0026-895X/96/020236-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:236–242 (1996).

Treatment of Glioblastoma U-87 by Systemic Administration of an Antisense Protein Kinase C-α Phosphorothioate Oligodeoxynucleotide

TAKAHITO YAZAKI,[1] SHAKEEL AHMAD, ALI CHAHLAVI, ESTER ZYLBER-KATZ,[2] NICHOLAS M. DEAN, SAMUEL D. RABKIN, ROBERT L. MARTUZA, and ROBERT I. GLAZER

Departments of Neurosurgery (T.Y., A.C., S.D.R., R.L.M.) and Pharmacology (S.A., E.Z.-K., R.I.G.), Georgetown Brain Tumor Center, Georgetown University Medical Center, Washington, D.C. 20007, and ISIS Pharmaceuticals, Inc., Carlsbad, California 92008 (N.M.D.)

Received February 14, 1996; Accepted April 8, 1996

SUMMARY

Glioblastoma multiforme is the most common form of malignant brain cancer in adults and, unfortunately, is not amenable to treatment with current therapeutic modalities. Human glioblastoma U-87 has many of the distinguishing phenotypic features of primary glioblastoma, including an autocrine form of proliferation, high levels of protein kinase Cα (PKCα), and infiltration via white matter tracts. We show that treatment of mice bearing U-87 xenografts with an antisense phosphorothioate oligodeoxynucleotide (S-oligodeoxynucleotide) against the 3′-untranslated region of PKCα mRNA results in suppression of tumor growth. Growth was inhibited in both subcutaneous and intracranial tumors, and in the latter instance, treatment with the antisense PKCα S-oligodeoxynucleotide resulted in a doubling in median survival time (>80 days), with 40% long term survivors. The antisense S-oligodeoxynucleotide did not produce systemic toxicity in mice with subcutaneous or intracranial tumors after daily intraperitoneal injection for 21 or 80 days, respectively, and a scrambled S-oligodeoxynucleotide with the same nucleotide composition as the antisense S-oligodeoxynucleotide did not produce an antitumor effect. The intratumoral levels of both antisense and scrambled S-oligodeoxynucleotide in subcutaneous tumors were 2 μM after 21 daily doses of 20 mg/kg S-oligodeoxynucleotide. The antisense S-oligodeoxynucleotide selectively reduced the levels of PKCα in subcutaneous tumors but not those of protein kinase Cε or protein kinase Cζ. This is the first demonstration that the growth of glioblastoma multiforme can be suppressed by an antisense PKCα S-oligodeoxynucleotide and suggests that this may represent an effective therapy for this type of malignancy.

Glioblastoma multiforme, the most common form of malignant brain tumor in adults, results in a median survival of 1 year from the time of diagnosis due to its refractoriness to traditional therapeutic modalities such as surgery, radiotherapy, and chemotherapy (1). To develop a more selective approach for the therapy of malignant gliomas, antisense oligonucleotides have been used to target and selectively inhibit the expression of specific gene products (2). One molecular target for glioblastoma multiforme that we have begun to explore is PKCα (3). The PKC family represents at least 11 closely related genes that are involved in signal transduction for processes such as proliferation, angiogenesis, inflammation, and the immune response (4, 5). Although the specific functions of the individual isozymes are still unclear, it is evident that one or more PKC isoforms may play a role in cell cycle progression and transformation (6).

PKC has been proposed as a target for the development of anticancer drugs (7) and as a therapeutic target for treating malignant gliomas (8). We have shown previously (3) that suppression of PKCα in glioblastoma U-87 after transfection with an antisense PKCα cDNA resulted in a reduction in proliferation, serum dependence, and tumorigenicity, as well as a decreased sensitivity to the selective PKC inhibitor Ro 31–8220 (3). Although these results suggested that the inhibition of PKCα may be an important cellular target for arresting tumor growth, *in vivo* transfection techniques in current use are neither practical nor feasible for clinical studies.

Recently, antisense S-oligodeoxynucleotides have been developed for selectively inhibiting human PKCα in A549 lung carcinoma cells (9). The therapeutic advantage of these ana-

This work was supported by National Institutes of Health Grants CA60176 (R.L.M) and NS34431 (R.I.G.), a grant from Neurofibromatosis Inc. (Massachusetts Bay area) (R.L.M.), and a grant from the American Brain Tumor Association (A.C.).

T.Y. and S.A. contributed equally to this study.

[1] Current affiliation: Departments of Neurosurgery and Physiology, School of Medicine, Keio University, Tokyo 160, Japan.

[2] Current affiliation: Clinical Pharmacology Unit, Hadassah University Hospital, Jerusalem, Israel.

