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Pharmacokinetics of Intrathecal Oligodeoxynucleotides

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Background: **Intrathecal administration of antisense oligonucleotides is a frequently used technique to alter gene expression for research purposes. However, in the future, antisense oligonucleotides will likely be administered intrathecally to humans for therapeutic purposes. To date, there have been no systematic studies of the pharmacokinetics of intrathecal oligonucleotides. This study was designed to fill that knowledge gap.**

Methods: **Microdialysis probes were placed intrathecally at the L4, L1, and T11 vertebral levels and epidurally at the L4 vertebral level in pigs. One of the study oligodeoxynucleotides (10-, 18-, or 30-nucleotide-long sequences of the human MDR-1 gene) was injected intrathecally at the L4 level at time 0. Microdialysis samples were obtained for measurement of oligodeoxynucleotide samples at 5-min intervals until 20 min, 10-min intervals until 60 min, and 20-min intervals until 180 min. Noncompartmental pharmacokinetic analysis was performed using PK Solutions software.**

Results: **Mean residence time and terminal elimination half-life did not differ significantly among the three oligodeoxynucleotides at any sampling site. In contrast, area under the concentration–time curve differed significantly among the oligodeoxynucleotides at all sampling sites and was inversely related to oligodeoxynucleotide length at the L4 and L1 intrathecal sites but not the T11 or epidural sampling sites. Similarly, clearance and volumes of distribution at the L4 level differed significantly among the oligodeoxynucleotides and were directly related to oligodeoxynucleotide length.**

Conclusion: **The intrathecal pharmacokinetics of oligodeoxynucleotides are largely determined by oligodeoxynucleotide length. This contrasts with smaller drug molecules, such as opioids, for which intrathecal and epidural pharmacokinetics are largely determined by lipid solubility, not size. The potential clinical utility of this information is that oligodeoxynucleotide distribution within the central nervous system may be controllable to some degree by varying oligodeoxynucleotide length.**

THE development of molecular biology techniques for manipulating gene expression has proven to be an invaluable research tool and holds promise as an important medical therapy for the not-too-distant future. One particularly powerful technique is the administration of short DNA strands, *i.e.*, oligodeoxynucleotides that are complimentary to messenger RNA (mRNA) coding for a particular protein that one desires to "eliminate." The antisense DNA strand binds to a portion of the target mRNA and thereby blocks its translation into protein. Consequently, the amount of target protein present is decreased (*i.e.*, "knocked down"), and the function it subserves is correspondingly diminished.

Many hundreds of *in vitro* and *in vivo* studies have documented the ability of antisense techniques to decrease production of a targeted protein and, in so doing, to alter cellular function. These techniques have been widely used in animal models to create disease states for the purpose of studying pathophysiology. In addition, they have been used as a means of effectively treating a variety of diseases. Perhaps the most graphic evidence of the potential for use of antisense oligonucleotides for medical purposes is the publication of a journal devoted solely to this issue, *Antisense and Nucleic Acid Drug Development*, which was first published in 1990.

Importantly, antisense therapy has reached the stage of human clinical trials.[1–5] In all of these trials, antisense oligodeoxynucleotides have been administered systemically. However, because of their large size and charge, antisense oligodeoxynucleotides do not readily cross the blood–brain barrier. Consequently, their use for treating central nervous system (CNS) disease will require their direct injection into the CNS. The most likely route of administration (and the one used almost exclusively in animal studies to date) is injection into the cerebrospinal fluid (CSF). In fact, multiple animal studies have clearly shown the ability of intrathecally administered oligodeoxynucleotides to knock down targeted proteins in both the spinal cord and the brain.[6–9]

Although intrathecal antisense oligodeoxynucleotides have been used to decrease expression of targeted proteins in the CNS, their application has largely been empiric. Critical pharmacokinetic issues, such as half-life, mean residence time, optimal dosing interval, oligodeoxynucleotide distribution within the CNS, extent of oligodeoxynucleotide redistribution out of the CNS, and others, have not been studied. This lack of basic pharmacokinetic information is an important barrier to the rational development of oligonucleotides as drugs for intrathecal use. Consequently, the goal of this study was to determine the basic pharmacokinetics of intrathecally administered oligodeoxynucleotides. To achieve this goal, we used a previously described pig model[10,11] in which microdialysis techniques were used to sample the intrathecal and epidural spaces after administration of three differently sized oligodeoxynucleotides (10, 18, and 30 bases) into the lumbar CSF of anesthetized pigs.

Materials and Methods

Studies were approved by the University of Washington Institutional Animal Care and Use Committee (Seattle, Washington). Twenty-one farm-bred pigs of both sexes, weighing 13.5 ± 2.6 kg, were used. Each of three

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oligodeoxynucleotides was administered to seven animals.

