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Perinatal Toxicity and Carcinogenicity Studies of Styrene – Acrylonitrile Trimer, A Ground Water Contaminant

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Abstract

Styrene Acrylonitrile (SAN) Trimer is a by-product in the production of acrylonitrile styrene plastics. Following a report of a childhood cancer cluster in the Toms River section of Dover Township, New Jersey, SAN Trimer was identified as one of the groundwater contaminants at Reich Farm Superfund site in the township. The contaminants from the Reich Farm site's ground water plume impacted two wells at the Parkway well field. The National Toxicology Program (NTP) studied the toxicity and carcinogenicity of SAN Trimer in rats exposed during their perinatal developmental period and adulthood. The chronic toxicity and carcinogenicity studies in F344/N rats were preceded by 7- and 18-week perinatal toxicity studies to determine the exposure concentrations for the 2-year studies. Subsequently, Fisher 344 pregnant dams were exposed to SAN Trimer containing diet at 400, 800, or 1600 ppm concentrations during gestation, nursing and weaning periods of offspring followed by two year of adult exposures to both male and female pups. There was no statistically significant evidence of carcinogenic activity following SAN-Trimer exposure; however, rare neoplasms in the brain and spinal cord were observed in males and to lesser extent in female rats. These incidences were considered within the range of historical background in the animal model used in the current studies. Therefore, the presence of a few rarely occurring CNS tumors in the treated groups were not judged to be associated with the SAN Trimer exposure. The major finding was a dose-related peripheral neuropathy associated with the sciatic nerves in females and spinal nerve roots in males and females thereby suggesting that SAN trimer is potentially a nervous system toxicant.

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CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

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Keywords

styrene acrylonitrile trimer; cancer; toxicity; rats; developmental

1. INTRODUCTION

Styrene-Acrylonitrile Trimer (SAN Trimer) was a ground water contaminant found at the Reich Farm Superfund site located in the Toms River section of Dover Township in New Jersey (NTP 2012). Its presence in the ground water was directly related to the dumping of process streams of polymers of styrene and acrylonitrile compounds manufactured by the Union Carbide Corporation; SAN Trimer was a component of these process streams. Although other chemicals were also identified in the well-water, SAN Trimer appeared consistently in the parts per billion range and was determined to be a site contaminant (Richardson *et al.*, 1999).

Chemically, SAN Trimer is a mixture of isomers formed by the condensation of two moles of acrylonitrile and one mole of styrene and has a molecular weight of 210 (Figure 1). The mixture is composed of two structural forms; 4-cyano-1,2,3,4-tetrahydro-a-methyl-1-naphthalene acetonitrile (THNA, CAS No. 57964-39-3) and 4-cyano-1,2,3,4-tetrahydro-1-naphthalene-propionitrile (THNP, CAS No. 57964-40-6). It is a by-product in the production of acrylonitrile styrene plastics (Union Carbide Corporation, 2001). According to Union Carbide Corporation, SAN Trimer is only created in specific manufacturing processes for polymers of acrylonitrile and styrene, and is still used by a few manufacturers.

The New Jersey Department of Health and Senior Services (NJDHSS) reported that childhood cancer incidence rates were greater than expected in Dover Township and in the Toms River section between 1979 and 1995 (NJDHSS 1997). The relative risk of cancer incidence was high for females under the age of 5 years in Toms River, NJ for acute lymphocytic leukemia (SIR=9.7, 95% CI 2.6 to 125 based on four observed cases compared to 0.4 expected) and for brain and central nervous system cancer (SIR=11.6, 95% CI 2.3 to 34 based on three observed cases compared to 0.3 expected). Dover Township, NJ, was the only municipality in Ocean County, NJ in which overall childhood cancer incidence was statistically significantly elevated over that time period.

In response to this finding, the NJDHSS, in cooperation with the Agency for Toxic Substances and Disease Registry (ATSDR), undertook epidemiologic analyses of various potential causes of the elevated cancer rates. The study examined exposures from 10 public well water fields and private wells between 1962 and 1996. Generally, no association was found between well water and cancer risk. However, prenatal exposure to water from one well-field (Parkway) from 1982 to 1996 (the period in which the wells were suspected to have been contaminated) was associated with an increased risk of leukemia among females (SIR=6.0, 95% CI 1.1 to 32), but not among males (SIR=0.0).

Samples collected in 1997 from the groundwater within the Reich Farm Superfund site's groundwater plume had concentrations of SAN-Trimer ranging from 1 to 20 ppb. Between the years 1990 and 1994, it was estimated that concentrations in the groundwater plume

typically ranged from 1 to 100 ppb (Richardson et al., 1999). A public health partnership that included the NTP was established to address the possible link of SAN-Trimer to childhood cancer in the Toms River section of the Dover Township in New Jersey (Maslia *et al.*, 2005). At the request of the SAN-Trimer Interagency Workgroup (NTP, 2012), the NTP conducted perinatal toxicology and carcinogenicity studies in Fischer 344/N rats. Since SAN-trimer has limited water solubility, feed was used as the preferred mode of oral administration in order to achieve maximal bioavailability.

The objective of these studies was to characterize the toxicity and carcinogenicity of SAN-Trimer in rats. The study design and dose selection for both, the prechronic and chronic phases were reviewed and approved by the SAN-Trimer Interagency Workgroup.

This paper summarizes the major findings of the NTP studies. Details including background information on SAN-Trimer exposure, selection, synthesis of the material used for the NTP studies and the remedial actions taken by the regulatory agencies are provided in the report published by the NTP (NTP 2012).

