CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

217026Orig1s000

NON-CLINICAL REVIEW(S)

MEMORANDUM

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Food and Drug Administration
Center for Drug Evaluation and Research

Date: March 10, 2023

From: Lois M. Freed, Ph.D.
Director, Division of Pharmacology/Toxicology-Neuroscience
Office of Neuroscience

Subject: NDA 217026 (Daybue, trofinetide)

NDA 217026 was submitted by the sponsor (Acadia Pharmaceuticals) on July 12, 2022, to support marketing of trofinetide for the treatment of Rett syndrome in adults and pediatric patients 2 years of age and older. Trofinetide is to be administered orally twice daily, with dose based on patient weight.

The nonclinical studies conducted on trofinetide to support clinical development and an NDA were reviewed in detail by Dr. Siarey (Pharmacology/Toxicology NDA Review and Evaluation, Richard Siarey, Ph.D., March 10, 2023). Based on his review, Dr. Siarey has concluded that the nonclinical data support approval of the NDA.

Selected nonclinical data are summarized in this memo.

Trofinetide is a chemically modified analog of GPE (glycine-proline-glutamate), an endogenous peptide produced by N-terminal cleavage of IGF-1. The mechanism by which trofinetide exerts therapeutic effects in patients with Rett syndrome is unknown.

The pharmacology of trofinetide was characterized in in vitro and in vivo studies, including one in vivo study in an animal model of Rett syndrome (*MeCP2* knockout mouse). In that study, trofinetide (0 or 20 mg/kg) was administered by intraperitoneal injection daily for 5 or 20 weeks. An increase in long-term potentiation in the CA1 region of the hippocampus, accompanied by increased dendritic length and arborization, was observed after 5 weeks of dosing. After 20 weeks of dosing, there was an increase in 50% survival to 15.5 weeks, compared to 13.5 weeks in controls; however, trofinetide had no functional effects (locomotor activity, grip strength, or general condition).

The PK/ADME of trofinetide was assessed in in vitro and in vivo studies. In in vitro metabolism studies in rat, dog, and human liver microsomes, trofinetide was relative stable for at least 30 min. No major metabolites were detected in human plasma.

Following administration of a single oral dose of ^{14}C-trofinetide (200 mg/kg) to male Sprague Dawley rats, tissue distribution of radioactivity was widespread, with the highest concentrations in gastrointestinal tissue (small and large intestine wall), kidney, and liver; concentrations in brain and spinal cord were below the quantitation limit at all time points (0.25-168 hrs post dose) and only above the quantitation limit in csf at one time point (6 hrs post dose).

The toxicity of trofinetide was tested in Sprague Dawley rat (4- and 26-week) and beagle dog (39-week); trofinetide was administered by oral gavage. The 4-week study was conducted in adult rats. The 26- and 39-week studies were conducted in juvenile animals; dosing was initiated at 2 weeks (postnatal days 13-14) and 5-6 months of age, respectively.

Rat: in the 4-week (+2-week recovery) study, trofinetide was administered at doses of 0, 150, 400, and 700 mg/kg TID (0, 450, 1200, and 2100 mg/kg/day). The only drug-related findings were changes in hematology (decreased hgb and MCHC; increased reticulocyte count) and clinical chemistry (decreased chloride and total protein; increased ALT and cholesterol) parameters at the highest dose tested; no microscopic correlates were detected.

In the 26-week study, trofinetide was administered at doses of 0, 150, 300, and 1000 mg/kg BID (0, 300, 600, and 2000 mg/kg/day). Growth (bone length and density) and neurobehavioral function (locomotor activity, Morris water maze) were assessed, in addition to the standard safety parameters and toxicokinetic analysis. No adverse effects were observed. Plasma exposure (AUC) at the highest dose tested was similar to that those in pediatric patients at recommended doses.

A separate toxicity study was conducted in juvenile rat in order to evaluate potential adverse effects on sexual maturation and reproductive function. In this study, trofinetide was administered at doses of 0, 150, 300, and 1000 mg/kg BID (0, 300, 600, and 2000 mg/kg/day) beginning on postnatal day (PND) 13 or 14 and continuing through PND 70. Sexual maturation (preputial separation or vaginal opening) was assessed beginning on approximately PND 27. On approximately PND 86, offspring were selected for evaluation of reproductive function; selected animals were mated (with untreated animals) approximately 4 weeks after the last dose. Male offspring were sacrificed after the mating period; females were sacrificed on gestation day 14. No adverse effects on sexual maturation or reproductive function were observed. Plasma exposures (AUC) at

the highest dose tested were similar to those in pediatric patients at recommended doses.

Dog: in the 39-week (+ 4-week recovery) study, trofinetide was administered at doses of 0, 25, 150, and 500 mg/kg BID (0, 50, 300, and 1000 mg/kg/day). Although the study was conducted in juvenile dog, no developmental parameters were evaluated. The only notable finding was an increase in watery or soft feces at the highest dose tested, which was associated with plasma exposures (AUC) less than those in pediatric and adult patients at the recommended doses.

A standard battery of reproductive and developmental toxicology studies was conducted in Sprague Dawley rat and New Zealand White rabbit. (Studies in juvenile animals are discussed in the previous section.)

A fertility and early embryonic development study was conducted in rat. Trofinetide was administered orally at doses of 0, 150, 450, and 1000 mg/kg BID (0, 300, 900, and 2000 mg/kg/day) to male and female rats, prior to and throughout mating (with untreated females and males, respectively) and continuing in females through gestation day 7. No adverse effects on fertility or reproductive function were observed. At the highest dose tested, plasma exposures (AUC) were less than that in humans at the maximum recommended (adult) human dose (12000 mg BID or 24000 mg/day).

Embryofetal development studies were conducted in rat and rabbit. Trofinetide was administered orally during the period of organogenesis at doses of 0, 150, 450, and 1000 mg/kg BID (0, 300, 900, and 2000 mg/kg/day) to rats and at doses of 0, 75, 150, and 300 mg/kg BID (0, 150, 300, and 600 mg/kg/day) to rabbits. No adverse effects on embryofetal development were observed in either species. Plasma exposures (AUC) at the highest doses tested were less than that at in humans at the maximum recommended (adult) human dose.

In the pre- and postnatal development study in rat, trofinetide was administered orally throughout pregnancy and lactation at doses of 0, 150, 450, and 1000 mg/kg BID (0, 300, 900, and 2000 mg/kg/day). No adverse effects were observed on pre- and postnatal development. Plasma exposure (AUC) at the highest dose tested was less than that in humans at the maximum recommended (adult) human dose.

A standard battery of genetic toxicology studies was conducted for trofinetide. Trofinetide was negative in in vitro (bacterial reverse mutation, chromosomal aberration in Chinese hamster ovary cells) and in vivo (mouse micronucleus) assays.

Carcinogenicity studies of trofinetide have not been conducted for the NDA, as previously agreed to by the division (Type C meeting minutes, June 22, 2015; email communication, November 10, 2021).

Recommendations

The nonclinical data submitted to the NDA support approval of trofinetide for the proposed indication, with appropriate labeling and post-marketing requirements for carcinogenicity studies in mouse and rat.

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/s/

LOIS M FREED
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DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY NDA REVIEW AND EVALUATION

Application number:	217-026
Supporting document:	001
Applicant's letter date:	July 12, 2022
CDER stamp date:	July 12, 2022
Product:	Trofinetide
Indication:	Rett Syndrome
Applicant:	Acadia Pharmaceuticals Inc
Therapeutic area:	Neurology
Clinical Review Division:	Division of Neurology I
Pharm/Tox Division	Division of Pharm/Tox for Neuroscience (DPT-N)
Reviewer:	Richard Siarey
Supervisor:	Lois Freed
Division Director:	Lois Freed
Project Manager:	Brenda Reggettz

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1 Executive Summary

1.1 Introduction

Trofinetide (NNZ-2566) is a 2-methylproline-substituted analogue of glycyl-L-prolyl-L-glutamate (GPE), a chemically modified peptide of the N-terminal tripeptide cleavage product of insulin-like growth factor-1 (IGF-1). The sponsor has developed trofinetide for oral treatment of adult and pediatric (aged 2 years and older) patients with Rett syndrome.

1.2 Brief Discussion of Nonclinical Findings

No clear mechanism of action has been determined for trofinetide. No significant binding was observed at any target in binding assays or inhibitory effect on of any kinases tested in functional assays. The sponsor hypothesized that trofinetide enhances neuronal synaptic function and morphology based, in part, on data from a mouse model of Rett syndrome. However, although hippocampal synaptic plasticity increased, only a trend for increased dendritic complexity and spine length was observed, and there was no effect on locomotor or respiratory functions.

Safety pharmacology studies were conducted only using IV administration of trofinetide. In rats, no adverse effects on the CNS (up to 350 mg/kg) or respiratory system (up to 700 mg/kg) were observed after IV trofinetide dosing. In dogs, IV infusion of trofinetide (800 mg/kg) had no effects on heart rate or arterial blood pressure; however, the QTcV interval was slightly prolonged by 19 to 33 ms at ≥ 400 mg/kg. The IC_{50} value of trofinetide on hERG currents was estimated at ≥ 20 mM.

Absorption of trofinetide in animals after oral administration was relatively rapid, with T_{max} of approximately 2 hours. In rats, plasma exposure to trofinetide was similar between sexes, with no accumulation observed. [^{14}C]-Trofinetide distributed to most tissues in rats, including the brain. No metabolites were detected in rat bile, feces, urine, plasma, or brain samples after oral administration of [^{14}C]-trofinetide. [^{14}C]-Trofinetide was excreted equally in the urine and feces, but with minimal levels detected in the bile.

In toxicology studies, oral trofinetide was well tolerated up to the high dose, 2000 (1000 mg/kg BID) and 1000 (500 mg/kg BID) mg/kg/day in 26- and 39-week studies in rat and dog, respectively, with no trofinetide-related deaths, minimal clinical signs, no adverse change in body weight, and few microscopic findings noted. In dog, only a decrease in uterine weight, likely due to a tendency for females to be in anestrus, and a stress-related effect on the estrus cycle were noted and considered adverse at the HD (1000 mg/kg/day). These data resulted in NOAELs of 2000 mg/kg/day (1000 mg/kg BID) in juvenile rats, 1000 mg/kg/day (500 mg/kg BID) in M dogs, and 300 mg/kg/day (150 mg/kg BID) in F dogs.

In genetic toxicology studies, trofinetide did not demonstrate mutagenic potential in the Ames assay, structural chromosomal abnormalities in the chromosomal aberration assay, or clastogenic potential at oral doses up to 2000 mg/kg/day in the *in vivo* mouse micronucleus assay.

Fertility was not affected in female or male rats at oral doses up to 2000 mg/kg/day (1000 mg/kg BID). In the rat embryofetal development study (0, 150, 450, and 1000 mg/kg BID, PO), trofinetide was well tolerated, with no unscheduled deaths, adverse clinical signs, changes in body weight and food consumption, or adverse findings on ovarian, uterine, or litter parameters, and no increase in malformations. The fetal NOAEL was established at 2000 mg/kg/day. In the rabbit embryofetal development study (0, 75, 150, and 300 mg/kg BID), abortions occurred in 1 control and 2 HD dams and were associated with severe reductions in food consumption and body weight loss. In dams, slight increases in early resorptions and post-implantation loss were noted at the HD, but with a minimal decrease in live births, no dead fetuses, and similar incidences of malformations, suggesting no adverse findings were observed on ovarian, uterine, or litter parameters resulting in a fetal NOAEL at 600 mg/kg/day. In the rat pre- and postnatal development study (0, 150, 450, and 1000 mg/kg BID), no trofinetide-related deaths, adverse clinical signs, or changes in body weight were observed, and reproductive parameters were comparable among all groups. In F_1 animals, all endpoints post-weaning were comparable among groups. Therefore, trofinetide was well tolerated, and the NOAEL for postnatal development of offspring and reproductive performance was 2000 mg/kg/day.

In two 13-week toxicity studies to qualify impurities, there were no substantial differences between the groups dosed with trofinetide with or without impurities, suggesting that the impurities at the levels dosed did not cause toxicity. However, impurities, (b) (4) were not qualified at the sponsor's initially proposed acceptance levels. Subsequently in a later submission (January 29, 2023) the sponsor reduced the acceptance criteria to acceptable levels. Four extractable/leachable compounds have limits of detection (LOD) above the analytical evaluation thresholds (AETs) which could results in a dose higher than the threshold for an impurity with genotoxic concern. The sponsor demonstrated these compounds were negative for mutagenicity based on citations and (Q)SAR analyses, which was confirmed by the Agency's Computational Toxicology Consultation Service (CTCS).

1.3 Recommendations

1.3.1 Approvability

Studies submitted to support the NDA for trofinetide included 4-, 26- and 39-week oral and IV toxicology studies in juvenile rat and dog, and reproductive and developmental studies in rats and rabbits were adequate. Few toxicity signals were observed with trofinetide administration. Therefore, after review of the studies submitted in the original NDA, it is recommended, from a nonclinical perspective, that trofinetide be approved.

1.3.2 Additional Nonclinical Recommendations

PMRs/PMCs

As previously communicated with the sponsor (correspondence dated November 10, 2021), carcinogenicity in two species will be needed but may be conducted post-approval.

1.3.3 Labeling

Suggested edits to the sponsors proposed nonclinical sections of the label were communicated with the sponsor.

2 Drug Information

2.1 Drug

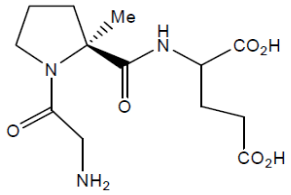
Generic Name: Trofinetide

Code Name: trofinetide

Chemical Name: Glycyl-L-2-methylprolyl-L-glutamic acid

Molecular Formula/Molecular Weight: $C_{13}H_{21}N_3O_6$ / 315.3 g/mol

Structure or Biochemical Description:



Pharmacologic Class: Unknown.

2.2 Relevant INDs, NDAs, BLAs and DMFs

(b) (4)

IND 114,319 (DN1, to improve functional outcomes in patients with Rett Syndrome; active)

(b) (4)

2.3 Drug Formulation

The formulation of trofinetide is an oral solution.

2.4 Comments on Novel Excipients

There are no issues with the excipients.

2.5 Comments on Impurities/Degradants of Concern

The Product Quality reviewers noted that the proposed specification of 9 impurities have acceptance limits above the (b) (4)% qualification threshold, and 4 extractable/leachable compounds had limits of detection (LOD) above the analytical evaluation thresholds (AETs). The sponsor claimed that the 9 impurities with acceptance limits above (b) (4)% were qualified at the proposed levels based on margins calculated using two 13-week toxicology studies using batches of trofinetide spiked with the impurities. However, the sponsor calculated margins of the 9 impurities normalized to body weight (mg/kg) instead of normalized to body surface area (mg/m^2). When margins are normalized to body surface area the following impurities, (b) (4), are not qualified at the proposed acceptance levels. The sponsor demonstrated that the 4 extractable/leachable compounds were negative for mutagenicity, which was confirmed by the Agency's CTCS.

2.6 Proposed Clinical Population and Dosing Regimen

The proposed clinical population for trofinetide are adult and pediatric (aged 2 years and older) individuals with Rett syndrome. The recommended dose is BID according to body weight set out in the sponsor's table below using an oral solution of 200 mg/mL.

Patient Weight	DAYBUE Dose
9 kg to <12 kg	25 mL twice daily
≥12 kg to <20 kg	30 mL twice daily
≥20 kg to <35 kg	40 mL twice daily
≥35 kg to <50 kg	50 mL twice daily
≥50 kg	60 mL twice daily

2.7 Regulatory Background

A pre-IND meeting for IND 114319 was held with the sponsor on May 1, 2012 (meeting minutes; May 10, 2012), with the original IND received on November 21, 2012, and allowed to proceed (Agency Letter, February 8, 2013). Fast Track designation was granted (Agency Letter, June 4, 2013); however, Breakthrough Therapy designation was denied (Agency Letters, February 24, 2015, June 20, 2017, and September 29, 2017).

3 Studies Submitted

3.1 Studies Reviewed

Pharmacology, safety pharmacology, PK/ADME, toxicology, and genetic toxicity studies were submitted to the NDA. Pivotal toxicology and genetic toxicity studies are listed below.

Toxicology - Repeat-Dose Toxicity

- Study №: 0621-16031. 26-Week GLP toxicity study of trofinetide (NNZ-2566) in rats dosed orally from 2 to 28 weeks of age.
- Study №: 0621-16032. Thirty-nine week oral GLP toxicity study of trofinetide in dogs with a four-week recovery period.

Toxicology - Genotoxicity

- Study №: AMS00605. Evaluation of trofinetide in the salmonella typhimurium-Escherichia coli reverse mutation (Ames) assay.
- Study №: CAB00505. In vitro mammalian chromosome aberration assay in Chinese hamster ovary cells challenged with trofinetide.
- Study №: MNA00506. Evaluation of trofinetide in the mouse bone marrow micronucleus assay.
- Study №: MNA00207. Evaluation of trofinetide in the mouse bone marrow micronucleus assay.

Toxicology - Developmental, Reproductive, and Juvenile Toxicity

- Study №: 20202588. An oral (gavage) fertility and early embryonic development study of trofinetide in rats.
- Study №: 0621-16030. GLP fertility and early embryonic development study of trofinetide (NNZ-2566) in rats dosed orally from 2 to 12 weeks of age.
- Study №: 20222415. An oral (gavage) embryo-fetal development study of trofinetide in rats.
- Study №: 20222417. An oral (gavage) embryo-fetal development study of trofinetide in rabbits.
- Study №: 20222418. An oral (gavage) developmental and perinatal/postnatal reproduction study of trofinetide in rats, including a postnatal behavioral/functional evaluation.

3.2 Studies Not Reviewed

Pharmacokinetics (ADME)

- Study №: XT180155. LC-MS/MS method qualification of trofinetide for in vitro studies.
- Study №: 1000-161920-1. Quantification of trofinetide (NNZ-2566) in stablized lithium heparinized rat whole blood by LC-MS/MS.
- Study №: 1000-202181-1. Bioanalytical method validation report for the quantification of trofinetide in lithium heparinized treated rabbit lysed whole blood by LC-MS/MS.

- Study №: 1000-05856-4. Quantification of trofinetide (NNZ-2566) in stabilized lithium heparinized dog whole blood by LC-MS/MS.

3.3 Previous Reviews Referenced

Application	Reviewer	Date in DARRTS
(b) (4)		

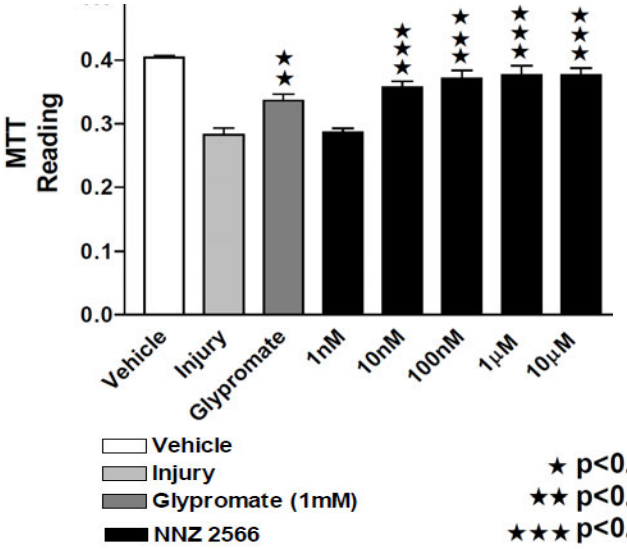
4 Pharmacology

Trofinetide displayed no significant binding at any target (> 80) in in vitro binding assays or inhibitory effects on kinases (including Akt, CaMK2α, IKKα, IRK, MAPK, mTOR, and RAF-1) or histone-deacetylase enzymes (HDAC1-4) in functional assays. Trofinetide is an analog of the cleavage product of IGF-1, therefore, an interaction with the IGF-1 receptor may be expected, but glycyl-L-prolyl-L-glutamate (GPE) does not bind to the IGF-1 receptor (Sara et al. 1993, Annals NY Acad of Sci 692, 183–191), and the sponsor states that GPE and analogs of GPE do not interact with the IGF-1 receptor. However, in vitro binding of trofinetide at the IGF-1 receptor was not assessed.

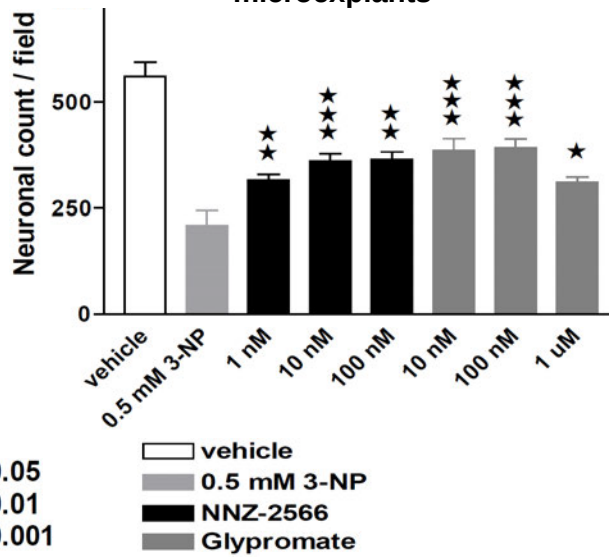
In vitro assays were conducted to investigate whether trofinetide may have neuroprotective effects. Okadaic acid- and 3-nitropropionic acid-induced cell death of striatal cultures and cerebellar microexplants, respectively were reduced in the presence of trofinetide. The neuroprotective effect of trofinetide was observed between 1 nM to 10 µM.

Figure: trofinetide effects on induced injury (Sponsor's)

Oxidative acid injury of striatal cultures



3-Nitropropionic acid injury of cerebellar microexplants



In vivo: Effect of trofinetide in MeCP2-null mutant mice (model of Rett syndrome)

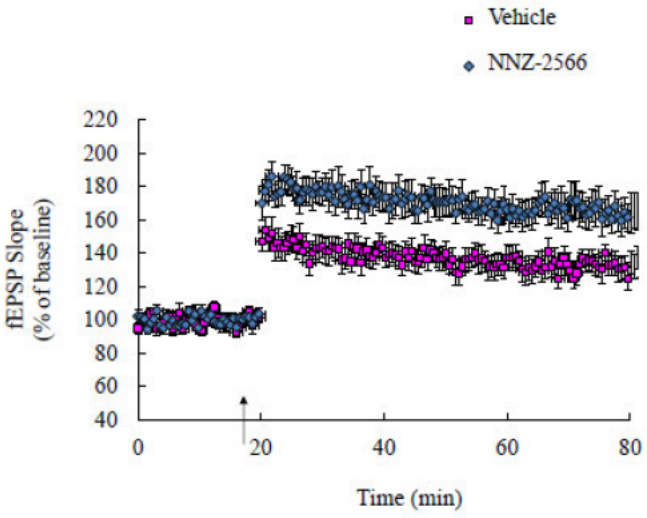
The effects of trofinetide (20 mg/kg/day IP) were investigated in a model of Rett syndrome the MeCP2$^{-/y}$ knockout mouse. Dosing was initiated in animals at 4 weeks of age; animals were sacrificed at 9 weeks of age, and basal transmission and CA1 long-term potentiation (LTP; induced by θ-burst stimulation) were assessed in the hippocampus. Golgi staining was used to examine dendritic spine density, spine length, and arborization. In a second experiment, animals were dosed for 20 weeks, with functional assessments of the respiratory system conducted in Weeks 7, 9, 11, 13, 15, and 17 and motor function tested in Week 13.

Neuronal connectivity

After 5 weeks of trofinetide dosing, hippocampal CA1 LTP was increased. Changes in numbers of dendritic spines were not significant, but a trend for increased dendritic complexity and spine length was observed.

Figure: changes in hippocampal LTP and dendrites after trofinetide dosing (Sponsor's)

LTP



Dendrite Morphology

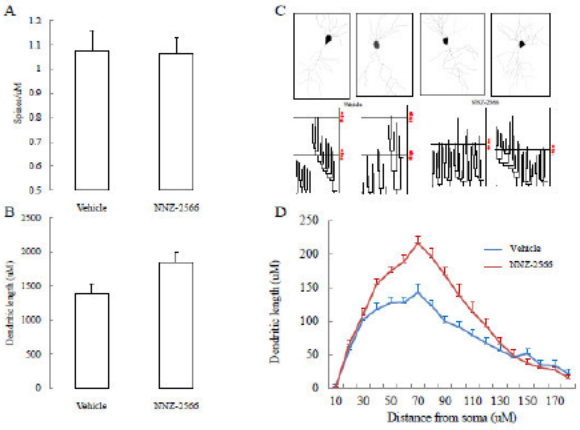


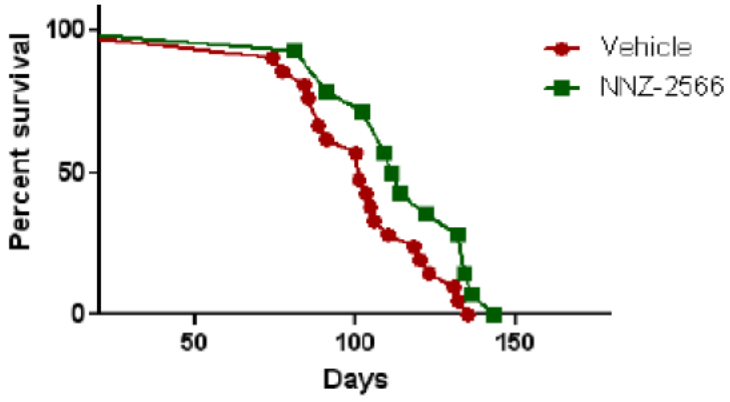
Figure 2. Effect of administration of NNZ-2566 20 mg/kg i.p. for 9 weeks in male *MeCP2* mice. N = 3 to 10 per group. Data are mean ± sem for (A) spine density, (B) spine length and (D) spine length plotted against distance from soma. Dendritic arborisation (C) is slightly improved after treatment.

Survival and motor function

In the 20-week study, survival was slightly increased, and tremors were significantly increased. At 13 weeks, locomotor activity was decreased in MeCP2 knockout mice compared to wild type mice. Trofinetide had no effect on the decreased locomotor activity observed in MeCP2 knockout mice or on scores for general condition, gait, or grip strength.

