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Enhanced activity of an antisense oligonucleotide targeting murine protein kinase C-α by the incorporation of 2′-*O*-propyl modifications

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ABSTRACT

We have previously described the characterization of a 20mer phosphorothioate oligodeoxynucleotide (ISIS 4189) which inhibits murine protein kinase C-α (PKC-α) gene expression, both *in vitro* and *in vivo*. In an effort to increase the antisense activity of this oligonucleotide, 2′-*O*-propyl modifications have been incorporated into the 5′- and 3′-ends of the oligonucleotide, with the eight central bases left as phosphorothioate oligodeoxynucleotides. Hybridization analysis demonstrated that these modifications increased affinity by ~8 and 6°C per oligonucleotide for the phosphodiester (ISIS 7815) and phosphorothioate (ISIS 7817) respectively when hybridized to an RNA complement. In addition, 2′-*O*-propyl incorporation greatly enhanced the nuclease resistance of the oligonucleotides to snake venom phosphodiesterase or intracellular nucleases *in vivo*. The increase in affinity and nuclease stability of ISIS 7817 resulted in a 5-fold increase in the ability of the oligonucleotide to inhibit PKC-α gene

(except the 3′-end residue, which cannot be 2′ modified due to synthesis constraints), with either phosphorothioate or phosphodiester backbones. These incorporations greatly increased the nuclease resistance of the molecule to snake venom 3′-exonuclease and intracellular nucleases in cell culture. This increased stability, combined with improved target affinity of the oligonucleotide, resulted in a 5-fold increase in activity in tissue culture for the inhibition of PKC-α mRNA.

MATERIALS AND METHODS

Cell culture

Murine C127 mammary epithelial cells were obtained from the American Type Culture Collection (Bethesda, MD) and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum and 5 mg/ml penicillin/streptimycin. The cells were routinely passaged at 80–95% confluency.

Oligonucleotide synthesis

2′-*O*-Propyl monomers were synthesized as previously described (14). Phosphorothioate (deoxynucleotide and 2′-*O*-propyl nucleotides) and phosphodiester were synthesized in 1 µmol lots using an Applied Biosystems 380B automated DNA synthesizer and purified as previously described (14,18). Purified oligonucleotides were >90% full-length material as assessed by polyacrylamide gel electrophoresis and capillary gel electrophoresis. The sequence of ISIS 4189 and its analogs, complementary to positions 243–262 of murine PKC-α mRNA, is 5′-CAGCCATGGTTCCCCCCAAC-3′.

Melting curves

The melting temperature for each oligonucleotide was determined in buffer containing 100 mM Na^+, 10 mM PO_4, 0.1 mM EDTA, pH 7.0, as previously described (14). Melting temperatures listed are an average of at least three experiments, with strands at a total concentration of 8 µM.

Treatment of C127 cells with oligonucleotides

Murine C127 cells were grown to 70–80% confluence in T-75 flasks. The cells were washed once with DMEM and then 5 ml DMEM containing 20 µg/ml *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride/dioleoylphosphatidylethanolamine (DOTMA/DOPE) (Gibco-BRL) solution was added to flasks. Oligonucleotides were then added to the required final concentration from a 100 µM stock solution and the flask swirled to mix. The cells were incubated at 37°C for 4 h and then the DOTMA/DOPE/oligonucleotide mixture was aspirated off and replaced with serum-containing medium for the indicated time.

Immunoblotting of PKC isozymes

PKC protein expression was evaluated as described (1). Briefly, C127 cells were treated with oligonucleotides, washed with cold phosphate-buffered saline and protein extracted in a Triton X-100 lysis buffer at 4°C. Protein levels were quantified using a BioRad DC protein assay with bovine serum albumin as a standard. Samples were electrophoresed through a 10% acrylamide gel and then immunoblotted as described previously (1).

Measurement of PKC-α mRNA levels

PKC mRNA expression in C127 cells was evaluated as described (1). Briefly, total RNA (10–20 µg) was resolved on a 1.0% agarose gel containing 1.1% formaldehyde and transferred by overnight capillary blotting to a nylon membrane (BioRad). The blot was then prehybridized in Quikhyb solution (Stratagene) for 1 h at 68°C. A bovine PKC-α cDNA probe (ATCC) was ^{32}P-radiolabeled with [α-^{32}P]dCTP by random priming (Promega) as per the manufacturer's protocol. The membranes were routinely stripped (boiling in 0.1% SSC/0.1% SDS for 2 min) and then reprobed with a radiolabeled human glycerol-3-phosphate dehydrogenase (G3PDH) probe to confirm equal loading.