ABBREVIATIONS: PKC, protein kinase C; HBSS, Hanks' balanced salt solution; S-oligodeoxynucleotide, phosphorothioate oligodeoxynucleotide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

logs is that they are relatively nontoxic after systemic administration (10) and are target selective (9). In this report, we demonstrate that a systemically administered antisense S-oligodeoxynucleotide against the 3′-untranslated region of human PKCα mRNA produces significant antitumor activity against glioblastoma U-87 xenografts without nonspecific cytotoxicity. Our results suggest a novel molecular target and an alternate therapeutic approach for the treatment of malignant gliomas.

Materials and Methods

S-oligodeoxynucleotide design. ISIS 3521 and ISIS 4559 were synthesized at ISIS Pharmaceuticals, Inc., Carlsbad, CA. ISIS 3521 hybridizes to the 3′-untranslated sequence of human PKCα beginning at the TGA codon and contains the sequence 5′-GTTCTCGCTGGTGAGTTTCA-3′. ISIS 4559 is a scrambled control S-oligodeoxynucleotide with the same base composition as ISIS 3521 and contains the sequence 5′-GGTTTTACCATCGGTTCTGG-3′ (9), which does not recognize any known human mRNA sequence, as based on a Blast search.

Cytotoxicity. For *in vitro* studies, a 24-well plate was seeded with 5000 cells/well in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50 μg/ml gentamicin. Cells were allowed to attach for 24 hr, medium was removed, and cells were washed twice with 0.5 ml of serum-free medium. Varying concentrations of S-oligodeoxynucleotide were diluted in sterile phosphate-buffered saline and added in a volume of 15 μl to each well containing 0.5 ml of Dulbecco's modified Eagle's medium and Lipofectin reagent (10 μg/ml; GIBCO-BRL, Gaithersburg, MD) (11). Controls contained either phosphate-buffered saline or Lipofectin alone, which was not found to be cytotoxic under these conditions. Cells were incubated at 37° for 4 hr, at which time the medium was discarded and replaced with complete medium containing the same concentration of S-oligodeoxynucleotide. Cells were incubated for 3–4 days, fixed at 4° for 1 hr by the addition of 50% trichloroacetic acid to a final concentration of 10%, washed five times with deionized water, air dried, and stained for 30 min at room temperature with 0.5 ml of 0.4% sulforhodamine B dissolved in 1% acetic acid (12). Absorbance was read at 560 nm in a microplate reader.

Immunoblotting. Mice with subcutaneous U-87 tumors were killed on days 21 and 35 after initiation of S-oligodeoxynucleotide treatment. Tumors were washed once in phosphate-buffered saline and homogenized at 4° in 200 μl of lysis buffer containing 20 mM Tris·HCl, pH 7.4, 1% Triton X-100, 5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 50 mM sodium fluoride, 10 mM sodium phosphate, 2 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (3). Tumor extracts were separated by electrophoresis in 8% sodium dodecyl sulfate-polyacrylamide gels (Novex, San Diego, CA) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 0.2% gelatin in 50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween-20 and reacted with monoclonal antibodies against PKCα, PKCβ, PKCγ, PKCδ, PKCε, and PKCζ (Transduction Laboratories, Lexington, KY). After incubation with an alkaline phosphatase-conjugated secondary antibody, PKC was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (3). U-87 tumors did not contain detectable amounts of PKCβ or PKCγ.[3]

Animal studies. Six-week-old female athymic BALB/c nu/nu mice weighing 22–25 g were purchased from Taconic Farms (Germantown, NY). Mice were housed in groups of five and had free access to autoclaved food and water. All animal procedures were approved by the Georgetown University Animal Care and Use Committee. Each mouse was anesthetized with an intraperitoneal injection of 0.25 ml of a solution containing 0.84% bacteriostatic saline, 10% sodium pentobarbital, and 6% ethyl alcohol (Abbott Laboratories, Chicago, IL) at a dose of 1 mg/kg. Tumors were implanted subcutaneously into the flank region as described previously (13). After 1 week, mice were injected intraperitoneally daily with ISIS 3521 or 4559 dissolved in HBSS at doses of 2 or 20 mg/kg or with HBSS alone (100 μl/25 g body weight). Tumor volume was estimated by caliper measurements of length × width × height. Statistical differences in tumor size were assessed by use of the unpaired *t* test.

U-87 cells (2×10^5 cells/50 μl) were also implanted intracranially into the right frontal lobe of nude mice as described previously (14). Seven days after implantation, animals were divided randomly into three groups of 10 mice each and treated with HBSS or ISIS 4559 or 3521 as described above, except that animals were treated daily until the end of the experiment on day 82 (approximately twice the survival time of control animals) or until death ensued. Statistical differences in tumor size were assessed by use of the Wilcoxon test.

