Stability, clearance, and disposition of intraventricularly administered oligodeoxynucleotides: Implications for therapeutic application within the central nervous system

(antisense/phosphorothioate oligonucleotide/brain tumor/chemotherapeutic)

LUKE WHITESELL†*, DANIEL GESelowITZ*, CHRISTINE CHAVANY*, BRIGID FAYMY*, STUART WALBRIDGE‡, J. R. ALGER§, AND L. M. NECKERS*

*Tumor Cell Biology Section, Clinical Pharmacology Branch, National Cancer Institute, and ‡Surgical Neurology Branch and ‡Neuroimaging Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD 20892

Communicated by Gilbert Stork, February 8, 1993

ABSTRACT We report experiments in the rat demonstrating the feasibility of intraventricular administration of oligodeoxynucleotides (ODNs) as a regional treatment approach to disorders within the central nervous system (CNS). Although we find little intrinsic nuclease activity in cerebrospinal fluid (CSF), phosphodiester ODNs are rapidly degraded by brain-associated a-exonuclease activity. Phosphorothioate ODNs, however, appear resistant to degradation in the CNS and, after intraventricular administration, they are cleared in a manner consistent with CSF bulk flow. Continuous infusion of ODN at 1.5 mmol/hr by miniosmotic pump can maintain micromolar concentrations of intact phosphorothioate ODN in CSF for at least 1 week without obvious neurologic or systemic toxicity. After infusion, extensive brain penetration and marked cellular uptake, especially by astrocytic cells, is demonstrated.

In the laboratory, antisense oligodeoxynucleotides (ODNs) have repeatedly demonstrated efficacy in modulating the expression of various genes, thus providing important insights into their roles in tumorgenesis or normal growth and development (1–3). Although attention has been focused recently on the development of antisense ODNs as therapeutics for a variety of diseases (4), data on systemic application of ODNs have been slow to accumulate.

To avoid obstacles associated with systemic administration, we and others have focused on regional therapeutic strategies to evaluate ODN actions in vivo (5, 6). We now describe the stability, disposition, and clearance of ODNs within a clinically important, physiologically well-defined biologic compartment—namely, the cerebrospinal fluid (CSF) space of the rat. We report that potentially therapeutic concentrations of intact phosphorothioate ODN can be maintained within the CSF without gross toxicity to the animal. Extensive penetration into brain parenchyma can be demonstrated. These findings support the feasibility of ODN-based therapeutic approaches to viral and neoplastic disorders within the central nervous system (CNS).

MATERIALS AND METHODS

ODN Synthesis, Modification, and Purification. Lyophilized preparations of several unmodified 15-base phosphodiester ODNs were obtained from Gilead Sciences (Foster City, CA) or Synthecell (Gaithersburg, MD). The sequences used in this study were as follows: 5'-ATG CGC AGC TGC TCC-3' (ODN 1) and 5'G GA GCA GCT CCG CAT-3' (ODN 2). Both ODNs were used interchangeably and behaved identically in all cases. These ODNs were synthesized by standard cyanoethyl phosphoramidite chemistry and were chromatographically purified. Corresponding 15-base phosphorothioate ODNs were prepared on an Applied Biosystems 380B automated synthesizer using phosphoramidite chemistry and Beaucage sulfuration reagent (Glen Research). Fluorescein-labeled ODNs were used either undiluted or diluted up to 1:3 with nonlabeled ODN of the same sequence.