2. MATERIALS AND METHODS

2.1. Procurement and Characterization of SAN-Trimer

Styrene-acrylonitrile trimer (SAN Trimer) was obtained from Union Carbide Corporation (South Charleston, WV). Identity and purity analyses were conducted by Battelle Memorial Institute Columbus Operations (Columbus, OH) and the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC). Karl Fischer titration and elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). The chemical, a thick brown gel, was identified as SAN Trimer by infrared and proton and carbon-13 nuclear magnetic resonance spectroscopy. All spectra were consistent with the manufacturer's spectra for SAN Trimer and with the structure of the test chemical. A Karl Fischer titration was used to determine the moisture content. The purity was determined by elemental analyses, gas chromatography (GC), and high-performance liquid chromatography (HPLC). Additional characterization of the major components of the bulk chemical profiled by GC and HPLC was obtained by coupling each of these analyses with mass spectrometry (MS). Elemental analyses for carbon, hydrogen, and nitrogen were in agreement with the manufacturer's data and the theoretical values for SAN Trimer. GC indicated six major peaks with areas of 22.2%, 16.3%, 12.6%, 28.3%, 7.8%, and 8.5% (cumulatively 95.7% of the total peak area); 13 smaller peaks had areas greater than or equal to 0.1% of the total peak area. HPLC detected six major peaks with areas of 18.1%, 20.4%, 14.8%, 7.3%, 8.9%, and 25.9% (cumulatively 95.4% of the total peak area) and 15 smaller peaks with areas greater than or equal to 0.1% of the total peak area. Results of GC/MS and HPLC/MS analyses supported the composition of the bulk chemical as a mixture of trimers of styrene and acrylonitrile, and the results of the HPLC/MS analyses were consistent with data from the manufacturer's analyses. Subsequent analysis of the test article for the presence of styrene and acrylonitrile monomers indicated that styrene was present at an average concentration of 0.0111% (4.5% RSD, n=4). Acrylonitrile was not present above the limit of detection (0.008%).

2.2. Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing SAN Trimer with NIH-07 or NTP-2000 feed. Homogeneity, dose formulations and stability studies in NIH-07 and NTP-2000 feed were performed by the analytical chemistry laboratory using GC. Homogeneity studies were performed on the 100, 250, 400, 1,600, and 4,000 ppm dose formulations and stability studies of the 100 ppm dose formulations in NIH-07 and NTP-2000 feed; all of these were confirmed by GC.

2.3. Animals and Exposures

The studies were conducted at Battelle Memorial Institute (Columbus, OH). Male and female F344/N rats (F0) were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt the rats were 9 to 10 weeks old. Rats were quarantined for 13 days and were 11 to 12 weeks old on the first day of the breeding period.

Pregnant F0 females were fed NIH-07 diet containing various concentrations of SAN-Trimer (details in Study Design section below). After weaning, the F1 rats were fed NTP-2000 diet containing the same exposure concentrations that were fed to their respective dams. Tap water and diet were made available *ad libitum*. The care of animals on this study was according to NIH procedures as described in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 1996). These studies were conducted in compliance with the Food and Drug Administration Good Laboratory Practice Regulations (Food and Drug Administration 1987).

2.4. Study Design

The schematic representation of the experimental design is provided in Figure 2. All three studies in the F₁ generations were exposed to SAN-Trimer in utero for two weeks and during weaning for 20 days. The postnatal exposures were for 15 days in 7-week studies, 14 weeks in the 18-week studies and 104 weeks for 2-Year studies. Further details are described below.

7-week studies—Groups of seven or eight pregnant F₀ females were fed diets (NIH-07) containing 0, 250, 500, 1,000, 2,000, or 4,000 ppm SAN Trimer beginning on gestation day 7, and groups of six to eight dams with litters continued in the same exposure groups until postnatal day 20 of the last litter delivered. Feed and water were available *ad libitum*. F₀ females were observed twice daily; parturition checks were conducted twice daily from gestation days 18 through 23, and the day of pup delivery was designated postnatal day 0. Dam body weights and clinical findings were recorded on gestation days 1, 7, 14, and 18 and on postnatal days 1, 7, 14, and 20. Feed consumption by dams/dams and pups was recorded from gestation day 7 through postnatal day 20. Necropsies were performed on all F₀ dams that delivered.

On postnatal day 1, animals were counted, weighed, and sexed. On postnatal day 4, litters were randomly selected for culling to a maximum of eight pups (four males and four females); culled pups were discarded without further examination. Several pups from large litters were fostered to smaller litters delivered on the same day and in the same exposure

group to equalize the lactation demand on the dams. Pups (F₁) were weighed on postnatal days 1, 4, 7, 14, and 20; clinical findings were recorded on postnatal days 4, 7, 14, and 20. The selection of pups for the remainder of the study occurred the day the last litter born had reached postnatal day 20. At this time, two male and two female pups were randomly selected from each litter, and their dams were subjected to a complete necropsy. Litters of only a single pup of either sex and foster pups were excluded from the study.

After weaning, groups of 10 male or 10 female F₁ rats, housed five per cage by sex, were fed diets (NTP-2000) containing the same exposure concentration that was fed to their respective dams. The animals were exposed to SAN-Trimer in the diet for 15 days after weaning for 7-week studies. Feed and water were available *ad libitum*. Animals were observed twice daily, weighed on days 1 and 8 after weaning, and at the end of the study; feed consumption was recorded on days 1, 4, 8, and 11 and at the end of the study; clinical findings were recorded weekly. Necropsies were performed on all F₁ rats that were selected to continue on study postweaning. When gross lesions were observed at necropsy, the tissue was examined in controls and to a no-effect level in exposed rats.

18-Week studies—Groups of eight or nine pregnant females were fed diets (NIH-07) containing 0, 100, 200, 400, 800, or 1,600 ppm SAN Trimer beginning on gestation day 7, and groups of four to eight dams with litters continued in the same exposure groups until postnatal day 20 of the last litter delivered. Other details are similar to the description in the section above with the exception that these animals were necropsied at 14-weeks postweaning as shown in Figure 2. Necropsies were performed on all F₁ rats after termination of the studies. The brain, heart, right kidney, liver, lung, spleen, right testis, thymus, and uterus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (eyes were first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on F₁ control and 1,600 ppm rats.