Histology. Mice containing intracranial tumors were killed on day 82 or used immediately at their time of death. Animals were perfused with 2% paraformaldehyde/5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid/2 mM magnesium chloride in 0.1 M 1,4-piperazinediethanesulfonic acid buffer, pH 7.3, and brains were removed with the skull intact and placed in 10% formalin. Samples were decalcified for 6 hr in a solution of 8% HCl and 10% formic acid, and microtome sections (5 μm) were prepared, mounted on glass slides, and stained with hematoxylin and eosin.

Capillary gel electrophoresis. Mice with subcutaneous U-87 tumors that were treated with HBSS or 20 mg/kg ISIS 3521 or ISIS 4559 were killed either 1 day or 2 weeks after the cessation of treatment on day 21 as described above. Tumor tissue (100 mg) was extracted in a Dounce homogenizer with 0.5 ml of buffer containing 0.5% (v/v) Nonidet P-40, 25 mM Tris·HCl, pH 8.0, 25 mM EDTA, and 100 mM NaCl (15). After disruption, proteinase K was added to a final concentration of 10 mg/ml, and 10 μM polyuridine (27-mer) was added to each sample as an internal control to measure recovery. Samples were incubated overnight at 37° and extracted with 1 ml of phenol/isoamyl alcohol/chloroform (24:1:24). The phases were separated by centrifugation, the organic phase was reextracted with 500 μl of water, and the two aqueous phases were combined and lyophilized in a Savant Speed-Vac concentrator. Samples were analyzed with a Beckman PA/CE System Gold 5010 capillary electrophoresis system with the detector set at 260 nm. Samples were loaded at 5–10 kV for 5 sec and separated at 10–15 kV constant voltage at 50° for 6 min (15).

Results

***In vitro* cytotoxicity of ISIS 3521 against U-87 cells.** The cytotoxicity of the antisense PKCα S-oligodeoxynucleotide ISIS 3521 was first evaluated against U-87 cells *in vitro* (Fig. 1). Continuous exposure of U-87 cells to ISIS 3521 for 3–4 days resulted in marked growth inhibition with an IC_{50} of ~100 nM (Fig. 1, *left*). Similar treatment with the scrambled S-oligodeoxynucleotide ISIS 4559 did not produce cytotoxicity. U-87 cells treated with ISIS 3521 exhibited a diminished number of processes and reduced cell number compared with cells treated with ISIS 4559 (Fig. 1, *right*) or untreated cells (results not shown).

Inhibition of U-87 xenografts by ISIS 3521. The activity of ISIS 3521 *in vivo* was examined in nude mice with subcutaneous U-87 xenografts. One week after tumor implantation, mice were administered daily doses of 2 or 20 mg/kg of ISIS 3521 or 4559 intraperitoneally for 21 days (Fig. 2). Significant inhibition of tumor growth was produced by both doses of ISIS 3521, and the respective tumor growth rates (mm^3/day) in mice treated with 2 or 20 mg/kg of ISIS 3521 during the first 28 days of tumor growth were 28% and

[3] S. Ahmad and R. I. Glazer, unpublished observations.

