employed, only bcl-2 is down-regulated in both T24 and PC3 cells at 50 nM oligonucleotide concentration. At 100 nM, both bcl-2 and PKC-α expression are down-regulated, and only at this concentration can "chemosensitization" to paclitaxel and carboplatin be observed. In contrast, the down-regulation of bcl-2 seems to be linked with that of RelA (p65). However, this too is also not sufficient for chemosensitization, even though it leads to the loss of expression of genes under the putative control of nuclear factor-κB and to detachment of the cells from plastic surfaces. These results underscore the complexity of the intracellular requirements for the initiation of chemosensitization to anti-neoplastic agents.

An emerging strategy to circumvent the development of clinical resistance to cytotoxic agents is to specifically target intracellular proteins that block the process of apoptosis. One example of such a protein is bcl-2, which seems to play a critical role in the delay or prevention of apoptosis by a variety of death-promoting signals, suggesting that it interacts with multiple components of the death signaling pathway (de Moissac et al., 1999).

The critical nature of bcl-2 in blocking apoptosis seems to

have manifested in recent clinical trials in advanced melanoma of G3139 (Jansen et al., 1998, 2000), an 18-mer phosphorothioate oligonucleotide targeted to the initiation codon region of the bcl-2 mRNA. These phosphorothioate oligonucleotides contain a sulfur atom substituted for an oxygen atom at a nonbridging site at each phosphorus atom in the oligonucleotide chain. This renders the oligonucleotide nuclease resistant, although it is by no means nuclease-proof. The substitution also retains the property of charge, aqueous solubility, and the ability to support RNase H activity (Stein et al., 1988). RNase H is a ubiquitous cellular enzyme that cleaves the mRNA strand of an RNA/DNA duplex and seems

to be critical for antisense activity (Walder and Walder, 1988). However, although capable of binding to their mRNA targets, binding affinities of phosphorothioates are somewhat lower than for the parent phosphodiester oligonucleotides (Stein et al., 1988). In addition, the specificity of phosphorothioate oligonucleotides in tissue culture is always suspect because of their ability to bind to a wide variety of proteins. These proteins are predominately heparin binding and include, for example, vascular endothelial growth factor (Guvakova et al., 1995), basic fibroblast growth factor (Fennewald and Rando, 1995; Guvakova et al., 1995) and epidermal growth factor receptor (Rockwell et al., 1997). This non–sequence-specific binding may produce numerous biological effects in addition to any sequence-specific, antisense effects that may be observed (Stein, 1999).

G3139 has been shown to down-regulate bcl-2 expression in numerous preclinical tumor models, including breast (Chi et al., 2000), bladder (Miyake et al., 1998), and prostate (Miyake et al., 2000), frequently leading to sensitization to a variety of cytotoxic agents (Jansen et al., 1998). In the advanced melanoma trial, in combination with the anti-neoplastic agent dacarbazine, G3139 induced objective responses in six of 14 heavily pretreated patients (Jansen et al., 2000). Significantly, bcl-2 down-regulation could be observed in at least one patient biopsy specimen. The compound is currently in phase III clinical trials in advanced melanoma.

Another potentially important anti-apoptotic protein to which antisense oligonucleotides have been directed is PKC-α, which belongs to a family of serine/threonine kinases containing at least 11 members. Conventional PKCs, whose activation is dependent on calcium, diacylglycerol, and phosphatidylserine, include PKC-α, -βI, -βII, and -γ. The novel forms, which are calcium-independent, include PKC-δ, -ϵ, -θ, and -η, whereas the atypical forms, which contain only a single cysteine-rich motif in the C1 domain, include PKC-ζ and -λ (Li et al., 1999).

PKC-α seems to play a major role in apoptotic signaling pathways, and its overexpression has been shown, by stimulating Akt activity, to suppress apoptosis in IL3-dependent T32 myeloid progenitor cells (Li et al., 1999). Several years ago, Isis 3521, a 20-mer antisense PKC-α phosphorothioate oligonucleotide, was developed (Dean et al., 1994; Dean and McKay, 1994) to inhibit PKC-α expression and was also employed by Yazaki et al. (1996) to inhibit the growth of the U-87 PKC-α-overexpressing glioma line in experimental animals. Significant prolongation of the life of the animals was observed. Subsequently, others (Shen et al., 1999) demonstrated induction of wild-type p53 and insulin-like growth factor-binding protein-3 after treatment of A172 glioma cells with Isis 3521. Levels of other apoptosis-related proteins, such as bcl-xL and bax, were unchanged. This oligonucleotide, in combination with paclitaxel and carboplatin, has induced responses in five of six heavily pretreated nonsmall lung cancer patients (Sikic et al., 1999). The combination is currently in phase III clinical trials for this tumor and is being studied in others (Yuen et al., 1999).

However, despite these encouraging data, the nonspecific effects of the phosphorothioate backbone interdict too fine a discernment of whether the "chemosensitive" phenotype occurs as a direct result of PKC-α or bcl-2 down-regulation and to what extent it is a combination of sequence- and non–sequence-specific events. Similarly, it is important to under-

stand whether down-regulation of the basal expression of PKC-α and bcl-2 are necessary or are both necessary and sufficient to produce diminished cellular viability, as measured by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay.

In this study, we have further examined the mechanism of action of Isis 3521 and G3139 by, in the first case, determining the ability of 3$'$-truncation mutants of Isis 3521 to inhibit PKC-α protein and mRNA expression. We show that down-regulation of PKC-α protein expression by Isis 3521 may be necessary but is insufficient to diminish cellular viability in response to cytotoxic agents. We also demonstrate that G3139-induced down-regulation of bcl-2, which is apparently linked to that of RelA (p65), is also insufficient to decrease cellular viability. However, we show that G3139 also down-regulates PKC-α expression, which seems to generate sufficiency.

Materials and Methods

Cells. Cells were obtained from the American Type Culture Collection (Manassas, VA). T24 cells were grown in McCoy's 5A medium (Invitrogen, Carlsbad, CA), containing 10% (v/v) heat-inactivated (56°C) fetal bovine serum (Invitrogen), supplemented with 25 mM HEPES, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. PC-3 cells were grown in RPMI plus 10% fetal bovine serum, supplemented by 1% nonessential amino acids, 1% pyruvate, and antibiotics as above. Stock cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

Reagents. The anti-PKC-α monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and the anti-PKC-ζ, -β1, and -ϵ polyclonal antibodies from Invitrogen. An anti-bcl-2 monoclonal antibody was purchased from Dako (Carpinteria, CA). The p65, p50, bax bcl-xL, IκBα, and MMP-9 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The XIAP and c-IAP-1 antibodies were from R & D Systems (Minneapolis, MN). The human PKC-α cDNA for Northern analysis was a generous gift of Dr. I. B. Weinstein (Columbia University, New York, NY). Lipofectin was purchased from Invitrogen and Eufectin 5 from Promega (San Luis Obispo, CA). Tetra(meso)4-methylpyridyl porphine (TMP) is a product of Porphyrin Products, Inc. (Logan, UT). Paclitaxel and carboplatin are products of Bristol-Myers Squibb (Princeton, NJ). Lactacystin and MG132 were purchased from Calbiochem (La Jolla, CA).

Synthesis of Oligonucleotides. The all-random mixture of phosphorothioate oligonucleotides used in these studies were synthesized on an Applied Biosystems (Foster City, CA) model 380B DNA synthesizer by standard methods. Sulfurization was performed using tetraethylthiuram disulfide/acetonitrile. After cleavage from controlled pore glass support, oligodeoxynucleotides were base deblocked in ammonium hydroxide at 60°C for 8 h and purified by reversed-phase high-performance liquid chromatography (0.1 M triethylammonium bicarbonate/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 of the full-length species were determined by UV spectroscopy. The 3$'$-Sp oligonucleotides were synthesized as described previously (Stec et al., 1998; Wilk et al., 2000).

The sequences were: 1) Isis 3521 (targeted to the 3$'$ region of the PKC-α mRNA), 5$'$-GTTCTCGCTGGTGAGTTTCA-3$'$; 2) Isis 3522 (targeted to the AUG codon region of the PKC-α mRNA), 5$'$-AAAACGTCAGCCATGGTCCC-3$'$; 3) G3139 (targeted to the first six codons of the human bcl-2 mRNA open reading frame), 5$'$-TCTC-CCAGCGTGCGCCAT-3$'$; 4) 2009 (targeted to bcl-2 mRNA codons 141–147; Ziegler et al., 1997; Zangemeister-Wittke et al., 1998),

5′-AATCCTCCCCCAGTTCACCC-3′; and 5) Isis 4559 (control, 5′-GGTTTTTACCATCGGTTCTGG-3′).