Figure: increased survival and tremors after trofinetide dosing (Sponsor's)

Survival



Tremors

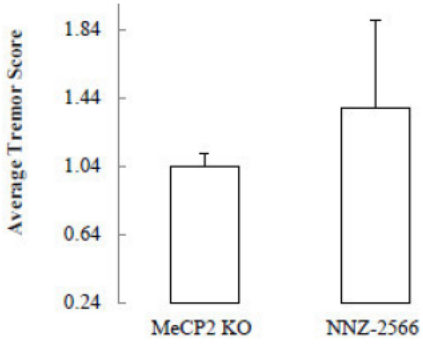
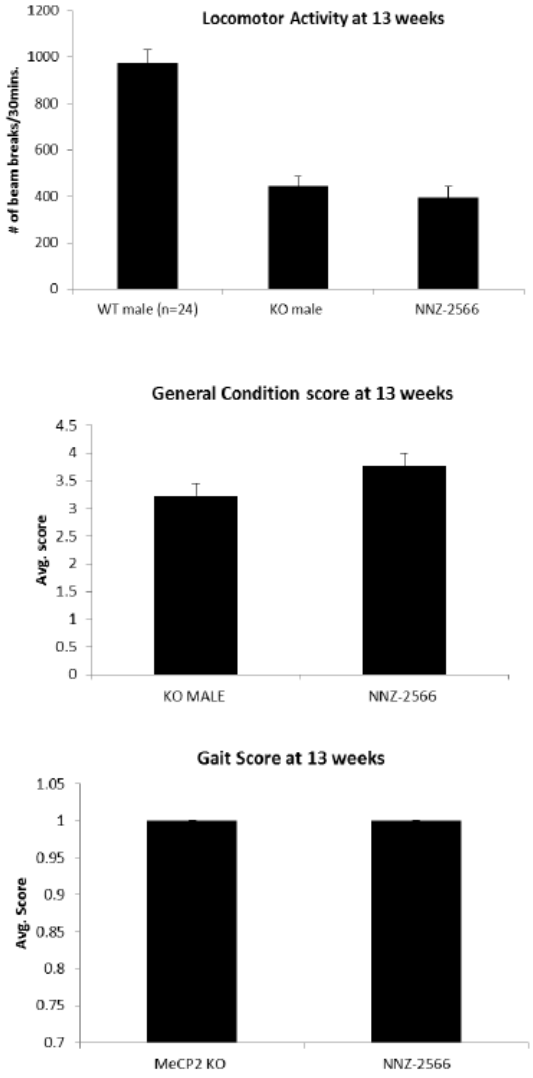


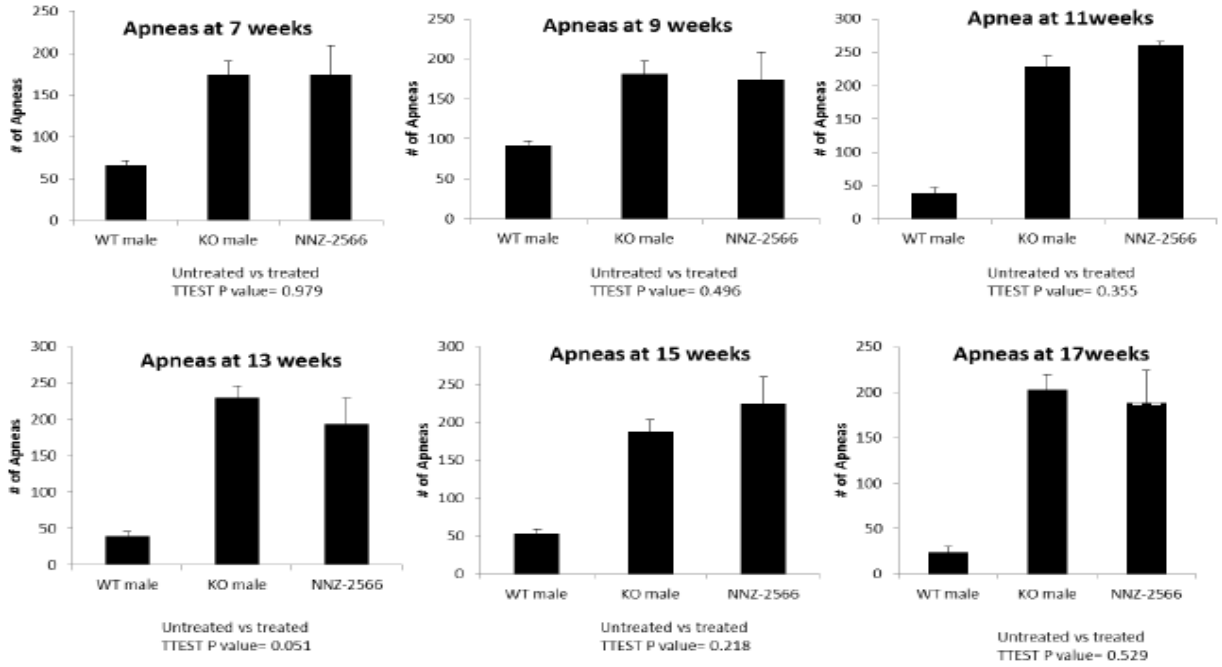
Figure: no change in motor function after trofinetide dosing to MeCP2 knockout mice (Sponsor's)



Respiratory function

In the 20-week study, trofinetide had no effect on apneas.

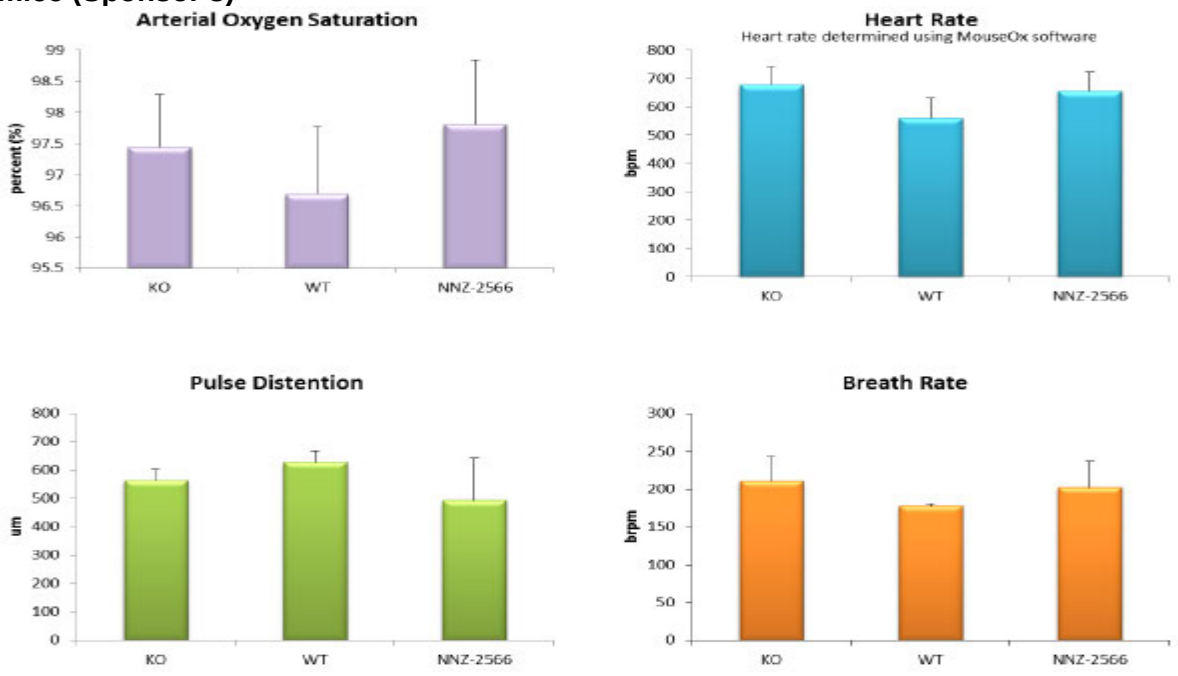
Figure: no change in respiratory function after trofinetide dosing to MeCP2 knockout mice (Sponsor's)



Autonomic function

No change was observed in arterial oxygen saturation, heart rate, pulse distension, or respiratory rate.

Figure: no change in autonomic function after trofinetide dosing to MeCP2 knockout mice (Sponsor's)



4.3 Safety Pharmacology

Neurological

The Effects of NNZ-2566 in the Irwin Test in Male Sprague Dawley Rats Given Single Intravenous (Bolus) Doses. (*Study №: KCM-2005-1428-SPC [SP105-023]*)
M Sprague-Dawley rats (6/group) were assessed for neurofunctional change using the Irwin test, after a single IV bolus injection of 0, 100, 200, or 350 mg/kg trofinetide or 2 mg/kg chlorpromazine (positive control). Observations were conducted at 0-5, 20, 60, and 120 minutes post-dose.

No effects on Irwin test parameters were noted at the MD. Animals dosed with chlorpromazine displayed the expected behavioral effects including decreased locomotor activity, alertness, startle response, touch response, body tone, and grip strength. Trofinetide displayed no significant adverse effects on functional neurological parameters.

Cardiovascular

Effects of NNZ-2566 on Cloned hERG Channels Expressed in Mammalian Cells. (*Study №: 050627.OQM*)
Investigation of the effect of trofinetide on the hERG current was conducted on hERG channels expressed in HEK293 cells. Trofinetide was tested at 9.86 and 98.56 µM (n = 3 cells/concentration). Terfenadine (0.06 µM; 2 cells) was the positive control.

Trofinetide did not inhibited the hERG current greater than 2%. Therefore, IC_{50} values were not calculated. Terfenedine inhibited the hERG current by 87% with results consistent with historical control data.

Effects of NNZ-2566 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells. (*Study №: 061211.OQM*)
Investigation of the effect of trofinetide on the hERG current was conducted on hERG channels expressed in HEK-293 cells. Trofinetide was tested at 20 mM (n = 4 cells). Terfenadine (0.06 µM; 2 cells) was used as the positive control.

Trofinetide at 20 mM inhibited the hERG current by 6%. The IC_{50} value was estimated at ≥ 20 mM. Terfenedine inhibited the hERG current by 83%.

Cardiovascular Effects of NNZ-2566 in Conscious, Telemetered Beagle Dogs. (*Study №: GBH5000*)
The effect of IV infusion (over 2 hours) of trofinetide (0, 120, 240, 400, or 800 mg/kg) on cardiovascular function was assessed in 6 M Beagle dogs, with a washout period of 7 days between doses. Blood pressure, heart rate, and ECG parameters were analyzed from 30 minutes pre-dosing and continued to 22 hours post-dose. Blood samples were collected to determine plasma trofinetide exposure at -5, 10, 30, 60, 90, 120, 150, 180, 210, and 240 minutes after start of the infusion.

Clinical signs included lip licking and tremors at all dose levels, subdued behavior at the HMD and HD, and pupil dilation at the HD. The sponsor suggested that the clinical

signs were due to hyperosmolarity of the drug solution. No effects on heart rate, arterial blood pressure, RR interval, PR interval, and QRS duration were observed. At 400 and 800 mg/kg, the QTcV interval (Van de Water method) was prolonged by 19-33 ms (7-14%) at 90 to 120 minutes post-dose. Mean peak plasma levels of trofinetide for these findings was 568 and 933 µg/mL, respectively.

Table: summary of trofinetide TK (Sponsor's)

Day	Dose (mg/kg/hr)	C_{inf} (µg/mL)	C_{max} (µg/mL)	t_{max}~ (hr)	AUC_{0-t} (hr*µg/mL)	AUC (hr*µg/mL)	$t_{1/2}$ (hr)
8	60	155 (22.6)	158 (20.5)	1.99 (1.05-2.02)	329 (14.1)	358 (16.4)	0.802 (12.4)
15	200	568 (14.6)	568 (14.6)	2.00 (2.00-2.02)	1190 (15.2)	1250 (10.4) [5]	0.843 (5.64) [5]
22	120	335 (14.2)	335 (14.2)	2.02 (2.00-2.03)	714 (11.9)	764 (12.5)	0.712 (29.1)
29	400	828 (31.4)	933 (13.9)	1.99 (1.50-2.00)	2000 (15.7)	2190 (18.2)	0.833 (11.5)

N = 6; unless otherwise stated [N].
~ median (range).

Respiration
Effects of NNZ-2566 on Respiration Rate and Tidal Volume in Conscious Rats. (*Study №: ZNA21053.001*)
The effect of an IV dose of trofinetide (0, 175, 350, and 700 mg/kg) or 10 mg/kg morphine (positive control) on respiration was assessed in M Sprague-Dawley rats (8/group). Respiratory parameters were measured pre-dose, and at 15 and 30 minutes post-dose using plethysmograph chambers.

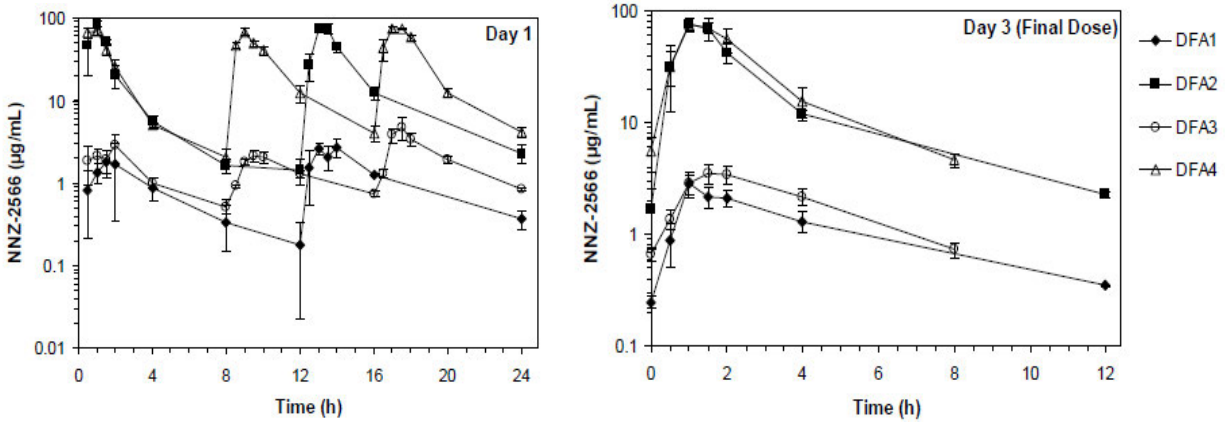
No effects on tidal volume or respiratory rate were observed up to 30 minutes post-dose at any dose of trofinetide, with the NOAEL determined as 700 mg/kg. The positive control induced a decrease in respiration rate at 15 minutes post-dose and decrease in tidal volume at 15 and 30 minutes post-dose, relative to the negative control.

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

Absorption
Repeat dose oral GLP pharmacokinetic study of NNZ-2566 in the rat. (*Study №: 0621-11118*) (b) (4)
PK evaluation of BID (12 hours apart) and TID (8 hours apart) oral doses of trofinetide (30 and 300 mg/kg; i.e., 60 and 600 mg/kg/day and 90 and 900 mg/kg/day) was assessed in rats (6/group). For BID administration, blood samples were taken pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 8, and 12 hours post-dose for both doses on Day 1 (the pre-dose sample for the second dose was the 12 hour post-dose sample of the first dose), and at the same sampling times after the final (sixth dose) on Day 3. For TID administration, blood samples were taken pre-dose and at 0.5, 1, 1.5, 2, 3, 4, and 8 hours post-dose for all three doses on Day 1 (the pre-dose sample for the second and third doses was the 8 hour post-dose sample of the previous dose), and at the same sampling times after the final (ninth dose) on Day 3.

Figure: blood trofinetide concentrations (Sponsor's)



T_{max} occurred by 2 hours at all doses, suggesting that trofinetide was rapidly absorbed. The regimen of dosing did not influence C_{max}, as C_{max} did not differ between BID and TID dosing, or with repeated doses. An initial accumulation occurred, as AUC values increased after the first dose but were similar between the second and last dose, for both dosing regimens. Both C_{max} and AUC values increased more than dose-proportionally. As expected, the AUC value on Day 1 was higher with TID dosing (90 and 900 mg/kg/day) than with BID dosing (60 and 600 mg/kg/day), but increased dose-proportionally.

Table: PK parameters of trofinetide after BID administration (Sponsor's)

Group	Parameter	Day 1			Day 3
		Dose 1 0-12 h	Dose 2 12-24 h	Total 0-24 h	Dose 6 0-12 h
DFA1 30 mg/kg q12h	Cmax (µg/mL)	1.83	2.72	2.72	2.87
	Tmax (h)	1.5	2	14	1
	AUC (µg*h/mL)	8.43	14.5	22.9	13.5
	t½ (h)				3.96
	R^2				0.995
DFA2 300 mg/kg q12h	Cmax (µg/mL)	84.7	75.4	84.7	76.1
	Tmax (h)	1	1.5	1	1
	AUC (µg*h/mL)	143	217	360	211
	t½ (h)				2.57
	R^2				0.940

AUC interval is defined by dose and day: on Day 1, AUC(0-12) for Dose 1, AUC(12-24) for Dose 2, and AUC(0-24) for total; on Day 3 AUC(0-12) for Dose 6

Table: PK parameters of trofinetide after TID administration (Sponsor's)

Group	Parameter	Day 1				Day 3
		Dose 1 0-8 h	Dose 2 8-16 h	Dose 3 16-24 h	Total 0-24 h	Dose 9 0-8 h
DFA3 30 mg/kg q8h	Cmax (µg/mL)	2.84	2.12	4.77	4.77	3.51
	Tmax (h)	2	1.5	1.5	17.5	1.5
	AUC (µg*h/mL)	10.6	10.6	17.0	38.2	16.3
	t½ (h)					2.66
	R^2					0.999
DFA4 300 mg/kg q8h	Cmax (µg/mL)	70.6	67.5	74	74	74.6
	Tmax (h)	1	1	1.5	17.5	1
	AUC (µg*h/mL)	141	178	215	534	213
	t½ (h)					1.75
	R^2					0.958

AUC interval is defined by dose and day: on Day 1, AUC(0-8) for Dose 1, AUC(8-16) for Dose 2, AUC(16-24) for Dose 3, and AUC(0-24) for total; on Day 3 AUC(0-8) for Dose 9

Distribution:

Minimal protein binding of trofinetide was detected in human plasma (4-6%) and to human serum albumin (4%-10%). In Sprague-Dawley rats, a single oral dose of 200 mg/kg trofinetide resulted in wide tissue distribution, with highest radioactivity levels detected in the small intestine wall, large intestine wall, and kidney. In Long-Evans rats, a single IV dose of 20 mg/kg [^{14}C]-trofinetide had no apparent preferential affinity for pigmented tissues. Low levels of radioactivity was detected in cerebrospinal fluid after a single oral dose of 200 mg/kg [^{14}C]-trofinetide, suggesting that trofinetide crosses the blood brain barrier, although at much lower levels than in plasma and other body organs.

Metabolism:

In vitro

The metabolic stability of trofinetide was evaluated in rat, dog, and human liver microsomes, and rat, dog, and human blood. In rat, dog, and human microsomes, $t_{½}$ was greater than 30 minutes with 74%, 83%, and 77% trofinetide remaining, respectively after a 30-minute incubation in 10 µM trofinetide. In rat, dog, and human blood, $t_{½}$ was greater than 60 minutes with 91%, 75%, and 44% trofinetide remaining, respectively after a 60-minute incubation in 20 µM trofinetide. In hepatocytes, no metabolites were observed after 4-hour incubation in 5 µM trofinetide.

In vivo

In a study with intact and bile duct canulated rats, no metabolites were detected in bile, feces, urine, plasma, or brain samples after a single oral dose of 200 mg/kg [^{14}C]-trofinetide, with only trofinetide detected in the samples.

Excretion:

In rats, a single oral dose of 200 mg/kg [^{14}C]-trofinetide was excreted approximately equally in urine (55%) and feces (44%), with minimal (< 0.2%) detected in bile.

Effects on CYP enzyme activity:
No appreciable effects of trofinetide on any CYP enzyme activity were observed, with IC_{50} values of CYPs 1A2, 2B6, 2C8, 2C19, 2D6, and 3A4/5 (by testosterone hydroxylation) from human liver microsomes greater than 4800 µM. In human hepatocytes, trofinetide treatment was associated with 2- to 3-fold increases in mRNA levels for CYPs 1A2, 2B6, and 3A4.

6 General Toxicology

Several IV toxicology studies were conducted in rats and dogs. These studies were conducted mainly for other INDs for other indications (see Section 2.2). Only those studies that are relevant for this application are included here.

6.1 Single-Dose Toxicity

6.1.1 Study Title: NNZ-2566: An oral dose range-finding de-escalation toxicity and toxicokinetic study in dogs (non-GLP)

Study no.: 2084-004
Drug, lot, and % purity: trofinetide (Lot No. 8007-A-R0-02-48-01, 99.5%).
Vehicle: Sterile Water for Injection
GLP compliance: No

Methods:

Doses: 0, 2000, and 600 (300 BID) mg/kg
Species/strain: Dog/Beagle
Route: Oral
Number/sex/group or time point (main study): 2/sex/group.
Duration/regimen: All dogs received control and both doses, with 1-3 days washout between doses.

Results:

- No animals were found dead or euthanized early.
- At 2000 mg/kg, panting and/or emesis were noted on Day 1, and fecal discoloration, feces soft/mucoid, loss of skin elasticity, and skin discoloration were noted on Day 2. At the LD, skin discoloration was noted after the first dose in M. Discoloration was also noted on Day 2 in both sexes.
- The MTD with oral administration was between 600 and 2000 mg/kg/day

6.2 Repeat-Dose Toxicity

6.2.1 Study Title: Repeat dose oral toxicity bridging study of NNZ-2566 in rats.

(b) (4)

Study no.:	2329-001
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	June 28, 2011
GLP compliance:	Yes
Drug, lot #, and % purity:	Trofinetide, Lot №'s: 6007-B-R1-03-93-01, 99.2% and 6007-B-R1-04-93-01, 99.1%

Key Study Findings

- Trofinetide was well tolerated at all doses, with no deaths or clinical signs.
- Histopathological findings in these tissues included mild liver necrosis and vacuolation, and minimal-mild hyperplasia of mucous-associated lymphoid tissue of the GI tract in control and dosed animals.
- The HD is the NOAEL as no clinical signs were observed and few histopathological changes detected. Plasma exposure ($AUC_{0\text{-}8h}$) following a single trofinetide dose at 700 mg/kg on Day 28 was 380 and 570 µg*h/mL in M and F, respectively.

Methods

Doses:	0, 150, 300, and 700 mg/kg
Frequency of dosing:	TID (every 8 hours)
Route of administration:	Oral gavage
Dose volume:	5 mL/kg
Formulation/Vehicle:	Sterile water for injection
Species/Strain:	Rats Crl:CD(SD)
Number/Sex/Group	
Main:	10
Toxicokinetic:	3 control and 9 LD, MD and HD
Recovery:	5 control and HD
Age:	7-8 weeks of age at initiation of dosing
Weight:	Males 157-175 g; Females 134-157 g

6.2.2 Study Title: 26-Week GLP toxicity study of trofinetide (NNZ-2566) in rats dosed orally from 2 to 28 weeks of age.

Study no.:	0621-16031
Study report location:	EDR
Study initiation date:	April 24, 2018
Conducting laboratory and location:	(b) (4)
Duration:	26
Duration Units:	weeks
GLP compliance:	Y, US 21 CFR, Part 58
Drug, lot #, and % purity:	NNZ-2566, Lot №: R0006475, 97.3%; NOR-C-94-1, 99.1%

Key Study Findings

- Several deaths occurred in all groups, none were related to trofinetide, and no adverse clinical signs were observed.
- Trofinetide led to faster growth (increased mean body weight) and increased food consumption at all doses in M and in MDF and HDF.
- No adverse histopathological changes were observed.
- No adverse effects were observed in locomotor activity, spatial learning and memory, and auditory startle reflex.
- There was an increase in somatotropin and a decrease in IGF binding protein 3 (IGFBP-3) in HDM. No increase in IGF-1 was observed.
- In the femur, there was no reduction in bone mineral density (BMD) and content (BMC), or bone area (BA), compared to controls, an increase in BMD and BMC occurred in HDM and HDF in the proximal and whole bone and, generally, increases in trabecular bone parameters were noted in HDM and HDF.
- The HD (2000 mg/kg/day) was the NOAEL, associated with plasma exposures (AUC) to trofinetide on Day 183 of 1690 and 1710 h*µg/mL in M and F, respectively.

Methods

Doses:	0, 150, 300, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Number/Sex/Group	
Main:	15
Recovery:	15
Toxicokinetics:	6 control and 24 LD, MD, and HD
Dose volume:	8 mL/kg/dose
Formulation/Vehicle:	Deionized water
Route of administration:	ORAL GAVAGE
Species:	RAT
Strain:	SPRAGUE-DAWLEY
Age / Sexual Maturity:	14 Days at first dose (20-57 g)
Comment on Study Design and Conduct:	On Day 15 (~PND 28), group sizes were reduced to 32/sex/group. On Day 86, group sizes were reduced to 30/sex/group by removing 2/sex/group, except for HDF. An attempt was made to cull rats equally among litters. The HD is above the recommended limit dose.

Observations and Results

Mortality

Observations for mortality and morbidity were conducted twice daily.

Several early deaths (1 control M, 5 control F, 2 LDM, 3 LDF, 1 HDM, and 2 HDF) occurred, as outlined in the table below. No deaths were considered related to trofinetide.

Table: mortalities (Sponsor's)

Noteworthy Findings in Rats Where Death was Unrelated to Trofinetide					
Animal No.	**Total Daily Dosage / BID Dosage (mg/kg)**	**Study Day**	**Status**	**Postmortem Findings**	**Likely Contributing Cause of Death**
1F186	0 (vehicle) / 0 (vehicle)	2	FD	None	Not determined
1F188	0 (vehicle) / 0 (vehicle)	6	FD	Not recorded	Esophageal perforation
1M006	0 (vehicle) / 0 (vehicle)	6	FD	White clotted material in thoracic cavity	Esophageal perforation
1F179	0 (vehicle) / 0 (vehicle)	7	FD	Esophageal perforation; White clotted material in thoracic cavity	Esophageal perforation
1F193	0 (vehicle) / 0 (vehicle)	7	Missing	Carcass cannibalized	Unknown
1F170	0 (vehicle) / 0 (vehicle)	105	NMS	Discolored right cervical lymph node; Mass, right neck	Sacrificed for humane reasons; malignant basal cell carcinoma
2F226	300 / 150	2	Moribund	Dark lung lobes	Esophageal perforation
2F211	300 / 150	6	FD	Most of carcass cannibalized	Not determined
2F216	300 / 150	8	FD	Esophageal perforation	Esophageal perforation
2M048	300 / 150	120	FD	None	Not determined
2M069	300 / 150	165	FD	None	Not determined
4F301	2000 / 1000	17	Moribund	Foamy material in lumen of trachea; dilated ventricles of cerebrum; discolored mottled lungs	Not determined[a]

Noteworthy Findings in Rats Where Death was Unrelated to Trofinetide					
Animal No.	Total Daily Dosage / BID Dosage (mg/kg)	Study Day	Status	Postmortem Findings	Likely Contributing Cause of Death
4F315	2000 / 1000	85	NMS	Malocclusion; thickened hard palette	Not determined[b]
4M137	2000 / 1000	157	Moribund	Discolored right lung and left caudal lung lobe	Misdosing or aspiration[c]
5M322	0 (vehicle) / 0 (vehicle)	178	FD	None	Unknown

BID = Twice daily dosing

FD = Found Dead; SM = Sacrificed Moribund; NMS = Non-moribund sacrifice

[a] Female 4F301 was evaluated for decreased activity and unkempt appearance. Examination revealed delayed righting reflex and difficulty rising and ambulating on forelimbs bilaterally (gait was unsteady). The rat was reluctant to move. A blood sample taken at euthanasia revealed the following noteworthy findings: severe dehydration (hematocrit = 73%), low lymphocyte count (2460/µL), high reticulocyte count (1253 x 10^3/µL)

[b] For female 4F315, the only noteworthy finding in a blood sample taken at euthanasia was neutrophilia (4050/µL). Sacrificed for humane reasons; unkempt appearance and malocclusion of the teeth.

