CITATION: Handa R, 2014. Terbuthylazine: An oral (gavage) study to assess the effects on the hormone-induced luteinizing hormone surge in ovariectomized female Wistar rats. College of Medicine, The University of Arizona Phoenix, 425 N. 5th Street, Building ABC1, Room 422, Phoenix, AZ 85004-2157, USA. Laboratory Report No. UA4205870-2 (Amendment 1), 17 September 2014. Unpublished. (Syngenta File No. GS13529_50056).

GUIDELINES: This is an investigative study with no applicable guidelines.

GLP: This non-guideline investigative study was not conducted in compliance with the current US EPA 40 CFR Part 160 GLP regulations. Analytical method validation and dose formulation analyses were performed under EPA 40 CFR Part 160 GLP.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a preferred test species. The number of animals used was considered to be the minimum required to meet the scientific and regulatory objectives of the study.

Previous studies have shown that the chlorotriazine herbicide, atrazine, can block the oestrogen and/or progesterone induced luteinizing hormone (LH) surge in ovariectomized Wistar, Sprague-Dawley (SD), or Long Evans (LE) rats when treated with high doses (***Foradori et al., 2009, McMullin et al., 2004***). Consequently, the objective of this study was to determine if terbuthylazine (TBA), a chlorotriazine with a similar chemical structure to atrazine, could block the hormone-induced LH surge in Wistar rats. The preovulatory LH surge was induced in adult ovariectomized female rats by the timed administration of estradiol (E) and progesterone (P). Previous studies have demonstrated that this is an effective procedure that induces a preovulatory–like surge of LH (***Foradori et al., 2009, 2011***).

EXECUTIVE SUMMARY

In a study to assess the effects on the hormone-induced luteinizing hormone (LH) surge in ovariectomized female rats, three groups of 8, 11 and 10 ovariectomized female Wistar rats were dosed via oral gavage with 1% carboxymethylcellulose sodium salt in saline (vehicle control group), 100 mg atrazine/kg/day (positive control group) or 106.5 mg terbuthylazine /kg/day (molar equivalent of 100 mg/kg atrazine), respectively. The animals were ovariectomized and stimulated with estradiol + progesterone to induce an afternoon LH surge. On the fourth day following ovariectomy, plasma samples were taken (once hourly for 8 hours) and analysed for LH levels.

Treatment of ovariectomized female Wistar rats with either terbuthylazine or atrazine resulted in a statistically significant reduction in the LH surge. The peak LH level of the terbuthylazine treated group was reduced to approximately 26% of the control levels while the peak LH level of the atrazine treated group was reduced to approximately 33% of the control level. In addition, treatment with terbuthylazine or atrazine resulted in a significant reduction in body weight.

Conclusion: At the dose level tested (106.5 mg/kg/day), terbuthylazine reduced the magnitude of the LH surge in female Wistar rats, analogous to the effects shown with molar equivalents of atrazine.

MATERIALS AND METHODS
Materials:

Test Material:	Terbuthylazine
Description:	Technical, white powder
Source:	Syngenta Crop Protection Inc., St. Gabriel, LA, USA
Lot/Batch number:	Batch ID Number 544309, Lot GBL6K26
Purity:	97.4 % a.i
CAS#:	Not reported
Stability of test compound:	Recertification date end May 2015 (stored at <30°C)

Positive Control Material:	Atrazine
Description:	Technical, white powder
Source:	Syngenta Crop Protection Inc., St. Gabriel, LA, USA
Lot/Batch number:	Batch Number 428162, Lot GBL3B17EB6
Purity:	97.6 % a.i
CAS#:	Not reported
Stability of test compound:	Confirmed 23 April 2014

Vehicle and/or positive control: 1% carboxymethylcellulose (CMC) in saline

Test Animals:	
Species	Rat
Strain	Wistar
Age/weight at dosing	60-70 days old on arrival
Source	Charles River Laboratories, San Diego, CA, USA.
Housing	Individually in individually-ventilated, disposable cages
Acclimatisation period	1 Week
Diet	Purina® Rat Chow® *ad libitum*
Water	Reversed-osmosis purified drinking water *ad libitum*
Environmental conditions	Temperature: 22±2°C Humidity: 50±15% Air changes: Not reported Photoperiod: 12 hours light, 12 hours dark

Study Design and Methods:
In-life dates: Start: 06 February 2014 End: 18 April 2014

Ovariectomization: All animals were bilaterally ovariectomized under isoflurane anaesthesia following the acclimation period.