Analysis of ODN Stability in Vitro. Solutions of 20 μM fluorescein-labeled ODN (both phosphodiester and phosphorothioate) were prepared in human CSF containing 100 units of penicillin per ml and 100 μg of streptomycin per ml (Biofluids, Rockville, MD). A vol of 0.75 ml was added per well in standard 24-well tissue culture plates (Costar). To some wells a 5-mm-thick axial cross-section of freshly isolated rat brain weighing 20–30 mg was added to the ODN/CSF solutions. The plate was incubated at 37°C in a humidified incubator. At 1, 6, and 24 hr postplating, CSF was harvested for analysis. A 50-μl aliquot was analyzed directly on an automated fluorescence plate reader (Fluoroskan 2; Flow Laboratories). Readings were compared to a standard curve prepared on the same plate by serial dilution of fluorescein-labeled ODN in CSF. The remainder of the CSF was extracted with an equal volume of water-saturated phenol followed by phenol/chloroform (1:1), chloroform, and ethanol precipitation. After a 70% ethanol wash, the pellet was dissolved in urea loading buffer [8 M urea/1× TBE (100 mM Tris/90 mM boric acid/1 mM EDTA)], heated to 90°C, and electrophoresed on a 15% polyacrylamide/8 M urea/1× TBE gel. After electrophoresis, fluorescein-containing bands were identified by long-wave UV transillumination.

Tissue accumulation of ODN was analyzed by digestion of rinsed brain sections in 1 ml of lysis buffer (200 mM Tris, pH 8.5/100 mM EDTA/1% SDS/1 mg of proteinase K per ml) at

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IVC, intracerebroventricular catheter; ODN, oligodeoxynucleotide.

†To whom reprint requests should be addressed: Clinical Pharmacology Branch, National Cancer Institute, Building 10, Room 13N262, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
In Vitro ODN Stability and Tissue Uptake. Both phosphodiester and phosphorothioate ODNs are quite stable in CSF after up to 24 hr of incubation at 37°C (Fig. 1 B and C, lanes 5–8). The total concentration of fluorescein label in CSF also remains relatively constant during incubation independent of the type of ODN labeled and of whether or not tissue is present (data not shown). However, addition of brain tissue results in near complete loss of intact phosphodiester ODN by 24 hr (Fig. 1 C, lanes 1–4). Phosphorothioate ODN stability, on the other hand, is insensitive to the presence of tissue and ODN remains intact for at least 24 hr (Fig. 1 B, lanes 1–4). Consistent with these degradation findings, very little fluorescein label can be recovered from the brain slice when phosphodiester ODN is used (Fig. 1D) and no intact phosphodiester ODN can be seen on gel electrophoresis (data not shown). When phosphorothioate ODN is used, however, a significant accumulation of label by the slice (almost 10% of the total incubated material) can be detected by 24 hr (Fig. 1D). Moreover, as Fig. 1A demonstrates, the label recovered represents intact phosphorothioate material.

Intracerebroventricular Bolus Kinetics. To standardize experiments and allow for meaningful comparison among ODN modifications, we coinjected $^{3}$H]inulin, a well-recognized marker of extracellular space sharing the same approximate molecular mass (5 kDa) as the ODNs under investigation (9). The level of $^{3}$H]inulin marker measured in CSF after bolus injection behaves quite consistently in this rat model. Generally, ~5 min after completion of a 50-μl injection, inulin
concentration in a CSF sample is 20–30% that of the injected material. During the next 2 hr, concentration falls with non-first-order kinetics (Fig. 2A). Inulin is thought to be cleared mainly by bulk flow; we believe the initial apparent $t_1/2$ observed reflects clearance of inulin from the ventricle, while the secondary $t_1/2$ represents dispersion and bulk flow from the total CSF. However, for several reasons determination of clearance $t_1/2$ values from such bolus data is subject to experimental artifact. First, distortion of CSF volume can occur due to an initial administration of 10% of total volume, followed by repeated sampling of up to 4% of that volume. Second, the level of radioactivity sampled at later time points is quite low and subject to error. Third, normal circulation of CSF in the rat depends in part on animal movement (data not shown) and the animals are anesthetized during the course of the bolus experiments. Therefore, we have not used bolus kinetic data to estimate clearance $t_1/2$ values. Instead, these values have been estimated from the steady-state concentration of ODN and inulin obtained during Alzet pump infusion (see below).