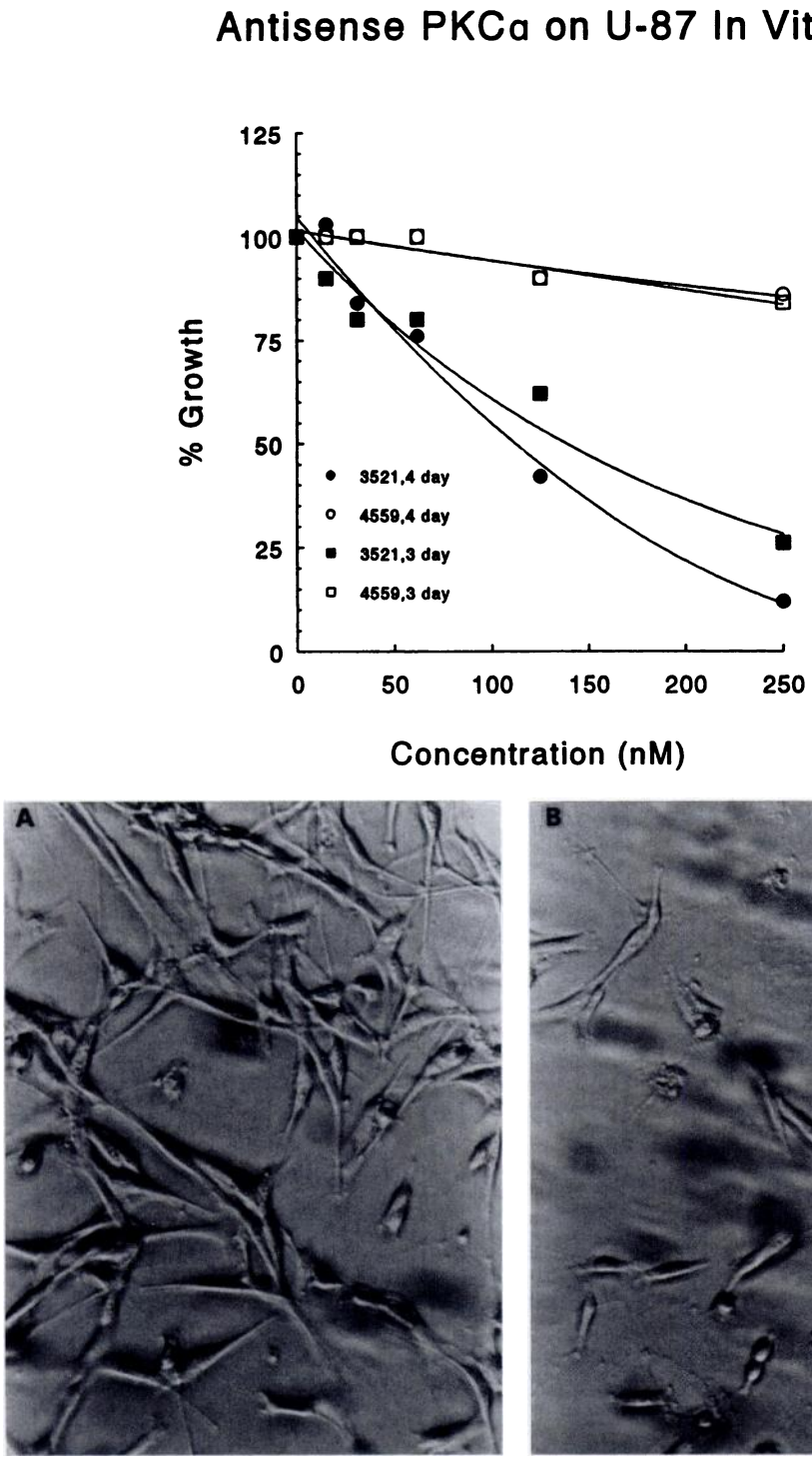

Fig. 1. Effect of ISIS 3521 on the growth of U-87 cells *in vitro*. *Top*, cells were incubated in serum-free medium for 4 hr with either PBS, Lipofectin, ISIS 3521, or ISIS 4559 mixed with Lipofectin. Cells were then washed and incubated in complete medium for 3–4 days with the indicated concentration of S-oligodeoxynucleotide. Cell growth was determined by staining with sulforhodamine B (12). *Bottom*, photomicrograph of U-87 cells treated for 3 days with 125 nM ISIS 4559 (A) or 3521 (B). Note the marked reduction in the number of processes in U-87 cells treated with ISIS 3521.

19% of control mice treated with HBSS alone ($p < 0.05$ and $p < 0.001$, respectively; t test). The respective tumor size in mice treated with 2 or 20 mg/kg of ISIS 3521 on day 28 was 18% and 11% of HBSS-treated mice.

Due to tumor burden, control and ISIS 4559-treated mice were killed on day 28. The two remaining groups of mice that had been treated with ISIS 3521 received extended treatment to determine whether inhibition of tumor growth could be reinduced in animals with a high tumor burden. On day 30, mice treated previously with 2 mg/kg ISIS 3521 were switched to receive treatment with 20 mg/kg ISIS 3521, and mice treated previously with 20 mg/kg ISIS 3521 were switched to receive treatment with HBSS (Fig. 2). Suppression of tumor growth was again noted in animals treated with 20 mg/kg ISIS 3521 ($p < 0.05$, t test), whereas mice receiving HBSS exhibited tumor progression.

Tumor levels of ISIS 3521 and ISIS 4559. Tumors were removed from mice at 1 day and 2 weeks after the last of 21 daily intraperitoneal injections of ISIS 3521 or ISIS 4559, and S-oligodeoxynucleotide concentrations were determined by capillary gel electrophoresis (Fig. 3). The intratumoral concentrations of ISIS 3521 and ISIS 4559 were 2.3 and 2.0

