Intrathecal Substance P-Saporin in the Dog

Distribution, Safety, and Spinal Neurokinin-1 Receptor Ablation


ABSTRACT

Background: Neurokinin-1 receptors (NK1-rs) located on superficial dorsal horn neurons are essential for integration of nociceptive input. Intrathecal injection of substance P-saporin (SP-SAP) leads to local loss of spinal NK1-r (+) neurons suggesting its potential as a therapeutic agent for chronic pain. The authors determined, in a canine model, effects of lumbar intrathecal SP-SAP.

Methods: Distribution of SP-SAP and Saporin was determined in plasma, lumbar cerebrospinal fluid, and tissue. Safety of intrathecal SP-SAP was determined in four groups (six dogs each) administered 0 (0.9% saline), 1.5, 15, or 150 µg SP-SAP through lumbar intrathecal catheters. Behavioral, physiologic, and biochemical variables were assessed. Spinal tissues were collected at 7 and approximately 90 days, or earlier if significant morbidity developed, and analyzed for NK1-r (+) neuron loss and histopathology.

Results: SP-SAP and Saporin were detectable in lumbar cerebrospinal fluid for up to 4 and 24 h, respectively. Animals receiving intrathecal saline, 1.5, or 15 µg of SP-SAP showed no persistent neurologic deficits. Three animals receiving 150 µg of SP-SAP developed pelvic limb paraparesis and were euthanized prematurely. Immunohistochemistry and in situ hybridization cell counts confirmed a significant reduction in NK1-r (+) in superficial dorsal horn neurons from lumbar spinal cord after intrathecal administration of 15 and 150 µg of SP-SAP. A significant loss of NK1-r neurons in the lumbar ventral horn occurred only with 150-µg SP-SAP.

Conclusion: Intrathecal 15-µg SP-SAP reduced dorsal, but not ventral, NK1-r (+) neurons at the spinal level of delivery with minimal side effects, whereas 150-µg SP-SAP resulted in motor neuron toxicity.

What We Already Know about This Topic

- Injection of substance P conjugated to the cell toxin Saporin in animals destroys cells in the spinal cord that express receptors for substance P and participate in pain transmission, but could also destroy other neurons which also express this receptor.
- The preclinical safety of this approach to pain relief has not been examined.

What This Article Tells Us That Is New

- Lumbar intrathecal injection of substance P-saporin, 15 µg, resulted in loss of neurons in the spinal cord dorsal horn expressing the neurokinin-1 receptor.
- A larger dose, 150 µg, resulted in progressive lower limb paraparesis and loss of motor neurons in the ventral horn expressing the neurokinin-1 receptor.

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NEUROKININ-1 receptors (NK1-r), activated by substance P (SP), are distributed throughout spinal cord and brainstem and are elements in local circuitry involved in sensory transmission and modulation of cardiovascular and respiratory chemoreceptor responses. At the spinal level, activation of NK1-r(+) neurons by SP released from high-threshold primary afferents has been identified on second-order dorsal horn nociceptive neurons, some of which are projection neurons. Preclinical work with NK1-r antagonists demonstrated modest analgesic efficacy in models of facilitated processing and inhalant anesthetic-sparing effects in dogs. In humans, NK1-r antagonists have been generally ineffective in acute and chronic pain syndromes, finding variously attributed to pharmacokinetic and brain penetration. Furthermore, although nociceptive neurons express the NK1-r, excitation of these neurons is characterized by a diverse pharmacology, one emphasizing the importance of additional receptors including notably those for glutamate. This suggests that although present on second-order nociceptive neurons, the NK1-rs are the only one mechanism whereby the primary afferent excites these neurons and that NK1-r blockade alone is insufficient to prevent second-order neuron excitation.

The close association of the NK1-r with dorsal horn nociceptive neurons, suggested by the above observations, provides an alternative strategy for modulating the nociceptive processing mediated by these neurons. The NK1-r is a metabotropic G-protein–coupled receptor that when occupied by a ligand (e.g., SP) internalizes the ligand–receptor complex. Saporin is a 31-kDa protein derived from the plant Saponaria officinalis which blocks ribosomal function, prevents protein synthesis, and leads to cell death. Importantly, the Saporin molecule alone is not taken up into cells. Coupling of this molecule to SP, however, yields a construct that binds to the G-protein–coupled NK1-r. The agonist binding to the NK1-r results in an internalization of the substance P–saporin (SP-SAP) complex into lysosomes. The acidic environment in lysosomes leads to hydrolysis of the complex, freeing Saporin into the intracellular milieu. Accordingly, the toxicity of the complex is limited to those cell membranes expressing NK1-r. Furthermore, once SP is cleaved, any residual Saporin loses its ability to alter cellular function. Lumbar intrathecal administration of SP-SAP reduces, over days to weeks, the number of lumbar spinal NK1-r–bearing neurons in rats and dogs. Loss of lamina I projection neurons by SP-SAP destruction produces profound, long-lasting attenuation of facilitated pain states without affecting acute nociception. Neurotoxic effects were anticipated given the mechanism of targeted cell death; however, these effects should be delimited to local region of delivery and isolated to neurons bearing the target receptor, NK1-r. Ventral horn motor neurons have been shown to express the NK1-r and thus might also be susceptible to the neurotoxic effects of SP-SAP.

We hypothesized that intrathecal SP-SAP would produce dose-dependent and selective loss of NK1-r–bearing neurons with distribution and effects limited to the region of lumbar delivery. The aims of this study were (1) to determine the spinal distribution and potential systemic uptake of intrathecal SP-SAP and (2) to define the systemic and neurotoxic effects of three concentrations of intrathecal SP-SAP and a saline control in dogs. The efficacy of this therapeutic intervention in the canine model is presented in the companion article.

Materials and Methods

The studies were conducted with the approval of the Institutional Animal Care and Use Committee of the University of California, San Diego, California.

Animals

Thirty purpose-bred Beagle dogs (Marshall BioResources, North Rose, NY) (8–12 months old; weighing 8–12 kg) were used. Animals were judged to be in good health on the basis of a physical examination conducted by a licensed veterinarian before inclusion into the study. Dogs were individually housed in runs on a 12-h light/dark cycle with wood chip shavings as bedding. Temperature was maintained between 65° and 82°F (30–70% relative humidity). Food and water were available ad libitum with the exception of a 16-h fast (water still available) before induction of anesthesia or blood sample collection.

