5.8.1 TOXICITY TO FERTILITY

Species	:	Rat
Sex	:	Male and female
Strain	:	Sprague Dawley
Test substance	:	as prescribed by 1.1 - 1.4
Reliability	:	(1) valid without restriction
Route of Administration	:	Oral gavage
Type	:	Reproductive/Developmental Toxicity Screening Test, OECD 421
Doses	:	0, 100, 300, and 1000 mg/kg/day
Animals per dose	:	10 male and 10 female
Frequency of Treatment	:	Daily
Exposure Period	:	Male 29 days; females 54 days.
Control group	:	Yes; concurrent vehicle
NOEAL	:	1000 mg/kg/day; highest dose tested.
Year	:	2012
GLP	:	Yes
Method	:	Groups of 10 male and 10 female Crl:CD(SD) rats were administered the test material daily, by gavage, at dose levels of 0 (control), 100, 300, or 1000 mg/kg/day. Female rats were dosed once daily for approximately two weeks prior to breeding, through breeding (up to two weeks), gestation (three weeks), and lactation (four days) up to termination. Male rats were dosed for two weeks prior to breeding and continuing through breeding (two weeks) until necropsy (test day 30). Effects on gonadal function, mating behavior, conception, development of the conceptus, parturition and early postnatal growth and survival were evaluated. In addition, a gross necropsy and histopathology of the adults were conducted with an emphasis on organs of the reproductive system.

Daily In-Life Observations
A cage-side examination was conducted at least twice daily. This examination was typically performed with the animals in their cages and was designed to detect significant clinical abnormalities that were clearly visible upon a limited examination, and to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary. Significant abnormalities that could have been observed included, but were not limited to: decreased/increased activity, repetitive behavior, vocalization, incoordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in fecal consistency, and fecal/urinary quantity. In addition, all animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily. Cage-side examinations were also conducted on dams and their litters at least twice daily. These examinations were conducted as described above.

Clinical Observations
Animals were observed approximately one hour after dosing, or at the anticipated time of peak effects, if known. Females were observed for signs of parturition beginning on or about GD 20 (see litter data). Clinical observations included a careful evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucous membranes,

as well as an assessment of general behavior, injuries, or palpable mass/swellings.

Body Weights/Body Weight Gains
All rats were weighed at least once during the pre-exposure period and on the first day of dosing. Male body weights continued to be recorded weekly throughout the study. Females were weighed weekly during the pre-breeding and breeding periods. During gestation, females were weighed on GD 0, 7, 14, 17, and 20. Females that delivered litters were weighed on LD 1 and 4. Females that failed to mate or deliver a litter were weighed at least weekly until termination. Body weight analyses were conducted for the following days: GD 0, 7, 14, 20, and LD 1 and 4. Body weight gains were determined for the following intervals: GD 0-7, 7-14, 14-20, 0-20, and LD 1-4.

Feed Consumption:
Feed consumed was determined weekly during the two week pre-breeding period for males and females by weighing feed crocks at the start and end of a measurement cycle. Feed consumption was not measured for males or females due to co-housing during breeding. Following breeding, feed consumption was not measured for males. For females during gestation, feed consumption was measured on GD 0, 7, 14, and 20. After parturition, feed consumption was measured on LD 1 and 4. Feed consumption was not recorded for females that failed to mate or deliver a litter. Feed consumption was calculated using the following equation: Feed consumption (g/day) = (initial weight of crock - final weight of crock) (# of days in measurement cycle)

Breeding Procedure
Breeding of the adults commenced after approximately two weeks of treatment. Each female was placed with a single male from the same dose level (1:1 mating) until pregnancy occurred or two weeks had elapsed. During the breeding period, daily vaginal lavage samples were evaluated for the presence of sperm as an indication of mating. The day on which sperm was detected or a vaginal copulatory plug was observed in situ was considered GD 0. The sperm- or plug-positive (presumed pregnant) females were then separated from the males and returned to their home cages.

