Combined Fertility and Embryofetal Developmental Toxicity Study of Cytisine in Rats

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ABSTRACT

Cytisine [(1*R*,5*S*)-1,2,3,4,5,6-hexahydro-1,5-methano-8*H*-pyrido[1,2a][1,5]diazocin-8-one] is a plant alkaloid used in Eastern Europe for smoking cessation. Through its activity as a partial agonist of α4-β2 nicotinic acetylcholine receptors, cytisine may attenuate symptoms of nicotine withdrawal. The present study was performed to assess the potential reproductive and developmental toxicity of cytisine in rats. Groups of 22 CD rats/sex received daily oral (gavage) administration of cytisine (in sterile water) at doses of 0 [vehicle control], 0.4, 2.0, or 10.0 mg/kg/day beginning 4 weeks (males) or 2 weeks (females) prior to mating. Dosing was continued in males until Day 72 and in females until scheduled cesarean sections on Gestation Day 20. Six additional rats/sex/cytisine groups were included for toxicokinetics (TK). Endpoints included plasma drug levels; TK; *in vivo* toxicity; estrus cyclicity; mating/fertility/fecundity; reproductive organ weights; semen analysis; reproduction parameters; fetal body weights; and fetal examinations to identify developmental toxicity. Plasma levels of cytisine (AUC) increased in approximate proportion to dose; systemic exposure was generally higher in females than in males. In the high dose group, ½ was 5.1 h in males and 6.6 h in females. No mortality or evidence of treatment-related gross toxicity was seen in any rat receiving cytisine. Statistically significant decreases in body weight gain were identified in both sexes in the high dose group (10 mg/kg/day); sporadic reductions in food consumption were seen in the high dose (10 mg/kg/day) and mid dose (2mg/kg/day) groups. Cytisine had no effect on estrus cyclicity; sperm counts, morphology, or motility; reproductive organ weights in either sex; mating indices; or any other parameter associated with fecundity or reproductive function. Fetal body weights were comparable in all study groups; no evidence of cytisine developmental toxicity (increased incidences of malformations or variations) was identified in external, visceral, cephalic, or skeletal evaluations of fetuses. Cytisine doses of up to 10 mg/kg/day induced no evidence of developmental or reproductive toxicity in rats; cytisine toxicity was limited to reduced body weight gain seen in the high dose group (10 mg/kg/day). [Supported by HHSN261201600015I from the NCI]

INTRODUCTION

Cytisine is a plant-based alkaloid found in members of the Leguminosae family. Cytisine is a partial agonist of nicotinic acetylcholine receptors (nAChRs), with an affinity for the α4β2 receptor subtype. Cytisine has been available for smoking cessation since the 1960s, largely in Eastern Europe. Cytisine has been reported to be superior to placebo for short-term and long-term abstinence.

Cytisine remains relatively unknown outside Eastern Europe despite calls for licensing world-wide because of its proven benefits, low cost as compared with other cessation medications (nicotine-replacement therapy, varenicline). Thus the NCI has funded a number of preclinical toxicity studies, with the ultimate goal of obtaining regulatory approval through submission of an Investigational New Drug (IND) application to the U.S. Food and Drug Administration (FDA) for the entry of cytisine into Phase I and Phase II clinical trials in the U.S.

The objectives of this study were to provide general information on the potential of cytisine to alter male and female fertility, implantation and early embryonic development in the rat and to determine the potential developmental toxicity of cytisine in the rat, including effects on the development of gametes, the estrous cycle, sperm transport, fertilization, implantation and fetal development.

MATERIALS AND METHODS

Experimental Design: The study was conducted using four groups. Rats in three groups were administered the test article via oral gavage. The fourth group served as a vehicle control and was gavaged with vehicle alone. Male rats were dosed for four weeks and females for two weeks prior to mating. During the two-week mating period, one female rat was cohabited with one male rat. Treatment continued through mating for both male and females, and subsequently through Gestation Day 17 for females and through Day 72 for males. The males were dosed for at least 10 weeks. Females were euthanized on Gestation Day 20 and males were euthanized on Day 73.