Oligonucleotide Transfection. Cells were seeded the day before the experiment in 6-well plates at a density of 25×10^4 cells per well to be 60 to 70% confluent on the day of the experiment. All transfections were performed in Opti-MEM medium (Invitrogen) per the manufacturer's instructions. The appropriate quantities of reagents were diluted in 100 μl of Opti-MEM medium to give a final concentration of delivery agent (usually Lipofectin) and 1 μM oligonucleotide. The solutions were mixed gently and preincubated at room temperature for 30 min to allow complexes to form. Then, 800 μl of Opti-MEM medium was added, and the solution was mixed and overlaid on the cells that had been prewashed with Opti-MEM. Complexes of oligonucleotides and TMP were prepared as described previously (Benimetskaya et al., 1998). When Eufectins were used as carriers, the oligonucleotide and lipid were diluted in 0.5 ml of Opti-MEM to the final concentrations described under *Results*. The solutions were then mixed gently and overlaid on cells that were prewashed with Opti-MEM. The incubation time for oligonucleotide/TMP or Lipofection complexes was 5 h, and for the Eufectins 24 h. The total incubation times before cell lysis and protein isolation were 19 h for Isis 3521 and 2009 and 72 h (37°C) for G3139.

Western Blot Analysis. Cells treated with oligonucleotide-lipid complexes were washed in PBS and then extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 50 μg/ml Pefabloc SC, 15 μg/ml aprotinin, leupeptin, chymostatin, and pepstatin A, 1 mM Na_3VO_4, and 1 mM NaF) at 4°C for 1 h. Cell debris was removed by centrifugation at 14,000g for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Richmond, CA). Aliquots of cell extracts, containing 25 to 50 μg of protein, were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to Hybond ECL filter paper (Amersham Pharmacia Biotech, Piscataway, NJ), and the filters were incubated at room temperature for 1 to 2 h. For PKC-α, -ζ, and -β1, p50, p65 bcl-xL, MMP-9, bax, XIAP, c-IAP-1, and IκBα, the filters were blocked in 5% nonfat dry milk and TBS-T and probed with corresponding primary antibodies at 4°C overnight. To detect bcl-2, blocking was performed in 5% bovine serum albumin in PBS containing 0.5% Tween 20. The filters were then probed with 1:500 dilutions of the anti bcl-2 antibody in 5% bovine serum albumin in PBS containing 0.5% Tween 20 at 4°C overnight. After washing in TBS-T buffer or in PBS containing 0.5% Tween 20 (for bcl-2), the filters were incubated for 1 h at room temperature in 5% nonfat dry milk in TBS-T (for PKC-α), or 5% nonfat dry milk in PBS containing 0.5% Tween 20 (for bcl-2 and other PKC isoforms) with a 1:3,000 dilution of a peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). After washing (3×10 min), ECL was performed according to the manufacturer's instructions.

Northern Blot Analysis. Total cellular RNA was isolated using TRIzol Reagent (Invitrogen). Twenty-five to 30 μg was resolved on 1.2% agarose gel containing 1.1% formaldehyde and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech). Human PKC-α and p65 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, $5\times$ SSC, $5\times$ 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 $2\times$ SSC and 0.1% SDS, once for 20 min in $1\times$ SSC and 0.1% SDS, and finally twice for 15 min in $0.1\times$ SSC and 0.1% SDS at 65°C. The filters were exposed to Kodak X-ray film for 12 to 48 h with intensifying screens at −70°C, and then developed.

Cell Viability. The effects on cellular viability of the combination of chemotherapeutic agents with antisense oligonucleotides were determined by MTT assay. Briefly, 0.6 to 1×10^4 T24 or PC3 cells were seeded in 96-well microtiter plates and allowed to attach over-

night. Cells were then treated with oligonucleotides (1 μM) complexed to 10 μg/ml Lipofectin for 5 h at 37°C, and then medium was replaced with 100 μl of complete medium containing various concentrations of drugs. After 3 days of incubation at 37°C, 10 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) in PBS was added to each well, followed by incubation for 4 h at 37°C. Then, 100 μl of 10% SDS in 0.04 M HCl was added to each well, followed by incubation overnight at 37°C to dissolve the formazan crystals. Absorbance was determined at 540 nm with a MR600 Microplate reader (Dynatech Labs, Chantilly, VA). Absorbance values were normalized to the controls to determine the percentage of survival. Each assay was performed in quadruplicate. Statistical analysis of the results was performed using the Analysis ToolPack provided by Microsoft Excel (Microsoft Corp., Redmond, WA). A Student's two-sample t test assuming unequal variances was used to determine the equality of the means of two samples. The confidence level (a) was 0.05. In some experiments, cellular viability was evaluated by MTT assay separately for adherent and nonadherent cells, as described by Khaled et al. (1996).

Electrophoretic Mobility-Shift Assay. T24 or PC3 cells were treated with G3139 or Isis 4559 control (1 μM) complexed to Lipofectin (10 and 12.5 μg/ml) for 5 h. The, medium was then replaced with fresh complete medium. After 3 days of incubation, the cells were treated with 50 ng/ml TPA for 4 h, then nuclear extracts were isolated, and electrophoretic mobility-shift assay were performed as described previously (Benimetskaya et al., 1997). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). The NF-κB, Sp1, and AP1 consensus probe sequences were, respectively, 5′- GTAGGGGACTTTCCGAGCTCGAGATCCTATG-3′, 5′-TCGAATTCGATCGGGGCGGGGCGAGAGC-3′, and 5′-AGCTTA-AAAAAGCATGAGTCAGACACCTG-3′. The probes were labeled with [^{32}P]dNTP as described previously (Benimetskaya et al., 1997). Briefly, oligonucleotides complementary to the 3′ end of the sequences listed above were annealed and extended with reverse transcriptase and 20 μCi of each [^{32}P]dNTP, followed by the addition of 500 μM unlabeled dNTPs to ensure complete reverse transcription. Unincorporated nucleotides were removed by column chromatography over a Sephadex G-25. Binding reactions were performed at room temperature for 20 min using 10 to 15 μg of nuclear extract and ^{32}P-labeled probes ($\sim$0.5 ng; 50,000 cpm) in 20 μl of binding buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 5% glycerol, 1 mg/ml BSA, and 2 μg of poly dI/dC. DNA-protein complexes were separated from unbound probe on nondenaturated 4% polyacrylamide gel in $0.5\times$ Tris-borate-EDTA and autoradiographed. Supershifts were performed with p65, p50, Sp1, or c-fos antibodies. The antibody (1 μl) was added to the binding mixture immediately after the addition of the radiolabeled probe. The reaction mixture was incubated for 20 min and the complexes resolved as described above.

Results

Isis 3521 and G3139 Decrease Cellular Viability as a Function of Paclitaxel Treatment. Both 20-mer phosphorothioate oligonucleotides, Isis 3521 and 3522, are excellent down-regulators of the expression of PKC-α protein in T24 and PC3 cells, but as single agents at a 1 μM concentration cause no decrease in cellular viability. As shown in Fig. 1, each (both 1 μM, delivered in complex with 10 μg/ml Lipofectin) decreased T24 cell viability, as measured by MTT assay, as a function of treatment with increasing concentrations of paclitaxel. However, Isis 3521 was more effective than Isis 3522 over a wider paclitaxel concentration range. The control phosphorothioate oligomer, Isis 4559, a 20-mer scrambled version of Isis 3521, was ineffective. Likewise, the 18-mer phosphorothioate oligonucleotide G3139, which is targeted to the initiation codon region of the bcl-2 mRNA,

also diminished cellular viability as a function of paclitaxel treatment. However, as determined by Student's two-sample t test, there was no significant difference in the diminished cellular viability at any paclitaxel concentration between cells treated with Isis 3521 or G3139; both oligonucleotides were essentially equally effective.

