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Study Title: Pharmacokinetics of Isradipine in Mice – Gavage

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1.0 ESTIMATED TIME LINE

- ***Pre-study***:
  - Transfer up to 10 wild-type and 44 transgenic mice from the alpha-synuclein transgenic colony (IACUC 2314) to this study (IACUC 3104).
  - Provide individual animal identification (tail marking).
  - On a prearranged schedule, collect blood and brain from up to ten wild type mice (untreated). Freeze plasma and brain hemispheres at -80°C, and deliver to the study chemist for analytical methods development and sample stability studies.
- ***Assignment of Mice to Necropsy Day and Terminal Time Points***:
  - Weigh and observe mice for clinical condition. Distribute mice across necropsy days and post-dosing time points with reasonably equal representation by age, sex, clinical status, and body weight, as below.
  - Day 1 - Two untreated mice will be terminated at the zero time point. Ten mice (2/time point) will be terminated after the first dose of isradipine, specifically, 20 min, 1, 2, 4, and 6 hrs post-dose. Ten additional mice (2/time point) will be given a first dose and then 6 hrs later a second, and terminated at 20 min, 1, 2, 4, and 6 hrs after the second dose. The concentration of isradipine in vehicle will be 0.25 mg/mL, and the dose volume will be 10 mL/kg/dose.
  - Day 1-20 - The remaining 22 mice will be dosed by gavage with isradipine at 2.5 mg/kg twice daily, ~6 hrs apart on Days 1-20. The concentration of isradipine in vehicle will be 0.25 mg/mL, and the dose volume will be 10 mL/kg/dose.
  - Day 21 - Two mice will be terminated prior to the morning dose (i.e., not dosed on Day 21). Ten mice (2/time point) will be terminated after the forty-first dose of isradipine, specifically, 20 min, 1, 2, 4, and 6 hrs post-dose. Ten additional mice (2/time point) will be given the forty-first dose and then 6 hrs later the forty-second dose, and terminated at 20 min, 1, 2, 4, and 6 hrs after the forty-second dose. The concentration of isradipine in vehicle will be 0.25 mg/mL, and the dose volume will be 10 mL/kg/dose.
  - If unscheduled deaths occur, then individual mice remaining on study may be reassigned to specific termination time points at the discretion of the study director in so far as this improves attainment of study objectives.
- ***In-Life Observations***:
  - Body weight will be recorded on assignment to study, and on the mornings of Day 1, Day 8, Day 15 and Day 21 (prior to the first dose of the day).
  - Clinical observations (daily, morning) and morbidity/mortality checks (daily, afternoon) will be recorded.

- ***Procedures at Scheduled Termination***:
    - At scheduled terminal time points, take body weight and euthanize under CO_2. Collect whole blood by cardiac puncture, process to plasma and store frozen (-80°C). Remove the brain, bisect on the mid-sagittal plane, flash freeze (liquid nitrogen) halves in separate cryovials, and store frozen (-80°C). Take tail snip for genotyping, place in a cryovial and store frozen (-80°C). Following tissue collection, place carcasses in refrigerated storage and contact Toxicology staff members to pick up for gross necropsy examinations.
- ***Unscheduled Termination***:
    - If an animal becomes moribund and/or requires unscheduled euthanasia, proceed with the same evaluations as if the animal had been terminated on schedule.
    - If an animal is found dead, place the carcass in refrigerated storage (if in DMPK area) and notify appropriate Toxicology staff for gross necropsy. Each found dead animal will have a gross examination with any findings recorded. Body weight, blood and brain samples will not be collected from animals found dead.
- ***Genotyping***: Save tail snips for genotyping from every animal on study whether found dead or terminated (scheduled or unscheduled) so that genotype can be verified for each animal. Store specimens for genotyping according to standard procedures (cryovial at -80°C).

