Reproductive and Developmental Toxicity, and Neurotoxicity of a Tropomyosin-Related Kinase A Inhibitor, ASP7962, in Rats

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Abstract

Background: Osteoarthritis (OA) is a cartilage degenerative disease and the most common joint disorder. The expression levels of both nerve growth factor (NGF) and tropomyosin-related kinase A (TrkA) are induced in synovial fluid of knee joint in painful OA condition. TrkA plays a role of nociceptors in the dorsal root ganglion by binding with NGF. The blockade of NGF-TrkA transduction is expected to be a novel therapeutic application to treat OA. ASP7962 was identified and is under development for treatment of painful knee OA as a possible selective inhibitor of TrkA, TrkB and TrkC kinase activities with 50% inhibition concentration (IC_{50}) values of 0.155, 1.41 and 1.09 μmol/L, respectively. As Trk family regulates neuronal development or maintenance in central and peripheral nervous systems, it is considered that ASP7962 possibly affects these nervous systems in developmental and adult phases.

Objectives: For neuronal development, effects of ASP7962 on embryo-fatal and pre-/postnatal development were evaluated from implantation until closure hard palate, delivery or weaning in rats. For neuronal maintenance in adult phase, effects of ASP7962 on sympathetic and sensory nervous systems were evaluated for 4 and 13 weeks in rats.

Methods: Reproductive and developmental toxicity studies were conducted following oral ASP7962 administration to pregnant Sprague Dawley (SD) rats from implantation until closure of the hard palate (from Day 7 to 17 of gestation), from implantation until before delivery (from Day 7 to 21 of gestation) or from implantation to weaning (from Day 7 of gestation to 20 days after delivery). Neurotoxicity studies investigating effects on sympathetic and sensory nervous

systems were conducted following oral ASP7962 administration to SD rats for 4 and 13 weeks.

Results: Reproductive and developmental toxicity studies showed corneal abnormalities, reduced milk uptake, loss of pain responses, and decreased reflexes in pups. Neurotoxicity studies showed reversible neuronal atrophy without neuronal death in the sympathetic ganglia (cervicothoracic ganglion, cranial mesenteric ganglion or superior (cranial) cervical ganglion) in pathology, and reversible decreased ganglion volume and/or neuron size in the superior (cranial) cervical ganglion in stereology.

Conclusion: ASP7962 had inhibitory effects on neuronal development in corneal innervation as well as facial motor neuron, sensory neuron, and spinal motor neuron. These effects were almost similar to phenotypes in knockout mice lacking TrkA, B, or C. Neuronal atrophy in the sympathetic ganglia including superior (cranial) cervical, cranial mesenteric and cervicothoracic ganglia was noted in rats treated with ASP7962 for 4 or 13 weeks. This atrophic change was reversible during the recovery period. ASP7962 did not induce neuronal death or detectable dysfunction of the sympathetic and sensory nervous systems in functional observational battery (FOB) assessment.

Contents

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
AUC_{24}	Area under the plasma concentration-time curve
BDNF	Brain derived neurotrophic factor
C_{max}	Peak plasma concentration
FOB	Functional observational battery
GMA	Glycol methacrylate
IC_{50}	50% inhibition condition
NGF	Nerve growth factor
NOAEL	No observed adverse effects level
NSAIDs	Non-steroidal anti-inflammatory drugs
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
NT-5	Neurotrophin 5
OA	Osteoarthritis
SD	Sprague Dawley
TK	Toxicokinetics
t_{max}	Time of maximum concentration
TrkA	Tropomyosin-related kinase A
TrkB	Tropomyosin-related kinase B
TrkC	Tropomyosin-related kinase C

General introduction

OA is a cartilage degenerative disease and the most commonly affecting joints resulting in decreased quality of life in patients (Briggs et al., 1999). In OA patients, articular cartilage destruction gradually progresses, and disabling pain and joint dysfunction are also induced (Crawford et al., 2013). Aging and obesity are considered as major risk factors of OA (Bijlsma et al., 2013). Symptoms of OA include pain, stiffness, crepitus with motion, joint effusion and limitation of movement in joints (Martin & Buckwalter, 2002). These symptoms need medical treatments such as pharmacological pain relief and surgery. Non-steroidal anti-inflammatory drugs (NSAIDs) or opioids are prescribed to OA patients to relieve pain. These drugs, however, have various side effects and often show insufficient analgesic effects in OA patients. Therefore, new medications with novel pharmacological actions have been desired to fulfill unmet medical needs in OA pains.

NGF is one of the neurotrophic factors, which also include brain derived neurotrophic factor (BDNF), neurotrophin-3, 4 and 5 (NT-3, NT-4 and NT-5). These neurotrophic factors play a role in survival, development and function of neurons in central or peripheral nervous system via receptors such as TrkA, B or C (Skaper, 2012, 2018). TrkA receptor regulates the nociceptive actions of NGF on nociceptive neurons in dorsal root ganglia (Fang et al., 2005). Thus, NGF exerts its nociceptive action via TrkA. The role of NGF-TrkA signaling in the nociceptive transduction is demonstrated by the analgesic effects of NGF-TrkA inhibitors. Various blocking agents of the NGF-TrkA signaling ameliorate hyperalgesic conditions in both animals and humans (Lane et al., 2010; Ugolini et al., 2007). NGF-TrkA signaling

blockade in the peripheral tissues is therefore considered an attractive target for analgesic drugs (Hefiti et al., 2006).

The expression levels of both NGF and TrkA are induced in synovial fluid of knee joint in painful OA condition (Montagnoli et al., 2017), suggesting an important role of NGF-TrkA signaling in pathophysiology of OA pain (Iannone et al., 2002). Anti-NGF antibody and TrkA inhibitors demonstrate analgesic effects in rat models of arthritis (Shelton et al., 2005, Nwosu et al., 2016). Anti-NGF monoclonal antibody, tanezumab, shows analgesic effects in clinical proof-of-concept study for OA pain (Lane et al., 2010). These scientific evidences suggest that inhibition of NGF-TrkA signaling has therapeutic potential against OA pain. However, a small molecule pharmaceutical product with the mechanism of NGF-TrkA signaling pathway inhibition has not been developed to treat neurogenic pain in OA patients. TrkA plays a role of nociceptors in the dorsal root ganglion by binding with NGF (Fang et al., 2005). The blockade of NGF-TrkA signal transduction is expected to be a novel therapeutic application to treat pain in OA patients. ASP7962 was identified as a possible selective inhibitor of TrkA, TrkB and TrkC kinase activities with IC_{50} values of 0.155, 1.41 and 1.09 μmol/L, respectively (Watt et al., 2019). ASP7962 is under development as an orally active medication for the treatment of painful knee OA. ASP7962 is a small molecule which has the advantage of oral availability for patients compared to anti-NGF antibody. Therefore, ASP7962 is expected to be a novel therapeutic drug for OA patients. It is necessary to clarify the toxicological profile of ASP7962 to proceed clinical development. Especially, it is worthwhile to investigate effects of ASP7962 on the nervous system based on the theoretical risk due to the blockade of NGF-

TrkA signal transduction.

NGF is a regulator in the developing sympathetic and sensory neurons (Bueker, 1948). NGF, BDNF, NT-3, 4 and 5 regulate the survival, development and function of neurons in both the central and peripheral nervous systems. These neurotrophic factors are activated by binding a family of three tropomyosin-related kinases such as TrkA, TrkB, and TrkC, respectively (Skaper, 2012, 2018). Mutant mice lacking TrkA, TrkB or TrkC show neuronal deficiencies in the central and peripheral nervous systems (Smeyne et al., 1994, Klein et al., 1993, 1994) indicating that Trk family plays an important role for promoting nervous system in embryo-fetal development. On the other hand, in adult phase, these neurotrophic factors are related to regulation of synaptic transmission and plasticity in nervous systems by signaling via its receptors (Valle et al., 2017). As each of Trk family receptors has roles for developmental and adult phases, it is worthwhile to evaluate the effects of ASP7962 on both embryo-fetal development and on sympathetic and sensory nervous systems in adult phase. In Chapter 1, I investigated the reproductive and developmental toxicity of ASP7962 in rats. In Chapter 2, I investigated the neurotoxicity of ASP7962 on the sympathetic and sensory nervous systems in rats.

Chapter 1: Reproductive and developmental toxicity studies of a tropomyosin-related kinase A inhibitor, ASP7962, in rats

Introduction

A family of three tropomyosin-related kinases (TrkA, TrkB, and TrkC) regulates the survival, development and function of neurons in both the central and peripheral nervous systems, and activated by NGF, BDNF, NT-3, 4 and 5 (Skaper, 2012, 2018). Inhibition of NGF induces the loss of these neurons during development due to cell death (Gorin & Johnson, 1979). Anti-NGF antibody induces morphological atrophy of the superior cervical ganglion with reduced neuronal number in rats (Gorin & Johnson, 1980). NGF binds to TrkA with high affinity and p75 neurotrophin receptor with low affinity (Kaplan et al., 1991). NGF supports the survival of sensory and sympathetic neurons. Mice lacking NGF show neuronal cell loss in the sensory and sympathetic ganglia (Crowley et al., 1994). In TrkA lacking mice, severe sensory and sympathetic neuropathies, and neuronal cell loss in the trigeminal, sympathetic and dorsal root ganglia were observed (Smeyne et al., 1994). BDNF-TrkB signaling regulates the synaptic plasticity in the brain. There is also a role of TrkB in hippocampal long-term potentiation and learning (Minichiello, 2009). Mutant mice lacking BDNF show deficits in coordination and balance which are related to the degeneration in the sensory ganglia including the vestibular ganglion (Ernfors et al., 1994). TrkB null mice show neuronal deficiencies in the central (facial motor neucleus and spinal cord) and peripheral (trigeminal and dorsal root ganglia) nervous systems (Klein

et al., 1993). NT-3 has the functional roles in the developing and mature sympathetic nervous system. NT-3-TrkC signaling regulates gangliogenesis by promoting of mitosis in migratory neural crest cells (Zhou et al., 1996). TrkC regulates the number of sensory neurons during dorsal root ganglia development (Menard et al., 2018). Mice defective of TrkC display the deficiency of Ia muscle afferent projections to spinal motor neurons and have fewer large myelinated axons in the dorsal root and posterior columns of the spinal cord, which result in abnormal movements and postures (Klein et al., 1994). These evidences, which are shown in mutant mice lacking TrkA, TrkB or TrkC, indicate that Trk family receptors play the essential roles in developing central and peripheral nervous systems.

ASP7962 is an inhibitor of TrkA which ameliorates the impairment of vertical activity in a rat model for knee OA, and inhibits kinase activities of TrkA, TrkB and TrkC with IC_{50} values of 0.155, 1.41 and 1.09 μmol/L, respectively (Watt et al., 2019). The effects on embryo-fetal and pre-/postnatal development by Trk inhibition have not been clarified. Based on the theoretical effects of Trk inhibition on the neural development, it is important to investigate the effects of ASP7962 on nervous development. To evaluate the effects of ASP7962 on embryo-fetal and pre-/postnatal development, reproductive and developmental toxicity studies were conducted in rats.

Materials and Methods

Test article

ASP7962 is a novel selective inhibitor of human TrkA for the treatment of OA. The inhibitory effects of ASP7962 on adenosine 5'-triphosphate (ATP) induced substrate phosphorylation in human TrkA, B, and C were assessed using the mobility shift assay method. Each Trk (purified cytoplasmic or catalytic domain) and substrate was incubated with ATP to induce substrate phosphorylation. Reactions were conducted in the presence of various concentration of ASP7962 to calculate IC_{50} values. It showed that ASP7962 inhibited ATP-induced substrate phosphorylation in human TrkA, B, and C with IC_{50} value of 0.155 μmol/L, 1.41 μmol/L, and 1.09 μmol/L, respectively (Watt et al., 2019).

To evaluate the effects of ASP7962 on embryo-fetal and pre-/postnatal development, reproductive and developmental toxicity studies were conducted in rats. ASP7962 was created by Astellas Pharma Inc. (Tokyo, Japan). The vehicle used in preparation of the test article formulations and for administration to the control group was 0.5% w/v methylcellulose in deionized water.

Animal and care

The first study for effects of the oral administration on embryo-fetal and postnatal development in rats were conducted at the Astellas Pharma Inc. Crl:CD(SD) rats were used as the test system for this study. Crl:CD(SD) rats (51 males at 11 weeks and 101 females at 10 weeks) were received from Charles River Laboratories. The subsequent study for effects of the oral administration on

pre- and postnatal development in rats were conducted at Nihon Bioresearch Inc. (Gifu, Japan) in compliance with Good Laboratory Practice. Crl:CD(SD) rats were used as the test system for this study. Crl:CD(SD) rats (73 males at 11 weeks and 155 females at 10 weeks) were received from Charles River Laboratories. The animals were allowed free access to pellet diet (CRF-1; Oriental Yeast Co., Ltd.). Drinking water was provided ad libitum. All animals were housed throughout acclimation and during the study in an environmentally controlled room. The room temperature and relative humidity controls were set to maintain environmental conditions of 20 to 26°C and 40 to 70%, respectively. The animals were selected for use based on the results obtained for general signs and body weights during acclimation period. The females were mated with males on a one-to-one basis overnight to obtain pregnant animals. The females confirmed to have copulated were allocated to groups using a computer program so that the mean body weight of each group on Day 0 of gestation was approximately equal.

In the first study, cesarean section groups for embryo-fetal development and delivery groups for postnatal development, the 0, 100, 300 and 600 mg/kg/day groups (Control, Low, Middle and High group, respectively) each consisted of 6 dams (Table 1-1). For the toxicokinetic analysis, the 100, 300 and 600 mg/kg groups each consisted of 8 dams were set (Table 1-1).

In the subsequent study, for pre- and postnatal development groups, the 0, 30, 100 and 200 mg/kg/day groups (Control, Low, Middle and High group, respectively) each consisted of 20 dams (Table 1-2). For the toxicokinetic analysis, the 0 mg/kg group consisted of 4 dams, and the 30, 100 and 200 mg/kg groups each consisted of 8 dams were set (Table 1-2).

ASP7962 administration and dosage levels

The vehicle and test article formulations were administered orally by gavage via gastric tube once daily. The dose volume for all groups was 5 mL/kg. Individual doses were based on the most recently recorded body weights to provide the correct mg/kg/day dosage. The day when the vaginal plug or sperm in the vaginal smear was found was defined Day 0 of gestation, and the day of gestation was counted from that day. The lactation period was Day 0 to 21 after birth.

In the first study, 600 mg/kg/day was selected as the high dose, and the middle and low doses were set at 300 and 100 mg/kg/day.

Dosage levels in the subsequent study were selected based on the results of the first study for the effects on embryo-fetal and postnatal development in rats. In delivery groups of the first study for postnatal development, all pups at 600 mg/kg died 1 day after birth, and corneal opacity and decreased weaning index 21 days after delivery were noted at 300 mg/kg. No adverse effects were noted in pups at 100 mg/kg. Therefore, in the subsequent study, 200 mg/kg/day was selected as the high dose, and the middle and low doses were set at 100 and 30 mg/kg/day.

Clinical sign, body weight, and food consumption for dams

The dams were observed for clinical signs before dosing and 2 or 3 times after administration during dosing period, and once or twice during pre- and post-dosing periods. In the first study, body weight and food consumption were recorded on Days 0, 4, 7 to 18, and 20 of gestation for cesarean section groups

and on Days 0, 4, and 7 to 21 or 22 of gestation (until before 24 delivery) and Days 0, 4, 7, 10, 14, 17, and 21 of lactation for delivery groups. In the subsequent study, Body weight was recorded on Days 0 and 4 of gestation, daily from Days 6 to 20 of gestation, and 0, 4, 7, 11, 14, 17, and 21 days after delivery. Food consumption was recorded on Days 0, 3, 6 to 19, and 20 of gestation and 0, 4, 7, 11, 14, 17, and 21 days after delivery.

Toxicokinetics for dams

In the first study, blood samples for toxicokinetics were collected on Days 7 and 20 of gestation from 4 animals/group/time points except for dead animals in all groups prior to dose administration (Day 20 of gestation only) and at approximately 0.5, 1, 2, 4, 8, and 24 hours after dose administration.

In the subsequent study, blood samples for toxicokinetics were collected on Days 7 of gestation and 20 days after delivery from 4 animals/sex in control group at approximately 0.5 hours following dose administration, and from 4 animals/sex/group/time point in low, middle and high groups prior to dose administration (20 days after delivery only) and at approximately 0.5, 1, 2, 4, 8, and 24 hours after dose administration.

- **First study:** schedule of procedure (Table 1-3)

Caesarean section group

- **Observation at Cesarean section**

In the first study, the numbers of embryo-fetal deaths and live fetuses were determined upon necropsy of the dams on Day 20 of gestation (embryo-fetal deaths

were classified as one of the following: implantation site, resorption, or dead fetus). Each live fetus was examined for external abnormalities (including the hard palate), and its sex and weight were determined. Each exterior of placenta was examined, and then its weight was determined.

The post-implantation loss rate, sex ratio, and incidence rate of fetuses with external abnormalities were calculated as follows:

Post-implantation loss rate (%)

= No. of embryo-fetal deaths/No. of implantations × 100

Sex ratio (%)

= No. of live male fetuses/No. of live male and female fetuses × 100

Incidence rate of fetuses with external abnormalities (%)

= No. of fetuses with external abnormalities/No. of fetuses examined × 100

- **Visceral observation**

Approximately half of the live fetuses from each dam were fixed in a mixture of neutral buffered 10% formalin and acetic acid. The thoracic viscera of the fetuses were examined with a stereomicroscope using a micro-dissection method, and the cephalic and abdominal viscera were examined using Wilson's free-hand razor method. Since no significant differences were observed in the incidence rate of fetuses with visceral abnormalities or variations between the control group and high dose group, the fetuses from the middle and low dose groups were not examined. Specimens subjected to examination were preserved in 10% neutral buffered formalin, and all other specimens were preserved in a mixture of 10% neutral buffered formalin and acetic acid.