Litter Data
Females were observed for signs of parturition beginning on or about GD 20. In so far as possible, parturition was observed for signs of difficulty or unusual duration. The day of parturition was recorded as the first day the presence of the litter was noted and was designated as LD 0. All litters were examined as soon as possible after delivery. The following information was recorded on each litter: date of parturition, litter size on the day of parturition (LD 0), the number of live and dead pups on days 0, 1, and 4, and the sex and the weight of each pup on LD 1 and 4. Any visible physical abnormalities or demeanor changes in the neonates were recorded as they were observed during the lactation period (see Daily In-Life Observations). In addition, pup clinical observations were recorded on each litter on PND 0 through 4. Any pups found dead were sexed and examined grossly, if possible, for external and visual defects and then discarded.

Anatomic Pathology

Adult Necropsy
Adult males (fasted) were submitted for necropsy after at least four weeks of exposure. Adult females (fasted) were terminated between lactation days 5 and 8, or at least 24 days after the end of the mating period for females not producing a litter. On the morning of the scheduled necropsy the animals were weighed. The animals were anesthetized by the inhalation of CO2, their tracheas were exposed and clamped, and the animals were euthanized by decapitation.

A complete necropsy was conducted on all animals by a veterinary pathologist assisted by a team of trained individuals. The necropsy included an examination of the external tissues and all orifices. The head was removed, the cranial cavity opened and the brain, pituitary and adjacent cervical tissues were examined. The eyes were examined in situ by application of a moistened microscope slide to each cornea. The skin was reflected from the carcass, the thoracic and abdominal cavities were opened and the viscera examined. All visceral tissues were dissected from the carcass, reexamined and selected tissues were incised.

The uteri of all females were stained with an aqueous solution of 10% sodium sulfide stain for approximately one minute and were examined for the presence and number of implantation sites. After evaluation, uteri were gently rinsed with saline and preserved in neutral phosphate-buffered 10% formalin.

Weights of the epididymides, kidneys, liver, and testes were recorded, and organ:body weight ratios calculated. Representative samples of tissues listed in Table 2 were collected and preserved in neutral, phosphate-buffered 10% formalin, with the exception of the testes and epididymides which were fixed in Bouin's. Transponders were removed and placed in jars with the tissues.

Offspring Necropsy
All pups surviving to LD 4 were euthanized by intraperitoneal administration of sodium pentobarbital solution, examined for gross external alterations, and then discarded. Any pups found dead or which are euthanized in moribund condition were examined to the extent possible and discarded.

Histopathology
Histologic examination of the tissues indicated in Table 2 was conducted on all control and high-dose adult rats. Examination of tissues from the remaining groups was limited to relevant gross lesions and those tissues that demonstrated treatment related histologic effects at the high dose (stomach). Paraffin embedded tissues were sectioned approximately 6 µm thick, stained with hematoxylin and eosin and examined by a veterinary pathologist using a light microscope.

Histopathological examination of the testes included a qualitative assessment of stages of spermatogenesis. A cross section through the approximate center of both testes of control and high-dose males was embedded in paraffin, sectioned at 5 µm and stained with modified periodic acid Schiffs-hematoxylin. The presence and integrity of the stages of spermatogenesis were qualitatively evaluated. Microscopic evaluation included a qualitative assessment of the relationships between

spermatogonia, spermatocytes, spermatids, and spermatozoa seen in cross sections of the seminiferous tubules. The progression of these cellular associations defines the cycle of spermatogenesis. In addition, sections of both testes were examined for the presence of degenerative changes (e.g., vacuolation of the germinal epithelium, a preponderance of Sertoli cells, sperm stasis, inflammatory changes, mineralization, and fibrosis).