Table 1. Experimental Design

Group	Test Article Dose Level (mg/kg/day) Oral Gavage	Number of Animals			
		Main Study		Toxicokinetic	
		Males[a]	Females[b]	Males	Females
1	0	22	22	3	3
2	0.4	22	22	6	6
3	2.0	22	22	6	6
4	10.0	22	22	6	6

[a]Terminal sacrifice after at least 10 weeks of dosing
[b]Terminal sacrifice and Cesarean section on Gestation Day 20

Parameter	Description
Animals	120 male (M) and 120 female (F) Sprague-Dawley [Crl:CD(SD)IGS BR] rats were obtained from Charles River Laboratories (Raleigh, NC).
Administration	• Single daily doses of the test article formulation or vehicle were administered by oral gavage at a constant dosing volume of 10 mL/kg.
Mortalty/Moribundity Checks	• Twice daily
Body Weights	• M: twice weekly throughout the study and at necropsy; F: GD 0, 3, 6, 9, 12, 15, 18, 20
Clinical Obs (Cage-Side)	• Once daily
Physical Exams (Hand-Held)	• Once weekly

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Parameter	Description
Mortalty/Moribundity Checks:	Twice daily
Body Weights:	Males; twice weekly throughout the study and at necropsy; Females – GD 0, 3, 6, 9, 12, 15, 18 and 20
Clinical Obs (Cage-Side):	Once daily
Physical Exams (Hand-Held):	Once weekly
Food Consumption	Concurrent with body weights for both males and females. No measurements during cohabitation (mating) period.
Vaginal Cyclicity (Estrous Cycle) and Mating	Vaginal smears were collected daily for 2 weeks prior to initiation of dosing and daily for 2 weeks prior to mating to evaluate cyclicity, ending when the presence of sperm/copulatory plug was detected or at the end of the 14-day cohabitation period. The presence of sperm in the smear or a copulatory plug indicated a positive mating and that day was designated as Gestation Day 0. A one-to-one mating regimen was used during the two-week cohabitation period (i.e., a single female rat was randomly paired with a single male rat from the same dose level for up to two weeks).
Blood Collection for Plasma Drug Analysis	Blood samples (approximately 0.5 mL each) for the determination of plasma drug levels were collected from the retro-orbital plexus of all Toxicokinetic (TK) animals. Samples were collected from the test article-treated (Groups 2-4) TK animals on Study Day 43 (males) and Gestation Day 17 (females) at six time points (approximately 0.5, 1, 2, 4, 8 and 24 hours post-dose).
Toxicokinetic (TK) Modeling	Mean plasma concentration-time profiles in rats at scheduled (nominal) sampling times were analyzed by noncompartmental toxicokinetic methods using Phoenix WinNonlin® Version 6.3 (Pharsight Corporation, Mountain View, CA).
Necropsy and Postmortem Examinations	Main Study animals were euthanized via CO_2 asphyxiation on Gestation Day 20 (females) or Day 73 (males). Gross necropsies were conducted on all males and all Cesarean section females.
Main Study Females (Scheduled Necropsy	The uterus was removed and weighed with the ovaries prior to the removal of fetuses from the uterine horns. The uterus was weighed The corpora lutea were counted for the left and right ovaries.
Gross Fetal Examination	Each uterine horn was opened and inspected for implantations; contents were recorded and classified: • Early resorption (placenta only with unrecognizable fetal tissue) • Late resorption (placenta with autolyzed, recognizable fetus) • Dead fetus (fetus with no signs of autolysis) • Live fetus (fetus pink in color and responds to touch)
Fetal External Examination	Fetuses were removed, counted, weighed and given a gross external morphological examination for variances and malformations.
Fetal Visceral Examination	Visceral examinations were performed on approximately one-half of the fetuses from each litter using a modified Staples' technique.
Cephalic Examination	Decapitated fetal heads from approximately one-half of each litter (those selected for visceral examination) were fixed in Bouin's solution for a minimum of one week prior to an examination using a modified Wilson's razor blade technique.
Skeletal Examination	Approximately one-half of the fetuses from each litter (those not selected for visceral examination) were processed for skeletal examination. All skeletal abnormalities were recorded and classified as variations or malformations.
Main Study Males (Scheduled Necropsy):	The reproductive organs (testes, epididymides, seminal vesicles, and prostate) were removed and weighed. Epididymal sperm counts were performed (10 per group), and slides for sperm morphology were prepared. The reproductive organs (left testis, left epididymis, seminal vesicles) were fixed (testis and epididymis in Bouin's solution and other tissues in 10% NBF) for possible histopathological evaluation.
Semen Analysis	The first ten surviving males in each group were designated for sperm analysis. The left epididymis was used for analysis of percent motility. Homogenized samples of the right caudal epididymis and the right testis were stained and sperm counts made using a Hamilton Thorne IVOS sperm analyzer. Additionally, two sperm morphology slides were prepared from the caudal epididymis total count preparation, stained with eosin, and then evaluated for morphological development.
Statistical Evaluations	Continuous data were compared via analysis of variance with post-hoc comparisons made using Dunnett's test; nonparametric data were compared via Mann-Whitney U test with post-hoc comparisons using Dunn's test. Incidence data were evaluated using Chi-square analysis and/or Fisher's Exact test with the litter as the experimental unit. The total number of litters with malformations and the total number of litters with variations were also statistically compared.