Surgical Preparation

Anesthesia was induced by intramuscular injection of ketamine, the animal was paralyzed with intramuscular succinylcholine (200 mg), the trachea was intubated, and the animal was mechanically ventilated to maintain end-tidal carbon dioxide at 34–40 mmHg (Datex Airway Gas Analyzer Type GAO; Datex, Helsinki, Finland). Anesthesia was maintained throughout with isoflurane (1.5 – 2.0%) in oxygen. Femoral arterial and venous catheters were placed *via* cutdown, and a continuous infusion of normal saline containing 0.05 mg/ml pancuronium bromide was infused through the venous catheter at 4 $ml \cdot kg^{-1} \cdot h^{-1}$ throughout the study.

Laminotomies (approximately 0.5 cm^2) were performed in the L4, L1, and T11 vertebrae to expose the spinal cord. At each of these vertebral levels, an incision of approximately 1.0 mm was made through the dura and arachnoid mater to permit insertion of custom-made microdialysis probes into the subarachnoid space (see below for description of microdialysis probes). The microdialysis probe at the L4 level was attached to an epidural catheter (Perifix; B. Braun, Bethlehem, PA) to permit intrathecal oligodeoxynucleotide injection. The incision through the spinal meninges was sealed by means of an epoxy cone plug at the base of the dialysis probe, and cyanoacrylate glue was used to hold the probes in place. A fourth microdialysis probe was inserted into the epidural space opposite the L4 intrathecal probe. The laminotomies were sealed with cyanoacrylate glue to restore the integrity of the epidural space.

Microdialysis probes were perfused with bicarbonate-buffered mock CSF (140 mEq NaCl, 25 mEq $NaHCO_3$, 0.4 mEq $MgCl_2$, 3.5 mEq urea, 4.0 mEq glucose, 2.0 mEq $CaCl_2$; pH = 7.38–7.42; 292–298 mOsm). A syringe pump (model 22; Harvard Apparatus Inc., Holliston, MA) was used to pump mock CSF through the dialysis probes at the rate of 10 μl/min.

Microdialysis Probe Manufacture

Microdialysis probe manufacture has been previously described. Briefly, polysulfone dialysis fibers (MicroKros; Spectrum Laboratories, Inc., Rancho Dominguez, CA) with a diameter of 0.5 mm and a pore size of 50 nm were coated with epoxy over all but the center 20 mm, thereby creating a 31.4-mm^2 dialysis window. A 16.5-μm-diameter tungsten wire (Hamilton Company, Reno, NV) was inserted through the lumen of the dialysis fiber, and the fiber was bent in half to form a dialysis loop. The intraluminal wire prevented kinking of the dialysis probe, thereby maintaining luminal patency. The nondialyzing body of the dialysis probe was coated with a cone-shaped bead of epoxy to fix the shape of the probe and to seal the meningeal hole through which the dialysis probe was inserted. Probes were allowed to "cure" for a minimum of 18 h before use.

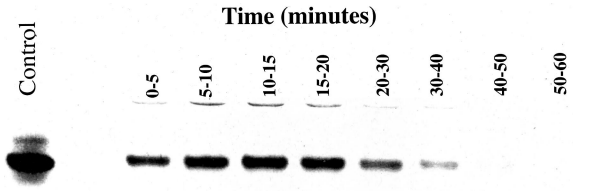

Fig. 1. Autoradiograph of a representative polyacrylamide–urea gel of dialysate samples from 0 to 60 min obtained from a single experiment with the intermediate-length oligodeoxynucleotide. The *control lane* is an aliquot of the parent radiolabeled oligodeoxynucleotide injectate. The bands gradually fade over time, indicating the decreasing concentration of oligodeoxynucleotide in cerebrospinal fluid. The absence of additional bands representing portions of a metabolic product of the parent oligodeoxynucleotide shows the absence of discernible metabolism within the cerebrospinal fluid.

Dialysis probes were tested *in vitro* to determine whether there was any difference in recovery efficiency for the differently sized oligodeoxynucleotides. Specifically, a dialysis probe was placed in a solution with a known concentration of one of the oligodeoxynucleotides, and the solution was dialyzed for 10 min. The oligodeoxynucleotide concentration in the dialysate was divided by the concentration in the dialyzed solution to determine the "recovery efficiency." Seven probes were tested against each of the three oligodeoxynucleotides.

Oligodeoxynucleotides

The three oligodeoxynucleotides used were complimentary to the portion of the human multiple-drug resistance (MDR-1) mRNA beginning at the start codon and extending 10, 18, or 30 bases into the coding sequence. The specific oligodeoxynucleotide sequences, CAAGATCCAT, GTCCCCTTCAAGATCCAT, and TCCTCCATTGCGGTCCCCTTCAAGATCCAT, were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). The oligodeoxynucleotides were radiolabeled according to the manufacturer's protocol using the RTS T_4 Kinase Labeling System (Invitrogen Life Technologies) and [γ-thio-^{35}S]adenosine triphosphate (4.63×10^{13} Bq/mM; NEN Life Science Products, Inc., Boston, MA; specific activity). For the 30-base oligodeoxynucleotide, unincorporated [^{35}S]adenosine triphosphate was removed using the QIAquick Nucleotide Removal Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. For the 18- and 10-base-long oligodeoxynucleotides, unincorporated [^{35}S]adenosine triphosphate was removed using Microspin G-25 columns (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol.