[c] Male 4M137 had wetness around the mouth and labored breathing on Day 157. A blood sample taken at euthanasia revealed the following noteworthy findings: dehydration (hematocrit = 59%), low lymphocyte count (2170/µL), high reticulocyte count (363 x 10^3/µL)

Clinical Signs

Detailed observations were conducted at least once daily from Day 1 until the day of necropsy.

Clinical observations included soft/watery feces in 14 HDM and 2 HDF and unkempt appearance in 2 HDF. These clinical signs recovered during the recovery period.

Body Weights

Body weights were recorded twice weekly prior to initiation of dosing (Days -2 and -1), twice weekly until Day 91, and weekly thereafter.

Mean body weights increased in all trofinetide dosed groups, with 10% and 18% increases in the HDM and HDF groups. Body weight in HD animals was still increased at the end of the recovery period.

Table: body weights (Sponsor's)

	Males				Females			
Dose (mg/kg/day)	0	300	600	2000	0	300	600	2000
Dosing period – all rats								
Day 7 (g)	39	42	41	40	37	40	40	40
Day 182 (g)	704	751	749	776*	386	396	410	455**
Vs. control group	---	**+7%**	**+6%**	**+10%**	---	+3%	**+6%**	**+18%**
Change								
Absolute (g)	+665	+709	+708	+736*	+348	+356	+370	+415**
Vs. control group	---	**+7%**	**+7%**	**+11%**	---	+2%	**+6%**	**+19%**
Recovery period – recovery rats								
Day 182 (g)	702	733	730	808**	386	387	410	457**
Day 210 (g)	734	761	761	850**	393	402	418	474**
Vs. control group	---	+4%	+4%	**+16%**	---	+2%	+6%	**+21%**
Change (g)								
Absolute	+32	+28	+31	**+42**	+7	+15	+8	**+17**
Vs. control group	---	-13%	-3%	**+27%**	---	+103%	+5%	**+143%**

* $p < 0.05$; ** $p < 0.01$

Food Consumption

Food consumption was recorded weekly from day of weaning until the end of the study.

Food consumption was increased during the dosing period by 11% and 13% in HDM and HDF, respectively.

Ophthalmoscopy
Examinations of animals were conducted during dosing on Day 183 and at the end of the recovery period on Day 205.

No adverse ophthalmoscopic changes were noted.

Hematology
Blood samples were taken at scheduled euthanasia.

In F, there was a decrease in RBC count and increases in MCV and MCH at the MD and HD and an increase in reticulocytes at the HD. These changes were not considered adverse.

Table: hematology changes (Sponsor's)

	Males				Females			
Dose (mg/kg/day)	**0**	**300**	**600**	**2000**	**0**	**300**	**600**	**2000**
RBC count								
End of dosing								
Mean ($X10^6$/µL)	9.11	9.08	8.71	8.75	8.67	8.76	8.19*	8.25*
Vs. control group	---	0%	**-4%**	**-4%**	---	+1%	**-6%**	**-5%**
End of recovery								
Mean ($X10^6$/µL)	9.59	9.34	9.25	9.70	8.78	8.50*	8.44**	8.50*
Vs. control group	---	-3%	-4%	+1%	---	-3%	**-4%**	**-3%**
MCV								
End of dosing								
Mean (fL)	52.5	52.8	54.0	54.3*	56.0	55.5	57.1	56.9
Vs. control group	---	1%	**+3%**	**+3%**	---	-1%	**+2%**	**+2%**
End of recovery								
Mean (fL)	51.8	53.2	52.7	52.6	55.8	55.7	56.7	56.5
Vs. control group	---	+3%	+2%	+2%	---	0%	+2%	+1%
MCH								
End of dosing								
Mean (pg)	17.2	17.4	17.9*	17.9*	18.6	18.7	19.2**	19.4**
Vs. control group	---	+1%	**+4%**	**+4%**	---	+1%	**+3%**	**+4%**
End of recovery								
Mean (pg)	16.3	16.7	16.7	16.4	18.0	18.0	18.4	18.2
Vs. control group	---	+2%	+2%	0%	---	0%	+2%	+1%
Reticulocyte count								
End of dosing								
Mean ($X10^3$/µL)	219	236	229	211	166	174	175	190
Vs. control group	---	+8%	+5%	–4%	---	+5%	+5%	**+14%**
End of recovery								
Mean ($X10^3$/µL)	231	233	234	223	163	182	172	187
Vs. control group	---	+1%	+1%	-3%	---	+11%	+6%	**+15%**

* $p < 0.05$; ** $p < 0.01$

Clinical Chemistry
Blood samples were taken at scheduled euthanasia.

There were increases in ALT, AST, ALP, glucose, and triglycerides at the HD.

Table: clinical chemistry changes (Sponsor's)

	Males				Females			
Dose (mg/kg/day)	0	300	600	2000	0	300	600	2000
ALT								
End of dosing								
Mean (U/L)	43	51	53	78**	37	53	37	65
Vs. control group	---	1.2x	1.2x	**1.8x**	---	1.4x	1.0x	**1.8x**
End of recovery								
Mean (U/L)	60	60	40	57	37	40	40	32
Vs. control group	---	0.0x	0.7x	1.0x	---	1.0x	1.0x	0.8x
AST								
End of dosing								
Mean (U/L)	81	91	91	96	80	88	69	101
Vs. control group	---	1.1x	1.1x	1.2x	---	1.1x	0.9x	**1.3x**
End of recovery								
Mean (U/L)	91	102	75	99	82	85	86	64
Vs. control group	---	1.1x	0.8x	1.1x	---	1.0x	1.0x	0.8x
Alkaline phosphatase								
End of dosing								
Mean (U/L)	66	67	72	86*	33	30	29	39
Vs. control group	---	1.0x	1.1x	**1.3x**	---	0.9x	0.9x	1.2x
End of recovery								
Mean (U/L)	82	85	72	64	31	34	34	26
Vs. control group	---	1.0x	0.9x	0.8x	---	1.1x	1.1x	0.8x

	Males				Females			
Dose (mg/kg/day)	0	300	600	2000	0	300	600	2000
Glucose								
End of dosing								
Mean (mg/dL)	252	264	268	306	238	248	238	235
Vs. control group	---	+5%	+6%	**+21%**	---	+4%	±0%	–1%
End of recovery								
Mean (mg/dL)	286	307	284	331	261	255	249	257
Vs. control group	---	+7%	+1%	**+16%**	---	-2%	-5%	-2%
Triglycerides								
End of dosing								
Mean (mg/dL)	88	94	116	142*	107	101	120	131
Vs. control group	---	+7%	**+32%**	**+61%**	---	–6%	+12%	**+22%**
End of recovery								
Mean (mg/dL)	162	155	142	177	126	115	96	224
Vs. control group	---	-4%	-12%	+10%	---	-9%	-23%	**+78%**

* $p < 0.05$; ** $p < 0.01$

Urinalysis

Urine was collected from the first 5/sex surviving main-study animals, from each dose group, prior to their scheduled euthanasia.

There was an increase in specific gravity in HDM (1.9%) and HDF (1.5%).

Gross Pathology
Gross pathology was assessed in all animals.

There were no adverse macroscopic findings.

Organ Weights
At necropsy, the following organs, as listed in the study report (see table in histopathology section), were weighed.

Generally, there was a dose-dependent increase in kidney, liver, and spleen weight in M and F.

Table: organ changes in absolute weight compared to control animals

	Adrenal	Brain	Epididymis	Heart	Kidney	Liver	Ovary	Spleen	Testes/ Uterus	Thymus	Thyroid
Male											
LD:	-6% (0%)	+5%* (+1%)	+8% (-1%)	+8% (+5%)	+16%* (+1%)	+14% (+3%)	-	+21%* (+5%)	-2% (-1%)	+13% (+17%)	-14% (+19%)
MD:	-1% (0%)	+4%* (+1%)	+11% (+1%)	+7% (+5%)	+17%* (+3%)	+17% (+5%)	-	+28%* (16%*)	-5% (-3%)	+18% (+3%)	+4% (+12%)
HD:	-5% (0%)	-4%* (+2%)	0% (+6%)	+9% (+15%*)	+27%* (+19%*)	+29%* (+23%*)	-	+10% (+26%*)	-9% (-1%)	+8% (+46%*)	0% (+15%)
Female											
LD:	-4% (-17%)	+1% (-1%)	-	+5% (+1%)	+12%* (0%)	+6% (+1%)	+10% (-18%)	+8% (0%)	-25% (+2%)	-5% (0%)	+5% (+10%)
MD:	0% (-5%)	+6%* (-3%)	-	+7% (+1%)	+19%* (+1%)	+8% (+3%)	0% (-7%)	+15% (+15%*)	-9% (-3%)	-2% (+1%)	+5% (+5%)
HD:	+10% (-7%)	-3% (-1%)	-	+11% (+11%*)	+26%* (+20%*)	+18%* (+29%*)	+14% (+10%)	+25%* (+19%*)	-30% (+11%)	+13% (+23%*)	+5% (+5%)

Recovery values in parenthesis. * Statistically significant.

Table: organ changes in weight (relative to body weight) compared to control animals

	Adrenal	Brain	Epididymis	Heart	Kidney	Liver	Ovary	Spleen	Testes/ Uterus	Thymus	Thyroid
Male											
LD:	-17% (0%)	-6% (-2%)	-3% (-4%)	-2% (+3%)	+4% (-3%)	+3% (0%)	-	+10% (+1%)	-12% (-5%)	+2% (+13%)	-25%* (0%)
MD:	-8% (0%)	-6% (-2%)	0% (-3%)	-3% (+1%)	+5% (-1%)	+6% (+1%)	-	+16% (+11%)	-14%* (-7%)	+9% (0%)	0% (0%)
HD:	-8% (-10%)	-8% (-11%)	-3% (-8%)	+3% (0%)	+20%* (+3%)	+21%* (+6%)	-	+5% (+7%)	-12% (-16%*)	0% (+25%*)	0% (0%)
Female											
LD:	-5% (-21%*)	-4% (-4%)	-	0% (-2%)	+7% (-4%)	+1% (-2%)	+3% (-20%)	+4% (-2%)	-28% (-2%)	-10% (-1%)	0% (0%)
MD:	-5% (-12%)	0% (-9%)	-	+1% (-6%)	+13%* (-6%)	+2% (-3%)	-7% (-14%)	+9% (+8%)	-12% (-9%)	-8% (-6%)	0% (0%)
HD:	-18% (-25%*)	-14%* (-17%*)	-	-4% (-9%*)	+11% (-1%)	+3% (+6%)	-2% (-7%)	+9% (-3%)	-37%* (-7%)	-5% (+3%)	-17% (-17%)

Recovery values in parenthesis. * Statistically significant.

Histopathology
At necropsy, the following organs, as listed in the study report, were collected. Microscopic examination was conducted on all tissues from control and HD animals,

any animal that died unexpectedly, and target organs (heart and kidneys in M and liver and lungs in both sexes at LD and MD).

Tissue	Organ Weight Taken	Collected and Preserved in 10% NBF	Microscopic Examination
Adrenal glands*	X	X	X
Aorta (thoracic)		X	X
Brain (7 sections)	X	X	X
Cecum		X	X
Cervix		X	X
Colon		X	X[a]
Duodenum		X	X
Epididymides*	X	X	X
Esophagus		X	X
Eyes		XX	X
Femur, proximal		X	
Heart	X	X	X
Identification		X	
Ileum		X	X
Jejunum		X	X
Kidneys*	X	X	X
Lacrimal gland		X	X
Lesion(s)		X	X
Liver	X	X	X
Lungs		X	X
Lymph node - cervical		X	X
Lymph node - mesenteric		X	X
Mammary gland (region)		X	X
Muscle *(biceps femoris)*		X	X
Optic nerves		XX	X
Ovaries*	X	X	X
Pancreas		X	X
Parathyroids (with thyroids)	With Thyroids	X	X (at least 1 parathyroid)
Peyer's Patch with jejunum and/or ileum		X	X
Pituitary		X	X
Prostate		X	X

Tissue	Organ Weight Taken	Collected and Preserved in 10% NBF	Microscopic Examination
Rectum		X	X
Salivary gland, mandibular		X	X
Sciatic nerves (right and left)		X	X
Seminal vesicles		X	X
Skin		X	X
Spinal cord – cervical, thoracic, lumbar		X	X
Spleen	X	X	X
Sternum		X	X (bone and marrow)
Stomach		X	X
Testes*	X	XX	X
Thymus (region)	X	X	X
Thyroids*	X	X	X
Tongue		X	
Trachea		X	X
Urinary bladder		X	X
Trigeminal ganglion and nerves		X	X
Uterus	X	X	X
Vagina		X	X

Paired organs (designated by *) were weighed together
XX = modified Davidson's solution
[a] Examination included evaluation of the autonomic plexus

Adequate Battery: Yes.
Peer Review: Yes, by John E Dillberger, DVM, PhD, DACVP, DABT, FIATP.
Signed Pathology report: Yes, by H C Thomas, DVM, PhD, DACVP.

Histological Findings

Few histopathological findings were noted. Findings included an increase in cardiomyocyte necrosis and inflammatory cell infiltration in M and is consistent with an exacerbation of progressive cardiomyopathy; this finding was reversible. An increase in chronic progressive nephropathy (CPN) was noted in MDM and HDM, CPN can be exacerbated with increased food consumption. An increase in incidence and severity of hepatocellular vacuolation was observed in M and HDF. These microscopic changes were not considered adverse.

Table: summary of selected microscopic findings (Sponsor's)
Main

Tissue/finding Sex	Males				Females			
NNZ-2566 Dose (mg/kg/day)	0	300	600	2000	0	300	600	2000
Heart No. examined:	16	15	15	15	18	2	0	17
Necrosis/Inflammatory cell infiltrate, cardiomyocyte								
Total number affected	2	5	7	9	1	0	0	2
Minimal	2	4	7	9	1	0	0	2
Mild	0	1	0	0	0	0	0	0
Liver No. examined:	16	15	15	15	18	17	15	17
Vacuolation, hepatocellular								
Total number affected	12	14	15	14	10	8	9	14
Minimal	9	8	6	6	10	8	9	9
Mild	3	5	7	5	0	0	0	4
Moderate	0	1	2	3	0	0	0	1
Kidney No. examined:	16	15	15	15	18	2	2	17
Chronic progressive nephropathy								
Total number affected	4	2	7	12	0	0	0	1
Minimal	4	2	7	9	0	0	0	1
Mild	0	0	0	2	0	0	0	0
Moderate	0	0	0	1	0	0	0	0

Recovery

Tissue/finding Sex	Males				Females			
NNZ-2566 Dose (mg/kg/day)	0	300	600	2000	0	300	600	2000
Heart No. examined:	15	15	15	15	15	0	0	15
Necrosis/Inflammatory cell infiltrate, cardiomyocyte								
Total number affected	5	4	1	7	0	0	0	1
Minimal	5	4	1	6	0	0	0	1
Mild	0	0	0	1	0	0	0	0
Liver No. examined:	15	15	15	15	15	15	15	15
Vacuolation, hepatocellular								
Total number affected	14	14	14	15	14	8	11	14
Minimal	10	10	9	4	13	8	11	13
Mild	4	4	5	7	0	0	0	1
Moderate	0	0	0	4	1	0	0	0
Kidney No. examined:	15	15	15	15	15	1	0	15
Chronic progressive nephropathy								
Total number affected	3	8	6	13	0	0	0	3
Minimal	3	7	5	12	0	0	0	2
Mild	0	1	1	1	0	0	0	1

Special Evaluation

Locomotor Activity Evaluation

Locomotor activity was evaluated by measuring distance traveled during test periods (5 mins) in a Force Plate Actimeter (FPA). Up to 15/sex/group (main-study animals) were evaluated near the end of the 26-week dosing period. The remaining rats were evaluated near the end of the recovery period.

No adverse effects were observed.

Spatial Learning and Memory Evaluation
Spatial learning and memory were evaluated using a Morris water maze. Up to 15/sex/group (main-study animals) were evaluated near the end of the 26-week dosing period. The remaining rats were evaluated near the end of the recovery period.

No adverse effects were observed.

Auditory Startle Reflex Assessment
Auditory startle reflex was assessed using a standard clap test. Up to 15/sex/group (main-study animals) were evaluated near the end of the 26-week dosing period. The remaining rats were evaluated near the end of the recovery period.

No adverse effects were observed.

Insulin-like growth factor-1 (IGF-1), IGF binding protein 3 (IGFBP3), and growth hormone (GH) Analysis
Blood samples were taken from control and HD animals at scheduled euthanasia.

There was an increase in somatotropin and a decrease in IGF binding protein 3 (IGFBP-3) in HDM. No increase in IGF-1 was observed.

Table: IGF-1, IGFBP-3, and somatostatin changes (Sponsor's)

		Male		Female	
		G 1 / M	G 4 / M	G 1 / F	G 4 / F
IGF1 [µg/dL] day 184	Mean	2,772 n	2,298	1,343 n	1,286
	S.d.	1,071	681	253	410
	N	15	14	13	14
SOMATRO [ng/mL] day 184	Mean	18.75 n	60.06 *	30.52 n	19.85
	S.d.	19.10	74.93	17.41	12.22
	N	15	14	13	14
IGFBP3 [mg/L] day 184	Mean	4.8 n	4.2 **	2.4 n	2.4
	S.d.	0.6	0.5	1.7	0.5
	N	15	14	13	14

Femur Bone Analysis
At scheduled necropsies, femurs were collected from all surviving main-study animals. A subset of femurs (10/sex/group) from all groups was submitted for analysis of:

- total femur length using sliding calipers.
- dual energy x-ray absorptiometry (DEXA) scans and analysis of the following endpoints:
  - bone mineral density (BMD)
  - bone mineral content (BMC)
  - bone area (BA) from,
    - whole femur
    - the distal 25% of the femur
    - the middle 50% of the femur
    - proximal 25% of the femur.
- micro-computed tomography (µCT) scans and analysis of the distal femoral metaphysis with the following endpoints:
  - total volume (TV)

- o bone volume (BV)
- o bone volume fraction (BV/TV)
- o trabecular number (Tb.N)
- o trabecular thickness (Tb.Th)
- o trabecular separation (Tb.Sp)
- o structure model index (SMI).

There was no reduction in BMD, BMC, or BA, compared to controls. Differences detected in femur that included an increase in proximal and whole bone BMD in HDM and at all doses in F that was still increased in recovery HD animals. An increase in whole bone BMC was observed at all doses in F. These increases were generally not greater than 10% and not dose dependent. In trabecular bone, there was an increase in volume, volume fraction, and number, and decreases in separation and SMI at the HD.

Table: bone analysis of main animals

ROI of femur	parameter	Males				Females			
		0	300	600	2000	0	300	600	2000
Distal	BMD	0.235	0.247	0.232	0.264	0.213	0.228	0.226	0.235
	BMC	0.207	0.229	0.209	0.226	0.137	0.153	0.152	0.150
	BA	0.882	0.926	0.904	0.856	0.642	0.674	0.672	0.637
Middle	BMD	0.231	0.245	0.229	0.247	0.184	0.194	0.191	0.195
	BMC	0.333	0.358	0.327	0.338	0.176	0.187	0.185	0.187
	BA	1.441	1.459	1.430	1.367	0.954	0.966	0.972	0.959
Proximal	BMD	0.219	0.234	0.218	0.244* (+11%)	0.196	0.211* (+8%)	0.209* (+7%)	0.211* (+8%)
	BMC	0.197	0.212	0.196	0.212	0.123	0.136* (+11%)	0.137* (+11%)	0.132
	BA	0.900	0.908	0.898	0.865	0.629	0.643	0.655	0.628
Whole	BMD	0.229	0.243	0.227	0.251* (+10%)	0.196	0.209* (+7%)	0.206* (+5%)	0.211* (+8%)
	BMC	0.735	0.797	0.732	0.774	0.435	0.474* (+9%)	0.472* (+9%)	0.468* (+8%)
	BA	3.210	3.284	3.228	3.081	2.220	2.271	2.291	2.217

*** Statistically significant; percent change in parenthesis.**

Table: bone analysis of recovery animals

ROI of femur	parameter	Males				Females			
		0	300	600	2000	0	300	600	2000
Distal	BMD	0.240	0.239	0.235	0.254	0.223	0.221	0.224	0.238
	BMC	0.221	0.214	0.212	0.243* (+10%)	0.145	0.146	0.154	0.166* (+14%)
	BA	0.919	0.896	0.904	0.959	0.651	0.660	0.685	0.697* (+7%)
Middle	BMD	0.236	0.242	0.239	0.260* (+10%)	0.188	0.191	0.197	0.210* (+12%)
	BMC	0.363	0.355	0.345	0.410* (+13%)	0.184	0.187	0.194	0.222* (+21%)
	BA	1.537	1.466	1.440	1.576	0.979	0.978	0.987	1.057
Proximal	BMD	0.226	0.229	0.224	0.246* (+9%)	0.206	0.205	0.209	0.223* (+8%)

ROI of femur	parameter	Males				Females			
		0	300	600	2000	0	300	600	2000
Proximal	BMC	0.201	0.204	0.202	0.235* (+17%)	0.131	0.130	0.138	0.153* (+17%)
	BA	0.890	0.892	0.902	0.954	0.637	0.634	0.661	0.687* (+8%)
Whole	BMD	0.235	0.238	0.234	0.254* (+8%)	0.203	0.204	0.208	0.222* (+9%)
	BMC	0.782	0.770	0.755	0.886* (+13%)	0.459	0.462	0.486	0.541* (+18%)
	BA	3.333	3.241	3.233	3.477	2.261	2.264	2.329	2.436* (+8%)

*** Statistically significant; percent change in parenthesis.**

Table: trabecular bone analysis of main animals

Parameter	Males				Females			
	0	300	600	2000	0	300	600	2000
Tb.TV	29.58	34.16	32.02	30.79	17.38	18.11	18.69	17.91
Tb.BV	3.39	4.84	3.70	7.37* (+117%)	3.15	4.72	4.50	5.30* (+68%)
Tb.BV/TV	11.28	14.15	11.53	24.07* (+113%)	18.12	25.86	24.10	29.79* (+64%)
Tb.Th	70.87	68.60	65.58	70.32	66.79	77.23	72.40	75.62
Tb.Sp	360.3	321.3	384.3	191.5* (-47%)	298.0	206.1	189.4* (-36%)	167.9* (-44%)
Tb.N	1.59	2.10	1.76	3.40* (+113%)	2.63	3.30	3.32	3.92* (+49%)
Tb.SMI	2.39	2.23	2.21	1.90* (-21%)	2.07	1.93	2.05	1.77
FL	41.81	42.43	42.62	41.04	34.81	35.94* (+3%)	36.05* (+4%)	35.12
FW	3.55	3.70	3.57	3.60	2.84	2.89	2.91	2.86

*** Statistically significant; percent change in parenthesis.**

Table: trabecular bone analysis of recovery animals

Parameter	Males				Females			
	0	300	600	2000	0	300	600	2000
Tb.TV	33.10	31.83	32.51	36.62	18.25	18.06	19.57	20.33
Tb.BV	4.26	3.77	3.39	5.55	4.17	4.14	4.29	5.20
Tb.BV/TV	12.86	11.71	10.73	15.10	23.28	22.45	21.49	25.58
Tb.Th	71.59	64.32* (-10%)	64.59* (-10%)	64.78* (-10%)	74.92	69.70	66.62	74.34
Tb.Sp	340.6	307.9	406.1	243.6* (-28%)	242.7	224.8	194.4	189.2
Tb.N	1.83	1.82	1.66	2.33	3.04	3.15	3.15	3.36
Tb.SMI	2.28	2.34	2.36	2.28	1.99	1.95	2.12	2.01
FL	42.82	42.36	42.67	43.49	35.68	35.80	36.57	36.86* (+3%)
FW	3.69	3.71	3.72	3.90	3.03	3.01	2.99	3.10

*** Statistically significant; percent change in parenthesis.**

Toxicokinetics
Blood samples were taken on Days 1, 66, and 183 at 2 hours post-dose from control animals and pre-dose (on Days 66 and 183) and at 1.5, 3, 6 (prior to second dose), 7.5, 9, and 24 hours post-dose from trofinetide-dosed animals.

On Day 1, the increase in plasma exposure (C_{max} and AUC) in F was approximately dose-proportional between the LD and MD and greater than dose-proportional between the MD and HD, in M, the increase in plasma exposure was approximately dose-proportional. On Days 66 and 183 plasma exposure was less than on Day 1 and with a T_{max} that was approximately 1.5 hours post-second dose.

Table: toxicokinetic data of trofinetide (Sponsor's)

Group	Total Daily Dosage (mg/kg)	Day	Gender	T_{max}[a] (hr)	C_{max} (µg/mL)	SE C_{max} (µg/mL)	AUC_{last} (hr*µg/mL)	SE AUC_{last} (hr*µg/mL)	AUC_{0-24} (hr*µg/mL)
6	300	1	Female	7.5	69.3	3.25	561	13.7	561
6	300	1	Male	7.5	61.5	6.58	668	82.4	668
6	300	66	Female	7.5	27.9	1.27	137	0.658	137
6	300	66	Male	7.5	31.3	3.10	184	6.70	184
6	300	183	Female	7.5	36.1	4.24	204	18.6	204
6	300	183	Male	7.5	33.1	3.38	211	8.97	211
7	600	1	Female	7.5	103	37.1	1150	132	1150
7	600	1	Male	9.0	108	9.65	1330	105	1330
7	600	66	Female	7.5	61.7	8.06	323	17.0	323
7	600	66	Male	7.5	69.5	1.28	371	26.2	371
7	600	183	Female	7.5	88.7	7.17	482	38.4	482
7	600	183	Male	7.5	82.6	5.02	416	24.5	416
8	2000	1	Female	9.0	405	26.6	5080	234	5080
8	2000	1	Male	9.0	301	54.9	4340	462	4340
8	2000	66	Female	7.5	170	26.2	1230	203	1230
8	2000	66	Male	7.5	150	20.2	1660	336	1660
8	2000	183	Female	7.5	183	1.86	1710	222	1710
8	2000	183	Male	7.5	164	18.5	1690	140	1690

[a]Time normalized to the first dose.

Oral studies

6.2.3 Study Title: Seven-day oral range-finding study of NNZ-2566 in dogs.

Study no.:	0621-15160
Study report location:	EDR
Study initiation date:	June 1, 2015
Duration:	7
Duration Units:	days
GLP compliance:	N
Drug, lot #, and % purity:	Trofinetide, Lot №: 02149996 #1, not provided

Key Study Findings

- No deaths or adverse effects were noted.
- Trofinetide was tolerated at 500 mg/kg BID.

Methods

Doses:	500 mg/kg
Frequency of dosing:	BID
Number/Sex/Group:	2
Dose volume:	5 mL/kg/dose
Formulation/Vehicle:	De-ionized water
Route of administration:	ORAL
Species:	DOG
Strain:	BEAGLE
Age / Sexual Maturity:	18-21 months

6.2.4 Study Title: Seven-day oral range-finding study of NNZ-2566 in dogs.

Study no.:	0621-15233
Study report location:	EDR
Study initiation date:	August 11, 2015
Duration:	7
Duration Units:	days
GLP compliance:	N
Drug, lot #, and % purity:	Trofinetide, Lot №: NOR-C-84-8, not provided

Key Study Findings

- No deaths or adverse effects were noted.
- Trofinetide was tolerated at 1000 mg/kg BID.