2-year studies—One hundred breeding cohorts of one male and two female F₀ rats were housed together during a 9-day breeding period instead of 7 days to provide a sufficient number of F₁ animals in the 2-year studies. Groups of 41 or 42 pregnant F₀ females were fed diets (NIH-07) containing 0, 400, 800, or 1,600 ppm SAN Trimer beginning on gestation day 7, and groups of 24 to 27 dams with litters continued in the same exposure groups until postnatal day 20 of the last litter delivered. F₁ pups were counted, weighed, and sexed on postnatal day 1. Litters were randomly selected for culling to a maximum of 10 pups (five males and five females) on postnatal day 7. As each litter reached postnatal day 20, up to three pups per sex per litter were randomly selected and assigned to the study. Weaning occurred the day the last litter born had reached postnatal day 21. Groups of 50 male and 50 female F₁ rats (22 to 26 litters) were fed diets (NTP-2000) containing the same exposure concentrations as their dams for 2 years.

All rats were observed twice daily for mortality and morbidity. For F₀ females, body weights and clinical findings were recorded on gestation days 1, 7, 14, and 20 and postnatal days 1, 7, 14, and 20. Other reproductive parameters that were assessed included but were

not limited to implantations per dam, fertility index, gestational index, number of litters, mean litter size, and the number of pups moribund or dead (see NTP 2012 for further details). For F₁ study rats, body weights were recorded initially, weekly for the first 13 weeks, monthly thereafter, and at study termination; clinical findings were recorded on postnatal days 7, 14, 20, and 29 and monthly thereafter; feed consumption was recorded weekly for the first 13 weeks after weaning and monthly thereafter.

Complete necropsies were performed on all F₀ females that delivered, and all F₁ study rats as described in the 18-week section above. Complete microscopic examinations were performed on all core study rats. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from both the sides were examined.

For the expanded evaluation of the central and peripheral nervous systems, the wet tissue was examined for the presence of any gross lesions; an additional three sections of paraffin-embedded brain from the superficial, middle, and deep aspects of each block were evaluated (resulting in the examination of nine sections of brain not previously examined); transverse, and oblique sections of the mid cervical intumescence, mid-thoracic, and mid-lumbar intumescence regions of the spinal cord were evaluated; and transverse and longitudinal (1 cm) sections of both the right and left sciatic nerves were evaluated. Sections of dorsal and ventral spinal nerve roots were also evaluated. Although not specifically collected for evaluation, the spinal nerve roots were present on most sections of lumbar spinal cord and, less commonly, the cervical and thoracic sections of spinal cord (hence the variation in the denominator as seen in Table 8). There were typically two longitudinal sections of nerve present from each animal (right and left). The severity scores for the nerve fiber degeneration ranged from minimal to mild in all animals. Criteria for grading were based on an approximate percentage of the nerve root fibers that were affected, rather than an absolute number, in order to compensate for variability in amount of nerve root available for examination. A lesion graded as 'minimal' had fewer than 10% of the nerve fibers in the section affected, and a lesion graded as mild had between 10 and 20%. No animal had more than 20% of the nerve fibers affected.

Sciatic nerve fiber degeneration was graded from minimal to mild. If two foci were essentially adjacent in the tissue and, in the pathologist's opinion, they likely represented the same degenerating fiber; they were only counted as one focus of degeneration. If an average of three or fewer foci of degeneration were present per longitudinal nerve section, the lesion was graded as 'minimal'. If an average of between 4 and 12 foci were present per longitudinal section of nerve, the lesion was graded as 'mild'. An average of 12 foci of degeneration was not exceeded in the study. There were typically two longitudinal sections of nerve present from each animal (right and left). The above grading system was based on the system reported in Cotard-Bartley et al. (1981).

2.5. Statistical Methods

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). The incidences of neoplasms or nonneoplastic lesions are presented as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance,

the incidences of most neoplasms and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. Because up to three pups per sex per litter were in the study, litter effects were taken into account in assessing neoplasm prevalence using weighted mixed effects logistic regression models (as implemented in SAS Proc GLIMMIX) with litter as a random effect. The survival-adjusted rate (based on the Poly-3 method) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal sacrifice (Bailer and Portier, 1988). Organ and body weight data, which historically have approximately normal distributions, were analyzed with mixed effects linear models using litter as the random effect, where technically possible, to take litter effects into account and with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972).

Significance of trends in gestational and fertility indices across dose groups was tested using Cochran-Armitage trend tests. Pairwise comparisons of each dosed group with the control group were conducted using the Fisher exact test.

3. RESULTS

3.1. 7-week Studies

There was no effect on survival except for early death of one male in the 4000 ppm group. Final mean body weights of 1,000, 2,000, and 4,000-ppm males and 2,000 and 4,000-ppm females were significantly less than those of the controls; weaning mean body weights were reduced in the 4,000 ppm males and females, and in the 2,000 ppm females. Feed consumption by 2,000 and 4,000 ppm males and females was less than that of the control groups (Table 1). There were no apparent SAN-Trimer-related effects on length of gestation, fertility, live birth/implantation ratio, litter size, or gender number (NTP, 2012). It is unclear if SAN-Trimer affected the gestational index because only the 4000-ppm group had a female that had implantation sites but no pups; slight increase in the moribundity could not conclusively be linked to exposure (Table 2). In adults, thinness in 4,000 ppm male rats was the only clinical finding related to SAN Trimer exposure. Nonneoplastic lesions were noted in the brain, thymus, spleen, liver, kidney, and reproductive organs of males and females. However, all histopathological findings were considered secondary to overt toxicity and associated with severe caloric deficiency.