In vitro nuclease stability

Oligonucleotide resistance to snake venom 3′ phosphodiesterase was determined as described previously (17). Briefly, the oligonucleotides were gel purified and 5′-end-labeled with HPLC-purified [γ-^{32}P]ATP (ICN). Oligonucleotides were then incubated with snake venom phosphodiesterase for the indicated times. Oligonucleotide metabolites were analyzed by separation on a 20% denaturing polyacrylamide gel followed by quantitation by PhosphorImager (Molecular Dynamics) analysis.

Oligonucleotide metabolism in C127 cells

Oligonucleotide metabolites were recovered from C127 cells and analyzed by capillary gel electrophoresis (CGE) as described previously (19). Briefly, C127 cells were treated with 500 nM oligonucleotide as described in Table 1 and allowed to recover for the indicated time. After digestion with proteinase K oligonucleotide metoblites were recovered by sequential passages through an anion exchange column and a reverse phase column. Analysis of the samples by capillary gel electrophoresis was performed on a Beckman 5010 P/ACE capillary electrophoresis unit.

Table 1. Hybridization thermodynamic properties of oligonucleotides designed to hybridize to murine PKC-α

Oligonucleotide	Sequence and modification	T_m (°C)	ΔG^o_{37} (kcal/mol)
4189	CsAsGsCsCsAsTsGsGsTsTsCsCsCsCsCsCsAsAsC	66.4	–16.9
8798	CoAoGoCoCoAoToGoGoToToCoCoCoCoCoCoCoAoAoC	72.8	–18.9
7817	**CsAsGsCsCsA**sTsGsGsTsTs**CsCsCsCsCsCsAsA**sC	71.2	–19.4
7815	**CoAoGoCoCoA**sTsGsGsTsTsCsCsCs**CoCoCoAoA**oC	73.9	–21.3

Upper case (A,G,T,C) represents the oligonucleotide base sequence. Lower case (o,s) represents oligonucleotide backbone structure (either phosphodiester or phosphorothioate respectively). The bases in bold are the 2′-*O*-propyl oligonucleotide.

RESULTS

Characterization of ISIS 4189 in cell culture

ISIS 4189 is a 20mer phosphorothioate oligodeoxynucleotide targeting the AUG initiation of protein synthesis codon on murine PKC-α and is a potent inhibitor of PKC-α mRNA and protein expression (1). In order to determine the mechanism of action for ISIS 4189 the oligonucleotide was synthesized as a 2′-*O*-methyl phosphorothioate, ISIS 4999. This modification does not support RNase H-mediated cleavage of hybridized mRNA (12) and, therefore, should not reduce PKC-α mRNA expression if RNase H is the mechanism of action. While ISIS 4189 reduced the amount of PKC-α mRNA by >90%, ISIS 4999 was completely without effect when cells were treated with 400 nM oligonucleotide (data not shown). While these data suggest that ISIS 4189 reduces PKC-α mRNA by a mechanism consistent with RNase H-mediated hydrolysis, they do not preclude an additional mechanism (i.e. translational arrest) which might inhibit PKC-α protein synthesis. ISIS 4999 was, however, also ineffective at reducing PKC-α protein synthesis at concentrations as high as 400 nM (data not shown). This strongly suggests that a reduction in PKC-α mRNA is required for ISIS 4189 to reduce PKC-α protein expression.