The incidence rate of fetuses with visceral abnormalities or variations was calculated as follows:

Incidence rate of fetuses with visceral abnormalities (%)

= No. of fetuses with visceral abnormalities/No. of fetuses examined × 100

Incidence rate of fetuses with visceral variations (%)

= No. of fetuses with visceral variations/No. of fetuses examined × 100

- **Skeletal observation**

Approximately half of the live fetuses from each dam were skinned and fixed in ethyl alcohol (at least 99%), followed by staining with alizarin red S using a modified version of Dawson's method. Specimens were examined for skeletal abnormalities, skeletal variations, and degree of ossification (number of ossified sternebrae and caudal vertebrae) using a stereomicroscope. Since there were significant differences in the incidence rate of fetuses with skeletal variations between the control group and high dose group, fetal specimens in the middle and low dose groups were also examined. Skeletal specimens were preserved in concentrated glycerol solution.

The incidence rate of fetuses with skeletal abnormalities or variations was calculated as follows:

Incidence rate of fetuses with skeletal abnormalities (%)

= No. of fetuses with skeletal abnormalities/No. of fetuses examined × 100

Incidence rate of fetuses with skeletal variations (%)

= No. of fetuses with skeletal variations/No. of fetuses examined × 100

- **Statistical analysis**

Data were statistically analyzed by the following procedures. The litter served as the unit of statistical analysis for litter data.

1. Body weight, food consumption, numbers of corpora lutea, implantations, embryo-fetal deaths (implantation sites, resorptions, and dead fetuses), live fetuses, sex ratio of live fetuses, live fetal weight, placental weight, and numbers of ossified sternebrae and caudal vertebrae:

   When the number of groups was 3 or more, homogeneity of variance was analyzed using Bartlett's test (significance level of the two sided test at 1%). When no significance was observed, Dunnett's test for multiple comparisons with the control group was used (significance level of the two sided test at 1% or 5%). When significance was observed, rank-transformed data were analyzed in the same manner using Dunnett's multiple comparison test (significance level of the two sided test at 1% or 5%).

2. Post-implantation loss rate, incidence rate of fetuses with visceral abnormalities or variations and incidence rate of fetuses with skeletal variations:

   The data were tested using the Wilcoxon rank sum test (Mann-Whitney U test) with the control group (significance level of the two sided test at 1 % or 5%).

3. Incidence rate of dams with fetal abnormalities or variations:

   When the expected frequency in any cell was 5 or less, Fisher's exact probability test was used (significance level of the two sided test at 1% or 5%).

Delivery group

- **Observation at birth**

After completion of birth, the number of live pups and number of pups that died at birth were counted, and the pups were sexed and observed for external abnormalities.

Live birth index was calculated as follows:

Live birth index (%) = No. of live pups/No. of implantations × 100

- **Clinical sign and body weight**

Pups were observed for clinical signs twice a day (in the morning and in the afternoon) during the lactation period, and once a day during the post-weaning period. The number of pups having milk in the stomach was recorded on Day 0 and 1 after birth. For the 300 mg/kg group, the number of pups having milk in the stomach was recorded afterwards until Day 10 after birth (except for Day 2).

Viability index on Day 4 after birth was calculated as follows:

Viability index on Day 4 after birth (%)

= No. of live pups (before cull)/No. of pups delivered × 100

Pups were measured for body weight on Days 0, 4, 7, 10, 14, 17, and 21 after birth. On Day 0, all pups from each litter were measured with male and female pups together. On Day 4 and later, each pup was measured separately.

- **Reflex functions and pain response**

On Day 21 after birth, pups were examined for pain response (against pinching the tail base with scissors-like forceps), auditory response (against the sound of a tuning fork), light reflex (against the light after mydriasis under a dark room),

surface righting reflex, floor walking, negative geotaxis reflex (on a slope at an angle of 45 degree), and mid-air righting reflex (from a position 30 cm high).

- **Necropsy at weaning on Day 21 after birth**

Three male pups and three female pups for each litter in the control and 100 mg/kg groups, other than those considered likely to affect the proper evaluation of the motor coordination test, were selected. For litters with fewer than three male or female pups, all respective male or female pups were selected. Pups in the 300 mg/kg group were not selected because their decreased body weights and abnormalities in the corneas prevented accurate evaluation of the motor coordination test.

Weaning index on Day 21 after birth and incidence rate of fetuses with internal abnormalities were calculated as follows:

Weaning index on Day 21 after birth (%)

= No. of live pups at weaning/No. of live pups (after cull) × 100

Incidence rate of pups with internal abnormalities (%)

= No. of liveborn pups with internal abnormalities

/No. of liveborn pups examined × 100

- **Motor coordination**

Pups were examined on Day 28 after birth for motor coordination with a rota-rod by the total number of incidences of falling off of the rotating bar for 3 min.

- **Necropsy and histopathology**

In the 300 mg/kg and control group, pups were sacrificed by exsanguination under ether anesthesia and necropsied on Day 21 after birth. In the 100 mg/kg and control group, after motor coordination test, pups were sacrificed by exsanguination under ether anesthesia and necropsied on Day 31 or 32 after birth.

The Harderian gland and extraorbital lacrimal gland of all pups in the control and 300 mg/kg groups and the eyeballs of all pups in the control group and pups with abnormalities in the cornea in the 300 mg/kg group were stained with hematoxylin and eosin at weaning on Day 21 after birth and examined for histopathology.

- **Statistical analysis**

Data were analyzed statistically with the following procedures. The litter served as the unit of statistical analysis for litter data.

1. Body weight, food consumption, numbers of implantation sites, pups delivered, liveborn pups, duration of gestation, result of motor coordination:

   When the number of groups was 3 or more, homogeneity of variance was analyzed using Bartlett's test (significance level of the two sided test at 1%). When no significance was observed, Dunnett's test for multiple comparisons with the control group was used (significance level of the two sided test at 1% or 5%). When significance was observed, rank-transformed data were analyzed in the same manner using Dunnett's multiple comparison test (significance level of the two sided test at 1% or 5%).

   When the number of groups compared was 2, homogeneity of variance was

analyzed using the F test (significance level of the two sided test at 5%). When a set of variances was homogenous, Student's t-test was used (significance level of the two sided test at 1% or 5%). Otherwise, Welch's t-test was used (significance level of the two sided test at 1% or 5%)

2. Live birth index, viability index on Day 4 after birth, weaning index on Day 21 after birth:

   The rank-transformed data were tested using Dunnett's test for multiple comparison with the control group (significance level of the two sided test at 1 % or 5%).

3. Incidence rate of pups with external or internal abnormalities and gestation index:

   When the expected frequency in any cell was 5 or less, Fisher's exact probability test was used (significance level of the two sided test at 1% or 5%).

4. Results of development of sense organs and behavior:

   The data were tested using the Wilcoxon rank sum test (Mann-Whitney U test) with the control group (significance level of the two sided test at 1 % or 5%).

➢ **Subsequent study:** schedule of procedure (Table 1-4)

F1 Pups

- **Observation at birth**

After completion of birth, the number of live pups and number of pups that died at birth were counted, and the pups were sexed and observed for external abnormalities.

Live birth index was calculated as follows:

Live birth index (%) = No. of live pups/No. of implantations × 100

- **Clinical sign, body weight and food consumption**

The pups were observed for clinical signs and mortality twice a day in the morning and afternoon from the day of birth to 4 days after birth. The pups were also observed for milk in the stomach twice a day, once in the morning and again in the afternoon, during this period. The pups were observed for clinical signs once a day thereafter.

Body weights of pups were recorded 0 (day of birth), 4 (before culling), 7, 11, 14, 17, 21, 28, 35, 42, 49, 56, 63, and 70 days after birth.

Food consumption was calculated from the weight of feed supplied 21, 28, 35, 42, 49, 56, 63, and 70 days after birth and the weight of feed remaining on each following day.

- **Morphological differentiation**

All pups were observed for separation of the auricles 2 days after birth, eruption of the incisors 10 days after birth, separation of the eyelids 14 days after birth, opening of the vaginal orifice 33 days after birth, and cleavage of the balanopreputial gland 39 days after birth. When a morphological differentiation was not completed in a pup within the designated period of time, the pup was observed for this differentiation daily until it was completed.

- **Reflex functions and pain response**

All pups were observed for the surface righting reflex 6 days after birth, negative geotaxis (using a slanting board) 10 days after birth, and air righting reflex, corneal reflex, pupillary reflex, and pinna reflex 17 days after birth. When a reflex function was not seen in a pup within the designated period of time, the pup was observed for this function daily until it appeared or until the day of gross pathology. Pain response was tested 17 days after birth by pinching the tail base with a pair of forceps, and reflex ability (turning, biting, and uttering a distress sound) was observed.

- **Emotionality**

Emotionality was tested by the open-field test (open field used: 100 × 100 cm, 5 × 5 divisions, M's Studio) 4 weeks after birth. The test was performed for 3 minutes each for 3 consecutive days (1:00 p.m. to 5:00 p.m.), and each pup was observed for latency of first movement, ambulation, rearing, face-washing, grooming, urination, and the number of fecal pellets. Data were totaled for each of the 3 days.

- **Motor coordination**

Motor coordination was tested by the Rotarod method (8 r.p.m., 3 minutes, Rotarod used: Natsume Seisakusho Co., Ltd.) 4 weeks after birth (the day after completion of examinations for emotionality). The animals were trained not to fall off the rotating bar for 30 seconds. The number of falls during a period of 3 minutes was counted.

- **Learning ability**

Learning ability was tested by the water multiple T-maze test (water multiple T-maze used: 120 × 135 cm, Natsume Seisakusho Co., Ltd.) 6 to 7 weeks after birth. The animals were trained in a straight waterway 3 times on the first day (data obtained were regarded as reference data and were not tabulated) and then tested in a water maze 4 times a day for the next 3 days. Each trial was continued for 3 minutes at the longest. The time necessary to reach the goal, selection errors, intra-zonal errors, and backing errors were recorded, and the total errors were calculated. The data were totaled for each day. When an animal failed to reach the goal within 3 minutes, the time necessary to reach the goal was recorded as being 181 seconds.

- **Necropsy**

Pups in all groups were sacrificed by exsanguination under ether anesthesia and necropsied on Day 21 after birth. All pups were sacrificed in the 200 mg/kg group on Day 21 after birth. In the 30 mg/kg, 100 mg/kg and control group, after emotionality, motor coordination, and learning ability, pups were sacrificed by exsanguination under ether anesthesia and necropsied on Day 56 after birth.

- **Reproductive function**

For estrous cycles, one female in each litter was examined for estrous cycles by vaginal smear test once a day for 14 days from 10 weeks after birth to the day

before the start of pairing and then until the day before confirmation of copulation.

For mating methods and confirmation of copulation, one pup of each sex in each litter was used for mating. Non-sibling males and females within the same groups were paired from 12 weeks after birth onward on a one-to-one basis from the evening to the next morning for a maximum period of 14 days.

Copulation was confirmed at about the same time every morning. Females that had a vaginal plug or sperm in the vaginal smear were regarded as having copulated. The day when the vaginal plug or sperm in the vaginal smear was found was defined as Day 0 of gestation, and the day of gestation was counted from that day.

F1 Dams

- **Clinical signs and body weights**

The dams were observed for clinical signs and mortality once a day in the morning from Day 0 of gestation to the day of necropsy. The dams were weighed on Days 0, 4, 7, 10, and 13 of gestation.

- **Observation at Cesarean section**

Dams that copulated were euthanized 13 days after copulation by bleeding from the abdominal aorta under 20% isoflurane anesthesia, necropsied, and examined for pregnancy. Regarding the dams in which pregnancy was confirmed, the number of corpora lutea and number of implantation sites were counted.

The number of embryonic deaths and number of live embryos were counted

for each dam; embryonic mortality was observed macroscopically. Embryonic deaths were classified as follows: a) only implantation scars found; b) resorption (not in embryonic shape); and c) dead embryos (in embryonic shape).

- **Statistical analysis**

Data were analyzed following the statistical methods described below.

Bartlett's test was performed at a significance level of 1%, and the other tests were performed at a two-tailed significance level of 5% (indices are shown at both $p<0.05$ and $p<0.01$).

1. Body weight, body weight gain, and food consumption and gestation duration, number of implantation scars, number of pups, number of stillbirths, number of dead pups at birth, sex ratio, body weights of both sexes of pups, food consumption of pups, emotionality, motor coordination, learning ability, number of estrous cases during the pre-mating period, number of days till copulation after pairing, number of corpora lutea, number of implantation sites, number of pre-implantation losses, number of embryonic deaths, and number of live embryos, the value obtained from each dam or the mean value obtained from each dam served as the unit for analysis, and group mean values and standard deviations were calculated for these parameters.

   Then the obtained data were tested by Bartlett's test for homogeneity of variance. When the variances were homogeneous, Dunnett's test was performed to compare the mean in the control group with that in each of the groups treated with the test article. When the variances were heterogeneous, the data were transformed to ranked data, and a Dunnett type test was performed to compare

the mean rank in the control group with that in each of the groups treated with the test article (a Dunnett-type test).

2. Birth index, viability index, weaning index, incidence of external abnormalities, incidence of pups with milk in the stomach, morphological differentiation, reflex functions, pain response, implantation rate, pre-implantation loss rate, and post-implantation loss rate, the index or rate obtained from each dam served as the unit for analysis, and group mean values and standard deviations were calculated for these parameters.

   Then the data were transformed to ranked data, and Wilcoxon's rank-sum test was performed to compare the mean rank in the control group with that in each of the groups treated with the test article.

3. Gestation index of F0 dams, number of F0 dams having stillbirths, number of F0 dams having dead pups at birth, number of F0 dams having pups with external abnormalities, and F1 pups' copulation index and fertility index, a chi-square test was performed to compare the control group with each of the groups treated with the test article. Fisher's exact test was performed to compare the control group with each of the groups treated with the test article, because the number of F0 dams that had stillbirths, dead pups at birth, or pups with external abnormalities was 5 or less.

Results

- **First study**

Caesarean section group

Test article related effects are summarized in Table 1-5.

No test article-related effects were noted in any group with regard to numbers of corpora lutea, implantation sites, or live fetuses, or for post-implantation loss rate, sex ratio, placental weight, or external and visceral findings. Fetal body weight was decreased in the 600 mg/kg group. Delayed ossification was noted in the 300 mg/kg group and higher, suggesting suppressed fetal development. No lethal effects on embryo-fetal development or teratogenicity were observed.

Delivery group

Test article related effects are summarized in Table 1-6.

- ***Effect on dams***

Decreased spontaneous motility, incomplete eyelid opening, and loss of hair in the 600 mg/kg group were observed. Suppressed body weight gain in the 600 mg/kg group and decreased food consumption in the 300 mg/kg group and higher were also noted. No test article-related effects were noted in any group with regard to duration of gestation, parturition, gestation index, number of implantation sites, or necropsy findings.

- **Toxicokinetics**

As shown in Table 1-7 and Figure 1-1, t_{max} values were 1.0 to 2.0 hours on Day 7 of gestation and 0.5 to 4.0 on Day 20 of gestation. Dose-related, but not dose-proportionate, increases in the peak plasma concentration (C_{max}) and area under the plasma concentration-time curve (AUC_{24}) were confirmed on Days 7 and 20 of gestation.

✓ ***Effect on pups***

- **Viability from birth to weaning**

The numbers of pups delivered or liveborn pups did not differ significantly in any treatment group. The number of liveborn pups in the 600 mg/kg group was low compared to the control group, although not to a statistically significant degree. Live birth index and viability index on Day 4 after birth were significantly decreased in the 600 mg/kg group. Viability index on Day 4 after birth was 0.0 % in the 600 mg/kg group. Viability index on Day 4 after birth was also decreased at 300 mg/kg, although not to a statistically significant degree. Weaning index on Day 21 after birth was significantly decreased at 300 mg/kg.

- **Clinical sign, milk uptake, and body weight**

In the 600 mg/kg group, all of the pups died by Day 1 after birth. Trauma (abdominal region, tail, and left hindlimb) was observed at Day 0. Clinical signs observed in the 300 mg/kg group were death (Days 0 to 20 after birth), trauma (tail, left and right hindlimbs, left and right forelimbs, and head) (Days 5 to 19), poor growth (Days 18 to 21), opacity of the cornea (Days 16 to 21), and ulcer of the

cornea (Days 20 to 21). Decreased rate of pups having milk in the stomach and suppressed body weight gain were noted in the 300 mg/kg group and higher.

- **Reflex functions and Pain response**

No differences were observed in the results of surface righting reflex, floor walking, negative geotaxis reflex, mid-air righting reflex, auditory response, or light reflex tests. No abnormalities were noted in the results of the light reflex test for any pups examined.

In the pain response test, the rate of pups with normal responses was significantly lower in the 300 mg/kg group (12.5 %) than in the control group (100.0 %).

- **Motor Coordination**

No differences were noted in the result of the motor coordination test in either the control or 100 mg/kg group.

- **Necropsy and Histopathology**

Necropsy of terminal-sacrificed pups on Day 21 (at weaning) showed a small Harderian gland, small extraorbital lacrimal gland, opaqueness of the cornea, and an ulcer of the cornea in the 300 mg/kg group. Examination for histopathology in the 300 mg/kg group showed inflammation of the cornea in the eye ball, atrophy and single-cell necrosis of acinus in the Harderian gland, and atrophy of the acinus in the extraorbital lacrimal gland, whereas no changes were found in the control group.

➢ **Subsequent study**

Test article related effects are summarized in Table 1-8.

✓ ***Effect on dams***

In the 200 mg/kg group, transiently decreased food consumption was sporadically noted during the gestation period. No treatment-related changes were noted in the clinical signs, body weight, body weight gain, gross pathological findings, gestation duration, number of implantation scars, gestation index, delivery conditions, or nursing conditions in any test article group.