Selected histopathologic findings were graded to reflect the severity of specific lesions to evaluate: 1) the contribution of a specific lesion to the health status of an animal, 2) exacerbation of common naturally occurring lesions as a result of the test material, and 3) dose-response relationships for treatment-related effects. Very slight and slight grades were used for conditions that were altered from the normal textbook appearance of an organ/tissue, but were of minimal severity and usually with less than 25% involvement of the parenchyma. This type of change would neither be expected to significantly affect the function of the specific organ/tissue nor have a significant effect on the overall health of the animal. A moderate grade was used for conditions that were of sufficient severity and/or extent (up to 50% of the parenchyma) that the function of the organ/tissue may have been adversely affected, but not to the point of organ failure. The health status of the animal may or may not be affected, depending on the organ/tissue involved, but generally lesions graded as moderate would not be life threatening. A severe grade would have been used if lesions were extensive enough to cause significant organ/tissue dysfunction or failure. This degree of change in a critical organ/tissue may be life threatening.

STATISTICS AND CALCULATIONS

Parental body weights and gestation and lactation body weight gains, litter mean body weights, feed consumption, and organ weights (absolute and relative) was first evaluated by Bartlett's test (alpha = 0.01) for equality of variances. Based upon the outcome of Bartlett's test, either a parametric or non-parametric analysis of variance (ANOVA) was performed. If the ANOVA was significant at alpha = 0.05, a Dunnett's test (alpha =0.05) or the Wilcoxon Rank-Sum (alpha = 0.05) test with Bonferroni's correction was performed. Feed consumption values were excluded from analysis if the feed is spilled or scratched. Gestation length, average time to mating, and litter size were analyzed using a nonparametric ANOVA. If the ANOVA was significant, the Wilcoxon Rank-Sum test with Bonferroni's correction was performed. Statistical outliers (alpha = 0.02) were identified and only excluded from analysis for documented, scientifically sound reasons. The mating, conception, fertility and gestation indices were analyzed by the Fisher exact probability test (alpha = 0.05) with Bonferroni's correction. Evaluation of the neonatal sex ratio on postnatal day 1 was performed by the binomial distribution test (alpha = 0.05). Gender was determined for pups found dead on postnatal day 0 and these data was included in sex ratio calculations.

Survival indices, post-implantation loss, and other incidence data among neonates was analyzed using the litter as the experimental unit by the censored Wilcoxon test (alpha = 0.05) with Bonferroni's correction. Non-pregnant females, females with resorptions only, or females found to be pregnant after staining of their uteri were excluded from the appropriate analyses. Both the Dunnett's test and Bonferroni's correction corrects for

multiple comparisons to the control to keep the experiment-wise error rate at 0.05. Both were reported at the experiment-wise alpha level.
Reproductive indices were calculated for all dose level groups as follows:
• Female mating index = (No. females with evidence of mating/No. paired) x 100
• Male mating index = (No. males with evidence of mating/No. paired) x 100
• Female conception index = (No. females with evidence of pregnancy/No. mated) x 100
• Male conception index = (No. males siring a litter/No. mated) x 100
• Female fertility index = (No. females with evidence of pregnancy/No. paired) x 100
• Male fertility index = (No. males siring a litter/No. paired) x 100
• Gestation index = (No. females delivering a viable litter/No. females delivering a litter) x 100
• Gestation survival index = percentage of delivered pups alive at birth
• Post-implantation loss = (No. implants – No. viable offspring)/(No. implants) x 100
• Day 1 or 4 pup survival index = (No. viable pups on day 1 or 4/No. born live) x 100

Result : Treatment-related parental toxicity was limited to point of contact irritation in the stomach of animals given 300 and 1000 mg/kg/day. Treatment-related gross pathological findings were limited to a thickened limiting ridge of the forestomach in males and females in the 1000 mg/kg/day group. Treatment-related histopathological changes consistent with point of contact irritation in the stomach were observed in the 300 and 1000 mg/kg/day groups. These findings consisted of: 1) hyperplasia of the stratified squamous epithelium at the limiting ridge of the forestomach with inflammation in males given 300 mg/kg/day (very slight) or males and females given 1000 mg/kg/day (very slight to slight) and 2) subacute to chronic, multifocal inflammation of the submucosa of the glandular stomach of males (slight) or females (very slight to slight) given 300 or 1000 mg/kg/day. All these treatment-related changes were interpreted to be localized irritation effects on the stomach due to repeated oral gavage of the test material. There were no treatment-related histopathologic changes in the stomach of males and females given 100 mg/kg/day. There were no effects on any parameter of reproductive performance or offspring survival at any dose level tested.