Animal assignment: Animals were randomly assigned to each of the following three groups:

Table 1: Study design

Test group	Dose (mg/kg bw/day)	Number of female rats
Vehicle control	0	8
Atrazine (positive control)	100	11
Terbuthylazine	106.5	10

Dosing preparations and analysis: Terbuthylazine and atrazine were formulated in 1% carboxymethylcellulose (CMC) in saline for dosing. Test substances were formulated the day prior to gavage treatment, stored at 4°C overnight and stirred continuously during dosing. Terbuthylazine and atrazine dose solutions were analyzed for concentration verification by WIL Research Laboratories.

Analysis results: Dose formulations for terbuthylazine and atrazine were 89.1% and 96.6% of nominal concentration, respectively.

Calibration standards of the test substance formulations stored at room temperature for a minimum of 3 days met the acceptance criteria for stability (post-storage concentration >90% of the pre-storage value).

Test substance homogeneity in 0.1 and 125 mg terbuthylazine/mL formulations met the acceptance criteria for homogeneity (RSD for the mean concentration was ≤10% at a concentration within the acceptable limits (85% to 115% of target). Assessment of test substance resuspension homogeneity and stability in formulations prepared at target concentrations of 0.1 and 125 mg terbuthylazine/mL following a minimum of 24 hours storage at room temperature met the acceptance criteria for resuspension homogeneity (RSD for the mean concentration was ≤10%) and met the acceptance criteria for stability. Assessment of test substance stability in formulations prepared at target concentrations of 0.1 and 125 mg terbuthylazine/mL following a minimum of 14 days of frozen (-20°C) storage met the acceptance criteria for stability.

Treatment: The test group animals were given an oral gavage dose of 106.5 mg terbuthylazine/kg body weight (the molar equivalent of 100 mg/kg atrazine) for four consecutive days once daily between 1000 and 1100h. Vehicle control rats were dosed with vehicle only at a dose volume of 5 mL/kg bw and the positive controls were dosed with 100 mg atrazine/kg bw.

On the second day of treatment, intra-atrial cannulae were implanted (***Foradori et al., 2009***) for blood sampling.

An LH surge was induced by giving a subcutaneous (SC) injection to each animal of 40 µg 17β-estradiol-3-benzoate/kg bw between 1000 and 1100h for three consecutive days, beginning on the second day of treatment. On the third day of injections and the final day of terbuthylazine treatment, the animals were given a SC injection of 2 mg/animal progesterone at 1100h to induce an afternoon LH surge. On the afternoon of the fourth day of treatment, blood samples (300 µL per sample) were taken at one hour intervals from conscious freely moving animals commencing at 1400h (lights out at 1900h) and extending to 2100h. Following each sample, the animal's blood volume and haematocrit were maintained by infusing each animal with an equivalent amount of red blood cells (that had been obtained from the previous sample) in saline. Plasma was removed from each blood sample by centrifugation and frozen for later analysis of LH by radioimmunoassay.

All animals were examined daily. Body weight measurements were recorded prior to the first treatment with terbuthylazine or atrazine and at the time of blood sampling.

Blood collection and processing: All blood samples were collected within ±10 minutes of the target collection time into chilled tubes containing 10 µL 1 M EDTA as the anticoagulant. Plasma samples were obtained by centrifugation and stored frozen at approximately -70°C until analysis.