Design and hybridization thermodynamics of 2′-*O*-propyl modified oligonucleotides

In order to incorporate the benefits of the 2′-*O*-alkyl modification yet retain the ability to cleave hybridized mRNA through an RNase H-mediated mechanism oligonucleotides have been synthesized as 2′-*O*-propyl/deoxy chimeric oligonucleotides. These consist of 2′-*O*-propyl modified sequences flanking an eight base deoxynucleotide sequence. The following analogs were synthesized: ISIS 7817, a full phosphorothioate with eight 2′-deoxy residues in the center (position 7–14) and six and five 2′-*O*-propyl modified residues at the 5′- and 3′-ends respectively (positions 1–6 and 15–19); ISIS 7815, a chimeric oligonucleotide with eight phosphorothioate 2′-deoxy residues flanked by six and five 2′-*O*-propyl residues linked with phophodiester linkages at positions 1–6 and 15–19. A full phosphodiester analog of ISIS 4189 (ISIS 8798) was also synthesized (Table 1). Free energies of duplex formation and T_m values listed in Table 1 were obtained from melting experiments. The inclusion of the 2′-*O*-propyl modifications resulted in an increase in oligonucleotide T_m of ~0.5°C per base as a phosphorothioate backbone.

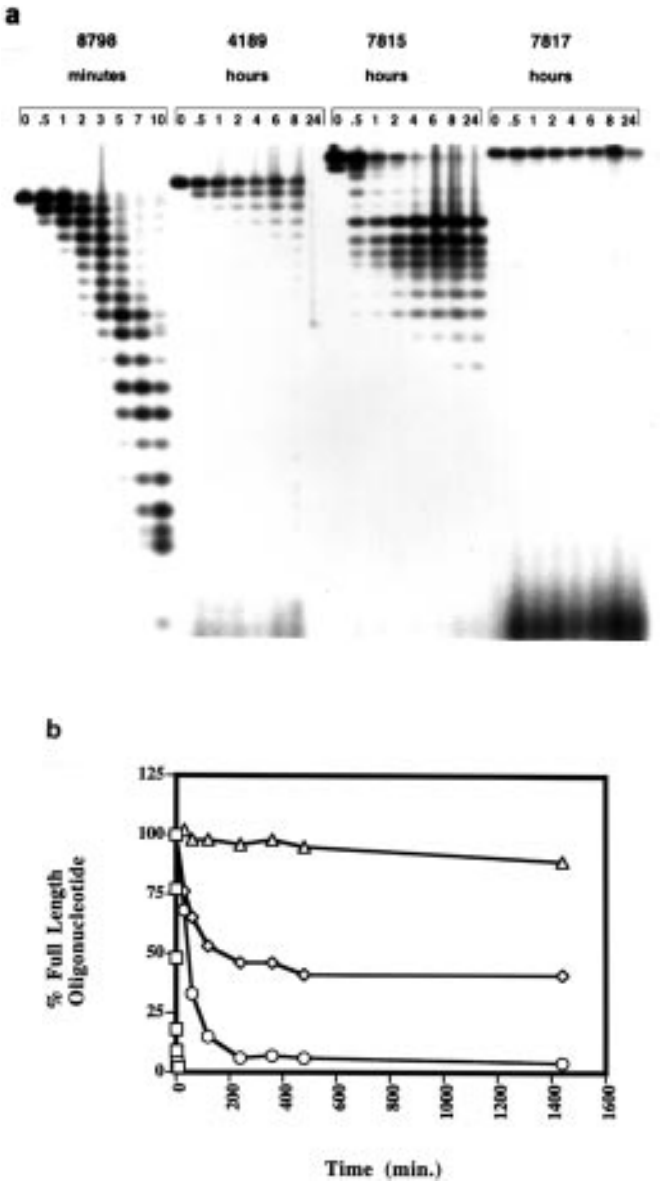
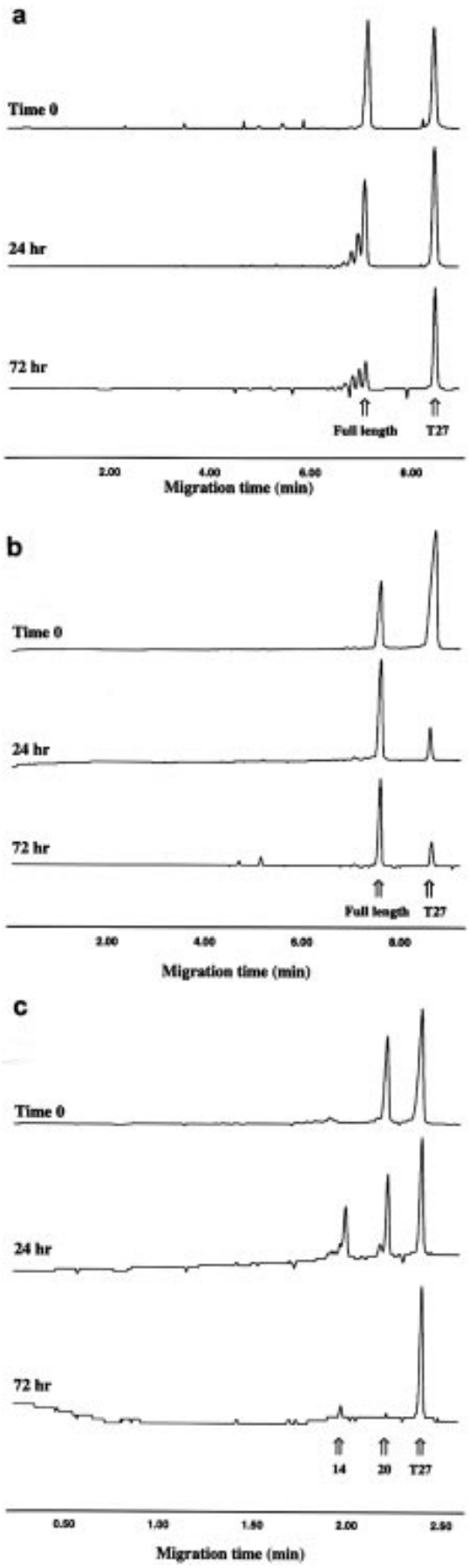