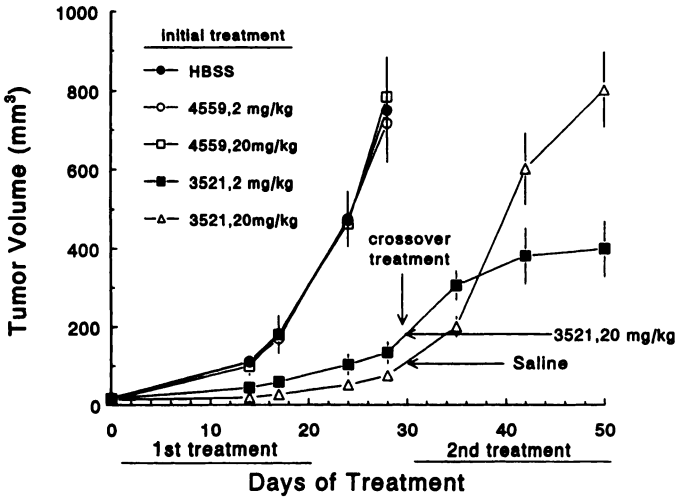

Fig. 2. Effect of ISIS 3521 on subcutaneous U-87 tumor growth in nude mice. *Bold lines on the abscissa*, first and second treatment intervals. For the initial treatment regimen, five groups of mice with subcutaneous U-87 tumors were administered daily intraperitoneal doses of 2 or 20 mg/kg ISIS 3521, ISIS 4559, or HBSS from days 0–20 as indicated in the figure legend, and tumor size was determined by caliper measurement. For the *crossover treatment*, the two surviving groups of mice treated initially with ISIS 3521 were treated from day 30 to day 50 as follows: mice treated initially with 2 mg/kg ISIS 3521 were switched to a dose of 20 mg/kg ISIS 3521, and mice treated initially with 20 mg/kg ISIS 3521 were switched to HBSS.

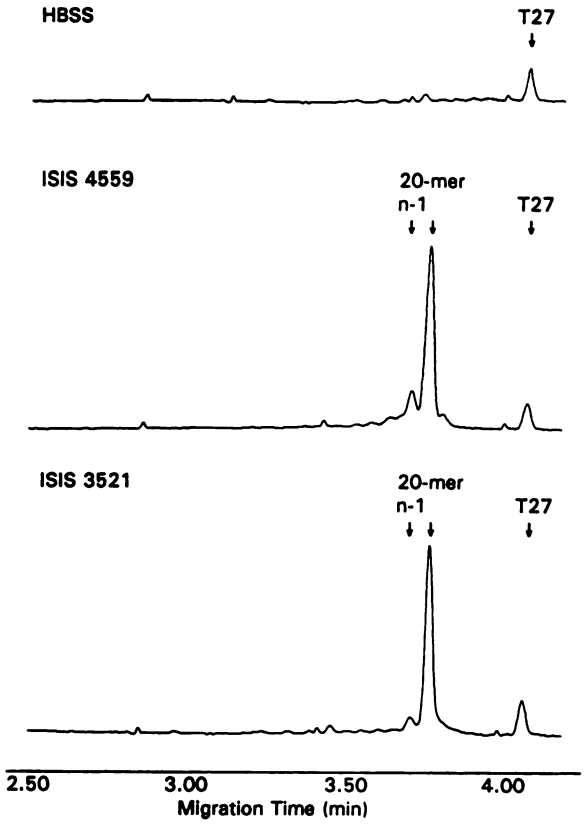

Fig. 3. Capillary gel electrophoresis of ISIS 3521 and ISIS 4559 in extracts of subcutaneous U-87 tumors. Nude mice with subcutaneous U-87 tumors were treated daily for 21 days with 20 mg/kg ISIS 3521 or ISIS 4559. One day after the last injection, residual tumor was removed, and S-oligodeoxynucleotide levels were determined in tumor extracts by capillary gel electrophoresis (15). A 27-mer polyuridine (*T27*) was added as an internal control to each extract to assess S-oligodeoxynucleotide recovery from tumor tissue. The retention time of intact ISIS 3521 or ISIS 4559 (*20-mer*) and their respective metabolites (*n-1*) was established by comparison with standards.

μM, respectively, 1 day after the last administered dose. No S-oligodeoxynucleotide was detected in tumor samples 2 weeks after the last dose of S-oligodeoxynucleotide (results not shown), and no absorbance was detected at a migration time of 3.8 min where the intact 20-mer S-oligodeoxynucleotide migrated in HBSS-treated animals. Minimal metabolism of either S-oligodeoxynucleotide was detected in tumor extracts, and the major metabolite, the n-1 oligomer, represented only 5% of the total S-oligodeoxynucleotide concentration.