Truncated Mutants of Isis 3521 Still Decrease PKC-α Protein but Not Necessarily mRNA Expression. We wanted to determine the minimal length of Isis 3521 that could still support RNase H cleavage of the targeted PKC-α mRNA. Accordingly, we synthesized a series of mutant oligonucleotides progressively truncated at the 3′ terminus. Surprisingly, all oligonucleotides tested, down to the level of a 13-mer (1 μM, variable concentrations of Lipofectin, as shown in Fig. 2), were essentially equal in their ability to down-regulate the expression of PKC-α protein. However, the 13-mer 3′-truncation mutant based on the sequence of Isis 4559 was completely ineffective. Some (about 30–50%) down-regulation of PKC-α protein expression could be observed even at the level of an Isis 3521 10-mer 3′-truncation mutant (1 μM; not shown), but no diminution was observed when the cells were treated with a 9-mer. Similar results were obtained with a series of Isis 3521 5′-truncation mu-
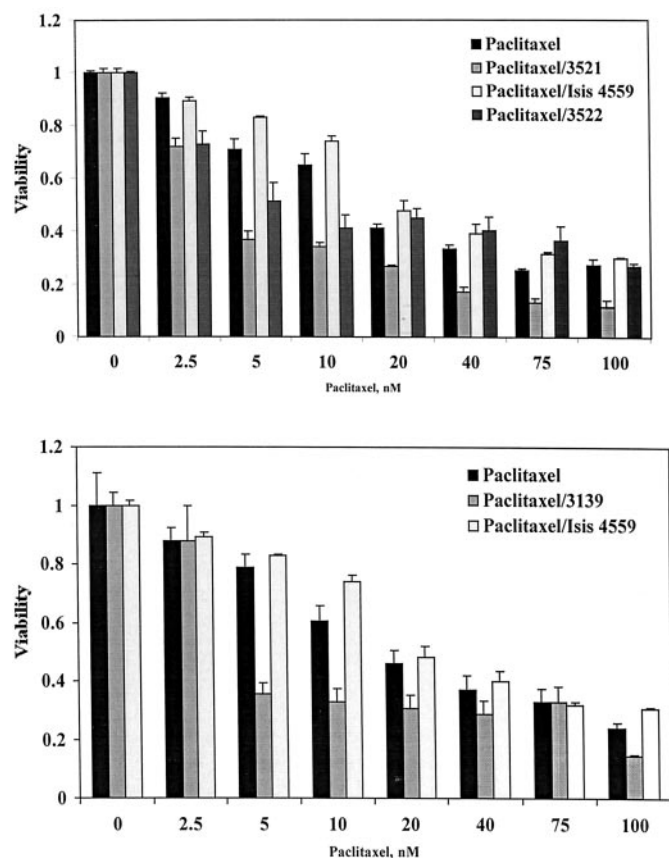

Fig. 1. Diminution in T24 cellular viabilities induced by the antisense oligonucleotides (top) Isis 3521, 3522, the control oligomer Isis 4559 (A) and G3139 and Isis 4559 (B) in response to increasing concentrations of paclitaxel. Cells were treated with oligonucleotides complexed to Lipofectin for 5 h at 37°C, after which the medium was replaced with medium containing various concentrations of paclitaxel. After 3 days of incubation, an MTT assay was performed. The absorbance at 540 nm was normalized to the controls to determine cellular viability. A Student's two-sample t test assuming unequal variances was used to determine the equality of the means of two samples. The confidence level was 0.05.

tants. Excellent suppression of PKC-α protein expression was observed down to the level of an 11-mer, but this effect disappeared when cells were treated with a 9-mer.

By comparison (Fig. 2C), neither bcl-2 nor α-tubulin expression (as assayed by Western blotting) were altered after treatment of cells with either the 20-, 15-, or 13-mer, demonstrating that the protein loading for each lane was equivalent. The percentage down-regulation of PKC-α expression (versus the control cells) by laser scanning densitometry was 84, 82, and 80% for the 13-, 15-, and 20-mer, respectively.

However, somewhat different results were observed when levels of PKC-α mRNA were examined by Northern blotting (Fig. 3). The Isis 3521 13-, 15-, and 16-mer 3′-truncation mutants were all capable of down-regulating the expression of PKC-α protein. Furthermore, the kinetics of down-regulation were similar for each oligonucleotide; after 5 h of incubation with oligonucleotide/Lipofectin complex in Opti-MEM and subsequent replacement with complete medium not containing complex, the rate of loss of PKC-α protein expression was essentially identical for the 20-mer and the 15-mer (data not shown). In addition, both the 20- and 15-mer suppressed PKC-α protein expression to the same extent for a 3-day period (data not shown). Nevertheless, a different picture emerged when expression of mRNA was examined (Fig. 3). Treatment of the cells only with 16-mer (and longer) antisense PKC-α oligonucleotides led to a diminution in mRNA levels; no consistent decrease in mRNA expression could be observed with the 13- or 15-mers. (It should be noted that the 8.5-kilobase mRNA transcript is the predominant form observed in the Northern blot. The 4.2-kilobase transcript is present at lower levels and is frequently difficult to visualize.) These data indicate that two distinct mechanisms inhibit the expression of PKC-α protein; one is RNase H-dependent and seems to occur at oligomer length of 16-mer or greater, whereas the other is RNase H-independent and may occur at oligomer lengths of 15-mer and less.

Treatment of T24 cells with the proteasome inhibitors 10 μM lactacystin or 10 μM MG132, commencing after 5 h of incubation of the cells with either the 13-mer 3′-truncation mutant or the 20-mer parental, Isis 3521 antisense PKC-α oligonucleotides, completely blocked the effect of either oligomer on PKC-α protein expression (Fig. 4A). Identical results were observed in PC3 cells, despite, in both cell lines, an appropriate diminution in PKC-α mRNA levels after treatment with the 20-mer (Fig. 4B). Furthermore, no translocation of PKC-α protein from cytosol to membrane was observed after treatment with any of the oligonucleotide/Lipofectin complexes. These data indicate that even "inactivated" PKC-α and activated PKC-α (Lu et al., 1998; Lee et al., 1997) are degraded via the ubiquitin-proteasome pathway, which can be observed when the inflow of PKC-α protein to the pathway is eliminated by antisense oligonucleotide inhibition.

Decreased Cellular Viability Is Correlated with the Length of the 3′-Truncation Mutation and Hence Its Ability to Support an RNase H-Based Mechanism. In addition to the parental Isis 3521 20-mer, as shown in Fig. 5, both the 17- and 16-mers (1 μM, 10 μg/ml Lipofectin) decrease cellular viability as a function of increasing paclitaxel concentration. To maximize this effect, and recognizing the phosphorothioate oligonucleotides are nuclease-resistant but by no means nuclease-proof, we then reasoned that increas-

ing oligonucleotide stability to 3′-nucleolytic activity would be useful. To accomplish this, we employed 3′-truncation mutant phosphorothioates that contained a single, 3′-Sp phosphorothioate diastereomer. This stereoregular modification was made because only the Sp diastereomer is highly nuclease-resistant (Stec et al., 1991; Koziolkiewicz et al., 1997). Addition of the 3′-stereoregular linkage did not alter the oligomers' ability to decrease PKC-α protein expression (not shown). In fact, it improved the ability of the 17- and 16-mer 3′-truncation mutants to decrease cellular viability as a function of increasing paclitaxel concentration (Fig. 5) versus the machine-synthesized random mixture of 3′-diastereomers (denoted Rp/Sp). However, the ability to decrease cellular viability was virtually entirely lost for the 15-mer 3′-truncation mutant, despite the fact that PKC-α protein expression was almost completely eliminated. However, the 15-mer 3′-truncation mutant, as shown above, does not support an RNase H-based mechanism. These data suggest that, whereas PKC-α protein expression may be necessary for the chemosensitive phenotype in this case, it is probably not sufficient. As a corollary, the data further suggest that the activity of the parental 20-mer results from a summation of mechanisms, only one of which is inhibition of PKC-α protein expression.

Down-Regulation of bcl-2 Expression Is Associated with the Down-Regulation of PKC-α but Not Other PKC Isoforms. The Western blot data in Fig. 6 demonstrate that the treatment T24 cells with G3139 caused not only down-regulation of bcl-2 protein expression, but an almost total down-regulation of PKC-α protein expression. The decrease in PKC-α protein expression was carrier-independent, because the carriers Lipofectin, TMP (Benimetskaya et al.,

1998), and Eufectin 5 all provided essentially identical results (not shown). Similar results were obtained in PC-3 prostate carcinoma cells. The down-regulation was highly isoform specific as no change was observed in the expression of PKC-β1, -ζ, or -ε proteins (the latter of which is, like PKC-α, degraded by the ubiquitin/proteasome pathway). Interestingly, addition of 10 μM lactacystin subsequent to the 5-h incubation of the cells with complexes of G3139 and Lipofectin completely blocked down-regulation of both PKC-α and bcl-2 protein expression. Down-regulation of PKC-α mRNA levels (Fig. 6) occurred congruently with down-regulation of protein expression. However, treatment of cells with Isis 3521, which is targeted to the PKC-α mRNA, did not decrease bcl-2 protein expression.