Figure 1. Stability of oligonucleotides towards snake venom phosphodiesterase. (**a**) Oligonucleotides were incubated with snake venom phosphodiesterase for the indicated times as described in Materials and Methods. The digested oligonucleotides were resolved on 20% polyacrylamide gels. (**b**) Quantitation of full-length (20mer) oligonucleotides in (a). ISIS 7817 (Δ), ISIS 4189 (◇), ISIS 7815 (○) and ISIS 8798 (□).

Nuclease resistance of modified oligonucleotides *in vitro*

Using an *in vitro* nuclease assay the resistance of each oligonucleotide to 3′ exonuclease degradation by snake venom phosphodiesterase was determined. ISIS 4189, the phosphorothioate oligodeoxynucleotide, was processively degraded at a rate such that 50% of the compound was still intact at 4 h (Fig. 1). This is typical for phosphorothioate oligodeoxynucleotides previously tested (Cummins, unpublished data). ISIS 8798, the phosphodiester oligodeoxynucleotide analog of ISIS 4189 exhibited a half-life of 15 min under the same conditions (Fig. 1). This increase in nuclease resistance obtained with the phosphorothioate backbone modification, both *in vitro* and *in vivo*, has been characterized previously for other sequences (20–22). ISIS 7817, the full phosphorothioate 2′-*O*-propyl chimeric oligonucleotide, displayed a substantially enhanced nuclease resistance. After 24 h incubation with snake venom phosphodiesterase >90% of the starting material remained full length (Fig. 1). In contrast, the half-life of ISIS 7815 (1 h) was shorter than that detected with the phosphorothioates, but substantially greater than the unmodified phosphodiester oligodeoxynucleotide (Fig. 1). It should be noted that there was an apparent accumulation of a 14mer product with time from ISIS 7815, which could be explained by the oligonucleotide being initially hydrolyzed 3′→5′ through the 2-*O*-propyl phosphodiester sequence until the phosphorothioate oligodeoxynucleotide sequences are encountered. At that point degradation occured at a rate characteristic of a phosphorothioate oligodeoxynucleotide.



Nuclease stability of modified oligonucleotides *in vivo*

We next performed experiments to determine whether any of the incorporated modifications provided enhanced nuclease resistance to intracellular nucleases in tissue culture. C127 cells were treated with oligonucleotide (500 nM in the presence of cationic lipid) and the oligonucleotide metabolites extracted from cells either 24 or 72 h later. The metabolites were resolved by CGE. CGE analysis of oligonucleotide metabolites is an extremely sensitive method for resolving full-length oligonucleotides from *n* – 1 oligonucleotides and metabolites, with a sensitivity as low as 10 nM from biological samples (23; Cummins, unpublished data).