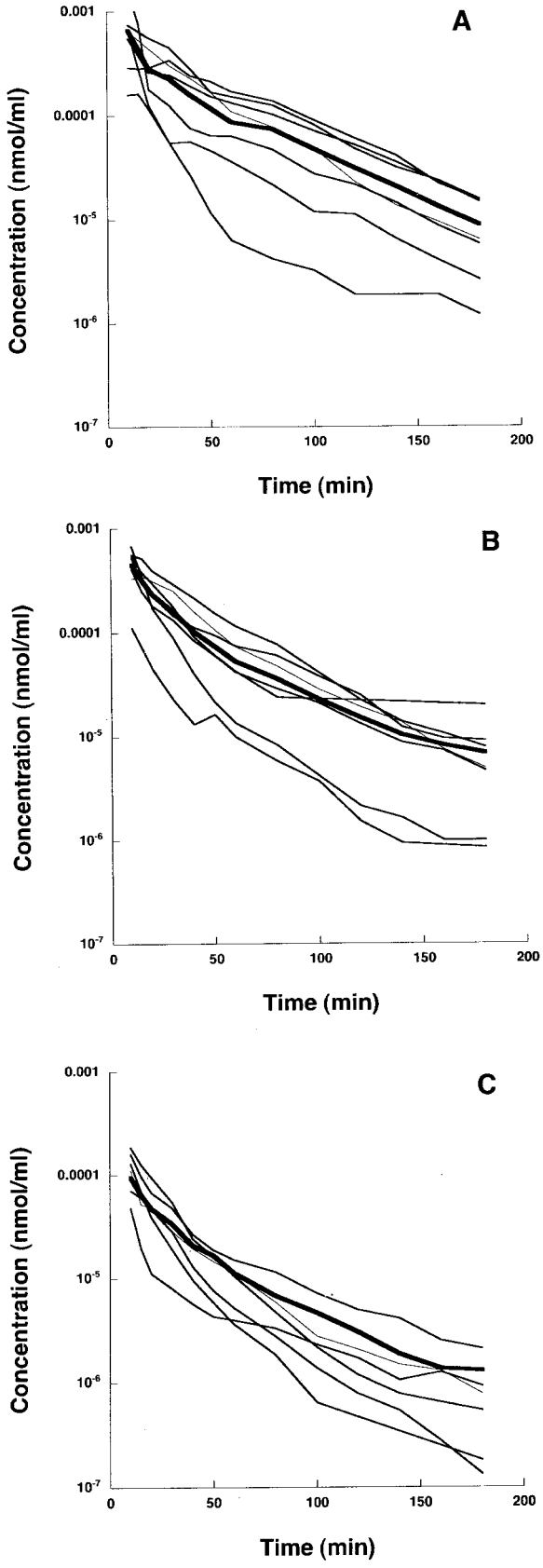

Fig. 2. Concentration-*versus*-time plots for the short (*A*), intermediate (*B*), and long (*C*) oligodeoxynucleotides at the L4 intrathecal injection site.

Study Paradigm

After completion of the surgical preparation, baseline dialysate samples were collected. The study oligodeoxynucleotide was diluted in mock CSF to a final volume of 250 μl and injected into the CSF over 1 min. The doses administered averaged 263 nmol for the 10-base oligodeoxynucleotide, 79 nmol for the 18-base oligodeoxynucleotide, and 50 nmol for the 30-base oligodeoxynucleotide. Differences in the molar dose injected reflect the fact that a constant mass of oligodeoxynucleotide was injected.

Dialysate samples were collected into sterile 1.5-ml microcentrifuge tubes over 5-min intervals for 20 min (50-μl samples), over 10-min intervals until 60 min (100-μl samples), and over 20-min intervals until 180 min (200-μl samples).

Oligodeoxynucleotide Analysis

Ten milliliters of scintillation fluid (Ecoscint; National Diagnostics, Atlanta, GA) was added to each aliquot of the dialysate sample, and the amount of ^{35}S-labeled oligodeoxynucleotide present was quantified in a Tri-Carb 2002 (Packard Instruments, Downer's Grove, IL) liquid scintillation counter. All samples were counted for 5 min or until the SD of the disintegrations per minute was less than 2%. All samples were corrected for background disintegrations per minute.

To determine whether significant metabolism of the oligodeoxynucleotides occurred in the CSF, aliquots of representative sets of microdialysate samples for each of the three oligodeoxynucleotides were analyzed using polyacrylamide–urea gel electrophoresis. As a control, the "parent" radiolabeled oligodeoxynucleotide was also loaded on each gel. After removing urea by soaking gels in 10% aqueous acetic acid, they were dried *in vacuo* at 80°C and autoradiographed.