- **Toxicokinetics**

As shown in Table 1-9 and Figure 1-2, t_{max} values were 0.5 to 1.0 hours both on Day 7 of gestation and 20 days after delivery. C_{max} values increased less than dose-proportionally, and AUC_{24} values increased almost dose-proportionally between 30 and 100 mg/kg/day both on Day 7 of gestation and 20 days after delivery. C_{max} and AUC_{24} values increased less than dose-proportionally between 100 and 200 mg/kg/day both on Day 7 of gestation and 20 days after delivery.

✓ ***Effect on pups***

- **Viability from birth to weaning**

In the 200 mg/kg group, compared with the control group, the viability index and weaning index were significantly low. No significant difference from the control group was seen in sex ratio, number of live pups at birth, number of

stillbirths, number of dead pups at birth, number of dams having stillbirths, number of dams having dead pups at birth, or birth index in the 200 mg/kg group.

- **Clinical sign, Milk uptake, and Body weight**

From the day at birth to the day of weaning, in the 200 mg/kg group, death (0 to 17 days after birth), decreased locomotor activity (17 and 18 days after birth), abdominal distension (16 to 21 days after birth), hypothermia (0 to 20 days after birth), and moribundity (3 to 20 days after birth) were noted until the day of weaning, and corneal drying (14 to 21 days after birth), corneal opacity (15 to 21 days after birth), and red eyeballs (17 to 21 days after birth) were noted after eye-opening. In the 100 and 30 mg/kg groups, the number of animals that died was similar to that in the control groups. Therefore, this finding is not considered to be an effect of the test article. After weaning, no abnormal signs were noted in the 100 mg/kg, 30 mg/kg, or control group.

No test article related effect was observed in the incidence of pups with milk in the stomach.

In the 200 mg/kg group, body weight before weaning was low.

- **Morphological differentiation**

No test article related effects were observed in any test article group.

- **Reflex functions and Pain response**

In the 200 mg/kg group, compared with the control group, the following reflex functions were significantly lower: the surface righting reflex 6 to 8 days after birth,

negative geotaxis 10 to 13 days after birth, the air righting reflex 17 and 18 days after birth, the corneal reflex 17 to 21 days after birth, and the pinna reflex 17 to 20 days after birth. In this group, no significant difference from the control group was seen in the pupillary reflex function or pain response. In the 200 mg/kg group, observation for pupillary reflex was not done in 52 pups due to eye abnormalities.

No significant difference from the control group was seen in any reflex function or pain response in the 100 or 30 mg/kg group.

- **Emotionality, Motor coordination, Learning ability, and Reproductive function**

No test article related effects were observed in any test article group in the 100 or 30 mg/kg group

- **Necropsy**

In the 200 mg/kg group, corneal drying, corneal opacity, red eyeballs, and distension of the digestive tract were noted in gross pathology at 21 days after birth. No abnormal gross pathological findings were noted in the 100 or 30 mg/kg group.

✓ ***Effect on F1 dams***

No test article related effects were observed in any test article group in the 100 or 30 mg/kg group.

Discussion

The primary effects of ASP7962 on embryo-fatal and pre-/postnatal development in these studies were considered to be corneal innervation as well as facial motor neuron, sensory neuron, or spinal motor neuron. Characteristic findings possibly related to TrkA, B, or C receptor inhibition were corneal abnormality, lack of milk in the stomach, loss of pain response, and decreased reflexes in pups at the higher dose levels in these studies (Table 1-10).

Corneal opacity has been reported in the eye of mice lacking TrkA, which indicates the effect on the blink response due to sensory deficiency (Smeyne et al., 1994). Mice lacking TrkA show that the blink response against noxious stimuli is reduced in sensitivity (De Castro et al., 1998). Corneal abnormality observed in these studies of ASP7962 is therefore considered to be related to drying and opacity of the cornea after eye-opening through TrkA receptor inhibition. Although skin ulceration is observed in TrkA null mice (Smeyne et al., 1994), this finding is not noted in these conducted studies. After birth, mice lacking TrkB were found to be without milk in their stomach, and this phenomenon is considered to be caused by no feeding activity due to the abnormality in facial motor neuron of swallowing because of the lack of TrkB receptor (Klein et al., 1993). The lack of milk in the stomach was also observed in pups in the conducted studies, and it was considered to be related to the inhibition of TrkB receptor. For pain response, mice lacking TrkB do not react to sharp pinpricks in the vibrissae (Klein et al., 1993), and TrkA null mice stay longer time on hot plate due to lack of sensory function compared to wild type or heterozygous mice (Smeyne et al., 1994). Loss of pain response observed in these studies of ASP7962 is therefore considered to be related to the

inhibition of TrkA and TrkB receptors. For behavior or motor function, mice lacking BDNF have been reported defective coordination of movements and balance (Ernfors et al., 1994). BDNF maintains the survival of motor neuron via TrkB receptor (Sendtner et al., 1992). Mice lacking TrkC do not move normally such as athetotic movements when walking, and show abnormal behavior without upright posture. It indicates that TrkC signaling regulates the development of spinal motor neurons (Klein et al., 1994). Although proprioception is observed in TrkC null mice (Klein et al., 1994), this finding is not noted in these conducted studies. In the conducted embryo-fetal and pre/post-natal development studies in rat, changes in reflective functions such as decreased reflexes in surface righting reflex, negative geotaxis or air righting reflex are considered to be related to the inhibition of TrkB or TrkC.

There are no available references which show the effects of small molecule Trk inhibitors on the development of pups in animals. On the other hand, anti-NGF monoclonal antibody, tanezumab, was administered to pregnant cynomolgus monkeys from the organogenesis at beginning of gestation day 20 to parturition to investigate the developmental effects on pups. As a result, tanezumab increased stillbirth and mortality in pups, and there was decreased number of neurons in the sympathetic and sensory nervous systems (Bowman CJ et al., 2015). These changes indicate developmental neurotoxicity of tanezumab in cynomolgus monkey pups. Mortality and reduced viability were observed in the conducted studies of ASP7962.

Given the results of these reproductive and developmental toxicity studies, it was considered that ASP7962 had inhibitory effects on neuronal development on corneal innervation as well as facial motor neuron, sensory neuron, or spinal motor

neuron. These effects were almost similar to phenotypes in knockout mice lacking Trk A, B, or C.

Conclusion

Reproductive and developmental toxicity studies of ASP7962 showed corneal abnormality, reduction in the level of milk present in the stomach, loss of pain response, and decreased reflex in rat pups. These deficiencies in corneal innervation as well as facial motor neuron, sensory neuron, and spinal motor neuron development are also seen in knockout mice lacking Trk A, B, or C, and thought to be attributed to the lack of function of corresponding kinases.

Table 1-1. Study group assignment - First study -

Cesarean Section Groups for Embryo-fetal Development

Group	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Dams
Control	Vehicle	0	5	6
Low	ASP7962	100	5	6
Middle	ASP7962	300	5	6
High	ASP7962	600	5	6

Delivery Groups for Postnatal Development

Group	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Dams
Control	Vehicle	0	5	6
Low	ASP7962	100	5	6
Middle	ASP7962	300	5	6
High	ASP7962	600	5	6

Toxicokinetic Groups

Group	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Dams
Low	ASP7962	100	5	8
Middle	ASP7962	300	5	8
High	ASP7962	600	5	8

Table 1-2. Study group assignment - Subsequent study -

Delivery Groups for Pre- and postnatal Development

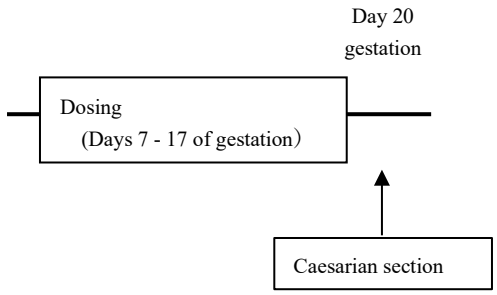
Group	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Dams
Control	Vehicle	0	5	20
Low	ASP7962	30	5	20
Middle	ASP7962	100	5	20
High	ASP7962	200	5	20

Toxicokinetic Groups

Group	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Dams
Control	Vehicle	0	5	4
Low	ASP7962	30	5	8
Middle	ASP7962	100	5	8
High	ASP7962	200	5	8

Table 1-3. Schedule of procedure – First study -

Caesarean group



Delivery group

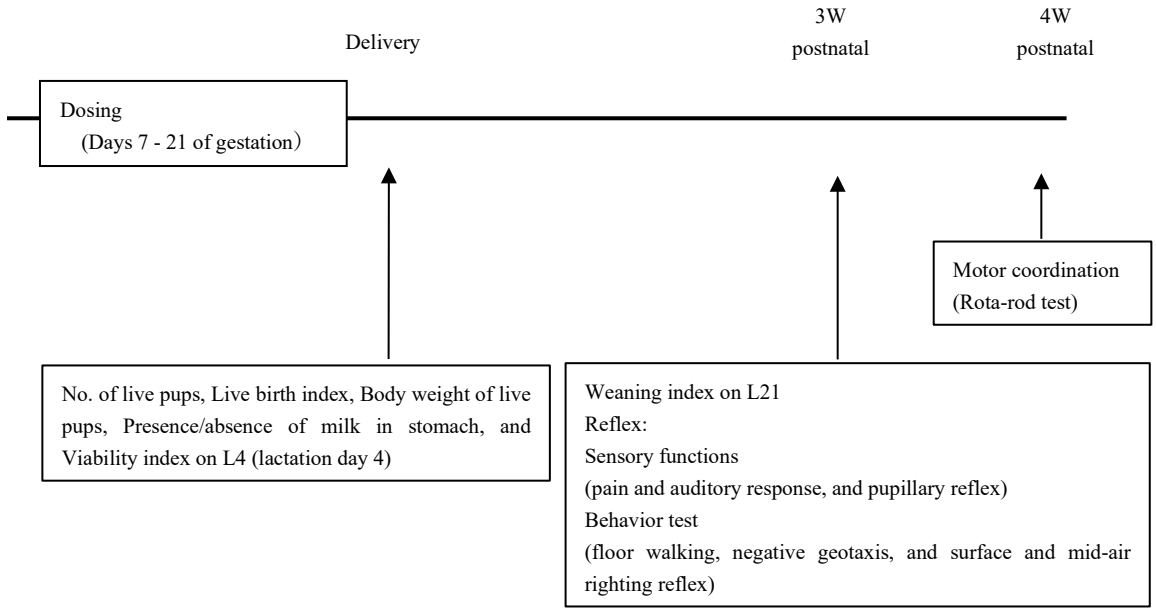
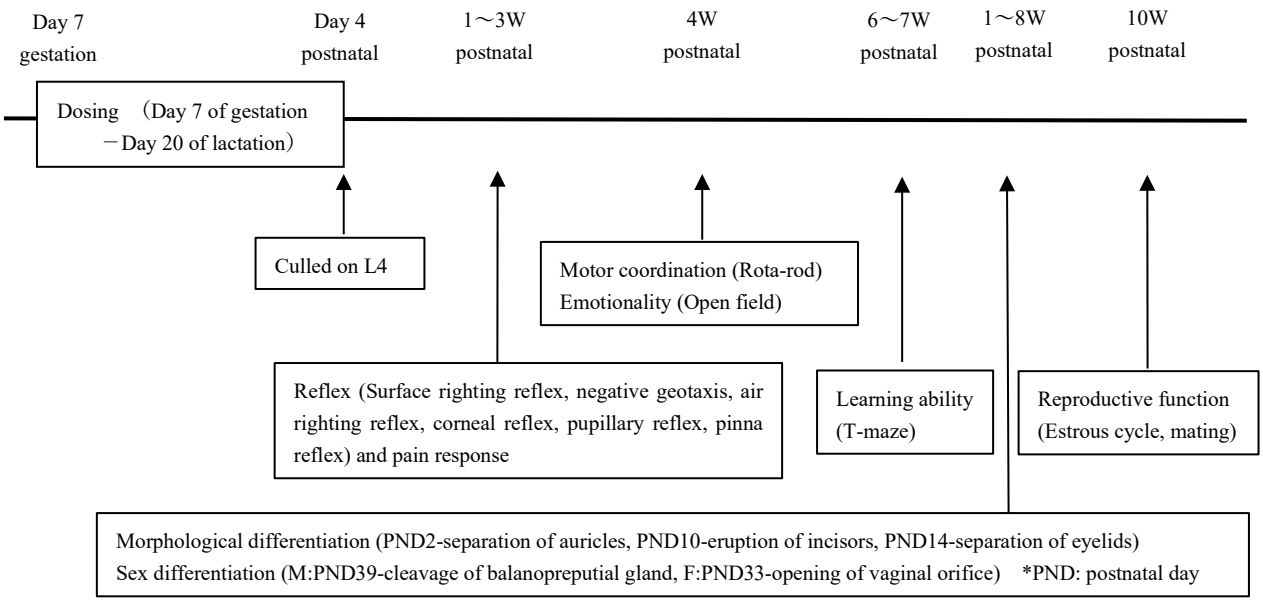


Table 1-4. Schedule of procedure - Subsequent study -



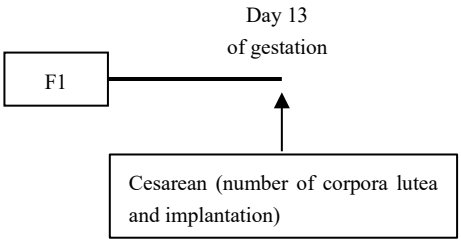


Table 1-5. Summary table of cesarean section group - First study -

Dose (mg/kg/day)		0	100	300	600
Number of litters evaluated		6	6	6	5
Fetus	No of live fetuses (mean)	85 (14.2)	87 (14.5)	74 (12.3)	61 (12.2)
	Fetal body weight				
	Male	3.77	3.77	3.63	2.89**
	Female	3.52	3.58	3.44	2.89**
	Skeletal ossification				
	Mean No. of sternebrae	5.6	5.5	5.4	3.7**
	Mean No. of caudal vertebrae	4.0	4.1	4.0	3.5

**p<0.01

Table 1-6. Summary table of delivery group - First study -

Dose (mg/kg/day)			0	100	300	600
Number of pregnant animals			6	6	6	6
Dam	Clinical observation		---	---	---	Incomplete eyelid opening, Decreased spontaneous motility, Loss of hair
	Body weight, Food consumption		---	---	FC↓	BWG↓ FC↓
	Gestation duration, Gestation index, Delivery condition		---	---	---	---
	Gross pathology		---	---	---	---
Pup (pre-weaning)	No of live pups (mean)		73 (12.2)	68 (11.3)	81 (13.5)	58 (9.7)
	Live birth index		93.6	97.4	96.4	70.7*
	Viability index on L4 (%)		100.0	98.8	79.0	0.0**
	Weaning index on L21 (%)		100.0	97.9	52.1*	NA
	Clinical observation		---	---	Trauma, Poor growth, Opacity/Ulcer of cornea	Trauma
	Milk in stomach (%(N/N))	L0	41.5 (28/73)	100.0 (68/68)	32.5 (26/81)	3.7 (2/53)
		L1	100.0 (73/73)	100.0 (68/68)	90.8 (69/77)	NA
	Body weight		---	---	BW↓	BW↓
	Reflex, Pain response		---	---	Pain response↓	NA
	Gross pathology on L21		---	---	Harderian gland small, Extraorbital lacrimal gland small	NA
Pup (post-weaning)	Motor coordination		---	---	NE	NA

L: Lactation day, ---: No noteworthy findings, BW: Body weight gain, FC: Food consumption, NA: Not applicable, NE: Not examined,
↓ : Decrease, * $p<0.05$, ** $p<0.01$

Table 1-7. Toxicokinetic parameters - First study -

Dosage	AUC_{24} (ng•hr/mL)		C_{max} (ng/mL)		t_{max} (hr)	
	GD 7	GD 20	GD 7	GD 20	GD 0	GD 20
100 mg/kg	149056.0	206006.6	13686.5	14154.5	2.0	4.0
300 mg/kg	442081.7	530691.0	26231.4	34897.2	1.0	0.5
600 mg/kg	760939.1	660173.1	45050.5	45107.7	2.0	1.0

GD: Gestation day

Table 1-8. Summary table - Subsequent study -

Dose (mg/kg/day)		0	30	100	200
Number of pregnant animals		19	19	18	19
Dam	Clinical observation, Body weight	---	---	---	---
	Food consumption	---	---	---	FC↓
	Gross pathology, Gestation duration and index, Delivery and nursing condition	---	---	---	---
Pup (pre-weaning)	No of live pups, Sex ratio, Live birth index, Milk in stomach	---	---	---	---
	Viability index on L4 (%)	93.7	96.1	97.7	86.1*
	Weaning index on L21 (%)	100.0	100.0	100.0	86.1**
	Clinical observations	---	---	---	Decreased in locomotor activity, Corneal drying and opacity, Eyeball red in color, Abdominal distention hypothermia, Moribundity
	Body weight	---	---	---	BW↓
	Morphological differentiation	---	---	---	
	Reflex, Pain response	---	---	---	Surface righting reflex, Negative geotaxis, Air righting reflex, Corneal reflex, Pinna response↓
	Gross pathology on L21	---	---	---	Eye ball: Dry cornea, Corneal opacity, Red in color, Digestive tract: Distention
Pup (post-weaning)	All examinations	---	---	---	NA[#]
F2 embryo	All examinations	---	---	---	NA[#]

L: Lactation day, ---: No noteworthy findings, BW: Body weight, FC: Food consumption, NA: Not applicable,
↓: Decrease, * $p<0.05$, ** $p<0.01$, # All F1 pups were necropsied on day21 after birth.