Surgical Preparation

Six dogs were implanted with two single-lumen lumbar intrathecal catheters for drug delivery and cerebrospinal fluid (CSF) sampling to determine pharmacokinetic values in the spinal distribution study. Twenty-four dogs were prepared with one single-lumen intrathecal catheter for drug delivery in the spinal safety study. Catheters (polyurethane, 0.61-mm outer diameter) were inserted at the atlantooccipital joint with the tip terminating at L1-3. Catheter implantation was performed aseptically during anesthesia with isoflurane as described in detail elsewhere. In the spinal safety study, the single intrathecal catheter was removed during brief anesthesia with isoflurane, 4 days after SP-SAP injection. Dogs were administered carprofen (4.5 mg/kg subcutaneous) postoperatively.

Drugs

Sterile SP-SAP (Advanced Targeting Systems, San Diego, CA), 600 μg/ml, was diluted in sterile saline (0.9% weight/volume; Sodium Chloride Injection USP; Abbott Laboratories, North Chicago, IL) to a final concentration of 300 μg/ml. In the spinal distribution study, 150 μg of SP-SAP in 0.5 ml was injected. In the spinal safety study, vehicle (saline), and 1.5, 15, or 150 μg of SP-SAP in 0.5 ml was injected intrathecally (six dogs in each group). A 0.3-ml volume was used to flush the catheter following each injection effectively clearing the 0.15-ml volume catheter.
**Drug Safety Study**

Twenty-four dogs were prepared with lumbar intrathecal catheters on day 3. On day 1, 0.5-ml lidocaine hydrochloride, 2%, was injected through the intrathecal catheter to confirm placement of the intrathecal catheter in the mid-lumbar region. On day 0, six dogs (three males and three females) were randomly assigned to receive one of four intrathecal treatments: 0.9% saline, 1.5, 15, or 150 μg SP-SAP in a volume of 0.5 ml. Injections were performed in awake dogs. The intrathecal catheter was removed 4 days after injection. Animals were then followed at least 90 days after intrathecal injection or until the development of morbidity that necessitated early euthanasia. In the spinal safety study, observations were carried out according to the schedule listed in table 1 and detailed below. Animals that completed the study were then anesthetized and cisternal CSF, blood, and urine samples collected, followed by terminal perfusion fixation and necropsy on the scheduled necropsy day or earlier if necessitated by development of morbidity.

**Spinal Distribution Study**

Six dogs were administered SP-SAP (150 μg/0.5 ml) intrathecally. Postinjection CSF samples were collected 20 min after injection and then 1, 2, 4, 8, and 24 h and then 2, 4, and 7 days after injection. CSF samples were collected from the proximal intrathecal catheter whenever possible. The distal catheter was used if sample collection was not possible from the proximal catheter. A volume of 0.15 ml (equivalent to the dead volume of the intrathecal catheter) CSF was collected and discarded before collecting 0.3 ml of CSF for sample analysis. Samples were placed on dry ice and then stored at −65°C until sample analysis. Catheter placements were confirmed at necropsy.

**Study Design**

**Clinical Observations.** Daily observations included assessment of appetite, urine and stool output, arousal, muscle tone, and motor coordination. Behavioral pain assessments were performed on animals in the spinal safety study twice-daily beginning on day 0. The pain scoring system assessing both spontaneous and evoked pain was used in this study is provided in Supplemental Digital Content 1, http://links.lww.com/ALN/A986. Dogs were assigned an individual score for each spontaneous and evoked pain assessment; however, for the purpose of data analysis, a score other than 0 constituted the criterion for presence of pain.

**Physiologic Parameters.** Body weight, auricular body temperature, heart rate, respiratory rate, and tail-base oscillometric blood pressure (Dinamap 8100, Criticon; GE Healthcare, Waukesha, WI) were recorded at predetermined times in accordance with the schedule of data collection table 1.

**Neurologic and Ophthalmologic Examination.** A licensed veterinarian (P. J. R.) performed neurologic and ophthalmologic examinations on dogs in the spinal safety study before intrathecal dosing and just before euthanasia using criteria described in the table, Supplemental Digital Content 2, http://links.lww.com/ALN/A987. Neurologic examination included assessments of attitude, gait, muscle tone, posture, cranial nerve and limb reflexes, and sensation. Ophthalmologic examination included evaluation of external ocular structures, direct ophthalmoscopy including retinal exam, and tonometry.

**Clinical Chemical Analysis.** A complete blood count, serum biochemical profile, coagulation profile, and urinalysis were performed before implantation of the intrathecal catheter(s) and after intrathecal dosing, just before euthanasia.

**Drug Analysis.** Enzyme-linked immunosorbent assay (using a Biomek 2000 robot) was used to determine SP-SAP and Saporin concentrations in CSF, plasma, spinal cord, and brain tissue. Samples were analyzed for both SP-SAP and free Saporin content in separate enzyme-linked immunosorbent assay. Biochemical analysis included complete blood count, serum biochemical profile, CSF chemistry, coagulation profile, and urinalysis. Pre- and postinjection postmortem samples were analyzed for SP-SAP and Saporin concentrations in CSF, plasma, spinal cord, and brain tissue. Samples were analyzed for both SP-SAP and free Saporin content in separate enzyme-linked immunosorbent assay.

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**Table 1. Schedule of Data Collection for Spinal Distribution and Spinal Safety Studies**

<table>
<thead>
<tr>
<th></th>
<th>Phase 1: Spinal Distribution</th>
<th>Phase 2: Spinal Safety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily observations*</td>
<td>Twice-daily through day 7</td>
<td>Twice-daily through day 11, once-daily until necropsy†</td>
</tr>
<tr>
<td>Clinical chemical analysis†</td>
<td>Approximately 1 week before catheter implant and on day 7</td>
<td>Approximately 1 week before catheter implant and on day of necropsy‡</td>
</tr>
<tr>
<td>CSF and blood for SP-SAP and Saporin assay</td>
<td>Days 0 (−60 min, 20 min, then 1, 2, 4, and 8 h), 1, 2, 4, 7</td>
<td>NA</td>
</tr>
<tr>
<td>Physiologic parameters</td>
<td>NA</td>
<td>Day 0 (before and after dosing), 4, and 6, then weekly until necropsy‡</td>
</tr>
<tr>
<td>Body temperature</td>
<td>NA</td>
<td>Daily through day 7, then weekly until necropsy‡</td>
</tr>
<tr>
<td>Body weight</td>
<td>NA</td>
<td>Day 0, 4, 6, then weekly until necropsy‡</td>
</tr>
<tr>
<td>Neurologic and ophthalmologic examination</td>
<td>NA</td>
<td>Approximately 1 week before catheter implant and 1 week before day of necropsy‡</td>
</tr>
</tbody>
</table>

*Daily observations: appetite, urine and stool output, arousal, muscle tone, motor coordination, and in phase 2, pain behaviors; †Clinical chemical analysis: complete blood count, serum biochemical profile, CSF chemistry, coagulation profile, and urinalysis; ‡Day of necropsy was day 90–95 unless otherwise noted in results.