Methods

Doses:	1000 mg/kg
Frequency of dosing:	BID
Number/Sex/Group:	2
Dose volume:	5 mL/kg/dose
Formulation/Vehicle:	De-ionized water
Route of administration:	ORAL GAVAGE
Species:	DOG
Strain:	BEAGLE

6.2.5 Study Title: Thirty-nine week oral GLP toxicity study of NNZ-2566 in dogs with a four-Week recovery period.

Study no.:	0621-16032
Study report location:	EDR
Study initiation date:	March 28, 2016
Conducting laboratory and location:	(b) (4)
Duration:	39
Duration Units:	weeks
GLP compliance:	Y, US 21 CFR, Part 58
Drug, lot #, and % purity:	NNZ-2566, Lot №s: E150651, 93.6%; E150652, 93.6%; 2A01R, 93.5%; NOR-C-84-8, 98.8%; NOR-C-84-9, 98.9%; NOR-C-84-10, 98.9%; NOR-C-84-11, 98.9%; R0006475 97.3%

Key Study Findings

- No deaths, clinical signs, body weight changes, or adverse microscopic findings were observed.
- There was a decrease in ovary and uterine weight in all F trofinetide-dosed groups.
- The sponsor considered the MD the NOAEL due to a decrease in uterine weight that is likely due to a tendency for F to be in anestrus and a stress-related effect on the estrus cycle in F. The NOAELs were 1000 mg/kg/day for M and 300 mg/kg/day for F, associated with plasma exposures (AUC) to trofinetide on Day 267 of 1475 and 343 µg*h/mL in M and F, respectively.

Methods

Doses:	0, 25, 150, 500 mg/kg
Frequency of dosing:	BID
Number/Sex/Group	
Main:	4
Recovery:	2 control and HD
Dose volume:	5 mL/kg/dose
Formulation/Vehicle:	Deionized water
Route of administration:	ORAL GAVAGE
Species:	DOG
Strain:	BEAGLE
Age / Sexual Maturity:	5-6 Months
Comment on Study Design and Conduct:	The HD is the recommended limit dose.

Observations and Results

Mortality

Observations for mortality and morbidity were conducted twice daily.

No unscheduled deaths occurred.

Clinical Signs
Detailed observations were conducted at least once daily from Day 1 until the day of necropsy.

Clinical observations included fecal changes, abnormal feces included soft feces, watery, mucoid and/or red-discolored diarrhea. These clinical signs recovered during the recovery period.

Table: selected clinical signs (Sponsors)

	Males				Females			
Dose (mg/kg/d) =	**0**	**50**	**300**	**1000**	**0**	**50**	**300**	**1000**
Any abnormal feces	83%	75%	100%	100%	83%	100%	75%	100%
Incidence (by type)								
Soft feces	67%	75%	75%	**100%**	50%	75%	75%	**100%**
Watery diarrhea	33%	25%	50%	**100%**	17%	75%	0%	**100%**
Mucoid diarrhea	33%	50%	25%	**100%**	33%	50%	25%	**83%**
Material, mucous	17%	25%	0%	**50%**	17%	0%	0%	**50%**
Red-discolored feces	17%	25%	0%	33%	0%	25%	0%	0%
Days recorded (by type)								
Soft feces	10	11	41	**192**	5	5	10	**161**
Watery diarrhea	2	2	14	**152**	4	3	0	**68**
Mucoid diarrhea	4	5	4	**52**	5	4	2	**15**
Material, mucous	1	1	---	8	1	---	---	3
Red-discolored feces	1	1	---	2	---	1	---	---
Frequency (by type)[a]								
Soft feces	0.6%	1.0%	3.7%	**11.7%**	0.3%	0.5%	0.9%	**9.7%**
Watery diarrhea	0.1%	0.2%	1.3%	**9.2%**	0.2%	0.3%	0%	**4.1%**
Mucoid diarrhea	0.2%	0.5%	0.4%	**3.2%**	0.3%	0.4%	0.2%	**9.1%**

[a] Number of days recorded divided by number of possible days, which was 1644 for the control and high-dose groups and 1096 for the low- and mid-dose groups.

Body Weights
Body weights were recorded once prior to initiation of dosing and weekly thereafter.

No adverse change in body weight was noted.

Food Consumption
Food consumption was recorded daily until the end of the study.

No adverse change in mean food consumption was noted.

Ophthalmoscopy
Examinations of animals were conducted prior to initiation of dosing, during dosing on Day 268/269, and at the end of the recovery period on Day 296/297.

No adverse ophthalmoscopic changes were noted.

ECG
Examinations of all animals were conducted once prior to initiation of dosing and at approximately 1-hour post-dose at the end of dosing and at the end of recovery. Corrected QT intervals (QTc) were calculated using the van de Water's correction method.

No drug-related changes in ECG were noted.

Hematology
Blood samples were taken prior to initiation of dosing, on Days 87, 178, and 268/269 during dosing, and Day 296/297 during recovery.

No adverse changes in hematology parameters were noted.

Clinical Chemistry
Blood samples were taken prior to initiation of dosing, on Days 87, 178, and 268/269 during dosing, and Day 296/297 during recovery.

No adverse changes in clinical chemistry parameters were noted.

Urinalysis
Urine samples were taken prior to initiation of dosing, on Days 268/269 during dosing, and Day 296/297 during recovery.

There was a slight decrease in pH and increase in specific gravity in HDF. Otherwise, there were no notable changes in urinalysis.

Gross Pathology
Gross pathology was assessed in all animals.

No adverse macroscopic findings were noted.

Organ Weights
At necropsy, the following organs, as listed in the study report (see table in histopathology section), were weighed.

There was a decrease in ovary weight in LDF (17%), MDF (41%), and HDF (34%) and uterus weight in LDF (33%), MDF (62%), and HDF (74%). Recovery was observed in organ weights, but uterus weight partially recovered (HDF decreased by 40%).

Histopathology
At necropsy, the following organs, as listed in the study report, were collected. Microscopic examination included all tissues from all animals.

Tissue	Organ Weight Taken	Collected and Preserved in 10% NBF	Microscopic Examination
Adrenal glands*	X	X	X
Aorta (thoracic)		X	X
Brain	X	X	X
Cecum		X	X
Cervix		X	X
Colon		X	X
Duodenum		X	X
Epididymides*	X	X	X
Esophagus		X	X
Eyes		XX	X
Femur, proximal		X	X
Gallbladder		X	X
Heart	X	X	X
Identification		X	
Ileum		X	X
Jejunum		X	X
Kidneys*	X	X	X
Lacrimal gland (accessory)		X	X
Lesion(s)		X	X
Liver	X	X	X
Lungs		X	X

Tissue	Organ Weight Taken	Collected and Preserved in 10% NBF	Microscopic Examination
Lymph node - cervical		X	X
Lymph node - mesenteric		X	X
Mammary gland (region)		X	X
Muscle (biceps femoris)		X	X
Optic nerves		XX	X
Ovaries*	X	X	X
Pancreas		X	X
Parathyroids (with thyroids)	With Thyroids	X	X (at least 1 parathyroid
Peyer's Patch with jejunum and/or ileum		X	X
Pituitary		X	X
Prostate		X	X
Rectum		X	X
Salivary glands, mandibular		X	X
Sciatic nerve		X	X
Skin		X	X
Spinal cord – cervical, thoracic		X	X
Spleen	X	X	X
Sternum		X	X (bone and marrow)
Stomach		X	X
Testes*	X	XX	X
Thymus (region)	X	X	X
Thyroids*	X	X	X
Tongue		X	
Trachea		X	X
Urinary bladder		X	X
Uterus	X	X	X
Vagina		X	X
Paired organs (designated by *) were weighed together XX = modified Davidson's solution			

Adequate Battery: Yes.
Peer Review: No.
Signed Pathology report: Yes, by Heath C Thomas, DVM, PhD, DACVP

Histological Findings

Few histopathological findings were noted. Findings included renal tubule degeneration in 1 HDM, Kupffer cell pigment in 2 HDM and 2 HDF, and thyroid findings of decreased colloid in HDM and HDF, C-cell hyperplasia in M at all doses, and follicular hypertrophy/hyperplasia in HDM and HDF. These findings were not adverse and were generally not observed in recovery animals.

Table: summary of selected microscopic findings

		MALE						FEMALE					
	(n):	4	4	4	4	2	2	4	4	4	4	2	2
Tissue	**Finding (mg/kg):**	**0**	**50**	**300**	**1000**	**0**	**1000**	**0**	**50**	**300**	**1000**	**0**	**1000**
Kidney	Cortical tubule degeneration												
	Grade 1:	0	0	0	1	0	0	0	0	0	0	1	0
Liver	Kupffer cell pigment												
	Grade 1:	0	0	0	2	0	0	0	0	0	2/4	0	1
Thyroid	Decrease colloid												
	Grade 1:	0	0	0	3	0	0	0	0	0	4	0	0
	Grade 2:	0	0	0	1	0	0	0	0	0	0	0	0
	C-cell hyperplasia												
	Grade 1:	0	2	1	2	0	0	0	1	0	0	1	0
	Grade 2:	0	2	0	2	0	0	1	0	0	0	0	0
	Grade 3:	0	0	1	0	0	0	0	0	0	0	0	0
	Follicular hypertrophy/hyperplasia												
	Grade 1:	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2:	0	0	0	4	0	0	0	0	0	4	0	0

Toxicokinetics

Blood samples were taken on Days 1, 87, and 267 pre-dose (for Days 87 and 267) and at 1, 2, 4, and 6 hours post-first dose and 2 hours post-second dose.

In general, plasma exposure (C_{max} and AUC) to trofinetide increased greater than dose-proportionally, was similar between the sexes, and showed no accumulation.

Table: toxicokinetic data of trofinetide (Sponsor's)

Dose (mg/kg /day)	Study Day	Parameter	Male			Female			Male + Female		
			Mean	SD	n	Mean	SD	n	Mean	SD	n
50	1	Cmax (µg/mL)	4.09	1.88	4	4.13	1.50	4	4.11	1.57	8
		Tmax (h)	3.00	3.37	4	4.50	4.04	4	3.75	3.54	8
		AUC(0-t) (µg*h/mL)	14.3	5.38	4	16.0	6.05	4	15.1	5.37	8
		AUC(0-24) (µg*h/mL)	30.3	8.92	4	44.0	20.3	4	37.1	16.3	8
		Cmax /Dose	0.0817	0.0374	4	0.0826	0.0298	4	0.0821	0.0313	8
		AUC(0-t) /Dose	0.286	0.107	4	0.319	0.121	4	0.302	0.107	8
		AUC(0-24) /Dose	0.605	0.178	4	0.880	0.406	4	0.742	0.326	8
	87	Cmax (µg/mL)	2.26	0.688	4	2.01	0.986	4	2.14	0.799	8
		Tmax (h)	6.25	3.50	4	4.75	3.78	4	5.50	3.46	8
		AUC(0-t) (µg*h/mL)	10.2	3.74	4	10.6	4.22	4	10.4	3.69	8
		AUC(0-24) (µg*h/mL)	30.9	9.52	4	28.4	10.4	4	29.6	9.33	8
		Cmax /Dose	0.0453	0.0138	4	0.0402	0.0197	4	0.0427	0.0160	8
		AUC(0-t) /Dose	0.205	0.0748	4	0.211	0.0843	4	0.208	0.0738	8
		AUC(0-24) /Dose	0.617	0.190	4	0.568	0.209	4	0.593	0.187	8
	267	Cmax (µg/mL)	4.27	2.69	4	2.36	1.01	4	3.31	2.14	8
		Tmax (h)	4.75	3.78	4	2.75	3.50	4	3.75	3.54	8
		AUC(0-t) (µg*h/mL)	16.3	10.1	4	11.5	3.82	4	13.9	7.51	8
		AUC(0-24) (µg*h/mL)	43.7	19.1	4	28.7	8.37	4	36.2	15.8	8
		Cmax /Dose	0.0854	0.0539	4	0.0472	0.0201	4	0.0663	0.0428	8
		AUC(0-t) /Dose	0.325	0.202	4	0.230	0.0763	4	0.277	0.150	8
		AUC(0-24) /Dose	0.873	0.382	4	0.574	0.167	4	0.723	0.316	8

Dose (mg/kg)	Study Day	Parameter	Male			Female			Male + Female		
			Mean	SD	n	Mean	SD	n	Mean	SD	n
300	1	Cmax (µg/mL)	48.8	27.6	4	38.2	6.63	4	43.5	19.4	8
		Tmax (h)	1.50	0.58	4	1.25	0.50	4	1.38	0.52	8
		AUC(0-t) (µg*h/mL)	127	66.5	4	117	2.52	4	122	43.9	8
		AUC(0-24) (µg*h/mL)	243	94.2	4	360	67.4	4	301	98.2	8
		Cmax /Dose	0.163	0.0919	4	0.128	0.0220	4	0.145	0.0646	8
		AUC(0-t) /Dose	0.424	0.222	4	0.389	0.00835	4	0.406	0.146	8
		AUC(0-24) /Dose	0.810	0.314	4	1.20	0.225	4	1.00	0.327	8
	87	Cmax (µg/mL)	15.0	4.81	4	20.1	3.77	4	17.6	4.84	8
		Tmax (h)	8.00	0.00	4	6.25	3.50	4	7.13	2.48	8
		AUC(0-t) (µg*h/mL)	55.5	19.2	4	76.5	10.5	4	66.0	18.2	8
		AUC(0-24) (µg*h/mL)	191	54.8	4	242	38.6	4	217	51.7	8
		Cmax /Dose	0.0500	0.0160	4	0.0670	0.0126	4	0.0585	0.0162	8
		AUC(0-t) /Dose	0.185	0.0642	4	0.255	0.0349	4	0.220	0.0607	8
		AUC(0-24) /Dose	0.637	0.183	4	0.808	0.129	4	0.722	0.172	8
	267	Cmax (µg/mL)	17.0	7.45	4	48.1	6.75	4	32.5	17.8	8
		Tmax (h)	6.25	3.50	4	1.00	0.00	4	3.63	3.62	8
		AUC(0-t) (µg*h/mL)	55.7	25.6	4	139	24.6	4	97.1	50.0	8
		AUC(0-24) (µg*h/mL)	198	66.2	4	343	56.7	4	270	96.2	8
		Cmax /Dose	0.0568	0.0249	4	0.160	0.0226	4	0.108	0.0594	8
		AUC(0-t) /Dose	0.186	0.0854	4	0.462	0.0818	4	0.324	0.167	8
		AUC(0-24) /Dose	0.658	0.221	4	1.14	0.189	4	0.900	0.321	8

Dose (mg/kg)	Study Day	Parameter	Male			Female			Male + Female		
			Mean	SD	n	Mean	SD	n	Mean	SD	n
1000	1	Cmax (µg/mL)	213	16.6	6	190	49.6	6	201	37.2	12
		Tmax (h)	1.83	0.408	6	2.83	2.56	6	2.33	1.83	12
		AUC(0-t) (µg*h/mL)	647	51.5	6	581	150	6	614	113	12
		AUC(0-24) (µg*h/mL)	1518	159	6	1533	165	6	1526	155	12
		Cmax /Dose	0.213	0.0166	6	0.190	0.0496	6	0.201	0.0372	12
		AUC(0-t) /Dose	0.647	0.0515	6	0.581	0.150	6	0.614	0.113	12
		AUC(0-24) /Dose	1.52	0.159	6	1.53	0.165	6	1.53	0.155	12
	87	Cmax (µg/mL)	103	49.5	6	117	26.5	6	110	38.6	12
		Tmax (h)	6.00	3.10	6	6.00	3.10	6	6.00	2.95	12
		AUC(0-t) (µg*h/mL)	356	201	6	426	104	6	391	157	12
		AUC(0-24) (µg*h/mL)	1057	337	6	1348	272.7	6	1203	329	12
		Cmax /Dose	0.103	0.0495	6	0.117	0.0265	6	0.110	0.0386	12
		AUC(0-t) /Dose	0.356	0.201	6	0.426	0.104	6	0.391	0.157	12
		AUC(0-24) /Dose	1.06	0.337	6	1.35	0.273	6	1.20	0.329	12
	267	Cmax (µg/mL)	136	45.5	6	142	51.7	6	139	46.5	12
		Tmax (h)	6.83	2.86	6	4.67	3.67	6	5.75	3.33	12
		AUC(0-t) (µg*h/mL)	416	140	6	557	156	6	486	159	12
		AUC(0-24) (µg*h/mL)	1475	381	6	1500	191.2	6	1488	287	12
		Cmax /Dose	0.136	0.0455	6	0.142	0.0517	6	0.139	0.0465	12
		AUC(0-t) /Dose	0.416	0.140	6	0.557	0.156	6	0.486	0.159	12
		AUC(0-24) /Dose	1.48	0.381	6	1.50	0.191	6	1.49	0.287	12

Intravenous studies

6.2.6 Study Title: 28 Day 4-cycle intravenous infusion GLP toxicity study in Beagle dogs

Study no.: 0621-06038
Study report location: EDR
Study initiation date: May 11, 2007
Conducting laboratory and location: (b) (4)
Duration: 28
Duration Units: days
GLP compliance: Y, US 21 CFR, Part 58
Drug, lot #, and % purity: Trofinetide, Lot №'s: 07-031-22-31, 98.23%, 07-031-21-34, 98.14%, 07-031-26-27, 98.44%, 07-031-37-23, 98.30%

Key Study Findings:

- Several dogs died, were sacrificed early, or removed early from the study, due to local toxicities or catheter plugging. Therefore, no drug related deaths.
- Histopathological findings included thyroid hyperplasia and local inflammation, at the infusion catheter tip.
- Although the LD produced minimal to slight thyroid hyperplasia, this is not adverse, and the NOAEL is the LD due to moderate findings of thyroid hyperplasia at the MD and HD.
- Plasma exposure ($AUC_{0\text{-}96\ h}$) to trofinetide at the NOAEL, for Cycle 4 was 21500 and 25100 µg*h/mL in M and F, respectively that results in an $AUC_{0\text{-}24\ h}$ of 5375 and 6275 µg*h/mL in M and F, respectively.

Methods

Doses:	0, 480, 1440, and 3600 mg/kg/day
Frequency of dosing:	Continuous infusion for 7 days/cycle with 3 days between cycles for a total of 4 cycles (total of 28 days of infusion)
Number/Sex/Group	
Main:	3
Recovery:	2 control and HD
Dose volume:	10 mL/kg/day
Formulation/Vehicle:	Lactated Ringers Solution
Route of administration:	INTRAVENOUS
Species:	DOG
Strain:	BEAGLE
Age / Sexual Maturity:	12-13 Months
Comment on Study Design and Conduct:	The HD is above the recommended limit dose.

Observations and Results

Mortality

Early deaths were reported for 3 HD animals. One HDM was dosed for 19 days and euthanized moribund 1 day after infusion was terminated. Postmortem evaluation indicated that the cause of morbidity was inflammation at the catheter tip. A second HDM was dosed for 25 days and found dead 8 days after the end of infusion. Death was due to ruptured vena cava secondary to catheter inflammation. One HDF was dosed for 21 days and euthanized moribund 2 days after infusion was terminated. Cause of morbidity was determined to be catheter inflammation. The sponsor suggested that the apparent increased severity of inflammation at the catheter tip in HD animals was due to an extremely hyperosmolar dosing solution.

Three dogs (1 MDM, 1 HDM, and 1 HDF) were removed early from the study due to plugged catheters. One MDM was removed after 2 days of infusion and replaced. One HDM was dosed for 4 days and removed with no postmortem evaluation and replaced. One HDF was infused for 14 days, removed from the study, and necropsied on Day 22 (5 days post-end of dosing), with inflammation noted at the catheter tip and pulmonary artery thrombosis and mild pneumonia.

Clinical Signs

Observations appeared dose-related and included thin or dehydrated appearance, decreased appetite, decreased activity, limping, lameness, labored breathing, and recumbency. These findings may be related to the general ill health caused by the inflammation at the catheter tip or the SC venous access port. One control F displayed decreased appetite. The route of administration may have contributed to the adverse clinical effects and combined with the increased osmolarity with increasing dose, may have confounded the results.

Body Weights
There was no adverse change in body weight.

Food Consumption
There was no adverse change in food consumption.

Ophthalmoscopy
No adverse ophthalmoscopic findings were noted.

ECG
No adverse ECG findings were noted

Hematology
There was an increase in WBC and neutrophils; otherwise, there were no adverse effects on hematology parameters noted.

Clinical Chemistry
No adverse effects on clinical chemistry parameters were noted.

Urinalysis
No adverse effects on urinalysis were noted.

Gross Pathology
Macroscopic observations included 1 HDF with mottled and irregular surface of the spleen, HD animals sacrificed early displayed ruptured vena cava and thickened veins at the tip of the catheter, and animals euthanized at the end of the study displayed thickened or distended veins at the catheter tip.

Organ Weights
No adverse changes in organ weights were noted.

Histopathology
At necropsy organs were collected and microscopic evaluation was conducted.

Adequate Battery: Yes
Peer Review: No
Signed Pathology report: Yes, by Dean Barnett, DVM, PhD, DACVP (Page 796)

Histological Findings
Few histopathological findings were noted. Findings included thyroid hyperplasia in animals from all dosed groups and increased incidence and severity of local inflammation at the catheter tip at the HD.

Table: selected systemic microscopic findings (Sponsor's)

Dose (mg/kg/d)	# Days Dosed	# Days, Last Dose to Death	Thyroid Hyperplasia Grade	Animal Number(s)
0				
	28	2	---	M1, M2, M3, F17, F18, F19
	28	16	---	M4, M5, F20, F21
480				
	28	2	---	M6, F22
	28	2	1	M8, F23
	28	2	2	M7, F24
1440				
	28	2	1	F26
	27-28	2	2	M11, F25, F27
	26-28	2	3	M9, M34
3600				
	14	5	---	F31
	25	8	2	M16
	19-28	1	2	M33
	19-28	2	2	M12, M13, F29
	21-28	2	3	F28, F30
	21-28	16	---	M14, F32

--- = normal thyroid gland

Table: selected local microscopic findings (Sponsor's)

Dose (mg/kg/h)	Calculated Osmolarity (mOsm)	Incidence		Grade		Incidence x Grade
		No.	%	Individual	Mean	
0	294	4	67%	2, 3, 3, 3	2.75	184
20	436	2	33%	2, 2	2.00	66
60	751	4	67%	2, 2, 3, 4	2.75	184
150	1436	6	86%	1, 3, 4, 4, 4, 4	3.33	286

Toxicokinetics

Blood samples were taken for each dosing cycle just before infusion and at 0.5, 1, 2, 4, 24, and 96 hours following infusion initiation, and at 2 and 4 hours post-end of infusion.

Systemic exposure at the end of infusion was generally similar in M and F and was approximately dose-proportional with $t_{½}$ approximately 1 hour. There was no accumulation between the cycles and not between the first and last cycles.

Table: toxicokinetic data of trofinetide (Sponsor's)
Cycle 1

Sex	Dose (mg/kg/hr)	C_{ss} (µg/mL)	AUC_{0-96} (µg*hr/mL)	AUC_{0-168} (µg*hr/mL)	$t_{1/2}$ (hr)	CL (mL/hr/kg)	V_z (mL/kg)	R_o
Male	20	38.6 (16.4)	3650 (14.4)	6440 (13.1)	0.924 (8.67)	517 (13.1)	689 (16.7)	NA
Female	20	39.9 (23.4)	3620 (33.6)	6500 (28.9)	0.812 (11.6)	513 (28.9)	601 (25.0)	NA
Male	60	84.6[4] (40.1)	7210[2] (30.1)	12000[2] (34.3)	0.871[2] (23.0)	832[2] (34.2)	1050[2] (60.5)	NA
Female	60	131 (23.3)	11400 (23.8)	20900 (19.4)	0.875[2] (2.49)	456[2] (25.1)	576[2] (27.7)	NA
Male	150	232[6] (29.3)	23100[6] (18.7)	39900[6] (22.7)	0.922 (11.1)	660 (20.8)	878 (31.4)	NA
Female	150	232 (38.2)	25500 (22.1)	42300 (28.0)	1.02 (30.3)	591 (28.2)	870 (22.1)	NA

N = 3 (20 and 60 mg/kg/hr) or 5 (150 mg/kg/hr), unless stated otherwise [n].
NA = Not applicable.

Cycle 2

Sex	Dose (mg/kg/hr)	C_{ss} (µg/mL)	AUC_{0-96} (µg*hr/mL)	AUC_{0-168} (µg*hr/mL)	$t_{1/2}$ (hr)	CL (mL/hr/kg)	V_z (mL/kg)	R_o
Male	20	34.4	3400	5880	0.927[2]	574[2]	767[2]	0.930
		(11.8)	(16.5)	(14.2)	(2.75)	(19.9)	(22.7)	(8.86)
Female	20	39.3	3720	6570	0.815[2]	610[2]	717[2]	1.03
		(43.1)	(30.6)	(34.4)	(7.36)	(14.9)	(22.4)	(3.21)
Male	60	114	10100	18300	0.819[2]	488[2]	577[2]	1.59[2]
		(27.0)	(31.6)	(29.4)	(5.35)	(30.2)	(24.6)	(64.8)
Female	60	134	12700	22400	0.909	447	586	1.11
		(5.34)	(12.5)	(5.81)	(11.3)	(5.68)	(15.0)	(11.1)
Male	150	245	23700	44600[4]	1.07[4]	559[4]	864[4]	1.07
		(28.3)	(19.6)	(17.7)	(16.9)	(17.5)	(34.5)	(9.42)
Female	150	288	25100	45900	0.980[4]	544[4]	769[4]	0.987
		(14.1)	(13.5)	(13.3)	(27.4)	(15.5)	(20.3)	(9.72)

N = 3 (20 and 60 mg/kg/hr) or 5 (150 mg/kg/hr), unless stated otherwise [n].

Cycle 3

Sex	Dose (mg/kg/hr)	C_{ss} (µg/mL)	AUC_{0-96} (µg*hr/mL)	AUC_{0-168} (µg*hr/mL)	$t_{1/2}$ (hr)	CL (mL/hr/kg)	V_z (mL/kg)	R_o
Male	20	32.8	3140	5510	0.917[2]	567[2]	751[2]	0.859
		(26.9)	(17.2)	(20.8)	(12.6)	(24.9)	(38.4)	(3.47)
Female	20	35.8	3370	5950	0.902	560	729	0.932
		(36.3)	(32.0)	(33.8)	(8.44)	(33.7)	(40.9)	(10.6)
Male	60	94.8	9250	16100	0.865	621	775	1.35
		(16.1)	(22.5)	(18.9)	(2.12)	(18.9)	(18.4)	(43.5)
Female	60	116	11300	19600	0.930	510	684	0.988
		(25.8)	(20.4)	(22.6)	(9.65)	(22.5)	(31.4)	(22.4)
Male	150	227	21500	38000[4]	1.41[3]	613[3]	1240[3]	0.972
		(20.6)	(23.2)	(25.0)	(38.9)	(25.5)	(64.5)	(12.2)
Female	150	266[3]	24600[3]	43800[3]	1.09[3]	570[3]	898[3]	0.905[3]
		(19.6)	(13.9)	(16.4)	(22.1)	(16.3)	(34.2)	(17.9)

N = 3 (20 and 60 mg/kg/hr) or 5 (150 mg/kg/hr), unless stated otherwise [n].