Pharmacokinetic Analysis

Noncompartmental analysis of the oligodeoxynucleotide concentration–time data was performed using PK Solutions 2.0 software (Summit Research Services, Montrose, CO). PK Solutions 2.0 uses the trapezoidal rule to determine the area under the concentration-*versus*-time data and from the area calculates area under the curve (AUC), mean residence time, volume of distribution (V_d), volume of distribution at steady state (V_{ss}), and clearance. For all data relying on AUC, we used the AUC extrapolated to infinity. However, the extrapolation was small and accounted for less than 5% of the total area in nearly all cases. Terminal elimination half-lives and the initial volume of the central compartment (V_c) were obtained by stripping the concentration–time plots and fitting an exponential curve using the method of least squares in the PK Solutions software.

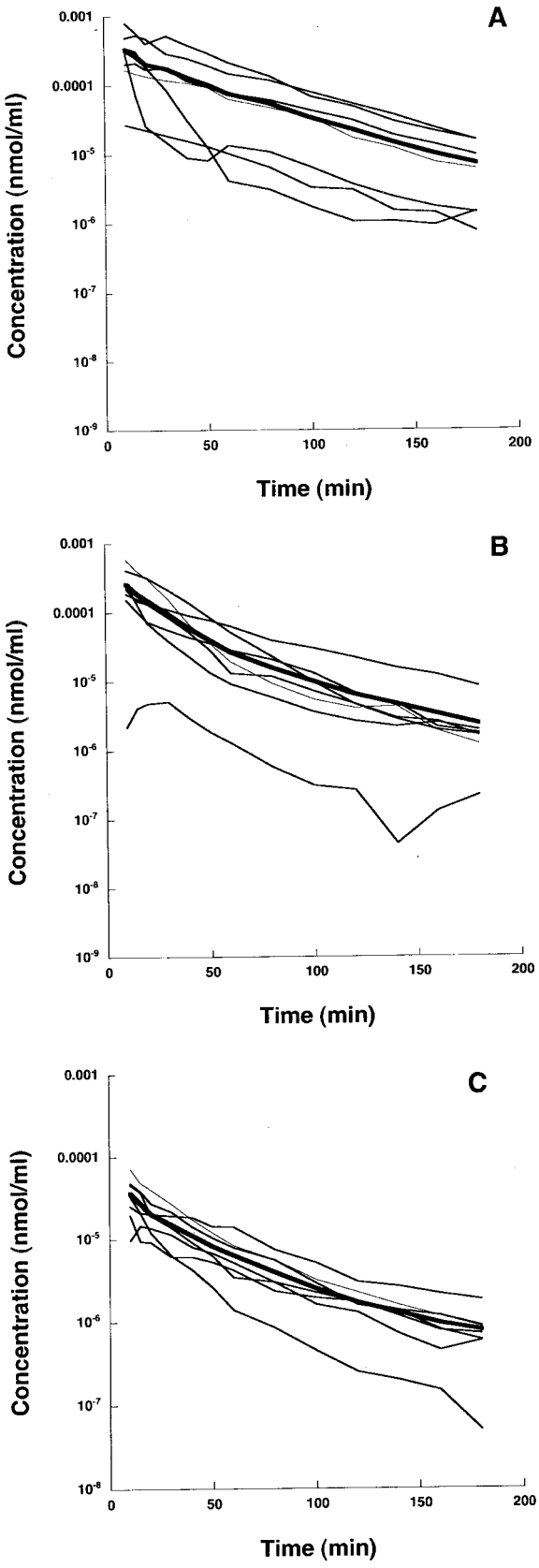

Fig. 3. Concentration-*versus*-time plots for the short (*A*), intermediate (*B*), and long (*C*) oligodeoxynucleotides at the L1 intrathecal sampling site.

Statistical Analysis

Statview 5 software (SAS Institute Inc., Cary, NC) was used for all statistical analyses. Differences in pharmacokinetic parameters were assessed for statistical significance using two-way analysis of variance with oligodeoxynucleotide and sampling site as the independent variables. The Fisher protected least significant difference test was used for *post hoc* testing. A P value of 0.05 was considered statistically significant.

Results

The *in vitro* studies performed to determine whether there was a difference in the ability of the dialysis probes to recover the different-length oligodeoxynucleotides showed that the dialysate concentration of the short oligodeoxynucleotide averaged 22 ± 7% of the concentration in the dialyzed solution. For the intermediate-length oligodeoxynucleotide, the dialysate concentration averaged 18 ± 8% of the concentration in the dialyzed solution, and for the long-length oligodeoxynucleotide, the dialysate concentration averaged 21 ± 10% of the concentration in the dialyzed solution. These differences were not statistically significant; therefore, oligodeoxynucleotide concentration was not corrected for differences in recovery.