CSF = cerebrospinal fluid; NA = not applicable; SP-SAP = substance P-saporin.
immunosorbent assay procedures. Standard curves were prepared using SP-SAP or cys-SAP as references. The effective range of the standard curve for these assays was defined as being the linear portion of the curve. Test samples were stored at −20°C before assay and diluted at the time of assay. Tissue samples were homogenized incorporating detergents, high pH, protease inhibitors using a Dounce homogenizer. Homogenization was followed by high-speed centrifugation and testing of supernatant. The typical effective range of the standard curve as established during these assays was 0.8–50 ng/ml for Saporin and 0.4–25 ng/ml for SP-SAP. Test samples were measured in six replicates.

**Tissue Harvest**

Dogs in the spinal distribution study were anesthetized with propofol (up to 10 mg/kg IV for induction and then 0.6–1 mg kg⁻¹ min⁻¹ IV for maintenance) for blood, urine, with propofol (up to 10 mg/kg IV for induction and then 0.6–1 mg kg⁻¹ min⁻¹ IV for maintenance) for blood, urine, and CSF collection 7 days after intrathecal SP-SAP. Dogs in the spinal safety study were anesthetized in a similar manner at 90 days, or earlier, if considered necessary. Blood samples were collected from a cephalic vein. CSF was collected by percutaneous puncture of the cisterna magna using a 22-gauge spinal needle and sterile procedures.

All dogs were euthanized with pentobarbital sodium (Beuthanasia®-D; Schering-Plough, Union, NJ) while still anesthetized with propofol after sample collection in the spinal distribution study. Methylene blue (0.1 ml) was injected into the distal intrathecal catheter of these dogs to confirm catheter location. Dogs in the spinal safety study were euthanized by perfusion of 4 l of saline, 0.9%, at 80–160 mmHg of pressure followed by 4 l of neutral buffered formalin, 10%, during propofol anesthesia.

After euthanasia, the spinal cord and brain were exposed and inspected for gross pathology. Spinal cord tissue samples were collected for SP-SAP and Saporin assay in the spinal distribution study and for microscopic, immunofluorescent, and *in situ* hybridization analyses in the spinal safety study. Spinal cord sections were divided into blocks A (spinal segment C3), B (spinal segment T9), C (spinal segment L4), and D (spinal segment L6-7). The brain was harvested *en bloc* for microscopic analysis or divided into brainstem, cerebellum, and forebrain for drug SP-SAP and Saporin assays in the spinal distribution study. Tissues were placed on dry ice and then stored at −65°C until the time of sample analysis for the spinal distribution study. Tissues for microscopic analysis were placed in 10% buffered formalin.

**Histopathologic Analysis**

Tissues for microscopic analysis were paraffin-embedded and sectioned at 3–5 μm and stained with hematoxylin and eosin. Cross sections of spinal cord stained with hematoxylin and eosin were examined for evidence of inflammation, reactive gliosis, and other signs of injury. The severity of observed changes was scored on a scale of 1–5 with 5 representing the most severe pathology. Microscopic examination was limited to brain sections, spinal cord sections, dorsal root ganglia, trigeminal ganglion, peripheral nerves, eye, and any tissue found to be abnormal at gross necropsy. The study pathologist (M. T. B.) was blinded (during the initial microscopic examination) as to the treatment. Treatment status was subsequently unblinded, data organized into treatment groups, and statistically analyzed.

**Spinal NK1-r Assessment: Immunofluorescence**

NK1-r neuronal cell loss was quantified using immunofluorescent staining and confirmed with *in situ* hybridization. Spinal cord samples were transferred from formalin to 30% (weight/volume) sucrose in phosphate buffer saline (PBS). Cryoprotected spinal segments were sectioned (30 μm) using a sliding microtome. Sections were labeled with primary antisera, rabbit anti-NK1-r (1:4,000; Advanced Targeting Systems), and visualized in spinal cord sections using a biotinylated secondary antibody and streptavidin-fluorescent dye conjugate amplification system. Neuronal Nuclei (NeuN) were colabeled using an indirect immunofluorescent detection system for counting NK1-r-immunoreactive cell bodies. Tissue incubations and rinses were carried out on a tissue shaker in multiwell plates using the free-floating staining method. All primary and secondary antibody and fluorescent dye conjugate dilutions were prepared in 1% bovine serum albumin in PBS. Tissue sections were rinsed three times for 10 min each in PBS and then permeabilized in a solution of 0.3% Triton X-100 (Sigma Chemicals, St. Louis, MO) in PBS (PBS-TX) for 10 min. Nonspecific binding was blocked with 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) and 5% normal dog serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) that were diluted in PBS, and sections were incubated for 1 h at room temperature.

A Streptavidin/Biotin Blocking Kit (Vector) was also used to block any nonspecific binding of streptavidin or biotin to the tissue per manufacturers instructions. The rabbit anti-NK1-r (1:4,000) and mouse anti-NeuN (1:1,000; Millipore, Temecula, CA) primary antibodies were prepared in bovine serum albumin in PBS and applied to tissue for 48 h at 4°C. After three rinses for 10 min each in PBS, biotinylated anti-rabbit IgG (1:500; Vector) was applied to the tissue and allowed to incubate for 1 h at room temperature. Tissue sections again were rinsed three times in PBS for 10 min each. Tissue was incubated in Alexa Fluor 488 Streptavidin (1:1,000; Molecular Probes, Eugene, OR) and Alexa Fluor 594 goat anti-mouse IgG (1:1,000; Molecular Probes) for 1 h followed by three final PBS rinses for 10 min each. A minimum of four spinal cord sections per block were transferred to slides, air-dried, and cover slipped using ProLong Gold Antifade Reagent (Molecular Probes). Primary antibody specificity was confirmed by including...
an isotype control. Nonimmune rabbit IgG (Vector) was diluted to the same concentration as the NK1-r antibody, and some tissue samples were incubated in this solution in lieu of the primary antibody application.