Cycle 4

Sex	Dose (mg/kg/hr)	C_{ss} (µg/mL)	AUC_{0-96} (µg*hr/mL)	AUC_{0-168} (µg*hr/mL)	$t_{1/2}$ (hr)	CL (mL/hr/kg)	V_z (mL/kg)	R_o
Male	20	42.8	4220	7300	0.942	456	620	1.15
		(21.5)	(18.4)	(19.6)	(5.32)	(19.5)	(24.4)	(6.94)
Female	20	45.3	4220	7480	0.888	445	571	1.17
		(11.0)	(5.59)	(7.96)	(0.616)	(7.97)	(8.46)	(28.7)
Male	60	104	9810	17300	1.06	577	884	1.43
		(9.67)	(6.05)	(1.91)	(7.73)	(1.92)	(9.28)	(16.7)
Female	60	141	12400	22600	0.898	442	573	1.09
		(8.38)	(8.96)	(6.00)	(0.300)	(5.99)	(6.28)	(16.0)
Male	150	244[3]	21500[3]	39100[3]	1.05[3]	637[3]	969[3]	0.903[3]
		(35.3)	(30.4)	(32.5)	(17.3)	(32.3)	(51.3)	(16.6)
Female	150	260[3]	25100[3]	43900[3]	0.968[3]	569[3]	795[3]	0.923[3]
		(12.5)	(11.5)	(11.6)	(19.7)	(11.8)	(9.65)	(9.48)

N = 3 (20 and 60 mg/kg/hr) or 5 (150 mg/kg/hr), unless stated otherwise [n].

7 Genetic Toxicology

7.1 *In Vitro* Reverse Mutation Assay in Bacterial Cells (Ames)

7.1.1 Study title: Evaluation of NNZ-2566 in the Salmonella Typhimurium-Escherichia Coli Reverse mutation assay (AMES).

Study no.:	AMS00605
Study report location:	EDR
Date of study initiation:	September 8, 2005
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	NNZ-2566, Lot № SNB-VII-168-2, 95%

Key Study Findings: The study was valid, with trofinetide causing no increase in revertant numbers in any strain up to 3500 µg/plate.

Methods

Strains:	TA98, TA100, TA1535, TA1537, and WP2*uvr*A.
Concentrations in definitive study:	218.8, 437.5, 875, 1750, and 3500 µg/plate
Basis of concentration selection:	The concentrations of trofinetide were chosen from the preliminary assay results.
Negative control:	See table below
Positive control:	See table below
Formulation/Vehicle:	0.9% Saline
Incubation & sampling time:	48-72 hours at 37°C

	TA98	TA100	TA1535	TA1537	WP2*uvr*A
+ve control					
Without S-9:	2-NF 20 µg	NaAzide 5 µg	NaAzide 5 µg	9-AA 50 µg	4-NQO 1 µg
With S-9*:	2-AA 2.5 µg	2-AA 2.5 µg	2-AA 2.5 µg	2-AA 2.5 µg	2-AA 25 µg
-ve control	Saline 0.9%	Saline 0.9%	Saline 0.9%	Saline 0.9%	Saline 0.9%

2-AA is 2-aminoanthracene, 2-NF is 2-nitrofluorene, NaAzide is sodium azide, 9-AA is 9-aminoacridine, 4-NQO is 4-nitroquinoline; *rat liver S9 prepared from Aroclor™ 1254-induced M Sprague-Dawley rats.

Study Validity

Five concentrations of trofinetide were chosen for the definitive assay, based on a preliminary range-finding test, with all the strains. In the definitive study, concentrations were assayed in triplicate, using the plate incorporation method; 2-aminoanthracene was used as the positive control with S9 for all strains.

Results

In the range-finding test, concentrations of trofinetide were 0.35, 3.5, 35, 350, and 3500 µg/plate. No precipitation or cytotoxicity was observed up to 3500 µg/plate.

In the definitive assay, the sponsor stated that "*the vehicle control was out of range for the WP2uvrA strain without activation; therefore, that strain was rerun.*" Therefore, the

test with *Escherichia coli* WP2*uvrA* without metabolic activation was repeated. No cytotoxicity was observed for any strain up to 3500 µg/plate in the absence and presence of S9. No positive mutagenic response was observed with any of the strains.

7.2 *In Vitro* Assays in Mammalian Cells

7.2.1 Study title: In vitro mammalian chromosome aberration assay in Chinese hamster ovary cells challenged with NNZ-2566

Study no.:	CAB00505
Study report location:	EDR
Date of study initiation:	September 22, 2005
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	NNZ-2566, Lot № SNB-VII-168-2, 95%

Key Study Findings: No increase in chromosomal aberrations was observed, either in the absence or presence of S9.

Methods

Cell line:	Chinese hamster ovary (CHO) cells
Concentrations in definitive study	
4 hours in absence of S9:	109.38, 218.75, 437.5, 875, 1750, and 3500 µg/mL
20 hours in absence of S9:	109.38, 218.75, 437.5, 875, and 1750 µg/mL
4 hours in presence of S9 mix:	437.5, 875, 1750, 2500, and 3500 µg/mL
Basis of concentration selection:	The concentrations were selected to yield cultures for aberration scoring, with at least 1 concentration demonstrating ~40-60% relative growth.
Negative control:	0.9% Saline
Positive control:	15 µg/mL Cyclophosphamide monohydrate 0.3 µg/mL Mitomycin C
Formulation/Vehicle:	0.9% Saline
Incubation & sampling time:	4 and 19 hours in the absence of S9* and 4 hours in the presence of S9*; 37°C

*** Rat liver S9 prepared from Aroclor™ 1254-induced M Sprague-Dawley rats**

Study Validity

The assay was a valid study. Three doses of trofinetide were analyzed in duplicate and a minimum of 200 metaphase spreads examined per dose.

Results

In the cytotoxicity test in the presence of S9, cell numbers were reduced by 40-60% between 218.75 and 3500 µg/mL, and 875 and 3500 µg/mL after 4 and 19 hour incubation, respectively. Structural and numerical aberrations were not statistically

increased in the absence or presence of S9. In contrast, the positive controls (cyclophosphamide and mitomycin C) caused significant structural damaged to cells.

7.3 *In Vivo* Clastogenicity Assay in Rodent (Micronucleus Assay)

7.3.1 Study Title: Evaluation of NNZ-2566 in the mouse bone marrow micronucleus assay

Study no:	MNA00506
Study report location:	EDR
Date of study initiation:	March 25, 2006
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	NNZ-2566, Lot № SR-11-94H, 99.6%

Key Study Findings: Oral trofinetide was negative for induction of micronuclei in mice in vivo up to 2000 mg/kg in M and F, respectively.

Methods

Doses in definitive study:	0, 500, 1000, and 2000 mg/kg
Frequency of dosing:	Daily for 2 days
Route of administration:	Oral gavage
Dose volume:	20 mL/kg
Formulation/Vehicle:	0.9% Saline
Species/Strain:	Mice/CD-1
Number/Sex/Group:	5
Basis of dose selection:	Dose range-finding study (0, 250, 500, 1000, and 2000 mg/kg/day PO, 5/sex/group)
Negative control:	Vehicle
Positive control:	50 mg/kg/day Cyclophosphamide administered orally once only
Study design:	Bone marrow was harvested approximately 24 hours after the second dose

Study Validity

The assay was a valid study. The HD was selected based on data from a dose range-finding study. The time between the last dose and tissue (bone marrow) harvest was 24 hours.

Results

In the definitive study, no abnormal clinical signs were observed. Micronucleus frequencies were 0.2, 0, 0.6, and 1.0 in control M, LDM, MDM, and HDM and 0.8, 0.4, 1.0, and 0.4 in control F, LDF, MDF, and HDF, respectively. There was no change in micronuclei in polychromatic erythrocytes up to a dose of 2000 mg/kg in M and F, respectively. Cyclophosphamide produced a significant increase in micronucleus frequency of 21 and 17 in M and F, respectively.

7.3.2 Study Title: Evaluation of NNZ-2566 in the mouse bone marrow micronucleus assay

Study no:	MNA00207
Study report location:	EDR
Date of study initiation:	March 23, 2007
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	NNZ-2566, Lot № 1003519, 99.4%

Key Study Findings: IV trofinetide was negative for induction of micronuclei in mice in vivo up to 825 and 600 mg/kg in M and F, respectively.

Methods

Doses in definitive study	
M:	0, 206.25, 412.5, and 825 mg/kg
F:	0, 300, 600, and 1200 mg/kg
Frequency of dosing:	Daily for 2 days
Route of administration:	IV
Dose volume:	10 mL/kg
Formulation/Vehicle:	0.9% Saline
Species/Strain:	Mice/CD-1
Number/Sex/Group:	5
Basis of dose selection:	Dose range-finding study (0, 250, 500, 1000, and 2000 mg/kg/day IV, 5/sex/group)
Negative control:	Vehicle
Positive control:	50 mg/kg/day Cyclophosphamide administered orally once only
Study design:	Bone marrow was harvested approximately 24 hours after the second dose

Study Validity

The assay was valid. The HD was selected based on data from a dose range-finding study, and an MTD was achieved in the definitive study. The time between the last dose and tissue (bone marrow) harvest was 24 hours.

Results

In the dose range-finding study, all HD animals and 2 HMDM died. No clinical signs were observed in surviving animals. In the definitive study, 1 HDM and 3 HDF died. Micronucleus frequencies were 0.6, 0.2, 0, and 0 in control M, LDM, MDM, and HDM and 0.6, 0.6, 0.2, and 0 in control F, LDF, MDF, and HDF, respectively. There was no change in micronuclei in polychromatic erythrocytes up to a dose of 825 and 600 mg/kg in M and F, respectively. Cyclophosphamide produced a significant increase in MPCE frequency of 26 and 16 in M and F, respectively.

9 Reproductive and Developmental Toxicology

9.1 Fertility and Early Embryonic Development

The initial fertility studies (Nos. 2282-001 and 2282-002) were submitted to (b) (4) and reviewed. The studies were found to be invalid, and the following communication sent to the original sponsor (Neuren Pharmaceuticals) on February 22, 2017.

> Please refer to SDN 18, dated 04/22/2016, specifically to the rat Segment I fertility studies Nos. 2282-001 and 2282-002. Due to the excessive incidence of premature deaths that occurred in controls as well as in all drug groups, these studies are considered invalid. The high mortality and the systemic inflammation associated with the catheter and IV infusion technique could have masked or altered potential drug-related toxicities.
>
> We recommend that you conduct a new Segment I fertility as well as other reproductive and developmental toxicity studies using the intended clinical route of administration (oral for this indication), and ensure adequate drug exposure. Use of alternative routes of administration other than the intended clinical route should be adequately justified. We also remind you that the high dose should be selected appropriately based on data from all available studies. Refer to ICHS5A, ICHS5B, and ICHS5(R2) for more guidance.

9.1.1 Study Title: An Oral (Gavage) Fertility and Early Embryonic Development Study of Trofinetide in Rats

Study no.:	20202588
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	August 2, 2019
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	Trofinetide, BX1003214, 98.4%

Key Study Findings

- There were no drug-related deaths.
- No adverse findings were observed on fertility and reproduction parameters (mating, sperm motility, spermatid density, sperm morphology, estrous cycle evaluations, and ovarian and uterine examinations).
- Trofinetide was well tolerated. The NOAEL for development of reproductive performance was the HD (2000 mg/kg/day).

Methods

Doses:	0, 150, 450, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Dose volume:	8 mL/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	Reverse Osmosis Deionized Water
Species/Strain:	Rat / Sprague-Dawley
Number/Sex/Group:	22
Study design:	M were dosed from 28 days before cohabitation, during cohabitation, and through to the day before euthanasia, a total of 66 days. F were dosed beginning 15 days before cohabitation, during, cohabitation, and through to GD 7.

Observations and Results

Mortality

Observations for mortality and morbidity were conducted twice daily.

One MDM was found dead on Day 42, with no adverse clinical signs or changes. Another MDM was euthanized early on Day 6; the early euthanasia was due to adverse clinical signs and with a perforation in the esophagus that suggests a gavage error. Therefore, the deaths were not considered related to trofinetide.

Clinical Signs

M and F were observed for clinical signs daily prior to initiation of dosing and twice daily during dosing (1 to 2 hours post-dose).

No adverse clinical signs in M and F were noted.

Body Weight

M body weights were recorded once prior to initiation of dosing and twice weekly during dosing. F body weights were recorded once prior to initiation of dosing, twice weekly until mating, and on GDs 0, 3, 7, 10, and 13.

No adverse change in body weight in M or F was noted.

Food Consumption

In M, food consumption was recorded weekly during dosing until cohabitation. In F, food consumption was recorded weekly during dosing and on GDs 0, 3, 7, 10, and 13.

No adverse change in food consumption in M or F was noted.

Reproduction Function Parameters

Estrous cycle

Vaginal lavages were collected daily, the stage of estrous determined, and continued until spermatozoa and/or copulatory plug were observed.

There were no adverse changes in estrous cycling.

Mating

Mating was for 14 days, 1 M was co-housed with 1 F. F not mated within the first 6 or 7 days of cohabitation were assigned an alternate M that had mated (same dose group) for the remaining days. Signs of mating were examined until mating was confirmed.

There were no adverse changes in mating parameters.

Ovarian and Uterine Examinations

F were sacrificed on GD 13 and the reproductive tract examined for number of corpora lutea, implantation sites, number of live and dead fetuses, resorptions, and placentae.

There were no adverse changes in reproduction parameters in dosed F.

Sperm analysis

Epididymides were collected and sperm motility, concentration, morphology, and counts analyzed.

There was no adverse change in sperm parameters.

Necropsy

Gross examination was conducted on all animals.

There were no adverse macroscopic findings noted in surviving animals.

Organ Weights

At necropsy, the following organs, as listed in the study report, were weighed.

Tissue	Weighed	Collected	Microscopically Evaluated	Comment
Animal Identification	-	X	-	All Animals
Cervix	-	X	-	Collected with uterus.
Epididymides	X	X	-	Individual weight.
Epididymis, left cauda	X	-	-	Weighed for all males at scheduled euthanasia
Esophagus		X	-	Infused with 10% neutral buffered formalin. Unscheduled deaths.
Gland, mammary	-	X	-	Collected with inguinal skin. Females only.
Gland, prostate	X	X	-	-
Gland, seminal vesicle	X	X	-	Paired weight with fluid.
Gross lesions/masses	-	X	-	-
Heart	-	X	-	Unscheduled deaths.
Kidney	-	X	-	Unscheduled deaths.
Liver	-	X	-	Unscheduled deaths.
Lung	-	X	-	Infused with 10% neutral buffered formalin. Unscheduled deaths.
Ovaries	-	X	-	-
Spleen	-	X	-	Unscheduled deaths.
Stomach	-	X	-	Unscheduled deaths.
Testes	X	X	-	Scheduled euthanasia: Individual weight. Left testis was weighed with and without tunica and then homogenized for spermatid concentration counts. Right testis was fixed in Bouin's solution for 48 to 96 hours before being retained in 10% neutral buffered formalin (See Appendix 1). Unscheduled deaths: Testes were fixed in Bouin's solution for 48 to 96 hours before being retained in 10% neutral buffered formalin (See Appendix 1).
Trachea	-	X	-	Infused with 10% neutral buffered formalin. Unscheduled deaths.
Uterus	-	X	-	Collected with cervix.
Vagina	-	X	-	-

X = procedure conducted; - = not applicable.

No adverse change in organ weights were noted.

9.1.2 Study Title: GLP fertility and early embryonic development study of trofinetide (NNZ-2566) in rats dosed orally from 2 to 12 weeks of age

Study no.: 0621-16030
Study report location: EDR
GLP compliance: Yes
Drug, lot #, and % purity: NNZ-2566, Lot № R0006475, 97.3%

Key Study Findings

- No drug-related deaths.
- Slight increase in pre-coital interval in mating of dosed HDM and decrease in pregnant HDF were noted.
- A non-statistically significant higher rate of implantation loss for the undosed F paired with the dosed HDM was noted with a lower mean number of viable fetuses; however, the differences were considered partly due to differences in ovulation rate of the undosed F.
- Trofinetide was well tolerated with the HD the NOAEL.

Methods

Doses:	0, 150, 300, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Number/Sex/Group:	20
Dose volume:	8 mL/kg/dose
Formulation/Vehicle:	Deionized water
Route of administration:	ORAL GAVAGE
Species:	RAT
Strain:	SPRAGUE-DAWLEY
Comment on Study Design and Conduct:	Dosing began on PND 13 or 14. Pups were weaned 7 days later. On Day 86 groups were culled to 20/sex/group. Approximately 4 weeks post-last dose, animals were mated for up to 15 days.

Observations and Results

Mortality

Observations for mortality and morbidity were conducted twice daily.

Several early deaths (1 control M, 2 control F, 1 LDM, 1 MDM, and 2 HDF) occurred, as outlined in the table below. No deaths were considered related to trofinetide, with signs of esophageal rupture and aspiration, suggesting gavage errors.

Table: mortalities (Sponsors)

Males

Animal No.	Study Day	Status	Postmortem Findings	Likely Contributing Cause of Death
1M22	46	FD	Dark lung lobes; Colorless fluid in thoracic cavity; Fluid in all lung lobes	Esophageal rupture
2M42	64	FD	No macroscopic findings	Unknown*
3M57	41	FD	Dark lung lobes; White fluid in thoracic cavity	Esophageal rupture

FD = Found Dead
*No trofinetide deaths were believed to occur during this study, so the death was considered incidental and unrelated to trofinetide since all other deaths were likely caused by accidental aspiration. Deaths occurred in the control, low and mid dose groups. No deaths occurred in the high dose group.

Females

Animal No.	Study Day	Status	Postmortem Findings	Likely Contributing Cause of Death
1F104	11	SM	Distended intestines and cecum	Accidental aspiration
1F112	2	SM	Dark lung lobes	Accidental aspiration
4F190	3	FD	Dark lung lobes	Accidental aspiration
4F198	3	SM	Dark lung lobes	Accidental aspiration

FD = Found Dead
SM = Sacrificed Moribund

Necropsy

Gross examination was conducted on all animals.

There were no adverse macroscopic findings noted in surviving animals.

Physical development
Vaginal opening and preputial separation were assessed from PND 27.

There were no effects on sexual maturation.

Reproduction Function Parameters
Estrous cycle
Vaginal smears were collected daily and the stage of estrous determined, beginning 10 days prior to co-housing and continued until mating was confirmed.

There were no effects on estrous cycling.

Mating
Approximately 4 weeks post-last dose, each dosed rat was co-housed with an undosed rat of the opposite sex for up to 15 days. Signs of mating were examined until mating was confirmed.

There was no statistically significant change in pre-coital interval

Dosed males and undosed females
After mating, M were sacrificed. Dosed F were maintained until GD 14 and the reproductive tract examined for number of corpora lutea, implantation sites, number of fetuses, and resorptions.

There was a non-statistically significant higher rate of implantation loss for dosed HDM, reflected by a lower mean number of implantations and lower mean number of viable fetuses. The differences were considered partly due to differences in ovulation rate of undosed F, as mean corpora lutea were 14.6 and 13.2 for F mated to control M and HDM, respectively.

Table: mean reproduction parameters

Group	Pre-coital interval (d)	Pregnant rats	Corpora lutea	Implant sites			Early resorption	Implantation Loss		
				Total	Viable	Non-viable		Pre	Post	Total
Control	3.4	14	14.6 (206)	14.6 (205)	13.9 (195)	0.1 (1)	0.6 (9)	0.1 (1)	0.7 (10)	0.8 (11)
LD	4.9	14	13.9 (194)	12.6 (176)	11.6 (162)	0 (0)	1.0 (14)	1.3 (18)	1.0 (14)	2.3 (32)
MD	3.7	16	14.3 (228)	13.7 (219)	12.9 (206)	0 (0)	0.8 (13)	0.6 (9)	0.8 (13)	1.4 (22)
HD	5.4	15	13.2 (198)	11.6 (174)	10.4 (156)	0 (0)	1.2 (18)	1.6 (24)	1.2 (18)	2.8 (42)

Total numbers are in parenthesis.

Dosed females and undosed males
After mating, M were sacrificed and discarded. Dosed F were maintained until GD 14 and the reproductive tract examined for number of corpora lutea, implantation sites, number of fetuses, and resorptions.

There were no adverse changes in reproduction parameters in dosed F.

Table: mean reproduction parameters

Group	Pre-coital interval (d)	Pregnant rats	Corpora lutea	Implant sites			Early resorption	Implantation Loss		
				Total	Viable	Non-viable		Pre	Post	Total
Control	3.5	14	14.3 (200)	13.7 (192)	13.1 (183)	0 (0)	0.6 (9)	0.6 (8)	0.6 (9)	1.2 (17)
LD	3.0	14	13.3 (186)	12.3 (172)	11.6 (163)	0.1 (1)	0.6 (8)	1.0 (14)	0.6 (9)	1.6 (23)
MD	3.4	15	15.9 (239)	15.9 (239)	15.3 (229)	0 (0)	0.7 (10)	0 (0)	0.7 (10)	0.7 (10)
HD	3.6	10	15.3 (153)	15.2 (152)	13.8 (138)	0 (0)	1.4 (14)	0.1 (1)	1.4 (14)	1.5 (15)

Total numbers are in parenthesis.

Sperm analysis of dosed males

Epididymides were collected from 10 dosed M/group and sperm motility and counts analyzed.

There was no change in sperm motility or counts.

9.2 Embryonic Fetal Development

9.2.1 Study Title: An Oral (Gavage) Embryo-Fetal Development Study of Trofinetide in Rats

Study no.: 20222415
Study report location: EDR
Conducting laboratory and location: (b) (4)
Date of study initiation: March 5, 2020
GLP compliance: Yes, US
QA statement: Yes
Drug, lot #, and % purity: Trofinetide, 201911120218, 94.6%

Key Study Findings

- No unscheduled deaths, adverse clinical signs, or adverse changes in body weight or food consumption were noted.
- No adverse findings were observed on ovarian, uterine, or litter parameters.
- Malformations were observed in 1, 1, 1, and 2 control, LD, MD, and HD fetuses.
- Trofinetide was well tolerated. The HD (2000 mg/kg/day) was the maternal and fetal NOAELs. The trofinetide plasma exposures (AUC) in dams on GD 17 associated with 2000 mg/kg/day was 1420 µg*h/mL.

Methods

Doses:	0, 150, 450, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Dose volume:	8 mL/kg
Route of administration:	ORAL GAVAGE
Formulation/Vehicle:	Deionized water
Species:	RAT
Strain:	SPRAGUE-DAWLEY
Number/Pregnant F/Group	
Main:	20
Toxicokinetic:	3 control and 6 LD, MD, and HD
Study design:	Trofinetide was administered daily on GDs 7-17

Observations and Results

Mortality

Observations for mortality and morbidity were conducted twice daily.

No unscheduled deaths occurred during the study.

Clinical Signs

F were observed for clinical signs daily prior to initiation of dosing, twice daily during dosing (1 to 2 hours after each dose), and daily during recovery.

No adverse clinical signs were noted.

Body Weight

Body weights were recorded on GD 0 prior to initiation of dosing and on GDs 7, 10, 12, 15, 18, and 21.

No adverse change in body weight was noted.

Food Consumption

Food consumption was measured on GDs 7, 10, 12, 15, 18, and 21.

No adverse change in food consumption was noted.

Necropsy

On GD 21, F were sacrificed, and external features, orifices, and visceral organs and placentas were examined macroscopically. The uterus was removed for assessment of fertility parameters. The cervix, ovaries, and uterus were weighed.

No adverse macroscopic findings were noted.

Cesarean Section Data

The number of corpora lutea, implantation sites, live and dead fetus, and early and late resorptions were recorded. Viable fetuses were sexed and weighed.

No adverse changes were noted on any ovarian, uterine, or litter parameters.

Offspring (Malformations, Variations, etc.)
All viable fetuses were examined for external findings. A visceral examination was conducted on approximately one-half of each litter. A skeletal examination was conducted on the other half of fetuses.

External
No adverse findings were noted.

Visceral
Malformations were observed 1, 1, 1, and 2 control, LD, MD, and HD fetuses.

Skeletal
No adverse findings were noted.

Toxicokinetics
Blood samples were taken on GDs 7 and 17 pre-dose (for GD 17 only) and at 1.5, 3, 7.5, 9, and 24 hours post-first dose for trofinetide dosed animals and at 1.5 hours post-first dose for control animals.

The increase in plasma exposure (C_{max} and AUC) was approximately dose-proportional. T_{max} was approximately 1.5 hours post-second dose and no accumulation was noted between GDs 7 and 17.

Table: toxicokinetic data of trofinetide (Sponsor's)

Day	Group	Dose Level (mg/kg/day)	T_{max} (hr)	C_{max} (µg/mL)	SE C_{max} (µg/mL)	T_{last} (hr)	AUC_{last} (hr*µg/mL)	SE AUC_{last} (hr*µg/mL)	RA C_{max}	RA AUC_{last}
GD 7	2	300	7.5	26.4	2.41	24	181	5.74	-	-
GD 7	3	900	7.5	115	16.0	24	588	47.6	-	-
GD 7	4	2000	7.5	160	2.96	24	1520	239	-	-
GD 17	2	300	7.5	29.8	5.06	24	182	23.8	1.13	1.01
GD 17	3	900	7.5	106	4.33	24	671	97.4	0.925	1.14
GD 17	4	2000	7.5	214	28.4	24	1420	119	1.34	0.933

9.2.2 Study Title: An Oral (Stomach Tube) Dose Range-finding Embryo-Fetal Development Study of Trofinetide in Pregnant Rabbits, including a Preliminary Evaluation in Non-Pregnant Rabbits

Study no.:	20222416
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	February 17, 2020
GLP compliance:	No
QA statement:	No
Drug, lot #, and % purity:	Trofinetide, 201911120218, 94.6%

Key Study Findings

- 1 control F was sacrificed early, and 1 HDF was found dead.
- Clinical signs included soft feces.
- Decreases in body weight and food consumption were observed in MD and HD F.
- Increased resorptions were observed in MDF and HDF, with an increase in post-implantation losses. Live fetuses were reduced at the HD, and fetal weight was reduced in MD and HD F.
- No malformations were observed.
- Trofinetide was generally not well tolerated at the MD and HD. The LD (300 mg/kg/day) was the maternal NOAEL due to effects on body weight and food consumption. The trofinetide plasma exposures (AUC) in dams on GD 19 associated with 300 mg/kg/day was 129 µg*h/mL.

Methods - Part A

Doses:	0, 150, 450, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Dose volume:	8 mL/kg
Route of administration:	ORAL
Formulation/Vehicle:	Deionized water
Species:	RABBIT
Strain:	NEW ZEALAND
Number/F/Group:	2
Study design:	Trofinetide was administered on Days 1-7

Observations and Results – Part A

Mortality

Observations for mortality and morbidity were conducted twice daily.