RESULTS

Mortality: All animals survived to their scheduled sacrifice time point (GD 20 or Day 73).

Clinical Observations/Physical Examinations: Transient observations of scab, sore and alopecia were noted in both males and females; however, none of these observations were considered treatment-related.

Body Weights: Statistically significant decreases in mean body weight gain were seen in the Group 4 (10.0 mg/kg) females for the GD 3-6 and 15-18 intervals, while a statistically significant decrease (5%) in mean body weight was seen in the Group 4 females on GD 18. A statistically significant decrease in body weight gain (12%) was present in the Group 4 males at the end of the dosing period. These decreases were considered treatment-related.

Food Consumption: Statistically significant, treatment-related decreases in food consumption were seen in the Group 4 (10.0 mg/kg) females during the gestation period.

Estrous Cycle: Administration of cytisine had no adverse impact on the estrous cycle.

Cohabitation: Administration of cytisine had no effect on mating, fertility or fecundity indices. All indices were 100% in the 10 mg/kg dose group.

Uterus Weight, Adjusted Body Weight and Weight Gain: No statistically significant differences in mean uterus weight, adjusted body weight or adjusted total weight gain were observed at cesarean section on GD 20 (Table 3).

Maternal Reproduction and Litter Viability: No statistically significant differences in mean number of corpora lutea, total number of implants, % pre- and post-implantation loss, total number of live implants, number of dead fetuses per dam, or number of resorptions were observed in any of the females at cesarean section on GD 20 (Table 4).

Fetal Body Weight: Using the litter as the experimental unit, statistically significant increases in comparison to the control group were seen in the mean fetal body weight for Group 4 (10.0 mg/kg) pups for male weight and combined sexes weight (Table 5).

Fetal External Gross External, Visceral and Cephalic Evaluations:

- No increase in the incidence of any gross external abnormalities (malformations or variations) was observed in the cytisine-treated groups compared to the control group. The only spontaneous malformations seen in any of the cytisine-treated groups were exencephaly, anopthalmia and absent upper jaw in one fetus in Group 3 (2.0 mg/kg) and tail agenesis in one fetus in Group 4 (10.0 mg/kg).
- No increase in the incidence of any visceral abnormalities (malformations or variations) was observed in the cytisine-treated groups compared to the control group. The most common observation was pigmentation (mottled, red, dark focus) in the lungs or adrenal glands. None of these changes were considered related to administration of the test article.
- No statistically significant increase in the incidence of any cephalic abnormalities (malformations or variations) was observed in the cytisine-treated groups compared to the control group. Cephalic findings consisted of microencephaly, dilatation of the third ventricle in the brain, absence of the cerebral hemisphere of the brain, microphthalmia, retinal fold and anopthalmia. Since these abnormalities were only seen in one to five pups from one to three litters, or occurred only in or with the greatest incidence in the control group, they were not considered treatment-related.

Fetal Skeletal Evaluations: No statistically significant increase in the incidence of skeletal malformations was observed. Skeletal abnormalities consisted primarily of signs of delayed ossification of the interparietal, parietal and supraoccipital bones of the skull; bipartite or dumbbell shaped thoracic centra; and lack of ossification of the 5th and/or 6th sternebrae; these observations were seen in all groups, including the control group. All observed skeletal abnormalities were considered variations rather than malformations, and based on the litter as the experimental unit, these variations were not statistically significant (Table 6).