Nevertheless, it can be demonstrated that bcl-2 is not upstream of PKC. As shown in Fig. 7A, the down-regulation of bcl-2 and PKC-α protein expression can be separated as a function of oligonucleotide concentration. This was achieved in both T24 and PC3 by employing the carrier Eufectin 5, which is a much more efficient oligonucleotide delivery reagent than Lipofectin. At a 50 nM concentration of G3139 (4.75 μg/ml Eufectin 5), only bcl-2 protein expression was down-regulated. At 100 nM (9.5 μg/ml Eufectin 5), both bcl-2 and PKC-α protein expression were dramatically down-regulated. These differences in protein expression as a function of oligomer concentration find perhaps further expression in the decrease in the viability (as measured by MTT assay) of G3139-treated cells as a function of increasing paclitaxel and carboplatin concentrations (Fig. 7B). At a G3139 concentration of 50 nM (only bcl-2 expression down-regulated), no augmentation in the drug-induced diminution of cellular viability could be observed for either cytotoxic agent. In con-
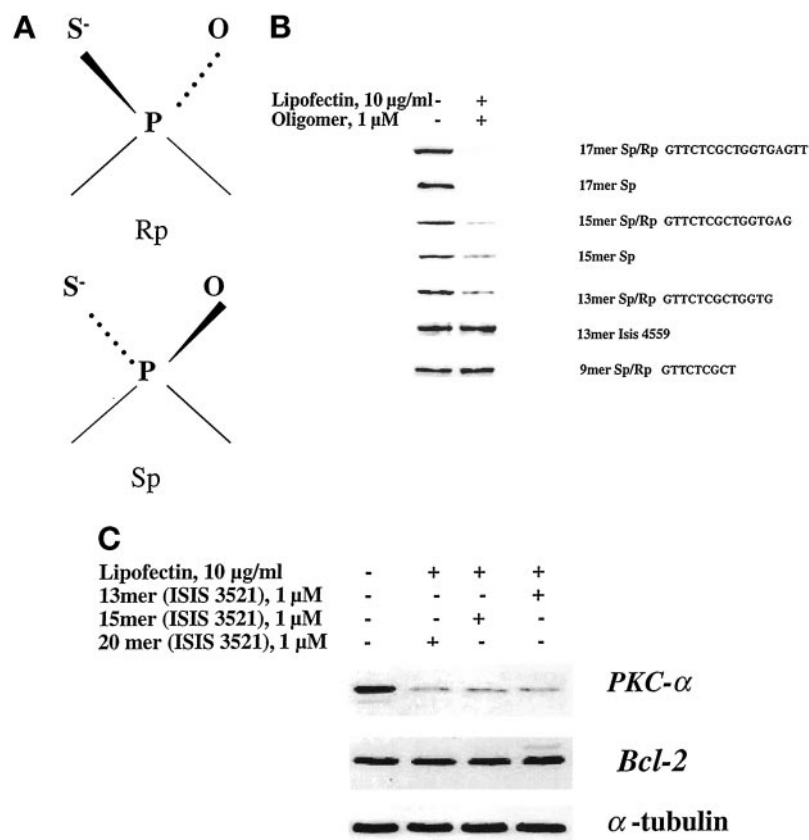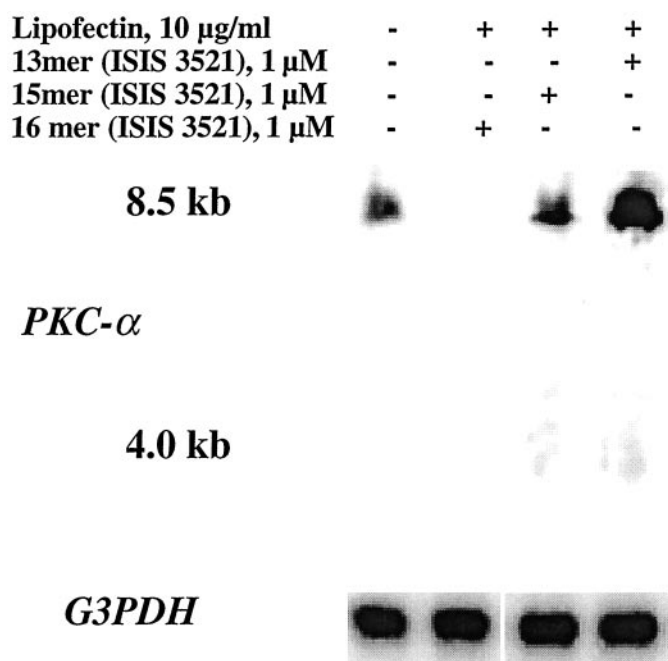
Fig. 2. A, stereochemical representation of 3′-Rp and Sp phosphorothioate diastereomers. B, effects of the Isis 3521 3′-truncation mutants on PKC-α protein expression in T24 cells. The cells were treated with complexes of oligonucleotides and Lipofectin for 5 h at 37°C, and 19 h later, total protein was harvested. Western blot analysis (25–30 μg of protein/lane) was performed as described under *Materials and Methods*. C, quantitation of down-regulation of PKC-α protein expression by the parental 20-mer Isis 3521 and the 15-mer and 13-mer 3′-truncation mutations. By comparison, neither bcl-2 nor α-tubulin expression is altered, demonstrating that PKC-α down-regulation does not affect bcl-2 expression, and that the protein loading for each lane is equivalent. The percentage down-regulation of PKC-α expression (versus the control cells) by laser scanning densitometry = 84, 82, and 80% for the 13-, 15-, and 20-mer, respectively. However, it should be noted that 10% experimental variation in the percentage down-regulation is not uncommon.

trast, at a 100 nM G3139 concentration (both bcl-2 and PKC-α protein expression down-regulated), cellular viability was substantially decreased as a function of cytotoxic agent concentration. These data strongly suggest that the elimination of bcl-2 may be necessary but is insufficient to significantly decrease cellular viability in response to cytotoxic agents.
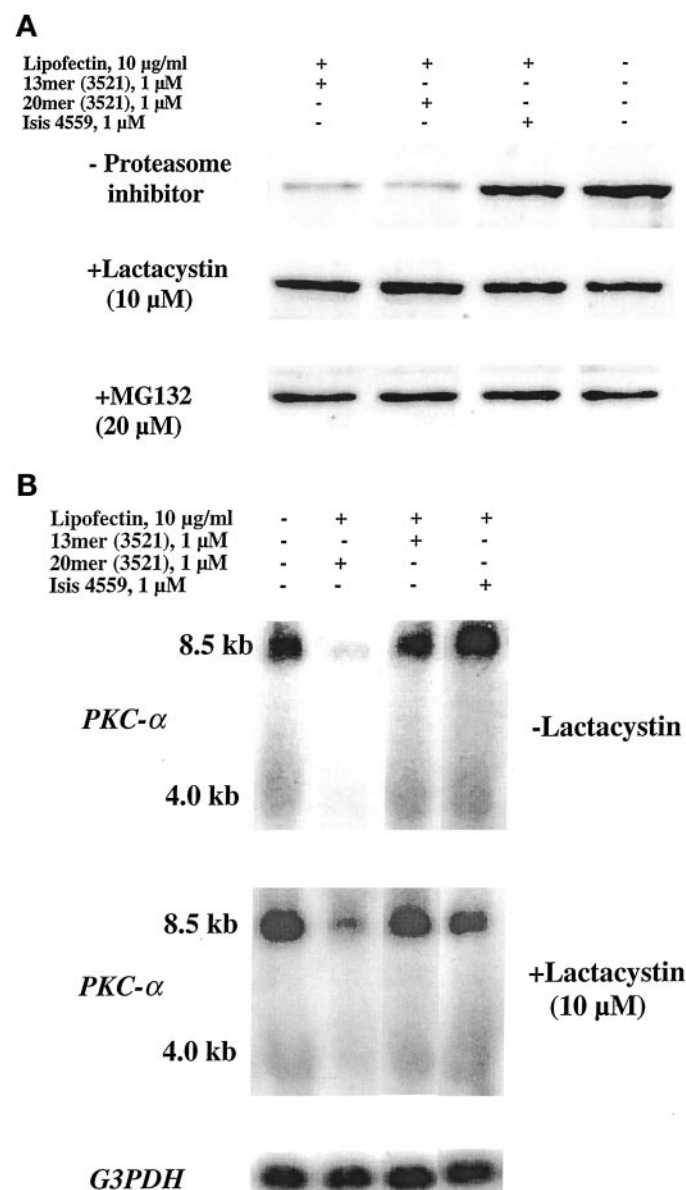
G3139 Down-Regulates the Expression of RelA (p65) and RelA Responsive Genes. As shown in the gel shift experiments performed with monoclonal antibody supershifting in Fig. 8, G3139 dramatically down-regulates the nuclear expression of RelA (p65) but completely spares that of NFκB1 (p50). Some nonspecific down-regulation can be seen with the control oligomer Isis 4559; Isis 3521 does not affect the nuclear expression of p65 (not shown). The nuclear expression of other transcriptional factors, such as Sp1 and c-fos, were unchanged. In addition, as shown in Fig. 10, the expression of p65 at the protein level was markedly diminished by treatment with 1 μM G3139, whereas it was only slightly affected by Isis 4559 and by two additional phosphorothioate oligonucleotide 18-mers. This down-regulation was kinetically linked to the down-regulation of bcl-2 protein expression. In both T24 and PC3 cells, it takes 3 days for maximal down-regulation of bcl-2 expression, and the same time for maximal down-regulation of p65 protein expression. Interestingly, Western analysis (Fig. 9) did show a diminution in the cellular expression of p50 protein in response to G3139 treatment. The reason for this is not clear but may be due to down-regulation of the expression of the protein that sequesters it in the cytoplasm, IκB-α (see below). In addition, Northern blotting revealed a significant diminution in p65 mRNA

expression compared with control, although some decrease after treatment with Isis 4559 was also observed.