2.0 EXPERIMENTAL SUBJECTS

Transgenic (Tg) donor males (alpha-synuclein A53T mice) for a rederivation were provided by MK Lee, Johns Hopkins University. Tg males (R9 and R10) were among the live born offspring produced from the rederivation project at Taconic (Project No. 009289) and these males sired the next generation of offspring during colony expansion at Taconic. The breeding program was continued at Taconic and at RTI. Counting Tg males R9 and R10 as the first generation, this study will use the third generation offspring (>4 months of age) born in the RTI transgenic breeding colony at RTI [i.e., offspring from cohabitation of alpha-synuclein (A53T) transgenic males cohabited with C57BL/6 females].

Initially, up to 10 wild-type mice will be used to collect blood and brain tissue for analytical methods development and sample stability studies. Up to forty-four (44) transgenic mice will be assigned to the PK study.

Offspring born in RTI's transgenic colony are identified by cage number, identity of sire and dam, and pup number within the litter. Upon assignment to this study, a unique individual animal number will be marked on the tail of each animal with indelible ink. Records will be maintained in order to allow continuity of individual identity throughout the lifetime of each mouse and for any biological specimens collected from each mouse.

Initial genotyping has already been completed for these offspring (tail biopsies collected and analyzed at RTI). Records of tail sample submission and genotyping results will be

maintained in the study records. Furthermore, genotype will be verified based on tail snips to be collected when the animal is found dead or terminated (scheduled or unscheduled) regardless of the cause of death or reason for termination.

Alpha-synuclein (A53T) mice display a progressive phenotype that includes abnormal behavior, neurological decline and early mortality (Lee et al., 2002; Smith et al., 2010). The behavioral phenotype and indices of neuropathology appear normal through ~4 months of age. In the preclinical phase (~5-7 months of age), abnormal behavior includes hyperactivity (horizontal and vertical), sustained posturing, bradykinesia, mild ataxia and dystonia. In the later clinical stages (>8 months), mice develop progressive movement dysfunction leading to paralysis and death (Lee et al., 2002; Smith et al., 2010). To be more specific, another laboratory reported onset of clinical symptoms at ~8 months of age with ~50% survival of A53T mice at ~9 months of age (263 ± 10 days; Smith et al. 2010). In the absence of pharmacological intervention, none of the A53T mice survived beyond ~13 months of age (Smith et al., 2010). Depending on the age span of mice on this PK study, it is possible that some of them will show clinical signs characteristic of the alpha-synuclein (A53T) phenotype.

3.0 SENTINELS AND ANIMAL HEALTH SURVEILLANCE

Sentinels are already available in the transgenic colony room, which will also be the study room. Extra wild-type offspring born at RTI and/or commercially purchased young adult C57Bl/6 mice will serve as sentinels. Health surveillance screening will follow RTI Animal Research Facility (ARF) Standard Operating Procedures (SOPs). Results will be maintained with the study records.

4.0 ANIMAL HUSBANDRY

4.1 Animal Housing and Environmental Enrichment

Study animals will be housed in the transgenic colony room, which will also serve as the study room. Study animals will be singly housed in solid-bottom polycarbonate cages (5” x 11.5” x 7”) with water bottles and certified Irradiated Sani-Chips® cage litter from P.J. Murphy Forest Products. Copies of the bedding certification will be maintained in the study record book. Assigned personnel will change cages at least weekly according to ARF SOPs.

Autoclaved Nestlets™ (Ancare, Bellmore, NY) will be furnished in all cages. Feed will be placed in the bedding for food foraging, which is another form of environmental enrichment (see *4.3, Feed*, below). If approved by the veterinarian, other forms of physical manipulanda may be introduced into the cage as long as all animals have equal access to the same type of environmental enrichment. Any physical manipulanda will be noted in the study records.

Animals scheduled for termination on day 1 and day 21 of the study will be transferred to room 608 in the ARF, and will be dosed on day 1 or 21 for collection of blood samples at specific times following dosing.