Table 1-9. Toxicokinetic parameters - Subsequent study -

Dosage	AUC_{24} (ng•hr/mL)		C_{max} (ng/mL)		t_{max} (hr)	
	GD 7	LD 20	GD 7	LD 20	GD 7	LD 20
30 mg/kg	47687.8	21022.5	10674.1	8334.6	1.0	0.5
100 mg/kg	189917.2	105145.7	19552.2	14967.3	1.0	0.5
200 mg/kg	309751.4	142853.2	21421.5	17041.9	0.5	0.5

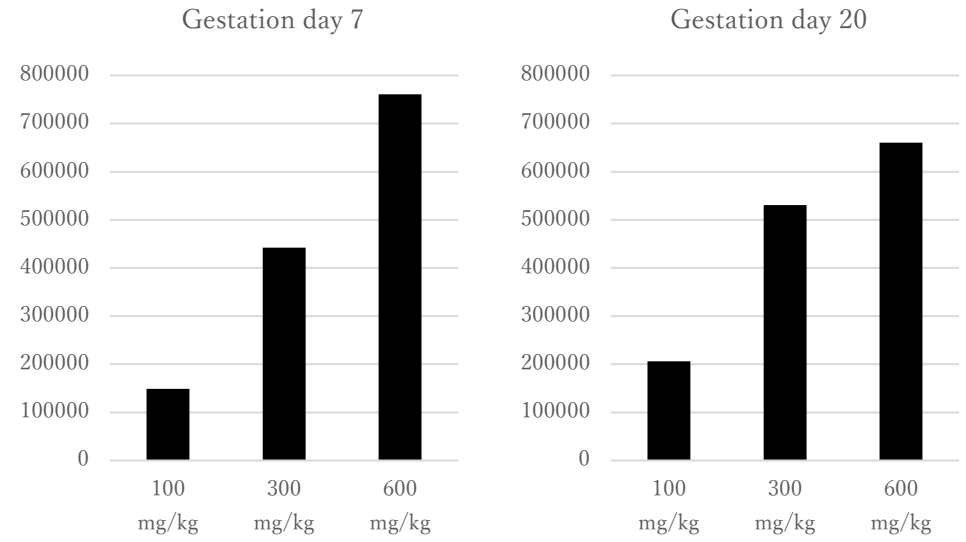
GD: Gestation day, LD: Lactation day

Table 1-10. TrkA/B/C related effects on pups after delivery

Study type		First study			Subsequent study		
Dose (mg/kg/day)		100	300	600	30	100	200
Knockout mice phenotype							
TrkA	Corneal abnormality		✓	NA#			✓
	Skin ulceration			NA#			
TrkB	Reduced milk uptake		✓	✓			
TrkC	Proprioception			NA#			
TrkA/B	Loss of pain response		✓	NA#			
TrkB/C	Decreased reflex			NA#			✓
TrkA/B/C	Reduced viability		✓	✓			✓

✓: Finding observed in first and subsequent studies, NA: Not applicable, # All pups died on lactation day 1.

(A) AUC_{24} (ng•hr/mL)



(B) C_{max} (ng/mL)

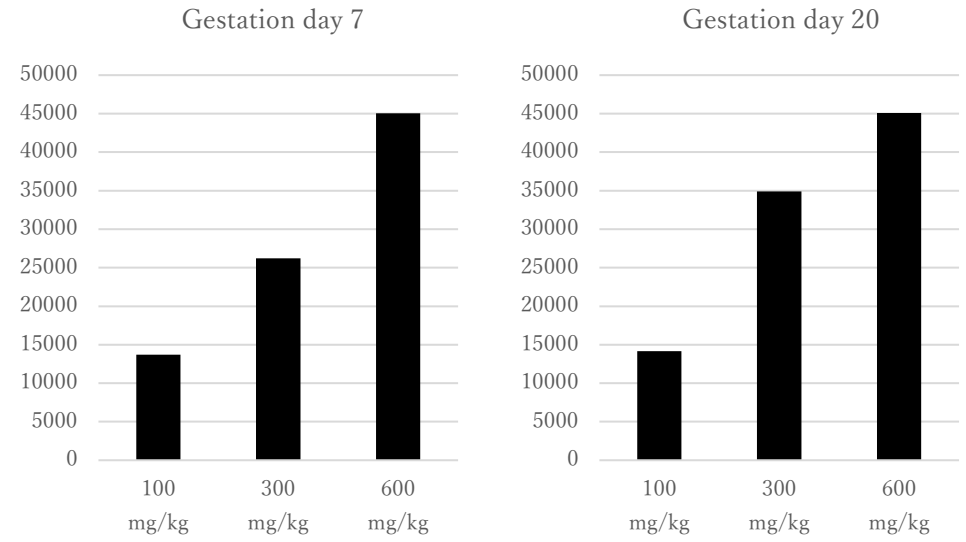
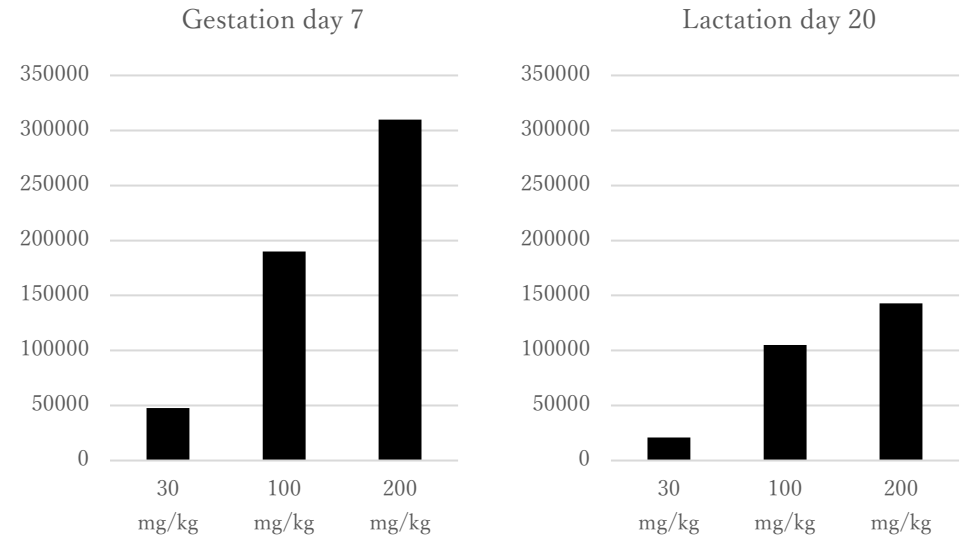


Figure 1-1. Toxicokinetic parameters - First study -
(A) AUC_{24} (B) C_{max}

(A) AUC_{24} (ng•hr/mL)



(B) C_{max} (ng/mL)

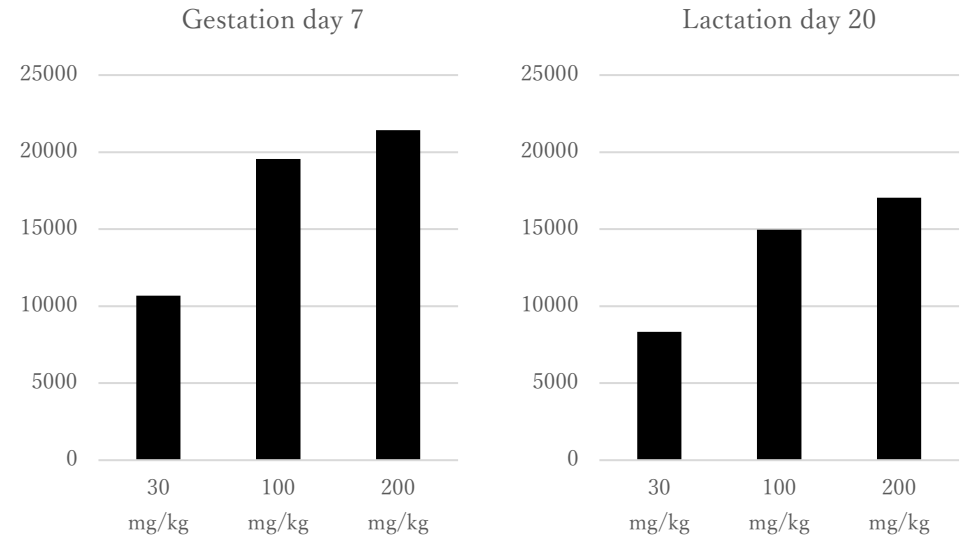


Figure 1-2. Toxicokinetic parameters - Subsequent study -
(A) AUC_{24} (B) C_{max}

Chapter 2: Neurotoxicity studies of a tropomyosin-related kinase A inhibitor, ASP7962, on the sympathetic and sensory nervous systems in rats

Introduction

NGF, BDNF, NT-3, 4 and 5 regulate the survival, development and function of neurons in both the central and peripheral nervous systems, and are activated by binding a family of three tropomyosin-related kinases (TrkA, TrkB, and TrkC), respectively (Skaper, 2012, 2018). NGF show the action in development of sympathetic and sensory neurons (Bueker, 1948). Inhibition of NGF induces the loss of these neurons during development due to cell death (Gorin & Johnson, 1979). On the other hand, once the sympathetic and sensory neurons enter into the post mitotic phase, the apoptotic pathways are highly restricted. In adult sensory and sympathetic neurons, the role of NGF-TrkA signaling is linked to regulation and sensitization of neurotransmitters, receptors, and ion channels such as nociceptors (Mantyh et al., 2011). BDNF-TrkB signaling regulates the synaptic plasticity in the brain. TrkB plays a role in hippocampal long-term potentiation and learning (Minichiello, 2009). NGF and BDNF control synaptic transmission and plasticity in neurons of superior cervical ganglion through TrkA and TrkB, respectively (Valle-Leija et al., 2017). NT-3 has functional roles in the development and maturation of sympathetic nervous system, which are involved in gangliogenesis in migratory neural crest cell via TrkC and induction of neuronal mitosis (Zhou et al., 1996). It is shown that TrkC signal transduction regulates the number of sensory neurons during dorsal root ganglia development

(Menard et al., 2018). Anti-human NGF antibodies such as tanezumab and fuluranumab showed decreased ganglion volume and neuronal size and increased glial cell density in the superior cervical ganglion, which were reversible, in cynomolgus monkeys but did not cause any neuronal death or necrosis in the sympathetic neural tissues (Belanger et al., 2017, Evans et al., 2018, Rocca et al., 2019).

ASP7962 is a small molecule which inhibits kinase activities of TrkA, TrkB and TrkC with IC_{50} values of 0.155, 1.41 and 1.09 μmol/L, respectively (Watt et al., 2019). Based on the information summarized above, the inhibition of TrkA, TrkB and TrkC signal transduction may induce impairment for neuronal survival and maintenance of neuronal function. It has not been known what effects are observed in sympathetic and sensory neurons, and their reversibility after a long-lasting inhibition of TrkA. It is important to evaluate the functional consequences which are related to the effects on sympathetic and sensory nervous systems by broad inhibition of all Trk family members. To evaluate the functional and morphological effects of ASP7962 on the sympathetic and sensory nervous systems, 4-week and 13-week repeated oral dose studies followed by recovery period were conducted in rats.

Materials and Methods

Test article

To evaluate the effects of ASP7962 on the sympathetic and sensory nervous systems, 4-week and 13-week repeated oral dose studies (with recovery periods) in rats were conducted with ASP7962, a TrkA inhibitor. ASP7962 was created at Astellas Pharma Inc. (Tokyo, Japan). The vehicle used in preparation of the test article formulations and for administration to the control group was 0.5% w/v methylcellulose solution in deionized water.

Animal and care

The in-life phase of 4-week and 13-week repeated dose studies in rats were conducted at the Charles River Laboratories (Ashland, OH) in compliance with Good Laboratory Practice. Crl:CD(SD) rats were used for these studies. The Crl:CD(SD) rats were 5–6 weeks old at receipt and were received in good health from Charles River Laboratories. PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 was provided for the diet ad libitum unless fasted for study procedures. Drinking water, delivered by an automatic watering system, was provided ad libitum. All animals were housed throughout acclimation and during the study in an environmentally controlled room. The room temperature and relative humidity controls were set to maintain environmental conditions of 19 to 26°C and 30 to 70%, respectively.

In a 4-week repeated dose study followed by an 8-week recovery period, as shown in Table 2-1, the control and 300 mg/kg/day toxicology groups (Groups 1 and 4, respectively) each consisted of 16 males and 16 females, and the 30 and

100 mg/kg/day groups (Groups 2 and 3, respectively) each consisted of 8 males and 8 females. For the toxicokinetic analysis, the control group (Group 1) consisted of 3 males and 3 females, and the 30, 100, and 300 mg/kg/day groups (Groups 2, 3, and 4, respectively) each consisted of 9 males and 9 females. At the initiation of dose administration, animals were approximately 9 weeks old.

In a 13-week repeated dose study followed by a 13-week recovery period, as shown in Table 2-2, the control and 300 mg/kg/day toxicology groups (Groups 1 and 4, respectively) each consisted of 16 males and 16 females, and the 3 and 30 mg/kg/day groups (Groups 2 and 3, respectively) each consisted of 8 males and 8 females. For the toxicokinetic groups, the control group consisted of 3 males and 3 females (Group 1), and the 3, 30, and 300 mg/kg/day groups (Groups 2, 3, and 4, respectively) each consisted of 9 males and 9 females. The animals were approximately 8–9 weeks old at the initiation of dose administration.

ASP7962 administration and dosage levels

The vehicle and test article formulations were administered orally by gavage once daily for 4 weeks via an appropriately sized, flexible, Teflon-shafted, stainless steel ball-tipped dosing cannula (Natsume, Tokyo, Japan), or once daily for 13 weeks using an approximately sized flexible plastic ball-tipped dosing cannula (Instech Solomon, Plymouth Meeting, PA, USA). Dosing was continued through the day prior to the primary necropsy (toxicology groups) or scheduled euthanasia (toxicokinetic groups). The dose volume for all groups was 5 mL/kg. The first day of dosing was designated as study day 0; the first week of dosing as study week 0.

In the 4-week repeated dose study, the highest dosage level was set at 300 mg/kg/day and lower dosage levels were set at 100 and 30 mg/kg/day. In the 13-week repeated dose study, the highest dosage level was set at 300 mg/kg/day. Lower dosage levels were set at 30 and 3 mg/kg/day.

Clinical observations, body weights, food consumptions and clinical pathology

Clinical observations were performed at the time of dose administration and at 1–2 hours and approximately 4 hours following dose administration. During the recovery period, the animals were observed once daily. Body weights were recorded weekly and on the day prior to each day of the scheduled necropsies. Food consumption was recorded weekly throughout the study period. Blood samples for clinical pathology evaluations (hematology and serum chemistry) were collected from all animals assigned to the scheduled necropsies (Weeks 4 and 12 in the 4-week repeated dose study, and Weeks 13 and 26 in the 13-week repeated dose study). The animals were fasted overnight prior to blood collection. The schedule of procedures during administration and recovery period is shown in Table 2-3.

FOB (Functional observational battery)

FOB assessments were recorded at Week 2/3 in the 4-week repeated dose study, and Weeks 6 and 12 in the 13-week repeated dose study, as well as at Week 25 during the recovery period in the 13-week repeated dose study. Assessments were conducted approximately 2 hours after dose administration. Animals were observed for home cage observations, handling observations, open field

observations, sensory observations, neuromuscular observations and physiological observations, as shown in Table 2-4.

Motor activity

Motor activity was assessed near the end of the dosing period (Week 2/3 in the 4-week repeated dose study, and Weeks 6 and 12 in the 13-week repeated dose study), and during the last week of the recovery period (Week 10/11 in the 4-week repeated dose study, and Week 25 [recovery period] in the 13-week repeated dose study). Motor activity, after the completion of the FOB assessment, was conducted using a personal computer-controlled system that utilized a series of infrared photobeams surrounding an amber, plastic rectangular cage to quantify each animal's motor activity. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Pathology

Animals at the scheduled necropsies were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital and perfused in situ using 4% paraformaldehyde fixative. Animals were fasted overnight prior to the scheduled necropsies. After completion of perfusion, organs and tissues for microscopic examination and stereological analysis were collected. Brain weight and size were recorded. Microscopic examination was performed on all tissues listed in Table 2-5 in the control and 300 mg/kg/day groups at the primary necropsy. The superior (cranial) cervical, cranial mesenteric, and cervicothoracic ganglia were

also examined from all animals in the 30 and 100 mg/kg/day groups in the 4-week repeated dose study and in the 3 and 30 mg/kg/day groups in the 13-week repeated dose study at the primary necropsies and all animals at the recovery necropsies.

Stereology

Stereological analysis was performed on superior (cranial) cervical ganglion (unilateral) of all animals in all dose groups at the primary and recovery necropsies and L5 dorsal root ganglion (unilateral) from all animals in the control and 300 mg/kg/day groups at the primary necropsy. Left and right ganglia were kept separated and appropriately labeled; however, only 1 side was used for stereology. The other side was taken to the blocking step and retained as a back-up. Animals from all groups were randomized and assigned random numbers to determine animal order. The random numbers were used for all animals throughout the process. A coin toss was used to determine whether to use the left or right side for the first animal. The side was then alternated for all subsequent animals (i.e., right, left, right, left, etc) throughout the study.

Ganglia were removed from fixative, blotted, and weighed. The ganglia were dehydrated and processed in glycol methacrylate (GMA) until the blocking step. After final infiltration with GMA, the ganglia were removed from the bath, blotted of excess resin and weighed. The largest diameter perpendicular to the long axis was measured with a Vernier caliper and recorded to the nearest 0.1 mm. The ganglia were rotated randomly along the long axis within semi-hard GMA and then processing resumed. The histology microtome was calibrated to obtain

30 μm sections (optical disectors) and the ganglia were exhaustively sectioned using systematic uniform random sampling, with a goal of obtaining 8 to 10 optical disectors per animal. Two backup disectors were taken at each sampling interval. In addition, a single 3-5 μm section (as well as backups) from the superior (cranial) cervical ganglion was taken from the center of the ganglion for routine H&E staining. Disectors were stained with cresyl violet acetate to outline the Nissl substance within neurons. The total ganglion volume was estimated by the Cavalieri method using a point probe (Gundersen & Jensen, 1987). Neurons were counted using fractionator sampling and an unbiased counting frame and neuron size was estimated using a rotator probe. For the L5 dorsal root ganglia, A and B neurons were counted separately (Tandrup, 1993).