To further confirm the at least partial linkage between the down-regulation of bcl-2 and p65 expression, we employed a different antisense bcl-2 oligonucleotide (2009), which is targeted to the open reading frame of the bcl-2 mRNA and not the initiation codon region, as is G3139. As shown by Western blotting, this oligonucleotide (1 μM), when complexed with increasing concentrations of Lipofectin, also effectively down-regulated p65 protein expression. Isis 4559, as above, was entirely ineffective in complex with Lipofectin. To rule out the possibility that the Lipofectin carrier was responsible

A

Lipofectin, 10 μg/ml	+	+	+	-
13mer (3521), 1 μM	+	-	-	-
20mer (3521), 1 μM	-	+	-	-
Isis 4559, 1 μM	-	-	+	-

- Proteasome
inhibitor

+Lactacystin
(10 μM)

+MG132
(20 μM)

B

Lipofectin, 10 μg/ml	-	+	+	+
13mer (3521), 1 μM	-	-	+	+
20mer (3521), 1 μM	-	+	-	-
Isis 4559, 1 μM	-	-	-	+

8.5 kb

PKC-α

4.0 kb

-Lactacystin

8.5 kb

PKC-α

4.0 kb

+Lactacystin
(10 μM)

G3PDH

Fig. 4. Effect of proteasome inhibitors on PKC-α expression in T24 cells treated with Isis 3521. The cells were treated with Isis 3521, the 13-mer 3′-truncation mutant or with 1 μM Isis 4559 complexed to 10 μM Lipofectin for 5 h. The medium was then replaced with medium containing 10 μM lactacystin or 10 μM MG132. After 14 h of incubation at 37°C in the presence or absence of the inhibitors, total protein was harvested, and Western blotting was performed as described in the text (A). PKC-α and G3PDH mRNA levels were determined by Northern blotting with the PKC-α and G3PDH probes as described under *Materials and Methods* (B).

Lipofectin, 10 μg/ml	-	+	+	+
13mer (ISIS 3521), 1 μM	-	-	-	+
15mer (ISIS 3521), 1 μM	-	-	+	-
16 mer (ISIS 3521), 1 μM	-	+	-	-

8.5 kb

PKC-α

4.0 kb

G3PDH

Fig. 3. The Isis 3521 16-mer 3′-truncation mutant down-regulates PKC-α mRNA expression in T24 cells, whereas the 13- and 15-mers do not. The cells were treated with complexes of oligonucleotides and Lipofectin for 5 h at 37°C. Nineteen hours later, total mRNA was isolated, and Northern blot analysis was performed as described in the text. Twenty-five micrograms of mRNA was loaded in each lane of the 1.2% denaturing agarose gel, transferred to Hybond-N nylon membranes, and probed with either PKC-α or control G3PDH cDNA probe.

for the down-regulation of p65 protein expression, we delivered 2 μM G3139 with a novel peptide carrier (denoted R-peptide-NLS). Similar to what we observed with the lipid carriers, p65 protein expression was substantially decreased, as determined by Western blotting.

Down-regulation of the expression of genes putatively controlled by the NF-κB transcription factor could also be demonstrated in T24 and PC3 cells after standard treatment with G3139 (Fig. 10A). These genes include the antiapoptotic bcl-xL (Chen et al., 2000), XIAP (Stehlik et al., 1998), and c-IAP-1 (Wang et al., 1998), the matrix metalloprotease inhibitor MMP-9 (Ricca et al., 2000), and IκB-α (Liou and

Baltimore, 1993). However, expression of the pro-apoptotic bax protein was essentially unchanged. Furthermore, because NF-κB has long been known to control cellular adhesion to surfaces (Narayanan et al., 1993), we evaluated the extent to which attached PC3 cells lifted off the surface of the plastic culture-dish wells to which they were attached. Approximately 50% of the previously attached cells, after treatment with G3139, detached from a plastic surface, compared with only about 10% of control cells treated with either Isis 3521 or 4559. Furthermore, as determined by MTT assay, these detached, floating cells produced copious amounts of formazan crystals and were thus alive (Fig. 10C).

Discussion

In this work, we have revealed at least a small part of the complicated series of events that occurs when cells are treated with phosphorothioate antisense oligonucleotides. Isis 3521, for example, potentiates the effects of paclitaxel on cellular viability. However, the data suggest that the ability of Isis 3521 to do this is only in part related to its ability to down-regulate PKC-α protein expression. Indeed, our data indicates that down-regulation of PKC-α protein expression, although perhaps necessary, is insufficient for decreased cellular viability after treatment with cytotoxic agents. This statement is most strongly supported by the ability of the shorter (15- and 13-mer) Isis 3521 3'-truncation mutations to still effect PKC-α protein down-regulation in the absence of any increment in loss of cellular viability after treatment with paclitaxel.

As shown in Fig. 5, Isis 3521 3'-truncation mutants containing a 3'-terminal Sp linkage, which are more nuclease resistant than the machine-made molecules that contain a 3' random mixture of diastereomers, are also, relative to the latter, superior enhancers of the effects of cytotoxic agents. This is true even though the ability of both the partially stereoregular and entirely stereorandom oligomers are identical in their ability to down-regulate PKC-α protein expression. These results also suggest that several mechanisms are involved in the observed decrease in cellular viability in response to paclitaxel in Isis 3521-treated T24 cells.
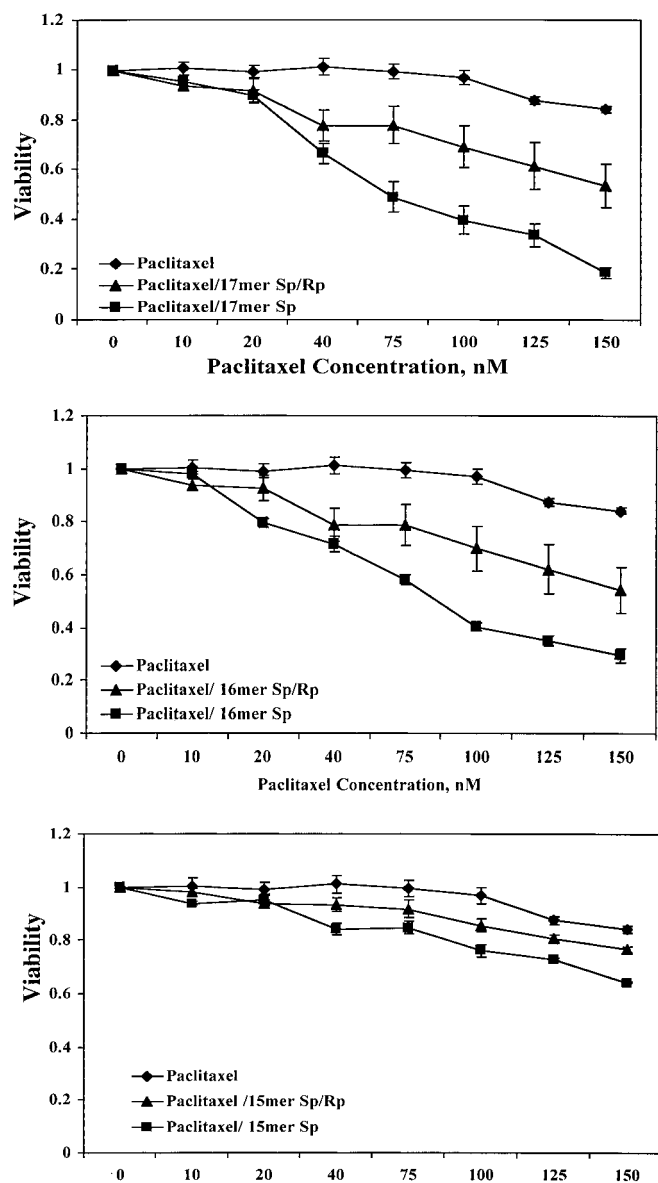