3.2. 18-week Studies in Rats

The incidence of sperm positive females was 83% (50/60); of the 50 females that were sperm positive, 39 (78%) delivered live pups (NTP 2012). It is unknown if the females that did not deliver pups were pregnant or if resorption had occurred because no examination for implantation sites was performed. There was evidence of an effect of SAN Trimer exposure on the fertility index, number of litters, and litter size in the 1,600 ppm group with no effect on pup growth. Smaller changes in litter size (reduced from 12 to 10 in the 400 and 800 ppm groups) accompanied by a lower fertility index may also have been related to exposure to SAN Trimer, and were consistent with the effects noted in the 1,600 ppm group (Table 3).

All rats survived to the end of the study. Final mean body weights of males exposed to 1,600 ppm and females exposed to 200 ppm were significantly less than those of the controls (Table 4). The liver weights of all exposed groups of males and the spleen weights of 800 and 1,600 ppm males and 1,600 ppm females were significantly greater than those of the controls (Table 5). There were no significant differences in sperm parameters of male rats or the estrous cyclicity of female rats administered 400, 800, or 1,600 ppm in the diet when compared to the control group. No exposure-related histopathologic lesions were observed.

3.3. 2-Year Studies

In general, there was a relatively low fertility index seen in all groups including controls (~60–64%) with no apparent treatment related differences in fertility index, gestational index or number of litters produced (Table 6). The smaller litter sizes as evidenced in the 18-week studies did not recapitulate in the 2-year studies. In adults, survival of exposed groups of male and female rats was similar to that of the control groups. Final mean body weights of 1,600 ppm males were more than 10% less than those of the controls after week 1; mean body weights of 800 and 1,600 ppm females were more than 10% less after weeks 41 and 13, respectively (Figure 3). Feed consumption by exposed groups of males and females was generally similar to that by the control groups.

There was a marginal and statistically insignificant increase in the incidence of neoplasms in the central nervous system (CNS; brain and spinal cord) as noted in Table 7. Astrocytomas occurred in the brains of one 800 ppm male and one 1,600 ppm male; granular cell tumors also occurred in the brains of one 800 ppm male and one 1,600 ppm male. All these tumors occurred in different animals. One granular cell tumor occurred in the brain of a 400 ppm female and mixed cell gliomas occurred in the brains of one female in the control, 400 ppm, and 800 ppm groups. Of these brain tumors, the astrocytoma in the 1,600 ppm male and the mixed cell glioma in the control female were identified grossly. In the spinal cord of males, a single astrocytoma occurred in a 1,600 ppm animal and a granular cell tumor occurred in a 400 ppm animal. One meningioma occurred in the spinal cord of an 800 ppm female. The glial tumors (astrocytomas and mixed gliomas) are considered of similar neuroepithelial origin arising from the CNS parenchyma and therefore were evaluated individually and in combination. Since meningiomas and granular cell tumors of the CNS are believed to arise from the arachnoid cells of the meninges they were also compared individually and in combination with each other, but they are not combined with glial tumors because the two groups arise from distinct origins (McConnell *et al.*, 1986; Solleveld and Boorman, 1990; Brix *et al.*, 2010).

Amongst non-neoplastic lesions in the peripheral nervous system, the main findings were statistically significant and dose-dependent increases in the incidence of sciatic nerve degeneration in the 800 and 1600 ppm females and increased incidence (males only) and severity (males and females) in spinal nerve root degeneration (Tables 7, 8). The sciatic nerve degeneration best appreciated and graded on the longitudinal sections of nerve. It was characterized by single to multiple vacuoles, typically arranged in short chains, containing small amounts of myelin and/or axonal debris. Less frequently, foamy macrophages were present within the vacuoles (Fig 4). Spinal nerve root degeneration was principally noted in

the lumbar nerve roots, particularly the ventral roots and was characterized histologically by a combination of features, including marked dilatation of the myelin sheath, infiltration of the myelin sheath by foamy macrophages, and vacuolation and fragmentation of the myelin sheath and/or axon forming variably sized fragments of myelin and axonal debris (Figures 5–8).

In addition to the CNS, there were significantly increased incidences of bone marrow hyperplasia in 1,600 ppm males and females and 800 ppm females. Incidences of bone marrow granulomatous inflammation were increased in 1,600 ppm males and 800 and 1,600 ppm females, and the increase in the 800 ppm females was significant. Because this lesion is very rare and did not occur in control animals, it was considered biologically significant. In the liver, the incidence of eosinophilic focus was significantly increased in 1,600 ppm males and the incidences of mixed cell focus were significantly increased in 400 and 1,600 ppm males. Exposure to SAN-Trimer also resulted in decreased incidences of pituitary gland adenomas in males (16/50, 10/50, 13/50, 4/50) and females (22/50, 12/50, 19/50, 9/50), mononuclear cell leukemia in males (15/50, 7/50, 5/50, 3/50) and females (13/50, 2/50, 3/50, 2/50) and mammary gland fibroadenoma in females (36/50, 31/50, 26/50, 20/50).

4. DISCUSSION

The NTP, in a public health partnership, conducted perinatal-postnatal studies based on concerns of increased incidences of childhood cancer in Dover Township between 1979 and 1995 (Maslia *et al.*, 2005). Since the primary concern with the exposure was brain cancer, the rationale for conducting studies on rats was based on reports indicating increased susceptibility to the induction of CNS tumors in rats compared with mice, thereby making it the more sensitive species (Ward and Rice, 1982; Rice and Wilbourn, 2000). The chronic toxicity and carcinogenicity studies were preceded by 7- and 18-week toxicity studies to determine the exposure concentrations for the 2-year studies. Animals were exposed during their *in utero* and neonatal developmental periods, which are generally considered to be especially susceptible to CNS toxicity and carcinogenicity compared to the adult phase of life (Rice and Wilbourn, 2000). This design is further strengthened by the Union Carbide Corporation-sponsored study results that showed that SAN Trimer was transported across the placenta to the developing fetus and that neonates are also exposed through their mother's milk (Gargas *et al.*, 2008).