Statistical analysis

Body weight, body weight change, food consumption, continuous FOB, clinical pathology, and brain weight and size data were subjected to a parametric one-way analysis of variance (ANOVA) to determine intergroup differences. If the ANOVA revealed statistically significant ($p<0.05$) intergroup variance, Dunnett's test was used to compare the test article-treated groups to the control group. FOB parameters that yielded scalar or descriptive data were analyzed using Fisher's Exact Test. Stereological data (estimated total number of neurons, neuron size, and total ganglion volume) were subjected to a parametric one-way ANOVA to determine intergroup differences between the control and test article-treated groups. If the ANOVA revealed significant ($p<0.05$) intergroup variance, a two-sample t-test was used to compare the test article-treated group and the control

group for the dorsal root ganglion analysis and recovery superior (cranial) cervical ganglion analysis, and Dunnett's test was used to compare each test article-treated group to the control group for the primary superior (cranial) cervical ganglion analysis since stereological data of superior (cranial) cervical ganglion were obtained at three test article-related groups because of target tissue. For motor activity, each analysis endpoint (total and ambulatory activity counts) was analyzed, by sex and session, with a repeated-measure analysis of variance.

Toxicokinetics

Blood samples for toxicokinetics were collected on Days 0 and 27 in the 4-week repeated dose study and on Days 0, 48, and 90 in the 13-week repeated dose study from 3 animals/sex in Group 1 at approximately 0.5 hours following dose administration, and from 3 animals/sex/group/time point in Groups 2-4 prior to dose administration (Day 27 only) and at approximately 0.5, 1, 2, 4, 8, and 24 hours after dose administration.

Results

Clinical observations, body weights, food consumption, clinical pathology, FOB and motor activity

✓ ***4-week repeated dose study***

Test article related effects are summarized in Table 2-6.

No test article-related mortality was observed in this study. There were no test article-related effects on body weights, food consumption, FOB, hematology or serum chemistry parameters.

Lower overall total and ambulatory motor activity counts were noted in the 100 and 300 mg/kg/day group males and females near the end of the dosing period (Week 3). These findings were attributed to a reduction in initial exploratory activity when the animals were put in a cage. Total and ambulatory motor activity counts in the 300 mg/kg/day group males and females were similar to those in the concurrent control group values at the last week of the recovery period (Week 11). There were no abnormalities in motor activity counts in the 30 mg/kg/day group.

✓ ***13-week repeated dose study***

Test article related effects are summarized in Table 2-7.

No test article-related mortality was observed in this study. There were no test article-related changes in the 3 or 30 mg/kg/day groups in any test items. At 300 mg/kg/day, there were no test article-related effects on food consumption, home cage observations, sensory observations, neuromuscular observations, or hematology parameters. No test article related changes were noted on autonomic function including sympathetic and parasympathetic systems which were examined

in palpebral closure, lacrimation, piloerection, salivation, respiratory rate, respiratory character, urination and defecation at any dose levels. No test article related changes were also noted on sensory function which were examined in approach response, startle response, pupil response, forelimb extension, air righting reflex, touch response, tail pinch, eyeblink response, hindlimb extension and olfactory orientation in any dose levels.

At 300 mg/kg/day, there were test article-related effects in clinical observations, body weights, handling observations, open field observations, physiological observations, motor activity and serum chemistry (phosphorus).

Increased incidence of red material around the eyes, nose, and/or mouth was observed in the 300 mg/kg/day group males and females. This correlated with an increased incidence of red deposits around the nose or mouth in the 300 mg/kg/day group males and/or females during the FOB assessments at Weeks 6 and 12.

Lower body weights and lower body weight gains were noted in the 300 mg/kg/day group males and females. During the dosing period, lower cumulative body weight gains were noted beginning at Week 4 and continued through Week 12. These lower body weights were also noted during the physiological observations of the FOB assessment at each interval. Lower body weights and body weight gains continued in the 300 mg/kg/day group males and females during the recovery period.

Increased phosphorus values were noted in the 300 mg/kg/day group males and females at Week 13.

Lower overall total motor activity counts were noted in the 300 mg/kg/day group males at Week 6 and lower overall total and ambulatory motor activity counts

were noted in the 300 mg/kg/day group females at Weeks 6 and 12. The lower motor activity counts also correlated to a lower occurrence of rearing in the 300 mg/kg/day group females during the FOB assessments at Weeks 6 and 12. These changes were not clear by the end of the recovery period (Week 25).

Pathology

✓ ***4-week repeated dose study***

Microscopic findings consisted of decreased size/atrophy in neurons and diffuse increased density of glial cells in the cervicothoracic ganglia, cranial mesenteric ganglia, and/or the superior (cranial) cervical ganglia (all sympathetic ganglia) at 300 mg/kg/day group males and in the 30, 100, and 300 mg/kg/day group females (Table 2-8) (Figure 2-1). Representative microscopic images of decreased neuron size and increased glial cell density in the superior (cranial) cervical ganglia at the primary necropsy from the 4-week repeated dose study are shown in Figure 2-2. In the 300 mg/kg/day group males and the 100 and 300 mg/kg/day group females, decreased size/atrophy of neurons in the superior (cranial) cervical ganglion directly correlated to a statistically significant decrease in average neuron size noted in the stereology evaluation. All test article-related morphologic changes in the 300 mg/kg/day males and females resolved after the recovery period. There were no changes in brain weights or measurements and microscopic changes in the brain, spinal cord, spinal nerve roots/ganglia, trigeminal ganglia, peripheral nerves including the vagus nerve, or parasympathetic ganglia in the 300 mg/kg/day group males and females.

✓ ***13-week repeated dose study***

Microscopic findings consisted of neuronal atrophy and increased density of glial cells in the cervicothoracic ganglia, cranial mesenteric ganglia, and superior (cranial) cervical ganglia (all sympathetic ganglia) in the 300 mg/kg/day group males and females at the primary necropsy (Table 2-9) (Figure 2-3). There were no test article-related changes in the ganglia from the 300 mg/kg/day group males or females at the recovery necropsy, which indicated resolution of the neuronal atrophy and increased density of glial cells during the recovery period. There were no changes in brain weights or measurements and microscopic changes in the brain, spinal cord, spinal nerve roots/dorsal root ganglia, trigeminal ganglia, peripheral nerves including the vagus nerve, or parasympathetic ganglia in the 300 mg/kg/day group males and females.

Stereology

✓ ***4-week repeated dose study***

Decreased superior (cranial) cervical ganglion neuron size, with secondary effects on total superior (cranial) cervical ganglion volume in the 30, 100, and 300 mg/kg/day group males and in the 100 and 300 mg/kg/day females were observed (Table 2-10) (Figure 2-4). These changes were recovered after the 8-week recovery period (Table 2-11) (Figure 2-5). On the other hand, there was no changes in ganglion volume, neuron number, or neuron size of the dorsal root ganglion at 300 mg/kg/day.

✓ ***13-week repeated dose study***

In stereological analysis, decreased neuron size in the superior (cranial) cervical ganglion was noted in the 300 mg/kg/day group females at the primary necropsy (Table 2-12) (Figure 2-6), which was consistent with neuronal atrophy at microscopic examination (Table 2-9). These changes were recovered after the 13-week recovery period (Table 2-13) (Figure 2-7). Lower neuron numbers were also observed in the 300 mg/kg/day group females at the primary necropsy with partial recovery noted at the recovery necropsy (Table 2-13) (Figure 2-7). This partial recovery of neuron numbers at the recovery necropsy indicated that the decreased counts at the primary necropsy were due to undercounting of atrophic neurons, not to actual neuron loss because there was no evidence of neuronal death in any sympathetic ganglia. An actual loss of neurons would not be expected to even partially resolve. This interpretation is supported by published reports of similar changes in studies of anti-human NGF antibodies (Belanger et al., 2017, Rocca et al. 2019). Lower total ganglion volume was observed in the 300 mg/kg/day group males at the recovery necropsy (Table 2-13) (Figure 2-7). This change was not observed at the primary necropsy, and not accompanied with any of the changes in neuron number or neuron size. Therefore, this change was not considered to be related to test article administration. There were no changes in total volume, neuron number, or neuron size in the dorsal root ganglion.

Toxicokinetics

✓ ***4-week repeated dose study***

As shown in Table 2-14 and Figure 2-8, t_{max} ranged from 0.5 to 4.0 hours on study Day 0 and 0.5 to 1.0 hours on study Day 27, showing no remarkable

difference between the single and repeated doses. On study Day 0, while C_{max} increased dose-dependently from 30 to 300 mg/kg, AUC_{24} increased more than dose proportionally from 30 to 100 mg/kg. AUC_{24} increased dose-dependently from 100 to 300 mg/kg. Values of C_{max} and AUC_{24} in females were higher than those in males, particularly at 30 mg/kg. C_{max} and AUC_{24} were comparable between study Days 0 and 27 in both sexes.

✓ ***13-week repeated dose study***

As shown in Table 2-15 and Figure 2-9, t_{max} ranged from 0.5 to 2.0 hours in all cases, with no remarkable difference between single and repeated doses. C_{max} increased almost dose-proportionally between 3 and 30 mg/kg and less than dose proportionally between 30 and 300 mg/kg in all cases. AUC_{24} increased more than dose-proportionally between 3 and 30 mg/kg/day. Those values increased slightly more than dose-proportionally in males and almost dose-proportionally in females between 30 and 300 mg/kg. Values of C_{max} and AUC_{24} in females tended to be higher than those in males. C_{max} and AUC_{24} were comparable among Days 0, 48, and 90 in both sexes.

Discussion

The primary effect in these studies was neuronal atrophy, which was observed in histopathology and stereology. Atrophic changes included decreased size/atrophy in neurons and diffusely increased density of glial cells in the cervicothoracic ganglia, cranial mesenteric ganglia, and/or the superior (cranial) cervical ganglia (all sympathetic ganglia). Atrophy of sympathetic ganglion neurons was reported to occur in association with anti-nerve growth factor states in rodents (Angeletti et al., 1971, Ruit et al., 1990). The observed increased glial cell density in the conducted studies was considered as a secondary effect, as the glial cells were generally more tightly packed due to the reduction in neuron size. These morphologic changes were reversible after the recovery period and were not associated with clinical signs or detectable dysfunction of the autonomic nervous system based on normal FOB parameters.

Lower neuron numbers were observed in the 300 mg/kg/day group females at primary necropsy after 13-week administration, and partial recovery was noted after the 13-week recovery period. The persistent decrease of neuron counts in the 300 mg/kg/day group females was considered to be due to under recognition of atrophic neurons, and not actual loss of neurons, because of the lack of any morphologic observation of neuronal death (or reaction to prior neuronal death) in the sympathetic ganglia. Neurons in ganglia are well-known to not regenerate, therefore, a partial recovery of the neuron counts supports this interpretation. Neuronal atrophy, which was similar to the results in studies using other anti-NGF antibodies, was considered non-adverse, because it was not related to associated

with any clinical signs or neuronal death, and the change was recovered after the recovery period (Belanger et al., 2017; Rocca et al., 2019).

Although decreased neuron size was noted at 30 mg/kg/day or more in males and 100 mg/kg/day or more in females in the 4-week repeated dose study, it was only noted at the 300 mg/kg/day in females in the 13-week repeated dose study despite the prolonged and higher plasma drug exposure levels. Based on these results, it was considered that neuronal atrophy did not progress time-dependently after reaching a plateau. The sex difference in decreased neuron size (not in males but in females at 300 mg/kg/day) after 13-week treatment was considered to be due to the higher plasma drug exposure level in females when compared with males throughout the administration period.

Lower body weights and lower body weight gains were observed at 300 mg/kg/day in the 13-week repeated dose study. These findings were considered to be toxicologically insignificant because they were almost within the historical control reference range. Increased phosphorous values in blood chemistry were observed at 300 mg/kg/day in the 13-week repeated dose study This was also considered to be toxicologically insignificant because the differences were slight and within the historical control reference range and there were no other correlating changes in serum chemistry parameters such as calcium, alkaline phosphatase, or other electrolytes.

In motor activity assessment, lower overall total and ambulatory motor activity counts were noted in the 100 and 300 mg/kg/day group at Week 3 in the 4-week repeated dose study, and also in the 300 mg/kg/day group at Weeks 6 and/or 12 in the 13-week study. These changes were not apparent by the end of

recovery period, therefore, were considered to be reversible. The lower motor activity counts were attributed to a reduction in initial exploration when animals were put in a cage at the start of the examination. Neuronal atrophy was observed in the sympathetic ganglia at the same dose levels in which lower motor activity were observed. However, motor activity assessments of these studies are not known to detect changes related to motor neurons, which direct autonomic actions in the sympathetic ganglia. Therefore, it was considered that the decreased motor activity was unrelated to neuronal atrophy in the sympathetic ganglia. The lower motor activity did not seem to be related to the neuronal atrophy by TrkA inhibition. However, the underlying cause of the initial decreased exploratory activity remains to be determined.

It was shown that ASP7962 inhibits TrkB/C receptors as well as TrkA receptor as the small molecule inhibitor of kinase activities for Trk family. TrkB/C signaling regulates the survival and maintenance of the central and peripheral nervous systems (Ménard et al. 2018; Minichiello 2009). However, there were no microscopic findings in any tissues or functional deficits in these nervous systems except neuronal atrophy in the sympathetic ganglia in 4-week and 13-week repeated dose studies. The reason of no abnormality in central nervous system is considered to be based on the low penetration of the drug into the brain.

Given the results of the 4-week and 13-week repeated dose studies, ASP7962 had no progressive or irreversible neuronal atrophy and did not induce neuronal cell death in the sympathetic ganglia in rats.

Conclusion

Stereological analysis revealed neuronal atrophy such as decreased ganglion volume and decreased neuron size in superior (cranial) cervical ganglion at ≥30 mg/kg/day in 4-week and at 300 mg/kg/day in 13-week repeated dose studies. The neuronal atrophy in the sympathetic ganglia including superior (cranial) cervical ganglion was also noted in pathology; however, no cell deaths were observed in any neuronal tissues. The reversibility of the neuronal atrophy was confirmed in sympathetic ganglia. ASP7962 induced reversible neuronal atrophy in the sympathetic ganglia which was not accompanied with cell deaths.

Table 2-1. Study group assignment - 4-week repeated dose study -

Group Number	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Animals Toxicology Groups [a]		Number of Animals Toxicokinetic Groups [b]	
				Males	Females	Males	Females
1	Vehicle	0	5	16	16	3	3
2	ASP7962	30	5	8	8	9	9
3	ASP7962	100	5	8	8	9	9
4	ASP7962	300	5	16	16	9	9

[a] Eight animals/sex/group were euthanized following 4 weeks of dose administration; the remaining eight animals/sex/group in Groups 1 and 4 were euthanized following an 8-week nondosing (recovery) period.
[b] All animals were euthanized and discarded following the final blood collection.

Table 2-2. Study group assignment - 13-week repeated dose study -

Group Number	Treatment	Dosage Level (mg/kg/day)	Dose Volume (mL/kg)	Number of Animals Toxicology Groups [a]		Number of Animals Toxicokinetic Groups [b]	
				Males	Females	Males	Females
1	Vehicle	0	5	16	16	3	3
2	ASP7962	3	5	8	8	9	9
3	ASP7962	30	5	8	8	9	9
4	ASP7962	300	5	16	16	9	9

[a] Eight animals/sex/group were euthanized following 13 weeks of dose administration; the remaining ≤8 animals/sex/group in Groups 1 and 4 were euthanized following a 13-week nondosing (recovery) period.
[b] All animals were euthanized and discarded following the final blood collection.