**Spinal NK1-r Assessment: In Situ Hybridization**

To confirm the NK-r immunofluorescence observations, in situ hybridization was accomplished in lumbar spinal blocks from saline and SP-SAP-treated dogs. Total messenger RNA (mRNA) was extracted from 50 mg of dog lumbar spinal cord (snap-frozen) using TRizol Reagent (Life Technologies, Grand Island, NY). Complementary DNA was prepared using the SuperScript III RT-PCR kit (Invitrogen, Life Technologies, Grand Island, NY). Full-length coding sequence of dog NK1-r (NM_001012619.1) was amplified (forward primer: CTAGGAGAGCATGTTGTGGAGTAG and reverse primer: ATGGATAACGCCTCC-CAGGTAG). PCR fragments (1.2 kb) were cloned into the TOPO-PCR II vector, in between a T7 and an SP6 promoters. Complementary DNA insertion was confirmed by sequencing. Digoxigenin-labeled antisense and sense probe were made from the TOPO-NK1-r vector by T7 and SP6 RNA polymerases by an in vitro transcription kit (Roche Applied Science, Indianapolis, IN). Spinal cord sections (30 µm) were mounted on slides for in situ hybridization. On day 1, sections were incubated in the following sequence: (1) 4% paraformaldehyde in PBS for 20 min; (2) PBS, three times for 5 min each; (3) proteinase K solution (Sigma) for 2 min; (4) PBS, twice for 5 min each; (5) 4% paraformaldehyde in PBS for 5 min; (6) PBS for 5 min; (7) 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min; and (8) x2 saline-sodium citrate (SSC), twice for 5 min each. Next, sections were incubated in prehybridization solution (50% formamide, 0.3 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, pH 8.0, 500 µg/ml salmon sperm DNA, and 500 µg/ml yeast transfer RNA) at 55°C in a chamber containing towels moistened with x4 SSC and 50% formamide. After incubation for 2 h, the prehybridization solution was drained, and sections were hybridized with digoxigenin/digoxin labeled antisense or sense probes for NK1-r (1:1,000), coverslipped, and placed in a 55°C oven overnight. On day 2, coverslips were removed and the hybridized, spinal cord sections were sequentially incubated in the following: (1) ×5 SSC at 55°C for 10 min; (2) 50% formamide in ×2 SSC at 55°C for 20 min; (3) RNase buffer at 37°C, twice for 5 min each; (4) RNase A (50 µg/ml; Sigma) at 37°C for 30 min; (5) RNase buffer at 37°C for 15 min; (6) 50% formamide, ×2 SSC at 55°C for 20 min; (7) ×2 SSC, twice for 15 min each; (8) washing buffer for 10 min; (9) blocking solution for 30 min; (10) anti-digoxigenin/digoxin antiserum conjugated to alkaline phosphatase (1:500) for 2 h; (11) washing buffer, twice for 15 min each; (12) detection buffer for 5 min; and (13) nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate overnight (DIG Nucleic Acid Detection Kit; Roche). On day 3, slides were rinsed in PBS and dehydrated through a graded ethanol series, CitriSolv, and coverslipped in DPX mounting media (Sigma). Neurons displaying NK1-r mRNA were counted as described for immunolabeled tissues.

Neuronal cells bearing NK1-r immunofluorescence were quantified in superficial cervical, thoracic, lumbar, and lumbosacral spinal cord segments. In separate lumbar sections, cell counts of NK1-r-mRNA (+) neurons were made. At the lumbar levels, NK1-r-(-) and NK1-r-mRNA (+) neurons were quantified from the superficial dorsal and ventral horn in all 30 dogs. The areas counted are shown in figure 1 and were obtained by averaging cell counts from four right and left representative sections for each area within each segment. Counting was performed without knowledge as to animal treatment. All treatment groups had six animals, but several animals had spinal blocks that could not be stained for unknown reasons, despite repeated efforts and therefore were not included in the counts.

**Statistical Analysis**

Unless otherwise noted, normal, continuous data were grouped by dose and reported as mean ± SD (for data reported in tables) or SEM (for data reported in graphs). Categorical data were grouped by dose and reported as median (interquartile). For repeated physiological observations, day 0 was used as the baseline observation and compared with all subsequent data were grouped by dose and reported as median (interquartile). For repeated physiological observations, day 0 was used as the baseline observation and compared with all subsequent data were grouped by dose and reported as median (interquartile). For repeated physiological observations, day 0 was used as the baseline observation and compared with all subsequent
observations. Initial inspection revealed no sex-related trends, thus analysis was carried out on pooled data (male and female) for each dose group. Normality of physiologic parameters were tested using the Shapiro–Wilks test and analyzed using a two-way repeated measures ANOVA with time and dose as the dependent variables. A Bonferroni multiple comparisons post hoc test was performed when significance was detected. To compare NK1-r(+) cell counts determined by immunohistochemistry and in situ hybridization analyses, a least squares linear regression was undertaken with slope and $r^2$ values determined; 95% CIs of slope and $r^2$ were determined. Dogs euthanized prematurely in the 150-$\mu$g dosing group were excluded from statistical analysis due to the presence of missing data points. Data from these animals were included as descriptive statistics (mean ± SD) and in graphs. NK1-r (+) immunofluorescent cell counts in cervical, thoracic, lumbar spinal cord segments for the three SP-SAP treatments were compared with saline control (saline) using a Kruskal–Wallis test and Dunn post hoc test. A similar analysis was carried out for the lumbar sections examined with in situ hybridization. Categorical data including clinical observations (muscle tone, motor coordination, arousal, appetite, stool and urine production, and pain score) were compiled as total number of abnormal observations compared among treatment groups using the Kruskal–Wallis test and Dunn multiple comparisons when significance was detected. Comparisons for categorical data were not parallel because of the early euthanasia of three dogs in the 150-$\mu$g group resulting in fewer total days of observation. Furthermore, several histological blocks would not take stains for unknown reasons and could not be counted. Pre- and postinjection clinical laboratory analyses were compared using a Student $t$ test for individual treatment groups. A $P$ value of less than 0.05 was considered significant. Statistical analyses were carried out using Prism 5 for Mac OS X, version 5.0d. (Graphpad Software, La Jolla, CA).

**Results**

**Catheter Placement**

All dogs tolerated intrathecal catheterization without observable changes in motor function. All criteria for proper lumbar intrathecal catheter placement were met: visual insertion in the subarachnoid space, unhindered passage of the catheter 45 cm, and uninterrupted aspiration of CSF from the inserted catheter. The intrathecal administration of 2% lidocaine (0.5 ml) 1 day before test article injection resulted in a rapid and transient (<30 min) pelvic limb paraplegia without evidence of sympathetic blockade indicating an absence of cranial catheter coiling in all dogs in the spinal safety. Correct catheter placement in dogs in the spinal distribution study was visually confirmed at the time of necropsy.