Our findings revealed chemical-related effects in both, the 7-week and 18-week studies in the 1,600 ppm groups as indicated by initial decreases in mean feed consumption; decreased mean body weight; however survival in the exposed groups was equal to or greater than that of the controls. The initial decrease in feed consumption was more severe and more prolonged in 1,600 ppm males than in 1,600 ppm females in the 18-week and 2-year studies. In the 2-year studies the overall feed consumption in the 1,600 ppm groups were 94% (males) and 92% (females) of the controls. The low initial consumption of feed could possibly be due to decreased palatability; however, there were no significant differences in food consumption after week 4 in the 18-week study and week 6 in the 2-year study (NTP 2012).

The nervous system was considered the major potential target of SAN Trimer-associated toxicity in the current studies as indicated by exposure concentration-related exacerbation of spinal nerve root fiber degeneration and sciatic nerve fiber degeneration in the peripheral nervous system (PNS) of males and females. The sciatic nerve degeneration was characterized by single to multiple vacuoles, typically arranged in short chains, containing small amounts of myelin and/or axonal debris. Spinal nerve root degeneration was principally noted in the lumbar nerve roots, particularly the ventral roots. It may be noteworthy that the histopathological changes did not correlate with clinical signs of toxicity. There were no behavioral changes noted based on the clinical observations recorded during the course of studies. A separate functional observational battery was not performed in the current studies to confirm the lack of correlation.

Due to the concerns of the potential relationship between SAN Trimer exposure and CNS neoplasms in children, the NTP conducted an expanded evaluation of the CNS to include nine additional brain sections as well as the spinal cord. Findings showed that in the SAN Trimer-exposed male rats there were two astrocytomas and two granular cell tumors of the brain, and one astrocytoma and one granular cell tumor of the spinal cord, distributed among the three exposed groups; with no background incidence in the controls. In females, there were two gliomas and two granular cell tumors in the exposed animals with a background incidence of one glioma in the controls. Due to the additional special pathology for this study, as well as the prolonged exposure period, these findings could not be directly compared with the NTP historical control database, which is typically in adult animals with more limited pathology and does not typically include the spinal cord. However, in spite of the expanded pathology as well as the extended exposure window to capture the developmental phase in these animals, incidences of neoplasms appeared to be within the range of historical background for this animal model. Although these studies cannot directly be compared with other NTP studies due to the lack of an appropriate historical control, it may be noteworthy in the traditional historical control database, the incidence of spontaneous brain neoplasms in F344/N rats is less than 0.5% (Sills et al., 1999), indicating the occurrence of these tumors in rats is uncommon or rare; the induction of which appear to be chemical specific. Out of approximately 550 carcinogenicity studies reported by the NTP during a period of 30 years, 11 studies showed any evidence of increases in the incidences of brain neoplasms in F344/N rats (<<http://ntp.niehs.nih.gov/go/SA-10>>). Of these studies, glycidol was clearly carcinogenic, but all other responses were considered equivocal because the incidences were marginal, did not have clear dose response relationships, latency periods were not reduced, and/or mortality was not affected.

Interestingly, a stand-alone genetic toxicology study was conducted by the NTP with the same batch of SAN Trimer as used in the current bioassay confirmed the lack of mutagenicity seen in the earlier bacterial and human studies, but found that SAN Trimer, administered once daily for 4 days by gavage to male and female F344/N juvenile rats was associated with significantly increased levels of DNA damage in brain cells from the cerebrum and cerebellum, measured by the Comet assay, and chromosomal damage in peripheral blood reticulocytes, measured by the micronucleus assay (NTP 2012, Hobb et al, 2012). Additional evidence of DNA damage, measured by the Comet assay, was seen in

liver cells and peripheral blood leukocytes of exposed male and female rats. *In vivo* assays for genotoxicity are generally less sensitive than *in vitro* assays, and positive results in the *in vivo* peripheral blood rodent micronucleus assay have been shown to have a high predictability for rodent carcinogenicity (Witt *et al.*, 2000).

Other findings from this study included nonneoplastic lesions in the liver and bone marrow in males and females, and urinary bladder in females, which were statistically significant only at the high dose and generally lacked a dose-response relationship. Additionally, there were decreases in the incidences of pituitary gland adenomas and mononuclear cell leukemia in males and females, and in the incidences of mammary gland tumors in females following SAN-Trimer exposure (NTP 2012).

To summarize, the nervous system appears to be a primary target of SAN Trimer-mediated toxicity in F344/N rats. Although there were sporadic incidences of rarely occurring neoplasms in the CNS following SAN-trimer exposure that were more prevalent in males than females, the occurrence of these tumors was considered within the range of historical background. The presence of these few rarely occurring CNS tumors in the treated groups was not judged to be associated with the SAN Trimer exposure (NTP 2012). In the PNS, there was a dose-dependent increase in incidence and severity of sciatic nerve and spinal nerve-root degeneration; the pathogenesis of these spontaneous and compound-induced lesions remains unknown.

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Figure 1.

The SAN-Trimer mixture is typically composed of two structural forms; 4-cyano-1,2,3,4-tetrahydro- α -methyl-1-naphthalene-acetonitrile (THNA, CAS No. 57964-393) and 4-cyano-1,2,3,4-tetrahydro-1-naphthalene-propionitrile (THNP, CAS No. 57964-406). This figure shows the four stereoisomeric forms of THNA and the two stereoisomers of THNP.