No unscheduled deaths occurred during the study.

Clinical Signs
F were observed for clinical signs at least once prior to initiation of dosing, post-dose at 1, 2, 3, and 4 hours post-dose for the first dose and then 1 and 2 hours post-dose for the second dose for the first 2 days and then daily (between 1 and 2 hours post-dose) if no adverse signs are observed.

Soft feces were observed in the MDF and HDF, and 1 HDF had a thin appearance on Day 8.

Body Weight
Body weights were recorded daily.

There was a decrease in body weight in MDF (30 g) and HDF (176 g).

Food Consumption
Food consumption was measured daily.

There was a decrease in food consumption in MDF (18%) and HDF (55%).

Methods - Part B

Doses:	0, 150, 300, and 500 mg/kg
Frequency of dosing:	BID, 6 hours apart
Dose volume:	8 mL/kg
Route of administration:	ORAL
Formulation/Vehicle:	Deionized water
Species:	RABBIT
Strain:	NEW ZEALAND
Number/Pregnant F/Group	
Main:	6
Toxicokinetic:	3
Study design:	Trofinetide was administered daily on GDs 7-19

Observations and Results – Part B
Mortality
Observations for mortality and morbidity were conducted twice daily.

One control F was sacrificed early on GD 28 due to reduced food consumption and 1 HDF found dead on GD 14, with food consumption reduced between GDs 9 and 13. The cause of death in the HDF was undetermined and not considered drug related.

Clinical Signs
F were observed for clinical signs once prior to initiation of dosing and daily between 1 and 2 hours post-dose.

Dose-dependent increases in incidences of soft feces and ungroomed fur were observed at the MD and HD.

Body Weight
Body weights were recorded on GD 0 prior to initiation of dosing and daily thereafter.

There was a decrease in body weight gain from GDs 7 to 29 in MDF (26%) and HDF (87%).

Food Consumption
Food consumption was measured daily.

Decrease in food consumption was noted from GDs 7 to 29 in MDF (20%) and HDF (43%).

Necropsy
On GD 29, F were sacrificed, and external features, orifices, and visceral organs and placentas were examined macroscopically. The uterus was removed for assessment of fertility parameters.

No adverse macroscopic findings were noted in animals that died early.

Cesarean Section Data
The number of corpora lutea, implantation sites, live and dead fetus, and early and late resorptions were recorded. Viable fetuses were sexed and weighed.

There was an increase in early and late resorptions in MDF and HDF, with increased post implantation losses in MDF and HDF and live fetuses reduced at the HD. Fetal weight was reduced at the MD and HD. Litter means for corpora lutea, implantations, dead fetuses, and fetal sex ratio were similar across the 4 groups.

Table: summary of ovarian and uterine examinations (Sponsor's)

Sex: Female		0 mg/kg/day	300 mg/kg/day	600 mg/kg/day	1000 mg/kg/day
Day(s) Relative to Mating (Litter: A)		Group 5	Group 6	Group 7	Group 8
Female with Live Fetuses	N+ve	5	6	6	5
	%	100.0	100.0	100.0	100.0
Number of Corpora Lutea	Mean	11.4	11.0	13.5	11.4
	SD	1.8	0.9	1.6	1.7
	N	5	6	6	5
	%Diff	-	-3.5	18.4	0.0
Number of Implantations	Mean	10.4	9.8	12.7	9.6
	SD	1.1	2.0	2.1	0.5
	N	5	6	6	5
	%Diff	-	-5.4	21.8	-7.7
Pre-implantation Loss (%)	Mean	8.16	9.85	6.28	13.96
	SD	5.14	20.00	9.00	15.88
	N	5	6	6	5
	%Diff	-	20.69	-23.08	71.09
Total Number of Resorptions	Mean	0.8	0.7	2.2	1.8
	SD	1.3	0.8	3.1	2.7
	N	5	6	6	5
	%Diff	-	-16.7	170.8	125.0
Number of Early Resorptions	Mean	0.4	0.2	1.5	0.8
	SD	0.5	0.4	2.3	1.8
	N	5	6	6	5
	%Diff	-	-58.3	275.0	100.0
Number of Late Resorptions	Mean	0.4	0.5	0.7	1.0
	SD	0.9	0.8	0.8	1.4
	N	5	6	6	5
	%Diff	-	25.0	66.7	150.0
Total Number of Fetuses	Mean	9.6	9.2	10.5	7.8
	SD	1.1	2.0	2.3	2.9
	N	5	6	6	5
	%Diff	-	-4.5	9.4	-18.8
Number of Live Fetuses	Mean	9.6	9.0	10.5	7.8
	SD	1.1	2.2	2.3	2.9
	N	5	6	6	5
	%Diff	-	-6.3	9.4	-18.8

Sex: Female Day(s) Relative to Mating (Litter: A)		0 mg/kg/day Group 5	300 mg/kg/day Group 6	600 mg/kg/day Group 7	1000 mg/kg/day Group 8
Number of Live Male Fetuses	Mean	4.2	4.2	4.8	3.6
	SD	2.3	2.5	1.7	1.1
	N	5	6	6	5
	%Diff	-	-0.8	15.1	-14.3
Number of Dead Fetuses	Mean	0.0	0.2	0.0	0.0
	SD	0.0	0.4	0.0	0.0
	N	5	6	6	5
	%Diff	-	-	-	-
Post-implantation Loss (%)	Mean	7.12	8.18	15.30	19.33
	SD	11.83	11.67	21.43	29.48
	N	5	6	6	5
	%Diff	-	14.89	114.84	171.49
Live Male Fetus/Litter (%)	Mean	43.21	44.03	45.73	48.79
	SD	19.77	24.12	13.46	10.63
	N	5	6	6	5
	%Diff	-	1.89	5.82	12.92
Number of Live Female Fetuses	Mean	5.4	4.8	5.7	4.2
	SD	1.9	2.3	1.9	1.9
	N	5	6	6	5
	%Diff	-	-10.5	4.9	-22.2
Mean Fetal Weight (both) (g)	Mean	45.24	43.16	39.75	36.97
	SD	4.34	7.41	2.39	8.79
	N	5	6	6	5
	%Diff	-	-4.59	-12.13	-18.29
Mean Fetal Weight (M) (g)	Mean	45.66	44.74	39.60	40.10
	SD	3.95	9.43	2.72	8.23
	N	5	6	6	5
	%Diff	-	-2.01	-13.29	-12.18
Mean Fetal Weight (F) (g)	Mean	44.07	42.88	39.81	32.97
	SD	4.92	7.19	2.37	13.00
	N	5	6	6	5
	%Diff	-	-2.70	-9.67	-25.17

Offspring (Malformations, Variations, etc.)
All viable fetuses were examined for external and visceral changes.

External
One control fetus had brachydactyly (shortened digits) of both fore- and hind-paws. Otherwise, no malfunctions were noted.

Visceral
No visceral malformations were noted.

Toxicokinetics
Blood samples were taken on GDs 7 and 19 pre-dose (for GD 19 only) and at 1, 3, 6, 8, 12, 16, and 24 hours post-first dose for trofinetide dosed animals and at 1 hour post-first dose for control animals.

The increase in plasma exposure (C_{max} and AUC) was generally dose-proportional. T_{max} ranged from 1 to 24 hours post-first dose, and approximately 6-fold accumulation was noted between GDs 7 and 19.

Table: toxicokinetic data of trofinetide (Sponsor's)

Day	Group	Dose Level (mg/kg/day)	C_{max} (µg/mL)	SE C_{max} (µg/mL)	T_{max} (hr)	AUC_{last} (hr*µg/mL)	SE AUC_{last} (hr*µg/mL)	T_{last} (hr)	RA C_{max}	RA AUC
GD 7	6	300	1.01	0.183	12	19.7	1.69	24	-	-
GD 7	7	600	3.24	0.890	24	51.8	7.35	24	-	-
GD 7	8	1000	4.42	0.536	24	78.6	5.72	24	-	-
GD 19	6	300	7.91	2.84	24	129	32.0	24	7.86	6.51
GD 19	7	600	15.4	2.61	8.0	326	48.8	24	4.75	6.28
GD 19	8	1000	23.7	0.100	1.0	470	38.6	24	5.36	5.97

9.2.3 Study Title: An Oral (Gavage) Embryo-Fetal Development Study of Trofinetide in Rabbits

Study no.: 20222417
Study report location: EDR
Conducting laboratory and location: (b) (4)
Date of study initiation: May 6, 2020
GLP compliance: Yes, US
QA statement: Yes
Drug, lot #, and % purity: Trofinetide, 201911120218, 94.6%

Key Study Findings

- Abortions associated with severe reductions in food consumption and body weight loss with subsequently euthanized occurred in 1 control and 2 HD dams.
- There were no adverse clinical signs, but decreases in maternal body weight and food consumption was observed at the MD and HD.
- Slight increases in early resorptions and post-implantation loss were noted in HDF, but with minimal decrease in live births and no dead fetuses; otherwise, no adverse findings were observed on ovarian, uterine, or litter parameters.
- Malformations were observed in 2, 8, 2, and 4 control, LD, MD, and HD fetuses.
- The LD (150 mg/kg/day) was the maternal NOAEL, due to reduced body weight and food consumption and the HD (600 mg/kg/day) the fetal NOAEL. The trofinetide plasma exposures (AUC) in dams on GD 19 associated with 150 mg/kg/day was 57.5 µg*h/mL and with 600 mg/kg/day was 425 µg*h/mL.

Methods

Doses: 0, 75, 150, and 300 mg/kg
Frequency of dosing: BID, 6 hours apart
Dose volume: 8 mL/kg
Route of administration: ORAL GAVAGE
Formulation/Vehicle: Deionized water
Species: RABBIT
Strain: NEW ZEALAND
Number/Pregnant F/Group
Main: 20
Toxicokinetic: 3
Study design: Trofinetide was administered daily on GDs 7-19

Observations and Results
Mortality
Observations for mortality and morbidity were conducted twice daily.

Abortions were observed in 1 and 2 control and HD animals on GD 22, 22, and 26, respectively, with the animals subsequently euthanized. The abortions of all animals were associated with severe reductions in food consumption, with a loss of body weight also noted in the 2 HD animals. A third HD animal was euthanized early on GD 20 due to a protruding eyeball.

Clinical Signs
F were observed for clinical signs daily prior to initiation of dosing, twice daily during dosing prior to each dose, once 1 to 2 hours post-dose, and daily during recovery.

Dose-dependent increases in incidences of soft feces and ungroomed fur were observed at the MD and HD.

Body Weight
Body weights were recorded on GD 0 prior to initiation of dosing and daily during the dosing and recovery periods.

There was a decrease in body weight gain from GDs 7 to 29 in LDF (6%), MDF (14%), and HDF (6%).

Food Consumption
Food consumption was measured daily during the study.

There was a dose-dependent decrease in food consumption from GDs 7 to 29 in LDF (9%), MDF (13%), and HDF (26%).

Necropsy
On GD 29, F were sacrificed, and thoracic, abdominal, and pelvic visceral organs and placentas examined macroscopically. The uterus was removed for assessment of fertility parameters.

No adverse macroscopic findings were noted in animals that survived to termination.

Cesarean Section Data
The number of corpora lutea, implantation sites, live and dead fetus, and early and late resorptions were recorded. Viable fetuses were sexed and weighed.

There were slight increases in early resorptions and post-implantation loss in HDF, but with a minimal decrease in live births and no dead fetuses.

Table: summary of ovarian and uterine examinations (Sponsor's)

Sex: Female		0 mg/kg/day	150 mg/kg/day	300 mg/kg/day	600 mg/kg/day
Day(s) Relative to Mating (Litter: A)		Group 1	Group 2	Group 3	Group 4
Female with Live Fetuses [f]	N+ve	18	18	18	17
	%	100.0	100.0	100.0	100.0
Number of Corpora Lutea [k]	Mean	13.4	13.5	13.1	13.2
	SD	2.2	3.5	1.6	2.1
	N	18	18	18	17
	%Diff	-	0.8	-2.5	-1.6
Number of Implantations [k]	Mean	10.1	11.2	10.5	10.1
	SD	2.7	2.7	1.5	1.9
	N	18	18	18	17
	%Diff	-	11.0	4.4	0.0
Pre-implantation Loss (%) [k]	Mean	24.22	15.87	18.99	22.86
	SD	19.57	14.61	11.06	13.49
	N	18	18	18	17
	%Diff	-	-34.48	-21.61	-5.63
Total Number of Resorptions [k]	Mean	0.5	0.6	0.5	0.9
	SD	0.9	0.8	1.0	1.3
	N	18	18	18	17
	%Diff	-	22.2	0.0	88.2
Number of Early Resorptions [k]	Mean	0.1	0.3	0.2	0.6
	SD	0.3	0.6	0.5	0.9
	N	18	18	18	17
	%Diff	-	150.0	50.0	429.4
Number of Late Resorptions [k]	Mean	0.4	0.3	0.3	0.4
	SD	0.8	0.6	1.0	1.1
	N	18	18	18	17
	%Diff	-	-14.3	-14.3	-9.2
Total Number of Fetuses [k]	Mean	9.6	10.6	10.0	9.1
	SD	2.4	2.7	1.3	1.6
	N	18	18	18	17
	%Diff	-	10.5	4.7	-4.6
Number of Live Fetuses [k]	Mean	9.6	10.5	10.0	9.1
	SD	2.4	2.8	1.3	1.6
	N	18	18	18	17
	%Diff	-	9.9	4.7	-4.6

Sex: Female		0 mg/kg/day	150 mg/kg/day	300 mg/kg/day	600 mg/kg/day
Day(s) Relative to Mating (Litter: A)		Group 1	Group 2	Group 3	Group 4
Number of Live Male Fetuses [k]	Mean	4.3	5.3	5.9*	4.2
	SD	1.7	1.7	2.0	1.6
	N	18	18	18	17
	%Diff	-	24.7	39.0	-2.4
Number of Live Female Fetuses [k]	Mean	5.3	5.2	4.1	4.9
	SD	2.1	2.1	1.7	1.6
	N	18	18	18	17
	%Diff	-	-2.1	-23.2	-6.4
Number of Dead Fetuses [k]	Mean	0.0	0.1	0.0	0.0
	SD	0.0	0.2	0.0	0.0
	N	18	18	18	17
	%Diff	-	-	-	-
Post-implantation Loss (%) [k]	Mean	4.11	6.17	4.22	8.37
	SD	6.80	8.09	8.22	11.11
	N	18	18	18	17
	%Diff	-	50.22	2.79	103.68
Live Male Fetus/Litter (%) [k]	Mean	46.01	50.90	59.06*	45.77
	SD	15.89	12.52	15.44	14.19
	N	18	18	18	17
	%Diff	-	10.62	28.36	-0.51
Mean Fetal Weight (both) (g) [G]	Mean	39.05	37.04	37.80	39.18
	SD	4.79	7.04	4.46	4.27
	N	18	18	18	17
	%Diff	-	-5.15	-3.20	0.32
Mean Fetal Weight (M) (g) [G]	Mean	39.52	37.51	38.26	39.72
	SD	6.12	7.07	4.38	5.10
	N	18	18	18	17
	%Diff	-	-5.08	-3.18	0.51
Mean Fetal Weight (F) (g) [G]	Mean	38.73	36.81	36.84	38.89
	SD	4.71	7.71	6.13	4.15
	N	18	18	18	17
	%Diff	-	-4.95	-4.88	0.40

Offspring (Malformations, Variations, etc.)
All viable fetuses were examined for external, visceral, and skeletal changes.

External
Malformations included an absent head with a misshapen right pinna and adactyly and brachydactyly in 1 LD fetus, hyperflexion of the forepaws in 1 MD fetus, umbilical hernia in 1 MD fetus, and a domed head in 1 HD fetus. These incidences were not dose dependent and fell within the historical control range. Therefore, these are not considered drug related.

Table: summary of external fetal abnormalities (Sponsor's)

Exam Type: External	0 mg/kg/day Group 1	150 mg/kg/day Group 2	300 mg/kg/day Group 3	600 mg/kg/day Group 4
Number of Fetuses Examined:	172	189	180	155
Number of Fetuses Evaluated:	172	190	180	155
Number of Litters Examined:	18	18	18	17
Number of Litters Evaluated:	18	18	18	17
Malformation				
Number of Fetuses	0	1	2	1
Litter % of Fetuses [k]	0.00	0.56	1.11	0.74
Number of Litters	0	1	2	1
All classifications				
Number of Fetuses	0	1	2	1
Litter % of Fetuses [k]	0.00	0.56	1.11	0.74
Number of Litters	0	1	2	1

Visceral
Malformations were observed in 2, 3, and 3 control, LD, and HD fetuses, respectively, and included persistent truncus arteriosus in 1, 2, and 3 control, LD, and HD fetuses, respectively and ventricular septal defect in 1, 3, and 3 control, LD, and HD fetuses, respectively, and retroesophageal right subclavian artery in 1 LD and 1 HD fetus, respectively. The absence of brain, eyes, tongue, and teeth was noted in 1 LD fetus, misshapen heart and small lung in 1 LD fetus, aortic arch dilatation and narrow pulmonary truck in 1 LD fetus, and small brain with severe lateral ventricle dilatation, large right eye with a misshapen ocular orbit in 1 HD fetus.

Table: summary of visceral fetal abnormalities (Sponsor's)

Exam Type: FreshVis	0 mg/kg/day Group 1	150 mg/kg/day Group 2	300 mg/kg/day Group 3	600 mg/kg/day Group 4
Number of Fetuses Examined:	172	189	180	155
Number of Fetuses Evaluated:	172	190	180	155
Number of Litters Examined:	18	18	18	17
Number of Litters Evaluated:	18	18	18	17
Variation				
Number of Fetuses	0	2	1	4
Litter % of Fetuses [k]	0.00	0.95	0.56	2.61
Number of Litters	0	2	1	3
Malformation				
Number of Fetuses	2	3	0	4
Litter % of Fetuses [k]	1.39	1.35	0.00	2.61
Number of Litters	1	3	0	4
All classifications				
Number of Fetuses	2	3	1	6
Litter % of Fetuses [k]	1.39	1.35	0.56	4.00
Number of Litters	1	3	1	4

Skeletal
No adverse findings were noted, with no malformations in HD fetuses.

Toxicokinetics
Blood samples were taken on GDs 7 and 19 pre-dose (for GD 19 only) and at 1, 3, 6, 8, 12, 16, and 24 hours post-first dose for trofinetide dosed animals and at 1 hour post-first dose for control animals.

The increase in plasma exposure (C_{max} and AUC) was generally dose-proportional. T_{max} was approximately 24 hours post-second dose, and accumulation was noted between GDs 7 and 19.

Table: toxicokinetic data of trofinetide (Sponsor's)

Day	Group	Dose Level (mg/kg/day)	Animal ID	T_{max} (hr)	C_{max} (µg/mL)	T_{last} (hr)	AUC_{last} (hr*µg/mL)	RA C_{max}	RA AUC_{last}
GD 7	2	150	5104	12	0.583	24	11.7	-	-
			5105	8.0	0.671	24	13.3	-	-
			5106	1.0	0.781	24	14.1	-	-
			N	**3**	**3**	**3**	**3**	**0**	**0**
			Mean	**7.00**	**0.678**	**24.0**	**13.0**	**NA**	**NA**
			SD	**5.57**	**0.0992**	**0.00**	**1.18**	**NA**	**NA**
			CV%	**79.5**	**14.6**	**0.0**	**9.1**	**NA**	**NA**
GD 7	3	300	5107	24	1.84	24	35.7	-	-
			5108	8.0	1.24	24	25.2	-	-
			5109	8.0	1.37	24	26.9	-	-
			N	**3**	**3**	**3**	**3**	**0**	**0**
			Mean	**13.3**	**1.48**	**24.0**	**29.3**	**NA**	**NA**
			SD	**9.24**	**0.316**	**0.00**	**5.66**	**NA**	**NA**
			CV%	**69.3**	**21.3**	**0.0**	**19.3**	**NA**	**NA**
GD 7	4	600	5110	24	3.19	24	59.1	-	-
			5111	24	5.95	24	84.4	-	-
			5112	24	2.81	24	48.3	-	-
			N	**3**	**3**	**3**	**3**	**0**	**0**
			Mean	**24.0**	**3.98**	**24.0**	**63.9**	**NA**	**NA**
			SD	**0.00**	**1.71**	**0.00**	**18.5**	**NA**	**NA**
			CV%	**0.0**	**43.0**	**0.0**	**29.0**	**NA**	**NA**

Day	Group	Dose Level (mg/kg/day)	Animal ID	T_{max} (hr)	C_{max} (µg/mL)	T_{last} (hr)	AUC_{last} (hr*µg/mL)	RA C_{max}	RA AUC_{last}
GD 19	2	150	5104	1.0	1.90	24	35.7	3.26	3.04
			5105	1.0	1.90	24	40.2	2.83	3.03
			5106	1.0	4.32	24	96.7	5.53	6.87
			N	**3**	**3**	**3**	**3**	**3**	**3**
			Mean	**1.00**	**2.71**	**24.0**	**57.5**	**3.87**	**4.31**
			SD	**0.00**	**1.40**	**0.00**	**34.0**	**1.45**	**2.22**
			CV%	**0.0**	**51.6**	**0.0**	**59.1**	**37.5**	**51.4**
GD 19	3	300	5107	8.0	11.0	24	248	5.98	6.94
			5108	16	16.6	24	315	13.4	12.5
			5109	8.0	11.4	24	199	8.32	7.40
			N	**3**	**3**	**3**	**3**	**3**	**3**
			Mean	**10.7**	**13.0**	**24.0**	**254**	**9.23**	**8.95**
			SD	**4.62**	**3.12**	**0.00**	**58.1**	**3.79**	**3.09**
			CV%	**43.3**	**24.0**	**0.0**	**22.9**	**41.0**	**34.5**
GD 19	4	600	5110	8.0	13.0	24	286	4.08	4.84
			5111	0.0	35.6	24	667	5.98	7.91
			5112	3.0	14.8	24	321	5.27	6.64
			N	**3**	**3**	**3**	**3**	**3**	**3**
			Mean	**3.67**	**21.1**	**24.0**	**425**	**5.11**	**6.46**
			SD	**4.04**	**12.6**	**0.00**	**211**	**0.964**	**1.54**
			CV%	**110.2**	**59.4**	**0.0**	**49.7**	**18.9**	**23.9**

Note: GD=Gestation Day

9.3 Prenatal and Postnatal Development

9.3.1 Study Title: An Oral (Gavage) Developmental and Perinatal/Postnatal Reproduction Study of Trofinetide in Rats, Including a Postnatal Behavioral/Functional Evaluation

Study no.:	20222418
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	April 8, 2020
GLP compliance:	Yes, US
QA statement:	Yes
Drug, lot #, and % purity:	Trofinetide, 201911120218, 89.78%

Key Study Findings

- No drug-related deaths, adverse clinical signs, or changes in body weight were observed in F_0 HD dams. One control F_0 dam was euthanized on lactation day 9, with all the pups culled.
- F_0 dam reproductive parameters, gestation length and index, implantation sites, and live embryos and live birth index were comparable among all the groups. Findings in F_0 dams included a slight increase in stillborn pups at the HD, which is likely due natural variation and not drug related because of the higher number of implantation sites in these animals and the data fell within the historical control range.
- Post-weaning, all recorded endpoints were comparable among all the groups for F_1 animals.
- Trofinetide was well tolerated in F_0 dams. The NOAEL for pre- and postnatal development and reproductive performance in F_1 was the HD. The maternal trofinetide plasma exposures (AUC) on lactation day 20 at 2000 mg/kg/day was 860 µg*h/mL.

Methods

Doses:	0, 150, 450, and 1000 mg/kg
Frequency of dosing:	BID, 6 hours apart
Dose volume:	8 mL/kg
Route of administration:	ORAL GAVAGE
Formulation/Vehicle:	Deionized water
Species:	RAT
Strain:	SPRAGUE-DAWLEY
Number/Pregnant F/Group:	22
Study design:	Trofinetide was administered daily on GDs 7 to Day 20 postpartum

Observations and Results for F_0 Dams
Mortality
Observations were conducted twice daily.

No drug-related deaths were observed.

Clinical Signs
F_0 dams were observed for clinical signs daily (1 to 2 hours post-dose).

No adverse clinical signs were observed during the study.

Body Weight
Body weight recordings for F_0 dams were taken on GD 0 and daily from GD 7 and during the lactation period.

No adverse changes in body weight gain were noted, with body weight gain comparable across the groups during gestation and lactation.

Food Consumption
Food consumption in F_0 dams was recorded on GDs 7, 10, 12, 15, 18, and 20 and on lactation days 1, 4, 7, 10, and 12.

There was no adverse change in food consumption during gestation or lactation.

Delivery Observations
The following natural delivery parameters were evaluated in F: adverse clinical signs, duration of gestation, litter size, and pup viability at birth.

Mean gestation length, gestation index, implantation site numbers, live embryos, and live birth index were comparable between all the groups. However, there was an increase in stillborn pups of 1, 2, 2, and 9 control, LD, MD, and HD, respectively in 1, 2, 2, and 4 control, LD, MD, and HD F_0 F groups, respectively. The increase in stillborn pups at the HD was due to 2 HDF and is likely due to the higher number of implantation sites in these animals, and it did not influence live births or body weight and fell within the historical control data range of values. Therefore, the increase is unlikely to be drug related. There were no adverse effects on maternal behavior observations during lactation with all F_0 F grooming the pups and removing the amniotic sac, placenta, and umbilicus following completion of parturition. Normal nesting and nursing behaviors were generally observed throughout the lactation period.

Table: summary of maternal F_0 reproduction parameters (Sponsor's)

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day Group 1	300 mg/kg/day Group 2	900 mg/kg/day Group 3	2000 mg/kg/day Group 4
Group Size - Females		22	22	22	22
Number of Females Pregnant [f]	N+ve	22	22	22	22
	%	100.0	100.0	100.0	100.0
Gestation Length (Days) [k]	Mean	21.5	21.1	21.5	21.5
	SD	0.6	0.4	0.5	0.5
	N	22	22	22	22
	%Diff	-	-1.7	-0.2	0.0
Gestation Index [f]	%	100.0	100.0	100.0	100.0
	ProA	22/22	22/22	22/22	22/22
Females Completing Delivery [f]	N+ve	22	22	22	22
Females with Liveborn [f]	N+ve	22	22	22	22
Female with no Liveborn Pups [f]	N+ve	0	0	0	0
Fem w/ Stillborn Pups [f]	N+ve	1	2	2	4
Stillborn Pups/Litter [k]	Mean	0.45	0.73	0.92	3.10
	SD	2.13	2.36	2.99	8.70
	N	22	22	22	22
	%Diff	-	60.26	102.02	581.32
Number Pups Stillborn	Sum	1	2	2	9
Number Live Newborn Pups [k]	Mean	10.3	11.5	11.0	10.8
	SD	2.8	1.9	1.6	1.6
	N	22	22	22	22
	%Diff	-	11.9	6.6	4.4
	Sum	227	254	242	237
Live Birth Index (%) [k]	Mean	99.55	99.27	99.08	96.90
	SD	2.13	2.36	2.99	8.70
	N	22	22	22	22
	%Diff	-	-0.28	-0.47	-2.65
Live Male Pups/Litter (%) Birth [G]	Mean	46.39	47.80	47.18	54.11
	SD	18.93	16.23	15.45	15.24
	N	22	22	22	22
	%Diff	-	3.05	1.71	16.64

Sex: Female Day(s) Relative to Littering (Litter: A)		0 mg/kg/day Group 1	300 mg/kg/day Group 2	900 mg/kg/day Group 3	2000 mg/kg/day Group 4
Implantation Sites - Total [k]	Mean	11.0	12.5	12.0	11.5
	SD	2.9	1.8	1.7	1.8
	N	21	22	22	22
	%Diff	-	12.7	8.6	3.7
Post-implant Loss/Litter (%) [k]	Mean	6.26	6.68	7.16	2.31
	SD	7.31	6.69	8.27	4.80
	N	21	22	22	22
	%Diff	-	6.55	14.32	-63.14

Necropsy
F_0 dams were sacrificed on PND 21, and a gross examination of the thoracic, abdominal, and pelvic viscera conducted.