Polyacrylamide–urea gel electrophoresis of representative samples of all three oligodeoxynucleotides showed a single, discrete band of the same molecular weight as the parent radiolabeled oligodeoxynucleotide (control lane) at each time point seen (fig. 1). The single band of the same molecular weight as the starting oligodeoxynucleotide indicates the absence of autoradiographic evidence of oligodeoxynucleotide degradation or metabolism within the CSF.

Figures 2–5 show both the individual and the average concentration-*versus*-time data for each oligodeoxynucleotide at the L4, L1, and T11 intrathecal and the L4 epidural sampling sites, respectively. Table 1 lists the descriptive pharmacokinetic parameters that were derived from the individual concentration-*versus*-time plots.

There were significant differences in dose-normalized AUC among the three oligodeoxynucleotides at all sample sites. At the L4 and L1 sites, there was a significant and strong inverse linear relation between oligodeoxynucleotide length and AUC (fig. 6). At the more rostral T11 and the epidural sampling sites, there was not a simple relation between oligodeoxynucleotide length and AUC.

At the intrathecal injection site (L4), there were significant differences in V_c, V_{ss}, V_d, and clearance among the three oligodeoxynucleotides. There was also a strong and statistically significant linear relation between oligodeoxynucleotide length and V_c and V_{ss} (fig. 7) and an exponential relation between V_d (fig. 8) and clearance (fig. 9).

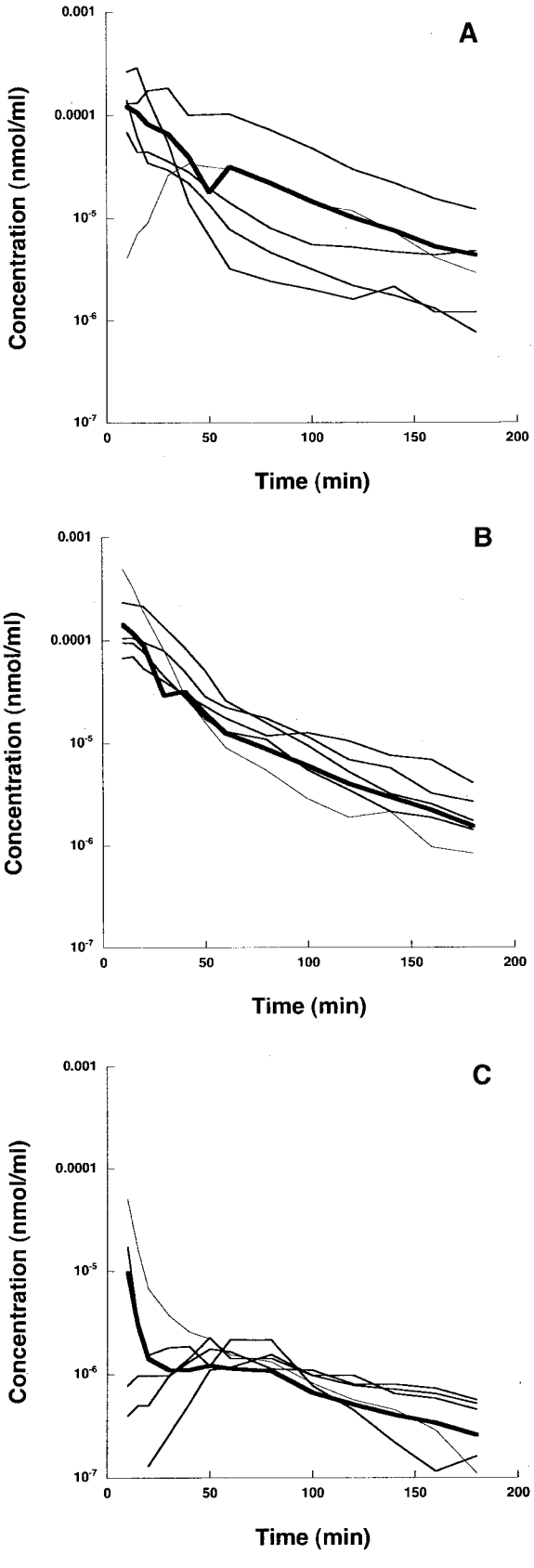

Fig. 4. Concentration *versus* time plots for the short (*A*), intermediate (*B*), and long (*C*) oligodeoxynucleotides at the T11 intrathecal sampling site.