All Studies	F ₀	GD 7 to Delivery	PND 1 to PND 20	
7-Week Study	F ₁	GD 7 to Delivery	PND 1 to PND 20	PND 21 to PND 35
18-Week Study	F ₁	GD 7 to Delivery	PND 1 to PND 20	PND 21 to PND 118 (PND 21+14 weeks)
2-Year Study	F ₁	GD 7 to Delivery	PND 1 to PND 20	PND 21 to PND 728 (PND 21+104 weeks; core study group) PND 21 to PND 546 (PND 21+27 weeks, PND 21+52 weeks, PND 21+78 weeks; special study groups)

GD=gestation day; PND=postnatal day; PND 20 of the last litter delivered

Dietary exposure
 In utero exposure
 Nursing and dietary exposure

Figure 2.
Study Design for 7-Week, 18-Week, and 2-Year Feed Studies of Styrene-Acrylonitrile Trimer

Figure 3.
Growth Curves for F1 Rats Perinatally and Postnatally exposed to SAN-Trimer in Feed for 2 Years

Figure 4. High dose male, Sciatic Nerve (2-year studies): Note the degeneration of nerve fibers characterized by clusters of vacuoles arranged in linear chains and containing fragments of myelin and/or axonal debris (arrows). Several macrophages are also present within one focus of degeneration (arrowhead). (40X) (H&E)

Figure 5.

Low dose female, Lumbar Spinal Nerve Root (2-year studies). Section of normal spinal nerve root; compare to figures 6 and 7 which are at the same magnification. Note the lack of degenerative changes and cellular infiltrates. A minimal amount of artifactual myelin vacuolation is occasionally present. (20X) (H&E)

Figure 6.

Low dose male, Lumbar Spinal Nerve Root (2-year studies): Note the minimal degeneration, characterized by marked dilatation of the myelin sheath (arrow) and small linearly arranged aggregates of macrophages (arrowheads) occasionally containing myelin and/or axonal debris. A grade of minimal was assigned if fewer than 10% of nerve fibers in the root were affected. (20X) (H&E)

Figure 7. Low dose male, Lumbar Spinal Nerve Root (2-year studies): Note the mild degeneration, characterized by multifocal dilatation of the myelin sheaths (arrows) and multifocal aggregates of macrophages (arrowheads). A grade of mild was assigned if 10–20% of the nerve fibers of the root were affected (20X) (H&E).

Figure 8. High dose female, Lumbar Spinal Nerve Root (2-year studies): High magnification image showing mild degenerative changes characterized by dilatation of myelin sheaths (arrows) and macrophage aggregates (arrowheads). (40X) (H&E).

Survival, Body Weights, and Feed Consumption of F₁ Rats in the 7-Week Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

Table 1

Concentration (ppm)	Survival ^b	Weaning Body Weight ^a (g)	Final Body Weight ^a (g)	Change in Body Weight ^a (g)	Final Weight Relative to Controls (g)	Feed Consumption Week 1	Feed Consumption Week 3
Males							
0	10/10	50 ± 4	118 ± 6	68 ± 2		10	16
250	10/10	48 ± 3	114 ± 4	66 ± 2	97	10	15
500	10/10	46 ± 3	109 ± 4	63 ± 2*	92	9	15
1,000	10/10	47 ± 2	106 ± 2*	59 ± 1**	90	9	14
2,000	10/10	42 ± 3	74 ± 3**	32 ± 1**	63	6	8
4,000	9/10 ^c	24 ± 1**	34 ± 2**	10 ± 1**	29	2	4
Females							
0	10/10	50 ± 4	106 ± 5	56 ± 1		10	13
250	10/10	48 ± 2	103 ± 3	56 ± 1	97	10	13
500	10/10	45 ± 3	101 ± 3	56 ± 1	95	10	13
1,000	10/10	45 ± 2	102 ± 2	56 ± 1	96	10	13
2,000	10/10	40 ± 3**	72 ± 3**	33 ± 1**	68	7	7
4,000	10/10	23 ± 1**	32 ± 1**	9 ± 1**	30	2	6

* Significantly different (P 0.05) from the control group by Williams' test

** P 0.01

^aWeights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day. Subsequent calculations are based on animals surviving to the end of the study.^bNumber of animals surviving 2 weeks after weaning/number in group at weaning^cDay of death: 3

Table 2
Mean Body Weights of F₁ Pups to Postnatal Day 20 in the 7-Week Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

Concentration (ppm)	Postnatal Day 1			Postnatal Day 4			Postnatal Day 7			Postnatal Day 14			Postnatal Day 20		
	No. ^b	Body Weight (g)	Weight Relative to Controls (%)	No. ^c	Body Weight (g)	Weight Relative to Controls (%)	Body Weight (g)	Weight Relative to Controls (%)	Body Weight (g)	Weight Relative to Controls (%)	Body Weight (g)	Weight Relative to Controls (%)	Body Weight (g)	Weight Relative to Controls (%)	
Males															
0	39	5.8		10	8.8		13.5		25.7		35				
250	30	5.9	102	10	9	102	13.6	101	26.2	102	35.8	102			
500	33	6	103	10	8.6	98	13	96	25.1	98	34.7	98	99		
1,000	31	5.8	100	10	8.4	96	13	96	24.9	97	34.5	97	99		
2,000	38	5.8	100	10	8.8	100	12.9	96	24.6	96	31.6**	96	90		
4,000	27	5.3**	91	10	7.5**	85	10.4**	77	16.8**	65	19.8**	65	57		
Females															
0	23	5.4		10	8.2		12.7		24.7		33.6				
250	34	5.6	104	10	8.5	104	12.9	102	24.7	100	33.9	100	101		
500	32	5.4	100	10	8.2	100	12.7	100	25	101	33.6	101	100		
1,000	40	5.5	102	10	8	98	12.4	98	24.2	98	32.7	98	97		
2,000	49	5.3	98	10	8.2	100	12.4	98	23.7	96	30.3**	96	90		
4,000	31	5.0**	93	10	7.3*	89	9.9**	78	16.1**	65	18.8**	65	56		

* Significantly different (P 0.05) from the control group by Dunnett's test

** P 0.01

^aWeights are given as group means.