Fig. 5. A 3'-Sp-stereoregular phosphorothioate of the 17- and 16-mer 3'-truncation mutants increases the chemosensitization of T24 cells to paclitaxel modification versus the isosequential machine-synthesized random mixture (Sp/Rp). However, no increase in paclitaxel cytotoxicity is observed with the 15-mer (Sp or Sp/Rp) 3'-truncation mutants. The cells were treated with the 17-mer (Sp and Sp/Rp) (A), 16-mer (Sp and Sp/Rp) (B), and 15-mer (Sp and Sp/Rp) (C) 3'-truncation mutants complexed to Lipofectin for 5 h at 37°C. Then medium was replaced with media containing various concentrations of paclitaxel. After 3 days of incubation, MTT assay was performed. Absorbance at 540 nm was normalized to the control, untreated cells to determine viability.
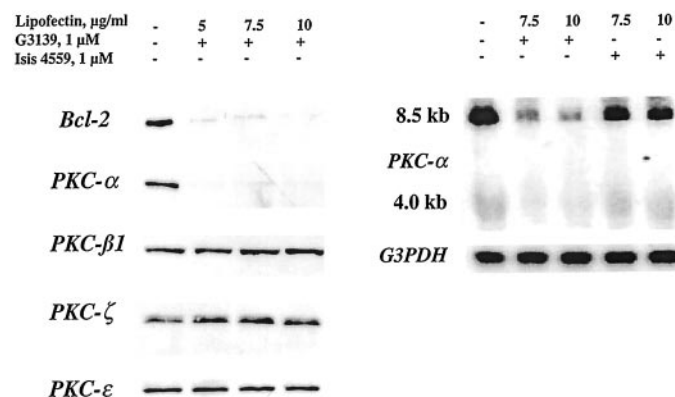

Fig. 6. Down-regulation of bcl-2 protein expression is associated with the down-regulation of PKC-α but not of other PKC isoforms. T24 cells were treated with complexes of G3139 or control Isis 4559 and Lipofectin for 5 h at 37°C. Nineteen hours later, total protein and mRNA were harvested. Western blotting was performed with anti-bcl-2 and anti-PKC-α, -β1, -ϵ, and -ζ antibodies. PKC-α and G3PDH mRNA levels were determined by Northern blotting with PKC-α and G3PDH probes as described under *Materials and Methods*.

As indicated earlier, phosphorothioate oligonucleotides possess significant non–sequence-specific activity, believed in large measure to be due to high-affinity binding to heparin-binding proteins (Guvakova et al., 1995). It is possible that this effect contributes to the net loss in cellular viability, in the context of the down-regulation of the expression of

PKC-α. However, it is perhaps more likely that Isis 3521, because it can support RNase H activity, also participates in the cleavage of nontargeted mRNAs (a process that has been
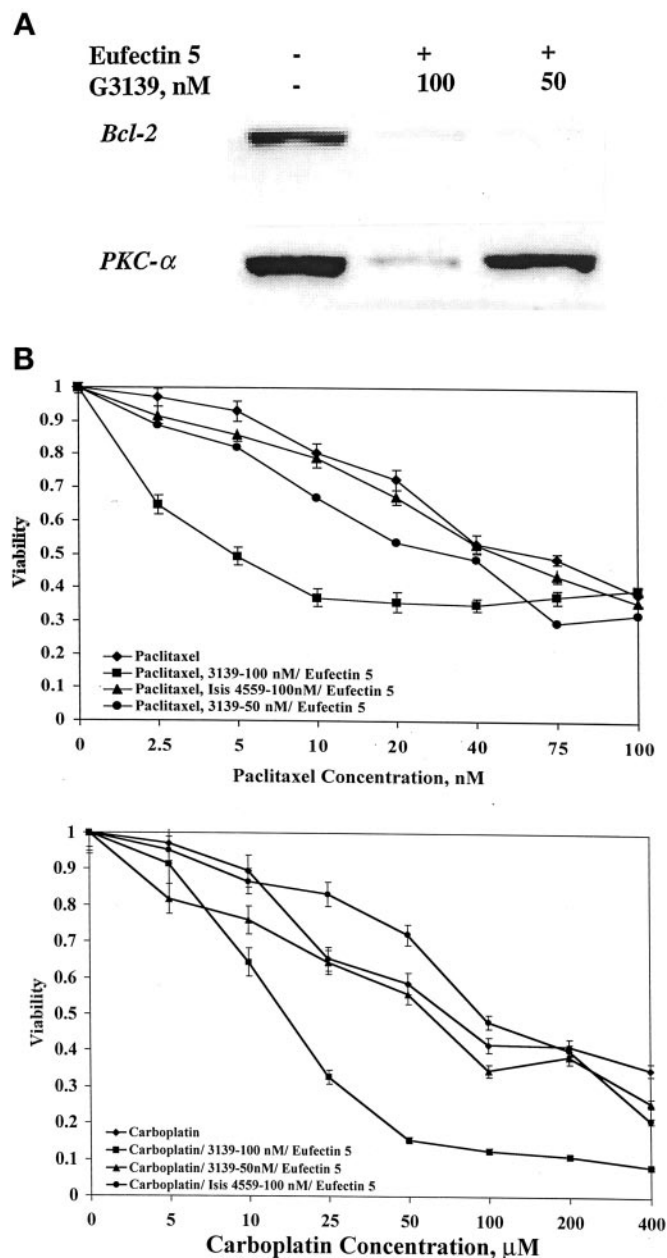
Fig. 7. A, the down-regulation of bcl-2 and PKC-α protein expression can separated as a function of oligonucleotide concentration by employing the carrier Eufectin 5. T24 cells were treated with G3139 (100 and 50 nM) complexed to Eufectin 5 (9.5 and 4.75 μg/ml, respectively) for 24 h at 37°C. Forty-eight hours later, total protein was harvested. Western blotting was performed with anti-bcl-2 and anti-PKC-α antibodies as described. B, diminished T24 cell viabilities induced by G3139 (100 and 50 nM) and versus Isis 4559 as a function of paclitaxel (top) and carboplatin (bottom) concentrations. Cells were treated with oligonucleotides complexed to Eufectin 5 (9.5 and 4.75 μg/ml, respectively) for 24 h at 37°C, and then the Opti-MEM was replaced with complete media containing various concentrations of drugs. After 3 days of incubation, MTT assay was performed. Absorbance at 540 nm was normalized to the control, untreated cells to determine cellular viability.
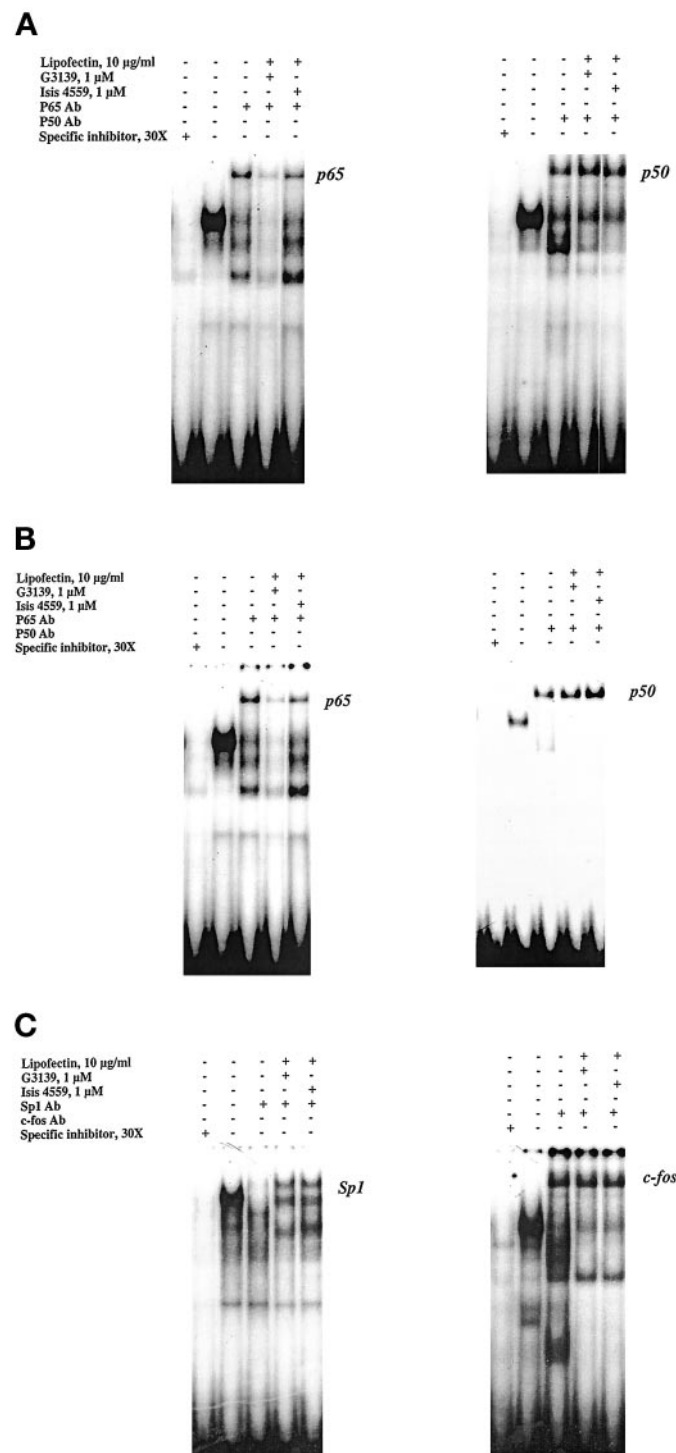
Fig. 8. G3139 down-regulates the nuclear expression of RelA (p65) but not NF-κB1 (p50), Sp1, and c-fos in T24 (A) and PC3 (B and C) cells. The cells were treated with complexes of G3139 or Isis 4559 and Lipofectin for 5 h at 37°C. Three days later, nuclear extracts were isolated and treated with ^{32}P-labeled NF-κB, Sp1, or Ap1 consensus sequence oligonucleotides (equal loading of probe in each lane). Electrophoretic mobility-shift assay was then performed as described under *Materials and Methods*, and the proteins visualized as monoclonal antibody supershifted bands. Free probe is seen at the bottom of the gel.