Conclusion : Based on the histopathologic stomach effects, the no-observed-effect level (NOEL) for parental toxicity was 100 mg/kg/day. The NOEL for reproductive effects was 1000 mg/kg/day, the highest dose tested.

Reliability : (1) valid without restriction

5.8.2 DEVELOPMENTAL TOXICITY/TERATOGENICITY

Species	:	Rat
Sex	:	Male and female
Strain	:	Sprague Dawley
Test substance	:	as prescribed by 1.1 - 1.4
Reliability	:	(1) valid without restriction
Route of Administration	:	Oral gavage
Type	:	Reproductive/Developmental Toxicity Screening Test, OECD 421
Doses	:	0, 100, 300, and 1000 mg/kg/day
Animals per dose	:	10 male and 10 female
Frequency of Treatment	:	Daily
Exposure Period	:	Male 29 days; females 54 days.
Control group	:	Yes; concurrent vehicle
NOEAL	:	1000 mg/kg/day; highest dose tested.
Year	:	2012
GLP	:	Yes
Method	:	Groups of 10 male and 10 female Crl:CD(SD) rats were administered the test material daily, by gavage, at dose levels of 0 (control), 100, 300, or 1000 mg/kg/day. Female rats were dosed once daily for approximately two weeks prior to breeding, through breeding (up to two weeks), gestation (three weeks), and lactation (four days) up to termination. Male rats were dosed for two weeks prior to breeding and continuing through breeding (two weeks) until necropsy (test day 30). Effects on gonadal function, mating behavior, conception, development of the conceptus, parturition and early postnatal growth and survival were evaluated. In addition, a gross necropsy and histopathology of the adults were conducted with an emphasis on organs of the reproductive system. Daily In-Life Observations A cage-side examination was conducted at least twice daily. This examination was typically performed with the animals in their cages and was designed to detect significant clinical abnormalities that were clearly visible upon a limited examination, and to monitor the general health of the animals. The animals were not hand-held for these observations unless deemed necessary. Significant abnormalities that could have been observed included, but were not limited to: decreased/increased activity, repetitive behavior, vocalization, incoordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in fecal consistency, and fecal/urinary quantity. In addition, all animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily. Cage-side examinations were also conducted on dams and their litters at least twice daily. These examinations were conducted as described above. Clinical Observations Animals were observed approximately one hour after dosing, or at the anticipated time of peak effects, if known. Females were observed for signs of parturition beginning on or about GD 20 (see litter data). Clinical observations included a careful evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucous membranes, as well as an assessment of general behavior, injuries, or palpable

mass/swellings.

Body Weights/Body Weight Gains
All rats were weighed at least once during the pre-exposure period and on the first day of dosing. Male body weights continued to be recorded weekly throughout the study. Females were weighed weekly during the pre-breeding and breeding periods. During gestation, females were weighed on GD 0, 7, 14, 17, and 20. Females that delivered litters were weighed on LD 1 and 4. Females that failed to mate or deliver a litter were weighed at least weekly until termination. Body weight analyses were conducted for the following days: GD 0, 7, 14, 20, and LD 1 and 4. Body weight gains were determined for the following intervals: GD 0-7, 7-14, 14-20, 0-20, and LD 1-4.

Feed Consumption:
Feed consumed was determined weekly during the two week pre-breeding period for males and females by weighing feed crocks at the start and end of a measurement cycle. Feed consumption was not measured for males or females due to co-housing during breeding. Following breeding, feed consumption was not measured for males. For females during gestation, feed consumption was measured on GD 0, 7, 14, and 20. After parturition, feed consumption was measured on LD 1 and 4. Feed consumption was not recorded for females that failed to mate or deliver a litter. Feed consumption was calculated using the following equation: Feed consumption (g/day) = (initial weight of crock - final weight of crock) (# of days in measurement cycle)

Breeding Procedure
Breeding of the adults commenced after approximately two weeks of treatment. Each female was placed with a single male from the same dose level (1:1 mating) until pregnancy occurred or two weeks had elapsed. During the breeding period, daily vaginal lavage samples were evaluated for the presence of sperm as an indication of mating. The day on which sperm was detected or a vaginal copulatory plug was observed in situ was considered GD 0. The sperm- or plug-positive (presumed pregnant) females were then separated from the males and returned to their home cages.