**Intrathecal SP-SAP: Behavioral Signs**

**Acute Behavioral Phenotype.** No reactions were observed during the injection of saline or the 1.5 or 15 $\mu$g dose of SP-SAP. One of six dogs displayed transient signs of pain (vocalization) that resolved after approximately 15 s during injection of 150 $\mu$g SP-SAP in the spinal distribution study. Dogs administered 15 or 150 $\mu$g SP-SAP but not saline or 1.5 $\mu$g SP-SAP developed persistent dose-dependent twitching of the ventral neck musculature (cleidocervicals, sternocleidomastoideus, sternothyroideus, and/or sternothyroides muscles) within 30 min to 2 h after test article administration. Twitching became more severe when the neck was put through a range of motion and persisted for up to 2 days in dogs administered 150 $\mu$g SP-SAP. The behavioral expression of this discomfort was reduced by carprofen 25 mg per os twice-daily administered for 2 days.

**Chronic Behavioral Phenotype.** All of the dogs administered saline, 1.5, and 15 $\mu$g survived until termination of the study (90 days) without behavioral changes. Three of six dogs administered 150 $\mu$g SP-SAP were euthanized before the scheduled 90 days (two: day 6, one: day 33) due to the development of progressive hind limb paraparesis. The onset of paresis began to develop 5 days after intrathecal SP-SAP administration in all three dogs. None of the six animals in the spinal distribution study (euthanized at day 7) displayed signs involving hind limb motor dysfunction, a phenomenon which we attributed to the repeated sampling of CSF after injection resulting in a decrease in local test article concentrations.

**Clinical Observations.** As summarized in table 2, incidence of abnormal muscle tone was significantly greater in dogs administered 150 $\mu$g SP-SAP compared with all other treatment groups. Acute changes included increased muscle tone or stiffness that corresponded with pain behaviors. Dogs that developed pelvic limb paraparesis initially developed increased muscle tone that lasted 2–4 days followed by reduced muscle tone and weakness in their pelvic limbs that persisted until the time of euthanasia. Abnormal motor coordination was observed only in the three dogs that developed pelvic limb paraparesis. Spontaneous pain behaviors were of greater frequency in the 150-$\mu$g group compared with the saline and 1.5-$\mu$g groups but did not differ from the 15-$\mu$g group (table 2). There were no significant differences among treatment groups for urine or fecal output, appetite, or arousal. No ophthalmologic changes attributable to SP-SAP could be identified.

**Physiologic Parameters**

There were no differences in physiologic parameters among treatment groups before or after test article administration (data not shown). Body temperature, body weight, and heart rate changed significantly over the course of 90+ days, but post hoc analyses showed no treatment-dependent differences among groups. All dogs lost weight over the first 4 days after intrathecal test article administration and then body weight increased significantly over the remainder of the safety study for dogs that survived to necropsy. Body temperature increased significantly over the course of the safety study but remained within normal limits. Respiratory rates were normal throughout the study.
Neurologic examination at the pre- and postinjection assessment period was normal in all dogs in the saline, 1.5-µg, 15-µg groups, and at the preinjection exam for dogs administered 150-µg SP-SAP. Four of six dogs (two female and two male) administered 150-µg SP-SAP developed neurologic abnormalities that persisted to the time of euthanasia. One dog (male) developed an abnormal (crooked) tail carriage that persisted until the time of euthanasia (93 days). Three dogs (two female and one male) developed pelvic limb paraparesis that became evident on day 5. Paresis progressed rapidly in two of the three dogs to the point of inability to ambulate with the pelvic limbs, and they were euthanized on day 6. One of these dogs (male) did not urinate on day 5; therefore a urinary catheter was passed for urine evacuation. Appetite was reduced in both dogs euthanized on day 6, and both dogs retained bowel function. One of the three dogs had an insidious progression of pelvic limb paresis that progressed over 3 weeks and then acutely worsened over 24 h until she was euthanized on day 33. This dog had loss of tail tone and developed urinary incontinence during the week before she was euthanized. All three dogs that developed pelvic limb paresis had loss of tail tone, absent pelvic limb proprioceptive reflexes, and withdrawal reflexes. Deep pain sensation was intact at the time of euthanasia.

Table 2. Clinical Observations of Intrathecal SP-SAP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>1.5 µg</th>
<th>15 µg</th>
<th>150 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. animals</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Muscle tone</td>
<td>0/642 (0%)</td>
<td>2/648 (&lt;1%)</td>
<td>5/648 (1%)</td>
<td>42/396 (11%)*†‡</td>
</tr>
<tr>
<td>Motor coordination</td>
<td>0/642 (0%)</td>
<td>0/648 (0%)</td>
<td>0/648 (0%)</td>
<td>5/396 (1%)</td>
</tr>
<tr>
<td>Arousal</td>
<td>0/642 (0%)</td>
<td>1/648 (&lt;1%)</td>
<td>1/648 (&lt;1%)</td>
<td>0/396 (0%)</td>
</tr>
<tr>
<td>Appetite</td>
<td>7/630 (1%)</td>
<td>9/636 (1%)</td>
<td>14/636 (2%)</td>
<td>24/387 (6%)</td>
</tr>
<tr>
<td>Stool</td>
<td>42/642 (7%)</td>
<td>41/648 (6%)</td>
<td>62/648 (10%)</td>
<td>34/396 (9%)</td>
</tr>
<tr>
<td>Urine</td>
<td>21/642 (3%)</td>
<td>20/648 (3%)</td>
<td>20/648 (3%)</td>
<td>26/396 (7%)</td>
</tr>
<tr>
<td>Pain</td>
<td>2/636 (&lt;1%)</td>
<td>11/642 (2%)</td>
<td>16/642 (2%)</td>
<td>53/393 (13%)†</td>
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Incidence of abnormal observations in muscle tone, motor coordination, arousal, appetite, stool production, urine production, or pain behaviors out of total number of possible observations (% occurrence) in animals that received intrathecal saline, 1.5, 15, or 150 µg SP-SAP. Number of animals refers to total number of animals surviving to the intended 90-day endpoint. Within a row, values in the 150 µg dosing group with different symbols are significantly different from (*) saline values, (†) 1.5 µg values, or (‡) 15 µg values.

SP-SAP = substance P-saporin.