called irrelevant cleavage) (Stein, 2000). Simply put, this occurs because RNase H does not require full-length homology between the antisense oligonucleotide and its target to recognize a cleavable DNA/mRNA duplex. Under some restricted circumstances, as small a region as a six-base duplex may suffice (Monia et al., 1993), and in any given 20-mer, there are a large number of nested 6-mers. Thus, it is perhaps no coincidence that when the Isis 3521 3′-truncation mutants lose their ability to support RNase H cleavage of the PKC-α mRNA, they also lose their ability to diminish cellular viability in response to increasing paclitaxel concentration.

However, it remains unclear how the shorter (e.g., 15- and 13-mer) Isis 3521 3′-truncation mutants, which do not apparently support RNase H activity, can still down-regulate the expression of PKC-α protein. In the case of the 13-mer, the melting point of the duplex formed is well below 37°C, and it could be anticipated that the unwinding activity of the 80S ribosome would be sufficient to destroy any residual partial duplex character (Liebhaber et al., 1984). One possible scenario is that binding of an antisense oligonucleotide, even transiently, to the Isis 3521 site on the mRNA, can cause a conformational change in the mRNA that renders it untranslatable. This is speculative and difficult to prove, and other possibilities certainly exist.

The use of the antisense biotechnology to easily and relatively specifically down-regulate basal gene expression allows for the straightforward examination of protein degradative pathways without interference by continuous protein replenishment. Both PKC-α (Lee et al., 1997; Lu et al., 1998) and bcl-2 (Wall et al., 2000) have been reported to undergo degradation by the ubiquitin/proteasome pathway, the former after activation and translocation. Our data suggest that this process can also be slow and perhaps constitutive; nevertheless, it can result in the complete elimination of

PKC-α protein when that protein is not replenished. Similarly, the effects of G3139 on bcl-2 expression may be eliminated if cells are treated with proteasome inhibitors, indicating that bcl-2, too, also undergoes degradation predominantly via the ubiquitin/proteasome pathway.

We have also demonstrated that G3139, when used in the appropriate concentration, down-regulates PKC-α protein and mRNA expression. This effect is highly isoform specific, as it does not affect PKC-β1, -ϵ, or -ζ. Interestingly, phosphodiester oligonucleotides targeted to PKC-α have been shown to down-regulate bcl-2 and NF-κB nuclear activation in GP7TB hepatic cells (Lin et al., 2000). However, these oligonucleotides are highly unstable with respect to nuclease digestion, and were delivered naked at high concentration (10 μM). In our hands, Isis 3521 did not down-regulate bcl-2 expression at all.

It is possible that the co-down-regulation of PKC-α and bcl-2 is due to irrelevant cleavage, because a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) revealed partial homology between G3139 and the PKC-α mRNA. Furthermore, Fig. 7A demonstrates that PKC-α is clearly not downstream of bcl-2. Regardless, as shown in Fig. 7B, the down-regulation of PKC-α protein expression seems to be important for diminished cellular viability in G3139-treated T24 cells in response to increasing concentrations of cytotoxic agents. Yet, a point of caution must be raised, as the down-regulation of PKC-α may only be a marker for the down-regulation of other, as-yet-unknown genes by G3139. Therefore, although the data are suggestive, it cannot yet be firmly concluded that PKC-α down-regulation is either necessary or sufficient for the decrease in cellular viabilities observed in Fig. 7B.

RelA (p65) seems to be the most important member of the NF-κB family of proteins. For example, p65 knockout mice die as embryos from massive hepatic necrosis, whereas p50
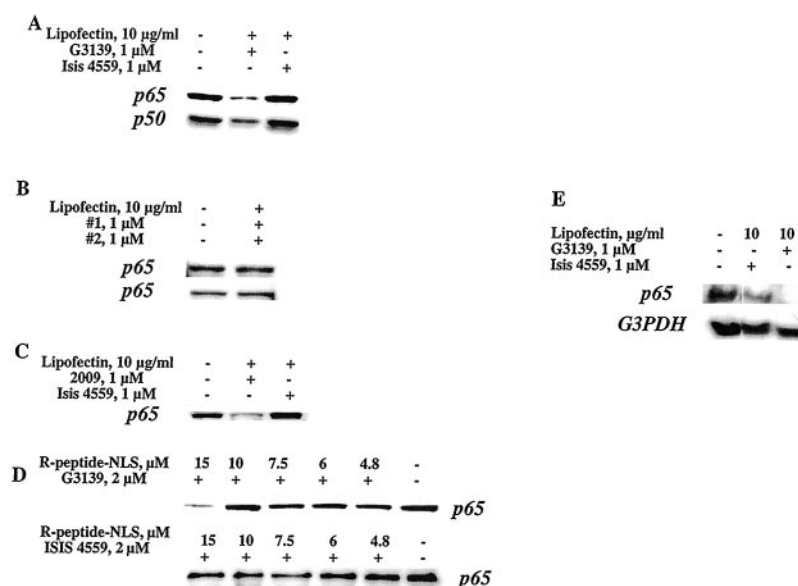
Fig. 9. A, G3139 but not Isis 4559 down-regulates the expression of RelA (p65) protein and, to a lesser extent, p50 protein, as demonstrated by Western blotting. B, two control 20-mer phosphorothioate oligonucleotides also do not down-regulate p65 protein expression. C, not only G3139 (targeted to the initiation codon region of the bcl-2 mRNA), but also 2009 (targeted to the open reading frame) down-regulate p65 protein expression, as demonstrated by Western blotting. D, the down-regulation of p65 protein expression is not dependent on the lipid carrier, but occurs also with a peptide carrier of G3139. T24 cells were treated with complexes of the G3139, 2009, or Isis 4559 oligonucleotides and either Lipofectin or a peptide carrier (D) for 5 h at 37°C. Three days later, total protein and mRNA were harvested. Western blotting was performed with anti-p50 or -p65 antibodies. E, G3139 decreases p65 mRNA expression as does Isis 4559, which does so to a lesser extent. p65 and G3PDH mRNA levels were determined by Northern blotting with probes as described under *Materials and Methods*.

knockout mice survive without appreciable harm. A large number of recent articles have described the anti-apoptotic function of the nuclear activation of p65 (e.g., (Wang et al., 1996, 1999; Duffey et al., 1999), but other work (e.g., Bentires-Alj et al., 1999) describes pro-apoptotic events that seem to be based on the same nuclear activation. In T24 and PC3 cells, down-regulation of bcl-2 protein expression also leads to the down-regulation of RelA (p65) protein and mRNA expression, but in this case, as opposed to the G3139-induced down-regulation of PKC-α protein expression, the events seem to be linked. Several lines of evidence support this, including the fact that the kinetics of down-regulation of bcl-2 and p65 proteins are virtually identical. In addition, unlike observations with G3139-induced PKC-α down-regulation, the down-regulation of bcl-2 and RelA cannot be separated as a function of oligonucleotide concentration. Finally, an oligomer (2009) with a completely different sequence than G3139, yet still targeted to and able to down-regulate bcl-2 protein expression, can also down-regulate p65 protein expression. Importantly, these effects are not carrier-dependent. These results are congruent with observations that forced increased expression of bcl-2 can lead to increased

NF-κB activation (de Moissac et al., 1998, 1999), although this may not necessarily be the case in prostate cancer cells (Herrmann et al., 1997).