Table 2-3. Schedule of procedure during administration and recovery period

Procedure	Frequency	
	4-week repeated dose study	13-week repeated dose study
Clinical observations	3 times per day during dosing period, 1 time per day during recovery period	
Body weights	Weekly and on the day prior to each day of the scheduled necropsies	
Food consumptions	Weekly	
Clinical pathology	Week 4 during dosing period, and Week 12 during recovery period	Week 13 during dosing period, and Week 26 during recovery period
Functional observational battery (FOB)	Week 2/3 during dosing period	Weeks 6 and 12 during dosing period, and Week 25 during recovery period
Motor activity	Week 2/3 during dosing period, and Week 10/11 during recovery period	Weeks 6 and 12 during dosing period, and Week 25 during recovery period
Toxicokinetics	Days 0 and 27	Days 0, 48 and 90

Table 2-4. FOB parameters

Procedure	Parameter
Home cage observations	Posture, convulsions, tremors, feces consistency, biting and palpebral closure
Handling observations	Ease of removal from cage, lacrimation, chromodacryorrhea, piloerection, palpebral closure, red deposits, crusty deposits, eye prominence, ease of handling animal in hand, salivation, fur appearance, respiratory rate, respiratory character, mucous membranes/eye/skin color and muscle tone
Open field observations	Mobility, rearing, convulsions, tremors, grooming, bizarre/stereotypic behavior, time to first step, gait, arousal, urination, defecation, gait score and backing
Sensory observations	Approach response, startle response, pupil response, forelimb extension, air righting reflex, touch response, tail pinch response, eyeblink response, hindlimb extension and olfactory orientation
Neuromuscular observations	Hindlimb extensor strength, hindlimb foot splay, grip strength and rotarod performance
Physiological observations	Catalepsy, body temperature and body weight

Table 2-5. Processing and staining for tissues

Tissues	Processing and Staining
Sympathetic nervous system	
Cervicothoracic ganglia (right and left)	Left: Paraffin/H&E; at least four levels (longitudinal to maximize the quantity of ganglion to be examined) through the ganglia (levels to be at least 40 microns apart); Right: processed if suitable sections not available from the left
Cranial mesenteric ganglion	Paraffin/H&E; at least four levels through the ganglia (levels to be at least 40 microns apart)
Superior (cranial) cervical ganglia	GMA/stereology, one single section for H&E
Spinal cord	Paraffin/H&E, Luxol Fast Blue, Bielschowsky's silver stains; three levels (caudal cervical, mid thoracic, caudal lumbar); oblique and transverse at each level
Parasympathetic nervous system	
Parasympathetic ganglia	Paraffin/H&E, duodenum, jejunum, ileum, cecum, colon, rectum
Sensory system	
Dorsal root ganglia with spinal nerve roots	Paraffin/H&E from 3 levels; GMA/stereology from L5 (dorsal root ganglia)
Trigeminal ganglion/nerve	Paraffin/H&E; Longitudinal section to include ganglia and trigeminal nerve branches
Peripheral nerve	
Peripheral nerves (sciatic, tibial, sural and vagus nerves)	Paraffin/H&E stained longitudinal section; Osmicated/resin embedded/toluidine blue stained cross section
Eyes with optic nerves	Paraffin/H&E
Central nervous system	
Brain (at least seven full coronal sections)	Paraffin/H&E, Luxol Fast Blue, Bielschowsky's silver stains

Table 2-6. Clinical observation, body weights, food consumption, clinical pathology, FOB and motor activity - 4-week repeated dose study -

Dosing period

Dose (mg/kg/day)		Dosing period							
		Males				Females			
		0	30	100	300	0	30	100	300
Number of animals		16	8	8	16	16	8	8	16
Clinical observation		---	---	---	---	---	---	---	---
Body weights		---	---	---	---	---	---	---	---
Food consumption		---	---	---	---	---	---	---	---
Clinical pathology		---	---	---	---	---	---	---	---
FOB		---	---	---	---	---	---	---	---
Motor Activity									
Mean total counts	Pretest	2082	1810	1874	2152	2588	2360	2131	2215
	Week 3	2287	1956	1755*	1712*	2691	2456	1919*	1901*
Mean ambulatory counts	Pretest	489	471	442	526	727	660	610	626
	Week 3	420	391	319	328*	646	656	472	459*

---: no noteworthy findings, *: $p<0.05$

Recovery period

Dose (mg/kg/day)	Recovery period			
	Males		Females	
	0	300	0	300
Number of animals	8	8	8	8
Clinical observation	---	---	---	---
Body weights	---	---	---	---
Food consumption	---	---	---	---
Clinical pathology	---	---	---	---
FOB	---	---	---	---
Motor Activity	---	---	---	---

---: no noteworthy findings

Table 2-7. Clinical observation, body weights, food consumption, clinical pathology, FOB and motor activity - 13-week repeated dose study –

Dosing period

Dose (mg/kg/day)		Dosing period							
		Males				Females			
		0	3	30	300	0	3	30	300
Number of animals		16	8	8	16	16	8	8	16
Clinical observation									
Increased incidence of red material around the eyes/nose/mouth		---	---	---	P	---	---	---	P
Body weights									
Predose (Day 0)		306	314	310	301	218	221	220	211
Week 13		621	623	608	574	323	334	343	310
Body weight gain	Week 0-13	316	310	299	273	106	113	123	100
Food consumption		---	---	---	---	---	---	---	---
Clinical pathology									
Phosphorus (mg/dL)		6.5	6.7	6.8	7.2**	5.4	5.4	5.4	6.1*
FOB									
Red deposits - nose (number of animals with observation during handling observations)									
Week 6 - present		3	0	0	2	0	0	1	5*
Week 12 - present		2	1	0	4	0	0	0	1
Red deposits - nose (number of animals with observation during handling observations)									
Week 6 - present		0	0	0	0	0	0	0	9*
Week 12 - present		0	0	0	5*	0	0	0	0
Rearing (mean number of animals with observation)									
Week 6		5.2	2.8	3.6	3.4	8.7	8.3	8.3	4.6**
Week 12		4.1	4.6	3.5	3.4	11.9	10.4	11.1	8.3*
Motor Activity									
Mean total counts	Pretest	1911	1788	1871	2091	2147	1953	2329	2208
	Week 6	2270	2126	2673	1714	2548	2647	2309	1620**
	Week 12	2275	1760	2430	1782	2454	1878	2291	1234**
Mean ambulatory counts	Pretest	465	458	494	527	625	617	700	677
	Week 6	369	375	452	341	593	591	524	412**
	Week 12	314	250	403	301	521	403	454	272**

---: no noteworthy findings, P: present (test article-related findings), *: $p<0.05$, **: $p<0.01$

Table 2-7. Clinical observation, body weights, food consumption, clinical pathology, FOB and motor activity - 13-week repeated dose study – (Continued)

Recovery period

Dose (mg/kg/day)		Recovery period			
		Males		Females	
		0	300	0	300
Number of animals		8	8	8	8
Clinical observation		---	---	---	---
Body weights					
Week 14		641	558**	343	311
Week 26		736	638*	389	337*
Body weight gain	Week 13-26	108	83	54	29**
Food consumption		---	---	---	---
Clinical pathology		---	---	---	---
FOB		---	---	---	---
Motor Activity		---	---	---	---

---: no noteworthy findings, *: $p<0.05$, **: $p<0.01$

Table 2-8. Microscopic findings in sympathetic ganglia - 4-week repeated dose study -

Tissue / Site / Diagnosis	Primary Necropsy								Recovery Necropsy			
	Males[1] (mg/kg/day)				Females[1] (mg/kg/day)				Males[1] (mg/kg/day)		Females[1] (mg/kg/day)	
	0	30	100	300	0	30	100	300	0	300	0	300
Cervicothoracic Ganglion												
Glial Cell / Increased Density	0/8	0/8	0/8	7/8	0/8	0/8	1/8	7/8	0/8	0/8	0/8	0/8
Neuron / Atrophy	0/8	0/8	0/8	7/8	0/8	0/8	0/8	7/8	0/8	0/8	0/8	0/8
Cranial Mesenteric Ganglion												
Glial Cell / Increased Density	0/8	0/8	0/8	2/8	0/8	0/8	3/8	7/8	0/8	0/8	0/8	0/8
Neuron / Atrophy	0/8	0/8	0/8	0/8	0/8	0/8	3/8	6/8	0/8	0/8	0/8	0/8
Superior (Cranial) Cervical Ganglion												
Glial Cell / Increased Density	0/8	0/8	0/8	8/8	0/8	2/8	3/8	8/8	0/8	0/8	1/8	0/8
Neuron / Atrophy	0/8	0/8	0/8	8/8	0/8	2/8	3/8	6/8	0/8	0/8	1/8	0/8

[1] The values listed indicate the incidence of findings out of the total number of animals in that dose group.

Table 2-9. Microscopic findings in sympathetic ganglia - 13-week repeated dose study -

Tissue Site / Diagnosis	Primary Necropsy								Recovery Necropsy			
	Males[1] (mg/kg/day)				Females[1] (mg/kg/day)				Males[1] (mg/kg/day)		Females[1] (mg/kg/day)	
	0	3	30	300	0	3	30	300	0	300	0	300
Cervicothoracic Ganglion												
Glial Cell / Increased Density	0/8	0/8	0/8	7/8	0/8	0/8	0/8	8/8	0/8	0/8	0/7	0/8
Neuron / Atrophy	0/8	0/8	0/8	7/8	0/8	0/8	1/8	8/8	0/8	0/8	0/7	0/8
Cranial Mesenteric Ganglion												
Glial Cell / Increased Density	0/7	0/8	0/8	6/8	0/7	0/8	0/8	5/7	0/8	0/8	0/7	0/8
Neuron / Atrophy	0/7	0/8	0/8	6/8	0/7	0/8	0/8	7/7	0/8	0/8	0/7	0/8
Superior (Cranial) Cervical Ganglion												
Glial Cell / Increased Density	0/8	0/8	0/8	6/8	1/8	0/8	0/8	8/8	0/8	0/8	0/7	0/8
Neuron / Atrophy	0/8	0/8	0/8	5/8	0/8	0/8	0/8	8/8	0/8	0/8	0/7	0/8

[1] The values listed indicate the incidence of findings out of the total number of animals in that dose group.

Table 2-10. Stereology data of superior (cranial) cervical ganglion at primary necropsy - 4-week repeated dose study –

Dose (mg/kg/day)	Males				Females			
	0	30	100	300	0	30	100	300
Total Volume (mm^3)	1.19	0.83**	0.80**	0.88**	0.93	0.80	0.78	0.71
STD	0.31	0.16	0.10	0.24	0.24	0.22	0.19	0.12
CE	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
CV	0.26	0.19	0.13	0.27	0.26	0.28	0.25	0.17
Neuron Number	26945	23573	20836	24824	22606	24950	22743	16935
STD	8518	8729	5326	4305	8350	4938	5983	4859
CE	0.08	0.10	0.09	0.08	0.10	0.09	0.09	0.10
CV	0.32	0.37	0.26	0.17	0.37	0.20	0.26	0.29
Neuron Size (μm^3)	9706	6925**	6400**	5894**	8959	6480	5007**	4359**
STD	2099	556	1677	1602	3698	1490	1273	1124
CV	0.22	0.08	0.26	0.27	0.41	0.23	0.25	0.26

** Significantly different from the control group at $p<0.01$.
STD: Standard deviation
CE: Coefficient of error
CV: Coefficient of variation

Table 2-11. Stereology data of superior (cranial) cervical ganglion at recovery necropsy - 4-week repeated dose study -

Dose (mg/kg/day)	Males		Females	
	0	300	0	300
Total Volume (mm^3)	0.91	0.92	0.82	0.84
STD	0.21	0.23	0.25	0.21
CE	0.01	0.02	0.02	0.02
CV	0.23	0.25	0.31	0.25
Neuron Number	18084	20594	19204	19618
STD	2952	3320	1588	4289
CE	0.10	0.09	0.09	0.09
CV	0.16	0.16	0.08	0.22
Neuron Size (μm^3)	8352	7969	6585	6336
STD	2860	2418	1609	1167
CV	0.34	0.30	0.24	0.18

-: Not applicable
STD: Standard deviation
CE: Coefficient of error
CV: Coefficient of variation

Table 2-12. Stereology data of superior (cranial) cervical ganglion at primary necropsy - 13-week repeated dose study –

Dose (mg/kg/day)	Males				Females			
	0	3	30	300	0	3	30	300
Total Volume (mm^3)	1.02	1.01	1.13	0.94	1.09	0.83	0.88	0.68
STD	0.25	0.28	0.23	0.16	0.36	0.16	0.40	0.40
CE	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
CV	0.24	0.28	0.23	0.17	0.33	0.20	0.45	0.58
Neuron Number	15016	16829	19318	15219	17671	20339	17597	11995*
STD	4526	4544	3859	2508	2209	4736	2745	5696
CE	0.07	0.07	0.07	0.07	0.06	0.07	0.07	0.08
CV	0.30	0.27	0.20	0.16	0.12	0.23	0.16	0.47
Neuron Size (μm^3)	13565	11941	13405	12175	12516	9069	9597	6385*
STD	1847	3074	4457	2566	3696	2010	3843	3030
CV	0.14	0.26	0.33	0.21	0.30	0.22	0.40	0.47

* Significantly different from the control group at $p<0.05$.
STD: Standard deviation
CE: Coefficient of error
CV: Coefficient of variation

Table 2-13. Stereology data of superior (cranial) cervical ganglion at recovery necropsy - 13-week repeated dose study -

Dose (mg/kg/day)	Males		Females	
	0	300	0	300
Total Volume (mm^3)	0.99	0.73**	0.83	0.76
STD	0.18	0.17	0.27	0.19
CE	0.01	0.01	0.02	0.02
CV	0.19	0.24	0.33	0.25
Neuron Number	17642	17530	17628	13097*
STD	4281	3473	3865	3450
CE	0.06	0.07	0.07	0.07
CV	0.24	0.20	0.22	0.26
Neuron Size (μm^3)	10607	8362	7703	8682
STD	4105	1345	1300	1284
CV	0.39	0.16	0.17	0.15

* Significantly different from the control group at $p<0.05$.
** Significantly different from the control group at $p<0.01$.
-: Not applicable
STD: Standard deviation
CE: Coefficient of error
CV: Coefficient of variation

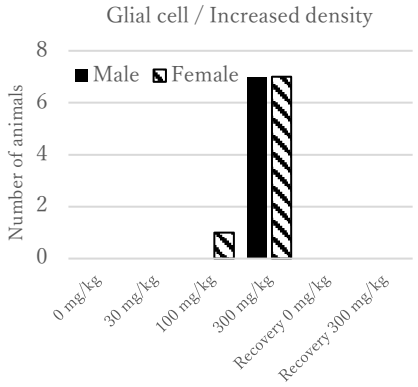
Table 2-14. Toxicokinetic parameters - 4-week repeated dose study -

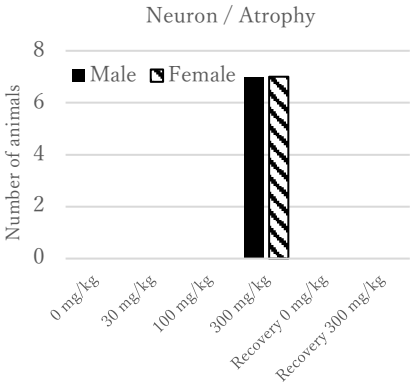
Dosage	AUC_{24} (ng•hr/mL)		C_{max} (ng/mL)		t_{max} (hr)	
	Day 0	Day 27	Day 0	Day 27	Day 0	Day 27
Males						
30 mg/kg/day	15623.0	15798.7	5210.1	4513.7	1.0	1.0
100 mg/kg/day	119121.4	101669.1	16161.1	12511.0	0.5	1.0
300 mg/kg/day	358724.7	365517.8	22333.0	29624.2	4.0	1.0
Females						
30 mg/kg/day	48851.9	45414.1	13107.1	10024.1	0.5	0.5
100 mg/kg/day	293784.7	174166.2	26172.6	21403.6	4.0	0.5
300 mg/kg/day	582315.5	376266.3	32856.0	44457.4	2.0	0.5

Table 2-15. Toxicokinetic parameters - 13-week repeated dose study -

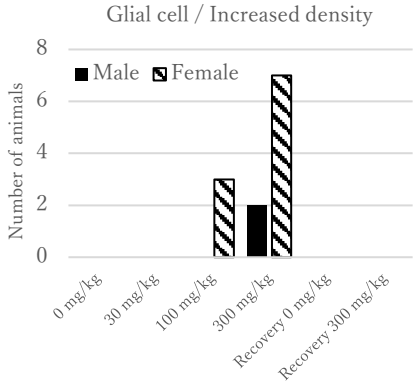
Dosage	AUC_{24} (ng•hr/mL)			C_{max} (ng/mL)			t_{max} (hr)		
	Day 0	Day 48	Day 90	Day 0	Day 48	Day 90	Day 0	Day 48	Day 90
Males									
3 mg/kg/day	617.3	1031.8	807.6	278.0	402.1	356.3	0.5	0.5	0.5
30 mg/kg/day	15782.5	18196.1	14855.4	5296.7	4496.5	4522.5	0.5	0.5	0.5
300 mg/kg/day	295590.3	330772.4	232145.0	19738.9	23404.9	22405.2	1.0	0.5	0.5
Females									
3 mg/kg/day	1417.9	1838.8	2095.3	683.2	921.4	703.4	0.5	0.5	1.0
30 mg/kg/day	33225.6	47991.1	31903.6	9291.3	8624.1	7247.9	0.5	0.5	0.5
300 mg/kg/day	318065.6	476680.8	357483.1	27703.9	43878.5	34000.0	0.5	0.5	2.0

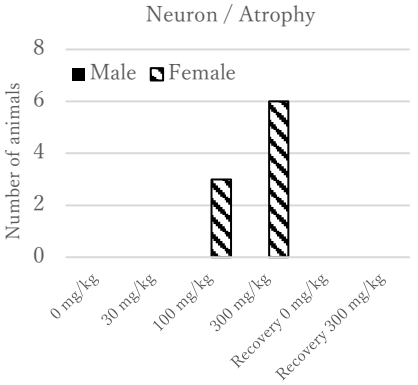
(A) Cervicothoracic ganglion



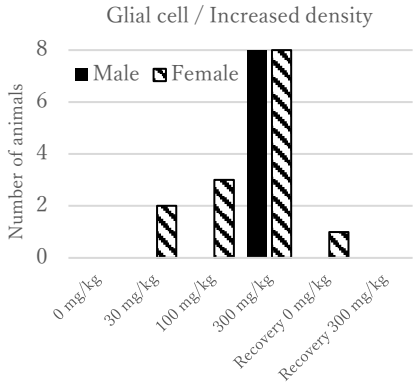


(B) Cranial mesenteric ganglion





(C) Superior (cranial) cervical ganglion



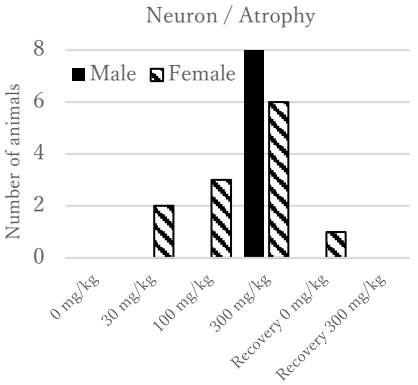
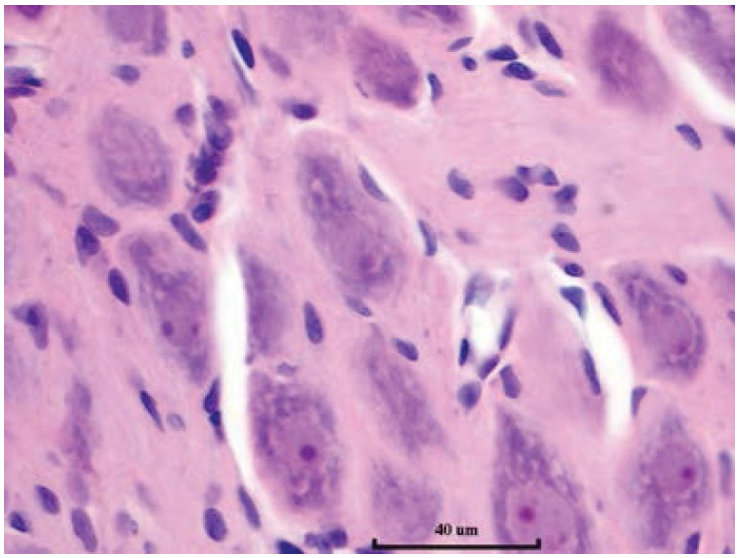