Radioimmunoassay for luteinizing hormone: Plasma LH levels were measured using reagents provided by the National Hormone and Peptide Program (Torrance, CA, USA). Rat LH-RP3 was used to create a standard curve (range:102.5 - 0.0049 ng). Tracer LH was iodinated using the chloramine-T method by the Colorado State University peptide assay core. Samples (10-25 µL per animal/time) were incubated overnight at 4°C with antiserum (NIDDK-Anti-rLH-SII; diluted 1:160,000). Following incubation, iodinated ovine LH (oLH, NHPP) was added to each tube (~10,000 cpm/tube) and incubated overnight at 4°C. Bound LH was separated from free LH by incubation with goat anti-rabbit γ-globulin (Calbiochem, cat#539845; 1:1000) in a 5% polyethylene glycol (Fisher Biotech; BP233-1) solution. The intra-assay variance, as measured by internal quality controls, was 8.9%.

Statistics: All LH data were initially analyzed by two-way ANOVA. Additionally, the peak amplitude of the LH surge was calculated by determining the highest concentration of LH achieved during the

sampling period, regardless of timing. The LH levels were plotted using all time points, and the area under the curve (AUC) for each hormone was determined using Prism 5 software (GraphPad Software, San Diego, CA, USA). A one-way ANOVA was used for analysis of peak amplitude and AUC data. The level of statistical significance was set at $p \leq 0.05$ for all studies.

RESULTS AND DISCUSSION

Effects on the luteinizing hormone surge: Terbuthylazine or atrazine caused a significant reduction in the estradiol + progesterone-induced LH surge. The results of a two-way ANOVA (treatment x time) analysis of plasma LH levels revealed a significant main effect of treatment [$F_{2,206} = 50.12$; $p < 0.0001$], time [$F_{7,206} = 12.49$, $p < 0.0001$] and interaction effect [$F_{14,206} = 3.77$, $p < 0.001$]. Post-hoc analysis showed that there was a significant difference between the vehicle and TBA groups at 1700h ($p < 0.05$) and between 1800h to 2100h (all $p < 0.001$). Similarly, when vehicle control and atrazine groups were compared, there were significant differences at 1700h ($p<0.05$), 1800-2000h ($p < 0.001$), and 2100h ($p < 0.01$).

Table 2: Summary of plasma leuteinizing hormone levels (ng/mL)

Treatment group		Time of day							
		14:00	**15:00**	**16:00**	**17:00**	**18:00**	**19:00**	**20:00**	**21:00**
Vehicle	Mean	1.52	1.85	3.47	13.36	25.24	22.74	25.08	20.44
	SEM	0.38	0.50	0.81	3.75	4.83	5.83	6.53	4.95
	N	8	8	8	8	8	8	7	7
100 mg/kg bw atrazine	Mean	0.75	1.66	1.90	2.88	6.60***	7.20***	6.41***	6.32**
	SEM	0.27	0.46	0.47	0.87	1.98	2.47	2.12	2.17
	N	11	11	11	11	11	11	11	11
106.5 mg/kg bw terbuthylazine	Mean	0.96	0.45	0.69	1.56*	5.37***	6.20***	3.60***	3.66**
	SEM	0.50	0.22	0.30	0.47	3.90	3.22	1.72	1.28
	N	10	10	10	10	10	10	10	10

* statistically significant ($p<0.05$), **statistically significant ($p < 0.01$), *** statistically significant ($p<0.001$)

Peak LH level of the terbuthylazine and atrazine-treated groups was approximately 26% and 33% of the vehicle control group values, respectively. There was a significant reduction in peak LH amplitude between treatment groups compared to vehicle controls group [$F_{2,26} = 8.51$, $p = 0.0014$]. Post hoc analysis using Bonferonni's multiple comparison test showed significant reductions ($p < 0.01$) in the terbuthylazine and atrazine groups compared to vehicle controls but no difference between the terbuthylazine and atrazine groups. Similarly, both terbuthylazine and atrazine reduced the LH AUC compared to the vehicle group [$F_{2,26} = 11.63$, $p = 0.0002$]. Post-hoc analysis with Bonferroni's multiple comparison test showed significant reductions in LH AUC in the terbuthylazine and atrazine groups versus controls ($p < 0.01$) with no difference between the terbuthylazine and atrazine groups.