On the other hand, whereas p50 expression in the nucleus is unchanged, total cellular p50 levels decrease, although they never disappear (on Western blot) even after G3139 treatment. It can thus be posited that there is a redistribution of p50 protein, favoring nuclear homeostasis. This would make sense given that p50 has no known cytoplasmic function and that the cells treated with G3139 have just lost most of their p65. However, nuclear p50 cannot compensate for the loss of p65, especially with respect to cellular adhesion (see below).

Simultaneously, we have shown that the expression of genes that are believed to be regulated by NF-κB are also diminished, as shown in Fig. 9A. These genes include the anti-apoptotic proteins bcl-xL (which also seems to be partially phosphorylated) c-IAP-1, XIAP, and MMP-9. On the other hand, no down-regulation of bax protein expression was observed, demonstrating that these effects are not due to generalized down-regulation of translation.

NF-κB also controls the expression of several cellular ad-
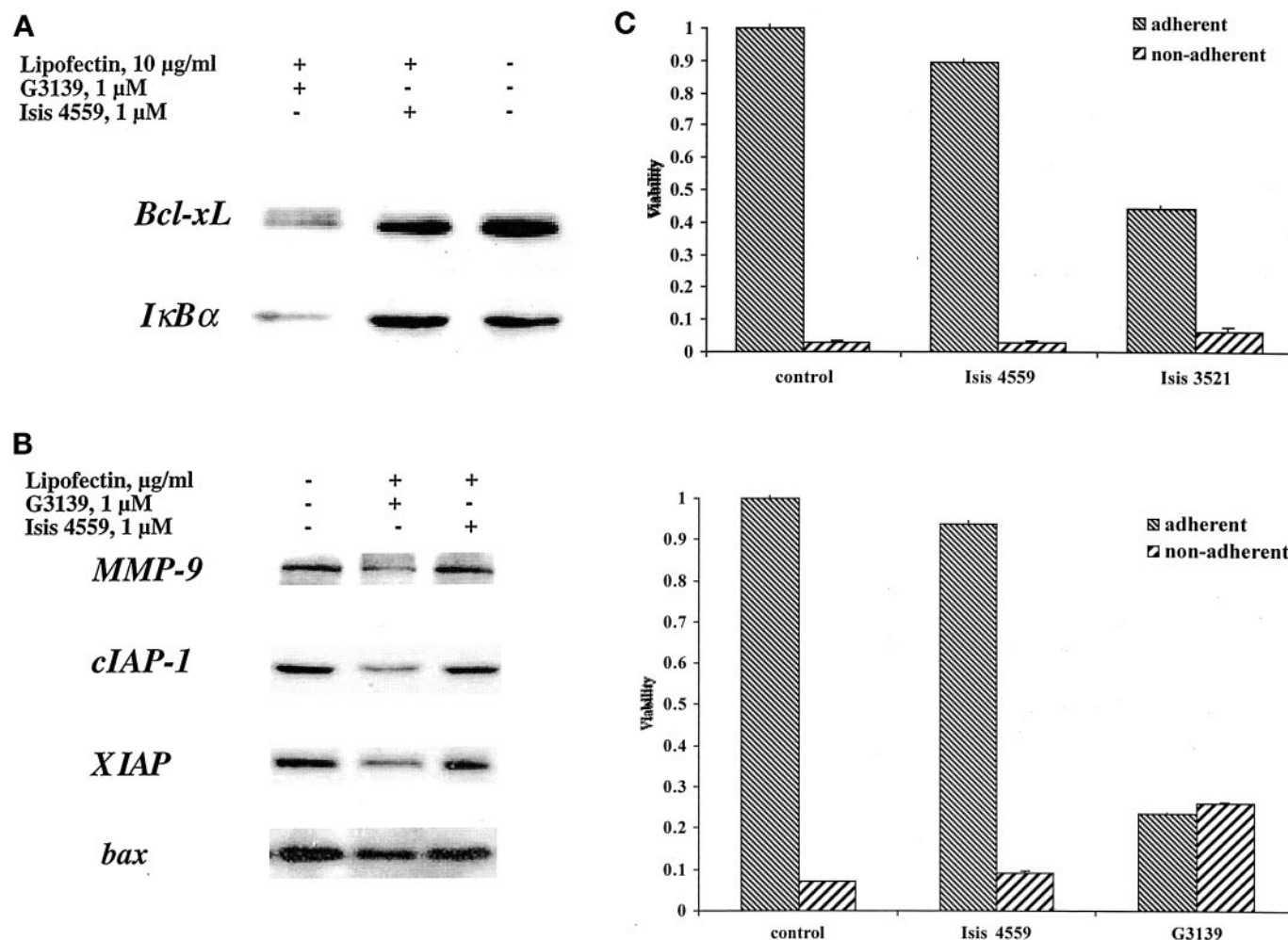
Fig. 10. In T24 cells, G3139 but not Isis 4559 down-regulates the expression of genes putatively regulated by NF-κB. These include bcl-xL and IκBα (A), and MMP-9, cIAP-1, and XIAP (B). In contrast, bax protein expression is not changed (B). Cells were treated with complexes of G3139 or Isis 4559 and Lipofectin for 5 h at 37°C, and 3 days later, total protein was harvested. Western blot analysis (25–30 μg of protein/lane) was performed with the appropriate antibodies as described in the text. C, G3139 but not Isis 3521 or Isis 4559 causes detachment of T24 cells from plastic surfaces. The viabilities of adherent and nonadherent cells were assessed by MTT assay as described in the text. For Isis 3521 (top), an incubation time of 1 day was employed. For G3139 (bottom), an incubation time of 3 days was employed.

hesive proteins, including VCAM, ICAM-1 (Neish et al., 1992), and ELAM-1 (Whelan et al., 1991). In addition, stable expression of inducible antisense RNA to p65 also caused inhibition of cell adhesion (Narayanan et al., 1993). Treatment of T24 or PC3 cells with G3139 causes the dissociation of approximately 50% of the cells from the plate (versus about 10% for the control oligomers, Isis 4559 and Isis 3521), but these cells, as assessed by MTT assay, remain viable.

The control oligomer, Isis 4559, does seem to have some effect on the nuclear activation of p65, although, in contrast, it does not seem to have any effect on the expression of p65 protein. In addition, with the exception of some slight effects on the expression of IκB-α protein expression, it also does not affect the expression of any of the putatively NF-κB-controlled genes in Fig. 10, and does not affect cellular attachment. In addition, three other control oligonucleotides also do not affect p65 protein expression in either T24 or PC3, thus leading to the conclusion that although there is a component of nonsequence specificity, the bulk of the effects of G3139 on p65 expression are indeed specific.

As described, the down-regulation of bcl-2 and p65 seem to be linked. This means it is also true that the down-regulation of RelA and the putatively NF-κB-regulated genes, at least in the cell lines employed in this study, are also insufficient to decrease cellular viability in response to treatment with cytotoxic agents. This result is somewhat surprising in light of the fact that antisense oligonucleotide down-regulation of bcl-xL has been associated with chemosensitization (Lebedeva et al., 2000). However, in that study, too, nonspecific effects probably contributed to the observed biological effects.

In summary, we have demonstrated that the inhibition of PKC-α protein expression by Isis 3521 and its 3′-truncation mutations can proceed by both RNase H-dependent and -independent pathways, depending on oligomer length. However, down-regulation of PKC-α protein, although perhaps necessary, is not sufficient for chemosensitization. G3139 down-regulates the expression of bcl-2, p65, and several NF-κB-controlled genes (including bcl-xL, c-IAP-1, and XIAP), but none of these effects seem to be sufficient for chemosensitization, although they all may be necessary. However, our data suggests the ability of G3139 to specifically (relative to other PKC isoforms) down-regulate PKC-α protein expression is closely associated with chemosensitization. These results, which should not be extrapolated to other tumor cell lines, highlight the complexity of the cell-death process, and the myriad of intracellular changes that may be required to achieve it. This, in turn, may shed some additional light on the cellular requirements necessary for the development of effective, specific therapeutic strategies.

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