Figure 2-1. Microscopic findings in sympathetic ganglia - 4-week repeated dose study -
(A) Cervicothoracic ganglion (B) Cranial mesenteric ganglion (C) Superior (cranial) cervical ganglion

(A) Normal neuron size and cellularity



(B) Decreased neuron size and increased glial cell density

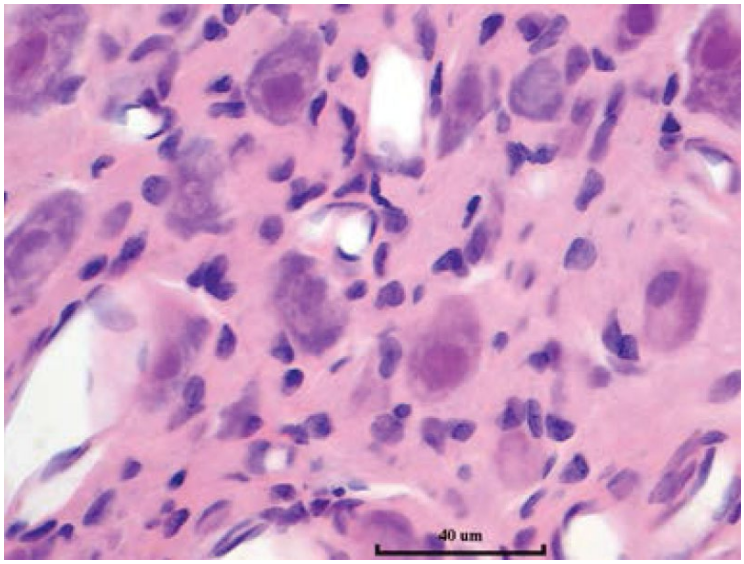
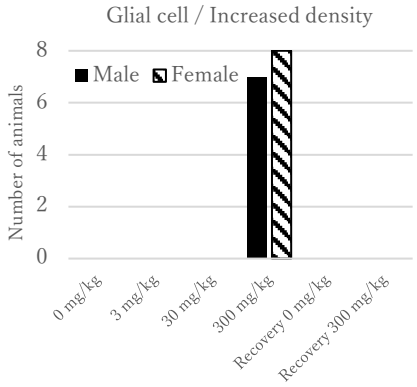
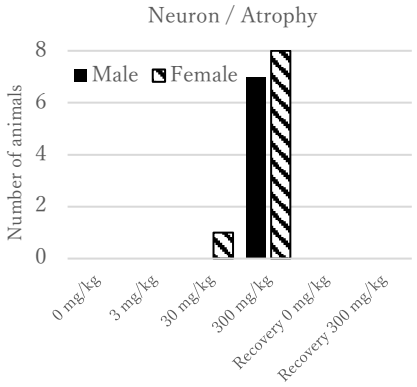


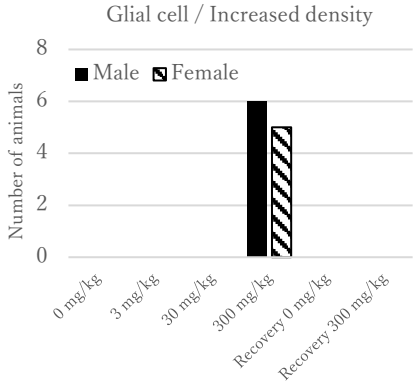
Figure 2-2. Representative microscopic images (magnification 40x) of superior (cranial) cervical ganglia at primary necropsy - 4-week repeated dose study -
(A) Normal neuron size and cellularity in the control group male (B) Decreased neuron size and increased glial cell density in the 300 mg/kg group male

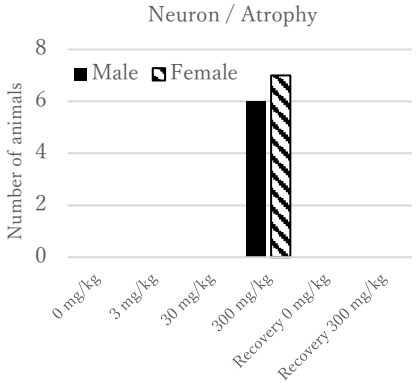
(A) Cervicothoracic ganglion



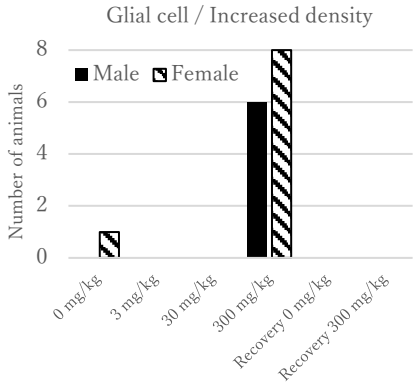


(B) Cranial mesenteric ganglion





(C) Superior (cranial) cervical ganglion



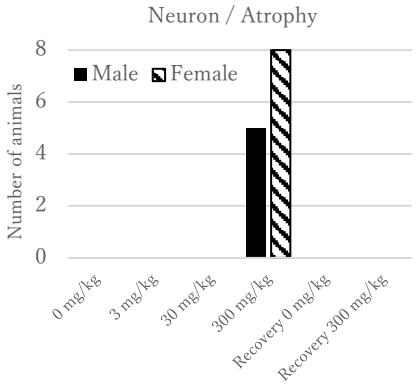


Figure 2-3. Microscopic findings in sympathetic ganglia - 13-week repeated dose study -
(A) Cervicothoracic ganglion (B) Cranial mesenteric ganglion (C) Superior (cranial) cervical ganglion

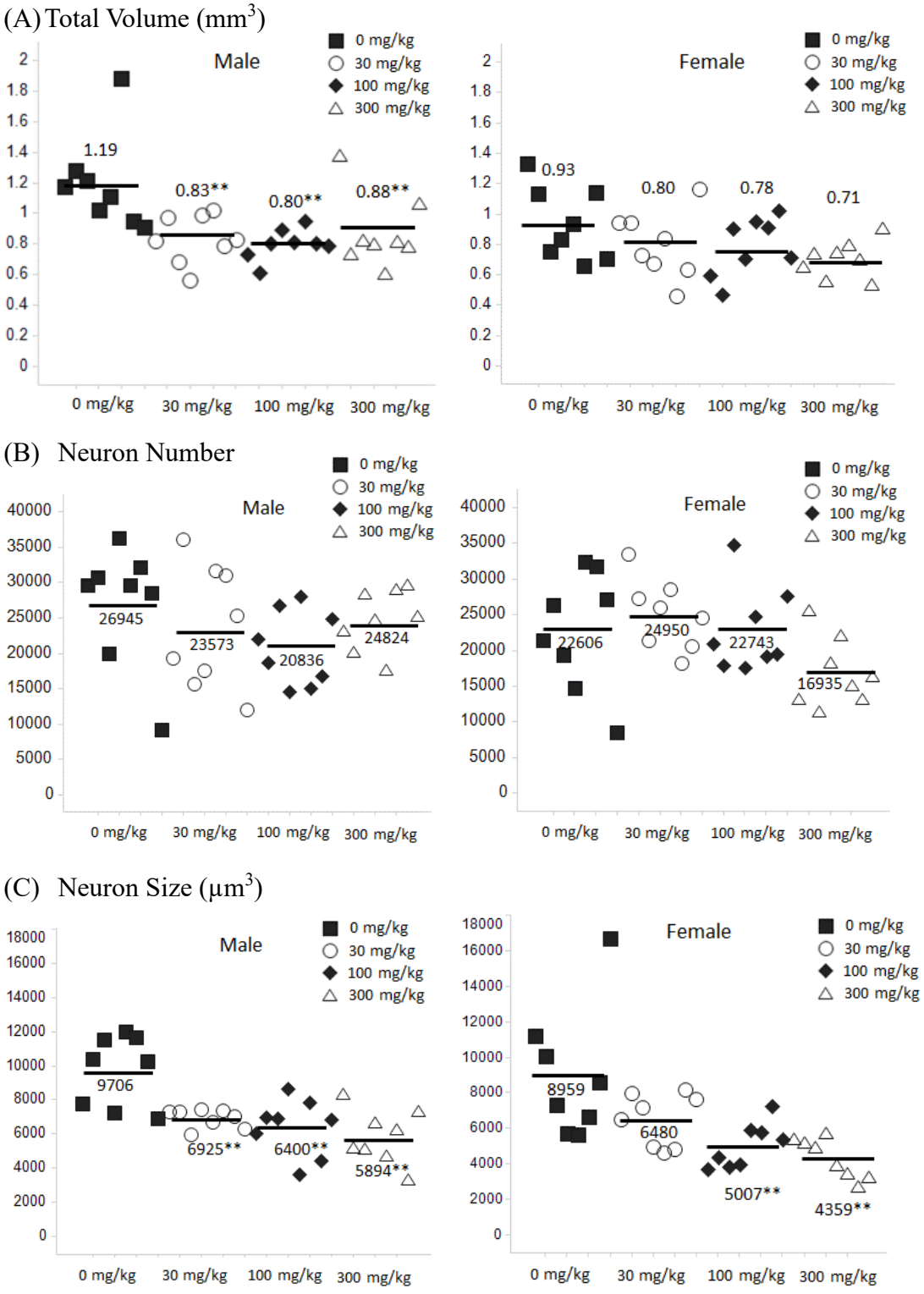
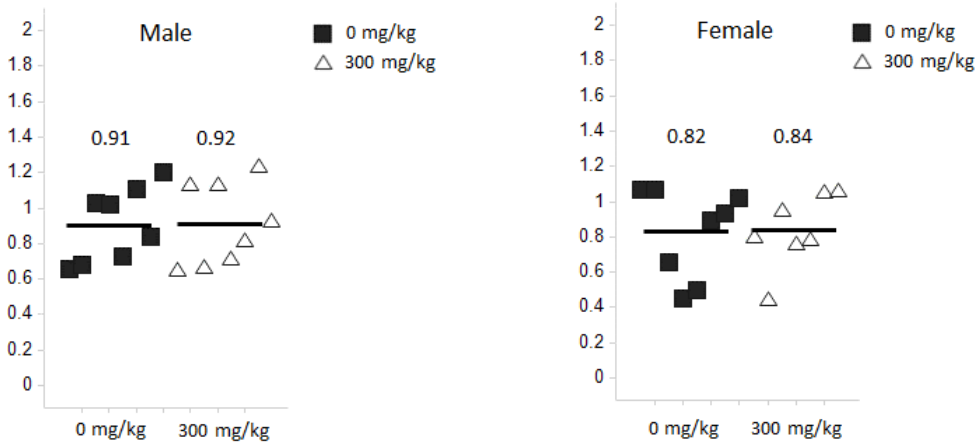


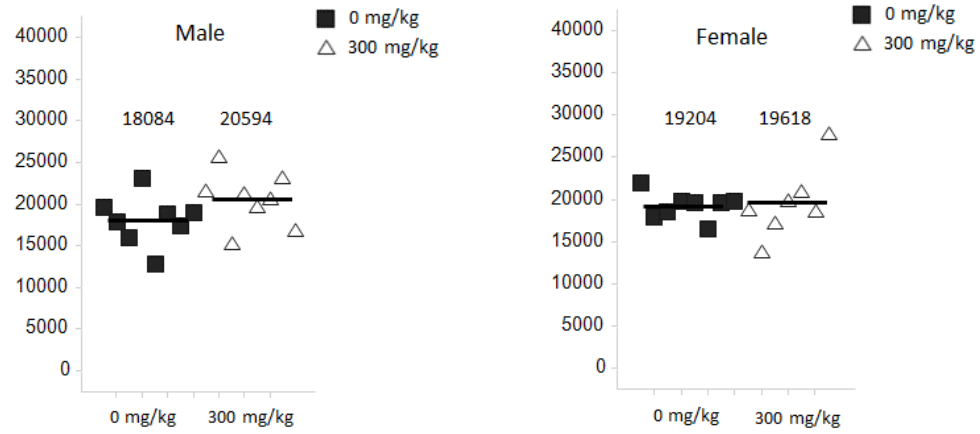
Figure 2-4. Superior (cranial) cervical ganglion data at primary necropsy - 4-week repeated dose study –

(A) Total Volume (B) Neuron Number (C) Neuron Size. Individual; ■ 0 mg/kg, ○ 30 mg/kg, ◆ 100 mg/kg, △ 300 mg/kg. Means; line. n=8 animals per group. ** Significantly different from the control group at $p<0.01$.

(A) Total Volume (mm^3)



(B) Neuron Number



(C) Neuron Size (μm^3)

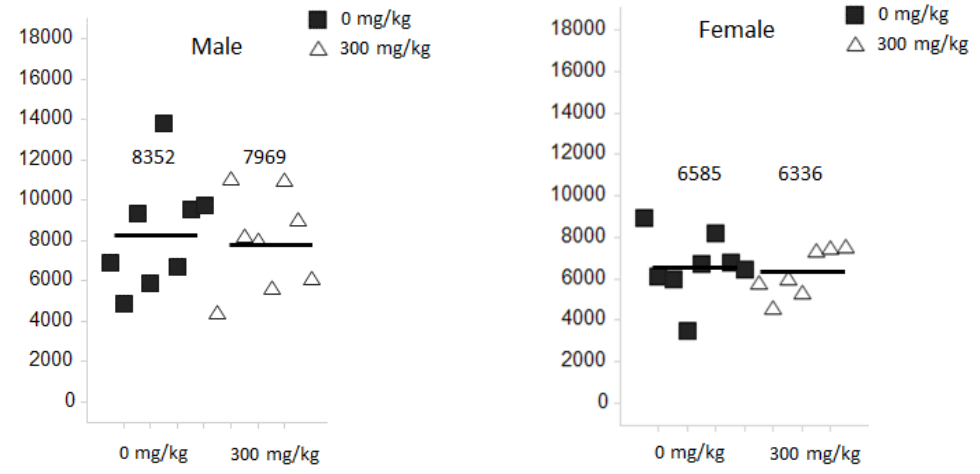


Figure 2-5. Superior (cranial) cervical ganglion data at recovery necropsy - 4-week repeated dose study –

(A) Total Volume (B) Neuron Number (C) Neuron Size. Individual; ■ 0 mg/kg, △ 300 mg/kg. Means; line. n=7-8 animals per group.

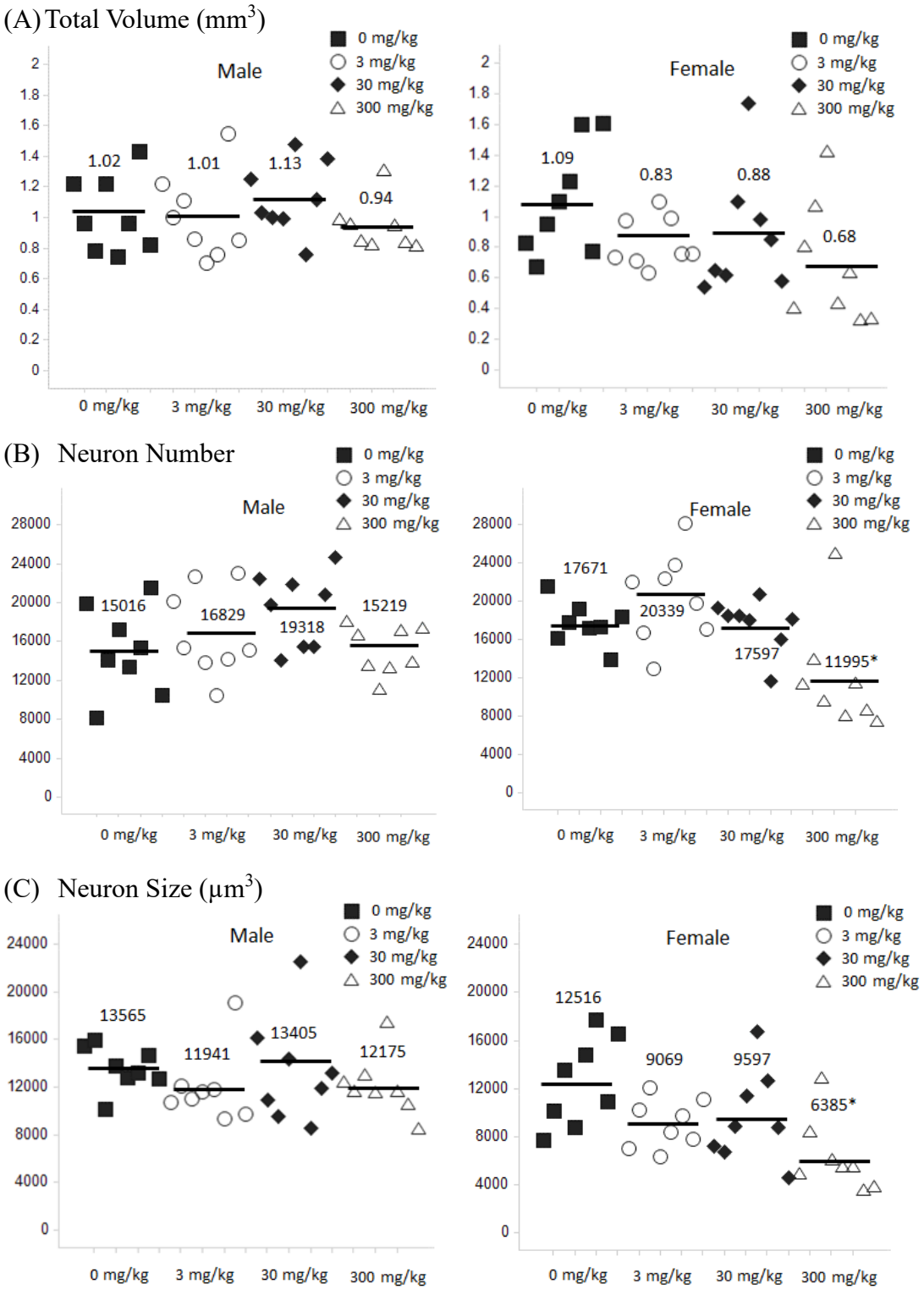
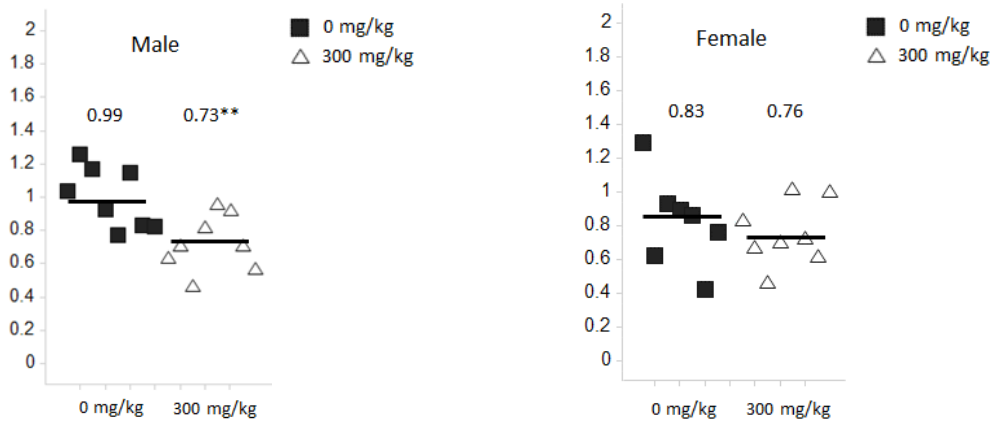