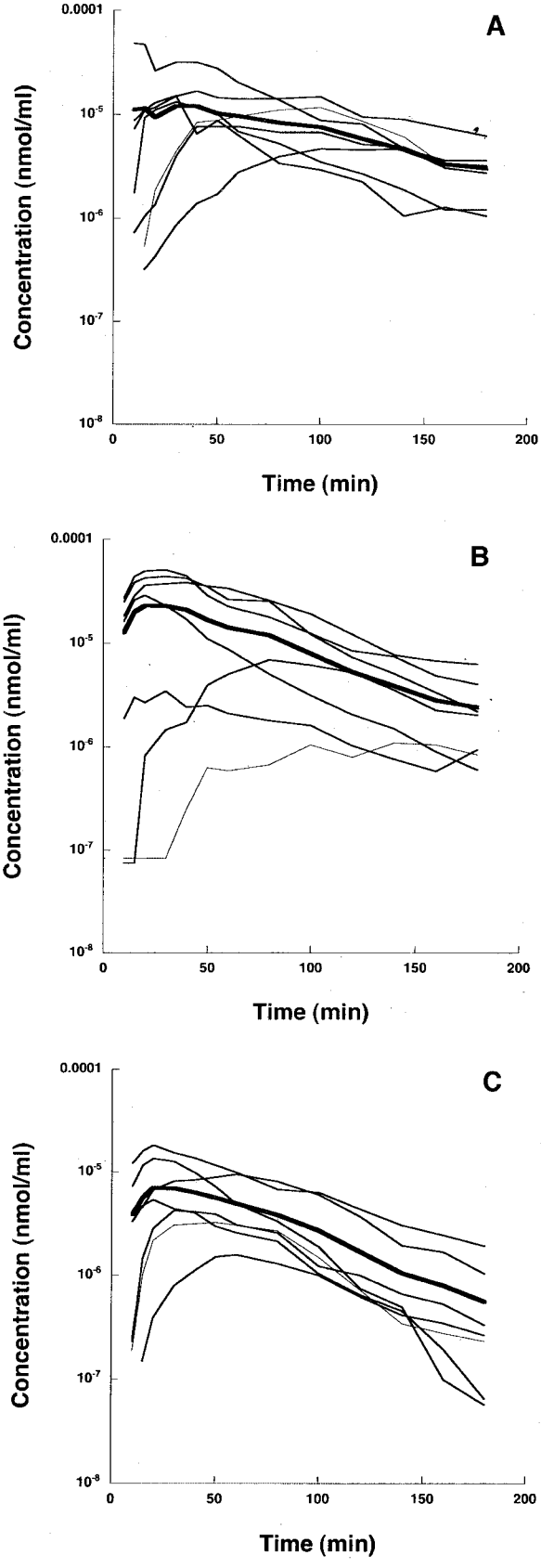

Fig. 5. Concentration-*versus*-time plots for the short (*A*), intermediate (*B*), and long (*C*) oligodeoxynucleotides at the L4 epidural sampling site opposite the intrathecal injection site.

Table 1. Pharmacokinetic Parameters

Sampling Site	Oligodeoxynucleotide		
	Short	Intermediate	Long
L4 intrathecal			
AUC/dose, min/ml × 10^3	27 ± 12* (I, L)	17 ± 7* (S, L)	3.5 ± 1.5* (S, I)
MRT, min	34 ± 17	33 ± 11	28 ± 10
V_c, l	1.1 ± 0.7* (L)	2.2 ± 2.0* (L)	7.4 ± 4.2* (S, I)
V_d, l	3.2 ± 3.8* (L)	6.9 ± 7.0* (L)	22 ± 14* (S, I)
V_{ss}, l	1.2 ± 0.6* (L)	3.7 ± 3.4* (L)	10.3 ± 8.5* (S, L)
Clearance, ml/min	50 ± 40* (L)	96 ± 91* (L)	351 ± 192* (S, I)
Terminal elimination $t_{1/2}$, min	39 ± 11	49 ± 12	47 ± 15
L1 intrathecal			
AUC/dose, min/ml × 10^3	17 ± 12* (L)	10 ± 7* (L)	1.5 ± 0.7* (S, I)
MRT, min	39 ± 17	33 ± 13	45 ± 41
Terminal elimination $t_{1/2}$, min	42 ± 18	53 ± 17	53 ± 20
T11 intrathecal			
AUC/dose, min/ml × 10^3	6.6 ± 4* (L)	8 ± 4* (L)	0.5 ± 0.6* (S, I)
MRT, min	62 ± 37	36 ± 18	105 ± 66
Terminal elimination $t_{1/2}$, min	62 ± 46	53 ± 17	65 ± 30
L4 epidural			
AUC/dose, min/ml × 10^3	1.5 ± 0.9* (I)	14 ± 12* (S)	9 ± 8
MRT, min	117 ± 48	103 ± 43	82 ± 39
Terminal elimination $t_{1/2}$, min	65 ± 28	59 ± 27	50 ± 51

* $P < 0.05$ (short [S], intermediate [I], or long [L]): indicates which oligodeoxynucleotides are significantly different.

AUC = area under the concentration-*vs.*-time curve; MRT = mean residence time; V_c = central compartment volume; V_d = volume of distribution; V_{ss} = volume of distribution at steady state.

Neither mean residence time nor terminal elimination half-life differed significantly among the three oligodeoxynucleotides at any of the four sampling sites, although the power for these analyses was relatively low (0.09–0.48).