Male Reproductive Organ Weight: No effects of cytisine were seen on the weight of any male reproductive organ.

Sperm Analysis: No statistically significant or cytisine-related changes in percent motile sperm, epididymal or testicular [homogenization-resistant spermatid (HRS)] sperm count, or percent abnormal sperm were seen.

Gross Necropsy Observations: No treatment-related gross pathology observations were identified in either sex.

Toxicokinetics: Systemic exposure was generally higher in females than in males regardless of dose. Over all dose levels and parameters, the female to male ratio averaged 1.29. After oral administration of cytisine on both Gestation Day 17 in females and Day 43 in males, exposure increased with dose for both sexes. For shorter periods of exposure (e.g., Cmax and AUC0-8), this increase was less than dose proportional, whereas for longer exposure (e.g., AUClast and AUCINF), the increase was nearly dose proportional (Table 2).

Table 2. Summary of Toxicokinetic Parameters in Female Rats on Gestation Day 17 and Male Rats on Day 43

Sex	Dose mg/kg	t_{max} (hr)	C_{max} (ng/mL)	AUC_{0-8} (hr*ng/mL)	AUC_{last} (hr*ng/mL)	AUC_{INF} (hr*ng/mL)	$t_{1/2}$ (hr)	CL/F (L/hr/kg)	Vz/F (L/kg)
Female	0.4	0.5	127	376	376	403	1.95	0.99	2.79
	2.0	1	271	1496	2105	2159	4.43	0.93	5.92
	10.0	0.5	807	4532	8148	8905	6.56	1.12	10.6
Male	0.4	1	108	319	319	366	2.70	1.09	4.25
	2.0	1	306	1155	1155	1281	2.29	1.56	5.15
	10.0	4	593	3812	6456	6766	5.11	1.48	10.9

Table 3. Summary of Dam Uterus Weight and Adjusted Body Weight/Weight Change

Dose Group	N	Uterus Weight (g) Mean (SD)	Adjusted Body Weight[a] (g) Mean (SD)	Adjusted Body Weight Change[b] (g) Mean (SD)
Group 1 (0 mg/kg)	21	88.2 (16.57)	326.3 (28.88)	63.6 (15.97)
Group 2 (0.4 mg/kg)	19	86.6 (10.57)	332.9 (27.06)	59.6 (19.61)
Group 3 (2.0 mg/kg)	19	85.5 (11.28)	328.6 (23.75)	63.9 (14.26)
Group 4 (10.0 mg/kg)	22	80.6 (17.61)	326.7 (22.23)	62.3 (12.10)

[a] Adjusted body weight = final (Gestation Day 20) body weight (g) - uterus weight (g)
[b] Adjusted total weight gain = total gain (g) - uterus weight (g)

CONCLUSIONS

- Administration of cytisine to male and female rats at levels of 0.4, 2.0 and 10.0 mg/kg for four (males) or two (females) weeks prior to mating, during the two-week mating period (both sexes), through Gestation Day 17 (females) and until sacrifice on Day 73 (males) did not result in any treatment-related mortality or clinical signs of toxicity.
- A slight treatment-related effect on body weight/body weight gain was seen at the 10.0 mg/kg dose level in both males and females.
- No treatment-related effects on mating, cyclicity, male or female fertility, implantation or early embryonic development were observed.
- No treatment-related embryofetal developmental effects were seen at any dose level, and no treatment-related effects on male reproduction were observed.
- Administration of cytisine under the conditions of this study at a dose up to 10.0 mg/kg did not result in any reproductive toxicity in rats.