The degradation pattern of the different oligonucleotides in C127 cells was similar to that observed with snake venom phosphodiesterase. ISIS 7817 remained almost completely intact in cells for up to 72 h (Fig. 2b). In contrast, ISIS 4189 and 7815 showed distinct profiles of degradation (Fig. 2a and c). ISIS 4189 appeared to be processively hydrolyzed by exonuclease degradation, resulting in the increased appearance over time of *n* – 1 to *n* – 10 metabolites. ISIS 7815 is more rapidly degraded, with the immediate accumulation of a 14mer product, consistent with the hypothesis that one end of the phosphodiester/2′-*O*-propyl portion of the molecule is being rapidly degraded until the phosphorothioate oligodeoxynucleotide sequence is reached. Metabolites of the phophodiester oligonucleotide ISIS 8798 could not be recovered by this procedure, presumably due to rapid metabolism.

Effect of 2′-*O*-propyl modified oligonucleotides on PKC-α mRNA expression in C127 cells

Both the increase in affinity and enhanced nuclease resistance seen in the previous experiments suggest that 2′-*O*-propyl modifications may greatly increase the activity of ISIS 4189 *in vivo*. The ability of the modified oligonucleotides in reducing PKC-α mRNA expression was determined 24 h after oligonucleotide addition to C127 cells. ISIS 7817 reduced PKC-α mRNA expression with an IC_{50} of ~75 nM and therefore was 2–3 times more active than ISIS 4189, which had an IC_{50} of 200 nM (Fig. 3). ISIS 7815 was nearly equipotent with ISIS 4189 with an IC_{50} of ~200 nM (Fig. 3). ISIS 8798 was completely inactive at any dose, presumably due to a lack of nuclease resistance.

The kinetics for the reduction in PKC-α mRNA were determined after a single treatment with a maximal (400 nM) concentration of oligonucleotide. Each of the oligonucleotides (except ISIS 8798) produced a maximum reduction in PKC-α mRNA (>80%) at between 15 and 24 h (Fig. 4). However, only ISIS 7817 maintained this level of inhibition to 72 h. In cells treated with ISIS 4189 and 7815 levels of PKC-α mRNA had returned to 60% and 100% of control respectively after 72 h (Fig. 4).

Since kinetic studies on PKC-α mRNA inhibition indicate that there are large differences in oligonucleotide activity at later time points (>48 h). We have compared the concentrations required to reduce PKC-α mRNA expression 72 h after oligonucleotide

Figure 2. Oligonucleotide metabolism in C127 cells. C127 cells were treated with oligonucleotides (ISIS 4189, 7815 or 7817) for either 0, 24 or 72 h as indicated. At specified times metabolites were extracted and resolved by capillary gel electrophoresis as described in Materials and Methods. (**a**) ISIS 4189. (**b**) ISIS 7817. (**c**) ISIS 7815.