When phosphodiester ODNs end-labeled with $^{32}$P at the 5' or 3' end are co-injected with inulin, the ratio of $^{32}$P in the CSF relative to injected solution is reduced compared to inulin by $\approx 20\%$ at 5 min, and this relative depletion of $^{32}$P typically increases during the course of experiments. For both labeling schemes, the mean ratio of inulin to $^{32}$P compared to injected material is 7:1 after 2 hr (0.078 $\pm$ 0.008 vs. 0.011 $\pm$ 0.001; $P = 0.001$). Systemic administration of a dose of probenecid reported to completely inhibit the choroid plexus small anion efflux pump (10) has no effect on the kinetics of label disappearance (data not shown). When phosphodiester ODN internally labeled with fluorescein is used and total CSF fluorescence is monitored, the ratio of $^{3}$H to fluorescence is typically reduced to $\approx 2:1$ after 2 hr. This variant behavior is readily apparent in Fig. 2A, which plots the various inulin/ODN ratios obtained in a representative experiment with dual-labeled ODN carrying both internal fluorescein and 5' $^{32}$P. Regression analysis confirms that the ODN disappearance curves generated by monitoring either $^{32}$P or fluorescence are significantly different from each other ($P < 0.05$).

Both patterns of apparent clearance are observed depending on which label is monitored.

This apparent discrepancy in clearance rates is most readily explained by degradation of phosphodiester ODN within the CSF space after injection. Electrophoresis of CSF samples after injection of fluorescein-labeled material demonstrates extensive degradation of material recovered after 100 min (Fig. 2B). Such a finding is consistent with the behavior of phosphodiester material observed in vitro in the presence of brain tissue. When 5'-labeled ODN is used, no size ladder of degradation products is seen on autoradiographs, although intact ODN rapidly disappears (when equivalent cpm are loaded per lane; data not shown). When a 5' label is used, a ladder similar to that seen with internal fluorescein-labeled material (Fig. 2B) can be seen. These results suggest that degradation of phosphodiester ODN is initiated primarily at the 3' end. The more rapid apparent clearance of 5' $^{32}$P label shown in Fig. 2A compared to fluorescein label also suggests the presence of significant brain-associated phosphatase activity in addition to nuclease activity.

When phosphorothioate ODN with internal fluorescein label is injected as an IVC bolus, the level of fluorescence in CSF closely matches the $^{3}$H level over 100 min (Fig. 3A). In contrast to the findings with phosphodiester material, electrophoretic analysis of CSF samples after phosphorothioate bolus injection reveals only intact ODN even after 100 min (Fig. 3B). Again, these findings are consistent with the pattern of stability observed in vitro in the presence of fresh brain slices.

**Continuous IVC Infusion.** Given the poor distribution (data not shown) and relatively rapid clearance demonstrated for ODNs after bolus administration, we explored the potential of a continuous infusion technique to maintain steady-state levels of ODN within the CSF of free-roaming rats. Concentrations up to 15 mM phosphodiester ODN are tolerated at a rate of 1 $\mu$L/hr without obvious neurologic or systemic toxicity after 7 days. Using 5'-end-labeled material, a total $^{32}$P steady-state level of $\approx 0.01\%$ of the concentration of the infused solution is seen (data not shown). This level is $\approx 1/100$th the $^{3}$Hinulin level we generally observe during

**FIG. 2.** Phosphodiester ODN clearance and degradation after IVC bolus injection of fluorescein/$^{32}$P dual-labeled ODN and [H]$^{3}$Hinulin. Concentrations are presented relative to injected material. (A) $\circ$, Concentration of inulin determined by liquid scintillation counting of $^{3}$H cpm in CSF samples obtained at intervals after injection; $\blacksquare$, concentration of ODN as determined by fluorescence intensity of CSF samples; $\blacktriangle$, concentration of ODN as determined by $^{32}$P cpm in CSF samples. (B) Electrophoretic analysis of ODN degradation after bolus injection. Lanes 1–5, CSF obtained at 105, 72, 50, 22, and 12 min, respectively, after injection of dual-labeled material as described above. ODN visualized by UV transillumination. This dual label experiment is representative of six animals in which $^{32}$P-labeled ODN was used and three animals in which fluoresceinated ODN was used to measure the kinetics of bolus phosphodiester ODN clearance.