There were no adverse macroscopic findings in F_0 F.

Toxicokinetics
Maternal blood samples were taken on lactation day 20 pre-dose and at 1.5, 3, 6, 7.5, 9, and 24 hours post-first dose for trofinetide dosed animals and at 1.5 hours post-first dose for control animals.

The increase in plasma exposure (C_{max} and AUC) was approximately dose-proportional, with T_{max} at 7.5 hours post-first dose.

Table: maternal toxicokinetic data for trofinetide (Sponsor's)

Day	Group	Dose Level (mg/kg/day)	T_{max} (hr)	C_{max} (µg/mL)	SE C_{max} (µg/mL)	T_{last} (hr)	AUC_{last} (hr*µg/mL)	SE AUC_{last} (hr*µg/mL)
LD 20	2	300	7.5	15.9	0.745	24	112	9.17
LD 20	3	900	7.5	68.1	8.84	24	424	48.6
LD 20	4	2000	7.5	147	13.6	24	860	70.4

Observations and Results for F_1 Generation (pre-weaning)
Litters were not culled during the lactation period.

Mortality and Clinical signs
Mortality was assessed in F_1 pups twice daily from birth, and observation of clinical signs were conducted daily.

There were 4, 7, 3, and 14 control, LD, MD, and HD pup deaths (stillborn, found dead, and euthanized) noted and the litter from the control F_0 dam that was euthanized on lactation Day 9. Clinical signs in pups included cold to touch on a single day in 2 LD (from 1 litter) and 8 HD (from 1 litter) pups, discolored skin (purple ventral aspect, generalized, muzzle, cranium, or hind paw) in 1 control (from 1 litter), 2 LD (from 2 litters), 2 MD (from 2 litters) and 3 HD (from 3 litters) pups, and observations of the eyes (closed, abnormal size, and/or opacity) in 3 HD pups (from 3 litters). These abnormalities were not considered to be related to trofinetide because they occurred in single pups and did not persist postweaning.

Body Weight
Body weight recordings for F_1 pups were taken on PNDs 1, 4, 7, 10, 14, 17, and 21.

No adverse changes in body weight gain were noted.

Necropsy
On PND 21, animal not to be continued in the study were sacrificed and a necropsy was conducted on F_1 pups, and any abnormalities recorded.

Macroscopic findings included discolored kidney in 1 HD pup, 1 small kidney in 1 HD pup, severe renal papilla in 1 HD pup, a fluid filled trachea in 1 HD pup, dilatation of the ureter in 1 HD pup, and absent stomach content in 2 LD, 1 MD, and 3 HD pups.

Observations and Results for F_1 Generation (post-weaning)
Placement of F_1 adults (post-weaning animals)
At weaning on PND 21 postpartum, at least 1 M and 1 F pup per litter, when possible, was selected to continue resulting in 22 M and 22 F pups per group

Mortality
Observations for mortality were conducted twice daily for F_1 animals.

One HDM was found dead on Day 76 with no observation of adverse clinical signs, decrease in body weight, or adverse macroscopic findings. The cause of death was undetermined.

Clinical signs
Observations were conducted at least once weekly.

No adverse clinical signs were observed.

Body Weight
Body weight recordings for M F_1 pups were taken at least once weekly and for F_1 F pups once weekly during premating and mating periods and on GDs 0, 3, 7, 10, and 13.

No adverse changes in body weight gain were noted in M and F.

Food Consumption
Food consumption was measured weekly until cohabitation and on GDs 0, 3, 7, 10, and 13 for F.

No adverse changes in food consumption were noted in M or F.

Physical development
Vaginal opening was assessed from PND 24 and balanopreputial separation was assessed from PND 39.

No adverse changes in balanopreputial separation or vaginal opening were noted.

Behavioral assessment
Tests were conducted on 1 M and 1 F from each litter and included locomotor activity on PND 60, auditory startle habituation on PND 65, and water maze (Morris Maze) on PNDs 70 and 90.

Motor activity: Ambulation and fine movements were comparable among groups in M and F, with no statistically significant increase in any dose group.

Auditory startle habituation: Auditory startle habituation was comparable among groups in M and F, with no statistically significant increase in any dose group.

Morris water maze: Performance in the Morris water maze was comparable among groups in M and F, with no statistically significant increase in any dose group.

Reproduction parameters – male and female trofinetide offspring
At PND 90 at least, mating pairs (1 M and 1 F) were set up for up to 2 weeks. F not mated within the first 7 days were assigned an alternate M that had successfully mated with a F.

Mating, fertility, and pregnancy indices and mean number of days mating were comparable between all the groups.

Ovarian and Uterine Examinations
On GD 13, animals were euthanized, and the reproductive tract examined for number of corpora lutea, implantation sites, and number of live and dead embryos.

Number of corpora lutea, implantation sites, live and dead embryos, early resorptions, and pre- and post-implantation losses were comparable between all the groups.

Necropsy
Gross examination was conducted on all animals; M were sacrificed after mating and F were sacrificed on GD 13. At necropsy, the following organs, as listed in the study report, were collected.

Tissue	Collected	Comment
Animal Identification	X	All rats with tissues retained.
Epididymides	X	All males.
Gross lesions/masses	X	All rats.
Heart	X	Rat that was found dead.
Kidneys	X	Rat that was found dead.
Liver	X	Rat that was found dead.
Lungs	X	Rat that was found dead.
Ovaries	X	Collect with oviducts. All females.
Spleen	X	Rat that was found dead.
Stomach	X	Rat that was found dead.
Testes	X	All males.
Trachea/Esophagus	X	Rat that was found dead; retained but not required per protocol or SOP.
Uterus	X	Collect with cervix. All nonpregnant rats.

X = Procedure conducted.

No adverse macroscopic findings were noted.

9.4 Juvenile Animal Studies

9.4.1 Study Title: Fourteen day dose range finding (DRF) study of NNZ-2566 in juvenile male and female Sprague-Dawley rats

Study no:	NURN0002:2141
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	August 4, 2014
GLP compliance:	No
QA statement:	Yes (Dated: August 6, 2014)
Drug, lot #, and % purity:	NNZ-2566, Lot № 8007-A-R0-01-48-01, 99%

Key Study Findings:

- Trofinetide was well tolerated in juvenile rats up to 1000 mg/kg BID.

Methods

Doses:	0, 100, 300, and 1000 mg/kg
Frequency of dosing:	BID (8-hour interval) for 14 days
Dose volume:	5 mL/kg
Route of administration:	Oral (gavage)
Formulation/Vehicle:	Water for injections (WFI) BP
Species/Strain:	Rat/ Sprague-Dawley
Number/Sex/Group:	5
Age:	3 weeks
Weight:	Male 96-109 g; Female 90-102 g

Observations and Results

Mortality

Observations were conducted twice daily.

There were no deaths reported.

Clinical Signs

Observations were conducted once daily.

No adverse changes in clinical signs were noted.

Body Weight

Body weights were recorded daily.

There were no adverse changes in body weight.

Hematology
Blood samples were taken on Day 15 at necropsy.

In M, increases were observed in reticulocytes (17%, 26%, and 30%), lymphocytes (39%, 17%, and 19%), and monocytes (57%, 57%, and 71%) in LD, MD, and HD, respectively. In F, increases were observed in reticulocytes (5%, 19%, and 19%) and lymphocytes (8%, 18%, and 14%) in LD, MD, and HD, respectively.

Clinical Chemistry
Blood samples were taken on Day 15 at necropsy.

In HDM and HDF, a decrease in cholesterol (8% and 13%), an increase in potassium (18% and 11%), and a statistically significant decrease in sodium (2% and 1%) was observed.

Gross Pathology
Gross pathology of animals was conducted at necropsy on Day 15.

No adverse macroscopic findings were noted.

10 Special Toxicology Studies

10.1 Mechanistic Studies

10.1.1 Study Title: Assessment of IGF-1, IGFBP-2 and IGFBP-3 Following 28 Day Repeated Dosing with NNZ-2566 in Adult and Juvenile Sprague-Dawley Rats.

Study no.:	NURN0001:2106
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	May 5, 2014
GLP compliance:	No
QA statement:	Yes
Drug, lot #, and % purity:	Trofinetide, Lot № 8007-A-R0-01-48-01, 99%

Key Study Findings:

- No trofinetide related deaths, adverse clinical signs, or adverse body weight changes were noted.
- There was no effect on plasma and brain homogenate IGF-1 and IGFBP-3 levels in LDM, HDM, LDF, and HDF juvenile and adult rats.
- On Day 2 at 4 hours post-dose an increase in plasma IGFBP-2 levels were noted in LDM adult and HDF juvenile rats.

Methods

Doses:	0, 100, and 1000 mg/kg
Frequency of dosing:	BID (~8 hours apart)
Route of administration:	Oral (Gavage)
Dose volume:	5 mL/kg
Formulation/Vehicle:	Water for irrigation
Species/Strain:	Rats / Sprague-Dawley
Number/Sex/Group	
Juvenile:	4
Adult:	4
Age	
Juvenile:	3 Weeks
Adult:	8 Weeks
Weight	
Juvenile:	M 76-101 g; F 85-107 g
Adult:	M 303-378 g; F 181-230 g

Observations and Results

Mortality

Observations of morbidity and mortality were conducted twice daily during the study.

There was one 1 control adult M culled on Day 13 due to a dosing error.

Clinical Signs

Observations of clinical signs were recorded once daily.

No adverse clinical signs were observed.

Body Weights

Body weights were recorded twice weekly.

Generally, there were no adverse changes on body weight, although body weight gain was decreased by 20% in adult HDM on Day 28.

Insulin Growth Factor-1 (IGF-1), Insulin Growth Factor Binding Protein-2 (IGFBP-2), and Insulin Growth Factor Binding Protein-3 (IGFBP-3) analysis

Blood samples were taken pre-dose on Day 1 and at 4 hours post-dose on Days 2, 6, 10, 14, 18, 22, and 26. Blood and brain samples were assessed for IGF-1, IGFBP-2, and IGFBP-3 levels.

There was no significant effect on plasma and brain homogenate IGF-1 and IGFBP-3 levels in LDM, HDM, LDF, and HDF juvenile and adult rats. There was an increase in plasma IGFBP-2 in LDM adult and HDF juvenile rats on Day 2 at 4 hours post-dose. No other changes in IGFBP-2 levels were observed at other time points or in the brain homogenate.

Table: different IGF peptides levels in males after trofinetide administration in juveniles

	IGF-1 (ng/mL)			IGFBP-2 (ng/mL)			IGFBP-3 (ng/mL)		
Time-point	**Control**	**LD**	**HD**	**Control**	**LD**	**HD**	**Control**	**LD**	**HD**
Day 1 pre-dose	447	473	515	81.8	91.1	76.5	187	188	189
Day 2, 4 h	602	562	628	107.2	108.3	88.2	204	204	218
Day 6, 4 h	809	813	776	92.0	96.3	79.7	161	158	149
Day 10, 4 h	852	775	994	65.2	62.8	64.3	241	249	239
Day 14, 4 h	1259	1186	1117	53.7	50.5	45.9	197	269	271
Day 18, 4 h	1172	1103	1017	45.8	47.7	43.7	308	307	290
Day 22, 4 h	1402	1377	1585	41.6	39.5	34.1	294	285	271
Day 26, 4 h	1245	1186	1300	39.6	36.5	35.7	267	238	209
Brain	0.056	0.068	0.060	0.073	0.079	0.081	0.341	0.498	0.395

Table: different IGF peptides levels in males after trofinetide administration in adult.

	IGF-1 (ng/mL)			IGFBP-2 (ng/mL)			IGFBP-3 (ng/mL)		
Time-point	**C**	**LD**	**HD**	**C**	**LD**	**HD**	**C**	**LD**	**HD**
Day 1 pre-dose	1255	1449	1309	31.4	32.4	26.7	334	227	243
Day 2, 4 h	1155	1258	1007	31.1	53.2*	47.4	171	128	148
Day 6, 4 h	1431	1424	1271	34.8	31.8	33.0	253	263	258
Day 10, 4 h	1592	1576	1612	35.9	30.2	30.3	296	242	193
Day 14, 4 h	1521	1674	1558	30.9	29.7	32.8	271	260	218
Day 18, 4 h	1239	1085	1436	32.1	30.0	33.9	288	277	222
Day 22, 4 h	1520	1245	1105	26.4	30.0	32.1	223	238	199
Day 26, 4 h	1240	1295	1150	31.1	28.2	29.9	256	243	228
Brain	0.049	0.052	0.032	0.059	0.086	0.068	0.243	0.464	0.292

*** - Statistically significant**

Table: different IGF peptides levels in females after trofinetide administration in juveniles.

	IGF-1 (ng/mL)			IGFBP-2 (ng/mL)			IGFBP-3 (ng/mL)		
Time-point	**C**	**LD**	**HD**	**C**	**LD**	**HD**	**C**	**LD**	**HD**
Day 1 pre-dose	475	549	530	80.5	84.7	96.9	150	194	184
Day 2, 4 h	623	683	727	87.7	97.8	107.8*	209	199	173
Day 6, 4 h	850	848	845	82.2	86.9	88.5	165	152	138
Day 10, 4 h	953	823	1039	70.7	68.8	69.8	223	233	246
Day 14, 4 h	1155	1092	1041	50.0	51.1	55.0	179	214	207
Day 18, 4 h	828	765	735	45.1	45.4	48.5	212	229	246
Day 22, 4 h	1023	932	1017	31.8	35.1	36.3	169	172	175
Day 26, 4 h	805	794	799	38.2	41.4	42.2	134	134	127
Brain	0.055	0.057	0.044	0.093	0.068	0.078	0.405	0.418	0.412

*** - Statistically significant**

Table: different IGF peptides levels in females after trofinetide administration in adults.

	IGF-1 (ng/mL)			IGFBP-2 (ng/mL)			IGFBP-3 (ng/mL)		
Time-point	**C**	**LD**	**HD**	**C**	**LD**	**HD**	**C**	**LD**	**HD**
Day 1 pre-dose	933	800	963	46.5	40.8	31.3	197	160	158
Day 2, 4 h	1163	831	1280	26.4	30.7	36.3	201	158	167
Day 6, 4 h	1118	976	1017	52.7	56.3	51.0	211	195	220
Day 10, 4 h	1244	1049	1110	55.2	51.8	46.8	161	148	142
Day 14, 4 h	1283	964	1254	45.9	46.1	49.6	179	137	159
Day 18, 4 h	844	743	826	48.1	46.0	35.2	194	169	144
Day 22, 4 h	986	646	766	37.5	49.1	37.5	122	99	127
Day 26, 4 h	650	676	806	59.2	58.4	47.4	178	191	220
Brain	0.045	0.048	0.040	0.076	0.059	0.071	0.515	0.381	0.371

10.2 Impurities

Nine impurities were noted by the Product Quality reviewer to have acceptance limits above the (b) (4)% qualification threshold. The sponsor conducted two 13-week studies to qualify the impurities above the (b) (4)% qualification threshold, but the margins of the nine impurities were calculated by normalizing to body weight (mg/kg) instead of to body surface area (mg/m^2).

10.2.1 Study Title: Thirteen-Week (91-Day) repeated dose toxicity study of trofinetide in Sprague-Dawley rats via twice-daily gavage administration.

Study no.:	NURN0007:2252
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	April 6, 2016
GLP compliance:	Yes
QA statement:	Yes (dated February 13, 2017)
Drug, lot #, and % purity:	#1 trofinetide, Lot № E150651 (93.6%), #2 NNZ-3566, Lot № 2AO1R (93.5%)
Impurities	(b) (4)

Key Study Findings:

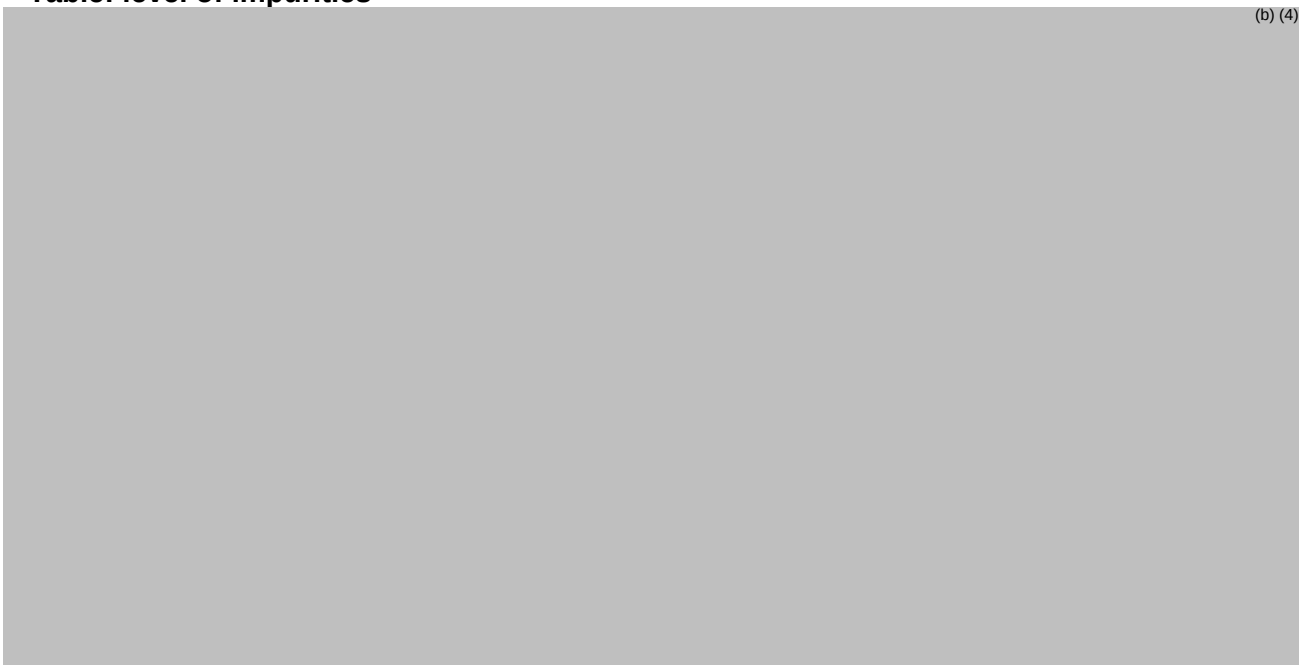
- One Group 3 M was found dead from unknown cause.
- No adverse clinical signs, body weight changes, or food consumption changes were observed.
- Non-adverse microscopic findings were observed and included increased incidences of basophilic tubules and interstitial lymphocytes in the kidney, vacuolation and centrilobular hypertrophy in the liver, alveolar macrophages in the lung, and dilated gastric glands in the stomach; kidney weights were increased.

- Changes in the 2 different trofinetide batch groups were generally similar compared to the control group.
- The sponsor considered 1200 mg/kg BID of both batches well tolerated, which appears appropriate.

Methods

Doses:	0, 1200 (#1 batch, Group 2), and 1200 (#2 batch, Group 3) mg/kg.
Frequency of dosing:	BID (6-8 hours apart).
Route of administration:	Oral (Gavage).
Dose volume:	8.5 mL/kg.
Formulation/Vehicle:	Water for irrigation
Species/Strain:	Rats / Sprague-Dawley.
Number/Sex/Group	
Main study:	10 for Groups 1-3
Toxicokinetic:	4 for Groups 2 and 3.
Age:	8-9 Weeks
Weight:	M 307-385 g; F 212-263 g

Table: level of impurities



Observations and Results

Mortality

Observations of morbidity and mortality were conducted twice daily.

One Group 3 M was found dead on Day 85 from unknown causes, as no adverse clinical signs were observed, and postmortem examination revealed no cause of death.

Clinical Signs
Observations of clinical signs were recorded twice daily during Week 1 and daily thereafter.

No adverse clinical signs were observed.

Body Weights
Body weights were recorded once prior to the initiation of dosing and then twice weekly.

There were no adverse changes in body weight.

Food Consumption
Food consumption was determined twice weekly.

There were no adverse changes in food consumption.

Hematology
Blood samples were taken prior to sacrifice on Day 92.

There were a few statistically significant changes from the vehicle group and between the 2 trofinetide groups. These are listed in the table below. The differences between the 2 trofinetide groups only included an increase in Group 3 M reticulocytes and an increase in Group 3 M and F LUC.

Table: statistically significant changes in hematology

		MALE			FEMALE		
Analyte	**Unit**	**Group 1**	**Group 2 (#1)**	**Group 3 (#2)**	**Group 1**	**Group 2 (#1)**	**Group 3 (#2)**
WBC	$x10^9$/L	10.33	12.04	12.03*	8.77	7.74	8.44
HCT	L/L	0.52	0.50*	0.50	0.47	0.44*	0.44*
MCHC	g/L	302	307	305	302	310*	316*
Reticulocytes	%	2.05	1.59*	2.00‡	2.02	1.99	1.96
Neutrophils	$x10^9$/L	1.67	1.91	1.85*	1.42	1.20	1.15
Lymphocytes	$x10^9$/L	8.15	9.48	9.47*	6.82	6.06	6.71
Monocytes	$x10^9$/L	0.22	0.35*	0.36*	0.21	0.28	0.31*
Eosinophils	$x10^9$/L	0.15	0.14	0.13	0.19	0.10*	0.11*
LUC	$x10^9$/L	0.07	0.08	0.12*‡	0.07	0.06	0.11*‡

*** Statistical significance from vehicle; ‡ statistical significance between Groups 2 (batch #1) and 3 (batch #2).**

Clinical Chemistry
Blood samples were taken prior to sacrifice on Day 92.

There were a few statistically significant changes from the vehicle group and between the 2 trofinetide groups. These are listed in the table below. The differences between the 2 trofinetide groups only included increases in Group 3 M calcium and F ALT and decreases in Group 3 F chloride and F urea.

Table: statistically significant changes in clinical chemistry

Analyte	Unit	MALE			FEMALE		
		Group 1	Group 2 (#1)	Group 3 (#2)	Group 1	Group 2 (#1)	Group 3 (#2)
ALT	U/L	55	63	66*	67	57	71‡
Calcium	mM	2.53	2.51	2.62*‡	2.67	2.68	2.74
Chloride	mM	104.5	102*	102.4*	105.7	105.3	103.3*‡
Triglycerides	mM	1.32	1.85	2.17*	NA	NA	NA
Cholesterol	mM	1.79	1.77	1.86	1.75	2.11	2.45*
Urea	mM	6.55	6.23	5.64	7.43	8.08	6.48‡

*** Statistical significance from vehicle; ‡ statistical significance between Groups 2 (batch #1) and 3 (batch #2).**

Urinalysis

Urine samples were taken from all animals on Day 84.

There appears to be no drug-related effects, although not all data were provided, as only positive findings and findings outside the “normal” ranges for specific gravity (1.010-1.025) and pH (6-7) were included.

Gross Pathology

Gross pathology was assessed in all animals.

In M, there was discoloration of liver in 1 Group 2 and 3 Group 3 animals, enlarged kidney in 1 Group 2 animal, and red popliteal lymph node in 1 Group 3 animal. In F, there was red popliteal lymph node in 1 Group 3 animal and discoloration of submandibular lymph node in 1 animal in Group 2 and 1 in Group 3.

Organ Weights

At necropsy, the following organs, adrenals, brain, epididymides, heart, kidneys, liver, lung, ovaries, prostate, seminal vesicles, spleen, testes, thymus, and uterus, were weighed.

In M, both trofinetide groups had an increase in kidney weight that was only significant in Group 3 M.

Table: organ changes in mean absolute weight compared to control organ weights

	Adrenal	Brain	Epididy/ Uterus	Heart	Kidney	Liver	Lung	Prostate	Seminal Vesicle	Spleen	Testes/ Ovary	Thymus
MALES												
GROUP 2:	-9%	+3%	+13%	+7%	+11%	+2%	+8%	+8%	+15%	-3%	+1%	0%
GROUP 3:	0%	+7%	+6%	+6%	+19%*	+8%	+4%	+15%	+8%	+8%	+6%	-3%
FEMALES												
GROUP 2:	+11%	-2%	-1%	+3%	+9%	+11%	+6%	-	-	+5%	0%	0%
GROUP 3:	+22%	-1%	+10%	+3%	+13%	+8%	+7%	-	-	-2%	-26%	+2%

Epididy - epididymides.

Table: organ changes in mean weight (relative to body weight) compared to control organ weights

	Adrenal	Brain	Epididy/ Uterus	Heart	Kidney	Liver	Lung	Prostate	Seminal Vesicle	Spleen	Testes/ Ovary	Thymus
MALES												
GROUP 2:	-6%	+3%	+14%	+8%	+12%	+3%	+9%	+7%	+13%	-3%	+1%	-1%
GROUP 3:	-6%	+1%	+2%	+2%	+14%	+4%	-2%	+8%	+2%	+3%	+1%	-7%
FEMALES												
GROUP 2:	+14%	-3%	-3%	+2%	+7%	+10%	+4%	-	-	+4%	0%	+1%
GROUP 3:	+21%	-3%	+10%	0%	+10%	+6%	+5%	-	-	-4%	-19%	+3%

Epididy - epididymides.

Histopathology

At necropsy, the following organs, adrenal, aorta, brain, cecum, colon, duodenum, epididymis, esophagus, femur marrow, heart, ileum, jejunum, kidney, liver, lung, mammary gland, lymph node (mandibular and popliteal), muscle, pancreas, pituitary, prostate, rectum, salivary gland (parotid and sublingual), sciatic nerve, seminal vesicle, skin and subcutis, spinal cord (cervical, lumbar, and thoracic), spleen, stomach, testis, thymus, thyroid, tongue, trachea, urinary bladder, and ureter, were prepared for histopathology examination. All tissues were examined in all animals.

Adequate Battery: Yes.
Peer Review: No.
Signed Pathology report: Yes, by Elizabeth McInnes BVSc, PhD, DACVP, FRCPath.

Histological Findings

Microscopic findings were observed in the kidney, liver, lungs, and stomach. Observations included increased incidences of basophilic tubules and interstitial lymphocytes in the kidney, vacuolation and centrilobular hypertrophy in the liver, alveolar macrophages in the lung, and dilated gastric glands in the stomach. These changes were generally similar in the 2 different trofinetide batch groups.