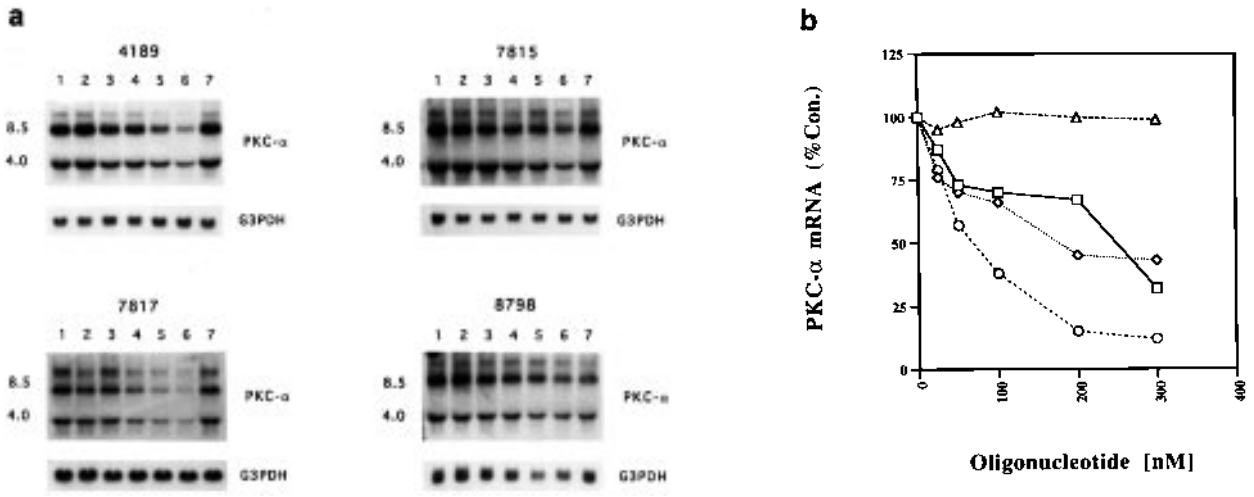


Figure 3. Dose–response characteristics for the reduction in PKC-α mRNA expression in C127 cells after 24 h exposure to oligonucleotides. (**a**) C127 cells were treated with the oligonucleotides indicated (either ISIS 4189, 7815, 7817 or 8798) for 24 h as described in Materials and Methods. Lane 1, no oligonucleotide; lane 2, 25 nM; lane 3, 50 nM; lane 4 100 nM; lane 5, 200 nM; lane 6, 300 nM; lane 7, no oligonucleotide. (Upper) PKC-α mRNA. (Lower) Same blot stripped and reprobed with G3PDH to demonstrate equal loading. (**b**) Levels of PKC-α mRNA from the above gel were quantified with a PhosphorImager and expressed as percent of control. ISIS 4189 (□), ISIS 7815 (◇), ISIS 7817 (○), ISIS 8798 (Δ).

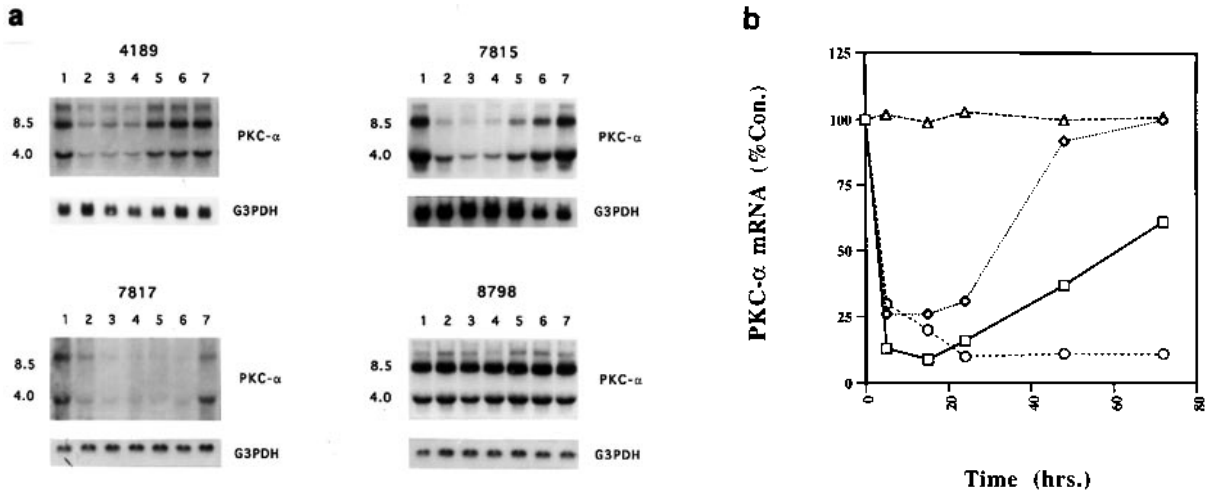


Figure 4. Kinetic analysis of the reduction in PKC-α mRNA by oligonucleotides. (**a**) C127 cells were treated with 400 nM indicated oligonucleotide (either ISIS 4189, 7815, 7817 or 8798) for different times as described in Materials and Methods. Treatment times were: lane 1, zero time; lane 2, 5 h; lane 3, 15 h; lane 4, 24 h; lane 5, 48 h; lane 6, 72 h; lane 7, zero time. (Upper) PKC-α mRNA. (Lower) Same blot stripped and reprobed with G3PDH to demonstrate equal RNA loading. (**b**) Levels of PKC-α mRNA from the above gels were quantified with a PhosphorImager and expressed as percent of control. ISIS 4189 (□), ISIS 7815 (◇), ISIS 7817 (○), ISIS 8798 (Δ).

treatment was initiated. ISIS 7817 was ~5 times more effective than ISIS 4189 (Fig. 5). ISIS 7815 and 8798 were inactive up to 300 nM (Fig. 5).