4.2 Environmental Conditions

Environmental conditions will be continuously monitored, controlled, and recorded (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal® Software, Siebe Environmental Controls, [SEC]/Barber-Colman Company, Loves Park, IL). Animal rooms will

be maintained on a 12:12 hour light:dark cycle. Set points and target ranges for temperature (°F) and relative humidity (RH) in the animal rooms were 72°F (69-75°F) and 50% RH (35-65% RH), respectively.

4.3 Feed

Rodent NIH-31 Modified Autoclavable diet (Zeigler Bros, Inc., Gardeners, PA) will be available *ad libitum*. [Note: RTI will purchase the diet through Taconic Farms, Germantown, NY and the diet will be autoclaved at Taconic prior to shipping to RTI]. Feed will be provided to mice through cage lid hoppers and scattered in bedding to promote foraging (a form of environmental enrichment). If an animal is debilitated (i.e., no longer foraging and/or unable to reach the feed hopper), feed will be moistened and placed within easy access on the cage floor and/or Nutra-Gel Diet (Bio-Serv Inc.) may be placed in the cage to supplement feed/water intake. Nutra-Gel Diet may be used at the discretion of the study staff and/or under direction of the facility veterinarian. Batch numbers and expiration dates for diet will be recorded per RTI SOP. Certified analysis of each NIH-31 feed lot will be provided by the manufacturer and included in the study records.

4.4 Water

Tap water from the Durham, NC, municipal water system will be available *ad libitum* and delivered via water bottles. Water bottles will be changed at least weekly. The city of Durham analyzes drinking water on a monthly basis for potential drinking water contaminants and publishes the results annually. A copy of each relevant annual report will be maintained in the study records. Any other relevant results of animal drinking water analyses (i.e., samples collected in the RTI Animal Research Facility) will be maintained in the facility records. As noted above (see *4.3, Feed*, above), Nutra-Gel Diet (Bio-Serv Inc.) may be used to supplement feed/water intake as warranted by the condition of an individual animal; batch numbers and expiration dates will be recorded per RTI SOP.

4.5 Animal Identification

Mice used on this study will be given a tail marking (indelible marker and/or tail tattoo).

4.6 Standards for Animal Care

RTI's Animal Research Facility (ARF) is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and operated in conformance with the *Guide for Care and Use of Laboratory Animals* (NRC, 1996). Animal use for this study will be reviewed and approved by RTI's Institutional Animal Care and Use Committee (IACUC) prior to study initiation.

5.0 EXPERIMENTAL DESIGN AND TREATMENT

5.1 Selection of Study Mice and Assignment to Terminal Time Points

For analytical methods development work and sample stability studies, select up to 10 wild-type mice (there are no specific age, body weight, or sex requirements for selection of these mice). These mice will either be untreated or, if requested by the pharmacokineticist, will be dosed with vehicle at a time point to be determined prior to sample collection. Wild-type mice

will be terminated for collection of blood and brain samples, as described below (see *6.2, Necropsy*).

For the PK study, select up to 44 Tg mice from the alpha-synuclein colony with a mean target body weight of 30 g on the first day of drug exposure. Assign two mice to each of 22 terminal time points (i.e., 11 time points on Day 1 and 11 time points on Day 21; see Table 1).

Distribute mice across necropsy days and time points with reasonably balanced representation by age, sex, clinical status, and body weight. Animals will be assigned manually rather than by automated randomization.

Based on the available population of mice, the number of mice per time point and/or the number of time points in the study design may be adjusted at the discretion of the study director.

5.2 Test Drug, Vehicle and Dose Formulation

Test Drug: Isradipine (CAS No. 75695-93-1) from Tecoland, Edison, NJ (Batch Number YP200901002). Purity (99.6% and 99.2%) is reported in vendor's Certificates of Analysis (COA) dated 1/15/2009 and 1/26/2011, respectively. The COAs recommend a retest date of 1/4/2013.