Table 3. Blood Chemical Analysis

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<td>Creatinine</td>
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<td></td>
<td>Post</td>
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<td>Potassium</td>
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<td>Post</td>
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<td></td>
<td>Post</td>
<td>150±4.56</td>
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<td>Pre</td>
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<tr>
<td></td>
<td>Post</td>
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<td></td>
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Serum biochemical and hematologic parameters that varied significantly from pre- to postinjection time points in dogs administered intrathecal saline, 1.5, 15, and 150 µg substance P-saporin.
Ophthalmologic Examination
No extra- or intraocular ophthalmologic abnormalities were observed during the pre- or postinjection assessment period in any of the treatment groups with the exception of changes in intraocular pressure. Increased intraocular pressure (>30 mmHg) was noted in dogs administered saline (two dogs preinjection, four dogs postinjection), 1.5 μg (one dog preinjection, three dogs postinjection), and 150 μg (one dog preinjection, two dogs postinjection) SP-SAP. One dog administered 150 μg SP-SAP had low intraocular pressure preinjection and was one of the two dogs with increased intraocular pressure in the postinjection period.

Blood Chemical Analysis
Preinjection blood and urine chemical analyses for all dogs were not different among groups and within normal limits for all dogs. Postinjection comparisons revealed changes in serum biochemical and hematologic values from preinjection time points in all treatment groups (table 3).

Gross and Microscopic Analysis
A veterinary pathologist, blinded to treatment groups, did not identify any gross abnormal findings attributable to test article administration aside from the presence of blood-tinted CSF in two of the three dogs euthanized early.

NK1-r Neuronal Quantification
Normal NK1-r Distribution. All treatment groups had six animals, but several animals had spinal blocks that could not be stained for unknown reasons, despite repeated efforts. These blocks were thus not included in the counts. NK1-r immunoreactivity was colocalized in NeuN (+) neurons throughout Rexed lamina I and to a lesser degree in outer lamina II dorsal horn and large motor neurons in the ventral horn at all levels of the spinal cord (fig. 1).

Effects of Intrathecal SP-SAP. Intrathecal SP-SAP resulted in a loss of NK1-r (+) cells in the superficial lumbar dorsal horn with little change in overall section appearance (fig. 2). Systematic counting of NK1-r (+) immunofluorescent cells revealed a dose-dependent and localized reduction in NK1-r (+)/NeuN (+) cell counts in the superficial dorsal horn of lumbar/lumbosacral spinal cord (proximate to the level of the intrathecal injection). There was a significant reduction in NK1-r (+) neurons in the cervical spinal cord in the 15-μg SP-SAP treatment group compared to the saline group (fig. 3). In situ hybridization was performed to confirm the identity of the NK1-r immunoreactivity in the lumbar sections of all dogs. Systematic counting of lumbar cords (proximate to the catheter tip) with in situ hybridization revealed a dose-dependent reduction in NK1-r mRNA (+) cells with the reduction in NK1-r (+) neurons in the superficial dorsal horn (lamina I/II) as compared with vehicle in both 15- and 150-μg SP-SAP treatment groups being statistically significant (fig. 4). Note that in the in situ, loss of cell counts was largely limited to the superficial Lam I and II and not the deeper laminae.

A significant reduction in the NK1-r (+) cell counts was observed in the ventral horn with the 150-μg (5.5 ± 3.4 for immunohistochemistry, 2.9 ± 1.7 for in situ hybridization) but

![Fig. 2. Low- and high (associated with the respective outlined areas)-magnification immunofluorescent-stained cervical (A–C) and lumbar (D–L) spinal cord sections of saline, 15-, and 150-μg intrathecal substance P-saporin–treated animals at 90 days with neurons labeled red and associated neurokinin-1 receptor labeled green.](Image)
not the 15-µg SP-SAP groups in both immunohistochemistry and in situ hybridization analyses. In a few sections, concurrent immunohistochemistry and in situ hybridization analyses were performed, illustrating the evident colocalization of the NK1-r immunoreactivity and NK1-r message hybridization product (fig. 5). Plotting the regression between NK1-r (+) immunohistochemistry and NK1-r mRNA cell counts revealed a significant covariance in the lumbar dorsal horn (fig. 5).

**Histopathologic Assessment**

Microscopic lesions were not observed in the saline, 1.5- or 15-µg SP-SAP groups. Test article–related microscopic abnormalities were present in the spinal cord of five of six dogs administered 150-µg SP-SAP. Histopathologic findings were categorized: (1) infiltrates present in the meninges, brain, and spinal cord. These infiltrates were predominantly mononuclear cells (2) vacuolation of the spinal gray and white matter; (3) spinal cord neuronal; and (4) nerve fiber degeneration in superficial long spinal tracts (fig. 6).

Microscopic histopathologic findings in the three dogs administered 150-µg SP-SAP were present in the lumbar and lumbosacral spinal cord gray matter (vacuolation, neuronal necrosis, and neuronal loss; table 4). The severity and incidence of changes decreased from the lumbar to cervical regions. Spinal cord white matter changes consisted of nerve fiber degeneration that was present in spinal cord tracts (dorsal, ventral, and lateral) in one dog euthanized prematurely and two other dogs in the 150-µg group that survived to scheduled euthanasia. Perivascular infiltrates were also observed in areas of damage. Occasional signs of vacuolation were noted in the brain (cerebellum). These changes were not noted in lower-dose or saline-treated animals.

**Spinal Distribution of SP-SAP**

All six dogs in the spinal distribution study survived the 7-day testing period. Sampling of lumbar CSF revealed no detectable immunoreactivity for either SP-SAP or Saporin alone in the preinjection samples. Peak SP-SAP concentrations after intrathecal administration of 150 µg SP-SAP, using an assay which detected only the SP-bound product, were observed at the first sample time point (20 min) and rapidly declined to undetectable levels by 4 h. CSF Saporin concentrations, using an assay that detected all Saporin (SP coupled or not), also peaked at 20 min and declined to undetectable concentrations by 24 h (fig. 7). Neither SP-SAP nor Saporin was detectable in the plasma, spinal cord, or brain tissue at any time point.