**FIG. 3.** Phosphorothioate ODN clearance and integrity following IVC bolus injection of fluorescein-labeled ODN and [H]$^{3}$Hinulin. Concentrations are presented relative to injected solution. (A) $\bullet$, Concentration of inulin determined by liquid scintillation counting of $^{3}$H cpm in CSF samples obtained at intervals after injection; $\triangle$, concentration of ODN as determined by fluorescence intensity of CSF samples. (B) Electrophoretic analysis of ODN degradation after bolus injection. Lanes: 1, $1\mu$L of injected solution as standard; 2–7, CSF samples obtained at 5, 12, 20, 48, 68, and 100 min, respectively, after injection. ODN was visualized by UV transillumination. This experiment was performed in three rats in which independent samples were drawn from CSF at the time points indicated. No significant differences were observed (by regression analysis) between the ODN and inulin clearance kinetics.
infusion and correlates well with the depletion of $^{32}$P relative to $^3$H we describe for late time points after bolus injection. Due to the extensive tissue-associated degradation of phosphodiester ODN we observe in bolus experiments and in vitro, we have not attempted to rigorously quantitate the level of intact phosphodiester material maintained during infusions. Gel electrophoresis of CSF samples, however, does reveal detectable, intact ODN. In light of degradation issues, we next examined the feasibility of continuous IVC infusion of fluorescein-labeled phosphorothioate ODN. Concentrations of infused solution that were >3 mM administered at 1 $\mu$l/hr are toxic. Global CNS depression followed by death occurs within 24 hr of infusion initiation. Concentrations up to 1.5 mM, however, are well tolerated during a 1-week period. ODN levels in the CSF of 0.1% of the infusion concentration can be readily maintained during infusion periods (Fig. 4). As might be expected from bolus observations, ODN levels correlate almost identically with the fractional level of $[^3]$H]inulin observed in a particular animal. The material recovered in CSF appears completely intact by gel electrophoresis (data not shown). We have calculated a clearance $\lambda$ for phosphorothioate ODN based on steady-state values ($n = 3$) to be 17.2 $\pm$ 4.7 min. The corresponding clearance $\lambda$ calculated for inulin ($n = 9$) is 23 $\pm$ 7.5 min. This value is consistent with previously published data on inulin clearance out of CSF (9).

**Tissue Penetration.** Given that phosphorothioate ODN appears stable in the presence of brain tissue, we examined phosphorothioate ODN penetration into brain by fluorescence microscopy. Paraformaldehyde-fixed frozen sections prepared from rat brains 30 min after bolus injection of 40 nmol of a fluorescein-labeled phosphorothioate ODN demonstrate readily detectable tissue signal (Fig. 5A). A gradient distribution is apparent with the highest signal noted at the ependymal surfaces, consistent with diffusion from in the surrounding CSF. The endothelium of small vessels also appears highly fluorescent, suggesting uptake of material that has been cleared into the vasculature. After continuous infusion of phosphorothioate ODN for 1 week (1.5 nmol/hr), a gradient distribution of ODN is less apparent, but marked uptake of fluorescent material is now observed in many cell bodies scattered throughout the tissue section (Fig. 5B). GFAP immunostaining of sections prepared from such a phosphorothioate-perfused brain demonstrates colocalization of ODN uptake and GFAP positivity within astrocytes (Fig. 5C and D). The identity of non-GFAP-positive cells that accumulate ODN during infusion remains to be determined.

**DISCUSSION**

We have sought to overcome many of the current obstacles associated with in vivo application of ODNs by avoiding the requirement for systemic administration. Because many drugs including ODNs show very poor penetration into the CNS after systemic administration (11), regional therapeutic strategies are particularly attractive in this organ system (12). The CSF space of the rat provides a practical, clinically relevant, physiologically well-defined compartment in which to systematically examine ODN actions in vivo.