Formulation: Isradipine prepared in Simple Syrup (0.25 mL/kg) will be used for dosing. The formulation procedure is based on MacDonald (1994). The dose volume is 10 mL/kg/dose (b.i.d. animals receive 20 mL/kg/day).

Procurement and Records: Verified copies of records [i.e., receipt and disposition, vendor's certificate of analysis (COA) for the bulk drug, Material Safety Data Sheet (MSDS), dose formulation, inventory and use logs] will be maintained for the study records.

Storage and Expiration Dates: The bulk drug and dose formulations will be stored refrigerated (~4°C) and protected from light and moisture when not in use. On each day of the dosing period, the formulations will be brought to room temperature, stirred on a stir plate prior to and continuously during dosing, then returned to refrigerated storage. Bulk drug and vehicle components will follow the manufacturer's expiration dates. Dose formulations will have an expiration date 35 days after formulation (MacDonald 1994).

Safety: A copy of the MSDS for isradipine will be maintained in all active work areas where isradipine is stored or in use. Procedures recommended in the MSDS for bulk chemical handling will be followed. Routine safety precautions, including PPE appropriate to the work area, will be followed for dosing.

Disposition of Bulk Drug and Dose Formulations: Any residual isradipine will be maintained in the MHF until disposition is approved by the Study Director. In the absence of specific instructions, the default disposal date will be not sooner than 30 days after the expiration date or not sooner than 30 days prior to completion date of the referenced prime contract (whichever comes first). Residual isradipine will be processed as waste for incineration.

5.3 Treatment Regimen

Color coding may be used to distinguish (Table 1) in order to facilitate accuracy of dosing and clarity of study records. Isradipine dose volume is 10 mL/kg/dose (b.i.d. animals receive 20 mL/kg/day).

Mice in the zero time point group (up to two) will be terminated on Day 1 without treatment (untreated). On Day 1 (start date for dosing), groups scheduled for Day 1 sampling will be transferred to Room 608 (DMPK), and will be dosed for collection of scheduled blood samples. The mice scheduled for day 21 samples will be dosed by volume based on body weight with isradipine twice daily for up to 20 days (Tox). On Day 21, the mice will be transferred to room 608 for final dosing and sample collection (DMPK).

Table 1: Number of Mice per Treatment Group and Sample Collection Time Point

		Transgenic Animals	
Time point (Day)	Time after First Dose	Tg Control	Tg Dosed
1	0 (predose)	2	0
1	20 min	0	2
1	1 h	0	2
1	2 h	0	2
1	4 h	0	2
1	6 h	0	2
1	6 h 20 min	0	2
1	7 h	0	2
1	8 h	0	2
1	10 h	0	2
1	12 h	0	2
21	0 (predose)	0	2
21	20 min	0	2
21	1 h	0	2
21	2 h	0	2
21	4 h	0	2
21	6 h	0	2
21	6 h 20 min	0	2
21	7 h	0	2
21	8 h	0	2
21	10 h	0	2
21	12 h	0	2

6.0 EVALUATION OF EXPERIMENTAL SUBJECTS

6.1 In-Life

- ***Untreated Mice Day 1***: Record body weight and clinical condition. Then euthanize as described below (see Section 6.2).
- ***Treated Mice Day 1-21***: Observe daily for clinical signs (morning) and cage-side morbidity/mortality checks (afternoon). Record body weight on Day 1, Day 8,

Day 15 and Day 21 (prior to termination). On the scheduled day of termination, euthanize and evaluate as described below (see Section 6.2).