Fig. 3. Cell counts with median and 25/75th % are presented for NK1-r (+) cells in (A) cervical dorsal horn, (B) thoracic, dorsal horn; (C) lumbar dorsal horn; and (D) lumbosacral dorsal horn spinal segments of dogs treated with 0 (saline), 1.5-, 15-, and 150-µg intrathecal SP-SAP. Each group should have six animals but for technical reasons immunohistochemistry failed in several blocks and counting could not be accomplished. Each data set (graph) was analyzed with a Kruskal–Wallis with the statistical significance for the analysis shown in each graph with a post hoc Dunn multiple comparison versus saline group. *P < 0.05; **P < 0.01. NK1-r = neurokinin-1 receptor; P1 = spinal distribution phase: 7-day sacrifice; P2 = spinal safety phase: maximum 90-day survival; SP-SAP = substance P-saporin.
Intrathecal Substance P-Saporin

Discussion

Spinal NK1-rs are involved in facilitated nociceptive processing as defined in preclinical models. Support for the hypothesized role of NK1-rs in spinal pain processing is in part based on the observation that the intrathecal administration of neurotoxins specifically targeting NK1-r–bearing neurons produces analgesic effects during injury-evoked facilitated states but have little effect on acute nociceptive processing in rats.

We have previously demonstrated that intrathecal administration of SP-SAP produces a significant, dose-dependent reduction in superficial dorsal horn NK1-r–bearing neurons. In the accompanying article, Brown et al. demonstrate the clinical relevance of this action by showing that intrathecal SP-SAP attenuates the pain states associated with osteosarcoma in companion dogs at comparable doses. Our experiments serve to define the safety profile and target effects of intrathecal SP-SAP over an extended period of time. We determined that intrathecal SP-SAP at doses of 15 µg or higher resulted in a reduction in NK1-r (+) spinal neurons in spinal cord segments proximate to the site of drug delivery and that SP-SAP at doses of 15 µg or less resulted in minimal pathology aside from the selective loss of superficial NK1-r (+) dorsal horn neurons.

Distribution of NK1-r Neurons

NK1-r immunoreactivity is distributed densely in superficial lamina I and II neurons in mouse, rat, guinea pig, and dog spinal cord with less dense expression in deeper dorsal horn. Although classically associated with dorsal horn neurons, NK1-rs have been identified on preganglionic sympathetic neurons and, in the present experiments, on somatomotor neuron cell bodies of dogs. Although less well characterized, a similar profile has been reported in human spinal tissue. An important issue is the verification of antibody specificity. This is particularly relevant given the observation in dogs, but not rodents, that NK1-rs are present on motor horn cells. In the current work, we confirmed this association by using in situ hybridization and showing that (1) there is colocalization of message and protein (as defined with the current antibody) and (2) there is close correlation between the two measures across intrathecal SP-SAP dosing in the lumbar cord of these animals.

Characteristics of the Effects of Intrathecal SP-SAP

Our results confirm and extend previous studies that have demonstrated that intrathecal SP-SAP produces a loss of NK1-r (+) neurons. This effect in the canine spinal cord has several characteristics.

1. NK1-r (+) neuron loss is detectable as early as 7 days after intrathecal SP-SAP.
2. NK1-r (+) neuron loss persisted over the next 3 months emphasizing that reduction of NK1-r protein and mRNA reflected a permanent state related to the hypothesized loss of targeted neurons.

Fig. 4. (A–C) Representative areas of quantification (dashed outlines) carried out for cells showing NK1-r (+) message (in situ hybridization) in superficial dorsal (lamina I-II), deep dorsal (lamina III-V), and ventral horns of dogs treated with saline, 15-, or 150-µg intrathecal substance P-saporin. (D–F) Presents superficial dorsal horn cell counts with median and 25/75th % of counts for in situ labeled NK1-r (+) cells in lumbar spinal segments. All treatment groups had six animals, but several animals had spinal blocks that could not take the in situ hybridization for unknown reasons, despite repeated efforts. These were not included in the counts. Each data set (graph) was analyzed with a Kruskal–Wallis with the statistical significance for the analysis shown in each graph with a post hoc Dunn multiple comparison versus saline group. *P < 0.05; **P < 0.01. NK1-r = neurokinin-1 receptor; P1 = spinal distribution phase: 7-day sacrifice; P2 = spinal safety phase: maximum 90-day survival.
3. Toxicity is limited to the SP-SAP conjugate. The SP neuropeptide is necessary for SP binding and internalization of the bound Saporin molecule. Thus, intrathecal Saporin alone fails to alter NK1-r counts in rats and dogs.

4. Target toxicity is limited to the NK1-r (+) cell populations. General cell death or gliosis was not observed in the dorsal horn as evidenced by unchanged NeuN cell counts across treatment groups (data not shown). Furthermore, systematic counting of NeuN (+) cells in Lamina I/II revealed no changes as compared with saline and SP-SAP doses up to 150 µg. NK1-r (+) cells are present in large motor neurons in the ventral horn, and ablation of these cells after intrathecal 150-µg SP-SAP likely contributed to the impaired motor function we observed.

5. Effects of intrathecal SP-SAP on NK1-r (+) neurons were most apparent in the lumbar sacral spinal cord proximate to the spinal level of drug delivery, a finding consistent with local distribution of injectate and its short half-life (see Spinal Distribution). The statistically significant reduction in cervical NK1-r (+) neurons of the 1.5-µg SP-SAP group may be a treatment-related effect. However, we believe that this is not likely, as no such difference was observed at other spinal levels. Moreover, this effect was not observed even with the 150-µg dose, e.g., there were no statistically significant differences at the cervical level as compared with the saline dose.

6. NK1-r (+) neuron loss was largely present in the superficial dorsal horn. Counting of NK1-r (+) motor horn cells showed a significant decrease when SP-SAP was administered at the highest dose, 150 µg.

**Fig. 5.** *In situ* hybridization (A, D, and G) and immunohistochemical (B, E, and H) product colabeling (F, I, merged) of lumbar superficial dorsal (lamina I-II) and ventral (motor) horn NK1-r (+) neurons in an animal at 90 days having received intrathecal saline. (D–F and G–I) Display enlargement of boxes in superficial dorsal horn and ventral horn, respectively. (C) Presents regression analysis (solid line) with 95% CIs (dashed line) between immunohistochemical and *in situ* cell counts in the superficial dorsal horn for all drug- and saline-treated animals as measured in tissues obtained at the time of sacrifice. The slope and its 95% CIs of the linear regression line (0.970: 0.394–1.547) were calculated and shown to be statistically different from zero ($P = 0.002$) with an $r^2 = 0.335$. IH = immunohistochemical analysis; *in situ* = *in situ* hybridization; NK1 = neurokinin-1; P1 = spinal distribution phase: 7-day sacrifice; P2 = spinal safety phase: maximum 90-day survival.
Intrathecal Substance P-Saporin

Behavioral Effects of Intrathecal SP-SAP

Intrathecal SP-SAP administered to awake animals resulted in an acute onset of a dose-dependent pain behavior that persisted for several days. In our previous work, intrathecal SP-SAP in dogs resulted in transient increases in heart rate, blood pressure, and body temperature. In that study, intrathecal SP-SAP injections were associated with mild repetitive contractions of the neck musculature and occasional truncal rigidity, particularly in dogs administered the highest dose, lasting up to 7 days.24 Interestingly, similar observations have not been previously reported in rodents. We believe that these phenomena reflect several mechanisms. Intrathecal SP evokes an acute, short-lasting hypersensitivity and hyperalgesia.41,42 Furthermore, spinal SP can initiate spinal prostaglandin release that is diminished by cyclooxygenase inhibition. 40,43 The pain behavior in the current study was effectively managed by administration of a cyclooxygenase-2 preferring inhibitor (carprofen). The persistence of pain behaviors for several days may reflect the ablation of NK1-r–bearing dorsal horn neurons and an associated local inflammatory reaction.