Discussion

The purpose of this study was to investigate the pharmacokinetics of intrathecally administered oligodeoxynucleotides in anticipation that these compounds are likely to be administered spinally in humans in the not-too-distant future. Evidence that intrathecally administered oligodeoxynucleotides will someday play a role in human medicine comes from multiple sources. The National Institute of Neurologic Disorders and Stroke recently solicited Research Funding Proposals for "Gene Expression Profiling in the Nervous System Following Traumatic Spinal Cord Injury" (BAA-RFP-NIH-NINDS-01-03). The stated objective of this Research Funding Pro-

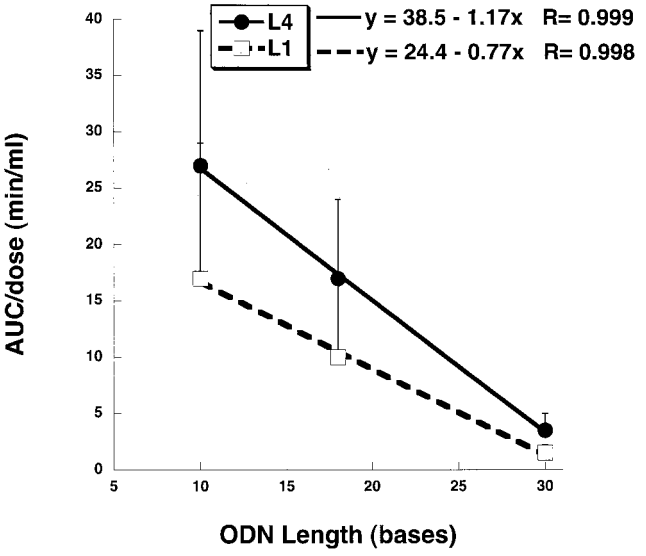

Fig. 6. Relation between oligodeoxynucleotide (ODN) length and dose-normalized area under the curve (AUC) at the L4 and L1 intrathecal sampling sites.

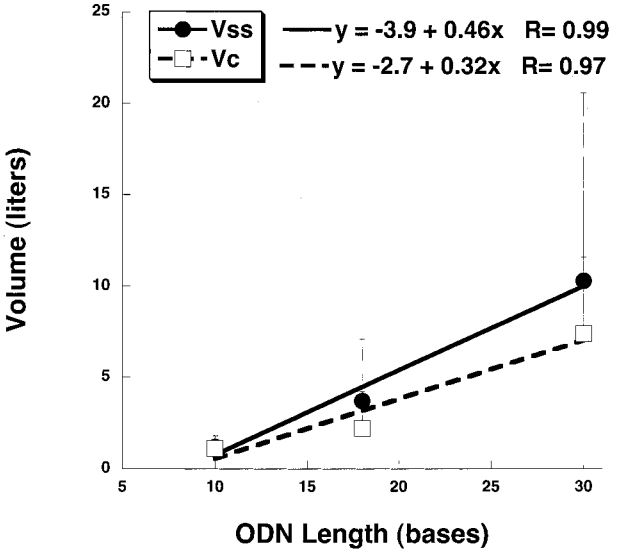

Fig. 7. Relation between oligodeoxynucleotide (ODN) length and volume of the central compartment (V_c) and volume of distribution at steady state (V_{ss}) at the L4 intrathecal injection site.

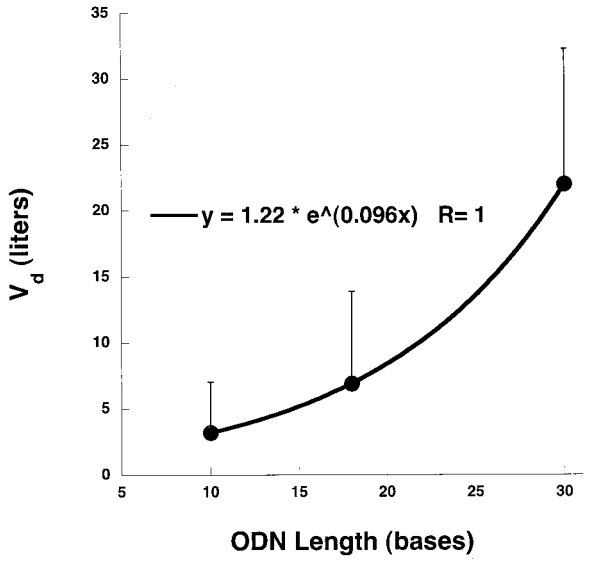

Fig. 8. Relation between oligodeoxynucleotide (ODN) length and volume of distribution (V_d) at the L4 intrathecal injection site.