6.2 PK Sample Collection/Gross Necropsy

- ***Unscheduled Deaths***: If an animal becomes moribund and/or requires an unscheduled euthanasia, the study director/study coordinator will be contacted (veterinarian may be contacted concurrently). The moribund animal will be euthanized and terminal PK samples (blood and brain) will be collected as noted for Scheduled Euthanasia animals below. Study groups may be rearranged as a result of unscheduled deaths (at the discretion of the study director or pharmacokineticist). Found dead animals will be subjected to necropsy (stored refrigerated and transferred to Tox staff, if necessary), but PK samples (blood and brain) will not be collected. A tail snip will be collected and stored in a cryovial at -80.
- ***Scheduled Euthanasia***: Animals will be euthanized at the appropriate time point (refer to Table 1) for terminal PK procedures and gross necropsy.
- ***Termination and Collection of Blood Samples:*** Mice will be euthanized by carbon dioxide asphyxiation, and blood collected by cardiac puncture. At termination, ~1 mL of whole blood will be collected via cardiac puncture (if necessary, trunk blood may be collected following decapitation). Whole blood will be processed per instructions from the PK chemist, but in general it is anticipated that whole blood (~1 mL) will be processed to plasma (~0.5 mL), and frozen in cryovials at -80°C. On a prearranged schedule, frozen plasma will be transferred to the assigned RTI chemist for analysis of isradipine concentration.
- ***Collection of Brain Samples:*** The whole brain will be removed and bisected on the mid-sagittal plane and each half flash frozen in liquid nitrogen and stored in separate cryovials at -80°C. On prearranged schedules, (a) the left brain hemisphere will be shipped on dry ice to MK Lee, Univeristy of Minnesota, and (b) the right brain hemisphere will to be transferred to the assigned RTI chemist for analysis of isradipine concentration.
- ***Gross Necrospy:*** Following tissue collection, place carcasses in refrigerated storage and contact Toxicology staff members to pick up for gross necropsy examinations.
- ***Genotyping Procedures:*** Following standard procedures, take tail snips (i.e., biopsy specimens) for genotyping from every animal on study whether found dead or terminated (scheduled or unscheduled) so that genotype can be verified for each animal. Store tail snips per standard procedures (cryovial at -80°C). On a prearranged schedule, tail snips will be transferred to Xinxin Zhang for genotyping.

6.3 Analysis of Isradipine in Plasma and Brain

- Storage stability of isradipine in relevant matrices (plasma and brain) at -80°C will be ***evaluated for up to 28*** days (e.g., 3, 7, 14 and 28 day time points) at three concentrations. Autosampler stability will be determined in extracts.
- Isradipine concentrations will be measured in plasma and brain of transgenic mice from the PK study using liquid chromatography-mass spectrometry (LC-MS/MS) with nifedipine as the internal standard. Samples will be analyzed within the period of proven stability when stored at -80°C, or on the day of sample collection if stability data are not available at that time. Any exceptions will be noted in the study record.
- Analytical methods for isradipine from other laboratories (e.g. Surmeier and collaborators, unpublished and confidential, and Anekonda et al., 2011) will be taken into consideration, and such analytical methods will be refined and adapted for optimal performance in our laboratories. Plasma and brain samples from wild-type mice will be used for analytical methods development work and sample stability studies. Standard curves will cover the anticipated concentration range of the samples to be analyzed. General guidance in estimating plasma and brain concentrations can be derived from recent work in other laboratories (e.g. Surmeier and collaborators, unpublished and confidential, and Anekonda et al., 2011). The concentrations of standards will be arranged so that each standard comes from an alternate stock standard. A six point calibration curve each made in duplicate will be employed for analyte quantitation. A simple linear regression model with or without weighting will be employed for curve fitting. If the analytical system is reproducibly non-linear, a non-linear regression model will be used. The precision (% relative standard deviation) and accuracy(% relative error) will be estimated. Six replicates of standard will be used to estimate the assay limit of quantitation (LOQ).
- The following estimates will be considered acceptable; the coefficient of determination, r2, of ≥ 0.98 for concentration-response relationship; an accuracy of ≤ ± 15% (for concentrations above LOQ) or ≤ ± 20% (for concentration at LOQ) of the nominal value; a precision of ≤ ± 15% (for concentrations above LOQ) or ≤ ± 20% (for concentration at LOQ) of the mean value.