Spinal Distribution

Bolus or slow infusion of isobaric solutions into the intrathecal space typically results in a segmentally localized spread of injectate.44,45 Sampling proximate to the catheter injection site reveals that intrathecal test articles show a rapid acute dilution which typically reflects a local dilution of the injectate in the CSF and a slow exponential decline in concentrations reflecting ongoing dilution and clearance from the CSF (into tissue and/or though the meninges) and metabolism (e.g., brain-derived nerve growth factor: 27 kDa; ziconotide: 3 kDa; and inulin: 6 kDa).46,47 We assessed lumbar CSF concentrations of the active 33-kDa SP-SAP and the total amount of Saporin (31 KDa) in lumbar CSF sampled proximate to the injection site. Peak lumbar CSF concentrations of both SP-SAP and Saporin occurred at the first-sampling time point (20 min). Concentrations of SP-SAP declined to minimally detectable levels at 4 h, whereas Saporin concentrations were detectable for up to 24 h. Examination of the SP-SAP:total Saporin ratio suggested that by 20 min after the injection of SP-SAP, the ratio had decreased to approximately 0.3 suggesting that the rapid decline in active toxin likely represented a rapid cleavage of the SP from the Saporin by CSF peptidases and proteases.48,49 Rapid cleavage, along with previous work showing that SAP alone failed to have any neurotoxic effects as measured by behavior or by NK1-r cell loss,6,24 is consistent with the anatomically delimited loss of NK1-r–bearing cells observed in the current study. Thus, localization and rapid conversion of Saporin to the inactive form minimize the likelihood of extensive neuraxial drug exposure.

Spinal Histopathology

Four criteria were used for interpreting test article–related microscopic changes: (1) an incidence clearly different from

Fig. 6. Representative sections of low- and high-magnification lumbosacral spinal cord showing histopathologic changes observed in animals euthanized 6 days after administration of intrathecal 150-µg substance P-saporin. White matter and gray matter inflammatory cell infiltrates were observed (A, A1). The arrow indicates a focus of perivascular infiltrates. The cells surrounding the blood vessel are predominantly lymphocytes with a few neutrophils present. Extensive vacuolation was observed in spinal cord grey and white matter (B, B1). The border of the gray matter is outlined (dorsal horn to the right; ventral horn to the left) with the arrow indicating an axon within a vacuolated area (a dilated myelin sheath). Spinal cord neuronal necrosis was identified in superficial long spinal tracts (C, C1). The upper arrow in the low magnification (C) and the arrow in the high magnification (C1) identify a necrotic neuron in the ventral horn of the gray matter, whereas the lower arrow in low magnification (C) shows a focus of perivascular infiltrates (primarily lymphocytes) in the meninges in the ventral fissure of the spinal cord. Nerve fiber/axonal degeneration was identified in the ascending tracts of the dorsal spinal cord (D, D1). The hashed line indicates the zone of degeneration. The arrows in the high magnification (D1) indicate dilated myelin sheaths devoid of axons. The arrows point to debris or macrophages containing debris. The majority of the nerve fibers in this zone are affected, but some dilated myelin sheaths do contain axons.
The dose of SP-SAP at which no clinical or microscopic histopathologic effects were identified during the in-life phase was 15 μg in 0.5 ml. Importantly, at this dose, the deep neurons and motor horn neurons at the level of the injection site were spared. Our experiments confirm the results of systematic reviews that the intrathecal administration of SP-SAP reduces pain through ablation of NK1-R-expressing neurons. Brown et al., 28 in

**Table 4.** Histopathology following Intrathecal SP-SAP

<table>
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<th>Pathology</th>
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Summary of treatment-related histopathologic findings in animals that received intrathecal (0) saline, 1.5, 15, or 150 µg SP-SAP. Number indicates the incidence of animals from each treatment group with histopathologic findings out of the total number of animals in each group (n = 6).

 Degeneration = long-tract neuronal degeneration; Infiltrates = inflammatory cell infiltrates (mononuclear cells ± granulocytes); L-S = lumbosacral; Necrosis = neuronal necrosis; SP-SAP = substance P-saporin; Vacuolation = vacuolation of degenerated neurons and/or neuronal processes.

**Fig. 7.** Time course for detection of substance P-saporin (SP-SAP) and Saporin in lumbar cerebrospinal fluid (LCSF) of dogs administered intrathecal 150-µg SP-SAP. The dotted line represents lower limit of detection by the assay. The solid line represents the line of best fit. SP-SAP and Saporin were detected after 17 h.
companion animal behavioral studies, show that doses similar to those studied here are likely responsible for a favorable response and therapeutic ratio for intrathecal SP-SAP in a well-defined large animal model. These studies constitute a necessary step in the preclinical development of this agent as a possible pain therapeutic.50–52

References


3. Nattie EE, Li A: Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity. J Physiol 2002; 544(Pt 2):603–16


27. Choi JI, Koehrn FJ, Sorkin LS: Carrageenan induced phosphorylation of Akt is dependent on neurokinin-1 expressing neurons in the superficial dorsal horn. Mol Pain 2012; 8:4


35. Li JL, Ding YQ, Xiong KH, Li JS, Shigemoto R, Mizuno N: Substance P receptor (NK1)-immunoreactive neurons projecting to the periaqueductal gray: Distribution in the spinal trigeminal nucleus and the spinal cord of the rat. Neurosci Res 1998; 30:219–25
50. Eisenach JC, Shafer SL, Yaksh T: The need for a journal policy on intrathecal, epidural, and perineural administration of non-approved drugs. Pain 2010; 149:417–9