^bNumber of animals weighed on postnatal day 1

^cNumber of animals weighed on postnatal days 4, 7, 14, and 20

Fertility, Gestational, Parturition, and F₁ Pup Survival Data to Postnatal Day 20 for Rats in the 18-Week Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

Table 3

	0 ppm	100 ppm	200 ppm	400 ppm	800 ppm	1,600 ppm
Fertility index ^a (%)	8/9 (89)	8/9 (89)	7/8 (88)	6/8 (75)	6/8 (75)	4/8# (50)
Gestational index ^b (%)	8/8 (100)	8/8 (100)	7/7 (100)	6/6 (100)	6/6 (100)	4/4 (100)
Number of litters with at least one pup surviving on postnatal day 4	8	8	7	6	6	3
Mean litter size on postnatal day 0	12.4	10.5	11.6	10.3	10	8.5#
Mean number of males born	6	6.3	6.3	4.5	4.5	4.3
Mean number of females born	6.4	4.3	5.3	5.8	5.5	4.3
Number of pups that died or were terminated moribund during the postnatal period (culls excluded)	1	8 ^c	0	0	0	1

^aNumber of pregnant females, which delivered pups/total copulation positive

^bNumber of females that delivered at least one live pup/total females having pups or evidence of pregnancy.

^cPups were euthanized due to dam's death (chyllothorax) on postnatal day 12.

Table 4Survival, Body Weights, and Feed Consumption of F₁ Rats in the 18-Week Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

Concentration (ppm)	Survival ^b	Initial Body Weight ^a (g)	Final Body Weight ^a (g)	Change in Body Weight ^a (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 13
Males							
0	10/10	45 ± 4	338 ± 7	294 ± 5		9	17
100	10/10	43 ± 3	344 ± 3	301 ± 3	102	8	18
200	10/10	48 ± 2	342 ± 4	294 ± 4	101	9	18
400	10/10	49 ± 2	337 ± 4	288 ± 3	100	9	18
800	10/10	44 ± 2	335 ± 6	291 ± 6	99	8	18
1,600	10/10	43 ± 1	302 ± 5**	259 ± 4**	89	7	17
Females							
0	10/10	47 ± 3	203 ± 4	156 ± 4		9	12
100	10/10	46 ± 3	202 ± 3	156 ± 3	100	8	12
200	10/10	48 ± 2	192 ± 2*	144 ± 2**	95	9	12
400	10/10	49 ± 2	196 ± 2*	147 ± 3**	97	8	12
800	10/10	47 ± 2	190 ± 1**	143 ± 2**	94	9	11
1,600	10/10	44 ± 1	184 ± 2**	140 ± 2**	91	7	11

* Significantly different (P 0.05) from the control group by Williams' test

** P 0.01

^a Weights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day.^b Number of animals surviving 3 months after weaning/number in group at weaning

Table 5

Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for F₁ Rats in the 18-Week Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

	0 ppm	100 ppm	200 ppm	400 ppm	800 ppm	1,600 ppm
No. Examined	10	10	10	10	10	10
Males						
Necropsy Body wt ^a	338 ± 7	344 ± 3	342 ± 4	337 ± 4	335 ± 6	302 ± 5 ^{**}
<i>Liver</i>						
Absolute	11.25 ± 0.21	11.96 ± 0.17 [*]	12.31 ± 0.21 ^{**}	12.53 ± 0.25 ^{**}	13.13 ± 0.34 ^{**}	12.68 ± 0.24 ^{**}
Relative	33.282 ± 0.37	34.754 ± 0.33 [*]	35.998 ± 0.45 ^{**}	37.195 ± 0.63 ^{**}	39.154 ± 0.54 ^{**}	41.972 ± 0.4 ^{**}
<i>Spleen</i>						
Absolute	0.733 ± 0.01	0.780 ± 0.02	0.764 ± 0.02	0.786 ± 0.02	0.799 ± 0.02 [*]	0.799 ± 0.01 [*]
Relative	2.172 ± 0.04	2.267 ± 0.04	2.232 ± 0.05	2.333 ± 0.04 ^{**}	2.383 ± 0.02 ^{**}	2.646 ± .04 ^{**}
<i>Right Testis</i>						
Absolute	1.460 ± 0.02	1.455 ± 0.02	1.448 ± 0.03	1.463 ± 0.01	1.457 ± 0.03	1.381 ± 0.01 [*]
Relative	4.326 ± 0.06	4.232 ± 0.06	4.240 ± 0.11	4.350 ± 0.07	4.350 ± 0.03	4.575 ± 0.05 [*]
Females						
Necropsy Body wt	203 ± 4	202 ± 3	192 ± 2 [*]	196 ± 2 [*]	190 ± 1 ^{**}	184 ± 2 ^{**}
<i>Spleen</i>						
Absolute	0.535 ± 0.01	0.523 ± 0.01	0.528 ± 0.02	0.533 ± 0.01	0.550 ± 0.01	0.579 ± 0.01 [*]
Relative	2.639 ± 0.03	2.596 ± 0.04	2.748 ± 0.1	2.712 ± 0.04	2.897 ± 0.06 ^{**}	3.147 ± 0.05 ^{**}

* Significantly different (P 0.05) from the control group by Williams' or Dunnett's test

** P 0.01

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-bodyweight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error)

Table 6

Fertility, Gestational, Parturition, and F₁ Pup Survival Data to Postnatal Day 20 for Rats in the 2-Year Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