Selectivity of ISIS 3521 on PKC isoform levels. PKCα levels were examined by immunoblotting in tumors 2 weeks after the initiation of treatment (day 14) or 2 weeks after cessation of treatment (day 35) (Fig. 4). There was a marked reduction in tumor PKCα levels 2 weeks after initiation of treatment with either dose of ISIS 3521, whereas tumor PKCα levels were unchanged in mice treated with either HBSS or ISIS 4559 or 2 weeks after the last injection of ISIS 3521. PKCε and PKCζ levels were unchanged during and after treatment with HBSS, ISIS 4559, or ISIS 3521. PKCβ and PKCγ isoforms were undetectable in tumors from all treatment groups.

Inhibition of intracranial U-87 xenografts by ISIS 3521. To determine whether ISIS 3521 would inhibit U-87 tumors implanted intracranially, mice were treated intraperitoneally daily for 82 days or until death ensued with either HBSS or 20 mg/kg ISIS 3521 or ISIS 4559 beginning 7 days after tumor implantation (Fig. 5). All animals treated with either HBSS or ISIS 4559 died by day 42. In contrast, 40% of the animals treated with ISIS 3521 were alive on day 82 ($p < 0.001$, Wilcoxon test). Brains were removed from five animals at the time of death in the HBSS- and ISIS 4559-treated groups as well as from the four surviving mice treated with ISIS 3521 on day 82 and were examined histologically. Representative sections from two animals treated with ISIS 4559 or ISIS 3521 are shown in Fig. 6. Large tumors were present in the brain of all saline- and ISIS 4559-treated mice (Fig. 6, A and B), whereas the brains from ISIS 3521-treated mice showed either a small tumor (three mice) or no tumor (one mouse) (Fig. 6, C and D). No pathological effect on the surrounding brain tissue was evident.

Discussion

PKC has been found to be an essential component in the signaling pathways for several growth factors that are involved in the proliferation of glioblastoma multiforme (16, 17). Treatment of glioblastoma cells with the nonselective protein kinase inhibitors staurosporine (18, 19) or H-7 (20) resulted in pronounced growth inhibition. Although the role of PKC in cell function has become increasingly complex due to the identification of multiple PKC isoforms, all studies of glioblastoma cell lines (3, 21–24) as well as of primary malignant gliomas (25) indicate that PKCα is the most abundant isoform. Our previous study showing pronounced inhibition of U-87 cell proliferation and tumorigenicity in U-87 cells after transfection with an antisense PKCα cDNA (3) also suggests that PKCα is an important regulatory factor in glioblastoma cell proliferation. In the current report, we ex-

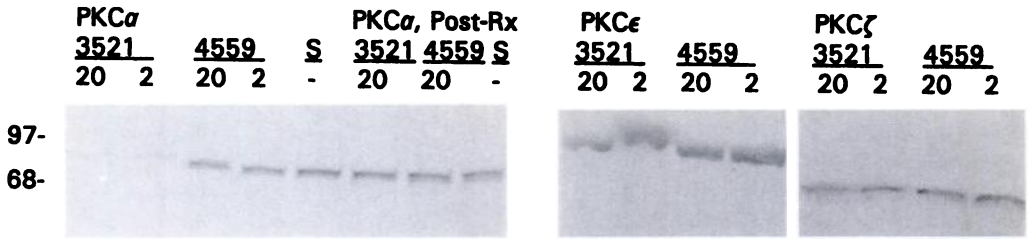

Fig. 4. Immunoblot of PKC isoforms in subcutaneous U-87 tumors after systemic administration of ISIS 3521. One group of mice was killed 2 weeks after receiving daily intraperitoneal injections of HBSS (*S*) or 2 (*2*) or 20 (*20*) mg/kg doses of ISIS 3521 (*3521*) or ISIS 4559 (*4559*). A second group of mice was killed 2 weeks after the last dose of S-oligodeoxynucleotide (*Post-Rx*). Tumor extracts were prepared, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. PKC was detected with isoform-specific monoclonal antibodies.

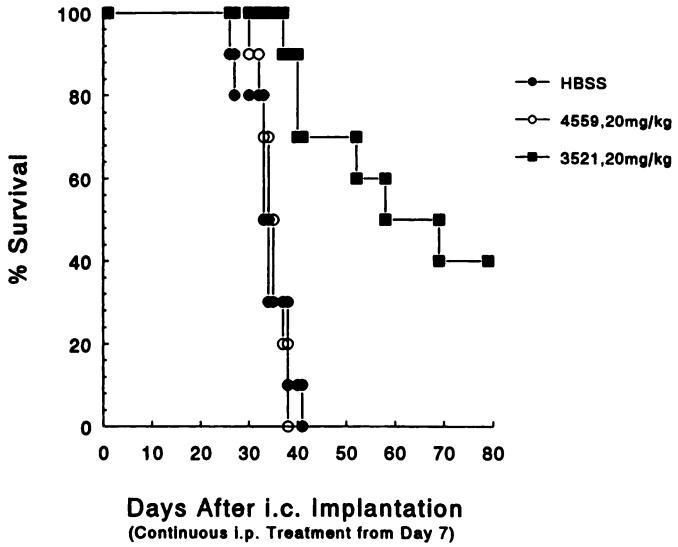

Fig. 5. Effect of ISIS 3521 on intracranial (*i.c.*) U-87 tumors. U-87 cells were implanted intracranially into the right frontal lobe of nude mice. One week after tumor implantation, mice were treated intraperitoneally (*i.p.*) daily with either HBSS or 20 mg/kg ISIS 3521 or ISIS 4559 for the duration of survival of each group of animals.