Table 3: Summary of plasma leuteinizing hormone levels (ng/mL): LH peak and LH AUC

Treatment group	Peak LH amplitude		LH area under the curve	
Vehicle	Mean	31.80	Mean	97.38
	SEM	5.51	SEM	18.91
	N	8	N	8
100 mg/kg bw atrazine	Mean	10.13**	Mean	30.17**
	SEM	2.49	SEM	7.36
	N	11	N	11
106.5 mg/kg bw terbuthylazine	Mean	8.19***	Mean	20.18***
	SEM	3.91	SEM	9.502
	N	10	N	10

* statistically significant (p<0.05), ** statistically significant (p<0.01), *** statistically significant (p<0.001)

Data were recalculated with the peak LH surge aligned to visualize the dynamics of the LH surge. Two-way ANOVA (treatment x time) showed a significant main effect of treatment ($F_{2,145} = 44.23$, $p < 0.0001$) and time ($F_{6,145} = 4.23$, $p = 0.0006$). There was no interaction effect ($p = 0.5007$). Bonferroni post-hoc analysis showed significant decreases at the -1 hr relative to peak ($p < 0.01$), 0 (designated as the peak; $p < 0.001$) and +1 hr relative to peak ($p < 0.01$) for terbuthylazine and atrazine treatment.

Table 4: Summary of plasma leuteinizing hormone levels (ng/mL): LH peak and LH AUC

Treatment group		Time relative to peak						
		-3	**-2**	**-1**	**0**	**1**	**2**	**3**
Vehicle	Mean	7.06	11.46	20.17	30.91	22.70	18.80	19.22
	SEM	4.08	3.96	5.36	5.67	5.30	4.18	5.83
	N	8	8	8	8	7	4	4
100 mg/kg bw atrazine	Mean	2.19	2.40	5.58**	9.37***	6.97**	4.70	3.97
	SEM	0.53	0.81	1.95	2.63	2.10	1.80	3.78
	N	11	11	11	11	9	3	3
106.5 mg/kg bw terbuthylazine	Mean	0.97	0.98	1.25***	6.29***	4.63***	3.11*	1.94*
	SEM	0.53	0.37	0.44	4.29	3.31	1.60	1.53
	N	9	9	9	9	9	9	6

* statistically significant (p<0.05), ** statistically significant (p<0.01), *** statistically significant (p<0.001)

Bodyweight: A significant effect of treatment on body weight change was observed when all treated animals were analysed ($F_{2,32} = 26.17$, $p < 0.0001$) and when only animals with a blood sample were included in the analysis ($F_{2,26} = 19.66$, $p < 0.0001$). Post-hoc analysis revealed significant decreases in body weight in the terbuthylazine and atrazine groups ($p < 0.001$), when compared to vehicle-treated animals.

Table 5: Summary of body weight changes (g)

Treatment group	Final body weight change: all animals		Final body weight change: sampled animals only	
Vehicle	Mean	-7.00	Mean	-5.63
	SEM	2.31	SEM	3.15
	N	12	N	8
100 mg/kg bw atrazine	Mean	-22.18**	Mean	-22.18**
	SEM	2.33	SEM	2.33
	N	11	N	11
106.5 mg/kg bw terbuthylazine	Mean	-26.43***	Mean	-26.30***
	SEM	1.29	SEM	1.56
	N	12	N	10

* statistically significant ($p<0.05$), ** statistically significant, ($p<0.01$), *** statistically significant ($p<0.001$)

CONCLUSION: At the dose level tested (106.5 mg/kg/day), terbuthylazine reduced the magnitude of the LH surge in female Wistar rats, analogous to the effects shown with molar equivalents of atrazine.

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(Handa R, 2014)