Effect of 2′-*O*-propyl modified oligonucleotides on PKC-α protein expression in C127 cells

The effects of the oligonucleotides on the steady-state levels of PKC-α protein in C127 cells were examined. We have previously reported that a substantial reduction in PKC-α protein expression with ISIS 4189 required repeated oligonucleotide treatments (1). This is likely due to the relatively long (6–24 h) half-life of PKC-α protein (24,25) and the loss of oligonucleotide due to degradation after 24–48 h. A single application of ISIS 4189 decreased PKC-α protein to levels of only 50–70% of control after 3 days (Fig. 6). In contrast, a single treatment of C127 cells with ISIS 7817 resulted in complete loss of PKC-α protein (Fig. 6). ISIS 7815 decreased PKC-α protein to ~80% of control at this time. An oligonucleotide with the same chemical structure as ISIS 7817 (targeting human PKC-η) was used as a control in this experiment and was without effect on PKC-α protein. The specificity for inhibition of PKC-α was examined by measuring the effect of the oligonucleotides on the levels of other isozymes of PKC. None of the oligonucleotides tested had any effect on the expression of PKC-δ or -ζ (Fig. 6).

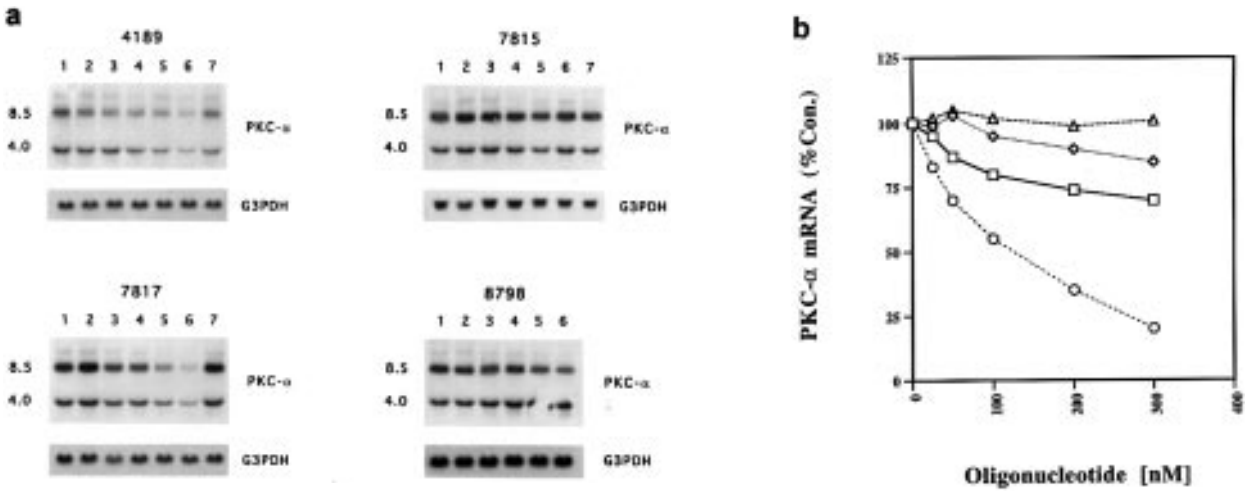


Figure 5. Dose–response characteristics for the reduction in PKC-α mRNA expression in C127 cells after 72 h exposure to oligonucleotides. (**a**) C127 cells were treated with the oligonucleotides indicated (either ISIS 4189, 7815, 7817 or 8798) for 72 h as described in Materials and Methods. Lane 1, no oligonucleotide; lane 2, 25 nM; lane 3, 50 nM; lane 4, 100 nM; lane 5, 200 nM; lane 6, 300 nM; lane 7, no oligonucleotide. (Upper) PKC-α mRNA. (Lower) Same blot stripped and reprobed with G3PDH to demonstrate equal RNA loading. (**b**) Levels of PKC-α mRNA from the above gels were quantified with a PhosphorImager and expressed as percent of control. ISIS 4189 (□), ISIS 7815 (◇), ISIS 7817 (○), ISIS 8798 (Δ).