Unlike blood and serum, CSF possesses little intrinsic nuclease activity. Unmodified phosphodiester ODNs remain undegraded after incubation in CSF ex vivo for up to 24 hr at 37$^\circ$C. This finding holds for both rat and human CSF, but care must be taken in small animals to avoid contamination with blood during the collection process or serum-associated nucleolytic activity can be inadvertently introduced. However, brain tissue ex vivo or in the living animal possesses abundant nuclease and phosphatase activity. The predominant mode of degradation as determined from end-label experiments is consistent with $3'$ attack by $\alpha$-exonuclease(s). Phosphorothioate-modified linkages, however, are resistant to this form of attack both ex vivo and after IV administration. Our findings underline the potential for significant artifacts in the study of ODNs in vivo that can be associated with common labeling strategies. In our hands, internal fluorescence has been the most convenient and robust label for in vivo studies.

After acute bolus administration, ODNs readily flow throughout the ventricular system as demonstrated by the rapid appearance of material in CSF sampled from the extracranial cisterna magna. CSF bulk flow is central to the distribution and clearance of IVC-administered ODN. When issues of degradation are eliminated by use of phosphorothioate material, the disappearance kinetics of ODNs are very similar to those reported for the bulk flow marker inulin (9).

Continuous infusion of $^{32}$P-labeled phosphodiester ODN results in detectable levels of material in CSF over the course of infusion, at least some of which is intact. Concentrations up to 15 mM delivering 15 nmol/hr are tolerated without gross evidence of toxicity. The finding that steady-state $^{32}$P levels are $\sim$1/10th those of $[^3]$Hinulin during confusion is consistent with overall clearance of phosphodiester material being composed of both bulk flow and tissue degradation. Continuous infusion of fluoresceinated phosphorothioate ODN also results in detectable levels of intact material in CSF. Unlike phosphodiester ODN, however, lethal toxicity occurs at infusion rates of $>3$ nmol/hr. The steady-state level of phosphorothioate achieved relative to infused solution can be maintained at $\sim$0.1%, closely matching the steady-state concentration of inulin. Consequently, the well-tolerated concentration of 1.5 mM results in steady-state phosphorothioate ODN concentrations of $>1$ $\mu$M. Unlike phosphodiester material, intact phosphorothioate can be recovered from brain tissue after both ex vivo incubation and IV injection. Fluorescence microscopy also reveals extensive brain uptake of phosphorothioate ODN. Although readily detected, the tissue uptake of phosphorothioate ODN during continuous infusion probably accounts for only a small fraction of the total material administered because it does not appreciably decrease ODN steady-state level in CSF compared to inulin. The striking gradient distribution seen in

![Fig. 4. Phosphorothioate ODN level in CSF during continuous IVC infusion of fluoresceinated ODN/$[^3]$H]inulin solution. Concentrations are presented relative to injected solution. o, Concentration of inulin determined by liquid scintillation counting of $[^3]$H cpm in CSF samples obtained during infusion period; m, concentration of ODN as determined by fluorescence intensity of CSF samples. Miniosmotic pump was disconnected from catheter at the infusion stop point indicated and reconnected at the infusion restart point. This is a representative experiment depicting data obtained at the independent time points shown from one animal. The experiment was repeated using the same time points (without pump shut off) in a total of three animals.](https://example.com/fig4.png)
subependymal brain shortly after bolus injection is probably related to very high transient concentrations of ODN in CSF. After continuous infusion, a more uniform distribution of material is seen with prominent uptake noted in the soma of many cells, especially those of presumed glial (GFAP positive) origin.

The rat model described in this report demonstrates the feasibility of continuously perfusing the CNS with potentially therapeutic concentrations of intact ODN. It clearly indicates that, although CSF itself is low in nuclease activity, ODN modifications that protect at least the 3′ base linkage from exonuclease attack are required to stabilize ODN in the CSF space and achieve tissue penetration of intact material. The penetration into brain we now report suggests the possibility of targeting both leptomeningeal and intraparenchymal disease processes.

We thank Dr. P. Henkart for assistance with fluorescence quantification. We also thank R. Dreyfuss for fluorescent photomicrography assistance. This work was supported in part by a Cooperative Research and Development Agreement with Gilead Sciences.