	0 ppm	400 ppm	800 ppm	1,600 ppm
Implantations per dama (n) ^b	10.098 ± 1.239 (41)	8.400 ± 1.219 (40)	9.725 ± 1.134 (40)	10.634 ± 1.105 (41)
Fertility index ^c (% copulation positive)	27/42 (64)	24/41 (59)	26/41 (63)	27/42 (64)
Total resorptions per littera	0.024 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Gestational index ^d (%)	27/27 (100)	24/24 (100)	26/26 (100)	27/27 (100)
Number of litters with at least one pup surviving on postnatal day 1	27	24	26	27
Mean litter size on postnatal day 0 ^a (n)	8.519 ± 0.568 (27)	8.292 ± 0.698 (24)	8.462 ± 0.691 (26)	8.333 ± 0.770 (27)
Mean number of males born	3.9	3.9	4	3.8
Proportion of male pups per dam ^a (n)	0.485 ± 0.036 (27)	0.473 ± 0.037 (24)	0.466 ± 0.036 (26)	0.456 ± 0.046 (27)
Mean number of females born	4.6	4.4	4.4	4.5
Number of males on postnatal day 20 (number needed)	96 (85)	87 (70)	101 (70)	93 (70)
Number of females on postnatal day 20 (number needed)	109 (85)	97 (70)	101 (70)	109 (70)
Number of pups that died or were terminated moribund from postnatal day 1 to postnatal day 6	14	6	5	5
Number of pups that died or were terminated moribund from postnatal day 7 to postnatal day 20	5	2	3	5
Number of litters with at least one pup surviving at postnatal day 21	26	24	25	26

^a Mean ± standard error; Differences from the control group are not significant by Dunn's test.

^b n=Number of dams

^c Number of pregnant females, which delivered pups/total copulation positive

^d Number of females that delivered at least one live pup/total females having pups

Table 7

Incidences of Neoplastic Lesions of the Central Nervous Systems in F₁ Rats in the 2-Year Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

	0 ppm	400ppm	800 ppm	1600 ppm
Males				
<u>Brain</u>^a	50 (25)	50 (21)	50 (22)	50 (25)
Astrocytoma ^{b,c}	0	0	1	1
Granular Cell Tumor ^d	0	0	1	1
<u>Spinal Cord</u>	50 (25)	50 (21)	50 (22)	50 (25)
Astrocytoma ^e	0	0	0	1
Granular Cell Tumor	0	1	0	0
<u>Brain and Spinal Cord</u>	50 (25)	50 (21)	50 (22)	50 (25)
Astrocytoma	0	0	1	2
Granular Cell Tumor	0	1	1	1
Females				
<u>Brain</u>	50 (21)	50 (22)	50 (22)	50 (22)
Glioma, Mixed Cellf	1	1	1	0
Granular Cell Tumor ^g	0	1	0	0
<u>Spinal Cord</u>	50 (21)	50 (22)	50 (22)	50 (22)
Meningioma	0	0	1	0
<u>Brain and Spinal Cord</u>	50 (21)	50 (22)	50 (22)	50 (22)
Glioma, Mixed Cell	1	1	1	0
Granular Cell Tumor or Meningioma	0	1	1	0

^aNumber of animals with tissue examined microscopically (number of litters with animals examined)

^bNumber of animals with neoplasm or lesion (lesions reported were from animals in separate litters)

^cHistorical incidence for 2-year feed studies with controls given NTP-2000 diet (mean $\pm$ standard deviation): 0/199; all routes: 3/1,297 (0.2% $\pm$ 0.7%), range 0%–2%

^dHistorical incidence for feed studies: 2/199 (1.0% $\pm$ 2.0%), range 0%–4%; all routes: 2/1,297 (0.2% $\pm$ 0.8%), range 0%–4%

^eHistorical incidence for feed studies: 0/200; all routes: 1/1,298 (0.1% $\pm$ 0.4%), range 0%–2%

^fHistorical incidence for feed studies: 0/150; all routes: 4/1,250 (0.3% $\pm$ 0.8%), range 0%–2%

^gHistorical incidence for feed studies: 0/150; all routes: 10/1,250 (0.1% $\pm$ 0.4%), range 0%–2%.

Table 8

Incidences of Non-Neoplastic Lesions of the Peripheral Nervous Systems in F₁ Rats in the 2-Year Perinatal and Postnatal Feed Study of Styrene-Acrylonitrile Trimer

	0 ppm	400 ppm	800 ppm	1,600 ppm
Male				
Original Evaluation				
Brain ^a	50 (25)	50 (21)	50 (22)	50 (25)
Astrocytoma ^b	0	0	1	1
Granular Cell Tumor	0	0	1	1
Spinal Cord	50 (25)	50 (21)	50 (22)	50 (25)
Astrocytoma	0	0	0	1
Granular Cell Tumor	0	1	0	0
Enhanced Evaluation				
Spinal Nerve Roots	47	48	50	50
Degeneration, Nerve Fiber	34 (1.0) ^c	37 (1.1)	37 (1.2)	43* (1.3)
Sciatic Nerve	50	50	50	50
Degeneration, Nerve Fiber	37 (1.1)	40 (1.2)	41 (1.3)	43 (1.3)
Spinal Cord	50 (25)	50 (21)	50 (22)	50 (25)
Granular Cell Tumor	0	1	0	0
Female				
Original Evaluation				
Brain	50 (21)	50 (22)	50 (22)	50 (22)
Glioma, Mixed Cell	1	1	1	0
Granular Cell Tumor	0	1	0	0
Enhanced Evaluation				
Spinal Nerve Roots	49	50	50	49
Degeneration, Nerve Fiber	43 (1.2)	40 (1.2)	42 (1.3)	45 (1.5)
Sciatic Nerve	49	49	49	50
Degeneration, Nerve Fiber	28 (1.0)	35 (1.1)	43** (1.1)	40* (1.1)
Spinal Cord	50 (21)	50 (22)	50 (22)	50 (22)
Meningioma	0	0	1	0

* Significantly different (P 0.05) from the control group by the Poly-3 test

** P 0.01

^a Number of animals with tissue examined microscopically (number of litters with animals examined)

^b Number of animals with neoplasm or lesion (number of litters having at least one animal with neoplasm)

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^d Number of animals with neoplasm per number of animals with tissue microscopically examined/number of animals examined.

^e Number of litters with neoplasm/number of litters examined

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

ⁱ Not applicable; no neoplasms in animal group

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