***Bulk Drugs for Analytical Chemistry*:**

- Isradipine (CAS No. 75695-93-1) from Tecoland, Edison, NJ (Batch Number YP200901002). Purity (99.6% and 99.2%) is reported in vendor's Certificates of Analysis (COA) dated 1/15/2009 and 1/26/2011, respectively. The COAs recommend a retest date of 1/4/2013.
- Nifedipine (CAS No. 21829-25-4) from Sigma-Aldrich (Lot Number 040M1912). Purity (100%) reported in vendor's COA dated 5/20/2010. No recommended retest date.

- Bulk drugs were procured, received and stored by RTI Materials Handling Facility (MHF). Verified copies of records [i.e., receipt and disposition, COA, Material Safety Data Sheet (MSDS), inventory and use logs] will be maintained in the study records.

Storage and Expiration Dates: Store refrigerated (~4°C) protected from light and moisture. Per COA, the vendor's recommended retest date for isradipine is 1/4/2013, and no retest date was recommended for nifedipine.

Safety: A copy of the MSDS for isradipine and/or nifedipine will be maintained in all active work areas where the bulk drugs are stored or in use. Recommendations of the MSDS will be followed for handling the bulk drugs and aliquots thereof. Routine safety precautions, including PPE appropriate to the work area, will also be applicable.

Post-Study Disposition of Bulk Drug: Bulk drugs will be maintained in the MHF until disposition is directed and approved by the Principal Investigator and Study Director.

7.0 DATA COLLECTION AND ANALYSIS

7.1 Data Collection

Body weights and clinical observations may be recorded in Provantis® Instem™ or recorded on paper using study-specific data collection sheets. Dosing and sample collection on Day 1 or Day 21 will be recorded in Debra (Lablogic Systems Limited) for those animals scheduled for sample collection, or recorded on paper. Tabulation of individual animal and summary data for these endpoints will be the responsibility of the respective Study Coordinators. The genotyping laboratory will provide results to the Principal Investigator, Study Director and Study Coordinator. Verified copies of laboratory notebooks and other related documentation will be maintained in the study records.

7.2 Analytical Chemistry

Results of sample analysis for isradipine concentration in plasma and brain will be provided in an appropriate format by the study chemist and interpretation will be provided by the study director and pharmacokineticist.

8.2 Collaborators and Consultants

Consultant for Alpha-synuclein Transgenics:	Michael K. Lee, Ph.D., U. Minnesota, Minneapolis, MN.
Consultant for Isradipine Neuroprotection:	D. James Surmeier, Ph.D., Northwestern University
Back-Up Veterinarians:	Donald B. Feldman, D.V.M., DACLAM
	David Kurtz, D.V.M., Ph.D., DACLAM

9.0 LITERATURE CITATIONS

Anekonda, TS, Quinn, JF, Harris, C, Frahler, K, Wadsworth, TL and Woltjer, RL. (2011). Ltype voltage-gated calcium channel blockade with isradipine as a therapeutic strategy for Alzheimer's disease. Neurobiol. Dis. 41(1), 62-70.

Lee, MK et al. (2002). Human α-synuclein-harboring familial Parkinson's disease-linked Ala-53 → Thr mutation causes neurodegenerative disease with α-synuclein aggregation in transgenic mice. PNAS, 99 (13), June 25, 2002.

MacDonald, JL et al. (1994). Stability of isradipine in an extemporaneously compounded oral liquid. Am J Hosp Pharm, Vol 51, October 1, 1994.

NRC (1996). National Research Council. Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC.

Smith, WW et al. (2010). Synphilin-1 attenuates neuronal degeneration in the A53T α-synuclein transgenic mouse model. Human Molecular Genetics (HMG Advanced Access March 20, 2010).