tended these observations by showing that an antisense S-oligodeoxynucleotide against PKCα may be a useful systemic therapeutic agent for the treatment of malignant gliomas.

ISIS 3521 was found to be a specific and potent inhibitor of PKCα expression in A549 lung carcinoma cells *in vitro* (9). We observed similar results with ISIS 3521 in U-87 cells in culture where it exhibited an IC_{50} of 100 nM. More striking, however, was the marked antitumor activity of ISIS 3521 *in vivo* against either subcutaneously or intracranially implanted tumors after daily intraperitoneal administration. Morphologically, subcutaneous tumors not only appeared smaller but also were hypovascular (results not shown). It is of interest that a member of the adhesion protein family, intercellular adhesion molecule-1, is also inhibited by ISIS 3521 in A549 lung carcinoma cells (9). However, it is unlikely that this mechanism is involved in our study because ISIS 3521 is specific for the human PKCα mRNA and angiogenesis in tumor xenografts is murine in origin. Therefore, it is more likely that the hypovascular appearance of ISIS 3521-treated tumors is a result of growth inhibition and cell death.

Capillary gel electrophoresis was used to determine the intratumoral concentration and metabolites of ISIS 3521 and ISIS 4559 in subcutaneous tumors 1 day and 2 weeks after

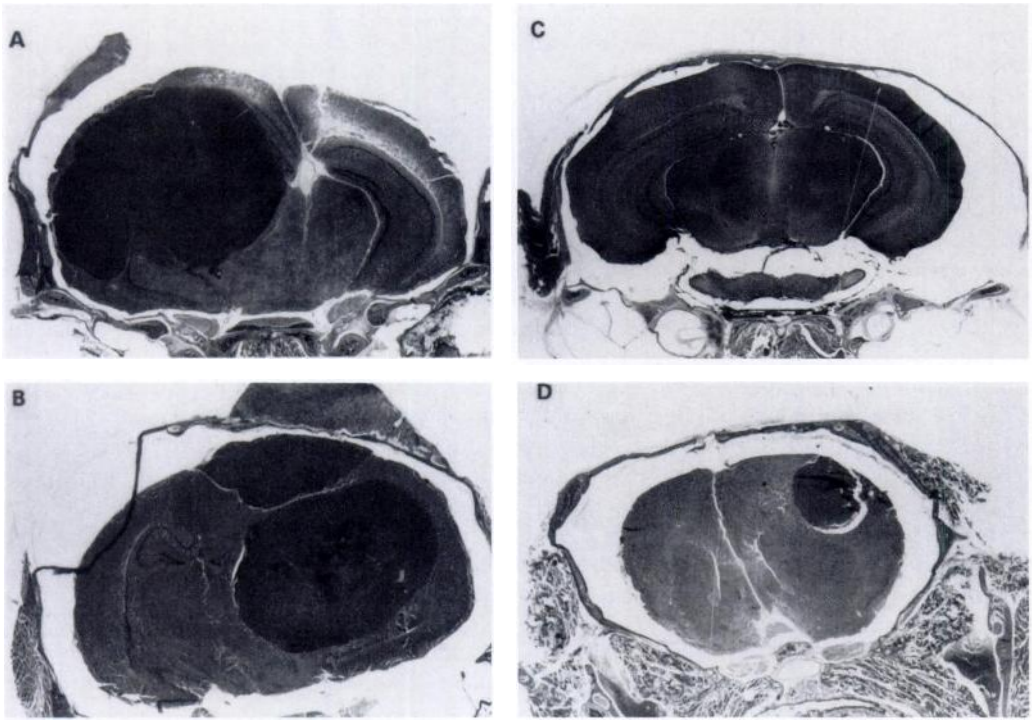

Fig. 6. Tissue sections of brains from animals implanted with intracranial U-87 tumors and treated with ISIS 3521. Cross sections of formalin-fixed brains were stained with hematoxylin and eosin. A and B, Brain tissue from mice treated with ISIS 4559 at the time of death on day 40. C and D, Brain tissue from control mice treated with ISIS 3521 and killed on day 82. All mice treated with either saline (not shown) or ISIS 4559 had large tumors, whereas mice treated with ISIS 3521 had either no tumor (one mouse) or small tumors (three mice). Two representative samples from the ISIS 4559 or ISIS 3521 groups are shown.