posal is to identify patterns of gene expression that have both negative and positive influences on outcome after spinal cord injury, with the expectation that future targets for oligodeoxynucleotide-based intervention will be identified. Another example of the future role of antisense oligodeoxynucleotide in treating CNS disease comes from the recent introduction of the term *molecular neurosurgery* to refer to the use of genetic techniques to treat surgical diseases, such as CNS cancer.[12–18] In addition, animal models suggest that antisense oligodeoxynucleotides may offer a means of treating human CNS diseases, such as multiple sclerosis.[9]

Perhaps the most striking finding from this study is the degree to which several pharmacokinetic variables are related to oligodeoxynucleotide length. Because nucleotide bases differ somewhat in size and molecular weight, the number of bases is not a perfect predictor of either size or weight. However, the fact that oligodeoxynucleotide length produced such strong correlations with AUC, apparent V_d, V_c, V_{ss} and clearance (r = 0.97–1.0) suggests that oligodeoxynucleotide length is likely a better predictor of these pharmacokinetic parameters than is molecular weight or molecular volume.

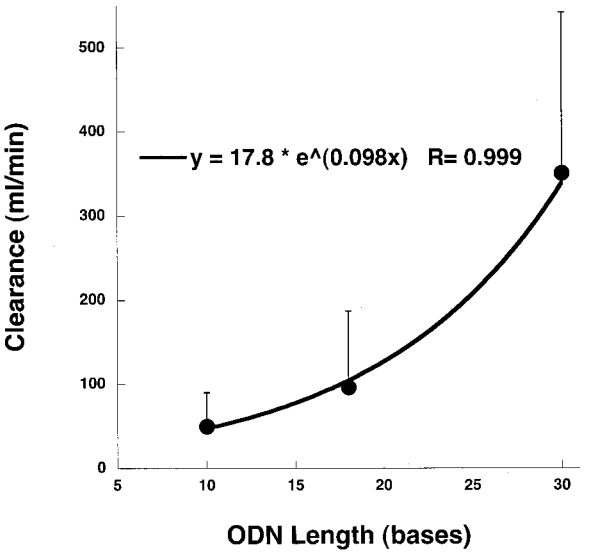

Fig. 9. Relation between oligodeoxynucleotide (ODN) length and clearance at the L4 intrathecal injection site.

Why length should be such a strong predictor of these pharmacokinetic parameters is not clear from the data. It may simply be that the rate at which the clearance process proceeds is related to the length of the oligodeoxynucleotide. Alternatively, the clearance mechanism may differ for oligodeoxynucleotides of different lengths, and the underlying processes may proceed at different rates. Because there was no evidence of oligodeoxynucleotide metabolism, size-related differences in metabolic rate cannot be invoked to explain differences in AUC, clearance, or apparent volumes of distribution. It is also unlikely that differences in diffusion rates explain the relation with oligodeoxynucleotide length because diffusion rate is inversely proportional to the square root of the molecular weight of a molecule. If differences in diffusion rate played a major role in oligodeoxynucleotide pharmacokinetics, one would expect variables such as clearance to be inversely related to oligodeoxynucleotide length, which was not the case.

Although the AUC was significantly greater for the shorter oligodeoxynucleotides compared with the long oligodeoxynucleotide at the more distant T11 sampling site, the relation between AUC and oligodeoxynucleotide length was not as strong as at the sampling sites closer to the site of oligodeoxynucleotide injection. The reason for this is not entirely clear but may reflect the fact that oligodeoxynucleotide concentration at this site is more dependent on the rate of oligodeoxynucleotide movement from the injection site to the sampling site than is the case with the L1 and L4 sampling sites. Regardless of the explanation, the important observation is still that the longest oligodeoxynucleotide has the lowest AUC at all sampling sites.

More striking was that in the epidural space the AUC was so much less for the short oligodeoxynucleotide than for the two longer oligodeoxynucleotides. Whether this reflects more rapid clearance of the smaller oligodeoxynucleotide from the epidural space or reduced penetration of the spinal meninges is unclear.

The potential clinical significance of the strong dependence of some pharmacokinetic variables on oligodeoxynucleotide length is that it offers the potential of manipulating oligodeoxynucleotide pharmacokinetics for clinical advantage. For example, given that shorter oligodeoxynucleotide lengths persisted longer in the CSF, the use of a shorter oligodeoxynucleotide would be expected to result in greater distribution in the CSF, with

correspondingly greater exposure of the underlying spinal cord to the oligodeoxynucleotide. Conversely, longer oligodeoxynucleotide sequences would be expected to have a more limited distribution. Of course, the antisense efficacy of oligodeoxynucleotides is related to their length; therefore there are limits to the extent that the length of any oligodeoxynucleotide can be manipulated without losing antisense efficacy. However, within those limits, which vary among oligodeoxynucleotides, changes in length may be an effective means to influence intrathecal distribution.

In summary, this is the first study to examine the pharmacokinetic behavior of oligodeoxynucleotides in the intrathecal space. The principal finding was that intrathecal but not epidural pharmacokinetics are largely determined by oligodeoxynucleotide length. This may be of significance in the future when designing oligodeoxynucleotides for clinical use.

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