Table: summary of selected microscopic findings

		MALE			FEMALE		
Tissue	**Finding**	**0**	**2400 (#1)**	**2400 (#2)**	**0**	**2400 (#1)**	**2400 (#2)**
Kidney	Basophilic tubules						
	minimal:	**0/10**	**3/10**	**3/10**	**0/10**	**0/10**	**0/10**
	slight:	**0/10**	**1/10**	**0/10**	**0/10**	**0/10**	**1/10**
	moderate:	**0/10**	**1/10**	**0/10**	**0/10**	**0/10**	**0/10**
	Tubular dilation						
	minimal:	**0/10**	**0/10**	**1/10**	**0/10**	**0/10**	**0/10**
	slight:	**0/10**	**0/10**	**0/10**	**0/10**	**0/10**	**1/10**
	Interstitial fibrosis						
	minimal:	**0/10**	**0/10**	**0/10**	**0/10**	**0/10**	**0/10**
	slight:	**0/10**	**1/10**	**0/10**	**0/10**	**0/10**	**0/10**
	Interstitial lymphocytes						
	minimal:	**1/10**	**2/10**	**3/10**	**0/10**	**1/10**	**1/10**
Liver	Vacuolation						
	minimal:	**0/10**	**2/10**	**2/10**	**0/10**	**1/10**	**0/10**

Tissue	Finding	MALE 0	MALE 2400 (#1)	MALE 2400 (#2)	FEMALE 0	FEMALE 2400 (#1)	FEMALE 2400 (#2)
Liver	Centrilobular hypertrophy						
	minimal:	**0/10**	**5/10**	**3/10**	**0/10**	**0/10**	**3/10**
Lung	Edema						
	minimal:	**0/10**	**0/10**	**0/9**	**0/10**	**0/10**	**0/10**
	slight:	**0/10**	**0/10**	**1/9**	**0/10**	**0/10**	**0/10**
	Alveolar macrophages						
	minimal:	**0/10**	**1/10**	**1/9**	**1/10**	**2/10**	**3/10**
	slight:	**0/10**	**0/10**	**0/9**	**0/10**	**0/10**	**0/10**
	moderate:	**0/10**	**0/10**	**0/9**	**0/10**	**0/10**	**1/10**
	Perivascular lymphocytes						
	minimal:	**4/10**	**4/10**	**2/9**	**2/10**	**3/10**	**5/10**
	slight:	**1/10**	**0/10**	**0/9**	**0/10**	**0/10**	**0/10**
Stomach	Dilated gastric glands						
	minimal:	**2/10**	**4/10**	**7/10**	**3/10**	**7/10**	**6/10**
Testes	Atrophy						
	minimal:	**0/10**	**1/10**	**0/10**	**-**	**-**	**-**
Urinary bladder	Urothelial hypertrophy						
	minimal:	**0/10**	**0/10**	**0/10**	**0/10**	**1/10**	**0/10**

Toxicokinetics

Blood samples were taken on Day 2 at 2-4 hours post-dose and on Day 3 pre-dose and at 2-4 hours post-dose.

Plasma levels of the two different trofinetide batches, measured 2-4 hours post-dose, were generally similar apart from Day 2 for Group 2 M. Pre-dose levels on Day 3 were also similar.

Table: toxicokinetic data of trofinetide (Sponsor's).

Time point	Trofinetide Concentration (µg/mL)			
	Male (µg/mL)		Female (µg/mL)	
	Group 2 (Trofinetide 1, 2400 mg/kg/day)	Group 3 (Trofinetide 2, 2400 mg/kg/day)	Group 2 (Trofinetide 1, 2400 mg/kg/day)	Group 3 (Trofinetide 2, 2400 mg/kg/day)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Day 2 (2-4 hours post-dose)	2.30 ± 1.06	126.75 ± 21.14	114.83 ± 18.63	126.84 ± 37.63
Day 3 (pre-dose)	3.00 ± 1.05	5.09 ± 3.06	3.39 ± 1.13	6.05 ± 4.76
Day 3 (2-4 hours post-dose)	137.44 ± 32.74	175.55 ± 8.02	147.94 ± 43.52[§]	152.63 ± 90.70

[§] No sample was collected from one female rat in Group 2.

10.2.2 Study Title: A 13-Week Qualification Study of Trofinetide Drug Product Impurities and Degradants by Oral Gavage in Sprague Dawley Rats.

Study no.:	00616060
Study report location:	EDR
Conducting laboratory and location:	(b) (4)
Date of study initiation:	April 6, 2020
GLP compliance:	Yes, US
QA statement:	Yes (dated June 18, 2021)
Drug, lot #, and % purity:	#1 Trofinetide, Lot №'s 097018-20002 (98.2%) first 58 days and Lot № 097018-20006 (98.2%) following 34 days
Lot with spiked impurities (see table):	#2 Trofinetide, Lot №'s 097018-20003 (96.8%) first 58 days and Lot № 097018-20007 (97.6%) following 34 days

Key Study Findings:

- One Group 2 F was sacrificed early for humane reasons due to acute clinical signs immediately after dosing that were not observed previously in the animal or any other animal. So, the sacrifice was not considered due to trofinetide.
- No adverse clinical signs, body weight and food consumption changes, or macroscopic findings were observed.
- Non-adverse microscopic findings were observed in the kidney and liver and included glomerulonephritis and chronic progressive nephropathy in the kidney and necrosis in the liver in F and was similar between the 2 trofinetide batches.
- Changes in the 2 different trofinetide batch groups were generally similar compared to the control group.

Methods

Doses:	0, 1000 (#1 batch, Group 2), and 1000 (#2 batch, Group 3) mg/kg.
Frequency of dosing:	BID (approximately 6 hours apart).
Route of administration:	Oral (Gavage).
Dose volume:	5 mL/kg.
Formulation/Vehicle:	Placebo (Lots 097018-20004P and 097018-20008P
Species/Strain:	Rats / Sprague-Dawley
Number/Sex/Group	
Main study:	10
Toxicokinetic:	3
Age:	8 Weeks
Weight:	180-283 g

Table: #1 batch, impurity profile of batch 097018-20006 (Sponsor's)

(b) (4)

Table: #2 batches impurity profile (Sponsor's)

(b) (4)

Table: sponsor's impurity qualification table (Table 3.2.S.3.2-3)

(b) (4)

However, the sponsor has the following statements (3.2.P.5.5.1)

- An acceptance criterion of NMT (b) (4) % w/w at release and NMT (b) (4) % w/w at shelf-life is proposed for the control of Impurity (b) (4)
- An acceptance criterion of NMT (b) (4) % w/w at release and NMT (b) (4) % w/w at shelf-life is proposed for the control of Impurity (b) (4)

Tables: level of impurities in #2 batch
Mean end level

(b) (4)

Observations and Results

Mortality

Observations of mortality were conducted twice daily (morning and afternoon).

One Group 2 F was sacrificed on Day 25 after the second dose for humane reasons due to acute clinical signs of labored breathing, abnormal breathing sounds, hunched posture, red fur staining around the mouth, abnormal gait, and decreased activity and moderate hemorrhage in lungs. Death was not considered due to trofinetide.

Clinical Signs
Observations of clinical signs were recorded once daily 1-3 hours post-dose. Detailed observations were conducted 4 times prior to initiation of dosing including on Day 1 prior to dosing and weekly thereafter.

No adverse clinical signs were observed.

Body Weights
Body weights were recorded 4 times prior to initiation of dosing including on Day 1 prior to dosing and weekly thereafter.

No adverse body weight changes were observed. Body weight changes at the end of dosing were -7% and -2% in Group 2 and 3 M, respectively and +12% and +5% in Group 2 and 3 F, respectively.

Food Consumption
Food consumption was determined once weekly during dosing.

No adverse food consumption changes were observed.

Ophthalmoscopy
Examinations of animals were conducted once prior to initiation of dosing and towards the end of the dosing period on Day 88.

There were no adverse ophthalmoscopy findings observed.

Hematology
Blood samples were taken prior to sacrifice on Day 92.

A few statistically significant changes were noted between the vehicle group and the trofinetide groups. These are listed in the table below and included decrease in reticulocytes in Group 2 and 3 M, in PT in and Group 2 and 3 M, and platelets in Group 3 F.

Table: statistically significant changes in hematology

		MALE			FEMALE		
Analyte	**Unit**	**Group 1**	**Group 2 (#1)**	**Group 3 (#2)**	**Group 1**	**Group 2 (#1)**	**Group 3 (#2)**
Platelets	$x10^3/\mu L$	1177	1000	969	1023	1041	905*‡
Reticulocytes	$x10^9/L$	259.53	210.34*	213.76*	165.58	177.57	187.01
WBC	$x10^3/\mu L$	7.503	7.087	8.376	4.645	5.487	4.367
Neutrophils	$x10^3/\mu L$	1.595	1.528	1.791	1.205	1.080	0.944
Lymphocytes	$x10^3/\mu L$	5.503	5.227	6.182	3.184	4.114	3.184
PT	sec	16.17	17.07*	16.81*	15.92	16.17	16.08
aPTT	sec	11.94	12.72 (+7%)	12.32 (+3%)	11.93	11.86 (-1%)	12.74 (+7%)

*** Statistical significance from vehicle; ‡ statistical significance between Groups 2 (#1) and 3 (#2).**

Clinical Chemistry
Blood samples were taken prior to sacrifice on Day 92.

There were a few statistically significant changes from the vehicle group and between the 2 trofinetide groups. These are listed in the table below. The differences between the 2 trofinetide groups included increases in Groups 2 and 3 M ALT.

Table: statistically significant changes in clinical chemistry

Analyte	Unit	MALE			FEMALE		
		Group 1	Group 2 (#1)	Group 3 (#2)	Group 1	Group 2 (#1)	Group 3 (#2)
AST	U/L	92.8	107.4	105.2	152.9	209.0	138.7
ALT	U/L	38.8	54.4*	55.5*	48.6	90.4	71.0
Triglycerides	mg/dL	90.1	85.0	100.9	64.3	88.4	71.8
Bilirubin	mg/dL	0.037	0.034	0.023	0.077	0.111	0.085
Cholesterol	mg/dL	82.5	63.4	59.0*	104.6	102.9	112.2

*** Statistical significance from vehicle.**

Urinalysis
Urine samples were taken prior to sacrifice on Day 92.

In M, there was an increase in specific gravity in Group 2 (4%) and a decrease in pH in Groups 2 and 3 (1.4 and 1.2, respectively) that was statistically significant from control animals. In F, there was an increase in specific gravity in Group 2 and 3 (2% and 2%, respectively) and a decrease in pH in Groups 2 and 3 (0.6 and 0.7, respectively) that was statistically significant from control animals.

Gross Pathology
Gross pathology was assessed in all animals.

There were no adverse macroscopic findings. Small testis and epididymides were observed in 1 Group 3 M.

Organ Weights
At necropsy, the following organs, as listed in the study report, were weighed.

Brain Epididymis Gland, adrenal Gland, pituitary Gland, prostate with gland, seminal vesicle Gland, thyroid with gland, parathyroid Heart Kidney	Liver Ovary with oviduct Spleen Testis Thymus Uterus with cervix

Increases in kidney and liver weights were noted in Group 2 and 3 F.

Table: organ changes in mean absolute weight compared to control organ weights

	Adrenal	Brain	Epididy	Heart	Kidney	Liver	Pit	Prostate/ Ovary	Spleen	Testes/ Uterus	Thyroid	Thymus
MALES												
GROUP 2:	-2%	0%	-1%	-7%	+7%	0%	-3%	+4%	+1%	-2%	-12%	-6%
GROUP 3:	-1%	-1%	-1%	-1%	+5%	+1%	-3%	+4%	-3%	-1%	0%	-17%
FEMALES												
GROUP 2:	+10%	-3%	-	+6%	+19%*	+11%	+11%	-4%	+8%	+9%	-8%	+11%
GROUP 3:	+9%	-1%	-	+7%	+17%*	+10%	+8%	+3%	+8%	+6%	+9%	+2%

*** Statistical significance from vehicle; Epididy – epididymides; Pit - pituitary.**

Table: organ changes in mean weight (relative to body weight) compared to control organ weights

	Adrenal	Brain	Epididy	Heart	Kidney	Liver	Pit	Prostate/ Ovary	Spleen	Testes/ Uterus	Thyroid	Thymus
MALES												
GROUP 2:	-2%	+1%	0%	-5%	+9%	+2%	-1%	+5%	+2%	-1%	-11%	-3%
GROUP 3:	-1%	-1%	-1%	-1%	+5%	+1%	-3%	+4%	-3%	0%	-1%	-16%
FEMALES												
GROUP 2:	+6%	-6%	-	+2%	+15%*	+7%	+6%	-7%	+5%	+7%	-12%	+8%
GROUP 3:	+10%	-1%	-	+8%	+17%*	+11%*	+8%	+4%	+10%	+6%	+9%	+2%

*** Statistical significance from vehicle; Epididy – epididymides; Pit - pituitary.**

Histopathology

At necropsy, the following organs, as listed in the study report, were prepared for histopathology examination. All tissues were examined in all animals.

Animal identification[a]	Large intestine, colon
Artery, aorta	Large intestine, rectum
Body cavity, nasal[a]	Larynx[a]
Bone marrow, sternum	Liver
Bone marrow, smear (from femur)[a,b]	Lung
Bone, femur	Lymph node, mandibular (2)[e]
Bone, sternum	Lymph node, mesenteric
Brain	Macroscopic abnormalities (gross lesions; when possible)
Epididymis (2)[c]	Muscle, skeletal (2)[e]
Esophagus	Nerve, optic (2)[d]
Eyes (2)[d]	Nerve, sciatic (2)[e]
Ganglion, dorsal root, lumbar[a]	Nerve, tibial (2)[a]
Gland, adrenal (2)	Ovary (2)
Gland, clitoral (2)[a]	Oviduct (2)[a]
Gland, lacrimal (extra-orbital [2])[a]	Pancreas
Gland, Harderian (2)[e]	Skin
Gland, mammary	Small intestine, duodenum
Gland, parathyroid (2)	Small intestine, ileum
Gland, pituitary	Small intestine, jejunum
Gland, preputial (2)[a]	Spinal cord
Gland, prostate	Spleen
Gland, salivary, submandibular (2)[e]	Stomach
Gland, salivary, sublingual (2)[a]	Testis (2)[c]
Gland, salivary, parotid (2)[a]	Thymus
Gland, seminal vesicle (2)	Tongue
Gland, thyroid (2)	Trachea
Gland, Zymbal's (2)[a]	Ureter (2)[a]
Gut-associated lymphoid tissue[f]	Urinary bladder
Heart	Uterus/Cervix
Joint, femorotibial	Vagina
Kidney (2)	
Large intestine, cecum	

[a] Not processed for histology or examined microscopically.
[b] Bone marrow smears were obtained at scheduled and from the animal euthanized in extremis and stored in the refrigerator, but not placed in formalin; slides were not examined.
[c] Fixed in modified Davidson's solution.
[d] Fixed in Davidson's solution.
[e] One of pair processed for histology and examined microscopically.
[f] From small intestine: Peyer's patch or solitary lymphoid follicle.

Adequate Battery: Yes.
Peer Review: No.
Signed Pathology report: Yes, by Michael Owston DVM, MS, DACVP.

Histological Findings

The main microscopic findings were observed in the kidney and liver. Glomerulonephritis, characterized by the presence of hyaline to granular eosinophilic material and increased cellularity within the glomerular tufts, casts, characterized by hyaline to granular eosinophilic material filling tubules, and chronic progressive nephropathy (CPN), characterized by the presence of basophilic tubules surrounded by thickened basement membranes were observed in kidney; no differences between the two trofinetide batches were observed apart from an increase in CPN in Group 3. Necrosis, characterized by multifocal foci of several hepatocytes with loss of nuclear detail, homogeneous cytoplasm, and loss of hepatocytes was observed in the liver in F and was similar between the two batches. Marked seminiferous tubule atrophy, with severe decreased cellularity, was observed in 1 Group 3 M and mild dilatation in the uterus of 1 Group 2 F.

Table: summary of selected microscopic findings

			MALE			FEMALE		
		(n)	10	10	10	10	9 (1)	10
Tissue	Finding	(mg/kg)	0	2000 (#1)	2000 (#2)	0	2000 (#1)	2000 (#2)
Adrenal	Cortical hypertrophy							
		minimal:	0	0	0	0	1 (0)	0
		mild:	0	0	0	0	1 (0)	0
Epididymides	Decreased cellularity							
		minimal:	0	0	0	-	-	-
		mild:	0	0	0	-	-	-
		moderate:	0	0	0	-	-	-
		marked:	0	0	0	-	-	-
		severe:	0	0	1	-	-	-
Heart	AV value fibrosis							
		minimal:	0	0	0	0	0 (0)	0
		mild:	0	1	0	0	0 (0)	0
Kidney	Glomerulonephritis							
		minimal:	0	0	0	0	0 (0)	0
		mild:	0	0	0	0	1 (0)	1
	Cast							
		Minimal:	1	0	0	1	1 (0)	2
		Mild:	0	0	0	0	1 (0)	1
	Chronic progressive nephropathy							
		Minimal:	4	1	0	2	0 (0)	2
		Mild:	0	1	5	0	1 (0)	0
Liver	Necrosis							
		minimal:	0	0	0	0	0 (0)	0
		mild:	0	0	0	0	2 (0)	1
	Vacuolation							
		minimal:	0	0	1	0	0 (0)	0
		mild:	0	1	0	0	0 (0)	0
	Peribiliary fibrosis							
		minimal:	0	0	0	0	1 (0)	0

			MALE			FEMALE	
		(n)	10	10	10	10	9 (1)	10
Tissue	Finding	(mg/kg)	0	2000 (#1)	2000 (#2)	0	2000 (#1)	2000 (#2)
Lung	Alveolar mixed cell infiltration							
		minimal:	0	3	2	0	0 (0)	1
	Macrophage aggregate							
		minimal:	0	2	0	0	0 (0)	0
Testes	Seminiferous tubule atrophy							
		minimal:	0	0	0	-	-	-
		mild:	0	0	0	-	-	-
		moderate:	0	0	0	-	-	-
		marked:	0	0	1	-	-	-
Uterus	Dilatation							
		minimal:	-	-	-	0	0 (0)	0
		mild:	-	-	-	0	1 (0)	0

Data from animal sacrificed early in parenthesis.

Toxicokinetics

Blood samples were taken on Day 91 at 1.5 and 7.5 hours post-dose.

On Day 91 plasma exposure for both trofinetide batches at 1.5 hours post-dose were similar.

Table: toxicokinetic data of both trofinetide batches.

Group	Study Day	Time (hours)	Male (µg/mL)	Female (µg/mL)
2 (Batch #1)	91	1.5	115	108
		7.5	106	149
3 (Batch #2)	91	1.5	139	148
		7.5	130	131

10.2.3 QSAR Report on Extractables and Leachables by CDER/OTS/OCP/DARS: Computational Toxicology Consultation Service (November 18, 2022)

The compounds (b) (4) all have a limit of detection (LOD) above the analytical evaluation threshold (AET, (b) (4) ng/mL all compounds):

(b) (4)

Using the LOD or detectable levels to calculate the highest possible level of these compounds in trofinetide all compounds would be above the maximum exposure threshold of 1.5 µg/day for an impurity with genotoxic concern:

(b) (4)

(b) (4)

From the above list, all compounds could be above the limit threshold for a genotoxic impurity. The sponsor justified the compounds with the following:

(b) (4)

The information above, that included the use of (Q)SAR models demonstrated these compounds were negative for mutagenicity. The Agency's Computational Toxicology Consultation Service was consulted and confirmed that the compounds were negative for mutagenic potential using three (Q)SAR models to evaluate each compound: Derek Nexus 6.2.1 (DX), Leadscope Model Applier 3.1.0-40 Bacterial Mut v2 model (LMA), and CASE Ultra 1.9.0.4 GT1_BMUT model 1.9.0.2 (CU).

11 Integrated Summary and Safety Evaluation

Trofinetide (NNZ-2566), a 2-methylproline-substituted analogue of glycyl-L-prolyl-L-glutamate (GPE), the N-terminal tripeptide cleavage product of insulin-like growth factor-1 (IGF-1), is intended for the treatment of patients with Rett syndrome.

Trofinetide had no significant binding at any target (> 80) in binding assays or inhibitory effect on the function of any kinase or histone-deacetylase enzymes tested in functional assays. No clear mechanism of action has been determined for trofinetide; however, the sponsor hypothesizes that trofinetide enhances neuronal synaptic function and morphology. This is not clear, as although survival was slightly increased and hippocampal CA1 LTP was increased after 5 weeks of trofinetide dosing in the *MeCP2*$^{-/y}$ knockout mouse (a model of Rett syndrome), only a trend for increased dendritic complexity and spine length, which was and not statistically significant, and no change in motor function, gait, respiration, or autonomic function was observed.

Safety pharmacology studies with trofinetide were not conducted using the oral route. In safety pharmacology studies, IV bolus administration of trofinetide to rats displayed no significant adverse effects on functional neurologic parameters up to 350 mg/kg and

no adverse effects on respiratory parameters up to 700 mg/kg. In dogs, IV infusion of trofinetide (800 mg/kg) had no effects on heart rate, arterial blood pressure, RR and PR intervals, and QRS duration; however, at 400 and 800 mg/kg, the QTcV interval was slightly prolonged at 90 to 120 minutes post-dose by between 19 and 33 ms. In in vitro hERG studies, the IC_{50} for trofenitide was estimated at ≥ 20 mM.

Absorption of trofinetide in animals after oral administration was relatively rapid, with T_{max} approximately 2 hours. In rats, plasma exposure to trofinetide was similar between sexes. No accumulation of trofinetide was observed. [^{14}C]-trofinetide distributed to most tissues in rats, with highest radioactivity levels found in the small intestine wall, large intestine wall, and kidney. No metabolites were detected in vivo after examination of rat bile, feces, urine, plasma, and brain samples after oral administration of [^{14}C]-trofinetide with [^{14}C]-trofinetide excreted equally in the urine and feces, but minimal [^{14}C]-trofinetide detected in the bile and no major or active metabolites detected in human blood or urine.

In toxicology studies, oral trofinetide was well tolerated up to a HD of 2000 (1000 mg/kg BID) and 1000 (500 mg/kg BID) mg/kg/day in chronic rat and dog studies (26- and 39-week, respectively) the recommended limit dose with NOAELs of 1000 and 150 mg/kg BID, respectively. No trofinetide-related deaths were observed, minimal adverse clinical signs, no adverse change in body weight, and few microscopic findings noted. Only a decrease in uterine weight, likely due to a tendency for F to be in anestrus and a stress-related effect on the estrus cycle in F dogs, was noted and considered adverse at the HD. These data resulted in NOAELs of 2000 mg/kg/day (1000 mg/kg BID), 1000 mg/kg/day (500 mg/kg BID), and 300 mg/kg/day (150 mg/kg BID) in juvenile rats (M and F) in the 26-week study, in M dogs in the 39-week study, and in F dogs in the 39-week study, respectively. In the 4-week continuous IV (7-day infusions for 4 cycles of infusions with 3 days between infusions) toxicology study in dogs, the HD (3600 mg/kg/day) was above the recommended dose limit and adverse findings of moderate (Grade 3) thyroid hyperplasia was observed at the MD (1440 mg/kg/day) and HD.

In genetic toxicology studies, trofinetide did not demonstrate mutagenic potential in the Ames assay or structural chromosomal aberrations in CHO cells, or clastogenic potential in the *in vivo* mouse micronucleus assay at oral doses up to 2000 mg/kg/day trofinetide. Carcinogenicity studies have not been conducted (with agreement from the Division) prior to submission of the NDA, but these studies will be required as a PMR.

Fertility was not affected in F or M with trofinetide well tolerated at doses up to 2000 mg/kg/day (1000 mg/kg BID), with no adverse findings on sperm motility, spermatid density, or sperm morphology; the NOAEL was determined as the HD, 2000 mg/kg/day (1000 mg/kg BID). Embryo-fetal development (EFD) studies were conducted in rat and rabbit. In the rat EFD study, trofinetide was well tolerated, with no unscheduled deaths, adverse clinical signs, changes in body weight and food consumption, or adverse findings in ovarian, uterine, or litter parameters, and no increase in malformations. The fetal NOAEL was established at the HD

(2000 mg/kg/day [1000 mg/kg BID]). In a rabbit dose range-finding study (0, 300, 900, 2000 mg/kg/day [0, 150, 450, and 1000 mg/kg BID]), there was an increase in early and late resorptions observed in MDF and HDF and increased post-implantation loss in MDF and HDF; live fetuses were reduced at the HD, the maternal NOAEL was established at 300 mg/kg/day (150 mg/kg BID) due to effects on body weight and food consumption at higher doses. In the pivotal rabbit EFD study (0, 150, 300, and 600 mg/kg/day [0, 75, 150, and 300 mg/kg BID]), abortions occurred in 1 control and 2 HD dams and were associated with severe reductions in food consumption and body weight loss suggesting the maternal NOAEL is 150 mg/kg/day (75 mg/kg BID). In dams, slight increases in early resorptions and post-implantation loss were noted at the HD, but with a minimal decrease in live births, no dead fetuses, and similar incidences of malformations, suggesting no adverse findings were observed in ovarian, uterine, or litter parameters resulting in a fetal NOAEL at 600 mg/kg/day (300 mg/kg BID).

In the pivotal pre- and postnatal development study (0, 300, 900, and 2000 mg/kg/day [0, 150, 450, and 1000 mg/kg BID]), no trofinetide-related deaths, adverse clinical signs, or changes in body weight were observed, and reproductive parameters were comparable among all groups. Findings in F_0 dams included a slight increase in stillborn pups in HD F_0 dams, but the increase is unlikely to be drug related as it did not influence live births or body weight, the animals had a higher number of implantation sites, and the data fell within the historical control range of values. All recorded endpoints post weaning were comparable among all the groups in F_1 animals. Therefore, trofinetide was well tolerated; the NOAEL for postnatal development of offspring and reproductive performance was 2000 mg/kg/day (1000 mg/kg BID).

Two 13-week toxicity studies were submitted to qualify impurities (b) (4) using a spiked drug product. In both studies, there was no substantial difference between the groups dosed with or without spiked impurities, suggesting that the impurities at the levels used did not cause toxicity. To qualify the impurities at the proposed acceptance levels above (b) (4)%, the sponsor calculated margins on body weight (mg/kg) basis instead of a body surface area (mg/m^2) basis. When the margins are calculated on body surface area basis the following impurities, (b) (4), are not qualified at the proposed acceptance levels. An information request (January 11, 20232) was sent to the sponsor to change the acceptance criteria for impurities (b) (4) the sponsor agreed to change the criteria to (b) (4)%, respectively (January 29, 2023). The extractable/leachable compounds (b) (4) all have an LOD above the AET which could result in an exposure higher than the threshold for an impurity with genotoxic concern. The information provided by the sponsor demonstrated these compounds were negative for mutagenicity.

Therefore, the studies submitted to support the NDA for trofinetide that included 4-, 26- and 39-week oral and IV toxicology studies in juvenile rat and dog, and reproductive and developmental studies in rats and rabbits were adequate and showed that few toxicity signals were observed with trofinetide administration.

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/s/

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03/10/2023 04:26:55 PM

LOIS M FREED
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