daily intraperitoneal treatment for 21 days. An intratumoral S-oligodeoxynucleotide concentration of 2 μM was detected after the 21-day regimen, but no S-oligodeoxynucleotide was detected 2 weeks after termination of treatment. In addition, 95% of either ISIS 4559 or ISIS 3521 was present as unmetabolized drug, indicating that 3′-exonuclease activity is extremely low in tumors, in contrast to normal organs such as liver and kidney (15). This phenotypic characteristic, especially if present in other tumor types, would be expected to enhance the therapeutic index of S-oligodeoxynucleotides as anticancer agents. Because equimolar tissue concentrations of ISIS 4559 did not produce cytotoxicity, these data indicate further that the antitumor activity of ISIS 3521 is due to its target specificity.

Even more remarkable was the antitumor activity of ISIS 3521 against intracranially implanted tumors, where 40% long term survival occurred. Although a 20-mer S-oligodeoxynucleotide was shown not to cross the blood-brain barrier in normal animals (15), the presence of a malignant glioma may sufficiently disrupt the blood-brain barrier to allow pharmacological concentrations of S-oligodeoxynucleotide to accumulate within the tumor. This is indeed the case in humans with glioblastoma multiforme.

For an antisense PKC S-oligodeoxynucleotide to have therapeutic efficacy, the PKC isoform pattern in the tumor versus normal tissue must be considered. Based on our previous results (3), PKCα was targeted because both human glioblastoma U-87 and other glioblastoma cell lines overexpress this isoform and are dependent on its expression for proliferation. Although PKCα is probably involved in signal transduction in normal tissues as well, the ability of ISIS 3521 to inhibit tumor growth in conjunction with PKCα selectivity and an apparent lack of nonspecific cytotoxicity indicates either a functional redundancy among PKC isoforms or a PKC isoform dependence for a specific growth factor signaling pathway in tumors that differs from that in normal tissue. The latter possibility is suggested by the demonstration that an antisense oligo against PKCδ inhibited platelet-derived growth factor-dependent but not epidermal growth factor-dependent proliferation of mouse 3T3 fibroblasts, whereas an antisense oligo against PKCα did not inhibit either pathway (26). Because U-87 cells are highly platelet-derived growth factor dependent (27, 28) and are markedly sensitive to inhibition of PKCα (3), our results suggest that the growth factor-dependent proliferation of malignant gliomas may differ from that of normal cells with respect to PKC isoform dependence and may also contribute to the selectivity of ISIS 3521.

The mechanism by which ISIS 3521 induces inhibition of tumor proliferation, cell death, or both is not known. One possibility is that inhibition of PKCα induces apoptosis, as has been found for glioblastoma and neuroblastoma cell lines treated with PKC inhibitors (29, 30). However, we have not found evidence of this process so far in subcutaneous U-87 tumors by light microscopy or TUNEL assay (31).[4] Another possibility is the interruption of cell adhesion or cell motility may result in loss of tumor viability. An antisense PKCα oligo blocked phorbol ester-mediated arachidonate release in canine Madin-Darby kidney cells (32). Arachidonate release is mediated by phospholipase A_2, which also leads to formation of lysophosphatidic acid, an activator of the Rho pathway leading to formation of stress fibers and focal contacts (33, 34). Whether this or another signaling pathway leads to inhibition of tumor proliferation or apoptosis remains to be determined. Regardless of the ultimate mechanism of action of ISIS 3521, it may represent a relatively selective therapy for the treatment of glioblastoma multiforme and probably other malignancies as well.

[4] N. M. Dean and R. I. Glazer, unpublished observations.

Acknowledgments

We thank Drs. William D. Hunter, Kent C. New, and Joseph T. Newsome for their helpful discussions and the Georgetown University Research Resource Facility staff for their technical assistance. We thank Dr. Herbert J. Manz for his evaluation of the morphology of U-87 tumors by light microscopy. We also thank Dr. Henri M. Sasmor and the ISIS Pharmaceuticals Oligonucleotide Synthesis Group for providing the S-oligodeoxynucleotides used in this study and Mark Graham and Robert McKay for performing the capillary gel electrophoretic analyses.

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Send reprint requests to: Dr. Robert I. Glazer, Department of Pharmacology, Georgetown University Medical Center, 3970 Reservoir Road NW, Research Building, Room W318, Washington, D.C. 20007. E-mail: glazzerr@gunet.georgetown.edu