Litter Data
Females were observed for signs of parturition beginning on or about GD 20. In so far as possible, parturition was observed for signs of difficulty or unusual duration. The day of parturition was recorded as the first day the presence of the litter was noted and was designated as LD 0. All litters were examined as soon as possible after delivery. The following information was recorded on each litter: date of parturition, litter size on the day of parturition (LD 0), the number of live and dead pups on days 0, 1, and 4, and the sex and the weight of each pup on LD 1 and 4. Any visible physical abnormalities or demeanor changes in the neonates were recorded as they were observed during the lactation period (see Daily In-Life Observations). In addition, pup clinical observations were recorded on each litter on PND 0 through 4. Any pups found dead were sexed and examined grossly, if possible, for external and visual defects and then discarded.

Anatomic Pathology
Adult Necropsy

Adult males (fasted) were submitted for necropsy after at least four weeks of exposure. Adult females (fasted) were terminated between lactation days 5 and 8, or at least 24 days after the end of the mating period for females not producing a litter. On the morning of the scheduled necropsy the animals were weighed. The animals were anesthetized by the inhalation of CO2, their tracheas were exposed and clamped, and the animals were euthanized by decapitation.

A complete necropsy was conducted on all animals by a veterinary pathologist assisted by a team of trained individuals. The necropsy included an examination of the external tissues and all orifices. The head was removed, the cranial cavity opened and the brain, pituitary and adjacent cervical tissues were examined. The eyes were examined in situ by application of a moistened microscope slide to each cornea. The skin was reflected from the carcass, the thoracic and abdominal cavities were opened and the viscera examined. All visceral tissues were dissected from the carcass, reexamined and selected tissues were incised.

The uteri of all females were stained with an aqueous solution of 10% sodium sulfide stain for approximately one minute and were examined for the presence and number of implantation sites. After evaluation, uteri were gently rinsed with saline and preserved in neutral phosphate-buffered 10% formalin.

Weights of the epididymides, kidneys, liver, and testes were recorded, and organ:body weight ratios calculated. Representative samples of tissues listed in Table 2 were collected and preserved in neutral, phosphate-buffered 10% formalin, with the exception of the testes and epididymides which were fixed in Bouin's. Transponders were removed and placed in jars with the tissues.

Offspring Necropsy
All pups surviving to LD 4 were euthanized by intraperitoneal administration of sodium pentobarbital solution, examined for gross external alterations, and then discarded. Any pups found dead or which are euthanized in moribund condition were examined to the extent possible and discarded.

Histopathology
Histologic examination of the tissues indicated in Table 2 was conducted on all control and high-dose adult rats. Examination of tissues from the remaining groups was limited to relevant gross lesions and those tissues that demonstrated treatment related histologic effects at the high dose (stomach). Paraffin embedded tissues were sectioned approximately 6 µm thick, stained with hematoxylin and eosin and examined by a veterinary pathologist using a light microscope.

Histopathological examination of the testes included a qualitative assessment of stages of spermatogenesis. A cross section through the approximate center of both testes of control and high-dose males was embedded in paraffin, sectioned at 5 µm and stained with modified periodic acid Schiffs-hematoxylin. The presence and integrity of the stages of spermatogenesis were qualitatively evaluated. Microscopic evaluation included a qualitative assessment of the relationships between spermatogonia, spermatocytes, spermatids, and spermatozoa seen in