Figure 2-6. Superior (cranial) cervical ganglion data at primary necropsy - 13-week repeated dose study –

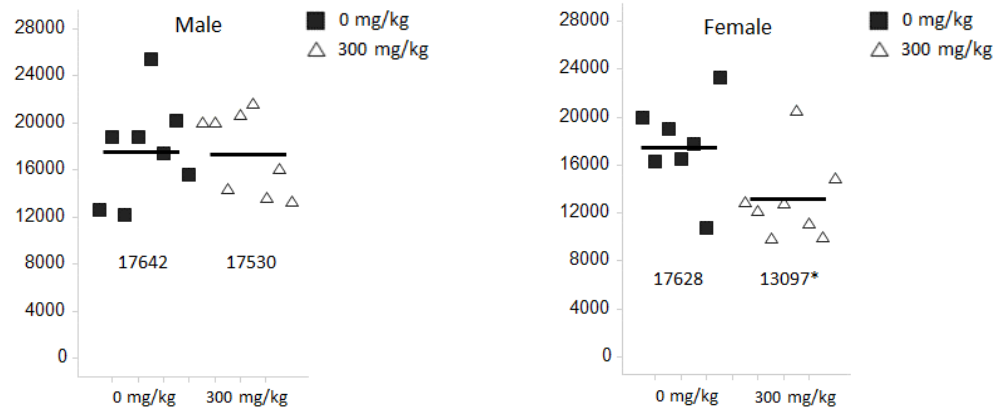
(A) Total Volume (B) Neuron Number (C) Neuron Size. Individual; ■ 0 mg/kg, ○ 3 mg/kg, ◆ 30 mg/kg, △ 300 mg/kg. Means; line. n=8 animals per group.

* Significantly different from the control group at $p<0.05$.

(A) Total Volume (mm^3)



(B) Neuron Number



(C) Neuron Size (μm^3)

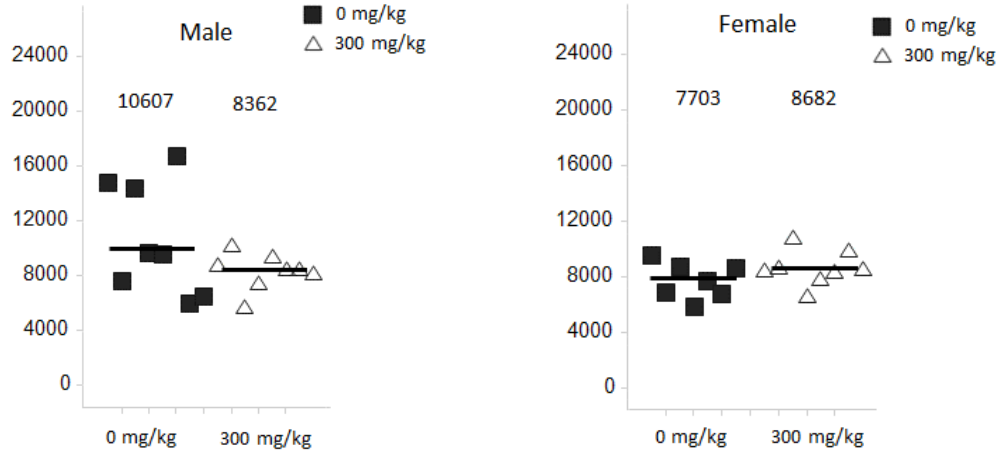
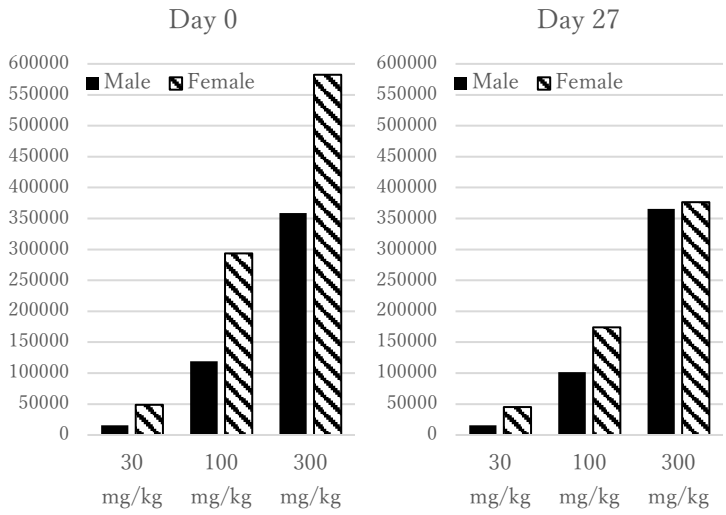


Figure 2-7. Superior (cranial) cervical ganglion data at recovery necropsy - 13-week repeated dose study –

(A) Total Volume (B) Neuron Number (C) Neuron Size. Individual; ■ 0 mg/kg, △ 300 mg/kg. Means; line. n=7-8 animals per group. * Significantly different from the control grop at $p<0.05$. ** Significantly different from the control group at $p<0.01$.

(A) AUC_{24} (ng•hr/mL)



(B) C_{max} (ng/mL)

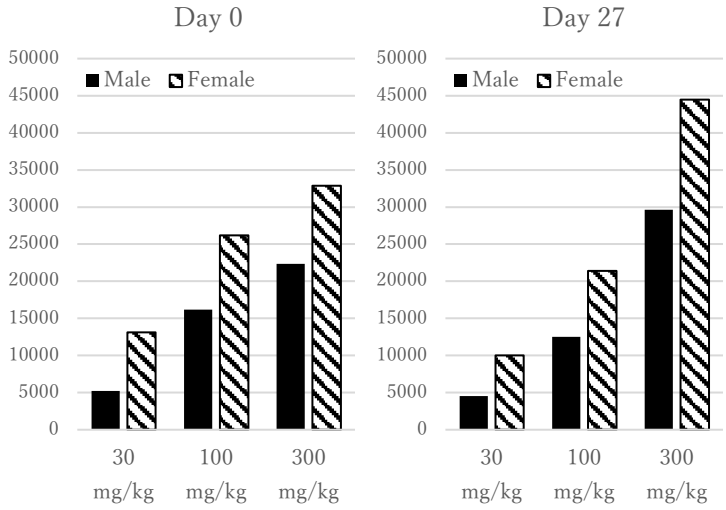
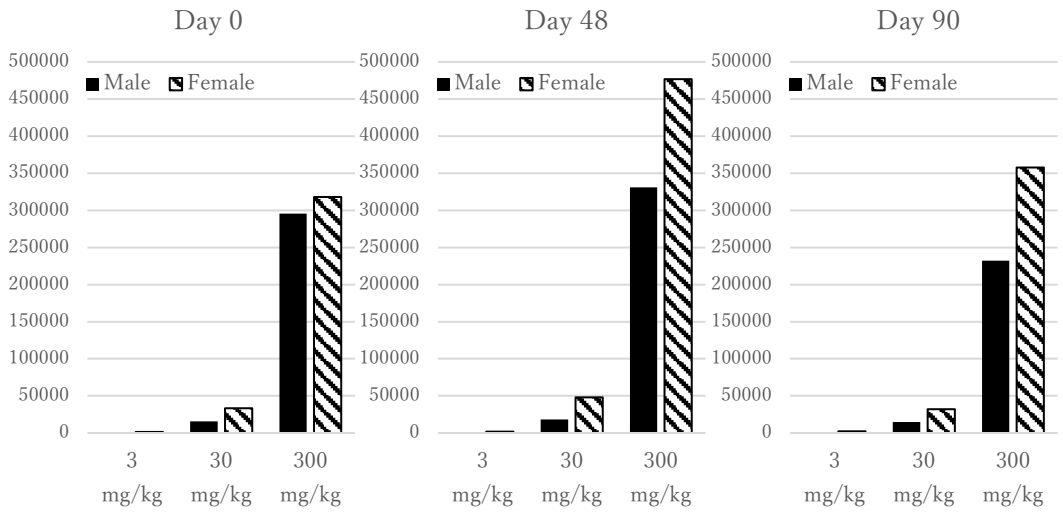


Figure 2-8. Toxicokinetic parameters - 4-week repeated dose study - (A) AUC_{24} (B) C_{max}

(A) AUC_{24} (ng•hr/mL)



(B) C_{max} (ng/mL)

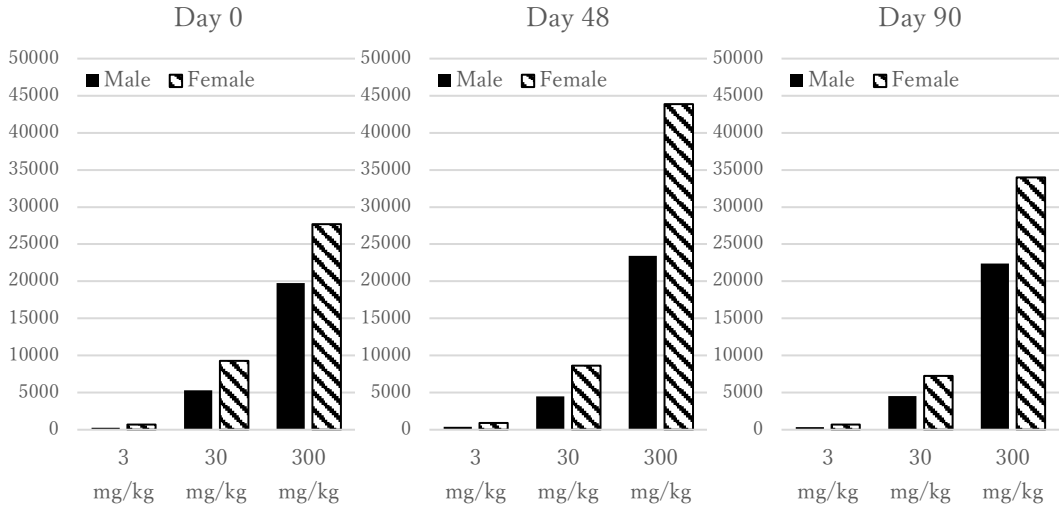


Figure 2-9. Toxicokinetic parameters - 13-week repeated dose study - (A) AUC_{24} (B) C_{max}

General discussion

ASP7962 was identified and is under development for treatment of painful knee OA as a possible selective inhibitor of TrkA, TrkB and TrkC. In order to develop ASP7962 as a drug to treat OA patients, it is essential to clarify the toxicological profile of ASP7962 in animals. Especially, it is worthwhile to investigate the effects of ASP7962 on the nervous system based on the theoretical risk due to blockade of neurotrophic factor-Trk family signal transduction. As each Trk family member has different roles in developmental phase and adult phase, it is important to evaluate the effects of ASP7962 on both embryo-fetal development, and on sympathetic and sensory nervous systems in adult phase. I investigated the potential effects of ASP7962 on embryo-fetal and pre-/postnatal development in rats (Chapter 1), and the potential effects on sympathetic and sensory nervous systems in rats (Chapter 2).

In Chapter 1, reproductive and developmental toxicity studies of ASP7962 showed corneal abnormality, reduced milk uptake, loss of pain response, and decreased reflex at the higher dose levels in rat pups (Table 1-10). It was considered that the primary test article related effects on embryo-fatal and pre-/postnatal development were corneal innervation as well as facial motor neuron, sensory neuron, or spinal motor neuron. For corneal innervation, corneal abnormality has been reported in the eye of mice lacking TrkA (Smeyne et al., 1994). This corneal change in the knockout mice was considered to be caused by the decreased blinking response due to sensory deficiency of corneal sensory neuron (De Castro et al., 1998). For facial motor neuron, mice lacking TrkB show reduced feeding activity

which is noted as reduced milk content in the stomach. This finding has been reported due to facial motor neuronal abnormality that impacts swallowing (Klein et al., 1993). For sensory neuron, mice lacking TrkB do not react to sharp pinpricks in the vibrissae (Klein et al., 1993), suggesting that pain response via sensory neuron was lacked in knockout mice. Mice lacking BDNF have been reported to have defective coordination of movement and balance (Ernfors et al., 1994), indicating decreased reflex. For spinal motor neuron, mice lacking TrkC display athetotic movement or abnormal posture with lack of upright (Klein et al., 1994). In embryo-fetal phase, ASP7962 induced changes, which were considered to be similar to changes observed in these deficient animals in cornea, facial motor neuron, pain response and reflex. These evidences suggested that the signaling of neurotrophins and its Trk receptors were possibly essential for neural development in sensory and motor neuron.

In Chapter 2, neurotoxicity studies of ASP7962 with 4-week and 13-week repeated oral dose in rats showed reversible neuronal atrophy without neuronal death in the sympathetic ganglia such as cervicothoracic ganglion, cranial mesenteric ganglion or superior (cranial) cervical ganglion in histopathology (Table 2-8, 2-9) (Figure 2-1, 2-3) and reversible decreased ganglion volume and/or decreased neuron size in the superior (cranial) cervical ganglion in stereology (Table 2-10, 2-11, 2-12, 2-13) (Figure 2-4, 2-5, 2-6, 2-7). It was considered that the primary test article related effects on sympathetic nervous system in adults were neuronal atrophy. Atrophy of sympathetic ganglion neurons has been reported to be induced in association with anti-nerve growth factor states in rodents (Angeletti et al., 1971, Ruit et al., 1990). In adult phase, although the neuronal atrophy was

observed in the sympathetic ganglia, this finding was not considered to be serious adverse effect because of the lack of neuronal loss, the lack of clinical signs and recovery of changes. These evidences indicated that the NGF-TrkA signal played a role of maintenance in sympathetic nervous system.

Considering the therapeutic application of Trk inhibitors such as ASP7962, the administration of Trk inhibitors should be avoided during pregnancy based on the neuronal deficiencies in cornea, facial motor neuron, pain response and reflex which are shown in pups in reproductive and developmental toxicity studies of ASP7962. It indicates that the inhibition of signaling pathway via Trk family receptors cause serious effects on the embryo-fetal development. On the other hand, in the adult phase, neuronal atrophy by TrkA inhibition is not considered to be a concern for sensory and sympathetic nervous systems because no neuronal cell death or functional deficiencies in these nervous systems were observed in neurotoxicity studies of ASP7962. In addition, anti-human NGF antibodies such as tanezumab and fuluranumab did not show any adverse effects on the sympathetic nervous system and did not cause any neuronal cell death or any apparent functional deficits of sympathetic neurons in cynomolgus monkeys (Belanger et al., 2017; Rocca et al., 2019). Although there are differences in mechanisms of action between anti-human NGF antibody and TrkA inhibitor, the effects on the sympathetic nervous system of this TrkA inhibitor in rats are similar to those induced by anti-human NGF antibodies in cynomolgus monkeys. It suggests that TrkA signal transduction is considered to be related to the size of sympathetic neurons, but not essential for maintenance of the function in the sympathetic nervous system. In the central nervous system, there were no

microscopic findings in the brain and no functional deficits in FOB in neurotoxicity studies with 4-week and 13-week repeated dose in rats. No abnormality in the central nervous system is considered to be based on the limited brain penetration of ASP7962. In addition, there was no treatment-related neurologic effect in the 4-week clinical trial of TrkA inhibitor, ASP7962 (Watt et al., 2019). Therefore, the risk of cognitive impairment or neurodegenerative effect due to ASP7962 administration is considered to be low in sub-chronic dosing period. However, as both nonclinical and clinical data were limited up to 4 or 13-week, it is necessary to further investigate the effect on central nervous system after longer dosing period for the use of ASP7962 in OA patients. ASP7962 is considered to have superiority compared to anti-human NGF antibodies in terms of quality of life for OA patients because ASP7962 can be orally dosed as small molecule drug while anti-human NGF antibodies are intravenously administered to patients in clinic.

Conclusion

Reproductive and developmental toxicity studies of ASP7962 showed adverse effects on sensory and motor neural development in rat pups. Therefore, ASP7962 should not be administered for pregnant women. Although the main target of OA is elderly patients, in order to expand therapeutic application for pediatric patients, juvenile animal toxicity study of ASP7962 will be conducted to investigate the effect on central and peripheral nervous systems because nervous system continues to develop in pediatric population.

Neurotoxicity studies of ASP7962 with 4-week and 13-week repeated oral dose in rats showed reversible neuronal atrophy without neuronal death in sympathetic nervous system. Although no severe neurotoxicity was noted up to 13-week repeated dose in these studies, the effects of ASP7962 on sympathetic and sensory nervous systems with the longer treatment period remain unknown. Therefore, based on the characteristics of target diseases, the long-term chronic neurotoxicity study will be conducted to secure the patient safety in longer treatment period. In addition, it is necessary to monitor the neurologic effects of ASP7962 in clinical trial.

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