Table 4. Summary of Maternal Reproduction and Litter Viability Data

Parameter	Group 1 (0 mg/kg)	Group 2 (0.4 mg/kg)	Group 3 (2.0 mg/kg)	Group 4 (10.0 mg/kg)
Initial group size (sperm positive)	21	19	19	22
Actual group size (gravid)	21	19	19	22
Number of viable litters (at least one live implant)	21	19	19	22
Number of non-viable litters (no live implants)	0	0	0	0
Total number of corpora lutea (mean ± SD)	362 17 ± 2.7	299 16 ± 3.1	308 16 ± 3.3	348 16 ± 2.1
Total number of implants (mean ± SD)	326 15 ± 2.7	288 15 ± 2.2	285 15 ± 2.5	304 14 ± 3.3
% pre-implantation loss (mean ± SD)	10 ± 12.1	6 ± 11.8	7 ± 8.4	13 ± 20.4
% post-implantation loss (mean ± SD)	6 ± 12.6	3 ± 7.0	6 ± 5.8	6 ± 8.0
Total number of live implants (fetuses) (mean ± SD)	307 15 ± 3.4	280 15 ± 2.5	268 14 ± 2.2	286 13 ± 2.2
% live implants (out of total implants)	94	97	94	94
Dead Fetuses (Number per Dam) (mean ± SD)	2 0 ± 0.4	1 0 ± 0.2	0 0 ± 0.0	0 0 ± 0.0
Number of resorptions (total)	17	7	17	18
Litters with resorptions	9	5	11	10
Total number of male fetuses	168	133	153	151
Total number of female fetuses	139	148	115	135
Sex ratio (male:female %)	121	90	133	112

Table 5. Summary of Fetal Body Weight Data

Sex	Group 1 (0 mg/kg) N=21	Group 2 (0.4 mg/kg) N=19	Group 3 (2.0 mg/kg) N=19	Group 4 (10.0 mg/kg) N=22
	Fetal Body Weight (g) (mean ± SD)			
Male	3.93 ± 0.326	3.96 ± 0.253	4.10 ± 0.273	4.19 ± 0.355*
Female	3.67 ± 0.379	3.78 ± 0.254	3.83 ± 0.250	3.97 ± 0.281
Combined Sexes	3.82 ± 0.347	3.86 ± 0.256	3.98 ± 0.243	4.09 ± 0.329*

Table 6. Summary of Fetal Skeletal Abnormalities by Individual Alteration

Skeletal Abnormality	Group 1 (0 mg/kg)	Group 2 (0.4 mg/kg)	Group 3 (2.0 mg/kg)	Group 4 (10.0 mg/kg)
	Number of Fetuses Affected (Number of Litters) [a]			
Number Examined:	**152 (21)**	**141 (19)**	**134 (19)**	**145 (22)**
SKULL				
Interparietal – reduced ossification	2 (2)	7 (6)	1 (1)	2 (2)
Parietal – reduced ossification	1 (1)	3 (3)	1 (1)	3 (3)
Supraoccipital – reduced ossification	1 (1)	4 (3)	1 (1)	1 (1)
Fontanel – enlarged	1 (1)	1 (1)	– (–)	3 (3)
Premaxilla – reduced ossification	– (–)	– (–)	1 (1)	– (–)
Maxilla – reduced ossification	– (–)	– (–)	1 (1)	– (–)
NECK				
Hyoid – unossified	1 (1)	1 (1)	– (–)	– (–)
VERTEBRAL COLUMN				
Thoracic Centra				
Bipartite	23 (10)	17 (11)	5 (4)	6 (5)
Dumbbell	3 (3)	2 (2)	7 (4)	4 (3)
Absent	1 (1)	– (–)	– (–)	– (–)
Misaligned	– (–)	– (–)	– (–)	1 (1)
Lumbar Centra – bipartite	– (–)	– (–)	– (–)	1 (1)
Sacral Centra – unossified	– (–)	1 (1)	– (–)	– (–)
Pubis/Ischium – unossified	– (–)	1 (1)	– (–)	– (–)
Pubis – absent	– (–)	– (–)	1 (1)	1 (1)
PECTORAL GIRDLE				
Sternebrae				
Unossified (1-4)	1 (1)	1 (1)	1 (1)	1 (1)
Unossified (5 and/or 6)	67 (20)	59 (18)	52 (14)	35 (16)
RIBS				
Rudimentary – right	2 (2)	– (–)	– (–)	– (–)
Rudimentary – left	1 (1)	1 (1)	– (–)	1 (1)
Extra – right	– (–)	1 (1)	– (–)	– (–)
Extra – left	– (–)	1 (1)	– (–)	– (–)