cross sections of the seminiferous tubules. The progression of these cellular associations defines the cycle of spermatogenesis. In addition, sections of both testes were examined for the presence of degenerative changes (e.g., vacuolation of the germinal epithelium, a preponderance of Sertoli cells, sperm stasis, inflammatory changes, mineralization, and fibrosis).Selected histopathologic findings were graded to reflect the severity of specific lesions to evaluate: 1) the contribution of a specific lesion to the health status of an animal, 2) exacerbation of common naturally occurring lesions as a result of the test material, and 3) dose-response relationships for treatment-related effects. Very slight and slight grades were used for conditions that were altered from the normal textbook appearance of an organ/tissue, but were of minimal severity and usually with less than 25% involvement of the parenchyma. This type of change would neither be expected to significantly affect the function of the specific organ/tissue nor have a significant effect on the overall health of the animal. A moderate grade was used for conditions that were of sufficient severity and/or extent (up to 50% of the parenchyma) that the function of the organ/tissue may have been adversely affected, but not to the point of organ failure. The health status of the animal may or may not be affected, depending on the organ/tissue involved, but generally lesions graded as moderate would not be life threatening. A severe grade would have been used if lesions were extensive enough to cause significant organ/tissue dysfunction or failure. This degree of change in a critical organ/tissue may be life threatening.

STATISTICS AND CALCULATIONS

Parental body weights and gestation and lactation body weight gains, litter mean body weights, feed consumption, and organ weights (absolute and relative) was first evaluated by Bartlett's test (alpha = 0.01) for equality of variances. Based upon the outcome of Bartlett's test, either a parametric or non-parametric analysis of variance (ANOVA) was performed. If the ANOVA was significant at alpha = 0.05, a Dunnett's test (alpha =0.05) or the Wilcoxon Rank-Sum (alpha = 0.05) test with Bonferroni's correction was performed. Feed consumption values were excluded from analysis if the feed is spilled or scratched. Gestation length, average time to mating, and litter size were analyzed using a nonparametric ANOVA. If the ANOVA was significant, the Wilcoxon Rank-Sum test with Bonferroni's correction was performed. Statistical outliers (alpha = 0.02) were identified and only excluded from analysis for documented, scientifically sound reasons. The mating, conception, fertility and gestation indices were analyzed by the Fisher exact probability test (alpha = 0.05) with Bonferroni's correction. Evaluation of the neonatal sex ratio on postnatal day 1 was performed by the binomial distribution test (alpha = 0.05). Gender was determined for pups found dead on postnatal day 0 and these data was included in sex ratio calculations.

Survival indices, post-implantation loss, and other incidence data among neonates was analyzed using the litter as the experimental unit by the censored Wilcoxon test (alpha = 0.05) with Bonferroni's correction. Non-pregnant females, females with resorptions only, or females found to be pregnant after staining of their uteri were excluded from the appropriate analyses. Both the Dunnett's test and Bonferroni's correction corrects for multiple comparisons to the control to keep the experiment-wise error rate at 0.05. Both were reported at the experiment-wise alpha level.

Result	:	Treatment-related parental toxicity was limited to point of contact irritation in the stomach of animals given 300 and 1000 mg/kg/day. Treatment-related gross pathological findings were limited to a thickened limiting ridge of the forestomach in males and females in the 1000 mg/kg/day group. Treatment-related histopathological changes consistent with point of contact irritation in the stomach were observed in the 300 and 1000 mg/kg/day groups. These findings consisted of: 1) hyperplasia of the stratified squamous epithelium at the limiting ridge of the forestomach with inflammation in males given 300 mg/kg/day (very slight) or males and females given 1000 mg/kg/day (very slight to slight) and 2) subacute to chronic, multifocal inflammation of the submucosa of the glandular stomach of males (slight) or females (very slight to slight) given 300 or 1000 mg/kg/day. All these treatment-related changes were interpreted to be localized irritation effects on the stomach due to repeated oral gavage of the test material. There were no treatment-related histopathologic changes in the stomach of males and females given 100 mg/kg/day. There were no effects on any parameter of reproductive performance or offspring survival at any dose level tested.
Conclusion	:	Based on the histopathologic stomach effects, the no-observed-effect level (NOEL) for parental toxicity was 100 mg/kg/day. The NOEL for offspring growth and survival was 1000 mg/kg/day, the highest dose tested.
Reliability	:	(1) valid without restriction