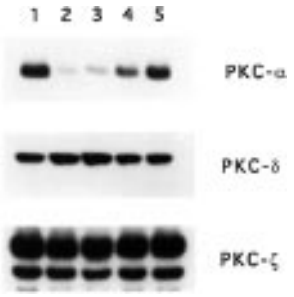


Figure 6. Effect of oligonucleotides on the expresssion of PKC isozymes in C127 cells. C127 cells were treated with oligonucleotides (400 nM) for 72 h. At this time proteins were extracted and immunoreactive PKC isozyme protein determined by immunoblotting (see Materials and Methods). Lane 1, no oligonucleotide; lane 2, ISIS 7817; lane 3, ISIS 4189; lane 4, ISIS 7815; lane 5, ISIS 8732 (control 2′-*O*-propyl oligonucleotide).

DISCUSSION

The identification of a 20mer phosphorothioate oligodeoxynucleotide (ISIS 4189) which specifically reduces murine PKC-α expression in tissue culture and in mice has been previously described (1). Additional chemical modifications have been incorporated into this sequence in an attempt to increase oligonucleotide activity by increasing affinity and nuclease resistance. While chemical modifications may be incorporated into an oligonucleotide to enhance a given characteristic (i.e. nuclease resistance), the modification may also posess an undesirable characteristic which could produce an overall decrease in antisense activity. Therefore, if chemical modifications are to be rationally incorporated in an attempt to increase antisense activity it is crucial to identify the mechanism of action for each individual oligonucleotide.

Chemical modifications incorporated into the 2′-sugar position of oligonucleotides offer the potential for increasing both duplex stability and nuclease resistance of an oligonucleotide. However, an undesirable characteristic of these modifications is an inability to support cleavage of hybridized mRNA by RNase H (13). This disadvantage may be overcome by creating a chimeric oligonucleotide composed of mixed 2′-*O*-modified sequences and oligodeoxynucleotides sequences. These molecules retain the ability to serve as a substrate for RNase H and incorporate some of the beneficial characteristics of 2′-*O* modifications (13) This strategy has been successfully used to combine internal phosphodiester and terminal methylphosphonodiesters (26,27). Similarly, chimeric phosphodiester/2′-*O*-methyl molecules have been shown to support RNase H cleavage of hybridized mRNA (12,28). A more extensive study published recently has characterized the effect of different oligodeoxynucleotide sequence sizes on the ability of an oligonucleotide to support RNase H cleavage *in vivo* and determined the effects of different 2′-*O* modifications on oligonucleotide/mRNA duplex formation (13). These studies have concluded that a minimum of four to five consecutive deoxy residues are required to support mammalian RNase H cleavage of hybridized mRNA.

This approach has been adopted to examine the effects of the incorporation of 2′-*O*-propyl modifications in the sequence of ISIS 4189. Previous work has shown that 2′-*O*-propyl modifications improve hybridization thermodynamics when compared with oligodeoxynucleotides (13,14) and are more nuclease resistant towards S-1 nuclease when incorporated uniformly into a phosphodiester or phosphorothioate oligonucleotide (17). However, neither intracellular nuclease stability nor the ability to decrease expression of a cellular targeted mRNA or protein have been previously reported for the 2′-*O*-propyl modification when tested in chimeric oligonucleotides.

Our results demonstrate that the duration of action for ISIS 4189 can be correlated with *in vivo* nuclease stability. The characteristic recovery with time of a target mRNA after initial inhibition by phosphorothioate oligodeoxynucleotides (1,29) can now be overcome by the incorporation of 2′-*O*-propyl nucleosides to enhance nuclease resistance. We have characterized the

2′-*O*-propyl modification and have shown that it is capable of enhancing hybridization thermodynamics and significantly increasing nuclease resistance of phosphorothioate oligodeoxynucleotides. This modification increased oligonucleotide activity 5-fold after 72 h in C127 cells, when compared with the parent oligodeoxynucleotide. In summary, these data suggest that 2′-*O*-propyl modified phosphorothioate chimeric oligonucleotides may be particularly useful for target mRNAs which produce proteins exhibiting long half-lives.

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